Origami Chips: paper-based Lab-on-a-Chip (LOC) devices for the rapid and cost-effective detection of drugs of abuse, including new psychoactive substances (NPS)

L F MCNEILL

PhD 2023

Origami Chips: paper-based Lab-on-a-Chip (LOC) devices for the rapid and cost-effective detection of drugs of abuse, including new psychoactive substances (NPS)

LAUREN FRANCES MCNEILL

A thesis submitted in partial fulfilment of the requirements of Manchester Metropolitan University for the degree of Doctor of Philosophy

Department of Natural Sciences

Manchester Metropolitan University

2023

Declaration

No part of this project has been submitted in support of an application for any other degree or qualification at this or any other institute of learning. Apart from those parts of the project containing citations to the work of others, this project is my own unaided work. This work has been carried out in accordance with the Manchester Metropolitan University research ethics procedures and has received ethical approval number (SE151633A1).

Abstract

New psychoactive substances and drugs of abuse are a major health risk globally. This research presents a simple, rapid, low-cost, and portable paper-based Lab-on-a-Chip (LOC) device for the selective multiplex detection of mephedrone (4-MMC; new psychoactive substance), its metabolite 4-methylephedrine (1-dihydromephedrone, 4-ME), and three commonly encountered drugs of abuse, amphetamine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA).

This LOC device has an 'origami' design that incorporates a competitive immunoassay using antibodies for the simultaneous multiplex detection of these controlled substances within three minutes at a cost of less than 50p per device.

These controlled substances could be detected down to clinically relevant levels. Cross reactivity of commonly encountered 'cutting agents', were investigated and shown to have no effect on the detection capabilities. Twenty seized drug samples (provided by Greater Manchester Police via MANchester DRug Analysis and Knowledge Exchange, MANDRAKE) were tested using the optimised LOC device to determine the reliability, specificity, and reproducibility of the LOC device, with 95% specificity and 100% sensitivity. The LOC device was also evaluated by non-scientifically trained individuals with 100% specificity and 100% sensitivity.

The results show that this LOC device can be used as a rapid and low-cost method to detect 4-MMC, 4-methylephedrine, amphetamine, methamphetamine and MDMA to clinically relevant levels. This portable device has the potential to provide on-site testing within forensic or clinical settings and therefore has wide global applicability.

Acknowledgements

I would like to express my thanks to my amazing supervisory team. Genuinely I have the most amazing supervisory team and I could not have done this without any of you. A special thanks to my Director of Studies (DoS), Dr Kirsty Shaw for your help, support, and guidance throughout my PhD. Honestly, you have been amazing, and I cannot thank you enough. You are extremely inspiring, and I will be eternally grateful. I think a special thank you, needs to be dedicated to your patience Kirsty from my waffling writing! I am also extremely grateful to my three other wonderful members of my supervisory team, Dr Oliver Sutcliffe, Dr David Megson and Dr Patricia Linton. You have all been supportive, patient, and helpful using your knowledge and expertise to allow me to enhance all elements of my research for my PhD. Ollie, you have provided support, many laughs and guidance from day 1. I still remember my first conversation with you when I was applying for this PhD, and if confirmed how much I wanted this PhD and to work with an amazing supervisory team. Dave, you were an amazing DoS during Kirsty's maternity leave, and ever since and became an invaluable member of my supervisory team. I cannot thank you enough for all the last-minute meetings to just discuss any little element of my research. You are brilliant at showing me all the goodness in everything. Thank you for your endless support. Trish, you are honestly a superwoman. You always make time to check on me and make sure that I am okay, which is extremely lovely (even when I know you have far too much to do already). Your lovely personality, support and guidance have been brilliant throughout. And an important note that you are the best at word count challenge for an abstract!

A special thanks to Dr Matthew Hulme, Dr Nicolas Gilbert and David Dickson, and all the MANDRAKE team for all their help and assistance in regard to the samples provided for my research to be undertaken. Honestly Matt, Nick and Dave, you have been super helpful over the years, and I cannot thank each of you enough!

I would like to say a special thanks to the Technicians on the 4th floor laboratory, especially Dr Ruth Shepherd, for your endless technical assistance throughout my PhD. To the Department of Life Sciences, for their kind words and support while I finished my PhD and undertook my role as a Life Sciences Tutor and Programme Support Tutor (PST) within the Department. You are all so lovely and supportive in helping me succeed. Chris, honestly thank you for all the support and endless chats, you are amazing.

To all the lovely friends that I have made whilst researching. Grace, I have enjoyed our endless office chats, I miss them so much! Tom, you have made our endless lab days fun and are honestly one of the most intelligent, hardworking, and inspiring scientists out there. Guuske, shark girl, you are an awe-inspiring Lab-on-a-Chip scientist, always remember that!

I am eternally grateful for the support of my family and friends, for all their love and support throughout my studies, in particular my parents, Allison and Michael, my mother-in-law (to be) Alison, and my other half Adam for putting up with my obsessive desire to learn. I dedicate this though to my lovely children, Mia and Lewis. I love you both to the moon and back and hope that you are very proud of your mummy.

Abbreviations

Ab	Antibody
ATR-FTIR	Fourier-transform infrared spectroscopy with attenuated total reflection
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CDTA	Chemical Diversion and Trafficking Act
CSA	Controlled Substances Act
DART	Direct analysis in real time
DEA	Drug Enforcement Administration
EEWS	European Early Warning System
ELISA	Enzyme linked immunosorbent assay
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EU	European Union
Fab	Antigen binding region
Fc	Constant region
GC	Gas chromatography
GC-MS-EI	Gas chromatography–mass spectrometry with electron ionisation
GHB	Gammahydroxybutrate
$\mu g\ mL^{-1}$	Microgram per millilitre
$HAuCL_4$	Gold (III) Chloride solution
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography

HPLC-ToFMS	high performance liquid chromatography coupled with time-of-flight mass spectrometry
IEC	International Electrotechnical Commission
ISO	International Organisation for Standardisation
LC-MS	Liquid chromatography mass spectrometry
LC-MS-MS	Liquid chromatography tandem mass spectrometry
LSD	Lysergic acid diethylamide
HRP	Horse Radish Peroxidase
µL	microlitre
L	Litre
LC-HR-QTOFMS	Liquid Chromatography-High Resolution-Quadrupole Time of Flight Mass Spectrometer
LC-MS-MS	Liquid Chromatography/Quadrupole Time-of-Flight Mass Spectrometry
LOC	Lab-on-a-Chip
LOD	Limit of Detection
LOQ	Limit of Quantification
M	Molar
MDA	3,4-Methylenedioxyamphetamine
mL	Millilitre
mM	Millimolar
MDMA	3,4-methylenedioxymethamphetamine
MDPHP	3,4-methylenedioxy-α-pyrrolidinohexanophenone
nm	Nanometre

mm	Millimetre
mg	Milligram
MS	Mass Spectrometry
NEP	N-ethylpentylone
ng mL^{-1}	Nanogram per millilitre
NPS	New Psychoactive Substances
NMR	Nuclear Magnetic Resonance Spectroscopy
ONDCP	Office of National Drug Control Policy
µPAD	Microfluidic Paper Analytical Device
PANI	Polyaniline
PBS	Phosphate Buffered Saline
PCP	Phencyclidine
PDMS	Polydimethylsiloxane
PEG	polyethylene glycol
PFTE	Polytetrafluoroethylene
pg mL^{-1}	Picogram per millilitre
pH	Potential of hydrogen
POC	Point of care
PDMS	polydimethylsiloxane
PU	Polyurethane
PVC	Polyvinyl chloride
p-BNC	Programmable bio-nano-chips
SERS	Surface-enhanced Raman Spectrometry

SWGDRUG	Scientific Working Group for the Analysis of Seized Drugs
Δ^9-THC	Tetrahydrocannabinol
TMB	3,3’,5,5’-tetramethylbenzidine
UHPLC-qToFMS	Ultra-high-performance liquid chromatography to quadrupole time-of-flight mass spectrometry
UN	United Nations
UNODC	United Nations Office on Drugs and Crime
UK	United Kingdom
USA	United States of America
3’-OOH-4-MC	normephedrone-ω-carboxylic acid
MDPV	3,4-methylenedioxypyrovalerone
4-CC	4-carboxycathinone (4-carboxynormephedrone)
4-CMC	4-carboxymethcathione (4’-carboxy-mephedrone)
4-CNE	4-carboxynorephedrine (4-carboxy-dihydro mephedrone)
4-MC	4-methylcathinone (normephedrone)
4-MC-SC	N-succinyl-nor-mephedrone
4-MMC	Mephedrone
4-MNE	4-methylnorephedrine (1-dihydro-nor-mephedrone)
4-OH-MC	4-hydroxymethylcathinone (hydroxytolylnormephedrone)
4-OH-MMC	4-hydroxymethylmethcathinone (hydroxytolylmephedrone)
6-MAM	6-monoacetylmorphine
^{o}C	Degrees Celsius

Contents

List of Figures

List of Tables

Chapter 1: Introduction

The data from this chapter has been published in the peer-reviewed journal Forensic Chemistry. As lead author for the manuscript, I was responsible for undertaking the systematic review and analysing the data obtained from the systematic review (L. McNeill, D. Megson, PE. Linton, J. Norrey, L. Bradley, et al. (2021). Lab-on-a-Chip approaches for the detection of controlled drugs, including new psychoactive substances: A systematic review. Forensic Chemistry. 26, pp.100370-100370 [1]).

Executive Summary

This chapter is largely based on the published systematic review investigating the available literature for the detection of controlled drugs, including new psychoactive substances (NPS) using Lab-on-a-Chip (LOC) technology [1]. According to the World Drugs Report (2019) from the United Nations (UN) on Drugs and Crime there were over half a million drug related deaths, 35 million people were treated for drug use disorders across the globe, and it is estimated that more than 270 million people used drugs during 2017 [2].

The published literature available on LOC methods for the detection of controlled drugs NPS was undertaken from January 1999 to March 2021 and identified 45 publications. From March 2021, using the same search criteria stated in the published systematic review (see Appendix 1.1) there have been an additional seven publications reporting the detection of drugs of abuse or NPS (see Appendix 1.2) [3-9].

A total of 28 different drugs of abuse were investigated, with cocaine the most widely studied (58%). The LOC devices were capable of accepting a wide range of biological and non-biological samples. A total of 18 countries have been involved in LOC research into detection of drugs of abuse, with locations generally following local trends in drug use. LOC devices employed a range of detection methods with immunoassays most commonly incorporated (34%). Recommendations are made for expanding the use of real-world samples, improved validation, and further analysis of practicality (in terms of providing information on cost, speed of analysis and ease of use). More than a third of all the publications included in this review

were published since 2019, representing a recent increase in research using LOC devices for the detection of drugs of abuse. There is currently an extensive range of LOC approaches available offering potential for these devices as cost-effective, rapid, and portable detection systems.

1.1. Introduction

Figures published by the United Nations on Drugs and Crime (UNODC) World Drugs Report (2019) stated that in 2017, there were around 585,000 drug related deaths as a result of 271 million people abusing drugs worldwide [2]. This highlights the significant numbers of individuals taking drugs, but also the significant number of deaths globally as a direct consequence of drug use. Well established drugs of abuse are very prevalent, with levels of global cocaine production at their highest to date, with 1976 tons reported in 2017 (a 25% increase from 2016) [2]. There has also been an increase in polydrug use and disorders, for example over 65% of cocaine drug users required treatment for other substances including cannabis and alcohol [2]. More recently, new psychoactive substances (NPS) are finding prominence as drugs of abuse. These issues highlight a requirement to have rapid detection methods for both NPS and drugs of abuse, for which there is currently no standardised approach.

Currently, the detection of seized drugs in forensic laboratories usually involves a two-step process of initial screening using a rapid presumptive test followed by a discriminatory technique(s), which are usually chromatographic [10]. Analytical methods are categorised according to the selectivity, with three classifications (A, B and C) according to the Scientific Working Group for the Analysis of Seized Drugs

(SWGDRUG). Category A techniques include Mass Spectrometry (MS), Nuclear Magnetic Resonance Spectroscopy (NMR), Raman Spectroscopy and Infrared Spectroscopy [11]. And the selectivity of the techniques for Category A are based on structural determination and are the most selective techniques [11]. Category B techniques are less selectivity than A, with techniques based on their selectivity through physical characterisation [11]. Examples of Category B techniques include Gas Chromatography (GC), Liquid Chromatography (LC), and Capillary Electrophoresis [11]. Category C are the least selective techniques that detect using general or class information, including colour tests and immunoassays [11].

Chromatographic techniques are the 'gold standard' discrimatory technique in forensic analysis as they specifically determine the number of compounds and also quantity present and subsequently the most selective [11, 12]. The combination of techniques such as GC and LC with MS, allows for more selective methods for the detection of drugs of abuse and NPS. GC-MS is a commonly used analytical technique for the detection of NPS and drugs of abuse in biological samples, and also from seized street samples [12-14]. However, it is worth noting that there are other techniques available for the detection of drugs of abuse and NPS.

Immunoassays are commonly used for initial screening of drugs of abuse especially within hospitals or as part of workplace drug testing programmes, as they are cost-effective, simple to perform and offer quick results when compared to more time and cost demanding analytical techniques, such as GC-MS and LC-MS [15-17]. Immunoassays are advantageous over other techniques as the antibody incorporated has the potential capability to cross-react with structurally similar

drugs of abuse and NPS. Highlighting the potential adaptability to the continually increasing number of NPS available within the recreational market, making a competitive immunoassay is ideal for analysis of NPS [12]. However, it is worth noting that immunoassays are only presumptive tests and therefore require a confirmatory test, such as GC-MS or LC-MS for the detection of drugs of abuse and NPS [11]. Another limitation of immunoassays is that they can also produce false-positive and false-negative results [11, 15, 18-20].

There are also alternative emerging technologies for the detection of drugs of abuse and NPS, including Surface-Enhanced Raman Spectroscopy (SERS), direct analysis in real time (DART)-MS and electrochemical sensing [12]. SERS offers a potentially cost-effective and rapid method for the detection of bulk and trace drug samples within forensics [9, 12, 21-23]. The use of DART-MS methodology coupled with high resolution time-of-flight mass spectrometry (HRToFMS), and also in-source collision-induced dissociation (CID) fragmentation for the detection of synthetic cathinones [24]. However, there are important disadvantages as this detection method is not portable, as it utilises benchtop equipment and the equipment required is extremely expensive. An alternative to the traditionally utilised laboratory-based methods is electrochemical sensing, which has the potential to be used for the low-cost, portable and disposable detection of drugs of abuse and NPS [12, 25]. However, a disadvantage of this method is that it is not as sensitive in comparison to other techniques, with higher limits of detection (LODs).

1.1.1. New Psychoactive Substances (NPS)

NPS produce a psychoactive effect when taken and are relatively new to the recreational drugs market. NPS are not collectively listed under the International Drug Control Conventions, with drug legislation varying from country to country, but they do present a significant risk to public health [26]. NPS exhibit similar biological and pharmacological activity to established drugs of abuse, such as cannabis and amphetamines, but less is known about the pharmacology and potential health risks [25, 27]. The most recent report from the UNODC published in October 2020 states that there were 1004 different NPS across 125 countries since NPS first emerged on the recreational drugs market [28]. An increase of 54 NPS and an additional 5 countries from the previous report published in 2019 [29], with NPS use exhibiting region-specific trends throughout the world [2, 30, 31].

In the World Drugs Report (2019), the largest group of NPS present in the global recreational drugs market were stimulants, comprising of 34% of those available, followed by opioids (29%), and then synthetic cannabinoids (24%) [2]. Stimulant NPS mimic the effects of established stimulant drugs of abuse such as amphetamine, cocaine, 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine. Mephedrone (4-MMC; 4-methylmethcathinone), methylone, and 3,4-methylenedioxypyrovalerone (MDPV) are examples that have successfully established a place on the recreational drugs market [31]. In Canada and the United States of America (USA) there is an issue with opioid use, in particular fentanyl derivatives of synthetic opioids [29], and this has led to an increase in the number of deaths reported due to overdose [30]. The third largest group of NPS is synthetic

cannabinoids, also known as synthetic cannabinoid receptor agonists, which act on the cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2) receptors and mimic the effects of cannabis [30, 32]. There are a number of synthetic cannabinoids on the recreational drugs market, such as JWH-018, AB-FUBINACA, and 5F-APINACA, which have been monitored by the UNODC since 2009 [32]. There are high levels of use within prisons and the homeless community worldwide, but particularly within the United Kingdom (UK) [32]. With the number of available NPS constantly increasing, this poses challenges in detecting these substances to keep up with determining the current trends. A previous review by Smith *et al.* discussed methods for the detection of different types of NPS [12]. Many of these methods involved the use of gas chromatography-mass spectrometry (GC-MS) [33, 34] or high-performance liquid chromatography (HPLC-MS) [35-37], as well as some more advanced tandem mass spectrometry (HPLC-HRqTOFMS) [38]. As a result, these detection methods are often non-portable, expensive and require specialist facilities and staff to operate.

Although legislation for NPS differs throughout the world [2], there is a current and timely global requirement for developing rapid drug detection tests that are accurate, portable, and cost effective to aid identifying NPS, and other drugs of abuse. This would be especially useful within Accident and Emergency (A&E) departments, prisons, police departments and for occupational drug testing. A LOC based testing system could meet these requirements compared to conventional laboratory techniques.

1.1.2. Legislation

The United Nations (UN) has three conventions to ensure international control on drugs of abuse: i) Single Convention on Narcotic Drugs (1961) that provides legislation to ensure that it is illegal to manufacture, produce, supply, trafficking, possess, import or export drugs controlled drugs; ii) Convention of Psychotropic Substances (1971) which provides international control of psychotropic drugs of abuse, as well as synthetic drugs; and iii) Convention against Illicit Traffic in Narcotic drugs and Psychotropic Substances (1988) that aims to internationally control the trafficking of drugs of abuse [39]. Stimulants that are under international control under the Single Convention on Narcotic Drugs (1961) or Convention of Psychotrophic Substances (1971) include amphetamine, cocaine, MDMA, and methamphetamine [31].

However, NPS are not all controlled under the Single Convention on Narcotic Drugs (1961) or Convention of Psychotropic Substances (1971) [31]. When an NPS has been deemed a serious health risk they are then placed under international control, such as 4-MMC and MDPV. According to the UNODC and their definition of a NPS, then these then are no longer classified as an NPS [31].

1.1.2.1 European Union

The three UN conventions discussed above provide a framework worldwide, but does not state how each country to enforce the punishments [40]. The European Union (EU) aims to achieve a balance, not only punishing according to the three UN conventions, but also to ensure effective treatment [40]. However, not all countries within the EU help the convicted drug users with treatment [40].

1.1.2.2. United Kingdom Legislation

In the United Kingdom (UK), the Misuse of Drugs Act (1971) was introduced to control drugs that posed a significant danger, enforcing legislation to ensure that it was illegal to produce, supply, possess, import or export controlled drugs within the UK [41]. The Misuse of Drugs Act (1971) is split into four classifications; Classes A to C, and also a temporary drugs classification (as shown in Table 1.1.) [41]. The classification is categorised according to the level of danger and harm the drug poses, and the punishments are in accordance to the potential danger and harm of the drug, with Class A drugs being the most harmful and therefore carrying the most severe punishment [41].

Table 1.1*. The Misuse of Drugs Act (1971) Drug Classification punishment for possession and the punishment of possession with the intent to supply (adapted from [42, 43])*

The Misuse of Drugs Act (1971) drug classification	**Examples**	**Punishment for possession**	**Punishment of possession with the intent to supply**
Class A controlled drugs	Fentanyl, morphine, heroin, LSD*, MDMA, oxycodone, methadone, cocaine, methamphetamine	Up to 7 years and/or an unlimited fine	Up to life and/or and limited fine
Class B controlled drugs	4-MMC, methcathinone, ketamine, codeine, ethylphenidate, and amphetamine	Up to 5 years and/or an unlimited fine	Up to 14 years and/or an unlimited fine
Class C controlled drugs	GHB*, clonazepam, diazepam, khat, cannabis, zopiclone, tramadol and testosterone	Up to 2 years and/or an unlimited fine	Up to 14 years and/or an unlimited fine

* *Lysergic acid diethylamide (LSD); Gammahydroxybutrate (GHB)*

More recently on the 26th of May 2016, the Psychoactive Substances Act 2016 came into force within the UK. This legislation made it illegal to produce, supply,

export or import an NPS, aimed at reducing the availability of NPS that were previously available via the internet, street dealers or in headshops [44, 45].

The latest Home Office report published in the UK reported that the prevalence in England and Wales has decreased from 0.8% from 2014-15 to 0.4% in 2017-18 in individuals aged 15-59 years old, which is perhaps a result of the introduction of the Psychoactive Substances Act 2016 [31, 46]. With the cost of NPS increasing due to this legislation being implemented, potentially a subsequent decrease in their use as a resultant [31]. In 16-24 year olds, the annual prevalence during 2017-18 has also seen a decrease to 1.2% from 2.8% [46]. However, there are survey's also reporting a lack of understanding relating to health risks associated with NPS use even since the introduction of the Psychoactive Substances Act 2016 [47]. A significant effect of the introduction of the Psychoactive Substances Act 2016 was the closure of 'head shops', which lead to a direct increase in NPS users having to use 'dark net' and street dealers [47, 48]. Thus, resulting in increased risks for NPS users, including exposure to drugs of abuse that have more harmful health risks and also the possibility that NPS users purchasing via the 'dark net' being prosecuted as this legislation made it illegal to produce, supply, export or import an NPS [47-49].

There has been an increase in the injecting of NPS reported in Scotland, which was linked to an increase in Hepatitis C Virus infections [50 51]. Another significant negative impact of the introduction of this legislation on the homeless communities, is the failure to reduce the use of synthetic cannabinoids within this vulnerable population [52].

Deen *et al* (2021) invested the effects of the Psychoactive Substances Act 2016 on the number of deaths in England, Wales and Northern Ireland [48]. There were 293 NPS-related deaths reported with an increase of over 220% deaths (at 222%), an increase in the average age of these individuals, and a significant increase in these individuals being from deprived areas, after the implementation of the Psychoactive Substances Act 2016 [48]. Demonstrating a significant shift in the not only the number of NPS-related deaths, but also in the shift to older individuals from deprived areas from previous recreational users of NPS prior to the introduction of this legislation [48].

1.1.2.3. United States Legislation

In the USA, the Comprehensive Drug Abuse Prevention and Control Act (1970) was passed which classifies drugs based on whether the drug has any medical use, a serious health risk, and the likelihood they will lead to either physical or psychological dependence from abuse [53, 54]. The Anti-Drug Abuse Act of 1986, has enabled the Controlled Substances Act (CSA) to include NPS, as well as provide required minimum sentencing for the trafficking of drugs [53, 55]. The Anti-Drug Abuse Act of 1988 was implemented to decrease the accessibility, but also the demand of drugs of abuse [53, 56]. The act importantly created the Office of National Drug Control Policy (ONDCP), and the Chemical Diversion and Trafficking Act (CDTA) that were formed to decrease the accessibility of the chemicals that are used to produce drugs of abuse [53, 57]. For example, 4-MMC is a Class B drug in the UK (Table 1.1.), whereas in the USA it is under Schedule I of the CSA (Table 1.2.). Demonstrating that the US has enforced the highest level of classification in

comparison to the UK where 4-MMC is under the second highest level of classification. It is worth noting, that each state in the USA have their own drug laws, which can vary largely between states with regard to the sentencing and consequences of possession.

Table 1.2. *Schedule I-V controlled substances of the Controlled Substances Act (CSA) (adapted from [57])*

The CSA (1970) drug scheduling classification	**Description**	**Examples**
Schedule I	This is the highest classification; with the drugs not being utilised in medical treatment, a serious health risk and having an increased likelihood to be ab(used)	Cathinone Cannabis 4-MMC MDMA MDPHP NEP Heroin
Schedule II	This is the second highest classification; controlled use of drugs for medical treatment within the USA, with the drugs still having an increased likelihood to be ab(used)	Amphetamine Fentanyl Morphine Cocaine Phencyclidine (PCP) Methamphetamine
Schedule III	This is the third highest classification; use of drugs for medical treatment within the USA, with their ab(use) having the potential to result in mild-moderate physical dependence, but also severe psychological dependence	Acetylsalicylic acid Codeine Paracetamol
Schedule IV	This is the fourth highest classification; use of drugs for medical treatment within the USA, lower likelihood to be abused than drugs classified within Schedules I-III	Clonazepam Diazepam Tramadol
Schedule V	This is the lowest classification; use of drugs for medical treatment within the USA, and having the lowest likelihood to be abused than Schedules I-IV	

Numerous synthetic cathinones have been placed under the Substance Control Act between 1973 and 2018. In 2011, the Drug Enforcement Administration

(DEA) placed 4-MMC, MDPV and methylone, three synthetic cathinones under the Substance Control Act [31, 58]. A recent report by the National Institute on Drug Abuse, stated that by 2018 there was a significant decrease in the use of synthetic cathinones by over 50% in 12th grade students, which could be a potential result of this legislation [31, 59]. In 2018, N-ethylpentylone (NEP) was placed under Schedule I of CSA, as this synthetic cathinone derivative was responsible for 151 overdose deaths [60].

1.1.3. Lab-on-a-Chip (LOC)

An LOC device allows for multiple laboratory-based analytical techniques to be miniaturised by incorporating microfluidic methodology. Microfluidics involves the manipulation of fluids within channels on a micrometre scale [61, 62]. This offers significant advantages over more traditional methodologies, but also enables new developments, which would not be possible on a larger scale. These wide-ranging advantages include: cost-effectiveness in terms of equipment needed and lack of specialist facilities/staff, reduced sample requirements, reagent consumption and waste effluent and sample requirements, faster reaction times (due to a larger surface area to volume ratio), and increased portability [62]. In recent years, the field of microfluidics has become an extremely multidisciplinary area of research. While the development of fully integrated 'sample in-answer out' LOC devices have focussed on fields such as clinical diagnostics [62]. An example of a completely integrated LOC for forensic purposes is the RapidHIT® ID System that analyses buccal swab samples for human identification purposes and can produce a DNA profile in just 90 minutes [63, 64]. The first research journal article reporting a fully

integrated LOC device capable of producing a DNA profile was published by Hopwood *et al.* in 2010 [65], and from this publication to the first commercially available LOC system it took approximately two years. Since the introduction of the Rapid DNA Act of 2017, such LOC technology has been used by law enforcement for the analysis of reference samples [63, 66]. This demonstrates that LOC systems have the potential to be used effectively as part of forensic investigations. However, in terms of using LOC systems for forensic analysis for legal use, it is essential that accreditation to ISO/IEC 17025 is undertaken [67, 68]. It is therefore essential that these LOC developed techniques are fit for purpose, including the ability to detect drugs of abuse and NPS (as well as their metabolites) in biological samples and also in terms of the detection window of the matrix [1]. With that in mind this review identifies the current state of LOC methods for the detection for drugs of abuse and NPS and the potential impact that these technologies could have within clinical and forensic settings.

This systematic review evaluated the use of LOC devices for the detection of controlled drugs. This has been achieved through: i) A review of the different drugs determined by LOC methods (chapter 1.3.1.); ii) A review of sample matrices and sample types analysed by LOC devices (chapter 1.3.2.); iii) Global trends on research into LOC use for drug detection (chapter 1.3.3.); iv) A comparison of LOC detection methods with regards to manufacturing materials, limits of detection (LOD) and analysis time (chapter 1.3.4.); and v) Identification of knowledge gaps and recommendations for future research (chapter 1.4.).

1.2. Methodology

Using the key criteria for a systematic review [69], a literature search of peer-reviewed articles published from between 1999 to March 2021 was conducted using the Scopus database. Due to the number of non-relevant publications exceeding 1000 for other search engines, such as Google Scholar and Web of Science, Scopus was the only one included in this systematic review. The following four search terms were used:

1. "LOC" OR "lab-on-a-*" OR "microfluidic*" AND "detection" AND "drug* of abuse" OR "new psychoactive substance*" OR "controlled drug*"
2. "lab-on-a*" OR "LOC" OR “microfluidic” AND "detection" AND “legal high*” OR “cathinone*” OR “cannabinoid*” OR "illegal drug*" OR "illicit drug*" OR "opiate*" OR "opioid*"
3. "portable" OR "handheld" OR "disposable" OR "presumptive*" AND "detection" AND "drug* of abuse" OR "controlled drug*" OR "illegal drug*" OR "illicit drug*" OR “legal high*” OR “cathinone*” OR “cannabinoid*”
4. "portable" OR "handheld" OR "disposable" OR "presumptive*" AND "detection" AND "new psychoactive substance*" OR "opiate*" OR "opioid*"

A total of 451 publications were identified. An initial suitability screen of titles and abstracts was performed with the following inclusion criteria; must be a publication containing primary research data incorporating an LOC device or a device with a microfluidic component for the detection of drugs of abuse and/or NPS. All other forms of literature, such as case reports, were not included, and the only literature

included had to be published in a peer-reviewed journal. The publications were considered from any country but needed to be published in English. This process resulted in the identification of a total of 87 manuscripts which were subject to a more rigorous full review. Duplicate manuscripts (from the 4 different combinations of search terms) were removed and the remaining papers screened for suitability. Each paper was reviewed blindly by two individuals and the following information gathered: LOC method detection, drug(s) investigated, LOC material, biological specimen(s) or sample, detection time, description, limits of detection (LOD) and limits of quantification (LOQ). Following all the essential steps of the method for this systematic review resulted in a total of 45 publications included in this study (Appendix 1. 3).

1.3. Results

1.3.1. Drugs of abuse and NPS analysed by LOC methods

1.3.1.1. Drugs of abuse and NPS

A total of 28 different drugs of abuse including NPS were reported in the 45 accepted publications. Fifty-five percent of publications reported more than one drug of abuse, reflecting the ability to perform multiplex detection. Four main drugs (cocaine, methamphetamine, morphine, amphetamine) have been detected in more than 11 of the publications, all of which have a high level of abuse which is reflected by the latest reports on drug trends [2, 31, 32] (Figure 1.1.). These were followed by drugs of abuse that were less commonly investigated (Δ^9-tetrahydrocannabinol, Δ^9-THC), codeine, ketamine, MDMA, heroin, benzoylecgonine), which were reported in between 9-16% of the accepted publications. Then the final group includes several drugs of abuse, metabolites, precursors and NPS that were reported in between 2-7% of publications. Importantly, it is worth noting that the existing literature has focused on the detection of the more established drugs of abuse, with only one of the publications reporting the detection of NPS (4-MMC and 4-ME) [61].

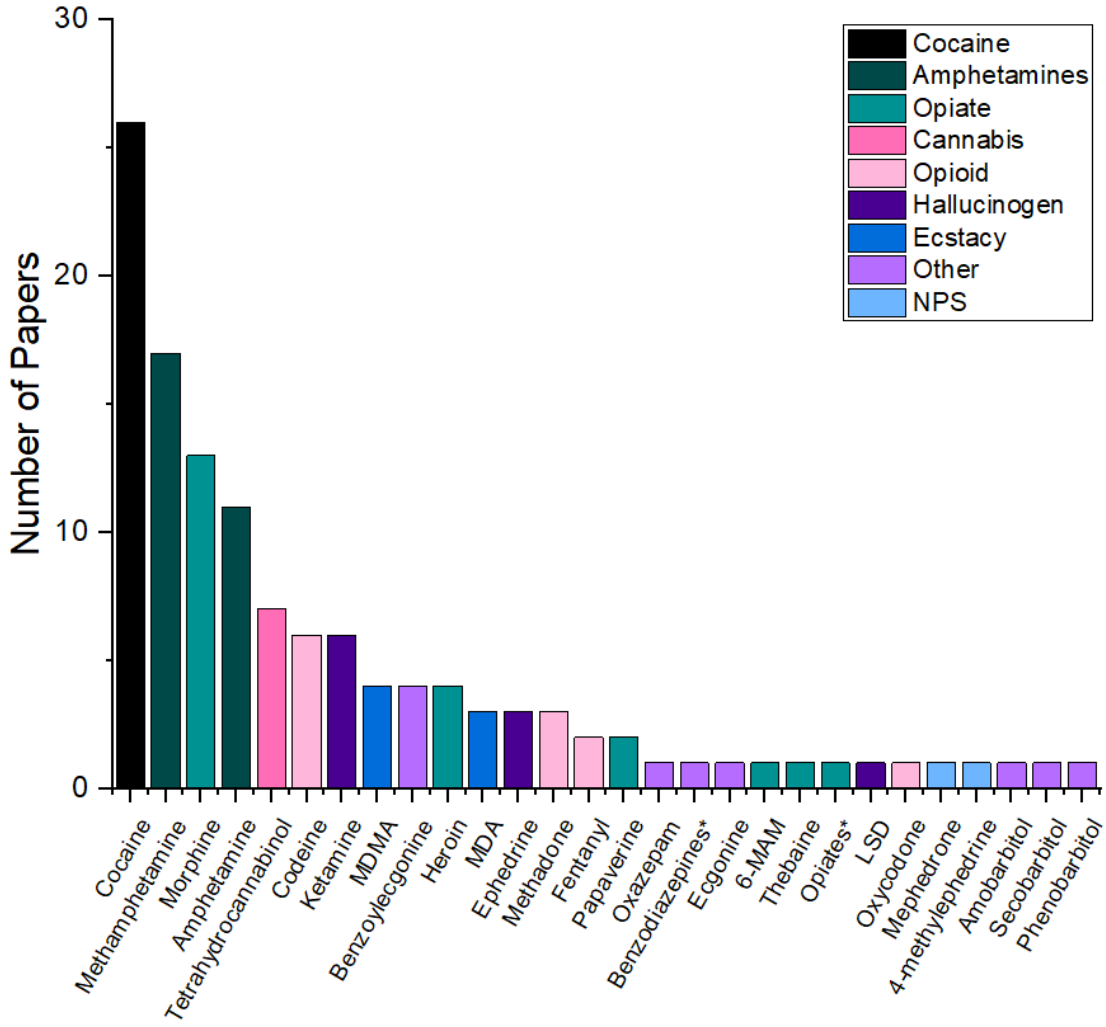


Figure 1.1. *Number of publications for each compound detected in the publications categorized according to the drug definitions used by the UNODC. *Li et al (2015) states opiates and benzodiazepines, however further clarification is not provided*

The most commonly investigated drugs of abuse using LOC devices reflected ongoing global trends in drug prevalence. Cocaine was the most investigated drug of abuse using LOC detection methods and was reported in 58% of the publications accepted in this review. This follows the global trends with cocaine being the main stimulant used in North and South America, as well as Central and

Western Europe, Australia and New Zealand, with 19 million users globally [70]. Twenty-seven million users of amphetamines were reported in the latest World Drug Report, globally encompassing the most popular group of stimulant drugs [70]. This correlates with methamphetamine and amphetamine being the second and fourth most reported drugs of abuse for LOC detection at 38% and 24% of publications, respectively. Methamphetamine dominates the manufacture amphetamines [31, 70] and this is reflected in the slightly higher proportion of publications detecting methamphetamine in comparison to amphetamine. Morphine was the most reported opiate and the third most commonly reported drug of abuse and was targeted for LOC detection by 29% of publications. Morphine continues to be one of the most abused opiates throughout the world [2, 30, 71]. However, with the increase in more potent opioids available on the recreational drug market, such as fentanyl and carfentanil, and the likely increase in resulting deaths globally [2, 30, 70, 72], it is probable that trends in the use of LOC detection methods will change in response.

For some drugs of abuse, their widespread global prevalence is not reflected in the number of publications related to their analysis using LOC devices. Δ^9-THC is the main psychoactive substance in cannabis, which was reported as the most commonly abused drug worldwide, with 192 million users estimated in 2018 [70], yet only 16% of articles reported THC detection. Codeine and ketamine were included in 14% of the accepted publications. Ketamine is not currently under international control but is a widely abused drug of abuse and is the main hallucinogen seized internationally accounting for 87% in the last five years,

mostly from East and South Asia. Heroin is one of the most commonly ab(used) established drugs of abuse worldwide since it emerged into the recreational drugs market in the 1960's and is still one of the most abused opiates throughout the world, but there were only 4 publications detecting heroin included in this review [73-76]. With over 66% of all globally reported drug-related deaths were related to opioid use [72], overall there were six opiates (36%) and four opioids examined, with the most commonly detected being morphine and codeine, respectively. With a distinct gap in the detection of Δ^9-THC, hallucinogens and opioids this indicates that perhaps research into detection using LOC do not necessarily follow the global drug use statistics, but more the societal impacts and health impacts.

A review of the existing literature shows that there is also significant gap in research using LOC technology for NPS detection with only 3 out of the 45 publications investigating NPS. Two of the articles detected fentanyl using SERS-based methods [74, 77], and one used a paper-based competitive immunoassay LOC device for the detection of 4-MMC and its metabolite, 4-ME [61]. As the articles were published between 2019-2021, this may hint at a potential increase in the number of future publications investigating the detection of NPS, as worldwide prevalence increases.

1.3.1.2. Cutting agents, diluents, adulterants and pro-drugs

Cross reactivity can have a significant effect on the accuracy of any drug detection methods, however, only 29% of the publications investigated the potential cross reactivity of cutting agents, diluents, adulterants or pro-drugs using an LOC device [61, 76, 78-86]. The most extensive research on cross reactivity has been carried out on

those devices which utilise colourimetric detection. Sixty-four compounds were investigated for their potential interference in the colourimetric detection of four different drugs of abuse (cocaine HCl, crack cocaine, heroin and methamphetamine) using a paper-based LOC device [76, 86]. Of these 64 compounds, there were only five that reacted, including levamisole, Xanax®, and procaine. Musile *et al* (2015), investigated the effects of four cutting agents, six diluents and eight common powders using an LOC device for multiplex detection of nine drugs of abuse using presumptive testing reagents [80, 86]. False positives for baking soda, caffeine, procaine and quinine were observed [80]. Da Silva *et al* (2018) recorded false positives for paracetamol when investigating the cross reactivity of the adulterant, phenacetin, as well as six commonly encountered cutting agents with a colourimetric LOC device for the detection of cocaine [79], as well as a 10% colour suppression for both procaine and aminopyrine [79].

When cross reactivity was examined in LOC devices that incorporated immunoassay-based detection systems, the results showed a lack of cross reactivity as would be expected due to the specificity of the antibody-antigen interaction. Krauss *et al* (2016) investigated the cutting agents, acetylsalicylic acid (aspirin), caffeine, dextrose and lidocaine and observed no cross reactivity with cocaine and methamphetamine [78]. While Bell and Hanes (2007) investigated the use of five commonly encountered cutting agents (aspirin, caffeine, dextrose, lidocaine, and starch) and showed no cross reactivity when detecting amphetamine, cocaine, methamphetamine and oxycodone [81]. A combination of 11 adulterants, interferents and cutting agents were investigated using a

competitive immunoassay by McNeill *et al* (2021) with no cross reactivity identified in detecting 4-MMC and its metabolite 4-methylephedrine [61].

In terms of electrochemical systems, Yehia *et al* (2020) investigated the effects of 6 interferents commonly encountered in beverages when developing a LOC device for detection of ketamine in spiked drinks and found that tryptamine and phenylethylamine affected potentiometric detection [85]. Ameku *et al* (2021) could successfully detect cocaine adulterated with 4-dimethy-aminoantipyrine using electrochemical detection but some cross reactivity was observed with other cutting agents such as lidocaine and levamisole [82]. A paper-based electrochemical LOC device used for the detection of LSD investigated interference testing with three compounds: methamphetamine showed no response, MDMA showed a well separated peak from LSD, but the structurally similar lysergic acid amide was indistinguishable due to similar voltametric peaks [83]. Wang *et al* (2018) developed an aptamer paper-based LOC device for the colourimetric detection of cocaine [84]. The authors investigated the effects of different interferents, including 8 common white powders, 4 diluents, 7 drugs of abuse and two metabolites of cocaine, with methamphetamine being the only substance to produce a reaction [87].

The publications that investigated the effects of cutting agents, diluents, and adulterants identified some degree of cross reactivity with the LOC devices. This is predominantly because the detection mechanisms investigated here were mostly colourimetric (59%), so can be influenced by coloured impurities and reactivity with reagents. Due to the potential issues with cross reactivity, it is

important that assessments are performed when validating a new LOC device, in order to avoid false positive or false negative responses. However, from reviewing the available literature it appears that this is not always the case across a wide variety of different detection methods and is something that should always be considered in such research to validate efficacy. These issues are not specific to the LOC devices though as standard colourimetric tests would also experience the same cross reactivity, yet the LOC devices offer the potential to include in-built controls, analysed in parallel, that could identify these and therefore enable the LOC device to be more reliable and accurate.

1.3.2. Sample Matrices

LOC devices have been used to detect drugs of abuse including NPS in five different biological matrices (urine, oral fluid, plasma/serum, sweat and hair), as well as in powder form or in aqueous solution (Figure 1.2.). Twenty-four percent of the publications studied more than one type of sample matrix, reflecting the adaptability of the LOC device to different types of sample matrices.

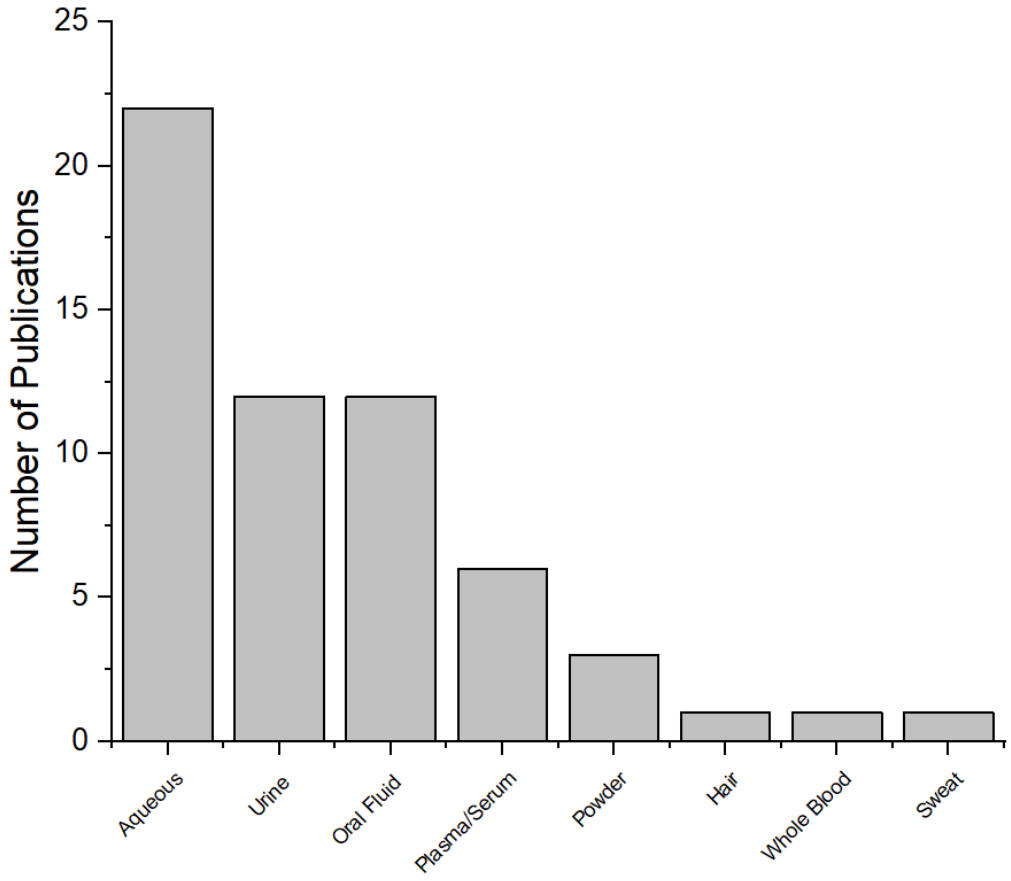


Figure 1.2. *The number of publications and the sample matrixes investigated*

1.3.2.1. Non-biological matrices

Aqueous solutions were the most used matrices in more than half of the publications (51%) and demonstrated using all the different types of detection methods discussed in this review. Aqueous samples offer advantages as these solutions can be representative of bulk or seized drug samples and can be easily prepared by dissolving the analyte in solution. A variety of non-biological matrices were reported including water [61, 80, 83, 88-92], acetonitrile [83], phosphate buffered saline (PBS) [82, 93-95], methanol [77, 83], combination of acetone and deionised water [80] and 2-(N-morpholino) ethanesulfonic acid (MES) buffer [96], as well as beverages

including energy drinks [85] and fruit juices [85] to represent 'spiked drink' samples. Only 7% of the publications using LOC devices tested powder drug samples. This is likely because the design of the sample interface with the LOC device is more complex for powdered samples compared to liquids that can be added to the device more easily. One disadvantage of this type of matrix is that they are not representative of biological samples, which may also include drug metabolites and additional interferants.

