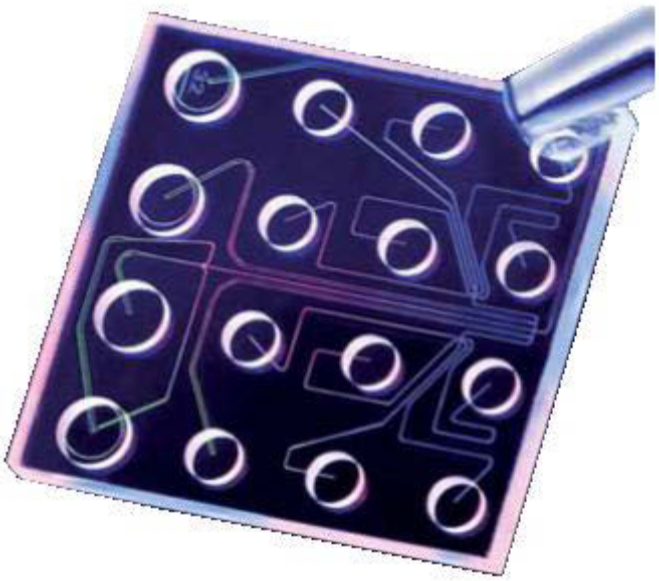
The analysis of amphetamine-type stimulants using microchip capillary electrophoresis

Aimee Lloyd



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Simplicity is the ultimate sophistication

Leonardo da Vinci

Certificate of authorship and originality

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

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Aimee Lloyd

31st July 2013

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Abbreviations

AFP	Australian Federal Police
AIC	Australian Institute of Criminology
AMP	amphetamine
ATR-FTIR	attenuated total reflection – fourier transform infrared
ATS	amphetamine-type stimulants
CE	capillary electrophoresis
CMC	critical micellar concentration
CNS	central nervous system
CZE	capillary zone electrophoresis
DNA	deoxyribonucleic acid
DTAF	5-([4,6-dichlorotriazin-2-yl]amino)fluorescein
ED	electrochemical detection
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EOF	electroosmotic flow
ESI	electrospray ionisation
ESR	institute of environmental science and research limited
FITC	fluorescein isothiocyanate isomer I

FTIR	fourier transform infrared
GC	gas chromatography
HPLC	high performance liquid chromatography
Hypo	hypophosphorus
IMS	ion mobility spectrometry
LC-MS	liquid chromatography-mass spectrometry
LED	light-emitting diode
LIF	laser-induced fluorescence
LOC	lab-on-a-chip
LOD	limit of detection
LOQ	limit of quantification
MA	methamphetamine
MCE	microchip capillary electrophoresis
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxyethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
3,4-MDP-2-P	3,4-methylenedioxyphenyl-2-propanone
MDPBP	3’,4’-methylenedioxy-α-pyrrolidinobutiophenone

4-MEC	4-methylethcathinone
MEKC	micellar electrokinetic chromatography
4-MMC	4-methylmethcathinone
MS	mass spectra
MS	mass spectrometry
NFSTC	National Forensic Science Technology Centre
NIR	near infra-red
OPA	o-phthalaldehyde
P2P	phenyl-2-propanone
PSE	pseudoephedrine
RSD	relative standard deviation
SDS	sodium dodecyl sulfate
SWGDRUG	scientific working group for the analysis of seized drugs
µ-TAS	µ-total analytical systems
TLC	thin layer chromatography
UN	United Nations
UNODC	United Nations Office on Drugs and Crime
β-PEA	β-phenethylamine

Abstract

The illicit drug trade, dominated by sophisticated trans-national criminal organisations, has put increasing demands on law enforcement bodies. Timely information concerning illegal activity is required to effectively combat the illicit drug problem. Rapid, if not real-time, identification tools would help direct investigators with sampling procedures and safety precaution measures at drug-related crime scenes. In addition to enhancing work-flow processes, for example the creation of *rapid laboratories* or intelligence units, a major focus rests on the miniaturisation of existing analytical techniques, predominantly spectroscopic-based, in order to create field portable tools for this purpose. Currently available techniques such as colour tests, Raman and infra-red spectrometers often have limitations associated with specificity, portability and sample preparation requirements. The diverse nature of exhibits present challenges for the in-field detection of controlled drugs and pre-cursors.

An emerging area of research, lab-on-a-chip (LOC), with its ability to integrate multiple functions on a microchip, has shown promising applications for in-field testing. The aim of this project was to evaluate a commercial portable microchip capillary electrophoresis (MCE) platform, the Agilent Bioanalyzer 2100, for the analysis of amphetamine-type stimulants (ATS). This device, although designed for the analysis of biological molecules, holds significant potential for the analysis of inorganic ions, explosives and illicit drugs. This project focused on developing and optimising a rapid, simple and inexpensive separation method. The method was adapted for the analysis of a wide range of casework exhibits including liquids, tablets and powders in order to test its in-field capabilities. The prospects, challenges and applications are discussed. This research has highlighted MCE as a competitive platform for the screening of ATS and has demonstrated its potential use in forensic drug analysis.

List of publications and presentations

Lloyd, A., Russell, M., Blanes, L., Doble, P. and Roux, C. Lab-on-a-chip screening of methamphetamine and pseudoephedrine in samples from clandestine laboratories. *Forensic Science International*. 2013; 228(1-3): 8-14.

Lloyd, A., Blanes, L., Beavis, A., Roux, C. and Doble, P. A rapid method for the in-field analysis of amphetamines employing the Agilent Bioanalyzer. *Analytical Methods*. 2011; 3(7): 1535-1539.

Lloyd, A., Russell, M., Somerville, R., Doble, P. and Roux, C. The use of portable microchip electrophoresis for the screening and comparative analysis of synthetic cathinone seizures. Submitted to *Forensic Science International (manuscript ID: FSI-S-13-01367).*

Lloyd, A., Russell, M., Blanes, L., Doble, P. and Roux, C. Rapid screening for pseudoephedrine and methamphetamine in clandestine laboratory samples using the Agilent 2100 Bioanalyzer [oral presentation]. 2012; *The 21st International Symposium on the Forensic Sciences of the Australian and New Zealand Forensic Science Society (ANZFSS) Hobart, Tasmania, Australia.*

Lloyd, A., Doble, P., Roux, C., Esseiva, P. and Delémont, O. Comparison between microchip capillary electrophoresis and capillary electrophoresis-mass spectrometry for the detection of amphetamines [oral presentation]. 2011; *19th International Association of Forensic Sciences (IAFS) world meeting, Funchal, Madeira, Portugal.*

Lloyd, A., Blanes, L., Beavis, A., Roux, C. and Doble, P. Analysis of amphetamine analogues using the portable Bioanalyzer 2100 lab-on-a-chip [poster presentation]. 2010; *17th International Symposium on Capillary electroseparation techniques (ITP 2010), Baltimore, Maryland, United States.*

Lloyd, A., Blanes, L., Beavis, A., Roux, C. and Doble, P. Analysis of amphetamine-type stimulants using the portable Bioanalyzer 2100 lab-on-a-chip [poster presentation].

