THE DETECTION OF NEW PSYCHOACTIVE SUBSTANCES USING MICROFLUIDIC DEVICES

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Abstract

Due to revisions in certain drugs laws and tightening in regulations, there has been a rise in the amount of new psychoactive substances entering the market. In order to combat this increase, investigations have begun into creating new and effective portable devices that are capable of producing rapid positive or negative responses for the presence of these types of drugs.

The main aim of this project was to convert a basic enzyme linked immunoassay onto a microfluidic device in order to create a system for detecting mephedrone and it metabolites. Small detection zones, capable of containing micro volumes of reagent, were created using liquid wax on analytical grade filter paper before being optimised using a glutaraldehyde activated chitosan surface. Using these wax printed microfluidic paper analytical devices (μPADs) and an enzyme-linked immunoassay it has been possible to detect a horseradish peroxidase (HRP) labelled cathinone sample within aqueous and biological media.

The same method was then applied to the detection of mephedrone and was successfully able to detect mephedrone in urine at clinically relevant concentrations, giving an LOD 4.078 μg/mL of and an LOQ of 1.597 μg/mL. This method has also shown positive results in detecting and distinguishing between mephedrone, methcathinone, cathinone, ephedrine and 4-methylephedrine. As well as being robust enough to be able to differentiate between the cathinones it also proved highly selective, producing negative responses for other illegal drugs, such as cocaine and ketamine, as well as legal drug and potential cutting agents, such as paracetamol and caffeine.

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Abbreviations

APTES	3-aminopropyl-treiethoxysilane
APTMS	3-aminopropyl-trimethylsilane
BZP	Benzylpiperazine
CBC	Cannabichromene
CBD	Cannabidiol
CBG	Cannabigerol
CBN	Cannabinol
Cl^-	Chlorine Ion
Cu^{2+}	Copper Ion
DBZP	1,4-Dibenzylpiperazine
EEWS	European Early Warning System
ELISA	Enzyme Linked Immunosorbent Assay
F^+	Fluorine Ion
F_{ab}	Antigen binding region
F_c	Constant region
GC	Gas Chromatography
GHB	Gammahydroxybutrate
µg/mL	Microgram per millilitre
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
IgG	Immunoglobulin
µL	microlitre
µL/min	microlitre per minute
LC-HR-QTOFMS	Liquid Chromatography-High Resolution-Quadrapole Time of Flight Mass Spectrometer
LOD	Limit of Detection
LOQ	Limit of Quantification
LSD	Lysergic Acid Diethylamide
M	Molar
mCPP	3-Chlorophenylpiperazine
mL	millilitre

mM	millimolar
MDMA	3,4-methylenedioxy-methamphetamine
mm	millimetre
MS	Mass Spectrometry
ng/mL	nano gram per millilitre
Na^+	Sodium Ion
NH_4^+	Ammonium Ion
nM	nanomolar
NO_3^-	Nitrate Ion
NP	Normal Phase
NPS	New Psychoactive Substances
µPAD	Microfluidic Paper Analytical Device
PBS	Phosphate Buffered Saline
PCP	Phencyclidine
PDMS	polydimethylsiloxane
PEG	polyethylene glycol
pg/mL	pico gram per millilitre
PEI	poly(ethylenemine)
PMMA	poly(methyl methacrylate)
PVA	poly(vinyl alcohol)
RP	Reverse Phase
THC	Tetrahydrocannabinol
TMB	3,3’,5,5’-tetramethylbenzidine
UNODC	United Nations Office on Drugs and Crime

Chapter 1– Introduction

1.1.Misuse of Drugs Act (1971)

In 1971 the UK Parliament passed the Misuse of Drugs Act in order to control the possession and supply of dangerous and harmful drugs, both those with a medicinal use, which are to some extent controlled under the Medicines Act (1968) but also those without a current medical use. The drugs that are classified under the Misuse of Drugs Act are known as controlled drugs and are grouped into three different classes based on their social harm, physical harm and the degree of dependence associated with long-term use of the drug. Each class carries a series of penalties that match the level of harm associated with that type of drug and potential offence attributed to it (Table 1.1).[1]

Table 1.1: Penalties for the supply, possession and trafficking of illegal substances.[1]

Class	Drugs Included	Possession	Supply and Possession with intent to supply
Class A	heroin, cocaine, MDMA, LSD, psilocybin mushrooms, methadone, any Class B drug that has been prepared to be injected	7 years imprisonment and an unlimited fine	Life imprisonment and an unlimited fine
Class B	cannabis, ketamine, amphetamine, codeine, barbiturates	5 years imprisonment and an unlimited fine	14 years imprisonment and an unlimited fine
Class C	GHB, anabolic steroids, minor tranquillisers, benzodiazepines, khat.	2 years imprisonment and an unlimited fine	14 years imprisonment and an unlimited fine

Due to the vast array of drugs classified under the Misuse of Drugs Act (1971) and their varying uses and abilities, the Misuse of Drugs Act was revised in 2001 in order to better regulate the possession and supply of drugs that carry useful medicinal abilities (Table 1.2).

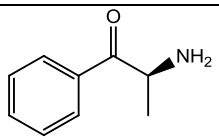
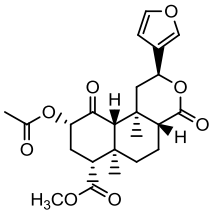
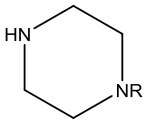
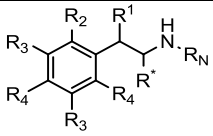
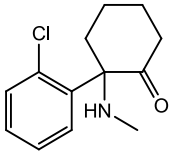
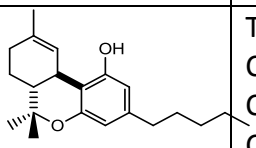
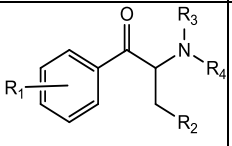
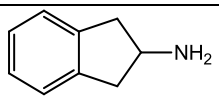
Table 1.2: Classification of illegal substances by schedule.[1]

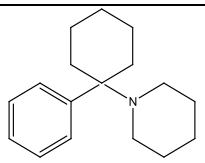
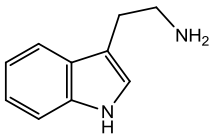
	Drugs Included	Controls Associated
Schedule 1	cannabis, raw opium, coca leaf, psilocin, LSD, ecstasy	Can only be supplied, possessed or administered in exceptional circumstance with a Home Office license.
Schedule 2	amphetamines, methadone, heroin, morphine, medicinal opium, cocaine, dihydrocodeine.	Possession is illegal unless prescribed by a doctor. Subject to strict record keeping and storage.
Schedule 3	barbiturates, rohypnol, temazepam	Subject to restrictions on prescription writing
Schedule 4	Part 1- minor tranquillisers Part 2- anabolic steroids	Part 1- Possession is illegal without a prescription Part 2- Possession is legal without a prescription
Schedule 5	mild painkillers, cough medicines	Sold over the counter without a prescription

1.2.Legal Highs-New Psychoactive Substances

Due to the revisions in the Misuse of Drugs Act (1971) access to certain precursor drugs became a lot more tightly regulated resulting in a decline in the quality of several substances. In order for drug users to induce the same effects they were either required to take a higher dose of the street form or find new or alternative ways of reaching the same high; both options could result in potentially fatal outcomes. In order for suppliers to meet these demands a range of new psychoactive substances began to appear on the market (Table 1.3).

Table 1.3: Drug structure, synthesis and pharmacology of the 'new psychoactive substances' as outlined by the United Nations Office on Drugs and Crime (UNODC).[2]

New Psychoactive Substances	Structure	Examples	References; Synthesis and Pharmacology
Plant Based Substances	 **1**  **2**	Khat (cathine) [3] *Salvia divinorum* (salvinorin A) [4]	Botany (khat) [5] Pharmacology (khat) [6] Botany (salvinorin A) [7] Pharmacology (salvinorin A) [8]
Piperazines	 **3**	Benzylpiperazine (BZP)[9] 1,4-Dibenzylpiperazine (DBZP) 3-Chlorophenylpiperazine (mCPP)	Synthesis (piperazines)[10] Pharmacology (piperazines)[11]
Phenethylamines[12]	 **4**		Synthesis [13] Pharmacology [14]
Ketamine [15]	 **5**		Synthesis [16] Pharmacology [17]
Synthetic Cannabinoids [18]	 **6**	Tetrahydrocannabinol (THC) Cannabinol (CBN) Cannabidiol (CBD) Cannabigerol (CBG) Cannabichromene (CBC)	Synthesis (cannabinol) [19] Pharmacology (cannabinol) [20]
Synthetic cathinones [21]	 **7**	4-Methylmethcathinone (Mephedrone) 4-Fluoromethcathinone (Flephedrone) Ethcathinone Benzedrone	Synthesis (mephedrone) [22, 23] Pharmacology (mephedrone) [24]
Stimulants	 **8**	Aminoindanes **[25]**	Synthesis [26] Pharmacology [26]

Hallucinogens	 **9**	Phencyclidine (PCP) [27]	Synthesis [29] Pharmacology [30]
	 **10**	Tryptamines [28]	Synthesis [31] Pharmacology [32]

1.3.Cathinones

The nature of drugs laws mean that the rules and regulations around a specific compound are often very specific and are based around the banning of a specific chemical structure. In order to get around these drugs laws, derivatives of already banned substances began to appear. In replacing one functional group for another, an already illegal substance would once again be classified as 'legal' without drastically changing the overall effect of the drug.[22] Cathinones are chemical derivatives of methcathinone, which is a psychoactive stimulant already classified under the Misuse of Drugs Act (1971) as a class B substance, and very closely related to methamphetamines.[1] In 2009 there was a significant rise in the popularity of new psychoactive substances (NPS) (formally known as "legal highs") as they were cheap and easily accessible however the results were often fatal.[33] In order to control the situation, methods were taken to identify, classify and ban these substances however this often lead to variations and adaptations being introduced (Figure 1.1).[34] With the advances in analytical techniques over the years it meant that the majority of the new substances on the market were quickly identified enabling the known substances to new substance ratio to change.[34]

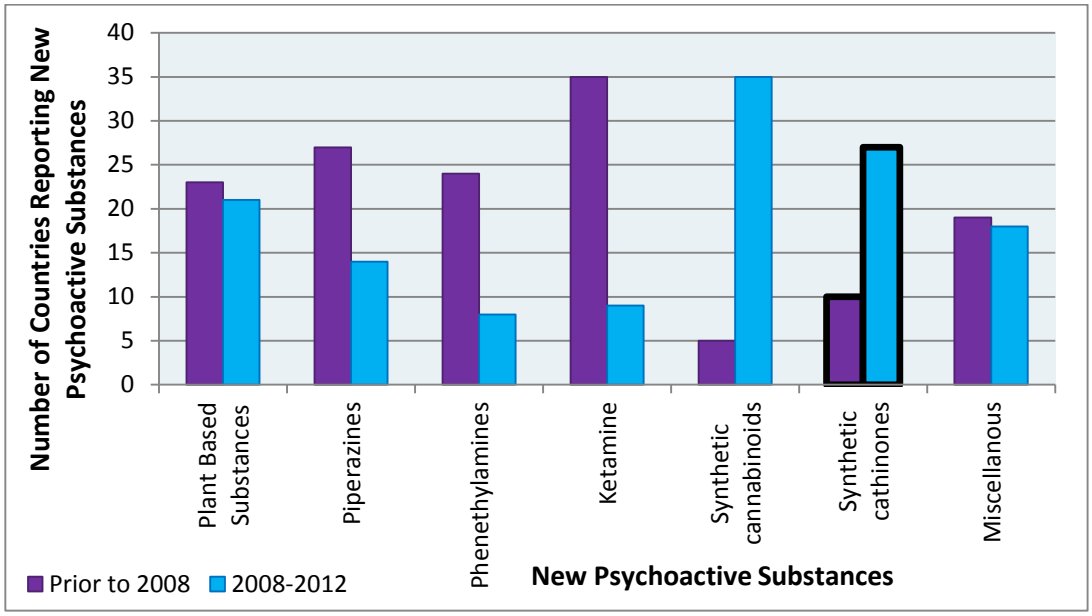
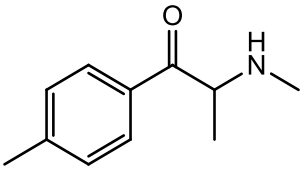


Figure 1.1: Representation of the data for the Global emergence of New Psychoactive substances according to the United Nations Office on Drugs and Crimes questionnaire on New Psychoactive Substances 2012 highlighting the increase in synthetic cathinones over recent years reproduced with permission.[34]

1.4. Mephedrone

Mephedrone (4-methylmethcathinone) is a powerful stimulant and is most commonly found as a white tablet or powder. Mephedrone (Figure 1.2) was first synthesised in 1929 but remained a product of academia until its rediscovery and appearance on the drug market in early 2003.[35] Though mephedrone was available on the market from 2003, there is limited data around its popularity and predominance prior to 2008 when it was first reported to the European Early Warning System (EEWS) due to increasing concerns over its effects.[35] As mephedrone was new on the market it was unregulated and therefore did not appear in any drug reports prior to 2012. The use of new psychoactive substances more than doubled between the period of 2009-2012 triggering investigations into substances such as mephedrone.[36] With mephedrone being categorised under the umbrella term New Psychoactive Substances (NPS) (formally known as “legal highs”) it quickly became one of the four most popular street drugs in the UK, due to it being relatively cheap and easily accessible, mephedrone was widely available online and in head shops being sold as ‘plant food’ and ‘bath salts’.[37]

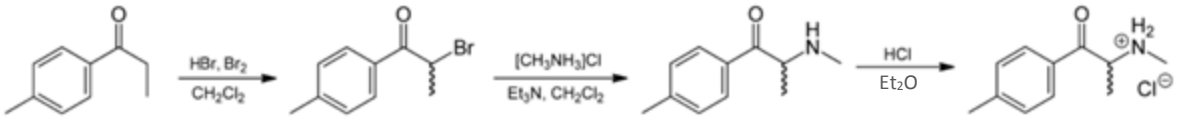


11

Figure 1.2: Mephedrone (4-methylmethcathinone, 4-MMC, 4-methylephedrone).[38]

1.4.1 Synthesis of mephedrone

Mephedrone (**11**) is synthesised (Figure 1.3) *via* an alpha-bromination of 4-methylpropiophenone (**12**) followed by a methamination of 4-methyl-2-bromopropriophenone producing 4-methylmethcathinone (**11**). The final product is isolated as it's corresponding hydrochloride salt, 4-methylmethcathinone hydrochloride (**14**).[39]



12 **13** **11** **14**

Figure 1.3: The synthesis scheme for 4-methylmethcathione as reported by Santali et al.[39]

1.4.2 Pharmacology of mephedrone

Mephedrone is considered a psychostimulatory drug similar to that of MDMA and cocaine, inducing effects such as increased energy and awareness, a sense of euphoria, heightened sensory experiences, improved mood and sociability.[40] Due to the relatively new nature of mephedrone, the short time between its appearance on the drugs market and its outlaw in 2010, few, if any, full clinical trials have been carried out into its pharmacology.[21] However, as it has a close structural similarity to amphetamines, MDMA and other cathinone derivatives, it is also believed to induce its effect through disruption of the central monoamine systems. It both stimulates and inhibits the monoamine neurotransmitters causing it to produce excessive amounts of serotonin, norepinephrine and dopamine but preventing the reuptake of these hormones resulting in increased synaptic concentrations.[41] As mephedrone has a chiral α-carbon it exists as both the *S*- and *R*-enantiomers but is generally found as a racemic mixture of the two. Recent studies have shown that the *R*-enantiomer of mephedrone, like amphetamine and cathinone, is more potent and results in a greater dopamine release. It has also been shown, that while

the *R*-enantiomer is largely responsible for the dopamine transmission the *S*-enantiomer is able to reduce this rewarding effect and decrease the withdrawal response.

1.5. Analytical Techniques

There are several different methods by which biological samples can be analysed for the presence of illicit substances. Routinely, presumptive drugs test are carried out on urine and saliva samples as they can be easily collected via a non-invasive process. The preferred method for initially testing a biological sample is to run an immunoassay,[42] this is due to the high selectivity and extremely low limits of detection allowing for even low concentrations of compounds to present a positive response. Another advantage of immunoassays is that the samples require little to no preparation prior to running the test allowing for a more precise determination of whether a specific compound is present.[43]

When carrying out tests involving illicit substance such as 'legal highs' there are two main categories of sample that can require analysis, dry samples such as powders or tablets and wet samples such as blood or urine samples. With either category it is possible to have either known, synthesised or spiked samples and unknown, seized samples. The sample must first be made aqueous in order to be able to undergo an immunoassay test. When screening unknown samples it is important to include a positive control in which to compare the result against, this will indicate that the test is working correctly allowing more confidence in the result achieved and a negative control to rule out contamination.

Generally immunoassays are considered presumptive tests and are the first step in testing seized samples. Due to the nature of these types of samples it is likely that there will be a number of other substances and impurities, such as other drugs and cutting agents, present within the sample.[24] Depending on the types of other compounds present it could be possible for them to react with in the immunoassay producing an inaccurate result.[44] Biological samples may also contain natural inhibitors that result in nonspecific protein binding leading to a false positive; they may also contain metabolites of both the drug in question and other possible substances present that due to structural similarities could also affect the result.

Previous studies into the pharmacokinetics of mephedrone using both plasma and urine samples in human and animals studies have shown that 4-methylmethcathinone (Mephedrone), 4-methcathinone (*nor*-mephedrone), 3-hydroxy-4-methylmethcathinone, 4-carboxylmethylmethcathinone, 4-hydroxymethylmethcathinone (Figure 1.4) and their isomers are the key compounds present after the metabolism of mephedrone.[45] Studies have shown that mephedrone has a relatively low bioavailability compared with other drugs of a similar nature, it is also subject to first pass metabolism.[46] When taken orally the drug is absorbed by the gastrointestinal tract and metabolised in the liver preventing it from being distributed throughout the body, further reducing its bioavailability and explaining why mephedrone is traditionally snorted as opposed to swallowed.[47] It is believed that mephedrone (**11**) undergoes phase I metabolism, proceeding via an *N*-demethylation to produce 4-methcathinone also known as nor-mephedrone (**15**). It also undergoes various oxidation reactions including aliphatic, aromatic and allylic hydroxylation to produce 3-hydroxy-4-methylmethcathinone (**16**), hydroxyl-4methylmethcathinone (**17**) and 4-hydroxymethylmethcathinone (**18**) respectively. Secondary metabolites have also been detected such as 4-carboxylmethylmethcathinone (**19**) which is produced from the oxidation of 4-hydroxymethylmethcathinone (**18**). Due to the minimal changes in the structures of the various compounds and the similarities in their chemical nature it is possible that the metabolites could cross-react with the mephedrone-antibody within the immunoassay presenting a stronger positive reaction and indicating a higher concentration of mephedrone than is truly present within the sample.