1.3.2.2. Biological matrices

While aqueous solutions were the most commonly used sample matrix, collectively biological matrices were included in the vast majority (58%) of publications testing drug of abuse including NPS using LOC devices (Figure 1.2.). Non-invasively collected samples (urine, oral fluid, sweat or hair) were predominantly analysed in over half of the publications (23 out of 45) as they are easier to collect, with urine and oral fluid in particular being compatible with current law enforcement practices [97]. Urine and oral fluid were the second most commonly used matrices included in 27% of the publications and were the most commonly encountered sample matrix in 50% of the immunoassay-based publications [61, 88, 94, 98-103]. Only two publications focussed solely on an invasively collected biological matrix of plasma [104, 105]. Three more publications demonstrated adaptability of their LOC devices in accepting multiple sample matrices including urine and plasma [98], oral fluid and plasma [99], and whole blood, plasma and urine [106]. The use of different matrices showed similar results for all these studies in terms of usability of the LOC device [98, 99, 106], for

example Far *et al*. (2005), showed clinically similar levels of amphetamine in plasma (6 ng mL^{-1}) and urine (20 ng mL^{-1}) [98].

The analysis of biological samples matrices provides the opportunity to examine both the parent drug and any metabolites which can provide additional information on how much of the drug has been administered to aid both clinical and forensic analysis [97]. In addition, the use of different biological samples allows for flexibility in testing with varying detection windows, with oral fluid (from hours), urine (days), sweat (weeks) to hair (months) [97]. For the drugs of abuse including NPS investigated, a total of eight metabolites were examined: two cocaine metabolites (benzoylecgonine, ecgonine methyl ester), benzodiazepine metabolite (oxazepam), three heroin metabolites (codeine, 6-monoacetylmorphine [6-MAM], and morphine), MDMA metabolite (3,4-Methylenedioxyamphetamine; MDA) and one of the metabolites of 4-MMC (4-ME). These eight metabolites were reported in over a third (40%) of the overall publications, with morphine being the most widely detected. Aqueous solution was the most encountered sample matrix in 39% of the publications. However, 22% of the publications that detected a metabolite included the use of urine as the biological matrix. Qiang *et al* (2009) detected eight drugs of abuse, including the parent drug heroin and two of its metabolites morphine and codeine in urine using capillary electrophoresis [73].

It is essential that when developing an LOC device for drug detection, that the time detection window is considered and appropriately matched to the sample matrices to be used. Across all drugs of abuse, the detection window for oral fluid is the shortest, while the detection window for hair is the longest (up to 90 days) [97]. For

example, in this review cocaine was the most widely included drug of abuse and the detection windows are; urine (2-4 days), oral fluid (1-36 hours), and hair (up to 90 days) [97]. Whereas, the metabolite of heroin, morphine was the third most widely used drugs of abuse and the detection windows are; urine (2-5 days), oral fluid (1-36 hours), and hair (up to 90 days). With the detection windows only differing for urine from the parent drug heroin (2-3 days), the metabolite offers the advantage of a slightly longer detection window. With all of the publications within this review detecting the drugs of abuse in 30 minutes or less, this highlights the advantageous speed of analysis of LOC detection for drugs of abuse including NPS.

LOC devices are available for a range of different sample matrices, but it is not possible to directly analyse all sample types with one universal device, as well as offering the opportunity for rapid analysis with short detection windows for some drugs that have a short half-life, such as 6-MAM. LOC devices are advantageous compared to traditional detection methods as they are portable which means they may encounter a range of sample matrices. Currently only 13% of publications that investigated the use of both biological and non-biological samples, and it would be beneficial to LOC devices using both to capture the potential of the LOC devices that are being researched.

1.3.3. Global Prevalence

There were 18 countries affiliated to publications on the detection of drugs of abuse including NPS using LOC devices (Figure 1.3.), with the largest number of papers were published in North America (44%). It is perhaps not unexpected that the majority of publications are from more wealthy countries in terms of research

capacity but here we explore links to specific drug prevalence's globally. Comparing the percentage of drugs of abuse in the six categories (amphetamines, cannabis, cocaine, ecstasy, opiates, and opioids) show a focus on amphetamines in North America (30%) whereas a focus on opiates in East and South-East Asia (29%).

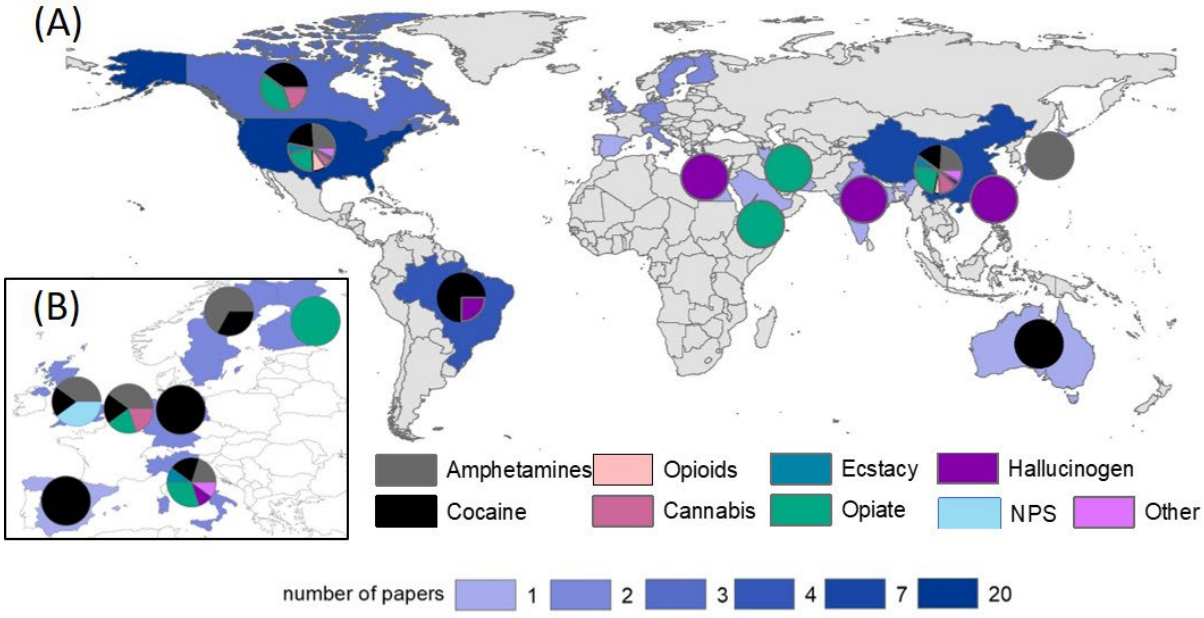


Figure 1.3. *Geological distribution of the accepted publications (all affiliations included) reporting the use of LOC for the detection of drugs of abuse including NPS, with pie charts illustrating the classifications of the drugs analysed (A) worldwide (B) Europe in more detail*

1.3.3.1. North America

North America accounted for the largest number at 41% of the total of publications in this review, with the second largest number of publications affiliated to solely to the USA (37%) (Figure 1.3.). The highest global annual prevalence of cocaine use was observed in North America [70]. Between 2013-2017 cocaine seizures were reported in over 140 countries worldwide, however this review shows LOC

technologies were only developed to detect cocaine in 11 different countries. In 2017, there were 238 tons of seizures in North America a dramatic increase from 94 tons in 2013 [2]. Half of the publications reporting the detection of cocaine affiliated USA and also 100% of the publications reporting the detection of its metabolite, benzoylecgonine. Highlighting that developing LOC devices that detect both the parent drug cocaine, and its main metabolite are invaluable to addressing the high levels of prevalence in North America.

North America has the second highest prevalence for amphetamines, ecstasy and opioids [70]. The trends for publications are well linked to geographical use, as 70% of the publications reporting the detection of amphetamine were published in the USA. An increase in the stimulant methamphetamine use in North America [2], is supported with a large number (73%) of the publications detecting methamphetamine affiliated to North America. The trends in the scientific literature appear to follow this increased drug use as 89% of the methamphetamine studies from the US were published between 2015-2019. North America had the highest number of the publications reporting the detection of MDMA (75%) and MDA (67%). Seventy-five percent of the publications reported the detection of an opioid in North America which correlates with the significant number of overdose deaths (~ 70,000) linked to opioid use in 2018 [70]. The highest annual global prevalence of cannabis use was observed in North America [70], which is reflected with 71% of the publications reporting the detection of Δ^9-THC published in the USA. However, it is worth noting that the overall number of publications in this review reporting the detection of Δ^9-THC is

relatively low in comparison to extremely global prevalence levels, which could be due to fewer health risks in comparison to other drugs of abuse.

1.3.3.2. Asia

Asia accounted for the joint second largest number of publications (at 18%) in this review, with the second largest number of publications affiliated to solely to China (16%) (Figure 1.3.). Publications were associated with the major drug trends/usage in these countries. For example, the main drug of abuse requiring treatment is methamphetamine and 57% of the publications in this review detecting methamphetamine were affiliated with Asia [2]. The highest prevalence for amphetamines was reported in Eastern and South-East Asia [70], reflected in the percentage of publications in China reporting the detection of amphetamines (57%). The second highest number of publications (14%) reporting the detection of Δ^9-THC were published in China (Figure 1.3.), with prevalence levels in East and Southeast Asia being the third highest globally. However, 97% of global morphine seizures were located to three countries (Iran, Afghanistan and India) [71] and there was only one publication affiliated to each of India [92] (reporting the detection of ketamine) and Iran [107] (reporting the detection of morphine, codeine and papaverine). The most recent of the two publications, Farahani *et al* (2020) addressed the requirement for the detection of morphine in relation to seizure trends [107].

1.3.3.3. Europe

Europe accounted for 28% of the total number of publications in this review, the joint second largest number of publications, with 7 countries represented. In the

World Drug Report (2020), Western and Central Europe reported the second highest annual prevalence of cocaine [70, 71]. There has also been an increase in the number of people requiring treatment for the first time as a result of cocaine use in Europe, with the large majority (75%) of these drug users requiring treatment in the UK, Spain and Italy [2]. Within Europe the combined contributions was 27% of the total of publications reporting the detection of cocaine. These trends show that LOC devices are predominantly being developed in westernised countries where cocaine is used recreationally.

Western and Central Europe have the third highest prevalence for amphetamines, and ecstasy [70], with amphetamines reported as the main stimulant in Europe [31]. The trends for publications are well linked to geographical use, reflected in the results as 42% of the publications reporting the detection of amphetamine were published in Europe. There has been no increase in research in European countries (25% of methamphetamine studies) where the levels of methamphetamine use have declined or remain at a stable level as the articles were published in prior to 2016 [31]. The second highest at 25% of the publications reporting the detection of MDMA were published in Europe, reflective of the prevalence levels. There was only one publication reporting the detection of Δ^9-THC using LOC devices, published in the Netherlands in 2009 with no further research in Europe, which is not reflective of current figures of cannabis use within Europe [70].

1.3.3.4. Rest of the world

South America accounted for 9% of the overall publications reviewed. Seventy-five percent of the accepted publications published in South America investigated cocaine detection using seized samples and were published recently (between 2018-2021), supporting their potential for use in examining seized drug samples with South America being one of the main trafficking routes of cocaine to North America [31, 70]. Both the Africa and Oceania were affiliated with single publications, based around detection of ketamine and cocaine.

1.3.4. Detection Methods

The detection methods utilised in the publications included in this review were grouped into 6 different categories; aptamers, capillary electrophoresis (CE), colourimetric, electrochemical, immunoassay, and spectrometry. However, it is worth noting that there were four publications that combined one or more detection method, including colourimetric and electrochemical [82], colourimetric and immunoassay [87], colourimetric, electrochemical and fluorimetric [85], and electrochemical and immunoassay [88]. Immunoassay-based detection techniques were the most common detection method used and accounted for 34% of the studies in this review (Figure 1.4.). This could be due to ease of manufacturing, simplicity of immunoassays as well as being relatively inexpensive. However, another important consideration is that immunoassays are the preferred initial screening tests in laboratories throughout the world for drugs of abuse, highlighting a beneficial advantage of this method for miniaturisation using microfluidics [97, 108-111]. A substantial number of studies also employed detection methods using

spectrometric (27%) or colourimetric tests (19%), whereas electrochemical (12%), CE (4%) and aptamer (2%) methods were used less (Figure 1.4.).

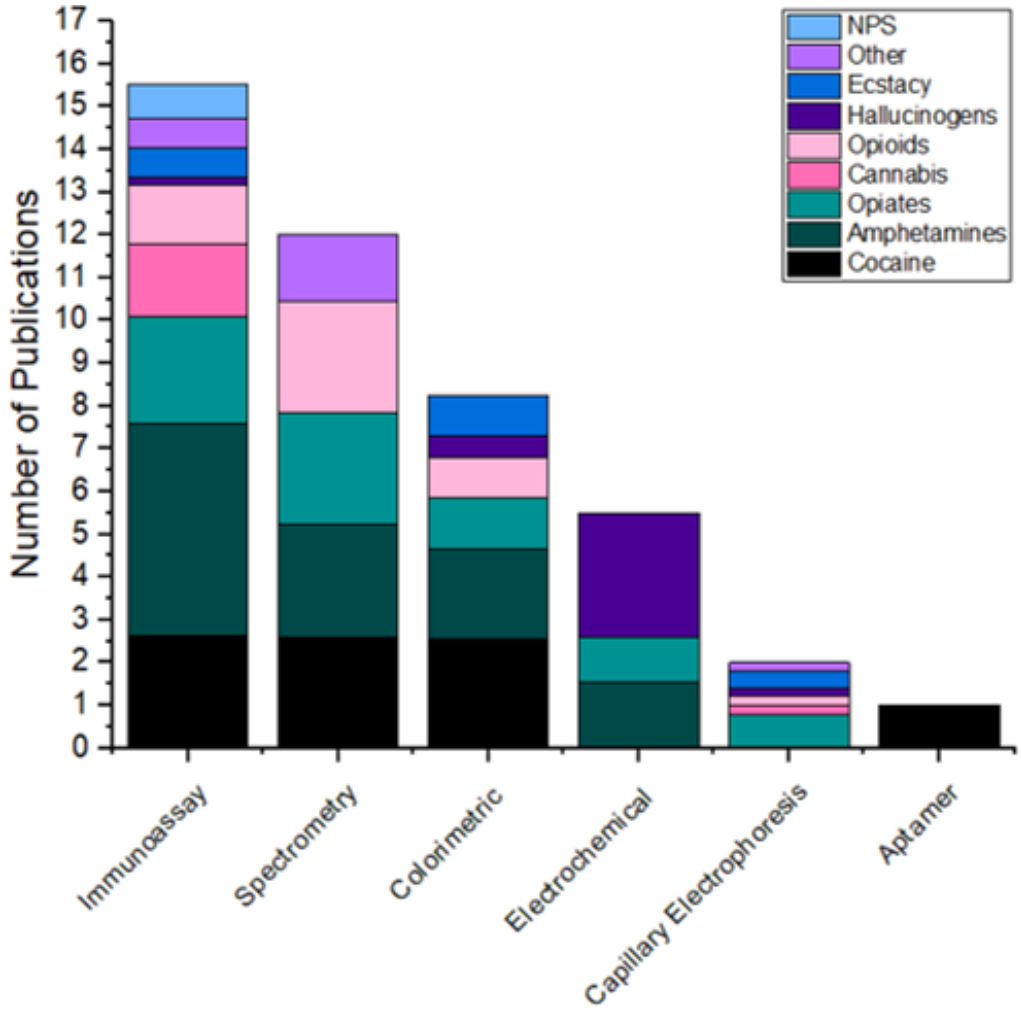


Figure 1.4. *The number of publications as analysed by different detection methods*

1.3.4.1. Multiplex detection

Multiplex detection was reported in more than half (51%) of the accepted publications. The most commonly encountered number of drugs for multiplex detection was 2 at 39% and the largest number of drugs detected using a multiplex LOC device was 12 using immunoassay-based programmable bio-nano-chips (p-BNC) [101]. One hundred percent of the publications using CE methods reported

multiplex detection [73, 96], followed by 79% of colourimetric detection [75, 78-81, 86] and 65% of the immunoassay-based publications [61, 93, 99, 101-103, 106, 112]. For the spectrometry techniques, 67% of the publications used multiplex detection [74, 77, 89, 113-115]. It is worth noting that there was no multiplex detection reported for the publications utilising either electrochemical or aptamer detection techniques. The World Drug report (2020) states an increasing trend in polydrug use, for example >65% of cocaine drug users requiring treatment for using with other substances [2], therefore multiplex detection would be invaluable, especially with a LOC device.

1.3.4.2. Limits of Detection (LODs)

An integral aspect of developing a detection device is to ensure that it is fit for purpose and able to detect to a both clinically and forensically relevant levels. It is worth noting the LODs were not clearly stated in 22% of the publications reviewed, but this is because the devices were designed to be quantitative. For some studies, the LOC device was designed to qualitatively identify a pure substance, however by including an indicative LOD it makes it much easier to identify the benefits of the device and establish if it may be applied to a wider range of applications. Where accepted publications clearly stated LODs, the majority of these were to clinically relevant levels (low ng level) highlighting the potential for LOC devices to be used to be used alongside (or replace) traditional laboratory-based methods. Immunoassay-based publications reported clinically relevant low LODs between 1-1000 ng mL^{-1} [61, 88, 94, 98, 99, 101, 103, 104], with one publication reporting levels to pg mL^{-1} [112]. The LODs for spectrometry techniques ranged from 0.0178-51 ng mL^{-1} and

offered similar level of sensitivity based on the LODs for drugs of abuse reported in the publications when compared to other LOC detection methods [77, 89, 90, 105, 107, 113, 114, 116-118]. All electrochemical techniques reported LODs, however, these were the least sensitive method ranging from 760.72 - 1.24 x 10^{16} ng mL^{-1} [83, 85, 91, 92, 119]. LOC devices utilizing CE also reported slightly higher LODs than the other LOC detection methods, with Qiang *et al* (2009) reporting LODs between 1150-2090 ng mL^{-1} for a range of drugs of abuse with the authors acknowledging further research needs to be undertaken to ensure that these are more clinically relevant [73]. Several colourimetric detection methods reported high LODs, ranging from 1200 ng mL^{-1} - 10 mg mL^{-1} [75, 78-80, 85]. With Musile *et al* (2015) reported a minimum quantity detectable (MQD) for both visual and instrumental analysis of 2500-1100 ng mL^{-1} and 1200-8700 ng mL^{-1}, respectively [80]. Whereas, Bell and Hanes (2007) reported positive results that were significantly lower than clinically relevant levels between 0.05-0.125 ng mL^{-1} [81].

The LOD was 0.659 x 10^{3} ng mL^{-1} for the aptamer detection method [95], which was higher than previously reported levels [120-124]. Due to the limited number within this detection method, it is difficult to provide further clarity using this aptamer detection. A paper by Yehia *et al* (2020) combined three different detections methods, electrochemical, colourimetric and fluorescence for the detection of ketamine with high varying LODs of 760.72 ng, 10 mg mL^{-1}, and 0.0475 mg mL^{-1} respectively [85].

1.3.4.3. Manufacturing materials

The LOC devices reported were manufactured from a range of different materials, categorized into polymers, glass, paper, and combined materials (Figure 1.5.). The most commonly utilized material category for the publications was polymers at 33%. Sixteen percent of publications included a combination of more than one manufacturing material categories.

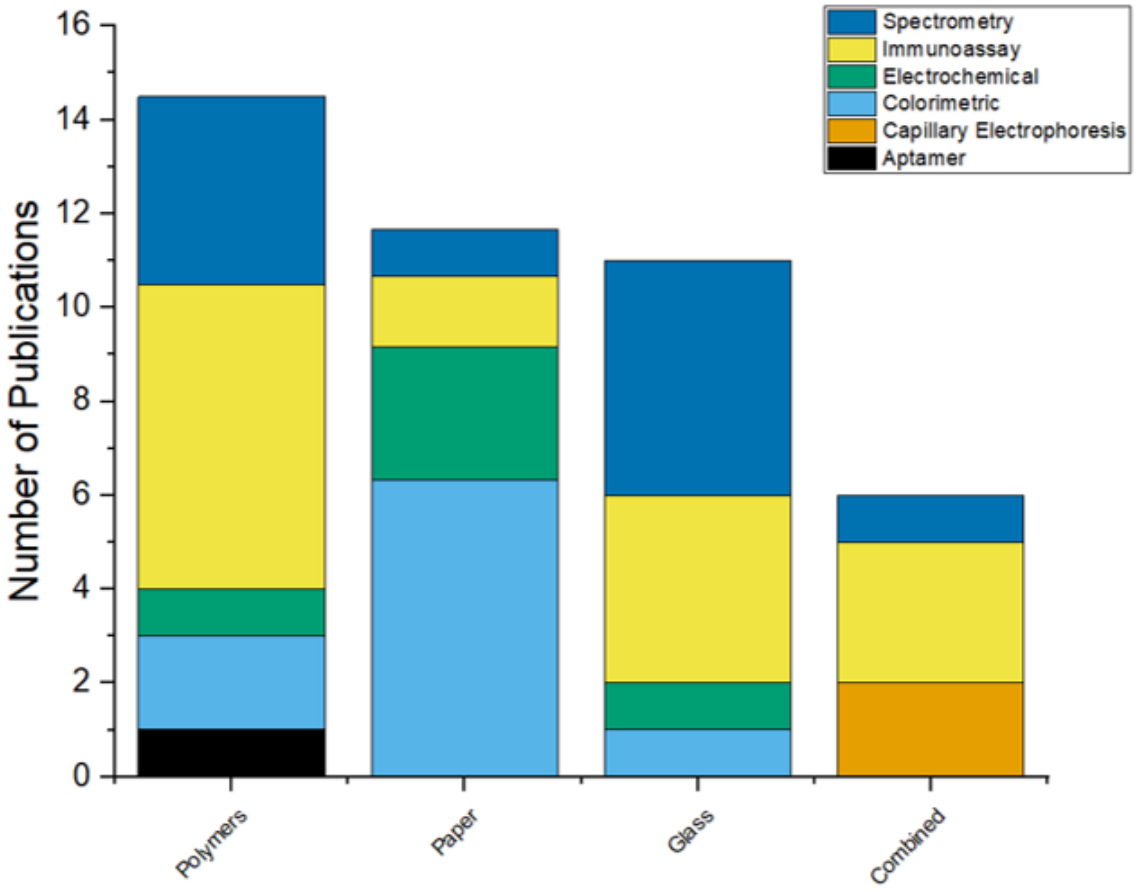


Figure 1.5. *Number of publications and manufacturing materials included categorised by the detection method incorporated within the LOC device. The polymers category includes polydimethylsiloxane (PDMS)* [77, 95, 103, 116]*, polyethylene glycol (PEG)* [104]*, polyvinyl chloride/ polytetrafluoroethylene (PVC/PFTE)* [117]*, polyurethane/polyaniline (PU/PANI)* [107]*, PDMS/PFTE* [88]*, polyester* [75, 78] *and plastic**

[98, 99]. *There were two publications that stated the inclusion of plastic as the LOC material, but did not clarify further. Silica-based LOC devices were included as part of the glass category*

The majority (43%) of the publications including a polymer as the manufacturing material incorporated immunological detection. The inclusion of polymers can be an advantageous in comparison to other LOC manufacturing materials, as they offer rapid prototyping, cost-effective in large numbers, combined with being extremely biocompatible therefore offering flexibility in potential detection methods, an ideal feature for a LOC detection device designed to test potential drugs of abuse in biological samples [125].

The second most commonly utilised manufacturing material was glass (24%) and was frequently combined with immunological detection methods (36%). Glass is not as biocompatible as other manufacturing materials but does have excellent optical properties, reflected by in the inclusion of glass using spectrometry detection methods (33%). A significant advantage of using glass is that there are certain reagents, such as Marquis and Mandelin's reagent used in a number of presumptive test reagents for colourimetric detection that use concentrated sulphuric acid, which are only compatible with glass [81].

Twenty-two percent of the publications reported the use of paper, with 60% of the total publications published since 2020 [61, 82, 83, 85, 86], and included the use of chromatography paper and office paper as example substrates. Ninety-one percent of the publications included sample in a liquid form (including aqueous solutions or

biological fluids). However, the publications that utilised paper as the manufacturing material only included two different biological matrixes (oral fluid and urine) in this review, even though paper is compatible with other biological fluids. However, paper does have limitations, as hair and powder samples require aqueous matrix for capillary action to take place. The use of paper-based LOC devices offers numerous advantages to their traditionally conventional counterparts: they are extremely cost-effective, environmentally friendly, and simple to manufacture for example using just a wax printer [125-129].

Seventeen percent of the publications reported the use of a combination of different materials which allows the LOC device to utilise the benefits the different material types, however due to the complexity of combining materials this has only been reported in 20% of the publication in the last five years. The combined materials category includes publications that reported a combination of different manufacturing materials [73, 90, 93, 96, 101], such as glass and PDMS [90, 96], or a programmable BNC for the detection of 12 drugs of abuse in oral fluid using agarose bead sensors [101]. For further information on fabrication methods for creation of LOC devices there are a number of detailed reviews, such as that by Scott and Ali (2021) [125].

1.3.4.4. Analysis time

One major advantage of LOC devices is their ability to provide a rapid analysis, however for a third (33%) of the publications the total analysis time was not clearly stated. The total analysis time for the publications that did report this ranged from seconds up to 30 minutes (Figure 1.6.). With more than half (51%) of publications

reporting total analysis times under 10 minutes [61, 75, 78, 80, 90, 91, 98, 99, 102, 103, 116, 130]. It is also worth noting that for the total analysis times that were stated, they were not always easy to obtain from the publications either due to timings given for individual processes rather than full analysis, lack of clarity or simply no discussion of the topic.

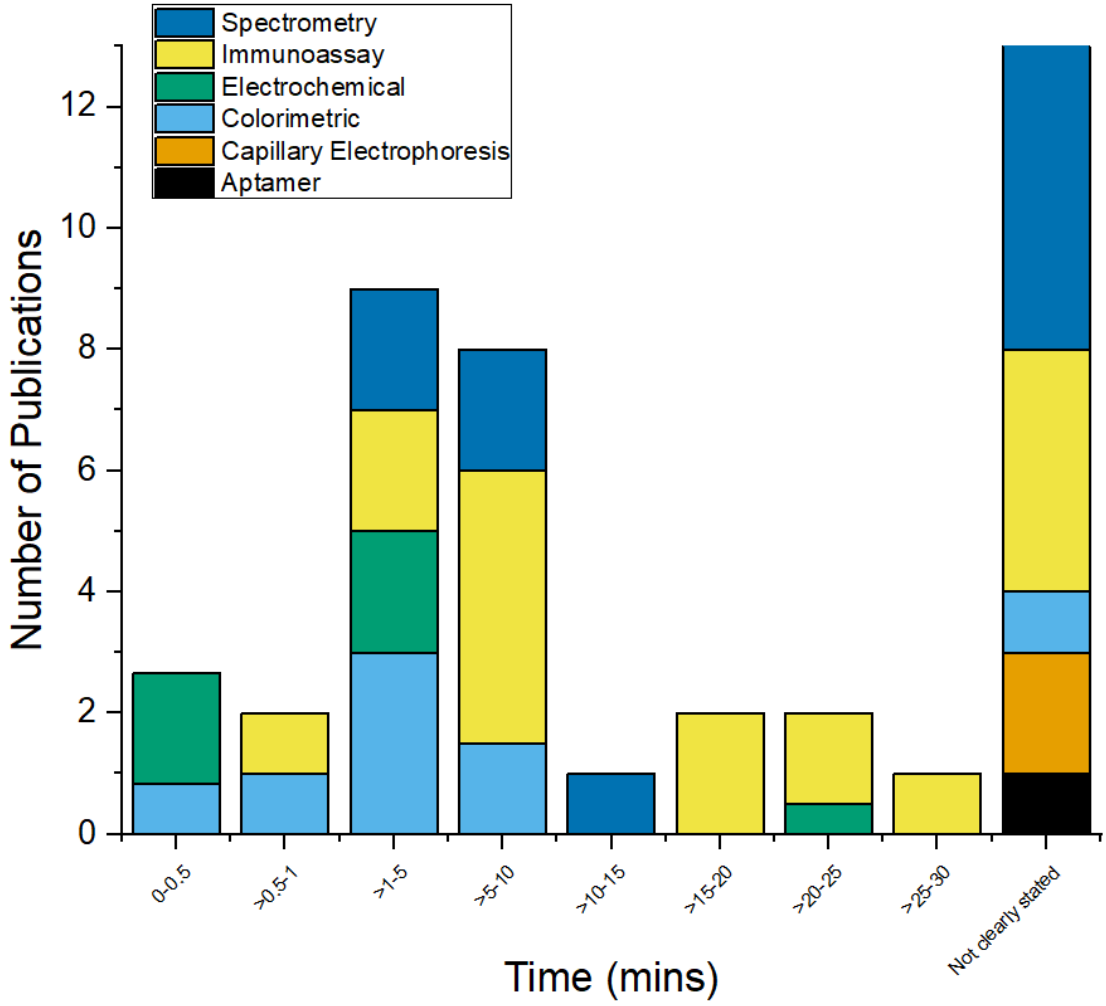


Figure 1.6. *The number of publications for the total analysis time included in the publications in this review*

All of the publications incorporating electrochemical techniques stated the total analysis times of 2.5 minutes and under [83, 91, 92], offering the fastest total analysis time of the detection methods in this review. One hundred percent of the colourimetric-based publications reported detection of the drugs of abuse in 6 minutes and under [75, 78-81, 84, 86]. Immunoassay-based techniques showed the largest variations in total analysis times from 1-30 minutes, with 62% of these publications 10 minutes and under. Less than half (46%) of the spectrometry detection methods clearly stated a total analysis time, however, those that did showed a range from 1 to 15 minutes. Statistical analysis was performed to investigate if there were associations between analysis time and either detection methods, manufacturing material or sample matrices. There was no significant difference observed across these three statistical tests using Kruskal-Wallis ($p > 0.05$). With low numbers in each category, in the future with more publications on LOC devices for the detection of drugs of abuse including NPS this could be investigated further in the future.

1.4. Knowledge gaps and recommendations

1.4.1. Sample types

This review has identified a number of knowledge gaps which could provide a direction for further research into the use of LOC devices for detection of drugs of abuse including NPS. Current statistics published by the UNODC [2, 31, 70] show high levels of prevalence of drugs of abuse including Δ^9-THC and also NPS (including synthetic cannabinoids), which are both under investigated globally. For example, 22 countries in Europe reporting a significant problem of synthetic cannabinoid use, observed in both prisons and homeless communities [70], however, there were no LOC devices reported for detection of synthetic cannabinoids in any of the publications to our knowledge. It is therefore recommended that existing LOC devices could be adapted to look at additional drugs of abuse. The rapid prototyping nature and relatively easy manufacture of LOC devices means that investigating emerging drug trends is feasible.

There were a limited number of publications that investigated real-world samples with whole blood and urine to investigate parent drugs and metabolites, 'spiked drinks', as well as seized drug samples. The inclusion of real-world samples offers an insight into the potential of the LOC device for the detection of drugs of abuse including NPS in the field and if they are fit for purpose as future commercial devices.

1.4.2. Quality assurance

Quality assurance and validation is an important element for any newly emerging techniques, particularly to enable comparison with conventional methodologies.

The most commonly encountered sample matrix was aqueous solution using spiked drugs of abuse, which is beneficial in assessing whether the LOC device can test bulk or seized samples. In addition to this, the inclusion of cutting agents or adulterants should be considered for investigation to check for any potential cross reactivity. However, another important aspect would be to analyse biological samples (rather than sample solutions) with the addition of investigating metabolites as well as parent drugs, and the effect of interferents to establish whether the device is fit for purpose in a wider range of scenarios. LODs need to be investigated if the LOC device offers quantitative analysis to ensure that the developed LOC device is fit for purpose and that they are applicable to real-world samples and can be easily used in the field to detect drugs of abuse and NPS to clinical and forensically relevant levels.

In addition, there are those LOC devices which offer semi-quantitative detection, such as work by our group which uses a paper-LOC device employing an immunoassay for the detection of cathinones [61]. These semi-quantitative methods can provide additional information compared to a simple presence/absence result but without the increased cost usually associated with quantitative methods. The use of positive and negative controls within the LOC devices should be considered to increase the reliability and integrity of each resulting test. While these controls are routinely applied in conventional laboratory settings, they are not always integrated onto LOC devices. This quality assurance is important for future validation of the LOC devices and acceptance within local criminal justice systems.

1.4.3. Practicality

Practicality of LOC devices is another important element as they need to be easy to use and so that potential viable portable detection methods can be used by non-scientifically trained individuals. This would include taking the devices out of the laboratory and conducting field tests to investigate the ruggedness and portability of the devices. Most studies (84%) claim that their LOC devices are “easy to use” but there was little / no quantitative data to help explain how easy these techniques are, which makes any comparison between detection methods difficult. A small number of these included end user testing to assess not only the accuracy of the devices but also if they were user friendly. This review has highlighted a lack of field testing and future publications should consider the inclusion of rigorous in-field test of LOC devices.

Storage is also an important element that is often overlooked, as this is vital requirement to produce a commercial device for the detection of drugs of abuse including NPS. Investigating a range of storage conditions over a period of time in order to determine if the device has the same level of sensitivity and selectivity in 6 months’ time, for example. An ideal LOC device will be able to be stored at room temperature to avoid additional storage requirements (e.g. access to a freezer) to simplify field deployment.

A major advantage to using LOC devices is that they are highly cost-effective in comparison to other traditional laboratory-based detection methods. They are relatively cheap to produce, require less reagents, and subsequently produce less waste, as well as lack a requirement for expensive, specialist laboratory space and

highly trained scientists. Whilst many studies stated the financial benefits of their device (86%) there was a lack of quantitative information that makes a comparison of cost of detection methods difficult.

Most studies used terms such as “low-cost”, “less expensive” or “inexpensive” to describe the costs of detection methods with only two stating the overall production cost of each LOC device, at 10 cents [84] and less than $2 to produce [86]. Therefore, it is recommended that this is included in future publications to enable comparison with traditional laboratory methods and between different detection methods and materials for the LOC devices.

Total analysis times, where reported, were all under 30 minutes (Figure 1.6.) and this highlights another potential advantage for portable testing compared to traditional laboratory-based methods. With a third of publications not reporting a total analysis time, it is recommended that future publications include this rather than giving more subjective statements. Evaluating these elements, cost, time and ease of use, will all aid in determining whether the LOC devices could be applicable for commercialisation.

1.5. Summary

Drugs of abuse including NPS are a continuing to be a worldwide challenge, therefore the development of new portable methods for their rapid detection is pertinent. This review has highlighted the wide range of detection methods, manufacturing materials, drugs of abuse that have been targeted, and the diverse range of sample matrices that can be incorporated into an LOC device.

Global trends in drug abuse were reflected in the number of publications which were aimed at detection of particularly drugs of abuse, for example, the majority of studies (58%) reported using LOC detection methods for cocaine detection, which reflects cocaine being the main stimulant used worldwide, with 18 million users. However, there were some exceptions to this such as cannabis as, although reported as the most commonly abused drug worldwide, only 16% of the publications reported the detection of Δ^9-tetrahydrocannabinol (Δ^9-THC).

Multiplex detection was common (55% of cases) which again is aligned with trends in poly drug use.

LOC devices are capable of accepting a wide range of sample types, both biological and non-biological, enabling all commonly encountered sample types to be analysed. This was linked to a range of detection methods, with immunoassays being the most commonly incorporated (34% of publications) due to their high sensitivity and specificity.

LOC devices for detection of drugs of abuse is still a rapidly evolving field, with 42% of articles published since 2019. Related technologies, without a microfluidic component, such as Lab-on-a-Glove for the electrochemical detection of fentanyl

[131] and Lab-on-a-screen-printed electrochemical cell for the detection of the "rohypnol" drug flunitrazepam [132] represent alternative portable detection methods that are of interest. Going forward this emerging scientific field could offer commercially viable detection, either qualitatively or quantitatively, for the portable and rapid detection of real-world drug samples.

The overall aim of this thesis is to address these gaps in knowledge by developing a rapid and cost-effective LOC device that incorporates a competitive immunoassay for the semi-quantitative multiplex detection of commonly encountered NPS and drugs of abuse. This will be achieved through the following objectives:

1. Undertake a systematic review investigating the available literature for the detection of controlled drugs, including NPS using LOC technology [1].
2. Design and evaluation of elements within the paper-based LOC device including, channel width and channel length (chapter 2).
3. Optimisation and evaluation of competitive antibody-based immunoassays incorporated within the LOC device, for the multiplex detection of 4-MMC and its metabolite 4-ME (chapter 3), amphetamine, methamphetamine and MDMA (chapter 4), and 3,4-methylenedioxy-α-pyrrolidinohexanophenone (MDPHP) and NEP (chapter 5).
4. Investigating the practicality of this LOC device in terms of cross reactivity with adulterants, cutting agents and interferents, investigating the use of aqueous and biological samples, storage, cost effectiveness, and total analysis time (chapter 3).

5. Development and evaluation of the ‘origami’ design of the LOC device for quality assurance purposes, with the inclusion of the positive and negative controls to increase the integrity and reliability of each result provided using the LOC device (chapter 4)
6. Testing the usability of the ‘origami’ LOC device by; blind testing seized drug samples, end-user testing and adaptability of the LOC device (chapter 5).

Chapter 2: Materials and Methods

The materials and methods from this chapter has been published in the peer-reviewed journal Forensic Chemistry. As lead author for the manuscript, I was responsible for preparing the LOC devices, freebasing of both cathinone hydrochloride and conjugation of cathinone to HRP and the freebasing of amphetamine sulphate and conjugation of amphetamine to HRP, method development of immunoassay parameters, aqueous and biological matrix investigations, and immunoassay investigations for the additional adaptability of the LOC device (L. McNeill, C. Pearson, D. Megson, J. Norrey, D. Watson, et al. (2021). Origami chips: Development and validation of a paper-based Lab-on-a-Chip device for the rapid and cost-effective detection of 4-methylamphetamine (4-MMC) and its metabolite, 4-methylephedrine in urine. Forensic Chemistry. 22, pp.100293-100293) [61].

2.1. Executive Summary

This chapter aims to provide a general overview of the materials and methods incorporated in this thesis. The subsequent chapters will discuss in more detail the specific experiments undertaken. Details on the equipment and reagents utilised to conduct experiments using the competitive immunoassay are detailed using the traditional 96 well microtiter plate and the LOC devices (96 well plate template and the optimised LOC device). This chapter will go through the preparation of reagents, the sample types used (biological and non-biological), optimisation processes, creation of the LOC device and testing. An overview of the methodology is shown below in Figure 2.1.

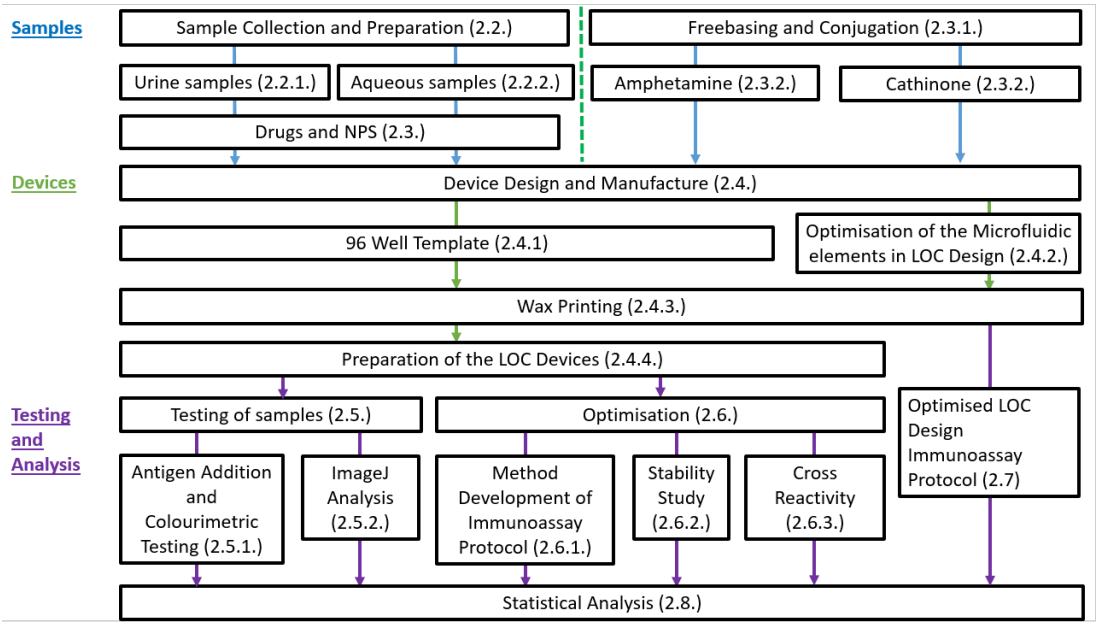


Figure 2.1. *Methodology flow chart*

2.2. Sample Collection and Preparation

The LOC device incorporates a competitive immunoassay for multiplex detection of 4-MMC and 4-ME (using the anti-methcathinone antibody) and amphetamine, methamphetamine and MDMA (using the anti-amphetamine antibody), in both urine and aqueous samples.

2.2.1. Urine Samples

Potential volunteers (inclusion criteria; healthy, over 18 years old, male or female, not a member of staff or a student at MMU, not taken any controlled or prescribed drugs in the last 48 hours, and not consumed alcohol in the last 24 hours) were provided with the Information Sheet and Consent Form (See Appendix 2.1.). The Information Sheet and Consent Form were treated confidentially and stored securely away from the sample(s). The human urine samples were self-collected in

a sterile container from healthy volunteers. Ethical approval for this study was obtained through the Research Ethics and Governance Committee at Manchester Metropolitan University (Ethics approval number: SE151633A1). Following collection, drug and alcohol-free urine samples were then spiked with target analytes and metabolites at clinically relevant concentrations [38], at varying concentrations ranging from (10 ng mL^{-1} – 0.0017825 ng mL^{-1}) to produce 7 dilutions. A negative control (distilled water) was also included in each experiment.

2.2.2. Aqueous

The controlled reference standards (cathinone hydrochloride, cocaine hydrochloride, amphetamine sulfate, MDMA, methamphetamine, and ketamine hydrochloride) for this research were obtained from either Sigma-Aldrich [Gillingham, UK] or Fluorochem Ltd [Hadfield, UK] under UK Home Office license, by authorized personnel and in compliance with both the UK Misuse of Drugs Act (1971) and UK Misuse of Drugs Regulations (2001). All controlled/restricted materials were stored, transferred, used and destroyed in compliance with the UK Misuse of Drugs Act (1971) and UK Misuse of Drugs Regulations (2001).

The synthesis of the racemic target compounds was achieved using the previously reported method by Mayer *et al.* [133]. The hydrochloride salts were obtained as stable, off-white powders and determined to be soluble (10.0 mg mL^{-1}) in distilled water, methanol and dimethyl sulphoxide (DMSO). To ensure the authenticity of the materials utilized in this study the synthesized samples were fully structurally characterized by nuclear magnetic resonance (^{1}H and ^{13}C NMR), GC-MS and Fourier-

transform infrared spectroscopy with attenuated total reflection (ATR-FTIR) and the purity of all samples confirmed by elemental analysis (>99.6% in all cases) [133]. Aqueous samples were prepared by dissolving the target analytes of varying concentrations (10 ng mL^{-1} – 0.156 ng mL^{-1}) in analytical grade to produce 7 dilutions. A negative control (distilled water) was also included in each experiment.

2.3. Drugs and NPS detected using this LOC device

The optimised LOC device designed in this thesis included two antibodies (anti-amphetamine antibody and anti-methcathinone) within the competitive immunoassay for the detection of 4-MMC, 4-ME, MDPHP and NEP (through the inclusion of the anti-methcathinone antibody), as well as amphetamine, methamphetamine and MDMA (through the inclusion of the anti-amphetamine antibody). The multiplex detection capabilities of both antibodies within the LOC device were investigated with structurally similar drugs of abuse and NPS.

The cross reactivity of the anti-methcathinone antibody was tested with 4-MMC and its metabolite 4-ME, as well as newer synthetic cathinones to the recreational market, MDPHP and NEP. The cross reactivity of anti-amphetamine antibody within the LOC device was tested using amphetamine, methamphetamine, and MDMA. Each drug of abuse and NPS was initially prepared in a 10 mg mL^{-1} solution of distilled water.

2.3.1. The drugs of abuse and NPS selected for conjugation to horseradish peroxidase (HRP)

The competitive immunoassay required two horseradish peroxidase (HRP) conjugated analytes to compete with the two target analytes, 4-MMC and

amphetamine in solution. HRP-conjugation was achieved through interaction of the HRP with a primary amine within a target molecule to be conjugated. 4-MMC does not have this functional group, therefore cathinone was chosen as the target molecule as it has significant (2200%) cross reactivity with the anti-methcathinone antibody [134]. To conjugate the HRP to the target molecule, the cathinone firstly needed to be converted into its freebase form to expose the amine and inducing a nucleophilic attack in the presence of HRP.

Unlike 4-MMC, amphetamine does have this functional group and was therefore used as the target molecule. To conjugate the HRP to the target molecule, the amphetamine firstly needed to be converted into its freebase form exposing the amine and thus inducing a nucleophilic attack in the presence of HRP.