2010; *The 20th International Symposium on the Forensic Sciences of the Australian and New Zealand Forensic Science Society (ANZFSS), Sydney, NSW, Australia*.

Chapter 1

Introduction

Chapter 1: Introduction

The possession, dealing, trafficking and manufacture of illicit drugs such as amphetamine-type stimulants (ATS) pose serious risks to national security. The association between chronic drug use and violent behaviour threatens public safety within local communities.[1,2] Drug trafficking can induce violent actions (e.g. assaults, homicides) within the hierarchy of established illicit drug supply chains.[3] The involvement of organised criminal groups in drug manufacture and trafficking is motivated by high profit margins.[4] This notion is acutely outlined by the Australian Crime Commission (ACC):

> Combatting the illicit drug market is at the forefront of our fight against serious and organised crime. Illicit drugs are a major source of profit for serious and organised crime, to the detriment of the Australian community – economically and socially.[5]

The manufacture and trafficking of ATS present unique challenges to law enforcement agencies. The highly profitable nature of illicit ATS manufacture is attributable to the ease of production, ready availability of starting materials and inherent flexibility of manufacturing processes.[6] They are the second most widely used class of illicit drugs behind cannabis. Global seizures increased threefold from 1998 to 2010, far outpacing the growth in heroin, cocaine and cannabis.[7] Further, the prominence of ATS is burgeoning due the synthetic nature of these psychoactive substances. The rapid emergence of new psychoactive substances in recent years, marketed as legal substitutes to controlled drugs, pose serious risks to public health and safety.[8]

The United Nations Office on Drugs and Crime (UNODC) recognise the serious nature of the illicit ATS market:

> The fast-paced nature of this market, the increased availability of these substances and the reports of increased and emerging use of and trade in

> such substances, have drawn concerns among the international community as there is the potential for transnational organized criminal groups to exploit the market for these substances.[8]

The analysis of drug exhibits plays a crucial role in the forensic investigation of drug-related crimes. The criminal justice system relies on the analysis of forensic exhibits to establish the presence or otherwise of a drug, in order to prove whether an offence has been committed.[9] Furthermore, strategic and tactical information on the illicit drug trade can be provided. In clandestine laboratory investigations the identification of drugs and substances related to their manufacture is essential in ascertaining the nature of the illicit activity that took place, if any. The relationship between the presence of controlled substances, seized materials and equipment, is used to determine if the clandestine laboratory site in question was established for the manufacture of illicit drugs.[10] Beyond routine analysis, more detailed information can be obtained from the profiling of illicit drugs to assist in the dismantling of drug trafficking organisations. Drug profiling involves the extraction of physical and chemical information from drug seizures. Chemical data can provide insight into the synthetic route of manufacture and the pre-cursor chemicals employed. Physical characteristics include the packaging materials, tablet colour, logo and dimensions. The combination of physical and chemical profiles can also be used to identify potential links between two drug seizure populations.[11,12]

This research aims at developing a rapid screening method for ATS using a novel separation technique. In applying the method through field testing it aims to identify specific areas of casework in which it could help combat the growing illicit drug market.

1.1 Amphetamine-type stimulants (ATS)

1.1.1 Classification

ATS are a group of substances that are predominantly synthetic in origin.[13] They are structurally related to the parent compound β-phenethylamine (β-PEA), a naturally

occurring neurotransmitter in the body. All ATS exhibit similar pharmacological properties.[14,15] ATS have powerful stimulating effects on the central nervous system, exaggerating the feeling of physical and mental wellbeing (euphoria).[16] They act by increasing the production of neurotransmitters such as dopamine (enhances sense of wellbeing and pleasure), norepinephrine (stimulator for wakefulness and motivation) and serotonin (mood enhancer).[9] The structure of β-PEA is presented in Figure 1.1, illustrating its defining characteristics: phenyl ring, carbonyl side-chain and amino moiety.

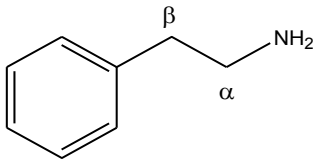


Figure 1.1 Structure of β-PEA.

Structural modifications of β-PEA create multiple synthetic derivatives known as analogues. The substitution positions on the side-chain (R1-R4) and aromatic ring (R5-R9) are illustrated in Figure 1.2.[13] Substitution at R1-R9 results in myriad structurally derived analogues. Structural modifications on the aromatic ring significantly alter the pharmacological properties.[16]

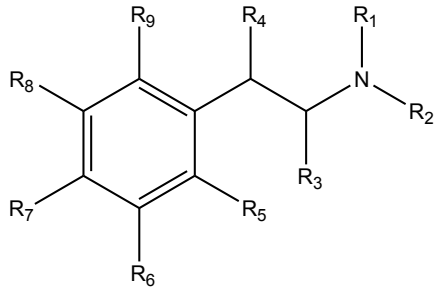


Figure 1.2 Possible substitution positions R1-R9. Adapted from [13]

For consistency and simplicity, ATS can be classified into two sub-groups defined by their substitution pattern on the aromatic ring.[17] To remain within the scope of this research only the most common stimulants from each sub-group will be reviewed in depth.

(1) *Amphetamines*: no aromatic ring substitution, include amphetamine and methamphetamine. Amphetamine, systematically named *N*,α-methylbenzeneethanamine, is an α-methyl derivative of β-PEA (Figure 1.3).[18]

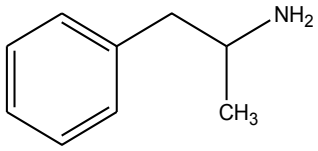


Figure 1.3 Molecular structure of amphetamine.

Amphetamine is typically seized as a sulphate salt in powder form. Its base form, often encountered at clandestine laboratories, is characterised by its unpleasant solvent odour. Methamphetamine or *N*,α-dimethylphenethylamine is an α-dimethyl derivative of β-PEA (Figure 1.4).[19]

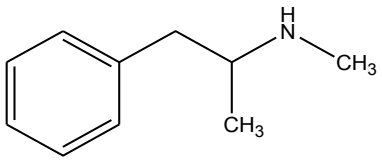


Figure 1.4 Molecular structure of methamphetamine.