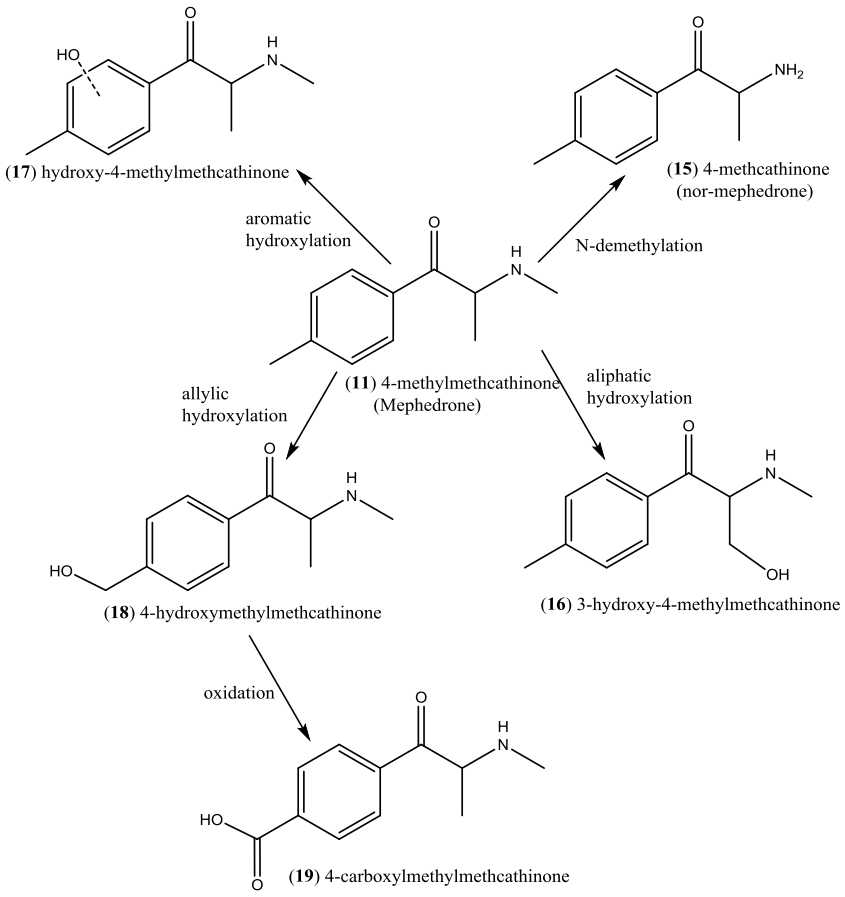


Figure 1.4: Metabolites and metabolic pathways of mephedrone.[46]

In order to establish the accuracy of the presumptive result and enable the determination of the exact compounds and concentrations present within the sample. Confirmatory tests such as GC-MS or HPLC would normally need to be performed.

1.5.1 Immunoassays

Immunoassays are a bioanalytical technique that measures the concentration of an analyte or antigen present within a sample through the use of specific antibodies. The antigen/antibody reaction is a mechanism that has been adapted from nature and works on a 'lock and key' principle (Figure 1.5).[47] Antibodies are produced by plasma cells within the body and recruited by the immune system in order to fight off any foreign objects present.[47] All antibodies have a similar Y shaped structure however each different type of antibody has a very unique binding site which is specific to its target.[42] Only the analyte or antigen specific to that antibody is able to bind to the active site (F_{ab} antigen binding region).

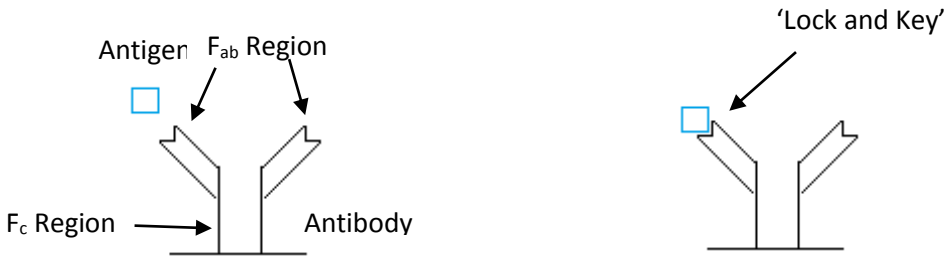


Figure 1.5: Antigen to antibody binding, 'lock and key' mechanism.

As antibodies are a biological response for when the body is under attack from foreign objects in order to create a useable supply of them the analyte must first be injected into a host triggering an immune system response and allowing a target specific antibody to be extracted.[48] Though antibodies are considered target specific it is often possible for them to cross-react, resulting in the binding site being able to accommodate the binding of similar types of analytes or metabolites, for instance the mephedrone specific antibody would be able to cross-react with other cathinone derivatives as well as binding to the mephedrone.[47] Though other cathinone derivatives would not be a perfect fit into the active site of the mephedrone antibody due to the similarity in size, structure and activity it will still successfully bind should it be present in the sample.[44] As the antibody is specific to the functional groups on the cathinone derivatives it allows for selectivity within this class enabling distinguishability between other drug classes.

1.5.1.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Though immunoassay is the preferred biological method of analysis there are several different types of immunoassays that can be adopted in order to detect specific analytes, the type of assay chosen is dependent on the type of analyte in question and the requirements of the analysis as certain methods will allow for a higher level of sensitivity.[47] Enzyme-Linked Immunoassays tend to be favoured over other types of assays such as those labelled with radioactive isotopes. Enzyme-Linked labels carry a longer shelf life and are more widely available than other variations; they also pose no risk to health and produce an almost instantaneous positive or negative response.[43] Though further testing is required in order to get a quantitative or qualitative result the instrumentation is relatively cheap and widely accessible compared to those required to detect radioactive isotopes or electrochemical tags.

1.5.1.2 Sandwich Immunoassay

Depending on the analyte and sample in question there are different methods that can be adopted, sandwich immunoassays are the most frequently used as they tend to be more sensitive and robust. Initially a highly selective primary antibody, specific to the antigen in question is adhered to the solid surface.[42] Often the second step involves blocking in order to prevent any unwanted analytes adhering to the solid surface. The sample containing the antigen or analyte is added followed by a second antibody, known as the detection antibody due to the presence of a chemically attached label (Figure 1.6).[49] Most commonly used labels are compounds such as horse radish peroxidase (HRP) which in the presence of 3,3',5,5'-tetramethylbenzidine (TMB) results in a colourimetric detection. The stronger the positive response the higher the concentration of antigen present.[42] Over recent years and with technological advances in certain types of plate readers chemiluminescent substrates and fluorophores are becoming more popular due to their increased sensitivity and ability to further amplify the signal.

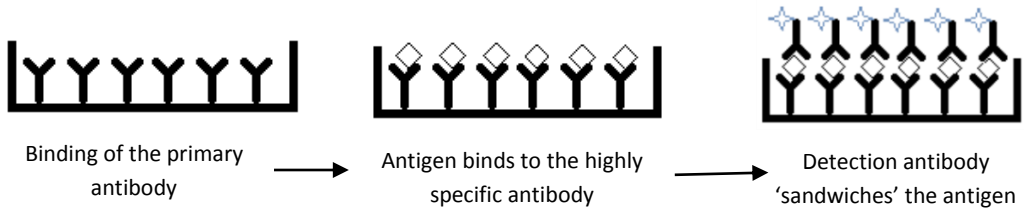


Figure 1.6: Schematic representation of a 'sandwich' immunoassay.[49]

1.5.1.3 Competitive Immunoassay

As a lot of the 'legal highs' on the market at the moment are relatively new and often subject to small structural changes, therefore they require specially manufacture antibodies, specific to that class. In cases where this occurs, a competitive immunoassay is often adopted as it only requires one form of the antibody and uses a sample that contains a known amount of labelled and a variable amount of unlabelled antigens (Figure 1.7). As the concentration of unlabelled antigens increases due to there being a limited number of binding sites the concentration of labelled antigens decreases resulting in a lower detection response.[49]

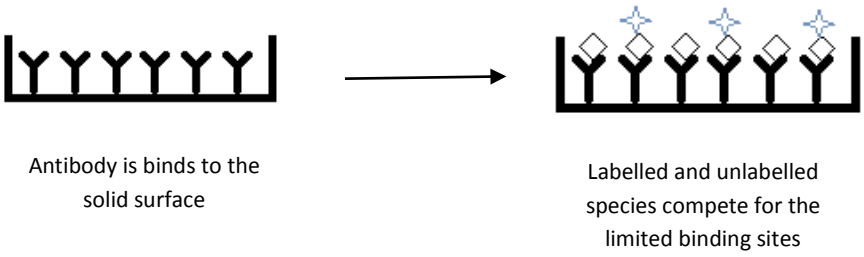


Figure 1.7: Schematic representation of a competitive immunoassay.[49]

Immunoassay tests are used as a means of screening samples for possible substances and are not relied upon to draw conclusions. In order to better understand the immunoassay result and develop a conclusion further confirmatory testing is required.[47]

1.5.2 Gas Chromatography Mass Spectrometry (GC-MS)

The most commonly used and highly reliable confirmation test is that of gas chromatography mass spectrometry (GC-MS). This technique has been applied to detection and quantification of various synthetic cathinones and new psychoactive substances both in street samples and biological matrices.[50-54] This is a combination of analytical methods, the GC is used to separate the compounds within the sample based on their chemical properties whereas the MS is used to determine the physical properties of the ion based on a specific molecular weight and will produce a fragmentation pattern for the molecule present. The sample is injected via a split/splitless injection port where it is vaporised by an inert carrier gas, the gas then carries the sample along the column. The type of the analyte will determine the choice of medium used to pack the stationary phase contained within the column. For a polar molecule such as mephedrone, a polar stationary phase would be employed, the more polar the compound the longer the retention time. The stationary phase of a GC is contained within an oven, the temperature of the oven is directly proportional to the rate at which the sample passes through, by varying the temperature of the oven or using a specific temperature programme this allows for elution of the compounds within an optimal range. Once all the compounds within the sample have been separated the sample is injected directly into the MS. The compounds are bombarded with ions in order to displace an electron and create positive ions, these ions are then accelerated towards a magnetic field.[55] The magnetic field will be set to select a specific mass to charge ratio depending on the analyte in question. Only the ions with this specific mass to charge ratio will pass through

the magnetic field all others will be deflected. The ions that successfully pass through the magnetic field will go on to the detector which records the charge induced or the current produced as the ions pass by it.[55]

Though GC is a widely used technique and a good method for the separation and analysis of certain compounds it is limited to those that are volatile or can be made volatile. Compounds such as mephedrone (and other cathinones) must first be derivatised prior to being injected. The derivatised forms of these substances are unstable and the high temperatures with in the GC oven can result in these compounds undergoing thermal degradation causing significant deformation of the peaks.[56]

In order to eliminate the problems associated with GC-MS other techniques such as high performance liquid chromatography mass spectrometry have been applied as the analytes do not require derivatisation prior to being injected.

1.5.3 High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS)

Another common and highly reliable technique in the separation of compounds within a sample is High Performance Liquid Chromatography (HPLC). This technique has been successfully applied, by several research groups, to the detection and quantification of a range of new psychoactive substances including several synthetic cathinones, with some particularly focusing on mephedrone.[50-53] There are a variety of different types of HPLC in which the stationary phase and mobile phase change depending on the chemical properties of the analyte in question (Table 1.4).[57] A high pressure pump is used to continuously pump the mobile phase through the system and a small amount of the sample is injected into the steam of mobile phase. The continuous flow of mobile phase allows the sample to be carried onto the column, the strength of the interactions between the column and analytes will determine the elution pattern and retention times of the analytes. In a similar way to a GC being coupled with an MS so can a HPLC, however in order for the separated sample to pass from the HPLC to the MS it must pass through a specialised interface in order eliminate the liquid medium. The solution is flowed through an electrospray needle that has a high potential difference applied across it, forcing a spray of charged droplets away from the needle and towards a counter electrode. As the solution passes from the needle to the electrode the solution is evaporated leaving only

the charged analytes.[58] The charged analytes are then bombarded with ions in order to create positive ions that can be accelerated through the magnetic field and onto the detector.

Table 1.4: Different separation modes available for high performance liquid chromatography.[57]

Separation Modes	Elution	System	Analytes
Normal Phase (NP)	The more polar the stationary phase the stronger the attraction to polar compounds. The least polar compound elutes first.	Polar Stationary Phase; Silica Non-polar Mobile Phase; Hexane	Non-polar; Hydrocarbons Aromatics Fluorinated
Reverse Phase (RP)	The less polar the stationary phase the stronger the attraction to non-polar compounds. The most polar elutes first.	Non-polar Stationary Phase; C_{18} ODS Polar Mobile Phase; Water	Polar; Alcohols Acids
Size Exclusion	Small molecules penetrate multiple pores as they pass through the column. The larger molecules elute first.	Polymeric gel	Compounds with varying molecular weights.
Ion Exchange	The greater the charge the stronger the attraction. The ions with the smallest charges will elute first.	Cationic; Sulphonic/Carboxylic acid Anionic; Quaternary ammonium/ secondary amine	Positive ions; Na^+ Cu^{2+} NH_4^+ Negative ions; F^- Cl^- NO_3^-
HILIC	The more polar the stationary phase the stronger the attraction to polar compounds. The least polar compound elutes first.	Polar Stationary Phase; Silica Mobile Phase; Acetonitrile and water	Non-polar; Hydrocarbons Aromatics Fluorinated

Though GC-MS is currently a more commonly used technique, with the changing face of the drugs market and the substances arising HPLC-MS has its advantages, as it is able to analyse a much wider range of compounds.[58]

1.6. Microfluidic Devices

Due to the effective nature of enzyme-linked immunoassays and the range of different industries that are able to apply it as an analytical technique there has been a great deal of research over recent years into ways that it can be further improved.[59] Microfluidics is a multidisciplinary application that generally involves taking well established techniques and scaling them down by applying them to microsized devices. Microfluidics has proven to be a powerful technological tool in improving the performance of immunoassays as they vastly reduce the volume of reagents required as everything is done on a microscale.[60] The reduction in the size of the device means that even though the volume of reagents being used is a lot smaller, the surface to volume ratio is greatly increased, resulting in much shorter incubation times and a reduction in the overall reaction time. This reduction in size provides a great deal of advantages over several already established techniques as well as some disadvantages (Table 1.5).

Table 1.5: Advantages and disadvantages of microfluidic devices. [61-63]

Advantages	Disadvantages
-Low fluid volumes consumption and fabrication costs. -Faster analysis and response times due to short diffusion distances, fast heating, high surface to volume ratios, small heat capacities. -Compactness of the systems due to integration of much functionality and small volumes. -Better process control (e.g. thermal control for exothermic chemical reactions). -Safer platform for chemical, radioactive or biological studies because of integration of functionality, smaller fluid volumes and stored energies.	-Novel technology, not yet fully developed. -Physical and chemical effects i.e. capillary forces, surface roughness, chemical interactions, become more dominant on small-scale. -Detection principles may not always scale down in a positive way i.e. low signal-to-noise ratios.

An example device is shown in Figure 1.8 (described in more detail in section 2.2.1), serpentine channels are adopted for efficient mixing, wider channels are used for detection and controls incorporated to ensure reliability. By utilising the flow of the device and pumping solutions through at low velocities the antigens are able to diffuse out of the solution and into the active sites of the antibodies with a lot more ease

achieved due to the reduction in space in which they have to interact. This allows for easy automation as solutions can be flowed through continuously.

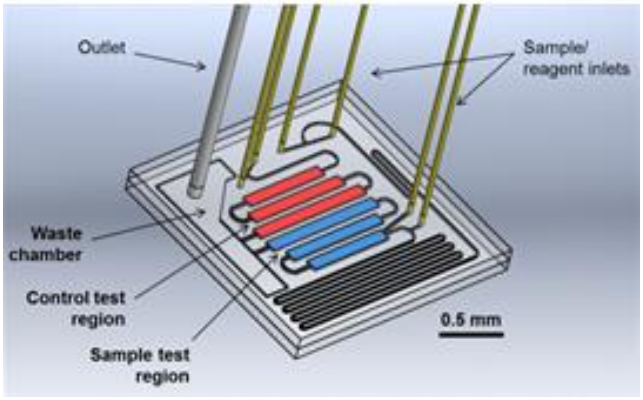


Figure 1.8: Graphical representation of the glass microfluidic device to be utilised in this study provided by the University of Hull.

Due to the advantages associated with microfluidic devices research into their analytical applications is becoming more widespread and in recent years investigations have begun to be more focused towards their possible uses as point-of-care tests. Biological tests, such as immunoassays, have successfully been applied to microfluidic devices and utilised as sensors for a range of applications. Sandwich immunoassays with fluorescent tags have been applied to PDMS microfluidic devices allowing for the detection of the C-reactive protein and other cardiac biomarkers in blood samples,[63] they have also been applied to protein analysis allowing for quantitative single cell measurements to be carried out.[64] Both sandwich and competitive immunoassays have been applied to microfluidic devices in the detection of natural and man-made pollutants such as algal toxins like saxitoxin[65] and herbicides like 2,4-dichlorophenoxyacetic acid.[66] Microfluidic devices have been used in drug based studies, the main focus of these studies have been around drug delivery systems and metabolic effects, however there has been several studies carried out into cancer drugs but they focus more on the effect of the drug. For example, Kim *et al.* created a way of generating uniform cancer cell spheroids for *in situ* cytotoxity studies of anticancer drugs.[67] Currently there has been very little research carried out into the use of microfluidic devices as point-of-care test for the detection of illicit drugs.

Overall microfluidic devices have shown high conversion rates in transferring from a standard ELISA format. Several examples of microfluidic devices have shown higher sensitivity when compared with conventional 96-well plate methods, such as in the immobilisation of prostate cancer biomarkers on polyethylene glycol (PEG) optimised

PDMS devices giving a sensitivity of 1.0 nM,[68] increased activation of enzymes displayed in the detection of anti-interferon-gamma (anti-IFN-y) on a tyrosine catalysed protein A surface[68, 69] and enhanced limit of detection demonstrated using a glutaraldehyde activated 3-aminopropyltriethoxysilane (APTES) surface.[70,71] Though investigations have recently begun into the application of microelectrodes for the detection of illegal drugs no work has previously been carried out into the use of an immunoassay based microfluidic techniques for the detection of NPS's.[72]

When transferring methods such as a sandwich immunoassay onto a microfluidic device, processes such as surface immobilisation need to be considered in order to ensure the selectivity and reproducibility are maintained.[71] There are a range of different materials from which a microfluidic device can be made: the most common of which are silicon, glass, polydimethylsiloxane (PDMS) and plastic. Based on the need for low cost, simple and fast acting point-of-care screening devices, a lot of research has recently been conducted into the use of paper as a medium for microfluidic devices. The highly absorbent nature of paper allows for sensitive and rapid multi-step immunoassays to be applied giving reproducible and reliable results. Paper itself is macroscopic however the absorption of the analytes on the surface is done via microscopic pores allowing for much easier immobilisation protocols.[71] The type of material used often depends on the required application. As each material contains different functional groups on the surface of the microfluidic channels, different immobilisation techniques have been tested in order to establish the most effective method in achieving an evenly coated surface.[71] The sensitivity of an immunoassay is dependent on the total activity of antibodies bound to the surface, by creating an optimal immobilisation surface it is possible to increase the number of binding site leading to a higher capture affinity. The more antibodies present on the surface the greater the chance of a reaction taking place allowing a lower limit of detection.[68, 69]

1.6.1 Physisorption

The simplest approach to carrying out immunoassays on microfluidic devices is via physisorption. The proteins adsorb to the surface of the device *via* intramolecular forces such as electrostatic, van der Waals or hydrogen bonding.[71] Depending on the type of device being used the functional groups present on the antibodies in question will affect

the type of bonding that occurs. Adsorbing antibodies to the surface of the device happens almost instantaneously and requires no external contributors in order for the process to occur, allowing for immunoassays that contain multiple steps to be carried out. However, although the process is relatively easy, the intramolecular forces between the surface of the device and the analyte are highly sensitive and are greatly dependent on their environmental conditions. Changes in temperature, pH or ionic strength can result in weakening of the bonds and loss of immobilisation of the analytes making reproducibility of results difficult.[71] Also as the analytes are adsorbing directly to the surface of the device immobilisation occurs randomly which can result in blocking of some active sites, uneven distribution of the analytes and incorrect orientation limiting the detection of the antibody.