2.3.2. Freebasing and Conjugation

Freebasing for both cathinone hydrochloride and amphetamine sulphate were undertaken separately but using the same methodology detailed below (Figure 2.2 A and B, respectively).

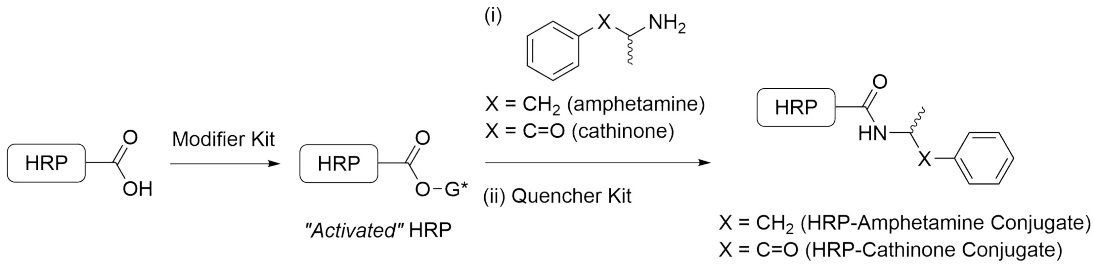


Figure 2.2. *Flow diagram representing the sequential steps of the freebasing and conjugation with HRP using cathinone and amphetamine; using the modifier kit HRP becomes "activated" HRP, (i) amphetamine and cathinone (X represents the functional group for each drug) and (ii) the quencher reagent was added to produce amphetamine-HRP and cathinone-HRP conjugates*

A 10.2 mg sample of cathinone hydrochloride [Fluorochem Ltd, UK] and a separate 10.0 mg sample of amphetamine sulphate [Sigma-Aldrich, UK] were each dissolved in 500 µL of distilled water before being added to a separate 10.0 mg mL^{-1} solution of sodium hydrogen carbonate [Fisher Scientific, UK]. Once mixed, each solution began to effervesce. After the reaction had ceased, the pH of each mixture was taken. Litmus paper test indicated a pH of 8 and confirmed that both the cathinone and the amphetamine were both in their freebase forms. The aqueous layers of the cathinone/amphetamine were washed with 500 µL diethyl ether six times [Fisher Scientific, UK] and the organic fractions were combined before the sample was evaporated to incipient dryness under nitrogen leaving a residue. The residue was reconstituted in 100 µL of DMSO [Sigma-Aldrich, UK] then 10 µL was removed and diluted using 990 µL of phosphate buffered saline [PBS; Oxoid Ltd, UK] to give a 1% DMSO/PBS solution. A HRP-conjugation kit [Abcam, UK] was then used as per the

manufacturer's standard protocol (Figure 2.2.). 10 µL solution of modifier reagent was added to 100 µL of the cathinone/amphetamine freebase solution and mixed thoroughly by vortexing for 1 minute. The cathinone/amphetamine freebase-modifier mixture was added to the LYNX lyophilized mix and left to incubate overnight at room temperature. After incubation, 10 µL of quencher reagent was added to the mixture and incubated at room temperature for 30 min prior to use and then stored in aliquots at -20 ◦C until required.

2.4. Paper-based Lab-on-a-Chip: device design and manufacture

2.4.1. 96 well template

The template used for the initial design of the paper-based LOC device was created in SolidWorks and was design to replicate the negative image of a 96 well enzyme linked immunosorbent assay (ELISA) plate (Figure 2.3. A). An image of the actual 96 well template is shown in Figure 2.3. B with a scale for reference.

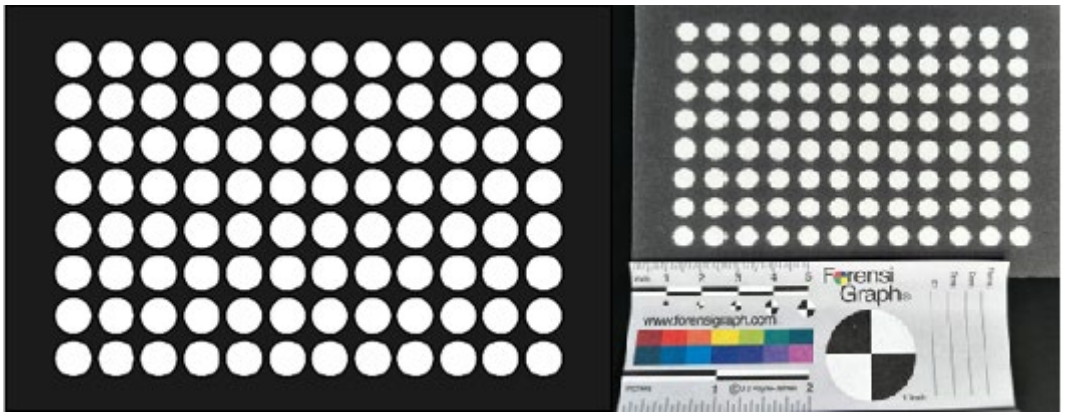


Figure 2.3. *(A) 96 well template. (B) Image of the actual 96 well template after it has been in the oven for at 130^{o}C for 180 seconds and scale*

2.4.2. Optimised LOC device

The optimised paper-based LOC device (Figure 2.4. A and B) has an origami design to incorporate a competitive antibody-based immunoassay adapted from Wang *et al.* (2012) [126], for the multiplex detection of 4-MMC, 4-ME, MDPHP and NEP (through the inclusion of the anti-methcathinone antibody), as well as amphetamine, methamphetamine and MDMA (through the inclusion of the anti-amphetamine antibody).

Figure 2.4 (A) is a schematic of the LOC design with central detection zone (black tab); sample tab (green (A)); wash tabs (blue (B) and purple (C); and detection reagents tab (yellow (D)). The location of the antibodies is shown using i) anti-methcathinone; ii) anti-amphetamine; iii) anti-horseradish peroxidase (HRP) and iv) negative control (distilled water) on the central detection zone (black tab). (E) Thin blotting filter paper to be attached under (B), (F) Thin blotting filter paper to be attached under (C). (G) and (H) additional filter paper to be attached under (E) and (F) for excess water waste; (B) Image of the printed LOC device with its 'origami design'. To allow the sequential steps of the immunoassay to take place the tabs are folded over the central detection zone (black tab) in order (from A to D).

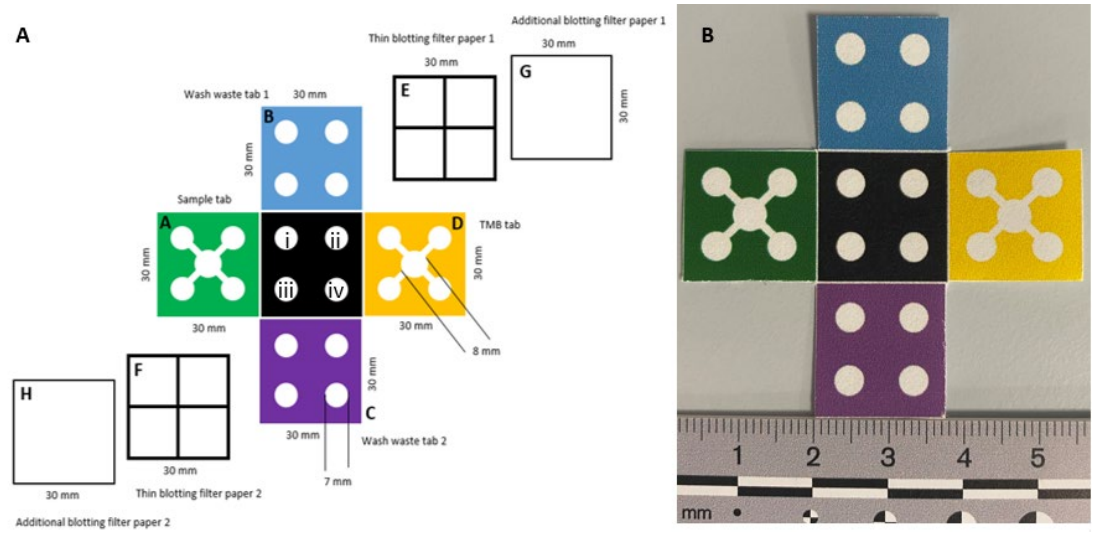


Figure 2.4. *(A). Schematic of the LOC design with central detection zone (black tab); sample tab (green (A)); wash tabs (blue (B) and purple (C); and detection reagents tab (yellow (D)); (B) Image of the printed LOC device with its 'origami design'. To allow the sequential steps of the immunoassay to take place the tabs are folded over the central detection zone (black tab) in order (from A to D)* [61]

2.4.3. Wax Printing

The LOC designs included two different designs; the 96 well template (Figure 2.3.) and the optimised LOC design (Figure 2.4.) were printed using a Xerox Phaser 8500 Solid Ink Printer. The cartridges placed within the printer contain solid wax, which was melted when printing the design onto chromatography paper [Fisherbrand, UK]. The printed designs were then placed into an oven at 130^{o}C for 180 seconds to create hydrophobic barriers within the design [135].

2.4.4. Preparation of the LOC devices

All the reaction wells for the LOC devices were prepared by adding 5 µL of 0.250 mg mL^{-1} chitosan [Sigma-Aldrich, UK] and allowed to dry at room temperature to activate the wells. Followed by 5 µL of 2.50% glutaraldehyde [Sigma-Aldrich, UK]

and left to incubate for 2 hours and then washed twice with 10 µL of distilled water (Figure 2.5.).

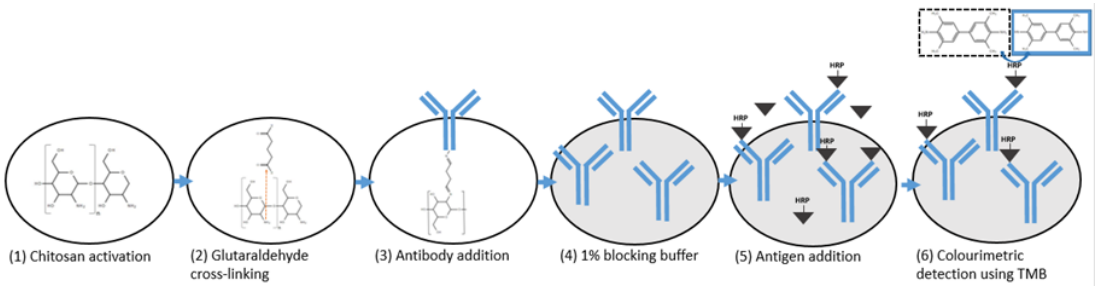


Figure 2.5. *Schematic of the preparation of the LOC device; (1) chitosan addition, (2) glutaraldehyde cross-linking. After 2 hours, this was washed twice with distilled water. (3) antibody addition (i-iv). After 30 minutes, this was then washed twice with distilled water. (4) 1% milk powder (blocking buffer) was then added. After 15 minutes, this was washed twice with 0.05% PBS-Tween. (5) Labelled and non-labelled antigen addition. This was then washed twice with distilled water and followed by (6) colourimetric detection using TMB* [61]

Antibody (4 µL) was then added to reaction well, permanently bound to the LOC device through the glutaraldehyde cross-linker. Following a 30-minute incubation, the reaction well was washed twice with 10 µL of distilled water to remove any unbound antibodies. Blocking was then achieved by adding 10 µL of 1 % milk powder (in PBS solution and incubated at room temperature for 15 minutes before washing twice with 10 µL of 0.05% PBS-Tween. A 50:50 dilution of 4 µL of the appropriate HRP-conjugate, either amphetamine-HRP or cathinone-HRP and the target sample was added to the reaction well for 210 seconds. This was then washed twice with distilled water and followed by colourimetric detection using 5 µL of 3,3',5,5' - tetramethylbenzidine (TMB).

2.5. Testing of Samples

2.5.1. Antigen addition and Colourimetric Testing

A 50:50 dilution of 4 µL of the cathinone-HRP and the target analyte was added to the reaction wells for 210 seconds. This was then washed twice with distilled water and followed by colourimetric detection using 5 µL of TMB.

2.5.2. ImageJ Analysis

To analyse the colour change reaction produced after the colourimetric immunoassay had been undertaken, photographic images were taken of the reaction wells (on the optimised LOC device and 96 well template) using an iPhone 11, which were then uploaded on to a computer for analysis. Using ImageJ software version 1.52a [http://imagej.nih.gov/ij/], a circle was placed onto the reaction well of the LOC device (either the 96 well template design or the optimised LOC device design) to analyse the average light intensity of each of the reaction wells. ImageJ measures the amount of white light present within the circle placed onto the reaction well of the photographic image, producing RGB values (blue component). The RGB values produced within ImageJ were then transferred into Origin 2015 to produce the graphical representations.

2.6. Optimisation of the LOC device

2.6.1. Method development of Immunoassay Parameters

In order to obtain the best results for all of the antibodies incorporated in this competitive immunoassay a series of experiments were undertaken to determine the optimum concentration of antibody and corresponding antigen using the 96 well template design (Figure 2.3.) and following Method 2.4.4.). Four microlitres of

each antibody stated and 4 microlitres of each corresponding labelled-antigen (both at varying concentrations) as detailed below.

A range of anti-methcathinone antibody concentrations ranging from 0.020 µg mL^{-1} to 2.04 mg mL^{-1}), and four labelled-antigen concentrations (cathinone-HRP) ranging from 0.200 ng mL^{-1} to 0.025 ng mL^{-1}, were evaluated in order to determine the best parameters for the competitive immunoassay.

A range of anti-HRP antibody concentrations ranging from 0.200 ng mL^{-1} to 0.050 ng mL^{-1}, and three labelled-antigen concentrations (cathinone-HRP) ranging from 0.200 ng mL^{-1} to 0.025 ng mL^{-1}, were also evaluated in order to determine the most effective concentration for this positive control.

A range of anti-amphetamine antibody concentrations (0.200 ng mL^{-1} to 0.050 ng mL^{-1}), and labelled-antigen concentrations (amphetamine-HRP) ranging from 0.200 ng mL^{-1} to 0.050 ng mL^{-1} were evaluated in order to provide a proof of concept for the adaptability of this LOC device for future testing of drugs of abuse or NPS.

2.6.2. Stability Study

The preparation of the LOC devices was undertaken using Method 2.4.4. Following these steps, the LOC devices were then stored in the four conditions; fridge (2-8 ^{o}C), freezer (-20 ^{o}C), in the dark at room temperature and in the light at room temperature. Each week from 1 to 8, the LOC devices were removed from the storage conditions and were tested using Method (2.5.1.). The signal intensity was recorded over an 8-week period in order to investigate the stability of the LOC device, which was subjected to four different storage conditions. However, it is

important to note that on week 0, the LOC devices were also tested using Method 2.5.1.

2.6.3. Cross reactivity using the paper-based LOC device

The cross reactivity of the anti-methcathinone antibody was investigated using the immunoassay protocol with adulterants (amphetamine, cocaine and ketamine), cutting agents (benzocaine, caffeine, lidocaine, paracetamol, procaine and taurine), and interferents (cornflour and flour) (Table 2.1). The cross reactivity of the anti-amphetamine antibody was investigated using the immunoassay protocol with methamphetamine and MDMA and MDA. A blank sample (no adulterant, cutting agent, or interferent present) and a concentration range (7 samples from concentrations 0.156 to 10.0 mg mL^{-1}) of commonly encountered adulterants, cutting agents, and interferents was spiked to an aqueous sample. To test the cross reactivity of these compounds, the preparation of the LOC devices was undertaken using Method 2.4.4. ensuring that the target analyte was the selected compound for the 50:50 dilution.

Table 2.1. *Summary table of the compounds and concentration ranges for the cross-reactivity compounds*

Adulterant/cutting agent/interferent	Compound	Concentration range *(mg mL^{-1})
Adulterant	Amphetamine	10 – 0.156
	Cocaine	10 – 0.156
	Ketamine	10 – 0.156
Cutting agents	Benzocaine	10 – 0.156
	Caffeine	2 – 0.13 200 – 0.13 µg mL^{-1}
	Lidocaine	300 – 4.69
	Paracetamol	2.5 – 0.07 200 – 6.5 µg mL^{-1}
	Procaine	10 – 0.156
	Sucrose	50 – 0.78 µg mL^{-1}
	Taurine	500 – 7.81
Interferents	Corn flour	10 – 0.156
	Flour	10 – 0.156

**mg mL-1 unless stated otherwise. Concentration ranges were determined after reviewing the values of each adulterant, cutting agent or interferents observed in literature*

2.7. Optimised LOC design Immunoassay Protocol

All the reaction wells for the LOC devices were prepared by adding 5 µL of 0.250 mg mL^{-1} chitosan and allowed to dry at room temperature [Sigma-Aldrich, UK] to activate the wells. Followed by 5 µL of 2.50% glutaraldehyde and left to incubate for 2 hours (Sigma-Aldrich, UK) and then washed twice with 10 µL of distilled water. The relevant antibody (4 µL) was then added to the appropriate well (black centre tab, Figure 2.6.): i) anti-methcathinone; ii) anti-amphetamine; and iii) anti-HRP (as a positive control). Four microlitres of distilled water was added to well iv) as a negative control.

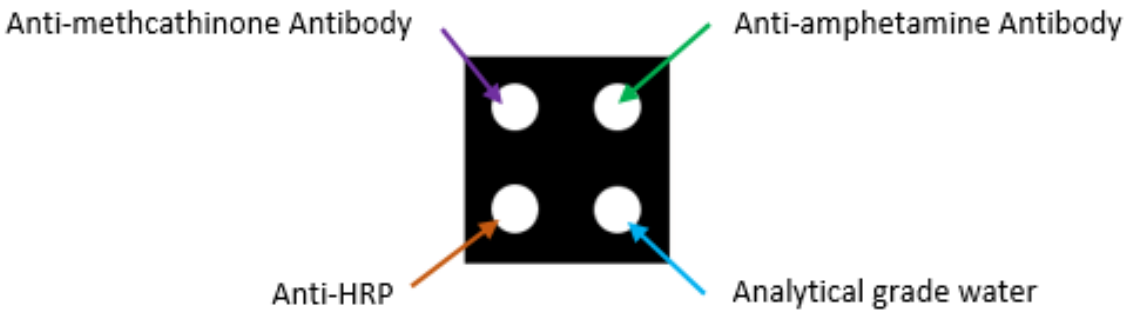


Figure 2.6. *Location of antibodies in the black central well of the optimised LOC device. Anti-methcathinone antibody for the detection of 4-MMC, 4-ME, MDPHP and NEP; anti-amphetamine antibody for the detection of amphetamine, methamphetamine and MDMA; Anti-HRP as a positive control and analytical grade water as a negative control*

The antibodies were permanently bound to the microfluidic device through the glutaraldehyde cross-linker. Following a 30-minute incubation, the wells were washed twice with 10 µL of distilled water in order to remove any unbound antibodies. Blocking was then achieved by adding 10 µL of 1 % milk powder (in PBS solution) and incubated at room temperature for 15 minutes before washing twice with 10 µL of 0.05% PBS-Tween.

A 25 µL solution at a 50:25:25 ratio of the 'sample', amphetamine-HRP, and cathinone-HRP was added to the green sample tab (Figure 2.7 (1)) and then folded directly onto the black central zone wells and kept in contact for at least 40 seconds using two pieces of glass (Figure 2.7 (2)). The sample tab was then removed (Figure 2.7 (3)). All four wells on the black centre tab (Figure 2.6.) were then washed with

10 µL of distilled water (Figure 2.7 (4)). The blue wash waste tab (B) was folded underneath the black central tab to absorb the excess water (Figure 2.7 (5)). This was held in place using two pieces of glass. The blue wash waste tab (B) was then removed. All four wells on the black centre tab (Figure 2.6.) were then washed again with 10 µL of distilled water. The purple wash waste tab (C) was folded underneath the black central tab to absorb the excess water (Figure 2.7 (6). This was held in place using two pieces of glass. The purple wash waste tab (C) was then removed. Then 25 µL of TMB was then added to the central well of the yellow TMB tab (Figure 2.7 (7)) and folded over to maintain contact with the central black tab (Figure 2.7 (8)). An image was taken and analysed using ImageJ (see Method 2.5.2.).

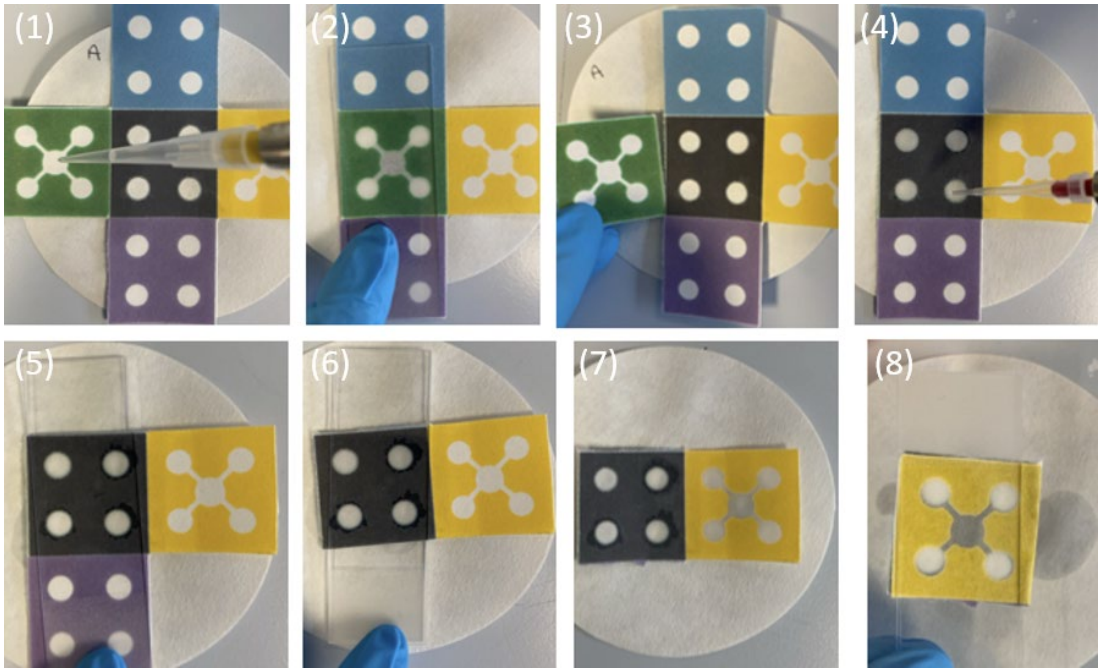


Figure 2.7. *Flow diagram demonstrating how to use the optimised paper-based LOC devices including (1) addition of the solution (containing 'sample', amphetamine-HRP and cathinone-HRP) to the green 'sample' tab, (2) folding directly onto the black centre tab, (3) removal of the green 'sample' tab, (4) the first wash with 10 µL of distilled water (5), the blue 'wash waste tab' (B) folded underneath the 'black central tab' to absorb the excess water, the second with 10 µL of distilled water, (6) the purple 'wash waste tab' (C) folded underneath the 'black central tab' to absorb the excess water, (7) the addition of TMB to the yellow 'TMB' tab, and (8) then folded over to maintain contact with the 'central black tab'.*

2.8. Statistical Analysis

SPSS (version 22) and R Studio (version 3.6.1) were used to perform statistical analysis. LOD were determined for the aqueous and biological samples. ANOVA was used to determine if there was any difference in signal intensity over an eight-week

period. The variation between devices was determined using Levene's test. Linear regression was used to determine if there was any cross reactivity with the anti-methcathinone antibody and any of the structurally similar NPS (4-MMC, 4-ME, MDPHP and NEP), as well as adulterants, cutting agents or interferents investigated. Linear regression was also used to determine if there was any cross reactivity with the anti-amphetamine antibody and the structurally similar drugs of abuse (methamphetamine, MDMA, MDA) investigated. Values of $P < 0.05$ were considered statistically significant. The LOD was reported at 3 times the standard deviation (SD) of the intercept (3x SD).

Chapter 3: Development of a paper-based Lab-on-a-Chip device for the detection of Mephedrone and its metabolite 4-methylephedrine

The data from this chapter has been published in the peer-reviewed journal Forensic Chemistry. As lead author for the manuscript, I was responsible for preparing the LOC devices, freebasing of cathinone hydrochloride and conjugation of cathinone to HRP, method development of immunoassay parameters, aqueous and biological matrix investigations (L. McNeill, C. Pearson, D. Megson, J. Norrey, D. Watson, et al. (2021). Origami chips: Development and validation of a paper-based Lab-on-a-Chip device for the rapid and cost-effective detection of 4-methylmethcathinone (4-MMC) and its metabolite, 4-methylephedrine in urine. Forensic Chemistry. 22, pp.100293-100293) [61].

Executive Summary

This chapter investigates the paper-based LOC device that incorporates a competitive immunoassay for the selective detection of synthetic cathinone, 4-MMC in urine (representative of an ingested sample) and in aqueous solution (representative of a bulk sample), as well as its metabolite, 4-ME. 4-MMC has emerged in drug seizures as a new psychoactive substance (NPS) causing a public health risk of global concern. This chapter has successfully developed a simple, low-cost, and portable paper-based LOC device for the detection of 4-MMC and its metabolite 4-methylephedrine (1-dihydromephedrone, 4-ME) within 3 minutes. The LOC device can detect 4-MMC to clinically relevant levels (2.51 ng mL^{-1} and 4.34 ng mL^{-1} for the aqueous 4-MMC and urine sample, respectively), with a higher degree of selectively observed for spiked urine samples. The cross reactivity of the

anti-methcathinone antibody and one of the main metabolites of 4-MMC, 4-ME was investigated and demonstrates that the LOC device is more selective for 4-MMC. There was no cross reactivity between the anti-methcathinone antibody and varying levels (Table 2.1.) of three commonly encountered adulterants (amphetamine, cocaine, ketamine), seven cutting agents (benzocaine, lidocaine, procaine, taurine, caffeine, paracetamol, and sucrose), as well as two interferents (corn flour and flour) that were investigated. Stability and reproducibility measurements showed no significant difference in signal intensity over eight weeks and no significant difference within or between devices. Therefore, this LOC device has the potential to provide cost-effective, rapid, on-site testing within forensic or clinical settings with global applicability.

3.1. Introduction

Mephedrone (4-methylmethcathinone, 4-MMC) (Figure 3.1. (a)) is a synthetic cathinone that was first synthesised in 1929 by Sanchez [136] and is structurally related to cathinone (Figure 3.1.). It was not until 2007 that 4-MMC emerged on the recreational drugs market in Israel, before spreading throughout the world and proceeding to become one of the most commonly (ab)used synthetic cathinones globally, with a focus across Europe [25, 31, 137-143], Australasia [137], Asia [31], and the USA [25, 27, 31, 137]. The EMCDDA issue risk assessments to assess the potential social and health risks of a drug [144]. Subsequently due to an increase in 4-MMC use, the EMCDDA reported on the risk assessment of 4-MMC to assess the risks associated with this drug [144].

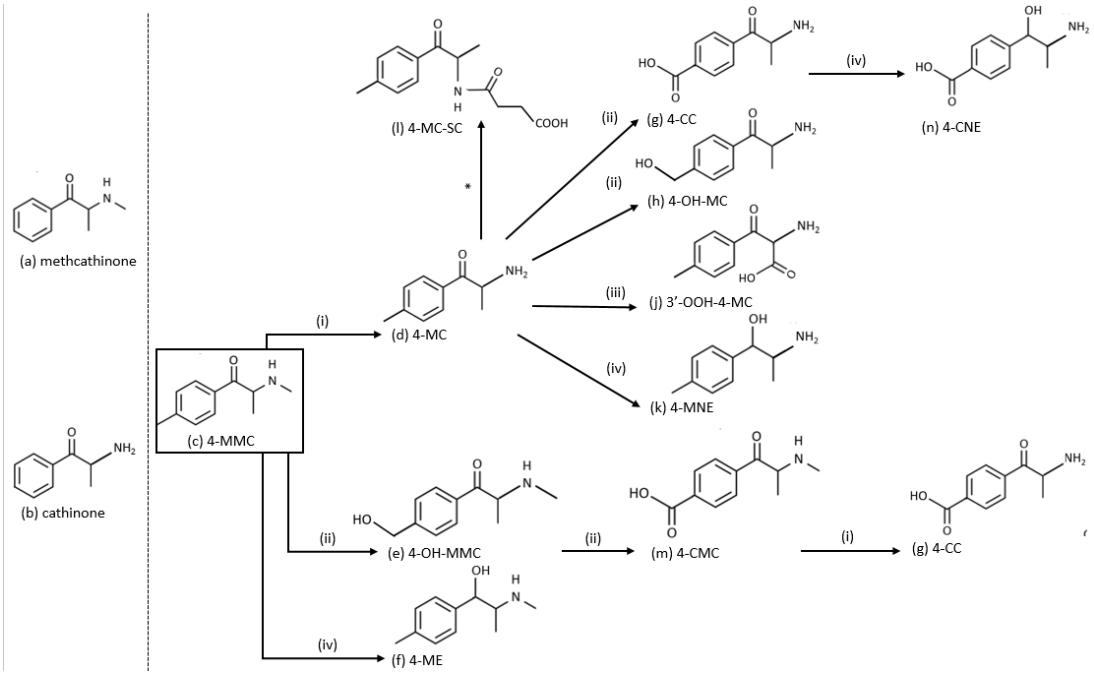


Figure 3.1. *Structures of (a) methcathinone, (b) cathinone (c) 4-MMC, (d) 4-MC, (e) 4-OH-MMC, (f) 4-ME, (g) 4-CC, (h) 4-OH-MC, (j) 3'-OOH-4-MC, (k) 4-MNE, (l) 4-MC-SC, (m) 4-CMC, (n) 4-CNE, and the proposed route for the Phase I metabolism of 4-MMC; (i) oxidative N-demethylation (ii) oxidation of the methyl group (iii) ω-oxidation at the position 3' (iv) carbonyl reduction (adapted from [17, 25, 145-156])*

4-MMC was initially (ab)used by drug users as a cheaper and "legal" alternative to illegal stimulant recreational drugs of abuse, such as methamphetamine, MDMA and cocaine [27, 31, 61, 137]. It is used by certain groups, including Chemsex (which refers to a group of users that administer certain substances directly prior to or during sexual activity) and predominantly observed in men who have sexual contact with other men [138, 157]. Desired effects of administering 4-MMC include euphoria, increased alertness, enhanced mood, and increased energy [139-142, 144]. Reported undesired adverse side effects of 4-MMC use can range from minor to life

threatening and are often associated with sustained use of 4-MMC and/or high dosage [142]. Adverse side effects include, sweating, blurred vision, insomnia, nausea, vomiting, palpitations, headache, agitation, vomiting, sweating, reduced appetite, teeth grinding, chest pain, increased blood pressure, seizures, psychosis, depression, palpitations, nausea, headaches, and less frequently death can be often observed [139-144, 158].

4-MMC is unstable in its freebase form so is usually found in the form of 4-MMC hydrochloride salt, which is a white powder, but also available less frequently in tablet form [143, 158, 159]. 4-MMC is most administered nasally (snorting), orally ("dapping" or "bombing"), injecting as it is water soluble or mixed in a drink [61, 141, 142, 159-161]. However, injecting intravenously, has recently increased in popularity [141, 160]. 4-MMC is commonly "cut" (diluted down with other less expensive chemical(s)) with paracetamol [159, 162], caffeine [24, 162-166], benzocaine [24, 164, 166] lidocaine [24, 164], MSG [166], procaine [164], sucrose [166], taurine [166] but also with other drugs of abuse, such as cocaine [159, 166] and ketamine [159, 162].

On the 16th of April 2010, 4-MMC was banned in the UK, and placed under Schedule 1 of the Misuse of Drugs Act 1971, as a Class B controlled drug [42]. 4-MMC was added to the Crime Survey for England and Wales in 2010-11 due to the high frequency of use and is still reported in the most recent report [167-169]. In 2015, at the 58th Commission on Narcotic Drugs (CND) in Vienna, 4-MMC was the first NPS to be listed in Schedule II of the UN 1971 Convention of Psychotropic Substances (decision 58/1), placing it under international control [170, 171]. This was due to recommendations from the UK and the Expert Committee on Drug Dependence

(ECDD) on the basis that there are no therapeutic benefits for 4-MMC and the risks to both public health and society [172].

4-MMC was easily obtainable via “head shops” (these were shops located on the high street that sold drug paraphernalia in addition to herbal remedies and ‘legal highs’ prior to the introduction of the blanket ban) or the internet, and as a cheaper alternative to the controlled drugs of abuse in which it imitates, this therefore successfully helped 4-MMC establish a place within the recreational drugs market [25, 44]. Although the supply and production of 4-MMC is also under international control, the current worldwide availability and popularity still represent a concern for society [44].

In 2008, the first death related to 4-MMC use was confirmed in Sweden through toxicological findings [173]. In the most recent report by the Office of National Statistics published in December 2022, there was a significant increase in the number of NPS-related deaths in England and Wales from 137 deaths in 2020 to 258 deaths in 2021 [167], even after the introduction of the Psychoactive Substances Act 2016 in the UK on the 26th of May 2016 which made it illegal to supply, possess, export or import NPS [25, 45, 61, 137, 160, 167]. Subsequently since this legislation has been in force, there has been a ‘crack down’ on street dealers, especially within Manchester to minimise the availability of NPS within the recreational drugs market.

The most recent report from the UNODC stated that there were 1,127 NPS that have been reported globally from 2006 to 2021, of which 201 were reported as synthetic cathinones, including 4-MMC [174]. There has been an increase in the

availability of NPS in prisons reported across Europe. This has resulted in increases in violence, bullying, aggressive behaviour, and debt because of high mark up prices, which is still a significant issue in UK prisons even after the introduction of the Psychoactive Substances Act 2016 [174]. Deaths directly related to NPS use within prisons can be complex and can often be under reported [175]. There are several reasons for this including a lack of reference standards for NPS, detecting low concentrations of NPS, and the commonly encountered issue that NPS are taken with other recreational drugs, known as poly-drug use [175].

3.1.1. Mephedrone Metabolites

Since 4-MMC emerged on the recreational drugs market in 2007, the metabolism of 4-MMC has been researched using animal models [145, 146], using spiked urine samples [147], as well as in clinical trials using humans [147]. Martínez-Clemente *et al* (2013) administered 4-MMC both orally (30 and 60 mg kg^{-1}), and also intravenously (10 mg kg^{-1}) to rats [145]. After the oral administration, the authors identified five phase I metabolites using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) [145]. 4-MMC and four of its metabolites were detected in rat urine, and also in human urine mainly 4-methylcathinone (normephedrone, 4-MC), but also 4-methylnorephedrine (1-dihydro-nor-mephedrone, 4-MNE) as well as 4-carboxynorephedrine (4-carboxy-dihydro mephedrone, 4-CNE), 4-hydroxymethylcathinone (hydroxytolylnormephedrone 4-OH-MC) and 4-hydroxymethylmethcathinone (hydroxytolylmephedrone, 4-OH-MMC) using gas chromatography coupled with mass spectrometry (GC-MS) [146]. Papaseit e*t al* (2016) conducted a randomised, double-blind, cross-over, placebo-controlled trial using 12

male participants (aged between 21-39 years old) to investigate the pharmacological effects of 4-MMC in direct comparison to MDMA [147]. The participants received either a placebo, 100 mg of MDMA or 200 mg of 4-MMC. Blood and urine samples were taken from the participants, with elimination half-life of 4-MMC to be significantly shorter than MDMA at 2.15 and 7.89 hours, respectively [147]. Pederson *et al* (2013) investigated the mephedrone metabolism using human liver microsomes, with 4-MC and 4-OH-MMC being the most abundant [155].

Torrance *et al* (2010) investigated four fatal forensic cases involving 4-MMC, using a range of biological specimens [17]. The authors used GC-MS with electron ionisation (GC-EI-MS-) and high-performance liquid chromatography coupled with time-of-flight mass spectrometry (HPLC-ToFMS) in order to detect 4-MMC metabolites, including 4-CNE and 4-MC. However, in 2010 with a lack of reference standards commercially available they were unable to provide complete identification of the metabolites of 4-MMC [17]. Six healthy volunteers administered 100 mg of mephedrone hydrochloride (nasally) to determine the pharmacokinetics of 4-MMC and its metabolites in plasma and whole blood taken [150]. LC-MS detected 4-MMC and five metabolites, including 4-MC, 4-ME, 4-MNE, 4-CMC, and 4-OH-MMC [150]. Ten metabolites of 4-MMC were detected in an *in vivo* human study using ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-qToFMS) after two healthy male volunteers had administered 200mg of 4-MMC [151]. Of these ten metabolites, six of these were phase I metabolites and four of these metabolites were phase II metabolites, including 4-

MC, 4-MNE, N-succinyl-nor-mephedrone (4-MC-SC), 4-ME [151]. Linhart *et al* (2016) detected a total of 15 metabolites in rats, ten phase I and five phase II using HPLC-MS, including; 4-MC, 4-carboxycathinone (4-carboxynormephedrone, 4-CC), 4-CNE, 4-OH-MNE, normephedrone-ω-carboxylic acid (3'-OOH-4-MC), 4-carboxymethcathione (4'-carboxy-mephedrone, 4-CMC), 4-OH-MMC, and 4-MC-SC [152]. Olesti *et al* (2017) published a fully validated LC-MS/MS method for the detection of 4-MMC and its metabolites in urine and plasma, using six healthy male subjects that had administered 150mg of 4-MMC [35]. The metabolite, 4-CMC was found to be the most abundant in urine, but the Authors also found 4-MC, N-succinyl nor-mephedrone (4-MC-SC) and 4-ME [35].

As discussed above (section 3.1.), the proposed route for the Phase I metabolism of 4-MMC metabolism producing metabolites; oxidative N-demethylation (Figure 3.1. i) to produce 4-MC, methyl group oxidation (Figure 3.1. ii) to produce 4-CC, 4-OH-MC, 4-OH-MMC and 4-CMC, position 3' ω-oxidation (Figure 3.1. iii) to produce 3'-OOH-4-MC and producing dihydro-metabolites such as 4-ME by the reduction of the carbonyl group (Figure 3.1. iv) [17, 25, 35, 145-156].

3.1.2. Lab-on-a-Chip detection

Even with 4-MMC under international control, the global prevalence is still causing a public health risk. There are currently no commercially available LOC devices for the 4-MMC and its metabolite, 4-ME in biological or aqueous samples. The inclusion of a competitive immunoassay within this LOC device is advantageous due to only requiring only one antibody for the detection of 4-MMC and 4-ME, due the cross reactivity of the anti-methcathinone antibody with structurally similar compounds

to varying degrees [61]. Especially with limited availability of the required reference standards for the detection of this commonly (ab)used NPS and its metabolite. This As previously stated, this chapter aims to investigate the optimisation and evaluation of competitive antibody-based immunoassay for the detection of 4-MMC and its metabolite 4-ME. As well as investigating the practicality of this LOC device. In terms of cross reactivity with adulterants, cutting agents and interferents, investigating the use of aqueous and biological samples, storage, cost effectiveness, and total analysis time. Thereby developing the first LOC device for the detection of 4-MMC and its metabolite 4-ME in both aqueous and urine samples [61]. Thus, offering a rapid, portable, and low-cost detection method to clinically relevant levels with the potential to provide on-site testing within forensic or clinical settings.

3.2. Experimental

3.2.1 Conventional Immunoassay Protocol

Prior to investigating the use of the paper-based LOC device (chapter 3.2.2) a conventional immunoassay using a traditional 96 well microtiter plate was used to set up the initial experiments. One hundred microlitres of 0.5% rat serum [Sigma, UK] in sodium carbonate buffer was added to each well of the microtiter plate and left to incubate overnight in the fridge (2-8°C). After the incubation, any unbound capture antibody solution was removed from the microtiter plate [Thermo Fisher Scientific, UK] by inverting the wells and vigorously slapping dry on absorbent paper to ensure all residual moisture was removed. The microtiter plate was then washed 3 times with 100 µL of distilled water. A range of concentrations for the anti-methcathinone antibody (between 0.1 to 0.00714 µg mL^{-1} followed by concentrations between 0.2 to 0.025 µg mL^{-1}) in PBS were investigated, with 60 µL of each concentration (including replicates) were added to the appropriate wells of the microtiter plate. This was left to incubate for 30 minutes at room temperature. Any unbound capture antibody solution was removed. The microtiter plate was then washed 3 times with 100 µL of distilled water. After the washing step was undertaken, 60 µL of blocking buffer (1% semi-skimmed milk powder in PBS) was added to each well and allowed to incubate the microtiter plate for 15 minutes at room temperature before being removed. The microtiter plate was washed 3 times with 100 µL of 0.05% PBS-Tween. Then 60 µL of cathinone-HRP was added to each well and allowed to incubate for 10 minutes at room temperature. The cathinone-HRP was removed from the microtiter plate before the wells were washed 3 times

with 100 µL of distilled water. TMB [100 µL; Thermo Scientific, UK] was added to each well and incubated for 30 minutes at room temperature in the dark, prior to microtiter plate being read on the microplate reader at 650 nm (blue wavelength) and record results. A stop solution of 1N sulphuric acid (100 µL) of was added to each well resulting in a colour change from blue to yellow. The microtiter plate was then read at 450 nm on the microplate reader and record results.

3.2.2. Preparation of paper-based microfluidic devices (96 well template design)

Initially a conventional immunoassay (chapter 2.4.1.) using a 96 well template (Figure 2.3.). The reaction wells (Figure 2.4 and Figure 2.5) were activated by adding 5 µL of 0.25 mg mL^{-1} chitosan [Sigma-Aldrich, UK] followed by 5 µL of 2.5% glutaraldehyde [Sigma-Aldrich, UK] and then washed twice with 10 µL of distilled water [61, 126]. The inclusion of the glutaraldehyde cross-linker enabled the anti-methcathinone antibody to become permanently bound to the LOC device. After washing, 4 µL of anti-methcathinone antibody was then added to each well (Figure 2.4). For each experiment, 4 µL of distilled water was added in replacement of the anti-methcathinone antibody to a series of wells a negative control. Following a 30-minute incubation, the wells were washed twice with 10 µL of distilled water to remove any unbound antibodies. Blocking was then achieved by adding 10 µL of 1% milk powder (in PBS) solution. This was left to incubate at room temperature for 15 minutes before the wells were washed twice with 10 µL of 0.05% PBS-Tween. The devices were either used immediately or stored for a period of up to 8 weeks for use in a stability study (chapter 3.2.5).

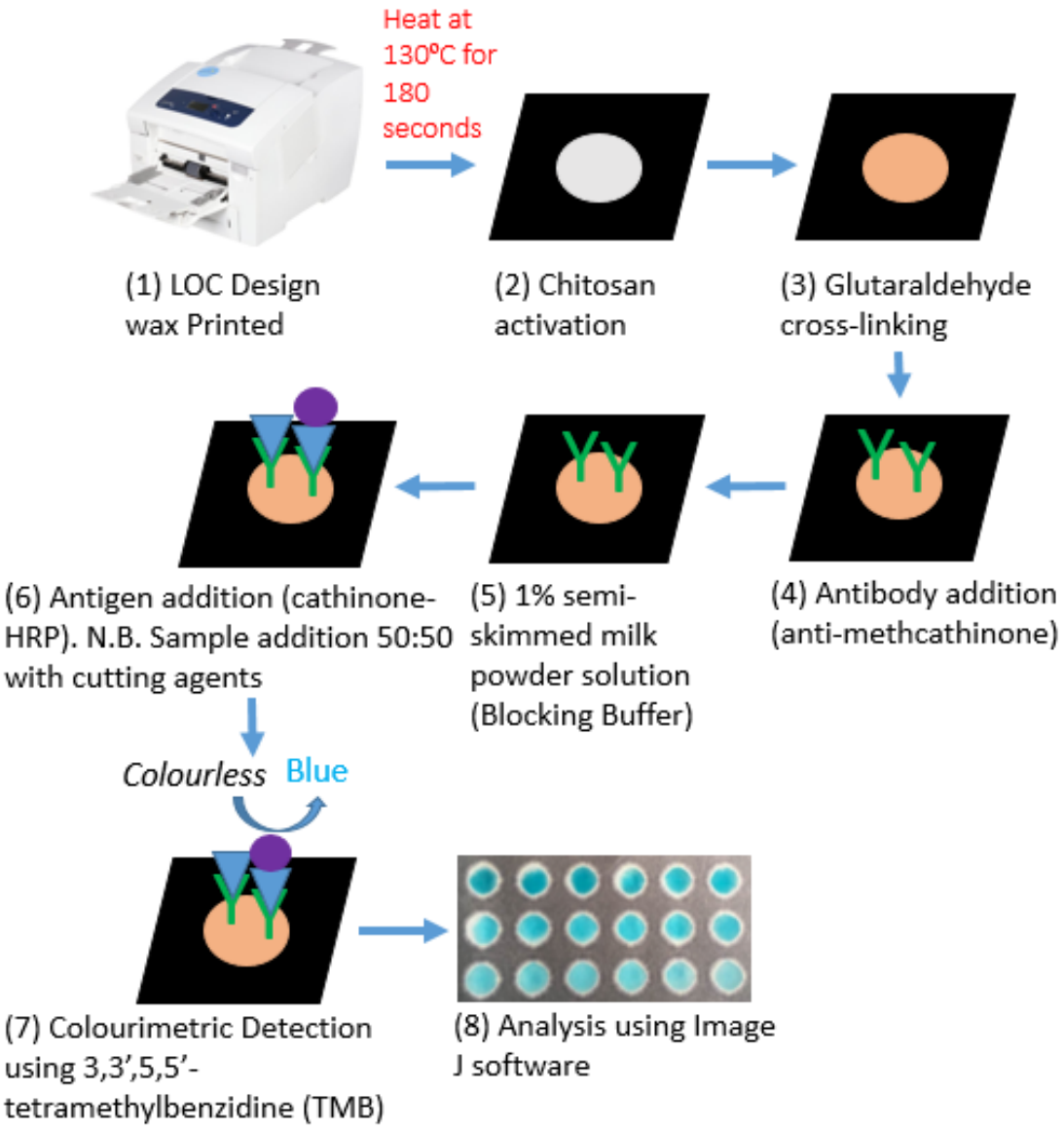


Figure 3.2. *Overall schematic showing preparation of the paper-based LOC devices including (1) wax printing, (4) antibody addition and (7) colourimetric detection prior to analysis using ImageJ (8). Design adapted from* [126]

3.2.3. Immunoassay protocol

The LOC device incorporates a competitive immunoassay for the selective detection of 4-MMC and 4-ME in both urine and aqueous samples. Aqueous samples were

prepared by dissolving the target analytes of varying concentrations (10.0 ng mL^{-1} – 0.137 ng mL^{-1}) in distilled water. Drug and alcohol-free urine samples were donated by healthy volunteers and spiked with target analytes and metabolites at clinically relevant concentrations [38, 61].