The common street form of methamphetamine is a hydrochloride salt frequently encountered as a powder, tablet or crystal. The latter, coined ice, is the most active and pure form of methamphetamine.[20] Amphetamines can be administered through nasal insufflations, oral ingestion or intravenous injection. The crystalline form of methamphetamine, with its sufficient volatility, can be smoked.

(2) *MDMA and analogues:* comprises 3,4-methylenedioxymethamphetamine (MDMA), a 3,4-methylene-dioxy derivative of methamphetamine, and its analogues. This group is characterised by a methylene-dioxysubstitution pattern on the aromatic ring (Figure 1.5).[7] Common analogues 3,4-methylenedioxyamphetamine (MDA) and 3,4-

methylenedioxyethylamphetamine (MDEA) are homologues of MDMA, differing in structure by a single methylene moiety.[6,21]

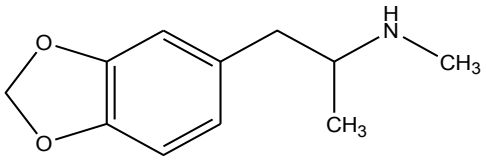


Figure 1.5 Molecular structure of MDMA.

Illicit MDMA, MDA and MDEA are usually encountered as tablets, in salt form (hydrochloride or phosphate), often coloured and stamped with distinctive logos. Powders, although less common, often contain a high concentration of the active ingredient.[13] The mode of use is typically via oral ingestion.[6] Note that during the 1980's, the term ecstasy encapsulated illicit preparations containing MDMA. Today, however, ecstasy refers to tablets or powders containing psychotropic active agents derived from β-PEA, often in combination.[14,22]

In the last decade a myriad of new psychoactive substances has emerged. Collectively defined as *'analogues, or chemical cousins, of controlled substances that are designed to produce effects similar to the controlled substances they mimic* [23]*'*. The literature, which is confusing and at times contradictory, has used a number of terms (e.g. designer drugs and legal highs) to classify these burgeoning, unregulated substances. A prominent branch, synthetic cathinones, encompasses structurally related derivatives of the parent compound cathinone. Synthetic cathinones are β-ketone derivatives of the phenethylamine family which includes amphetamine and MDMA.[24] They contain a ketone functional group at the β position on the side chain which is elucidated in Figure 1.6.[25] Synthetic cathinones are often sold in powder form and frequently insufflated.

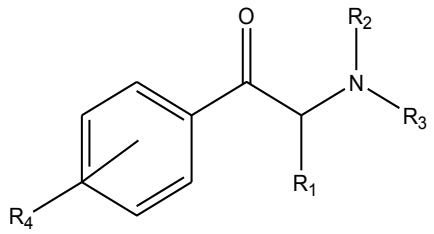


Figure 1.6 The generic structure of synthetic cathinones, showing α and β side-chain positions.

1.1.1.1 Terminology

As pointed out by Schloenhardt in an Australian Institute of Criminology (AIC) report, *'the terminology for illicit drugs is frequently confusing, highly technical and sometimes conflicting, and many expressions used overlap [6]'*. To avoid confusion the following terminology is utilised throughout this thesis:

Analogue: *'a drug whose structure is related to that of another drug but whose chemical and biological properties may be quite different [26]'*.

Controlled substance: *'a psychoactive substance and its pre-cursors whose availability are forbidden under the international drug control treaties or limited to medical and pharmaceutical channels [27]'.*

Designer drug: *'a substance which is structurally related to a control drug and which has been synthesised to produce certain psychoactive effects, but which is not covered by existing regulations on controlled substances [27]'*.

A drug is defined as *'any substance which when absorbed into a living organism may modify one or more of its functions. The term is generally accepted for a substance taken for therapeutic purpose, but is also commonly used for abused substances [26]'*.

Pre-cursor: *'a chemical substance that in the manufacturing process becomes incorporated in full or in part into the molecule of a narcotic drug or psychotropic substance [27]'.*

Stimulant: *'any agent that activates, enhances or increases neural activity. Also called psychostimulant [27]'*.

Synthetic (of a substance): *'made by chemical synthesis, especially to imitate a natural product [28]'*.

1.1.2 History

The origins of ATS use date back thousands of years.[29] Ephedrine, a pre-cursor of methamphetamine, is a naturally occurring alkaloid in certain species from genus *Ephedra* (family Ephedraceae).[30,31] More than 30 different species of the *Ephedra* genus exist. These species are widespread in the subtropical and temperate regions of Europe, Asia and the Americas.[30]

Ma huang, an umbrella term for *Ephedra* species native to the greater China region, has been utilised by Chinese native physicians since circa. 2,500 BC. Initially prepared as a natural herbal remedy for colds, asthma and allergies, the stimulant properties were not recognised until the Han Dynasty (circa. 207 BC – 220 AD).[6,29,32]

Literature documenting the ancient use of genus *Ephedra* is fragmented. However, records indicate that *Ephedra gerardiana* was a component of Indian folk medicine and was commonly used during the time of the Roman Empire.[29] Further, in Russia, an extract of *Ephedra vulgaris* was used in the 19th century to treat rheumatism, syphilis and gout.[30]

Ephedrine was chemically isolated from *Ephedra* species and characterised by Nagai in 1887. Soon after, in 1889, Ladenburg and Ölschlägel isolated its diastereoisomer pseudoephedrine.[29] The growing popularity of ephedrine during the late 19th century generated concern that its natural source would be depleted.[6] Subsequently, a search for alternatives and an investigation into the synthetic manufacture of ephedrine was initiated.[33,34]

1.1.2.1 Amphetamines

Amphetamine, the first ephedrine substitute to be discovered, was first synthesised in 1887 by German pharmacologist Lazar Edeleano.[6] The stimulant effects, however, were not discerned at this time.