In order to improve the reproducibility of the immunoassay results and increase the amount of analytes detectable, techniques have used pre-immobilisation in order to enhance the natural qualities of the devices surface. Already well-established techniques such as bioaffinity, covalent bonding and electrochemistry have been scaled down and applied to microfluidic devices across a range of applications.[71]

1.6.2 Bioaffinity Immobilisation

Bioaffinity interactions exploit specific types of binding that already exist in nature. These types of interactions tend to be stronger and more specific than physisorption and result in more accessible binding sites to allow for a more even distribution and better orientation of the immobilised analytes.[71] Avidin-biotin, protein A/G antibody, affinity capture ligands and aptamers have all been employed with microfluidic devices in order to enhance the immobilisation properties of the surface and each exhibited a range of strengths and weaknesses. Steptavidin-biotin is probably the most widely used bioaffinity immobilisation technique due to it's exceptional binding strength and highly specific nature, the addition of a streptavidin coating to the surface increases the number of binding sites allowing for a greater number of analyte-biotin conjugates to be immobilised on the surface resulting in better detectability due to lower nonspecific protein adsorption.[71] However, in order to link the functional groups of the analytes to the biotin, a costly binding reagent is required which is not ideal when attempting to create widely available reproducible devices. Aptamers have begun to draw attention

over the years, and are often used in conjunction with steptavidin-biotin for surface optimisation**.** They are acid biomolecules, that are like antibodies as they are able to bind specific molecules however, they are much smaller than antibodies and are able to provide greater surface coverage producing a larger binding capacity. Due to the highly specific nature of antibody binding it is still the more favoured capture reagent.[71] The use of streptavidin-biotin has been applied to the surface of magnetic beads to conjugate DNA specific aptamers in order to detect the C reactive protein in biosamples in a pneumatically driven microfluidic device.[63] The microfluidic system provided an enhancement of detection limit by one order of magnitude from 0.125 to 0.0125 mg/mL when compared to a standard ELISA as well as a 20% reduction in overall immunoassay time.[63] Protein A/G antibody is also a popular technique because of its affinity to bind to the F_c (constant) region leaving the F_{ab} (antigen binding) region accessible for binding (Figure 1.5). By creating an easily accessible surface it ensures that when the analytes bind they do so with the correct orientation making them easily detectable, also as protein A has multiple binding sites it has been shown to improve protein binding by 3 fold.[71] Protein A has been used in conjunction with tyrosinase in order to improve the immobilisation of anti-IFN-γ on a poly (methyl methacrylate) (PMMA) surface.[69] The optimisation of the PMMA surface saw a seven fold increase in antibody immobilisation, this indicated that the binding capacity of the surface had been enhanced due to the increase in active sites available on the surface. This increase in binding sites also lead to a decrease in the overall enzymatic reaction time as there were more antibodies available for the final immunoreaction. The optimisation of the surface using protein A also lead to a decrease in non-specific binding, wider dynamic range of 20 to 1,200 pg/mL and lower detection limit of 20 pg/mL when compared to a standard 96 well plate.[69] Though bioaffinity immobilisation creates a stronger bond between the surface and the antibody, they are often used in conjunction with other techniques such as covalent bonding in order to further enhance the strength of the bond, this often results in a far lengthier immobilisation process than is desired, leading to covalent bonding being a better initial choice.

1.6.3 Surface Covalent Bonds

In order for the characteristics of an immunoassay to be maintained, highly stable immobilisation bonds are required allowing for reproducible results. The surface of the

microfluidic device is activated using a reactive reagent allowing irreversible bonds to form between the reagent and the analyte.[71] Cross-linker molecules such as glutaraldehyde, *N*-hydroxysuccinimide, sulfhydryl-epoxide or isothiocyanate are covalently bonded to the surface of the microfluidic device via one end and covalently bonded to the analyte *via* the other.[71] Due to the nature of covalent bonding it is often possible for the cross-linkers to form bonds to the active sites reducing the proteins activity. In order to reduce this effect, spacer molecules such as poly(ethyleneimine) (PEI), 3-(aminopropyl)-triethoxysilane (APTES) and 3-(aminoproplyl)-trimethylsilane (APTMS) are used to minimise the steric hindrance and prevent conformational changes.[71] PEI has been used to not only provide an available amine group for protein binding but to serve as a spacer ensuring the protein is kept away from the hydrophobic surface thus preserving its activity and preventing denaturing.[68] Studies have shown that PEI has also been used to control the orientation of the antibodies binding, as it contains a positive NH_2 group it provides a more favourable binding site for IgG antibodies thus improving binding efficiency.[68] Similarly, APTES has been activated using glutaraldehyde on a silanised poly(dimethylsiloxane) (PDMS) surfaces in order to convert the surface amino groups into aldehyde group thus allowing for the covalent bonding of poly(vinyl alcohol) (PVA), a successful sandwich immunoassay was conducted on the optimised surface using anti-rabbit IgG.[70] The formation of the hydrophilic PVA layer reduced non-specific protein binding enhancing the amount of capture antibody able to bind to the surface leading to an increase in detection sensitivity and a reduced false positive response. The modification of the surface also led to a lower limit of detection of 15 ng/mL and wider dynamic range of 1.12 pg/mL to 11.2 pg/mL.[70] Though this type of bonding allows for a more stable and active immobilisation surface, in order for the covalent bond to form the incubation times required are often longer than that of other optimisation techniques.

1.6.4. Smart Immobilisation

With research into microfluidic devices becoming more advanced over recent years various smart immobilisation techniques have been applied in order to create stand-alone platforms.[71] Techniques such as light-activated immobilisation, thermally-activated immobilisation and electrochemically activated immobilisation have become more prominent. Light-activated immobilisation and thermally-activated immobilisation have advantages over electrochemically-activated immobilisation as they can be performed

after the device has been assembled, during the assay stage.[71] As most microfluidic devices are transparent**,** UV light or infrared radiation can penetrate the surface and initiate the immobilisation of the proteins on the surface.[71] Light-activation has been applied to acrylamide-based photoactive hydrophilic gel (LAVAgel) functionalised with benzophenone methacrylamide monomer in order to convert the molecular sieve into an immobilisation surface allowing for the formation of stable covalent linkages.[71] This method of immobilisation reduced non-specific protein adsorption and limited the need for multiple often time-consuming blocking and washing steps. This technique led to an increase in reactive sites and greater capture efficiency of two/three orders of magnitude than standard immunoassay formats, as well as a substantial reduction in precious biospecimens and costly antibodies.[73] Though light-activated immobilisation eliminates the need for long incubation times when compared to covalent bonding the strength of the bond achieved is weaker. Thermal-activation allows for quick and easy transitions between hydrophilic and hydrophobic surfaces making the immobilisation and release of proteins relatively rapid.[71] While, electrochemically-activated immobilisation allows for specific immobilisation of proteins on the surface, one major disadvantage is that it requires electrodes to be integrated onto the surface of the devices during assembly, resulting in complex device fabrication.[71] The use of electrodes in microfluidic devices have also been investigated. Platinum microelectrodes fabricated by photolithography based techniques, have been integrated onto the inner surface of a glass top layer.[74]The device consisted of three layers, the top layer containing the microelectrode, a silicon middle layer which forms the surface of the microfluidic channels and a poly(ethyl glycol)dimethacrylate coated bottom glass layer that would remain unaffected by the electrochemical activation and allow for site-specific immobilisation of the antibodies. [74]

Though research into smart immobilisation as a way of optimising the surfaces of microfluidic devices have yielded positive results they often require expensive specialised equipment, such as clean rooms, scanners for photolithography[75] and CD injection moulding, or complex processes, like photografting,[76, 77] to be applied before the device is ready to use.

1.7. Aims

Due to the prevalence and rise in ‘legal highs’ and new psychoactive substances over recent years this project aims to apply a microfluidic detection system in order to create an effective portable device capable of producing a rapid positive or negative response for the presence of mephedrone and/or its metabolites in both aqueous and biological media. The main aim of this study is to convert a basic enzyme linked immunoassay on to a microfluidic device in order to create an optimal and effective detection system. The study was broken down into sections, first focusing on deciding the most appropriate medium for the device itself as well as determining the most optimal surface conditions. Secondly looking specifically at the optimal concentrations for the anti-methcathinone antibody and the cathinone HRP as well as the method for the immunoassay before finally moving onto testing the developed method and device for the presence of mephedrone and/or its metabolites in both aqueous, urine and saliva samples.

Chapter 2- Experimental Section

2.1 Presumptive Colour Testing

In order to investigate the applicability of already established techniques to the detection of NPS's and to give a visual indication as to the similarity between the compounds several widely accepted reagents were tested (Section 3.1).

2.1.1 Marquis Reagent

A 10 mL solution of concentrated sulphuric acid (Fisher Scientific, UK) was dissolved in 1% formaldehyde solution (Sigma-Aldrich, UK).[78] For the test 1-2 drops of the test sample (10 mg/mL) was added to 2 drops of the test reagent on a white spotting tile and the reaction observed after 5 minutes.

2.1.2 Mandelin Reagent

A 1% ammonium metavanadate (Sigma-Aldrich, UK) solution was dissolved in 10 mL of concentrated sulphuric acid.[78] For the test 1-2 drops of the sample (10 mg/mL) was added to 2 drops of the test reagent on a white spotting tile and the reaction observed after 5 minutes.

2.1.3 Simon's Reagent

Reagent 1: 2% aqueous sodium carbonate (Sigma-Aldrich, UK) solution.
Reagent 2: 1% aqueous sodium nitroprusside (Sigma-Aldrich, UK) solution.
Reagent 3: 50:50 ethanol:acetaldehyde (Sigma-Aldrich, UK) solution.[78]
For the test 2 drops of reagents 1-3 were mixed sequentially before 1-2 drops of the test sample (10 mg/mL) was added to a white spotting tile and the reaction observed after 5 minutes.

2.1.4 Robadope Reagent

Reagent 1: 2% aqueous sodium carbonate solution.
Reagent 2: 1% aqueous sodium nitroprusside solution.
Reagent 3: 50:50 ethanol:acetone (VWR Chemicals, UK) solution.[78]
2 drops of reagents 1-3 were mixed sequentially before 1-2 drops of the test sample (10mg/mL) were added to a white spotting tile and the reaction observed after 5 minutes.

2.1.5 Scott Reagent

A 0.1002g sample of cobalt (II) thiocyanate (Sigma-Aldrich, UK) was dissolved in a 10 mL solution of glycerol (Fisher Scientific) in distilled water (1:1).[78] 1-2 drops of the test sample (10 mg/mL) was added to 2 drops of the test reagent on a white spotting tile and the reaction observed after 5 minutes.

2.1.6 Zimmerman Reagent

Reagent 1: 1% 1,3-dinitrobenzene (Alfa Aesar, UK) was dissolved in methanol (Fisher Scientific).

Reagent 2: 15% potassium hydroxide (Fisher Scientific, UK) in distilled water.[78]

For the test 2 drops of reagents 1 and 2 were mixed sequentially before 1-2 drops of the test sample (10 mg/mL) were added to a white spotting tile and the reaction observed after 5 minutes.

Table 2.1:Expected results for the Marquis, Mandelin, Simon's, Robadope, Scott and Zimmerman reagents with several common drug of abuse.[79]

Reagent	Opiates	Amphetamine	MDMA	Cannabis	Cathinones
Marquis	Purple	Red/Brown	Dark Red	No Reaction	No Reaction
Mandelin	Black	Green	Purple	No Reaction	No Reaction
Simon's	No Reaction	No Reaction	Blue	No Reaction	Dark Blue
Robadope	No Reaction	Brown	No Reaction	No Reaction	No Reaction
Scott	Blue-Pink-Blue	No Reaction	No Reaction	No Reaction	No Reaction
Zimmerman	No Reaction	No Reaction	No Reaction	No Reaction	Purple

2.2 Microfluidic Device Preparation

Both glass and paper microfluidic devices were initially investigated in order to establish which method would provide the best results and be most applicable for a point-of care-test.

2.2.1 Glass Microfluidic Devices

The bottom plate was etched using photolithography and wet etching in order to create both the serpentine and detection channels, holes were drilled in the top plate to provide access before the two sections were thermally bonded together (Figure 2.2.1).[80, 81] The completed devices (Figure 1.8) were provided by the University of Hull.

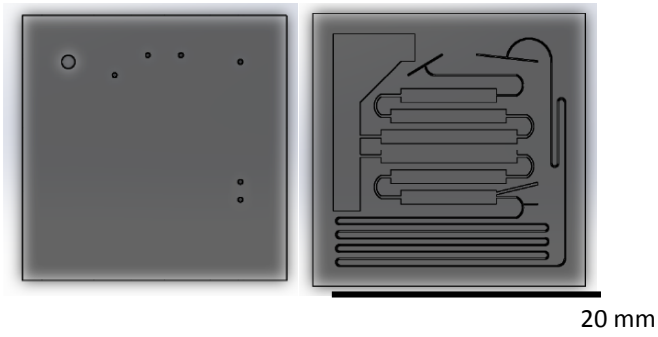


Figure 2.2.1: Grey Scale image of the top (Left Image) and bottom plate (Right Image) of the glass microfluidic device designed using SolidWorks 2014.

2.2.2 Paper-based Microfluidic Devices

Traditional Wax Printing

The initial design for the paper-based microfluidic device was based on a 96 well ELISA plate and a negative image was created using SolidWorks 2014 (Figure 2.2.2). Initial testing was carried out on plates printed using a traditional wax printing method. The SolidWorks design was applied to a 40 threads per cm polyester fabric screen that had been stretched over a 297 mm x 420 mm aluminium frame. A thin coat of light sensitive emulsion was applied in a dark room and left to dry for 6 hours. Once the emulsion has completely dried the negative image is placed on the back of the screen and placed within an exposure unit for 60 seconds. The dark areas of the image prevent light from passing through whereas the light areas allow the light to reach the screen causing the emulsion to harden. The screen was then washed thoroughly with water in order to remove the emulsion that was not exposed to light. Once the screen had dried, several different methods were applied to ensure the greatest degree of surface covering as well as the largest depth of wax absorption was achieved. [82]

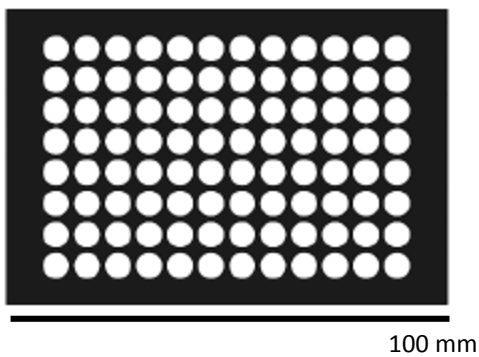


Figure 2.2.2.: Negative image of a 96 well ELISA plate designed using SolidWorks 2014.

From the various methods attempted the most successful design was achieved when a piece of filter paper was placed directly below the screen and a line of liquid wax was poured just above the design and squeegeed across the pattern. The paper was removed from the screen and placed on a hot plate at approximately 200 ^{o}C for several minutes to allow the wax to melt into the paper. The filter paper was then placed in an oven at 130 ˚C for 15 minutes.[82]

Wax Printer

Later tests were carried using the same SolidWorks design printed via a Xerox Phaser 8500 solid ink printer. Solid wax is loaded into the printer and melted within the device in order to create the image. Once the designs were printed onto the filter paper they were melted at 130^{o}C for 180 seconds.

2.3 Surface Preparation

2.3.1 Glass Microfluidic Device

In order to prepare the surface of the glass chip for covalent bonding of the glutaraldehyde (Sigma-Aldrich, UK) solution, the surface must first undergo silanisation. This process coats the hydroxyl groups on the surface with a silicone substance in order to make it chemically inert and increasing its functionality enabling the glutaraldehyde to bond. The microfluidic device was cleaned by sequentially washing with 1 mL each of 1M sodium hydroxide (Sigma-Aldrich, UK), deionised water and ethanol (Fisher Scientific, UK) at 20 µL/min before being dried over night at 110 ˚C. The channels were then immersed in a solution 1% APTES (100 µL 3-(aminopropyl)-triethoxysilane (Sigma-Aldrich, UK) in 10 mL of anhydrous acetone (Fisher Scientific, UK)) and pumped through at 5 µL/min for 1 hour. The microfluidic device was then washed sequentially with 1 mL each of deionised water and ethanol at 20 µL/min before being placed in an oven at 90 ^{o}C overnight. Following the silanisation process, the microfluidic channels were immersed in 1 mL of the 2.5% glutaraldehyde solution (100 µL of 50% glutaraldehyde solution in 1900 µL of analytical grade water) at 5 µL/min for 2 hours. The channels were then rinsed with deionised water and left in an over overnight at 110 ˚C to ensure the device was completely dry.

2.3.2 Paper-based Microfluidic Device

A 5 µL solution of 0.25 mg/mL chitosan (2.5 mg of chitosan (Sigma-Aldrich, UK) dissolved in 10 mL analytical grade water) was pipetted into each paper well and allowed to dry at room temperature before 5 µL of 0.01 mol/L solution of 2.5% glutaraldehyde (500 µL of 50% glutaraldehyde diluted with 9.5 mL of analytical grade water) was left for 2 hours in order to activate the wells. The paper based microfluidic device was placed on a piece of blotting paper in order to absorb the waste water from the bottom of the wells. Each individual well was then washed twice with 10 µL of analytical grade water.[82]

2.4 Immunoassay

2.4.1 Glass Microfluidic Device

Prior to any test being run, the microfluidic device was flushed sequentially with 1 mL each of ethanol (Sigma-Aldrich, UK) and analytical grade water before being left in an oven overnight at 90 ˚C to ensure that all of the antibodies from the previous immunoassay were removed from the surface and the device was completely dry.

Method Development

Prior to the application of 'legal highs' and new psychoactive substances preliminary studies were carried out using a well-established enzyme-linked immunoassay that was adapted and applied to a glass microfluidic device.

2.4.1.Method A

The glass chip was coated with 0.5% rat serum (Sigma-Aldrich, UK) solution (50 µL of anti-rat antibodies (Abcam,UK) in 10 mL of 0.05 M carbonate (Timstar Laboratories, UK)/bicarbonate (BDH Laboratory Supplies, UK) buffer solution) at 5 µL/min for approximately 6 hours in order to allow enough time for the primary antibodies to adsorb to the surface of the device, the microfluidic device was then stored for 3 days in a fridge at 4 ^{o}C. The microfluidic device was allowed to reach room temperature before being washed with PBS (Phosphate Buffered Saline) Tween (0.05%, BDH Laboratory Supplies, UK) at 5 µL/min for 10 minutes to remove any unbound antibodies from the surface of the device, the vacant sites on the surface were blocked with 1% semi-skimmed milk powder (Marvel, Supermarket, UK) solution at 1 µL/min for 10 minutes. In order to clean the surface and ensure only properly bonded antibodies remained on the device the

microfluidic chip was washed with PBS Tween (0.05%) at 5 µL/min for 10 minutes. The channels were then flushed with a 1:2000 dilution of anti-rat immunoglobulin horseradish peroxidase conjugate (Sigma-Aldrich, UK) at 1 µL/min for 40 minutes before being washed with PBS Tween (0.05%) at 5 µL/min for 10 minutes.

2.4.1.Method B

As for method A except the channels were flushed with a 1:1000 dilution of anti-rat immunoglobulin horseradish peroxidase conjugate was flushed through at 1 µL/min for 40 minutes. The microfluidic device was washed with PBS Tween (0.05%) at 5 µL/min for 10 minutes to remove any non-bound antibodies and excess horseradish peroxidase from the channels.

2.4.1.Method C

As for method A except the channels were flushed with a 1:1000 dilution of anti-rat immunoglobulin horseradish peroxidase conjugate at 20 µL/min for 20 minutes and left to incubate for 10 minutes to ensure the antibodies had time to properly adhere to the surface. A solution of PBS Tween (0.05%) was then run through the device at 5 µL/min for 10 minutes to remove any unbound antibodies from the channels.

2.4.1.Method D

A 1 µL solution of both the neat and 1:1000 dilutions of anti-rat immunoglobulin horseradish peroxidase conjugate was smeared onto two separate microscope slides and allowed to dry before the addition of the luminol (Fisher Scientific, UK) -hydrogen peroxide (Sigma-Aldrich, UK) solution (60 µM luminol, 2 mM H_2O_2, 0.1 M tris buffer (Fisher Scientific, UK))

2.4.1.Method E

A 1 µL solution of both the neat and 1:1000 dilutions of anti-rat immunoglobulin horseradish peroxidase conjugate was smeared onto two separate microscope slides and allowed to dry before the addition of the luminol-hydrogen peroxide (Figure 10-left image) and enhanced luminol-hydrogen peroxide solution (1.25 mM luminol, 0.41 mM p-iodophenol (Sigma-Aldrich, UK), 2.7 mM H_2O_2, 0.1 M tris buffer)[83]

2.4.1.Method F

A neat solution of anti-rat immunoglobulin horseradish peroxidase was pumped through one device and 1:1000 dilution of anti-rat immunoglobulin horseradish peroxidase was pumped through another, both were run at 1 µL/min for 30 minutes. The flow rate was then increased to 5 µL/min for 15 minutes in order to ensure that the antibodies had sufficient time to adhere to the surface. The surface of the microfluidic devices were then washed with PBS Tween (0.05%) at 1 µL/min for 5 minutes before being increased to 5 µL/min for 10 minutes. 0.5 µL of enhanced luminol solution was flushed manually through the microfluidic device.