Samples were mixed in a 50:50 ratio with the HRP-conjugated cathinone and 5 µL was added to each well on the LOC device (Figure 3.2). Samples were incubated for 210 seconds before washing twice with distilled water. Finally, 5 µL of TMB was added and an image of the colour change by taken on an iPhone 7 and measured using ImageJ analysis [version 1.52a; National Institute of Health, USA]. The colour change was proportional to the amount of HRP present.

Optimisation of the immunoassay was achieved by testing a range of antibody (anti-methcathinone) and labelled antigen (cathinone-HRP) concentrations initially between wider ranges of concentrations of the cathinone-HRP were investigated, ranging from 0.01 to 0.00714 µg mL^{-1}. A more defined range of concentrations between 0.2 to 0.025 µg mL^{-1} were then investigated, to provide the linear range.

3.2.4. Stability study

Investigating the stability of the LOC device was undertaken (chapter 2.6.2.) using a 96 well template (Figure 2.3.).

3.2.5. Cross reactivity using the paper-based LOC device

Investigating the cross reactivity of the LOC device was undertaken (chapter 2.6.3.) using the 96 well template (Figure 2.3.).

3.3. Results and Discussion

3.3.1. Optimisation of anti-methcathinone and cathinone-HRP

Initially a conventional immunoassay (chapter 3.2.1.) using a 96 well template was utilised to determine the optimum concentrations for both the anti-methcathinone antibody and the cathinone-HRP within the competitive immunoassay. For this competitive immunoassay incorporated within the LOC device, if 4-MMC is present, TMB will subsequently produce a lower response as the unlabeled target antigen (4-MMC) out competes the labelled cathinone-HRP for the active sites of the anti-methcathinone antibody. Therefore, when 4-MMC is at lower concentrations, then the TMB response will be greater. Therefore, if there is no 4-MMC present at all within the samples (either urine or aqueous) then the response from TMB is the most intense colour (blue) that can be observed by eye.

Initially, wider ranges of concentrations of the cathinone-HRP were investigated, ranging from 0.1 to 0.00714 μg mL^{-1}. Subsequently as a result on these investigations it was decided to narrow down the concentrations range to include concentrations between 0.2 to 0.025 μg mL^{-1}, in order to provide the linear range. Thus, ensuring that the anti-methcathinone antibody and the cathinone-HRP were not too dilute or too concentrated, effectively optimising the vital components of the competitive immunoassay, to ensure that when 4-MMC was added that it was sensitive and importantly detectable. Lower concentrations of the anti-methcathinone antibody ranging from 255.5-511 pg mL^{-1} were then investigated to completely establish the optimal concentration for both the cathinone-HRP and anti-methcathinone antibody.

Cathinone-HRP dilutions from 0.2 to 0.025 µg mL^{-1}were tested, with the 1:500 dilution provided highest response (0.2 ng mL^{-1}, Figure 3.3). Providing the optimum concentration for anti-methcathinone and cathinone-HRP are 0.000511 µg mL^{-1} and 0.2 ng mL^{-1}, respectively. Therefore, all further experiments used these conditions.

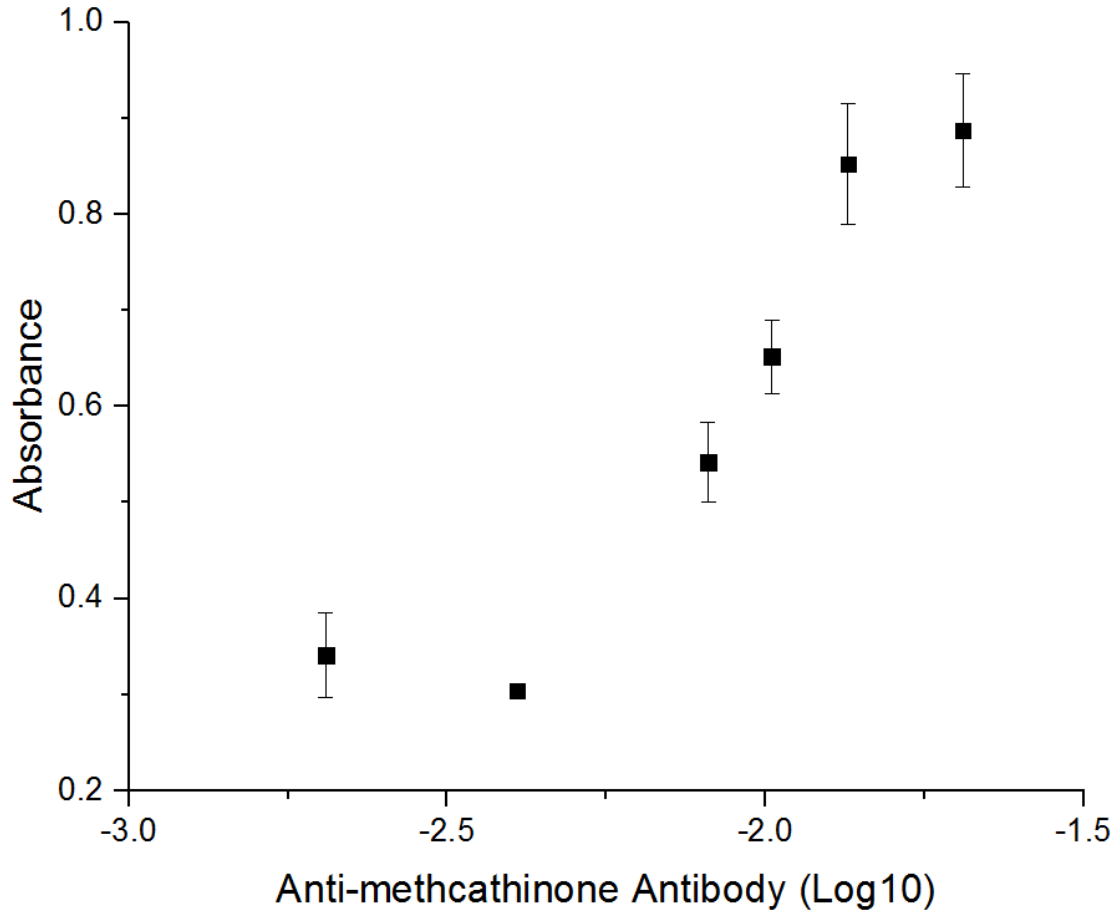


Figure 3.3. *Comparison calibration graph of the 1:500 cathinone-HRP (0.0002 mg mL^{-1}) and a concentration range of 0.02044-0.002044 µg mL^{-1} anti-methcathinone antibody and absorbance (n = 4)*

3.3.2. Optimisation and evaluation of anti-methcathinone antibody and cathinone HRP

A range of dilutions of the cathinone-HRP were used; 1:2000, 1:4000, 1:8000 and 1:14000 to establish the linear range. The resulting calibration curve with 1:2000 dilution provided the highest response (Figure 3.4). Providing the optimum concentration for anti-methcathinone and cathinone-HRP at 0.000511 µg mL^{-1} and 0.2 ng mL^{-1}, respectively. Therefore, for all future experiments these conditions were used.

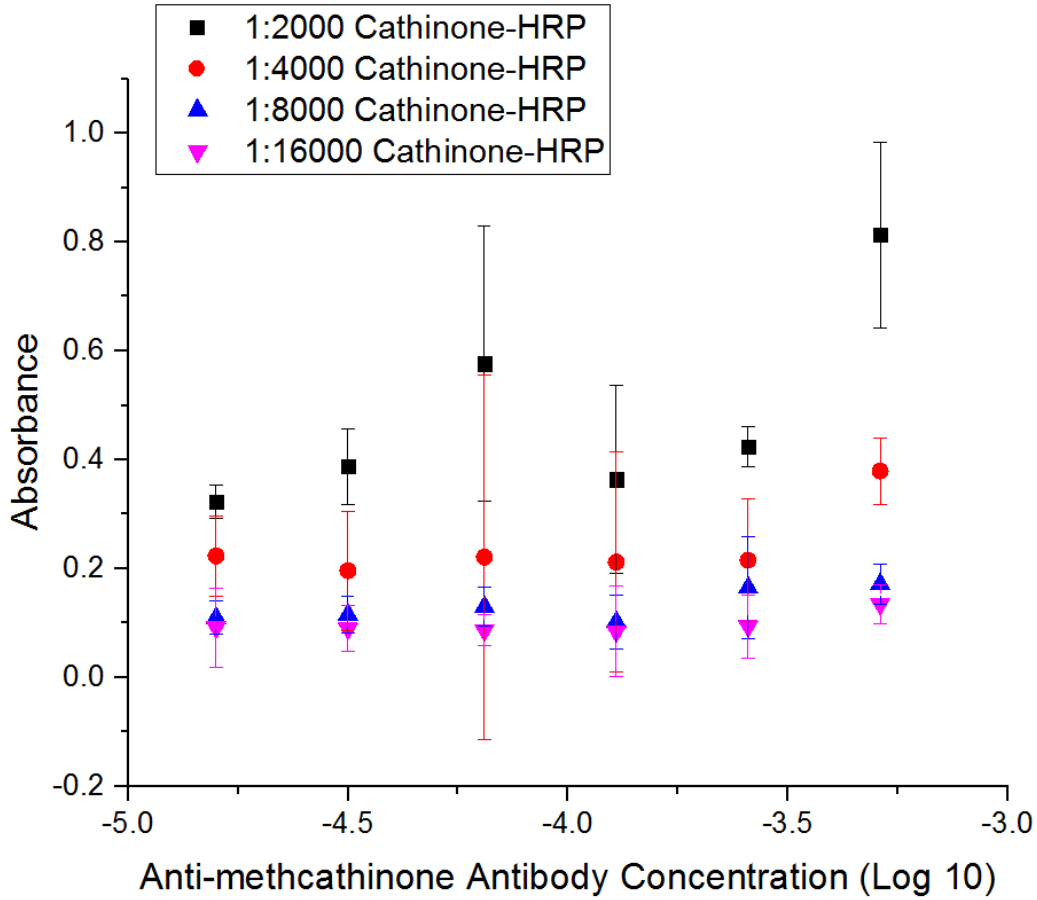


Figure 3.4. *Comparison calibration graph of the 1:2000, 1:4000, 1:8000 and 1:16000 dilutions of cathinone-HRP for the concentration range of 0.000511-0.0002555 µg mL^{-1} anti-methcathinone antibody plotted on a Log^{10} scale (n = 4)*

The 1:8000 and 1:16000 dilutions of cathinone-HRP demonstrated within the conventional immunoassay, these concentrations were not as effective as the 1:2000 dilution. The 1:8000 and 1:16000 dilutions of cathinone-HRP were therefore too dilute. Whereas, the 1:4000 dilution was more effective at detecting cathinone-HRP, demonstrating a higher response, which therefore meant that cathinone-HRP was therefore slightly more detectable using the conventional immunoassay at this more concentrated dilution. However, the 1:2000 provided the greatest response, effectively demonstrating that this would be the optimum dilution to utilise within the conventional immunoassay, as it would not inhibit but aid in detecting 4-MMC at small concentrations. Through the use of chitosan activation and glutaraldehyde cross-linking, the results to date when investigating this LOC device incorporating a competitive immunoassay, imply that the anti-methcathinone antibody has been effectively covalently immobilised to the well. Immobilising the anti-methcathinone antibody has shown that it can effectively detect 4-MMC [61, 126].

3.3.3. Evaluation of matrix effects

A vital aspect of this paper-based LOC device is the potential for point-of-care (POC) testing therefore it was essential that the LOC device is applicable not only to test aqueous (dissolved powder) samples, but also 4-MMC in non-invasively collected urine samples at clinically relevant levels. Therefore, samples of 4-MMC were prepared from both spiked urine and dissolved powder (aqueous) to represent both a clinical specimen and 4-MMC in bulk forensic sample.

Using the paper-based LOC protocol (chapter 2.4.4.) the LOD for 4-MMC in both urine and aqueous samples were determined. Initially 4-MMC in urine and aqueous

solutions between 0.15625-5 ng mL^{-1} were investigated, however, as the calibration curves were still showing a decrease at very low concentrations, further investigations were undertaken for lower concentrations.

The calibration curve for the 4-MMC in aqueous solution and the 4-MMC in urine samples at these lower concentrations were similar, showing effective detection at extremely low concentrations of 4-MMC (Figure 3.5). However, 4-MMC detected using this LOC device showed a higher degree of selectivity and specificity within spiked urine samples, than compared to aqueous samples.

Analysis of the data sets showed a linear range of 0.078 to 10.0 ng mL^{-1} for the aqueous and urine samples (Figure 3.5.). Regression analysis was used to determine the LOD of both the aqueous and urine samples of 4-MMC. An LOD of 2.51 ng mL^{-1} and 4.34 ng mL^{-1} was calculated for the aqueous 4-MMC and urine sample, respectively to clinically relevant concentrations of 4-MMC in urine samples that reported an LOD of 2 ng mL^{-1} [38]. It is worth noting that the standard deviation between replicates of the same 4-MMC concentration for both the urine and aqueous samples did show some variation within the data. Therefore, there have been some data points have deviated from the mean, indicating that the data obtained from the urine samples is more reliable and and replicates demonstrated a greater degree of consistency.

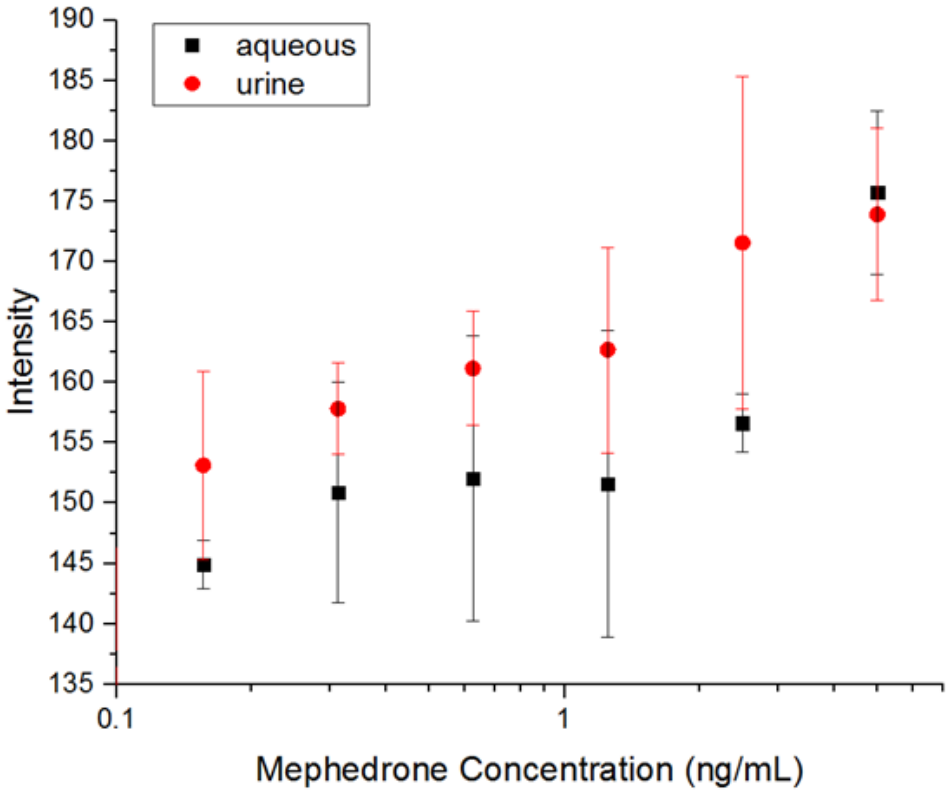


Figure 3.5. *Detection of 4-MMC in both spiked urine and aqueous samples (n = 6)*

3.3.4. Mephedrone Metabolites

Urine was selected as the biological sample type for this project due to it being the most appropriate specimen type that was suitable for the detection of 4-MMC using the LOC device. Initially, 4-MMC and the metabolite, 4-ME (Figure 3.6) were investigated using spiked urine samples, across 12 concentration ranges, between 0.00048825 - 1 ng mL^{-1}. However, as the calibration curves were still showing a decrease at very low concentrations, further investigations were undertaken to investigate the effectiveness of the LOC device at detecting 4-MMC and 4-ME at lower concentrations. The concentrations range for both 4-MMC and 4-ME were lowered to 0.04 - 0.000625 ng mL^{-1}, with both 4-MMC and 4-ME establishing linear

ranges (Figure 3.6). Both 4-ME and 4-MMC, effectively competed with the cathinone-HRP for the active sites of the anti-methcathinone antibody at both the higher and lower concentration ranges investigated. With 4-MMC showing a higher degree of selectivity and specificity for 4-MMC when compared to 4-ME, as the average light intensity measurements are slightly higher. In terms of the competitive immunoassay the binding affinity of 4-ME is not as effective as the binding affinity for 4-MMC for the active sites of the anti-methcathinone antibody, as the antibody has a greater degree of specificity for 4-MMC as expected [176].

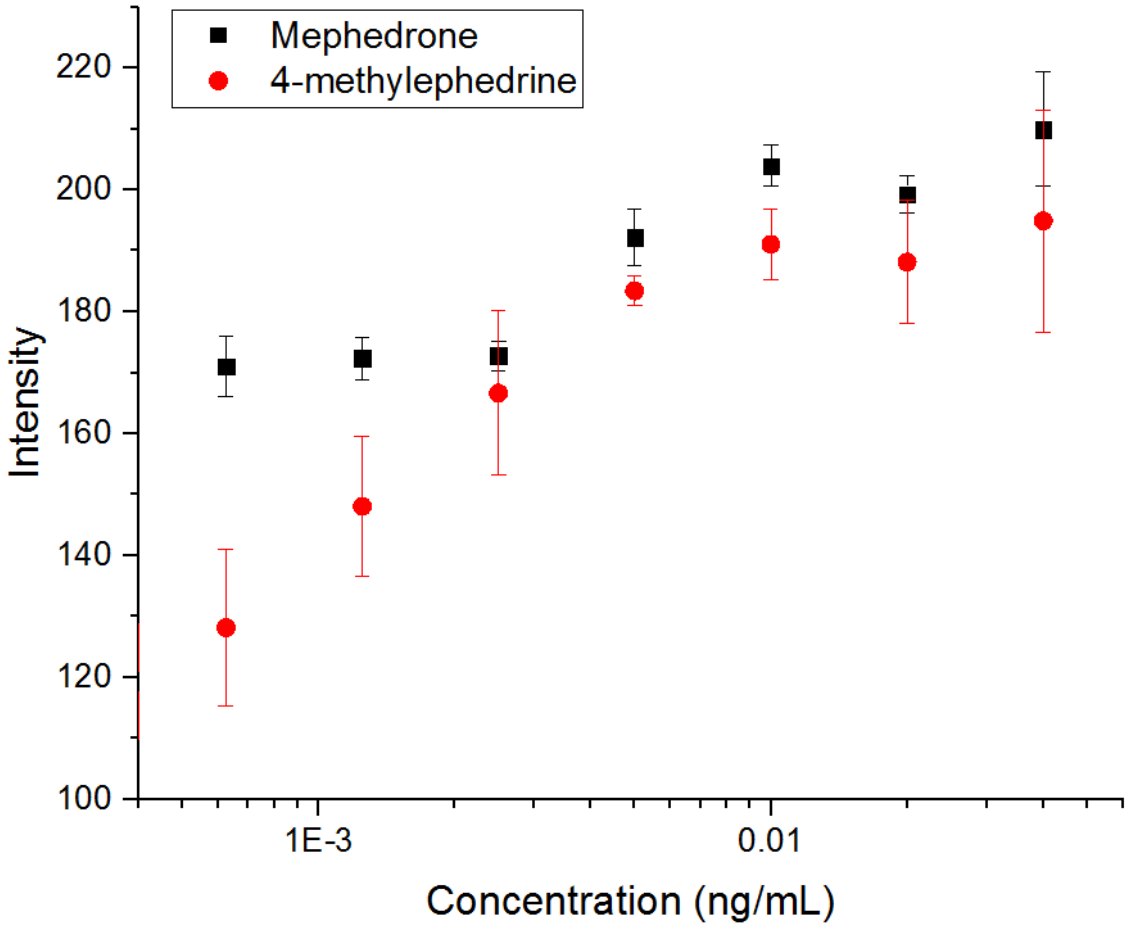


Figure 3.6. *Comparison of 4-MMC and its main metabolite, 4-ME in urine from 0.04 - 0.000625 ng mL^{-1} (n = 6)*

Statistical analysis was used to investigate the significance of 4-MMC and its metabolite 4-ME using SPSS. Regression analysis gave LOD for 4-MMC < 0.1 ng mL^{-1} and 4-ME < 0.2 ng mL^{-1}, respectively to clinically relevant concentrations of 4-MMC in urine samples, LOD of 2 ng mL^{-1} [38]. Showing that 4-MMC detected using this LOC device has a higher degree of selectivity and specificity for 4-MMC within urine samples, than compared to 4-ME within urine samples.

4-MMC and its metabolite, 4-ME demonstrate that they were both successful at competing with the cathinone-HRP for the active sites on the anti-methcathinone antibodies. By using low concentrations of 4-MMC and 4-ME, they were both still effective at competing with the cathinone-HRP. However, the average light intensity values were higher and the calibration curve response for 4-MMC was higher than 4-ME. Indicating that the binding affinity for 4-ME is not as effective as 4-MMC. However, this LOC device has clearly demonstrated that 4-MMC and its main metabolite, 4-ME were shown to be easily detectable in urine samples.

3.3.5. Stability

For the LOC device to be applicable and therefore fit for purpose in clinical and forensic settings, it is essential to determine the most effective storage conditions for the LOC devices, so that they can be used when they are required. Therefore, it was essential to investigate different storage conditions across a period of time to determine the stability of the LOC device for practicability purposes. Thus, determining whether the reagents and other components incorporated within the LOC device degrade over time under certain conditions.

A control immunoassay experiment was undertaken to investigate the stability of the LOC device by subjecting the LOC device to four different storage conditions: fridge (2–8 ^{o}C), freezer (-20 ^{o}C), in the dark at room temperature and in the light at room temperature (Figure 3.7).

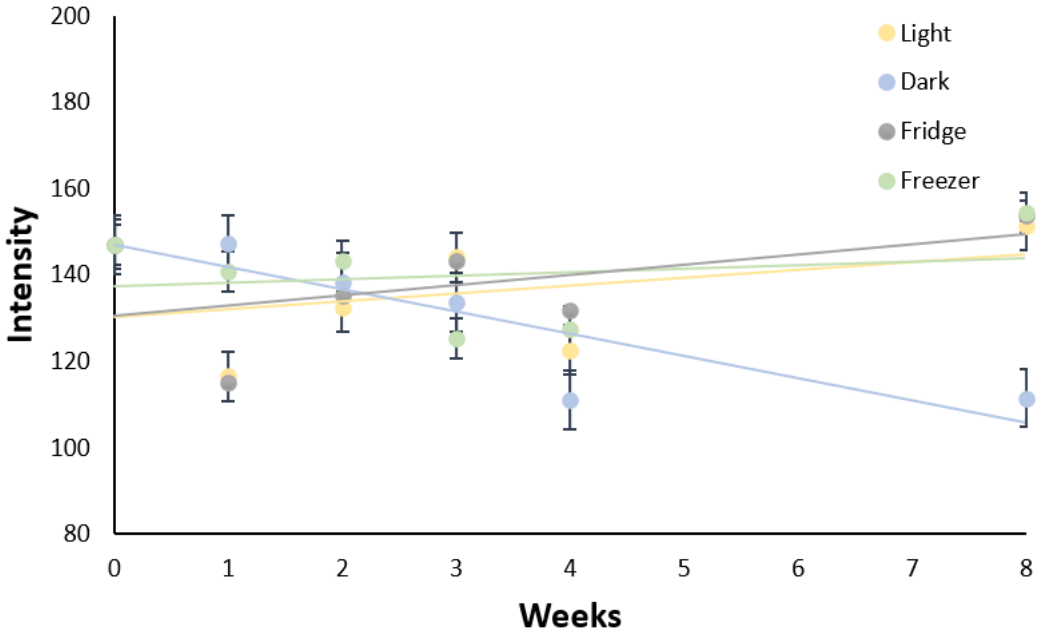


Figure 3.7. *Signal intensity recorded at weeks 0, 1, 2, 3, 4 and 8 after being stored in four different conditions (n = 12)*

Over a period of 8 weeks the signal intensity was recorded and statistical analysis showed that there was no significant difference between the values recorded during this time ($p = 0.146$) (Figure 3.7). However, analysis did show that there was a significant difference between the four conditions ($p < 0.001$). Three of the four conditions (fridge, freezer, and light at room temperature) were not significant indicating that these three conditions were stable for the 8-week period and could all therefore effectively used as suitable storage conditions. The significant

difference in the LOC devices stored in the dark at room temperature ($p < 0.001$) indicates that the LOC devices were not stable in this condition, and therefore this storage condition is not recommended.

3.3.6. Cross reactivity

Due to potential issues with cross reactivity of detection methods, it is important that this is investigated when validating a new LOC device, to avoid false positive or false negative responses. By investigating the cross reactivity of compounds with the anti-methcathinone antibody often found in combination with 4-MMC, it will aid in establishing the selectivity and specificity of the LOC device. Therefore, highlighting if there are any potential issues that could arise if the device was implemented in the either clinical or forensic settings. The cross reactivity of the anti-methcathinone antibody was investigated using the immunoassay protocol with a range of adulterants, cutting agents and interferents. Based on information described in the literature, the following compounds were selected to investigate the cross reactivity with the anti-methcathinone antibody: paracetamol [159, 162], caffeine [24, 162-166], benzocaine [24, 164], lidocaine [24, 164], MSG [166], procaine [164], sucrose [166], and taurine [166] but also with other drugs of abuse, such as cocaine [159, 162] and ketamine [159, 162]. None of these compounds demonstrated any cross reactivity with the anti-methcathinone antibody.

The anti-methcathinone antibody is polyclonal and therefore has the ability to cross-react with structurally similar compounds to varying degrees [61, 176]. Due to structural differences observed between caffeine and mephedrone (Figure 3.8), the competitive immunoassay utilising this polyclonal anti-methcathinone antibody

within this LOC device, would not be expected to cross-react with caffeine due to the specificity of the antibody [61, 176].

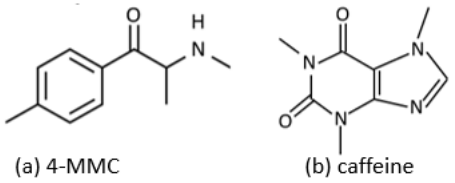


Figure 3.8. *Structures of (a) 4-MMC (b) caffeine*

Table 3.1 demonstrates that that caffeine did not cross-react with the anti-methcathinone antibody within these concentration ranges [61, 176]. This indicates that caffeine does not effectively compete with the cathinone-HRP for the active sites of the anti-methcathinone antibody. This data also indicates that caffeine did not interfere with any other elements incorporated within the competitive immunoassay [177, 178]. False positives for caffeine have been reported when using paper-based LOC devices incorporating modified Morris reagent [80]. Thus, highlighting that this LOC device is fit for purpose for on-site detection of 4-MMC and 4-ME within clinical and forensic settings, even in the presence of caffeine, which mephedrone is commonly 'cut' with [24, 162-166].

Table 3.1. *Summary table for linear regression analysis cross reactivity of cutting agents, adulterants and interferents*

Classification	Compound	CAS Number	P-value	Adjusted R-Squared	Concentration range investigated (mg mL^{-1})
Adulterants	Amphetamine	300-63-9	0.121	0.0651	10 – 0.156
	Cocaine	50-36-2	0.837	0.0902	10 – 0.156
	Ketamine	6740-88-1	0.610	0.0329	10 – 0.156
Cutting agents	Benzocaine	94-09-7	0.203	0.0308	10 – 0.156
	Caffeine	58-08-2	0.998	0.0217	2 – 0.13 0.2 – 0.00013
	Lidocaine	137-58-6	0.272	0.0001666	300 – 4.69
	MSG	142-47-2	0.353	0.0469	2.5 – 0.07 0.2 – 0.0065
	Paracetamol	103-90-2	0.0883	0.0866	10 – 0.156
	Procaine	59-46-1	0.304	0.00471	0.05 – 0.00078
	Sucrose	57-50-1	0.069	0.0692	500 – 7.81
	Taurine	107-35-7	0.0685	0.109	10 – 0.156
Interferents	Flour	130498-22-5	0.0983	0.00289	10 – 0.156
	Corn flour	9005-25-8	0.0789	0.00321	10 – 0.156

Concentration ranges were determined after reviewing the values of each adulterant, cutting agent or interferents observed in literature.

The cross reactivity of the anti-methcathinone antibody with all the adulterants, cutting agents and interferents discussed in Table 3.1 at varying concentrations were investigated. Linear regression analysis (Table 3.1) showed that there was no detrimental cross reactivity for any adulterants, cutting agents, or interferents investigated. There was no statistically significant increase between the response, and the concentration of the adulterants, cutting agents, or interferents in the spiked samples. This indicates that the level of cathinone-HRP binding to the antibody is consistent across the dilution ranges of the adulterants, as well as the cutting agents and interferents.

3.4. Conclusion

The data published in this chapter demonstrates that this paper-based LOC device can detect both 4-MMC and its metabolite, 4-ME in both aqueous (dissolved powder) and spiked urine samples (ingested sample) at clinically significant levels. With 4-MMC and 4-ME, effectively competed with the cathinone-HRP for the active sites of the anti-methcathinone antibody at both the higher and lower concentration ranges investigated. Overall, the anti-methcathinone antibody demonstrated a higher degree of selectivity and specificity for 4-MMC when compared to 4-ME, as the average light intensity measurements were slightly higher producing a better response. In terms of the competitive immunoassay the binding affinity of 4-ME is not as effective as the binding affinity for 4-MMC for the active sites of the anti-methcathinone antibody, as the antibody has a greater degree of specificity for 4-MMC as expected [61, 176]. The LOC device successfully demonstrated the detection of 4-MMC to clinically relevant limits of detection of the device of 2.51 ng mL^{-1} and 4.34 ng mL^{-1} for aqueous and urine samples, respectively. Thus, highlighting this paper-based LOC device for the detection of 4-MMC is highly comparable with traditional laboratory based analytical techniques. The data in this chapter highlights that the LOC has the potential to be used for on-site testing within clinical or forensic settings in both in aqueous samples that may have been 'cut' with diluents or administered with other drugs of abuse and non-invasive biological samples (urine), as well as highlighting its potential use in POC testing. As the anti-methcathinone antibody incorporated within the competitive immunoassay showed no cross reactivity in the presence of commonly encountered

cutting agents, adulterants and interferents. In conjunction with the data obtained in the stability studies, demonstrates the reliability of the LOC devices were stable for up to eight weeks when stored in different conditions.

Therefore, implying that this LOC device has the potential to offer rapid, portable, and on-site testing of 4-MMC and its metabolite, 4-ME in the future through the use of microfluidic investigations.

Chapter 4 goes on to investigate how the optimised LOC protocol presented here can be used for multiplex detection by including simultaneous detection of amphetamine alongside built-in positive and negative controls.

Chapter 4: Multiplex Detection of amphetamine, methamphetamine and MDMA, and the assessment of positive and negative controls

Proof of concept through the optimisation of the anti-amphetamine antibody and amphetamine-HRP for the additional testing well for the detection of amphetamine, as well as data relating to the positive and negative controls discussed in this chapter has been published in the peer-reviewed journal Forensic Chemistry. As lead author for the manuscript, I was responsible for preparing the LOC devices, freebasing of amphetamine sulphate and conjugation of amphetamine to HRP, method development of immunoassay parameters, and immunoassay investigations for the additional adaptability of the LOC device (L. McNeill, C. Pearson, D. Megson, J. Norrey, D. Watson, et al. (2021). Origami chips: Development and validation of a paper-based Lab-on-a-Chip device for the rapid and cost-effective detection of 4-methylamphetamine (4-MMC) and its metabolite, 4-methylephedrine in urine. Forensic Chemistry. 22, pp.100293-100293) [61]. Data relating to the detection of amphetamine, methamphetamine and 4-methylenedioxymethamphetamine (MDMA) using LOC technology has been published in the peer-reviewed journal Forensic Chemistry. As lead author for the manuscript, I was responsible for writing the manuscript, undertaking the systematic review and analysing the data obtained (L. McNeill, D. Megson, PE. Linton, J. Norrey, L. Bradley, et al. (2021). Lab-on-a-Chip approaches for the detection of controlled drugs, including new psychoactive substances: A systematic review. Forensic Chemistry. 26, pp.100370-100370) [1].

Executive Summary

This chapter investigates the optimised LOC design (Figure 2.5 A and B) for the multiplex detection of amphetamine, methamphetamine and MDMA to clinically relevant levels, by including an additional testing well (utilising anti-amphetamine antibody) to offer future flexibility of the LOC device (Figure 2.6.). The LOC device can detect amphetamine, methamphetamine and MDMA to clinically relevant levels (3.11 ng mL^{-1}, methamphetamine 3.93 ng mL^{-1}, and MDMA 4.84 ng mL^{-1} respectively), demonstrating that this developed LOC device is applicable for real-world analysis in a variety of clinical and forensic settings and therefore, fit for purpose. The anti-amphetamine antibody incorporated within this LOC device demonstrated a higher degree of selectivity and specificity for amphetamine but can also successfully detect structurally similar drugs of abuse, methamphetamine and MDMA.

The LOC device design was also modified to include positive and negative control wells for quality assurance purposes to enhance the reliability of the results produced when using the LOC device. With only 7% of publications for the detection of drugs of abuse and NPS, the inclusion of the positive and negative controls significantly increasing the reliability of using this developed LOC device [1]. Thus, addressing a significant gap in the quality assurance for future validation of the LOC devices and the potential acceptance within local criminal justice systems, that has been significantly overlooked in published literature to date on LOC technology.

4.1. Introduction

Amphetamine (α-methylphenethylamine) (Figure 4.1. (i)) is a synthetic substance first synthesised in 1887 by Lazar Edeleanu, however it was not until 1927 that Alles synthesised and patented both the L-amphetamine and D-amphetamine salts [179-181]. It is more commonly manufactured in clandestine laboratories for use as a recreational stimulant drug commonly referred to as 'speed' that affects the central nervous system [181]. The metabolism of amphetamine within the body can occur via two pathways [182, 183].

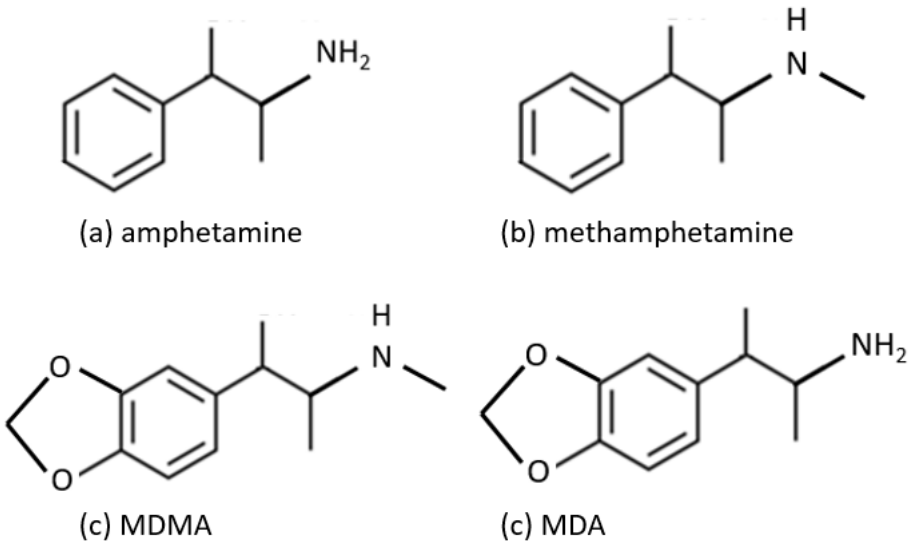


Figure 4.1. *Structure of (a) amphetamine and structurally similar drugs of abuse (b) methamphetamine, (c) MDMA and (d) MDA*

Desired effects of administering amphetamine and methamphetamine can include; an increase in confidence, energy, and alertness, as well as enhanced mood and euphoria [179, 184]. Reported undesired adverse side effects can include; lethargy, irritability, restlessness, sweating, insomnia, palpitations, agitation, reduced

appetite, and depression [179, 182]. It is rare for the administering of amphetamine to result in death [179, 181]. The reported fatal dose of amphetamine of non-addicts is estimated at 200 mg, but for naive users this can be significantly higher [181]. Amphetamine is most commonly reported as amphetamine sulphate often found in the form of 4-MMC hydrochloride salt, which is a white or off-white powder, but also available less frequently in tablet form [181]. Amphetamine is most commonly administered nasally (snorting), orally ("dapping" or "bombing") or by injecting as it is water soluble [185-187]. Amphetamine is commonly "cut" with caffeine [116, 185-190], creatine [190], lactose [189, 190], paracetamol [185, 190], and sucrose [157, 166, 185, 190], but also adulterated with other drugs of abuse.

Amphetamine, MDMA, MDA and methamphetamine are all internationally controlled and, in the UK, they are placed under Schedule 2 of the Misuse of Drugs Act 1971 as either Class A (for methamphetamine, MDMA and MDA) and Class B (for amphetamine) controlled drugs [41, 179] (Table 1.1.). It is worth noting that methamphetamine changed from a Class B to a Class A controlled drug in 2006 [42]. Since amphetamine emerged on to the recreational drugs market in the 70s, it has successfully established a place as one of the most prevalent drugs of abuse. The figures published in the most recent World Drug Report (2023) reported an estimated 27 million users of amphetamine and methamphetamine worldwide, with 20 million 'ecstasy' users worldwide during 2021 [1, 191, 192].

4.1.1. Detection of Amphetamine, Methamphetamine and MDMA using LOC technology

In the systematic review (published from the data in chapter 1), there were 10 articles reporting the detection of amphetamine as shown below in Table 4.1 [1]. One of the very first publications showcasing the use of an LOC device for the detection of drugs of abuse was Far *et al* (2005) detecting amphetamine in plasma and urine samples using an ELISA [98]. The publications reporting the detection of amphetamine in five different biological matrices (urine [98, 102, 113, 193], oral fluid [99, 101], whole blood [193], plasma/serum [98, 193], and sweat [112]), as well as in powder form [81] or in aqueous solution [80, 93]. Twenty-four percent of the publications studied more than one type of sample matrix, reflecting the adaptability of the LOC device to different types of sample matrices. Ninety per cent of the publications, reported multiplex detection of drugs of abuse [80, 81, 93, 99, 101, 102, 112, 113, 193], with the large majority (89%) of these articles detecting four or more drugs of abuse.

Table 4.1. *Publications reporting the use of LOC technology for amphetamine, methamphetamine and MDMA (adapted from [1])*

Methodology	Drug(s) investigated	LOC Material	Sample Matrix	Analysis Time (min)	Sensitivity	Reference
Immunoassay	**AMP**	Plastic	Plasma Urine	10	LODs: 20 ng mL^{-1} (urine); 6 ng mL^{-1} (plasma)	98
	AMP, MA, +3 DoA	Plastic	Plasma Oral fluid	1	LODs: states can detect to sub-nanogram per millilitre levels	99
	AMP, MA, MDMA, + 7 DoA (also two pro-drugs)	Agarose bead sensors	Oral fluid	~ 10	LODs: ranging from 0.14-7.4 ng mL^{-1}	101
	AMP, + 1 DoA	Graphene, PDMS, and PMMA	Aqueous	-	Not clearly stated	93
	MA	Glass	Hair	< 30	LOQ: ≤0.2 ng mg^{-1}	130
	AMP, MA, + 4 DoA	Glass	Blood Plasma Urine	3	Not clearly stated	193
	AMP, MA, + 4 DoA	Glass	Urine	-	Not clearly stated	102
	MA	Paper and PVC	Aqueous	10	LODs: 3.34×10−9 mol L^{-1}	194
	AMP, MA, + 2 DoA	Polystyrene	Sweat	16	LODs: ranging between 1.6-142 pg mL^{-1}	112
CE	**MDMA,** + 7 DoA	Quartz and PMMA	Urine	-	LODs: 1.94 µg mL^{-1} (MDA); 1.32 µg mL^{-1} (MDMA)	73
MS	**AMP, MA,** + 2 DoA	Paper	Urine **	-	LODs: 0.10-0.33 ng mL^{-1} (dry urine); 0.51-0.97 ng mL^{-1} (fresh urine)	113
SERS	**MA**	PDMS	Oral fluid	few minutes	LOD: 10 nM	116
	MA, + 2 DoA	Glass	Aqueous	-	LODs: 4.5 ng mL^{-1} (MA);	89
	MA		Oral fluid Urine	1***	Not clearly stated	118
Colourimetric	**MA,** + 2 DoA	Polyester	Aqueous	~ 6	LOD: 75 mg mL^{-1} (MA)	78
	MDMA, MA, + 4 DoA	Polyester	Aqueous	<1	LOD: 0.75 mg mL^{-1}(MA)	75
	MA, + 3 DoA	Paper	Powder	3	LODs: 55-100 µg	86
	AMP, MDMA, MA, + 6 DoA	Paper	Aqueous	5	MDQ for instrumentation ranging 1.2-8.7 µg and visual 2.5-11 µg	80
	AMP, MA, + 7 DoA	Glass	Powder	<0.25	LODs: 50–125 pg	81

polydimethylsiloxane (PDMS); poly(methyl methacrylate) (PMMA), surface-enhanced raman spectroscopy (SERS), mass spectrometry (MS), capillary electrophoresis (CE), minimum detectable quantity (MDQ). Drugs investigated will only refer to the either amphetamine (AMP), methamphetamine (MA) and/or MDMA. All other drugs of abuse (DoA) that the publications detected will be added as an additional number. ** (both fresh and dried). *(1 min for detection. However - pre-treatment to extract MA of 10 mins). ****Minimum detectable quantity (MDQ)*

The majority (70%) of LOC devices reporting the detection of amphetamine, used an immunoassay for the detection method [93, 98, 99, 101, 102, 112, 193]. This could be due to the selectivity of antibodies for the detection of amphetamine for the inclusion within an immunoassay, thus offering a detection method with limited cross reactivity. As well as the cost-effectiveness offered by using an immunoassay in comparison to other detection methods. However, the LOC device discussed in this chapter, is the first that includes the multiplex detection of both drugs of abuse (amphetamine, methamphetamine and MDMA) and NPS, including 4-MMC and its metabolite 4-ME. Being advantageous, especially as they are often found in combination in adulterated drug samples.

4.1.2. Quality Assurance

To ensure direct comparison with conventional technologies, quality assurance and validation are important elements that need to be considered for any newly emerging techniques, such as LOC devices for the detection of drugs of abuse and NPS. One key consideration is that the developed LOC device is applicable for their intended use by ensuring that the LOC devices can test bulk or seized samples. Another significant element of consideration would include testing the LOC device using biological samples with the inclusion of parent drugs and their metabolites to evaluate whether the device is fit for purpose in a wider range of scenarios. In addition to this, investigations into the cross reactivity of the LOC device with potential cutting agents and adulterants. In order to be directly comparable to conventional detection methods, LODs need to be determined to ensure that if the LOC device offers quantitative analysis it fit for purpose, applicable to real-world samples and can be easily used in the field to detect drugs of abuse and NPS to clinical and forensically relevant levels.

In addition, the use of positive and negative controls within the LOC devices should be considered to increase the reliability and integrity of each resulting test. While these controls are routinely applied in conventional laboratory settings, unfortunately they are not always integrated within LOC devices for the detection of drugs of abuse and NPS [1]. This quality assurance is important for future validation of the LOC devices and acceptance within local criminal justice systems.