Methamphetamine, in powder form, was initially synthesised in 1893 in Japan by Nagai Nagayoshi.[35] In 1919 Akira Ogata synthesised crystalline methamphetamine from the reduction of pseudoephedrine using red phosphorus and iodine.[36]

It was not until 1929 when it was re-synthesised by biochemist Gordon Alles that the sympathomimetic and adrenergic effects were revealed.[37,38] Simultaneously, and possibly inspired by Alles' discovery, the pharmaceutical company Smith, Kline and French (SKF) began to study the base form of amphetamine. Patented in 1933 by SKF (Benzedrine™), amphetamine was initially marketed as an inhaler for the treatment of asthma and congestion, given its broncho-dilating properties.[39] The American Medical Association (AMA) approved it for the treatment of narcolepsy and Parkinson's disease in 1937.[39,40]

Methamphetamine, patented in 1920, was later marketed for the treatment of obesity (Methedrine™) by the pharmaceutical company Burroughs Wellcome.[41] During World War II, in one of the earliest reports of widespread use, methamphetamine was distributed to American, British, German and Japanese troops to alleviate fatigue.[42]

After World War II, the surplus of methamphetamine from the Japanese military instigated the first methamphetamine epidemic in Japan which lasted from 1945 until 1957.[43]

The United States amphetamine epidemic (1929-1971) was fuelled by increased commercial drug development and competition in the burgeoning pharmaceutical industry.[39] Post World War II, medical practitioners were prescribing amphetamine

liberally for alcoholism, depression and weight reduction.[40] Amphetamine was marketed as an anti-depressant by SKF (Figure 1.7).

Figure 1.7 Benzedrine tablets in an early medical journal advertisement.[39]

The use of methamphetamine escalated during the 1960's in the United States and a trend of abuse via intravenous injections started in San Francisco.[44] The market grew and the user population expanded to include truck drivers, chefs, shift workers and students looking to relieve fatigue.

Smaller epidemics were experienced in other parts of the world, for example in the United Kingdom. Hawks *et al.* describe an epidemic of methamphetamine injecting over the summer of 1968 in London, at a time when licit methamphetamine was still readily available.[45]

Initial warnings of dependence and tolerance were not heeded. In 1938, Young and Scoville documented amphetamine-induced psychosis as a rare occurrence.[46,47] However, due to widespread consumption, the negative side-effects and addictive properties of amphetamines surfaced.[18] The phenomenon of amphetamine-induced psychosis was described in detail by Connell in 1958 who discovered the link to prolonged use.[48]

In the 1970's, tighter controls were introduced to restrict the availability of pharmaceutical preparations containing amphetamines.[39] The United Nations (UN) adopted an International Convention on Psychotropic Substances in 1971 to regulate the supply and scheduling of psychotropic drugs such as amphetamines.[9]

Following a decrease in supply and due to controls put on amphetamine prescriptions, users pursued alternative sources. The excessive supply of pharmaceutical grade amphetamines from earlier decades was diverted to supply the black market that had evolved.[43] Once the supply depleted (between 1959 and 1963), criminal motorcycle gangs in the United States became involved in manufacturing methamphetamine from the commercial supply of phenyl-2-propanone (P2P) which was not restricted at the time.[49]

The abuse of amphetamines developed into a global phenomenon between 1970-80.[6] There was a dramatic increase in the use of ATS in North America, Western Europe and Asia. International drug trade proliferated and illegal amphetamine laboratories appeared in new markets such as Australasia. The first illegal methamphetamine laboratory in Australia was discovered in New South Wales in 1976. While the first laboratory encountered by the New Zealand Police was in 1988.[6]

The predominant route of illicit manufacture was via P2P until it was added to the Controlled Substances Act in 1980.[50] Sophisticated trafficking methods, combined with strict controls on the pre-cursor chemicals, buoyed the production of illicit amphetamines.[50] In 1983, the possession of pre-cursor materials and equipment used in the production of methamphetamine was prohibited in the United States.[51]

1.1.2.2 3,4-Methylenedioxymethamphetamine (MDMA)

MDMA was first synthesised in 1912 by Köllisch at Merck, a German pharmaceutical company, as a pre-cursor in a new chemical pathway.[22] Its analogue MDA was synthesised and patented by SKF who investigated its potential as an appetite suppressant and antidepressant between 1949 and 1957.[22,52] However, shortly after in 1958, MDA was tested in human trials and abandoned due to its psychoactive properties.[53] MDA first emerged on the illicit drug scene in San Francisco during the mid-1960's as the *love drug* and quickly gained popularity. The first reported MDMA street seizure was recorded in 1970 in Chicago.[54] It was not until the 1980's that MDMA became popular in the music industry and the American street name *ecstasy* was devised. MDMA was never used for medicinal purposes due to its negative side effects.[9] These included nausea, increased body temperature, numbness, anxiety and loss of appetite.[55] The popularity of MDMA as a recreational drug evolved during the 1980's particularly among college students. MDMA and MDA became schedule I controlled substances under the Controlled Substances Act in 1985.[39]

1.1.3 Recent trends in Australasia

During the 1980s, following legislative control measures, amphetamine use began to decline. The popularity of methamphetamine, however, flourished in the United States and the black market evolved to serve a wider geographical area.[51,56]

Methamphetamine use and manufacture escalated in Australia during the late 1990s and early 2000s (Figure 1.8). A marked 6-fold increase in the number of illicit drug laboratories was observed from 58 in 1996 to 390 in 2006.[6] In New Zealand a similar trend was observed with only two laboratories in 1996 increasing to 211 in 2006.[57,58]

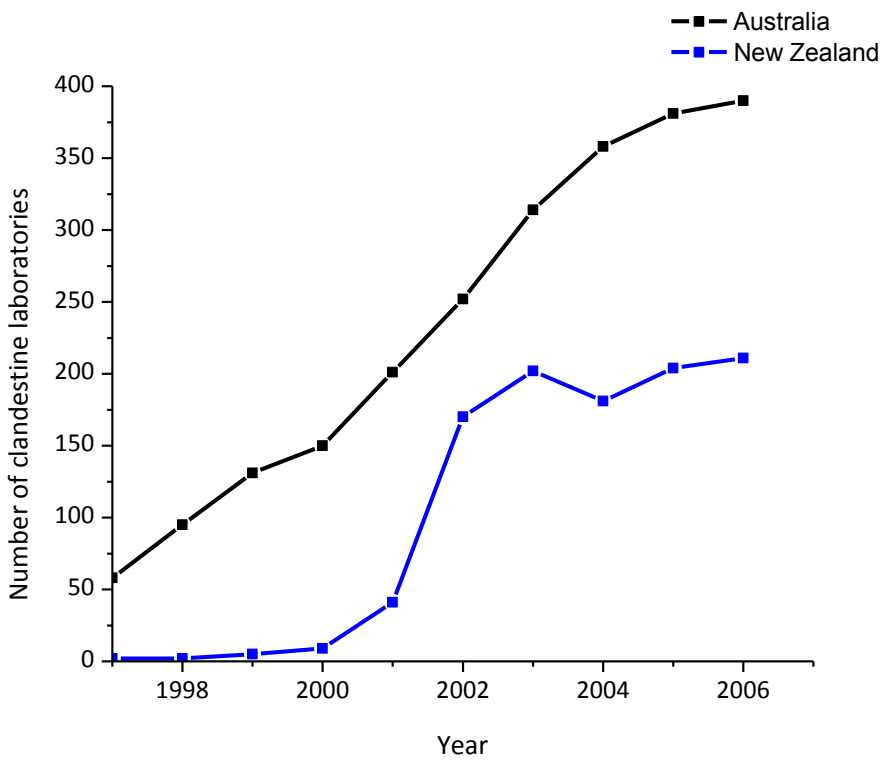


Figure 1.8 Number of clandestine laboratories detected in Australasia from 1997 to 2006.[6,57,58]