2.4.2 Paper-based Microfluidic Device

Method Development

2.4.2.Method A

A 4 µL 0.5% rat serum solution (50 µL in 10 mL of 0.05M carbonate/bicarbonate buffer solution) was added to each well and allowed to incubate for 30 minutes at room temperature and then washed twice with 10 µL of analytical grade water in order to remove any excess antibodies from the wells. 10 µL of blocking buffer solution (1% semi-skimmed milk powder solution) was then pipetted into each well and incubated at room temperature for 15 minutes. They were then washed twice with 10 µL of PBS Tween (0.05%).[84] A 4 µL solution of 1:1000 dilution of anti-rat immunoglobulin conjugate horseradish peroxidase was added to the wells and allowed to incubate for 210 seconds. They were then washed twice with 10 µL of analytical grade water. 4 µL of 3,3',5,5'-tetramethylbenzidine (TMB, Thermo Scientific, UK) was added to each well and left to dry.[84]

2.4.2.Method B

A 4 µL solution of 1:1000 dilution of anti-rat immunoglobulin conjugate horseradish peroxidase was added directly to the wells and allowed to incubate for 210 seconds before being washed twice with 10 µL of analytical grade water. 4 µL of 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and left to dry.[84]

2.4.2.Method C

As for method A except a 4 µL 0.5% rat serum solution (50 µL in 10 mL of 0.05M carbonate/bicarbonate buffer solution) was added to each well and allowed to incubate for 30 minutes at room temperature and then washed twice with 10 µL of analytical grade water in order to remove any excess antibodies from the wells. 10 µL of blocking buffer solution (1% semi-skimmed milk powder solution) was then pipetted into each well and incubated at room temperature for 15 minutes. They were then washed twice with 10 µL of PBS Tween (0.05%).[84] A serial dilution of 1:2000 dilution anti-rat immunoglobulin conjugate horseradish peroxidase were prepared. A 4 µL solution of each dilution was added in triplicate in chronological order down the wells and allowed to incubate for 210 seconds. They were then washed twice with 10 µL of analytical grade water. A 4 µL solution of 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and left to dry.[84]

2.4.2.Method D

As for method A except a 3 µL solution covering a dilution range of 1:2000-1:1048576000 of the anti-methcathinone antibody was added to each well and allowed to incubate for 30 minutes at room temperature and then washed twice with 10 µL of analytical grade water in order to remove any excess antibodies from the wells. 10 µL of blocking buffer solution (1% semi-skimmed milk powder solution) was then pipetted into each well and incubated at room temperature for 15 minutes. They were then washed twice with 10 µL of PBS Tween (0.05%).[84] A series of dilutions, ranging from 1:10,000-1:100,000, of cathinone-HRP were prepared. A 3 µL solution of each was added in triplicate down the wells and allowed to incubate for 210 seconds. They were then washed twice with 10 µL of analytical grade water. 4 µL of 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and left to dry.[84]

2.4.2.Method E

As for method A except a 3 µL solution of the 1:16384000 dilution of the anti-methcathinone antibody was added to each well and allowed to incubate for 30 minutes at room temperature and then washed twice with 10 µL of analytical grade water in order to remove any excess antibodies from the wells. 10 µL of blocking buffer solution (1% semi-skimmed milk powder solution) was then pipetted into each well and incubated at room temperature for 15 minutes. They were then washed twice with 10 µL of PBS

Tween (0.05%).[84] A series of dilutions, ranging from 70ng/mL-0.1367ng/mL, for each specific drug were prepared with the addition of an equivalent amount of cathinone-HRP (100% v/v). A 3 µL solution of each was added in triplicate down the wells and allowed to incubate for 210 seconds. They were then washed twice with 10 µL of analytical grade water. 4 µL of 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and left to dry.[84]

N.B. Each serial dilution was run in triplicate across three separate plates.

2.4.3. Stability Study

Week 0;

All four plates were treated with 4 µL of 0.5% rat serum solution (50 µL in 10 mL of 0.05M carbonate/bicarbonate buffer solution) which was added to each well and allowed to incubate for 30 minutes at room temperature. They were then washed twice with 20 µL of analytical grade water in order to remove any excess antibodies from the wells. 20 µL of blocking buffer solution (1% semi-skimmed milk powder solution) was then pipetted into each well and incubated at room temperature for 15 minutes before being washed twice with 20 µL of PBS Tween (0.05%).[84] Strips 2, 3 and 4 from all four plates were stored in the fridge at 4 ˚C.

A serial dilution of 1:2000 dilution anti-rat immunoglobulin conjugate horseradish peroxidase were prepared and applied to strip 1 from each of the plates; 4 µL of each solution was added in triplicate in chronological order down the wells and allowed to incubate for 210 seconds. They were then washed twice with 20 µL of analytical grade water. 4 µL of 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and left to dry.[84]

2.5 Detection

2.5.1. Colourimetric

A solution of 3,3',5,5'-tetramethylbenzidine (TMB) was added at 1 µL/min for 10 minutes to methods A, B and C for both glass and paper devices.

2.5.2 Chemiluminescense

Initial testing was carried out using an unenhanced luminol-hydrogen peroxide solution (60 µM luminol, 2 mM H_2O_2, 0.1 M tris buffer). This was later adapted and an enhanced luminol-hydrogen peroxide solution (1.25 mM luminol, 0.41 mM p-iodophenol, 2.7 mM H_2O_2, 0.1 M tris buffer) was used.

N.B. Due to the light sensitivity and instability of the luminol-hydrogen peroxide solution, both solutions were made fresh on the day and stored in the fridge at 4˚C when not in use.

2.6 Analysis

2.6.1 Microscopy

A Zeiss Primovert Microscope with monitor and integrated HD IP camera for transmitted-light brightfield and universal phase contrast with objectives 4x Ph0, 10x Ph1, LD 20x Ph1, LD 40X Ph2 was used to visualise and record the immunoassay immobilised on the glass microfluidic device.

2.6.2 ImageJ

Photographs of the paper microfluidic devices were taken using an Iphone 4S and uploaded on to a computer. Using imageJ, a circle was drawn around the smallest, most intense reaction well on a colour JPEG image. The area was analysed and a measurement of the average light intensity across the selected area was taken. ImageJ measures the degree of white light present within an image, the lower the values the more intense the reaction colour indicating a greater presence of HRP and therefore less competitor. The data was then transferred into Microsoft Excel and Minitab allowing calibration curves to be created and the significance of the data to be determined.

2.6.3 GeneSnap

A series of 10 images were taken of the chemiluminescense reactions at intervals of 5 seconds, 10 seconds, 20 seconds, 30 seconds, 45 seconds, 1 minute, 5 minutes, 10 minutes, using a Syngene G.BOX F3 and analysed using Genesnap software. The information from the previous image was added to the new image taken and the image was then loaded into imageJ in order to measure the intensity of the chemiluminescent reaction.

2.7 New Psychoactive Substances

In order to set up a competitive immunoassay for the detection of cathinones a labelled species was required (Figure 2.7.1). Due to the functional groups of the 4-methylmethcathinone it is not possible to attach a HRP label as a primary amine is required in order for the two to conjugate. However, due to the structural similarity between the cathinones and the large degree of cross-reactivity associated with the anti-methcathinone antibody it was possible to label the cathinone with HRP.

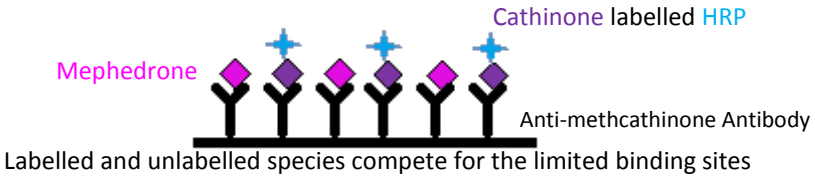


Figure 2.7.1; Schematic representation of the competitive immunoassay immobilised on the surface of the paper device using cathinone labelled HRP

Cathinone hydrochloride was purchased from (Fluorochem Ltd, UK), while the 4-methylmethcathinone (mephedrone) and methcathinone were synthesised at the University of Strathclyde, as their hydrochloride salts, using the method reported by Santali *et al.* prior to the legislation change.[39]

2.7.1 Freebasing the cathinone hydrochloride

In order to conjugate the HRP, the cathinone first needed to be returned to its freebase form, exposing the amine and inducing a nucleophilic attack in the presence of the HRP. A 0.0102 g sample of cathinone hydrochloride was dissolved in 500 µL of analytical grade water before being added to a 10 mg/mL solution of sodium hydrogen carbonate (0.0100 g in 1000 µL, Fisher Scientific, UK). Once mixed the solution began to effervesce. After the reaction has ceased the pH of the mixture was taken, a litmous paper test indicated a pH of 8 and confirmed that the cathinone was in its freebase form. The aqueous layer was washed with diethyl ether (6 x 500 µL, Fisher Scientific) and the organic fractions were combined before the diethyl ether was blown off with nitrogen to leave a pale residue in the bottom. The residue was reconstituted in 100 µL of dimethyl sulphoxide (DMSO, Fisher Scientific, UK) before 10 µL was removed and diluted using 990 µL of PBS (100x dilution, Oxoid LTD. UK) to give a 1% DMSO/PBS solution.

2.7.2 Labelling cathinone with HRP

A 10 µL solution of modifier reagent (Abcam, UK) was added to 100 µL of the cathinone freebase solution and mixed. The cathinone freebase-modifier mixture was pipetted directly onto the LYNX lyophilized mix (Abcam, UK) and gently pipetted up and down twice to re-suspend. The cap was replaced onto the vial and left to incubate over night at room temperature. After incubation 10 µL of quencher reagent (Abcam, UK) was added to a vial and left to stand for 30 minutes.

Chapter 3- Results and Discussion

Qualitative Testing

Several qualitative tests were carried out in order to determine the precise nature of the compounds being used.

3.1 Presumptive Colour Testing

Several common colour tests were applied to mephedrone, methcathinone and cathinone in order to establish whether presumptive tests already in use for long standing illegal substances could also be applied to the detection of New (or novel) Psychoactive Substances (Figure 3.1.1). Carrying out the same colour tests across the three cathinone derivatives simultaneously allowed for direct comparison between them and gave a visual indication as to the structural similarity between the compounds.

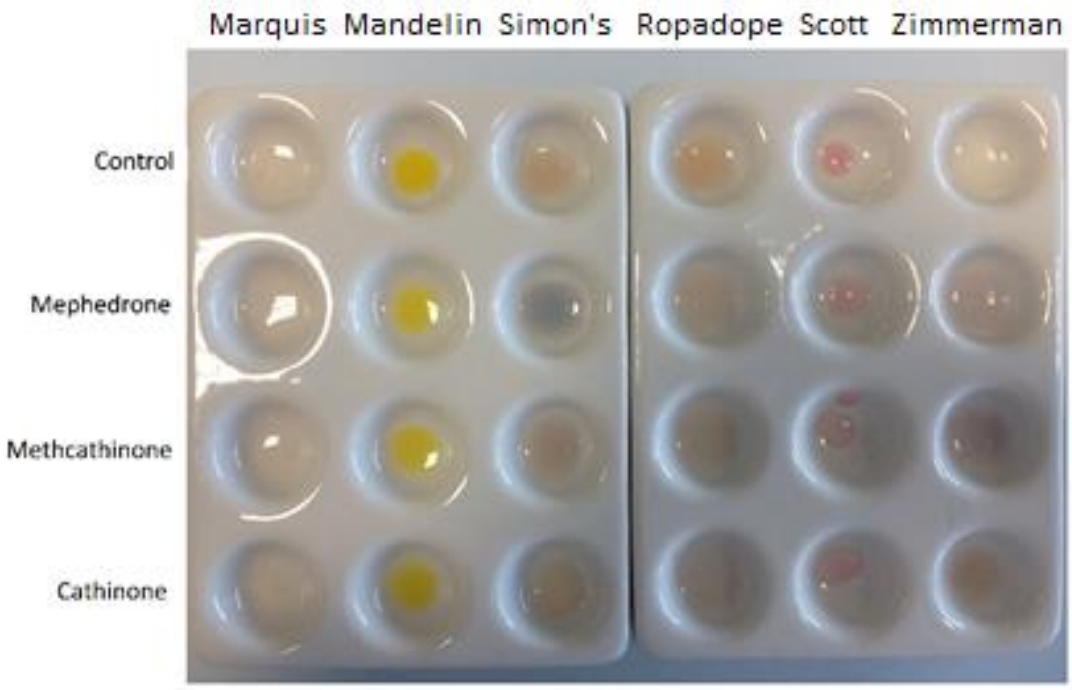


Figure 3.1.1: Spotting tile visualised under white light displaying the presumptive colour test results for pure samples of the compounds.

	Marquis	Mandelin	Simon's	Robadope	Scott	Zimmerman
Control	Colourless	Yellow	Orange	Orange	Pink	Clear
Mephedrone	- (Colourless)	- (Yellow)	+ (Dark Blue)	- (Orange)	- (Pink)	+ (Purple)
Methcathinone	- (Colourless)	- (Yellow)	+ (Dark Orange)	- (Orange)	- (Pink)	+ (Purple)
Cathinone	- (Colourless)	- (Yellow)	- (Orange)	- (Orange)	- (Pink)	+ (Purple)

Table 3.1.1: Table representing the observations made during the presumptive colour tests for the pure samples of the compounds (+ = positive result; - = negative result).

The colour changes observed for the neat compounds (Table 3.1.1) were consistent with the results expected. Strong positive results were observed using the Zimmerman reagent for all compounds as it is commonly used to test for cathinones. A strong positive result was seen between the mephedrone and the Simon's reagent, a weak positive result was seen between methcathinone and the Simon's reagent and a negative result was seen with the cathinone. This is as expected as the Simon's reagent is usually used to test for secondary amines, which is present on both the mephedrone and methcathinone structures but not on the cathinone. Negative results were obtain using the Marquis, Mandelin, Ropadope and Scott reagents as they are used to detect ring substituted amphetamines, tertiary amines, opiates and primary amines respectively, none of which are present within either the mephedrone, methcathinone or cathinone compounds.

Street samples often contain more than one novel psychoactive substance along with several other compounds, running the same presumptive tests on varying mixtures, 25:75, 50:50, 75:25, of the compounds as well as the pure samples gave an indication as to the likeliness of cross-reactivity within the group (Table 3.1.2, 3.1.3, 3.1.4).

	Marquis	Mandelin	Simon's	Robadope	Scott	Zimmerman
Control	Colourless	Yellow	Orange	Orange	Pink	Clear
25:75	- (Colourless)	- (Yellow)	+ (Dark Orange)	- (Orange)	- (Pink)	+ (Purple)
50:50	- (Colourless)	- (Yellow)	+ (Grey/Blue)	- (Orange)	- (Pink)	+ (Purple)
75:25	- (Colourless)	- (Yellow)	+ (Grey/Blue)	- (Orange)	- (Pink)	+ (Purple)

Table 3.1.2: Table representing the observations made during the presumptive colour tests for the different ratios of mephedrone and cathinone.

The biggest effect of the mephedrone and cathinone combination (Table 3.1.2) was observed for the Simon's reagent. When carried out with the pure samples the mephedrone produced a positive reaction whereas the cathinone produced a negative reaction. This result became more obvious when altering the ratios, as the colour change gradually became more predominant with larger amounts of mephedrone. The Zimmerman observations for the pure samples were consistent across all three compounds and though the mixtures produced a positive result it was not altered by the varying ratios.

	Marquis	Mandelin	Simon's	Robadope	Scott	Zimmerman
Control	Colourless	Yellow	Orange	Orange	Pink	Clear
25:75	- (Colourless)	- (Yellow)	- (Orange)	- (Orange)	- (Pink)	+ (Purple)
50:50	- (Colourless)	- (Yellow)	- (Orange	- (Orange)	- (Pink)	+ (Purple)
75:25	- (Colourless)	- (Yellow)	+ (Dark Orange)	- (Orange)	- (Pink)	+ (Purple)

Table 3.1.3: Table representing the observations made during the presumptive colour tests for the different ratios of methcathinone and cathinone.

The results observed for the methcathinone and cathinone combination (Table 3.1.3) for the Simon reagent were consistent with those seen with the neat samples, the weak positive result observed for the methcathinone became weaker the more cathinone that was present, due to the negative result of the cathinone compound. Due to the structural similarity between methcathinone and cathinone, and the weakness of the positive methcathinone result, the difference in the colour variations is somewhat harder to spot. The Zimmerman observations for the pure samples were consistent across all three compounds and though the mixtures produced a positive result it was not altered by the varying ratios.

	Marquis	Mandelin	Simon's	Robadope	Scott	Zimmerman
Control	Colourless	Yellow	Orange	Orange	Pink	Clear
25:75	- (Colourless)	- (Yellow)	+ (Grey Blue)	- (Orange)	- (Pink)	+ (Purple)
50:50	- (Colourless)	- (Yellow)	+ (Grey Blue)	- (Orange)	- (Pink)	+ (Purple)
75:25	- (Colourless)	- (Yellow)	+ (Dark Blue)	- (Orange)	- (Pink)	+ (Purple)

Table 3.1.4: Table representing the observations made during the presumptive colour tests for the different ratios of mephedrone and methcathinone.

For the mephedrone and methcathinone combination (Table 3.1.4), the observations made for the Simon's reagent were consistent with those previously observed, however, as both pure mephedrone and methcathinone gave a positive result with the Simon's reagent the reaction is more pronounced. A positive result, for the Simon's reagent, was seen across all three ratios, however the greater the amount of mephedrone present the stronger the colour change. The Zimmerman observations for the pure samples were consistent across all three compounds and though the mixtures produced a positive result it was not altered by the varying ratios.

In comparing the colour tests from the three different combinations, the Simon's reagent gave the biggest variations in results. The strongest positive result was always achieved with the highest concentrations of mephedrone is present and the weakest positive result is achieved with the highest concentration of cathinone is present. This demonstrates that the secondary amine group is more reactive towards the Simon's reagent than the primary amine group, this could be due to a greater number of electron donating groups surrounding the nitrogen. The two methyl groups present are able to push electron density onto the nitrogen making it more electron rich, resulting in a more nucleophilic amine with a greater ability to attack the reagent.[78]

All of the colour tests applied to the pure samples were also applied to the combinations in order to establish whether cross reactivity between the compounds would affect the negative results as well as the positive results. However this was not the case and the negative results remained so across all ratio's and combinations.

Quantitative Testing

3.2.Glass Microfluidic Device

A standard enzyme-linked immunoassay (Figure 3.2.1) was used in the early stages of method development and was the basis of the simplified protocol applied to the glass microfluidic device. Variations of the standard assay were tried (see Section 2.4.1 Method A) both pre and post optimisation, before an immunoassay was successfully established on the surface.

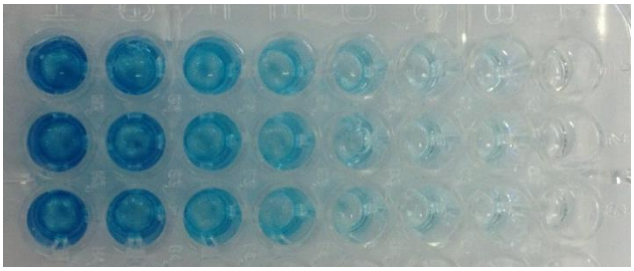


Figure 3.2.1: Standard enzyme-linked immunoassay using anti-rat immunoglobulin conjugate horseradish peroxidase carried out in a standard 96 well ELISA plate. Label concentrations/replicates in figure.

