UTS UNIVERSITY OF TECHNOLOGY SYDNEY

The development and validation of screening test methods for the presumptive detection of New Psychoactive Substances

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Thesis submitted in fulfilment of the requirements for the degree of **Doctor of Philosophy (Science)**

under the supervision of Prof. Shanlin Fu, Dr. Ronald Shimmon and Dr. Morgan Alonzo

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Certificate of authorship and originality

I, Laura Clancy, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Mathematical and Physical Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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Date

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Abbreviations

µPAD paper-based microfluidic analytical device

25B-NB4OMe 4-bromo-2,5-dimethoxy-*N*-[(4-methoxyphenyl)methyl]-benzeneethanamine

25B-NBF 4-bromo-*N*-[(2-fluorophenyl)methyl]-2,5-dimethoxy-benzeneethanamine

25B-NBOMe 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25C-NB3OMe 4-chloro-2,5-dimethoxy-*N*-[(3-methoxyphenyl)methyl]-benzeneethanamine

25C-NBOMe 2-(4-chloro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25D-NBOMe 2-(4-methyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25E-NBOMe 2-(4-ethyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25G-NBOMe 2-(3,4-dimethyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25H-NB4OMe 2,5-dimethoxy-*N*-[(4-methoxyphenyl)methyl]-benzeneethanamine

25H-NBOMe 2-(2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25I-NB3OMe 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-(3-methoxybenzyl)ethan-1-amine

25I-NBMD *N*-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethanamine

25I-NBOH 2-(((4-iodo-2,5-dimethoxyphenethyl)amino)methyl)phenol

25I-NBOMe 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25iP-NBOMe 2-(4-isopropyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25-NBOMe 2,5-dimethoxy-*N*-(2-methoxybenzyl) phenethylamine derivatives

25N-NBOMe 2-(4-nitro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25P-NBOMe 2-(4-propyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25T2-NBOMe 2-(4-ethylthio-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25T4-NBOMe 2-(4-isopropylthio-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25T7-NBOMe 2-(4-propylthio-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

25T-NBOMe 2-(4-methylthio-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

2C-B 2,5-dimethoxy-4-bromophenethylamine

2C-D 4-methyl-2,5-dimethoxy-benzeneethanamine

2C-E 4-ethyl-2,5-dimethoxy-benzeneethanamine

2C-H 2,5-dimethoxy-phenethylamine

2C-I 4-iodo-2,5-dimethoxy-benzeneethanamine

2C-T-2 4-ethylthio-2,5-dimethoxy-benzeneethanamine

2C-X 2,5-dimethoxy-phenethylamine derivatives

30C-NBOMe 2-(4-chloro-2,5-dimethoxyphenyl)-*N*-(3,4,5-trimethoxybenzyl)ethanamine

4-FMC 4-fluoromethcathinone

4-MEC 4-Methyl-*N*-ethylcathinone

4-MMC 4-methyl-*N*-methylcathinone

$5HT_{2A}$ 5-hydroxytryptamine 2A serotonin receptor

ACD acetaldehyde

ACN acetonitrile

AFP Australian Federal Police

ANOVA analysis of variance test

ATR attenuated total reflectance

ATS amphetamine-type substances

BZP 1-benzylpiperazine

conc. concentrated

CYMK cyan, yellow, magenta, black

DAD diode array detector

DART	direct analysis in real time
DCM	dichloromethane
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DEA	Drug Enforcement Administration
DIMS	Drug Information and Monitoring System
DPIC	Dutch Poisons Information Centre
EI	electron ionisation
ELISA	enzyme linked immunosorbent assays
EMCDDA	European Monitoring Centre on Drugs and Drug Addiction
EWA	Early Warning Advisory
FTIR	Fourier transform infrared spectroscopy
GC	gas chromatography
h	hours
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
HSB	hue, saturation, and brightness
HSL	hue, saturation, and lightness
HTML	hexadecimal colour
IR	infrared
JWH-018	1-naphthalenyl(1-pentyl-1*H*-indol-3-yl)-methanone
JWH-073	(1-butylindol-3-yl)-naphthalen-1-ylmethanone
LC	liquid chromatography
LOD	limit of detection
LSD	lysergic acid diethylamide
MANOVA	multivariate analysis of variance test

mCPP	1-(3-chlorophenyl)piperazine
MDMA	3,4-methylenedioxy-*N*-methylamphetamine
MDPBP	3,4-methylenedioxy-α-pyrrolidinobutiophenone
MDPV	methylenedioxypyrovalerone
MeOH	methanol
Mescaline-NBOMe	3,4,5-trimethoxy-*N*-[(2-methoxyphenyl)methyl]-benzeneethanamine
min	minutes
MPBP	4-methyl-α-pyrrolidinobutiophenone
MPPP	4-methyl-α-pyrrolidinopropiophenone
MS	mass spectrometry
NIJ	National Institute of Justice
NIR	near-infrared
NMR	nuclear magnetic resonance
NPF	Non-pharmaceutical fentanyls
NPS	new psychoactive substances
NQS	1,2-napthoquinone-4-sulphonate
***o*-TCBQ**	3,4,5,6-tetrachloro-1,2-benzoquinone
PIHKAL	'Phenethylamines I Have Known and Loved'
PMA	*para*-methoxyamphetamine
PMMA	*para*-methoxymethamphetamine
ppm	parts per million
QQQ-MS	triple quadrupole mass spectrometry
QTOF-MS	quadrupole time of flight mass spectrometry
RGB	red, green, blue
RT	room temperature

RYB	red, yellow, blue
SCRA	synthetic cannabinoid receptor agonists
SUSMP	Standard for the Uniform Scheduling of Medicines and Poisons
SWGDRUG	Scientific Working Group for the Analysis of Seized Drugs
TCBQ	2,3,5,6-tetrachloro-1,4-benzoquinone
TFMPP	1-(3-trifluoromethylphenyl)piperazine
TLC	thin layer chromatography
UK	United Kingdom
UN	United Nations
UNODC	United Nations Office on Drugs and Crime
UR-144	(1-pentyl-1*H*-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone
USA	United States of America
UV	ultraviolet
WHO	World Health Organisation
Δ^9-THC	delta-9-tetrahydrocannabinol

Abstract

The growing number of new psychoactive substances (NPS) necessitates the need to identify these drugs under any circumstance. Rapid, on-site presumptive tests are integral to identifying unknown substances in case work, healthcare settings, and harm reduction situations. There are currently a wide variety of colour tests available for the identification of traditional illicit drugs (e.g. cocaine, amphetamines), however, specific tests for the detection of NPS are limited. The inability of current presumptive test methods to selectively identify the large variety of NPS indicates the need for the development of such techniques that can provide information about the presence of these drugs in unknown materials. This research has developed a thoroughly validated colour test method utilising 2,3,5,6-tetrachloro-1,4-benzoquinone that is capable of selectively identifying NBOMe compounds, with significant potential implications for application in case work settings. This test method was then further investigated to improve the portability and the discrimination of the colour change results. Mobile phone applications were assessed for their ability to utilise the inbuilt camera to record RGB values which may be attributed to a drug, drug class, and even a concentration range. Statistical analysis of the RGB values was completed to further determine the applicability of this method. Promising results indicate that NBOMe analogues can be distinguished from other illicit compounds. The further optimisation of the predictive model would be of benefit for the extension of this study. The translation of the NBOMe test, along with three other colour test methods for the detection of synthetic cathinones (neocuproine-copper reagent), piperazines (1,2-naphthoquinone-5-sulphonate reagent), and fentanyl analogues (naphthoquinone reagents), to paper-based systems was assessed for ease of portability and the potential for development of a multiplexed device. The combination of these tests, and therefore their results combined into a single device, would assist in the identification process of a range of NPS without the need for multiple test kits. A proof-of-concept study was carried out to assess the possible visualisation of NBOMe compounds in oral fluid samples. The addition of colour test reagents to spiked oral fluid samples, or the inclusion of a paper chromatographic method, demonstrated the ability to detect NBOMe compounds. While there is still optimisation required, these methods showed great potential to be used in the presumptive identification of NPS and in particular, NBOMe analogues. This research demonstrates the promise of simple, affordable, and rapid methods to be utilised as on-site testing devices for NPS.

Chapter 1:

Introduction

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1.1 New Psychoactive substances

Since 2009, the illicit drug industry has developed with current research and technology helping to unveil new substances which have not previously been used recreationally. New psychoactive substances (NPS) are, according to the United Nations Office on Drugs and Crime (UNODC), emerging drugs of abuse which are not scheduled under the United Nations 1961 nor the 1971 Conventions, but may pose a public health threat [1, 2]. NPS encompass a wide variety of substances, with the most common effect types being synthetic cannabinoid receptor agonists (SCRA), stimulants, hallucinogens, and more recently, opioids [3]. NPS are primarily research drugs, or compounds first synthesised years ago which have only recently begun to be misused [4]. They can be derivatives or analogues of illicit drugs, misused pharmaceuticals, research chemicals, and naturally occurring compounds [5]. Every year identified NPS are reported to the UNODC Early Warning Advisory (EWA). Up to December 2020, 1047 different NPS had been reported to the EWA throughout 126 countries and territories since 2009 [6]. The last few years have shown a steadying of the NPS market in terms of the number of substances seen each year, remaining around 500. However, as observed with the increase in synthetic opioids, it is evident that even though the overall quantity of substances may not increase, the variety of substances is ever-changing [6, 7].

NPS can exhibit a wide range of desired effects depending on the drug type that they may be used as an alternative to, from stimulants like cocaine and 3,4-methylenedioxymethamphetamine (MDMA), to classic hallucinogens such as lysergic acid diethylamide (LSD) and even heroin [8]. It has been reported that NPS have functioned as market substitutes at times of low availability or poor quality of established illicit drugs [9-11]. There are many other reasons why drug users may choose NPS over traditional illicit drugs. Legal status in comparison to other traditional illicit drugs, availability, cost, and the desire to avoid detection due to the limited testing methods currently available were early motivations behind the use of NPS [12, 13]. In the UK, the legal status of NPS has not been shown to be the primary motivation for use over other illicit drugs. Their use is more related to experimentation, as users are often users of existing illicit drugs anyway [14-16]. Similar to that of many other substances, motives behind the use of NPS include curiosity, affordability, enjoyable effects, euphoria, creative thinking, and exploration [13, 17-21]. Some groups, such as

military personnel and athletes, may choose NPS as they are regularly drug tested and these substances may avoid detection [17].

Many NPS have additional, accompanying effects to those seen with corresponding traditional drugs. Furthermore, there is little research on the effects of NPS and possible interactions with other medications, illicit substances, and mental health disorders [22]. NPS use in conjunction, either intentionally or not, with other drugs is apparent, and with few studies completed on the effects, may raise some serious health concerns [23-25]. Unfortunately, trend information on NPS is limited, and evidence of the use of these substance is difficult to obtain due to their highly dynamic nature [26]. This has led to limitations on the information provided to drug users to allow for safer use of NPS in different settings [27]. Difficulties are also present within health services, with minimal information on effects and potential harms associated with the use and treatment of NPS abuse and overdose available for health care providers [3].

The internet has been an important driver for the development of the NPS market, particularly in the last decade. In 2013, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) identified hundreds of websites with 'legal highs' for sale [28]. There is some evidence to suggest many NPS users prefer to purchase drugs online as they view it as a safer alternative to meeting a dealer in person [13]. As these substances become legislated around the world, the move to dark net marketplaces is apparent. Here, vendors can avoid identification, raising issues for law enforcement and the control of these substances [22]. Some disruption of drug sales on the dark net was noticeable after the shutdown of several major sites in 2017. However, it is unknown if these results are ongoing as new sites have now likely filled this void [29].

1.1.1 Phenethylamines

Phenethylamines, including 2C-X and 25-NBOMe compounds, are becoming more prevalent among users of NPS. They are the third most prevalent NPS class, and use appears to be rising. Sutherland et al. [30] reported that in a group of previous drug users, eight percent had used phenethylamines in the six months before the study (2010). By 2015, when the study concluded, this had increased to over eighteen percent, making them the most commonly used NPS in these years. The UNODC, in their 2017 World Drug Report, also reported an increase in the use of hallucinogenic NPS, in particular 25-NBOMe compounds, in certain regions such as South America [3]. In South America, NBOMes are commonly sold on blotters as LSD or 'legal LSD' alternatives posing risks to users if they are not aware of what they are taking [31-33].

2C-X compounds are substituted hallucinogenic phenethylamines, with a similar structural backbone to amphetamine type substances [34]. They began to gain popularity in the 1980s when 2C-B became a choice of replacement for MDMA due to its highly stimulating and hallucinogenic properties [35, 36]. Shulgin's book, *Phenethylamines I Have Known and Loved (PIHKAL)*, described the synthesis of a range of psychoactive phenethylamines, including many of the 2C-X series, and helped continue their popularity into the 1990s [37]. Evidence suggests that 2C-X series compounds are still being used by young people today as a 'party drug' similar to MDMA [38]. 2C-X compounds are frequently present as powders or small tablets containing different quantities of the drug. 2C-X substances, and in particular 2C-B, have been reported as some of the most commonly used NPS by regular drug users in Australia [11, 39, 40]. In 2013, 2C-B was the most frequently reported 'new phenethylamine' in Europe [41].

The terminology '2C' originally referred to the number of carbons between the benzene ring and the primary amine group. However, now it more generally corresponds to hallucinogenic phenethylamines containing two methoxy groups, one at each of the 2- and 5- positions of the benzene ring, rather than the large number of phenethylamines included in the initial definition (see Figure 1-1) [34, 42]. Further substitution on the benzene ring, commonly at the 3- and/or 4-position, effects the potency of the drug and can consist of almost any functional group but are commonly halogens, alkyl, and alkyl thiol groups [43]. These substituents, along with the additional methoxy groups, greatly increase the hallucinogenic properties of these substances, particularly in comparison to other phenethylamine-type drugs [44].

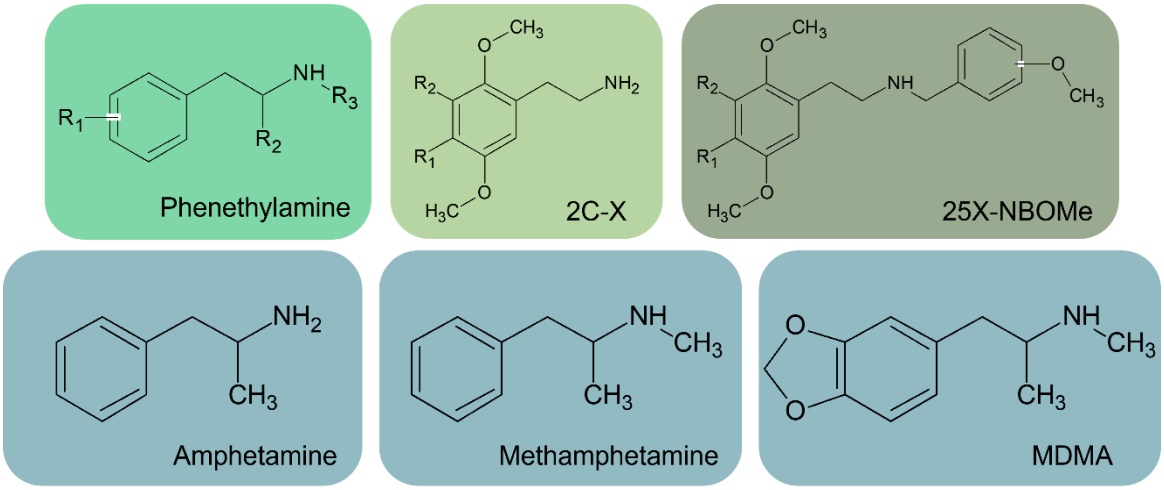

Figure 1-1: General structures of phenethylamine-type drugs

2C-X compounds are serotonergic hallucinogens that act as 5-HT_{2A} receptor agonists similar to other hallucinogens such as LSD [36]. They are highly potent, with typical dosage amounts of around 10-50 mg. Effects last up to five hours and after effects, those exhibited after the 'high' or during the 'coming down' stage, may last for up to four hours. As with many drugs, the route of administration determines the dose required for individual effects to be seen. Insufflation only requires approximately one-third of an oral administration dose [35]. The World Health Organisation (WHO) recommended international control of 2C-B in 2001 and subsequently placed it in Schedule II of the 1971 Convention on Psychotropic Substances [45, 46]. Other 2C-X compounds are specifically included in national legislation around the world [36, 47-50].

25-NBOMe substances are derived from the 2C-X series and display hallucinogenic properties from the addition of a 2-methoxybenzyl group on the amine (see Figure 1-1). 25-NBOMe compounds were first synthesised in 2003 by Ralf Heim as part of a PhD dissertation at the Freie Universität Berlin [51, 52]. The appearance on NBOMe compounds on the drug market, however, has only been reported in recent years and were initially often sold as 'legal LSD' [53]. Not until 2011 was 25I-NBOMe first seen on blotter papers in the designer drug market [54] and many countries such as Japan did not see 25-NBOMe substances until 2013 [55]. 25-NBOMe substances are primarily ingested as blotter papers, similar to LSD, though they are also sold as powders and liquids [56, 57].

NBOMe related compounds provide the user with stimulant and hallucinogenic or psychoactive effects, similar to that of LSD. This is due to them being highly potent agonists of the serotonin 5-HT_{2A} receptors with as little as a 50 microgram dose being an effective dose [58, 59]. The potency of 25-NBOMe compounds is increased significantly compared to phenethylamines without an N-benzyl moiety as this identifying feature provides a higher receptor affinity [60-64]. There are reports that the substituent present at the 4 position on the benzene ring may also affect the potency and the effects of 25-NBOMe compounds [65].

There have been few studies that report the effects of 25-NBOMes and most stated effects are recounts from users who have consumed a 25-NBOMe substance thinking it was another drug. Desired effects include euphoria, visual and auditory hallucinations, and feelings of love and empathy [66, 67]. Side and after effects such as panic attacks, loss of time and location, and nausea appear to be less common in comparison to other drugs. However, several effects have serious health risks for users, including tachycardia and hypertension [59, 67]. Due to their potency, ingesting only very small amounts, between 50 – 250 µg, of the drug is required to

provide the user with any effects [59, 68]. Generally, peak effect occurs one hour after consumption and the total action can be from four to ten hours when taken sublingually. Effects come about almost immediately after insufflation, with after effects possible for up to seven days after use [62]. It should be noted that there is limited information regarding the dose required for such effects and no specifics about acute vs chronic use of these drugs, with effects reported from those who have used these substances [62]. There are reports that 25-NBOMes are inactive when taken orally, and while this has not been proven, it may explain why they are so commonly found on blotter papers [62, 69]. A number of 25-NBOMe related deaths have also been reported. Due to these substances' high affinity and potency, overdoses are common, particularly when combined with other drugs such as alcohol [57, 69, 70]. Other cases indicate that acute toxicity from NBOMe related compounds were seen after taking what the user thought was LSD [57, 71, 72]. These cases resulted in severe side effects on those users, including tachycardia, hypertension, hyperthermia, agitation, seizures, and even kidney injury, which are rarely seen after ingestion of LSD [73]. Reports of self-harm after NBOMe ingestion [72, 74], along with fatalities caused directly from NBOMe use [75, 76], are common and provide insight into the increased risk of these drugs in comparison with LSD and other classic hallucinogens.

Prior to 2015, no 25-NBOMe compound was controlled by UN drug conventions, so they were technically ‘legal’ in countries with laws based on these treaties. In 2015, three 25-NBOMe compounds (25B, 25C, and 25I-NBOMe) were placed under international control and are now listed in Schedule I of the 1971 Convention on Psychotropic Substances [3, 45]. Some countries now include 25-NBOMes under their controlled substance legislation [3, 77]. Many countries, including Australia, have begun to modify their laws to account for NPS and their ever changing nature [48, 59]. Others have developed legislation which encompass a variety of NPS, including structurally similar compounds, derivatives or analogues of other listed drugs, those with comparable effects to existing controlled substances, those considered a serious drug alternative or that meet specific criteria e.g., psychoactivity [49, 59, 78, 79]. The UK’s Misuse of Drugs Act 1971 considers phenethylamine derivatives as ‘class A substances’, and the Advisory Council has expressed concerns about the high risk of overdose specifically of 25B-NBOMe [58, 80].

The need to differentiate the contents of blotter papers sold as LSD is therefore apparent, and the ability to do this on site would be advantageous. This, along with the apparent ease of accessibility, highlights the need to identify these substances and control both the sale and use to help prevent further incidents occurring.

1.1.2 Synthetic cathinones

Cathinone is a naturally occurring alkaloid (see Figure 1-2) found in the khat plant, a plant grown and used throughout Africa and the Arabian Peninsula often for the euphoric and stimulant effects when the leaves are chewed [81, 82]. Synthetic cathinones are beta-ketone derivatives of phenethylamines repeatedly characterised as amphetamine-like psychostimulants [83]. Commonly sold as "bath salts", synthetic cathinones became popular due to the stimulant and hallucinogenic effects that mimic those of illicit drugs such as cocaine, MDMA, and amphetamine [84].

The first synthetic cathinones were created in the early 20th century for potential clinical uses, though many were not used due to unfavourable side effects and the potential for abuse [85, 86]. Methcathinone was used throughout Russia and the Soviet Union in the 1930s and 1940s [87] and was the first so called designer cathinone reported in the USA in the early 1990s [88]. By the early 2000s, there was a reappearance of several cathinone derivatives with their use increasing and emerging as "legal highs" and "legal MDMA" [14, 89]. By 2009 the popularity of these drugs was surging, coinciding with the poor quality and low availability of MDMA and cocaine throughout Europe [14, 87]. The legal status of these drugs, notably mephedrone, may also have contributed to their popularity at this time [90].

Structurally, cathinones are similar to amphetamines and other phenethylamine compounds with only the addition of a carbonyl group at the β position of the alkyl side chain. They can be further distinguished into three main structural groups with modifications in three distinct regions (see Figure 1-2).

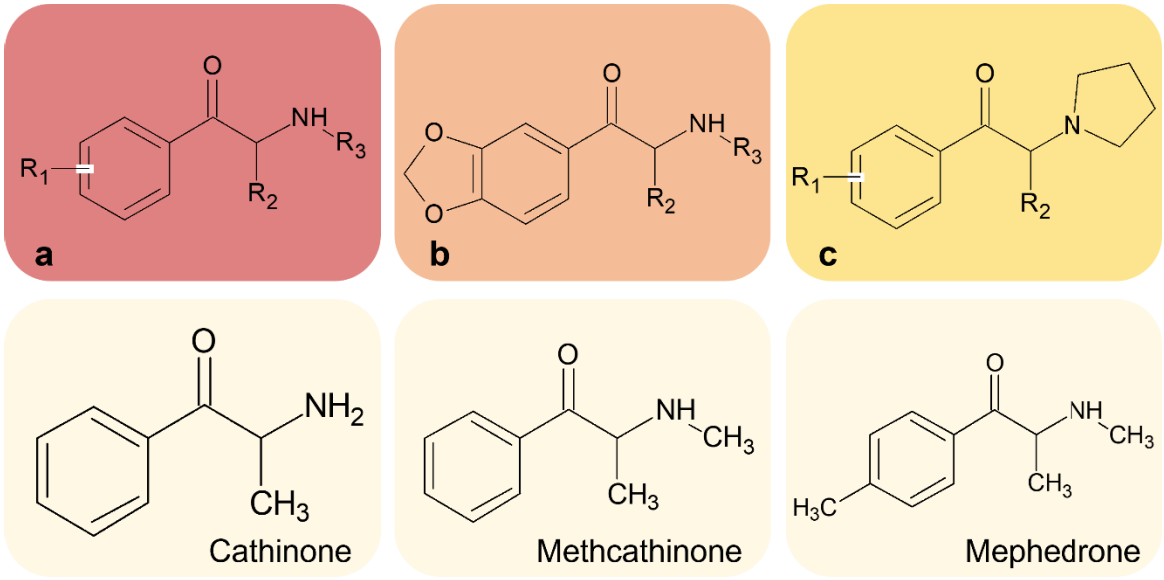

Figure 1-2: Top: a) cathinone general structure, b) methylenedioxy substituted cathinones, c) pyrrolidine substituted cathinones. Bottom: structures of common cathinones

Depending on the cathinone structure, the compound's overall effects can change based on the receptors upon which they act. For example, unlike amphetamine and methamphetamine, methcathinone binds to the $5HT_{2A}$ receptor producing hallucinogenic effects [91]. The addition of the carbonyl group on the alkyl chain also lowers the penetration of the blood brain barrier in comparison to amphetamine-type substances (ATS) [92]

The administration of synthetic cathinones appears to vary, however, common routes include nasal insufflation and oral ingestion [93], with both intravenous and intramuscular injection also having been reported [94]. Overall, reported desired effects include euphoria, increased energy, empathogenic effects, and pleasure, which may last for several hours [95-97]. Many acute adverse effects have also been seen and include anxiety, dysphoria, paranoia, nausea, aggression, delusion, and suicidal ideation [95, 96, 98, 99], lasting from hours to days. More serious health effects such as tachycardia, hypertension, hyperthermia, and even cardiac arrest have been described [100-102]. Severe intoxication and fatalities have been reported widely after consumption of synthetic cathinones on their own and in conjunction with other drugs [98, 101-103]. The causes of fatality range from medical issues [104, 105] to accidental deaths and suicide [95, 106].

1.1.3 Synthetic opioids

Novel synthetic opioids, including fentanyl analogues and non-fentanyl opioids, are becoming more prevalent throughout the illicit drug market. Fentanyl (*N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propenamide) was first synthesised in 1960 by Dr Paul Janssen as an analgesic and potential anaesthetic drug [107, 108]. It is an entirely synthetic and highly potent μ-opioid receptor agonist and was the first of this chemical class to be approved for human use [107]. These days, fentanyl is used to treat chronic pain, in particular for patients with cancer, through transdermal patches and transmucosal delivery systems [109]. It is also still used as the most common intravenous analgesic for complex medical procedures. Over the last few decades, the concern for other fentanyl analogues and their abuse potential has dramatically increased in parallel with the increase of overdose deaths and misuse of these drugs [107, 108, 110].

Fentanyl analogues, or non-pharmaceutical fentanyls (NPF), are chemically related to fentanyl and share a similar core structure. Their structure can be altered simply by changing one functional group or adding a new group in several positions, see Figure 1-3. NPFs vary in potency in relation to fentanyl, making it difficult for users to know what would be considered a standard dose and what could be fatal [9]. Analogues such as alpha-methylfentanyl have been attributed to overdose deaths from as early as 1979 [111]. More analogues have appeared throughout the years since and the internet plays a major role in how these drugs are obtained [9]. At the time of writing, the EWA has reported the presence of over 80 different synthetic opioids since 2009, with fentanyl analogues making up a large number [112]. Many of these analogues are cheap to synthesise using easily obtained precursor materials.

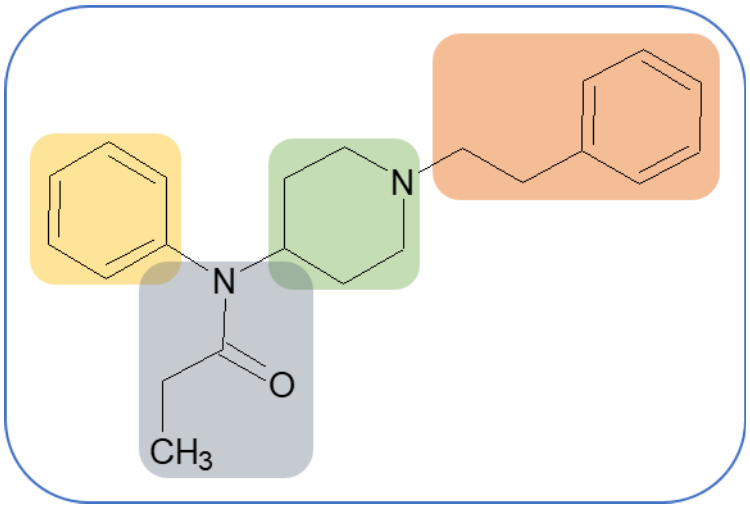

Figure 1-3: Fentanyl general structure with highlighted sections indicating where structural changes may be made

Fentanyl and its analogues are often found in samples of other illicit or pharmaceutical drugs, particularly heroin [95, 97, 100] and others, including, but not limited to, cocaine, MDMA, and hydrocodone [7, 113-119]. The appearance of these opioids in other drug samples, particularly stimulants, is concerning due to the increased risk of overdose and death when taken in combination [23]. Over the years, a huge increase in reported use, overdose deaths and heroin containing fentanyl analogues has been seen particularly across the USA [120]. The use of fentanyl and its analogues in non-medical settings is often not intentional, rather it is taken with the user believing it is another substance. Harm reduction services in British Columbia, Canada, reported that 73% of survey participants who tested positive for fentanyl did not report using it [121].

While fentanyl and some of its analogues have beneficial effects when administered appropriately in a medical environment, they can otherwise affect the central nervous system and lead to a vast array of side effects [122]. These may include drowsiness, nausea, dizziness, fatigue, headaches, and constipation [9, 107]. Overdose symptoms may include more severe presentations such as chest wall rigidity [123], respiratory depression, hypotension, bradycardia, and convulsions [124].

It is important to mention the other synthetic opioids including the U-series and AH-series, which are chemically different from the fentanyl analogues. These drugs are still potent opioid receptor agonists and can be substituted for other opioid drugs [125, 126] but are not a drug of focus in this thesis.

1.1.4 Piperazine analogues

Piperazine derivatives were studied for medical use throughout the second half of the 20th century, though the amphetamine-like effects and abuse potential made them unsuitable for many applications [127]. Derived from piperazine, the cyclic molecule containing a six membered ring with nitrogen replacing the carbons at positions 1 and 4, the most popular derivatives are benzyl and phenyl derivatives [128, 129] (see Figure 1-4). BZP is the most commonly found piperazine compound and was commonly detected initially, presumably due to its legal status compared to other illicit stimulants [130]. Reports suggest that piperazines are often sold as traditional illicit drugs [131] or found in combination with another drug, particularly ATS [132, 133].

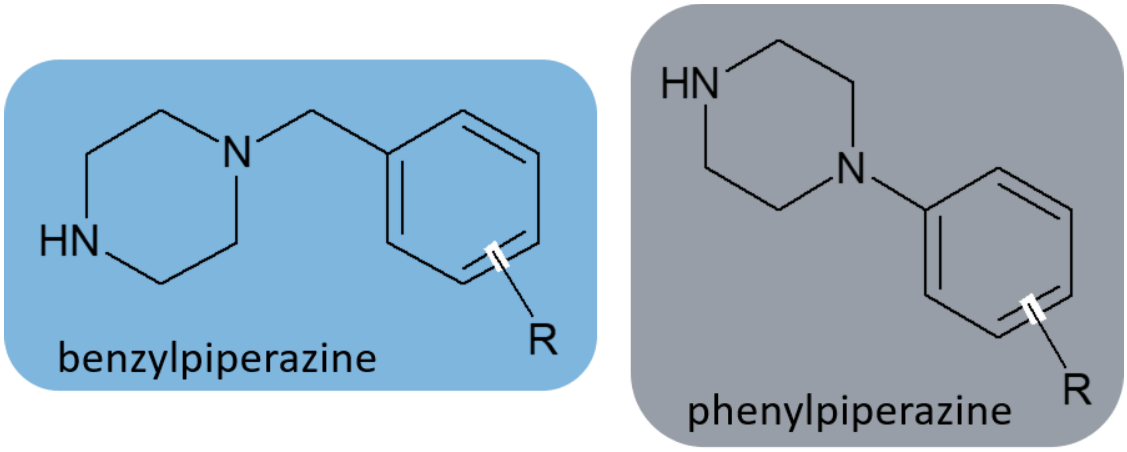

Figure 1-4: General structure of benzyl and phenyl piperazines

BZP has been available on internet sites since as early as 2000 [134]. It is known to have similar effects to that of amphetamines, while in combination with TFMPP, provides effects more similar to that of MDMA [131, 134, 135]. "Party pills" containing piperazine emerged as legal alternatives to illicit stimulant drugs in New Zealand. At the time, in 2007, as these compounds were legal, there was a substantial market for them, sold through the internet, at liquor, adult and convenience stores being marketed as safe alternatives to other illicit drugs [130].

Derivatives have been sought for their psychostimulant properties [128] however they can have many adverse effects and toxicity knowledge is still limited. No fatalities were reported up to 2011 involving piperazines alone. They were seen, however, when piperazines were taken in combination with other drugs [136]. At low doses, piperazines often display milder effects than most amphetamines but at higher doses the most commonly reported health effects include anxiety, confusion, agitation, headaches and dizziness, nausea and vomiting, seizures, tachycardia, and serotonin syndrome [127, 137].

1.2 Analysis of illicit drugs in seized samples

In 2018, over 8,000 tonnes of illicit drugs were seized globally [138] including samples found at borders, through customs, in the postal system and at crime scenes such as clandestine labs. Drug seizures have been increasing in both frequency and volume over the years with the contents changing rapidly, increasing pressure on law enforcement and forensic analysts [139, 140]. In Australia, unknown drug seizures have increased 95% over the last decade and include precursors, anaesthetics, NPS, pharmaceuticals, and drugs not elsewhere classified [141].

The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) outline recommendations for the techniques which may be used to identify seized substances [142]. These techniques are categorised by their discriminating power based on the level of information they can provide about an unknown substance. While SWGDRUG uses three categories, many techniques are used in combination, hence are outlined in only two subsections; those which provide structural information or are used alongside such techniques and those which provide information about the chemical or physical properties of an unknown substance. Many of these techniques have been used for the identification of NPS in seized material both in research and casework and are centred here.

1.2.1 Techniques for structural identification

The main techniques used to analyse illicit drugs in seized materials and those recommended by SWGDRUG to provide a high level of selectivity include mass spectrometry, NMR spectroscopy, IR spectroscopy, and Raman spectroscopy [142]. These techniques may be coupled with other methods to provide even more information about the seized material and its contents. It should be noted, that while gas chromatography (GC) and liquid chromatography (LC) are listed as Category B techniques by SWGDRUG, the analysis of illicit drugs using these methods is outlined here as they are commonly used in conjunction with mass spectrometry (MS) techniques.

Mass spectrometry is a useful drug analysis tool, especially when coupled with techniques such as gas or liquid chromatography and the analysis of seized materials is commonly performed using these methods. The growth in the number of NPS has led to research and development of methods that can differentiate these compounds where methods designed to identify more traditional illicit drugs may not be able to. GC-MS, a standard method for drug analysis in many forensic laboratories, has been used to identify a range of NPS in seized materials. From mixtures of piperazines and ATS in tablets [132] to 25-NBOMe compounds found on blotter papers [59, 143] GC-MS appears to be a useful technique for many matrices even though derivatisation may be necessary to differentiate analogues [144]. Due to the structural similarity of phenethylamines, similar base peaks and mass fragmentation patterns are commonly found to be present in 25-NBOMe analogue spectra along with a small molecular ion peak which can pose difficulties in the identification of specific substances [145].

A screening method using GC coupled with time of flight mass spectrometry (TOF-MS) for a range of NPS has been investigated [146]. The method was developed with more than fifty different NPS

and was able to detect NPS including synthetic cathinones, synthetic cannabinoids, and phenethylamine related drugs found across 63 judicial seizures at an Italian airport. This type of method proves useful in the detection of new substances that may not have previously been identified in a particular region.

Liquid chromatography (LC) is commonly used for the identification and quantification of illicit substances. LC methods coupled with high resolution mass spectrometry are able to provide exact molecular masses and include high performance liquid chromatography triple quadrupole mass spectrometry (HPLC-QQQ-MS) [56], quadrupole time-of-flight mass spectrometry LC-QTOF-MS [59], and orbitrap mass spectrometry often referred to as high resolution mass spectrometry [147]. These techniques can provide highly accurate mass determinations and may be used alongside IR and Raman spectroscopy and also NMR for further structural elucidation [147, 148]. This may help to overcome difficulties that arise due to the lack of complete library data and expensive references standards, even without the possibility for comparative or quantitative results in many instances [149]. LC coupled with a diode array detector (LC-DAD) has been utilised for the screening of NPS in casework and is able to distinguish between isomers which chromatography alone is unable to do [150, 151].

Direct Analysis in Real Time (DART) is a MS technique which has begun to gain recognition and application across many areas including forensic analysis [152]. The sample can be placed directly in the gas flow sample area, removing the need for extraction or derivatisation [153]. This technique has been applied to blotter papers containing 25-NBOMe compounds with no sample preparation required and has provided comparative results to those obtained using common GC and HPLC mass spectrometric techniques [56, 153, 154]. Due to the simple and rapid nature of this method, there may be potential for use as a drug screening method alongside other techniques. Further studies include the analysis as a screening method for the determination of 11 other NPS from seized samples and a comparison to results found with LC-QTOF-MS [155]. While promising results were obtained, the higher limits of detection and poor reproducibility compared to the LC method were a downfall of this analysis.

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) is a useful tool to assist in confirmation of an unknown substance or in the screening process for seized materials [156]. It has been shown to be useful in the identification of a range of NPS across several materials and substance classes [157-159]. Infrared spectroscopy is commonly used in conjunction with other analytical techniques for the identification and structural confirmation of unknown NPS

[149, 160, 161]. Near infrared spectroscopy (NIR) has also been utilised as a presumptive test for a range of NPS [162]. It has shown success in distinguishing between isomers of compounds, however, analogues with only small substitutional differences provide an almost identical spectrum. It is apparent that the carbon-halogen bonds only provide a small contribution to the spectrum in comparison to other bonds present in these compounds. This is particularly important when considering the differentiation of the common NBOMe analogues from one another.

Raman spectroscopy can be used to analyse samples through a variety of materials including samples contained in postal packaging, plastic bags, glass bottles, and even food matrices [163-165]. It also has the potential to be utilised in a handheld device for portable analysis of unknown substances which could play an important role in casework, drug checking, and border control scenarios [166-168]. Raman has been investigated for the analysis of a range of NPS and has shown good potential for the discrimination of NPS families [169] as well as the identification of particular substances within a family such as cathinone analogues [170, 171]. Raman does pose limitations including common spectral peaks seen with many drug species, the need for a detailed library for accurate determination of an unknown and instrumental issues such as fluorescence and low sensitivity [172, 173].

The detection of NPS on blotter papers has been extensively reported particularly in cases in South America [31-33, 44, 143, 158, 159, 174-178]. The detection methods often include a combination of several techniques including GC-MS [31, 33, 44, 143, 174, 178], LC coupled with a variety of detectors [32, 143, 175, 176, 178], IR [32, 158, 159, 175, 177], and NMR [32, 175]. The presence of substances other than LSD on blotter papers seized in Brazil has been increasing dramatically since 2011 when 100% of blotter papers contained LSD [33]. The range of other substances present in these blotter papers includes many traditional illicit drugs such as MDMA and many different NPS including NBOMe-type substances, ketamine, synthetic cannabinoids, and even fentanyl. In 2017, almost half the blotter papers that were determined positive to the presence of a substance by GC-MS, contained 25I-NBOH and 12% were positive for fentanyl. These results indicate both the risk in the unknown consumption of dangerous NPS when thought to be another drug and the need for a means to determine the contents before samples even get to the laboratory.

1.2.2 Techniques based on chemical characteristics

SWGDRUG outlines techniques which provide information about the chemical and physical characteristics of analytes or general and class information [142]. These often have lower levels of discriminating power than those previously described. Many of these are often referred to as presumptive or preliminary test methods and include colour tests, UV spectroscopy, melting point, capillary electrophoresis, and chromatographic methods.

Presumptive tests are named as such as they give an indication of the identity of a compound or compound class via simple and rapid procedures [179, 180]. The main purpose of presumptive tests is to screen and exclude suspect samples indicating the need for these tests to be reliable, highly sensitive and have broad group specificity [181]. The presumptive analysis of seized materials is critical to the investigative process as it provides quick information for the analyst to proceed onto further testing methods. Presumptive test data is limited for NPS due to the lack of library data, specific methods, expensive reference standards, and the structural similarities to traditional illicit drugs limiting the separation between these compounds. Several studies have been completed on the presumptive identification of 25-NBOMe compounds using some of the aforementioned techniques [62, 182].

Thin layer chromatography (TLC) is a separation technique that can be used as a presumptive test assisting in the identification of a range of substances. However, it requires the use of often harmful, flammable or corrosive solvents along with a lengthy preparation procedure and can provide detrimental results if the sample size is too large and is hence not an ideal presumptive testing method [183]. These limitations lead to it only being used if other chemical testing cannot give a clear indication of the identity of the substance or as a complementary method to such testing. High performance TLC has been used to identify and quantify 25-NBOMe compounds found on blotter papers [143, 176].

Traditionally field test methods do not require expensive, time-consuming instrumentation however, with advances in technology, the miniaturisation of these instruments have been widely studied. Portable methods are idyllic, and their development allows advanced equipment to be taken into the field. In particular, portable IR and Raman spectroscopic instruments have been broadly studied along with method development, validation, and coupling with a spectral library [162, 168, 184]. While these techniques have some major advantages, the following sections will discuss the possibilities of more simple and inexpensive presumptive methods.

1.2.2.1 *Colour testing*

Chemical colour tests are one of the most commonly used, and often preferred, presumptive methods for the analysis of suspected drug samples [185, 186]. They utilise chemical reactions between illicit drugs and simple reagents to provide the analyst with almost instant results. They provide a colour change which is visible to the naked eye when a reagent reacts with the test sample and different drugs or drug classes are indicated by a particular colour change [187]. The simplicity and inexpensive nature of these chemical tests makes them a desirable method for on-site detection of illicit substances [186] and there are a variety of commercial test-kits available [188-190]. Colour tests involve a range of chemical reactions to produce a colour change, differing between reagents and target drug classes (see Figure 1-5). Transition metal complexes where the drug chelates the molecule are common and produce highly coloured compounds when these structures are formed (e.g. Scott's, Chen-Kao). Other reagents involving organic species reactions (e.g. Marquis, Mecke, Liebermann's [191]) or a complex ion and organic species present together (e.g. Simon's, Mandelin's) are also common [187, 192].

There are many validated colour test methods for the identification of traditional and well-studied illicit drugs. These can aim to identify particular drugs such as cocaine or may provide broad-spectrum results for a range of other substances. For example, Marquis reagent is commonly performed at the start of the testing procedure and provides a range of colour changes with different drug classes [186]. These reagents are useful for general screening of an unknown substance and will be indicative of a drug group, however, further tests may be required for a specific drug to be identified. One disadvantage of such reagents is that as they are highly reactive with a range of compounds, they also produce colour changes with other licit materials potentially leading to false positives [193]. While these colour test methods are useful for the detection of traditional illicit drugs there is limited data regarding the use of colour tests for NPS.

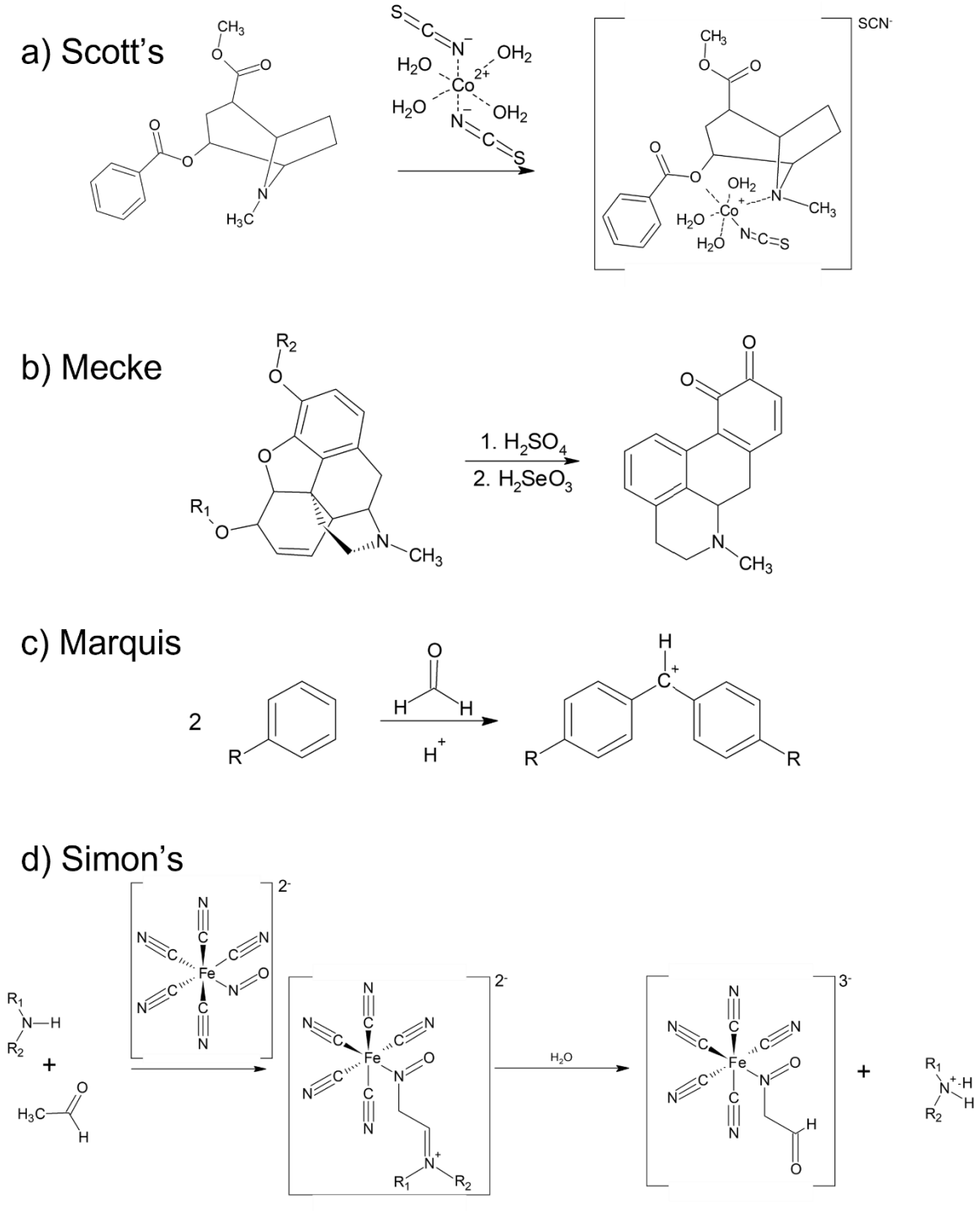

Figure 1-5: Examples of common colour test mechanisms a) Scott's test for cocaine, b) Mecke test for opiate drugs, c) Marquis test for aromatic compounds, and d) Simon's test for amine containing compounds

Studies have been conducted to assess the colour change results seen with a variety of NPS when they undergo a colour test created for the identification of other traditional illicit drugs [194, 195]. It was found that an advantage of the Marquis reagent is its ability to detect the presence of many NPS even if the colour change is not specific to that drug or drug class. The Mandelin reagent was

shown to distinguish some NBOMe compounds and other phenethylamine-type substances, as well as differentiating these substances from methamphetamine and amphetamine. Several 2C-X compounds react with tests established for other ATS which given the structural similarity with these compounds is not surprising. A trending issue appears to be the different colours seen between analogues and the similarity of colour changes with these NPS and other traditional substances. This suggests that, if these tests were to be used, more than one would be required for the selective identification of NPS classes.

A range of NPS including cathinones, piperazines, tryptamines, and phenethylamines, were studied by Toole et al. for the colour reactions seen with well-known colour tests and newly developed methods [195]. The tested compounds provided colour changes with many of the colour tests. Though, these changes were commonly non-specific or differing colours were seen within a common group of substances as was reported previously [194]. Two of the methods investigated were those developed and reported by Philp et al. [196, 197]. Methods for the detection of both synthetic cathinone analogues utilising a neocuproine-copper reagent and a 1,2-napthoquinone-4-sulphonate (NQS) reagent for the detection of piperazine analogues are reported. Both methods were fully validated and show high selectivity and specificity for the respective compounds. Drug forum sites provide information about drugs users experiences both taking substances and also the tests they perform before use. Data from these sites has shown how colour tests are used to try and differentiate drugs, such as 25-NBOMe from LSD using Ehrlich's and Marquis reagent [198]. There is not however, enough data from these tests to utilise this in a broad sense. Ehrlich's test has also shown to be potentially useful in the identification of some indole containing synthetic cannabinoids such as AB-001 [199]. While this shows benefits for synthetic cannabinoid detection, it may decrease the reliability of using such a test to distinguish between other drugs as colour changes may be interpreted incorrectly.

While colour tests are highly useful simple methods to presumptively detect unknown substances, they do come with limitations. Interpretation of colour changes can be difficult and weather conditions, limited visibility at night, analysis under street or flashlight, and general constraints of human vision variance can be detrimental [200]. Subjective interpretations of a colour change result are also prevalent especially when concentrations are low, or impurities are present. It can also be difficult for an analyst to instantaneously differentiate colours without direct comparison, which may be limited to test kit swatches or charts or may not be available at all.

1.2.3 Digital image analysis

With concerns surrounding the selectivity and interpretation of colour tests, several studies have attempted to circumvent these issues and suggest objective ways to record the colour change [200-203]. A simple solution appears to be the use of digital image analysis of the colour through photography and software such as Adobe® Photoshop® [202] or mobile phone applications based on basic red green blue (RGB) data obtained [203]. Reflected light from the sample is passed through three filters (red, green & blue) to be detected by image sensors, the final colour is then composed of additive data from the three filters and also adjustments based on capture conditions [203]. This can provide accurate colour identification and shows potential for fast and direct objective analysis. It may also increase the discriminative value of colour test results for use in forensic casework.

While there are several studies outlining the use of digital image and/or colour analysis for different analytes of interest, the use of these techniques for illicit drug detection is limited. Choodum et al. have completed substantial research in this area using well known colour test reagents such as the Marquis reagent and Simon test reagents for the detection and in some cases quantitation of illicit substances. Opiate drugs, morphine, codeine, and diamorphine were analysed using Marquis and nitric acid tests in combination with the image processing tool box in Matlab, providing semi-quantitative results [201]. Further, the use of Adobe Photoshop software was used in conjunction with Marquis and Simon reagents for the analysis of amphetamine and methamphetamine [202]. The analysis of explosives and other analytes of forensic interest have also been investigated using these techniques including the digital image analysis of colorimetric tests for TNT in soil [204].

Other less traditional colour-based tests have also utilised digital image analysis. The detection of codeine sulphate with gold nanoparticles both in solution and in post-mortem bone samples was completed with Adobe Photoshop to obtained RGB data [205]. TLC and a developed mobile phone application were employed to detect and quantify potentially falsified pharmaceuticals [206]. The authors claim that the application was able to analyse TLC spot intensity and retention factor to determine the presence and quantity of any UV-absorbing active pharmaceutical ingredient.

1.2.3.1 *Smartphone applications for enhanced colour detection*

The first recorded use of a smartphone application for colourimetric analysis of illicit drugs involved the Simon test for real time analysis of methamphetamine in 2014 [203]. The colour change was measured using the ColorAssist app (FTLapps Inc.) and the concentrations of methamphetamine in seized tablets could be conveniently determined. There has since been a range of methods investigated using similar applications for colour-based testing of illicit drugs and other analytes of interest. Elkins et al. compared the colour recordings between two mobile phone applications (ColorAssist® and Colorimeter®), on a number of devices and assessed the differences in colour output with and without the camera light [200]. These applications show potential to evaluate and quantify colour changes in a more objective environment than with the naked eye, removing variations in the colour result reports.

Several studies use solid sensors or other forms of detection techniques before inclusion of a mobile phone camera. Cocaine was able to be detected in a lateral flow assay with a biomimetic material using the red colour intensity changes relative to concentration [207]. It was analysed with the smartphone application Color Analysis and quantitative data on the amount of cocaine present was obtained. Further studies also focussed on cocaine analysis, including the detection of the impurity, phenacetin, in cocaine samples [208]. This analysis was completed with a paper-based sensor using a smartphone camera as the colour detector. Another paper based device was used to detect ketamine as an immunosorbent assay with image recording using a smartphone camera [209]. A single centrifugal microdevice in combination with smartphone analysis was developed to detect both cocaine and methamphetamine [210]. This device proved useful in the simple on-site detection of these drugs both in pure form and in the presence of cutting agents.

The only NPS-specific use of mobile phone applications focussed on the use of a modified Ehrlich reagent to identify the synthetic cannabinoid AB-001 [199]. The reagent, originally used for the detection of LSD interacts with the indole component of the synthetic cannabinoid. It was adsorbed onto silica for analysis and then RGB data was collected with a purpose developed Android app. This method focussed on the intensity of the green colour value and it could be used as a variable for quantification [199]. Other studies, outside the realm of illicit drugs have been completed on the development of new apps for colour determination [211, 212] and ways to limit light interferences, such as calibration to compensate for measurement errors, with the RGB values obtained from a mobile phone camera [213].

There is a lack of data for the detection of NPS using these techniques and also limited data in relation to simple chemical colour test methods for illicit drugs used in combination with smartphone devices. The use of these together may provide a simple way for more accurate and even quantitative analysis in the field when detecting unknown substances. This type of analysis may prove useful in differentiating the presence of NPS in unknown samples and even give an indication of the amount present, which may help decrease the risks of these drugs.

1.2.4 Microfluidic analysis

Microfluidics is the flow of liquids within channels of which at least one dimension is within a range of micrometres or tens of micrometres [214]. A microfluidic device, sometimes referred to as a chip, contains channels which may be set for a specific task such as mixing, sorting, pumping or analysing. These channels are created through etching, moulding or chemically modifying the substrate which is commonly glass, silicon, paper or a polymer. The device will also contain inlets/outlets where liquids will be introduced or removed dependent on the task. Many devices will also require a system to assist the flow of the liquid through the channels such as a push syringe or pump [215, 216].

Microfluidics is widely used in medical diagnostics and devices range from simple chromatographic strips, e.g. pregnancy tests, to complicated systems which require external instrumentation and expert training [217]. Recently, there has been a considerable amount of research into microfluidic devices which are portable and easy to use for point of care situations. These require no external instrumentation, power supply or specialised training and would be created from low cost and disposable materials. Target areas of use are varied, with some devices already available commercially such as at home pregnancy tests, diabetes, drugs of abuse and biomarkers of pathogens tests, detection of urine analytes, and blood analysis [217, 218]. Other possibilities include the use of these devices after environmental disasters and biological warfare attacks, and in resource limited areas such as health care situations in developing countries. These devices aim to be energy independent and utilise capillary forces such as those of paper to avoid the need for an external pumping system [219]. The use of paper, and hence the avoidance of pumping systems, greatly decreases the cost of the manufacture of these devices and makes them simple to use.

1.2.4.1 *Microfluidic paper-based analytical devices (µPADs)*

Paper-based microfluidic devices (µPADs) can be applied in a number of diverse areas. They are simply fabricated on filter paper and require no external forces to drive the analyte to the detection area based on the hydrophilic nature of paper. They are easy to manufacture, low cost, have high wicking properties and porosity and they are disposable, non-toxic, biodegradable, and biocompatible [220]. Certain parameters need to be assessed in the construction of a µPAD device including the concentration and volume of reagent, and the channel dimensions [221].

There has been an increased interest into µPADs in the forensic science area as they require low reagent and sample volumes, fast analysis, inexpensive materials, and there is increased potential for portability for onsite analysis [210]. It has become apparent that a range of sample types can be detected, from explosives to drugs in human serum, and the detection method is commonly colourimetric [218]. In a forensic sense, they have been extensively studied for the detection of a range of explosives often used in conjunction with a detection source [222-226]. The use of colourimetric detection methods on white paper is advantageous as it provides a clear background for the colour to be viewed [220, 227]. Reagents are applied to the paper in a detection area and as the drug solution moves through the channels; it may react with the reagent to form a colour change. In theory, this should be applicable to any analyte with a suitable reagent that can be applied to the detection area.

Similar to traditional colourimetric tests, colour determination can be difficult by the naked eye due to colour perception, lighting differences, and differences with dry and wetted paper [218]. These limitations may be overcome with the use of cameras, scanners or detectors. These systems can utilise a detection method such as UV spectroscopy to analyse and quantify the unknown analyte, use the comparison of colour intensity to that of a known compound for quantification [218] or simply visualise a colourimetric analysis. Similar to colour testing in solution, the use of smart phone analysis could be beneficial to these devices particularly in a portability aspect [228, 229]. Another approach may be to use multiple indicators for a single analyte to allow for a higher degree of discrimination. This is possible as simultaneous tests can be completed in parallel requiring less sample consumption, increasing the discriminative power and providing confidence in the identification while still being a simple and rapid presumptive test [221]. This type of device may be suitable to assist in the discrimination between structurally similar compounds, which may produce the same colour with one test reagent but a slightly different colour change with another.

This reduces both the time taken to perform multiple tests as well as limiting the sample required to complete the testing procedure.

There have been only a few studies relating to the development of paper-based devices capable to identify multiple analytes. These include the detection of a range of illicit drugs [230, 231] and also the detection of cutting agents present in illicit samples [232]. Musile et al. [230] developed a paper-based microfluidic device for the presumptive testing of seized illicit substances. The device contained multiple channels where different reagents could be placed so sequential reactions could occur (see Figure 1-6). This in turn means that multiple drugs can be detected using the same device as well as multiple reactions occurring with the particular analyte being detected. Many currently used drug colour test reagents contain concentrated sulphuric acid which would not be compatible on paper. These tests were modified, such as the removal of strong acids, to be more suitable for application on a paper substrate before development could occur. The separation of reagents or multi-step processes were shown to be applicable in this device. Two areas can be created within the channel to allow one reagent to combine with the drug solution first, followed by the final detection area. The development of a similar device but with parallel test channels has been created and utilised for both the detection a range of illicit drugs [231] as well as for detecting cutting agents such as lactose [232] (see Figure 1-6). The channels each contain reagents which will react to provide colour changes and hence information about the unknown substance. What may be a disadvantage of this design is that the substance must be in powder form and is swiped across the channels before the paper is placed in water. This limits the types of substances that may be analysed with this device and also appears to require quite a large amount of sample which may not be accessible. The use of these types of devices would greatly increase the discriminative power of these colourimetric tests when results from all reagents are combined. These device designs could provide inexpensive determination of a variety of analytes with results visible to the naked eye. The addition of smart phone analysis or spectroscopic analysis could also be incorporated to further enhance the specificity of the results.

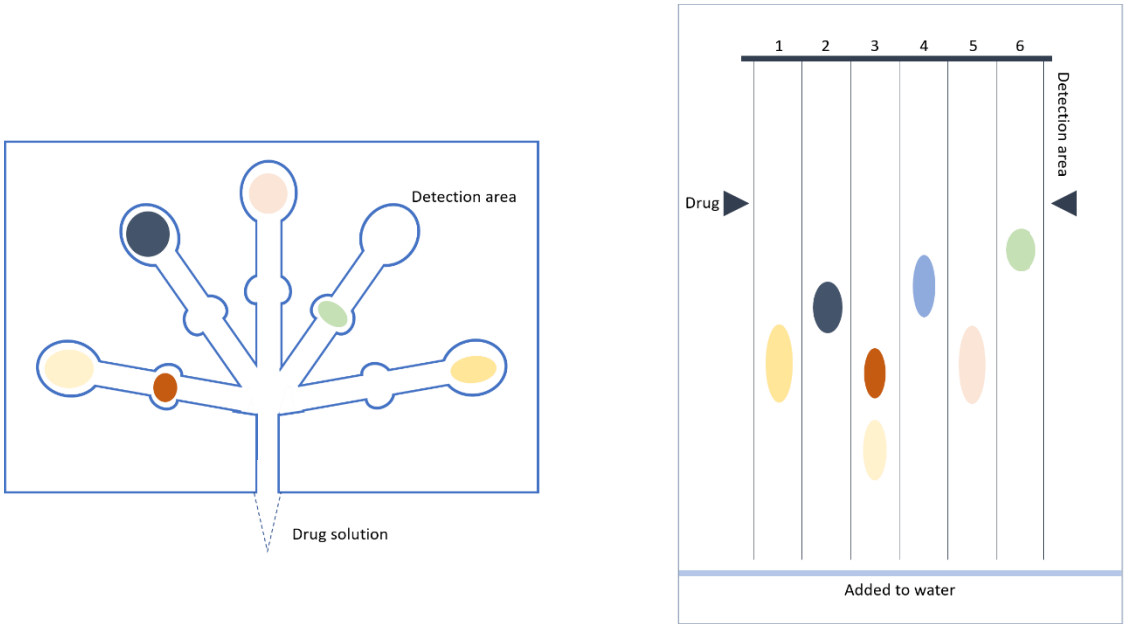

Figure 1-6: Representative diagrams of multiplex paper-based devices for colourimetric detection of illicit drugs

Integrating multiple tests into one device has many major benefits including the ability for detection of multiple drug analytes using the same device rather than the use of multiple test kits as is currently used. Multi-testing devices may also have the potential to indicate if the unknown analyte is a mixture of more than one drug or if that mixture contains impurities. This would require tests with the capability of detecting a large number of substances. While traditionally colourimetric tests are completed in a test tube, ampule or on a spot plate, applying them to other substrates may be advantageous [227]. Combining colour testing and paper may be a powerful tool as it is low cost and readily available but would also reduce the use of expensive lab equipment, requires minimal sample volumes, and may enable portability for these types of test methods. White paper specifically has the advantage of providing a strong contrast background for colour change results. Simple paper devices can be developed by creating isolated test zones which limit mixing of chemical components.