Global ATS seizures have steadily increased over the last decade (Figure 1.9), far exceeding the increase in heroin, cocaine and cannabis seizures.[7] The global prevalence of amphetamines is second only to cannabis. The World Drug Report published by the UNODC estimates that in 2010 between 0.3 and 1.2 percent of the world population were users of ATS. Oceania has a particularly high prevalence of ATS use, exceeding 1 percent.[7]

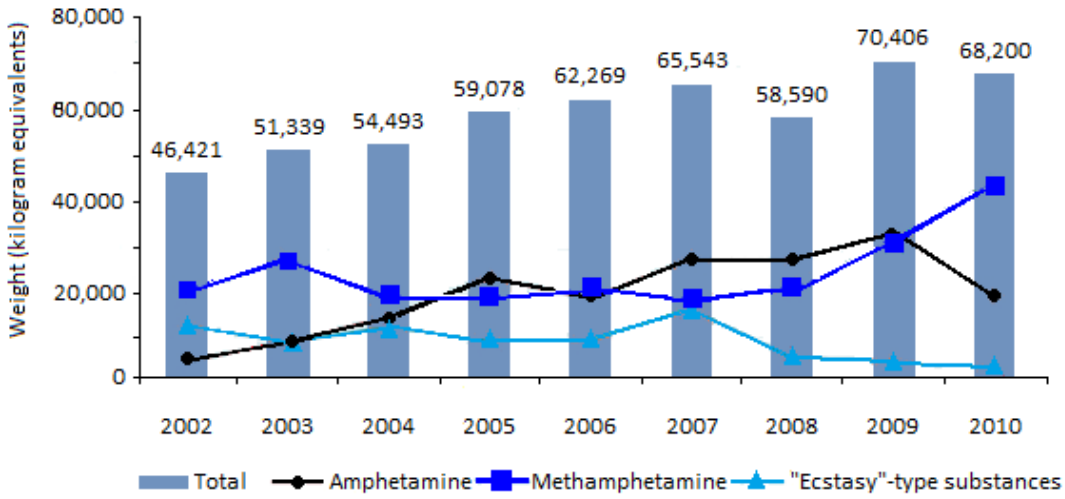


Figure 1.9 ATS seized worldwide, by weight, 2002-2010.[7]

The illicit drug market in Australasia is highly profitable and well-structured, making it a popular destination for international trafficking.[59] The prevalence of methamphetamine and MDMA use in Australasia is high and has remained relatively stable over the last few years.[6] The prevalence of amphetamine use from 1998 to 2008, for ages 15-45, ranged from 2.1 to 3.9 percent and 3 to 5 percent in Australia and New Zealand, respectively.[7]

Historically, in Australasia, ATS was predominantly sourced from Europe. Recently, however, the illicit manufacture of ATS, mostly methamphetamine, has shifted closer to consumer markets in Australia and New Zealand.[7]

Most methamphetamine, in powder form, is now supplied through the domestic market which continues to be robust.[59] Amphetamine is still primarily sourced from the European drug market.[7] The supply of crystal methamphetamine (ice), also sourced from overseas, typically originates from East Asia.[6]

The majority of the tablets and powders containing MDMA and analogues are imported.[59] The large scale manufacture of MDMA was, in the past, almost exclusively carried out in Europe. Since 2003-2004 operations established for the manufacture of MDMA have also been encountered in North America, South-East

Asia and Oceania. During 2010 and 2011 16 of the total 703 clandestine laboratories dismantled in Australia were MDMA laboratories.[6] An increase in the weight of MDMA pre-cursors (2570 litres of safrole) was also detected at the Australian border.[60]

Until recently, pseudoephedrine and ephedrine were found in over-the-counter pharmaceutical preparations such as, Sudafed and Coldral (Johnson&Johnson). As a result of their excessive use in the manufacture of methamphetamine, recent legislative measures have restricted supply. Pseudoephedrine-containing medicines became prescription only in New Zealand. In Australia, an initiative called *project STOP* was instigated in 2007 to allow Australian pharmacists to monitor dispensing. Subsequently, the importation of bulk pseudoephedrine has been observed at the border, predominantly originating from Vietnam, Singapore, Hong Kong and China.[61] A consequence of this change in legislation was the shift back to the phenyl-2-propanone method of synthesis.[62] Similarly, the global shortage of 3,4-MDP-2-P (3,4-methylenedioxyphenyl-2-propanone), a common pre-cursor of MDMA, lead to an increased effort in sourcing other pre-cursors such as safrole and isosafrole.[59]

The illicit drug market is dynamic and resilient. Organised criminal entities involved in the manufacture of methamphetamine quickly adapt to changes in the control of pre-cursors and essential chemicals. To remain undetected, alternative manufacturing methods are used which rely on sourcing alternative non-regulated substances.[59] Efficient methods of distributing ATS have been developed. International shipments of key pre-cursor chemicals have been concealed by chemically modifying them to form new legitimate chemicals. Following delivery, the pre-cursors are extracted.[63]

The emergence of new synthetic substances, marketed as ecstasy, has dominated the market over recent years.[7,59] Although first synthesised in 1928 the reappearance of analogues such as synthetic cathinones was most likely to circumvent legislative control.[25] The early warning system, a branch of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), has recorded the entry of each new psychoactive substance onto the European drug market. The marked increase in the

number of synthetic cathinones introduced from 2005 is illustrated in Figure 1.10. More recently, one of these, 4-methylmethcathinone (4-MMC), has attracted significant media attention following a number of fatalities.[64]