A simplified version of the immunoassay method (see Section 2.4.1.Method B) was applied to the microfluidic device (Figure 1.8). The device was left to incubate for a further 20 minutes to ensure the reaction had taken place, however no obvious colour change was observed within the channels. Upon closer inspection of the device, a distinct blue colour was observed around the connections between the syringe and the injector ports, indicating that the reaction was taking place and the substrate was successfully being converted by the horseradish peroxidase attached to the antibody. Though no distinct blue colour was observed, a faint blue tinge did appear to be present. The microfluidic device was observed under an inverted microscope with integrated camera and manual focus in order to gain a clear view of the channels (Figure 3.2.2).

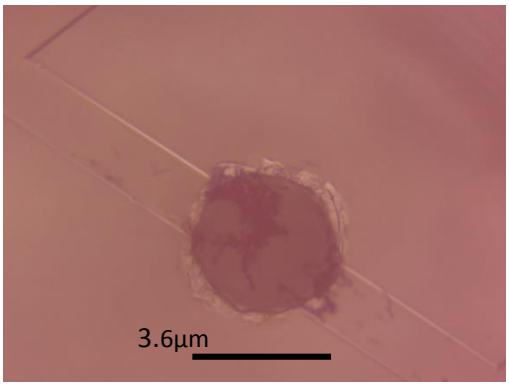


Figure 3.2.2: X 40 magnification of the injection point on a glass microfluidic device containing the 1:2000 solution of anti-rat immunoglobulin horseradish peroxidase (see Section 2.4.1.Method B).

Due to the 'sticky' nature of the hydrophilic surface of the glass chip, the majority of the antibodies within the 1:2000 dilution of the anti-rat immunoglobulin horseradish conjugate adhered to the surface of the device as soon as they came into contact with it, resulting in a large proportion of the antibodies bonding directly below the injection point (Figure 3.2.2). A very small number of antibodies were visually observed to be present further along the proceeding channels, however, due to the small number of antibodies

present in these sections they were below the limit of detection and unable to be visualised. Based on the observation both visually and from under the microscope it was hypothesised that the concentration of anti-rat immunoglobulin horseradish peroxidase was too low, limiting the reaction between the horseradish peroxidase and the TMB. Also it was thought that by the time the solution reached the later channels that all the antibodies present had already adhered to the surface upon initial contact leaving none left to be distributed throughout the remaining channels. Furthermore it was believed that as the antibodies were adhering as soon as they came into contact with the surface of the device, that they may not be adhering in the correct orientation and are therefore not able to react with the TMB substrate.

In order to improve the surface coverage of antibodies, a higher concentration of anti-rat immunoglobulin horseradish peroxidase was proposed (see Section 2.4.1.Method C). Increasing the concentration of antibodies present also increases the amount of horseradish peroxidase present resulting in a stronger reaction. As the surface remains unchanged the antibodies will still adhere to the surface at their first point of contact. By increasing the number of antibodies present once all of the active sites around the injection point are filled, the remaining antibodies will have to move further along the channels in order to find available binding sites.

Again the device was left to incubate and on this occasion the edges of the channels were more defined and faint areas of blue were visible. Upon microscopic inspection, a more even distribution of antibodies was inferred. Though the majority of the antibodies adhered to the surface were still focused in the beginning of the first channel (Figure 3.2.3 A), there was an increase in the number of the antibodies that made it to the proceeding channels (Figure 3.2.3 B). Increasing the concentration showed a slight improvement in the surface coverage however, antibodies are not only able to bind to the active sites on the surface but they are also able to form linkages with one another resulting in bunching and clusters of antibodies forming.

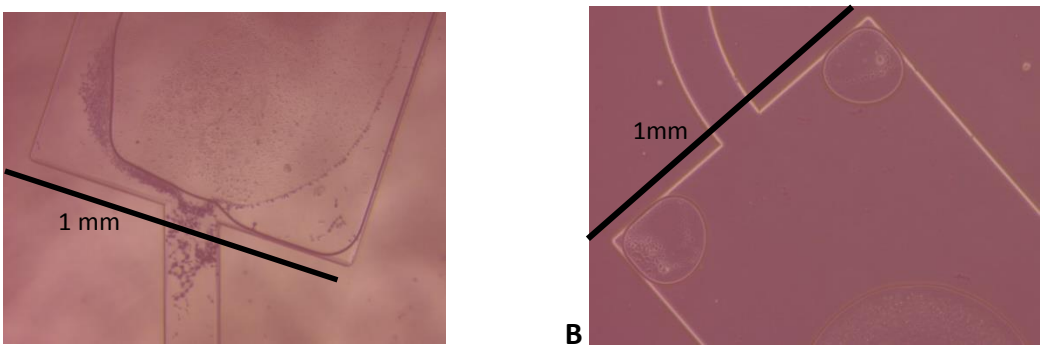


Figure 3.2.3: X 40 magnification of a glass microfluidic device containing the 1:1000 solution of anti-rat immunoglobulin horseradish peroxidase.

In order to enhance the immobilisation properties on the glass surface and improve antibody binding, pre-treatment of the device was carried out. Due to the hydrophilic nature of the surface of the glass microfluidic device it made eliminating water from the channels difficult resulting in pockets of water presenting within the channels, glutaraldehyde is a hydrophobic compound and creates a less favourable surface for the water and allowing for a more even coverage (see Surface Preparation 2.3.1). The success of the glutaraldehyde covering was observed due to a change in direction of the contact angle of the water, changing from concave in an uncoated channel to convex in a coated channel.

After optimisation (see Surface Preparation 2.3.1) the same higher concentration immunoassay method (see Section 2.4.1.Method C) was applied. As with the previous runs, the visual observations were limited and only became apparent under high magnification. Microscopic analysis of the microfluidic channels showed that, similarly to the results reported by *L.Yu et. al.,* the use of glutaraldehyde prior to the addition of the antibodies had increased the surface coverage in all three channels as well as generating a much more even distribution.

In order for the microfluidic device to function as a portable on site point-of-care kit the indication of a positive result needs to be easily observable. When a traditional enzyme-linked immunoassay is performed, the concentration of antibodies present within each sample is usually determined using a plate reader that measure the amount of light absorbed by each well. These values can then be compared to a calibration graph of known concentrations. However, research has recently begun into smart phone applications that use specially designed software in order to convert the colour intensity

of an image into a statistical R value that can then be compared against a calibration graph of known concentrations.[85] Though both applications measure the intensity of the colour of the sample in different ways, they are both dependent on a distinct blue colour being present. If the colour is too faint neither application will be able to detect it and will present an inaccurate limit of detection. Increasing the concentration of the HRP did not prove to increase the intensity of the blue colour that presents when it reacts with the TMB substrate and would be undetectable given its current intensity.

Several studies have been carried out into the use of chemiluminescence as a way of enhancing the signal of the horseradish peroxidase within enzyme-linked immunoassays.[83] Instead of using TMB as the substrate, a solution of luminol and hydrogen peroxide was used. Once the two compounds are mixed, the hydrogen peroxide deprotonates the nitrogen group on the luminol allowing a cyclic addition of dioxygen to take place and the subsequent removal of the dinitrogen. This reaction results in the creation of a radical which then reacts with the horseradish peroxidase to produce a photon of light.[86]

The test (see Section 2.4.1.Method D) clearly showed that the reaction between the horseradish peroxidase and luminol was working (Figure 3.2.4) as when applied to the neat solution it gave off a very prominent reaction, giving an average light intensity value of 15964. However when applied to the 1:1000 dilution the reaction was considerably weaker, giving an average light intensity value of 537.

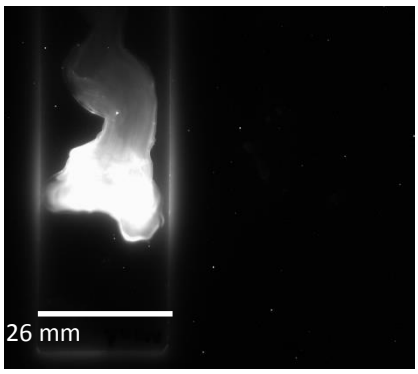


Figure 3.2.4: GeneSnap Image made up of a series of 5 images taken at set interval of over a period of 51 minutes of the neat anti-rat immunoglobulin horseradish peroxidase (left) and the 1:1000 dilution (right) using a luminol solution.

Initial observations of the image make it appear as though the concentration of horseradish peroxidase present in the 1:1000 dilution is too weak in order for the reaction to be monitored (Figure 3.2.4) and several studies have been carried out into the effect of phenols as enhancers for luminol. Research carried out into the use of enhanced luminol solution for similar applications implied that p-iodophenol was the most effective. A similar test (see Section 2.4.1.Method E) was run to allow a comparison to be carried out between luminol-hydrogen peroxide (Figure 3.2.5 left image) and enhanced luminol-hydrogen peroxide solutions[83] (Figure 3.2.5 right image). Visual observations showed a considerable enhancement of the reaction between the horseradish peroxidase and the enhanced luminol-hydrogen peroxide for the neat solution. However the reaction for the 1:1000 dilution appears unchanged, this is confirmed by ImageJ analysis of the images (Appendix 2, Figure A2.1).

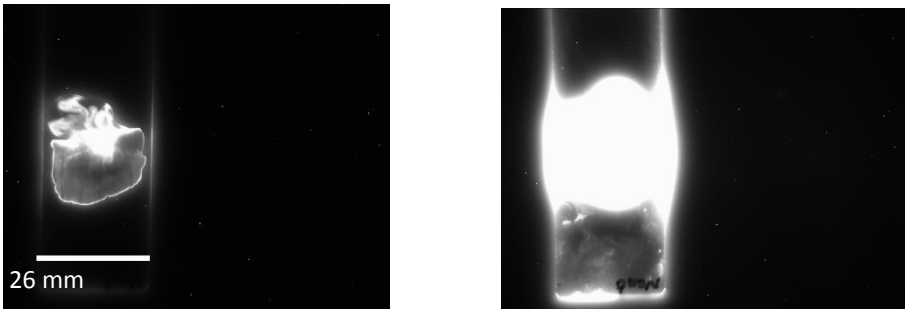


Figure 3.2.5: GeneSnap Image made up of a series of 5 images taken over a period of 51 minutes of the neat anti-rat immunoglobulin horseradish peroxidase and the 1:1000 dilution using a luminol-hydrogen peroxide solution (left image) and an enhanced luminol-hydrogen peroxide solution (right image).

Though the enhanced luminol-hydrogen peroxide solution doesn't visually appear to have had an effect on the 1:1000 dilution (Figure 3.2.5) from the data collected an increase in the light intensity of both the neat and 1:1000 solutions was achieved (Figure 3.2.6). However, as the intensity of the neat solution is so strong it is possible that it is contributing to the value detected for the 1:1000 solution.

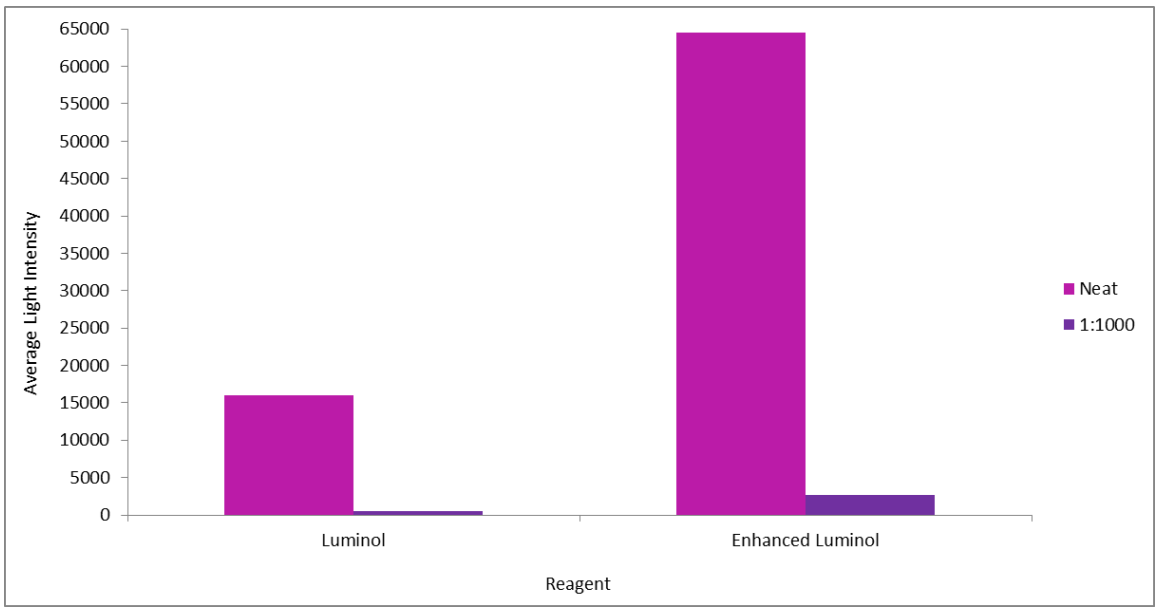


Figure 3.2.6: Graph comparing the data collected using ImageJ software from the GeneSnap images taken after 51 minutes of the luminol-hydrogen peroxide solution and the enhance luminol-hydrogen peroxide solution.

In order to better analyse the reaction between the enhanced luminol solution and the horseradish peroxidase present in the 1:1000 solution, the same protocol was run using a 1:500 dilution alongside the 1:1000. By comparing the two images it became apparent that the reaction occurring on the neat slide was affecting the results of the 1:1000 dilution as the light intensity values dropped when in the presence of the 1:500 dilution (Figure 3.2.7).

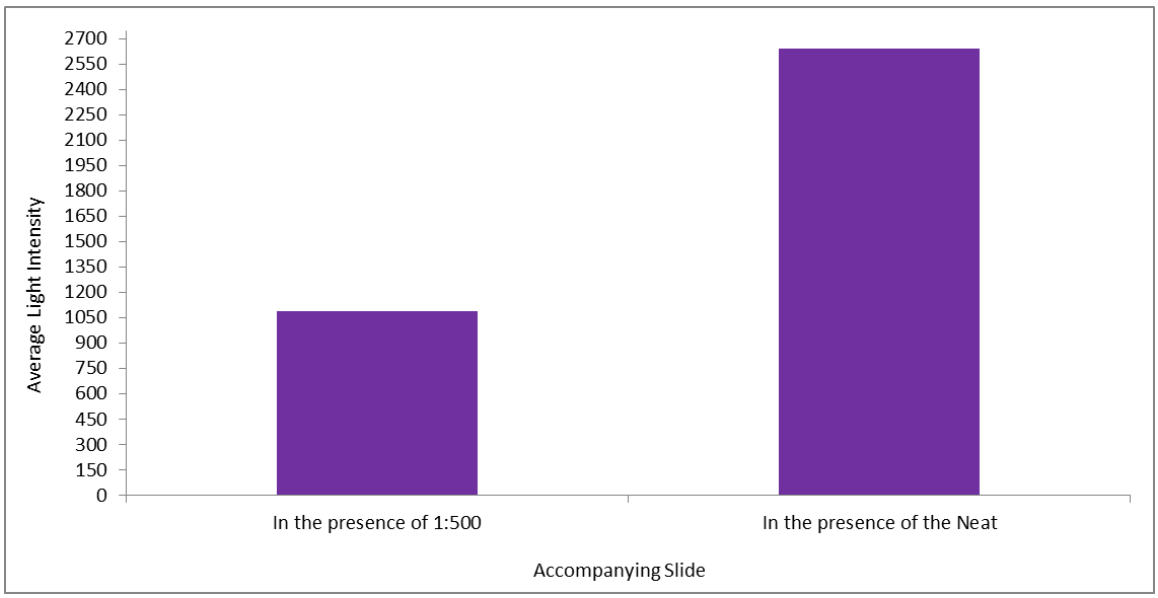


Figure 3.2.7: Graph comparing the data collected using ImageJ software of a 1.45mm area from the 1:1000 dilution in the presence of different accompanying slide.

In order to establish whether the same degree of luminescence could be observed when the reaction was carried out on a microfluidic scale, both the neat anti-rat immunoglobulin solution and the 1:1000 dilution were applied to the channels of the microfluidic device (see Section 2.4.1.Method F).

The enhanced luminol solution was added to each microfluidic device separately and the reaction monitored immediately after. The solution was manually pushed through the device until the appearance of waste solution from the outlet to ensure that the enhanced luminol-hydrogen peroxide solution had covered the entire length of the microfluidic channels.

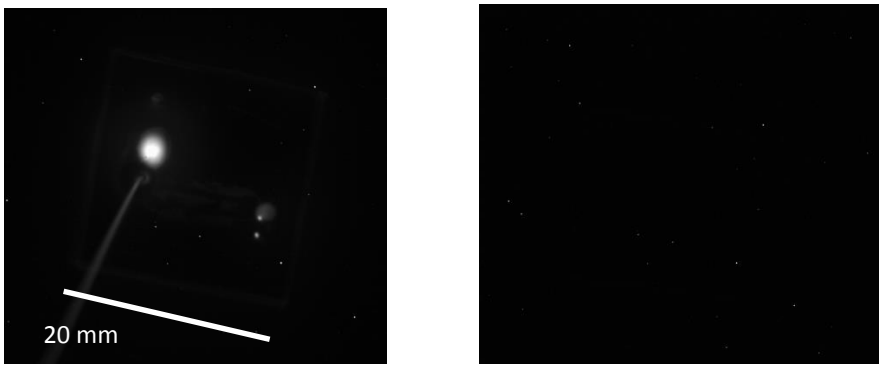


Figure 3.2.8: GeneSnap Image made up of a series of five images taken at set interval of over a period of 51 minutes of the neat anti-rat immunoglobulin horseradish peroxidase (left) and the 1:1000 dilution (right) applied to a microfluidic device using an enhance luminol solution.

In applying the solutions to the microfluidic devices, the volume of solution present was considerably decreased. Hence, carrying out the reactions on a micro scale proved to considerably decrease the luminescence of both solutions (Appendix 2). It reduced the light intensity value of the neat solution from 64523, when detected on the slide, to 2802 when detected on the microfluidic device. The 1:1000 dilution was reduced from 1090, on the slide, to 619 on the microfluidic device which was comparable to data collected when no device was present (Appendix 2 Figure A2.7).

When applied to the microfluidic device, the reaction between the enhanced luminol-hydrogen peroxide solution and neat anti-rat immunoglobulin was still visually observable at both the injection point and the waste outlet (Figure 3.2.8 left image), however the reaction was not observed within the channels. When the 1:1000 reaction was applied to the device no reaction was observed (Figure 3.2.8 right image) and in carrying out ImageJ

analysis on the corresponding image no light intensity was detected (Appendix 2, Figure A2.7).

Due to the micro volumes contained within the channels, even at the highest concentrations, the reaction occurring was too weak to be detectable. It was for this reason, and due to the time limitations of the project, that it was decided this type of microfluidic device coupled with an ELISA based competitive immunoassay would not be viable for drug detection. In order for a point-of-care test to be desirable it needs to be able to detect substances even at their lowest concentrations and this would not be possible with this method.

3.3 Paper Based Microfluidic Device

In order to test which of the methods previously applied to the glass microfluidic device provided the greatest degree of capture efficiency, revised methods (see Section 2.4.1.Method A and Section 2.4.1.Method B) were applied to the optimised paper-based device (see Surface Preparation 2.3.1).

Both a sandwich immunoassay, (Section 2.4.1 Method A (Appendix 3 Figure A3.1.1)) and direct immobilisation of the antibody, (Section 2.4.1 Method B (Appendix 3 Figure A3.1.2)) were carried out alongside each other. This was done in order to establish if the same difficulties in adhering the primary antibodies to the surface, as already established with the glass device, were also present using a paper medium. Visual observation of the result seemed to indicate that the opposite was in fact true for the paper medium. The sandwich immunoassay (see Section 2.4.1 Method A) provided a more reproducible result with a stronger positive reaction than when only the secondary antibodies were added to the surface indicating that more of the horseradish peroxidase is present and hence more of the secondary antibodies have adhered to the primary antibodies than to the surface itself. In order to test the applicability the paper based device a standard sandwich immunoassay practical (see Section 2.4.1 Method C) was scaled down in order to suit the parameters of the microfluidic device (Figure 3.3.1).