1.3 Analysis of illicit drugs in oral fluid

Oral fluid (OF) is an ideal matrix for the detection of illicit drugs and has gained more recognition over the past years as an alternative matrix in forensic and clinical settings [233]. Workplace drug testing and driving under the influence of drugs have been large areas of interest in regard to the use of oral fluid [234]. It is less invasive than blood and supervised collection is possible without specialised facilities or personnel which avoids the potential for adulteration such is the case with

urine samples [235, 236]. The dominant species in OF is also the parent drug, which can allow for easy determination of the drug present without the need to detect metabolites [234]. The concentration of a drug found in OF can also reflect the amount that is present in the blood [237].

Screening devices for OF vary in design and usually only test for a handful of drugs. Devices can be lateral flow based or can also utilise enzyme-linked immunosorbent assays (ELISA) as the technology to indicate the presence of an illicit drug. The collection techniques differing along with the number and variety of drugs in which they can detect. Commonly, these drugs include amphetamines, THC, cocaine, opiates, and benzodiazepines [238]. Many of these devices have been compared with one another and also to confirmatory testing, usually conducted using GC-MS [239-242] or LC-MS [243-246] techniques.

More recently, the new issue of being able to detect NPS in oral fluid via the current methods, particularly screening methods has become prominent, as no specific methods are available for the detection of NPS [247]. The high turnover of new substances, the low quantities consumed, and unknown metabolite pathways all contribute to the analytical problems arising with these drugs [248]. Limited method development has been completed to combat this issue, with most studies focussing on laboratory based analytical techniques. There has been some investigation for the identification of NPS using screening methods. New amphetamine-type substances were assessed based on the cross reactivity profile with an amphetamine-, methamphetamine-, and MDMA-specific ELISA device [249]. It was determined, however, that even at high concentrations (10 000 ng/mL) these kits are not able to detect these new drugs. An ELISA device was developed for the specific detection of several synthetic cannabinoids, most of which were structurally similar to JWH-018 [250]. Synthetic cannabinoids and cathinones appear to be the two main focus groups for the development of laboratory-based methods. A synthetic cannabinoid method using UHPLC-QTRAP MS was developed to detect 19 different compounds [236]. The same group has developed a similar method for the detection of cathinones and other phenethylamine-type NPS which included 32 different compounds [251]. Further, a GC-MS method for the detection of some cathinones was investigated, and included common metabolites such as ephedrine [252].

While methods for the presumptive detection of traditional illicit drugs in OF exist, few have been assessed or developed for the detection of new psychoactive substances. Many OF screening devices are not able to detect NPS and this has been confirmed by secondary testing, usually with LC-MS, which did find NPS present in the sample of the same participant [253, 254]. Some research has been completed into how these drugs may interact with currently used OF detection devices

[237, 255]. Positive results are apparent at higher concentrations (100 µg/mL) for certain drugs due to the cross reactivity as a result of structural similarities with other traditional drugs such as methamphetamine that the devices are designed to detect. Variations in these results are also seen particularly between devices.

1.4 Significance of presumptive test methods for illicit drug detection

Presumptive detection of illicit substances plays an important role in the forensic process. Not only is it timely and cost effective, but it also allows the laboratory to utilise appropriate confirmatory methods to identify and quantify the substances and, in some jurisdictions, positive results can even be grounds for search or arrest warrants. The need to detect NPS in seized material is crucial for both forensic and harm reduction purposes. The ability to do this in the field is highly beneficial and can provide almost instantaneous information to allow for better decision making from any party. Colourimetric test procedures have shown to be beneficial for this purpose and the adaptation of these methods to incorporate colour analysis and portable systems can remove a level of subjectivity and improve the discrimination of resulting colour changes. The application of simple presumptive test methods to oral fluid analysis is also valuable to assist in the detection of a variety of drugs in situations where impairment is problematic. This thesis aims to address the lack of presumptive methods for NPS with a focus on 25-NBOMe compounds. The ability to identify these compounds with a high level of selectivity and accuracy would be highly valuable across law enforcement, health care settings, and harm reduction situations.

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Chapter 2: Phenethylamine colour test development and optimisation

Chapter 2: Phenethylamine colour test development and optimisation

Summary

This chapter outlines the development and optimisation of a colour test method created for the presumptive detection of new phenethylamine compounds focusing on the 25-NBOMe class of substances with further testing completed for the 2C-X series.

A portion of this chapter includes a first author publication in the Wiley Journal, Drug Testing and Analysis. This publication describes the colour test developed for the detection of 25-NBOMe compounds. Further testing with this test method, including validation steps with 2C-X compounds, and optimisation steps have been completed and included in this chapter.

Development and validation of a colour spot test method for the presumptive detection of 25-NBOMe compounds

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Statement of contributions of joint authorship

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2.1 Introduction

The last decade has seen substantial change and development in the illicit drug industry with research and technology unveiling new substances. The occurrence of new psychoactive substances (NPS) has greatly increased since 2009 with 950 new substances reported to the United Nations Office on Drugs and Crime (UNODC) Early Warning Advisory up until December 2019, indicating the public health risks across many communities [256, 257]. NPS encompass a wide variety of substances, with synthetic cannabinoids, cathinones, phenethylamines, and opioids of significance in a highly dynamic market [3].

25-NBOMe substances are substituted hallucinogenic phenethylamines derived from the 2C-X series compounds with the addition of a 2-methoxybenzyl group on the amine functional group. They are highly potent agonists to the serotonin 5-HT2A receptors even in microgram doses [58, 258]. This gives them highly stimulant and hallucinogenic or psychoactive effects, similar to that of lysergic acid diethylamide (LSD). The 2-methoxybenzyl substituent, which identifies 25-NBOMe compounds, increases the potency significantly in comparison to the 2C-X substances [60-62]. The substituent commonly present at the para-position on the phenyl ring may also have an effect on the potency and effects of 25-NBOMe compounds [65]. Figure 2-1 and Table 2-1 show the structural relationship of 2C-X and 25-NBOMe compounds along with several 25-NBOMe related analogues.

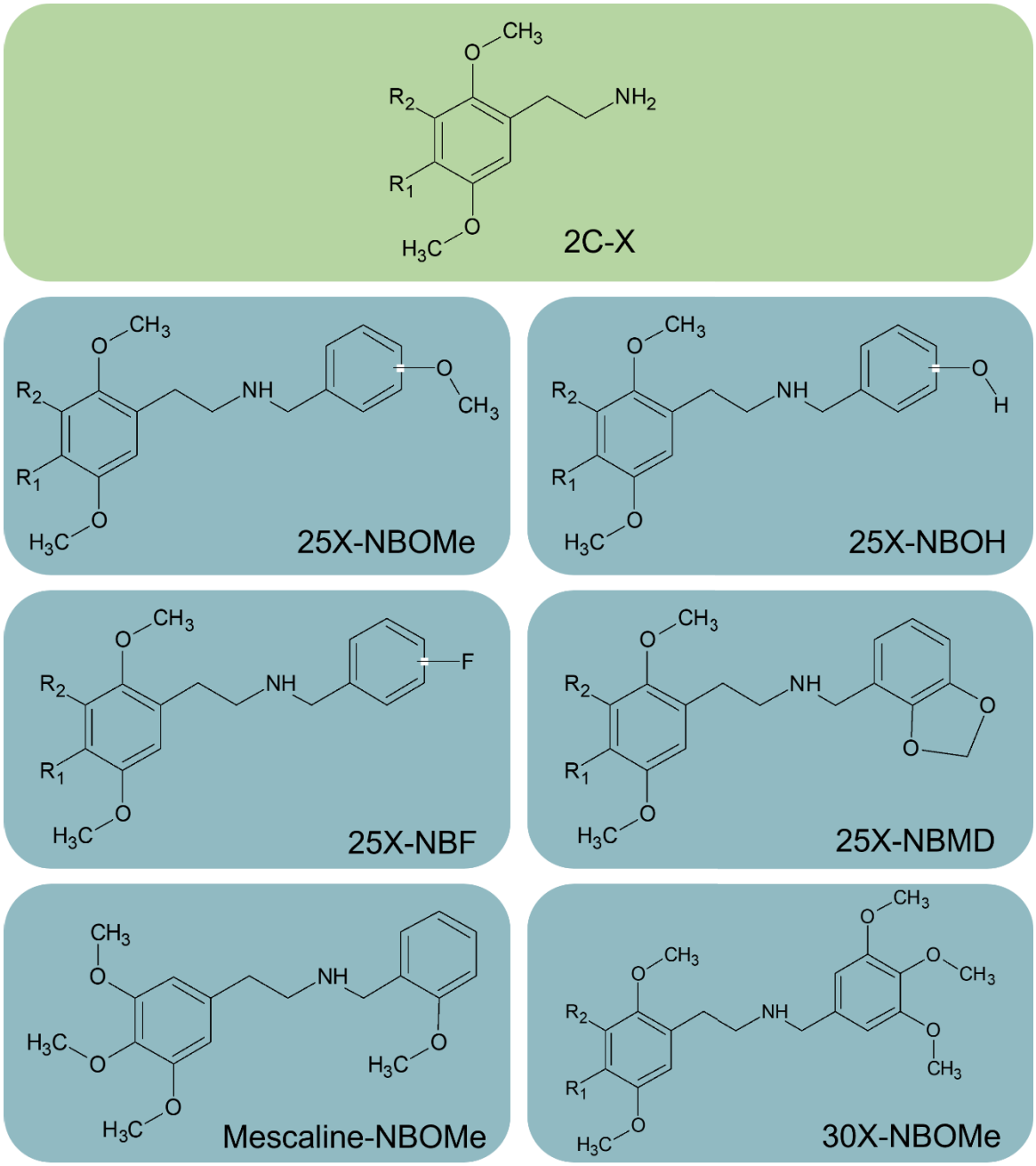

Figure 2-1: General structures of 2C-X, 25-NBOMe and its related analogues

Table 2-1: Associated substituents for compounds outlined in Figure 2-1*

Prefix (2C-X/25X/30X)	R1 substituent	R2 substituent
2C-B/25B/30B	-Br	-H
2C-C/25C/30C	-Cl	-H
2C-D/25D/30D	$-CH_3$	-H
2C-E/25E/30E	$-CH_2CH_3$	-H
2C-F/25F/30F	-F	-H
2C-G/25G/30G	$-CH_3$	$-CH_3$
2C-H/25H/30H	-H	-H
2C-I/25I/30I	-I	-H
2C-N/25N/30N	$-NO_2$	-H
2C-P/25P/30P	$-CH_2CH_2CH_3$	-H
2C-T/25T/30T	$-SCH_3$	-H
2C-T2/25T2/30T2	$-SCH_2CH$	-H
2C-T4/25T4/30T4	$-SCHCH_3CH_3$	-H
2C-T7/25T7/30T7	$-SCH_2CH_2CH_3$	-H

** This table does not apply to mescaline-NBOMe as it is a single analogue rather than a general structure that will be further substituted*

Usage and appearance on the drug market have only been reported in recent years, initially appearing as 'legal LSD' [53]. Evidence from the literature suggests that 25-NBOMe substances are some of the more recent NPS to appear on the market [59]. Notable syntheses of 25-NBOMe compounds were first reported in 1994 [259] and more recently in 2003 [51]. It wasn't until 2011 that 25I-NBOMe first appeared on blotter papers in the designer drug market [52, 54] while many countries, such as Japan, did not see 25-NBOMe substances until as late as 2013 [55]. There is data from several jurisdictions including Brazil, Columbia, and Portugal that indicates blotter papers sold as LSD can contain 25-NBOMe compounds [33, 260, 261]. Other case reports indicate that many people present to hospital after taking what they thought to be LSD or an unknown substance and have acquired acute toxicity from an NBOMe related compound [57, 71, 72]. These cases resulted in severe effects on those users such as tachycardia, hypertension, hyperthermia, agitation, seizures, and even kidney injury which are rarely seen after ingestion of LSD [73]. Reports of self-harm after NBOMe ingestion [72, 74] along with fatalities resulting from NBOMe use [75, 76] are common and provide insight into the increased risk of these drugs in comparison with LSD and other classic hallucinogens. The need to differentiate the contents of blotter papers sold as LSD is therefore apparent and the ability to do this on-site would be advantageous for police personnel, paramedics, and drug-checking facilities.

The analysis of 25-NBOMe compounds has been successful using gas or liquid chromatographic methods, often in conjunction with mass spectrometric techniques, and have been extensively reported in recent years [65, 144, 148, 154, 262, 263]. The analysis of blotter papers is commonly performed using these methods and typically requires an extraction or derivatisation step before the instrumental analysis. 25-NBOMe compounds on blotter papers have also been quantified using high-performance liquid chromatography methods with a range of detection methods [56, 176, 258]. Other methods, including ATR-FTIR, have been reported as a potential direct analysis solution [158, 159]. This said, there are few available methods for these samples to be analysed without any preparation required that can indicate the presence of 25-NBOMe compounds.

Colour tests provide analysts with preliminary information regarding the type or class of a substance. They are simple, rapid, and inexpensive chemical spot tests that provide a colour change visible to the naked eye when a reagent is added to an unknown substance [187]. The ease of use, portability, rapid results, and the lack of sample preparation and expensive equipment required makes colour tests ideal for on-site identification of substances [185, 186]. For these reasons, they are usually preferred by law enforcement and other forensic drug testing laboratories. They are also the most commonly used presumptive test methods with a range of commercial test kits available [188-190]. Over recent years there have been concerns regarding the selectivity of colour spot tests particularly with the appearance of many NPS. The increase in NPS availability has posed new challenges in drug detection for law enforcement, policymakers, drug testing services, and scientists [24]. Currently, no presumptive test data exists for 25-NBOMe compounds [62, 182].

Colour tests validated for traditional illicit drugs were studied by Cuypers et al. with a range of NPS including 25-NBOMe compounds [194]. 25-NBOMe compounds produced colour changes with some of these tests. For example, Scott's Reagent (cobalt thiocyanate), traditionally used for cocaine detection, gave a green colour change in the final step with several 25-NBOMe compounds [194]. Two main issues arose: 1) The analogues of the 25-NBOMe series produce a range of different colours, and 2) these observed colour changes cannot be distinguished from the colours seen when testing traditional illicit drugs and other NPS. Unpublished data from drug forum sites indicates that colour tests created for traditional drugs have been used in attempts to identify 25-NBOMe compounds [198]. Ehrlich's test, used to identify indoles such as LSD, and Marquis reagent have been utilised to detect 25-NBOMe compounds and potentially distinguish between LSD and 25-NBOMe compounds on blotter papers. The results, however, indicate they are not reliable enough to accurately identify 25-NBOMe compounds. The high potency of 25-

NBOMe compounds stresses the need for a test that can quickly and accurately identify these compounds or eliminate the possibility that one is present. A test that can identify 25-NBOMe compounds and distinguish these compounds from LSD would be highly valuable particularly directly from a blotter paper. The data indicating 25-NBOMe compounds are being sold as LSD signals the need to be able to differentiate between these samples.

This study aimed to develop and validate a specific colour test method for the rapid identification of 25-NBOMe compounds, in particular, to be able to differentiate these compounds from LSD. Such a method would need to produce an obvious colour change with 25-NBOMe compounds and have little to no cross-reactivity with other compounds that may be found in a suspected illicit drug sample.

2.2 Materials and methods

2.2.1 Reagents and chemicals

2,3,5,6-Tetrachloro-1,4-benzoquinone (TCBQ), acetaldehyde and propan-2-ol were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Ethyl acetate, 1,4-dioxane, methanol, acetonitrile, sodium carbonate (Na_2CO_3) and sodium hydroxide (NaOH) pellets were obtained from Chem-Supply (Gillman, SA, Australia). Disodium phosphate (Na_2HPO_4) and monosodium phosphate (NaH_2PO_4) were obtained from Ajax Finechem (Taren Point, NSW, Australia). Deionised water from a laboratory supply was used throughout the methods.

Quinones including 3,4,5,6-tetrachloro-1,2-benzoquinone (*o*-TCBQ), 2,5-dichloro-1,4-benzoquinone, 2,6-dichloro-1,4-benzoquinone, chloranilic acid, 2,3,5-trichloro-1,4-benzoquinone, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, 2,3,5,6-tetrafluoro-1,4-benzoquinone, 2,3,5,6-tetrabromo-1,4-benzoquinone, 2,5-dibromo-1,4-benzoquinone, 1-aminoisoquinoline, hydroquinone and 2,3-dicyanohydroquinone were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Acenaphthenequinone was obtained from Fluka.

Propionaldehyde, glycine, tetrabutylammonium hydroxide, tris(hydroxymethyl)aminomethane, *n*-propyl gallate and 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene (BHT)) were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). Butyraldehyde, ammonia solution and sodium tetraborate (borax) were obtained from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia). Ammonium chloride was obtained from Chem-Supply Pty Ltd (Gillman, SA, Australia),

and ascorbic acid was obtained from BDH Laboratory Chemicals (Poole, England). Hydrochloric acid (32%) was obtained from RCI Labscan (Taren Point, NSW, Australia).

2.2.2 Reference materials

Reference standards were purchased as hydrochloride salts including 2-(2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25H-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25C-NBOMe), 2-(4-methyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25D-NBOMe), 2-(4-ethyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25E-NBOMe), 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25B-NBOMe), 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe), 2-(3,4-dimethyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25G-NBOMe), 2-(4-methylthio-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25T-NBOMe), 4-bromo-2,5-dimethoxyphenethylamine (2C-B), 4-iodo-2,5-dimethoxyphenethylamine (2C-I), 4-ethyl-2,5-dimethoxyphenethylamine (2C-E), 4-ethylthio-2,5-dimethoxyphenethylamine (2C-T-2), 2,5-dimethoxy-4-methylphenethylamine (2C-D), (±)-*N*-methyl-3,4-methylenedioxyamphetamine (MDMA), (+)-*S*-methamphetamine and d-amphetamine sulphate from the National Measurement Institute (NMI, North Ryde, NSW, Australia). Also purchased were the following NBOMe analogues 2-(4-chloro-2,5-dimethoxyphenyl)-N-[(3,4,5-trimethoxyphenyl)methyl]ethanamine (30C-NBOMe), 2-(4-nitro-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25N-NBOMe), 2-(3,4,5-trimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (mescaline-NBOMe), 2-(4-bromo-2,5-dimethoxyphenyl)-N-[(4-methoxyphenyl)methyl]ethanamine (25B-NB4OMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-[(3-methoxyphenyl)methyl]ethanamine (25C-NB3OMe), 2-(4-Iodo-2,5-dimethoxyphenyl)-N-[(2-hydroxyphenyl)methyl]ethanamine (25I-NBOH), 2-(4-bromo-2,5-dimethoxyphenyl)-N-[(2-fluorophenyl)methyl]ethanamine (25B-NBF) and 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2,3-methylenedioxyphenyl)methyl]ethanamine (25I-NBMD) from Novachem (Heidelberg West, Victoria, Australia). 2-(4-isopropyl-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25iP-NBOMe), 2-(2,5-dimethoxy-4-nitrophenyl)-N-[(2-methoxyphenyl)methyl]ethanamine, monohydrochloride (25N-NBOMe), 2-(4-propyl-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25P-NBOMe), 2-(4-(ethylthio)-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25T2-NBOMe), 2-(4-(1-methylethyl)thio-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25T4-NBOMe), 2-(4-propylthio-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine

(25T7-NBOMe), 2-(2,5-dimethoxyphenyl)-N-[(4-methoxyphenyl)methyl]ethanamine (25H-NB4OMe), and 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(3-methoxyphenyl)methyl]ethanamine (25I-NB3OMe) were purchased from Sapphire Bioscience (Redfern, NSW, Australia) as hydrochloride salts. LSD and ergotamine-d-tartrate were purchased as 1 mg/mL solutions and powder standards respectively from Lipomed (Arlesheim, Switzerland). Fentanyl was purchased as a powder standard from PM separations (Capalaba, Queensland, Australia) and JWH-073 was synthesised in-house [264]. (1*S*,2*R*)-(+)-ephedrine hydrochloride and (1*S*,2*S*)-(+)-pseudoephedrine hydrochloride were obtained from Sigma Aldrich. Further pure drug reference standards were also obtained from NMI through the Australian Federal Police (AFP, Sydney, Australia). A complete list of these substances can be found in the results. All reference standards were obtained in powder or crystalline form.

Ibuprofen, paracetamol, 3,4-dimethoxyphenethylamine, caffeine, lidocaine, magnesium stearate, dimethyl sulfone, phenacetin, benzocaine, tetramisole hydrochloride, sorbitol, phenobarbital, salicylamide, 4-methoxyphenol (MEHQ), inositol, creatine and 4-aminophenazone were obtained from Sigma Aldrich Pty Ltd (Castle Hill, NSW, Australia). Aspirin, phenolphthalein, citric acid, benzoic acid and mannitol were obtained from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia) and L-ascorbic acid from VWR chemicals (Campbellfield, Vic, Australia). Thiaminium dichloride (thiamine) was obtained from Merck KGA (Castle Hill, NSW, Australia), and quinine was obtained from Fluka (Castle Hill, NSW, Australia). A range of sugars were also obtained, including cellulose and starch from Sigma Aldrich Pty Ltd (Castle Hill, NSW, Australia), lactose, D-fructose and D-glucose from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia), and sucrose from Mallinckrodt Pharmaceuticals (Kew, Vic, Australia). Several primary and secondary amines and amino acids were also obtained including L-threonine, L-phenyldiamine, L-valine, glycine, diphenylamine, L-phenylalanine, methylamine HCl, methoxylamine HCl, ethylenediamine, ethylenediamine-N,N'-diacetic acid, D-alanine and piperazine hexahydrate from Sigma Aldrich Pty Ltd (Castle Hill, NSW, Australia). Diphenylamine and aniline were obtained from VWR chemicals (Campbellfield, Vic, Australia).

2.2.3 Preparation of solutions

A 3×10^{-3} M TCBQ solution was prepared by dissolving 0.73 g in 100 mL ethyl acetate. The phosphate buffer solution was prepared to contain sodium hydroxide (NaOH) and sodium hydrogen phosphate (Na_2HPO_4). The buffer was prepared to pH 11.4 using NaOH (0.1 M, 9.1 mL) and Na_2HPO_4 (0.05 M, 50 mL) made up to 100 mL in deionised water.

A methanolic solution of the synthesised 25H-NBOMe was prepared at a concentration of 0.3 mg/mL to be used for method validation analyses. 25H-NBOMe (0.025 mmol, 7.5 mg) was dissolved in methanol (25 mL) and when required, aliquots of this solution were added to a micro well-plate and placed in the fume hood to allow the methanol to evaporate. Testing then proceeded with the addition of 100 µL of each reagent solution. Solutions of other compounds and reagents were prepared as necessary using methods similar to those described.

2.2.4 Colour test method development

2.2.4.1 *Preliminary testing*

The initial method was based upon a published method by Walash et al. describing the spectrophotometric identification of phenylpropanolamine [265]. Several adaptations to the method were incorporated, including the removal of the extraction step and the use of the phosphate buffer solution. Initial tests were completed with 25H-NBOMe in test tubes. Approximately 1 mg of the drug was added followed by 1 mL of each reagent in the order:

1. Phosphate buffer solution (pH 11.4)
2. TCBQ in 1,4-dioxane solution (0.003 M)
3. Acetaldehyde in propanol solution (8% v/v)

Following these tests, further adaptations were made. The reagent (TCBQ) concentration was reduced from 3×10^{-2} M to 3×10^{-3} M and prepared in ethyl acetate rather than 1,4-dioxane. Acetaldehyde replaced the original 8% acetaldehyde in propanol solution after testing showed greater colour changes. A reduction in the reagent volumes was also favourable to perform this test on a micro-scale. Tests were completed on white porcelain spot plates with 3 drops of each adapted reagent added to approximately 1 mg of 25H-NBOMe.

2.2.4.2 *Reagent optimisation*

Twelve solutions of TCBQ in ethyl acetate were prepared at concentrations 8×10^{-5}, 1×10^{-4}, 3×10^{-4}, 5×10^{-4}, 8×10^{-4}, 1×10^{-3}, 3×10^{-3}, 5×10^{-3}, 8×10^{-3}, 1×10^{-2}, 3×10^{-2} and 5×10^{-2} M to assess the effect on the colour change results across a simple within log scale. This testing was completed in triplicate in a micro well-plate by the addition of the previously prepared solution of 25H-NBOMe (0.3 mg/mL, 500 µL) to each well and the methanol allowed to evaporate before testing. A set of

methanol control blanks were also prepared using 500 µL methanol in each well. 100 µL of each reagent solution was added to the blank and 25H-NBOMe containing wells and the colour changes recorded. An appropriate concentration could then be identified for future testing. The method was repeated with 2C-H (0.5 mg/mL, 500 µL) to determine if these substances could also use the same concentration of TCBQ to obtain a positive result.

2.2.4.3 *Buffer solution optimisation*

Five buffer solutions were prepared to assess the effect of changing the pH and buffer composition on the final colour change result. Table 2-2 outlines the composition and pH of each buffer. These buffers were tested on 25H-NBOMe for comparison with TCBQ in ethyl acetate (3×10^{-3} M) and acetaldehyde.

Table 2-2: Composition of buffer solutions prepared for analysis

pH value	Buffer composition	Final volume
8.0	NaH_2PO_4 (5.3 mL, 0.2 M) & Na_2HPO_4 (94.7 mL, 0.2 M)	200 mL
10.9	Na_2HPO_4 (100 mL, 0.05 M) & NaOH (6.6 mL, 0.1 M)	200 mL
11.0	$NaHCO_3$ (100 mL, 0.05 M) & NaOH (45.4 mL, 0.1 M)	200 mL
11.4	Na_2HPO_4 (100 mL, 0.05 M) & NaOH (18.2 mL, 0.1 M)	200 mL
12.0	Na_2HPO_4 (100 mL, 0.05 M) & NaOH (53.8 mL, 0.1 M)	200 mL

A further six buffer solutions were prepared to assess the effect of changing the entire composition. These buffer solutions were pH 9 or greater and, compared to the phosphate and carbonate combinations used previously, could be considered more organic solutions. The phosphate buffer was replaced by each of these buffer solutions with blank, 2C-H, and 25H-NBOMe samples. Table 2-3 outlines the composition of the prepared solutions.

Table 2-3: Composition of new buffer solutions

pH value	Buffer composition	Final volume
9.0	tris(hydroxymethyl)aminomethane (100 mL, 0.1M) & hydrochloric acid (11.4 mL, 0.1 M)	200 mL
9.1	borax (100 mL, 0.025 M) & hydrochloric acid (4.0 mL, 0.1 M)	200 mL
10.0	tetrabutylammonium hydroxide (1.0 M in methanol)	n/a
10.6	glycine (25 mL, 0.2 M) & sodium hydroxide (22.75 mL, 0.2 M)	100 mL
10.7	ammonium chloride (6.75 g) & aqueous ammonia (57 mL)	100 mL
10.8	borax (100 mL, 0.025 M) & sodium hydroxide (48.5 mL, 0.1 M)	200 mL

2.2.4.4 *Reactions with other quinone reagents*

Thirteen other related quinone compounds were investigated to observe any changes or similarities of the colour change result when the structure of the primary reagent differed slightly. All quinones were prepared to mimic the original reagent (3×10^{-3} M in ethyl acetate) and were applied to blank, 2C-H, and 25H-NBOMe samples with the phosphate buffer solution and acetaldehyde.

2.2.4.5 *Effect of other aldehydes*

Due to the toxic and volatile nature of acetaldehyde, alternatives to its use were investigated. Simply removing this reagent did not appear possible as an aldehyde seems to be an essential part of this reaction. Therefore, the homologous alkyl aldehydes, propionaldehyde, and butyraldehyde were tested by replacing the acetaldehyde and comparing the colour change results to the original method.

2.2.5 Blotter paper analysis

Two blotter paper tabs (perforated squares) were prepared with 25B-NBOMe HCl for comparative analysis. A solution of acetonitrile containing 500 µg of the compound was applied to the two blotter papers and the solvent allowed to evaporate so that each tab contained approximately 250 µg of 25B-NBOMe. One tab was placed in 500 µL of methanol and soaked for 1 h simulating an extraction process. The paper tab was removed, and the methanol evaporated. The other blotter paper tab was tested with the reagents applied directly to the paper in a microcentrifuge tube. To both samples, the buffer, TCBQ in ethyl acetate, and acetaldehyde were added (100 µL

each), the tubes agitated gently, and the colour change observed. A blank sample of the blotter paper was also tested as a control.

Further repetitions were completed using other NBOMe analogues and drug amounts applied. Solutions of 1 mg/mL in methanol 25D-NBOMe, 25G-NBOMe, 25I-NBOMe, 25C-NBOMe, 25T-NBOMe, and 30C-NBOMe were pipetted on single blotter paper tabs from 50 – 200 µL, exact volumes tested for each drug can be found in the results (Table 2-7). The solvent was evaporated before the papers were placed in microcentrifuge tubes for testing. To each tube, 100 µL of each buffer, TCBQ solution, and acetaldehyde were added, the tubes agitated gently, and colour changes observed.

2.2.6 Method validation

After optimisation of the general method, validation was completed with procedures based upon guidelines outlined by the UNODC and National Institute of Justice (NIJ) [266, 267]. This included assessment of specificity and selectivity, limit of detection (LOD), precision, stability, and impurity testing.

2.2.6.1 *Specificity and selectivity*

A comprehensive range of drug compounds, analogues, and cutting agents were tested to assess the specificity and selectivity of this test method. These included NBOMe analogues and derivatives, illicit compounds from other drug classes, illicit drug precursors, pharmaceuticals, common adulterants, and diluents along with other amine-containing chemicals.

These tests were completed with no sample preparation: a pinhead-sized amount of each powder (or one drop from a Pasteur pipette if in liquid form) was added to a porcelain spot plate followed by the reagents outlined in the general procedure (see 2.3.2). These tests were completed as though they would be in the field with no accurate measurement of the amount of drug being tested. The colour test was performed in duplicate with the colour change recorded twice: immediately after addition of final reagent, and again five minutes later.

2.2.6.2 *Limit of detection*

The LOD was determined through a modified version of the method outlined by the NIJ Colour Test Standard [267]. Twelve aliquots (0, 10, 25, 50, 75, 100, 150, 200, 300, 350, and 500 µL) of methanolic 25H-NBOMe (0.3 mg/mL) solution were pipetted into a micro well-plate in triplicate. The methanol was evaporated in the fume hood before 100 µL of each reagent solution was added. The smallest sample at which the colour change was still differentiable from the reagent blank and considered a positive result was determined as the LOD. This was decided by the consensus of two analysts who visualised the colour change results with the naked eye. The NIJ guidelines suggest that this quantity would be multiplied by 10 to find the operational detection limit.

This method was repeated to determine the limit of detection for 2C compounds with a 2C-H solution (0.5 mg/mL) in methanol. The same volumes of drug solution were added to wells in triplicate and the solvent evaporated before addition of each reagent solution (100 µL).

2.2.6.3 *Purity testing*

Twelve aliquots of 25H-NBOMe (100 µL, 1 mg/mL) were added to a microwell plate and the solvent allowed to evaporate. To all but one of these wells, 100 µL aliquots of eleven adulterant solutions (1 mg/mL) were added and the solvent evaporated once again. These included methamphetamine, MDMA, ephedrine, paracetamol, pseudoephedrine, caffeine, ibuprofen, aspirin, lidocaine, benzocaine, and creatine. 100 µL of the three reagents were then added and any colour change observed. A reagent blank was also performed with methanol for comparison to the mixtures.

The assessment of the effects of impurities on 2C-X compounds was completed with eight commonly found adulterants. These included caffeine, paracetamol, lactose, glucose, salt, artificial sweetener, aspirin, crushed Codral tablet, cornflour, starch, cellulose, and mannitol. A small amount (~1 mg) of each was added to two wells and 2C-H in a similar quantity was added to one of these followed by the reagents buffer, TCBQ, and acetaldehyde (3 drops each).

2.2.6.4 *Precision analysis*

The UNODC guidelines suggest completing at least 10 replicates at concentrations between 1.25× and 2× the LOD value [266]. Using this, in a microwell plate, twelve replicates were completed in duplicate at amounts just below 1.25× LOD and 2× the LOD value. A 1 mg/mL methanolic solution of 25H-NBOMe was used for this analysis and the methanol evaporated before 100 µL of each reagent was added to the wells in the recommended order (see 2.3.2). Further to this, tests were completed to assess the intra- and inter-day repeatability of the test method. Over ten days, tests with 25H-NBOMe were completed in triplicate at three separate occasions throughout each day.

The reproducibility of the test was assessed by changing the conditions in which the reaction was performed. This included testing two sets of the three reagents using reagent solutions which were prepared at different times. These tests were all carried out on porcelain spot plates using 1 mg 25H-NBOMe with 3 drops of each reagent solution from a Pasteur pipette in the order: buffer, TCBQ, acetaldehyde. A number of certified reference standards were tested in duplicate at the AFP laboratory (Sydney, Australia) with a freshly prepared reagent. Several of the NBOMe and ATS reference materials (different samples) were also tested in the UTS laboratory representing an inter-laboratory investigation. These samples are identified in the selectivity results (Table 2-8).

2.2.6.5 *Blind tests*

Blind tests were prepared in 24 wells of a 96 well plate with the addition of a selection of NBOMe, 2C-X, ATS solutions (100 µL, 1 mg/mL), and methanol blanks by a second analyst. The solvent was evaporated before 100 µL of each reagent solution was added to each well and the colour change recorded. Along with the colour changes, a determination of what the colour change identified was also recorded. After all tests had been completed and colour changes recorded, the results were compared to the compounds which had been added to each well.

2.2.6.6 *Stability*

The prepared TCBQ in ethyl acetate reagent (3×10^{-3} M) was stored in three different environments for eight weeks. One vial of the reagent was left on the laboratory bench for the study, a second wrapped in foil and stored in the laboratory cupboard and a third wrapped in foil and stored in the refrigerator (4 °C). The reagents were tested with 25H-NBOMe on porcelain spot plates over

the eight-week period comparing the stored solutions each time to a freshly prepared reagent solution and colour changes observed. The coloured compound formed was also assessed for stability to determine how long the positive colour change could be seen. Photos were taken at regular time intervals over 48 hr to record the colour changes over time. The coloured product was monitored in total for a period of one week.

2.2.6.7 *Stability of reagent mixtures*

For a mixture of these reagents to be used, stability tests needed to be completed to ensure the same colour change would be seen if the reagents have been stored together. Vials were prepared containing equal quantities of each reagent solution (2 mL) and stored under three different conditions 1) on the lab bench, 2) in the lab cupboard wrapped in foil and 3) in the laboratory fridge wrapped in foil (~4 °C).

Further stability tests were prepared with mixtures of two of the reagents. Three different mixtures were prepared, (TCBQ and acetaldehyde, TCBQ and buffer, acetaldehyde and buffer) and were stored in the same conditions as the previous study. These mixtures were applied to a reagent blank and 25H-NBOMe with the third reagent solution added separately. These were also compared to the mixture of all three reagents over the same time.

Three commercial antioxidants, ascorbic acid, propyl gallate, and 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene (BHT)), were assessed for their potential to improve the stability of the combined reagent. Initially, these antioxidants were tested to ensure they did not interfere with the colour change reaction of the reagents and the test substance. A preliminary test was completed where a small amount of antioxidant was combined with the reagents in a vial before addition to the drug. Another stability study was prepared as per the previous study, with the three antioxidants added to each combination described above. These solutions were stored in the laboratory cupboard for this study.

2.3 Results & Discussion

2.3.1 Colour test method development

2.3.1.1 *Preliminary testing*

Initial testing with 25H-NBOMe produced a light green colour change. Once the outlined adaptations had been implemented, the blue colour, characteristic of this test, was seen with the 25H-NBOMe.

2.3.1.2 *Reagent optimisation*

This testing was completed to determine firstly, how much the concentration would affect the final colour change result and secondly, if there was an optimal concentration which could be used to perform the test procedure. The greatest difference in colour development appeared between the concentrations of 1×10^{-3} and 3×10^{-3} M (see Figure 2-2). A concentration of 3×10^{-3} M was chosen (see Figure 2-2g) for all further testing as it was the lowest concentration that had the most distinguishable colour change from the reagent blank.

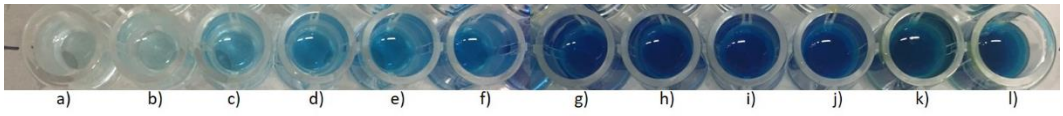

Figure 2-2: TCBQ reagent concentration study results with 25H-NBOMe. a) 8×10^{-5} M, b) 1×10^{-4} M, c) 3×10^{-4} M, d) 5×10^{-4} M, e) 8×10^{-4} M, f) 1×10^{-3} M, g) 3×10^{-3} M, h) 5×10^{-3} M, i) 8×10^{-3} M, j) 1×10^{-2} M, k) 3×10^{-2} M, l) 5×10^{-2} M TCBQ in ethyl acetate solutions

The ideal concentration for the detection of 2C compounds was the same as that for 25-NBOMe compounds, 3×10^{-3} M TCBQ, and it was deemed suitable for further testing and validation procedures see Figure 2-3g.

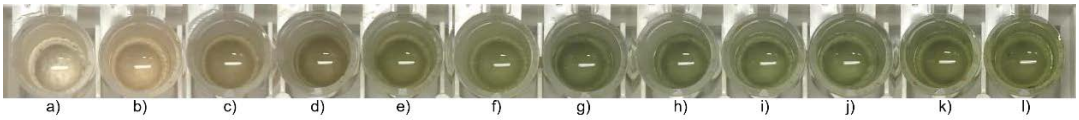

Figure 2-3: TCBQ reagent concentration study results with 2C-H. a) 8×10^{-5} M, b) 1×10^{-4} M, c) 3×10^{-4} M, d) 5×10^{-4} M, e) 8×10^{-4} M, f) 1×10^{-3} M, g) 3×10^{-3} M, h) 5×10^{-3} M, i) 8×10^{-3} M, j) 1×10^{-2} M, k) 3×10^{-2} M, l) 5×10^{-2} M TCBQ in ethyl acetate solutions

2.3.1.3 *Buffer solution optimisation*

The tested buffer solutions showed colour change results with little variation between pH values of the phosphate-based solutions. The buffer that had the most different colour change with 25H-NBOMe was the pH 11 carbonate-based buffer solution, indicating that this may play a part in the reaction occurring even though the pH value may not be too significant. The pH 11.4 buffer solution was chosen for analysis to ensure reaction with the drug without increasing the pH unnecessarily, reducing the volume of corrosive chemicals required.

Several of these new buffer solutions produced a similar colour change to the original phosphate buffer solution shown in Table 2-4. In particular, the pH 10.80 buffer containing borax and NaOH produced results considerably similar to those seen with the original PO_4 buffer solution, blue colour with the 25H-NBOMe, and a pale blue-green with the 2C-H. Many other buffer solutions produced shades of blue or green with the 25H-NBOMe and could potentially be used for this test. To assess this further, the buffer solutions, which did produce a colour change, were prepared with the other reagents into mixtures for stability assessment. These tests involved a mixture of all three reagents and were completed over a two-week period, after which, no colour changes were seen.

Table 2-4: Results of new buffer solution tests with TCBQ and acetaldehyde

Buffer composition	**Final volume**	**pH**	**Colour change result [a]**		
			Blank	**2C-E**	**25H-NBOMe**
borax (100 mL, 0.025 M) HCl (4.0 mL, 0.1 M)	200 mL	9.10	colourless	lt. green	d. green-blue
borax (100 mL, 0.025 M) NaOH (48.5 mL, 0.1 M)	200 mL	10.80	colourless	p. blue-green	blue
tris(hydroxymethyl)aminomethane (100 mL, 0.1M) HCl (11.4 mL, 0.1 M)	200 mL	9.0	colourless	p. blue	green-blue
ammonium chloride (6.75 g) aqueous ammonia (57 mL)	100 mL	10.7	v. pale green	brown-yellow	p. green
glycine (25 mL, 0.2 M) NaOH (22.75 mL, 0.2 M)	100 mL	10.6	v. pale yellow	green	lt. blue
tetrabutylammonium hydroxide (1.0 M in methanol)	n/a	10	colourless	brown-green	d. brown-green

[a] *v. = very; lt. = light; p. = pale; d. = dark*

2.3.1.4 *Reactions with other quinone reagents*

A number of the quinone compounds tested afforded a colour change with the compounds of interest. Three compounds: 2,5-dichloro-1,4-benzoquinone, 2,6-dichloro-1,4-benzoquinone, and 2,3,5,6-tetrabromo-1,4-benzoquinone, showed a colour change with 25H-NBOMe that is highly similar to the colour produced with the p-TCBQ reagent. Table 2-5 outlines the reagents tested and the results of the quinone reagents with both 2C-H and 25H-NBOMe. These results indicate that the test method reagent could be interchanged with other compounds and yield positive results. The interactions occurring with these other related compounds would be highly similar, resulting in the similar final coloured compound.

Table 2-5: Colour change results of tests completed with a range of quinone reagents

Reagent	**Colour change** [a]		
	Blank	**2C-H**	**25H-NBOMe**
2,3,5,6-tetrachloro-1,4-benzoquinone (*p*-TCBQ)	colourless	green	bright blue
3,4,5,6-tetrachloro-1,2-benzoquinone (*o*-TCBQ)	yellow	bright red	p. orange
2,5-dichloro-1,4-benzoquinone	v. pale pink	p. blue	d. blue
2,6-dichloro-1,4-benzoquinone	v. pale yellow-pink	lt. blue	bright blue
chloranilic acid	lt. pink-purple	lt. pink-purple	lt. yellow-pink
2,3,5-trichloro-1,4-benzoquinone	colourless	colourless	p. yellow
2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)	lt. orange-red	red	red-brown
2,3,5,6-tetrafluoro-1,4-benzoquinone	lt. purple	pink-purple	d. purple
2,3,5,6-tetrabromo-1,4-benzoquinone	v. pale yellow	d. green	bright blue
2,5-dibromo-1,4-benzoquinone	colourless	p. orange	blue-green
acenaphthenequinone	colourless	colourless	p. yellow
1-aminoisoquinoline	colourless	colourless	p. yellow
hydroquinone	colourless	colourless	p. yellow
2,3-dicyanohydroquinone	colourless	colourless	p. yellow

[a] lt. = light; v. = very, p. = pale, d. = dark

Some literature suggests the reaction of amines with DDQ is a charge transfer interaction [268]. This may explain the opposing colours seen with the DDQ compared to the other chlorine containing quinone compounds. Upon testing without an aldehyde, 2C-H, and 25H-NBOMe still produced a colour change with DDQ indicating this is not necessary for reaction to occur. Hence,

as this does not occur with other quinone compounds, the reaction mechanism is likely to be different.

2.3.1.5 *Effect of other aldehydes*

Propionaldehyde and butyraldehyde were tested, replacing the acetaldehyde. These aldehydes show some benefits over the use of acetaldehyde as they are less toxic and less volatile, so that it would be more ideal in a field setting. However, these aldehydes did not produce the same intense colour changes with the drugs as seen with the acetaldehyde (Table 2-6).

Table 2-6: Colour change results of 2C-H and 25H-NBOMe with changing aldehydes

Aldehyde	Colour change [a]		
	Blank	**2C-H**	**25H-NBOMe**
acetaldehyde	colourless	green	bright blue
propionaldehyde	colourless	lt. grey-purple	v. pale green
butyraldehyde	colourless	grey	lt. bright green

[a] lt. = light; v. = very

2.3.2 General recommended procedure

At this stage of development, a general recommended procedure was acquired and further used for all remaining testing including validation procedures, limiting variations throughout. The general procedure for this colour test method, when performed on a ceramic spot plate is:

To 1 mg of a solid sample or 1-2 drops of liquid sample add:

1. 3 drops pH 11.4 phosphate buffer solution
2. 3 drops 3×10^{-3} M TCBQ in ethyl acetate solution
3. 3 drops acetaldehyde
4. Observe colour change immediately and after 5 minutes

2.3.3 Blotter paper analysis

The blotter paper test methods were compared based on the observed colour change and the time taken for this change to occur. Both samples, extracted and direct application, showed a bright blue colour change similar to that expected from 25-NBOMe compounds in the general test method. The great similarity in the colour change of these two samples indicates that the blotter

paper has no major matrix effect on the overall reaction and colour change. It is also important to note that the blotter paper used had a yellow and orange coloured print on one side and this played no effect on the colour change seen. The application of this test directly to the blotter paper is ideal for presumptive testing. While some agitation of the sample tube was required, the direct application still provided a colour change result almost immediately (<1 min) (see Figure 2-4).

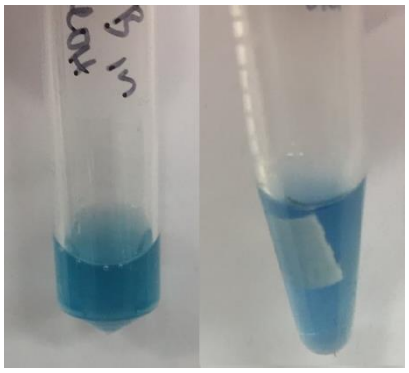

Figure 2-4: Comparison of blotter paper test methods. Left: Colour change result of the extracted blotter paper tab; Right: Colour change result of direct application to the blotter paper tab

The repetitions of other analogues and concentrations showed similar results. Drug amounts of 150 µg and above (per blotter) all successfully showed the blue colour change expected with this test method. The 50 µg samples which were tested, produced a pale or light blue colour more indicative of a secondary amine being present and the 100 µg samples differed between analogues (see Table 2-7). The values are also below the commonly found amounts of NBOMe compounds on a single blotter paper, as further referred to in Section 2.3.5.2, upwards of 250 µg [56]. These differences indicate a concentration dependent colour change and while this may appear to be a limitation of this test, generally LSD is the only other drug commonly found on blotter papers [269]. LSD does not react with this test to produce a colour change similar to that of NBOMe compounds or other compounds containing a secondary amine. This would indicate that in most circumstances where a blotter paper was tested, and produced a blue colour change, the most likely conclusion would be the presence of an NBOMe-related compound. The positive results seen with this test on blotter paper samples illustrate the real-world application of this method for the detection of NBOMe related compounds.

Table 2-7: Results of blotter paper analysis

Compound	Amount added to blotter (µg)	Colour change result [a]
25D-NBOMe	50	lt. blue
	100	lt. blue
	100	blue
	150	bright blue
	200	bright blue
25C-NBOMe	100	p. blue
	200	blue
25G-NBOMe	50	p. blue
	100	blue
	150	bright blue
	200	bright blue
25I-NBOMe	100	blue
	200	blue
25T-NBOMe	100	lt. blue
	200	blue
30C-NBOMe	100	lt. blue
	200	blue

[a] *lt. = light; v. = very; p. = pale*

2.3.4 Proposed reaction mechanism

The mechanism of this type of reaction has been proposed in the chemical literature to produce a vinylamino-substituted benzoquinone product [265]. The product of this test with NBOMe compounds has not successfully been isolated and analysed however a general proposed reaction between any primary or secondary amine and the reagents is shown in Figure 2-5.

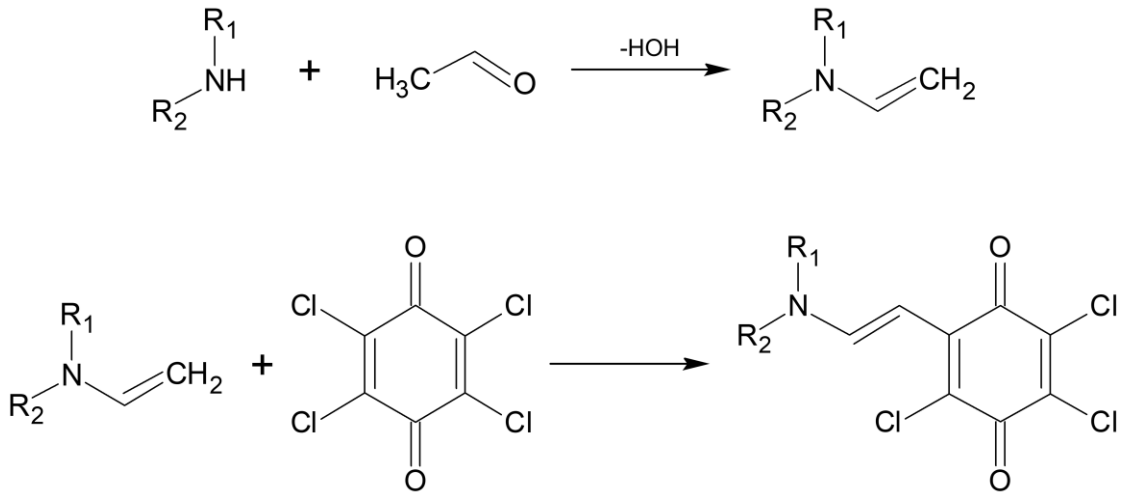

Figure 2-5: Proposed mechanism of reaction of TCBQ test with primary or secondary amines containing compounds

2.3.5 Method validation

2.3.5.1 *Specificity and selectivity*

A total of 112 illicit substances and precursor chemicals along with 42 common cutting agents, sugars, amines, and amino acids were tested to assess the selectivity of the method. Sixteen 25-NBOMe analogues were tested to assess the specificity of the method.

Seven 25-NBOMe analogues resulted in a blue/bright blue colour change almost immediately. Another seven analogues produced the same colour change up to five minutes after the reagents were applied. There were only two analogues, 25I-NBOH and 25I-NBMD, which resulted in a light blue and green-blue colour change respectively even after five minutes. This is most likely due to the small structural differences compared to the other NBOMe compounds (see Figure 2-1). Both the hydroxy (25I-NBOH) and methylenedioxy (25I-NBMD) groups are more strongly activating than the methoxy group present on the other NBOMe analogues. It appears that the change in this substituent, specifically a change to a more electron donating substituent, will have an effect on the final colour change. However, the change in the substituent on the dimethoxy substituted benzene ring does not seem to influence the colour change result. This indicates that these compounds may not be able to be distinguished from other secondary amine-containing compounds using this test. However, overall, the method proved to be specific for the majority of NBOMe type compounds.

Of the other illicit drugs and precursors tested, only one other compound, 4-hydroxymethcathinone (Figure 2-6), gave a blue colour change that would be considered a positive result (see Table 2-8). While cathinones are generally known to be more prevalent than the 25-NBOMe compounds, this particular analogue has not been reported in the literature for its occurrence or use in the way more common cathinones such as methylone have been [270, 271]. This indicates that this cathinone would not appear to be a problematic interferant. Other secondary amine-containing compounds, particularly of the amphetamine-type substances, resulted in a light blue colour change indicating that this reagent is selective for amine-containing compounds. These results could still be differentiated from the positive colour change of the 25-NBOMe compounds at this concentration. Methamphetamine in this set of tests gave a light blue colour change indicative of the secondary amine, however, in other tests it resulted in a false positive blue colour change which could not be distinguished from the NBOMe compounds. It

should also be noted that if the concentration of the NBOMe compound is below the LOD specified in Section 2.3.5.2, these colour changes may no longer be able to be distinguished as an NBOMe and would simply confirm the presence of a secondary amine. Even considering this information, the ability for this test to distinguish between 25-NBOMe and LSD is apparent, as no colour change like that of the NBOMe positive result was seen with LSD.

Table 2-8: Resulting colour changes of illicit substances tested initially and after 5 minutes

Compound Class	Compound	Initial Colour change [a]	5 min colour change [a]	Test result [b]
NBOMe analogues and derivatives	25G-NBOMe HCl [c]	green-blue	blue	+
	25D-NBOMe HCl [c]	blue	NC	+
	25B-NBOMe HCl [c]	blue	NC	+
	25H-NBOMe HCl [c]	d. blue	NC	+
	25I-NBOMe HCl [c]	green-blue	blue	+
	25E NBOMe HCl [c]	blue	NC	+
	25C-NBOMe HCl [c]	green-blue	blue	+
	25B-NBF	bright blue	d. blue	+
	25I-NBOH	p. blue	lt. blue	+/-
	25I-NBMD	lt. green-blue	green-blue	+/-
	25T-NBOMe	lt. blue	blue	+
	25N-NBOMe	lt. green-blue	blue	+
	30C-NBOMe	blue	bright blue	+
	mescaline-NBOMe	lt. blue	blue	+
	25C-NB3OMe	green-blue	d. blue	+
	25B-NB4OMe	blue	d. blue	+
	25iP-NBOMe	blue	d. blue	+
	25P-NBOMe	bright blue	NC	+
	25T2-NBOMe	blue	NC	+
	25T4-NBOMe	blue	NC	+
	25T7-NBOMe	blue	NC	+
	25H-NB4OMe	blue	d. blue	+
	25I-NB3OMe	blue	d.blue	+
2C-x series	2C-H HCl	lt. yellow-green	yellow-green	-
	2C-T-7 HCl	lt. yellow-green	yellow-green	-
	2C-I HCl [c]	NC	lt. yellow-green	-
	2C-E HCl [c]	lt. yellow-green	bright green	-
	2C-B HCl [c]	lt. green	bright green	-

	2C-D HCl [c]	lt. yellow-green	bright green	-
	2C-T-2 HCl	lt. green	NC	-
Amphetamine type substances	(+)-*S*-methamphetamine HCl [c]	p. blue	lt. blue [d]	+/-
	(±)-*N*-methyl-3,4-methylenedioxyamphetamine HCl (MDMA) [c]	pale blue	lt. blue	+/-
	d-amphetamine	lt. green	NC	-
	3-fluoroamphetamine HCl	v. pale yellow	lt. yellow-green	-
	3,4-dimethoxymethamphetamine HCl	v. pale blue	lt. blue	+/-
	4-hydroxyamphetamine	p. green	lt. yellow	-
	2-fluoromethamphetamine HCl	p. blue	NC	-
	4-methoxymethamphetamine	lt. blue	NC	+/-
	4-fluoromethamphetamine HCl	lt. blue	NC	+/-
	2-methylamphetamine (oretamine) HCl	lt. yellow-green	NC	-
	(+/-)-3-methylamphetamine HCl	NC	lt. yellow-green	-
	(+/-)-3-methylmethamphetamine HCl	p. blue	NC	-
	(+/-)-3-methoxymethamphetamine	p. blue	colourless	-
	(+/-)-2-methylmethamphetamine HCl	NC	NC	-
	(+/-)-3-methoxyamphetamine HCl	NC	NC	-
	(+)-3-fluoromethamphetamine HCl	v. pale blue	blue-grey	-
	4-methylmethamphetamine HCl	lt. blue	lt. blue	+/-
	(+/-)-2-methoxyamphetamine HCl	p. green	lt. green	-
	(+/-)-3,4-dimethoxyamphetamine HCl	lt. yellow-green	NC	-
	(+/-)-4-methylthioamphetamine HCl	lt. yellow-green	NC	-
	(+/-)-4-bromo-2,5-dimethoxyamphetamine HCl	lt. yellow-green	NC	-
	(+/-)-N,N-dimethyl-3,4-methylenedioxyamphetamine HCl	NC	NC	-
	(+/-)-N,N-dimethylamphetamine HCl	NC	NC	-
	(+/-)-N-methyl-1-(3,4-methylenedioxyphenyl)-2-butylamine HCl	lt. blue	NC	+/-
	2,5-dimethoxy-4-methylamphetamine HCl	lt. green	NC	-
	(+/-)-2-chloroamphetamine HCl	NC	lt. green	-
	(+/-)-4-chloroamphetamine HCl	NC	lt. green	-
	(+/-)-2-bromoamphetamine HCl	NC	lt. green	-
	(+/-)-2-bromomethamphetamine HCl	lt. blue	NC	+/-
	4-isopropoxy-2,5-dimethoxyphenethylamine HCl	lt. yellow	lt. yellow-green	-

	(+/-)-3-bromomethamphetamine HCl	lt. blue	NC	+/-
	(+/-)-3-bromoamphetamine HCl	NC	p. green	-
	(+/-)-bromo-dragonFLY HCl	p. yellow-green	NC	-
	(+/-)-4-chloro-2,5-DMA HCl	lt. green	bright green	-
	(+/-)-N-ethyl-3,4-methylenedioxyamphetamine HCl	NC	NC	-
	(+/-)-2,5-dimethoxyamphetamine HCl	lt. green	bright green	-
	(+/-)-3,4,5-trimethoxyamphetamine HCl	lt. green	NC	-
	N-ethylamphetamine HCl	v. pale blue	NC	-
	(+/-)-4-methoxyamphetamine HCl	lt. green	NC	-
	(+/-)-4-methylamphetamine HCl	lt. green	bright green	-
	3-chloromethamphetamine HCl	lt. blue	NC	+/-
	(+/-)-3-chloroamphetamine HCl	v. pale green	NC	-
	(+/-)-4-bromomethamphetamine HCl	lt. blue	NC	+/-
	2-chloromethamphetamine HCl	lt. blue	NC	+/-
	4-chloromethamphetamine HCl	lt. blue	NC	+/-
	(+/-)-4-bromoamphetamine HCl	lt. green	bright green	-
	(+/-)-4-fluoroamphetamine HCl	v. pale green	NC	-
	2-fluoroamphetamine HCl	lt. yellow	NC	-
Cathinones	3,4-methylenedioxy-N,N-dimethylcathinone HCl	NC	NC	-
	4-methyl-a-pyrrolidinobutiophenone HCl	NC	NC	-
	iso-ethcathinone HCl	NC	NC	-
	2,4,5-trimethylmethcathinone HCl	v. pale blue	lt. blue	+/-
	3,4-dimethylmethcathinone HCl	NC	NC	-
	(+/-)-N,N-diethylcathinone HCl	NC	lt. brown	-
	4-fluoromethcathinone HCl	NC	NC	-
	(+/-)-N,N-dimethylcathinone HCl	NC	NC	-
	4-methylethylcathinone HCl	NC	NC	-
	2,4-dimethylmethcathinone HCl	lt. blue	NC	+/-
	2,3-dimethylmethcathinone HCl	lt. blue	NC	+/-
	(+/-)-3-bromomethcathinone HCl	p. blue-green	NC	-
	4-methoxy-a-pyrrolidinopropiophenone HCl	NC	NC	-
	4-methyl-N-benzylcathinone HCl	lt. green-blue	NC	-
	(+/-)-3-fluoromethcathinone HCl	NC	NC	-
	3,4-methylenedioxymethcathinone HCl	NC	NC	-
	(+/-)-4-methylmethcathinone HCl [c]	NC	NC	-
	butylone HCl	lt. green-blue	NC	-

	3,4-methylenedioxypyrovalerone HCl [c]	NC	NC	-
	4-methoxymethcathinone HCl	NC	NC	-
	3-methylmethcathinone HCl	NC	NC	-
	2-methylmethcathinone HCl	lt. green-blue	NC	-
	4-bromomethcathinone HCl	lt. green-blue	NC	-
	(+/-)-a-pyrrolidinopentiophenone HCl	NC	NC	-
	pyrovalerone HCl	NC	NC	-
	(+)-cathinone HCl	NC	NC	-
	4-hydroxymethcathinone	lt. blue	blue	+
	2-fluoromethcathinone HCl	NC	NC	-
Tryptamines	5-methoxy-N-methyl-N-isopropyltryptamine HCl	NC	NC	-
	5-methoxy-N,N-diallyltryptamine	p. purple	NC	-
	5-methoxy-N,N-dimethyltryptamine	NC	blue-grey	-
Piperazines	1-(4-chlorophenyl)-piperazine.2HCl	lt. blue	NC	+/-
	Piperazine hexahydrate	green-brown	brown	-
	methylbenzylpiperazine.2HCl	NC	NC	-
	1-(4-fluorophenyl)-piperazine.2HCl	p. blue	NC	-
Other illicit substances and precursors	LSD	d. green	NC	-
	ergotamine tartarte	NC	NC	-
	fentanyl	NC	NC	-
	JWH-073	NC	NC	-
	methylamine HCl	p. green	lt. green	-
	Pseudoephedrine	p. green	green-blue	+/-
	ephedrine	v. pale blue	lt. green-blue	-

[a] lt. = light; d. = dark; p. = pale; v. = very; NC = no colour change observed
[b] (+) = positive, (-) = negative, (+/-) = positive for the presence of a secondary amine
[c] compounds tested at both UTS and AFP laboratories
[d] methamphetamine resulted in some blue colour changes that could not be distinguished from NBOMe compounds

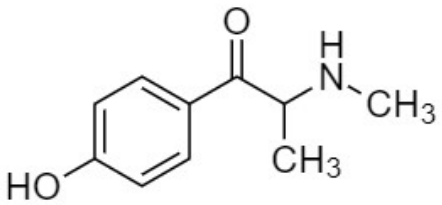

Figure 2-6: Chemical structure of 4-hydroxymethcathinone

Of the adulterants and non-drug compounds tested, no false positives were identified and many of these tested compounds produced little or no colour change with the reagents. For ease, those compounds which did not produce a colour change have not been included in Table 2-9.