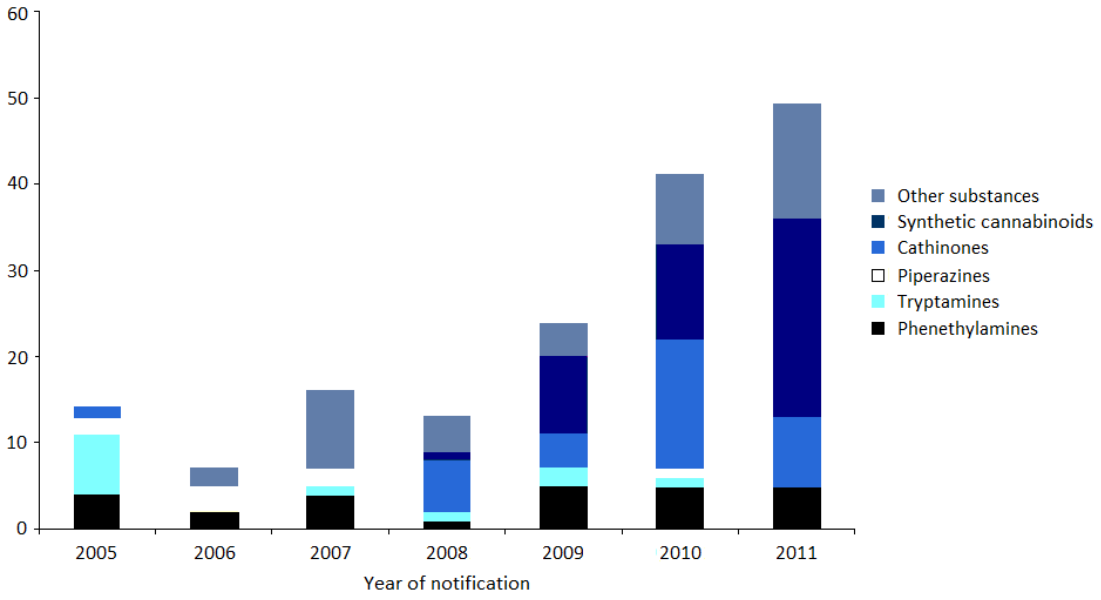


Figure 1.10 New psychoactive substances notified to the European early-warning system from 2005 to 2011.[65]

1.1.4 Physiological and behavioral effects

The unknown quality, strength and identity of ATS street drugs pose serious risks to users. The origins of street drugs are typically not known by buyers and sellers in the illicit drug market. Poor quality drugs can cause infections, tissue damage, overdose or life-threatening interactions.[61] Users that administer ATS via injection are also at risk of contracting viral infections such as HIV and Hepatitis.[19]

Today, there are very few medicinal uses for ATS. Exceptions include: the dextro form of amphetamine sulfate (Dexedrine) for the treatment of narcolepsy, a chronic sleep disorder. Methedrine and Desoxyn, containing methamphetamine HCl, and Adderall, containing mixed amphetamine salts, are marketed for the treatment of attention deficit hyperactivity disorder (ADHD) and in rare cases, obesity.[51]

Table 1.1 presents a summary of the effects amphetamines and MDMA-related substances have on the brain, how they lead to tolerance and withdrawal and some

side effects, following prolonged use. The effects observed are typically dose dependent.

Substance	Primary mechanism of action	Tolerance and withdrawal	Prolonged use
Amphetamines	Increases release of dopamine from nerve terminals and inhibits the reuptake of dopamine and related transmitters.	Tolerance develops rapidly to behavioural and physiological effects. Withdrawal is characterised by fatigue, depression, anxiety and intense craving for the drug.	Sleep disturbances, anxiety, decreased appetite: alterations in brain dopamine receptors, regional metabolic changes, motor and cognitive impairments [66,67]
MDMA-related	Increased serotonin release and blockade of reuptake.	Tolerance may develop in some individuals. Most common withdrawal symptoms are depression and insomnia.	Damages brain serotonin systems, leads to behavioural and physiological consequences. Long-term psychiatric and physical problems such as impairments of memory, decision-making and self-control, paranoia, depression and panic attacks [68,69]

Table 1.1 Summary of the behavioural effects, development of tolerance and effects of prolonged use for amphetamines and MDMA-related stimulants.[70]

1.1.4.1 Amphetamines

Amphetamines are central nervous system (CNS) stimulants that increase the release of dopamine from nerve terminals. This induces such sensations as euphoria, alertness, wakefulness and increased energy. The associated increase in endurance was abused by students, truck drivers and athletes in the 1960's. The onset period depends on the route of administration. If injected or smoked, effects are instantaneous; 5 minutes if through nasal administration; or 20 minutes if taken orally.[43] The crystalline form of methamphetamine quickly enters the bloodstream and is sent directly to the brain and effects can be more pronounced. Effects typically last for 4-24 hours.[20] Physical effects observed during this time can include constriction of blood vessels, dilation of pupils, an increase in heart rate, breathing rate and blood pressure. Amphetamines are particularly susceptible to the

development of tolerance to behavioural and physiological effects which may develop in a few weeks. Withdrawal symptoms can include fatigue, depression, anxiety and agitation and an intense craving for the drug.[70] Chronic stimulation often results in sleep disturbances, anxiety and decreased appetite. Some users have a tendency to engage in criminal activity. When coupled with the paranoia and delusions associated with the drug, this often results in violence.[41] The cognitive impairments following prolonged use will impact on daily tasks such as driving and therefore criminal liability. Large doses can induce stimulant psychosis which has comparable symptoms to paranoid schizophrenia. The National Drug Strategy Household Survey in 2007 noted that *'physiological distress and diagnoses or treatment for a mental illness are high among recent users of meth/amphetamines and MDMA-related stimulants [71]'*.

1.1.4.2 MDMA and analogues

The substitution pattern on the aromatic ring of MDMA-related stimulants changes the pharmacological properties of the drug.[21] Following ingestion of MDMA the increased serotonin release causes a sense of euphoria, increase in physical and emotional energy and increased sociability.[41] Some MDMA-related substances are mildly hallucinogenic. The words *empathogenic* and *entactoenic* are often used to describe its effects on sociability. In contrast to amphetamines, the effects of MDMA-related substances only last for 4 to 6 hours.[72] Physical effects can be muscular, for example jaw clenching and deep tendon reflexes. In addition, increased body temperature and blood pressure are often observed.[72] These effects generally last for up to 24 hours. Tolerance can develop but is normally not conducive to addiction as tolerance develops to the positive effects and the negative effects become exacerbated. This accumulative tolerance occurs regardless of the period of time between doses.[53] Withdrawal symptoms can include depression and insomnia.[41] MDMA is a popular club drug and is particularly dangerous in night clubs with high ambient temperatures in combination with physical exertion and dehydration.[20] In these situations, hyperthermia can develop following the disruption of central neurotransmitter-mediated thermoregulatory control.[73,74] The most common result

of chronic MDMA use is cognitive impairment e.g. memory loss. There is evidence to suggest that the prolonged use of MDMA damages the hippocampus which is the part of the brain involved in short-term memory.[75]