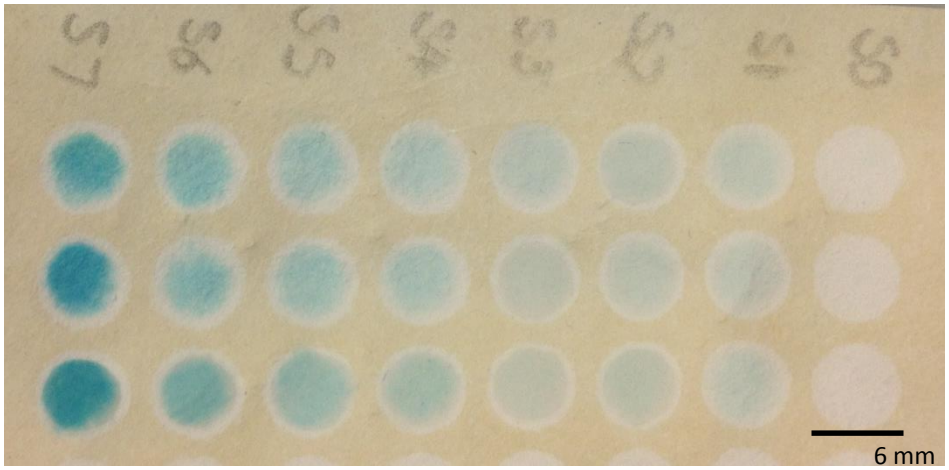


Figure 3.3.1: Sandwich immunoassay of the serial dilutions of the 1:2000 dilution of the anti-rat immunoglobulin conjugate horseradish peroxidase reacted with TMB. N.B. Truncated for simplicity. Specify S1-S7.

Based on the results collected from the glass microfluidic device, chemiluminescence had proved the most effective form of detection. However, when the sandwich immunoassay was carried out on the paper based device this was not the case. From the results collected, it was apparent colourimetric detection was going to be the most effective method for the paper based device and, though several other methods of detection have been reported for paper based microfluidic device,[87] it was believed that colourimetric would deliver the most instantaneous response and provided the quickest and easiest method of detection.

3.3.1 Intra/Inter Variation Study

In order to test the reliability and reproducibility of the results collected utilising the sandwich immunoassay (see Section 2.4.1.Method C) an intra/inter variation study was carried out (see Section 2.3.1.Method D) using four plates across four weeks.

The light intensity values from the different concentrations of anti-rat IgG-HRP were collected using ImageJ and analysed using both Microsoft Excel and Minitab, a general linear model using the Tukey method with a 95% confidence level was run in order to establish the significance of the values (Appendix 4.2). The general trend across the plates showed that there was no significant difference in the light intensity results collected, indicating that the inter-plate variability is low, and that the method of device production leads to consistent results between the plates. However, analysis of the results across the weeks showed there was a significant difference in the light intensity results collected. Though the results varied slightly for the different concentrations, the general trend

showed that week's 0, 1 and 2 produced significantly better result than those collected in week 4, with week 2 consistently producing good results across all concentrations (Figure 3.3.2).

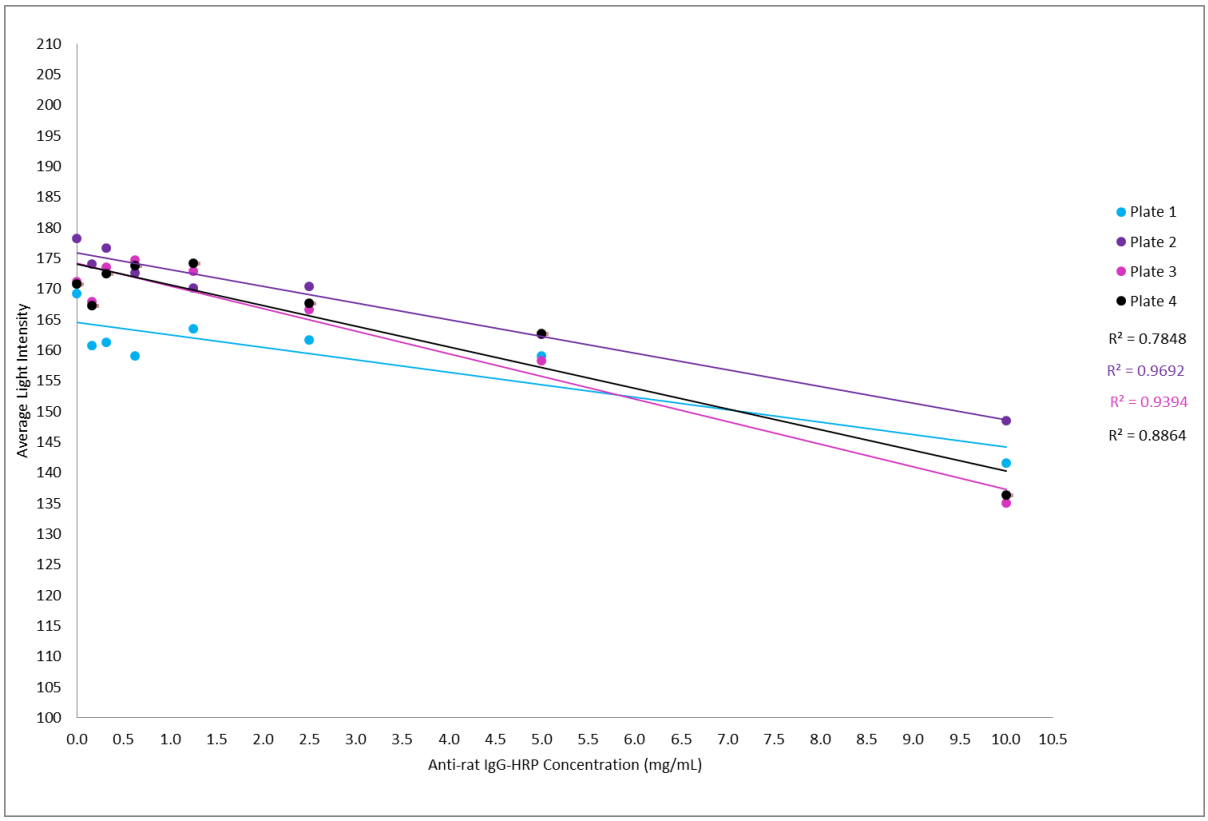


Figure 3.3.2: A calibration graph displaying all the average results from all four plates of week 2 against concentration.

This was supported by plotting the average light intensities against concentration, the calibration graph produced for week 2 (Figure 3.3.2) displayed the trendlines with the highest R^2 values as well as containing the smallest standard deviation error bars with the least overlap. The significant difference from week 2 to week 4 suggests that the stability of the plate drops considerably between week 2 and 4, indicating that in order to achieve optimal results testing should be carried out no later than week 2.

By plotting an inter variation graph of the average light intensities from the individual weeks against concentration (Figure 3.3.3) the drop from week 2 to week 4 can be seen more clearly. Though the linearity is maintained within each week there is an obvious drop in the light intensity values obtained from week 2 to week 4. From the literature it had been reported that similar devices kept under the equivalent storage condition have shown responses after 40 days that are comparable to those seen at day 1.[82] Though good results were still achieved in week 4 the drop in the response seen over the weeks

would led us to conclude that prolong storage of the device could affect the accuracy of the result being achieved.

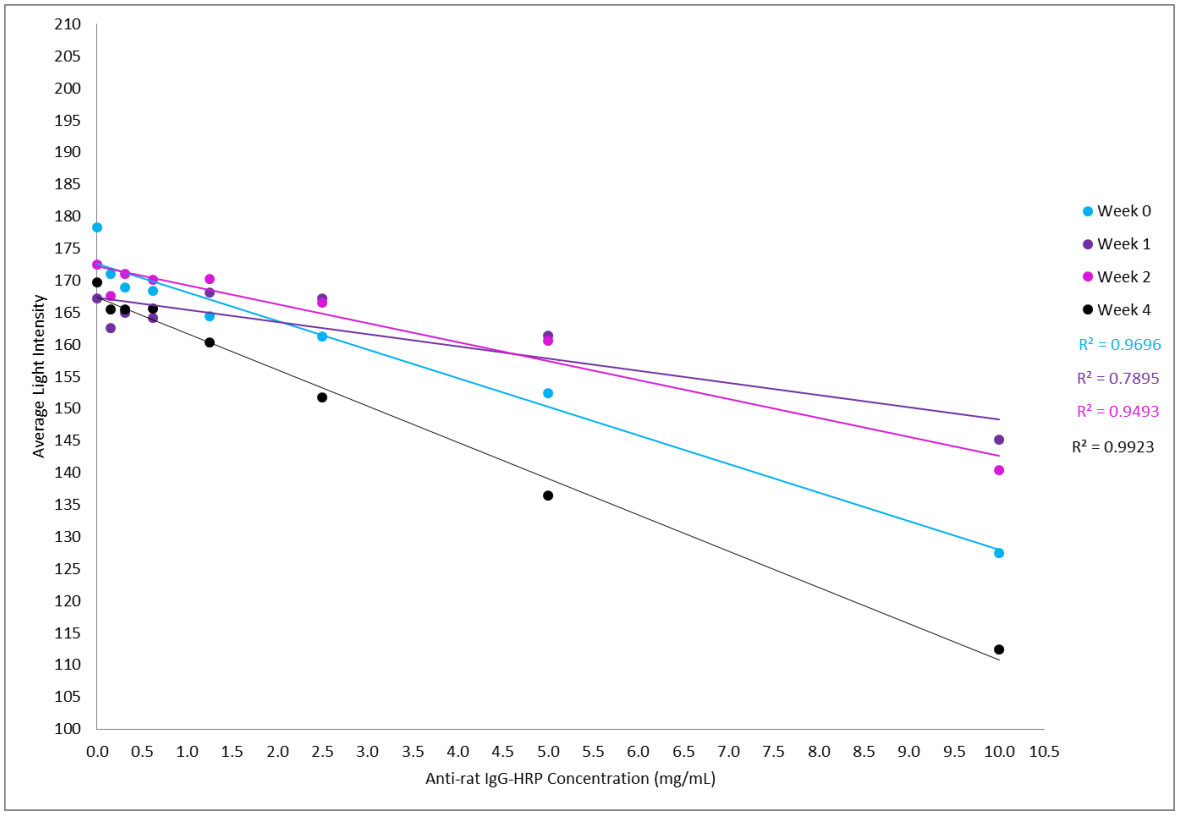


Figure 3.3.3: A calibration graph displaying the inter plate results from all four weeks against concentration.

3.3.2. Printing Method

Investigations into paper based microfluidics has grown in popularity over recent years and with wax printing being the most widely used method of printing, wax printers have become more and more widely available. Though a traditional printing technique was used to create the majority of the devices utilised within this study, access to a wax printer became available. Several studies, initially carried out on the traditionally printed plates were applied to the computer printed plates in order to compare the data. The results observed using the new method followed the same trend as previously established giving good indication that the techniques are comparable. The key difference noticed between the plates was the reproducibility of the wells themselves and in order to establish the consistency of the well area created for each method analysis was carried out (Figure 3.3.4).

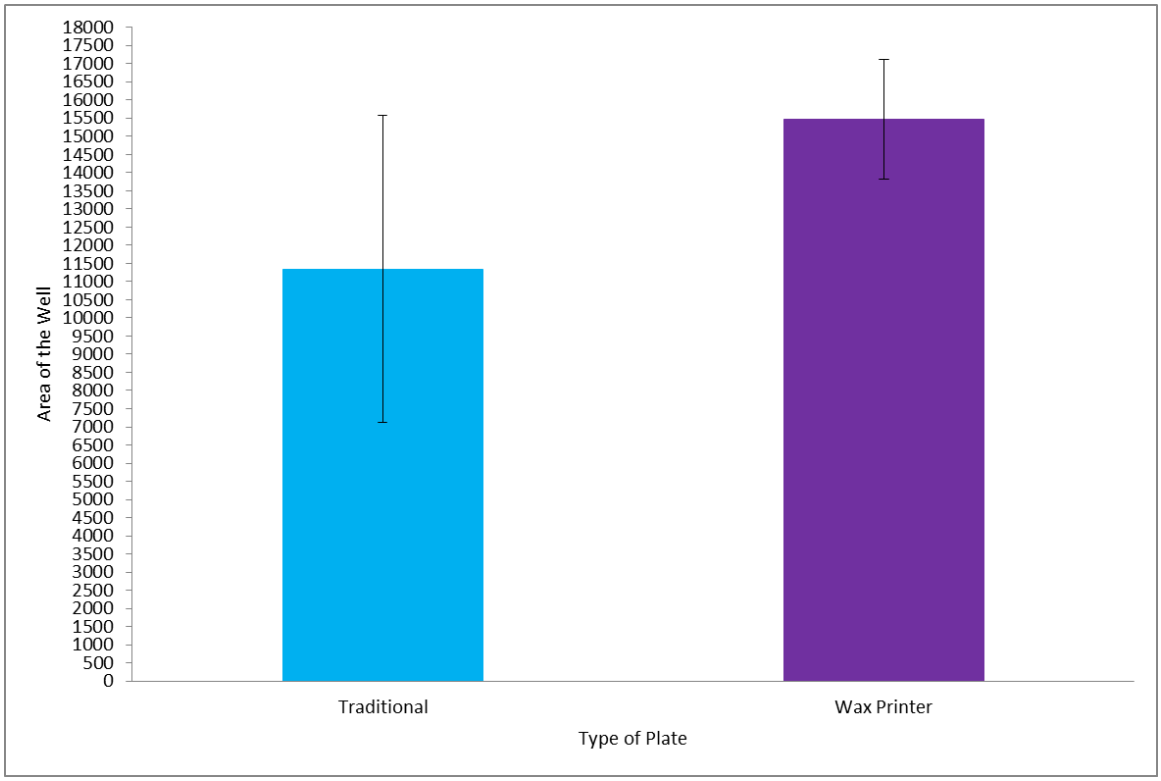


Figure 3.3.4: A comparison between the consistency of the well area created using the traditional printing method and the wax printer.

As this method is intended for use as a portable point-of-care test, it is vital that the process used to manufacture the devices is consistent and therefore allowing reproducible reliable results to be generated. The uniformity achieved using the wax printer was obvious during testing as adsorption of the microvolumes was consistent across all the individual test zones. A random selection of both types of plates were analysed. By applying standard deviation error bars to the data sets it shows the variation in the size of the well created using the traditional method is considerably greater than that of the wax printer. As well as providing more consistent and reproducible plates the acquisition of the wax printer also reduced the time, cost and labour required in order to produce the plates. Taking into account all of these variables it is clear that the wax printer was a more suitable technique and was carried forward and applied to any further testing.

3.3.3.Optimisation

A similar method (see Section 2.4.1.Method D) to the one developed for the intra/inter variation study was applied in order to determine the optimum dilution for both the anti-methcathinone antibody and the cathinone-HRP (Figure 2.7.1). Initially, a variety of dilutions of the cathinone-HRP were tried, ranging from 1:10,000 to 1:100,000. It was

established that 1:10,000 was too concentrated; preventing subtle changes in the amount of drug present from affecting the amount of cathinone-HRP, but anything above 1:20,000 was too dilute and therefore undetectable. This allowed a narrower dilution range of between 1:12,000 to 1:20,000 to be established. From the dilution range established, it was observed that the 1:12,000 1:14,000 and 1:16,000 dilutions of cathinone-HRP provided the best calibration curves and R^2 values (Appendix A5.2) as well as producing values within a similar range to those seen in the previous immunoassays.

The manufacturers recommended anti-methcathinone antibody concentration was 0.625 µg/mL. Due to the increase in surface area to volume ratio associated with microfluidic devices a much larger volume of antibodies were adhering to the surface and thus a much lower dilution was required. The dilutions of cathinone-HRP were then applied to a series of antibody concentrations, ranging from 5.79 µg/mL to $1.104x10^{-5}$ µg/mL (Appendix 5, Figure A5.1.1/2/3).

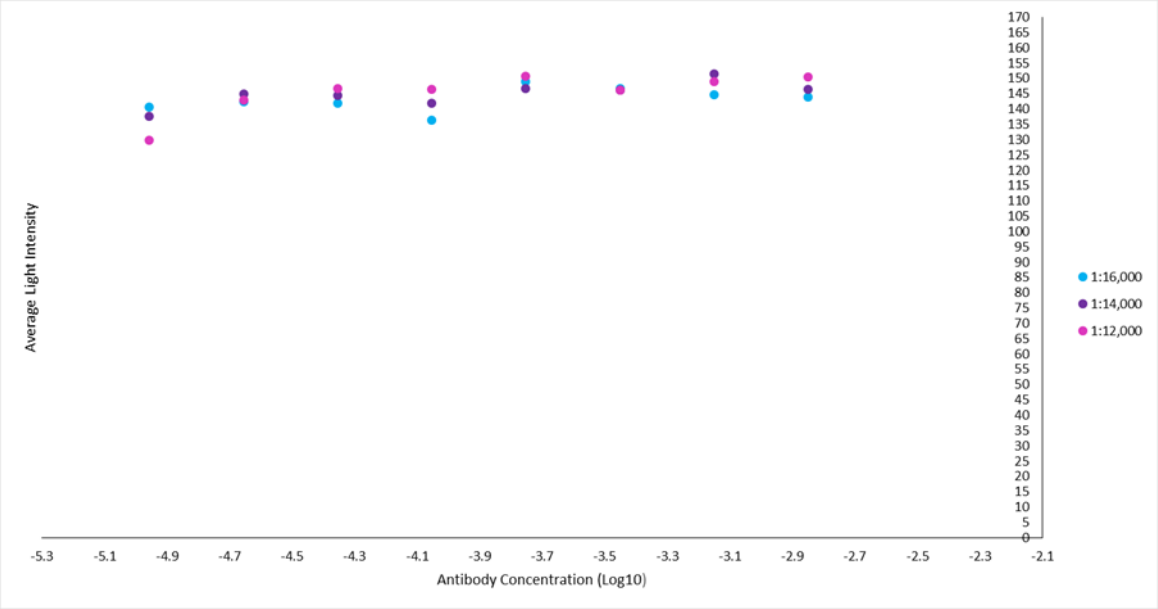


Figure 3.3.5: Comparison calibration graph of the 1:16,000 1:14,000 and 1:12,000 dilutions of cathinone-HRP for the concentration range of 0.00141-$1.104x10^{-5}$ µg/mL anti-methcathinone antibody plotted on a Log10 scale.

From the graphs plotted of the anti-methcathinone antibody, the concentration range of 0.00141 µg/mL to $1.104x10^{-5}$ µg/mL (Figure 3.3.5) appeared to display a cut-off point as the average light intensity values appeared to plateau at $8.835x10^{-5}$ µg/mL and the light intensity values were within the range achieved when no sample was present within the well. This was later confirmed by regression analysis (Appendix 5 Figure A5.2.3), as the

concentration range of 0.00141 µg/mL to $1.104x10^{-5}$ µg/mL provided the best R^2 values across all three dilutions. Analysing the three dilutions separately showed that the 1:12,000 dilution of cathinone-HRP (Figure 3.3.6) maintained the best calibration curve up to the cut-off point and was therefore the most suitable dilution for setting up the immunoassay (Figure 2.7.1).