Table 2-9: Resulting colour change of common cutting agents, sugars and other amines and amino acids

Compound class	Compound [a]	Initial Colour change [b]	5 min colour change [b]	Test result [c]
Common adulterants	lidocaine	p. green	NC	-
	paracetamol	p. yellow	v. pale orange	-
	ibuprofen	NC	p. yellow	-
	caffeine	p. yellow	p. yellow	-
	tetramisole HCl	p. yellow	v. pale orange	-
	4-methoxy phenol (MEHQ)	p. purple	purple	-
	3,4-dimethoxyphenethylamine	brown	NC	-
	magnesium stearate	p. yellow	NC	-
	benzocaine	NC	v. pale purple	-
	phenobarbital	brown-yellow	purple	-
	salicylamide	p. yellow	NC	-
	aspirin	p. yellow	NC	-
	creatine	p. yellow-orange	NC	-
	4-aminophenazone	brown-purple	brown	-
	quinine	p. yellow	pale grey-brown	-
Sugars	cellulose	p. yellow	NC	-
Amines and amino acids	aniline	d. brown	NC	-
	glycine	p. yellow	yellow	-
	ethylenediamine-N,N'-diacetic acid	NC	p. orange	-
	diphenylamine	p. purple-blue	lt. blue	+/-
Pharmaceuticals	nortriptyline	black-blue	blue-black	-
	protriptyline	black-blue	purple-black	-

[a] Compounds which did not react are not listed in this table for ease of reading
[b] lt. = light; d. = dark; v. = very; p. = pale; NC = no change
[c] (+) = positive, (-) = negative, (+/-) = positive for the presence of a secondary amine

2.3.5.2 *Limit of detection*

The limit of detection study of the TCBQ method was completed by assessing the colour change of a range of amounts of 25H-NBOMe and determining the point where the colour becomes indistinguishable from the blank. The lowest concentration at which the colour change was differential from the reagent blank and provided what would still be classed as a positive result was 22.5 µg of 25H-NBOMe (0.075 mg/mL, in 300 µL). This correlates to Figure 2-7e) where 75 µL of the 0.3 mg/mL 25H-NBOMe solution was added. Below this, the colour would be described as light or pale blue rather than the bright or dark blue associated with the positive interaction and colour change result. The guidelines from NIJ's Colour Test Standard suggest a working LOD ten times this value. This working value, 225 µg, would in many cases be appropriate as a single blotter paper containing an NBOMe compound will often contain upwards of 250 µg [56]. For those analogues, notably NBOH and NBMD, which at amounts of 100 µg only produced light blue colour changes, they would not be able to be determined from this test as containing an NBOMe like structure. Particularly if the concentrations were lower, the result could only indicate the presence of a compound containing a secondary amine and further testing would be required to determine the presence of these compounds. If a compound containing a secondary amine was at a much higher concentration, there is the possibility it could be misidentified as an NBOMe. Through the completed testing, it appears only 4-hydroxymethcathinone and methamphetamine would potentially be interpreted as an NBOMe containing compound.

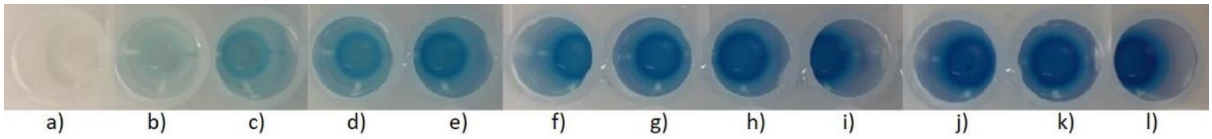

Figure 2-7: LOD colour change results: a) reagent blank, b) 3, c) 7.5, d) 15, e) 22.5, f) 30, g) 45, h) 60, i) 75, j) 90, k) 105, l) 150 µg 25H-NBOMe

The LOD for 2C-H was determined to be equivalent to 50 µg (see Figure 2-8f)), which is a relatively small quantity assuming an average dose of 10 mg. The working LOD value would equate to 500 µg, which is still substantially lower than a regular single dose of these drugs.

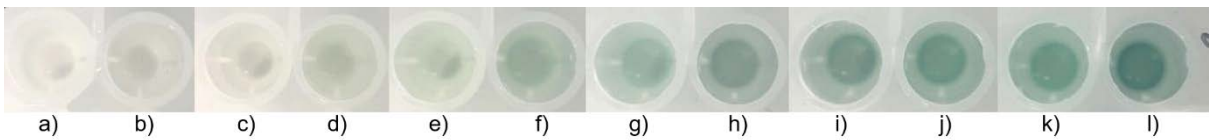

Figure 2-8: LOD colour change results with 2C-H. a) reagent blank, b) 5, c) 25, d) 25, e) 37.5, f) 50, g) 75, h) 100, i) 125, j) 150, k) 175, and l) 250 µg 2C-H

2.3.5.3 *Purity testing*

All eleven mixtures tested were differentiable from the reagent blank (not shown in Figure 2-9). Of these, only two mixtures showed differences from the 25H-NBOMe control (ephedrine and aspirin). Ephedrine alone gave a pale green-blue colour change with the method and has been shown to react with a similar method [272]. This may indicate why there was some interference when combined with the 25H-NBOMe. The result with aspirin, however, was not expected as it did not react to produce a colour change with the reagents on its own. It is unclear why, when mixed with the NBOMe drug, the presence would influence the final colour change result. Literature has shown that some compounds with similar functional groups (carboxylic acids and alcohols) may interfere with the reaction between the amine group of the drug and the benzoquinone [273, 274]. Even with these small differences, the colour changes seen would still indicate the presence of a drug, potentially 25-NBOMe, and would require further testing.

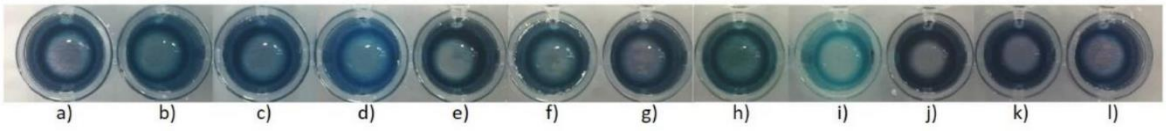

Figure 2-9: Results of purity tests with 25H-NBOMe and adulterants in 1:1 ratio. A) 25H-NBOMe blank, b) methamphetamine, c) MDMA, d) ephedrine, e) paracetamol, f) pseudoephedrine, g) caffeine, h) ibuprofen, i) aspirin, j) lidocaine, k) benzocaine, l) creatine

Individually, the adulterants gave no colour changes, and a green or yellow-green colour change was seen for all tests containing 2C-H. It appears that these cutting agents had minimal to no effect on the colour change seen with 2C-H when present in combination.

2.3.5.4 *Precision analysis*

The repeatability of this test was determined by assessing the similarity or differences in colour change across multiple 25H-NBOMe samples using the same reagents under the same laboratory conditions. The replicates at concentrations 1.25× – 2× the LOD all produced blue colour change results consistent with a positive result across the replicates. These amounts were from 28 – 45 µg of 25H-NBOMe in 300 µL of reagents (0.093 – 0.15 mg/mL). It is also noteworthy that the time of day had minimal impact on the colour change, and no trends were seen for a certain result occurring at a particular time of day.

Reagent solutions that were prepared independently were tested in combinations with the other reagents. Changing the reagent solutions and the combinations had a limited effect on the final colour change result with 25H-NBOMe. Most tests with 25H-NBOMe produced a blue colour change representative of a positive result. There were some small differences between the TCBQ solutions, the older solution seeing less consistent positive results with the 25H-NBOMe in comparison to a more recently prepared reagent solution. This reagent solution was almost two months old indicating that even though some repetitions still produced a positive colour change result, the reagent may not be suitable to be used after this time.

The results seen between the compounds tested at both UTS and the AFP laboratories were also comparative. Repetitions of the colourimetric reactions of these compounds gave highly similar colour changes at both laboratories, with positive blue colour changes seen for all 25-NBOMe compounds tested at both locations.

2.3.5.5 *Blind tests*

The results of the blind tests are outlined in Table 2-10. Overall, these results showed a high percentage of true positives for 25-NBOMe related compounds. Some analogues such as 25I-NBOH and 25I-NBMD, previously identified to produce a light blue colour change, were only able to be identified as containing a secondary amine. Another, 25T-NBOMe, which had previously shown a light blue colour change initially, was also only able to be determined as a secondary amine-containing sample, even after several minutes. Methamphetamine was present in four of the blind samples and twice was interpreted as a 25-NBOMe compound producing a blue colour that was indistinguishable from that associated with the positive colour change. This indicates the potential for some interference of methamphetamine with this test, however, it is unlikely that methamphetamine would be found on a blotter paper. In Brazil, there have been reports of many drugs found on blotters other than LSD yet methamphetamine was not reported in any samples over a six year period [33]. All methanol blanks and 2C samples were determined as negative. Overall, the identified false negatives were classed as being positive for containing a secondary amine and would be able to be identified through further testing.

Table 2-10: Resulting colour change and interpretations of blind tests with corresponding compounds

Sample No.	Colour change [a]	Result interpretation	Actual compound
1	lt. green	- (1° amine)	2C-T2
2	d. blue	+ (NBOMe)	25I-NBOMe
3	NC	-	methanol
4	bright blue	+ (NBOMe)	25B-NBOMe
5	blue	+ (NBOMe)	mescaline-NBOMe
6	blue	+ (NBOMe)	methamphetamine
7	blue	+ (NBOMe)	methamphetamine
8	lt. blue	+/- (2° amine)	25I-NBMD
9	d. blue	+ (NBOMe)	25D-NBOMe
10	lt. blue	+/- (2° amine)	methamphetamine
11	bright blue	+ (NBOMe)	25E-NBOMe
12	lt. blue	+/- (2° amine)	25I-NBOH
13	NC	-	methanol
14	lt. green	- (1° amine)	2C-D
15	lt. blue	+/- (2° amine)	methamphetamine
16	lt. blue	+/- (2° amine)	25I-NBOH
17	blue	+ (NBOMe)	25H-NB4OMe
18	blue	+ (NBOMe)	30C-NBOMe
19	lt. green	- (1° amine)	2C-I
20	NC	-	methanol
21	lt. blue	+/- (2° amine)	25T-NBOMe
22	blue	+ (NBOMe)	25G-NBOMe
23	blue	+ (NBOMe)	25I-NBMD
24	bright blue	+ (NBOMe)	25T2-NBOMe

[a] lt. = light; d. = dark; v. = very; p. = pale; NC = no change

[b] (+) = positive, (-) = negative, (+/-) = positive for the presence of a secondary (2°) amine

2.3.5.6 *Stability*

The stability of the reagents was tested and compared by applying the testing method using reagent solutions which were prepared and stored in different conditions. Figure 2-10 shows the colour change results of the three storage conditions in comparison to a freshly prepared reagent after eight weeks of storage. It can be seen that no distinct differences were observed after the storage of the reagent solution over this time nor differences between storage conditions. Based on these results and also the results of the precision analysis, it would be recommended that the TCBQ solutions are not used for this test after two months of storage.

Figure 2-10: Comparison of storage conditions with 8 week old reagent (left to right fresh, fridge, cupboard, laboratory bench)

Outside of this stability study, there were some instances where the reagent solution was not suitable for use after 8 weeks. As mentioned above, it was noted during precision analysis that there may have been some degradation of the reagent solution as it no longer provided the expected blue colour change result. Freshly prepared solutions would be the most suitable for this test method and storage in darkness preferred to assist in prolonging the shelf life of the solution. The solutions used throughout other testing were prepared in large batches (100-500 mL) and the consistent opening (3× per week on average) of the reagent bottle for use may have contributed to the poor results seen in the precision study. Whereas the reagents used in the stability study were only opened once per week over the eight weeks.

The stability of the coloured compound was observed for 48 hr as after this time the colour change was not considered a positive colour. There were no significant changes in the colour for the first 24 hr. Only after this time did the blue colour become much darker than the initial test result and by 48 hr was a blue-black colour which would be too dark to be considered positive. While the regular observations stopped at this point, the vial was kept and after one week, the blue colour had completely gone, and the solution was yellow-brown. It is appropriate that the positive blue colour remains similar to the initial change for several hours so the result can potentially be observed by another analyst or device to further reinforce the result without being there at the time of the test.

The complete results of this study are shown in Table 2-11. These results suggest that, if stored in a closed container, the results of this colour test would not have to be reported immediately or could be confirmed by a second analyst at a later time on the same day of testing. This may also allow for documentation to occur at a later time in a more appropriate environment. Delayed documentation may be necessary for situations where the lighting is not ideal for capturing the final colour change result of the test on the scene.

Table 2-11: Stability of coloured compounds over 1 week

Time elapsed	Colour with 2C-H [a]	Colour with 25H-NBOMe [a]
0 minutes	lt. green	bright blue
20 minutes	lt. green	bright blue
40 minutes	green	bright blue
1 hour	green (slightly darker)	bright blue
2 hours	brown-green	bright blue (slightly darker)
20 hours	yellow-brown	d. blue
24 hours	yellow-brown	d. blue
44 hours	orange-brown	black-blue
48 hours	orange-brown	black-blue
1 week	orange-brown	brown-orange

[a] *lt. = light; d. = dark*

2.3.5.7 *Stability of reagent mixtures*

Completing this test with a combined reagent could provide a number of benefits. It simplifies the test, reduces the procedure time, allows for only one sample chamber to be required, limits the contact between the analyser and harmful chemicals, and increases the potential for use in the field. As the current general recommended procedure requires three separate solutions to be added to the seized substances this method is only suitable for use in the laboratory.

Over nine weeks, the most promising reagent mixtures appeared to be the acetaldehyde and buffer mixture and the TCBQ and buffer mixture (see Table 2-12 below). The combination of TCBQ and acetaldehyde gave no colour change results after the first week of storage. It is possible these reagents interact with one another over time, making it impossible for the same colour change reaction to occur. These reagents are also light, air, and temperature sensitive, which may have played a role in the lack of stability over time. Unfortunately, the mixture of all three reagents also did not provide good results over the course of the study. After approximately one week there was no longer a colour change seen with the 25H-NBOMe. The storage of the solutions in the fridge did not have a major effect on the stability. Surprisingly those mixtures stored at room temperature often gave better colour change results with the 25H-NBOMe.

Table 2-12: Stability study colour change results with 25H-NBOMe

Time	Mixture	Colour change with 25H-NBOMe [a]		
		Bench	**Cupboard**	**Fridge**
1 day	TCBQ & acetaldehyde	p. brown	p. orange	p. green
	TCBQ & buffer	p. yellow	green	p. yellow
	acetaldehyde & buffer	green-blue	green-blue	blue-green
	TCBQ, acetaldehyde & buffer	green-blue	lt. brown	brown
1 week	TCBQ & acetaldehyde	yellow	yellow-pink	p. yellow
	TCBQ & buffer	green-blue	lt. green-blue	yellow-brown
	acetaldehyde & buffer	d. green-blue	green-blue	green-blue
	TCBQ, acetaldehyde & buffer	yellow-brown	yellow	p. brown
3 weeks	TCBQ & acetaldehyde	yellow	yellow	p. yellow
	TCBQ & buffer	blue	green-blue	brown-yellow
	acetaldehyde & buffer	green-blue	green-blue	blue-green
	TCBQ, acetaldehyde & buffer	yellow	yellow	yellow
5 weeks	TCBQ & acetaldehyde	p. yellow	p. yellow	p. yellow
	TCBQ & buffer	grey-green	lt. green	green-blue
	acetaldehyde & buffer	blue	green-blue	black-blue
	TCBQ, acetaldehyde & buffer	yellow	yellow	yellow
7 weeks	TCBQ & acetaldehyde	colourless	p. yellow	p. yellow
	TCBQ & buffer	green-blue	blue	blue
	acetaldehyde & buffer	d. green-blue	green-blue	lt. green-blue
	TCBQ, acetaldehyde & buffer	yellow	yellow	yellow
9 weeks	TCBQ & acetaldehyde	yellow	yellow	p. yellow
	TCBQ & buffer	green	d. blue	blue-green
	acetaldehyde & buffer	d. yellow-green	d. yellow-green	d. green
	TCBQ, acetaldehyde & buffer	yellow	yellow	yellow

[a] *lt. = light; d. = dark; p. = pale*

While the storage of the three reagents together does not appear possible long term, this study has shown the potential for two of the three reagents to be stored together, limiting the mixing of reagents required before testing and increasing the potential for a less complicated portable device. Many currently available colour test kits contain multiple ampules to hold reagent solutions. The reagents are then only combined after the drug has been added removing the need for them to have long term stability as a mixture. The TCBQ reagent has previously been shown to be stable over an eight-week testing period in different storage conditions while the buffer solution and acetaldehyde have long shelf lives when appropriately stored.

The addition of antioxidants did not improve the stability of these reagent mixtures. The reagent mixtures containing BHT showed the best colour change reactions with the 2C-H and 25H-NBOMe.

A blue-green colour change was seen with the two reagent combination reactions with the 25H-NBOMe. These antioxidants, however, did not improve the stability of the combination of all three reagents and the colour change was no longer seen after approximately one week.

2.4 Conclusions

A simple colour spot test method was developed and sufficiently validated for the rapid and selective detection of 25-NBOMe analogues. Almost all tested 25-NBOMe analogues and derivatives produced a bright blue colour change, with the exception of those NBOH and NBMD compounds. Only two other illicit substances produced a similar result in some tests. The working limit of detection for 25H-NBOMe, of 225 µg, is sufficient for this type of test with these compounds. 2C-X analogues and other primary amine containing compounds produce a light green colour change with this test and this result may be valuable in a colour testing scheme. This method has the capacity to directly detect 25-NBOMe compounds impregnated onto blotter papers, a common matrix which they are sold, and differentiate these compounds from LSD. While no real case samples have been available for this study, this test shows the potential to be applied to these samples with no significant interferences identified. The results of the work in this chapter gave rise to a patent application.

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Chapter 3: Investigation of mobile phone applications for colour detection and identification of NBOMe analogues

Chapter 3: Investigation of mobile phone applications for colour detection and identification of NBOMe analogues

3.1 Introduction

The interpretation of colour-based test results remains a subjective measure as results are typically analysed with the naked eye and analysis takes place in uncontrolled environmental conditions (e.g. lighting). The addition of digital image analysis has proven to enhance the discrimination of colour changes, when compared to the naked eye, and hence provides more objective and accurate results. Colour values, most commonly RGB values, can be used to identify small differences in colour changes to indicate a particular result. Using a camera, reflected light is detected by three filters: red, green, and blue, each providing a numerical data point (0-255). These values determine the precise hue, saturation, and lightness of the test results which can in turn be used to detect the presence, or even an approximation of the quantity, of a drug. Digital image analysis has been completed employing common chemical colour tests, including the Marquis and Simon test, for the analysis of amphetamines and opiates [201, 202]. The Scott test has also been evaluated using digital image analysis to discriminate true positive results for cocaine and possible false positive results [275]. These methods utilised Adobe Photoshop® and MATLAB® respectively to obtain the RGB values.

The use of smartphones for this type of analysis effectively removes the need for a computer and software analysis and therefore allows for almost immediate colour values to be obtained. The digital camera of a smartphone can be utilised along with an application to provide colour analysis. In recent years, the use of smartphone applications for this purpose has been of interest and shows promise for combination with colour-based drug testing methods. Methods have included the simple use of smartphone colour analysis directly on the colour test solution of seized drugs [200, 203], while others have created new devices or sensors to utilise smartphone applications [207, 210, 276]. Further expansion to other matrices, such as oral fluid testing, is evident with the development of an ELISA test for ketamine which can be analysed with a developed smartphone application [209] and the use of a smartphone for analysis of a lateral flow device for detecting drugs of abuse [277]. Smartphone algorithms have been investigated for use in conjunction with

a range of colourimetric based testing [212]. The combination of the smartphone camera with a developed application, which involves automatic calibration procedures and comparison to reference images or colour values, shows promise to be applied to any colourimetric test device to provide the analyst with timely results, particularly in resource-limited areas. There has been minimal investigation into these methods for the analysis of NPS. The synthetic cannabinoid, AB-001, was analysed using a modified Ehrlich reagent on a solid sensor and RGB values were used for detection and quantification [199].

Investigation into the use of smartphone applications for a more discriminative analysis of the developed colour test for the detection of NBOMe compounds is outlined in this chapter. The importance of differentiation between NBOMe compounds and other drugs, as well as concentration analysis and the effect of impurities, are explored. The tests were analysed using three smartphone applications, and the results from these applications were compared. To determine the usefulness of these applications, the resulting colour values were visualised and analysed using a number of statistical tests and data plots. Further to this, predictive analysis was completed to assess the potential for a drug or drug class to be accurately determined.

3.2 Methods and materials

3.2.1 Materials and reference standards

2,3,5,6-Tetrachloro-1,4-benzoquinone (TCBQ) and acetaldehyde were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Ethyl acetate, methanol, acetonitrile, and sodium hydroxide (NaOH) pellets were obtained from Chem-Supply (Gillman, SA, Australia). Disodium phosphate (Na_2HPO_4) was obtained from Ajax Finechem (Taren Point, NSW, Australia). Deionised water from a laboratory supply was used throughout the methods.

Pure reference standards including hydrochloride salts of 25B-NBOMe, 25C-NBOMe, 25D-NBOMe, 25E-NBOMe, 25G-NBOMe, 25H-NBOMe, 25I-NBOMe, 25T-NBOMe, 2C-B, 2C-I, 2C-E, 2C-T-2, 2C-D, 2C-H, MDMA and d-methamphetamine along with d-amphetamine sulphate and were obtained from the National Measurement Institute (NMI, North Ryde, NSW Australia. Other standards were obtained as HCl salts from Novachem (Heidelberg West, Victoria, Australia), including 30C-NBOMe, 25B-NB4OMe, 25C-NB3OMe, mescaline-NBOMe, 25B-NBF, 25I-NBMD and 25I-NBOH. 25iP-NBOMe, 25N-NBOMe, 25P-NBOMe, 25T2-NBOMe, 25T4-NBOMe, 25T7-NBOMe, 25H-

NB4OMe, and 25I-NB3OMe were purchased from Sapphire Bioscience (Redfern, NSW, Australia) as HCl salts. (1*S*,2*S*)-(+)-Pseudoephedrine HCl, (1*S*,2*R*)-(+)-ephedrine HCl, cellulose, methylamine HCl and paracetamol were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). Citric acid, urea and aspirin were obtained from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia)

3.2.2 Mobile phone applications for RGB capture

Data acquisition was completed using three smartphone applications that have the capability to record colour values using the smartphone camera. The applications chosen were ColorAssist (© FTLapps, Inc.), Colorimeter (© Chris Becker), and What a Color? (© Oleg Brailean) and were downloaded from the app store at no cost. An iPhone 6 (Apple Inc.) was used for the analysis of the colour change results. This smartphone camera has an 8-megapixel sensor with 1.5 µm pixels and ƒ/2.2 aperture. Each of these applications utilise slightly different methods in analysing the colour change and provide a range of colour index values (see Table 3-1). It should be noted that for some later tests the application Colorimeter had to be used on a new device (iPhone XS) due to software issues and device availability.

Table 3-1: Information that can be obtained by each application

Outputs and functions [a]		**ColorAssist**	**Colorimeter**	**What a Color?**
Index values	RGB	✓	✓	✓
	HTML	✓	✓	✓
	HSL/HSB	✓	×	✓
	CYMK	✓	×	✓
	RYB	✓	×	×
Names	HTML name	✓	×	×
	Colour name	✓	×	✓
	Simple colour name	✓	×	×
Functionalities	Light option	✓	×	✓ (camera flash)
	Sample aperture size	✓	×	×

[a] RGB = red, green, blue; HTML = hexadecimal colour; HSL = hue, saturation, lightness; HSB = hue, saturation, brightness; CYMK = cyan, yellow, magenta, black; RYB = red, yellow, blue

3.2.3 TCBQ colour test method

Reagent solutions including TCBQ in ethyl acetate and a phosphate buffer solution were prepared as previously described in Section 2.2.3. Acetaldehyde was used as purchased and stored at 4 °C in the absence of light. Tests were conducted in micro well plates obtained from Greiner Bio-One through Interpath Services Pty Ltd. (West Heidelberg, Victoria, Australia) and Agilent Technologies (Mulgrave, Victoria, Australia). Drug solutions were prepared in methanol (1 mg/mL) and pipetted into wells at the required concentration. The solvent was allowed to evaporate before the reagents (100 µL each) were added sequentially. From a height of approximately 15 cm above the well and under ambient fluorescent lighting in a laboratory, the colour was recorded using each of the applications outlined in the previous section.

3.2.4 Data analysis and visualisation

The complete data set was made up of reagent blanks, NBOMe analogues, 2C analogues, methamphetamine, MDMA, and amphetamine (see Table 3-2 and Table B. 1). Depending on availability, each drug sample was analysed for its colour values across at least twelve concentrations in duplicate. Data analysis was completed on the entire dataset and also subsets of this. The three applications were compared using all NBOMe analogues at a concentration of 100 µg and the reagent blanks. Concentrations studies were completed with all concentrations of the NBOMe analogues along with reagent blanks. To compare the drug classes, a data set of all the drugs and the reagent blanks was collated, this was further reduced to only include drug quantities of 100 µg and the reagent blanks. The analysis of the presence of impurities was completed using an additional data set containing samples of impurities at 100 µg and also mixed samples containing each 100 µg NBOMe and impurity. These data points were compared to the NBOMe samples at 100 µg and reagent blanks.

Table 3-2: Number of tests and data points obtained for this study

Drug	No. of analogues	No. individual tests	Total data points
Blank	-	60	182
NBOMe	22	548	2032
2C	6	157	604
Methamphetamine	-	154	572
MDMA	-	60	190
Amphetamine	-	24	72

Visual representation of the data obtained throughout the colour analysis was completed using 3D scatterplots and boxplots prepared using R Studio. These plots provided a simple method to observe and show relationships between data points and their distribution, and to identify any correlations present.

Kruskal-Wallis Rank Sum tests were completed with Wilcoxon Pairwise comparisons to determine where statistically significant differences were occurring in the data sets [278]. This method was chosen over ANOVA tests as it does not assume normality [279]. In comparison to an ANOVA test, Kruskal-Wallis analysis tests differences in ranks rather than the group means, thus it tests differences in how groups are distributed overall. This testing was conducted on a range of data sets to compare the colour values between the different applications, the use of a light source, different drug classes, the drug concentrations, and also the presence of impurities in NBOMe samples. Pairwise analysis allows for determination of where in a data set a difference may be occurring. It compares each pair, or two classes, to ascertain if they are different. For all tests, p-values <0.05 indicate that the null hypothesis, where the samples are the same, should be rejected and there is therefore a significant difference in these values. This data was also analysed using multivariate analysis to determine if there is a significant difference overall rather than just within each variable. The multivariate ANOVA (MANOVA) test was considered for this analysis, however, as with the traditional ANOVA it assumes that the data is normally distributed. While there are non-parametric MANOVA type tests, they are not commonly used and are often specific for datasets [280]. Therefore, as an alternative, this analysis was completed using the *npmv* R package

as outlined by Burchett et al [281]. While there are alternatives to this analysis which may have been appropriate [280], this was chosen as it provides simple but statistically correct results and allows for the comparison of variables to identify where differences are occurring within the data.

Further to this, the prediction of a sample into a class was assessed using multinomial logistic regression analysis [282, 283]. This method utilises the calculated log odds of a sample belonging to a group or class for prediction. In this case, the RGB values are used as the predictor variables for assigning samples to groups. A training set is randomly produced (66-70% of data) and then the remaining data is used as a test set to run against this model. The method provides a prediction of which class the data is most likely to belong to.

3.3 Results and discussion

3.3.1 Mobile phone applications and initial testing

The applications chosen for this study all proved to be useful in collecting RGB data. While these applications were able to obtain other colour values such as hexadecimal values and hue, saturation and lightness values (HSL), these other values were not used for comparison purposes. These values are often alternatives to RGB or as in the case of hexadecimal values, a direct correlation to the RGB value itself as these can be converted rather simply. RGB values appeared the most in literature regarding image analysis of illicit drug colour testing [200, 202, 203, 275] and so was chosen as the sole colour value in this study. Figure 3-1 provides an example of the output provided by each application.

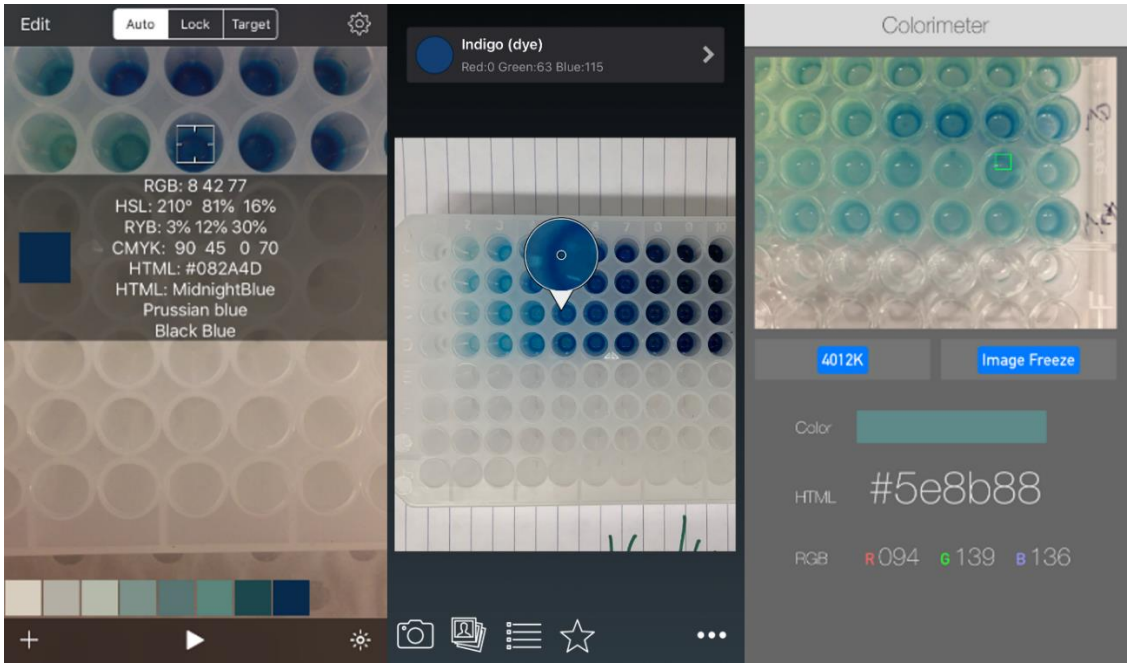

Figure 3-1: Example screenshots of colour recordings with ColorAssist (left), What a Color? (centre) and Colorimeter (right)

3.3.2 Data analysis and visualisation

The methods utilised for the analysis of these data sets were found to be effective in determining where differences were occurring and what samples may be grouped. Tests for normality were completed prior to analysis and included Shapiro-Wilk's method [284] and a skewness test [285] as well as visualising normality through histogram, density, and Q-Q plots using the ggpubr package (v0.4.0) [286]. It was identified that the data were not normally distributed across the three variables (see Table 3-3 and Figure 3-2), and so the non-parametric tests chosen for analysis would be the most appropriate.

Table 3-3: Normality test outputs of Shapiro-Wilks test and skewness test for the complete data set

Complete data set output		**R**	**G**	**B**
Shapiro-Wilk test	W (test statistic)	0.93549	0.93882	0.96667
	p-value [a]	2.2×10^{-16}	2.2×10^{-16}	2.2×10^{-16}
Skewness test	Value	-0.1874613	-0.6897262	-0.6759187
	Interpretation [b]	Approx. symmetric	Moderately skewed left	Moderately skewed left

[a] *significance level assessed at α = 0.05*

[b] *Value < -1 or > 1 = highly skewed; value between -1 and -.05 or 0.5 and 1 = moderately skewed; value between -0.5 and 0.5 = approximately symmetric*

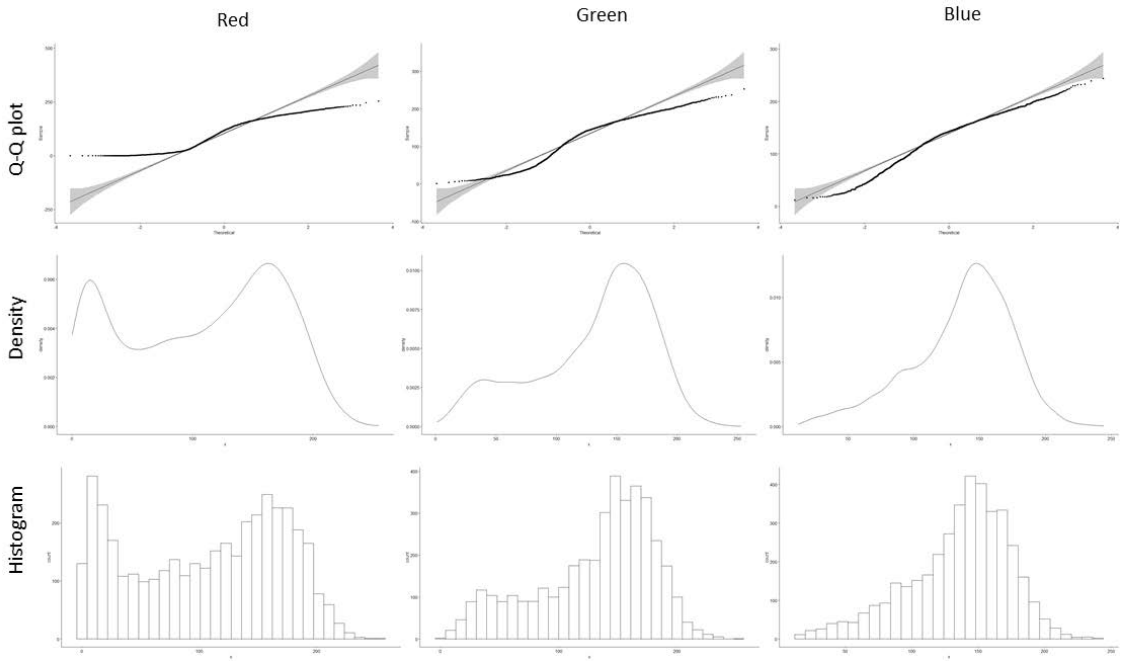

Figure 3-2: Normality test plots for the complete data set. Q-Q plot (top), density plot (middle) and histogram (bottom) for R, G and B values (left to right)

3.3.2.1 *Evaluation of applications*

The RGB output of the three mobile phone applications were compared using the same NBOMe analogue test samples, which contained 100 µg of the drug. Ideally, there would be minimal differences between each application, as they are analysing the same set of samples and therefore would be expected to obtain the same values. However, it was seen that there was a significant difference between the values obtained using Colorimeter and the other two applications, which appeared to arise from a difference in the R values in particular (see Table 3-4).

Table 3-4: Pairwise comparison results (p-values) between each application

	p-value [a]		
Colour values	**ColorAssist v Colorimeter**	**ColorAssist v What a Color?**	**What a Color? v Colorimeter**
R	0.0015	0.4163	0.0058
G	0.0015	0.9636	0.0018
B	0.023	0.895	0.023

[a] *significance level assessed at α = 0.05*

The boxplot (Figure 3-3) shows that the median blue values are similar across all three applications, and the distribution of values for ColorAssist and What a Color? are also similar. The same is true for these two applications and the other colour values. However, Colorimeter shows variation in the median value and the distribution of values for both green and red compared to the other applications.

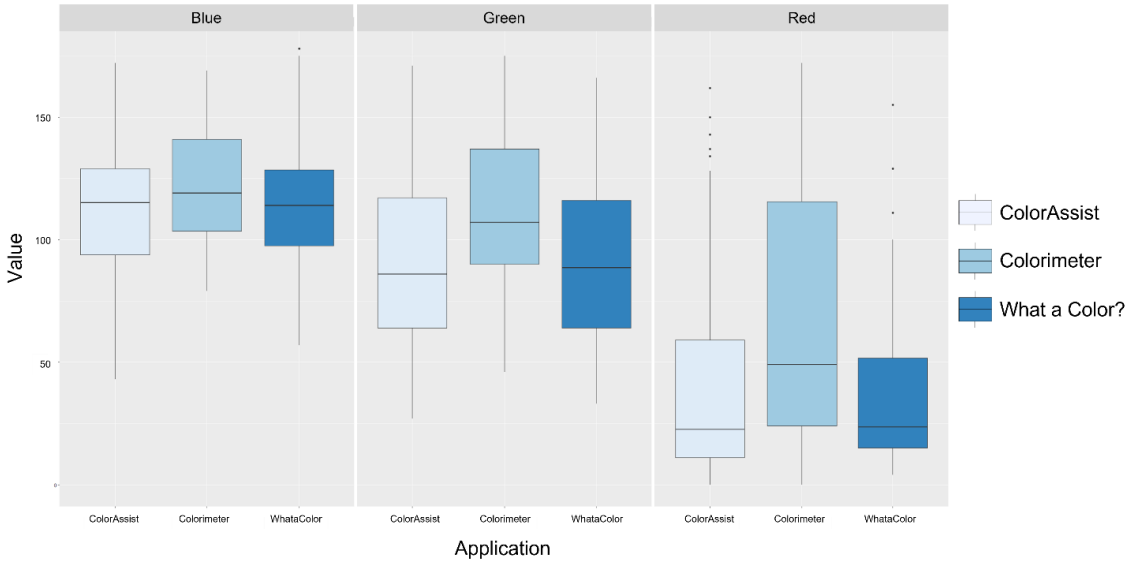

Figure 3-3: Boxplot showing data distribution for each application

The results here suggest that Colorimeter may not be a suitable application for use in conjunction with this colour test method. It was previously reported that Colorimeter was not the best application for colour analysis regarding ease of use and returning the correct colour based on comparison with Munsell colours [200]. The comparison to ColorAssist led the authors to use only ColorAssist for further testing as it has a more user-friendly platform and could analyse a larger portion of the colour test itself with the possibility of the aperture size being changed. This makes it a more versatile options for analysing colour tests. Due to these findings, in this study, data from each application was compared to assess the effect that this application had on the overall results. It should be mentioned that this variation was seen before the use of the new device with this application, and so it is assumed that the new device played no part in contributing to this variation. The results between the devices appeared to be comparable and it is hypothesised that any smart phone could be used with RGB values remaining consistent. It would be beneficial to thoroughly assess the effect different devices may have on the resulting colour values obtained with each application. This was not completed in this study to avoid any potential variation that may have been found and to therefore maintain consistent results for analysis.

Another area of interest regarding the applications was the comparison of values obtained when collected with and without the use of a light source. This comparison was only completed for ColorAssist and What a Color? as these applications have an inbuilt light function and previously showed to not have an observable difference in collected values. Figure 3-4 and Figure 3-5 below show that there is not an apparent separation in these values, and they appear to have a similar distribution. The statistics support this observation with the null hypothesis accepted with a p-value 0.367 (see Table 3-5). When compared individually, all three variables were demonstrated to be statistically similar. This is a promising result regarding this test, as interference from different light conditions would be problematic. One of the reasons to incorporate a detection source is to avoid the errors made when ambient light conditions are poor. The use of the inbuilt light function appears to have no effect on the RGB value recorded so it would be appropriate to use if required. Studies have involved the use of a custom-built detection box with an external light source attached [203, 276]. If this type of design were portable, it could also be useful for ensuring adequate light in the field.

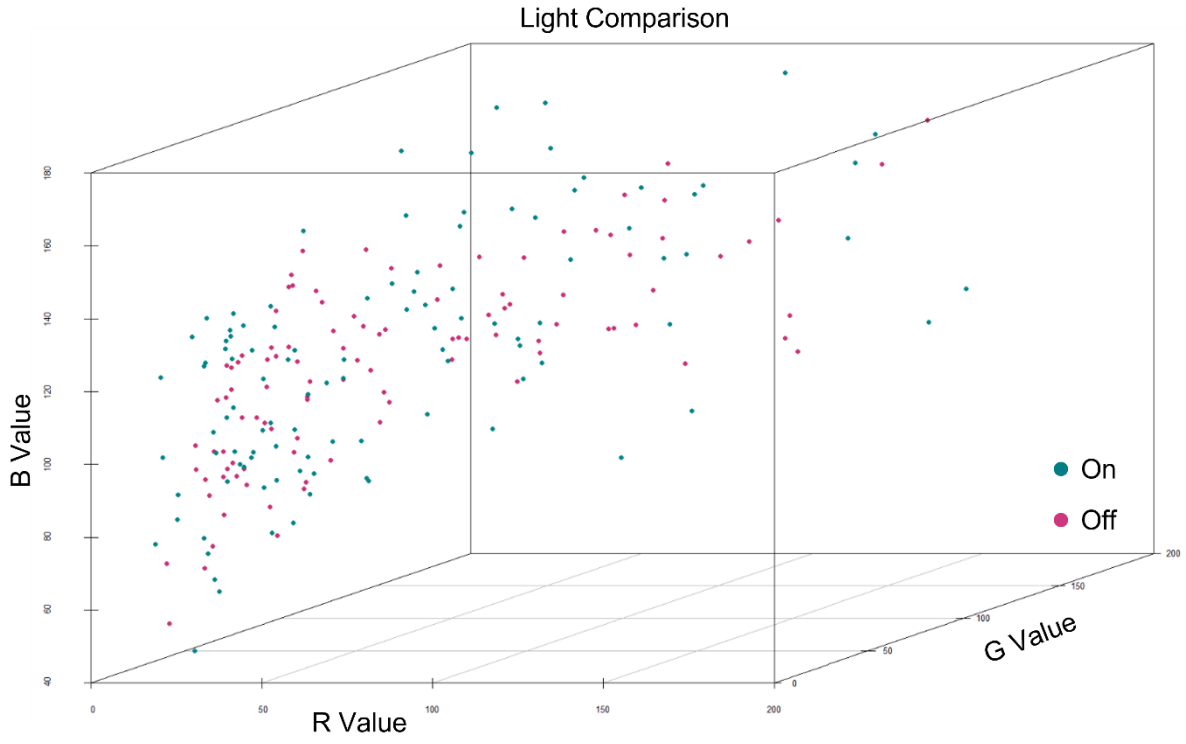

Figure 3-4: 3D Scatterplot comparing values obtained with (on) and without (off) a light source using ColorAssist and What a Color?

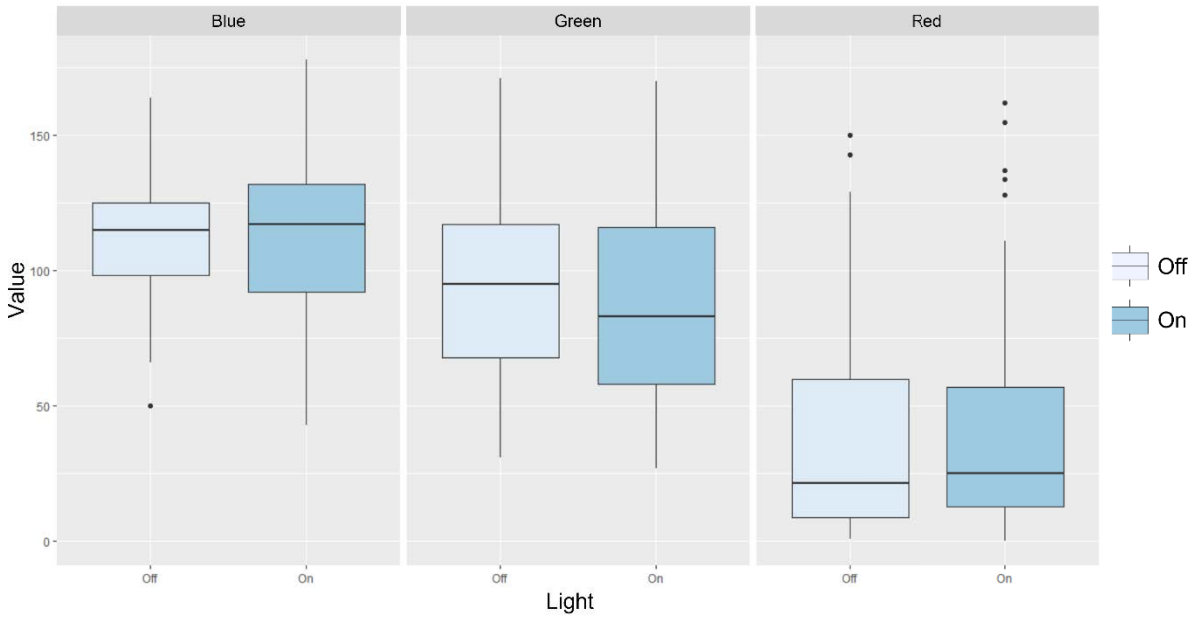

Figure 3-5: Boxplot showing distribution of colour values for samples analysed with (on) and without (off) a light source using ColorAssist and What a Color?

Table 3-5: p-values obtained for comparing the use of a light source (on vs off) with both ColorAssist and What a Color?

Colour value	p-value [a]
R	0.367
G	0.4767
B	0.1498
Multivariate (R, G and B)	0.6067

[a] *significance level assessed at α = 0.05*

3.3.2.2 *Concentration studies*

A range of NBOMe solutions at different concentrations were prepared and analysed for determination of a limit of detection but also to compare to other concentrations and the negative control. To prepare these tests, aliquots of the drug solution (1 mg/mL) were pipetted into a micro well plate to obtain quantities in the range of 0-500 µg as required. The solvent was removed by evaporation, reagent solutions were added (100 µL each), and colour change recordings were taken of each concentration using the three smartphone applications. The colour values obtained (n=2215) were analysed and compared to one another.

As the concentration of NBOMe increased, the average RGB values began to decrease, and Figure 3-6 shows a representative trend line of 25E-NBOMe. Once the quantity of the drug reached approximately 75 µg, a plateau in the red value was observed (see Figure 3-6). It was also observed that the decrease in the green and blue values slowed at around 150 µg. There was no clear trend in the correlation between the concentration of the NBOMe and RGB value. For this reason, further analysis was completed to assess the possibility of differentiating between concentrations.

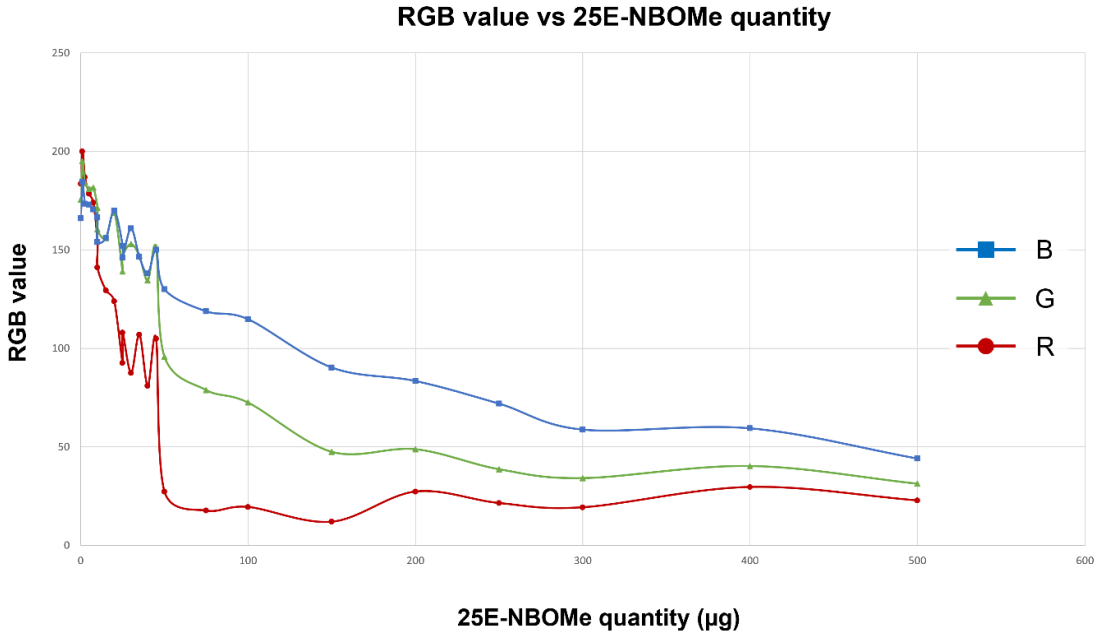

Figure 3-6: Average RGB values vs quantity of 25E-NBOMe

Analysis of the NBOMe concentrations, using Kruskal-Wallis testing and Wilcoxon pairwise analysis, indicated that quantities in the higher range (>50 µg) are easily distinguishable from the reagent blank with p-values < 5.5×10^{-8} for each variable. Unsurprisingly, the concentration values at the lower end appear to be more similar to the reagent blank based on the comparison of each variable. Quantities less than 20 µg do not appear to be differentiable from the reagent blank. A similar pattern was observed with other concentrations where similarities were seen between drug concentrations around the same range and at the highest quantities. This would indicate that quantification of a specific amount may not be possible, but an approximation of the amount of drug present could be completed based on the RGB value.

These trends can be visualised in the 3D scatterplot (Figure 3-7), where there is a lot of overlap between concentrations similar to one another, especially those at either end of the range. These similarities are likely occurring for a number of reasons. The colour changes of concentrations

close to one another are visually highly comparable and would result in similar RGB values, resulting in some overlap. Another possibility is the differences in colour change seen between NBOMe analogues. Some analogues, such as 25I-NBOH, have a much lighter blue colour change compared to others, for example, 25B-NBOMe as outlined in Section 2.3.5.1. This means that even at the same concentration, the colour values across various NBOMe analogues will not be consistent, which creates difficulties in differentiating between quantities.

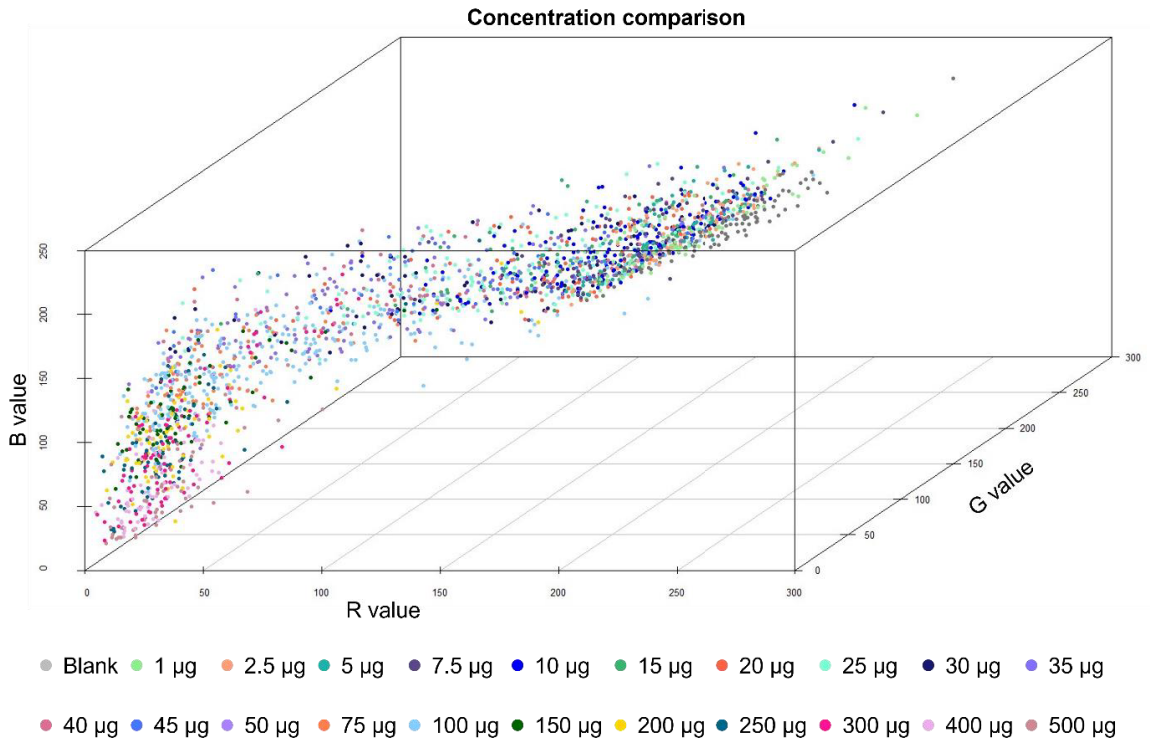

Figure 3-7: 3D scatterplot comparing NBOMe concentration values

This analysis was repeated with the data obtained from each application individually. The applications all displayed a significant difference across the dataset ($p < 0.05$). All three applications showed similar results regarding the quantity of the NBOMe analogues, which can be differentiated from the negative control (≥50 µg).

3.3.2.3 *Comparison of drug classes*

The NBOMe compound results as a class were compared to the four other drug classes and negative controls. This comparison was completed both with the entire dataset containing all concentrations and also with the drug quantity at 100 µg. The NBOMe analogues and the 2C-X analogues were each classed as a single drug class for the purpose of this analysis. Even with the potential discrepancies outlined in the previous section, it seemed beneficial to group the NBOMe

analogues to cover the range of possible colour values obtained for this group. Methamphetamine, MDMA, and amphetamine were also analysed as individual groups. Overall, it was found that there was a significant difference within these groups for both test sets. When visualising the data, it was observed that there was substantial overlap with the entire dataset (see Figure 3-8 and Figure 3-9).

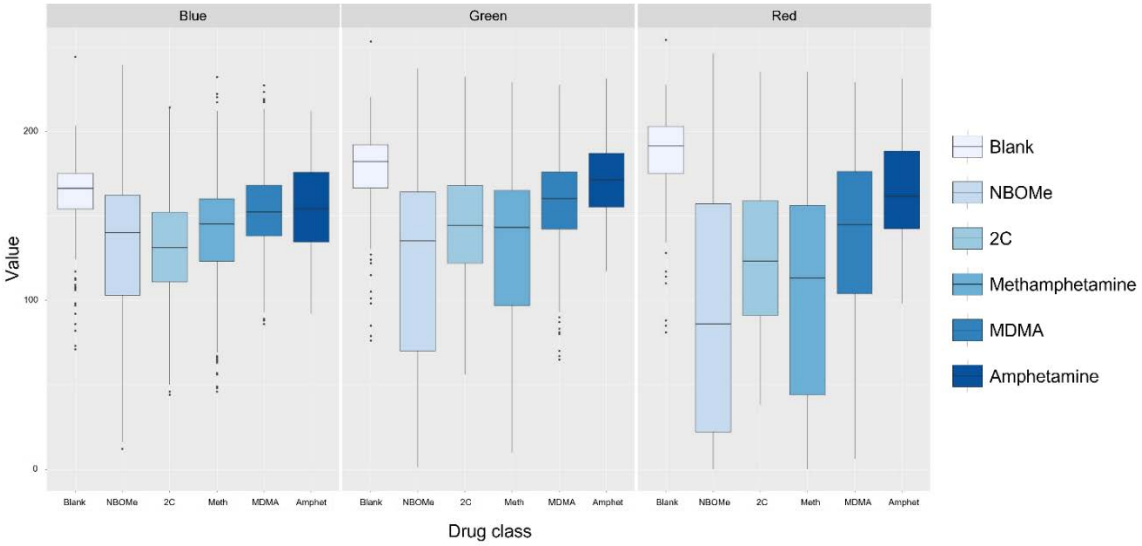

Figure 3-8: Boxplot comparing the drug classes and their distribution across all concentrations

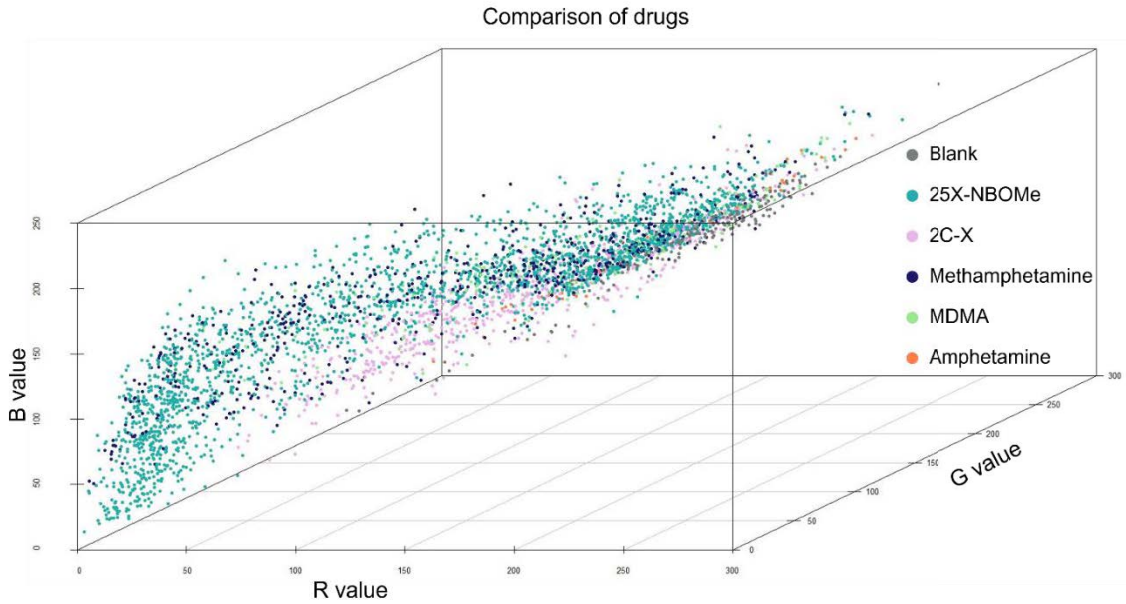

Figure 3-9: 3D scatterplot comparing the six drug classes across all concentrations

The comparison of the groups at 100 µg was able to show more clearly the differences in the values obtained from each drug class (see Figure 3-10). The difference identified in the data was broken down using pairwise comparison of each variable. This comparison indicated a significant

difference between each of the other drug types and the NBOMe analogues for all variables. All drugs except amphetamine showed a significant difference from the negative control. There were some similarities observed between the other drugs, but as this test is not validated to detect the amphetamine drugs, this would not appear to be a problem.