1.1.4.3 Cathinones

Over 30 synthetic cathinones have been reported to the *Early Warning System* at ECMDDA since 2005.[65] Cathinones that have no aromatic ring substitution have similar CNS actions to amphetamines.[76] The presence of a ketone group at the β carbon position reduces the potency relative to their amphetamine equivalent. It increases the polarity of the drug, thus reducing its ability to cross the blood-brain barrier.[25] Few studies have been conducted to determine the associated prevalence of the harmful physiological and behavioral effects associated with their ingestion. Available data is sourced from self-reports, internet discussion sites, surveys of users and individual case reports provided by healthcare facilities. Of the cathinones, 4-MMC (known as mephedrone) is by far the most common and is of particular concern due to its link to fatalities.[25] The most frequent adverse effects associated with mephedrone include: increased body temperature, chest pain, memory loss, anxiety, agitation, insomnia, hallucinations.[77] Users often consume several doses of mephedrone in succession as the associated *rush* is short-lived.[76] Given its recent appearance as a recreational drug on the market the effects due to prolonged use have not yet been examined.[77]

1.1.5 Legislation

1.1.5.1 International framework

In response to the proliferation of illicit production and abuse of synthetic substances in the 1960's the UN placed ATS and their pre-cursors under two international control treaties.[6] The 1971 *Convention on Psychotropic Substances* sought to limit the use of psychotropic substances to scientific and medical purposes.[78] Substances were scheduled according to their therapeutic value and abuse risk (Table 1.2).[62] Schedule I, the most restrictive, comprises substances that pose serious risk to public health

and have no known therapeutic value. Those listed in Schedules II and III have limited therapeutic value and thus are less stringently controlled.[6] The convention established a legal precedent for signatories and introduced control measures for the trade, manufacture and distribution of ATS.[79]

Schedule I	Schedule II	Schedule III
Cathinone MDA MDMA Methcathinone	Amphetamine Methamphetamine	N-Ethyl-amphetamine

Table 1.2 ATS scheduled in the *1971 Convention on Psychotropic Substances* as of May 2010. Adapted from.[80]

The 1988 *Convention against Illicit Traffic in Narcotic and Psychotropic Substances* was aimed at suppressing illicit trafficking and the escalating involvement of organised crime in the drug trade.[81] It categorised pre-cursors into two schedules (Table 1.3). Class I pre-cursors, predominantly key components of scheduled ATS substances, include ephedrine, phenyl-2-propanone and pseudoephedrine. They are largely confined to the pharmaceutical industry and thus more strictly controlled.[82,83] Class II pre-cursors, largely reagents and solvents used in illicit clandestine production, have widespread industrial and commercial applications.[83,84] The convention provides a clear mandate for signatories to criminalise activities associated with the manufacture, distribution, trafficking, supply and possession of scheduled substances and Class I and II pre-cursors.[6] Rigorous measures are adopted to counter money laundering and the appropriation of assets related to drug crime.[82] Further, it enforces a comprehensive framework for international cooperation encompassing extradition mechanisms, mutual legal assistance and joint tracing and seizure initiatives.[6,84,85]

Class I	Class II
Ephedrine	Acetone
Isosafrole	Hydrochloric acid
3,4-methylenedioxyphenyl-2-propanone	Methylethylketone
Norephedrine	Piperidine
Phenylacetic acid	Sulphuric acid
Piperonal	Toluene
Potassium permanganate	
Pseudoephedrine	
Safrole	

Table 1.3 ATS pre-cursors scheduled in the *1988 Convention against Illicit Traffic in Narcotic and Psychotropic Substances* as of January 2012. Adapted from.[86]

1.1.5.2 Australasian framework

Australia and New Zealand are signatories to the 1971 and 1988 UN conventions.[87,88] These treaties are not self-executing and obligate signatory countries to pass laws pertaining to the criminalisation of use, possession and supply of scheduled drugs and pre-cursors.[89,90]

Drug offences in Australia are regulated at both state and federal level.[91] Federal offences, which are consolidated in the *Criminal Code Act 1995*, aim to prevent the import and export of scheduled drugs.[6,90] The Act also codifies, concurrently with state laws, the trafficking, manufacture and possession of controlled drugs.[91,92] Controlled drugs and pre-cursors are defined in two sections:

- Division 314.1 includes the ATS amphetamine, methamphetamine, methcathinone, MDA, MDMA and 4-MMC
- Division 314.3 includes the ATS pre-cursors ephedrine, isosafrole, 3,4-methylenedioxyphenyl-2-propanone, 3,4-methylenedioxyphenylacetic acid, phenylacetic acid, piperonal, 1-phenyl-2-propanone, pseudoephedrine and safrole

Federal offences, and corresponding penalties, are tiered according to the quantity of the controlled drug involved (Table 1.4).

Controlled ATS Maximum Penalty	Trafficable Quantity (Grams)	Marketable Quantity (Grams)	Commercial Quantity (Grams)
Amphetamines	2	250	750
Methamphetamine	2	250	750
Methcathinone	2	250	750
MDA	0.5	100	750
MDMA	0.5	100	500
4-MMC	2	250	750
Maximum Penalty	10 years imprisonment	25 years imprisonment	Life imprisonment

Table 1.4 Controlled ATS quantities and associated maximum penalties under the Criminal Code Act 1995.[92]

Australian states and territories have ratified and implemented a host of offences relating to the production, trafficking and consumption of illicit drugs.[91] The controlled drugs listed in the schedules of relevant state laws are largely identical throughout Australia's eight jurisdictions.[6] All states and territories criminalise the unlawful possession, manufacturing, and trafficking of ATS.[90,91] Further, all ATS pre-cursors are scheduled and penalised at a state level. The scope of offences and penalties vary significantly between the jurisdictions.[93-95] A summary of the thresholds defined for trafficable quantities of selected ATS according to each jurisdiction are presented in Table 1.5.[96]

Jurisdiction	Amphetamine	Methamphetamine	MDMA
Vic *	3 g	3 g	3 g
Qld *	2 g	2 g	2 g
ACT *	2 g	2 g	0.5 g
Tas	25 g	25 g	25 g
NSW	3 g	3 g	0.75 g
SA	2 g	2 g	0.5 g
WA	2 g	2 g	2 g
NT	2 g	2 g	0.5 g

Table 1.5 Thresholds for trafficable quantities of amphetamine, methamphetamine and MDMA, by drug type and jurisdiction.[96] * Based on pure drug (excluding inert material).