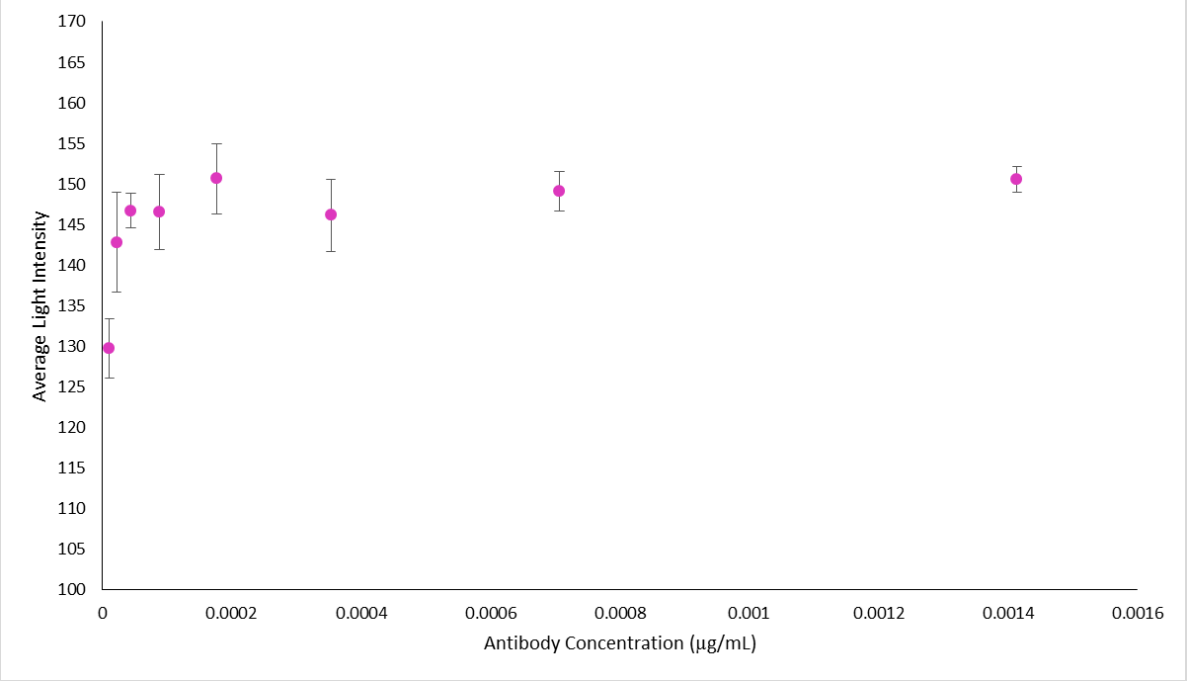


Figure 3.3.6: Calibration graph displaying the six lowest concentrations of the 1:12,000 dilution of cathinone-HRP for a concentration range of 0.00141-$1.104x10^{-5}$ µg/mL anti-methcathinone antibody.

As a final anti-methcathinone antibody dilution had yet to be established a competitive immunoassay using a mephedrone and cathinone-HRP serial dilution was applied (see Section 2.4.3.Method E). The mephedrone concentration ranged from 70 ng/mL to 0.137 ng/mL, and was run in triplicate on the four lowest antibody concentrations of the 0.00141 µg/mL to $1.104x10^{-5}$ µg/mL range. The results gathered (Appendix 5, Figure A5.2.8) demonstrated that the $7.068x10^{-4}$ µg/mL anti-methcathinone antibody concentration presented the most consistent results across the dilution range and led to the most ideal calibration curve (Figure 3.3.7).

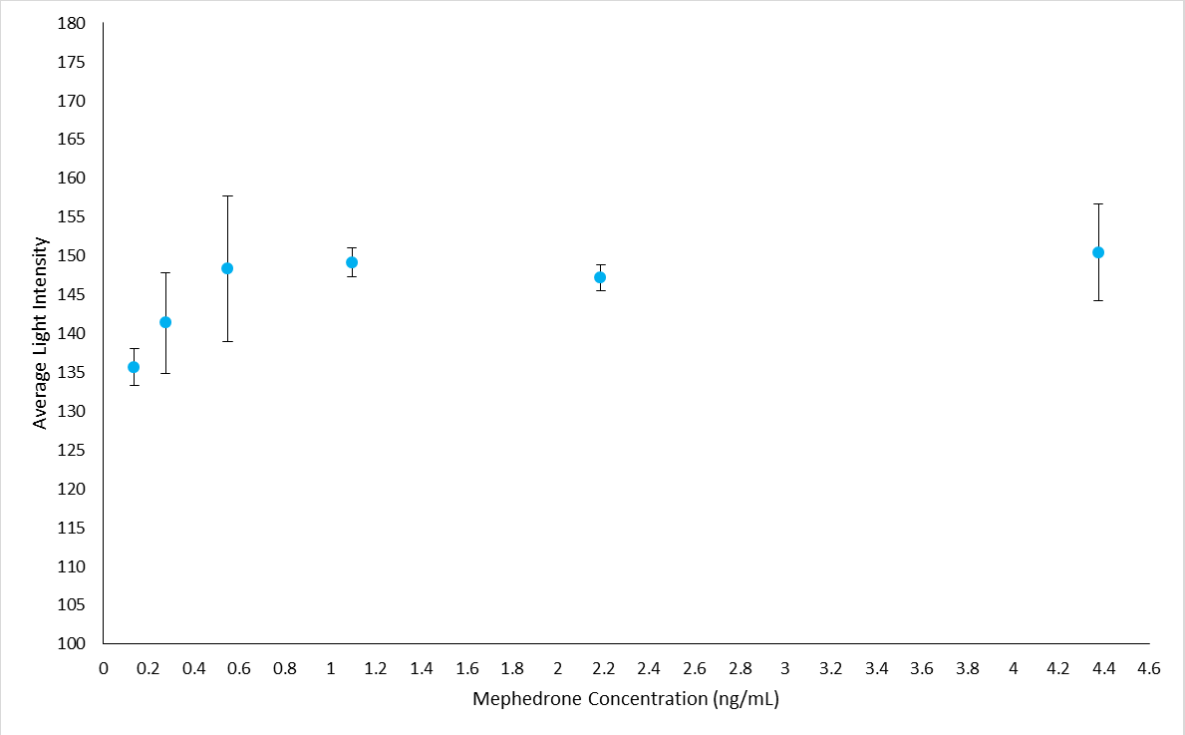


Figure 3.3.7: Calibration graph of the six lowest concentrations of mephedrone run on the 7.068x10^{-4} µg/mL concentration of the anti-methcathinone antibody.

3.3.4. Biological Samples

In order for this test to be viable as an on-site, point-of-care device the method needed to be compatible with biological samples. Urine and saliva were considered the most applicable to this study as they required no prior preparation and were least likely to interfere with the chose method of detection. Samples were donated by healthy volunteers and anonymised upon receipt. Initial observations indicated the results collected from the mephedrone spiked urine sample were comparable to those collected when mephedrone was carried out in aqueous media. In the presence of high concentrations of mephedrone the response generated by the TMB was less indicating that the mephedrone was successfully competing with the cathinone-HRP for the active sites of the antibody. However, this was not the case for the saliva sample as even at high concentrations of mephedrone a colour change presented indicating the presence of cathinone-HRP (Figure 3.3.8).

As the biological samples were tested without prior purification, it was hypothesised that there was potentially a naturally occurring enzyme present that could be affecting the results. Controls of both the neat samples and the samples with only cathinone-HRP were tested. The results for urine were as expected, no reaction was observed for the neat sample and the blue colour change reaction was observed for the sample with cathinone-

HRP. This was not the case for the saliva sample as both the neat and the sample with cathinone-HRP produced a blue colour change reaction. A simple test of equal parts of neat saliva to TMB produced a strong colour change, confirming that there was a naturally occurring enzyme present affecting the colourimetric test. It is believed that the presence of hydrogen peroxidase in the antibacterial enzymes and salivary lactoperoxidase in the antimicrobial enzymes are responsible for this reaction. [88]

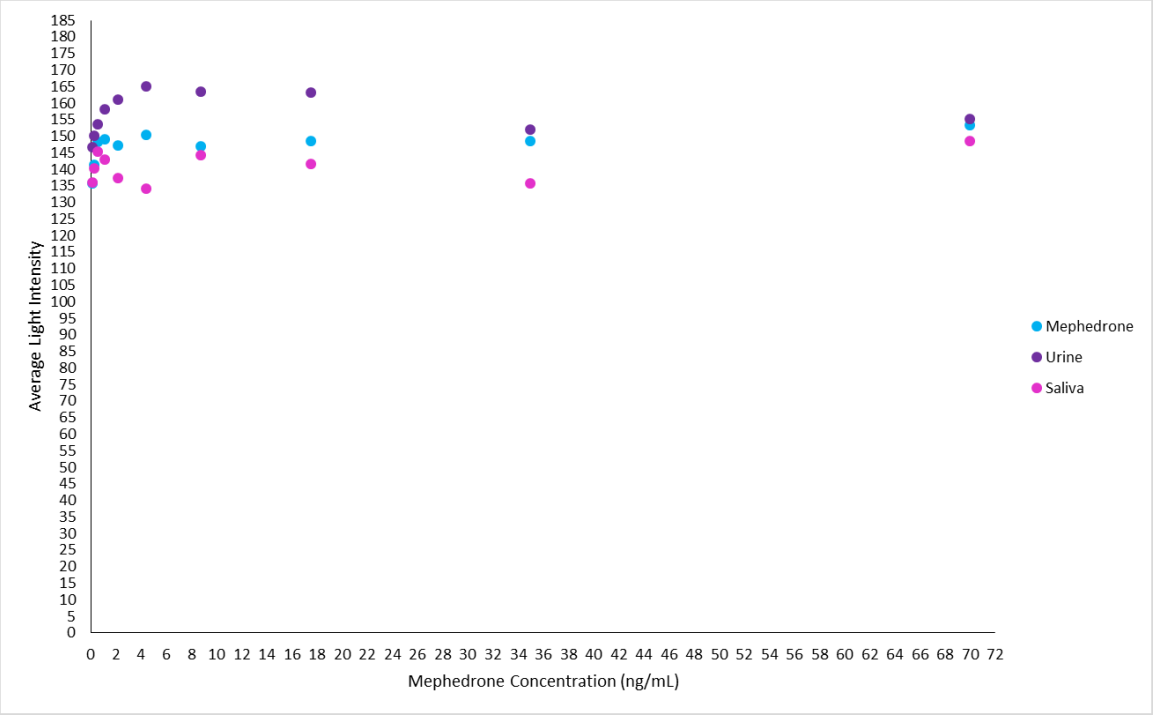


Figure 3.3.8: Comparison between the average light intensity results of a serial dilution of a 70ng/mL solution of mephedrone carried out in aqueous, urine and saliva samples.

The ImageJ data collected from the mephedrone spiked biological samples was compared to an aqueous dilution of mephedrone (Figure 3.2.8). As expected, based on initial observations, the calibration curve for the spiked saliva sample showed no trend. However, the calibration curve obtained for the spiked urine sample was comparable to that of the aqueous mephedrone and showed good distinguishability even at the lowest concentrations of mephedrone. As ImageJ analyses the degree of white light its make differentiating between the higher concentrations hard than that of the lower concentrations. Analysis of the three data sets showed that the linearity of both the urine and aqueous mephedrone solution became poor above 4.375 ng/mL (Appendix 6.2). As expected, the spiked saliva sample showed little to no correlation. This reason, along with the results collected from the controls, indicated that using saliva as a medium in which to detect mephedrone is not a viable option for this method.

In order to determine the limit of detection (LOD) and the limit of quantification (LOQ), regression analysis of the 4.375 μg/mL concentration of the aqueous mephedrone and the biological samples was carried out (Appendix 6.2). An LOD of 4.078 μg/mL and 1.597 μg/mL was calculated for the aqueous mephedrone and spiked urine sample, respectively. In comparing these values with the clinically relevant concentrations for mephedrone in urine (LOD= 2 μg/mL and LOQ= 4 μg/mL) it shows that this method has good sensitivity.[89] As well as being highly sensitivity for this specific application it also showed improved sensitivity to already reported methods such as streptavidin-biotin on magnetic beads which was only able to detect down to a mg/mL level. [63]

3.3.5.Cross-Reactivity

Due to the high degree of cross-reactivity associated with the anti-methcathinone antibody and the positive results obtained with both mephedrone and cathinone-HRP conjugate, the same concentration range was applied to samples of methcathinone, cathinone, 4-methylephedrine and ephedrine. Establishing whether this test is selective as well as sensitive is important, as it will allow for differentiation between powdered samples as well as providing a more robust method for detecting these substances within biological matrices. Analysis of the data collected (Figure 3.3.9) confirmed that the anti-methcathinone antibody did in fact cross-react, to varying degrees, with all of the members of the cathinone family tested.

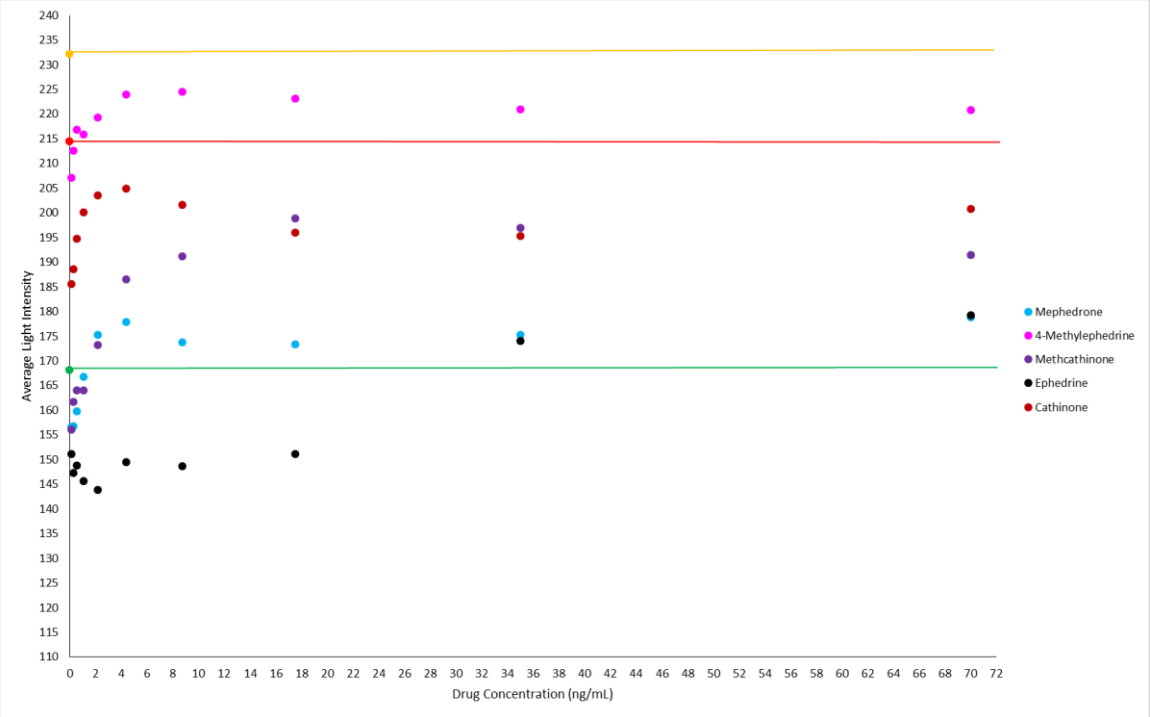


Figure 3.3.9: Comparison of the competitive immunoassays set up using a serial dilution of a 70ng/mL solution of mephedrone, methcathinone, cathinone and the corresponding metabolites 4-methylephedrine and ephedrine (yellow line = blank, red line = neat urine, green line = urine + HRP).

Mephedrone is metabolised by the body via two different processes, the main type of metabolism it undergoes is via a primary route of demethylation in order to form a cathinone product, it can also metabolise by a secondary route via the reduction of the ketone to form a 4-methylephedrine product. From Figure 3.3.9 it can be seen that both the cathinone and 4-methylephedrine produced positive light intensity results as well as good calibration curves. Thus demonstrating that both the 4-methylephedrine and the cathinone are successfully competing with the cathinone-HRP for the active sites on the antibodies and that even small changes in the drug concentration affect the amount of cathinone-HRP present. Positive results were also observed for another common cathinone derivative, methcathinone and its primary metabolite, ephedrine. However, both the light intensity values and the calibration curves were weaker than those previously seen indicating that even though they both successfully compete for the active sites on the antibodies their binding affinity is not as strong as the mephedrone metabolites, making them harder to detect.

Due to the concentration range over which the cathinones were tested, it was not possible to calculate the percentage cross-reactivity as the cathinones did not reach 50%

of their original concentration (B_0), which is required in order to calculate it. Instead a T-test was carried out on the data sets. From the statistical analysis (Appendix 7.2.1) it can be seen that there is significant difference between mephedrone and 4-methylmethcathinone and between mephedrone and cathinone. As both values are higher than zero it indicates that both 4-methylephidrine and cathinone have a greater degree of cross-reactivity to the antibody than mephedrone. On the other hand, the significant difference between mephedrone and ephedrine was observed to be lower than zero, implying that ephedrine has less cross-reactivity to the antibody. The analysis also showed that there was a significant difference between mephedrone and methcathinone, however this difference is minimal, suggesting that there was no difference in the affinity. Though it was not possible to calculate the cross-reactivity of this study, reported data showed that the methcathinone had a percentage cross-reactivity of around 50% supporting the findings of this study.[90] Data for the other cathinones used in this study could not be found.

In order to test the cross-reactivity of the anti-methcathinone antibody with other adulterants the same method (see Section 2.3.2 Method E) and concentration range was applied to samples of cocaine and ketamine. The data collected (Figure 3.3.10) showed that the level of cathinone-HRP binding to the antibody is consistent across the dilution range of both the cocaine and ketamine, suggesting that the cathinone-HRP is unaffected by the changing concentration of the drugs and that there is little cross-reactivity between neither cocaine nor the ketamine.

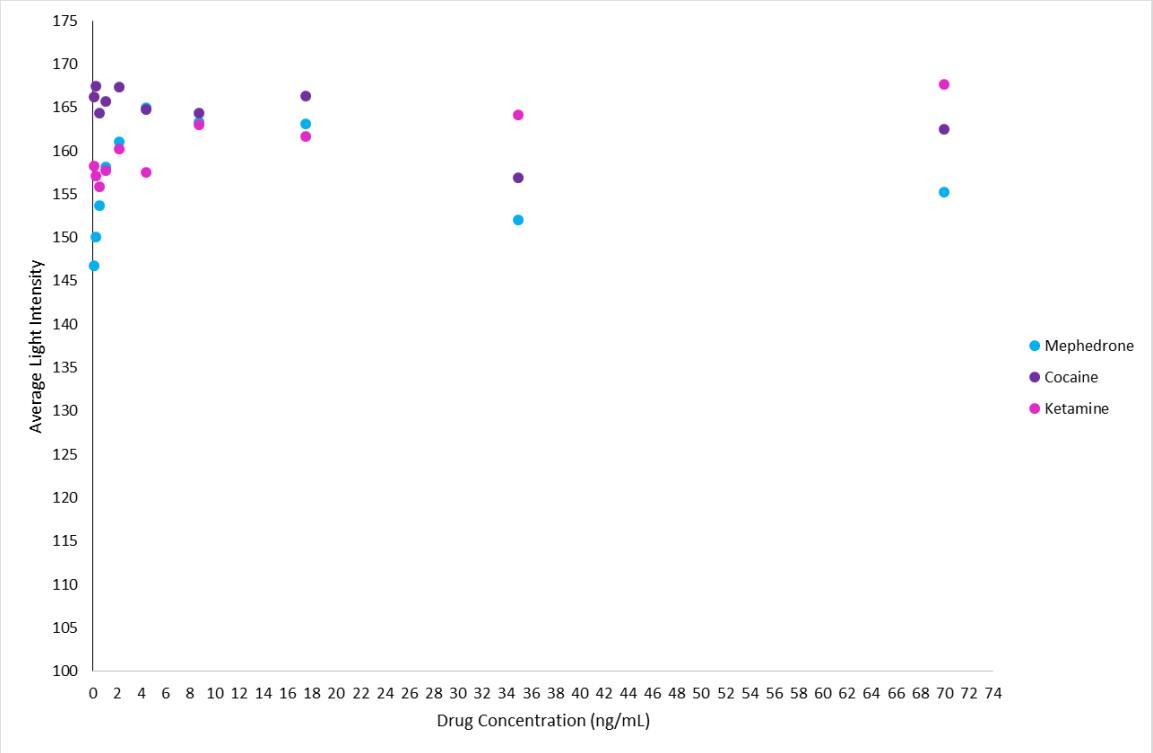


Figure 3.3.10: Comparison between the average light intensity results of a serial dilution of a 70ng/mL solution of mephedrone, cocaine and ketamine carried out in urine.