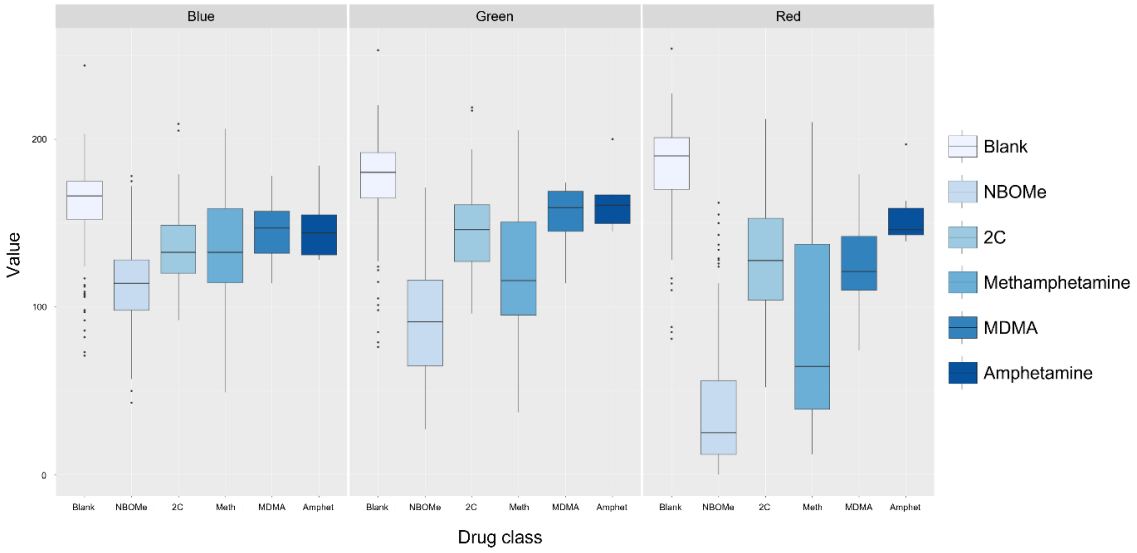

Figure 3-10: Boxplot comparing the distribution of the five drug classes at 100 µg and blank (negative control) samples

3.3.2.4 *Analysis of impurities*

Eight potential drug impurities including, pseudoephedrine HCl, ephedrine HCl, cellulose, methylamine HCl, paracetamol, citric acid, urea and aspirin were analysed with this method. Samples of the impurities alone and combined with 25D-NBOMe (1:1, 100 µg) were tested, and the colour changes were analysed using the three mobile phone applications. Twelve replicates of each impurity and each combination with the NBOMe were completed. These results were compared with positive controls (25-NBOMe, 100 µg) and negative controls (reagent blanks) to assess the impact they may impose on this method.

It was found that the colour values for the impurities were significantly different from all other samples. What is interesting is that the NBOMe values showed that they were different from the 25D-NBOMe combined with these impurities (Figure 3-11). This difference provides both potential advantages and disadvantages for this type of analysis. The differentiation between a pure drug and a drug containing adulterants can be beneficial knowledge for a drug user or an analyst. Even without knowing what the other compound is, this may change the way the sample is further analysed. On the other hand, this difference could be a barrier to detecting the drug if it is not

classified as a positive result. Unfortunately, there is no published data of other materials or compounds that may be found in NBOMe samples. It has been reported that blotter papers may contain multiple NBOMe derivatives, though their presence is likely due to synthetic procedures or contamination after synthesis, rather than being deliberately added [56]. This study still provides relevant information that when something other than a pure sample is present, it can be differentiated.

Further analysis into the ratio of NBOMe and impurity present should be considered to understand if there is a level at which these become different. It would also be beneficial to assess the impact of the presence of other potential interferents or cutting agents in an NBOMe sample and, as such, the colour value recorded.

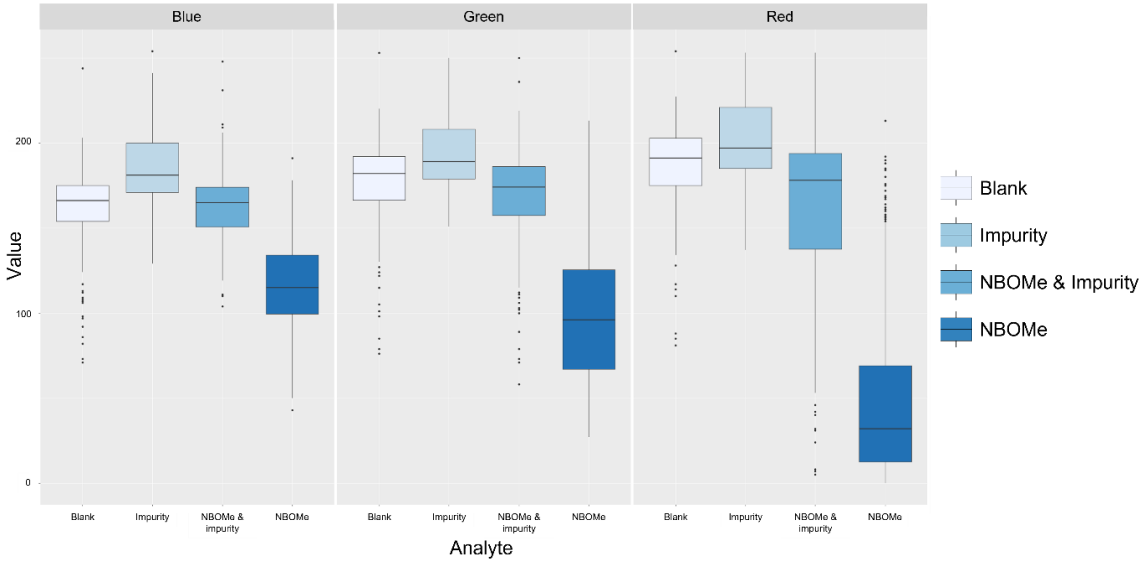

Figure 3-11: Boxplot comparing blank, impurity, NBOMe & impurity and NBOMe

3.3.3 Predictive modelling

Initial modelling with just the NBOMe samples and negative controls (Model 1) showed promising results. The model correctly predicted these samples with 97% accuracy (see Table 3-6). To assess this potential further, another three models were evaluated. The first was created with data from all six drug classes but separated into just two groups, NBOMe analogues, and all other samples (Model 2).

Table 3-6: Model 1 prediction results

		True class	
		NBOMe	**Blank**
Predicted class	**NBOMe**	645	11
	Blank	7	26
Total		652	37

In this second model, a number of samples were incorrectly predicted as belonging to the other group, with the accuracy lowered to 64% (see Table 3-7). When looking closer at the NBOMe samples, which were incorrectly predicted, the spread across concentration follows a similar trend with regard to the results from previous analyses. In the test set, 157 NBOMe samples were incorrectly predicted, and these were made up of mostly concentrations less than 20 µg (n = 94). This result corresponds well to the results seen with the previous analysis (Section 3.3.2.2), where concentrations below this value were not distinguishable from the reagent blank. This may indicate a reason as to why these samples were incorrectly predicted. A further 34 samples between 20 and 50 µg and 29 samples above 50 µg were also incorrectly predicted. These higher concentration values are more likely to result from some overlap between the NBOMe values and the values obtained from methamphetamine and MDMA, as these drugs provide a light blue colour change. In the training data set, it was noted that incorrectly predicted high concentration NBOMe samples contained various analogues, including those that provide a light blue colour change with this test (e.g., 25I-NBOH). The samples above 50 µg which were incorrectly predicted in the test set were 25H-NBOMe, 25E-NBOMe, and 25I-NBOMe, all of which are expected to provide a blue or bright blue colour change with this test.

When considering the other drugs that were predicted as NBOMe compounds in the test set, this was made up of two blank samples, four 2C-B samples, 54 MDMA, and 202 methamphetamine samples. The high number of methamphetamine samples further indicated the interference this drug has with this colour test. Previous analysis has shown that it can be challenging to differentiate methamphetamine from the NBOMe analogues due to the blue colour change seen, especially at high concentrations. It was expected that higher quantities of methamphetamine might be incorrectly predicted with this model however, it was observed that there was a range of quantities, including some <10 µg, that were predicted as containing an NBOMe.

Table 3-7: Model 2 prediction results

		True class	
		NBOMe	**Other**
Predicted class	**NBOMe**	421	262
	Other	157	339
Total		578	601

The results from Model 2 were further explored by the separation of each drug class, so possible predictions can be any one of the six drug classes. Model 3 was prepared using all six drug classes as part of the training set before testing with the remaining data points. Table 3-8 outlines the prediction outcomes of the test data set. While the previous analyses indicated significant differences between these drug classes, and in particular between NBOMe and the other drug classes, there were samples that were incorrectly predicted as an NBOMe. In fact, in the test set, none of the amphetamines were correctly predicted. Similar to the results above, many of the methamphetamine and MDMA samples were predicted as an NBOMe. However, it was observed that the number of NBOMe samples that were incorrectly predicted in this model (false negatives) is much lower than that of the previous model (n=12). Though, in turn, there is a much larger proportion of false positives arising from the other drug classes. The accuracy of this model is reduced again to 57%.

Table 3-8: Model 3 prediction results

		True class					
		Blank	**NBOMe**	**2C**	**Meth**	**MDMA**	**Amphetamine**
Predicted class	**Blank**	23	1	10	4	3	2
	NBOMe	5	566	54	272	92	5
	2C	5	11	95	17	8	15
	Meth	0	0	0	0	0	0
	MDMA	0	0	0	0	0	0
	Amphetamine	0	0	0	0	0	0
Total		33	578	159	293	103	22
% correctly identified		69.7	97.9	59.7	0	0	0

Model 4 was created somewhat differently from the previous three. A training set containing only NBOMe analogues and blank samples was prepared by selecting half of the NBOMe and blank samples to ensure the set included a range of concentrations and various NBOMe analogues. The testing data was made up of the remaining blank and NBOMe samples, and also all data from the other four drugs. This was completed because it seems appropriate to model this method around the results which this colour test is validated to provide. It was hypothesised that this method may help clarify which data points were being predicted as false positives and why this was occurring.

It was found that 98% of NBOMe samples were correctly predicted with this model (Table 3-9). However, there was a high number of false positives attributed to the other drug classes. While they may not show a high similarity with the NBOMe samples, these values were still determined to be significantly different from the blanks as well. This has led to them being predicted to the class they might be more similar to, which with this model is the NBOMe class. Overall, this model has the lowest accuracy at 44% and would not be recommended for this analysis.

Table 3-9: Model 4 prediction results

		True class	
		NBOMe	**Other**
Predicted class	**NBOMe**	1063	1521
	Other	14	164
Total		1077	164

This predictive analysis of the RGB values has both advantages and disadvantages. This analysis is able to correctly predict NBOMe samples based on their RGB values to a high level of accuracy, and very few false negative results are reported. However, the inclusion of other drugs, especially those which react with this colour test, results in a high proportion of false positive outcomes that would pose issues with the application of this method. This method would benefit from the addition of further data points, including those of other drugs and also licit compounds that may be present in illicit drug samples. It would be useful to analyse substances which are commonly found on blotter papers such as LSD, to further determine the accuracy of such a method. Due to the difference in colour change seen with LSD it would not be expected that it would be incorrectly predicted as a NBOMe analogue. This type of testing could be applied to any colour test method to potentially improve the discrimination of colour change results. It would be interesting to investigate analysing the RGB values of a combination of colour test results for a single sample. This would provide more variables which could increase the sensitivity of this analysis.

3.4 Conclusions

The investigation of smartphone applications for the improved colour analysis of the NBOMe colour test has the potential for the objective detection of NBOMe analogues in seized material. ColorAssist and What a Color? are two applications that provide true to eye colour results compared to the values obtained with Colorimeter. The comparison with other drugs and compounds found that NBOMe analogues are distinguishable from tested illicit drugs and impurities based on the RGB values. They are also easily differentiated from negative controls at quantities of 50 µg or greater. It has been shown that incorporating a predictive model could play a role in assisting in the identification of NBOMe analogues using this colour test.

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Chapter 4: Translation of colour test methods to paper-based systems for the detection of NPS

Chapter 4: Translation of colour test methods to paper-based systems for the detection of NPS

4.1 Introduction

Traditionally, colour-based drug tests have been completed on porcelain spot plates, in test tubes or as part of a test kit in ampules. While this is commonly useful, the incorporation of a paper-based technique may be advantageous for a number of reasons [227]. White paper provides a simple yet strong contrasting background for colourimetric analysis to occur so that colour changes can be easily seen and interpreted [220]. Paper-based devices are low cost and may also remove the need for volumes of hazardous solvent solutions being used for on-site analysis [287].

Paper-based analytical devices (µPADs) have been widely used in many areas of medical diagnostics, analyte analysis, and point of care situations. These devices do not require any specialised instrumentation and rely on the capillary forces of paper, making them cost-effective and easy to use [288]. Colourimetry is widely regarded as one of the most compatible detection methods with these types of devices and can be easily used alongside simple reporting systems [289]. The interest and development of paper-based devices in forensic science has focussed on the detection of explosives [222, 224, 225]. Many of these methods are based on a simple colour change result or fluorescence detection and have a detector or sensor incorporated [223, 224]. The application of equivalent concepts to the analysis of drugs of abuse shows potential for similar devices to be developed. In recent years, the analysis of drugs of abuse with µPADs has become a major area of interest. Typically, this has been in relation to the more traditional illicit drugs [209, 221, 290], indicating the need for similar methods for the detection of new psychoactive substances (NPS). The application of multiple detection areas, with a number of reagents, has also been shown to provide a potential single device to detect a range of explosives or illicit drugs [223, 226, 230-232]. Performing tests simultaneously reduces the amount of sample and the time required to perform multiple tests while potentially providing a more discriminative result [221]. Such a device allows inexpensive determination of a variety of analytes with results visible to the naked eye. The addition of smartphone analysis or spectroscopic analysis could also be incorporated to further enhance the specificity and sensitivity of the methods.

This chapter focusses on four previously developed colour test methods for the detection of 25-NBOMe compounds, synthetic cathinones, piperazines, and fentanyl analogues. These methods are all part of research undertaken at UTS by several researchers. This chapter aims to translate these colour test methods, which have been developed as solution-based tests, to paper-based systems. The first test method for the analysis of 25-NBOMe compounds is outlined in detail in Chapter 2 of this thesis. The test methods for synthetic cathinones and piperazine analogues are detailed by Philp et al. [196, 197]. Piperazine analogues can be identified using a 1,2-naphthoquinone-4-sulphonate (NQS) reagent in an alkaline environment, giving an orange-red colour change. The detection of synthetic cathinones involves copper(II) nitrate, neocuproine, and a sodium acetate buffer solution which in the presence of cathinones exhibits an orange colour change result. The test method for the detection of fentanyl and its analogues is still in the optimisation stages and has not yet been published. It utilises a naphthoquinone reagent solution, which in the presence of an alkaline buffer provides an initial green colour change that over time becomes blue. The speed of this reaction to the blue colour can be increased with the addition of a catalyst and UV light exposure. The developed colour tests had been previously validated through the assessment of selectivity and specificity, precision, limit of detection, robustness, and common adulterant analysis. The paper-based systems underwent some of these validation parameters and were also assessed for their potential to be used in both field work and case work situations. It was of particular interest to assess if they had potential to be combined to create a multiplexed device to detect a variety of NPS. The integration of several tests within one device is beneficial for fast and simple detection of NPS without needing to complete multiple individual tests or to have knowledge of the potential nature of the unknown substance.

4.2 Phenethylamine colour test

The phenethylamine test for the detection of 25-NBOMe and 2C-X compounds has been described in detail in Chapter 2 of this thesis. Here, the translation of this test to a paper-based system from a solution test is investigated.

4.2.1 Materials and methods

4.2.1.1 *Chemicals and reference standards*

2,3,5,6-Tetrachloro-1,4-benzoquinone (TCBQ), acetaldehyde (ACD), (1*S*,2*R*)-(+)-ephedrine hydrochloride, protriptyline HCl, paracetamol, and Whatman® Grade 1 Qualitative filter paper were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). Ethyl acetate, analytical grade methanol and sodium hydroxide (NaOH) pellets were obtained from Chem-supply (Gillman, SA, Australia). Sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), aspirin and acetic acid (glacial) were obtained from Ajax Finechem (Scoresby, Victoria, Australia). Deionized water from a laboratory supply was used throughout the procedure.

Pure reference standards including 25H-NBOMe, 25E-NBOMe, 25D-NBOMe, 25G-NBOMe, 25T-NBOMe, 25I-NBOMe, 2C-E, 2C-I, BZP, MDMA and methamphetamine were obtained from the National Measurement Institute, Lindfield, NSW Australia. 25iP-NBOMe, 25N-NBOMe, 25P-NBOMe, 25T7-NBOMe, 25H-NB4OMe, and 25I-NB3OMe were purchased from Sapphire Bioscience (Redfern, NSW, Australia) as HCl salts. 30C-NBOMe and mescaline-NBOMe were purchased as HCl salts from Novachem (Heidelberg West, Victoria, Australia). Fentanyl was purchased as a powder standard from PM separations (Capalaba, Queensland, Australia). 4-MMC, MDPV, and JWH-073 were synthesised in-house by the Centre for Forensic Science. 25H-NBOMe (freebase) was synthesised following literature methods [51, 291, 292] and product confirmed by gas chromatography-mass spectrometry (GCMS), nuclear magnetic resonance spectroscopy (NMR) and high resolution-mass spectrometry (HRMS).

4.2.1.2 *Preparation of test reagents*

Reagent solutions including TCBQ in ethyl acetate and a phosphate buffer solution were prepared as previously described in Section 2.2.3. TCBQ solutions were prepared at concentrations of

3×10^{-3} and 6×10^{-3} M and the buffer solution was prepared at pH 11.4. Acetaldehyde was used as purchased and stored at 4 °C in the absence of light. Filter paper was cut into strips 40 mm x 5 mm with a tapered end to indicate which side of the strip would be added to the drug solutions.

4.2.1.3 *Initial paper testing*

TCBQ was applied to the paper strips using three different application methods for comparison. A TCBQ paste (TCBQ powder with a minimal amount of ethyl acetate added to form a paste like mixture) was pressed into the paper strip with a flat spatula to the top 5 mm of the strips. A 3×10^{-3} M solution of TCBQ was spotted, with a Pasteur pipette, onto half of the paper strip. Finally, the paper strips were soaked in the 3×10^{-3} M solution of TCBQ for several minutes. These strips were tested both immediately (while still wet) and after being dried out. The paper strip was added to a solution of ACD, buffer solution, and the drug. The solution travelled onto the paper strip to the end containing TCBQ and any reaction was recorded. Tests were conducted in vials or micro well plates throughout the study.

Paper strips were also soaked in a combined solution of TCBQ and ACD in equal volumes. Similarly, these strips were used immediately and after being dried before testing. In this case, the drug was combined with the buffer only, in the vial, before the paper strip was added. These tests were all initially carried out using synthesised 25H-NBOMe (0.5 mL, 1 mg/mL in methanol). The soaked and dried TCBQ strips were used to assess three other illicit drugs (2C-E, MDMA, and methamphetamine) which were tested for comparative purposes.

4.2.1.4 *Stability of TCBQ on paper*

Several storage conditions and preparation method combinations were prepared to assess which would provide the best results. Paper strips were soaked in a 3×10^{-3} M solution of TCBQ for several hours. The strips were removed from the solution and placed on watch glasses in the fume hood to dry. Once dry the soaked strips were stored in scintillation vials in the laboratory cupboard. Another set of strips were prepared with this same method, however, were separated and stored in different environments. Paper test strips were stored on the laboratory bench (RT), in the cupboard (RT), in the fridge (4 °C), and in the freezer (-18 °C). Within each of these locations, three vials of strips were prepared so they could be stored as an open vial, a closed vial (added while still wet) and in a small volume of the reagent solution (closed vial).

Testing was also carried out using a 6×10^{-3} M solution of TCBQ, twice that of the usual method concentration. The application and storage of these papers was completed in two ways similar to the above. First, papers stored in a small amount of solution (darkness, 4 °C) and second, 10 drops of solution added to each paper strip, allowed to dry, and stored in the same conditions. Over time, strips were removed from the vials, placed in a mixed solution containing 25-NBOMe, ACD, and the buffer solution and the colour change recorded within five minutes.

4.2.1.5 *Selectivity and specificity*

For this study, alongside the NBOMe analogues, a selection of compounds including cathinones, piperazines, other NPS and illicit drugs, as well as some licit compounds were analysed. For each compound, 100 µL of solution (1 mg/mL) was added to the test well and the solvent evaporated. Addition of the buffer solution and acetaldehyde to the well was followed by a dried test paper containing 10 drops TCBQ (6×10^{-3} M). Any colour changes were observed and recorded immediately and 5 min after addition of the paper.

4.2.1.6 *LOD study*

The limit of detection was determined in a similar manner to the colour test in solution. Aliquots of 25D-NBOMe were added across twelve wells increasing the volumes. The paper-based test was then carried out using dried papers containing ten drops of TCBQ (6×10^{-3} M) added to a combined solution of 100 µL of each buffer and ACD. As previously described in Section 2.3.5.2, the LOD was considered the lowest concentration still distinguishable from the reagent blank.

4.2.1.7 *Precision analysis*

To complement the variety of tests completed throughout this study, a repeatability study was prepared with fifteen 25H-NBOMe samples. Aliquots were added to test wells in a range of 90-120 µg per well, following the National Institute of Justice (NIJ) guidelines [267] for precision testing conducted at 1.5-2 times the LOD. The solvent was evaporated before addition of the buffer solution and acetaldehyde to each well. Papers containing 10 drops TCBQ (6×10^{-3} M) were dried before addition to each well. Colour changes were observed and recorded immediately and 5 min after addition.

4.2.1.8 *Investigation of a wax-printed paper system and the e-Beagle device*

The use of a wax printed paper system was investigated, and several parameters needed to be considered in order to determine the best application method, sequence and volumes of reagent, and sample for the application of this test. Whatman No. 1 filter paper test sheets with wells created by wax printing were provided by Natasha Benson (UTS) for initial testing. A number of sequences were trialled (Table 4-1), initially on regular filter paper followed by the wax printed wells. Reagent and sample volume tests and ratio testing were also completed. Initial testing of sequences was conducted on filter paper squares with 20 µL of each reagent and 20 µL of 1000 ppm 25H-NBOMe in acetonitrile. Smaller volumes (10 and 2 µL) were also tested.

Table 4-1: Overview of tested sequences for addition of reagents and drug onto paper.

Sequence No.	Order of addition [a]			
	1st	2nd	3rd	4th
1	Drug	TCBQ	Buffer	Acetaldehyde
2	Drug	TCBQ	Acetaldehyde	Buffer
3	Drug	Acetaldehyde	TCBQ	Buffer
4	Drug	Acetaldehyde	Buffer	TCBQ
5	Drug	Buffer	Acetaldehyde	TCBQ
6	Drug	Buffer	TCBQ	Acetaldehyde
7	TCBQ	Buffer	Acetaldehyde	Drug
8	TCBQ	Buffer	Drug	Acetaldehyde
9	TCBQ	Acetaldehyde	Drug	Buffer
10	TCBQ	Acetaldehyde	Buffer	Drug
11	TCBQ	Drug	Acetaldehyde	Buffer
12	TCBQ	Drug	Buffer	Acetaldehyde
13	Acetaldehyde	Buffer	TCBQ	Drug
14	Acetaldehyde	Buffer	Drug	TCBQ
15	Acetaldehyde	TCBQ	Drug	Buffer
16	Acetaldehyde	TCBQ	Buffer	Drug
17	Acetaldehyde	Drug	TCBQ	Buffer
18	Acetaldehyde	Drug	Buffer	TCBQ
19	Buffer	TCBQ	Acetaldehyde	Drug
20	Buffer	TCBQ	Drug	Acetaldehyde
21	Buffer	Drug	TCBQ	Acetaldehyde
22	Buffer	Drug	Acetaldehyde	TCBQ
23	Buffer	Acetaldehyde	TCBQ	Drug
24	Buffer	Acetaldehyde	Drug	TCBQ

[a] Drug: 25H-NBOMe solution (1 mg/mL), TCBQ: solution in ethyl acetate (3 x 10^{-3} M), Acetaldehyde, Buffer: phosphate buffer solution (pH 11.4)

The volume of the reagents and samples were decreased to avoid leakage outside the wax printed cells. Two volumes, 10 and 2 µL, were tested and compared in the same manner as the 20 µL volume tests described. Along with this, ratio tests were also conducted. Doubling the volume of the drug solution in comparison to the reagents was completed (Table 4-2). A mixed solution test was also carried out with the combination of 100 µL of each reagent (TCBQ, ACD, and buffer) and an aliquot of that mixture applied to two different volumes of drug solution on the paper.

Table 4-2: Volumes and ratios of reagents and 25H-NBOMe tested on paper

<table>
<tr><th rowspan="2"></th><th colspan="4">Solution [a]</th></tr>
<tr><th>25H-NBOMe</th><th>TCBQ</th><th>ACD</th><th>Buffer</th></tr>
<tr><td rowspan="8">Volume of solution added (µL)</td><td>20</td><td>20</td><td>20</td><td>20</td></tr>
<tr><td>10</td><td>10</td><td>10</td><td>10</td></tr>
<tr><td>2</td><td>2</td><td>2</td><td>2</td></tr>
<tr><td>20</td><td>10</td><td>10</td><td>10</td></tr>
<tr><td>40</td><td>20</td><td>20</td><td>20</td></tr>
<tr><td>20</td><td colspan="3" rowspan="2">100 each mixed and then 50 added to paper after drug</td></tr>
<tr><td>50</td></tr>
</table>

[a] 25H-NBOMe: solution in methanol (1 mg/mL), TCBQ: solution in ethyl acetate (3 x 10^{-3} M), ACD: Acetaldehyde, Buffer: phosphate buffer solution (pH 11.4)

A device known as the e-Beagle was designed and created at UTS for the analysis of explosives and related materials on µPADs. A prototype of this device and how it functions is outlined by Taudte et al [224]. The paper chips are wax printed with the option to have a simple microfluidic system or simple two round wells for the application of samples (see Figure 4-1). Explosive detection has been validated using the fluorescence quenching of pyrene, using UV light as the excitation source. The combination of UV and white light detectors can then indicate the presence of an explosive with a programmable signal on screen. The current device has the potential to be programmed for other analytes using colour or fluorescence information. Due to the potential of this device for colour detection, a study was conducted to investigate its potential use for improving the value of the TCBQ colour test method results.

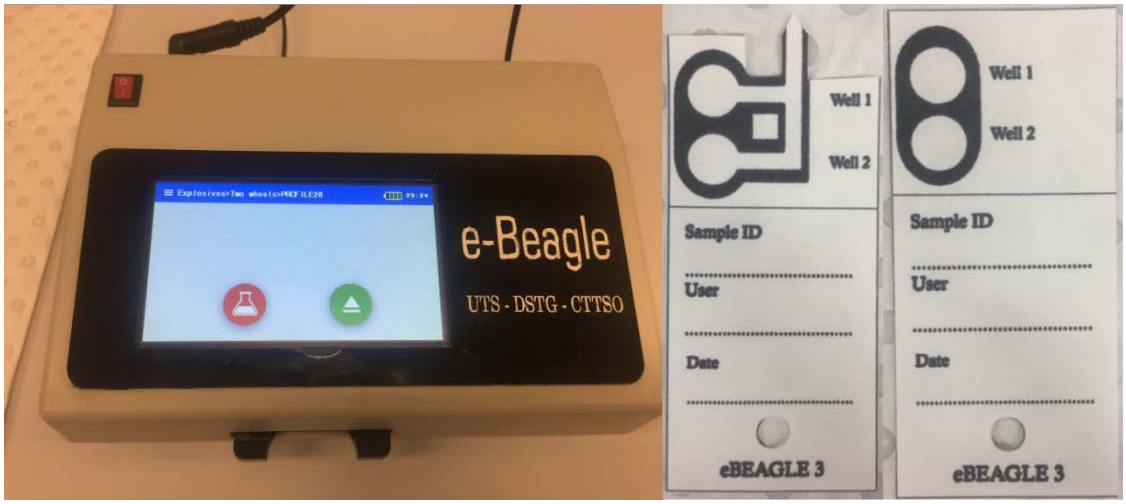

Figure 4-1: e-Beagle device (left) and the two PAD designs which are compatible with the system

The low volume tests described above were utilised for application to the wells for e-Beagle analysis. The colour test reagents and an NBOMe or blank sample were added directly into the wells of the paper chip. It was then inserted into the device and five data collections were completed on each sample. The system provides values for unfiltered, red, green, and blue light from both detectors. This data could then be analysed to determine if samples containing 25-NBOMe compounds could be identified and differentiated from reagent blanks. For the preliminary testing two NBOMe analogues and a range of negative control samples were analysed.

4.2.2 Results and discussion

4.2.2.1 *Initial paper testing*

All initial tests with papers containing TCBQ showed a colour change reaction with 25H-NBOMe. The TCBQ paste method provided a dark saturated green colour change rather than the blue colour changes usually seen for this test (see Figure 4-2). This is potentially due to a highly concentrated volume of TCBQ present at the reaction site and it being a bright yellow coloured powder. It was determined that this method of application was the least preferred.

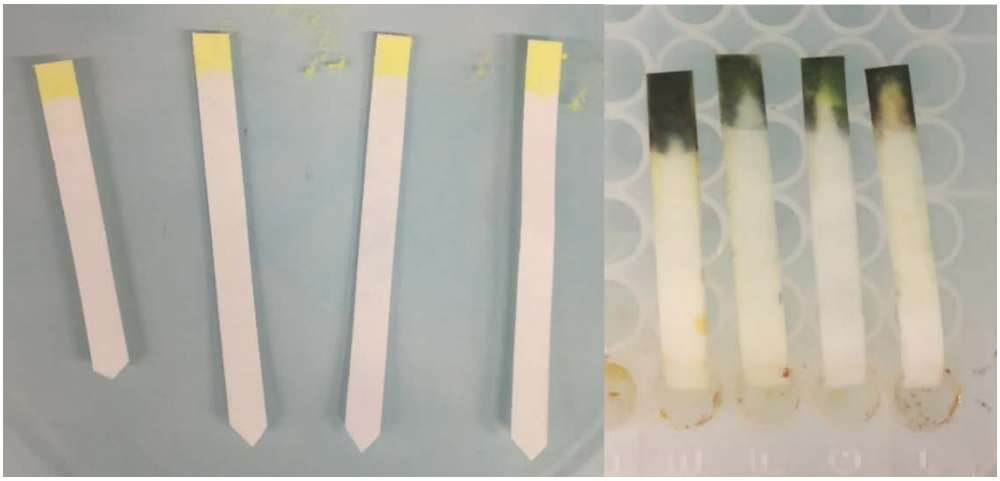

Figure 4-2: TCBQ paste applied to paper strips (left) and colour change seen after placed in reagent and drug solution (right)

The spotting and soaking methods of TCBQ addition, showed very comparable results to one another and to the positive colour expected from this test (see Figure 4-3). Once the drug and reagent solution reached the TCBQ it reacted to produce a blue colour change. It was noted that the blue coloured compound kept travelling up the paper strip via capillary action after reaction.

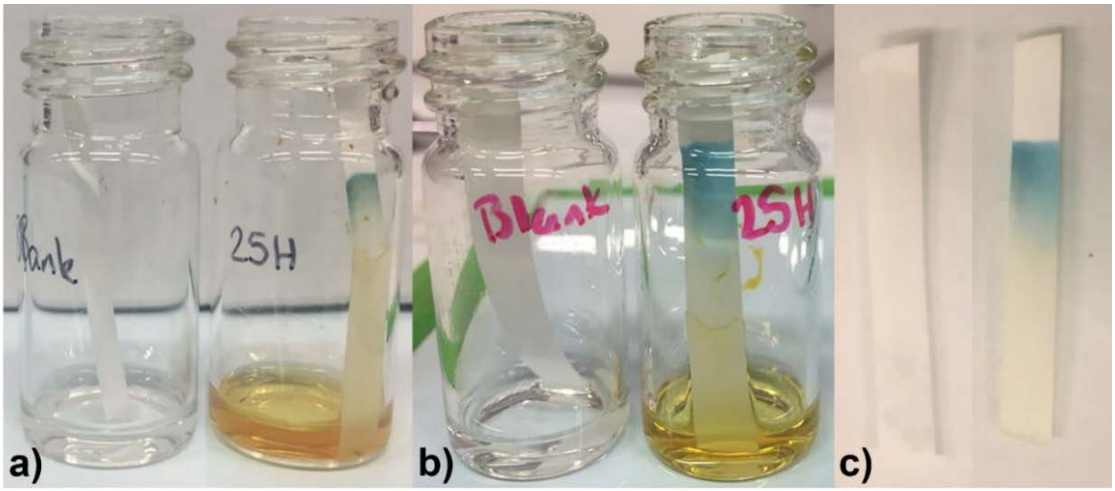

Figure 4-3: Resulting colour changes after papers were: a) soaked in TCBQ then tested immediately (left: blank, right: 25H-NBOMe), b) spotted with TCBQ and allowed to dry before testing (left: blank, right: 25H-NBOMe), and c) removed from test vial once colour change had occurred

The method completed with the TCBQ and acetaldehyde solution mixture did not show such promising results. The dry paper strips did not produce a colour change while the paper strips used immediately after being soaked produced a grey-blue colour change with some other discolouration on the paper (see Figure 4-4). The lack of bright blue colour changes with this method can likely be attributed to the volatility of the acetaldehyde. Particularly in the case of the dried papers, the acetaldehyde had likely evaporated from the paper leaving little to none remaining for the reaction with 25H-NBOMe.

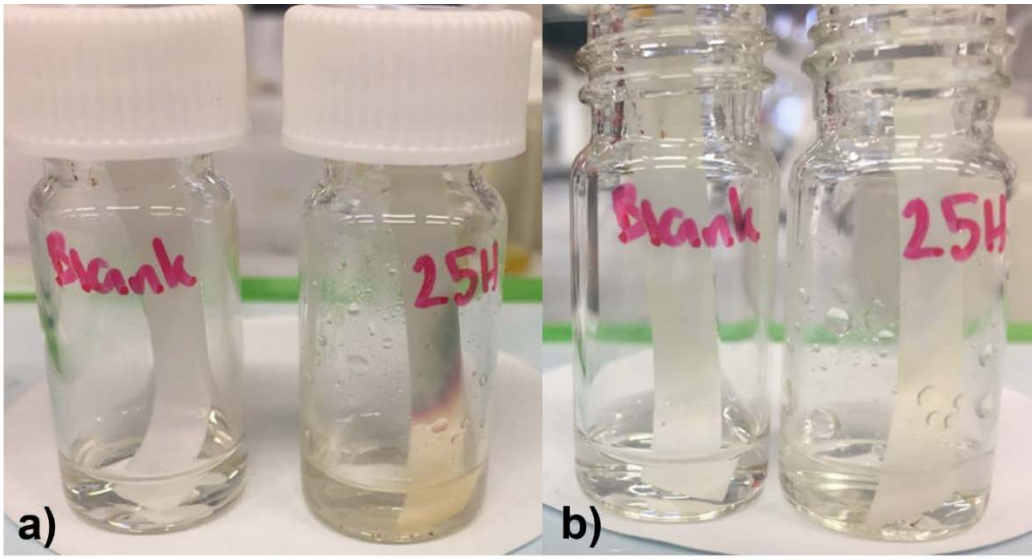

Figure 4-4: Resulting colour changes after paper was soaked in TCBQ and acetaldehyde then: a) tested immediately (left: blank, right: 25H-NBOMe) and b) allowed to dry before testing (left: blank, right: 25H-NBOMe)

These results indicate that the most appropriate method for a paper system is the addition of TCBQ solution, either via spotting or soaking of the papers. This method correlates well to the results seen previously from stability studies of combined reagent solutions (see Section 2.3.5.7). This work found that the most stable and most suitable mixed solution for use was an acetaldehyde and buffer solution. If the TCBQ is added through the use of a paper strip, it would remove the need for all three reagent solutions to be mixed for this test to be completed. This would provide a simple test kit design for this colour test to be performed in the field.

The tests with the three other illicit drugs (2C-E, MDMA, and methamphetamine) provided interesting results. While the same amount of each drug (0.5 mL, 1 mg/mL) was added to the solutions only the 25H-NBOMe produced a colour change (see Figure 4-5). The other drugs tested produced colour changes under traditional colour test conditions however, within this paper-based system provided no colour change. The paper-based system introduces new conditions to the test procedure and these conditions may influence the specificity and efficiency of the test. These conditions include the localised site of reaction on the test strip (concentrates the drug and reagents) and also the low volumes of reagents used in the paper-based method. The paper itself may also influence the reaction for several reasons. The polar nature of the cellulosic paper due to -OH groups may act as a barrier to the drug of interest. The interconnected microstructure of the cellulosic fibres in the paper could affect the mixing and transport of reactants during the testing process.

It would be beneficial to assess the causes of the lack of colour change with these drugs and the use of UV-vis spectroscopy for confirmation that a reaction is occurring would be of interest. This is a potentially advantageous result for the detection of 25-NBOMe compounds if colour changes with other drugs are not seen with this type of analysis. During this test, it was observed that the blue colour reacted first on the paper then dissipated into the solution in the vial. This would not pose significant issues as the colour change seen is still the required colour for a positive result.

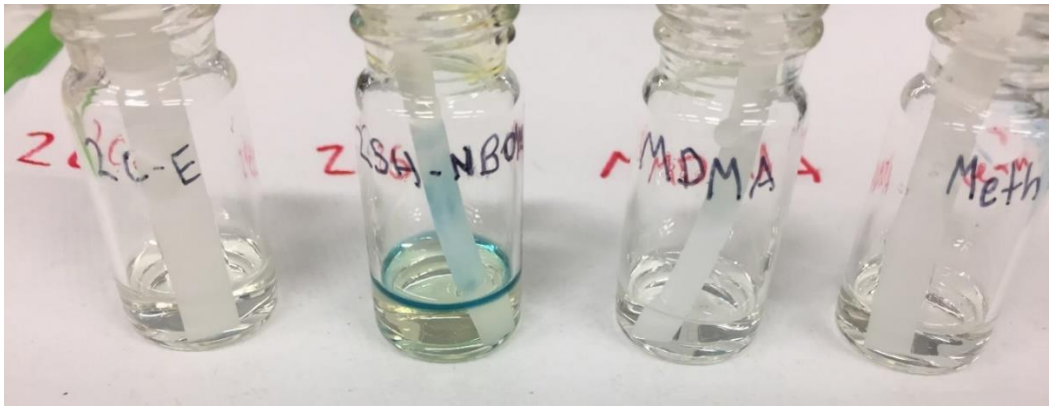

Figure 4-5: Drug testing results with 2C-E, 25H-NBOMe, MDMA and Methamphetamine using TCBQ soaked and dried paper strips.

4.2.2.2 *Stability of TCBQ on paper*

The stored, soaked papers were tested with a 25-NBOMe, ACD, and buffer solution alongside a blank. However, the papers did not produce a positive result after only one day of storage. It was hypothesised that there may not be enough TCBQ staying on the paper for the colour change to occur or to visibly see that colour change. This was the main reason that the concentration of the TCBQ solution was increased, and further tests were completed with the 6×10^{-3} M TCBQ solution. To ensure the lack of colour change was a consequence of the TCBQ and not another reagent, several drops of TCBQ solution were added to the reaction vial after the paper test showed no colour change. This resulted in a positive colour change within minutes that was not previously seen (see Figure 4-6).

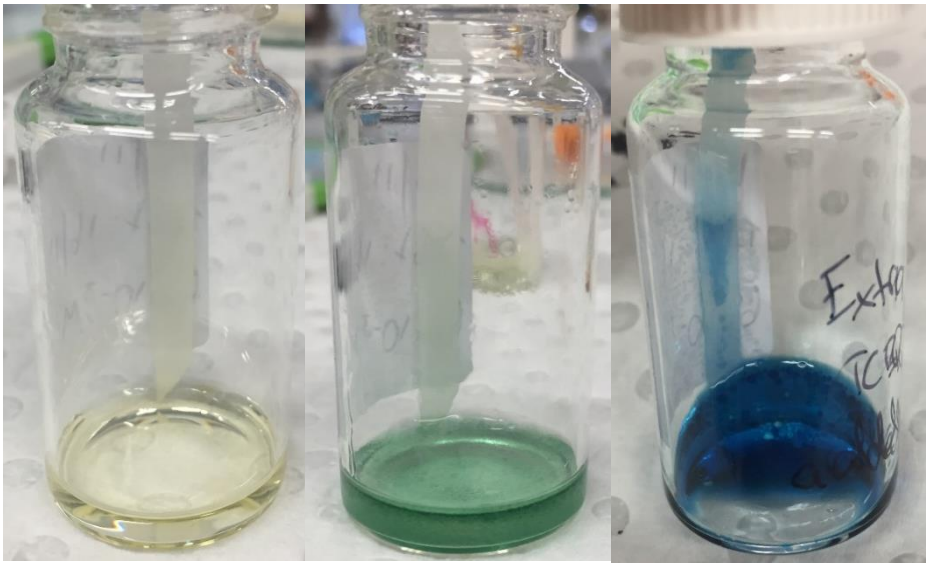

Figure 4-6: Colour development over time and addition of extra TCBQ. Left to right: initial test with TCBQ solution soaked paper, immediately after extra TCBQ was added and several minutes after extra TCBQ was added.

Another explanation for this lack in colour change after storage is that the TCBQ may require an amount of solvent to be present for the reaction to take place. Though, this theory is not supported by the results with the TCBQ paste, as those tests did show a colour change reaction, with the greener colour attributed to the large amount of yellow powder (TCBQ) present on the paper. TCBQ is a light sensitive compound, and this may also play some part in the instability on paper. Storage of paper strips in a solution and also in cold, dark conditions may be more beneficial for the success of this method even though this limits the portability of such a device.

The storage of the paper strips in varying environments produced a range of results. Generally, those stored in closed vials and in darkness showed reasonable storage times of up to a few weeks (Table 4-3). Storage in a small volume of the reagent solution appears to be the best choice for this test method however does pose issues in terms of practicality.

Table 4-3: Results from stability of TCBQ on paper study displayed as positive (+) or negative (-)

Day	Bench [a]			Cupboard [a]		
	Open	**Closed**	**Solution**	**Open**	**Closed**	**Solution**
1	+	+	+	-	+	+
3	-	+	+	-	+	+
9	-	+	+	-	+	+
18	-	-	-	-	+	+
25	-	-	-	-	-	+
Day	**Fridge [a]**			**Freezer [a]**		
	Open	**Closed**	**Solution**	**Open**	**Closed**	**Solution**
1	-	+	+	+	+	+
3	-	+	+	-	+	+
9	-	+	+	-	+	+
18	-	+	+	-	+	+
25	-	-	+	-	+	+

[a] *Open = papers dried and stored in an open vial; Closed = papers dried slightly (<5 min) and stored in a closed vial; Solution = papers stored in a small amount of TCBQ reagent solution*

The use of the increased concentration of TCBQ (6×10^{-3} M) did not increase the possible storage time of this reagent on paper. It was observed, however, that the papers containing 10 drops of solution could be dried before storage and a positive result could still be seen after three weeks of storage. Figure 4-7a) shows an example of the colour change after two weeks of storage. Tests were still run after this time and it was observed that when papers were left in the test solution for up to 24 h after addition, discolouration of the test paper was seen (see Figure 4-7b) and d)).

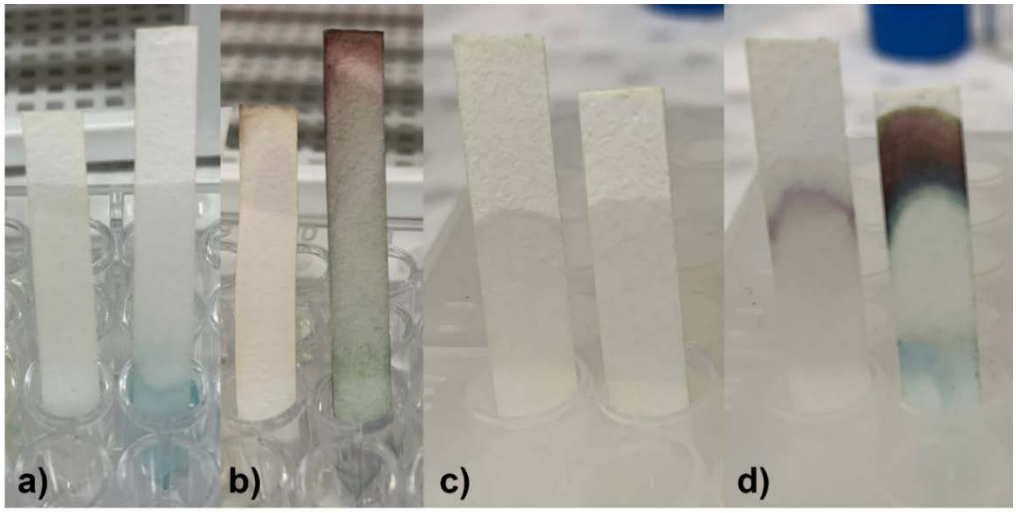

Figure 4-7: Resulting colour changes of blank and 25D-NBOMe after storage of papers containing 10 drops TCBQ (6×10^{-3} M) after: a) 15 days, 2 min after addition and b) 24 hr after addition, c) 6 weeks, 2 min after addition and d) 2 hr after addition

4.2.2.3 *Recommended test method*

A recommended method for the detection of 25-NBOMe analogues was determined based on tests completed to this point. This method was then used for all further testing procedures and is as follows.

To prepare test paper strips, add to a 4 cm x 5 mm strip of Whatman No. 1 filter paper:

1. 10 drops TCBQ solution (6×10^{-3} M in ethyl acetate)
2. Allow to dry before testing or storage*

To test an unknown substance:

3. Add 100 µL PO4 buffer to unknown substance, either in powder or liquid form
4. Add 100 µL acetaldehyde
5. Add prepared test paper strip to the solution
6. Record colour change immediately and after 5 min

*store prepared paper strips for no longer than 3 weeks (darkness, 4 °C)

4.2.2.4 *Selectivity and specificity*

The range of compounds tested as part of the selectivity study showed no unexpected results (see Table 4-4). Based on the results of this test in solution, there were some illicit drugs which provided a pale blue colour change with the paper-based method. No further colour reactions were noted for any of the tested compounds. This test method has shown to remain highly selective when transferred to a paper system.

Table 4-4: Colour change results from selectivity testing of the TCBQ reagent test

Compound	**Colour change [a]**
25D-NBOMe	blue
25E-NBOMe	blue
25H-NBOMe	blue
25H-NB4OMe	blue
25I-NB3OMe	lt. blue
25iP-NBOMe	blue
25N-NBOMe	green-blue
25P-NBOMe	blue
25T-NBOMe	lt. blue
25T7-NBOMe	lt. blue
30C-NBOMe	blue
Mescaline-NBOMe	green-blue
2C-E	p. green
2C-I	p. green
Methamphetamine	p. blue
MDMA	NC
Amphetamine	NC
BZP	NC
TFMPP	p. blue
4-MMC	p. blue
MDPV	NC
Fentanyl	NC
JWH-073	NC
Ephedrine	NC
Paracetamol	NC
Aspirin	NC
Protriptyline	p. purple-blue

[a] NC: no colour change observed; p. = pale, lt. = light

4.2.2.5 *Limit of detection study*

The LOD for this test on paper was determined to be 60 µg of 25D-NBOMe (see Figure 4-8i)). About an hour after the test, pale blue colour changes were seen on the edges of some lower concentrations (30-50 µg) however, these would not be considered a true positive result as they did not show an immediate colour change. The LOD value determined here is close to three times the LOD determined for this test in solution (see Section 2.3.5.2). The value of 60 appears quite low and would often be appropriate for the detection of NBOMe samples that are commonly found in amounts between 50-250 µg per dose. However, once the operational LOD is considered, this value becomes greater than the reported average dose amount now at 600 µg.

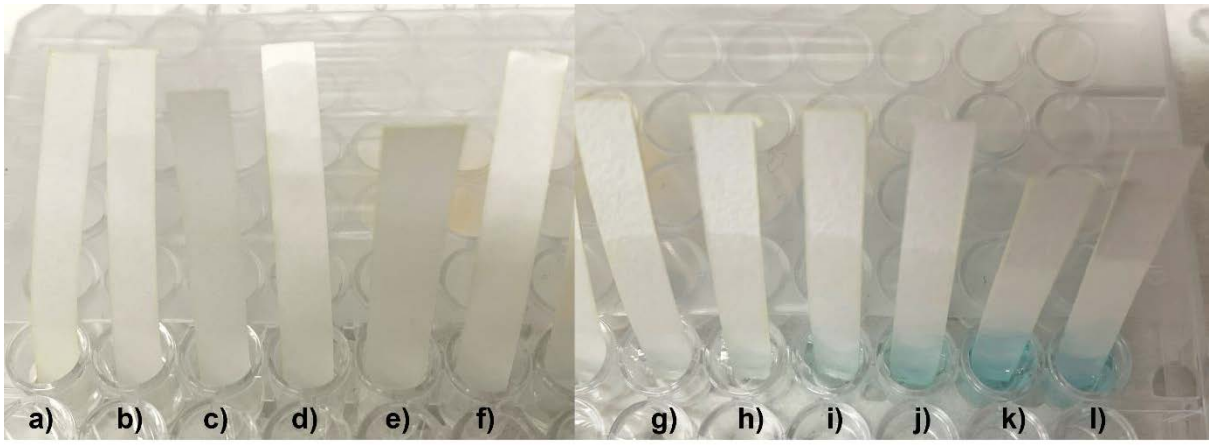

Figure 4-8: TCBQ paper-based LOD colour change results: a) reagent blank, b) 5 µg, c) 10 µg, d) 15 µg, e) 20 µg, f) 30 µg, g) 40 µg, h) 50 µg, i) 60 µg, j) 75 µg, k) 100 µg, l) 150 µg 25D-NBOMe

4.2.2.6 *Precision analysis*

Of the fifteen 25H-NBOMe tests completed, all but one showed a positive blue colour change within five minutes of addition of the test paper. This test provided a very pale green-blue colour change which would not be indicative of a positive result but may allow for identification of the presence of a secondary amine. These similar colour changes were observed for NBOMe samples in over 90% of tests indicating a high level of precision for this test.

4.2.2.7 *Investigation of a wax-printed paper system and the e-Beagle device*

The colour change results of the sequence testing with 20 µL of the drug and reagent solutions on filter paper can be seen in Table 4-5 and some examples of the colours seen in Figure 4-9. These results indicate that several sequence orders are suitable for this testing method, as shown in Table 4-5. The 2:1 ratio of drug and reagent volumes produced similar results to that seen with the 1:1 test at 20 and 10 µL. The mixed solution tests showed a blue-green colour change, rather than the expected bright blue colour, for both volumes.

Table 4-5: Colour change results for sequence testing (sequences outline in Table 4-1)

Sequence	Colour change [a]	Sequence	Colour change [a]
1	grey-blue	13	p. blue
2	blue	14	p. blue
3	yellow/blue	15	green-blue
4	brown	16	green-blue
5	blue	17	blue-green
6	brown/blue	18	NC
7	blue-green	19	yellow
8	brown/green	20	grey-blue
9	green-blue	21	blue
10	blue-green	22	grey-blue
11	brown/blue	23	p. blue
12	brown/blue	24	lt. blue

[a] NC: no colour change observed; p. = pale, lt. = light

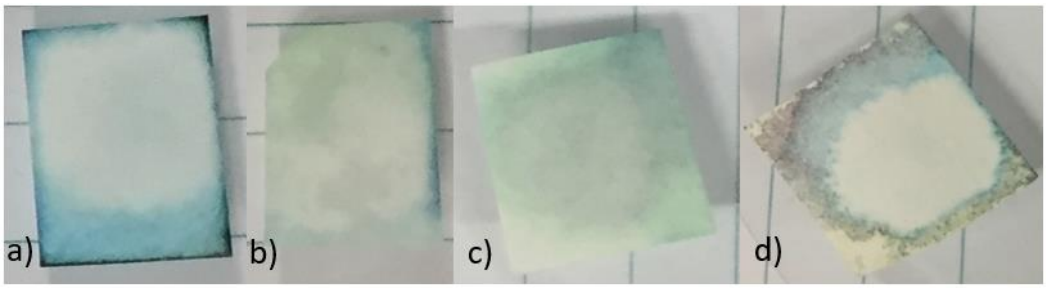

Figure 4-9: Sequence test results examples. a) Sequence 2, b) sequence 9, c) sequence 16, d) sequence 22

The 2 µL tests were completed as a small-scale test on the wax printed test sheets, as the previous volumes were too great. The wax printed wells can hold only very small volumes (up to 10 µL) of solution without leaking outside the barriers. Unfortunately, no visible colour changes were seen for these tests most likely due to the extremely small amounts of the solutions being present and the amount of drug being significantly below the determined limit of detection.

Initial tests showed that most samples could be differentiated from one another. However, 25H-NBOMe samples did not show any significant difference from both methanol controls and the TCBQ control tests. On the other hand, it was noted that between the NBOMe samples there were some differences seen. This could pose challenges for the identification of the NBOMe class but may potentially indicate that a light detection method may help differentiate between the NBOMe analogues themselves. The results from the UV detector provided even more variability and inconsistent results with several NBOMe tests only showing differences with the controls on some occasions. Table 4-6 outlines the number of p-values <0.05 for both the white and UV detectors which indicate a difference is present.

The increase in sample size also increased the amount of variation between tests. Differences were not identified between reagent blanks and many of the NBOMe analogues. This may be due to the low quantity of drug present in each test, a value well below the limit of detection for this colour test. It appears the analysis using this device requires a substantial amount of optimisation with these reagents and investigation into the use of a fluorescent detection source may be more beneficial than a simple colour change. While this device did show promise to be used in conjunction with this type of testing, further analysis was not completed as part of this thesis.

Table 4-6: Number of p-values <0.05, indicating significant differences between pairs of solutions analysed with e-Beagle

		Test completed [a]								
	White [b]	25H	25E	25H mix	25E mix	MeOH	MeOH & TCBQ	TCBQ	water	reagent
Test completed [a]	25E	0								
	25H mix	1	4							
	25E mix	0	0	4						
	MeOH	0	4	4	4					
	MeOH & TCBQ	4	4	4	4	4				
	TCBQ	0	4	4	4	4	4			
	Water	4	4	4	4	4	4	4		
	Reagent	4	4	4	4	4	4	4	4	
	mix reagent	4	4	4	4	4	4	4	4	0
		Test completed [a]								
	UV [c]	25H	25E	25H mix	25E mix	MeOH	MeOH & TCBQ	TCBQ	water	reagent
Test completed [a]	25E	2								
	25H mix	1	3							
	25E mix	4	4	4						
	MeOH	4	4	3	4					
	MeOH & TCBQ	4	3	2	4	4				
	TCBQ	4	4	4	0	4	4			
	water	2	4	2	4	4	4	4		
	reagent	3	2	3	4	4	1	4	4	
	mix reagent	4	3	2	4	4	4	4	4	2

[a] *25H = 25H-NBOMe reaction with all reagents added separately; 25H mix = 25H-NBOMe reaction with reagents combined prior to addition to paper; 25E = 25E-NBOMe reaction with all reagents added separately; 25E mix = 25E-NBOMe reaction with reagents combined prior to addition to paper; Reagent = all reagents added separately; Mix reagent = reagents combined prior to addition to paper*

[b] *White: results from white light detector*

[c] *UV: results from UV detector*

4.2.3 Conclusion

The TCBQ method has shown to be appropriate for use as a paper-based system. While the short stability time and higher LOD are limitations of this method, the precision and selectivity are equally as good as the test in solution.

4.3 Piperazine colour test

The developed method for the detection of piperazine analogues utilises the reaction with 1,2-naphthoquinone-4-sulphonate (NQS) in an alkaline environment [196]. A positive reaction is identified by the appearance of a red-orange colour change. This test also provides positive results with the presence of the surfactant triton X-100, however, this is not required when the test is carried out in solution. It has no significant effect on the final colour change result with the piperazine compounds when performed in solution.

4.3.1 Materials and methods

4.3.1.1 *Chemicals and reference standards*

Sodium 1,2-naphthoquinone-4-sulphonate (NQS) and triton-X-100 were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). Sodium hydrogen carbonate ($NaHCO_3$) was obtained from Chem-Supply (Gillman, SA, Australia). Pure reference standards 1-benzylpiperazine dihydrochloride (BZP), 1-(3-trifluoromethylphenyl)piperazine hydrochloride (TFMPP) and 1-(3-chlorophenyl)piperazine hydrochloride (mCPP), 2C-I HCl, 2C-B HCl, 25H-NBOMe HCl, cocaine HCl (±)-*N*-methyl-3,4-methylenedioxyamphetamine HCl (MDMA), (+)-*S*-methamphetamine HCl and d-amphetamine sulphate were obtained from National Measurement Institute (NMI, North Ryde, NSW, Australia). 4-Methyl-*N*-ethylcathinone (4-MEC) HCl was synthesised in-house by the Centre for Forensic Science. (1*S*,2*R*)-(+)-ephedrine HCL, (1*S*,2*S*)-(+)-pseudoephedrine HCl, paracetamol, cellulose, methylamine HCl, glycine and magnesium stearate were obtained from Sigma Aldrich Pty Ltd (Castle Hill, NSW, Australia). Aspirin, phenolphthalein, urea, and citric acid were obtained from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia). L-ascorbic acid was from VWR chemicals (Campbellfield, Vic, Australia).

4.3.1.2 *Preparation of test reagents*

A solution of 1,2-naphthoquinone-4-sulphonate was prepared to 6.4×10^{-3} M in deionised water. The carbonate buffer was prepared by adjusting the pH of a 0.1 M sodium hydrogen carbonate ($NaHCO_3$) solution to 10.8 with the addition of NaOH (5 M). A 0.1 % v/v solution of triton-X-100 was prepared in deionised water. Solutions of BZP, mCPP, and TFMPP (1 mg/mL) were prepared in methanol.

4.3.1.3 *Initial testing – recommended method in solution*

Tests were initially carried out in spot plates to provide a reference colour change and to also ensure all reagents were working appropriately. These tests were repeated regularly to test the stability of the reagent solutions as it has been previously identified that the NQS solution is no longer suitable for use after one week.

A blank test and three replicates at different amounts of BZP solution were tested on a ceramic spot plate. Reagents were added in the volumes provided in the general recommended method; 5 drops buffer solution, 4 drops NQS solution and colour change observed immediately and after 10 minutes. To assess the compatibility of a mixed reagent, equal volumes of the two reagents were combined and stored in 3 environments: lab bench, laboratory cupboard, and the laboratory fridge (4 °C). These were then tested with BZP across several days and the results compared to freshly prepared reagent solutions.

4.3.1.4 *Initial paper testing*

This test was applied directly to small filter papers to ensure all reagents were appropriate for application to paper. To a 2 cm filter paper, 1 drop of the drug solution (methanol for blank), NQS solution, and buffer solution were added. The results were recorded immediately and after 10 minutes.

4.3.1.5 *Storage and stability of reagents on paper*

Paper strips were cut to size 40 mm × 5 mm with a tapered end. Two sets of these strips were prepared to contain either NQS or the buffer solution. These were allowed to dry slightly (~10-20 min) before being stored in the fridge. These were tested with methanol blank and BZP periodically.

Three sets of paper strips were prepared differently and stored in sealed tubes in a laboratory cupboard for comparison to one another and to freshly prepared paper strips. Along with this, another set was prepared with 1 drop of each of the buffer and NQS solutions and stored in open tubes at 4 °C. The following list outlines how these sets of paper strips were prepared.

1. 1 drop each buffer & NQS next to one another (A-J)
2. 1 drop each buffer & NQS on the same spot (K-T)
3. 5 drops each buffer & NQS added to test tube, the paper added (U-CC)
4. 1 drop each buffer & NQS next to one another – stored in an open tube in fridge and darkness (DD-LL)
5. 1 drop each buffer & NQS next to one another prepared fresh before testing (MM-VV)

4.3.1.6 *Investigation of reagent conditions*

Different volumes and ratios were tested on the paper strips to determine if there was a particular amount most ideal for undertaking this method. In the published method, a 5:4 ratio of buffer to NQS was recommended so this was the starting ratio on paper. Simply 5 and 4 µL of these solutions were added to the paper respectively. Other ratios were also tested in the same way including 10:8, 5:5, 6:4, 3:4, 7:3, 4:3, 3:3, and 3:2.