In addition to quality assurance measures, it is essential that for research using LOC technology for quantitative (or semi-quantitative) analysis determine LODs. To ensure that the LOC device that has been developed is applicable is relevant to real-world analysis in clinical and forensic settings. Therefore, demonstrating that the LOC device is fit for purpose.

4.2. Experimental

4.2.1 Paper-based LOC device

The 96 well template was used to optimise the antibody (anti-amphetamine) and labelled antigen (amphetamine-HRP) concentrations, as well as testing a range of antibody (anti-HRP) and labelled antigen (cathinone-HRP) concentrations. Optimisation of the competitive immunoassay was achieved by investigating a range of antibody (anti-amphetamine) concentrations from 0.050 ng mL^{-1} to 0.200 ng mL^{-1} and labelled-antigen (amphetamine-HRP) concentrations from 0.050 ng mL^{-1} to 0.200 ng mL^{-1}. The most effective concentration of anti-amphetamine antibody was determined to be 1.75 ng mL^{-1} and 0.200 ng mL^{-1} of the labelled-antigen concentration which provided the greatest response [61].

The optimised LOC device incorporates an additional testing well that was incorporated for multiplex detection, using a competitive immunoassay for the selective detection of amphetamine in aqueous samples.

Investigating the cross reactivity of the anti-amphetamine antibody with varying concentrations (between 0.0156-10.0 ng mL^{-1}) of amphetamine, methamphetamine and MDMA, also used this 96 well template. Investigations for evaluating the selectivity and specificity of the antibodies incorporated within the LOC device used the optimised LOC device (Figure 2.3 A and B).

The reaction wells were prepared using the method in chapter 2.4.2 using anti-amphetamine antibody within the competitive immunoassay.

4.2.2. Cross reactivity using the paper-based LOC device

The cross reactivity of the anti-amphetamine antibody with investigated using the immunoassay protocol (chapter 2.6.3.) with three structurally similar drugs of abuse including, methamphetamine, MDMA and MDA. A blank sample (no drug present) and a

concentration range (7 samples from concentrations 0.156 to 10.0 ng mL^{-1}) of the three drugs of abuse were spiked to an aqueous sample and the signal intensity recorded. Aqueous samples were prepared by dissolving the target analytes of varying concentrations (10.0 ng mL^{-1} – 0.0156 ng mL^{-1}) in distilled water. Samples were mixed in a 50:50 ratio with the HRP-conjugated amphetamine and 5 µL was added to each well on the LOC device (Figure 3.2.). Samples were incubated for 210 seconds before washing twice with distilled water. Finally, 5 µL of TMB was added and an image of the colour change by taken on an iPhone 11 and measured using ImageJ analysis (version 1.52a). The colour change was proportional to the amount of HRP present.

4.3. Results and Discussion

4.3.1. Optimisation of anti-amphetamine and amphetamine-HRP

Initially a conventional immunoassay (chapter 2.4.1.) using a 96 well template (Figure 2.3.) was utilised to determine the optimum concentrations for both the anti-amphetamine antibody and the amphetamine-HRP within the competitive immunoassay.

Concentrations of the amphetamine-HRP were investigated, ranging from 0.200 to 0.050 ng mL^{-1} to establish the linear range. Thus, ensuring that the anti-amphetamine antibody and the amphetamine-HRP were not too dilute or too concentrated, to provide the best results for the vital components of the competitive immunoassay, to ensure that when amphetamine was added that it was sensitive and ultimately detectable. Lower concentrations of the anti-amphetamine antibody were then investigated to completely establish the optimal concentration for both the amphetamine-HRP and anti-amphetamine antibody.

Amphetamine-HRP dilution from 25 to 200 ng mL^{-1} were tested with anti-amphetamine antibody 0.200 to 0.050 ng mL^{-1} (Figure 4.2.). Providing the chosen concentration for the anti-amphetamine antibody and amphetamine-HRP are 1.75 ng mL^{-1} and 0.200 ng mL^{-1}, respectively.

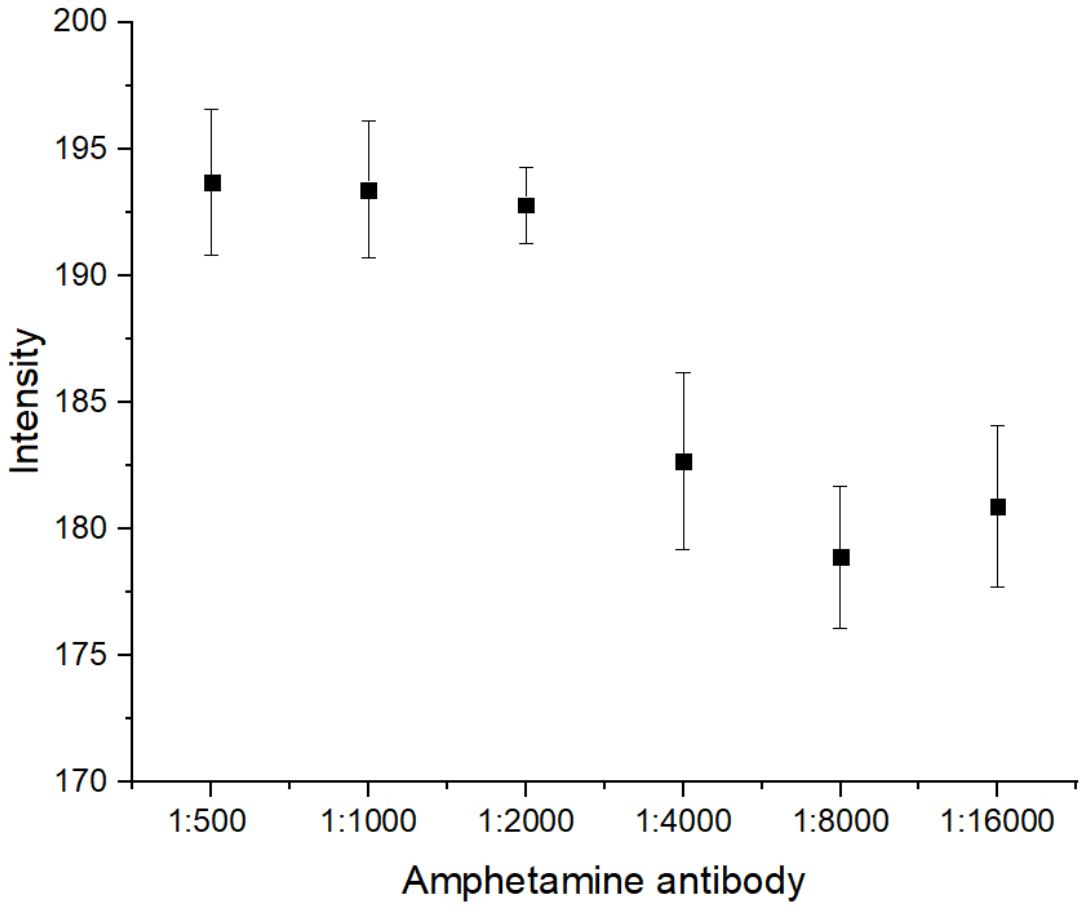


Figure 4.2. *Comparison calibration graph of the 1:500 amphetamine-HRP (200 ng mL^{-1}) and a concentration range of 0.02044-0.002044 µg mL^{-1} anti-amphetamine antibody and absorbance (n = 4)*

4.3.2. Optimisation and evaluation of anti-amphetamine antibody and amphetamine HRP

A range of dilutions of the amphetamine-HRP were used; 1:500, 1:1000, and 1:2000 to establish the linear range. The resulting calibration curve with 1:500 dilution provided the greatest response (Figure 4.3.). Providing the chosen concentration for anti-amphetamine antibody and amphetamine-HRP at 1.75 ng mL^{-1} and 200 ng mL^{-1}, respectively. Therefore, for all future experiments these conditions were used.

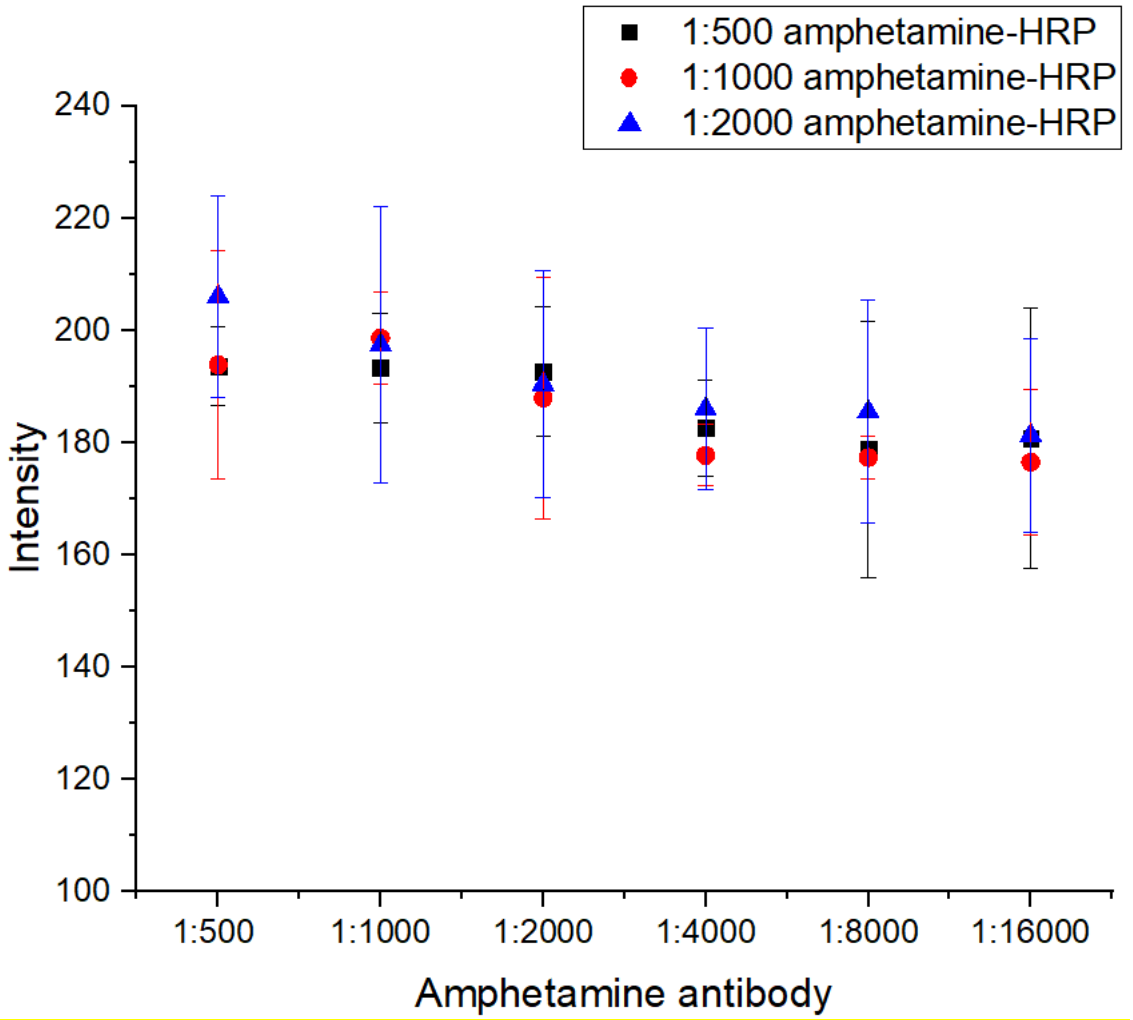


Figure 4.3. *Comparison calibration graph of the 1:500, 1:1000, and 1:2000 dilutions of amphetamine-HRP for the concentration range of 1.75 ng mL^{-1} anti-amphetamine antibody plotted on a Log^{10} scale (n = 4)*

The 1:1000 and 1:2000 dilution of amphetamine-HRP demonstrated within the LOC based immunoassay, these concentrations were not as effective as the 1:500 dilution. The 1:1000 and 1:2000 dilutions of amphetamine-HRP were therefore too dilute. Whereas the 1:500 dilution was more effective at detecting amphetamine-HRP, demonstrating a higher response, which therefore meant that amphetamine-HRP was therefore slightly more

detectable at this more concentrated dilution. As demonstrated in Chapter 3, the results demonstrate that using chitosan activation and glutaraldehyde cross-linking [126], implies that the anti-amphetamine antibody has been effectively covalently immobilised to the well, via the competitive immunoassay within this LOC device. By immobilising the anti-amphetamine antibody has shown that it can effectively detect amphetamine.

4.3.3. Optimisation and evaluation of anti-HRP antibody and cathinone HRP

A range of dilutions of the cathinone-HRP were used; 1:500, 1:1000, and 1:2000, for a concentration range of 0.200 to 0.050 ng mL^{-1} of anti-HRP antibody (dilutions at 1:5000, 1:10000, 1:15000, 1:20000) to provide the linear range. The resulting image was taken after the experiment took place (Figure 4.4.) and the calibration curve with the 1:500 dilution provided the greatest response (Figure 4.5.). Providing the optimum concentration for anti-HRP antibody and cathinone-HRP at 0.050 ng mL^{-1} and 0.200 ng mL^{-1}, respectively. Therefore, for all future experiments these conditions were used.

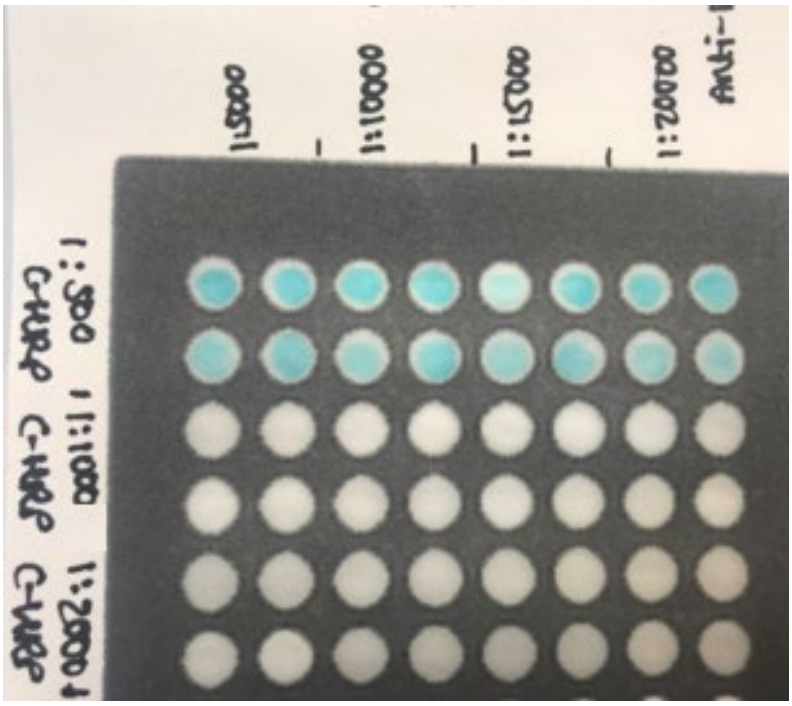


Figure 4.4. *Image of the printed 96 well template investigating the 1:500, 1:1000, and 1:2000 dilutions of cathinone-HRP against the concentration range of 0.0500-0.200 ng mL^{-1} anti-HRP antibody*

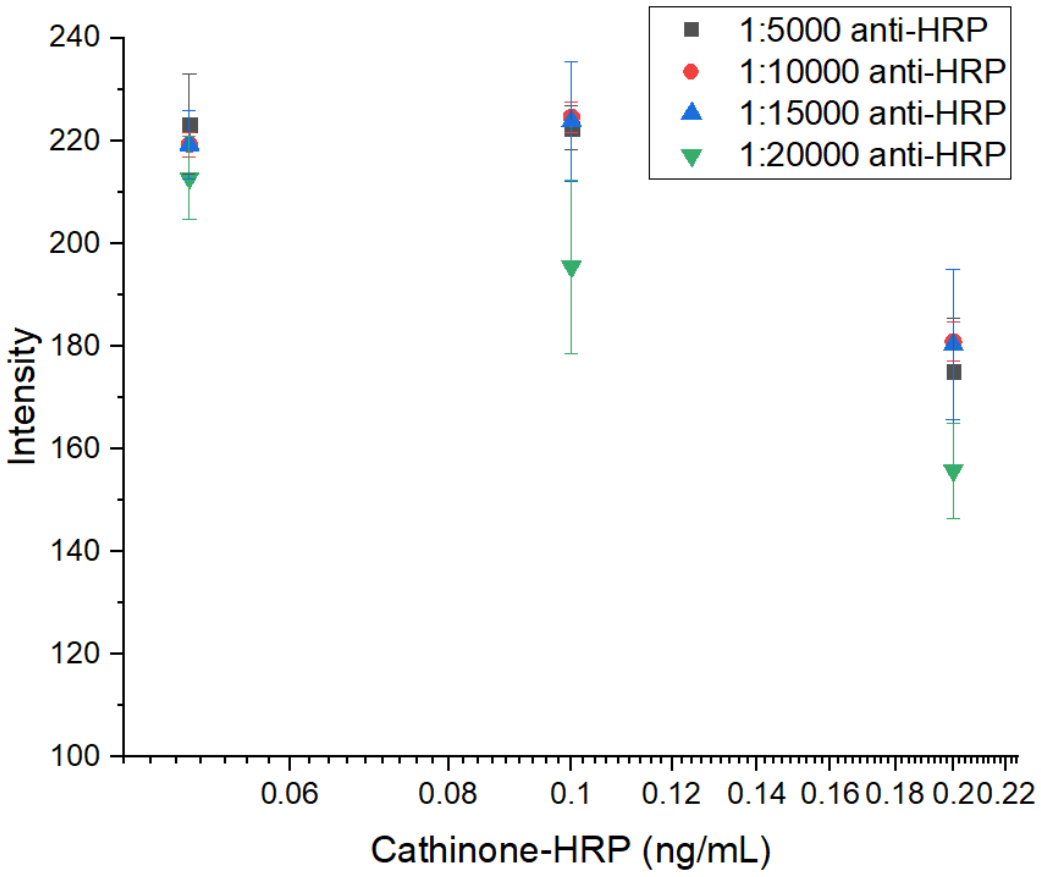


Figure 4.5. *Comparison calibration graph of the 1:500, 1:1000, and 1:2000 dilutions of cathinone-HRP for the concentration range of 0.0500-0.200 ng mL^{-1} anti-HRP antibody plotted on a Log^{10} scale (n = 4)*

The 1:1000 and 1:2000 dilutions of cathinone-HRP demonstrated within the LOC competitive immunoassay, these concentrations were not effective in comparison to the 1:500 dilution. The 1:1000 and 1:2000 dilutions of cathinone-HRP were therefore too dilute. Whereas, the 1:500 dilution effective at detecting cathinone-HRP, demonstrating a higher

response, which demonstrated that cathinone-HRP was therefore more detectable using the competitive immunoassay at this more concentrated dilution. Therefore, this optimum concentrations of anti-HRP and cathinone-HRP incorporated within this immunoassay, would aid in the detection of HRP at small concentrations, and therefore representing an effective positive control.

4.3.4. Cross reactivity of Structurally Similar Drugs of Abuse

Initially, amphetamine and methamphetamine were investigated using spiked aqueous samples, across 7 concentration ranges, between 0.0156-10 ng mL^{-1}. However, the calibration curve (Figure 4.6) showed that the recorded light intensity values were continuing to decrease as the concentration decreased. Therefore, further investigations were undertaken to investigate the effectiveness of the LOC device at detecting amphetamine and methamphetamine at lower concentrations. The concentrations range of both amphetamine and methamphetamine were lowered to 0.000488 - 1 ng mL^{-1}. Both amphetamine and methamphetamine effectively competed with the amphetamine-HRP for the active sites of the anti-amphetamine antibody at both the higher and lower concentration ranges investigated. With the anti-amphetamine antibody showing a higher degree of selectivity and specificity for amphetamine when compared to methamphetamine, due to slightly higher average light intensity measurements producing a better calibration curve. In terms of the competitive immunoassay, the binding affinity for methamphetamine is not as effective as the binding affinity for amphetamine for the active sites of the anti-amphetamine antibody, as the antibody has a greater degree of specificity for amphetamine as expected [176].

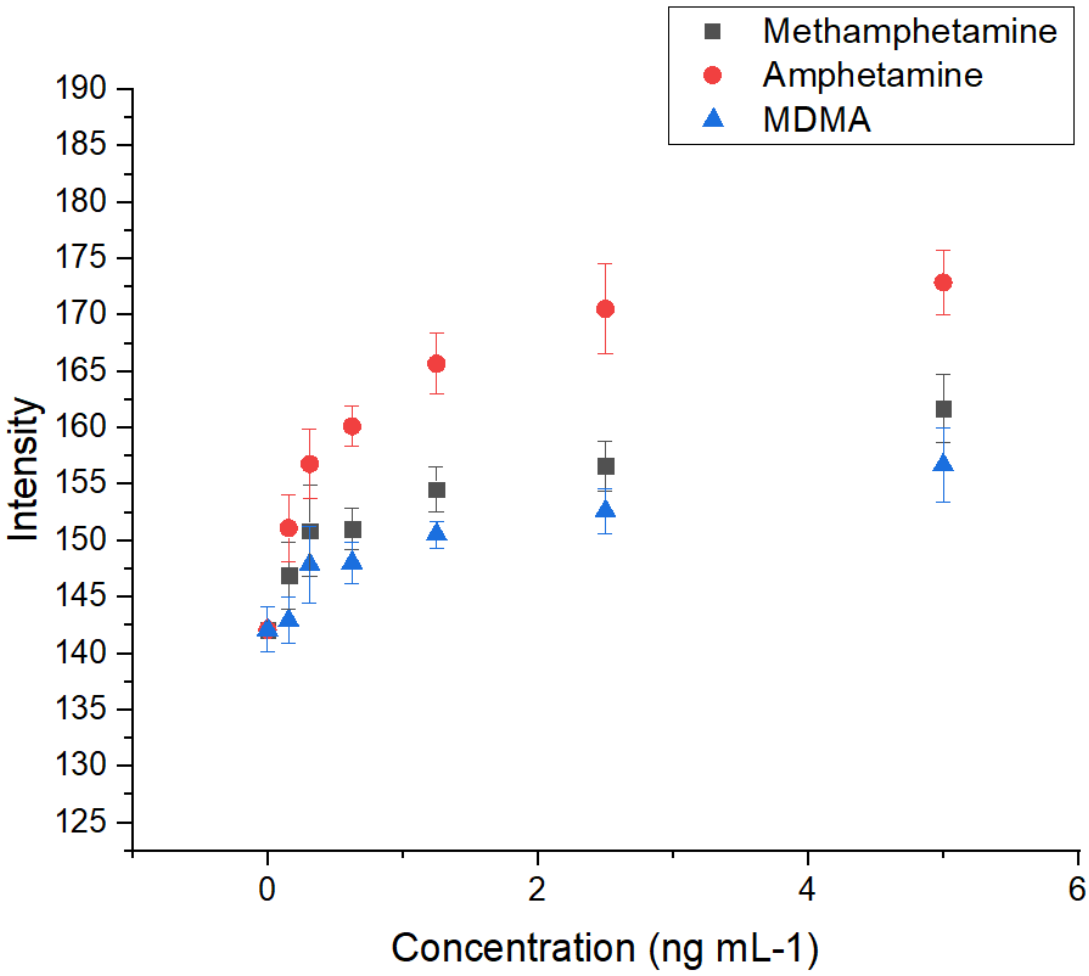


Figure 4.6. *Comparison of amphetamine, methamphetamine and MDMA in aqueous solution at concentrations from 0.00156-1 ng mL^{-1} (n = 6)*

The LOC device successfully demonstrated that ability to detect amphetamine, methamphetamine and MDMA to forensically relevant LODs of the device of 3.11 ng mL^{-1}, 3.93 ng mL^{-1} and 4.84 ng mL^{-1} for aqueous samples, respectively. Reported LODs for amphetamine [80, 81, 93, 98, 99, 101, 102, 112, 113, 193], methamphetamine [75, 78, 80, 81, 86, 89, 99, 101, 102, 112, 113, 116, 118, 130, 193] and MDMA [73, 75, 80, 101] ranging from picogram to milligram levels. Thus demonstrating that by incorporating the anti-amphetamine antibody within this LOC device, it offers multiplex detection of drugs of abuse amphetamine, methamphetamine and MDMA, in addition to the detection of 4-MMC and 4-ME (using the anti-methcathinone antibody) to

forensically relevant levels, which was the first LOC device to report the detection of an NPS, but offers flexibility in terms of being able to distinguish between compounds that 4-MMC can be adulterated with [61].

4.3.5. Quality Assurance

To increase the reliability of each result obtained when using the optimised LOC device positive and negative controls were integrated. A positive control was indicated by a blue colour change as the HRP (labelled antigen) successfully competed for the active sites on the anti-HRP antibody. Whereas the negative control was indicated by a white colour as there is no antibody present in the immunoassay. The positive (anti-HRP antibody) and negative (distilled water) controls were effective within the LOC device with signal intensity ranges between 122 - 139 and 178–198, respectively. If the positive or negative controls were ever out of these values, then the experiment undertaken would need to be repeated. Figure 4.7 demonstrates an example of negative and positive control observed when testing the optimised LOC device. The location of the positive control (anti-HRP) is shown below on the bottom left well (labelled iii) and the negative control is located on the bottom right well (labelled iv).

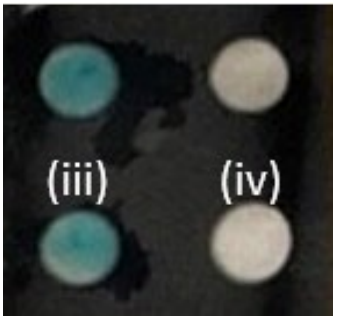


Figure 4.7. *Image of the central detection zone (black tab) of the optimised LOC device showing the (iii) positive control and (iv) is the negative control*

The inclusion of these in-built controls enables this LOC devices to analyse in parallel to sampling, increasing the reliability and accuracy of results provided when testing with this LOC device. The importance of this crucial in terms of working towards validation of this LOC device to offer the potential as a portable detection device for drugs of abuse and NPS in clinical or forensic settings. It appears that this is not always the case across a wide variety of different detection methods using LOC technology for the detection of drugs of abuse and NPS, which is something that should be always be considered in such research to validate efficacy.

4.4. Conclusion

The data in this chapter demonstrates that this paper-based LOC device offers multiplex detection of amphetamine, methamphetamine and MDMA (in addition to 4-MMC and 4-ME discussed in chapter 3) to forensically relevant levels. Amphetamine, methamphetamine and MDMA all effectively competed with the amphetamine-HRP for the active sites of the anti-amphetamine antibody at both the higher and lower concentration ranges investigated. Overall, the anti-amphetamine antibody demonstrated a higher degree of selectivity and specificity for amphetamine when compared to methamphetamine and MDMA, as the average light intensity measurements are slightly higher for amphetamine demonstrating a higher response. In terms of the competitive immunoassay, the binding affinity of methamphetamine and MDMA were not as effective as the binding affinity for amphetamine for the active sites of the anti-amphetamine antibody, as the antibody has a greater degree of specificity for amphetamine as expected [176]. However, the binding affinity of methamphetamine was more effective than MDMA for the active sites of the anti-amphetamine antibody. The LOC device successfully demonstrated forensically relevant LODs for the LOC device for amphetamine, methamphetamine and MDMA to 3.11 ng mL^{-1}, 3.93 ng mL^{-1} and 4.84 ng mL^{-1} for aqueous samples, respectively.

Thus, highlighting this paper-based LOC device for the detection of amphetamine, methamphetamine and MDMA is highly comparable with traditional laboratory based analytical techniques with its forensically relevant LODs and rapid total analysis time. However, it is worth noting that a limitation of this LOC device is that this LOC device cannot distinguish between these compounds at varying concentrations. But it's potential as a rapid and portable semi-quantitative detection method for amphetamine, methamphetamine,

MDMA, in addition to 4-MMC and 4-ME (discussed in chapter 3) for on-site testing within both clinical and forensic applications, on a global scale is advantageous.

Finally, with only 7% of publications for the detection of drugs of abuse and NPS using LOC technology including positive and negative controls, the inclusion of positive and negative controls within this LOC device significantly increases the reliability and integrity of using this developed LOC device [1]. Which is an extremely important quality assurance measure to for future validation of this LOC devices and the potential for acceptance within local criminal justice systems.

Chapter 5 goes on to investigate usability and adaptability of the optimised LOC device through the evaluation of 20 seized drug samples (provided by Greater Manchester Police via MANchester DRug Analysis and Knowledge Exchange, MANDRAKE) to determine its reliability, specificity, and reproducibility. As well as evaluating the optimised LOC device through end-user testing. In addition to investigating the adaptability of the optimised LOC design for the detection of new emerging NPS, such as MDPHP and NEP.

Chapter 5: Testing the usability and adaptability of the optimised LOC device

Recommendations discussed with regards to quality assurance and practicality using LOC technology have been published in the peer-reviewed journal Forensic Chemistry (L. McNeill, D. Megson, PE. Linton, J. Norrey, L. Bradley, et al. (2021). Lab-on-a-Chip approaches for the detection of controlled drugs, including new psychoactive substances: A systematic review. Forensic Chemistry. 26, pp.100370-100370) [61]*. The subsequent concluding chapter will discuss all the chapters within this thesis.*

Executive Summary

This chapter aims to investigate both the usability and adaptability of the optimised LOC device. In terms of usability the optimised LOC device was evaluated by; 20 seized drug samples (provided by Greater Manchester Police via MANDRAKE) were tested using the optimised LOC device to determine the reliability, specificity, and reproducibility of the LOC device. End-user testing of the optimised LOC device was also evaluated by non-scientifically trained individuals that tested three different compounds using a set of instructions (Appendix 5.1.). End users were asked to submit Anonymous End-user Testing Feedback Questionnaire (Appendix 5.2.) when using the LOC device. In terms of adaptability, this chapter investigates the adaptability of the optimised LOC design for the detection of new emerging NPS, MDPHP and NEP to clinically relevant levels.

This chapter has successfully developed a user-friendly LOC device that can successfully be used by non-scientifically trained individuals, who correctly identified three different compounds with 100% specificity and 100% sensitivity. In addition to this, this chapter highlights the significant impact that this optimised LOC device could offer if used for the detection of synthetic cathinones and amphetamines for on-site testing by non-scientifically trained individuals within a variety of clinical or forensic settings globally. By undertaking

'Blind Testing' of 20 seized suspected drug samples, in terms of practicality this optimised LOC device with 95% specificity and 100% sensitivity demonstrates its potential as a portable detection method for real-word samples. As well as demonstrating the reliability and reproducibility of the LOC device. This chapter importantly concludes with successfully detected new emerging NPS, MDPHP and NEP clinically relevant levels. LOD of 1.84 ng mL^{-1} and 1.23 ng mL^{-1}, respectively to clinically relevant concentrations of MDPHP in urine and aqueous samples. An LOD of 2.32 ng mL^{-1} and 2.11 ng mL^{-1} was calculated for NEP in urine and aqueous samples, respectively. Demonstrating the adaptability of the detection capabilities of this LOC device for the continuously increasing number of available NPS. Therefore, future proofing the potential of this LOC device in detecting these substances to keep up with the current trends of the recreational drugs market.

5.1. Introduction

LOC devices need to be developed and tested to ensure that they are user-friendly, but also to ensure that the potential LOC device is fit for purpose for its intended use. For example, an LOC device for the detection of drugs of abuse and NPS should be designed to either test biological or bulk samples. With an ever-increasing number of NPS available to the recreational drugs market, the LOC device should also offer adaptability to aid in the detection of these compounds [1, 2, 28-31]. With the latest World Drug Report stating an increase of 54 new NPS in one year, and over 1000 different NPS reported across 125 countries since NPS first emerged on the recreational drugs market [1, 2, 28-31]. This highlights the requirement for adaptability and portable detection methods to aid in addressing this issue.

5.1.1. Seized Drug Testing

The detection of seized drugs in forensic laboratories usually involves a two-step process of initial screening using a rapid presumptive test followed by a discriminatory technique(s), usually chromatographic [10]. It is worth noting that there are other techniques, however chromatographic techniques are the 'gold standard' discrimatory technique in forensic analysis as they specifically determine the number of compounds and also quantity present and subsequently the most selective [11, 12]. The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) provide required sensitivity levels to come to a scientifically supported conclusion and laboratories must ensure that they meet the criteria [11]. Analytical methods are categorised according to the selectivity, with three classifications according to their selectivity. Category A is the most selective and the selectivity of the techniques are based on structural determination, including MS, NMR, Raman Spectroscopy and Infrared Spectroscopy [11]. Category B has less selectivity than A, with the techniques basing their selectivity through physical characterisation, including GC, LC, and CE [11]. Category C are the least selective techniques that detect using general or class information, including colour tests and immunoassays. All forensic laboratories should have a protocol to ensure that the required level of selectivity is achieved to allow for the determination of drugs of abuse or NPS drawn from scientifically supported conclusion based on the analytical methods undertaken [11]. In terms of LOC technology for the use in forensic settings, it is also essential that considerations are made to assess whether the LOC device is suitable for presumptive initial screening or if it could potentially offer an alternative to traditionally used techniques as a discriminatory technique.

By combining techniques such as GC and LC with MS, this allows for more selective methods for the detection of drugs of abuse and NPS. GC-MS is a commonly used analytical

technique for the detection of NPS and drugs of abuse in biological samples, and also from seized street samples [12-14].

According to the SWGDRUG, immunoassays are amongst the least selective methods that can be utilised for the detection of drugs of abuse and NPS [11]. Therefore, immunoassays as are presumptive tests that require a confirmatory test, such as GC-MS or LC-MS. However, immunoassays are commonly used for initial screening of drugs of abuse especially within hospitals or as part of workplace drug testing programmes, as they are cost-effective, simple to perform and offer quick results when compared to more time and cost demanding analytical techniques, such as GC-MS and LC-MS [15-17]. With competitive immunoassays readily utilised for the detection of seized or bulk drugs of abuse as they only require one antibody, along with a labelled 'tracer' that is a known amount of labelled antigen [176]. A distinct advantage of utilising a competitive immunoassay is that the antibody incorporated has the potential capability to cross-react with the continually increasing number of NPS available within the recreational market, making a competitive immunoassay is ideal for analysis of NPS [12]. Another limitation of immunoassays is that they can also produce false-positive and false-negative results [11, 15, 18-20].

There is very limited research using LOC technology to detect seized drug samples. In 2015, a colourimetric paper-based semi-quantitative LOC device for the multiplex detection of seized drug samples was published [80]. This LOC device was designed with its intended use in mind, for the on-site testing of suspected drug samples either by the police, airport security and/or border control [1, 80]. Wang *et al* (2018) developed an aptamer paper-based LOC device paired with gold nanoparticles for the colourimetric detection designed to detect seized cocaine samples [84]. The authors investigated the effects of different interferents, including 8 common white powders, 4 diluents, 7 drugs of abuse and two metabolites of

cocaine, with methamphetamine being the only substance to produce a reaction [84]. However, they did not use the LOC device for real-world seized samples. There was also a publication from a research group in Brazil investigating LOC technology for the detection of seized cocaine samples [82]. Both colourimetric and electrochemical detection were incorporated to analyse seized cocaine that was adulterated with 4-dimethy-aminoantipyrine, lidocaine and levamisole [82]. Highlighting that these LOC devices were developed for their potential use in examining seized drug samples with South America, one of the main trafficking routes of cocaine to North America [31, 70, 82]

The inclusion of using real-world seized and bulk samples in this chapter was instrumental to investigating the applicability of the LOC device being used as an on-site detection method. By undertaking 'Blind Testing' of 20 seized suspected drug samples (Table 5.1), this optimised LOC device investigates the sensitivity and sensitivity for real-word samples. As well as investigating the reliability and reproducibility of the LOC device. This is the first research using a LOC device for the 'Blind Testing' of unknown samples highlights its novelty, demonstrating the reliability and robustness of this detection method.

5.1.2. End-user Testing

The field of LOC technology has evolved into a diverse multifaceted research area. There have been significant developments into clinical diagnostics [62], for fully integrated LOC devices that offer 'sample in-answer out' therefore making them user-friendly. However, in the field of forensics, there are only a limited number of commercialised devices that are currently available to the end-users that they were developed for. Examples of a completely integrated LOC, include the RapidHIT® ID System that was mentioned in chapter 1.1.3. This technology produces a DNA profile for human identification in just 90 minutes using a buccal swab sample [63]. Two other commercialised systems are ParaDNA (LGC Forensics)

and Rapid DNA (ANDE) both rapid DNA instruments for identification, which are systems that have been developed through the use of end-user testing to enable forensic DNA analysis [195-199].

The first research journal article reporting a fully integrated LOC device capable of producing a DNA profile was published by Hopwood *et al* in 2010 [65], and from this publication to the first commercially available LOC system it took approximately two years. Since the introduction of the Rapid DNA Act of 2017, such LOC technology has been used by law enforcement for the analysis of reference samples [63]. This demonstrates that LOC systems have the potential to be used effectively as part of forensic investigations. Another LOC device that has been successfully developed within healthcare is the commercially available electrophoresis-based LOC device called the Gazelle platform is available for the portable and cost-effective detection of haemoglobin disorders (such as sickle cell disease in new-borns), using a small drop of blood [200]. This LOC device was developed as a POC testing platform for the detection in low resource settings, using a step-by-step user guide [200]. This LOC device was tested across clinical sites by local users, including healthcare workers and clinical laboratory personnel testing 768 subjects at clinical sites in the USA, Central India, sub-Saharan Africa, and Southeast Asia. Importantly, research was undertaken into the validation studies, which took place in Bangkok and Thailand (for haemoglobin E testing), and in Chhattisgarh, India, and Nigeria (for haemoglobin S testing), where the sickle cell disease burden is the highest in the world [200].

Currently there are a limited number commercially available immunoassay kits for the detection of drugs of abuse and NPS that can be used with different biological specimens. Randox Toxicology has developed a range of ELISA kits for the detection of NPS and drugs of abuse, including the 'Mephedrone/Methcathinone (Bath Salts)' that can detect a range of

synthetic cathinones to varying degrees in different biological matrices, including urine, blood and oral fluid [201]. With LODs for 4-MMC/methcathinone of 0.4, 0.57 and 0.9 ng mL^{-1} urine, blood and oral fluid, respectively [201]. There is also literature investigating the cross-reactivity of these commercially kits with 4-MMC and other NPS. Torrance *et al* (2010) found that there was cross-reactivity of the in-house ELISA for methamphetamine using urine and blood samples that involved 4-MMC of approximately 1-3% [17]. A study by Swortwood *et al* (2014) assessed 16 different ELISA kits in order to establish the cross-reactivity of 30 NPS [202]. Two of the sixteen ELISA kits were the Randox MDPV ELISA and the 4-MMC/methcathinone ELISA kits that are designed to be selective for MDPV and 4-MMC/methcathinone, respectively [202]. Swortwood *et al* (2014) demonstrated that there was cross-reactivity with Bk-MBDB when testing the Randox Toxicology 'MDPV' kit, even down to a concentration of 150 ng mL^{-1}. Whereas there was no cross-reactivity for MDPV when testing the Randox 'mephedrone/methcathinone' kit [202]. Randox Toxicology has also developed for the commonly encountered drug of abuse amphetamine, has LODs of 11.7 and 108.2 ng mL^{-1} for blood and urine, respectively [201]. Bell *et al* (2011) investigated the cross-reactivity of eight NPS with the commercially available kits developed by Thermo Scientific 'CEDIA® Amphetamines/Ecstasy' immunoassay [203].

There are two organisations that work together in science and technology to ensure the development, maintenance, and also to promote standards within this field and thus increasing the reliability and accuracy of results through standardisation [204, 205]. These organisations are the International Organisation for Standardisation (ISO) and the International Electrotechnical Commission (IEC). For newly developed techniques, it is imperative that quality assurance and validation are undertaken to enable development and evaluation (in terms of accuracy and reliability) to be suitable for their intended purposes [1,

[67, 204, 205]. In terms of LOC technology for forensic analysis for legal use, accreditation to ISO/IEC 17025 is a fundamental component that must be considered [67, 68]. It is essential that newly developed techniques consider that the detection method is fit for purpose, such as the ability to detect drugs of abuse and NPS (and also the metabolites) in biological samples [1]. With an extensive array of different biological matrices, the design needs to understand the importance of whether the LOC device is aimed at invasive or non-invasive drug testing and depending on the chosen drugs of abuse or NPS to test the other implication that is important here is the half-life of the drug and therefore the detection window of the matrix (as discussed in Chapter 1).

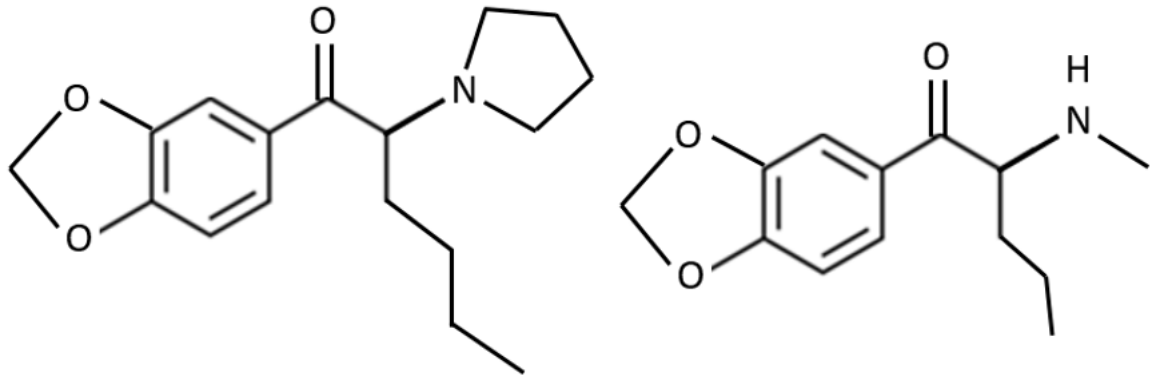
This chapter highlights the significant impact that this optimised LOC device could offer if used for the detection of synthetic cathinones and amphetamines for on-site testing by non-scientifically trained individuals within a variety of clinical or forensic settings globally. Therefore, evaluating the optimised LOC device to determine the reliability, specificity, ease of use and portability when being used by non-scientific individuals, as well as assessing if there were any potential issues during these investigations is essential. In the recent systematic review on LOC technology for the detection of drugs of abuse and NPS (discussed in chapter 1), it was highlighted that there is a lack of supporting data provided by the publications on the ease of use impeding comparison between different detection methods [1]. End-user testing has been undertaken was undertaken by a small number of publications reporting the detection of a range of drugs of abuse to support the reliability of the LOC devices as a potential detection method.

5.1.3. Adaptability of this LOC device for the detection of new synthetic cathinones

Synthetic cathinones are structurally related to cathinone, with methcathinone being the first synthetic cathinone to emerge in the USA in 1993 [1, 159]. However, it was not until the

mid-2000s when synthetic cathinones effectively established a place within the recreational drugs market globally [2, 25, 26]. In the latest World Drugs Report (2022) there were 201 synthetic cathinones reported [206], highlighting a timely requirement that there are adaptable portable detection methods in clinical and forensic environments, such as the LOC device discuss in this chapter to identify the presence of commonly abused synthetic cathinones.

Methylenedioxypyrrolidinohexiophenone (MDPHP, 3,4-methylenedioxy-α-pyrrolidinohexanophenone, "monkey dust") (Figure 5.1.a) a synthetic cathinone that was first synthesised in the 1960s [207-210]. However, it was not until the 6th of November 2014 that MDPHP was first reported in Sweden to the EMCDDA [210, 211]. The metabolism of MDPHP within the body can occur via Phase 1 or Phase 2 processes [210]. Deaths associated to the use of MDPHP are relatively rare, with the first report in 2018 of a foetal death in Poland from the associated complications of MDPHP and α-pyrrolidinopentiophenone (α-PVP) by the mother use [207] and the death of a 48-year-old male in Italy due to associated use of MDPHP [212]. The recreational use of MDPHP has been reported in Stoke-on-Trent (located in the UK), with around 950 reports within a 3-month period [213], highlighting a local surge in recreational use and requirement for portable detection methods within clinical and forensic settings. Tandem LC-MS is currently the most commonly reported technique for the detection of MDPHP in biological samples [210, 212, 214-219].



(a) MDPHP (b) N-ethylpentylone

Figure 5.1. *Structures of (a) MDPHP and (b) NEP*

N-ethylpentylone (NEP, 1-(2H-1,3-benzodioxol-5-yl)-2-(ethylamino)pentan-1-one) (Figure 5.1.b) is a synthetic cathinone, that is closely related to pentylone, ethylone, and mephedrone [220-222]. Similarly, to MDPHP, NEP was first synthesised in 1960s by Boehringer Pharmaceuticals [208, 209, 220, 222, 223], but the first report of NEP for recreational use was in the USA in 2014 [221, 222], then again in 2016 in the USA, but also in Europe by drug seizure forensic analysis [221]. There have been admissions to emergency departments globally [220, 222, 224-227], with fatalities as a result of NEP toxicity in Brazil [220, 225, 228] and the USA [224-227]. Thus, highlighting a significant impact that synthetic cathinones, including NEP are putting on public services, including the police and emergency departments on a global scale.