It is often found that street samples of illegal drugs are 'cut' with cheaper, legal drugs that are easily accessible, such as paracetamol or caffeine, in order increase the batch size and profit.[91] As these are everyday substances, even if they are not present in the drug sample, it is likely they could occur in a urine sample. From the literature it has been demonstrated that 1-4% of a 500 mg paracetamol tablet is present unmetabolised in urine.[92] Based on this assumption, a concentration range of 50 mg/mL – 0.048 mg/mL was established for paracetamol. The paracetamol concentration range was compared to that of mephedrone.

A general trend of linearity was observed across the concentration range of paracetamol, however some deviation was observed at the lower concentrations. From regression analysis (Appendix 7.2.2) it can be seen that the trend of linearity is constant across the entire range. Statistical analysis of the data (Appendix 7.2.2) gave a t-test value of 0.96 indicating that the values are not significant and that the paracetamol does not have an effect on the level of HRP being detected. The light intensity values detected and the trend observed suggests that paracetamol does not cross-react with the anti-methcathinone antibody and that the cathinone-HRP is unaffected by the change in its concentration and is able to bind to the active sites on the antibody regardless of the

amount of paracetamol present in the urine sample. The data collected gives a good indication that, whether it be as a cutting agent or self-administered, the presence of paracetamol in a urine sample would have little to no effect on the ability to detect the presence of mephedrone or other cathinone derivatives.

Chapter 4- Conclusion

Several different variations of microfluidic devices were trialled during this study. Though positive results were initially observed with the glass microfluidic device it was believed that due to the large amount of equipment required in order to analyse the device and the limits of detection being achieved that applying an assay to this medium was not a viable method for this type of test as adapting it into a portable, easy to use, point-of-care test would be challenging and not within the time frame allowed for this study. This conclusion allowed investigation into paper based devices to begin.

A variety of different methods were tested in order to find the optimal way in which to wax print the devices. The majority of the microfluidic plates were printed by a traditional screen printing technique and yielded a great deal of positive results. The application of an intra-inter stability study showed that there was no significant difference between different plates, this gave good indication that tests carried out across a range of different plates would in fact yield reproducible data. By carrying out the study over several weeks it allowed us to establish the stability of the surface antibodies and estimate how long the plates could be kept in cold storage, the study indicated that the plates were optimal up to two weeks. This would allow for more widespread use of the point-of-care test and enable optimisation and pre-coating with the primary antibody to occur prior to distribution.

A late stage acquisition of a wax printer provided a much simpler, quicker and more uniform method of printing plates, comparison between the two methods yielded similar results and provided good confirmatory evidence for the results previously established. The plates produced using the printer showed a lot less variation between individual plates and gave a more reproducible test area. In order to make a point-of-care test applicable in the field each one produced needs to be identical to ensure the most accurate and reproducible results possible. The combination all the positive factors associated with the wax printer led to the decision that it was the most appropriate method for producing the paper based devices.

Though development of the paper based device itself is still required in order for it to be utilised as a point-of-care test, this study has yielded positive results. A competitive immunoassay has successfully been immobilised on the surface of the device and has not only been able to detect mephedrone, but has been able to detect and distinguish between several different cathinones and their metabolites. The devised method has successfully encompassed many of the advantages associated with microfluidic devices; it is both cheap and easy to produce, the incubation and overall reaction time has been reduced from days on a standard 96 well plate to hours on the paper based device, both the reagent and sample volumes require have been reduced to microlitre quantities and the sensitivity has been increased, taking the LOD from 4 μg/L using a combined targeted/untargeted LC-HR-QTOFMS to 2 ng/mL using the paper based device.[89] As well as being able to distinguish between different cathinone derivatives, it has also proved successful in being able to differentiate between cathinones and other illegal and legal drugs, producing negative results in the presence of cocaine, ketamine and paracetamol.

Chapter 5- Future Work

In order to develop this method further, additional investigations would need to be carried out. Studies into a wider range of other illegal drugs such as amphetamines, NRG substances and cannabis, as well as cutting agents and everyday compounds such as caffeine, ascorbic acid and sugars, would need to be considered to ensure that the immunoassay was selective for cathinone derivatives.[91] Also, in order to fully understand the extent of cross-reactivity between the cathinones, a wider concentration range would need to be applied. The concentration range would need to be expanded to ensure that a value 50% of the original concentration (B_0) was reached in order for the data to be applied to the cross-reactivity calculation.

This method could also be applied to the already established presumptive test. As several of the colour tests require a mixture of reagents, they are applied to the well before the sample is added. This could allow for a microfluidic paper analytical device (µPAD) to be utilised as a cheaper alternative to current point-of-care presumptive colour tests. The reagents could be pre-applied to specific zones on the µPAD ready for activation and the application of the sample on-site.

In addition to method development, further consideration could be applied to device design. Factors such as well size and shape, as well as the overall schematic of the plate could be considered to increase the sensitivity as well as prevent un-necessary wax being applied creating the most cost-effective, optimal, user friendly design for infield testing. Studies have been carried out into the use of 'origami' style designs (Figure 5.1).[82] The use of different test sites would allow for the incorporation of both a positive and a negative control, and by utilising multiple tabs it could allow the devices to be expanded to detected a wider range of drugs. Also, as the plates are stable for around two weeks, pre-application of the primary antibodies would ensure minimal steps were required within the field, making it both a rapid and easy-to-use test.

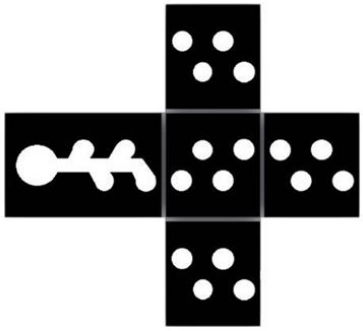


Figure 5.1: SolidWorks 2014 design of an 'origami' style paper microfluidic device.[82]

Once a fully optimised device was established the method would need to be fully validated. Factors such as robustness, selectivity and precision would need to be considered before this method could be used for forensic detection. As well as the device itself, consideration would need to be given to the method of detection. With the continuous improvement in technology, investigations have begun into the use of smart phone detectors.[93] Developing an 'app' that could analyse the colour change, measure the level of HRP and calculate the amount of drug present would allow for a more precise understanding of the result achieved and make this test both quantitative and qualitative.

References

1. DrugScope, Drugs Laws, http://www.drugscope.org.uk/resources/drugsearch/drugsearchpages/laws) (accessed 14 October 2014).
2. UNODC, New Psychoactive Substances Poster) (accessed 14 October 2014).
3. J. S. Chappell and M. M. Lee, *Forensic Science International*, 2009, **195**, 108-120.
4. D. Lee, Y.W., L. Yang, G. Deng, L. Guo and L.-Y. Liu-Chen, *Bioorganic and Medicinal Chemistry Letters*, 2010, **20**, 5749-5752.
5. G. Cox and H. Ramples, *Royal collage of psychiatrists*, 2003, **3**, 456-463.
6. P. Nencini and A. M. Ahmed, *Drug and Alcohol Dependence*, 1989, **23**, 19-29.
7. D. J. Siebert, *Annals of Botany*, 2004, **93**, 763-771.
8. Y. Zhang, E. R. Butelman, S. D. Schlussman, A. Ho and M. J. Kreek, *Psyychopharmacology*, 2005, **179**, 551-558.
9. A. Parkin, I. Oswald, D. H. and S. Parsons, *Acta. Crstallographica Section B*, 2004, **60**, 219-277.
10. D. W. Henry, *A Journal of Heterocyclic Chemistry*, 1966, **3**.
11. P. A. Mason and G. Sturman, *British Journal of Pharmacology*, 1972, **44**, 169-176.
12. J. Z. Min, Y. Shimizu, T. Toyo'oka, S. Inagaki, Kikura-Hanajiri and Y. Goda, *Journal of Chromatography B*, 2008, **873**, 187-194.
13. J. C. Robinson and H. R. Snyder, *Organic Synthesis*, 1955, **3**, 720.
14. E. M. Parker and L. X. Cubeddu, *The Journal of Pharmacology and Experimental Therapeutics*, 1988, **245**, 199-210.
15. T. Villevielle and G. Mion, *CNS Neuroscience and Theropeutics*, 2013, **19**, 370-380.
16. S. C.L., Synthesis of Ketamine, http://www.google.com/patents/US3254124) (accessed 9 Septemeber 2014).
17. C. Morgan, J. A. and H. V. Curran, *Addiction*, 2011, **107**, 27-38.
18. C. H. Ashton, *British Journal of Anaesthesia*, 1999, **83**, 637-649.
19. P. E. Harrington, I. A. Stergiades, J. Erickson, A. Makriyannis and M. A. Tius, *Journal of Organic Chemsitry*, 2000, **65**, 6576-6582.
20. M. Guzman, *Nature Reviews:Cancer*, 2003, **3**.
21. J. B. Zawilska and J. Wojcieszak, *Forensic Science International*, 2013, **231**, 42-53.
22. M. Coppola and R. Mondola, *Toxicology Letters*, 2012, **211**, 144-149.
23. C. L. German, A. E. Fleckenstein and G. R. Hanson, *Life Sciences*, 2014, **97**, 2-8.
24. O. I. G. Khreit, C. Irving, E. Schmidt, J. A. Parkinson, N. Nic Daeid and O. B. Sutcliffe, *Journal of Pharmaceutical and Biomedical Analysis*, 2012, **61**, 122-135.
25. M. Coppola and R. Mondola, *Toxicology Letters*, 2013, **218**, 24-29.
26. B. A. Hathaway, D. E. Nichols, M. B. Nichols and G. K. W. Yim, *The Journal of Medicinal Chemistry*, 1982, **25**, 535-538.
27. RSC, Phencyclidine, CSID:6224, http://www.chemspider.com/Chemical-Structure.6224.html (accessed 9 September, 2014).
28. S. D. Brandt, S. Freeman, I. A. Fleet, P. McGagh and J. F. Alder, *The Analyst*, 2004, **130**, 340-344.
29. A. T. Shulgin and D. E. MacLean, *Clinical Toxicolgy*, 1976, **9**, 553-560.
30. T. Bey and A. Patel, *The California Journal of Emergency Medicine*, 2007, **8**, 9-14.
31. R. A. Abramovitch and D. Shapiro, *Journal of the Chemical Society (Resumed)*, 1956, 4589-4592.
32. R. S. G. Jones, *Progress in Neurobiology*, 1982, **19**, 117-139.
33. P. Adamowicz, B. Tokarczyk, R. Stanaszek and M. Slopianka, *Journal of Analytical Toxicology*, 2012, 1-16.
34. UNODC, *World Drugs Report 2013*, United Nations, 2013.
35. UNODC, Early Warning Advisory on New Psycoactive Substances, https://www.unodc.org/LSS/SubstanceGroup/Details/67b1ba69-1253-4ae9-bd93-fed1ae8e6802, (accessed 14 October, 2014).

36. UNODC, *World Drug Report 2014*, United Nations, 2014.
37. J. M. Prosser and L. S. Nelson, *Journal of medical toxicology : official journal of the American College of Medical Toxicology*, 2012, **8**, 33-42.
38. S. Gibbons and M. Zloh, *Bioorganic and Medicinal Chemistry Letters*, 2010, **20**, 4135-4139.
39. E. Y. Santali, A.-K. Cadogan, N. N. Daeid, K. A. Savage and O. B. Sutcliffe, *Journal of Pharamaceutical and Biomedical Analysis*, 2011, 246-255.
40. L. Iversen, M. White and R. Treble, *Neuropharmacology*, 2014, **87**, 59-65.
41. L. A. Johnson, R. L. Johnson and R.-B. Portier, *The Journal of Emergency Medicine*, 2013, **44**, 1108-1115.
42. D. J. Newman and C. P. Price, *Principle and Practice of Immunoassay*, Macmillan Reference, Basingstoke, Second Edition edn., 1997.
43. E. I. Azhar, *Journal*.
44. M. J. Swortwood, W. L. Hearn and A. P. DeCaprio, *Drugs Testing and Analysis*, 2014, 716-727.
45. M. R. Meyer, J. Wilhelm, F. T. Peters and H. H. Maurer, *Analysis of Bioanalytical Chemistry*, 2010.
46. J. Martinez-Clemente, R. Lopez-Arnau, M. Carbo, D. Pubill, J. Camarasa and E. Escubedo, *The Journal of Psycopharmacology*, 2013.
47. D. W. Geoffrey, *Immunoassay Handbook-Theory and Applications of Ligand Binding, ELISA and Related Techniques*, Fourth Edition edn., 2013.
48. G. J. V. Nossal, *Scientific American*, 1964.
49. K. L. Cox, V. Devanarayan, A. Kriauciunas, J. Manetta, C. Montrose and S. Sittampalam, *Journal*, 1-35.
50. E. Y. Santali, A.-K. Cadogan, N. N. Daeid, K. A. Savage and O. B. Sutcliffe, *Journal of Pharmaceutical and Biomedical Analysis*, 2011, **56**, 246-255.
51. P. M. O'Bryne, P. V. Kavanagh, S. M. McNamara and S. M. Stokes, *Journal of Analytical Toxicology*, 2013, **37**, 64-73.
52. D. Ammann, J. M. McLaren, D. Gerostamoulos and J. Beyer, *Journal of Analytical Toxicolgy*, 2012, **36**, 381-389.
53. A. M. Leffler, P. B. Smith, A. De Armas and F. L. Dorman, *Forensic Science International*, 2014, **234**, 50-56.
54. J. P. Smith, O. B. Sutcliffe and C. E. Banks, *Royal Society of Chemistry*, 2015, **Analyst**.
55. K. Downard, *Mass Spectrometry; A Foundation Guide*, Royal Society of Chemistry, 2004.
56. R. P. Archer, *Forensic Science International*, 2009, **185**, 10-20.
57. K. Robards, P. R. Hoddad and P. E. Jackson, *Principles and practice of modern chromatographic methods*, Academic Press Limited, San Diego, CA, 1994.
58. D. Parriott, *A Practical Guide to HPLC detection*, Academic Press INC., San Diego, CA, 1993.
59. G. M. Whitesides, 2006, **442**, 368-373.
60. E. Al-Hetlani, *Electrophoresis*, 2013, 1262-1272.
61. D. Kim and A. E. Herr, *Journal of Biomicrofluidics*, 2013, **7**, 1-47.
62. S. Halldorsson, E. Lucumi, R. Gomez-Sjoberg and R. M. T. Fleming, *Biosensors and Bioelectronics*, 2014, 218-231.
63. Y.-N. Yang, H.-I. Lin, J.-H. Wang, S.-C. Shiesh and G.-B. Lee, *Biosensors and Bioelectronics*, 2009, **24**, 3091-3096.
64. A. Salehi-Reyhani, S. Sharma, E. Burgin, M. Barclay, A. Cass, M. A. A. Neil, O. Ces, K. R. Willison and D. R. Klug, 2013, 2066-2074.
65. J. Zhang, S. Liu, P. Yang and G. Sui, 2011, 3516-3522.
66. I. P. M. Wijaya, T. J. Nie, S. Gandhi, R. BORO, A. Palaniappan, G. W. Hau, I. Rodriguez, C. R. Suri and S. G. Mhaisalkar, 2009, 634-638.
67. C. Kim, J. H. Bang, Y. E. Kim, S. H. Lee and J. Y. Kang, *Lab on a Chip*, 2012, **12**, 4135-4142.
68. Y. Bai, C. G. Koh, M. Boreman, Y. J. Juang, I. C. Tang, L. J. Lee and S. T. Tang, *Langmuir*, 2006, **22**.
69. Y. Yuan, H. He and L. J. Lee, *Biotechnology and Bioengineering*, 2009, **102**.
70. L. Yu, C. M. Li, Q. Zhou and J. H. Luong, *Bioconjugate Chemistry*, 2007, **18**.

71. D. Kim and A. E. Herr, *Biomicrofluidics*, 2013, **7**, 1-47.
72. J. P. Smith, J. P. Metters, O. I. G. Khreit, O. B. Sutcliffe and C. E. Banks, *Analytical Chemistry*, 2014.
73. A. J. Hughes, R. K. Lin and D. M. Peehl, *Proceedings of the National Academy of Sciences U.S.A.*, 2012, **109**.
74. M. Hashimoto, H. Kaji and M. Nishizawa, *Biosensors and Bioelectronics*, 2009, **24**, 2892-2897.
75. Y. Wang, H. H. Lai, M. Bachman, C. E. Sims, G. Li and N. L. Allbritton, *Analytical Chemistry*, 2005, **77**.
76. R. P. Sebra, K. S. Masters, C. Y. Cheung, C. N. Bowman and K. S. Anseth, *Anlytical Chemistry*, 2006, **78**.
77. G. Sui, C. Wang, C. C. Lee, W. Lu, S. P. Lee, J. V. Leyton, A. M. Wu and H. R. Tseng, *Analytical Chemistry*, 2006, **78**.
78. G. Nagy, I. Szollosi and K. Szendrei, *Colour Test for Precursor Chemicals of Amphetamine-Type Substances*, Szeged University, Hungary, UNODC, 2005.
79. UNODC, *Rapid Testing Methods of Drug of Abuse*, 1994.
80. M. Rouse and M. Haughn, *Journal*, 2013.
81. M. A. Huff, *Journal*.
82. L. Ge, S. Wang, X. Song, S. Ge and J. Yu, *Lab on a Chip*, 2012, 3150-3158.
83. G. H. G. Thorpe, L. J. Kricks, S. B. Moseley and T. P. Whitehead, *Clinical Chemistry*, 1985, **31**, 1335-1341.
84. S. Wang, L. Ge, X. Song, J. Yu, S. Ge, J. Huang and F. Zeng, *Biosensors and Bioelectronics*, 2012, 212-218.
85. S. Wang, X. Zhao, I. Khimji, R. Akbas, W. Qiu, D. Edwards, D. W. Cramer, B. Ye and U. Demirci, 2011, 3411-3418.
86. F. Declan, The Chemiluminescence of Luminol, http://www.chm.bris.ac.uk/webprojects2002/fleming/index.htm, (accessed 9 November, 2014).
87. L. D, B. Raguse, J. Gooding and E. Chow, *Sensors*, 2012, **12**, 11505-11526.
88. P. D. Vigna de Almeida, A. M. T. Gregio, M. A. N. Machado, A. A. Soares de Lima and L. R. Azevedo, *Journal*, 2008, **9**, 1-11.
89. M. Paul, Josef, C. Herrmann, S. Guber and W. Schultis, *Analytical and Bioanalytical Chemistry*, 2014, **406**, 4425-4441.
90. K. N. Ellenfsen, S. Anizan, M. S. Castaneto, N. A. Desrosiers, T. M. Martin, K. L. Klette and M. A. Huestis, *Drug Testing and Analysis*, 2014.
91. C. Cole, L. Jones, J. McVeigh, A. Kicman, Q. Syed and M. A. Bellis, *A Guide to Adulterants, Bulking agents and other Contaminants found in illicit drugs*, Liverpool John Moores University, Centre for Public Health, 2012.
92. G. M. Pacifici and K. Allegaert, *Current Therapeutic Research*, 2015, 24-30.
93. S. T. Park, W. Li and J.-Y. Yoon, presented in part at the 16th International Conference on Miniaturized Systems for Chemistry and Life Science, Okinawa Japan, 2012.

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Appendix 1-Presumptive Testing

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Appendix 2- Glass Microfluidic Device Chemiluminescent Analysis

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Appendix 3- Anti-rat Immunoglobulin-HRP Immunoassay

3.1 Surface Optimisation

3.2 Raw Data

For data refer to USB drive Appendix 3

Appendix 4- Inter/Intra Variation Study

4.1 Raw Data

4.2 Statistical Analysis

For data refer to USB drive Appendix 4

Appendix 5- Optimisation

5.1 Raw Data

5.2 Statistical Analysis

For data refer to USB drive Appendix 5

Appendix 6- Biological Samples

6.1 Raw Data

6.2 Statistical Analysis

For data refer to USB drive Appendix 6

Appendix 7- Cross-reactivity

7.1 Raw Data

7.2 Statistical Analysis

For data refer to USB drive Appendix 7