Paper strips took considerable time to dry before testing and so new NQS solutions were prepared in 3 organic solvents to compare results to the solution in water. Ethyl acetate, methanol, and ethanol were chosen for this study due to their lower toxicity in relation to many solvents but increased volatility in comparison to water.

4.3.1.7 *Re-addition of triton-X-100 and its effect on stability*

Triton-X-100 was removed from the originally published method as it did not significantly affect colour change results. This study investigated the re-addition of the solution and its effect on the stability of the reagents on paper. Two sets of paper strips were prepared, one containing the buffer, NQS, and triton-X-100 solutions and the second containing only NQS and triton-X-100 solutions. Equal parts of each of the reagents were added to the paper and stored in sealed vials in darkness at 4 °C. These paper strips were tested periodically on blank and BZP samples. The papers containing only NQS, and triton-X-100 were added to BZP in the buffer solution (100 µL, 1 mg/mL) or buffer solution alone (blank).

4.3.1.8 *Limit of detection study*

The limit of detection (LOD) for the original method was determined to be 4 µg giving an operational limit of detection of 40 µg. To determine the limit of detection for the paper-based test, these values were considered and a range of BZP aliquots were added to wells in

concentrations from 0-40 µg. Paper strips were prepared with all three reagents applied and added to the wells containing BZP in the concentration range. Another set of LOD tests were conducted using paper strips containing only NQS and triton-X-100 with the drug dissolved in the buffer solution. A second set of papers was prepared with NQS and triton-X-100 to be added to BZP in the buffer solution after storage. The LOD was determined when the colour change on the paper was no longer considered positive in comparison to the reagent blank.

4.3.1.9 *Precision study*

To assess the precision of this method, two sets containing 10 test papers were prepared to test with BZP at 1.25 to 2x LOD as per the colour test guidelines from the NIJ [267]. One set was used after being freshly prepared while the other set was stored in darkness at 4 °C for two weeks prior to testing. Each set was tested following the same method, 100 µL CO3 buffer added to the drug followed by the test paper. Colour changes were observed and recorded immediately and after 5 min.

4.3.1.10 *Selectivity study*

Analogues of piperazines, other illicit drugs, precursors, and licit compounds were tested with the prepared NQS and triton X-100 papers, with the addition of the buffer to the analyte solution. Tests were completed on 100 µL analyte solution (1 mg/mL) with both freshly prepared and stored test papers in duplicate. Colour changes were observed and recorded within five minutes of addition.

4.3.1.11 *Blind testing*

A second analyst prepared 12 samples for analysis containing an unknown combination of drug solutions and methanol blanks. For each sample, 100 µL of the drug solution (1 mg/mL) or methanol was added to a well and the solvent allowed to evaporate. The method was then carried out as per usual, with the addition of the carbonate buffer to the well and using papers prepared with NQS and Triton X-100 earlier that day. Colour results were recorded, and an interpretation of each result completed. The possible interpretations included positive for piperazine (+), positive for an amine (+/-) or negative (-). This interpretation system had been used previously for the blind tests completed with the TCBQ method outlined in Section 2.3.5.5.

4.3.2 Results and discussion

4.3.2.1 *Initial testing – recommended method in solution*

The expected orange-red colour change was seen with BZP when the colour test reagents were added sequentially (Figure 4-10). As for the mixed reagent solution, after less than a week of storage, it was clear this would not be an appropriate method for use of this test. The colour changes were less vibrant, and the blank samples also provided colours very similar to the tests containing BZP (see Table 4-7).

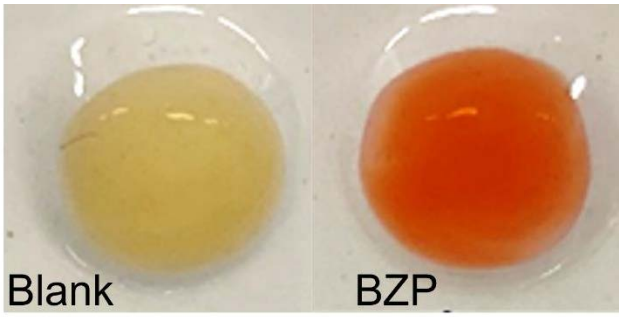

Figure 4-10: Colour change results seen from NQS test method: reagent blank (left) and BZP (right)

Table 4-7: Colour change results of mixed reagent solutions after storage

	Sample	Bench	Cupboard	Fridge	Unmixed fresh
Day 2	Blank	yellow-brown	brown	brown	yellow
	BZP	yellow-brown	brown	red-brown	orange
Day 6	Blank	yellow-brown	yellow-brown	orange-brown	orange-yellow
	BZP	yellow-brown	yellow-brown	orange-brown	orange-red

4.3.2.2 *Initial paper tests*

Upon addition to the filter paper, the colour change seen was lighter and less bright than what is seen on a spot plate or in a test tube, see Figure 4-11**Error! Reference source not found.**. This is likely due to the smaller amounts of both drug and reagents added to the paper. It was therefore concluded that for this test carried out on paper, an orange colour change will be considered positive for piperazines.

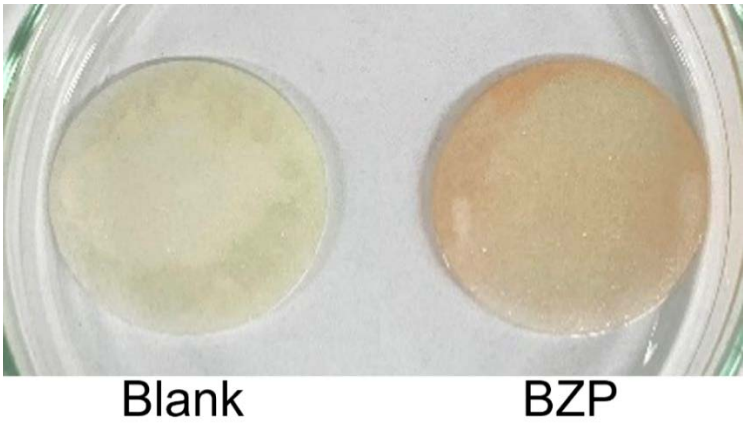

Figure 4-11: Colour change after application of the test reagents to paper with reagent blank (left) and BZP (right)

4.3.2.3 *Storage and stability of reagents on paper*

The stability of NQS on paper was only successful for around 24 h after application even with storage in darkness at 4 °C. This factor would hinder the possibility of this test method having the applicability to on-site testing and incorporation into a multiplex device, as paper strips would need to be prepared in advance and not the day that testing would occur. The buffer could be stored on paper with NQS added just prior to testing with BZP and a positive colour change seen. While this reduces the number of solutions required for testing, it does not assist in the prolonged shelf life of the NQS solution.

Table 4-8 outlines the colour change results over the several weeks these papers were stored. It was seen that upon storage of the combined reagent paper strips in a dark but not cold environment that they would no longer provide positive results after any amount of storage. Thus, storage in darkness at 4 °C, the recommended conditions for NQS, provided inconsistent results across the storage period and so would not be recommended for a paper-based system. The freshly prepared paper strips provided good results, giving an orange colour change consistently. It was here that an alternative solution for the storage of these paper strips was required, and the re-addition of triton-X-100 considered. Results for this study are outlined in Section 4.3.2.5.

Table 4-8: Colour change results of combined reagent paper strips storage and comparison to freshly prepared paper strips

	Colour change results [a]									
Set	**(1)**		**(2)**		**(3)**		**(4)**		**(5)**	
Day 1	A	yellow-grey	K	lt. brown	U	lt. brown	DD	brown-orange	MM	orange
Day 2	B	lt. grey	L	lt. brown-yellow	V	lt. brown-yellow	EE	NC	NN	orange
Day 3	C	grey	M	lt. yellow	W	brown-yellow	FF	lt. brown	OO	orange
Day 4	D	grey	N	p. yellow	X	p. brown-yellow	GG	Brown	PP	orange
Day 7	E	grey	O	yellow	Y	brown-yellow	HH	lt. orange-brown	QQ	orange
Day 8	F	p. orange-grey	P	lt. orange-brown	Z	yellow-brown	II	orange/ grey	RR	orange
Day 9	G	grey	Q	yellow	AA	yellow-brown	JJ	grey/ yellow-brown	SS	orange
Day 10	H	lt. orange/ grey	R	lt. orange-yellow	BB	brown-yellow	KK	brown/ grey	TT	orange
Day 14	I	p. orange/ grey	S	lt. brown	CC	lt. brown-yellow	LL	brown-yellow	UU	orange
Day 18	J	yellow/ grey	T	lt. brown-yellow	-	-	-	-	VV	orange

[a] *lt: light; p. = pale; NC = no colour change observed; (-) = not completed*

4.3.2.4 *Investigation of reagent conditions*

The colour change results did not appear to differ greatly with the volume ratio changes of the reagents. It was noted that 10 µl appeared to be too much liquid from only 1 reagent and 3 µl was the best volume for one reagent regarding application. Here the solution did not spread too much and could leave space on the paper for the second reagent if they could not be mixed. It was seen, however, that this may not have been enough reagent to get a clear result all the time. The chosen volume was 5 µl of each reagent and this volume was used for all further testing.

NQS was not soluble in ethyl acetate and was only slightly soluble in ethanol. It appeared to be soluble in methanol however this was only after substantial shaking of the solution to promote dissolution. Nonetheless, all three solutions were added to paper strips before testing with BZP,

those less soluble ensuring some particles were added along with the solvent. Water or methanol appeared to be the best options for NQS to be applied to the paper based on solubility and the final colour change results (see Figure 4-12). Further tests continued with water as the solvent for simplicity.

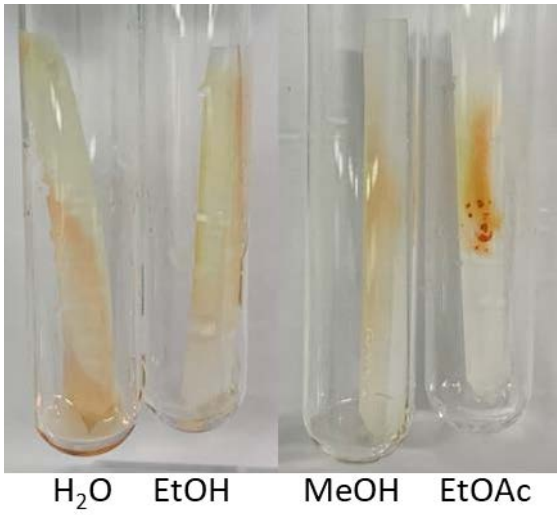

Figure 4-12: Comparison of solvents used for the preparation of NQS solution

4.3.2.5 *Re-addition of triton-X-100 to paper test and effect on stability*

After a lack of positive results from the previous stability study, the re-addition of triton-X-100 showed some success. Initial testing of the paper strips containing only NQS and triton-X-100 were then applied to BZP in the buffer solution, and showed positive colour changes after almost a week of storage. The same was not seen for the strips which also contained the buffer solution, with colour changes no longer seen after two days (see Table 4-9). These results led to the preparation of a much larger storage study to test the stability over a longer period. Across the course of this study, an orange colour change was still seen with these paper strips when reacting with BZP up to 10 weeks after preparation. Storage longer than this time has not been investigated here, though there were no signs that degradation of the reagents was occurring across the testing period and so it is hypothesised that this storage time may be increased with no impact on the colour changes seen.

Table 4-9: Colour change results after storage of paper strips with the re-addition of triton-X-100, up to 70 days after preparation

	Day	Sample	Combination	
			NQS & triton X-100	NQS, triton X-100 & buffer [b]
Colour change results [a]	1	Blank	NC	NC
		BZP	orange	p. yellow-orange
	2	Blank	NC	NC
		BZP	orange	p. yellow
	5	Blank	NC	NC
		BZP	orange	NC
	13	Blank	NC	-
		BZP	orange	-
	20	Blank	NC	-
		BZP	orange	-
	24	Blank	NC	-
		BZP	orange	-
	27	Blank	NC	-
		BZP	orange	-
	30	Blank	NC	-
		BZP	orange	-
	60	Blank	NC	-
		BZP	orange	-
	70	Blank	NC	-
		BZP	orange	-

[a] *NC = no colour change observed; p. = pale*

[b] *Due to lack of colour change, this combination was no longer tested after 5 days (-)*

4.3.2.6 *Recommended test method*

A recommended method for the detection of piperazine analogues was determined based on tests completed to this point. This method was then used for all further testing procedures and is as follows:

To prepare test paper strips, add to a 4 cm x 5 mm strip of Whatman No. 1 filter paper:

1. 5 µL triton X-100 (0.1% v/v)
2. 5 µL NQS solution (6.4×10^{-3} M in deionised water)
3. Allow to dry slightly before testing or storage

To test an unknown substance:

4. Add 100 µL carbonate buffer to unknown substance, either in powder or liquid form
5. Add prepared test paper strip to the solution
6. Record colour change immediately and after 5 min

4.3.2.7 *Limit of Detection study*

The freshly prepared paper strips showed good results in this study. The LOD was determined to be 6 µg, giving an operational LOD of 60 µg, results which are not considerably different from the previously published method. The stored paper strips containing only NQS, however, did not provide good LOD results with none of the concentrations showing a colour change.

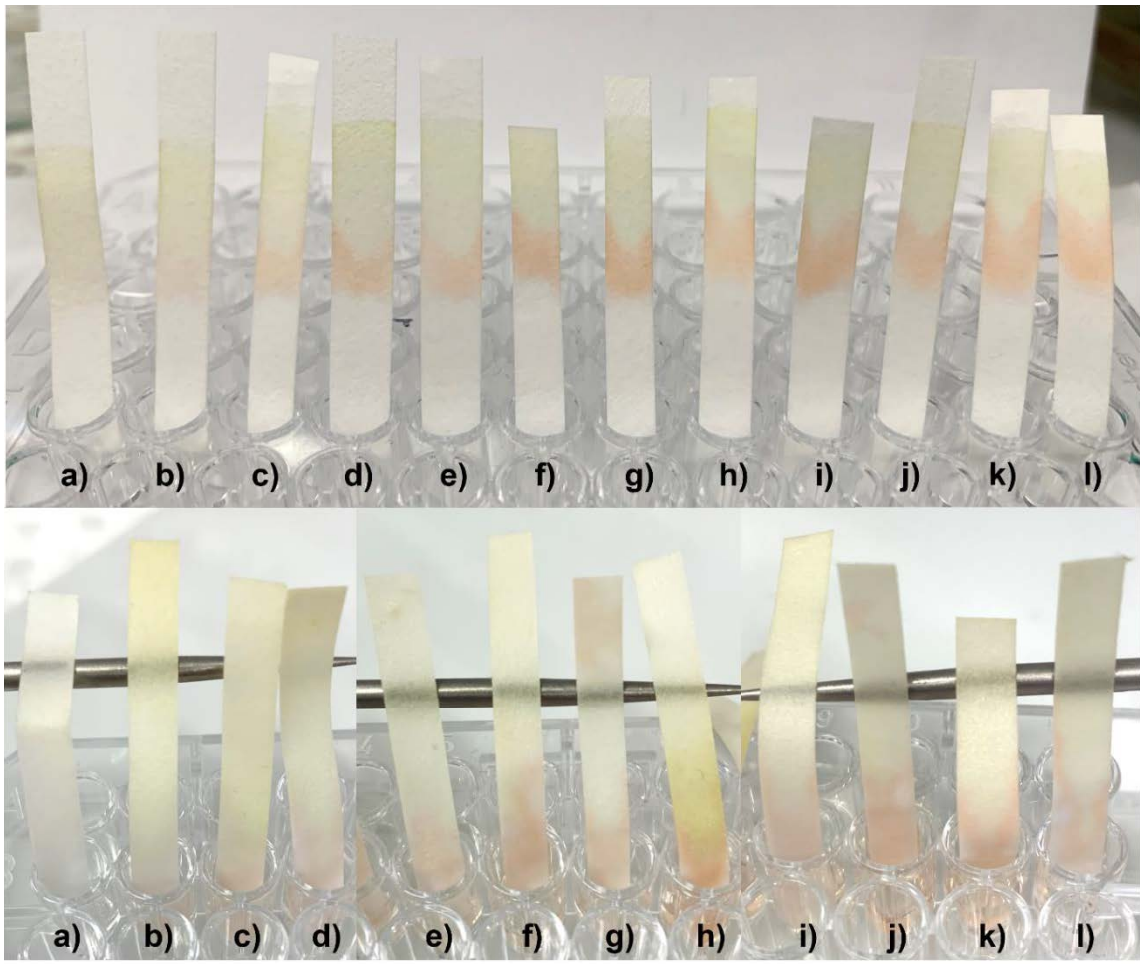

Figure 4-13: LOD results for freshly prepared paper strips containing NQS (top) and stored paper strips containing NQS and triton X-100 (bottom). Concentrations a) 0 µg, b) 2 µg, c) 4 µg, d) 6 µg, e) 8 µg, f) 10 µg, g) 15 µg, h) 20 µg, i) 25 µg, j) 30 µg, k) 35 µg, l) 40 µg

The lack of results for this study and the successful results after storing papers with the addition of triton X-100 meant a second study of the LOD was completed. Papers prepared with NQS and triton X-100 were stored for 5 days before completion of the LOD in the same method as above but with the drug in the buffer solution. Highly similar results were seen to the freshly completed LOD study as shown in Figure 4-13, with a LOD determined to be 6 µg and hence, once again, an operational LOD of 60 µg.

4.3.2.8 *Precision study*

All BZP samples provided an orange colour change within 5 min of addition of the test paper. It was observed that some stored papers showed a pale orange colour change in comparison to the bright orange seen with the fresh papers.

4.3.2.9 *Selectivity study*

This test method had been previously shown to be highly selective for piperazine analogues though it was noted it may also interact with other amine containing compounds [196]. A similar trend was observed in the analysis of selectivity here. The other compounds, and particularly the illicit compounds which also reacted with this test, show the potential to be interpreted as false positives due to the similar colour changes seen (see Table 4-10). A high concentration of one of these compounds, or in turn a low concentration of a piperazine, could result in the incorrect interpretation of the colour change.

Table 4-10: Selectivity results of the piperazine test on paper

Compound class	Compound	Colour change [a]
Piperazines	BZP HCl	orange
	TFMPP HCl	orange
	mCPP HCl	orange
Other illicit drugs	d-amphetamine	p. yellow-orange
	methamphetamine HCl	p. orange
	MDMA HCl	NC
	4-MEC HCl	NC
	2C-B HCl	NC
	2C-I HCl	pink-orange
	25H-NBOMe HCl	p. orange
	fentanyl HCl	p. orange
	cocaine HCl	p. orange
Cutting agents	ephedrine HCl	p. orange
	pseudoephedrine HCl	p. orange
	paracetamol	NC
	aspirin	NC
	methyl amine	grey-brown
	ascorbic acid	NC
	glycine	NC
	phenolphthalein	bright pink
	citric acid	NC
	magnesium stearate	NC
	urea	NC
	cellulose	NC

[a] *NC = no colour change observed; p. = pale*

4.3.2.10 *Blind testing*

Blind testing methods which involve interpretation of a result are a beneficial way to ensure the analyst is not developing the test with any bias, when they know a sample contains a certain analyte. Here the blind testing showed promising results for this test method to be used in field settings. The piperazine analogues were correctly identified and there were no false positives (see Table 4-11. Three tests showed a pale orange colour change which has been interpreted as a compound containing an amine functional group. As previously mentioned, some of these substances have shown to react with this colour test, and they could possibly be interpreted as false positives.

Table 4-11: Blind testing results of the piperazine test on paper

Sample No.	Colour change [a]	Result interpretation [b]	Actual compound
1	p. yellow	-	Methanol blank
2	orange	+ (Piperazine)	BZP
3	yellow-brown	-	4-MMC
4	p. yellow-orange	-	MDMA
5	lt. orange	+/-	Methamphetamine
6	orange	+ (Piperazine)	mCPP
7	p. yellow	-	Methanol blank
8	yellow-orange	+/-	25I-NBOMe
9	orange	+ (Piperazine)	TFMPP
10	lt. orange	+/-	25H-NBOMe
11	p. yellow	-	Methanol blank
12	Orange	+ (Piperazine)	BZP

[a] *lt. = light; p. = pale*
[b] *(+) = positive, (-) negative, (+/-) positive for amine containing compound*

4.3.3 Conclusions

Overall, the translation of this colour test to a paper-based system has been successful. While there appears to be some potential interferents, the orange colour change seen with the piperazines can be differentiated, particularly at higher concentrations. The stability and storage study shows promising results, as the papers are stable for at least 10 weeks, but should be continued so a recommended maximum storage time can be estimated.

4.4 Cathinone colour test

4.4.1 Materials and methods

The developed cathinone colour test method involves the formation of a copper complex through the reaction of copper (II) nitrate and neocuproine hemihydrate with a sodium acetate buffer. The colour change seen is from a pale blue (blank) to a bright orange colour with cathinone analogues. The original method required heat for the reaction to proceed within 10 minutes and further development was completed to remove this heating step. The addition of a catalyst and an organic solvent extraction was utilised so this test could be performed without the need for a heat source and a colour change still seen within 10 minutes.

4.4.1.1 *Chemicals and reference materials*

Neocuproine hemihydrate, copper(II) nitrate ($Cu(II)(NO_3)_2$) trihydrate were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). Analytical grade anhydrous sodium acetate obtained from Ajax Finechem (Scoresby, Victoria, Australia). Hydrochloric acid (32 % w/w) was obtained from RCI Labscan (Taren Point, NSW, Australia). The synthetic cathinones 4-MEC HCl, 4-methyl-*N*-methylcathinone (4-MMC) HCl, 4-fluoromethcathinone (4-FMC) HCl, methylone, methylenedioxypyrovalerone HCl (MDPV), pyrovalerone, 4-methyl-pyrrolidinopropiophenone (MPPP), 4-methyl-pyrrolidinobutiophenone (MPBP) and 3,4-Methylenedioxy-α-pyrrolidinobutiophenone (MDPBP) were synthesised in house at UTS by the Centre of Forensic Science. JWH-073 was also synthesised in-house. Fentanyl was purchased as hydrochloride (HCl) salts manufactured by Chiron Chemicals (Hawthorn, VIC, Australia) and purchased from PM Separations (Capalaba, QLD, Australia). 25T-NBOMe HCl, methamphetamine, d-amphetamine sulphate and BZP were purchased from NMI (North Ryde, NSW, Australia). Protriptyline HCl, nortriptyline HCl, (1*S*,2*R*)-(+)-ephedrine HCl and paracetamol were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Aspirin and urea were obtained from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia).

4.4.1.2 *Preparation of test reagents*

A neocuproine solution was prepared to 2.90×10^{-2} M in 0.1 M hydrochloric acid (HCl) and 5.12×10^{-3} M in 0.1 M HCl. Copper(II) nitrate solution (Cu(II)) was prepared in deionised water to 1.25×10^{-2} M and 5.00×10^{-3} M. A sodium acetate buffer was prepared to 2.00 M. 1 mg/mL solutions of synthetic cathinones were prepared in deionised water as needed.

4.4.1.3 *Initial testing*

Initial tests were completed with a solution of 4-MEC. The general recommended method was performed both with the three reagents added separately and also after they had been mixed in the appropriate ratio. When applied separately the method calls for five drops of Cu(II) solution, two drops of neocuproine solution and two drops of sodium acetate buffer. The plate is then heated on a hot plate set at 100 °C and the colour change is recorded within 10 minutes. The mixed solution was prepared in the same ratio (5:2:2) and 7 drops were added to the drug before heating.

The original studies with this test method included a paper-based test study involving the application of the reagents to a paper strip followed by the addition of this strip to a drug solution. The paper strip was then placed on a hot plate at 60 °C for several minutes before the colour change was interpreted. This method was followed here with both a deionised water blank and a sample containing 4-MEC.

4.4.1.4 *Use of the developed simple test protocol*

To remove the need for a heat source in this method, a simple test procedure was developed involving mixed reagent solution, a catalyst, sodium chloride (NaCl), and the organic solvent, dichloromethane (DCM) extraction of the coloured product [293]. The mixed reagent solution was added to the sample (7 drops), followed by NaCl (1 M, 2 drops) and DCM (5 drops) in a test tube. The tube was gently shaken, and colour changes recorded. This method was followed to ensure reagents were working appropriately before further testing was conducted.

A paper-based method was designed based on the simple test protocol previously described. The three reagents were added to the paper strips in a mixed solution (7 drops), followed by 2 drops NaCl (1 M) and 2 drops DCM. The strip was then added to 5 drops of a 1 mg/mL solution of 4-MEC

and a blank. This was repeated, this time including the DCM in the drug solution rather than adding it to the paper strip. The previous method had seen that the DCM did not soak into the paper as the previously applied reagents were aqueous based. An increased amount of NaCl was added by using a saturated solution in place of the 1 M and the same method repeated.

4.4.1.5 *Adaptation of reaction conditions to increase the speed of reaction*

New higher concentrations of Cu(II) and neocuproine solutions were prepared to 1.6×10^{-2} M and 3.2×10^{-2} M respectively. These were the highest concentrations deemed appropriate for this test method in solution, due to the reagents reacting without a cathinone at concentrations greater than this. A mixed reagent solution using these new solutions was prepared in the appropriate ratios before addition to paper strips that had been soaked in the catalyst solutions.

Other halide salts had been tested as catalysts in the simple protocol development. Here, eight other salts were tested in addition to NaCl to determine if one of these could increase the speed of the reaction on paper. Sodium iodide (NaI), sodium bromide (NaBr), sodium fluoride (NaF), potassium chloride (KCl), potassium iodide (KI), potassium bromide (KBr), potassium fluoride (KF), and lithium fluoride (LiF) were all tested and were prepared as saturated solutions. Initial tests indicated that both the iodine and bromine containing salts would not be appropriate as they afforded colour changes without the presence of a cathinone in solution. The five remaining salt solutions, including NaCl, were tested both on paper strips and in test tubes to assess the potential for one of them to be used as the catalyst.

KCl, KF, LiF, and NaF were all used in a repetition of the simple test protocol, replacing NaCl. These tests were performed in semi-micro test tubes with the mixed reagent (1 mL), catalyst solution (0.5 mL), and DCM (1 mL) added. The colour change was recorded after 10 minutes and then after the test tubes were left overnight at room temperature.

Saturated solutions of these salts (KCl, KF, LiF, and NaF) along with NaCl were added to paper strips (2 drops) before the addition of mixed reagents, with the paper allowed to dry between additions. Further paper strips were prepared by applying a slight excess (6-8 drops) of each of the saturated solutions and allowing the paper to dry before the addition of the mixed reagent. Tests were completed on 4-MEC and methanol blanks with these prepared papers containing the different salt solutions. Stability assessment was also performed as further described in Section 4.4.1.6.

The combination of these salt solutions with the 4-MEC (1 mg/mL) was also assessed and the paper containing the mixed reagent was added to this mixed solution for analysis. Equal volumes of the saturated salt solution and 4-MEC solution were combined to create a drug solution containing 0.5 mg/mL 4-MEC. Ten drops of this drug solution were then added to the wells before addition of the paper strip. Blanks were completed by the addition of only the saturated salt solution to the well.

4.4.1.6 *Stability testing*

Several stability studies were completed with different combinations of reagents and concentrations. These combinations are outlined in Table 4-12 below. They were then stored separately at room temperature.

Table 4-12: Cathinone test stability study combinations

Applied to paper	Copper(II) nitrate	Neocuproine	Acetate buffer
Mixed reagent A	5.00×10^{-3} M	5.12×10^{-3} M in 0.1 M HCl	2.00 M
Mixed reagent B	1.25×10^{-2} M	2.90×10^{-2} M in 0.1 M HCl	2.00 M
Mixed reagent C	1.60×10^{-2} M	3.20×10^{-2} M in 0.1 M HCl	2.00 M

These stability tests were applied to 4-MEC samples alongside deionised water blanks. These combinations, which do not contain a catalyst, were also applied to the 4-MEC and saturated catalyst combined solutions as previously described in 4.2.7. The resulting colour changes were compared between these tests, along with the time required for a colour change to be seen. It should be noted that mixed reagent C contains an increased concentration of both the copper and neocuproine solutions compared to previous tests. These concentrations are the highest recommended concentration of the reagents outlined in the published method.

Stability studies including the saturated salt solutions on paper strips were also carried out. 10 drops of the salt solution were added to paper strips and allowed to dry. These papers were either stored with only the salt solution or mixed reagent C was then added and the paper dried and stored in individual vials. These could then be added to blank and drug solutions at required time intervals. Further papers were prepared to assess the concentrations of the reagent mixtures with

KF as the catalyst. Saturated KF solution was added to the papers in excess (10 drops), and these were allowed to dry before addition of the mixed reagents A, B or C (see Table 4-12). The papers were allowed to dry fully and stored until testing was required.

4.4.1.7 *Separation of reagents across paper and in drug solution*

To help avoid reaction of the reagents without the presence of the cathinone, methods to both separate the reagents across the paper strip as well as combine with the drug solution were prepared. Ideally, the reagents in solution would be able to be kept in a single container to avoid mixing of reagents on site and therefore a reduction in steps to carry out the method. Table 4-13 outlines the combinations and concentrations of reagents which were prepared. The volumes of each reagent remained constant across all tests to ensure the ratio between the Cu(II) and neocuproine was also consistent. For each test 50 µL Cu(II), 20 µL neocuproine, 20 µL sodium acetate, and 50 µL potassium fluoride were added initially and the volume of potassium fluoride was increased to 100 µL in later testing.

Table 4-13: Outline of the combinations of separated reagent addition for the cathinone test

Combination number	Added to paper		Added to drug in solution	
	Reagent	**Concentration**	**Reagent**	**Concentration**
1	neocuproine	0.00512 M	copper (II) nitrate	0.005 M
	sodium acetate	2 M	potassium fluoride	saturated
2	neocuproine	0.00512 M	copper (II) nitrate	0.005 M
	sodium acetate	2 M		
	potassium fluoride	saturated		
3	copper (II) nitrate	0.005 M	neocuproine	0.00512 M
	sodium acetate	2 M	potassium fluoride	saturated
4	copper (II) nitrate	0.005 M	neocuproine	0.00512 M
	sodium acetate	2 M		
	potassium fluoride	saturated		
5	copper (II) nitrate	0.005 M	neocuproine	0.00512 M
			sodium acetate	2 M
			potassium fluoride	saturated
6	neocuproine	0.00512 M	copper (II) nitrate	0.005 M
			sodium acetate	2 M
			potassium fluoride	saturated
7	neocuproine	0.029 M	copper (II) nitrate	0.0125 M
	sodium acetate	2 M	potassium fluoride	saturated
8	neocuproine	0.029 M	copper (II) nitrate	0.0125 M
	sodium acetate	2 M		
	potassium fluoride	saturated		
9	copper (II) nitrate	0.0125 M	neocuproine	0.029 M

	sodium acetate	2 M	potassium fluoride	saturated
10	copper (II) nitrate	0.0125 M	neocuproine	0.029 M
	sodium acetate	2 M		
	potassium fluoride	saturated		
11	copper (II) nitrate	0.0125 M	neocuproine	0.029 M
			sodium acetate	2 M
			potassium fluoride	saturated
12	neocuproine	0.029 M	copper (II) nitrate	0.0125 M
			sodium acetate	2 M
			potassium fluoride	saturated
13	neocuproine	0.032 M	copper (II) nitrate	0.016 M
	sodium acetate	2 M	potassium fluoride	saturated
14	neocuproine	0.032 M	copper (II) nitrate	0.016 M
	sodium acetate	2 M		
	potassium fluoride	saturated		
15	copper (II) nitrate	0.016 M	neocuproine	0.032 M
	sodium acetate	2 M	potassium fluoride	saturated
16	copper (II) nitrate	0.016 M	neocuproine	0.032 M
	sodium acetate	2 M		
	potassium fluoride	saturated		
17	copper (II) nitrate	0.016 M	neocuproine	0.032 M
			sodium acetate	2 M
			potassium fluoride	saturated
18	neocuproine	0.032 M	copper (II) nitrate	0.016 M
			sodium acetate	2 M
			potassium fluoride	saturated

4.4.1.8 *Selectivity and specificity study*

In total, nine cathinone analogues and twelve other compounds were tested to assess the selectivity of this test method (for full list see Figure 4-25). For each compound, 100 µL of compound solution (1 mg/mL) was added to a well followed by Cu(II) solution (50 µL, 0.016 M), and potassium fluoride (100 µL, saturated). A test paper containing neocuproine (20 µL, 0.032 M) and sodium acetate (20 µL, 2 M) was then added to each well and any colour changes observed regularly for 1 hour.

4.4.1.9 *Limit of detection study*

To determine the limit of detection for this test method, aliquots of 4-MMC (1 mg/mL) were added to twelve wells in the range of 0 – 150 µL. To each well, Cu(II) (50 µL, 0.016 M) and potassium fluoride (100 µL, saturated) were added. This was followed by the addition of a test paper containing neocuproine (20 µL, 0.032 M) and sodium acetate (20 µL, 2 M). The test wells were observed for 1 hour and colour changes recorded, noting the time required for that colour change to be identified.

4.4.2 Results and discussion

4.4.2.1 *Initial testing*

The expected colour change results were seen with both mixed and unmixed reagents after application to the hot plate at 100 °C (see Figure 4-14). A bright orange colour change, considered to be positive is seen with 4-MEC and this test was carried out periodically to ensure reagents were working appropriately.

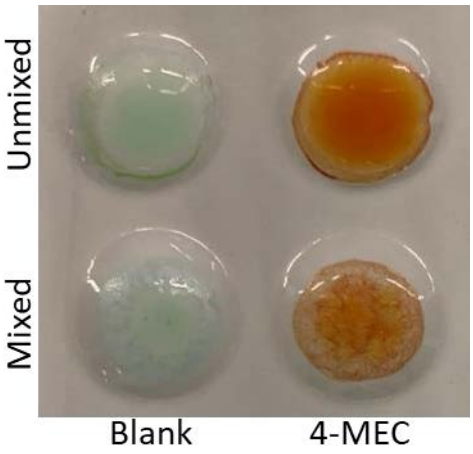

Figure 4-14: Colour change results of cathinone test after heating for 10 min as outlined in the published method

The expected result of a yellow-orange colour change on paper was successfully seen with 4-MEC after heating the paper strips (Figure 4-15). Part of the translation aim was to remove the need to use a heat source, and this is investigated in subsequent sections.

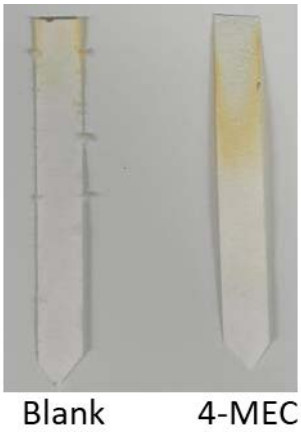

Figure 4-15: Colour change results of the paper-based method included as part of the original study

4.4.2.2 *Use of the developed simple test protocol*

This test method showed expected colour change results as previously reported (Figure 4-16). Yellow-orange colour changes were seen with 4-MEC at room temperature within a few minutes and no colour change was seen with the reagent blank.

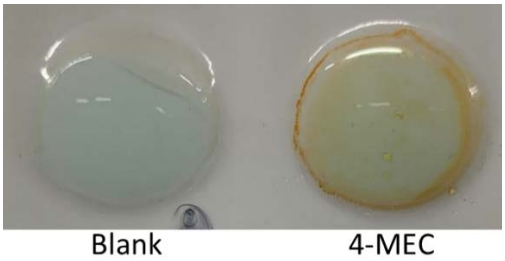

Figure 4-16: Colour change results with the simple test method

No colour change was seen with either paper strip within 20 min of addition. The papers were left overnight and after 24 h an orange colour change was seen with the 4-MEC sample. The method involving the DCM added to the drug solution showed similar results to the first attempt, with colour changes only seen overnight. It was clear that the test was working however not in a length of time appropriate for these types of test methods. The focus was turned to the catalyst, as this should increase the speed of the reaction. The original solution of NaCl was replaced with a saturated solution however no change was seen as per the two previous tests.

4.4.2.3 *Adaptation of reaction conditions to increase the speed of reaction*

As reported in the previous section, an orange colour change was seen after ~2 h for KF, LiF, and KCl, providing a promising result for these catalysts. The blanks did start to show an orange colour change after several hours (~3 h) indicating the concentrations may be too great for the test to require the presence of a cathinone to form the complex.

After addition of the saturated solutions to the paper strips, they were left to air-dry. In this time the iodine-containing salts oxidised before the other reagents were added (Figure 4-17) and also when the other reagents were added even without the drying time (Figure 4-18) and a brown colour seen. These salts were not tested further.

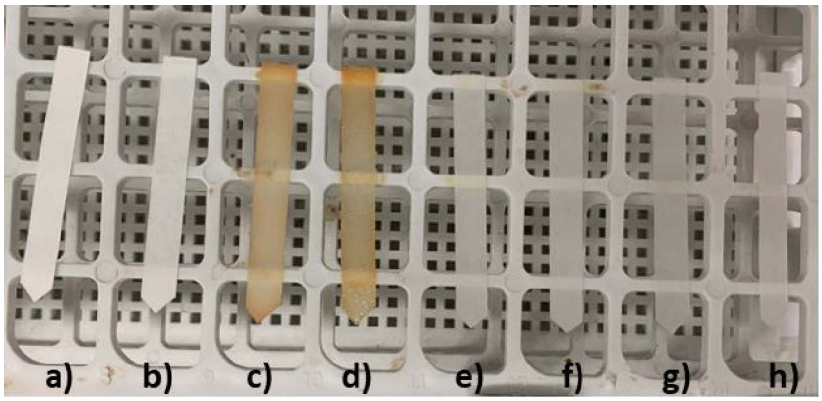

Figure 4-17: paper strips after addition of catalyst solutions, a) KCl, b) LiF, c) NaI, d) KI, e) KF, f) NaF, g) NaBr, and h) KBr

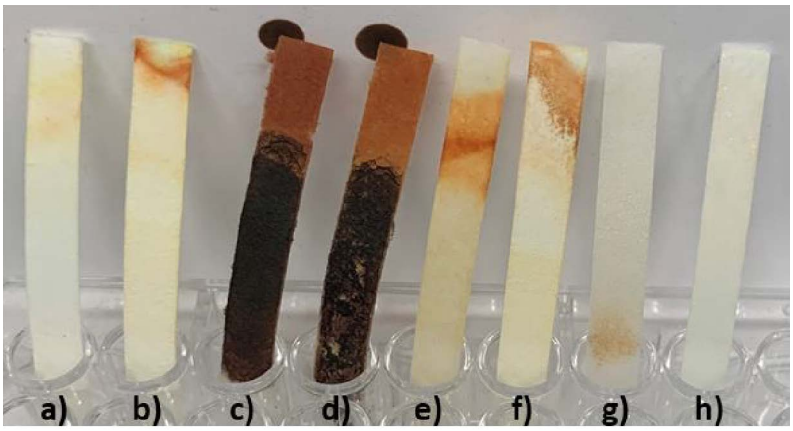

Figure 4-18: Colour change results with paper strips containing catalysts and mixed reagents added to 4-MEC solution. a) KCl, b) LiF, c) NaI, d) KI, e) KF, f) NaF, g) NaBr, and h) KBr

Figure 4-18 shows the colour changes on the paper strips with KCl, LiF, KF, and NaF after 24 h. These salts also gave the best results in test tubes after a similar amount of time (orange precipitate was seen after 24 h). The bromine-containing salts showed orange colour changes with the reagent blanks as well as with the cathinone samples, see Figure 4-19.

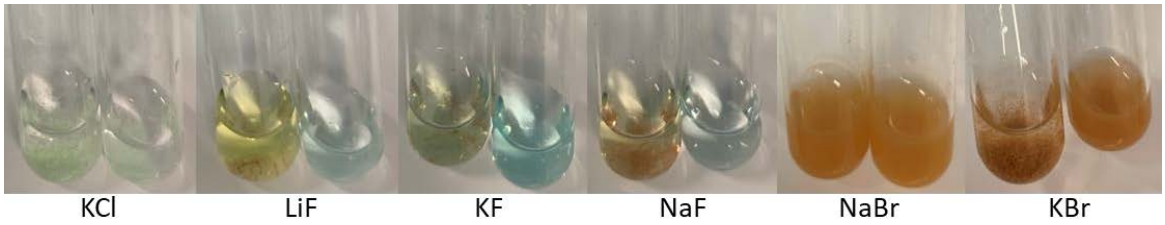

Figure 4-19: Colour changes seen in test tubes in the presence of the selected catalysts. 4-MEC (left tube), blank (right tube)

The results from the inclusion of the organic extraction step with these four catalysts are shown in Figure 4-20. After 10 minutes there was a light orange colour change seen, similar to that seen with NaCl. Overnight a bright red-orange colour change was seen in all four test solutions. At this stage, no further testing was completed using this method.

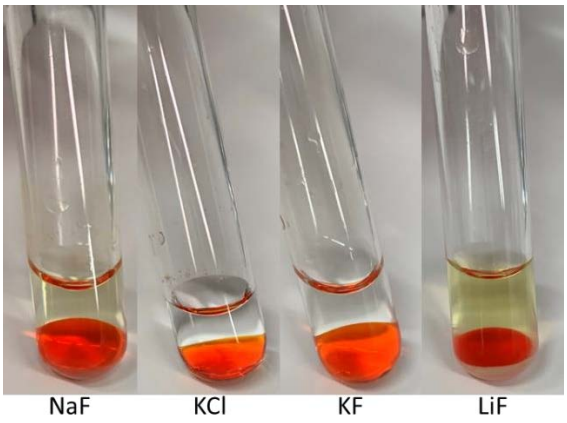

Figure 4-20: Colour change results after 24 h with the addition of DCM for colour complex extraction in the organic layer using different catalysts

The application of these saturated solutions to paper strips both in small volumes and in excess showed colour changes with 4-MEC after several hours. Sometimes it was not until 24 h later that an orange colour change was seen. It was also seen in some cases that the excess catalyst would also cause reagent blank paper strips to change colour after only ~2 h. Figure 4-21 shows a positive orange colour change with LiF, KCl, and KF after several hours.

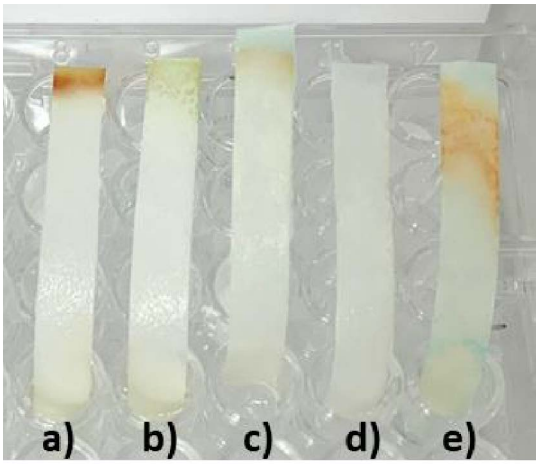

Figure 4-21: Results with 4-MEC with addition of a) LiF, b) NaF, c) KCl, d) NaCl, and e) KF after several hours

Figure 4-22 indicates an example where the reagent blanks also gave an orange colour change with the excess addition of KF and LiF. The orange colour changes seen with NaF, KF, and LiF appear to be the most promising and so testing was continued with a focus on these three as potential catalysts. The paper strips which were added to the mixture of saturated salt solutions and 4-MEC showed an orange colour change the following day.

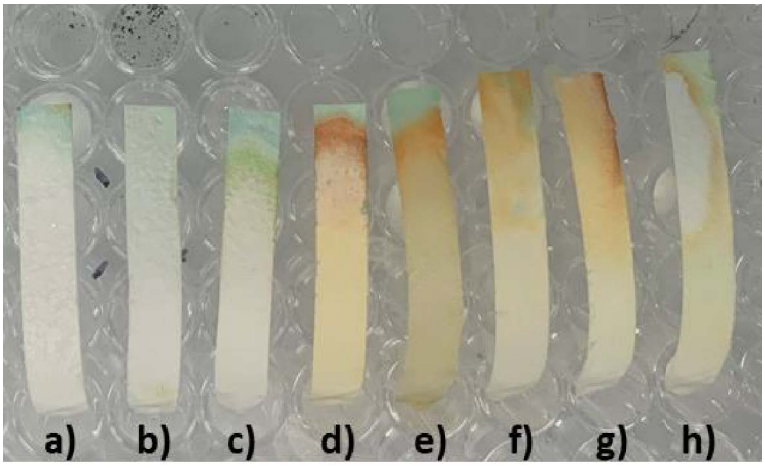

Figure 4-22: Colour change results after several hours with the addition of excess catalyst a) KCl blank, b) KCl, 4-MEC, c) NaF blank, d) NaF 4-MEC, e) KF blank, f) KF 4-MEC, g) LiF blank, and h) LiF 4-MEC

4.4.2.4 *Stability testing*

The storage of paper strips containing only the mixed reagents did not affect the resulting colour change when compared to freshly prepared paper strips. These tests did, however, take up to 24 h to show a colour change. When the 4-MEC was combined with the saturated salt solutions, there was still no colour change within 20 min of addition. However, after approximately 3 h for mixed

reagents A & B, both KF and LiF showed yellow-orange colour changes. Mixed reagent C showed orange colour changes within ~2 h with KF, LiF, and KCl as catalysts. However, soon after the blanks also started to show orange colour changes with these catalysts as well. This did indicate that the higher concentrations of reagents may be needed for this reaction to occur on paper. The papers containing both the saturated salts and the mixed reagent solutions initially showed promising results. It was observed however, that these papers began to change colour in storage after approximately two weeks. This meant that when applied to the test solutions, blank samples would also show an orange colour change.

The application of the mixed reagents prior to testing showed some promise, particularly with KF as the catalyst. Orange colour changes were seen within 30 minutes of addition to the drug solution. It was here that further test papers were prepared with KF as the catalyst for the reaction. Over the course of several weeks, there was variability in the resulting colour changes seen with stored papers containing mixed reagent C and KF separately and in combination, with false positive results a common occurrence. After 4 weeks of storage, a test completed with the combined KF, and mixed reagent paper showed a colour change with 4-MEC within 30 minutes of addition but provided no colour change with the reagent blank (see Figure 4-23). This showed promise for an optimised method, however later tests showed false positives occurring with the blank samples and so testing moved on to the separation of the reagents.

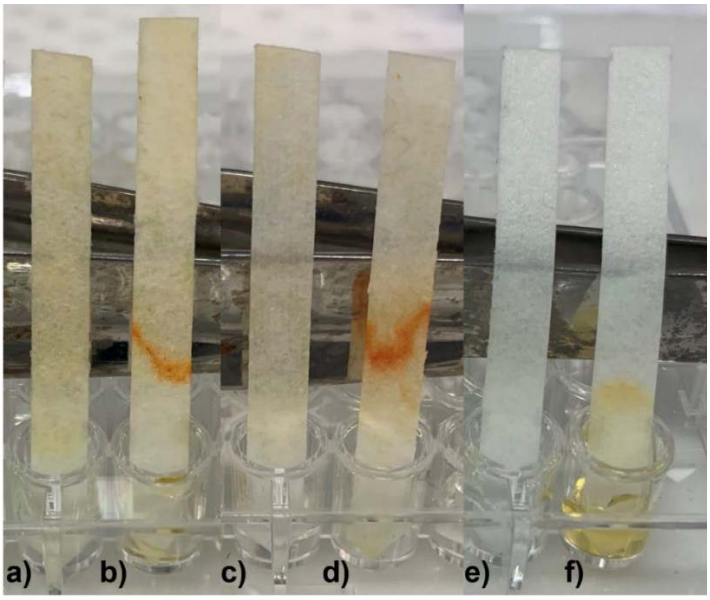

Figure 4-23: Colour change results of papers containing mixed reagent C and KF after 4 weeks storage with blank (a & c), 4-MEC (b & d) compared to papers prepared and tested the same day

4.4.2.5 *Separation of reagents across paper and in drug solution*

The resulting colour changes with 4-MEC for each combination of separated reagents is outlined in Table 4-14. Based on these initial results, combinations 5, 7, 9, 11, 13, 15, and 17 were investigated further. The similarity observed with these combinations was that the copper nitrate and neocuproine remained separate until testing occurred. This appears to be the most appropriate option for storage of these papers, as the reagents cannot undergo reaction prior to the cathinone being introduced to the system.

Table 4-14: Combinations of separated reagents on paper and in solution and their resulting colour changes with 4-MEC

Combination number [a]	**Colour change result** [b]	
	Blank	**4-MEC**
1	p. yellow	lt. orange
2	p. yellow	v. pale orange/yellow
3	p. yellow	v. pale orange/yellow
4	v. pale orange/yellow	v. pale orange/yellow
5	p. yellow	lt. orange
6	v. pale orange/yellow	lt. orange
7	v. pale orange/yellow	lt. orange
8	p. yellow	v. pale orange/yellow
9	p. yellow	lt. orange
10	p. yellow	v. pale orange/yellow
11	p. yellow	lt. orange
12	v. pale orange/yellow	lt. orange
13	p. yellow	lt. orange
14	p. yellow	v. pale orange/yellow
15	p. yellow	lt. orange
16	v. pale orange/yellow	v. pale orange/yellow
17	p. yellow	lt. orange
18	v. pale orange/yellow	lt. orange

[a] *refer to Table 4-13 for reagent details*

[b] *v. = very; p. = pale; lt. = light; NC = no colour change observed*

Further testing utilised an increased quantity of KF from 50 to 100 µL to assist in speeding up the reaction. Following this, it was found that combination 13 provided the best results and allowed for a colour change of light orange-yellow to be seen within 15-20 min. A darkening of this colour was seen over the following 60 min. Storage studies of these papers, containing neocuproine and sodium acetate, indicated stability for up to 8 weeks. The same can be said for the reagents remaining in solution, and these can be stored as a mixed solution or added to the reaction separately for a comparable colour change result. These papers were used for all further testing.

4.4.2.6 *Recommended test method*

The tests completed to this point indicated the most appropriate test conditions to analysed cathinone analogues in a paper-based system. This method was used for all further testing procedures and is as follows.

To prepare test paper strips, add to a 4 cm x 5 mm strip of Whatman No. 1 filter paper:

1. 20 µL sodium acetate (2 M in deionised water)
2. 20 µL neocuproine solution (0.032 M in 0.1 M HCl)
3. Allow to dry before testing or storage

To test an unknown substance:

4. Add the unknown substance, either in powder or liquid form, to a test vial or well
5. Add 100 µL saturated KF solution
6. Add 50 µL $Cu(NO_3)_2$ (0.016 M in deionised water)
7. Add prepared test paper strip to the solution
8. Record colour change after 5 min and observed further changes for at least 20 min

4.4.2.7 *Selectivity and specificity study*

The original developed cathinone test method displayed some variability in the reaction of different cathinone analogues. It was suggested that those analogues with greater carbon bond saturation do not show the yellow-orange colour change with this test. A similar trend was seen with the paper-based test system with analogues including MDPV, MDPBP, MPBP, and pyrovalerone not providing the same colour change as that seen with the other analogues (see Figure 4-24). There was also some inconsistency across tests with the same drug, with some false

negative results seen with analogues such as 4-MMC (Figure 4-24a), at a concentration which would be expected to provide a positive colour change. These results indicate the necessity for further optimisation of this test method and investigation into the occurrence of false negatives.

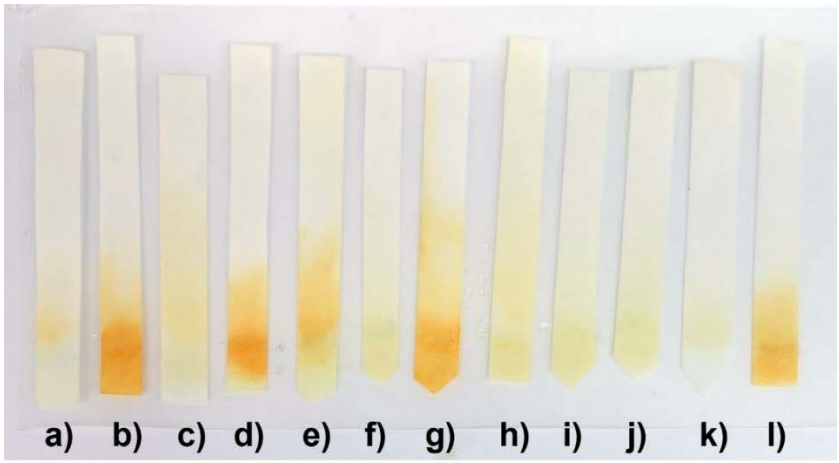

Figure 4-24: Colour change results of testing with cathinone analogues: a) 4-MMC (100 µg), b) 4-MMC (75 µg), c) MDPV (100 µg), d) methylone (100 µg), e) 4-FMC (100 µg), f) MDPBP (100 µg), g) MPPP (100 µg), h) MPBP (100 µg), i) pyrovalerone (100 µg), j) pyrovalerone (75 µg), k) MDPV (75 µg), and l) 4-FMC (75 µg)

The other drugs and licit compounds which were tested showed that only two compounds, the synthetic cannabinoid JWH-073 and paracetamol, reacted to give an orange colour change (see Figure 4-25). JWH-073 had not been previously tested with this test method and here provided a light orange colour change. The paracetamol gave an orange colour change, similar in intensity to some cathinone analogues, while when it was previously tested it showed a yellow colour change result. These results indicate the need to further investigate the reaction with other ketone containing compounds with a focus on synthetic cannabinoids which share a similar chemical structure to JWH-073. It was observed in the original test development that some ketone, carboxylic acid and amino acid containing compounds reacted to produce an orange colour change suggesting that this test may not be selective to only cathinone analogues [197].

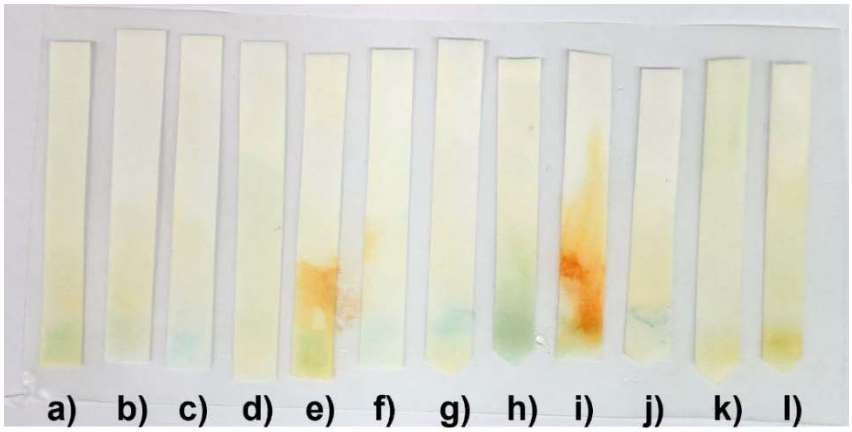

Figure 4-25: Colour change results of selectivity study for the cathinone test, each tested at 100 µg : a) 25T-NBOMe, b) methamphetamine, c) d-amphetamine, d) BZP, e) JWH-073, f) ephedrine, g) fentanyl, h) aspirin, i) paracetamol, j) nortriptyline, k) urea, and l) protriptyline

4.4.2.8 *Limit of detection study*

It has become apparent that this test method is not highly reproducible and there is often variation in the colour changes seen even for tests containing the same components. The results for the limit of detection were no different. The first six concentrations show a gradual increase in depth of the orange colour change (Figure 4-26 a – f), which would be expected with the increase in 4-MMC concentration. The sample containing 40 µg of 4-MMC however, showed little to no yellow-orange colour change, unlike the two concentrations either side of it.

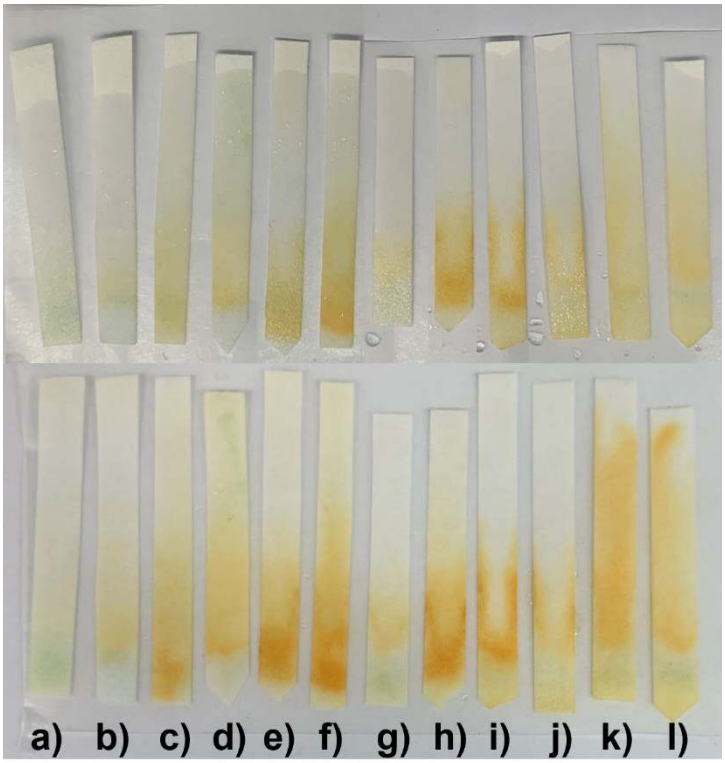

Figure 4-26: Colour change results of LOD study with 4-MMC after 15 min (top) and 60 min (bottom): a) reagent blank, b) 5 µg, c) 10 µg, d) 15 µg, e) 20 µg, f) 30 µg, g) 40 µg, h) 50 µg, i) 60 µg, j) 75 µg, k) 100 µg, and l) 150 µg

It appears that the detection of 4-MMC at a concentration of 10 µg is possible, which would provide an operational LOD of 100 µg. This value is much higher than the value determined for the test in solution of 4 µg and hence an operational limit of 40 µg. The variability in colour change between analogues is important to note here as the LOD would likely change for those other compounds.

4.4.3 Conclusion

Removal of the heating step and a timely reaction (<20 min) indicate that this test is applicable to a paper-based system. While the separation of reagents, and therefore a reagent solution, is required, the colour change with cathinones is obvious and highly selective.

4.5 Fentanyl colour test

A colour test to detect fentanyl and its analogues was developed by a previous researcher at UTS using a 1,2-naphthoquinone reagent solution in an alkaline environment. The developed test was performed in solution and utilises a copper (II) chloride catalyst and UV light to increase the rate of reaction. This method was investigated for its application to a paper-based system. Due to a variety of factors, other quinones were tested in the place of this reagent and another reagent 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone was also investigated thoroughly. These reagents have both advantages and disadvantages, and further research is required to determine the most appropriate method for analysis of fentanyl analogues in this way.

4.5.1 Materials and methods

4.5.1.1 *Chemicals and reference materials*

1,2-naphthoquinone (1,2-NQ), 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone, 2-hydroxy -1,4-naphthoquinone, 5,8-dihydroxy-1,4-naphthoquinone, chloranilic acid, 2,3-dichloronaphthoquinone, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, 1,4-dihydroxynapthalene, 1,4-dihydroxynapthoquinone, and Quinizarin were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). 1,4-anthraquinone was manufactured by TCI Chemicals (Kumagaya City, Japan) and purchased from ChemSupply (Gillman, SA, Australia). Monosodium phosphate (NaH2PO4), disodium phosphate (Na_2HPO_4), and copper (II) chloride dihydrate ($CuCl_2{\cdot}2H_2O$) were obtained from Ajax Finechem (Taren Point, NSW, Australia). Acetonitrile and methanol were of LC-MS grade and obtained from Honeywell International Inc (North Ryde, NSW, Australia). Deionised water was obtained from the laboratory supply.