This LOC device has the opportunity to provide rapid, on-site testing within a forensic or clinical setting, which could aid a potential reduction of the use of synthetic cathinones globally. These two synthetic cathinones represent the largely varying complications and side effects from recreational drug use, and the pressures that these can have on public services.

5.2. Experimental

To determine the reliability, specificity and reproducibility of the optimised LOC device, 20 seized drug samples (provided by Greater Manchester Police via MANDRAKE) were tested. The optimised LOC device was also evaluated by non-scientifically trained individuals using a set of instructions (Appendix 5.1.) who tested three samples A, B and C. The individuals used the optimised LOC devices to determine if there was a presence or absence of synthetic cathinones or amphetamine-type substances in any of these samples. In addition to this, the End-users testing the LOC device were asked to complete Anonymous End-user Testing Feedback Questionnaire (Appendix 5.2) on using the LOC device to evaluate practicability of the optimised LOC device to determine whether the LOC device is fit for purpose. Investigations for both the blind testing and the end-user testing were undertaken using the optimised LOC device and were prepared using the optimised LOC methodology (chapter 2.7.). All LOC devices were prepared in triplicate for each test for the blind testing and the end-user testing.

5.2.1. Blind Testing

Twenty seized suspected drug samples were tested in triplicate using the optimised LOC device. The 20 samples were anonymised by labelling 1-20 (Table 5.1.) by a member of the MANDRAKE team. The samples were of varying concentrations of different drugs of abuse, NPS, and or cutting agent. Aqueous samples were prepared by dissolving each of the 20 seized drug samples in 500 µL of distilled water. The identification of 20 seized suspected drug samples was undertaken to the selectivity and sensitivity of the optimised LOC device. As well as also investigating the variability between the replicates. It is worth noting that the list for the samples detailing what sample 1-20 was released after the experiments and subsequent analysis had taken place to ensure blind testing was undertaken.

5.2.2. End-user Testing

For End-user Testing, the optimised LOC devices were prepared for five non-scientifically trained individuals. These individuals then followed a set of instructions (Appendix 5.1.) to test three different samples, labelled A, B and C in triplicate. The instructions sheet included a clear protocol of how to test the three samples and how to correctly evaluate the results. Their anonymous feedback was also submitted onto an online survey system for the Anonymous End-user Testing Feedback Questionnaire (Appendix 5.2) when using the LOC device and images of the replicates was taken to compare reproducibility between the devices.

5.2.3 MDPHP and NEP

The immunoassay protocol (chapter 2.7.) using a 96 well template (Figure 2.3.) was used for these investigating the cross reactivity of the anti-methcathinone antibody with varying concentrations (from 0.15625-5 ng mL^{-1}) of MDPHP and NEP in both aqueous and urine. Aqueous samples were prepared by dissolving the target analytes in distilled water.

5.2.4 Data analysis

The data collected from the Anonymous End-user Testing Feedback Questionnaire (Appendix 5.2.) was transferred into Origin (version 2019) for the graphical representations, and SPSS (version 22) to allow statistical analysis to be performed. The numerical graded values in the Anonymous End-user Testing Feedback Questionnaire are classified as ranked data and were therefore analysed using non-parametric tests. As a non-parametric test uses the ranks of the data from the answers to the Questionnaire and does not assume that the data is normally distributed. Values of $P < 0.05$ were considered statistically significant.

5.3. Results and Discussion

5.3.1. Blind Testing

For the LOC device to be fit for purpose in clinical and forensic settings, it is essential ensure that the optimised LOC device can detect the presence or absence of synthetic cathinones and amphetamine within in seized or bulk samples. But to also investigate the variation between the replicates of the LOC devices for each of the 20 seized drug samples (provided by Greater Manchester Police via MANDRAKE) that were tested. Therefore, the blind testing was designed to evaluate both the variability and whether the LOC device is fit for purpose.

5.3.1.1. Identification

The sensitivity and specificity were determined using the LOC device. The sensitivity was calculated by the ability of the LOC device to correctly identify true positive results from the 20 blind samples. Whereas the specificity was calculated on the ability of the LOC device to correctly identify samples that the LOC device was design to detect. The LOC device demonstrated 95% sensitivity, as one of the twenty samples was a false-negative, and 100% specificity as the LOC device can correctly identify samples that contain amphetamine, MDMA, NEP, clephedrone (4-chloromethcathinone, 4-CMC; Figure 3.1) and MDPHP. However, the 4-CMC present in sample 1 was not detected, thus demonstrates the reduction in the sensitivity (95%) of the LOC device. This was due to one false negative result, as the LOC device did not correctly identify one of the three replicates for sample 1 that contained a mixture of MDMA and 4-CMC (Table 5.1.). 4-CMC is a synthetic cathinone that closely related to 4-MMC, and subsequently be likely to cross-react with the anti-methcathinone antibody to varying degrees (Figure 3.1.) [229, 230].

4-CMC only differs from 4-MMC by a chlorine substitution on the benzene ring in place of the methyl group [150, 153, 229, 230].

Table 5.1. *Summary table of the 20 anonymised seized drug samples (provided by Greater Manchester Police via MANDRAKE) that were tested using the optimised LOC device. These have been confirmed by GC-MS and FT-IR* [231, 232]

Sample Number	Concentration (mg mL^{-1})	Seized Drug Sample*	Synthetic Cathinones		Amphetamines	
			Positive	Negative	Positive	Negative
1	10.8	***MDMA*** + **4-CMC**	-	✓	✓	-
2	-	**NEP**	✓	-	-	✓
3	12.8	**NEP**	✓	-	-	✓
4	11.4	**4-CMC**	✓	-	-	✓
5	25.1	MDMB-4en-PINACA	-	✓	-	✓
6	10.8	Ketamine	-	✓	-	✓
7	10.4	Paracetamol	-	✓	-	✓
8	11.0	2C-B	-	✓	-	✓
9	10.5	***MDMA***	-	✓	✓	-
10	11.9	**4-MMC**	✓	-	-	✓
11	10.5	Caffeine	-	✓	-	✓
12	10.3	Zopiclone	-	✓	-	✓
13	10.1	Cocaine + Phenacetin	-	✓	-	✓
14	11.5	Cocaine	-	✓	-	✓
15	10.0	***MDMA***	-	✓	✓	-
16	10.5	**4-MMC**	✓	-	-	✓
17	10.2	***MDMA***	-	✓	✓	-
18	12.3	***Amphetamine*** + Caffeine	-	✓	✓	
19	9.7	Heroin, Caffeine, Acetylcodeine + Noscapine	-	✓	-	✓
20	10.6	**MDPHP**	✓	-	-	✓

**samples highlighted in bold and underlined only are synthetic cathinones that are expected to cross-react with the anti-methcathinone antibody located in the top left well located within the black central detection zone (Figure 2.2.). Samples highlighted in bold and italics are amphetamine-type stimulants that are expected to cross-react with the anti-amphetamine antibody located in the top right well located within the black central detection zone (Figure 2.2.).*

5.3.1.2. Amphetamines

Five out of the 20 seized samples for blind testing contained either MDMA or amphetamine that have been confirmed by confirmed by GC-MS and FT-IR [231, 232]. All five

samples for MDMA (sample 1, 9, 15 and 17) and amphetamine (sample 18) were correctly identified as positive using the LOC device (as shown in Table 5.1.). An example image using the LOC device for sample 18 is shown in Figure 5.2. Depicting the three replicates obtained for sample 18 for the correct identification of amphetamine.

Figure 5.2 *Image of replicates obtained for sample 18 (amphetamine) depicting the identification of amphetamine. The top left well on the black tab contains the anti-methcathinone antibody, as there is no 4-MMC present within sample 18 then the response from TMB is the most intense colour (blue) that can be observed by eye. The top right well on the black tab contains the anti-amphetamine antibody: as amphetamine is present within sample 18, the TMB subsequently produces a lower response as the unlabeled target antigen (amphetamine) out competes the labelled amphetamine-HRP for the active sites of the anti-amphetamine antibody*

As discussed in chapter 4, amphetamine and MDMA have been investigated, and both effectively competed with the amphetamine-HRP for the active sites of the anti-amphetamine antibody at both the higher and lower concentration ranges investigated. In samples 1, 9, 15 and 17, MDMA present in each sample has successfully competed with the amphetamine-HRP for the active sites of the anti-amphetamine antibody to produce a positive result using the LOC device. In sample 18, amphetamine present in the sample successfully competed with the amphetamine-HRP for the active sites of the anti-amphetamine antibody to produce a positive result using the LOC device. Figure 5.3 shows the data obtained from the three replicates for the samples 1, 9, 15 and 17 (MDMA) and sample 18 (amphetamine). There was some variation observed between replicates of sample (that were used for the detection of the same MDMA concentration) did show some variation within the data, but this was not statistically significant ($P > 0.05$) (Figure 5.3.). It is also worth noting that the caffeine present in sample 18 did not show any cross reactivity with the anti-amphetamine antibody.

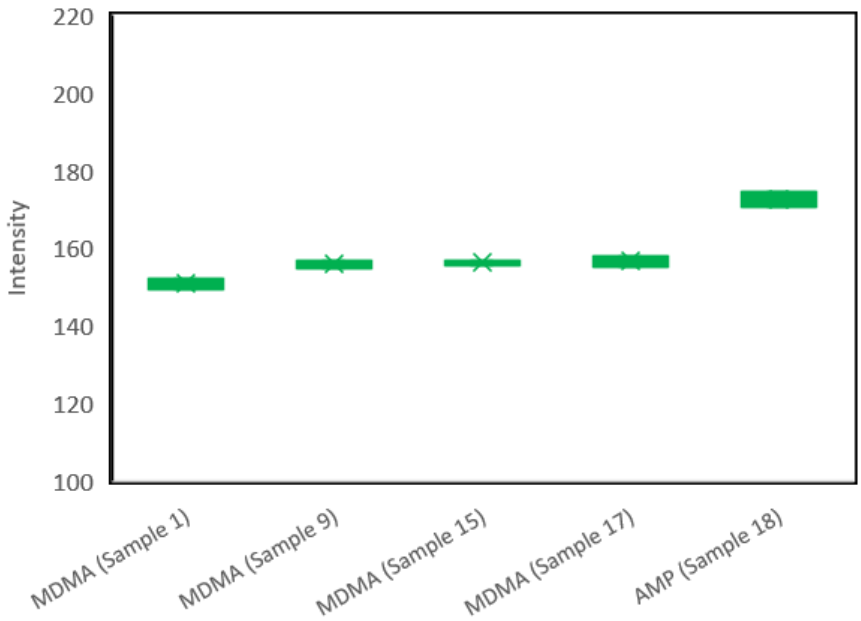


Figure 5.3. *Signal intensity recorded between replicates of the LOC devices for samples 1, 9, 15 and 17 (MDMA) and sample 18 (amphetamine*), *indicating a positive result when using the optimised LOC device (n = 3)*

It is also worth noting that in sample 1, contained a mixture of both MDMA and 4-CMC, and sample 18 contained amphetamine and the cutting agent caffeine. Whereas the samples 9, 15 and 17 only contained MDMA. As there was no significant statistical difference ($P > 0.05$) between the intensity recorded in these samples using regression analysis this indicates that there was no cross reactivity or interference observed when using the LOC device to detect MDMA (in sample 1) from 4-CMC. In addition to this no cross reactivity was observed with the other antibodies located within the black central detection zone (anti-methcathinone and anti-HRP) antibody from the presence of caffeine within the sample. This can also be observed in Figure 5.1, as there was no cross reactivity with the anti-methcathinone antibody, as this is the greatest intensity from TMB (bluest colour that can be observed).

5.3.1.3. Synthetic cathinones

Seven out of the 20 seized samples for blind testing contained synthetic cathinones, which were all expected to cross-react with the anti-methcathinone antibody incorporated within the LOC device. Six out of the seven samples for the synthetic cathinones were identified as positive when using the LOC device, with sample 1 showing a false-negative result for the 4-CMC for one of the three replicates. For this false-negative result, the 4-CMC has not successfully competed with the labelled cathinone-HRP for the active sites of the anti-methcathinone antibody, and subsequently TMB produced a greater response for this competitive immunoassay. The replicate that produced the false-negative result indicates that there is no presence of 4-CMC present as the response from TMB is the bluest colour that can be observed (producing the greatest response) (Appendix 5.3). It is worth noting that this could not be due to experimental errors (as each investigation is undertaken with a systematic check list of steps for the competitive immunoassay) or a heterogenous sample, as each sample is vortexed for each replicate to ensure a homogeneity.

As 4-CMC (Figure 3.1.m) is a synthetic cathinone structurally similar to 4-MMC (Figure 3.1. c) [150, 152, 153, 229, 230], 4-CMC was expected to cross-react with the anti-methcathinone antibody. As in terms of the competitive immunoassay, 4-CMC has a degree of binding affinity for the active sites of the anti-methcathinone antibody [176]. This false-negative result of one of the replicates demonstrates a potential limitation of incorporating an immunoassay for the detection of drugs of abuse and NPS. There are numerous explanations for false-negative results when using an immunoassay as the method of detection (as well as false-positive results should they occur). However false negative results are usually due to low concentration levels of the drug present, or due to

adulteration of a biological sample to avoid a positive result [11, 15, 18-20]. In terms of the false-negative result of one of the samples tested in triplicate was of 4-CMC at a concentration of 10.8 mg mL^{-1}. The concentration was significantly above the LOD reported in Chapter 3, in 2.51 ng mL^{-1} aqueous solution [61]. Therefore, as this drug was at a high concentration (10.8 mg mL^{-1}) then the false-negative could not be due to a low concentration. Especially as this LOC device successfully detecting two of the three replicates (for sample 1), in addition to sample 4. However, it is worth noting the LOD for 4-CMC using this LOC device has not been investigated.

In sample 4, the 4-CMC present in each sample successfully competed with the cathinone-HRP for the active sites of the anti-methcathinone antibody to produce a positive result using the LOC device. In samples 2 and 3, the 4-ethylpentylone present in each sample successfully competed with the cathinone-HRP for the active sites of the anti-methcathinone antibody to produce a positive result using the LOC device. In samples 10 and 15, the 4-MMC present in each sample successfully competed with the cathinone-HRP for the active sites of the anti-methcathinone antibody to produce a positive result using the LOC device. In sample 20, the MDPHP present in the sample has successfully competed with the cathinone-HRP for the active sites of the anti-methcathinone antibody to produce a positive result using the LOC device. Figure 5.4 shows the data obtained from the three replicates for the samples 1 and 4 (4-CMC), samples 2 and 3 (NEP), samples 10 and 16 (4-MMC) and 17 (MDMA) and sample 20 (MDPHP). The standard deviation between replicates of the same concentration of 4-CMC (Sample 1) did show some variation within the data due to the false-negative result.

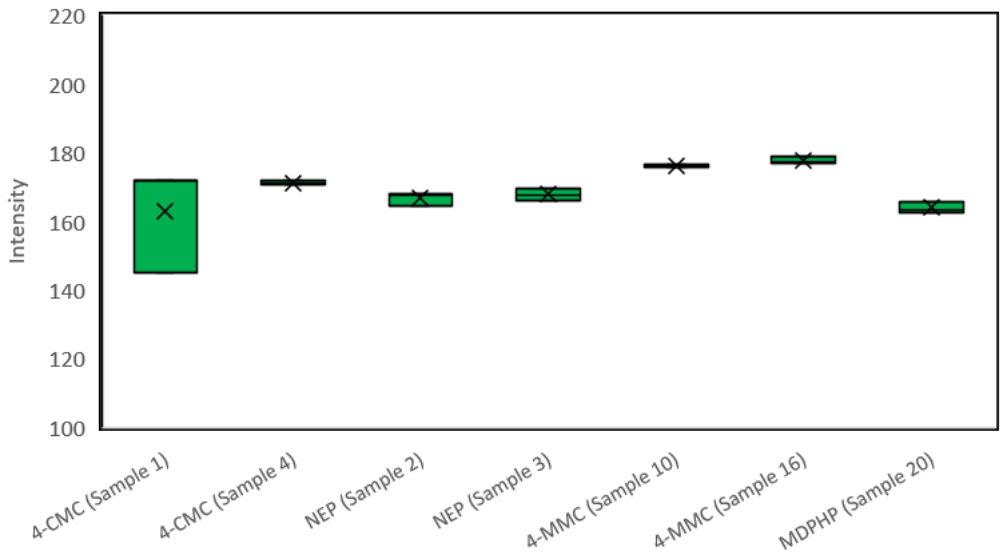


Figure 5.4. *Signal intensity recorded between replicates of the LOC devices for 4-CMC (samples 1 and 4), NEP (samples 2 and 3), 4-MMC (samples 10 and 16) and MDPHP (sample 20). The 4-CMC (sample 1) indicates a negative response, whereas 4-CMC (samples 4), NEP (samples 2 and 3), 4-MMC (samples 10 and 16) and MDPHP (sample 20) all indicate positive results using the LOC device (n = 3)*

5.3.1.4. Other samples

Nine out of the 20 seized drug for blind testing contained no synthetic cathinones or amphetamine that this LOC device has been developed to detect (samples 5-8, 11-14, and 19) (Table 5.1.). The compounds present were a range of other drugs of abuse, NPS, cutting agents, and also prescribed medication (Figure 5.2.). None of these compounds demonstrated any cross reactivity with the anti-methcathinone antibody, the anti-amphetamine antibody or the anti-HRP antibody.

5.3.2. End-user Testing

For the LOC device to be fit for purpose in clinical and forensic settings, it is essential to investigate end-user testing of the LOC devices by the non-scientifically trained

individuals, to investigate if these are fit for use in the field. Therefore, it was implicit that investigations assess whether all the non-scientifically trained individuals were able to successfully identify all three samples (A, B and C) using a set of instructions, investigate whether there was any variation between the LOC devices for each of the three samples (A, B, C) when tested by the non-scientifically trained individuals. As well as evaluating the anonymous feedback provided by the non-scientifically trained individuals, with both numerical results provided using a scale from not at all to extremely, and explanatory written feedback to provide more in-depth information to evaluate whether the LOC device is fit for purpose.

5.3.2.1. Identification of Samples A, B and C

Sample A was amphetamine at a concentration of 5 mg mL^{-1}. When amphetamine is present, the TMB will subsequently produce a lower response as the unlabelled target antigen (amphetamine) competes with the labelled amphetamine-HRP for the active sites of the anti-amphetamine antibody. Therefore, the higher the average light intensity values, the lower the response of the labelled antigen (amphetamine-HRP) and the whiter the colour.

Sample B was distilled water. When no 4-MMC or amphetamine is present, the TMB will subsequently produce a higher response as there is no unlabelled target antigen present (4-MMC or amphetamine) and is therefore outcompeted by the labelled cathinone-HRP for the active sites of the anti-methcathinone and anti-amphetamine antibodies. Therefore, the lower the average light intensity values, the more intense the reaction colour indicating a greater presence of HRP (labelled antigen). Therefore, as there is no target present then the response from TMB is the most intense colour (blue) that can be observed by eye.

Sample C was the synthetic cathinone, 4-MMC at a concentration of 5 mg mL^{-1}. When 4-MMC is present, the TMB will subsequently produce a lower response as the unlabelled target antigen (4-MMC) competes with the labelled cathinone-HRP for the active sites of the anti-methcathinone antibody. Therefore, the higher the average light intensity values, the lower the response of the labelled antigen and the whiter the colour.

Table 5.2. *Anonymous feedback evaluating the LOC device was evaluated by non-scientifically trained individuals after testing three different compounds*

End-user Testing					
Sample	**Tester 1**	**Tester 2**	**Tester 3**	**Tester 4**	**Tester 5**
A	amphetamine	amphetamine	amphetamine	amphetamine	amphetamine
B	negative	Water	blank – water – no drug	no drug present – water	water
C	synthetic cathinone	mephedrone	synthetic cathinone	synthetic cathinone	synthetic cathinone

Non-scientifically trained individuals tested three different suspected samples, labelled A, B, and C using a set of instructions (Appendix 5.1). The instructions sheet included a clear protocol of how to test the three samples and how to correctly evaluate the results. Their feedback was also submitted onto an online survey system to obtain feedback when using the LOC device (to investigate whether the LOC device was easy to use) and images of the replicates were taken to compare reproducibility between the devices. All five non-scientifically trained individuals successfully identified the 3 samples A to C (Table 5.2.). Figure 5.5 demonstrates an example of the three replicates collected from one of the non-scientifically trained individuals for sample C, correctly showing a positive result for 4-MMC.

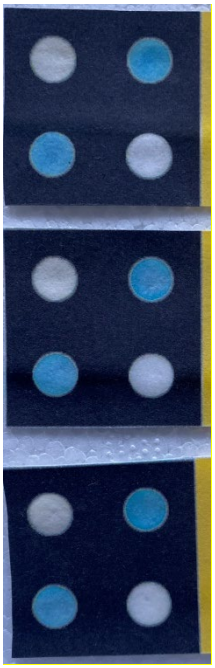


Figure 5.5. *Images collected from one the non-scientifically trained individuals during end-user testing Sample C (4-MMC)*

5.3.2.2. Reliability of the data from non-scientifically trained individuals

For the LOC device to be fit for purpose in clinical and forensic settings, it is essential to investigate the variation between the LOC devices tested by the non-scientifically trained individuals. Therefore, it was implicit that investigations were undertaken to assess the level of variation between the LOC devices tested for each of the three samples (A, B, C) by each of the non-scientifically trained individuals to ensure that when the LOC device is fit for purpose.

The non-scientifically trained individuals tested three different suspected samples, labelled A, B, and C using a set of instructions (Appendix 5.1.). The five non-scientifically trained individuals all successfully identified the 3 samples A to C (Appendix 5.2.) using the optimised LOC device demonstrating that this LOC device is fit for purpose by non-scientifically trained individuals. The variability between the LOC devices for the testing of

samples A (amphetamine) is shown in Figure 5.6. Tester 2 had the largest SD between the intensity values for Sample A (at 3.41) and therefore the largest variation between the replicates (Figure 5.6.). Tester 1 demonstrated the lowest SD at 0.52, and subsequently the smallest variation of all the non-scientifically trained individuals testing the three samples (A, B and C) using the LOC devices (Figure 5.6.).

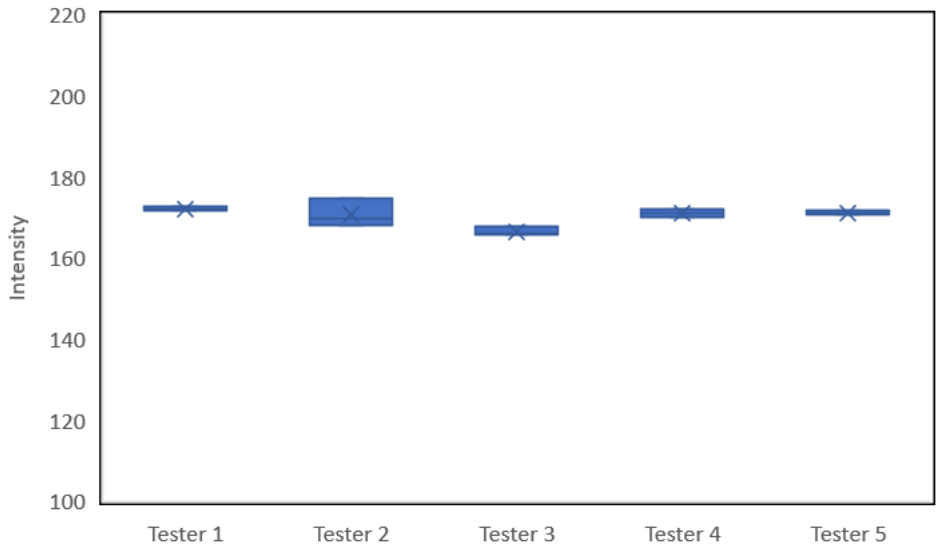


Figure 5.6. *Signal intensity recorded between the replicates of the LOC devices tested by the non-scientifically trained individuals (Testers 1-5) during end-user testing for sample (A) (amphetamine) (n = 3)*

5.3.2.3. Anonymous End-user Testing Feedback Questionnaire

For this optimised LOC device to be fit for purpose to be used by non-scientifically trained individuals in clinical and forensic settings, it is essential to evaluate the use of the LOC devices using a set of instructions to test real-world samples. Thus, determining whether the non-scientifically trained individuals can use the LOC device to correctly identify the three samples, but also to provide invaluable anonymous end-user testing feedback.

End-user testing was undertaken by five non-scientifically trained individuals who tested three different suspected samples, labelled A, B, and C using a set of instructions (Appendix 5.1) and submitted their feedback Anonymous End-user Testing Feedback Questionnaire (Appendix 5.2). The individuals provided feedback on questions using both a scale of 1-10, not at all to extremely, in addition to providing written feedback. The numerical results provided using a numerical scale are shown in Figure 5.7 and the written feedback submitted is shown in Appendix 5.2. The five non-scientifically trained individuals successfully identified the 3 samples A to C (Table 5.2.). Individual graphical representations for each of the numerical data obtained for Questions 2 to 12 obtained via the anonymous online survey system are also shown in Appendix 5.5.

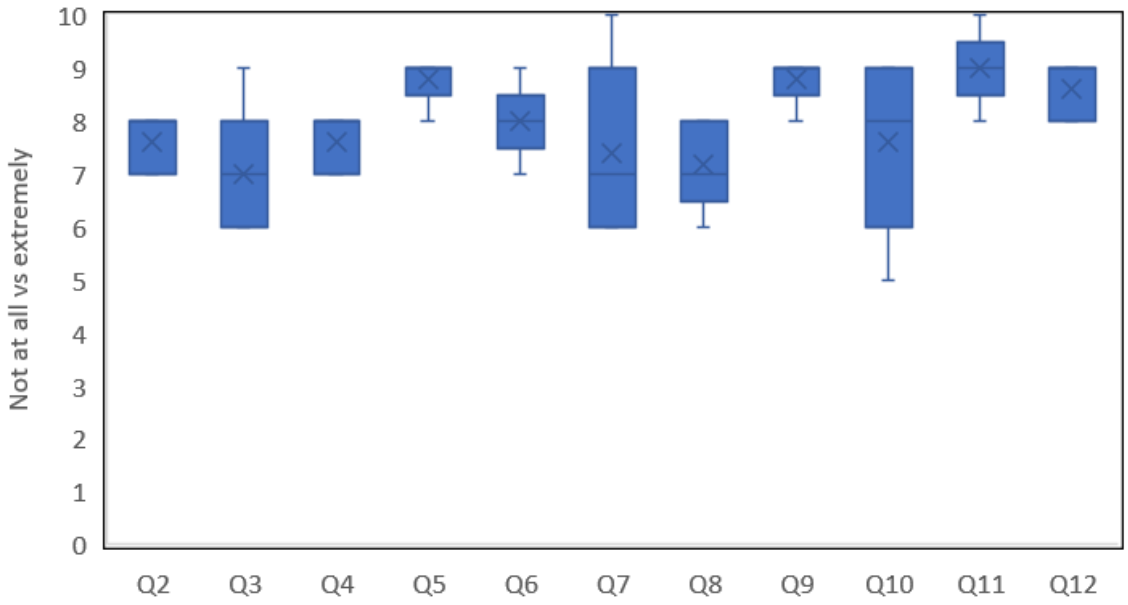


Figure 5.7. *Represents the numerical data obtained for Questions 2 to 12 obtained via the anonymous online survey system (Q2) Were the instructions easy to follow? (Q3) Was the 'background' section useful? (Q4) Was the 'brief overview' useful? (Q5) Was the 'Figure 1 - 'Origami' LOC design' useful? (Q6) Was 'Figure 2 - Black central detection well in the centre of the LOC design' useful? (Q7) Was the 'Equipment and reagents' section useful?*

(Q8) Was the 'Protocol' section useful? (Q9) Was 'Figure 3 - Positive and Negative controls' useful? (Q10) Was 'Figure 4 – Flow chart of protocol' easy to follow? (Q11) Was the 'Figure 5 – Identification' useful? (Q12) How confident did you feel identifying sample A, B and C?

The numerical data obtained (Figure 5.7) indicates that the LOC device was overall easy to use and was effective in identifying the three samples using the set of instructions provided. Representing all of the questions in the Anonymous End-user Testing Feedback Questionnaire received a positive response in relation to practical elements that the non-scientifically trained individuals evaluated. The data obtained from Question 9 (was 'Figure 3 - Positive and Negative controls' useful?), Question 11 (was the 'Figure 5 – Identification' useful?), and Question 12 (how confident did you feel identifying sample A, B and C?) reported the highest median value of 9/10, representing the highest overall values on Figure 5.7. Demonstrating that the non-scientifically trained individuals testing samples A to C reported that the 'Positive and Negative controls' and 'Figure 5 – Identification' were reported as the most useful.

Feedback obtained from the non-scientifically trained individuals for Questions 4, 6, 9, 10 and 11 (Appendix 5.2) highlight the requirement of a good visual guide to help identification, which are commonly used in literature for the detection of drugs of abuse and NPS using colourimetric detection [75, 78, 80, 81, 86]. This data indicates that this is a crucial element to the development of an LOC device for the detection of NPS and drugs of abuse.

Question 12 (how confident did you feel identifying sample A, B and C?), demonstrates that the non-scientifically trained individuals testing samples A to C feedback was

extremely positive in regard to identification. This is a crucial element to designing a LOC device for the detection of drugs of abuse and NPS by non-scientifically trained individuals in clinical and forensic settings.

Over 75% of the written feedback for the 11 questions stated that elements were helpful, especially in reference to: the non-scientifically trained individuals feeling confident in identifying sample A, B and C (Question 12). As well as the images included in the instructions (Questions 5, 6, 9, 11) and the inclusion of the equipment and reagents section (Question 7) in the instructions being helpful. For example, the written feedback received for question 9 (Appendix 5.2.) was positive with Tester 1 stating 'Yes it helped to explain what the results meant' and Tester 3 stating 'Definitely helped!'. Providing evidence that these sections were beneficial to the instructions for the non-scientifically trained individuals.

Question 7 (was the 'Equipment and reagents' section useful?), demonstrates that the non-scientifically trained individuals reported 6/10 (40%), 7/10 (20%) or 8/10 (20%) or 10/10 (20%). Varying from 6/10 to 10/10, representing a mixed consensus on whether the section on equipment and reagents was useful. Indicating that this section could potentially be removed or placed at the end of the instructions in an appendix for further information if required. Question 6 and Question 9 had the highest degree of variation from the data reported. This variation was directly reflected in the written feedback, with Tester 1 reporting 10/10 and 'perfect', to Testers 3 and 5 stating that it could be condensed or removed (Appendix 5.2.).

Some suggestions and improvements were reported in the feedback in terms of clarity of scientific terminology, condensing certain sections, and ensuring that the LOC device is suitable for all potential users. For example, the written feedback for Question 2,

Question 4, Question 6 and Question 8 all highlighted that these sections contained scientific terminology, which would aid in improving the ease of use. The feedback obtained from Question 3 (was the 'background' section useful) indicated that this section could have been more in-depth and offer clarity (Appendix 5.2.). This would improve this section to add more context to when using this LOC device for testing is applicable. Question 10 demonstrates that the 'Figure 4 – Flow chart of protocol' would require some improvements to be easy to follow as there was a mixed response in the feedback obtained. There were suggestions such as ‘less words and more pictures’ and also aid from being more concise. Question 5 (was the 'Figure 1 - ‘Origami’ LOC design' useful) highlighted that this was helpful and visually demonstrated how to orientate the LOC device, but an important suggestion of ensuring that the colours on the LOC device as inclusive to all users, such as colour blindness. This is valuable as it demonstrates how the instructions must be inclusive to ensure that it is easy to use.

Overall, the anonymised written feedback from the non-scientifically trained individuals on using the LOC device and the instructions provided to them was constructive and positive in their critique and evaluation. In terms of scientific writing, the instructions could have been less technical in the breadth of the scientific writing style, as well as being more descriptive in terms of the all the equipment but whilst reducing the word count and being as concise as possible. This could enable the LOC device to be more inclusive to all the non-scientifically trained individuals in the future. In addition to this, a visual guide with some descriptions would have aided their understanding, especially to some of the feedback obtained during the end-user testing. Overall, the written feedback offered invaluable insight into potential improvements and issues when the LOC devices were used by non-scientifically trained individuals.

5.3.3. Adaptability of the LOC device: the detection of MDPHP and NEP

Future proofing this LOC device by demonstrating its ability to adapt to be able to detect newer synthetic cathinones that become available to the recreational drugs market. that this LOC device can detect MDPHP and NEP in both aqueous (dissolved powder) samples and non-invasively collected urine samples to clinically relevant levels. Therefore, enhancing the potential for POC testing in both clinical and forensic environments, as both MDPHP and NEP were prepared from spiked urine and dissolved powder in aqueous solution to represent both a non-invasively collected clinical specimen and bulk forensic sample.

5.3.3.1. Evaluation of matrix effects (aqueous and urine)

Varying concentrations of MDPHP and NEP (0.15625-5 ng mL^{-1}) in both urine and aqueous solutions were investigated. Both MDPHP and NEP in aqueous solution and in urine samples demonstrated were effectively detected (Figure 5.8.). However, this LOC device showed a higher degree of selectivity and specificity within spiked urine samples, than compared to aqueous samples for both MDPHP and NEP.

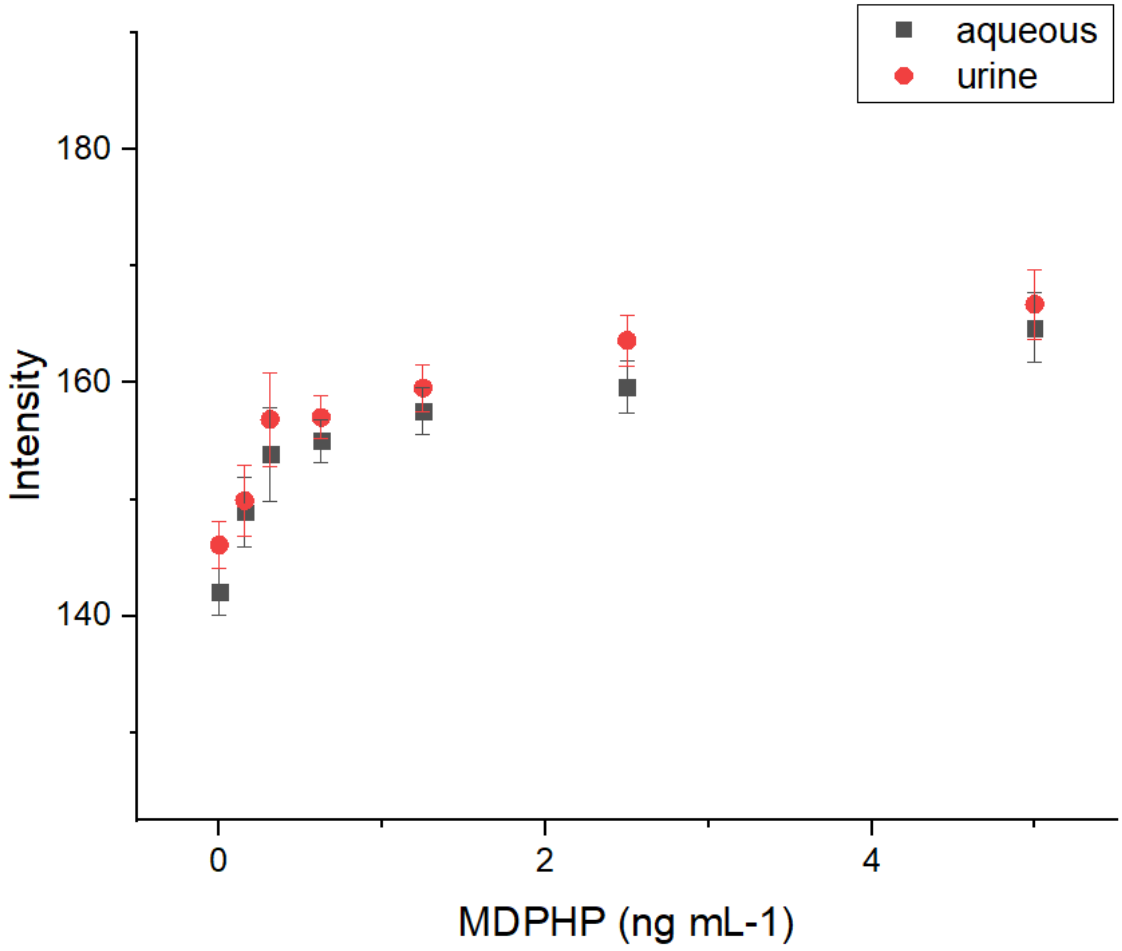


Figure 5.8. *Detection of MDPHP in both spiked urine and aqueous samples (n = 6)*

Analysis of the data sets showed a linear range of 0.15625-5 ng mL^{-1} for the aqueous and urine samples (Figure 5.8). LODs were determined for both MDPHP and NEP samples. LOD for MDPHP were reported at 1.84 ng mL^{-1} (urine) and 1.23 ng mL^{-1} (aqueous), respectively to clinically relevant concentrations of MDPHP reported to nanogram levels [210, 212, 214, 219, 230, 233]. LODs for NEP were reported at of 2.32 ng mL^{-1} (urine) and 2.11 ng mL^{-1} (aqueous) clinically relevant concentrations of NEP in the literature [220, 222, 224-228].

The anti-methcathinone antibody within this LOC device showed a higher degree of selectivity and specificity for 4-MMC, than compared to both MDPHP and NEP in urine

samples (Figure 5.9.). This was also observed for 4-MMC, when compared to both MDPHP and NEP in aqueous samples. With the LOC device showing a higher degree of selectivity and specificity for 4-MMC.

In addition, the cross reactivity (CR) values were calculated for MDPHP and NEP using the following equation [176, 234]:

$CR = IC_{50}$ (target analyte)/ IC_{50} (tested cross-reacting compound) x 100

With cross-reactivity percentage for MDPHP and NEP at 98.7% and 95.1%, respectively. With Figure 5.9 demonstrating this that the LOC device has a higher degree of selectivity and specificity for 4-MMC, when compared to MDPHP and NEP.

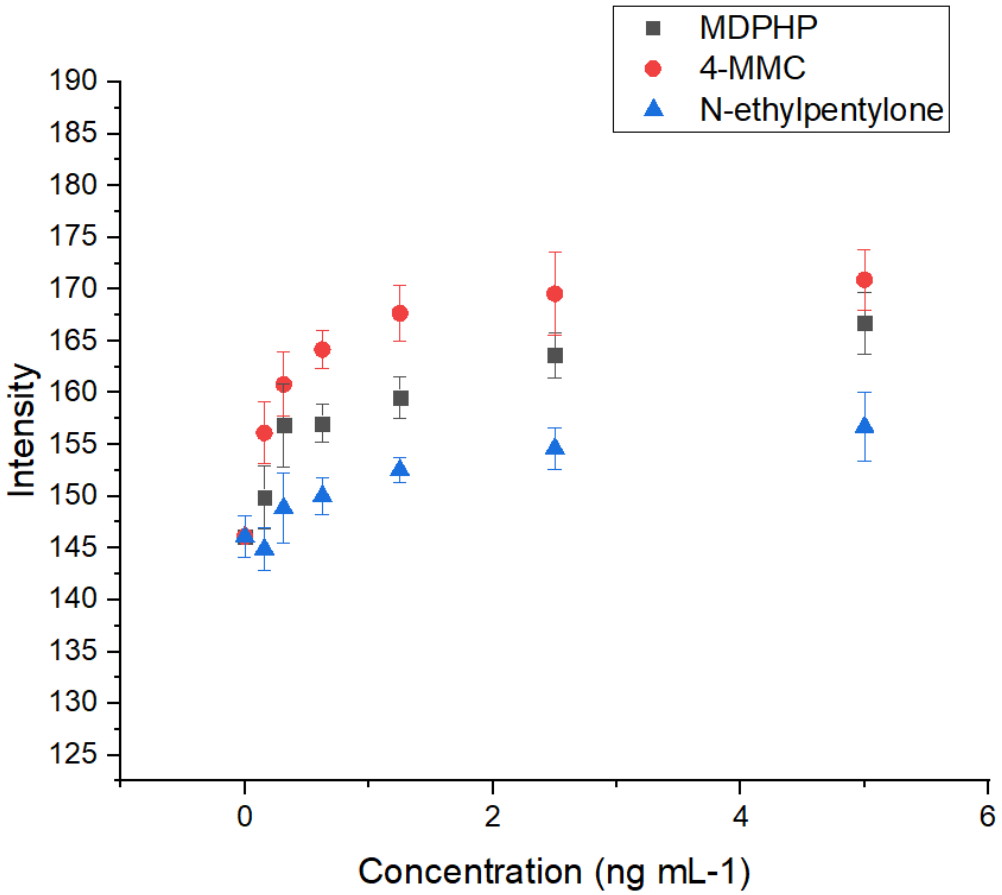


Figure 5.9. *Comparison of 4-MMC, MDPHP and NEP in urine solution at concentrations from 0.00156-5 ng mL^{-1} (n = 6)*

6.3.5. Inter-device variability

A fundamental component of developing a POC testing device is detects to a high level of sensitivity and sensitivity, but that the LOC device also provides results that have a high level of reproducibility and therefore the reliability of the LOC device. Therefore, the variation between five individual optimised LOC devices was determined using Levene's. Inter-device variation was investigated to observe differences between using the optimised LOC device on different days and thus assessing the reliability of the manufacturing of the LOC devices. This showed that there was no significant difference

between devices ($p > 0.05$). Intra-variability between devices was also investigated demonstrated equal variances ($p > 0.05$) supporting intra-device variability was observed. No significant difference was observed between the individual optimised LOC devices, thus demonstrating that the LOC device could successfully detect MDPHP across multiple LOC devices indicating that this LOC device offers a reliable detection method for the detection of MDPHP.

5.4. Conclusion

The data in this chapter demonstrates that this paper-based LOC device is fit for purpose in terms of usability with the analysis of 20 seized drug samples (provided by Greater Manchester Police via MANDRAKE) using the optimised LOC device with 95% specificity and 100% sensitivity. All non-scientifically trained individuals successfully evaluated the LOC device, with 100% specificity and 100% sensitivity. This chapter also demonstrates that this LOC device can detection new emerging NPS to the recreational drugs market, MDPHP and NEP to clinically relevant levels.

The inclusion of using real-world seized samples was fundamental to investigating the applicability of the LOC device being used as an on-site detection method. Five out of the twenty seized drug samples contained either MDMA or amphetamine. The LOC device successfully identified all of these five samples in triplicate. This demonstrates that both MDMA and amphetamine successfully competed with the amphetamine-HRP for the active sites of the anti-amphetamine antibody present within these seized drug samples. The anti-methcathinone antibody incorporated in this LOC device demonstrates its adaptability by offering the potential to cross-react with structurally similar compounds, including synthetic cathinones. Six out of the twenty seized drug samples contained synthetic cathinones including; 4-CMC, NEP, 4-MMC, and MDPHP. The LOC device successfully identified 5 out of 6 of the samples.

Overall, this LOC device demonstrated 95% specificity and 100% sensitivity for the blind testing of these samples. One-false-negative result was obtained as the LOC device did not successfully identify 4-CMC in just one of the three replicates. It is also important to note that that there were no false positive results, demonstrating no cross reactivity with either the anti-methcathinone or anti-amphetamine antibodies for samples.

The LOC device was also evaluated by five non-scientifically trained individuals who all correctly identified three samples with 100% specificity and 100% sensitivity. This was a novel approach as we were unable to identify any end-user testing previously reported in any publications. This is an important step to demonstrate that the device could be used effectively in the field by non-scientific experts and highlights its potential use in POC testing.

This chapter importantly concludes with successfully detected new emerging NPS, MDPHP and NEP clinically relevant levels. LOD for MDPHP were reported at 1.84 ng mL^{-1} (urine) and 1.23 ng mL^{-1} (aqueous), for NEP were reported at of 2.32 ng mL^{-1} (urine) and 2.11 ng mL^{-1} (aqueous). This demonstrates the adaptability of this LOC device for the continuously increasing number of available NPS and therefore future proofs the potential of this LOC device to keep up with the current trends of the recreational drugs market.

Chapter 6: Conclusions

Motivation for the Thesis

There is a significant global burden placed on public services, including A&E departments and prisons, as a direct result of NPS and drugs of abuse ab(use). The research undertaken investigates an easy-to-use, portable and cost-effective (cost of less than 50p per device) paper-based LOC device for semi-quantitative detection of 4-MMC, its metabolite 4-ME, and three commonly encountered drugs of abuse, amphetamine, methamphetamine and MDMA. The 'origami' design of the LOC device was developed to incorporate reagents of a competitive immunoassay in different locations, but to offer stability so that it can be used when required. This is possible by undertaking the sequential steps that are incorporated in the design for on-site analysis. Enabling them to be utilised by non-scientifically trained individuals in both clinical and forensic settings.

The overall aim of this thesis was to address the gaps in knowledge discussed in chapter 1 so this general discussion will relate directly to the objectives stated in this thesis (chapter 1.5) and will focus on the key findings and impact of these. Figure 6.1. demonstrates how this was achieved through objectives stated in chapter 1.