N-Phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide (fentanyl), 4-fluoroisobutyrylfentanyl, cyclopropylfentanyl, benzodioxolefentanyl, phenylpropionylfentanyl, β-hydroxyfentanyl, remifentanil and alfentanil were purchased as hydrochloride (HCl) salts manufactured by Chiron Chemicals (Hawthorn, VIC, Australia) and purchased from PM Separations (Capalaba, QLD, Australia) as neat standards. Heroin HCl was obtained from Lipomed (Arlesheim, Switzerland). Morphine HCl, mCPPP HCl, TFMPP HCl, BZP 2HCl, 2C-I HCl, 25D-NBOMe HCl, tetrahydrocannabinol, (+)-S-Methamphetamine HCl, and d-amphetamine sulphate were obtained from the National Measurement Institute (NMI, North Ryde, NSW, Australia). Protryptyline HCl,

nortryptaline HCl, ramipril, (1*S*,2*R*)-(+)-ephedrine HCl, paracetamol, magnesium stearate, glycine, and sodium chloride were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Codeine phosphate, phenolphthalein, aspirin, citric acid, D-glucose, and urea were obtained from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia). All synthetic cathinones were used as hydrochloride salts including pyrovalerone HCl, MPPP HCl, MPP HCl, MDPBP HCl, methylone, 4-MMC, 4-FMC, and 4-MEC which were synthesised in-house at UTS by the Centre for Forensic Science. The synthetic cannabinoids, JWH-073 and UR-144, were synthesised in house by Watanabe et al. [264, 294].

4.5.1.2 *Preparation of test reagents*

A solution of 1,2-NQ was prepared in ACN to 150 ppm. The copper chloride ($CuCl_2$) catalyst was prepared to 1.9 mM in deionised water. A phosphate buffer solution was prepared by the addition of NaH_2PO_4 and Na_2HPO_4 to pH 8. A mixed reagent solution was also prepared by combining the 1,2-naphthoquinone and $CuCl_2$ solutions in a 5:1 ratio.

Solutions of each quinone reagent were prepared in ACN to 150 ppm. Stock solutions of both 1,2-NQ and 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone were prepared to obtain the desired concentration of 2000 ppm. To obtain a 300 ppm solution, the stock solution was diluted accordingly. A 1.00 M Na_2HPO_4 solution was prepared volumetrically by dissolving 8.96 g in 25.00 mL deionised water. To aid with dissolution, the solution was gently heated on a hot plate. A 1.00 M solution of NaH_2PO_4 was prepared volumetrically by dissolving 3.92 g in 25.00 mL deionised water. To a 50 mL volumetric flask, 4.66 mL of Na_2HPO_4 1.00 M solution and 0.34 mL of NaH_2PO_4 1.00 M solution was added and brought to volume, yielding a 0.10 M phosphate buffer. A 1.90 mM solution of $CuCl_2$ was prepared volumetrically by dissolving 16.2 mg in 50.00 mL deionised water. A mixed reagent solution for each of 1,2-NQ and 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone was prepared by combining the 150 ppm and 300 ppm solution respectively with the $CuCl_2$ solution in a 5:1 ratio.

4.5.1.3 *Initial testing of 1,2-napthoquinone*

The method for the colour test was initially based on the solution-based fentanyl colour test method using the 1,2-NQ mixed reagent. To 100 µL of fentanyl HCl solution (1 mg/mL in methanol), 40 µL of buffer solution followed by 240 µL of the mixed reagent solution were added and exposed to UV light (365 nm, 6 W) for 10 min before the colour change was observed and

recorded. This method was reduced to a smaller scale for the addition to paper. 5 µL buffer solution and 30 µL mixed reagent was added to a paper strip before the strip was added to 50 µL of fentanyl solution (1 mg/mL) and exposed to UV light. This method was repeated with the buffer solution added to the drug solution rather than the paper and with the removal of the $CuCl_2$ catalyst. Depending on the reaction result seen, tests with other drugs, commonly heroin and morphine, were tested to observe similarities or differences in colour change. Prepared papers were stored in closed vials, after the solvent was allowed to evaporate, for two days to assess the short-term stability and hence the applicability of these papers to onsite testing.

4.5.1.4 *Effect of UV intensity on colour change*

Previous testing had reported that the type of UV light source, used to increase the speed of reaction, altered the final colour change when 1,2-NQ reacted with fentanyl. To further understand which UV light source would be the most ideal option for this test and other similar tests, several UV light sources were investigated to assess the effect they had on the colour change results of fentanyl samples. The use of the TLC light box (365 nm, 6 W) was suggested in the initial development of this test. Three other light sources were investigated including the Rofin Polilight™ PL550XL at three intensities (350 nm, P1, P4, and P7), a UV light box (365 nm, 12 W) and a UV torch (365 nm, 1 W). All tests were carried out with fentanyl in the test solution and were exposed to the UV light source for 10 mins. Colour changes were recorded immediately and monitored for changes for two hours post exposure.

4.5.1.5 *Other quinone reagent testing*

Nine other quinone reagents were tested in place of 1,2-NQ, both in solution and on paper. The phosphate buffer solution was included in all tests, and each was tested with and without the $CuCl_2$ catalyst for comparison. Reagent solutions were made up to 150 ppm in acetonitrile before being combined with the catalyst to create mixed reagent solutions. The individual reagents and the mixed reagents were analysed with fentanyl following the methods previously described for solution and paper-based tests. The results of these tests were recorded and those which afforded a colour change were investigated further.

4.5.1.6 *2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone colour test*

Paper-based tests involving 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone were initially completed in the same manner as the previously described test with the reagent concentrations and ratios remaining the same. Early testing involved comparison of freshly prepared paper strips with those which had been dried for varying lengths of time to assess the compatibility with the paper system. The UV exposure step was kept as the final step before colour analysis. Tests with and without the inclusion of the $CuCl_2$ solution were compared to assess the need for a catalyst with this reagent. To store prepared test papers, the solvent was allowed to evaporate from the papers for a minimum of 10 minutes before storing in a scintillation vial with a loosely screwed lid to allow further solvent evaporation. Papers were stored in the dark at room temperature for up to a week for all experimental work, other than stability studies. Before extensive studies were performed with this reagent, heroin was also tested to assess the potential to differentiate fentanyl from other opioids.

4.5.1.7 *Reagent optimisation*

Seven different solutions of 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone were prepared through dilution of the stock solution. The range of concentrations included 150, 350, 600, 800, 1000, 1250, and 1500 ppm and were tested to observe the effect on colour change. After testing each concentration on a blank and fentanyl sample, an optimal range was determined to include concentrations of 150, 300, and 350 ppm. Each concentration was then tested with or without copper chloride for comparison on freshly prepared papers as well as dried papers.

$CuCl_2$ concentrations of 0.5, 1.9, 5, and 10 mM were tested to observe the effect in a 5:1 mixed reagent solution. Various ratios of quinone to copper were trialled; including 9:1, 5:1, 3:1, and 3:2 ratios. For all these tests, the 0.10 M phosphate buffer solution was not added to the mixed reagent solution and was added to the papers separately.

4.5.1.8 *Stability and storage of test papers*

The stability of the stored test papers was completed indirectly through the preparation of papers which were then tested on another day, usually no longer than a week after preparation. To complement this, once a method had been established a set of eight test papers were dried

completely and split into 2 sets of 4, where each set underwent a light study to determine the stability under different storage conditions. Each set of 4 papers was stored in a sealed scintillation vial. One was placed on the laboratory bench for a period of 2 weeks, and the other was placed in a dark cupboard for the same amount of time. To test the stability, each set underwent one blank test and three fentanyl tests.

4.5.1.9 *Precision*

Paper testing was conducted with three to five replicates to determine repeatability. Replicates were produced across different days with mixed reagents that had been prepared at different times. Three replicates of the dried papers were prepared and tested on blanks and 50 µL of 1 mg/mL fentanyl solution. Further to this, a set of ten paper test strips were prepared with the three reagent solutions added separately. These were tested with 50 µL fentanyl solution and exposed to UV for 10 min after addition of the paper strip to the drug solution.

4.5.1.10 *Specificity and selectivity*

For comparative purposes, selectivity and specificity studies were carried out with both 1,2-NQ and 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone. The test method was carried out on a variety of other drug compounds, cutting agents, and fentanyl analogues to assess the specificity and selectivity. Such compounds included other illicit drugs and precursors, pharmaceutical drugs, and other miscellaneous licit compounds, many of which are used as cutting agents for a range of illicit drugs. A full list of these compounds can be found in Table 4-18 in the results. For each compound, 50 µL of a 1 mg/mL solution in methanol was added to a GC vial and underwent a singular test. Those compounds which indicated a colour change akin to the colour change of a positive result underwent repeat testing in triplicate.

4.5.1.11 *Limit of detection*

A range of 0-50 µg of fentanyl was used to determine the limit of detection (LOD) of the test with 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone, with intervals at every 5 µg. The appropriate amount of fentanyl solution was added to a vial and made up to a total volume of 50 µL. After an initial test set, the range was reduced to 0-30 µg with further replicates completed. Following this, fentanyl samples of 15, 20, and 25 µg underwent tests in triplicate to determine the LOD. In this

work, the lowest concentration at which the colour change was observed on a triplicate set on more than one set of papers was determined to be the LOD. The value was then multiplied by 10 according to NIJ guidelines [267] to provide the operational detection limit.

4.5.2 Results and discussion

4.5.2.1 *Initial testing of 1,2-napthoquinone*

The initial goal of the study was to translate the method outlined using the 1,2-naphthoquinone reagent onto a paper-based system, however, a number of difficulties arose. The quinone was able to produce a blue colour change with a freshly prepared paper which was no more than 30 minutes old, see Figure 4-27.

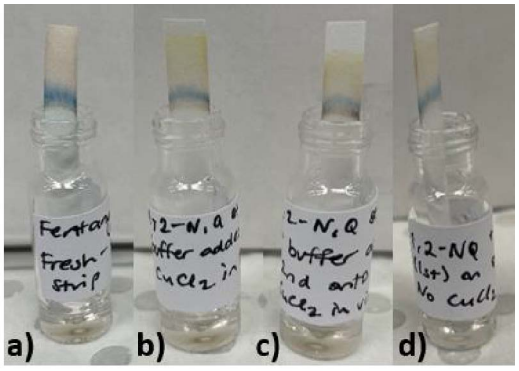

Figure 4-27: Resulting colour changes with fentanyl for a) 30 µL mixed reagent and 5 µL buffer on paper; b) 5 µL buffer followed by 25 µL 1,2-N,Q on paper, vial containing 5 µL $CuCl_2$ and 50µL drug; c) 25 µL 1,2-N,Q followed by 5 µL buffer, vial containing 5 µL $CuCl_2$ and 50 µL drug; d) 5 µL buffer followed by 25 µL 1,2-N,Q on paper, vial containing 50 µL drug

Ideally, the test paper would be dry for easy access in the field, however, the quinone appeared to degrade once dried, changing from yellow to a brown colour at which point the reagent lost its effectiveness. Dried papers did not produce a blue colour change with fentanyl, rather a brown line was seen and was not a selective colour change. Resulting colour changes of the initial assessment of the selectivity of the dried papers with heroin and morphine can be seen in Figure 4-28. Extensive attempts were made to rectify this issue, including altering storage conditions, quinone concentrations, solvent compatibility, variation in buffer additions, order of reagents, light source variations, and the use of a surfactant. Despite the efforts made, no changed variable resulted in a blue colour change with fentanyl using 1,2-naphthoquinone when the paper was dried.

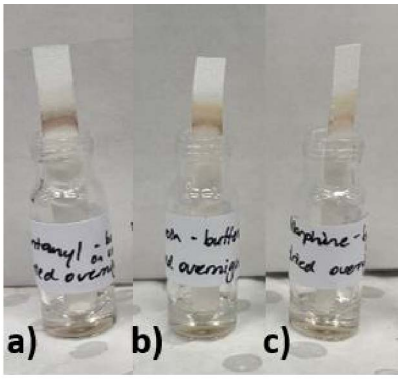

Figure 4-28: Resulting colour changes for a) fentanyl; b) heroin and c) morphine tests on dried overnight papers containing mixed reagent and buffer

4.5.2.2 *Effect of UV intensity on colour change*

The colour changes observed after exposure to the different UV light sources are shown in Table 4-15. The best results were seen with the 12 W light box and the 6 W TLC lamp. Both light sources provided similar blue colour changes within a similar time frame of less than 30 min. The tests conducted with the 1 W UV torch did not show a blue colour change though did show a green colour, more indicative of the reaction having not gone to completion.

The Polilight proved successful however only when used on a low power setting (P1). The two higher power settings resulted in no colour change with the fentanyl and also a loss of colour in both the fentanyl sample and the reagent blank solutions. Based on these results, all further testing utilised either the 12 or 6 W UV lights.

Table 4-15: Results of UV light sources on the colour change seen with fentanyl and the 1,2-naphthoquinone test method

		Results [a] – 10 min post UV exposure			
	Reaction condition	**Mixed reagent**		**Quinone only**	
	Sample	**Fentanyl**	**Blank**	**Fentanyl**	**Blank**
Light source	TLC light box (365 nm, 6 W)	blue	yellow	blue	yellow
	UV light box (365 nm, 12 W)	blue	yellow	blue	yellow
	UV torch (365 nm, 1 W)	green	yellow	green	yellow
	Polilight (350 nm, P1)	grey-blue	yellow	green-blue	yellow
	Polilight (350 nm, P4)	lt. pink-orange	lt. pink-orange	p. yellow	p. yellow
	Polilight (350 nm, P7)	p. pink-orange	p. pink-orange	p. yellow	p. yellow

[a] *lt. = light; p. = pale*

4.5.2.3 *Other quinone reagent testing*

The tested quinone reagents displayed no reaction with fentanyl in solution after 10 min exposure to UV. Four quinones (see Table 4-16) showed colour changes when applied to paper and tested with fentanyl. Of these, 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone showed the best colour change in terms of contrast with the reagent blank and so it was investigated further. Once again, with these tests, different results were observed between solution and paper-based test methods. As previously discussed, regarding the TCBQ test method in Section 4.2.2.1, the chemical or physical properties of the paper and chromatography technique may have influenced these differences. In particular for these quinone reactions, the speed of the reaction on paper vs in solution may be different and the intermolecular bonds to the paper substrate may have affected the reactions and therefore the colour changes that were seen.

Table 4-16: Colour change results of new tested quinone reagents with fentanyl in solutions and on paper

	Solution [a, b]		**Paper** [a, b]			
			Quinone only		**Mixed reagent**	
Quinone reagents	**Blank**	**10 min**	**0 min**	**10 min**	**0 min**	**10 min**
2-hydroxy-1,4-napthoquinone	yellow-orange	NC	NC	NC	NC	NC
5,8-Dihydroxy-1,4-napthoquinone	red	NC	NC	NC	NC	lt. orange
Chloranilic acid	p. pink	NC	NC	NC	NC	NC
2,3-dichloro-5,8-dihydroxy-1,4-napthoquinone	d. pink	NC	NC	NC	purple	dark blue
2,3-dichloronapthoquinone	white	NC	NC	NC	NC	NC
2,3-dichloro-5,6-dicyano-1,4-benzoquinone	pink-orange	NC	NC	NC	NC	pink-orange
1,4-anthroquinone	yellow	NC	NC	NC	NC	NC
1,4-dihydroxynapthalene	white	NC [c]	NC	NC	NC	NC
Quinizarin	yellow	NC	yellow-orange	NC	yellow-orange	NC
1,4-dihydroxynapthoquinone	yellow	NC	yellow-orange	NC	yellow-orange	NC

[a] *Indicated time is after 10 min UV exposure.*
[b] *NC = no colour change observed; lt. = light; p. = pale; d. = dark*
[c] *Fluoresced under UV*

4.5.2.4 *2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone colour test*

Testing with freshly prepared papers containing 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (150 ppm) and the $CuCl_2$ solution added separately, produced a blue colour change 10 min after exposure to UV light. The quinone did not react in the same manner without the $CuCl_2$ and remained pink (see Figure 4-29) similar to the reagent blank. Once the papers were dry, the quinone did not produce a strong blue colour, but rather a grey. As a result, the reagent required further optimisation to obtain a suitable result for the dried test, as discussed further in section 4.5.2.5.

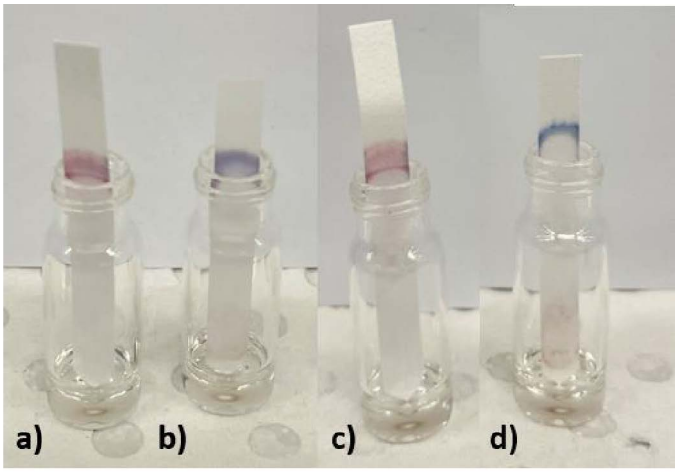

Figure 4-29: Results of 2,3-dichloro-5,8-dihdroxy-1,4-napthoquinone test with fentanyl; a) immediately after UV; b) with $CuCl_2$ immediately after UV; c) 10 min after UV; and d) with $CuCl_2$ 10 min after UV

The initial test to compare the fentanyl result to a reaction with heroin showed a purple colour change immediately after UV exposure for both tests. However, after 10 minutes, the colour change with the heroin had faded to a pink colour comparable to the reagent blank while fentanyl had darkened to the blue colour expected for a positive result (see Figure 4-30). This was positive sign in terms of the discrimination of fentanyl from other opioids using this reagent, so testing and optimisation were continued.

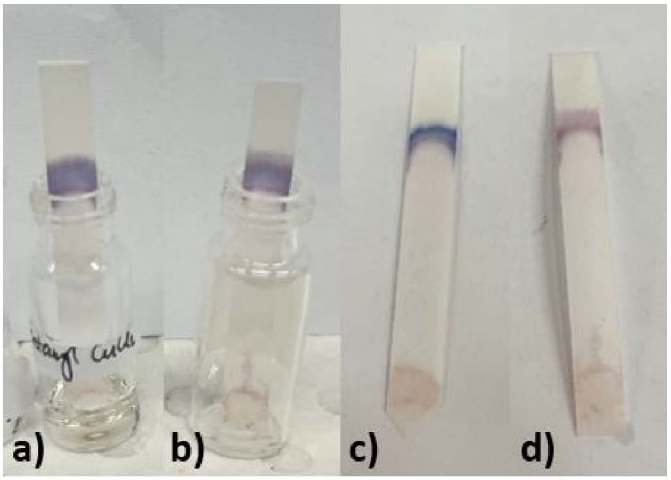

Figure 4-30: Colour change results of 2,3-dichloro-5,8-dihdroxy-1,4-napthoquinone, $CuCl_2$ and buffer with; a) fentanyl and b) heroin immediately after UV and c) fentanyl and d) heroin 10 min after UV

4.5.2.5 *Reagent optimisation*

After testing the range of concentrations of 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone on freshly prepared as well as dried papers, the quinone was found to perform best at a concentration of 300 ppm. While a positive result was observed with higher concentrations, the increased concentration results in a colour interference. The excess quinone spreads along the test paper and makes interpretation more difficult in comparison to the lower concentration results. This is demonstrated in Figure 4-31 when using freshly prepared test papers. The reagents were added to the test papers separately in the order buffer, copper chloride, and finally quinone solution. After drying the papers, this application method did not result in a prominent colour change. A very faint blue colour change was observed which was not ideal due to difficulties with interpretation.

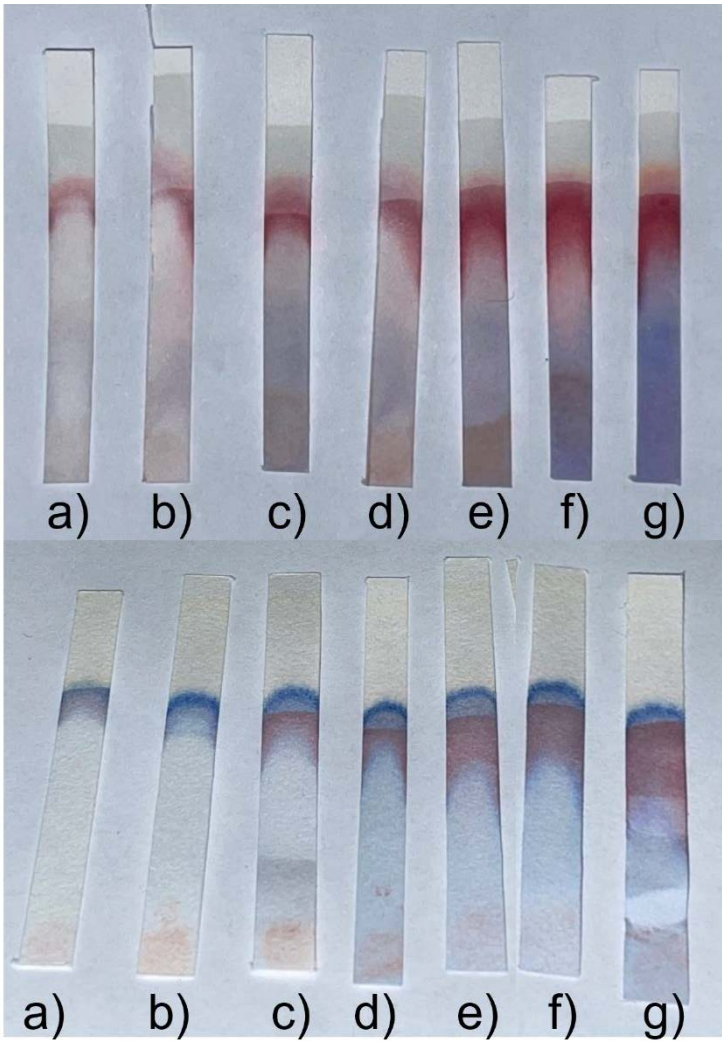

Figure 4-31: Resulting colouration of the test strips after addition of increasing concentrations of quinone reagent in increasing concentration order a) 300, b) 450, c) 600, d) 750, e) 1000, f) 1200, g) 1400 ppm with reagent blanks (top), and 50 µg fentanyl (bottom)

Further attempts were made to increase the prominence of the colour on dried papers. This was eventually achieved through the use of the 5:1 mixed reagent of the 300 ppm quinone solution, results of which are shown in Figure 4-32. A clear blue line is visible on the strips and is an improvement in comparison to separate reagent addition; the modified method involved buffer addition followed by addition of the mixed reagent. The papers were blue immediately after 10 minutes of UV exposure and lightened slightly after a period of 10 minutes.

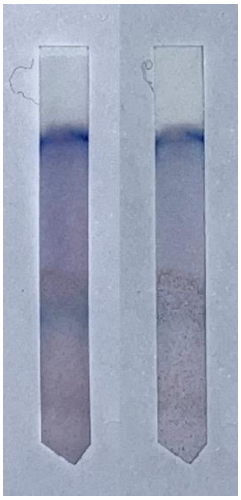

Figure 4-32: Dried 300 ppm mixed reagent and buffer (left) immediately after UV and (right) 10 minutes after UV

It was recognised that in the original 1,2-naphthoquinone test method, when testing 50 µg of fentanyl, the ratio of fentanyl to quinone is approximately 3:2. Due to an increase in molecular weight, this provides a theory as to why the 150 ppm 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone did not perform so well in comparison to the 300 ppm solution on dried papers. A 150 ppm solution of the 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone results in a ratio of 3:1, whereas increasing the concentration to 300 ppm results in a 3:2 ratio. Note that this ratio was not presented in the original work, but this observation may provide insight into the mechanism of action by which the quinone reacts with the fentanyl. Further investigations may focus on testing other quinone compounds. Various quinones were tested at a concentration of 150 ppm, though, if a fentanyl to quinone ratio of 3:2 is essential for a colour change these concentrations may need to be adjusted. Additional investigation into which quinone may be the most appropriate for the detection of fentanyl would be beneficial.

In terms of the various copper concentrations which were tested, it was observed that the 5 and 10 mM solutions produced pale blue results, as shown in Figure 4-33. In addition, it was observed that these reagents in solution had increased solid formation in solution in comparison to the 0.5 and 1.9 mM solutions. It was at this point that it was recognised that the copper was interacting with the quinone reagent in solution and possible substitution or complex formation was occurring. It was concluded that an increased copper concentration resulted in increased solid formation, thus reducing the effectiveness of the reagent. From this, it was concluded that the 1.9 mM solution was sufficient and the results were not significantly different from the 0.5 mM solution. The 1.9 mM solution was therefore used in the quinone mixed reagent solution.

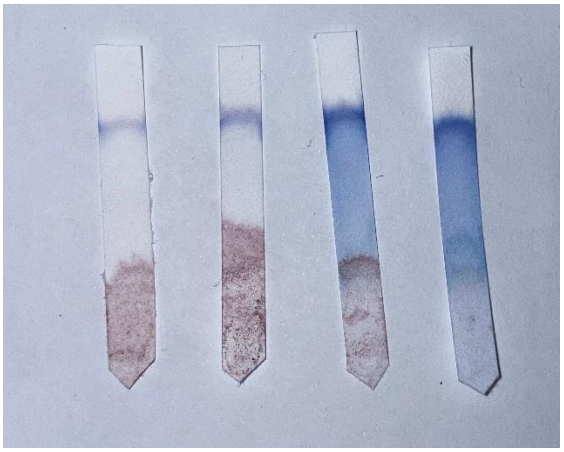

Figure 4-33: Fentanyl test paper results with varying copper (II) chloride concentrations left to right 10, 5, 1.9, and 0.5 mM

4.5.2.6 *Test paper conditions*

To aid with efficiency in the field, it is ideal to obtain a test method in which the papers could be dried and stored. While the reaction with fentanyl provides a stronger blue colour change using freshly prepared papers, this would not be feasible in a field setting. Therefore, dried test paper storage needed to be considered. Test papers which were prepared and stored in a loosely sealed scintillation vial for up to one week did not show signs of degradation or ineffectiveness of the reagent when tested with fentanyl and other various drug compounds. Additionally, papers which were dried for several days were also comparable to papers which were dried for a period of 2 hours. Thus, the storage conditions for the test papers seem to be reasonably simple; the papers do not need to be stored in conditions more complex than a sealed container. Further results of the storage and stability of these test papers is outlined in Section 4.5.2.8.

4.5.2.7 *Colour test in solution*

Initially, the colour test was performed in solution using the 150 ppm 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone solution. This did not result in a colour change even though the paper containing $CuCl_2$ exhibited a blue colour change after UV. After settling on 300 ppm as the optimal concentration, the solution test was reattempted. Colour changes were observed, thus supporting the theory that a fentanyl to quinone ratio of 3:2 is required for the colour change. A summary of the various solution test results is provided in Table 4-17. Solutions without buffer did not exhibit a colour change at all. Additionally, none of the solutions exhibited a colour change after UV exposure, i.e., any colour change happened before UV exposure. A solution containing the

quinone and the buffer immediately changed colour upon drug addition. This solution is of interest because no copper is present, yet an immediate colour change is observed. The reverse is true for the paper test in that copper is required for a colour change. Though it is unclear why the copper solution is required for a colour change on paper-based tests and not solution-based tests, it is possible that the copper ions assist in the absorption or stabilisation of the quinone to cellulose within the porous paper substrate. Quinones are commonly used as dyes, and it is reported that due to the polar nature of both cellulose fibres and many quinones bonding between the two is inhibited [295]. To combat this issue, metal ions are used as mordants which prevent the negatively charged cellulose surface and reagents from repelling each other. However, a similar solution with the addition of $CuCl_2$ solution was immediately blue before drug addition and remained blue after drug was added. Due to the turquoise colour of the $CuCl_2$ solution, the use of this solution may lead to false positive results when combined with the other reagents. This combination is therefore not suitable for the test in solution, however, can be utilised for the paper test.

Table 4-17: Colour observations before and after addition of fentanyl in various reagent solutions

Reagent solution	Colour before fentanyl addition	Colour after fentanyl addition
300 ppm quinone	red	red
300 ppm quinone + buffer	dark red	dark blue
300 ppm mixed reagent	red	red
300 ppm mixed reagent + buffer	dark blue	dark blue

4.5.2.8 *Stability and storage of test papers*

Dried papers which were stored in sealed containers under two lighting conditions underwent a blank and three fentanyl tests. Results of lighting conditions did not indicate significant differences between light or dark storage. Only very minimal degradation of the reagent was evident, if at all. In Figure 4-34, the minimal difference between the fentanyl tests for each storage condition can be observed.

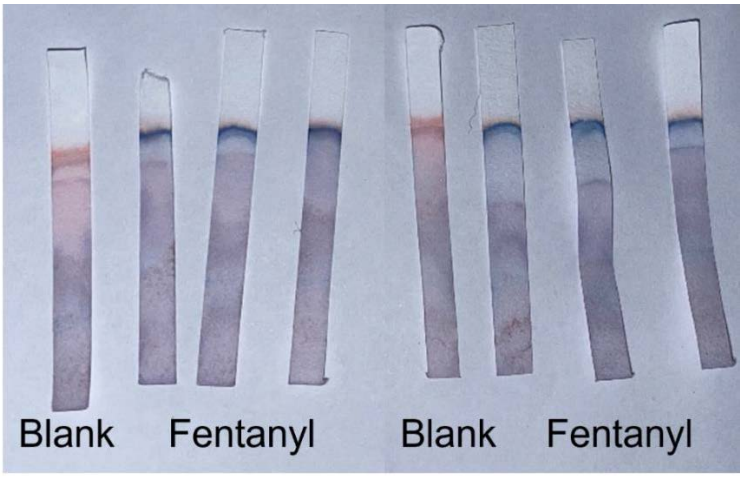

Figure 4-34: Storage test results after 2 weeks for paper stored in darkness (left) and light (right). A blank and three fentanyl replicates are shown for each condition

It could be said that the papers which were stored in the light may appear to be slightly paler than the tests which were stored in the dark. Neither of the blanks exhibited a colour change nor an unexpected result. For each lighting condition, all three fentanyl tests were strongly blue and did not exhibit inconsistencies in terms of the colour change.

While the test papers were shown to maintain their effectiveness, the product which was formed did not exhibit the same properties. Test papers that had been tested with fentanyl did not maintain the blue colour and rather faded to a blue-grey colour. Similarly, selectivity tests with other compounds also faded. However, they faded to various colours such as pink, grey, or purple-grey. It was observed that after fading, these fentanyl papers maintained more of a blue hue than other compounds.

4.5.2.9 *General recommended procedure*

After this stage of testing, a proposed method for fentanyl detection was determined and utilised throughout all further testing procedures including method validation procedures. The recommended method is as follows:

To prepare a dried test paper, add to the tip of a 4 cm x 5 mm rectangular strip of Whatman No. 1 filter paper:

1. 5 µL 0.1 M phosphate buffer
2. 30 µL of mixed reagent (2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (300 ppm) and $CuCl_2$ (1.9 mM) 5:1)
3. Dry papers in ambient conditions and store until required*

When required for testing:

4. Add 50 µL of a liquid or dissolved sample to a GC vial or similar
5. Add test paper to vial, ensuring paper is in contact with solution
6. Expose to 365 nm light for 10 minutes
7. Observe colour change

*Papers can also be used fresh, i.e., at any time after allowing excess solvent to evaporate for approximately 1 minute.

4.5.2.10 *Precision*

The results for each replicate were comparable to each other and no major differences were observed, as presented in Figure 4-35. Although the blanks showed no indication of a positive result, the intensities of the pink result were shown to vary. However, the intensity of the pink colour does not have any significance in terms of result interpretation. Apart from the test line on the paper, each paper differs in appearance along the length of the strip. This is due to the method of test paper preparation and the variability involved with non-automated work.

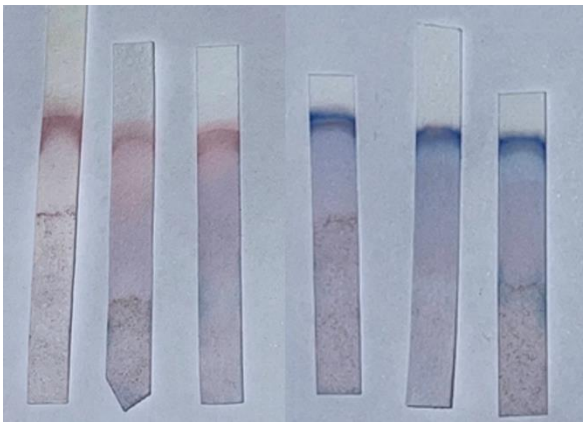

Figure 4-35: Colour change results of dried paper triplicates. Left: blank and right: fentanyl

Similar results were seen with the set of ten replicates prepared with separate reagent solutions. Immediately after UV exposure, half of the tests did not show the prominent blue colour change expected with this method. However, within 10 minutes, all replicates provided the blue coloured line on the paper, as has come to be known as a positive result. Over time these colours deepened to become darker and brighter blues as is shown in Figure 4-36c).

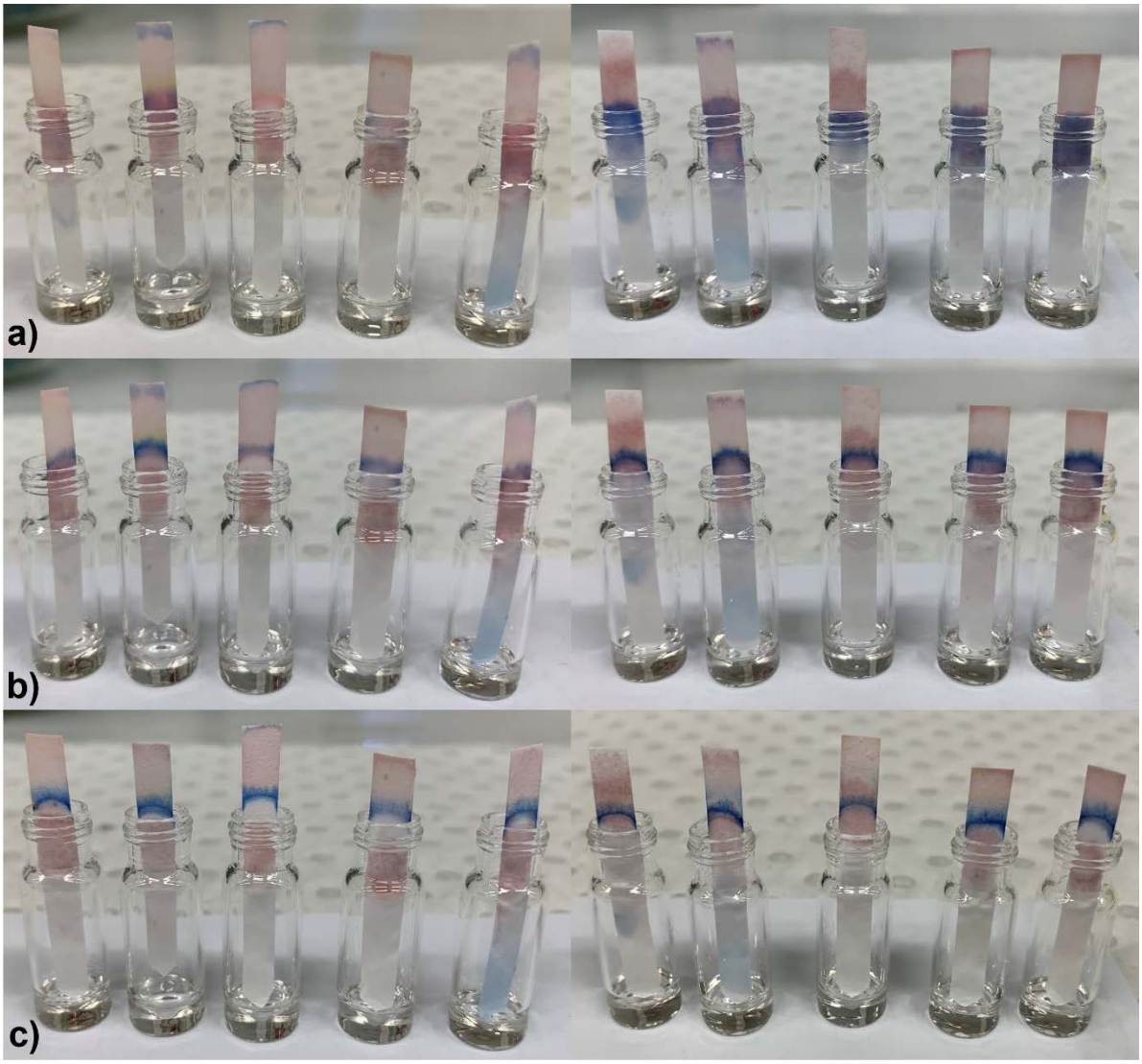

Figure 4-36: Colour change results of repetitions of 50 µg fentanyl with the 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone test method: a) immediately after 10 min of UV exposure, b) 10 min after removed from UV and c) 60 min after removed from UV

4.5.2.11 *Specificity and selectivity*

For comparison purposes, both quinone reagents underwent selectivity and specificity analysis. A variety of other drug compounds, cutting agents, and fentanyl analogues underwent specificity and selectivity testing with the mixed reagent. It was observed that compounds containing amine groups generally produced a positive result. Through a simple comparison, it was observed that 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone is not as selective as 1,2-napthoquinone. This is a limitation of 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone; while the reagent has the ability to test compounds using dried papers (unlike 1,2-naphthoquinone), there is an increased likelihood of false positives (see Table 4-18).

It was found that none of the tested opiates gave a positive blue colour change result. This is beneficial in that this test appears to be able to distinguish fentanyl from other opioids. Fentanyl-type substances have been known to be present in samples of other opioids such as heroin and synthetics [109], thus the detection of its presence would be valuable. The fentanyl analogues which were tested, all showed positive colour change results with the new quinone apart from the therapeutic fentanyls, remifentanil and alfentanil. This observation had been made in the original development of the test with 1,2-naphthoquinone in solution. It was theorised that this was potentially due to the substitution on the piperidine ring preventing the reaction from going ahead (see Figure 4-37) as most other fentanyl analogues do not contain this substitution. A predicted mechanism indicates that a hydrogen in this position is required for the product to be formed (see Figure 4-38) and so without it, no colour change would occur. It was also noted that β-hydroxyfentanyl did not react with 1,2-naphthoquinone, however this fentanyl analogue reacted with 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone.

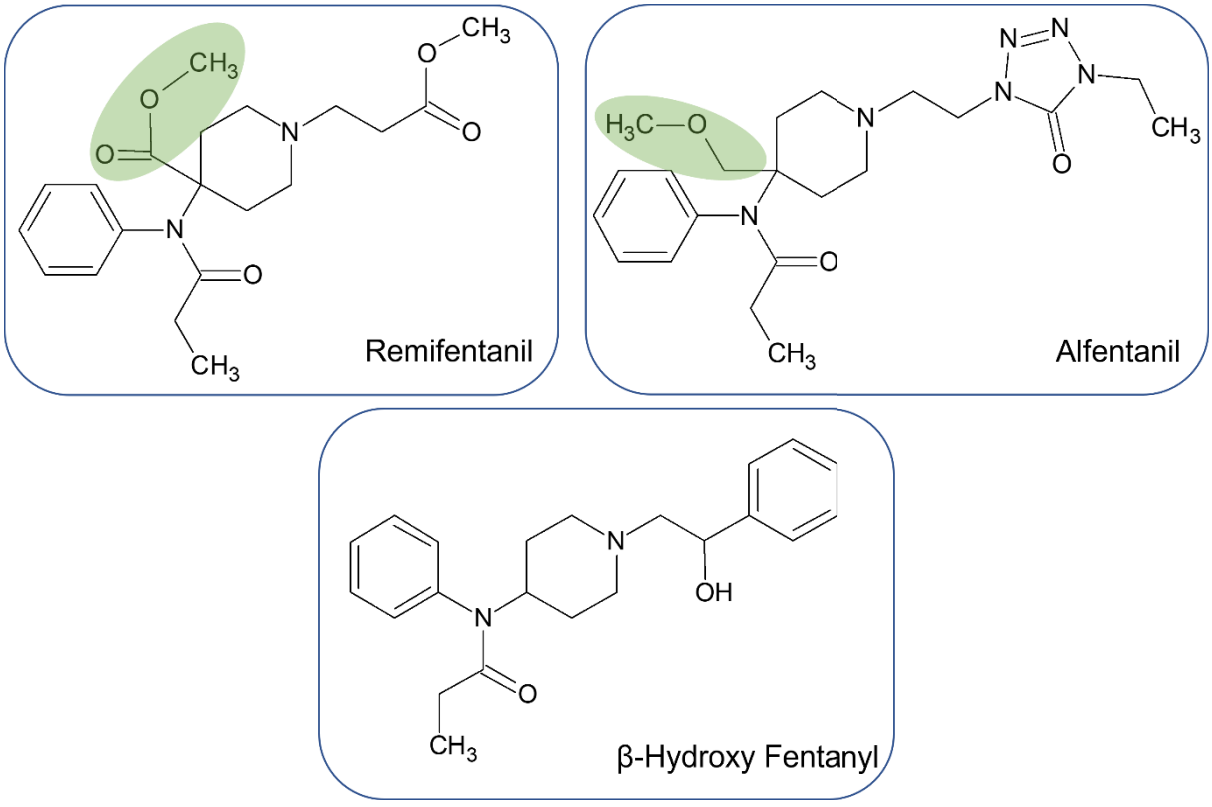

Figure 4-37: Structures of remifentanil and alfentanil (top) with piperidine ring substitution highlighted in green, and β-Hydroxy fentanyl (bottom) to show structural differences

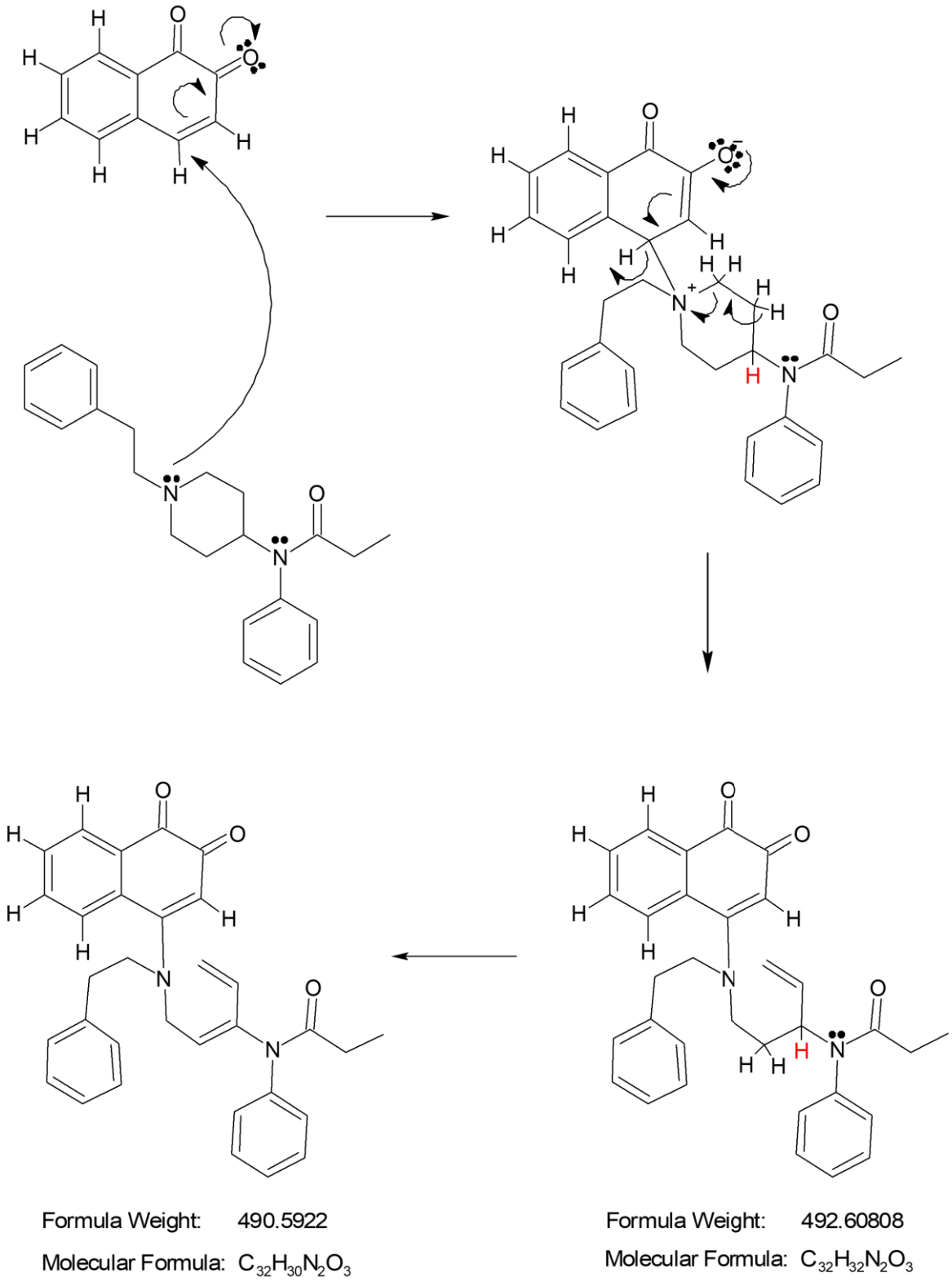

Figure 4-38: Predicted mechanism of reaction for 1,2-napthoquinone and fentanyl. The hydrogen likely involved in the formation of the product has been highlighted in red

Cathinone analogues appear to be the most reactive drug class with these reagents other than the fentanyls themselves. Four cathinones showed a colour change with 1,2-napthoquinone, and all but one exhibited a reaction with 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone. Of the eight cathinone analogues which were tested, only methylenedioxypyrrolidinobutiophenone HCl did not show a colour change with 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone. A trend was observed with the cathinones that reacted with 1,2-naphthoquinone. It appeared that the pyrrolidine ring containing cathinones including Pyrovalerone, 3,4-MDPBP, MPPP, and MPBP gave a grey or grey-blue colour change, which was the most similar to that seen with fentanyl than any other tested compound. It was theorised that the 1,2-naphthoquinone may react with fentanyl on the piperidine ring and therefore reacted comparably with the pyrrolidine ring containing cathinones.

The results with 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone, however, do not appear to follow the same trend. While the fentanyls showed little difference in reactivity between the quinones, the reaction of both other cathinone analogues and several other amine containing drugs indicates that the 1,2-naphthoquinone and 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone do not react with these compounds via the same mechanism, which also suggests a different reaction mechanism with the fentanyls.

The phenethylamine and amphetamine type drugs did not show a distinct trend in reactivity with 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone. The secondary amine containing NBOMe compounds provided a positive result as did methamphetamine, though dexamphetamine reacted while the 2C-I did not. Due to the limited amount of testing with this type of drug, not much can be suggested in terms of the reactivity of the quinone with these drugs overall. The other drug class which indicated reactivity with the 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone reagent was the piperazines. While only three analogues were tested, two indicated a positive result. Additional testing with piperazines would be required to make an informed decision on the reactivity with the quinone reagent. However, Philp et al. [196] proposed a reaction mechanism for piperazines in the development of colour spot method. It was suggested that the piperazine underwent product formation with the reagent through nucleophilic substitution with the amino group on the piperazine. The quinone reagent contains two chlorine substituents which could react via a nucleophilic substitution with the piperazine amino group. The lack of reaction for the piperazines with 1,2-napthoquinone may support this theory.

Table 4-18: Selectivity results with both quinone reagents investigated for the fentanyl test

		Colour change results [a]			
		1,2-Naphthoquinone		**2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone**	
Class	**Compound**	**Immediate result**	**10 mins post UV exposure**	**Immediate result**	**10 mins post UV exposure**
Fentanyl analogues	Fentanyl HCl	blue	blue	purple	blue
	Alfentanil	NC	NC	purple	purple
	Benzodioxole fentanyl	lt. blue	darkened blue	blue edges	blue edges
	Cyclopropyl fentanyl	p. blue	darkened blue	blue	blue
	4-fluoroisobutyrylfentanyl	p. blue	NC	blue	blue
	β-hydroxyfentanyl	NC	NC	blue	blue
	Phenylpropionyl fentanyl	strong blue	darkened blue	blue	blue
	Remifentanil	lt. brown	slight darkening	NC	NC
Cathinone analogues	4-FMC HCl	lt. brown	slight darkening	purple	grey
	4-MEC	NC	NC	blue edges	p. blue
	4-MMC	lt. brown	slight darkening	purple	grey
	3,4-MDPBP	lt. blue	lt. blue-grey	pink	pink
	Methylone HCl	lt. brown	slight darkening	blue	blue
	MPBP	grey	NC	blue	blue
	MPPP	grey	NC	blue	blue
	Pyrovalerone	grey	Pale brown	purple	grey
Piperazines	BZP	NC	NC	pink	pink
	1-(3-chlorophenyl) piperazine HCl	NC	NC	blue	p. blue
	1-(3-trifluoromethylphenyl) piperazine HCl	NC	NC	blue	p. blue
Phenethylamines	25D-NBOMe	lt. brown	NC	blue edges	p. blue
	25I-NBOMe	lt. brown	slight darkening	blue	NC
	25G-NBOMe	lt. brown	NC	blue	purple
	2C-I	lt. brown	slight darkening	pink	pink
	Cocaine HCl	lt. brown	slight darkening	blue	lt. purple

Other substances of abuse and precursors	Codeine phosphate	lt. brown	slight darkening	pink	pink
	Dexamphetamine sulphate	lt. brown	slight darkening	blue	blue
	Ephedrine	lt. brown	slight darkening	pink	pink
	Heroin	lt. brown	slight darkening	purple-grey	grey
	JWH-073	lt. brown	slight darkening	pink	pink
	Methamphetamine	lt. brown	slight darkening	blue	blue
	Methylamine	lt. brown	slight darkening	purple	pink
	Morphine HCl	lt. brown	slight darkening	pink	pink
	Pseudoephedrine	lt. brown	slight darkening	pink	pink
	THC	lt. brown	slight darkening	blue edges	blue edges
	UR-144	lt. brown	slight darkening	pink	pink
Licit compounds and cutting agents	Ascorbic acid	lt. brown	slight darkening	pink	pink
	Aspirin	lt. brown	slight darkening	pink	pink
	Citric acid	lt. brown	slight darkening	pink	pink
	Glucose	lt. brown	slight darkening	pink	pink
	Glycerine	lt. brown	slight darkening	blue edges	blue edges
	Magnesium stearate	lt. brown	slight darkening	pink	pink
	NaCl	lt. brown	slight darkening	pink	pink
	Nortryptaline HCl	lt. brown	slight darkening	blue	blue
	Paracetamol	lt. brown	slight darkening	pink	pink
	Phenolphthalein	lt. brown	slight darkening	pink	pink
	Protryptyline HCl	lt. brown	slight darkening	pink	pink
	Ramipril	lt. brown	slight darkening	pink	pink
	Urea	lt. brown	slight darkening	pink	pink

[a] *NC = no colour change observed; lt. = light; p. = pale*

While the selectivity of this test is a limiting factor, the bright blue colour change seen with fentanyl analogues and no other opioids is a promising result. Further optimisation of this test and investigation into the mechanism of reaction, could allow for development of a more selective test method.

4.5.2.12 *Limit of detection*

Initial tests indicated that the LOD may occur between 15-25 µg. Many tests were completed to try and obtain a consistent result. However, unlike the initial tests for repeatability which showed fairly consistent results, the same was not observed during LOD testing and interpretation was slightly difficult. Various tests of 15, 20, and 25 µg were attempted. Results are presented in Figure 4-39.

Figure 4-39 a) - c) depict the results of tests carried out on 15 µg of fentanyl. While blue colours were observed on several test papers, Figure 4-39 a) and c) demonstrate the inconsistencies with the test results. In these two sets, one of the three papers is a prominent blue colour while the other two papers exhibit very minimal blue colouring. Figure 4-39 a) shows an example of the variation in the blue colour which can be observed as the blue is more faint and greyer in colour. Similar inconsistencies were observed for the 20 µg tests where as in Figure 4-39 f), one test paper shows an extremely bright blue colour change, while the other two tests in that set show faint grey-blue colours. Figure 4-39 d) and e) differ in terms of an inter-test comparison, with a fainter blue colour change seen in d). However, within these two sets the colour changes are comparable to one another. It was seen that at 25 µg, the results appeared to be more consistent with two of three sets (Figure 4-39 g) and i)) indicating positive results across the three repetitions. Once again, however, this result is not observed in Figure 4-39 h) with a much fainter blue-grey colour change in comparison.

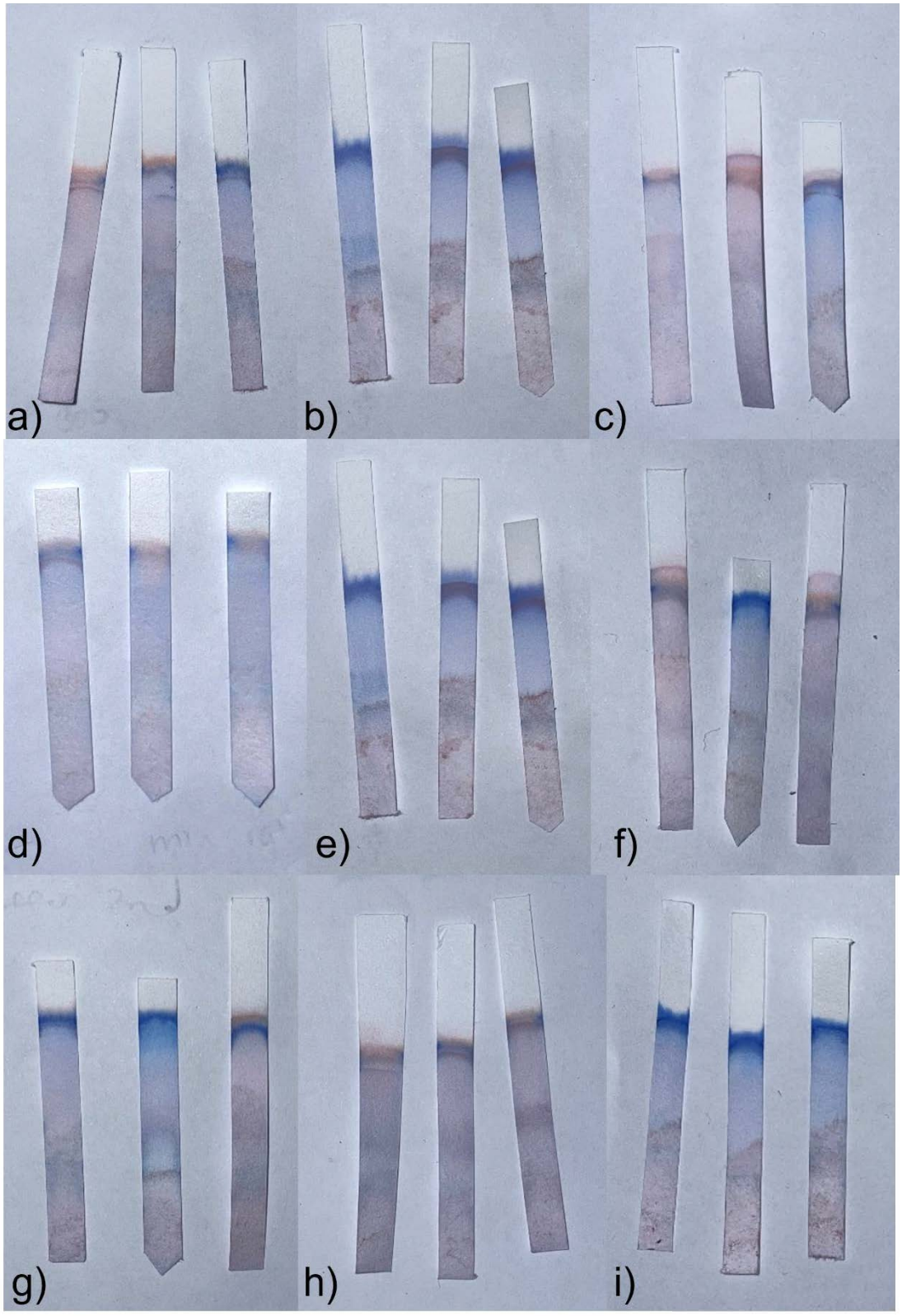

Figure 4-39: Colour change results of LOD testing repetitions with fentanyl at: 15 µg (a-c), 20 µg (d-f) and 25 µg (g-i)

From the results depicted in Figure 4-39, it was observed that the repeatability of the test requires improvement. While a definite LOD could not be determined due to both inter- and intra-test inconsistencies, it can be proposed that the LOD for fentanyl may exist around 20-25 µg, due to more prominent blue colour changes observed at these amounts.

The NIJ Colour Test Standard guidelines for the determination of drug detection limits recommends testing five samples of the same concentration to determine the LOD. Here, a minimum of nine tests were completed for these three concentrations. Even considering the inconsistencies across these tests, hence suggesting reproducibility needs improvement, there were five replicates at 25 µg which afforded the bright blue colour change result. Based on this, it would be suggested that the LOD be 25 µg of fentanyl and as such the operational detection limit at 250 µg. The illicit sale of fentanyl is typically in terms of "per gram" [296], thus this detection limit is suitable for law enforcement purposes. Further work is still required to improve the reproducibility of this colour test in a paper-based system which may in turn effect the LOD.

4.5.3 Conclusions

During this study, the effect of addition of buffer to the mixed reagent was not extensively studied. A brief test observed potential for this combination to still produce a colour change with fentanyl, therefore future research could investigate this to aid in step minimisation. While initial tests indicated that the reproducibility of this test was sufficient, further tests such as the LOD tests seemed to contradict this. A time study may also be beneficial in terms of reagent additions to the paper as well as extensive comparisons of results over various time intervals. Such a study may determine optimal time intervals during paper preparation, and a suitable time range between preparation and drug detection and thus may improve the reproducibility. A stability study should also be conducted over a longer time period. Finally, more investigations into 1,2-naphthoquinone may result in the development of a more selective test. While extensive tests were completed on dried papers of 1,2-naphthoquinone, no variables were shown to improve the colour change. Future works could focus on research into this compound and thus draw a conclusion as to its suitability for a paper-based test.

4.6 Discussion

The studies to date have shown the potential for paper-based systems to be used as a medium for the previously developed colour tests. Here, the simplest of microfluidic devices was utilised, however, a more advanced device could be produced to allow for portability and field use. Figure 4-40 shows a simple wax printed microfluidic system. It is labelled to reflect its use for the phenethylamine test method, however, could be applied to any of the tests outlined in this chapter. The use of such a system simply allows for a more focussed colour detection area, rather than the reaction occurring anywhere on a paper strip. This could assist in the correct interpretation of results. Reagent and solvent volumes would require optimisation to be used with this type of system, as was seen with the µPADs tested with the phenethylamine test.

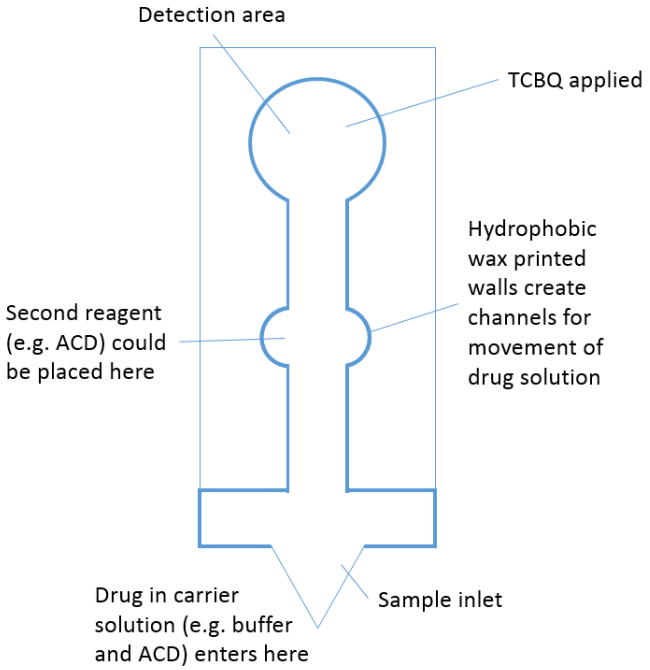

Figure 4-40: Microfluidic device design, individual channel for use with one reagent method

With further development these paper-based tests could provide an inexpensive way to bring these colour tests into the field, potentially in a single device. Further research and optimisation are required for all four tests in different areas. Each test would benefit from an advancement of the storage time of test papers to ensure they are field appropriate. This would allow for the methods to be used as part of testing kits for law enforcement, healthcare services, and harm minimisation settings. The incorporation of multiple tests in one device could provide a beneficial system to identify NPS in the field. Integrating multiple reagents, and hence test results, would

allow for not only a higher level of selectivity but also the ability to use a single device to detect a range of analytes. This has shown to be successful in the detection of other illicit drugs using paper-based systems [230, 231]. These methods indicate how simple colour tests can be combined to provide the analyst with a great deal of information about the sample. The device diagram in Figure 4-41 displays just one possibility for the design of a multiplexed system. The incorporation of multiple channels, each containing a different reagent test, allows for multiple colour change results which can be combined to gain information about the analyte.