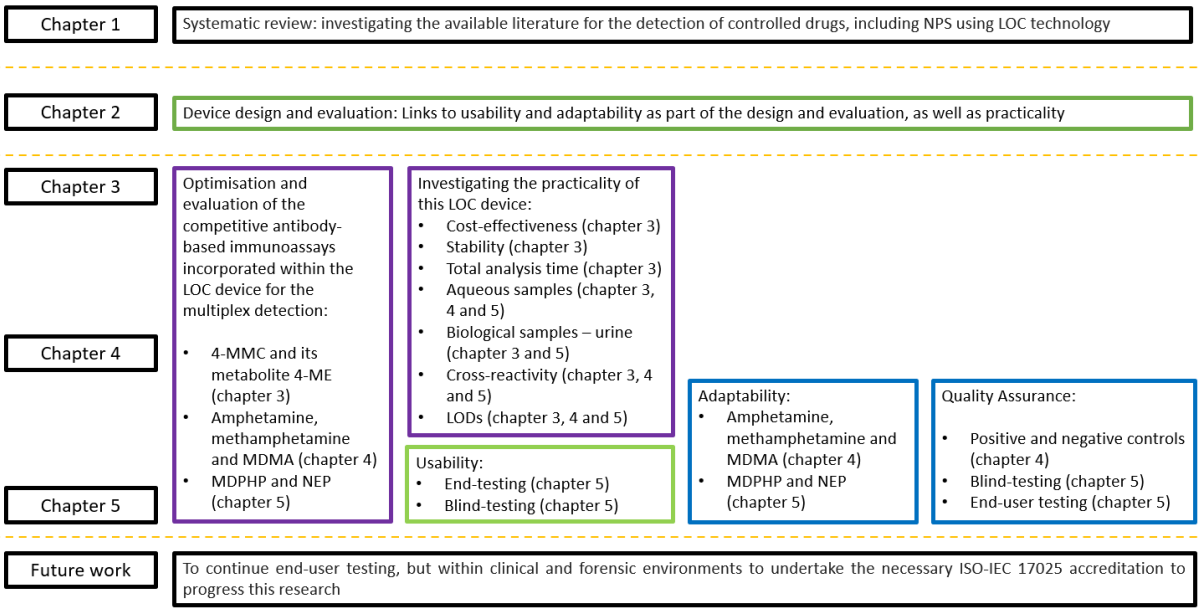


Figure 6.1. *Flow chart to demonstrate how this thesis addresses the aims and objectives stated in chapter 1*

6.1. Optimisation and evaluation of the competitive immunoassay

The optimisation and evaluation of the competitive antibody-based immunoassays incorporated within this LOC device (Figure 6.1.) enabled the semi-quantitative detection of 4-MMC, 4-ME, amphetamine, methamphetamine and MDMA. This was done by investigating a range of concentrations for both the anti-methcathinone antibody and cathinone-HRP, and also the anti-amphetamine antibody and amphetamine-HRP. The optimum concentration for anti-methcathinone antibody and cathinone-HRP was 0.000511 µg mL-^{1} and 0.2 ng mL^{-1}, respectively, and the optimum concentration for anti-amphetamine antibody and amphetamine-HRP at 1.75 ng mL-^{1} and 200 ng mL^{-1}, respectively. Through the use of chitosan activation and glutaraldehyde cross-linking, the antibodies incorporated within this LOC device have been effectively covalently immobilised to the well of this LOC device and can effectively detect the 4-MMC and 4-

ME (using the anti-methcathinone antibody), and amphetamine, methamphetamine and MDMA (using the anti-amphetamine antibody) [61]. The anti-methcathinone antibody could also successfully detect MDPHP and NEP (chapter 5) in addition to 4-MMC and 4-ME (chapter 3). This is also the first LOC device that has successfully detected synthetic cathinones, and also offers adaptability to be able to detect newer synthetic cathinones available to the recreational drugs market. Therefore, demonstrating this LOC device is fit for purpose.

6.2. Practicality

There are advantages and disadvantages to each biological matrix that can be used to detect drugs of abuse and NPS. This LOC device was designed and developed for testing of both biological sample (urine) and an aqueous solution (representing bulk samples) demonstrating the potential of this LOC device for on-site testing within clinical or forensic in both in aqueous samples and also non-invasive biological samples (urine), highlighting its potential use in POC testing.

This LOC device offers semi-quantitative detection, which can provide additional information compared to a simple presence/absence result, but without the increased cost usually associated with quantitative methods [1, 61]. The cost of using this LOC device is around 50p (Appendix 6.1. for cost breakdown), which demonstrates the potential impact of using this LOC device in low-income countries, giving it a wide global applicability, to support efforts to reduce NPS and recreational drug use.

By offering semi-quantitative detection, this LOC device has the ability to detect 4-MMC, 4-ME, amphetamine, methamphetamine and MDMA to sub-nanogram levels (ranging from 2.51-4.84 ng mL^{-1}). These LODs are comparable to published literature of these compounds using traditional laboratory methods discussed in this thesis. Thus, ensuring

that the developed LOC device is fit for purpose on a global scale and is applicable to real-world samples and can be easily used in the field to detect drugs of abuse and NPS to clinical and forensically relevant levels [1].

Due to the sequential steps of the competitive immunoassay that take place when using this LOC device, it can rapidly detect 4-MMC, 4-ME, amphetamine, methamphetamine and MDMA within 3 minutes, which is considerably quicker than conventional laboratory-based detection methods, such as GC-MS. This highlights a significant advantage of using this LOC device incorporating a competitive immunoassay as a rapid semi-quantitative detection method.

Cross reactivity of commonly encountered 'cutting agents', interferants and adulterants were investigated and shown to have no effect on the detection capabilities of this LOC device. Therefore, highlighting that this LOC device has the potential to be used for POC or on-site testing within clinical or forensic settings in both in aqueous samples that may have been 'cut' with diluents or administered with other drugs of abuse and non-invasive biological samples (urine).

Finally, the stability and reproducibility measurements in chapter 3 showed no significant difference in signal intensity over an 8-week period, and also no significant difference within or between devices. Therefore, demonstrating that this LOC device has been developed with the aim to work towards ISO/IEC 17025 accreditation to be fit for purpose.

6.3. Usability

Twenty seized drug samples (provided by Greater Manchester Police via MANDRAKE) were tested using the optimised LOC device to determine the reliability, specificity, and reproducibility of the LOC device, with 95% specificity and 100% sensitivity. This LOC

device was also evaluated through end-user testing (by non-scientifically trained individuals) with 100% specificity and 100% sensitivity. With all non-scientifically trained individuals able to correctly identify the three samples provided, highlighting that this LOC device does not need to be used by scientifically trained individuals [1].

6.4. Quality Assurance

While controls are routinely applied in conventional laboratory settings, they are not always integrated onto LOC devices, with only 7% of the literature stating the inclusion of controls [1]. The data in chapter 4, demonstrates that positive and negative controls were successfully integrated within this LOC device, increasing the reliability and integrity of each resulting test. This quality assurance is important for future validation of the LOC devices and acceptance within local criminal justice systems, as has been significantly overlooked in published literature to date on LOC technology. As well as offering clarity when this LOC device is being used by non-scientific trained individuals (chapter 5).

To date, there have been no publications using end-user testing for the detection of NPS or drugs of abuse using LOC technology previously reported. The data obtain in chapter 5 demonstrates that the end-user testing provided invaluable data on the ease of use, effectiveness at detection and the reproducibility of this LOC device when used by non-scientifically trained individuals. The majority of the anonymous feedback from using the LOC devices was positive (including ease of use and confidence in correctly identifying sample A, B and C) and constructive feedback has provided invaluable information regarding improvements and can aid any future work that could be undertaken using this LOC device.

6.5. Future work

Future work would include developing this LOC device in two areas, which would include improving usability of this LOC device and validation studies. In terms of improving the usability for non-scientifically trained individuals this would involve simplifying the terminology and instructions to make them easier to understand. In addition to this, altering the colours of the tab of this LOC device to ensure that it is as inclusive as possible. For example, selecting more suitable colours to ensure that the LOC device is more user friendly for colourblind individuals. Future work with validation studies would include working in external settings. For example, validation studies undertaken in an A&E department, GP surgery, and/or in a prison. This would provide data on real-world samples and continue working towards developing a commercially available LOC device with ISO/IEC 17025 accreditation. And finally, conducting a larger study of real-world samples from drug seizures to ensure that this LOC device is fit for purpose in terms of sensitivity and specificity. Thus, increasing the reliability of the results produced using the LOC device.

List of references

1. L. McNeill, D. Megson, P. E. Linton, J. Norrey, L. Bradley, O. B. Sutcliffe and K. J. Shaw, *Forensic Chemistry*, 2021, **26**, 100370.
2. W. D. Report, *World Drugs Report, Booklet 1: Executive Summary; Conclusions and Policy Implications, United Nations Office on Drugs and Crime,*, Vienna, 2019.
3. W. Zhou, E. Nazdrajić and J. Pawliszyn, *Anal Chem*, 2023, **95**, 6367-6373.
4. P.-C. Chen, W.-Z. Zhang, W.-R. Chen, Y.-C. Jair, Y.-H. Wu, Y.-H. Liu, P.-Z. Chen, L.-Y. Chen and P.-S. Chen, *Sensors and Actuators: B. Chemical*, 2022, **350**.
5. M. Paul, R. Tannenberg, G. Tscheuschner, M. Ponader and M. G. Weller, *Biosensors (Basel)*, 2021, **11**.
6. L. M. Dignan, M. S. Woolf, J. A. Ross, C. Baehr, C. P. Holstege, M. Pravetoni and J. P. Landers, *Anal Chem*, 2021, **93**, 16213-16221.
7. L. Zhang, X. Li, Y. Li and H. Z. Yu, *Analyst*, 2021, **146**, 538-546.
8. H. Martínez-Pérez-Cejuela, P. García-Atienza, E. Simó-Alfonso, J. Herrero-Martínez and A. S, *Mikrochim Acta*, 2023, **190**, 271.
9. R. Alder, J. Hong, E. Chow, J. Fang, F. Isa, B. Ashford, C. Comte, A. Bendavid, L. Xiao, K. K. Ostrikov, S. Fu and A. B. Murphy, *Sensors (Basel)*, 2021, **21**.
10. L. Harper, J. Powell and E. M. Pijl, *Journal*, 2017, **14**, 1-13.
11. SWGDRUG, *SCIENTIFIC WORKING GROUP FOR THE ANALYSIS OF SEIZED DRUGS (SWGDRUG) RECOMMENDATIONS* 2019.
12. J. P. Smith, O. B. Sutcliffe and C. E. Banks, *Journal*, 2015.
13. A. J. Dickson, S. P. Vorce, B. Levine and M. R. Past, *J Anal Toxicol*, 2010, **34**, 162-168.
14. A. M. Leffler, P. B. Smith, A. de Armas and F. L. Dorman, *Forensic Sci Int*, 2014, **234**, 50-56.
15. K. E. Moeller, K. C. Lee and J. C. Kissack, *Mayo Clinic proceedings*, 2008, **83**, 66-76.
16. D. A. Armbruster, R. H. Schwarzhoff, E. C. Hubster and M. K. Liserio, *Clin Chem*, 1993, **39**, 2137-2146.
17. H. Torrance and G. Cooper, *Forensic Sci Int*, 2010, **202**, e62-63.
18. D. J. Dietzen, K. Ecos, D. Friedman and S. Beason, *Journal*, 2001, **25(3)**, 174-178
19. S. D. Ferrara, L. Tedeschi, G. Frison, G. Brusini, F. Castagna, B. Bernardelli and D. Soregaroli, *J Anal Toxicol*, 1994, **18**, 278-291.
20. J. J. Fenton, *Journal*, 2001.
21. W. W. Lee, V. A. Silverson, L. E. Jones, Y. C. Ho, N. C. Fletcher, M. McNaul, K. L. Peters, S. J. Speers and S. E. Bell, *Chem Commun (Camb)*, 2016, **52**, 493-496.
22. V. Halouzka, B. Halouzkova, D. Jirovsky, D. Hemzal, P. Ondra, E. Siranidi, A. G. Kontos, P. Falaras and J. Hrbac, *Talanta*, 2017, **165**, 384-390.
23. H. Dies, J. Raveendran, C. Escobedo and A. Docoslis, *Sensors and Actuators B: Chemical*, 2018, **257**, 382-388.
24. A. D. Lesiak, R. A. Musah, R. B. Cody, M. A. Domin, A. J. Dane and J. R. Shepard, *Analyst*, 2013, **138**, 3424-3432.
25. J. P. Smith, J. P. Metters, O. I. G. Khreit, O. B. Sutcliffe and C. E. Banks, *Analytical chemistry*, 2014, **86**, 9985-9992.
26. E. M. C. f. D. a. D. addiction, Early warning system on NPSs https://www.emcdda.europa.eu/publications/topic-overviews/eu-early-warning-system_en).
27. UNODC, *World Drugs Report 2016*, Vienna, 2016.

28. UNODC, *Current NPS Treats Volume III October 2020*, Vienna, 2020.
29. E. a. Europol, *EU Drug Markets Report 2019*, 2019.
30. WHO, Opioid Overdose, https://www.who.int/news-room/fact-sheets/detail/opioid-overdose, (accessed January, 2022).
31. UNODC, *World Drugs Report, Booklet 4: Stimulants, United Nations Office on Drugs and Crime*, Vienna, 2019.
32. UNODC, *World Drugs Report, Booklet 5: Cannabis and Hallucinogens*, Vienna, 2019.
33. K. A. Alsenedi and C. Morrison, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2018, **1076**, 91-102.
34. K. A. Alsenedi and C. Morrison, *Analytical Methods*, 2018, **10**, 1431-1440.
35. E. l. Olesti, M. Farré, E. Papaseit, A. Krotonoulas, M. Pujadas, R. de la Torre and O. s. J. Pozo, *The AAPS Journal : An Official Journal of the American Association of Pharmaceutical Scientists*, 2017, **19**, 1767-1778.
36. M. Concheiro, M. Castaneto, R. Kronstrand and M. A. Huestis, *Journal of Chromatography A*, 2015, **1397**, 32-42.
37. M. Concheiro, S. Anizan, K. Ellefsen and M. A. Huestis, *Analytical and bioanalytical chemistry*, 2013, **405**, 9437-9448.
38. M. Paul, J. Ippisch, C. Herrmann, S. Guber and W. Schultis, *Anal Bioanal Chem*, 2014, **406**, 4425-4441.
39. UN, *The International Drug Control Conventions*, 2013.
40. EMCDDA, *Drugs in focus: Measuring prevalence and incidence of drug use*, 2002.
41. G. Legislation, *Journal*, 2010.
42. G. Legislation, *Journal*, 2010.
43. H. Office, List of commonly encountered drugs currently controlled by the misuse of drugs legislation, List of most commonly encountered drugs currently controlled under the misuse of drugs legislation - GOV.UK (www.gov.uk)).
44. *Journal*, 2016.
45. H. Office, *Journal*, 2018.
46. H. Office, *Journal*, 2018.
47. E. Deligianni, O. J. Daniel, J. M. Corkery, F. Schifano and L. A. Lione, *Br J Clin Pharmacol*, 2020, **86**, 505-516.
48. A. A. Deen, H. Claridge, R. D. Treble, H. J. Hamnett and C. S. Copeland, *J Psychopharmacol*, 2021, **35**, 1315-1323.
49. C. Miliano, G. Margiani, L. Fattore and M. A. De Luca, *Brain Sci*, 2018, **8**.
50. A. Shafi, A. J. Berry, H. Sumnall, D. M. Wood and D. K. Tracy, *Ther Adv Psychopharmacol*, 2020, **10**, 2045125320967197.
51. A. McAuley, A. Yeung, A. Taylor, S. J. Hutchinson, D. J. Goldberg and A. Munro, *Int J Drug Policy*, 2019, **66**, 30-37.
52. R. Ralphs, P. Gray and O. B. Sutcliffe, *Int J Drug Policy*, 2021, **97**, 103305.
53. L. Sacco, *Drug Enforcement in the United States: History, Policy, and Trends*, 2014.
54. M. Gabay, *Hospital Pharmacy*, 2013, **48**, 473-474.
55. U. Congress, **Address** and D. Washington , United States, *Journal*, 1986.
56. U. Congress, **Address** and D. Washington , United States, *Journal*, 1988.
57. DEA, *Drugs of Abuse: A DEA Resource Guide 2017 Edition*, 2017.
58. DEA, *Journal*, 2011.

59. N. I. o. D. Abuse, *Journal*.
60. DEA, DEA
acts against dangerous, deadly designer synthetic drug linked to 151 overdose deaths since 2015, https://www.dea.gov/press-releases/2018/08/31/dea-acts-against-dangerous-deadly-designer-synthetic-drug-linked-151).
61. L. McNeill, C. Pearson, D. Megson, J. Norrey, D. Watson, D. Ashworth, P. E. Linton, O. B. Sutcliffe and K. J. Shaw, *Forensic Chemistry*, 2021, **22**.
62. G. M. Whitesides, *Nature*, 2006, **442**, 368-373.
63. S. Jovanovich, G. Bogdan, R. Belcinski, J. Buscaino, D. Burgi, E. L. R. Butts, K. Chear, B. Ciopyk, D. Eberhart, O. El-Sissi, H. Franklin, S. Gangano, J. Gass, D. Harris, L. Hennessy, A. Kindwall, D. King, J. Klevenberg, Y. Li, N. Mehendale, R. McIntosh, B. Nielsen, C. Park, F. Pearson, R. Schueren, N. Stainton, C. Troup, P. M. Vallone, M. Vangbo, T. Woudenberg, D. Wyrick and S. Williams, *Forensic Science International: Genetics*, 2015, **16**, 181-194.
64. S. Verheij, L. Clarisse, M. van den Berge and T. Sijen, *Forensic Science International: Genetics Supplement Series*, 2013, **4**, e254-e255.
65. A. J. Hopwood, C. Hurth, J. Yang, Z. Cai, N. Moran, J. G. Lee-Edghill, A. Nordquist, R. Lenigk, M. D. Estes, J. P. Haley, C. R. McAlister, X. Chen, C. Brooks, S. Smith, K. Elliott, P. Koumi, F. Zenhausern and G. Tully, *Analytical chemistry*, 2010, **82**, 6991-6999.
66. U. Government, *Journal*, 2017.
67. UKAS, Laboratory Accreditation Forensics, https://www.ukas.com/accreditation/standards/laboratory-accreditation/forensics/?_gl=1*3n5zmg*_up*MQ..&gclid=CjwKCAjwjOunBhB4EiwA94JWsJHMZNmlg9xMbl6ieonSXzeMr0udjjrwzz45knuAJ10Ear5PPtSeSRoCVl8QAvD_BwE, (accessed June, 2023).
68. F. S. Regulator, *Journal*, 2021.
69. D. Moher, A. Liberati, J. Tetzlaff and D. G. Altman, *Journal of Clinical Epidemiology*, 2009, **62**, 1006-1012.
70. UNODC, *World Drugs Report, Booklet 1: Executive Summary; Impact of COVID-19 Policy Implications*, Vienna, 2020.
71. UNODC, *World Drugs Report, Booklet 2: Drug Use and Health Consequences*, Vienna, 2020.
72. UNODC, United Kingdom: ACMD report on the misuse of fentanyl and fentanyl analogues as global number of opioid NPS rises, https://www.unodc.org/LSS/Announcement/Details/94dc6286-16bb-4e7a-9429-65d28918b332, (accessed January, 2021).
73. W. Qiang, C. Zhai, J. Lei, C. Song, D. Zhang, J. Sheng and H. Ju, *The Analyst*, 2009, **134**, 1834-1839.
74. R. Salemmilani, M. Moskovits and C. D. Meinhart, *The Analyst*, 2019, **144**, 3080-3087.
75. S. T. Krauss, M. S. Woolf, K. C. Hadley, N. M. Collins, A. Q. Nauman and J. P. Landers, *Sensors & Actuators: B. Chemical*, 2019, **284**, 704-710.
76. T. X. Leong, S. L. Bliese, A. Helmke, A. Richard, G. Merga, J. Rorabeck and M. Lieberman, *Journal of Forensic Sciences*, 2020, **65**, 1289-1297.
77. R. Mirsafavi, M. Moskovits and C. Meinhart, *Analyst*, 2020, **145**, 3440-3446.
78. S. T. Krauss, T. P. Remcho, S. M. Lipes, R. t. Aranda, H. P. Maynard, 3rd, N. Shukla, J. Li, R. E. Tontarski, Jr. and J. P. Landers, *Analytical chemistry*, 2016, **88**, 8689-8697.

79. G. O. da Silva, W. R. de Araujo and T. R. L. C. Paixão, *Talanta*, 2018, **176**, 674-678.
80. G. Musile, L. Wang, J. Bottoms, F. Tagliaro and B. McCord, *Analytical Methods*, 2015, **7**, 8025-8033.
81. S. C. Bell and R. D. Hanes, *Journal of Forensic Sciences*, 2007, **52**, 884-888.
82. *Chemicals & Chemistry*, 2021, 6968.
83. M. F. M. Ribeiro, F. Bento, A. J. Ipólito and M. F. de Oliveira, *Journal of Forensic Sciences*, 2020, **65**, 2121-2128.
84. L. Wang, G. Musile and B. R. McCord, *ELECTROPHORESIS*, 2018, **39**, 470-475.
85. A. M. Yehia, M. A. Farag and M. A. Tantawy, *Analytica chimica acta*, 2020, **1104**, 95-104.
86. T.-L. E. Lockwood, T. X. Leong, S. L. Bliese, A. Helmke, A. Richard, G. Merga, J. Rorabeck and M. Lieberman, *Journal of forensic sciences*, 2020, **65**, 1289-1297.
87. C.-A. Chen, P.-W. Wang, Y.-C. Yen, H.-L. Lin, Y.-C. Fan, S.-M. Wu and C.-F. Chen, *Sensors & Actuators: B. Chemical*, 2019, **282**, 251-258.
88. N. A. Abdelshafi, J. r. m. Bell, K. Rurack and R. J. Schneider, *Drug Testing and Analysis*, 2019, **11**, 492-500.
89. N. D. Kline, A. Tripathi, R. Mirsafavi, I. Pardoe, M. Moskovits, C. Meinhart, J. A. Guicheteau, S. D. Christesen and A. W. Fountain, 3rd, *Analytical chemistry*, 2016, **88**, 10513-10522.
90. R. Y. Mirsafavi, K. Lai, N. D. Kline, A. W. Fountain, 3rd, C. D. Meinhart and M. Moskovits, *Analytical chemistry*, 2017, **89**, 1684-1688.
91. R. C. Moreira, B. M. C. Costa, M. C. Marra, M. H. P. Santana, A. O. Maldaner, É. D. Botelho, T. R. L. C. Paixão, E. M. Richter and W. K. T. Coltro, *Electrophoresis*, 2018, **39**, 2188-2194.
92. J. Narang, N. Malhotra, C. Singhal, A. Mathur, D. Chakraborty, A. Anil, A. Ingle and C. S. Pundir, *Biosensors and Bioelectronics*, 2017, **88**, 249-257.
93. K. Mikael, S. Carl, J. Johnny, Ö. Olof, P. Ingemar, E. Louise, D. Simon, F. Ying and W. Qin, *Journal*, 2019, **19**, 2214.
94. T. Teerinen, T. Lappalainen and T. Erho, *Analytical and Bioanalytical Chemistry*, 2014, **406**, 5955-5965.
95. J. Zhou, A. V. Ellis, H. Kobus and N. H. Voelcker, *Analytica Chimica Acta*, 2012, **719**, 76-81.
96. B.-Y. A. D, *IEEE Sensors journal*, 2009, **9**, 81-86.
97. S. E. Hadland and S. Levy, *Child Adolesc Psychiatr Clin N Am*, 2016, **25**, 549-565.
98. H. R. Mobini Far, F. Torabi, B. Danielsson and M. Khayyami, *J Anal Toxicol*, 2005, **29**, 790-793.
99. D. M. Bruls, T. H. Evers, J. A. H. Kahlman, P. J. W. van Lankvelt, M. Ovsyanko, E. G. M. Pelssers, J. J. H. B. Schleipen, F. K. de Theije, C. A. Verschuren, T. van der Wijk, J. B. A. van Zon, W. U. Dittmer, A. H. J. Immink, J. H. Nieuwenhuis and M. W. J. Prins, *Lab on a chip*, 2009, **9**, 3504-3510.
100. R. Chand, N. Mittal, S. Srinivasan and A. R. Rajabzadeh, *Analyst*, 2021, **146**, 574-580.
101. N. Christodoulides, R. De La Garza, G. W. Simmons, M. P. McRae, J. Wong, T. F. Newton, R. Smith, J. J. Mahoney Iii, J. Hohenstein, S. Gomez, P. N. Floriano, H. Talavera, D. J. Sloan, D. E. Moody, D. M. Andrenyak, T. R. Kosten, A. Haque and J. T. McDevitt, *Drug and Alcohol Dependence*, 2015, **153**, 306-313.
102. Y. Li, J. Xuan, T. Xia, X. Han, Y. Song, Z. Cao, X. Jiang, Y. Guo, P. Wang and L. Qin, *Analytical chemistry*, 2015, **87**, 3771-3777.

103. L. Zhang, X. Li, Y. Li, X. Shi and H.-Z. Yu, *Analytical chemistry*, 2015, **87**, 1896-1902.
104. S. Jafari, Y. Thillier, Y. H. Ajena, D. Shorty, J. Li, J. S. Huynh, B. M.-C. Pan, T. Pan, K. S. Lam and R. Liu, *Molecules (Basel, Switzerland)*, 2019, **24**.
105. X. Kong, X. Chong, K. Squire and A. X. Wang, *Sensors and Actuators: B. Chemical*, 2018, **259**, 587-595.
106. L. Ying, U. Uvaraj, H. Bangshun, W. Ping and Q. Lidong, *Analytical Chemistry*, 2017, **89**, 8273(8281).
107. A. Farahani and H. Sereshti, *Analytical and Bioanalytical Chemistry*, 2019, **412**, 129-138.
108. E. Al-Hetlani, *Electrophoresis*, 2013, **34**, 1262-1272.
109. Y. Xia, J. Si and Z. Li, *Biosensors and Bioelectronics*, 2016, **77**, 774-789.
110. D. M. Cate, J. A. Adkins, J. Mettakoonpitak and C. S. Henry, *Analytical chemistry*, 2015, **87**, 19-41.
111. A. K. Yetisen, M. S. Akram and C. R. Lowe, *Lab on a Chip*, 2013, **13**, 2210-2251.
112. W. Xue, X. Tan, M. K. Khaing Oo, G. Kulkarni, M. A. Ilgen and X. Fan, *The Analyst*, 2020, **145**, 1346-1354.
113. D. E. Damon, Y. S. Maher, M. Yin, F. P. M. Jjunju, I. S. Young, S. Taylor, S. Maher and A. K. Badu-Tawiah, *The Analyst*, 2016, **141**, 3866-3873.
114. A. E. Kirby, N. M. Lafrenière, B. Seale, P. I. Hendricks, R. G. Cooks and A. R. Wheeler, *Analytical chemistry*, 2014, **86**, 6121-6129.
115. C. Shende, C. Brouillette and S. Farquharson, *The Analyst*, 2019, **144**, 5449-5454.
116. C. Andreou, M. R. Hoonejani, M. R. Barmi, M. Moskovits and C. D. Meinhart, *ACS nano*, 2013, **7**, 7157-7164.
117. D. J. Cocovi-Solberg, F. A. Esteve-Turrillas, S. Armenta, M. de la Guardia and M. Miró, *Journal of Chromatography A*, 2017, **1512**, 43-50.
118. M. Su, Y. Jiang, F. Yu, T. Yu, S. Du, Y. Xu, L. Yang and H. Liu, *ACS applied bio materials*, 2019, **2**, 3828-3835.
119. E. Ollikainen, T. Aitta-aho, M. Koburg, R. Kostiainen and T. Sikanen, *Scientific Reports*, 2019, **9**, 1-9.
120. B. Shlyahovsky, D. Li, Y. Weizmann, R. Nowarski, M. Kotler and I. Willner, *Journal of the American Chemical Society*, 2007, **129**, 3814-3815.
121. J. Liu, J. H. Lee and Y. Lu, *Analytical chemistry*, 2007, **79**, 4120-4125.
122. B. R. Baker, R. Y. Lai, M. S. Wood, E. H. Doctor, A. J. Heeger and K. W. Plaxco, *Journal of the American Chemical Society*, 2006, **128**, 3138-3139.
123. J. Chen, J. Jiang, X. Gao, G. Liu, G. Shen and R. Yu, *Chemistry (Weinheim an der Bergstrasse, Germany)*, 2008, **14**, 8374-8382.
124. R. Freeman, Y. Li, R. Tel-Vered, E. Sharon, J. Elbaz and I. Willner, *The Analyst*, 2009, **134**, 653-656.
125. S. M. Scott and Z. Ali, *Micromachines (Basel)*, 2021, **12**.
126. S. Wang, L. Ge, X. Song, J. Yu, S. Ge, J. Huang and F. Zeng, *Biosens Bioelectron*, 2012, **31**, 212-218.
127. A. W. Martinez, S. T. Phillips, M. J. Butte and G. M. Whitesides, *Angew Chem Int Ed Engl*, 2007, **46**, 1318-1320.
128. A. W. Martinez, S. T. Phillips, E. Carrilho, S. W. Thomas, H. Sindi and G. M. Whitesides, *Anal Chem*, 2008, **80**, 3699-3707.
129. D. A. Bruzewicz, M. Reches and G. M. Whitesides, *Anal Chem*, 2008, **80**, 3387-3392.
130. H. Miyaguchi, H. Takahashi, T. Ohashi, K. Mawatari, Y. T. Iwata, H. Inoue and T. Kitamori, *Forensic Science International*, 2009, **184**, 1-5.

131. A. Barfidokht, R. K. Mishra, R. Seenivasan, S. Liu, L. J. Hubble, J. Wang and D. A. Hall, *Sens Actuators B Chem*, 2019, **296**.
132. F. Tseliou, P. Pappas, K. Spyrou, J. Hrbac and M. I. Prodromidis, *Biosens Bioelectron*, 2019, **132**, 136-142.
133. F. P. Mayer, L. Wimmer, O. Dillon-Carter, J. S. Partilla, N. V. Burchardt, M. D. Mihovilovic, M. H. Baumann and H. H. Sitte, *Br J Pharmacol*, 2016, **173**, 2657-2668.
134. Abcam, *Cross-reactivity of Anti-methcathinone Antibody*, USA, 2020
135. E. Carrilho, A. W. Martinez and G. M. Whitesides, *Anal Chem*, 2009, **81**, 7091-7095.
136. S. Sanchez, *Bull Soc Chimique*, 1929, **45**, 284-286.
137. A. L. Bretteville-Jensen, S. S. Tuv, O. R. Bilgrei, B. Fjeld and L. Bachs, *Forensic Sci Rev*, 2013, **25**, 7-26.
138. A. Bohn, D. Sander, T. Köhler, N. Hees, F. Oswald, N. Scherbaum, D. Deimel and H. Schecke, *Front Psychiatry*, 2020, **11**, 542301.
139. T. M. Brunt, A. Poortman, R. J. M. Niesink and W. van den Brink, *Journal of Psychopharmacology*, 2011, **25**, 1543-1547.
140. A. R. Winstock, L. R. Mitcheson, P. Deluca, Z. Davey, O. Corazza and F. Schifano, *Addiction (Abingdon, England)*, 2011, **106**, 154-161.
141. L. Karila, B. Megarbane, O. Cottencin and M. Lejoyeux, *Curr Neuropharmacol*, 2015, **13**, 12-20.
142. EMCDDA, *Europol–EMCDDA Joint Report on a new psychoactive substance: 4-methylmethcathinone (mephedrone).* 2010.
143. D. M. Wood, S. Davies, M. Puchnarewicz, J. Button, R. Archer, H. Ovaska, J. Ramsey, T. Lee, D. W. Holt and P. I. Dargan, *J Med Toxicol*, 2010, **6**, 327-330.
144. EMCDDA, *EMCDDA Risk Assessments: Report on the risk assessment of mephedrone in the framework of the Council Decision on new psychoactive substances*, Luxembourg, 2011.
145. J. Martínez-Clemente, R. López-Arnau, M. Carbó, D. Pubill, J. Camarasa and E. Escubedo, *Psychopharmacology*, 2013, **229**, 295-306.
146. M. R. Meyer, J. Wilhelm, F. T. Peters and H. H. Maurer, *Anal Bioanal Chem*, 2010, **397**, 1225-1233.
147. E. Papaseit, C. Pérez-Mañá, J. A. Mateus, M. Pujadas, F. Fonseca, M. Torrens, E. Olesti, R. de la Torre and M. Farré, *Neuropsychopharmacology*, 2016, **41**, 2704-2713.
148. H. M. Elbardisy, A. García-Miranda Ferrari, C. W. Foster, O. B. Sutcliffe, D. A. C. Brownson, T. S. Belal, W. Talaat, H. G. Daabees and C. E. Banks, *Journal*, 2019.
149. A. J. Pedersen, L. A. Reitzel, S. S. Johansen and K. Linnet, *Drug Test Anal*, 2013, **5**, 430-438.
150. J. Czerwinska, M. C. Parkin, C. George, A. T. Kicman, P. I. Dargan and V. Abbate, *J Anal Toxicol*, 2021, **45**, 730-738.
151. Ó. Pozo, M. Ibáñez, J. V. Sancho, J. Lahoz-Beneytez, M. Farré, E. Papaseit, R. de la Torre and F. Hernández, *Drug Metab Dispos*, 2015, **43**, 248-257.
152. I. Linhart, M. Himl, M. Židková, M. Balíková, E. Lhotková and T. Páleníček, *Toxicol Lett*, 2016, **240**, 114-121.
153. E. Olesti, M. Farré, E. Papaseit, A. Krotonoulas, M. Pujadas, R. de la Torre and Ó. Pozo, *AAPS J*, 2017, **19**, 1767-1778.
154. O. I. Khreit, C. Irving, E. Schmidt, J. A. Parkinson, N. Nic Daeid and O. B. Sutcliffe, *J Pharm Biomed Anal*, 2012, **61**, 122-135.

155. A. J. Pedersen, P. W. Dalsgaard, A. J. Rode, B. S. Rasmussen, I. B. Müller, S. S. Johansen and K. Linnet, *J Sep Sci*, 2013, **36**, 2081-2089.
156. P. I. Dargan, R. Sedefov, A. Gallegos and D. M. Wood, *Drug Test Anal*, 2011, **3**, 454-463.
157. A. Guirguis, J. M. Corkery, J. L. Stair, S. B. Kirton, M. Zloh and F. Schifano, *Hum Psychopharmacol*, 2017, **32**.
158. P. Adamowicz, B. Tokarczyk, R. Stanaszek and M. Slopianka, *J Anal Toxicol*, 2013, **37**, 37-42.
159. EMCDDA, *Synthetic Cathinones Drug Profile*, http://www.emcdda.europa.eu/publications/drug-profiles/synthetic-cathinones, (accessed 10th October, 2021).
160. EMCDDA, *Injection of Synthetic Cathinones*, http://www.emcdda.europa.eu/topics/pods/synthetic-cathinones-injection_en, (accessed 10th October, 2021).
161. F. Measham, K. Moore, R. Newcombe and Zoë, *Drugs and Alcohol Today*, 2010, **10**, 14-21.
162. Wedinos, http://www.wedinos.org/resources/downloads/Philtre_Issue_2.pdf, (accessed June, 2016).
163. S. Davies, D. M. Wood, G. Smith, J. Button, J. Ramsey, R. Archer, D. W. Holt and P. I. Dargan, *QJM*, 2010, **103**, 489-493.
164. S. D. Brandt, H. R. Sumnall, F. Measham and J. Cole, *Drug Test Anal*, 2010, **2**, 377-382.
165. H. A. Spiller, M. L. Ryan, R. G. Weston and J. Jansen, *Clinical Toxicology*, 2011, **49**, 499-505.
166. B. Miserez, O. Ayrton and J. Ramsey, *Forensic Toxicology*, 2014, **32**, 305-310.
167. O. f. N. Statistics, Drug misuse in England and Wales: year ending June 2022, Drug misuse in England and Wales year ending June 2022.pdf).
168. H. Office, *Drug Misuse: Findings from the 2014/15 Crime Survey for England and Wales*, 2015.
169. H. Office, *The 2010/11 British Crime Survey (England and Wales)*, 2011.
170. UNODC, *Decision 58/1: Inclusion of mephedrone (4-methylmethcathinone) in Schedule II of the Convention on Psychotropic Substances of 1971*, 2015.
171. U. Nations, *Commission on Narcotic Drugs Report on the fifty-eighth session (5 December 2014 and 9-17 March 2015)*, 2015.
172. W. H. O. (WHO), *Follow up on recommendations made by 36th ECDD: Expert Committee on Drug Dependence*, 2015.
173. D. Gustavsson and C. Escher, *Lakartidningen*, 2009, **106**, 2769-2771.
174. UNODC, *World Drugs Report 2022: Booklet 4 Drug Market Trends*, 2022.
175. EMCDDA, *New Psychoactive Substances in Prisons, EMCDDA Rapid Communication.* , Luxembourg, 2018.
176. D. Wild, *Journal*, 2013.
177. M. D. Krasowski, M. G. Siam, M. Iyer and S. Ekins, *Ther Drug Monit*, 2009, **31**, 337-344.
178. M. D. Krasowski and S. Ekins, *J Cheminform*, 2014, **6**, 22.

179. D. J. Heal, S. L. Smith, J. Gosden and D. J. Nutt, *Journal of Psychopharmacology*, 2013, **27**, 479-496.
180. UNODC, *AMPHETAMINE-TYPE STIMULANTS*
A GLOBAL REVIEW
Prepared by UNDCP at the request of the Commission on Narcotic Drugs.
181. EMCDDA, Amphetamine Drug Profile, https://www.emcdda.europa.eu/publications/drug-profiles/amphetamine_en).
182. M. Carvalho, H. Carmo, V. M. Costa, J. P. Capela, H. Pontes, F. Remião, F. Carvalho and M. e. L. Bastos, *Arch Toxicol*, 2012, **86**, 1167-1231.
183. T. Kraemer and H. H. Maurer, *Ther Drug Monit*, 2002, **24**, 277-289.
184. D. Luethi and M. E. Liechti, *Archives of Toxicology*, 2020, **94**, 1085-1133.
185. C. Cole, L. Jones, J. McVeigh, A. Kicman, Q. Syed and M. A. Bellis, *Cut, a Guide to Adulterants, Bulking Agents and Other Contaminants Found in Illicit Drugs*, 2010.
186. C. Cole, L. Jones, J. McVeigh, A. Kicman, Q. Syed and M. Bellis, *Drug Test Anal*, 2011, **3**, 89-96.
187. S. Pichini, F. P. Busardò, A. Gregori, P. Berretta, S. Gentili and R. Pacifici, *Drug Test Anal*, 2017, **9**, 485-490.
188. A. Żubrycka, A. Kwaśnica, M. Haczkiewicz, K. Sipa, K. Rudnicki, S. Skrzypek and L. Poltorak, *Talanta*, 2022, **237**, 122904.
189. E. Katainen, M. Elomaa, U. M. Laakkonen, E. Sippola, P. Niemelä, J. Suhonen and K. Järvinen, *J Forensic Sci*, 2007, **52**, 88-92.
190. M. F. Andreasen, C. Lindholst and E. Kaa, *The Open Forensic Science Journal*, 2009, **2**, 16-20.
191. UNODC, *World Drugs Report, Booklet 4: Drug Market Trends: Cocaine, Amphetamine Type Stimulants*, 2021.
192. UNODC, *Journal*, 2023.
193. Y. Li, U. Uddayasankar, B. He, P. Wang and L. Qin, *Anal Chem*, 2017, **89**, 8273-8281.
194. *Obesity, Fitness & Wellness Week*, 2021, 1044.
195. M. Li, R. Tao, W. Zhou, Y. Li, M. Meng, Y. Zhang, L. Yu, L. Chen, Y. Bian and C. Li, *Forensic Sci Res*, 2019, **6**, 84-91.
196. M. Ragazzo, S. Melchiorri, L. Manzo, V. Errichiello, G. Puleri, F. Nicastro and E. Giardina, *Genes (Basel)*, 2020, **11**.
197. N. Dawnay, B. Stafford-Allen, D. Moore, S. Blackman, P. Rendell, E. K. Hanson, J. Ballantyne, B. Kallifatidis, J. Mendel, D. K. Mills, R. Nagy and S. Wells, *Forensic Sci Int Genet*, 2014, **11**, 73-79.
198. S. Blackman, B. Stafford-Allen, E. K. Hanson, M. Panasiuk, A. L. Brooker, P. Rendell, J. Ballantyne and S. Wells, *Forensic Sci Int Genet*, 2018, **37**, 151-161.
199. B. Bruijns, J. Knotter and R. Tiggelaar, *Sensors (Basel)*, 2023, **23**.
200. *Health & Medicine Week*, 2021, 5741.
201. R. Toxicology, ELISA Solutions, https://dashboard.randox.com/wp-content/uploads/2018/01/Reagents-ELISA-Solutions.pdf, (accessed August, 2022).
202. M. J. Swortwood, W. L. Hearn and A. P. DeCaprio, *Drug Test Anal*, 2014, **6**, 716-727.
203. C. Bell, C. George, A. T. Kicman and A. Traynor, *Drug Test Anal*, 2011, **3**, 496-504.
204. IEC, What we do, https://iec.ch/what-we-do, (accessed June, 2023).

205. ISO, About us, https://www.iso.org/about-us.html, (accessed June, 2023).
206. U. N. O. o. D. a. Crime, *Booklet 4 - Drug market trends of Cocaine, Amphetamine-type stimulants and New Psychoactive Substances*, 2022.
207. P. Adamowicz and P. Hydzik, *Clinical toxicology (Philadelphia, Pa.)*, 2019, **57**, 112-116.
208. *Germany Pat.,* 1965.
209. *UK Pat.,* 1963.
210. P. Kavanagh, M. Gofenberg, V. Shevyrin, O. Dvorskaya, G. Dowling and A. Grigoryev, *Drug Testing and Analysis*, 2020, **12**, 1442-1451.
211. EMCDDA–Europol, *Annual Report on the implementation of Council Decision 20142005/387/JHA.*, 2014.
212. D. Di Candia, M. Boracchi, B. Ciprandi, G. Giordano and R. Zoja, *International Journal of Legal Medicine*, 2022, **136**, 1291-1296.
213. A. Redfern, Hooked on monkey dust: The £2-a-hit drug that has now reached 'epidemic levels' in Stoke-on-Trent, Hooked on monkey dust: The £2-a-hit drug that has now reached 'epidemic levels' in Stoke-on-Trent - Stoke-on-Trent Live (stokesentinel.co.uk), (accessed August, 2022).
214. A. Namera, S. Urabe, T. Saito, A. Torikoshi-Hatano, H. Shiraishi, Y. Arima and M. Nagao, *Forensic Toxicology*, 2013, **31**, 338-343.
215. M. Bäckberg, K.-H. Jönsson, O. Beck and A. Helander, *Drug Testing and Analysis*, 2018, **10**, 340-349.
216. A. Wurita, K. Hasegawa, K. Minakata, K. Gonmori, H. Nozawa, I. Yamagishi, O. Suzuki and K. Watanabe, *Legal Medicine*, 2014, **16**, 241-246.
217. V. A. Boumba, M. Di Rago, M. Peka, O. H. Drummer and D. Gerostamoulos, *Forensic science international*, 2017, **279**, 192-202.
218. A. Sorribes-Soriano, F. A. Esteve-Turrillas, S. Armenta, P. Amorós and J. M. Herrero-Martínez, *Analytica Chimica Acta*, 2019, **1052**, 73-83.
219. A. Niebel, F. Krumbiegel, S. Hartwig, M. K. Parr and M. Tsokos, *Forensic Science, Medicine and Pathology*, 2019, **16**, 32-42.
220. J. L. Costa, K. F. Cunha, R. Lanaro, R. L. Cunha, D. Walther and M. H. Baumann, *Drug Testing and Analysis*, 2019, **11**, 461-471.
221. S. Brandt, *N-Ethylnorpentylone: Critical Review Report. Technical Report.*, 2018.
222. G. Blanco, D. Vidler, C. Roper, D. M. Wood, P. I. Dargan, L. Keating, R. Macfarlane, S. Emmett, G. Johnson, M. Eddleston, S. L. Hill and S. H. L. Thomas, *Clinical Toxicology*, 2021, **59**, 1270-1273.
223. 1967.
224. *Life Science Weekly*, 2017, 5748.
225. I. Chisom, D. S. Charmian, M. C. Lyn and S. W. David, *Journal*, 2018, **8**, 307-310.
226. D. Atherton, D. Dye, C. A. Robinson and R. Beck, *Journal of Forensic Sciences*, 2019, **64**, 304-308.
227. Z. Marcin, N. Karolina and S. Paweł, *Journal*, 2019, 1-9.
228. A. J. Krotulski, D. M. Papsun, B. S. De Martinis, A. L. A. Mohr and B. K. Logan, *J Anal Toxicol*, 2018, **42**, 467-475.
229. M. Taschwer, J. A. Weiß, O. Kunert and M. G. Schmid, *Forensic Science International*, 2014, **244**, e56-e59.
230. M. K. Woźniak, L. Banaszkiewicz, M. Wiergowski, E. Tomczak, M. Kata, B. Szpiech, J. Namieśnik and M. Biziuk, *Forensic Toxicology*, 2019, **38**, 42-58.
231. L. H. Antonides, R. M. Brignall, A. Costello, J. Ellison, S. E. Firth, N. Gilbert, B. J. Groom, S. J. Hudson, M. C. Hulme, J. Marron, Z. A. Pullen, T. B. R. Robertson, C.