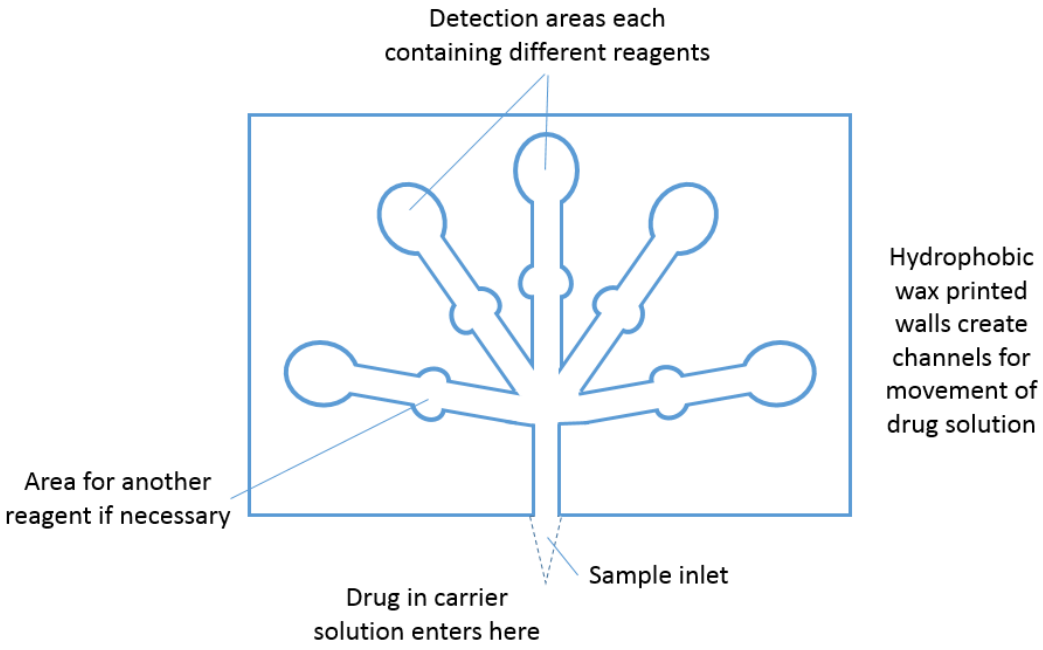

Figure 4-41: Multiplexed microfluidic device design, for testing with multiple reagents in multiple channels for a more discriminative result and more versatile design

Resulting colour codes could be identified for each drug or drug class to limit the issues associated with potential interferants and false positives. By combining the colour changes from each test for a particular drug, this could provide the analyst with a more discriminative result than what can be gained from a single colour test. This type of device and analysis is not only useful in a forensic sense but would be highly beneficial for harm minimisation purposes. Potential drug users would be able to gain more accurate information about the substance they possess before choosing to consume it. These colour codes, with much more investigation, could even include cutting agents and more complex mixtures of drugs and other compounds. Table 4-19 shows the possible colour codes for six NPS which were tested in this study. It can be seen that, by combining the results of all four tests, all six of these drugs could be easily distinguished from one another. Even those which provided the same or highly similar colour changes with one test, could be identified overall.

Of course, this is a small set of substances that are being compared and the analysis of a wide variety of compounds would be required for this type of analysis to become beneficial for real case samples. The inclusion of a simple detection system, such as a mobile phone application, should also be considered to provide a higher degree of discrimination between samples. It would be ideal for a detection system to be able to provide a suggestion of the composition of an unknown substance based on the colour codes provided. This would require extensive testing and analysis to be able to provide accurate results for a variety of illicit substances.

Table 4-19: Colour changes with all four colour tests for a variety of NPS

	Test and colour change [a]			
Drug	**TCBQ**	**NQS**	**Neocuproine**	**DDNQ**
25-NBOMe	blue	pale orange	NC	light blue
4-MMC	pale blue	NC	orange	purple/grey
BZP	NC	orange	NC	pale blue
Fentanyl	NC	pale orange	NC	blue
JWH-073	NC	NC	light orange	NC
2C-X	green	pink-orange	NC	NC

[a] *NC = no colour change observed*

4.7 Conclusions

The four colour test methods show potential to be utilised as paper-based systems. The phenethylamine test remains highly selective on paper and shows an adequate level of precision. This method does, however, require further optimisation of its storage as a paper-based system along with investigation into lowering the limit of detection. The stability of the reagents on paper for the piperazine test showed the best results and promise for longer storage times. The limit of detection for this method remained low though it was noted that the selectivity was diminished on paper. Both the cathinone and fentanyl test methods require more optimisation. While these methods provide strong colour changes for their target analytes, they both display some limitations as paper-based systems. The cathinone test shows inconsistencies across tests and at times will not provide the orange colour change within 20 minutes. The fentanyl test method requires further investigation into an appropriate quinone reagent, that is ideally highly selective

for these analogues. The lack of reproducibility of this test at lower concentrations is problematic and requires optimisation. The combination of these tests into a single device would reduce the impact of these limitations and potentially provide a more discriminative result to identify NPS in unknown samples.

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Chapter 5: Application of TCBQ colour test method for the detection of 25-NBOMe in oral fluid samples

Chapter 5: Application of TCBQ colour test method for the detection of 25-NBOMe in oral fluid samples

5.1 Introduction

Oral fluid (OF) testing has many advantages for drug analysis over other bodily fluids such as blood and urine. The non-invasive nature of the collection method, along with the added difficulty to adulterate, substitute or dilute samples makes oral fluid a desirable matrix for drug detection after recent usage [297, 298]. Roadside drug testing methods have changed in recent years and the interest in the use of oral fluid tests has increased as it shows effective implementation [299-301]. Depending on the jurisdiction, the range of drugs that can be analysed in oral fluid is often limited [300]. As with any presumptive on-site testing, confirmatory analysis is required for oral fluid samples collected in these settings [234].

There has been substantial evaluation into the capabilities of drug screening devices. Particularly in Europe, the performance of a range of commercially available devices has been compared and evaluated by utilising results of laboratory instrumentation [238, 302-304]. The most frequent devices are lateral flow-based and include the Dräger DrugTest 5000, the Securetec DrugWipe® 5, and the Mavand Rapid STAT® (shown in Figure 5-1). These, and other similar devices have been evaluated in several jurisdictions to help determine the applicability to an operational context. This is completed using consistent assessment of the sensitivity and reliability of the devices [245, 305, 306]. In Australia, Police jurisdictions use the DrugWipe® devices for roadside testing [307]. An early evaluation of this device for random roadside drug testing was conducted by Victorian Police in 2007 and determined sufficient sensitivity for recent use of both THC and methamphetamine [308]. The previously mentioned studies indicate the usefulness for these devices to identify those driving under the influence of drugs, though the sensitivity could be improved in many situations.

Figure 5-1: Commonly used oral fluid screening devices: a) Dräger DrugTest 5000 [309], b) Mavand Rapid STAT® [310], c) Securetec DrugWipe® 5 [307]

Laboratory based techniques including LC-MS/MS, have been shown to be able to identify NPS in oral fluid samples [248, 251, 311]. 25-NBOMe analogues are among these substances with some methods having detection limits at concentrations as low as 0.006 ng/mL [312]. Current onsite detection techniques and devices have validated methods for the analysis of traditional illicit drugs however, there is a lack of these methods which are able to selectively identify NPS [235]. The cross reactivity of several NPS has been investigated using screening devices. Novel amphetamine-type substances (n=39) were assessed with two devices. Two analytes (PMA and PMMA) demonstrated high levels of cross-reactivity for amphetamine/methamphetamine tests [237]. This indicates that the reliance on current devices is not feasible for the selective determination of NPS, not even for those which are structurally similar to the validated illicit drugs. Simple methods for the presumptive on-site detection of NPS and in particular, NBOMe compounds, would be beneficial to law enforcement and other testing situations. Currently these substances are not able to be detected, in oral fluid samples, outside a laboratory.

This chapter investigates the use of the described TCBQ colour test methods for hallucinogenic phenethylamines as a visualisation tool for the detection of these substances in oral fluid. The simplicity of this method is beneficial as it would require little expertise for analysis to take place. This is a proof-of-concept study, and the methods would require more optimisation to be appropriate for the detection of these drugs in case samples.

5.2 Materials and methods

5.2.1 Chemicals and reference materials

TCBQ, acetaldehyde, 1-butanol and Whatman No. 1 filter paper were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). Ethyl acetate, 1,4-dioxane, chloroform and methanol were from Chem-Supply (Gillman, SA, Australia). Glacial acetic acid was obtained from Scharlau Chemicals (Sentmenat, Barcelona, Spain) and aqueous ammonia solution (2 M) was obtained from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia). 25B-NBOMe, 25C-NBOMe, 25D-NBOMe, 25E-NBOMe, 25G-NBOMe, 25H-NBOMe, 25I-NBOMe and 25T-NBOMe, 2C-B, 2C-E, 2C-I, 2C-T-2, methamphetamine, MDMA were purchased as HCl salts from NMI (North Ryde, NSW, Australia). 30C-NBOMe and 25I-NBOH were obtained as HCl salts from Novachem (Heidelberg West, Victoria, Australia). 4-FMC, 4-MMC, methylone and JWH-073 were synthesised in house at UTS by the Centre of Forensic Science according to literature methods.

5.2.2 Oral fluid sample collection and preparation

Oral fluid samples were collected directly into a tube following approval from the UTS Human Research Ethics Committee (ETH18-2521). Analysis of oral fluid samples was always completed on the same day as collection. To prepare spiked oral fluid samples, the required volume of NBOMe solution (1 mg/mL in methanol) was added to a microcentrifuge tube and the solvent allowed to evaporate before addition of the oral fluid. Spiked samples were shaken thoroughly in an attempt to evenly distribute the drug throughout the oral fluid. Aliquots of this spiked sample could then be taken for analysis as required. The colour test reagents were prepared as outlined in Section 2.2.3. TCBQ was prepared at both 3×10^{-3} M and 6×10^{-3} M in ethyl acetate.

5.2.3 Direct analysis of spiked oral fluid samples

The application of colour test reagents directly to a spiked oral fluid sample was the first test completed. This was carried out using several NBOMe analogues (up to 100 µg) in 500 µL oral fluid by simply adding the reagents (100 µL each) as if it were a pure drug sample. The tube was shaken, and any colour change observed. This method was completed with both TCBQ concentrations for comparison, with NBOMe quantities outlined in Table 5-2.

5.2.4 Chromatographic analysis of spiked oral fluid samples

To assist in the analysis of these spiked samples, particularly at low concentrations, a simple chromatographic method was applied in an attempt to separate the drug from the oral fluid. Several mobile phase solutions were tested (see Table 5-1) to separate the analyte. These mobile phase solutions were prepared and allowed to equilibrate before analysis.

Table 5-1: Solvents tested with paper chromatographic system

Solvents	Ratio
1-butanol:acetic acid:water	4:1:5
1-butanol:acetic acid:water	78:10:12
chloroform:water	9:1
chloroform:ethyl actetate	1:1
1-butanol:dioxane:NH_4OH (2M)	4:1:5
chloroform:methanol	9:1
methanol	n/a
ethyl acetate	n/a

To determine the best system, the oral fluid samples (500 µL) were spiked with 25E-NBOMe (100 µL, 1 mg/mL) and 10 µL spotted onto a Whatman No. 1 filter paper strip (5 x 3 cm) alongside an oral fluid blank (see Figure 5-2). The paper strip was added to the solvent solution and removed once the solvent reached approximately 1 cm from the top of the strip. The system was then developed with a combined solution of the colour test reagents via capillary action. The results with each mobile phase were compared and the best performing was used for all further testing. As a comparative tool, the reagents were also applied through capillary action without the sample paper having the mobile phase step applied.

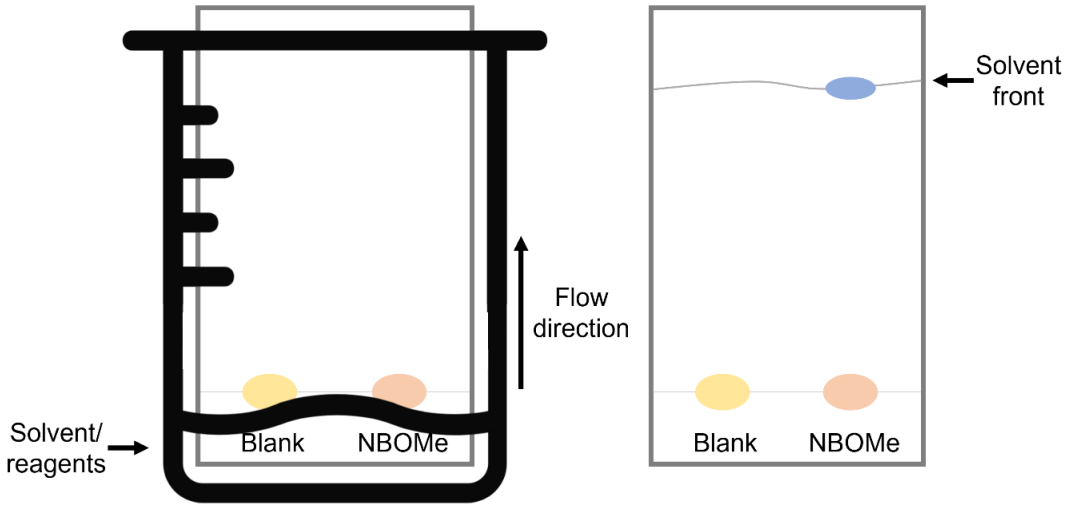

Figure 5-2: Diagram of chromatographic test setup (left) and a positive test result (right)

5.2.5 Method validation

To determine the potential usefulness of this method, several simple validation procedures were assessed including precision, selectivity and specificity, and the limit of detection. All validation procedures were carried out using the solvent step first before addition of the paper strip to the reagent solutions.

5.2.5.1 *Precision analysis*

The repeatability of this test was assessed through the analysis of 56 replicates at varying concentrations. Each test included both a blank sample and a NBOMe spiked sample at concentrations ranging from 400 – 2000 µg/mL. To the paper strip, 10 µL of either blank or spiked oral fluid was added before the chromatographic method was followed, and colour change results recorded.

5.2.5.2 *Selectivity and specificity*

The selectivity and specificity of this test were assessed through the analysis of 10 NBOMe analogues and a variety of other illicit substances including 2C-X analogues, cathinones, ATS, and a synthetic cannabinoid (see Table 5-3). Oral fluid samples were spiked as previously described to obtain concentrations of 500 µg/mL for each compound. For each test, 10 µL of the spiked oral

fluid sample was run alongside an oral fluid blank using the outlined chromatographic method. The colour change results were recorded and also compared to both true positive and true negative colour changes.

5.2.5.3 *Limit of detection*

The LOD was determined by the addition of decreasing amounts of 25H-NBOMe solution for spiked sample preparation. An aliquot of that sample could then be added to the paper strip for analysis using the chromatographic method and the colour change recorded. Final quantities in 10 µL of oral fluid ranged from 0.2 – 10 µg. The LOD would be assigned to the value where a colour change could be differentiated from the blank sample.

5.3 Results & discussion

5.3.1 Direct analysis of spiked oral fluid samples

Direct addition of the colour test reagents to spiked oral fluid samples did not initially provide a positive result with the 3×10^{-3} M TCBQ solution. A very pale blue tinge was observed for the two samples at 200 µg/mL but this was not seen with the lower concentrations. An increase of the TCBQ concentration to 6×10^{-3} M allowed for a bright blue colour change to be seen with 100 µg 25G-NBOMe, in 0.5 mL oral fluid, a value substantially higher than those able to be detected with the paper-based method.

Table 5-2: NBOMe analogues, their tested concentrations and the colour change results of direct oral fluid analysis

Drug	Drug amount (µg)	Equivalent concentration (µg/mL)	TCBQ concentration	Colour change
25H-NBOMe	100	200	3×10^{-3} M	v. pale blue
25E-NBOMe	100	200		v. pale blue
25I-NBOMe	50	100		NC
25I-NBOMe	5	10		NC
25G-NBOMe	100	200	6×10^{-3} M	bright blue
30C-NBOMe	65	130		lt. blue
25T-NBOMe	50	100		lt. green-blue
25I-NBOMe	5	10		NC

[a] *NC = no colour change observed; v. = very; lt. = light*

The blue colour sat as its own layer on top of the otherwise colourless oral fluid unless the tube was shaken (see Figure 5-3 a) and b)). Lighter blue and green-blue colour changes were seen with 30C-NBOMe (65 µg) and 25T-NBOMe (50 µg), both also in 0.5 mL oral fluid. It was observed that the colour change deepens after 5 min for these tests at lower concentrations.

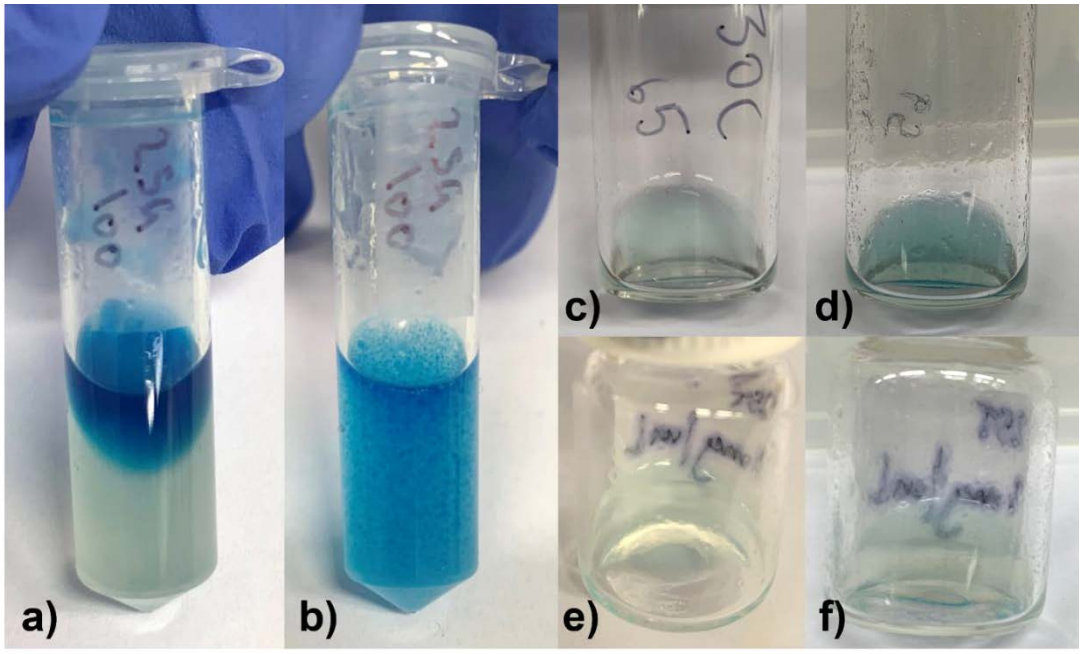

Figure 5-3: Colour change results of direct oral fluid tests with 6×10^{-3} M TCBQ: a) 25G-NBOMe immediately after addition of reagents, b) 25G-NBOMe 5 min after addition of reagents and solution shaken, c) 30C-NBOMe immediately after addition of reagents, d) 30C-NBOMe 5 min after addition of reagents, e) 25T-NBOMe immediately after addition of reagents, and f) 25T-NBOMe 5 min after addition of reagents

It is possible that lower concentrations of 25-NBOMe are not able to be visualised directly in oral fluid samples, due to the amount of dilution occurring and therefore very pale colour changes. Testing has not indicated that the presence of the oral fluid has any effect on the reaction of the colour test itself, so the dilution of the colour change seems to be a likely possibility. The same could be said regarding the lower TCBQ concentration, as lighter colour changes are seen with it in comparison to the 6×10^{-3} M solution.

It would be useful to assess the use of other collection methods, such as swabbing, in conjunction with this colour test. Considerations such as volume and as mentioned, concentration, would be required for it to become a valuable analysis method.

5.3.2 Chromatographic analysis of spiked oral fluid samples

It was determined that the chloroform and ethyl acetate (1:1) mobile phase was the most appropriate for this test method. It allowed for the drug to be moved up the paper strip and after addition of the test reagents, a blue spot was seen at the solvent front with the spiked sample. The blank sample showed no colour change (see Figure 5-4).

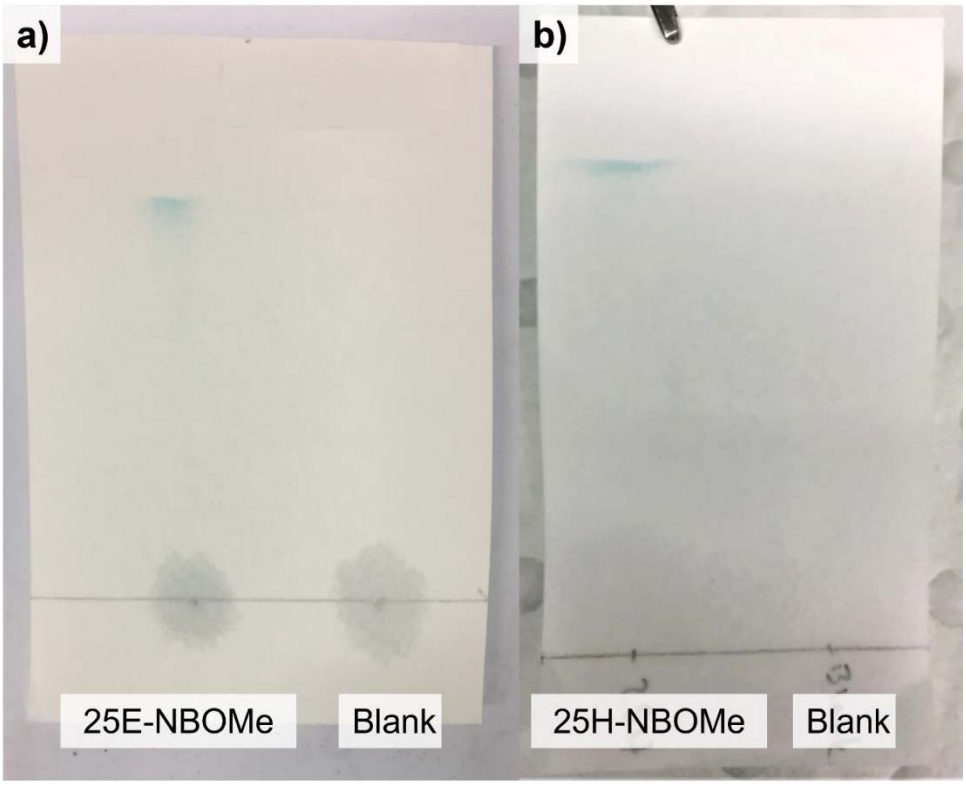

Figure 5-4: Results after application of chloroform/ethyl acetate followed by reagents with: a) 25E-NBOMe and b) 25H-NBOMe, both alongside blank oral fluid samples

Applying the reagents without the sample strip having the chloroform and ethyl acetate step applied, showed a slightly different result. A blue colour change was seen as a streak across the paper rather than as a clear spot at the solvent front (see Figure 5-5 a)). The application of the buffer to the oral fluid and then the addition of the paper into only a TCBQ and ACD solution mixture, showed a lighter green-blue colour change (see Figure 5-5 b)).

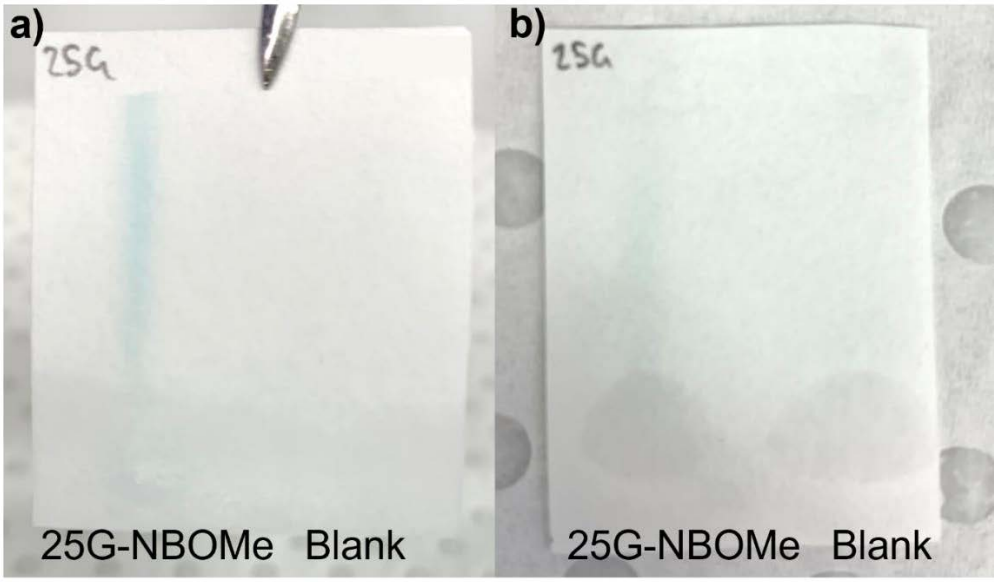

Figure 5-5: Colour change results of 25G-NBOMe spiked oral fluid comparing a) paper added to reagent mixture and b) buffer added to spiked oral fluid sample before addition to paper, paper added to TCBQ and ACD only

The use of the extra solvent step appears beneficial in terms of providing a clear colour change result. It should be noted however, that this step limits the practicality of this method regarding its use in the field as an on-site test method. Hazardous and volatile solvents are not ideal to be used outside the laboratory, particularly when considering effects from uncontrollable environmental factors. If a test such as this could be incorporated into a simple microfluidic-type device, the need for these solvents may be reduced or removed completely. The time required for this method to be completed should be further explored to make this a viable on-site testing method. While this study only completed testing of oral fluid samples, other biological matrices or even complex seized drug samples may also have the potential to be analysed using a similar method.

Oral fluid samples are known to contain primarily the parent drug for two reasons, passive diffusion allowing movement of small lipophilic molecules and also the direct absorption of a drug based on the consumption method [313]. To date there have been no studies on identifying 25-NBOMe metabolites in oral fluid samples and therefore this study was conducted using only the primary drug. 25-NBOMe metabolites have been identified using human hepatocytes and in authentic human samples, and this has shown that metabolism initially changes the substitution of the benzyl rings in the compound, including demethylation and hydroxylation [314]. This could indicate that even the presence of metabolites in an oral fluid sample would not change the results seen with this test method as they still contain the secondary amine and may also interact with the colour test method.

5.3.3 Method validation

5.3.3.1 *Precision analysis*

The results of the repeatability study showed promise. Of the 56 NBOMe spiked oral fluid samples analysed, 37 displayed a blue colour change and were determined to be true positives. This in turn lead to 19 false negatives of NBOMe samples not showing a blue colour change. These false negatives were noted to be those NBOMe analogues which provided the pale or light blue colour changes observed with the selectivity study. 25C-NBOMe and 25T-NBOMe were shown here to only provide a colour change above 600 μg/mL. No blank oral fluid samples showed a colour change with this test method, so no false positives were recorded.

5.3.3.2 *Selectivity and specificity*

In addition to the promising precision results, this test method did not show any false positives from the other substances tested. A pale blue and pale grey-blue colour change were seen for MDMA and methylone respectively, however these colour changes were too pale to be considered a positive result. It was observed however, that there was some variability between the NBOMe analogues tested and some of these would also not be considered true positive results.

Table 5-3: Selectivity results of oral fluid tests using the chromatography method

Drug	Colour change [a]	Result [b]
25B-NBOMe	lt. blue	+
25C-NBOMe	p. blue	+/-
25D-NBOMe	p. blue	+/-
25E-NBOMe	blue	+
25G-NBOMe	blue	+
25H-NBOMe	blue	+
25I-NBOMe	lt. blue	+
25T-NBOMe	p. blue	+/-
30C-NBOMe	blue	+
25I-NBOH	p. blue	+/-
2C-B	NC	-
2C-D	p. green	-
2C-E	NC	-
2C-I	NC	-
2C-T-2	NC	-
Methamphetamine	NC	-
MDMA	p. blue	+/-
4-FMC	NC	-
4-MMC	NC	-
Methylone	p. grey-blue	+/-
JWH-073	NC	-

[a] *lt. = light; p. = pale; NC = no colour change observed*
[b] *(+) = positive; (-) = negative; (+/-) = positive for secondary amine*

5.3.3.3 *Limit of detection*

Cut-off limits for commonly used screening devices such as Dräger DrugTest® 5000 and DrugWipe® 5/5+, range from 5 ng/mL up to 100 ng/mL depending on the drug of interest [302, 304, 315]. The limit of detection for this test method was determined to be 1.5 µg in a 10 µL spiked oral fluid sample or 150 µg/mL, a value significantly greater than the reported cut-off values for commercial screening tests. A pale blue colour change was still seen at this concentration (see Table 5-4).

Below this value, the colour changes are too pale to be considered a true positive result. While this value is appropriate for a colour-based drug test, it is substantially higher than the values often reported for illicit drugs detected in oral fluid.

Table 5-4: Limit of detection colour changes with 25H-NBOMe and resulting interpretation

Amount (µg) in 10 µL	Concentration (µg/mL)	Colour change [a]	Result [b]
10	1000	blue	+
8	800	blue	+
6	600	blue	+
4	400	blue	+
3	300	lt. blue	+
2	200	lt. blue	+
1.5	150	p. blue	+
1	100	v. pale blue	+/-
0.5	50	v. pale blue	+/-
0.2	20	NC	-

[a] *lt. = light; p. = pale; v. = very; NC = no colour change observed*
[b] *(+) = positive; (-) = negative; (+/-) = positive for secondary amine*

The small quantities of NBOMe compounds in a single dose [316] would suggest that the amount of drug that could be detected in oral fluid will be smaller than that of other drugs. There has been little investigation into the concentration of NBOMe compounds found in oral fluid samples, with analysis requiring the use of laboratory instrumentation. While values below 1 ng/mL are possible with techniques such as HPLC-MS/MS [312], this sensitivity is likely not possible with a screening device. Further research into the quantity, of not only 25-NBOMe compounds but other NPS as well, which exhibits impairment of a person is required to determine appropriate cut-off values for these drugs.

5.4 Conclusions

A simple paper-based system for the visualisation of 25-NBOMe compounds in oral fluid samples has been developed in early stages. The chromatographic system can provide a blue colour change with most 25-NBOMe analogues at a concentration of 150 µg/mL and above. While this is far above the expected cut-off values for illicit drugs in oral fluid, this study shows a simple

colourimetric method for these types of samples which has not been previously investigated. Further development to reduce the solvent usage and time required, also needs to be explored to make this a viable on-site testing method. The detection and interpretation of the colour change result using a light detection source may provide more discrimination for the identification of these compounds with this system.

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Chapter 6: Conclusions and future directions

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The rapid emergence and growth in the variety and number of NPS in the last decade demonstrates the need for fast and simple methods to detect these substances in the field. The need to identify these drugs and differentiate them from their traditional counterparts is particularly important from a forensic point of view, as well as from a health care, and harm minimization perspective. Colour testing remains, and will continue to be, the most commonly used presumptive test method for illicit drug analysis. The development of these methods for NPS analysis is therefore of great importance. The subjective nature of these tests also needs to be reevaluated, as there are ways to overcome this limitation without the need for expensive and complicated instrumentation. The main aim of this research was to develop a potential colour test method for the detection of 25-NBOMe compounds and investigate how this test could be incorporated into testing devices for field analysis. It was broadened to include the detection of other NPS for a multiplex device, and the visualisation of 25-NBOMe compounds in oral fluid.

A selective colour test method was developed using TCBQ and acetaldehyde for the detection of 25-NBOMe compounds, and the details of this test are outlined in Chapter 2. All NBOMe analogues gave a blue colour change, though there were some minor colour differences observed for 25I-NBOH and 25I-NBMD, light blue and green blue respectively. There were only two possible illicit interferences, 4-hydroxymethcathinone and methamphetamine, identified with this test method from a range of over 100 compounds. A noteworthy result was the lack of positive colour change when LSD is present, indicating that these substances can be easily differentiated using this test. This result is important due to the prospect of NBOMe analogues being sold as LSD, particularly on blotter papers. The ability to differentiate them plays an important role in healthcare and harm minimisation settings as a dose of an NBOMe analogue can provide a substantially different effect to a dose of LSD. The colour test is able to detect NBOMe substances directly from blotter papers, and the blotter paper appears to have no effect on the test even when a coloured pattern is present. It could be further explored to ensure other inks or paper types do not affect the resulting colour changes. This colour test is also able to indicate the presence of a range of amine containing compounds, such as methamphetamine derivatives and MDMA which provide pale or light blue colour changes, indicative of a secondary amine. The 2C-series substances were assessed and can be detected as they provide a light green colour change result with this method. However, this is not a selective colour change as many other amphetamine-type substances gave similar colour changes, likely due to the similarities in chemical structure. The light green colour change is

therefore an indicator of a primary amine containing compound. The results with compounds other than NBOMe analogues, could play a role in a colour testing scheme. The use of this test, alongside other colour tests such as the Marquis reagent, could provide the analyst with more information about the unknown substance.

The addition of a detection source, such as a smartphone application, has the potential to become a valuable addition to colour tests in a field setting. Chapter 3 investigated the use of three applications, ColorAssist, Colorimeter, and What a Color?, with the colour test described in Chapter 2. It was found that the resulting colour values were highly accurate for NBOMe analogues. It was clear however, that there is still the possibility of other compounds interfering based on their colour change. Further to this, a predictive model, using multinomial logistic regression analysis, was created and could correctly identify 25-NBOMe analogues to a high level of accuracy. It is noted that additional work focused on improving the sensitivity of predictive modelling is required as false positives were apparent, especially with methamphetamine. This may be achieved by further exploring the way data is collected and processed for analysis. The incorporation of more colour values than RGB may be considered and could improve the sensitivity of this test. Other predictive analyses may also be investigated with machine learning processes to enhance the classification of drug classes and concentration values. The incorporation of simple detection devices, such as mobile phones, could play a huge role in the on-site detection of many substances. This type of analysis should be further explored to assess its potential for not only the colour test outlined in this thesis, but for any colour test for an illicit drug. Applications could be designed and developed specifically for this purpose and the use of a detection box could be incorporated in the result calibration to help avoid interference from external factors. Controlling both the level of light available and the camera distance from the sample may assist in the accurate identification of substances using a mobile phone application. This type of system could be purpose built for field analysis so that this method could then be utilised in any circumstance.

Paper-based microfluidics has been shown to be a practical tool in the colourimetric analysis of a range of analytes including illicit drugs. The translation of four developed colour tests for NBOMe analogues, synthetic cathinones, piperazines, and fentanyl analogues into paper-based systems in Chapter 4 was successful with simple modifications completed as necessary. The tests could all identify the target compounds although, the overall selectivity for the piperazine and fentanyl tests was reduced in comparison to the original tests in solution. Due to the lighter orange colour change provided on paper, there were other illicit drugs which gave similar colour changes to the

piperazines. The assessment of the fentanyl test indicated that there were two possible test reagents both with advantages and disadvantages. 1,2-naphthoquinone is a selective reagent for fentanyl and its analogues, however, is not stable in a paper system. On the other hand, 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone is able to be stored on paper for testing, however, is more reactive with several illicit compounds such as cathinone analogues. These reagents, and other similar reagents should be further investigated to determine the most appropriate method for the detection of fentanyl analogues. Expansion of this test to other fentanyl analogues would also be beneficial to not only ensure the test can detect those substances but to also expand on the analysis of the reaction mechanism at play. The limit of detection of these four colour tests was not improved with this translation, however, it did remain in the microgram range (<100 μg) for all four methods. Further investigation to optimise each method for field use is still required. This would include limiting solvent solutions and extending the stability of the reagent papers. It would also be valuable to assess the effect of mixtures and seized samples on each test method. With further development, these methods show potential to be used simultaneously within a multiplexed device for improved detection of the targeted NPS. Development of a system which can integrate these tests into a single device will require substantial study. It would be ideal for the unknown substance to be added to the system in a single solution which can then be detected by each colour test. Currently, these four tests have slightly different application processes for both the reagents and the drug solutions. Volatile reagent solutions, such as acetaldehyde, require consideration in a portable system. These solutions cannot be stored on paper and may require a sealed and light protected container for storage within the device. Further to this, the stability of reagents in a range of conditions, such as high and low temperatures, would need to be assessed as this may not be possible to control in a field setting. The requirement of UV light, for the fentanyl test, also poses a challenge as this increases the complexity of the device and may also hinder the portability.

The potential to incorporate multiple tests into a single device would allow for easier and more rapid detection of different analytes. A device which can identify a range of NPS and even some more traditional illicit drugs in a matter of minutes would greatly improve the screening processes for unknown substances. Paper is a possible substrate for this type of device and has been shown to be useful for this type of analysis. The incorporation of a simple detector, such as a mobile phone, with this device would further improve its capability to detect a vast range of illicit substances.

Chapter 5 described a proof-of-concept study to visualise NBOMe analogues in oral fluid samples and straightforward methods were found to be possible using the TCBQ colour test method. A simple paper-based system can be used for this visualisation, and this improves the detection limit in comparison to applying the reagents directly to the oral fluid. While the detection limit is much greater than what is expected with current oral fluid testing devices, this research has demonstrated the possibility of utilising chemical methods without interference from the oral fluid. This type of testing currently does not have the required level of sensitivity for drug detection in oral fluid. Other chemical sensing techniques such as nanoparticles or molecularly imprinted polymers may be utilised for this purpose. ELISA test systems are often used in illicit drug detection and these types of systems should be investigated and further developed for the detection of NBOMe analogues and other NPS in oral fluid. The detection of illicit drugs, and particularly NPS in oral fluid, requires attention. The limitations of current devices regarding their inability to detect NPS is challenging as the extent of impairment of these drugs is not known. Detection methods, and eventually devices that can detect these substances in low quantities in a field setting, would be highly beneficial, particularly within workplaces and for ensuring safety on the road. Detection limits of these substances need to be further investigated to ensure a developed device can accurately detect the substance at an appropriate level.

This thesis provides a validated, rapid test for the detection of NBOMe analogues, which may be used in the field for law enforcement, healthcare, and harm reduction scenarios. The incorporation of a mobile phone application with the developed colour test presents a cost-effective solution to make colour testing a more objective analysis, particularly for substances that provide similar colour changes. Paper-based analysis delivers a simple substrate for the transportation of colour tests and appears to be appropriate for the analysis of several analytes with the creation of multiplexed devices. The expansion of the range of compounds would be highly beneficial for all studies included in this thesis. Improvement of the presumptive detection of NPS and other illicit substances, which cause harm in our society, would not only assist law enforcement to prevent the distribution of these substances, but also ease the burden on healthcare and harm reduction workers who help keep the public safe.

Appendices

Appendix A: Synthesis methods and characterisation of 2C-H and 25H-NBOMe

A.1 Synthesis of 2C-H and 25H-NBOMe

The synthesis of 25H-NBOMe involves a three step synthetic procedure, with 2C-H the product of the second step. The methods outlined below have been adapted from that outlined in the literature. Authority to synthesise controlled substances has been granted by the Pharmaceutical Services Branch, NSW Health. These compounds were used for the development and optimisation of test methods to avoid the excessive use of expensive reference standards.

A.1.1 Preparation of trans-2,5-dimethoxy-β-nitrostyrene.

The preparation of trans-2,5-dimethoxy-β-nitrostyrene involves a nitro aldol reaction procedure adapted from that outline by Heim [51] with 2,5-dimethoxybenzaldehye and nitromethane and the overall reaction can be seen in Figure A.1.

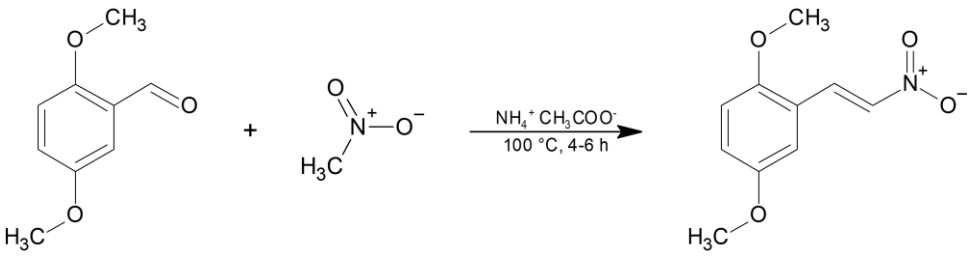

Figure A.1: Reaction scheme of nitro aldol reaction of nitromethane and 2,5-dimethoxybenzaldehyde to form trans-2,5-dimethoxy-β-nitrostyrene

2,5-Dimethoxybenzaldehyde (1.6 g, 10.0 mmol) and dry ammonium acetate (0.7 g, 10.0 mmol) were dissolved in glacial acetic acid (15 mL) in a round bottom flask. Under nitrogen atmosphere, nitromethane (12.5 mL, 230 mmol) was added via syringe and heated with stirring to 100 °C. When the reaction mixture became bright orange-red, it was stirred at 100 °C for 4-6 h, monitoring with TLC. After cooling to RT, the reaction was completed with 50 mL deionised water and the product extracted with dichloromethane (5 x 25 mL). The combined extracts were washed with water (2 x 25 mL) and saturated sodium chloride solution (1 x 25 mL) and dried over sodium sulphate. Remaining DCM was then removed by rotary evaporation. The liquid residue was cooled to room

temperature, orange crystals began to appear and the product, trans-2,5-dimethoxy-β-nitrostyrene, was dried under high vacuum overnight (yield 1.8 g, 89%). The dried product was analysed using GC-MS, NMR and QTOF-MS. (see Figure A. 2, Figure A. 3, Figure A. 4 and Figure A. 5)

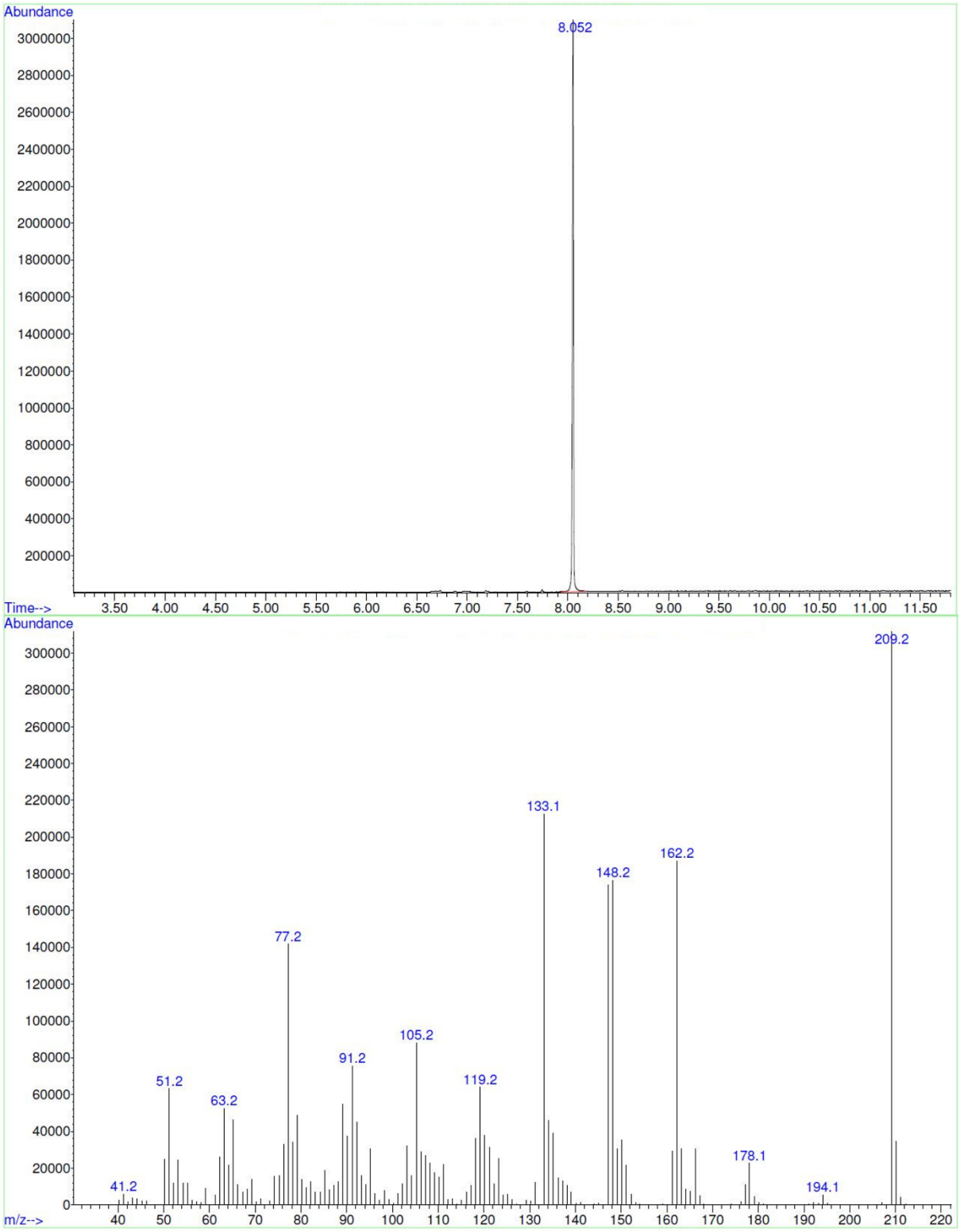

Figure A. 2: TIC of trans-2,5-dimethoxy-β-nitrostyrene (above) and associated mass spectrum (below)

Table A. 1: NMR data and peak assignment for trans-2,5-dimethoxy-β-nitrostyrene

Position	^{13}C NMR	^{1}H NMR		
	Chemical shift (δ in ppm)	**Chemical shift (δ in ppm)**	**Multiplicity**	**J coupling constant (J, Hz)**
1	119.145	-	-	-
2	112.408	6.9	d	3
3	138.498	-	-	-
4	116.303	7.0	dd	3.5, 9
5	119.501	6.8	d	9
6	135.247	-	-	-
7	55	3.8	s	-
8	55	3.7	s	-
9	153.531	7.8	d	13.5
10	153.949	8.1	d	13.5

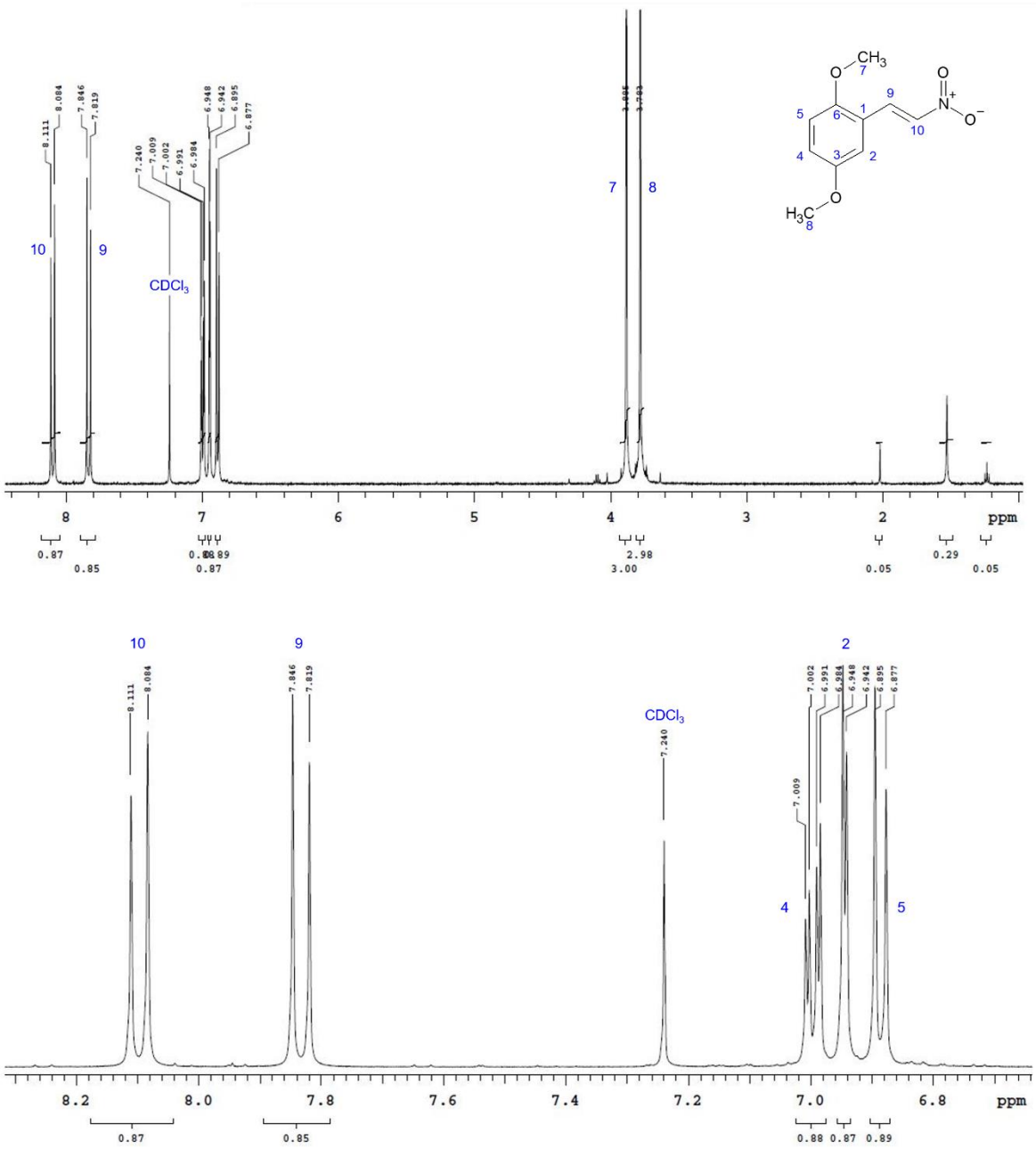

Figure A. 3: Annotated ^{1}H-NMR spectra of trans-2,5-dimethoxy-β-nitrostyrene measured in deuterated chloroform

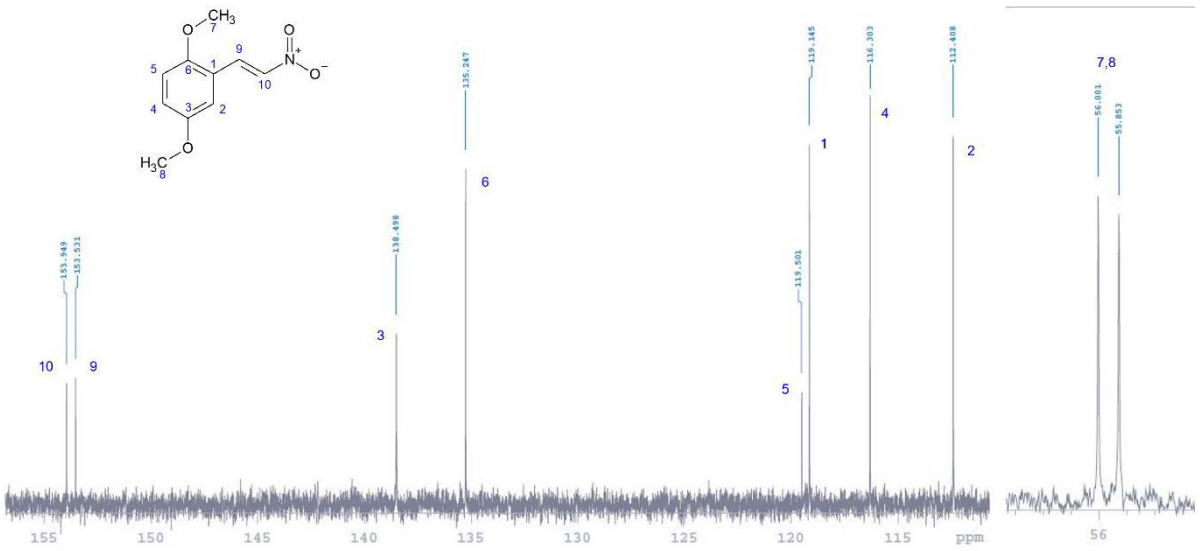

Figure A. 4: Annotated ^{13}C-NMR spectra of trans-2,5-dimethoxy-β-nitrostyrene measured in deuterated chloroform

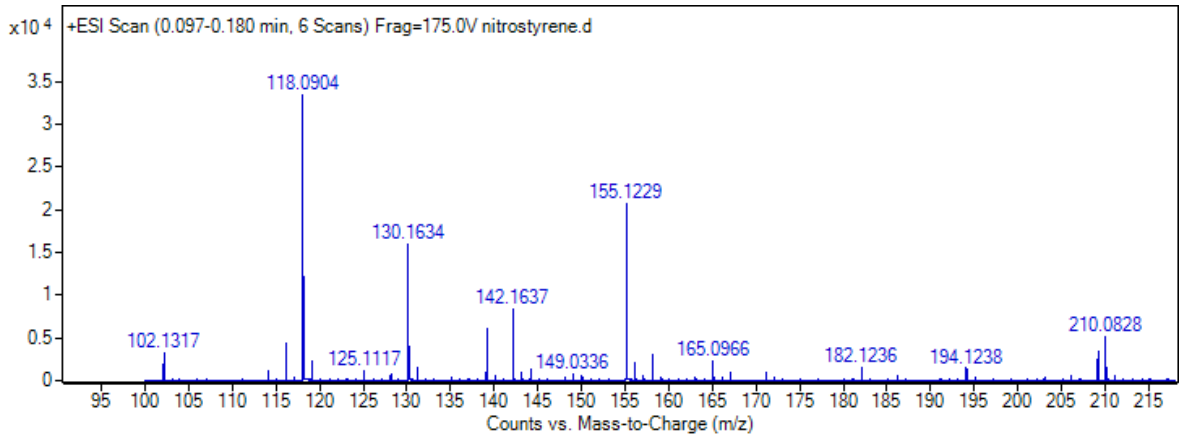

Figure A. 5: Mass spectrum of nitrostyrene in acetonitrile from QTOF-MS

A.1.2 Preparation of 2,5-dimethoxyphenethylamine (2C-H)

The chosen method for the reduction of trans-2,5-dimethoxy-β-nitrostyrene to 2C-H was adapted from a method outlined by Varma and Kabalka [292] and utilised sodium borohydride ($NaBH_4$) and boron trifluoride diethyl etherate ($BF_3{\cdot}Et_2O$) in THF. The in-situ formation of $BF_3{\cdot}$THF allows the reduction to proceed within 5-6 h (see Figure A. 6).

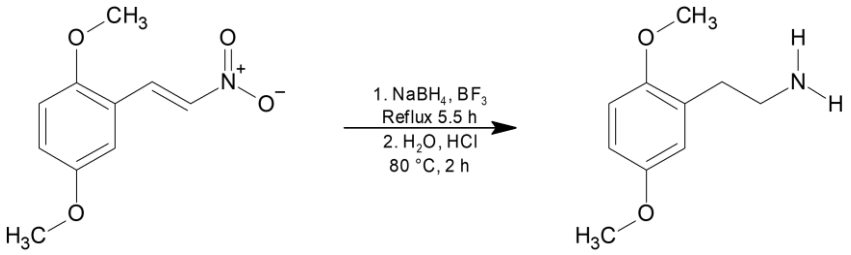

Figure A. 6: Reaction scheme of substituted nitrostyrene reduction to 2C-H

Sodium borohydride (0.37 g, 9.5 mmol) was placed in a cooled round bottom flask with reflux condenser and nitrogen inlet followed by the sequential addition of dry THF (10 mL) and $BF_3{\cdot}Et_2O$ (46.5% solution, 2.5 mL, 12 mmol) keeping the flask cool in an ice bath. The mixture was then stirred at room temperature for 15 min. A solution of trans-2,5-dimethoxy-β-nitrostyrene (0.42 g, 2 mmol in 10 mL THF) was injected via syringe dropwise to the reaction mixture. The solution was then refluxed on an oil bath for 5.5 h (60-70 °C). After cooling to room temperature, the reaction was (cautiously) quenched with water (25 mL) and acidified with HCl (25 mL, 1 M) and then heated again at 80 °C for 2 h. Once again the reaction was cooled to room temperature and the acidic layer was washed with Et_2O (2 x 15 mL). The amine was liberated with NaOH (3 M) until the solution reached approximately pH 11. A small amount of solid NaCl was added and gently shaken to dissolve. The product was then extracted into Et_2O (3 x 25 mL) and the combined extracts dried over $MgSO_4$. The ether was removed via rotary evaporation and the product, 2,5-dimethoxyphenethylamine remained (0.21g, 60%). The product was analysed using GC-MS, NMR and QTOF-MS (see Figure A. 7, Figure A. 8, Figure A. 9 and Figure A. 10).

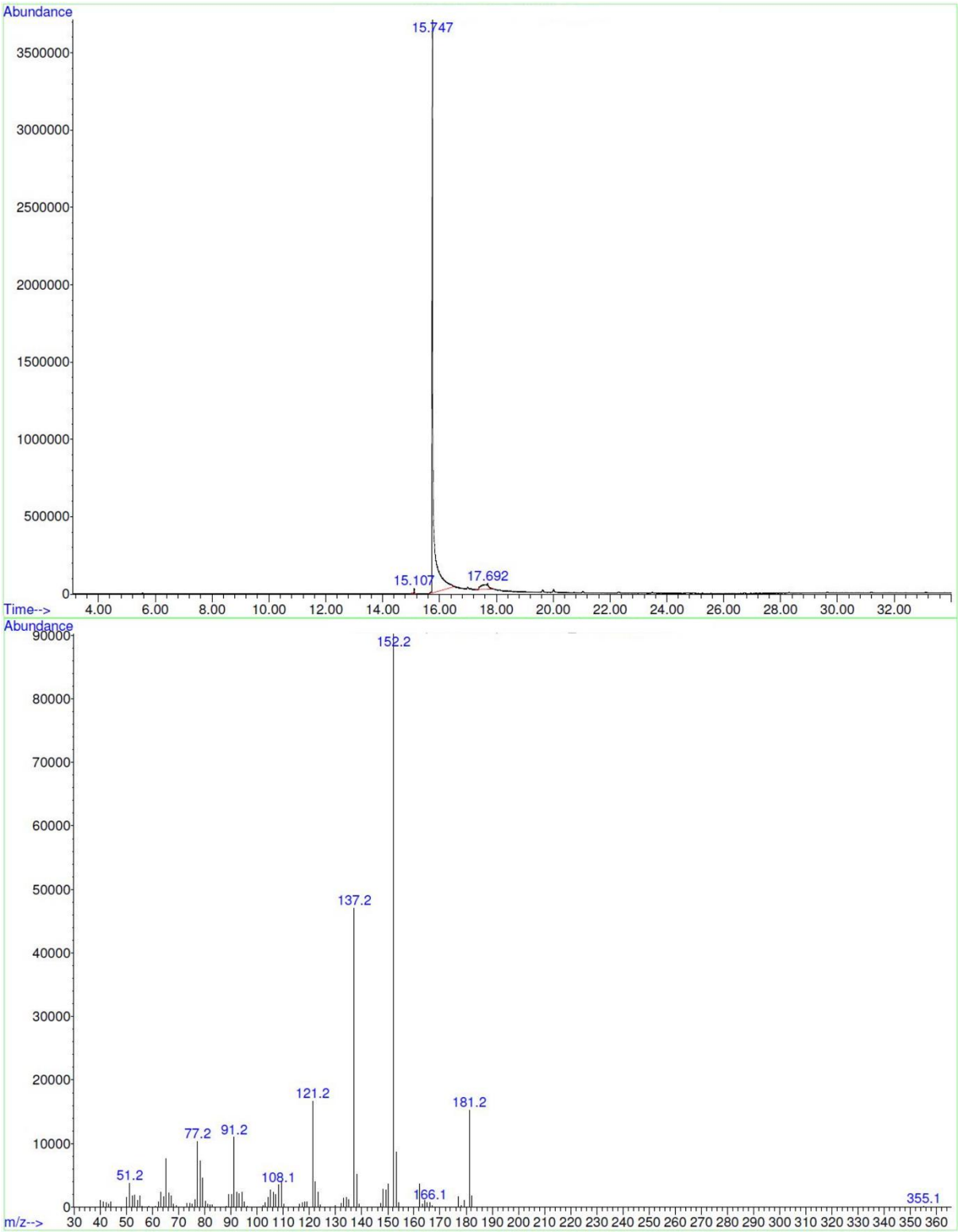

Figure A. 7: TIC of 2,5-dimethoxyphenethylamine (2C-H) (above) and associated mass spectrum (below)

Table A. 2: NMR data and peak assignment for 2,5-dimethoxyphenethylamine

Position	^{13}C NMR	^{1}H NMR		
	Chemical shift (δ in ppm)	**Chemical shift (δ in ppm)**	**Multiplicity**	**J coupling constant (J, Hz)**
1	129.499	-	-	-
2	111.344	6.70	d	3
3	152.035	-	-	-
4	111.295	6.73	dd	3, 7.5
5	116.973	6.77	d	8.5
6	153.457	-	-	-
7	55	3.7	s	-
8	55	3.7	s	-
9	35.064	2.9	t	7
10	42.354	2.7	t	7
11	-	-	-	-

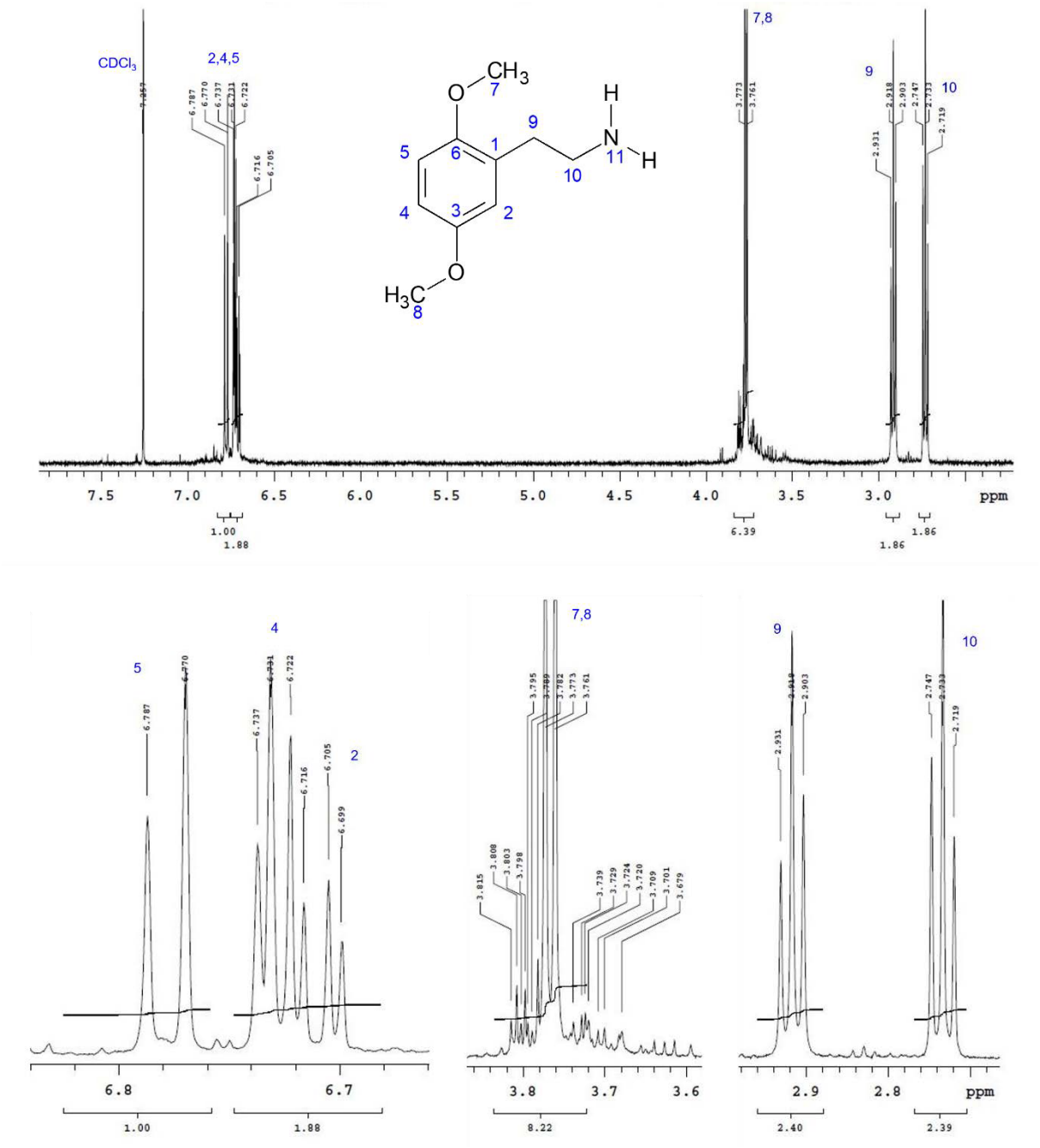

Figure A. 8: Annotated ^{1}H-NMR spectra of 2C-H measure in deuterated chloroform

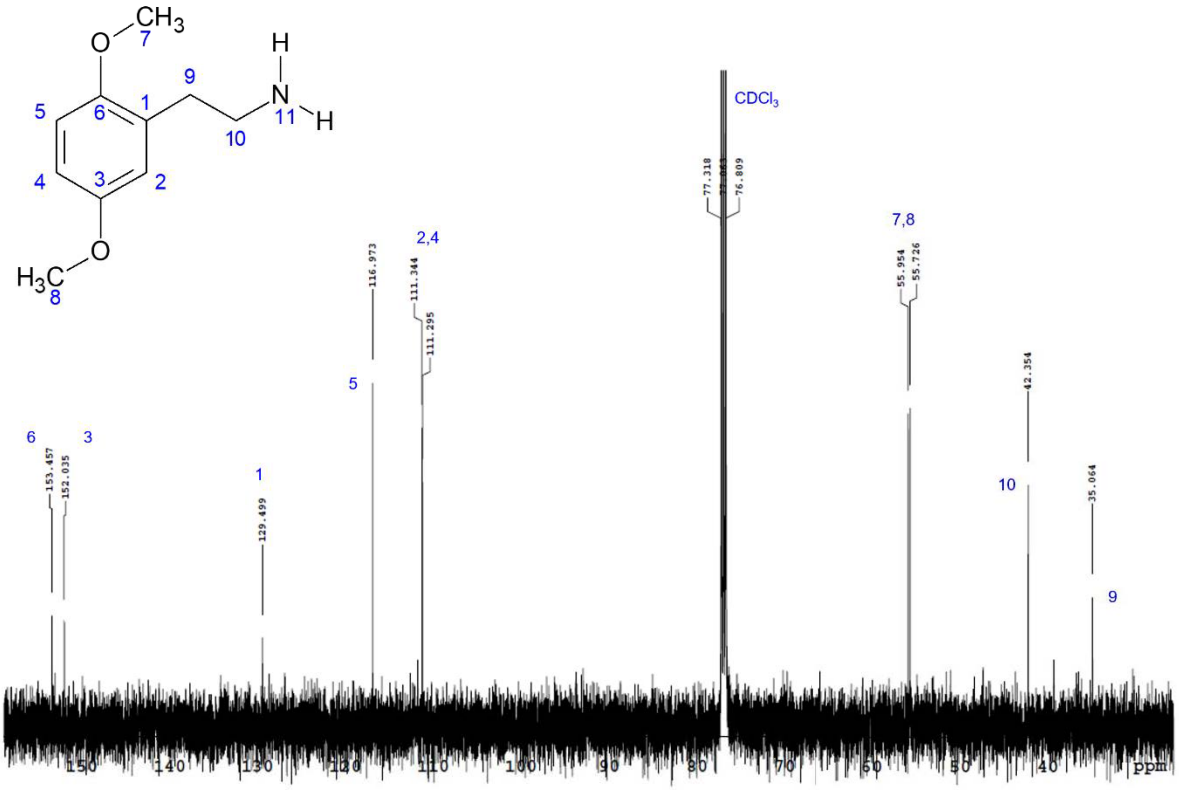

Figure A. 9: Annotated ^{13}H-NMR spectra of 2C-H measure in deuterated chloroform

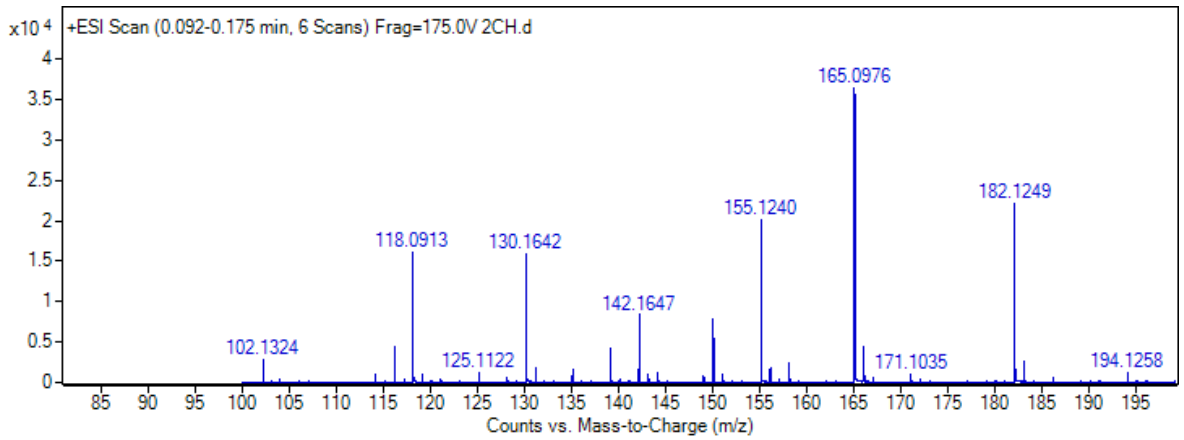

Figure A. 10: Mass spectrum of 2C-H in acetonitrile from QTOF-MS

A.1.3 Preparation of 2,5-dimethoxy-N-(2-methoxybenzyl) phenethylamine (25H-NBOMe)

The preparation of 25H-NBOMe was completed following the method outlined by Heim [51], again on a smaller scale. This method (method 1) produced low yields (0.18 g, 44%) and often a lack of reaction of the benzaldehyde. For this reason a second, very similar however with longer reaction times, method was also attempted [291] and larger yields were obtained (0.29 g, 65%). This method is outlined below and shown in Figure A. 11.

2C-H freebase (0.4393 g, 2.4 mmol) and 2,5-dimethoxybenzaldehyde (0.3634 g, 2.6 mmol) were dissolved in approximately 30 mL methanol and stirred overnight at room temperature. Powdered $NaBH_4$ (0.4202 g, 10.47 mmol) was added slowly in small portions over 30 minutes with good stirring. Reaction left to proceed for 24 h. The solvent was removed by rotary evaporation, and the remaining residue taken up in DCM (50 mL). The organic solution was washed with saturated $NaHCO_3$ solution (30 mL) and separated. The organic phase dried over Na_2SO_4, filtered and solvent removed by rotary evaporation. The product was analysed using GC-MS, NMR and QTOF-MS. (see Figure A. 12, Figure A. 13, Figure A. 14 and Figure A. 15)

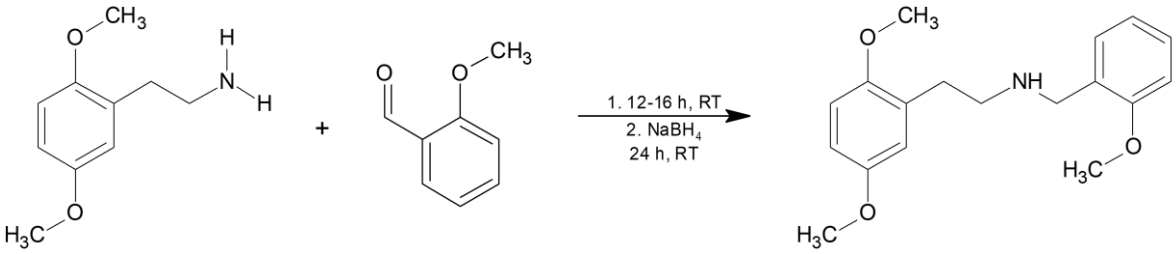

Figure A. 11: Reaction scheme of method 2 reductive alkylation of 2C-H and 2-methoxybenaldehyde to form 25H-NBOMe

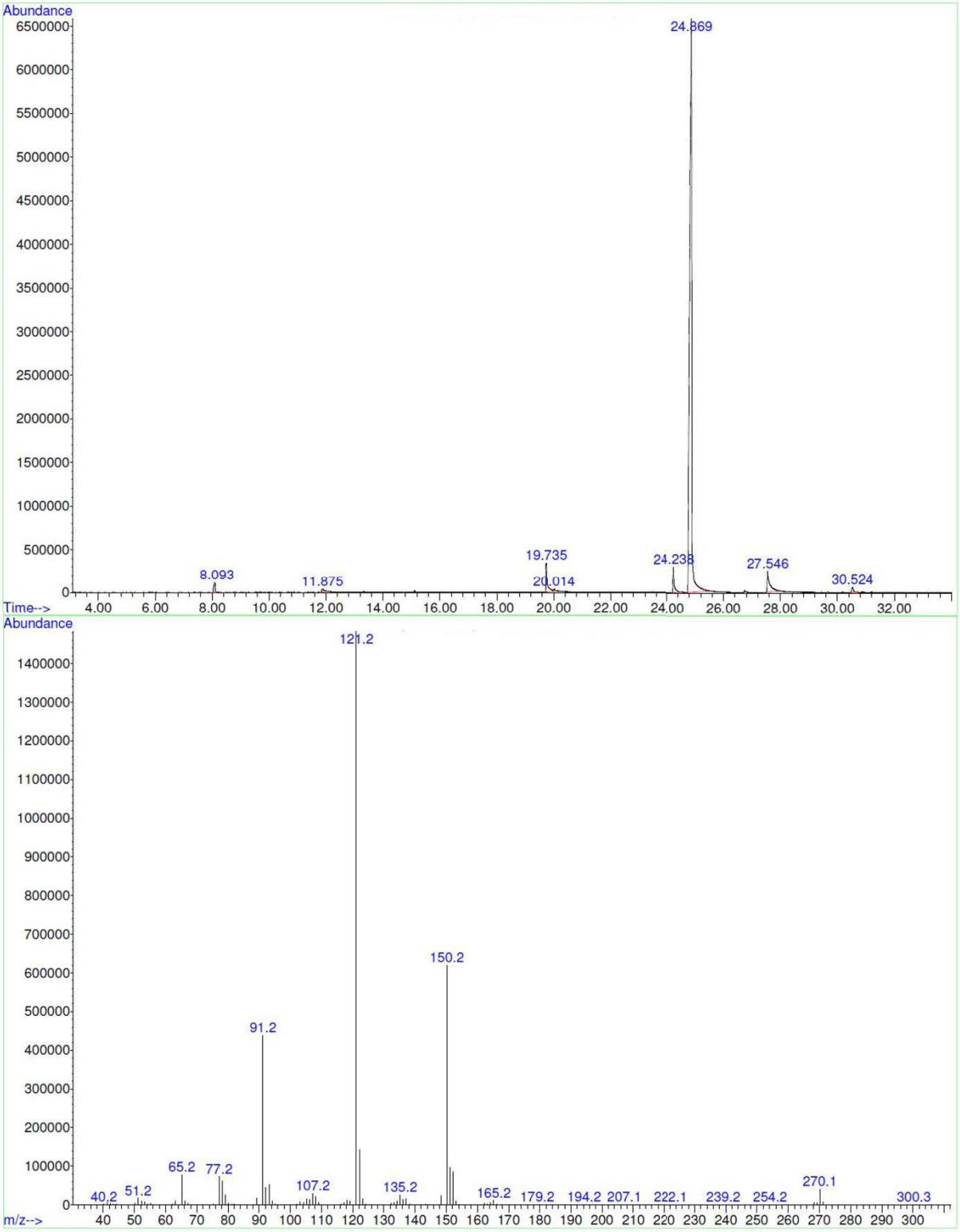

Figure A. 12: TIC of 25H-NBOMe (above) and associated mass spectrum (below)

Table A. 3: NMR data and peak assignment for 25H-NBOMe

Position	^{13}C NMR	^{1}H NMR		
	Chemical shift (δ in ppm)	Chemical shift (δ in ppm)	Multiplicity	J coupling constant (J, Hz)
1	129.586	-	-	-
2	111.136	6.709	s	-
3	153.219	-	-	-
4	109.910	6.72	d	2.5
5	116.416	6.7	d	3
6	151.770	-	-	-
7	55	3.89	s	-
8	55	3.89	s	-
9	30.743	3.096	s	-
10	48.728	3.72	s	-
11	-	4.161	s	-
12	48.978	3.72	s	-
13	120.099	-	-	-
14	129.586	7.38	d	6
15	127.838	6.928	t	7
16	128.786	7.299	t	8
17	111.041	6.80	d	8
18	157.407	-	-	-
19	55	3.621	s	-

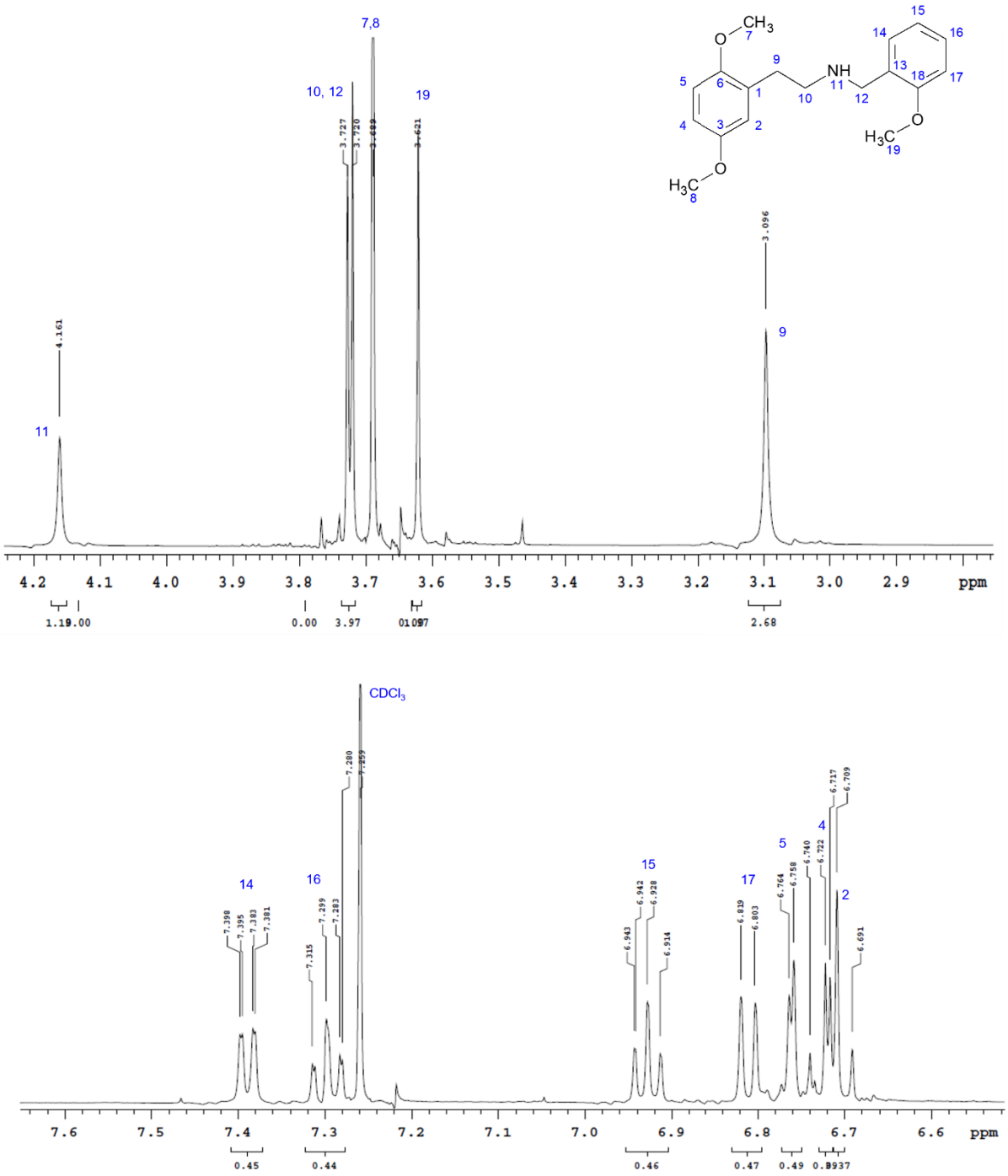

Figure A. 13: Annotated ^{1}H-NMR spectra of 25H-NBOMe measure in deuterated chloroform

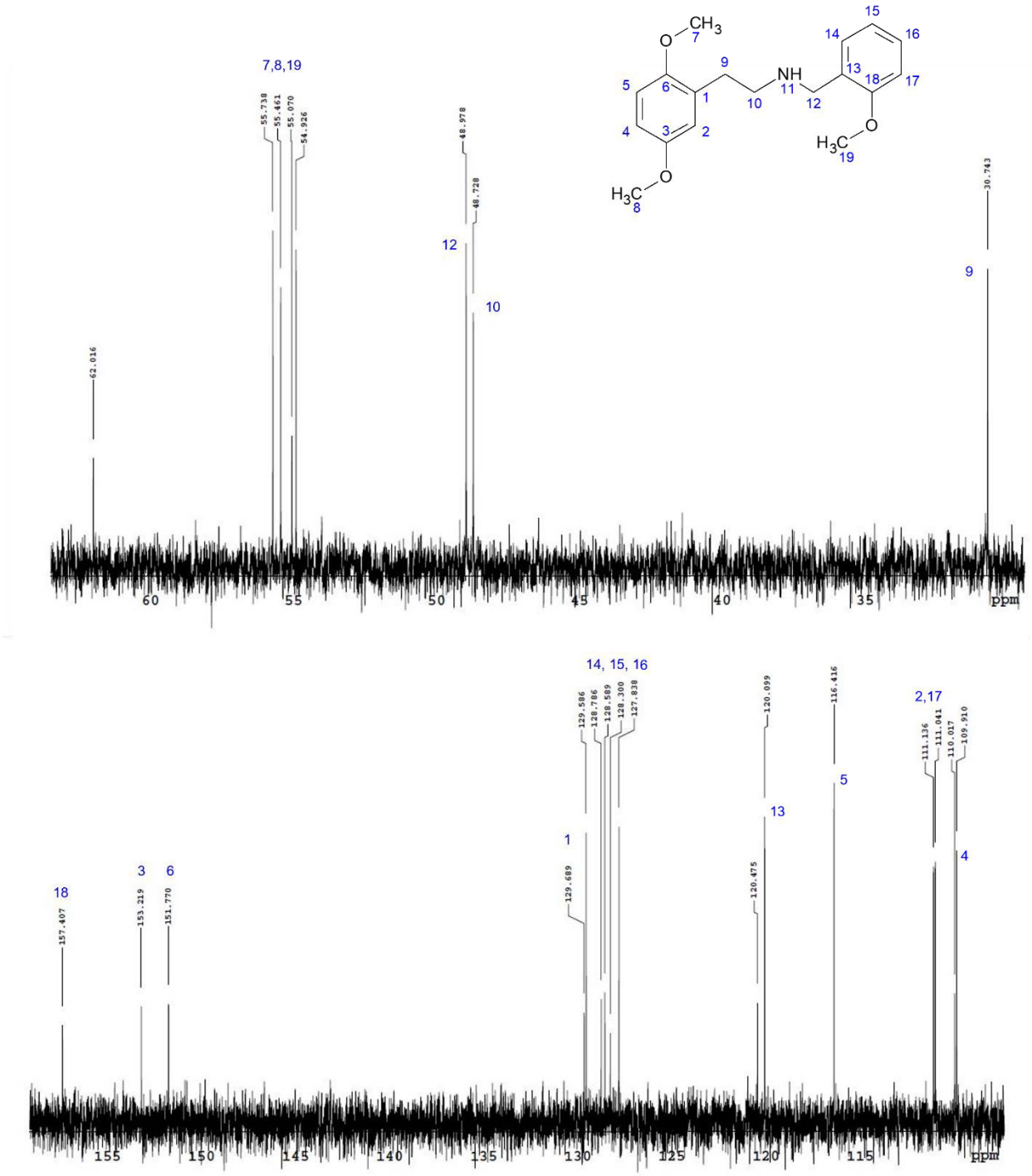

Figure A. 14: annotated 13C-NMR spectra of 25H-NBOMe measure in deuterated chloroform

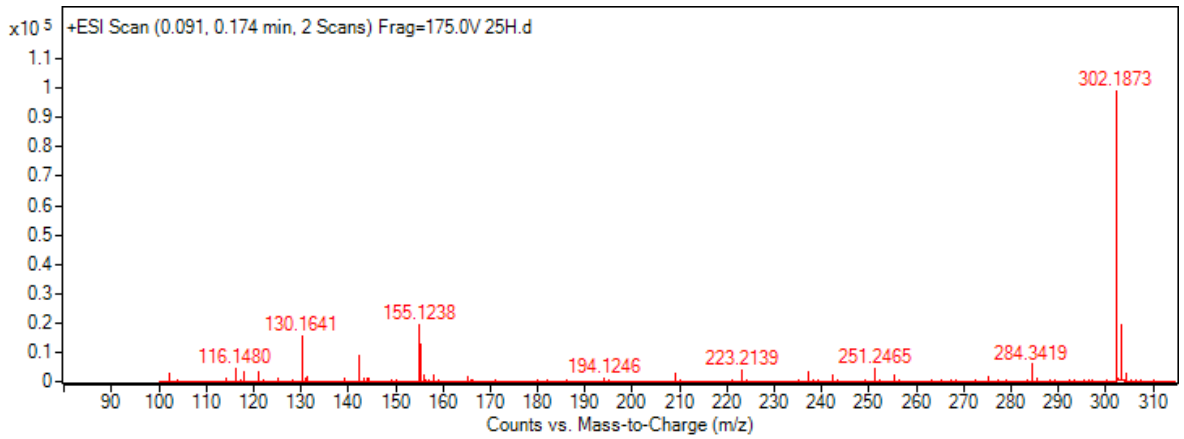

Figure A. 15: Mass spectrum of 25H-NBOMe from QTOF-MS

51. Heim, R., *Synthesis and pharmacology of potent 5-HT2A receptor agonists with N-2-Methoxybenzyl partial structure [Ph. D. thesis].* Free University, Berlin, Germany, 2003.

291. Tirapegui, C., Toro-Sazo, M. A., and Cassels, B. K., *Synthesis of N-(Halogenated) Benzyl Analogs of Superpotent Serotonin Ligands.* Journal of the Chilean Chemical Society, 2014. **59**(3): p. 2625-2627.

292. Varma, R. S. and Kabalka, G. W., *A simple route to alkylamines via the reduction of nitroalkenes.* Synthetic Communications, 1985. **15**(9): p. 843-847.

Appendix B: Mobile phone application data and code

B.1 Complete compound list for mobile phone application study

Table B. 1: List of compounds used for the analysis of colour by the mobile phone applications

Class	Compound
NBOMe analogues	25B-NBOMe
	25C-NBOMe
	25D-NBOMe
	25E-NBOMe
	25G-NBOMe
	25H-NBOMe
	25I-NBOMe
	25T-NBOMe
	30C-NBOMe
	25B-NB4OMe
	25C-NB3OMe
	mescaline-NBOMe
	25iP-NBOMe
	25N-NBOMe
	25P-NBOMe
	25B-NBF
	25I-NBMD
	25I-NBOH
	25T2-NBOMe
	25T4-NBOMe
	25T7-NBOMe
	25H-NB4OMe
	25I-NB3OMe
2C-X analogues	2C-B
	2C-I
	2C-E
	2C-T-2
	2C-D

	2C-H
Amphetamine type substances	MDMA
	d-methamphetamine
	d-amphetamine

B.2 General R code

```
## normality tests ##
ggqqplot(data$R)
shapiro.test(data$R)
ggdensity(data$R)
gghistogram(data$R)
skewness(data$R, na.rm = TRUE)

## Kruskal-wallis test ##
kruskalR <- kruskal.test(R ~ Drug, data = data)
pairwiseR <- pairwise.wilcox.test(data$R, data$Drug, p.adjust.method = "bonferroni")

## Facet box plots ##
ggplot(data2, aes(x=Drugclass, y=Value)) +
  geom_boxplot(aes(fill=Drugclass))+ facet_grid(. ~ Col)+
  scale_fill_brewer(palette="Blues")

## 3D Scatterplots ##
with(data, {scatterplot3d(R, G, B, main = "Drug comparison",
                          xlab = "R value", ylab = "G value", zlab = "B value", color = pcolor, pch = 20)})
```

B.2.1 Generic multivariate analysis:

```
## Nonparametric inference for multivariate data ##
install.packages("npmv")
library(npmv)
data <- nonpartest(R|G|B ~ Drug, data = data, permtest = TRUE, permreps = 10000, plots =
TRUE, tests = c(1,1,1,1), releffects = TRUE)

ssnonpartest(R|G|B ~ Drug, data = drata, test = c(1,0,0,0), alpha = 0.05, factors.and.variables =
FALSE)


## create dataframe of results ##
dataresults <- data.frame(data[["results"]])
datareleffectsresults <- data.frame(data[["releffects"]])
```

B.2.2 Multinomial regression:

```
library(dplyr)
View(dataset)
Drugclass <- c(1)
dataset[,Drugclass] <- lapply(dataset[,Drugclass],factor)
str(dataset)

## set training and testing data ##
training <- sample_frac(dataset, 0.7)
trainset <- as.numeric(rownames(training))

testing <- dataset[-trainset,]
#or#
testing <- read_excel("~/R/Data for upload/testset.xlsx")

## relevel data and run model ##
training$Drugclass <- relevel(training$Drugclass, ref = "1")
require(nnet)
multinomdata <- multinom(Drugclass~R + B + G -1, data = training)
summary(multinomdata)

## extracting coefficients from the model and exponentiate ##
expcoef <- exp(coef(multinomdata))
z <- summary(multinomdata)$coefficients/summary(multinomdata)$standard.errors
View(z)

## 2-tailed z-test ##
p <- (1 - pnorm(abs(z), 0, 1))*2
View(p)

## predicted probabilities ##
head(pp <- fitted(multinomdata))
#or#
head(probtable <- fitted(multinomdata))

## prediction for training set ##
training$predicted <- predict(multinomdrug, newdata = training, "class")

## prediction for test set ##
testing$predicted <- predict(multinomdrug, newdata = testing, "class")

## build classification table ##
classtabletrain <- table(training$Drugclass, training$predicted)
classtabletest <- table(testing$Drugclass, testing$predicted)

##calculating accuracy ##
round((sum(diag(classtabletrain))/sum(classtabletrain))*100,2)
```

round((sum(diag(classtabletest))/sum(classtabledtest))*100,2)

B.3 Raw data and plots

B.3.1 Normality test results

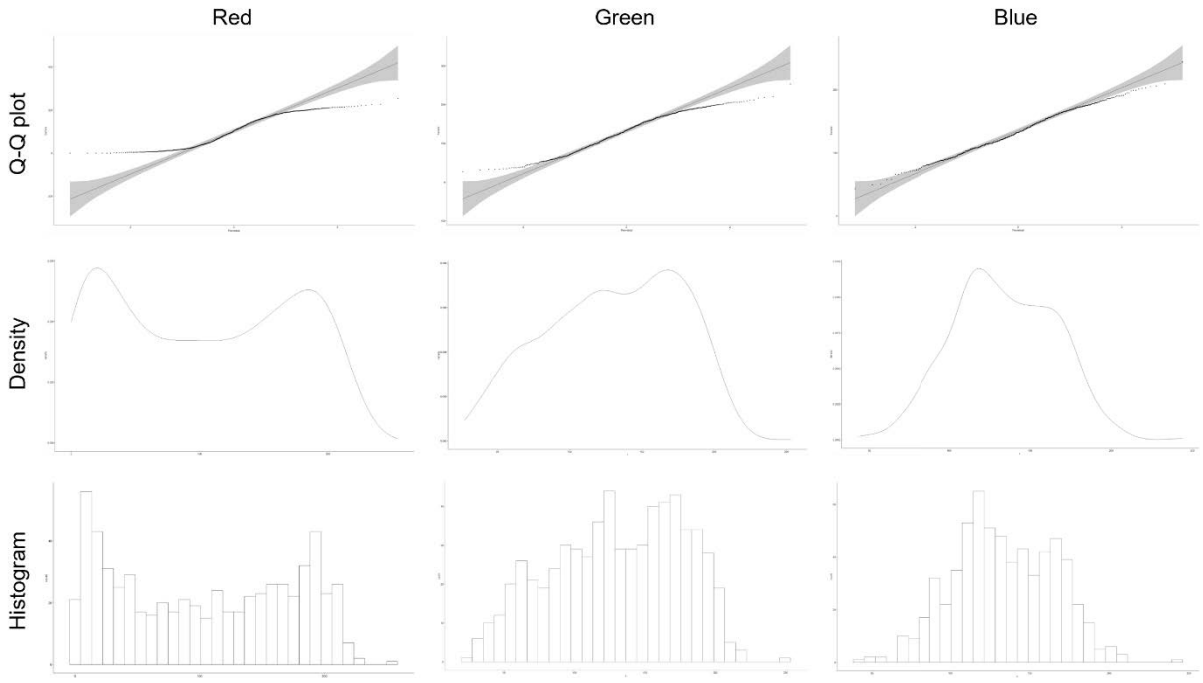

Figure B. 1: Normality test plots for the data set containing all drug classes at 100 µg and blank samples

Table B. 2: Normality test values for the data set containing all drug classes at 100 µg and blank samples

Drugs100		R	G	B
Shapiro-Wilk	W	0.92023	0.97267	0.99373
	p-value [a]	$2.2e^{-16}$	$1.446e^{-9}$	0.009127
Skewness	Value	0.04215594	-0.2292597	0.005988397
	Interpretation [b]	Approx. symmetric	Approx. symmetric	Approx. symmetric

[a] *significance level assessed at α = 0.05*

[b] *Value < -1 or > 1 = highly skewed; value between -1 and -.05 or 0.5 and 1 = moderately skewed; value between -0.5 and 0.5 = approximately symmetric*

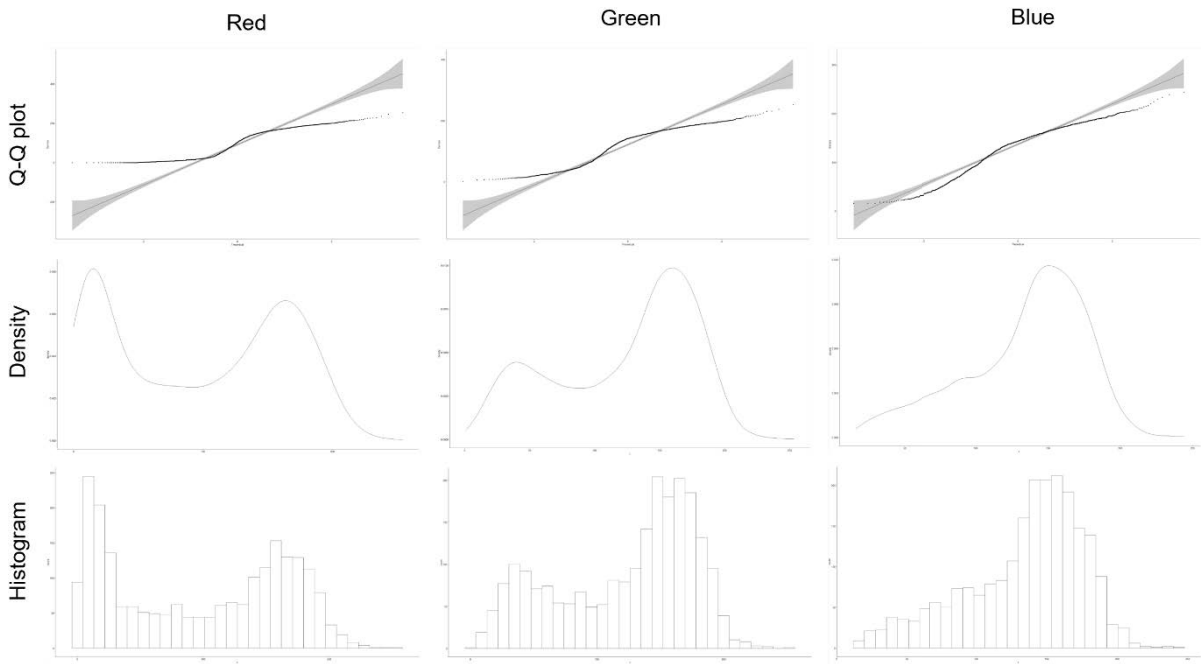

Figure B. 2: Normality test plots for the data set containing all concentrations of NBOMe solutions and blank samples

Table B. 3: Normality test values for the data set containing all concentrations of NBOMe solutions and blank samples

NBOMe conc		R	G	B
Shapiro-Wilk	W	0.89661	0.92298	0.99373
	p-value [a]	$2.2e^{-16}$	$2.2e^{-16}$	$2.2e^{-16}$
Skewness	Value	-0.007556013	-0.537692	-0.7419297
	Interpretation [b]	Approx. symmetric	Moderately skewed	Moderately skewed

[a] *significance level assessed at α = 0.05*

[b] *Value < -1 or > 1 = highly skewed; value between -1 and -.05 or 0.5 and 1 = moderately skewed; value between -0.5 and 0.5 = approximately symmetric*

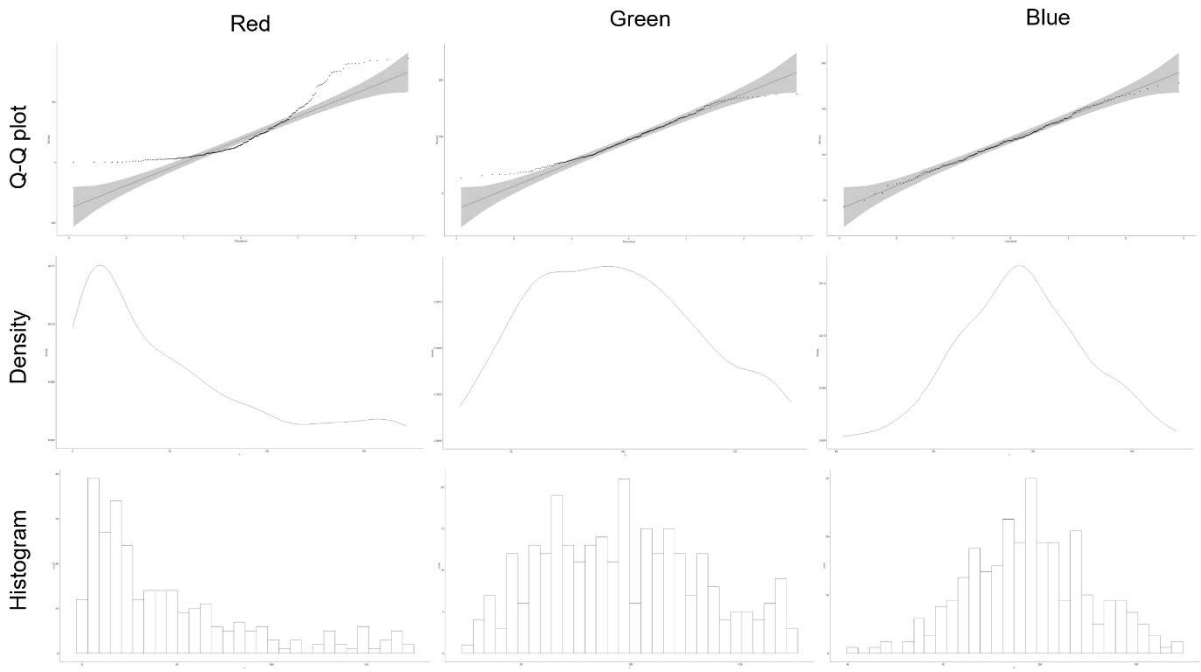

Figure B. 3: Normality test plots for the data set containing NBOMe analogues at 100 µg and blank samples to compare applications

Table B. 4: Normality test values for the data set containing NBOMe analogues at 100 µg and blank samples to compare applications

Apps		R	G	B
Shapiro-Wilk	W	0.83908	0.97606	0.99577
	p-value [a]	$2.2e^{-16}$	0.0001108	0.6429
Skewness	Value	1.30609	0.25308	0.02569464
	Interpretation [b]	Highly skewed	Approx. symmetric	Approx. symmetric

[a] *significance level assessed at α = 0.05*

[b] *Value < -1 or > 1 = highly skewed; value between -1 and -.05 or 0.5 and 1 = moderately skewed; value between -0.5 and 0.5 = approximately symmetric*

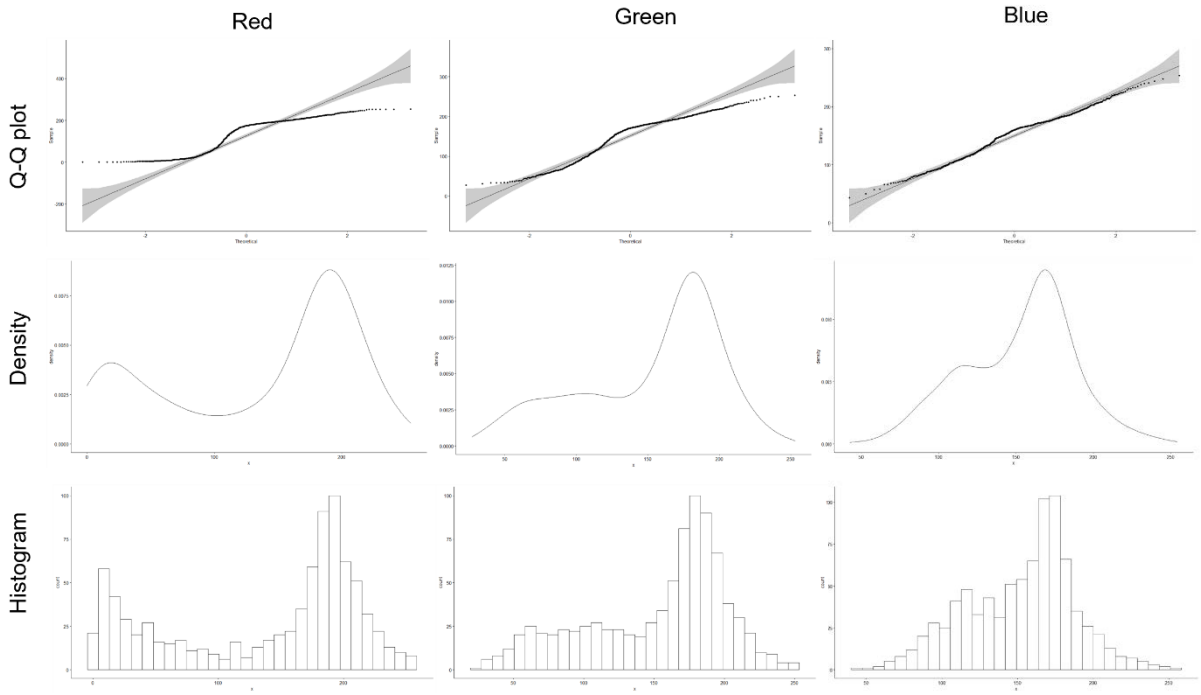

Figure B. 4: Normality test plots for the data set containing NBOMe analogues at 100 µg, impurities, combined impurity and NBOMe and blank samples to assess the effect of impurities

Table B. 5: Normality test values for the data set containing NBOMe analogues at 100 µg, impurities, combined impurity and NBOMe and blank samples to assess the effect of impurities

Impurities		R	G	B
Shapiro-Wilk	W	0.8653	0.92758	0.98257
	p-value [a]	$2.2e^{-16}$	$2.2e^{-16}$	$1.315e^{-8}$
Skewness	Value	-0.5818066	-0.6732149	-0.2771495
	Interpretation [b]	Moderately skewed	Moderately skewed	Approx. symmetric

[a] *significance level assessed at α = 0.05*

[b] *Value < -1 or > 1 = highly skewed; value between -1 and -.05 or 0.5 and 1 = moderately skewed; value between -0.5 and 0.5 = approximately symmetric*

B.3.2 Application evaluation

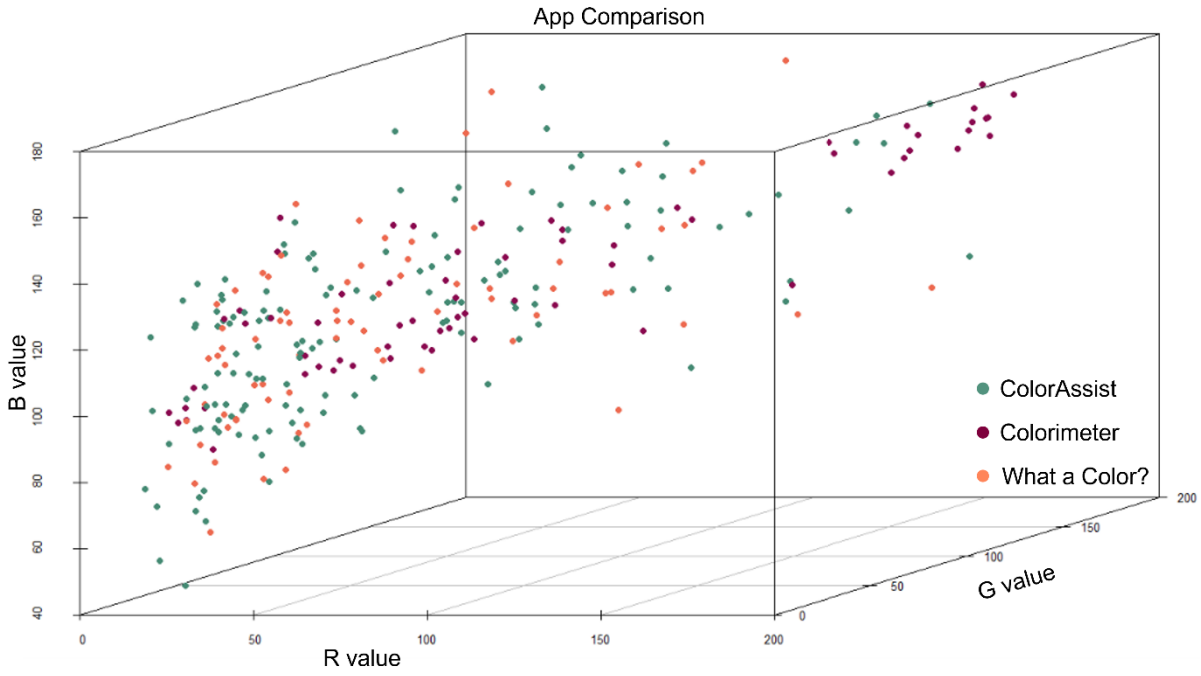

Figure B. 5: 3D scatterplot comparing the three applications

Table B. 6: p-values obtained for comparison of applications using the Kruskal-Wallis test

	R	G	B
P-value [a]	0.001134	0.0008262	0.01719

[a] *significance level assessed at α = 0.05*

Table B. 7: p-values obtained for light comparison using the Kruskal-Wallis test

	R	G	B
P-value [a]	0.4767	0.1498	0.6067

[a] *significance level assessed at α = 0.05*

B.3.3 Impurities

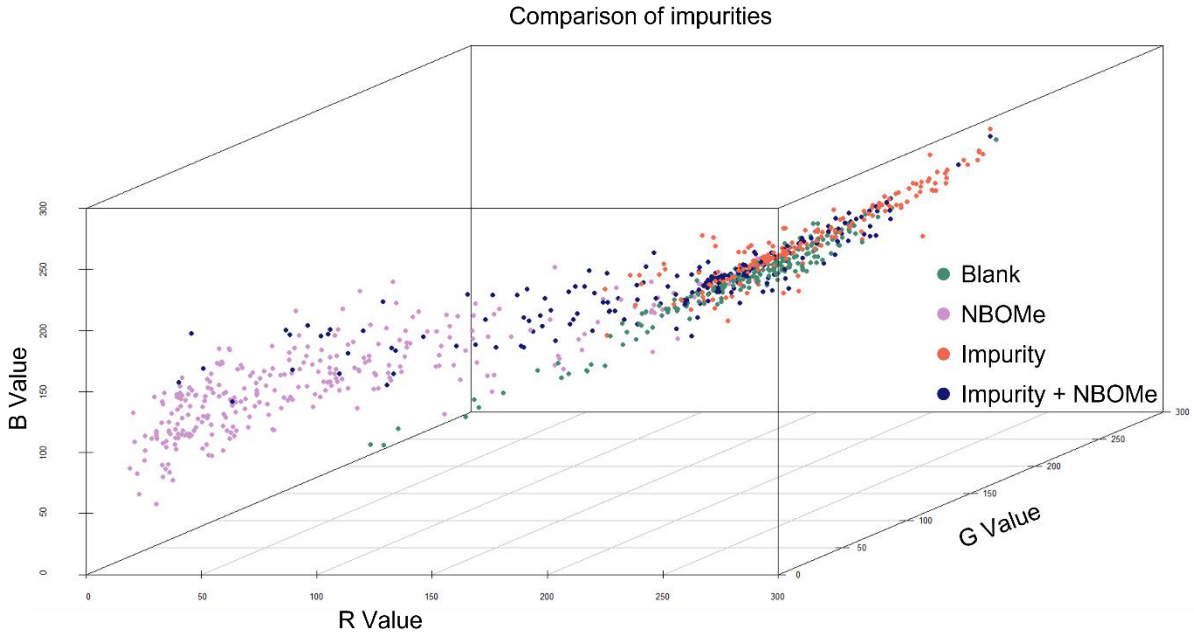

Figure B. 6: 3D scatterplot comparing the impurity tests to NBOMe and blanks

Table B. 8: p-values obtained for impurity testing using the Kruskal-Wallis test

	R	G	B
P-value [a]	2.2e-16	2.2e-16	2.2e-16

[a] *significance level assessed at α = 0.05*

Table B. 9: p-values [a] obtained for impurity testing, pairwise comparison with Wilcoxon test

	Blank			NBOMe			Impurity		
	R	G	B	R	G	B	R	G	B
NBOMe	2e-16	2e-16	2e-16	-	-	-	-	-	-
Impurity	4.3e-06	1.1e-08	2e-16	2e-16	2e-16	2e-16	-	-	-
Impurity + NBOMe	2.1e-06	0.0058	0.75	2e-16	2e-16	2e-16	2e-16	2e-16	2e-16

[a] *significance level assessed at α = 0.05*

B.3.4 Concentration comparisons

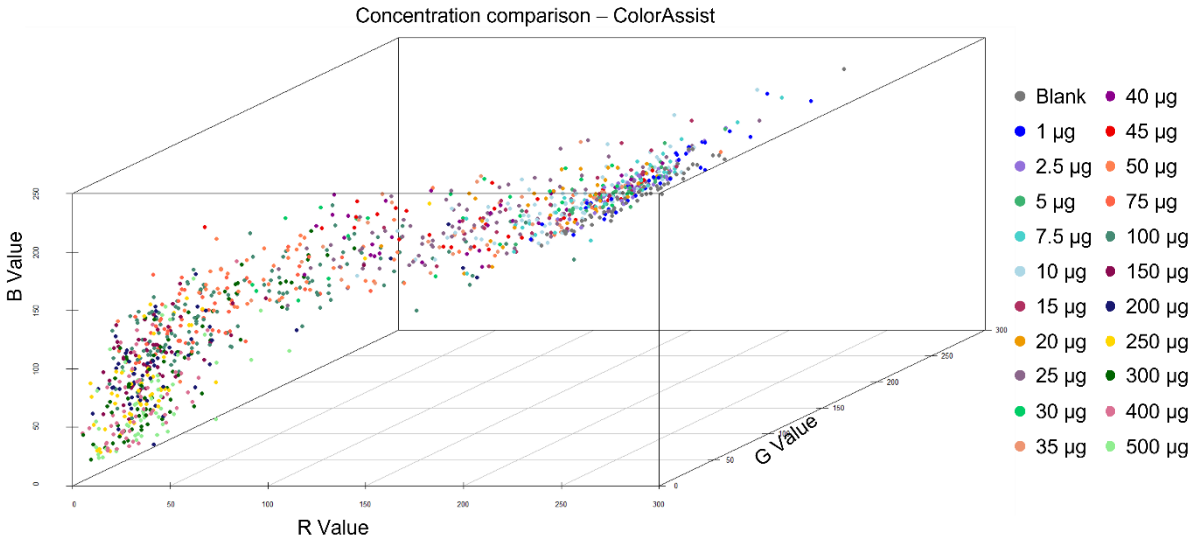

Figure B. 7: 3D scatterplot comparing NBOMe concentrations with values obtained from ColorAssist

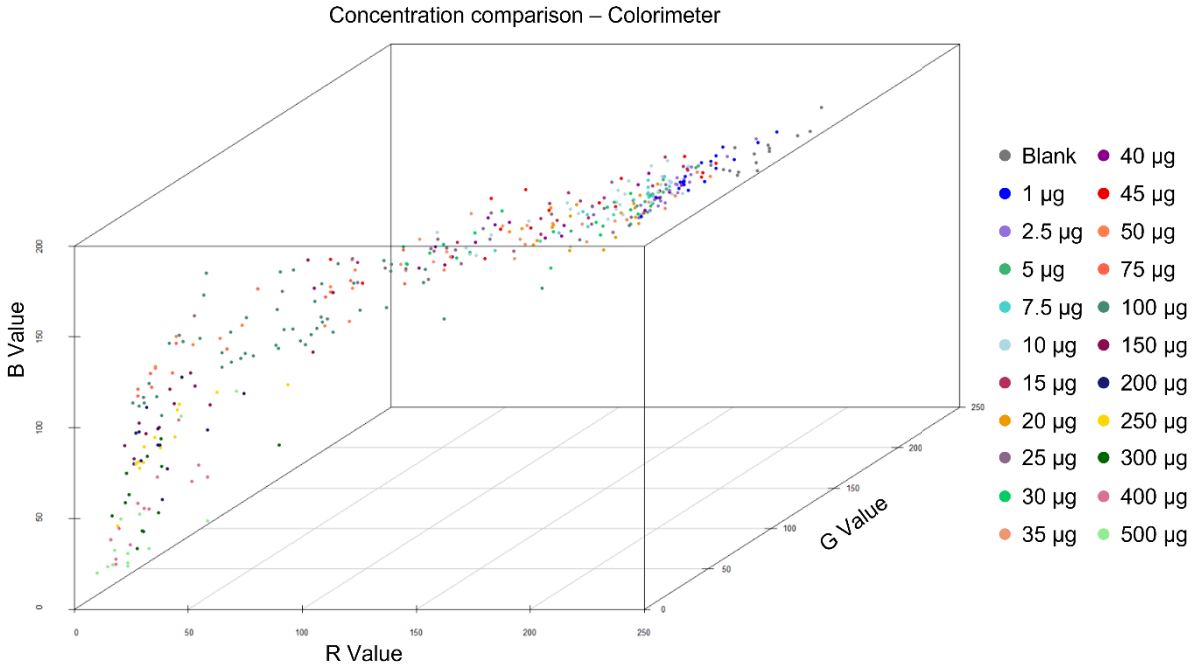

Figure B. 8: 3D scatterplot comparing NBOMe concentrations with values obtained from Colorimeter

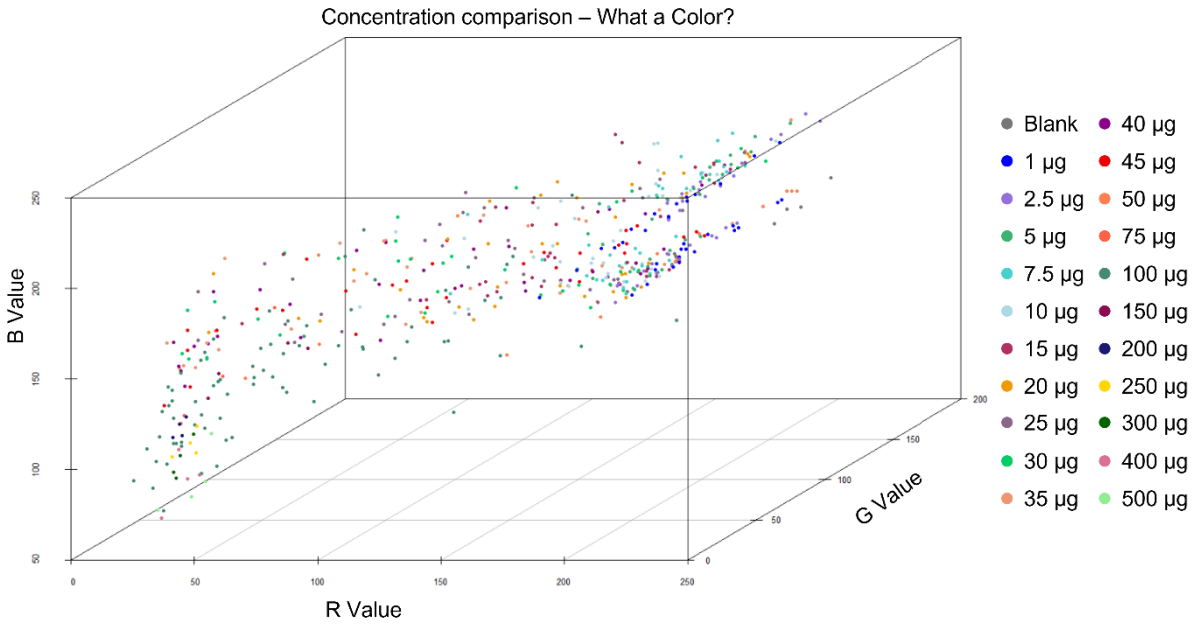

Figure B. 9: 3D scatterplot comparing NBOMe concentrations with values obtained from What a Color?

B.3.5 Multinomal logistic regression

B.3.5.1 Model 1:

Table B. 10: Results from the prediction results of the testing dataset with Model 1

Accuracy	Precision	Sensitivity	Specificity
0.973875	0.983232	0.989264	0.702703

B.3.5.2 Model 2:

Table B. 11: Results from the prediction results of the testing dataset with Model 2

Accuracy	Precision	Sensitivity	Specificity
0.644614	0.616398	0.728374	0.56406

Table B. 12: Prediction results of the testing dataset with Model 2, outlining the specific drug groups each was predicted

		True class					
		NBOMe	**Blank**	**2C**	**Meth**	**MDMA**	**Amphetamine**
Predicted class	**NBOMe**	421	2	4	202	54	0
	Other	157	31	155	82	49	22

B.3.5.3 Model 3:

Table B. 13: Results from the prediction results of the testing dataset with Model 3

Accuracy	Precision	Sensitivity	Specificity
0.575758	0.569416	0.979239	0.216117

B.3.5.4 Model 4:

Table B. 14: Results from the prediction results of the testing dataset with Model 4

Accuracy	Precision	Sensitivity	Specificity
0.444243	0.411378	0.987001	0.097329

Appendix C: Colour chart

Table C. 1: Colour chart of colour names used to describe results of the colour tests

Pale blue	Light yellow-green	Dark pink
Light blue	Pale yellow	Bright pink
Blue	Light yellow	Pale pink
Bright blue	Yellow	Pale purple
Dark blue	Yellow-orange	Purple
Blue-grey	Light yellow-orange	Pale purple-blue
Green-blue	Pale orange-yellow	Pink-orange
Light green-blue	Pale orange	Light pink-orange
Pale green-blue	Light orange	Pale pink-orange
Pale green	orange	Orange-brown
Light green	Bright orange	Red-brown
Green	Orange-red	Grey-brown
Bright green	Bright red-orange	Yellow-brown
Dark green	Red	Green-brown
Yellow-green	Dark red	Brown