J. Schofield, D. C. Williamson, E. K. Kemsley, O. B. Sutcliffe and R. E. Mewis, *ACS Omega*, 2019, **4**, 7103-7112.
232. D. I. Dixon, L. H. Antonides, A. Costello, B. Crane, A. Embleton, M. L. Fletcher, N. Gilbert, M. C. Hulme, M. J. James, M. A. Lever, C. J. Maccallum, M. F. Millea, J. L. Pimlott, T. B. R. Robertson, N. E. Rudge, C. J. Schofield, F. Zukowicz, E. K. Kemsley, O. B. Sutcliffe and R. E. Mewis, *J Pharm Biomed Anal*, 2022, **219**, 114950.
233. A. Carlsson, V. Sandgren, S. Svensson, P. Konradsson, S. Dunne, M. Josefsson and J. Dahlén, *Drug Test Anal*, 2018.
234. D. V. Sotnikov, A. V. Zherdev, E. A. Zvereva, S. A. Eremin and B. B. Dzantiev, *Journal*, 2021, **11**.

Appendix

Appendix 1.1.

The following four search terms were used:

1. “LOC” OR “lab-on-a-*” OR “microfluidic*” AND “detection” AND “drug* of abuse” OR “new psychoactive substance*” OR “controlled drug*”

2. “lab-on-a*” OR “LOC” OR “microfluidic” AND “detection” AND “legal high*” OR “cathinone*” OR “cannabinoid*” OR “illegal drug*” OR “illicit drug*” OR “opiate*” OR “opioid*”

3. “portable” OR “handheld” OR “disposable” OR “presumptive*” AND “detection” AND “drug* of abuse” OR “controlled drug*” OR “illegal drug*” OR “illicit drug*” OR “legal high*” OR “cathinone*” OR “cannabinoid*”

4. “portable” OR “handheld” OR “disposable” OR “presumptive*” AND “detection” AND “new psychoactive substance*” OR “opiate*” OR “opioid*”

Appendix 1.2.

Results from the four search criteria from March 2021 after the systematic review was undertaken (Table 1.1.).

Table 1.1: Summary information on all articles published from March 2021.

Search Criteria	Methodology	Drug(s) Investigated	Reference	Country
1	MS	Cocaine Codeine Morphine	Zhou *et al* 2023	Canada
1	LC-MS	10 drugs of abuse 2 metabolites	Chen *et al* 2022	Taiwan
2	immunoassay	Cocaine	Paul *et al* 2021	Germany
2 and 4	Immunoassay	Opiates	Dignan *et al* 2021	USA
2	Immunoassay	Amphetamine Cocaine Morphine	Zhang *et al* 2021	China/Canada
3	SERS	Cocaine	Alder *et al* 2021	Australia
3	Spectrometry	Synthetic cannabinoids	Martínez-Pérez-Cejuela *et al* 2023	Spain

Appendix 1.3.

Results from the published systematic review (Table 1.2.).

Table 1.2: Summary information on all articles included in the systematic review, as categorised by detection methodology.

Methodology	Drug(s) Investigated	LOC Material	Sample Matrix	Analysis Time (minutes)	Sensitivity	Reference	Country

Immunoassay	Amphetamine	Plastic	Plasma Urine	10	LOD: 20 ng/mL (urine); 6 ng/mL (plasma)	Far *et al* (2005)	Sweden
	Cocaine	PDMS and PTFE	Aqueous (water) Oral fluid Urine	25	LOD: 0.15 ng/L	Abdelshaf *et al* (2018) [38]	Germany
	Amphetamine Methamphetamine Cocaine Morphine THC	Plastic	Plasma Oral fluid	1	LOD: states can detect to sub-nanogram per millilitre levels (3 pM)	Bruls *et al* (2009) [50]	The Netherlands
	THC	Glass and cotton fibres	Oral fluid	20	LOD: 2 ng mL^{-1}	Chand *et al* (2021) [70]	Canada
	Cocaine, Benzoylecgonine MethamphetamineAmphetamine Methadone THC Morphine Benzodiazepines MDA MDMA (also two pro-drugs)	Agarose bead sensors	Oral fluid	~ 10	LODs: ranging from 0.14-7.4 ng/mL	Christodoulides *et al* (2015) [28]	USA
	Morphine	PEG	Plasma	Not clearly stated	LOD: 0.35 M (100 ng/mL)	Jafari *et al* 2019 [52]	USA
	Amphetamine Cocaine	Graphene, PDMS, and PMMA	Aqueous (PBS)	Not clearly stated	Not clearly stated	Karlsson *et al* [44] (2019)	Sweden
	Methamphetamine	Glass	Hair	< 30	Lowest limit of quantitation (≤0.2 ng/mg for each compound)	Miyaguchi *et al* (2009) [68]	Japan
	Amphetamine Cocaine Methamphetamine	Glass	Whole blood Plasma Urine	3	Not clearly stated	Li *et al* (2017) [64]	USA China

	Methadone Morphine Oxazepam THC						
	Amphetamine Methamphetamine Cocaine Opiates Benzodiazepines THC	Glass	Urine	Not clearly stated	Not clearly stated	Li *et al* (2015) [65]	USA China
	Methamphetamine	Paper and PVC	Aqueous	10	LODs: 3.34×10 −9 mol/L	Liang *et al* (2020) [42]	China
	Morphine	Paper, PAE and Plastic	Aqueous (PBS) Oral fluid	Not clearly stated	LOD: 1 ng/mL	Teerinan *et al* (2014) [45]	Finland
	Morphine Cocaine	PDMS	Oral fluid	5-10	LODs: Cocaine 5.0 ng/mL; Morphine 1.0 ng/mL	Zhang *et al* (2015) [51]	Canada China
	Mephedrone 4-methylephedrine	Paper	Aqueous (water) Urine	3	LOD: Mephedrone 4.34 ng mL^{-1} and 4-methylephedrine 2.51 ng mL^{-1}	McNeill *et al* (2021) [20]	UK
	Amphetamine Methadone MethamphetamineTHC	Polystyrene	Sweat	16	LODs: ranging between 1.6-142 pg mL^{-1}	Xue *et al* (2020) [69]	USA
Aptamer	Cocaine	PDMS	Aqueous (PBS)	Not clearly stated	LOD: 0.2 µM = 0.659 x 10^3 ng mL^{-1}	Zhou *et al* (2012) [46]	Australia
Capillary electrophoresis (CE)	Morphine Codeine	PDMS and Glass	Aqueous (MES)	Not clear	LODs: Codeine and morphine showed separation voltage and	Bani-Yaseen *et al* (2009) [48]	Kingdom of Saudi Arabia

					detection potential at 150 V/cm and 1.3 V, respectively.		
	Ephedrine Heroin Ketamine MDA MDMA Morphine 6-MAM THC	Quartz and PMMA	Urine	Not clearly stated	LODs: 2.09 µg/mL (ephedrine); 1.96 µg/mL (heroin); 1.62 µg/mL (ketamine); 1.94 µg/mL (MDA); 1.32 µg/mL (MDMA); 1.15 µg/mL (morphine); 1.12 µg/mL (6-MAM); 1.69 µg/mL (THC)	Qiang *et al* (2009)	China
Spectrometry – Ion mobility spectrometry (IMS)	Cocaine Ecgonine methyl ester	PVC and PFTE	Oral fluid	7.5	LODs: Cocaine 0.3 g/mL; ecgonine methyl ester 0.14 µg/L; LOQs: Cocaine 0.9 g/mL; ecgonine methyl ester 0.4 µg/L	Cocovi-Solberg *et al* (2017) [66]	Spain
Spectrometry - Fourier transform infrared (FTIR)	Codeine Morphine Papaverine	PU and PANI	Urine	Not clearly stated	LODs: MOR, COD, and PAP were 4–240, 4–	Farahani and Sereshti (2020)	Iran

					210, and 1–150 ng mL^{-1}		
Spectrometry - Mass spectrometry (MS)	Amphetamine Cocaine Benzoylecgonine Methamphetamine	Paper	Urine (both fresh and dried)	Not clearly stated	LOD's ranging from: 0.10-0.33 ng/mL for dry urine; 0.51-0.97 ng/mL for fresh urine. LOQ's ranging from: 0.38-0.87 ng/mL for dry urine; 1.40-2.50 ng/mL for fresh urine.	Damon *et al* 2016	USA UK
Spectrometry-Mass spectrometry (MS)	Cocaine Benzoylecgonine Codeine	Glass	Urine (dried)	<15	LOQ for cocaine is 40 ng/mL; LODs are 51 ng/mL, 21 ng/mL, and 39 ng/mL for cocaine, BZE, and codeine, respectively	Kirby *et al* 2014	Canada USA
Spectrometry - Surface-enhanced raman spectroscopy (SERS)	Methamphetamine	PDMS	Oral fluid	States a few minutes	LOD: 10 nM	Andreou *et al* (2013)	USA
	Morphine Cocaine Methamphetamine	Glass	Aqueous (water)	Not stated	LODs: 4.5 ng/mL (metham	Kline *et al* (2016)	USA

					phetamine); 4.6 ng/mL (codeine); 13 ng/mL (morphine)		
	Cocaine	Silica	Plasma	Not stated	LODs: 10 ppb (parts per billion) 10 ng/mL	Kong *et al* (2018) [72]	China USA
	Fentanyl Heroin	Glass	States non-aqueous solution	Not clearly stated	States that it can detect very low levels of fentanyl at (1:10000 mol/mol (fentanyl:heroin ratio)	Salemmilani *et al* (2019) [24]	USA
	Cocaine Amobarbitol Secobarbitol, Phenobarbitol Benzoylecgonine	Glass	Oral fluid	10	Not clearly stated	Shende *et al* (2005) [86]	USA
	Methamphetamine		Oral fluid Urine	1* (1 min for detection. However - pre-treatment to extract MA of 10 mins)	Not clearly stated	Su *et al* (2019) [74]	China
	Papaverine	PDMS and Glass	Aqueous (water) Powder	~ 2.5	6 µM = 0.018 ng/mL	Mirsafavi *et al* (2017) [40]	USA
	Fentanyl (and two precursors - chemical precursors, despropionylfentanyl	PDMS	Aqueous (methanol)	Not clearly stated	100 uM	Mirsafavi *et al* (2020) [27]	USA

	(4ANPP) and N-phenethyl-4-piperidinone (NPP))						
Electrochemical detection	Morphine	Polymer	Plasma and brain homogenate samples (Mouse)		LLOQ - 0.05–0.5 µM and 5 and 20 µM in plasma	Ollikainen (2019) [75]	Finland
	Cocaine	Glass	Aqueous (water)	2	LOD: 41 µmol/L; LOQ: 136 µmol/L	Moreira *et al* (2018) [41]	Brazil
	Ketamine	Paper	Aqueous (water and fruit juice) Urine	2 seconds	LOD: 0.001 nM/mL	Narang *et al* (2016) [47]	India
	LSD	Paper	Aqueous (acetonitrile, water, methanol)	2.5	LODs: 0.38 and LOQ 1.27 µmol/L,	Ribeiro *et al* (2020) [34]	Brazil
Colourimetric	Cocaine Methamphetamine	Polyester	Aqueous	~ 6	LODs: 0.25 mg/mL (cocaine); 0.75 mg/mL (methamphetamine)	Krauss *et al* (2016) [29]	USA
	Codeine Cocaine Heroin MDA MDMA Methamphetamine	Polyester	Aqueous	<1	LOD: 0.75 mg/mL (methamphetamine)	Krauss *et al* (2019) [25]	USA
	Cocaine HCl Crack cocaine Heroin Methamphetamine	Paper	Powder	3	LODs: 55-100 µg	Lockwood *et al* (2020) [35]	USA
	Phenacetin Cocaine	Paper	Aqueous	Not clearly stated	LOD: 3.5 µg/mL (phenacetin); LOQ: 12 µg/mL (phenacetin)	Da Silva *et al* (2018) [30]	Brazil
	Amphetamine Cocaine Codeine	Paper	Aqueous	5	Minimum detectab	Musile *et al* (2015) [31]	USA Italy

	Ephedrine Ketamine MDMA Methamphetamine Morphine Thebaine		(50% acetone/50% water)		le quantity (MDQ) for instrumentation ranging from 1.2-8.7 µg and visual 2.5-11 µg		
	Amphetamine Cocaine Methamphetamine Oxycodone	Glass	Powder	<0.25	LODs: 50–125 pg	Bell and Hanes (2007) [32]	USA
	Cocaine	Paper	Aqueous (water)	5	LODs: The visual LOD for the method was 2.5 µg and the camera based LOD was 2.36 µg.	Wang *et al* (2018) [36]	USA/Italy
Combined Colourimetric and Electrochemical	Cocaine	Paper	Aqueous (PBS)	Not clearly stated	Not clearly stated	Ameku *et al* (2021) [33]	Brazil
Colourimetric, Electrochemical and Fluorimetric	Ketamine	Paper	Aqueous (spiked beverages)	<1	LODs: 10 mg mL^{-1}, 760.72 ng and 0.0475 mg mL^{-1}	Yehia *et al* (2020) [37]	Egypt
Colourimetric and Immunoassay	Ketamine	Paper	Oral fluid	6	Not clearly stated	Chen *et al* (2019) [87]	Taiwan
Electrochemical and Immunoassay	Cocaine	PDMS and PTFE	Aqueous (water) Oral fluid Urine	25	LODs: 0.15 ng/L in water and 1 ng/L in urine/oral fluid	Abdelshafi *et al* (2019) [43]	Germany

*polydimethylsiloxane (PDMS); polytetrafluoroethylene (PTFE); poly(methyl methacrylate) (PMMA); Polyethylene glycol (PEG); poly(aminoamide)-epichlorohydrin (PAE); Polyurethane/polyaniline (PU/PANI); 2-(N- ethanesulfonic acid (MES) buffer

Appendix 2.1.

Information Sheet and Consent Form (See Appendix 2.1.).

Faculty of Science and Engineering

School of Science and the Environment

Information Sheet

Name of Researcher: Lauren McNeill (PhD Student)

Name of Supervisors: Dr Kirsty Shaw (Lecturer), Dr Oliver Sutcliffe (Senior Lecturer), Dr Patricia Linton (Deputy Head of Department) and Dr David Megson (Senior Lecturer)

Title of Study: Origami Chips: paper-based Lab-on-a-Chip devices for the rapid and cost-effective detection of New Psychoactive Substances (NPS).

1. What is the purpose of this research?

For my PhD research project, I am developing a portable Lab-on-a-Chip (LOC) device for the detection of NPS and drugs of abuse, which can be used in clinical or forensic settings. In order to do this, we require urine samples that will be spiked with relevant drugs of abuse in order to test the device. There will be no information relating to health undertaken for this research. If you have any further questions, please contact myself (Lauren McNeill) via email on lauren.mcneill@stu.mmu.ac.uk.

2. What is involved?

You will be asked to voluntarily donate drug and alcohol free urine at MMU toilet facilities. You will be provided with a sterile container to deposit your urine sample in. The sample(s) will all be anonymous and ONLY used for research purposes. There will be NO health investigations undertaken with your sample(s).

3. Who can take part?

Any healthy male or female aged over 18 years old.

4. Do I need to provide consent?

By returning your completed Information Sheet and Consent Form you are giving consent for the urine provided to be used in this study. The Information Sheet and Consent Form will be treated confidentially and will be stored securely away from the sample(s). There will be a cooling off period of 24 hours between giving consent and providing a sample. You have the right to withdraw from the study or decline to donate a sample at any time, without having to provide a reason. If you decide to withdraw from the study, this will be done with immediate effect. The urine sample(s) already collected with consent will be retained and used in the study. No further data or sample(s) will be collected or any other research procedures carried out on or in relation to the participant.

5. Data Protection Act (1998) and storage

All sample(s) are confidential and anonymous. The completed Information Sheet and Consent Form will be locked away securely.

Have you read and understood the Information Sheet provided:

Name of Participant:..... **Signature:**..... **Date:** .....

Name of Researcher:.....**Signature:** ..... **Date:**.....

Investigators include: Lauren McNeill (PhD Student), Dr Kirsty Shaw (Lecturer), Dr Oliver Sutcliffe (Senior Lecturer), Dr Patricia Linton (Deputy Head of Department) and Dr David Megson (Senior Lecturer)

Faculty of Science and Engineering

School of Science and the Environment

Consent Form

Please complete both the Information Sheet and Consent Form prior to voluntarily donating a urine sample. All Information Sheets and Consent Forms will be treated confidentially. All sample(s) are confidential and anonymous. The completed Information Sheet and Consent Form will be locked away securely and will be kept separate from the urine sample to ensure anonymity. At the end of the study, the completed Information Sheet and Consent Form will be destroyed by confidential waste.

Section A: ABOUT YOU *(please **initial** in the box if true)*

1. Are you aged over 18 years old: ☐
2. I confirm that I have not taken any drugs within the last 48 hours: ☐
3. I confirm that I have not consumed alcohol within the last 24 hours: ☐
4. Are you healthy: ☐
5. I am not a member of staff or a student at MMU ☐

Section B: INFORMED CONSENT *(please initial in the box if true)*

6. Have you read the information sheet provided? ☐
7. Are you happy to donate a drug and alcohol free urine sample? ☐

Section C: INFORMED CONSENT STATEMENT

My concerns regarding this study have been answered and any further concerns I have during the time of this study will be responded to. It has been clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, that I should inform the Chair of the Ethics Committee of the School of Science and the Environment, Manchester Metropolitan University, Oxford Road, Manchester, M1 5GD.

I give my consent for the collection of urine during the course of this study.

If you have initialled in the boxes in agreement to Questions 1-6, and happy with the above statement, please sign below.

Name of Participant:..... **Signature:**..... **Date:**.....

Name of Researcher:.....**Signature:** ..... **Date:**.....

Investigators include: Lauren McNeill (PhD Student), Dr Kirsty Shaw (Lecturer) and Dr Oliver Sutcliffe (Senior Lecturer)

Appendix 5.1.

Set of instructions:

Appendix 5.2.

The written feedback from the Anonymous End-user Testing Feedback obtained from using the online survey system (Table 5.1.).

Table 5.1 *Written feedback from the non-scientifically trained individuals on using the LOC device and the instructions provided to them (their feedback was anonymised by submitting onto an online survey system)*

Question		**Numerical result (scale from 1 to 10 - not at all vs extremely)**	**Written explanation for each answer provided by each tester**
2	**Were the instructions easy to follow?**	8	Clear and detailed - although could be more concise. Ideally one sheet of A4 (front and back) rather than 3 pages.
		8	Yes the were clear to follow, but being someone that has never worked in science I did not know what certain items were. For example, an eppendorf tube - might be helpful to have a little image with the checklist to help.
		7	Yes but a bit too scientific
		8	the instructions in general were easy to follow
		7	On the whole - really good. I felt that there was still a lot of scientific wording and jargon and would be improved by a re-edit. However, the images were super clear

3	**Was the 'background' section useful?**	7	Could offer more on the different environments and situations that it can be used in
		9	Yes, gave an interesting concise background
		6	Yes but no info on amphetamine or what this device is for. I felt this would be important here.
		6	Gave background but should have stated why the actual testing was important
		7	Felt that this could have been clearer and stated more regarding the use of the lab-on-a-chip
4	**Was the 'brief overview' useful?**	8	Yes, but ideally shorter. But it did make it easier to understand the device
		7	Yes, but a little too scientific for me
		7	Yes, relevant and easy to follow
		8	Yes!
		8	yes - clear and concise
5	**Was the 'Figure 1 - 'Origami' LOC design' useful?**	9	Yes - nice and easy to follow. Could add the written bits just to this figure and condense the information from the section
		9	Yes - I understood which way round the device should orientate
		8	Really like the design, explanations and use of colour. Although it might be worth noting if the colours chosen are inclusive to all potential users, such as people that are colour blind
		9	Yes! The pic helped me orientate the chip
		9	Helpful

6	**Was 'Figure 2 - Black central detection well in the centre of the LOC design' useful?**	8	Helped me to understand a little more, but this could be explained a bit more in non-scientific language
		8	Helped me to understand a little more
		8	Helpful
		7	Yes, but some very scientific wording in the paragraph above
		9	Made me realise that there is an important in how the centre of the lab-on-a-chip works
7	**Was the 'Equipment and reagents' section useful?**	10	Perfect - cannot fault (really helped me to check what I needed and had in order to proceed)
		7	Yes but pictures/images of the equipment would have helped. But nice to be able to check I had everything
		6	This makes the instruction document longer. I get that it is helpful but perhaps this could have been condensed more.
		7	Helpful from an organisation aspect
		8	I think that removing this would make the instructions smaller (as quite long). However, on the plus side it made it easier to tally everything up
8	**Was the 'Protocol' section useful?**	8	easy to follow, but long (14 points)
		7	Yes - clear but keep having to double check everything as complicated
		6	Yes, but this could be merged with the flow chart. The use of pictures is more helpful and visual.
		7	Yes but some scientific things I didn't understand such as vortex so I had to google

		8	Yes, clear steps. Some wording was a bit too technical though
9	**Was 'Figure 3 - Positive and Negative controls' useful?**	9	Yes it helped to explain what the results meant
		9	Yes - I needed this!
		8	Definitely helped!
		9	Yes made me understand what to look for
		9	Loved. Pictures and less text definitely make it easier
10	**Was 'Figure 4 – Flow chart of protocol' easy to follow?**	8	*No answer entered*
		9	Loved - this made it so much easier
		7	Yes, but this could be merged with the protocol. The images definitely helped!
		5	On the whole I didn't think that this offered much more to the document and may be worth reducing the document and excluding this section
		9	Yes. Same as above. It makes it easy to picture. Less words and more pictures
11	**Was the 'Figure 5 – Identification' useful?**	8	helped me identify
		9	I feel confident in my answer with this helpful part
		9	Great
		9	Yes, as a clear visual guide
		10	100% clear
12	**How confident did you feel identifying sample A, B and C?**	8	I feel that as a non-scientific person - it was interesting, clear and helpful for me to identify the samples
		9	*No answer entered*
		9	I feel pretty confident with the help of the identification images
		9	*No answer entered*

		8	Confident but worry as there are so many steps that I might have done something wrong

Appendix 5.3.

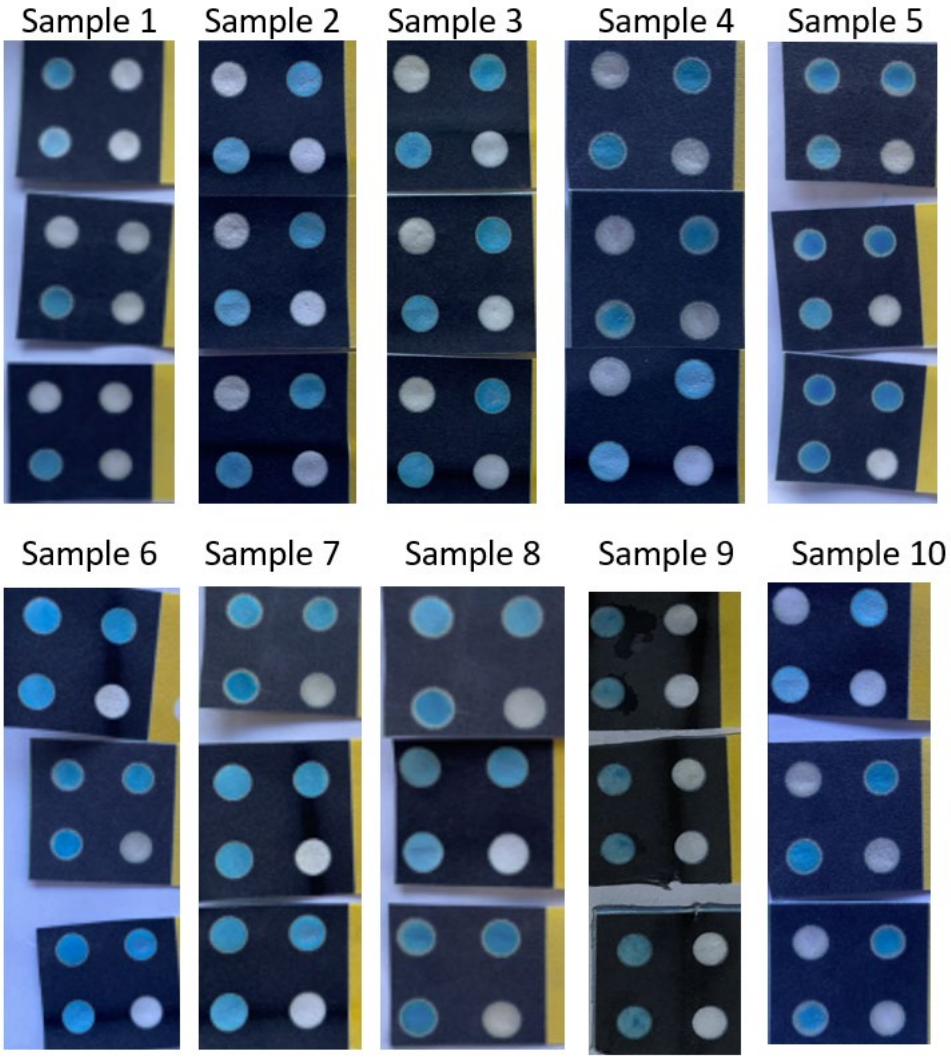
The results from testing sample 1 shows a false-negative result for the 4-CMC for one of the three replicates (shown below on Figure 5.3. top replicate is blue). For this false-negative result, the 4-CMC has not successfully competed with the labelled cathinone-HRP for the active sites of the anti-methcathinone antibody, and subsequently TMB produced a greater response for this competitive immunoassay. The replicate that produced the false-negative result indicates that there is no presence of 4-CMC present as the response from TMB is the bluest colour that can be observed (producing the greatest response).

Figure 5.3. *Image of replicates obtained for sample 1 (MDMA and 4-CMC) depicting the identification of MDMA on all three replicates and one false negative result for 4-CMC*

Appendix 5.4.

The images of the three replicates for each of the 20 seized drug samples (provided by Greater Manchester Police via MANDRAKE) that were tested using this LOC device are shown in the Figure below (Figure 5.4.A and 5.4.B).

Figure 5.4.A: *Images collected from samples 1-10 of the blind testing samples (in triplicate)*



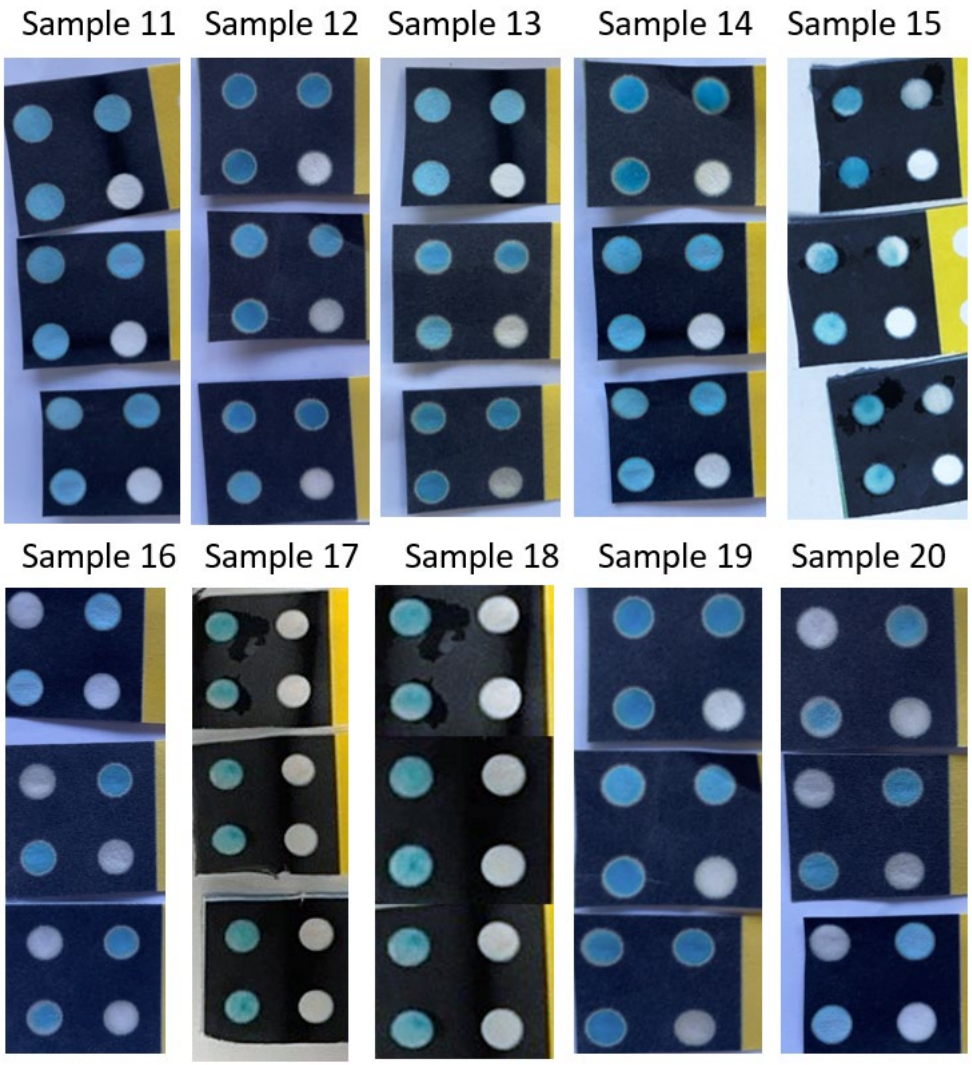
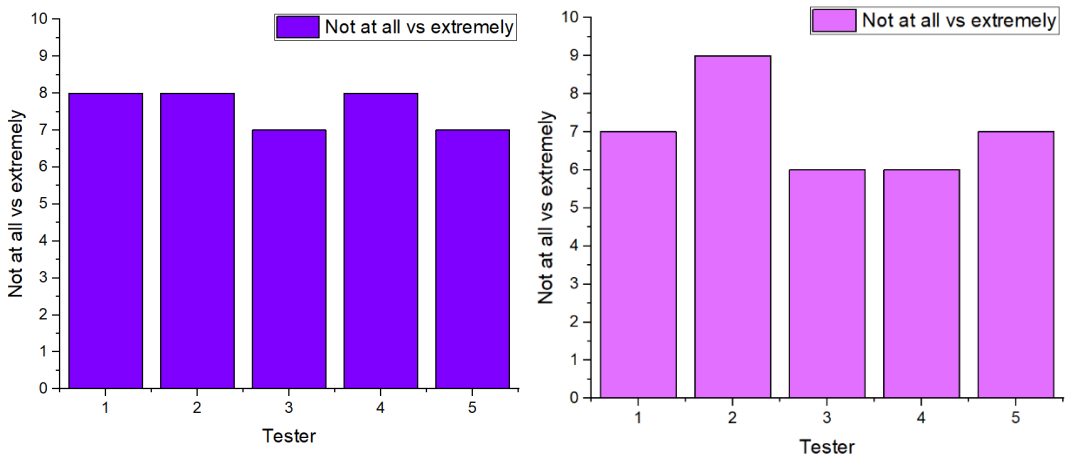


Figure 5.4.B: *Images collected from samples 11-20 of the blind testing samples (in triplicate*

Appendix 5.5.

Figures for each question asked in the Anonymous End-user Testing Questionnaire (Figure 5.5A-5.5C).



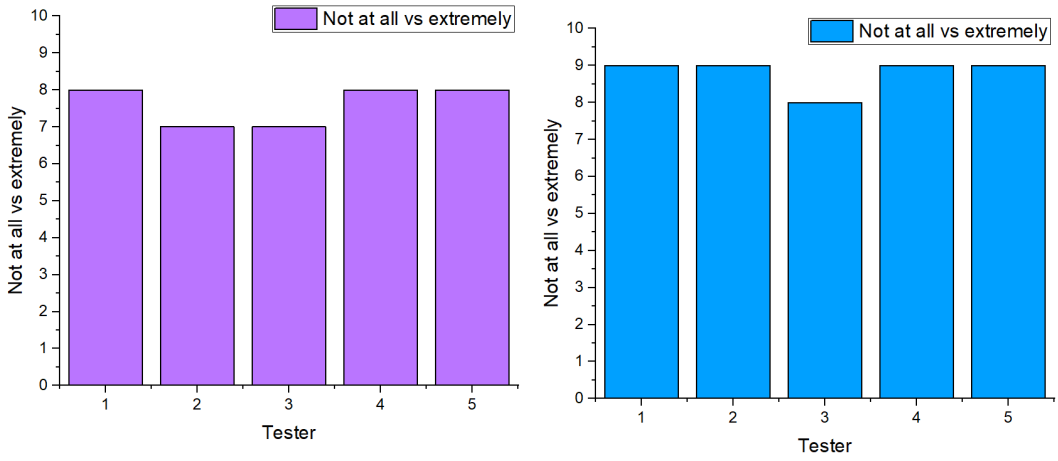


Figure 5.5.A. *Represents the numerical data obtained for Questions 1 to 4 obtained via the anonymous online survey system (A) Question 2. Were the instructions easy to follow? (B) Question 3. Was the 'background' section useful? (C) Question 4. Was the 'brief overview' useful? (D) Question 5. Was the 'Figure 1 - 'Origami' LOC design' useful?*

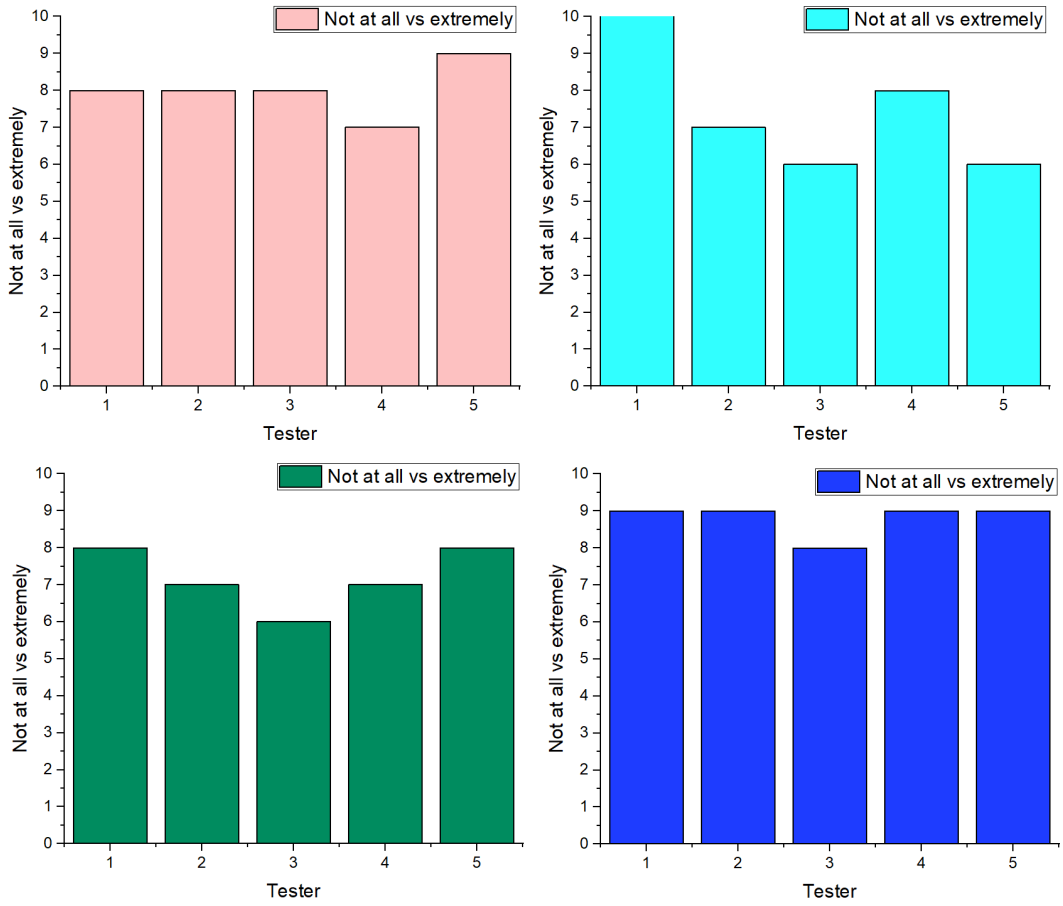


Figure 5.5.B. *Represents the numerical data obtained for Questions 5 to 8 obtained via the anonymous online survey system (A) Question 6. Was 'Figure 2 - Black central detection well in the centre of the LOC design' useful? (B) Question 7. Was the 'Equipment and reagents' section useful? (C) Question 8. Was the 'Protocol' section useful? (D) Question 9. Was 'Figure 3 - Positive and Negative controls' useful?*

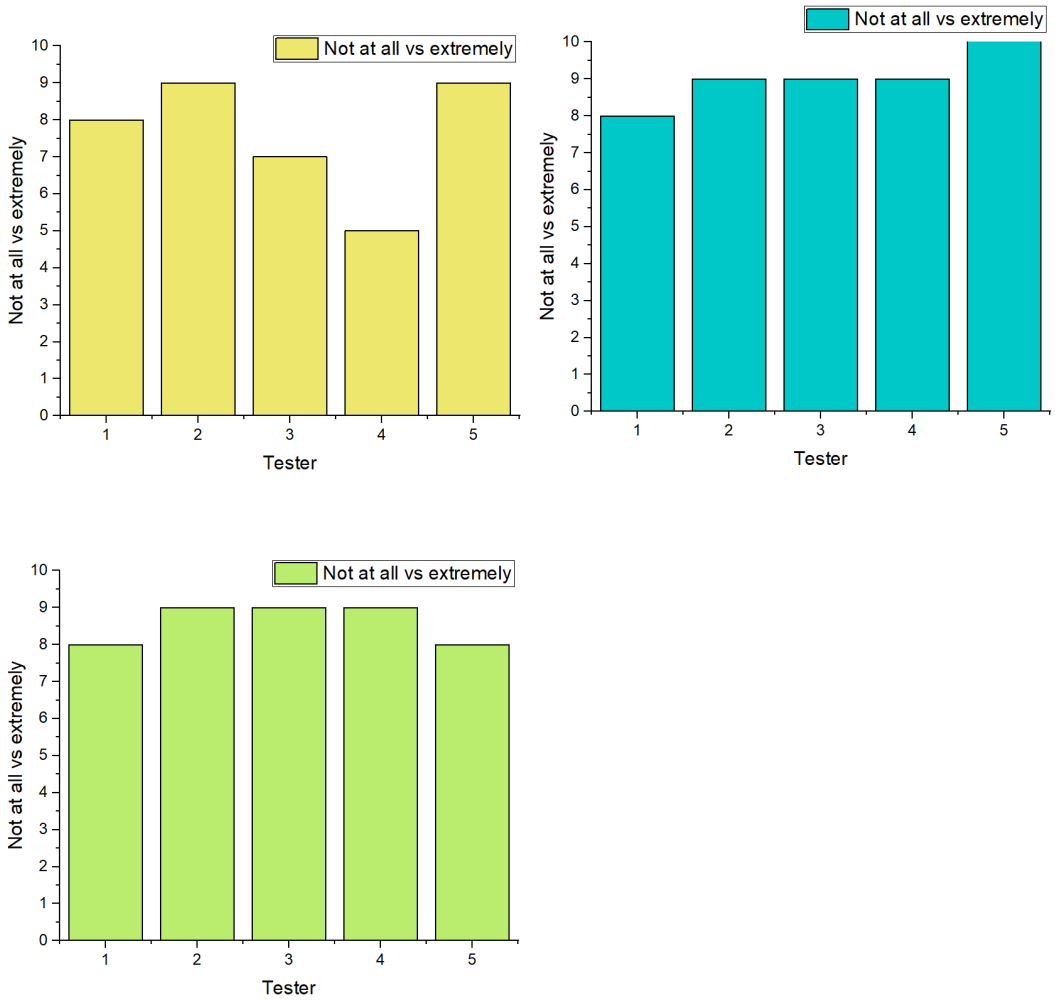


Figure 5.5.C. *Represents the numerical data obtained for Questions 9 to 11 obtained via the anonymous online survey system (A) Question 10. Was 'Figure 4 – Flow chart of protocol' easy to follow? (B) Question 11. Was the 'Figure 5 – Identification' useful? (C) Question 12. How confident did you feel identifying sample A, B and C?*

Appendix 6.1.

The cost of using this LOC device is less than 50p (approximately 47p) per test. The cost breakdown for this is provided in Table 6.1. The LOC designs were printed using a Xerox Phaser 8500 Solid Ink printer. The wax cartridges were melted when printing the design onto chromatography paper [Fisherbrand, UK]. All the reagents and components have been included.

Table 6.1: Summary information on costings and breakdown of price for test using the LOC device.

Manufacturing component of LOC device or materials	Component	Costing and notes	Cost per device
Paper	Whatman Grade 501 200 x 200 mm paper	£98.05 for 100 sheets	0.245125p (however rounded to 25p per LOC device)
	Wax cartridges	Black: £104.01 (4 pack) 8600 pages at 1.21p per page. Blue, Magenta and Yellow: £117.99 for each colour (2 pack) 4400 pages at 2.68p per page. Two LOC devices printed per page using a combination of colours.	4.625p (however rounded to 5p per LOC device)
	Chitosan	£59.40 for 25g - 5 µL of 0.250 mg mL^{-1} chitosan. 25g = 25000 mg. Therefore, 0.25 mg mL^{-1} chitosan. Due to dilution and extremely small quantities per LOC device required the cost per LOC device is less than 1p per LOC device. Only 5 µL x 4 for each of the black central wells for each device.	<1p (rounded to 1p for costings)
	Glutaraldehyde	£27.50 for 25 mL - 5 µL of 2.50% glutaraldehyde. Due to dilution and extremely small quantities per LOC device required. Only 5 µL x 4 (of a 2.5% solution) for each of the black central wells for each device.	<1p (rounded to 1p for costings)
	PBS	£73.20 for 50 tablet – 1 tablet per L (at £1.46 per litre). Per LOC device, extremely small quantities of PBS required the cost per LOC device is less than 1p per LOC device.	<1p (rounded to 1p for costings)
	Tween	£19.2 for 10 mL - 0.05% PBS-Tween solution. Due to dilution and extremely small quantities per LOC device required the cost per LOC device is less than 1p per LOC device.	<1p (rounded to 1p for costings)
	Milk powder	£4 for 175g. 1% semi-skimmed milk powder solution required, which was made in small quantities for each use. Due to dilution and extremely small quantities per LOC device required the cost per LOC device is less than 1p per LOC device.	<1p (rounded to 1p for costings)
	TMB	£96 per 100 mL. 20 µL of TMB required for each LOC device – therefore 100 mL = 100000 µL, (96/100000)/20 (per LOC device) = 0.0192 x 100 from pounds to pence.	1.92p (rounded to 2p for costings)
Antibodies	Anti-methcathinone antibody	£299 for 50 µL – 1:1250 dilution. 50 x 1250 = 62500. (299/62500)x100 (pounds into pence) = 0.4784p x 4 (µL) = 1.9136 p	1.9136 p (rounded to 2p for costings)
	Anti-amphetamine antibody	£299 for 500 µL -1:16000 dilution. 500 x 16000 = 8000000. (299/8000000)x100 (pounds into pence) = 0.0037375p x 4 (µL) = 0.01495 p	<1p (rounded to 1p for costings)
	Anti-HRP antibody	£299 for 50 µL- 1:20000 dilution. 50 x 20000 = 1000000. (299/1000000)x100 (pounds into pence) = 0.0299p x 4 (µL) = 0.1196 p	<1p (rounded to 1p for costings)

HRP conjugation	HRP kit	£299 for 3 kits. Only 2 x kits required – one for amphetamine-HRP and one for cathinone-HRP conjugations. Amphetamine-HRP used at a concentration of 1:1000 dilution. Cathinone-HRP used at a concentration of 1:250. Each of the 3 kits is equivalent to £100 that gives ~100 µL of concentrated conjugate solution. For cathinone-HRP (dilution 1:250): 100 µL x 250 = 25000. £100/25000 = 0.004 x 100 (into pence) = 0.4p x 12 = 4.8p For amphetamine-HRP (dilution 1:1000): 100 µL x 1000 = 100000. £100/100000 = 0.001 x 100 (into pence) = 0.1p x 4 = 0.4p	4.8p (rounded to 5p for costings) for cathinone-HRP 0.4p (rounded to 1p for costings) for amphetamine-HRP
Total			**47p in total per device**