Drug offences in New Zealand are amalgamated in the *Misuse of Drugs Act 1975*. Drugs are classified *'A, B or C based on a broad assessment of the risk of harm they pose to individuals, or to society, by their misuse* [97]*'*. Class B and C drugs are further segmented *'to regulate matters such as prescribing, storage and record-keeping by persons authorised to deal in controlled drugs* [97]*'*.

- Class A drugs are those that pose a very high risk of harm. Includes the ATS methamphetamine and MDA.
- Class B drugs are those that pose a high risk of harm. Include amphetamine, MDMA and methcathinone.
- Class C drugs are those that pose a moderate risk of harm. Include amphetamine analogues such as MDEA.

Note that isomers and salts of scheduled drugs are included. Pre-cursors for scheduled drugs are controlled in Schedule 4 of the Act. Similar to the 1988 UN convention, New Zealand lists ATS pre-cursors such as ephedrine and pseudoephedrine.

The Act imposes different maximum penalties based on the classification of the substance in question.[98] Offences involving manufacture, importation or supply are

strictly controlled and *'punishable by imprisonment for life where the drug dealt with is Class A, imprisonment for a term exceeding 14 years for Class B controlled drugs and imprisonment for a term not exceeding 8 years in any other case [99]'.* Presumption of supply is defined according to drug specific thresholds (for example 5 grams for amphetamine, methamphetamine, MDMA, MDA, and MDEA).[98] Possession offences incur notably lower penalties.[99]

1.1.6 Manufacture

The illicit manufacture of ATS encompasses the production, extraction or processing of drugs and their pre-cursors. Sites established for criminal activities related to the manufacture of illicit drugs are commonly referred to as *Clandestine Laboratories.* The joint Australian Standard/New Zealand Standard: Handling and Destruction of Drugs defines a clandestine laboratory as '*an illicit operation consisting of apparatus and/or chemicals that either have been or could be used in the manufacture or synthesis of drugs. This includes premises and/or sites* [100]'. Clandestine laboratories range from small-scale, makeshift operations to highly sophisticated laboratories involved in large scale manufacturing processes.[6,101] They are often located in private residences, hotel or motel rooms, garages or commercial establishments. Mobile laboratories have also been discovered in trailers, trucks, caravans and houseboats or in remote rural areas. These smaller laboratories can be easily relocated following market shifts or to remain undetected by law enforcement bodies.[70] Some highly sophisticated operations have been concealed behind legitimate businesses. Multiple sites can be involved in the overall manufacturing process whereby each site carries out a single step, for example the extraction of pseudoephedrine from pharmaceutical tablets.[102,103]

The ready availability of manufacturing methods sourced from the internet or *underground* literature, such as Uncle Fester[104] and Otto Snow,[105] has increased accessibility to the general population. The clandestine production of ATS, predominantly methamphetamine, is often attempted by individuals without the knowledge or expertise in chemistry. According to a bulletin published by the FBI in

2000 less than 10 percent of the clandestine methamphetamine *cooks* were trained chemists in the United States.[106] The situation is similar in Australasia where many unskilled amateurs attempt manufacture.[6] They often take uncalculated risks, make use of inappropriate equipment and operate in crude conditions which creates many hazards.[103]

Methamphetamine is relatively easy to synthesise and the manufacturing routes are flexible.[6] Reagents and equipment can be easily obtained from local hardware stores or household products can be used, such as lighter fluid or drain cleaner.[57] In addition, there is a high demand for methamphetamine and large profit margins particularly in Australasia.[7] For these reasons, the small scale manufacture of methamphetamine is particularly widespread. Clandestine laboratories are often located in close proximity to consumer markets to meet local demand.[6]

The clandestine manufacture of amphetamine is largely concentrated in Europe and North America.[107] In 2008 only 45 amphetamine laboratories and 3866 methamphetamine synthesis laboratories were dismantled, most of which were in West and Central Europe.[108]

Illicit MDMA manufacture is generally concentrated in larger more refined laboratories that distribute to wider geographical areas.[6] Until early 2000, the clandestine manufacture of MDMA-related stimulants was almost exclusively carried out in Western Europe (primarily in the Netherlands).[107] It is now manufactured worldwide but MDMA-related laboratories are not as widespread due to the greater level of skill and sophisticated equipment required.[62] MDMA-related substances in Australasia have been predominantly sourced from overseas. In 2004, 78% was sourced from Europe, 14% from North America and 5% from South East Asia.[108] Recently, however, the illicit manufacture of MDMA has spread to areas such as Oceania. In Australia during the period 2010-11 a total of 16 out of 703 clandestine laboratories dismantled were involved in the manufacture of MDMA.[108]

1.1.6.1 Synthetic routes

The knowledge of illicit manufacturing routes of ATS is important for the interpretation of analytical results.[13] Multiple synthetic routes can be used to manufacture ATS which have been described in detail in the literature.[109,110] The chosen synthetic route for the clandestine manufacture of ATS is largely dependent on the availability of pre-cursors, other chemicals, the simplicity of the process, availability of equipment and the chemical hazards associated.[13,111] Two methods common to most ATS, the Leuckart reaction and reductive amination, are illustrated in Figure 1.11. Both start with the pre-cursor P2P or one of its analogues, such as 3,4-MDP-2-P. The former can be defined as *reductive alkylation* in which the pre-cursor (e.g. P2P) acts as an alkylating agent of an amine (ammonia or methylamine) and formic acid is the reducing agent. The resulting intermediate, the N-formyl derivative of the ATS, is hydrolysed in aqueous acid to produce the desired ATS. Reductive amination involves treatment with an amine (ammonia or methylamine) followed by reduction with sodium cyanoborahydride, sodium borahydride or Al/$HgCl_2$.[111,112]

1.1.6.2 Methamphetamine

The illicit manufacture of methamphetamine is relatively simple, involving only a few steps, and pseudoephedrine is predominantly used as a starting product.[6] The methods are flexible as for each method a variety of substitutes can be obtained. A range of different solvents, catalysts and pre-cursors can be used.[103] These factors contribute to the large variation in final products seen on the illicit market. Pseudoephedrine and ephedrine are commonly extracted from cold and flu pharmaceutical preparations some of which are available over-the-counter. The supply of these pharmaceutical preparations is strictly controlled and hence they are often trafficked into the country from Asia.[7] P2P, the predominant pre-cursor of amphetamine, is not manufactured in Australasia and is a prohibited import, thus is sourced through illicit means.[111] P2P can be manufactured from phenylacetic acid or phenyl-2-nitropropene, however, phenylacetic acid is strictly regulated. The most

common clandestine routes used in the manufacture of amphetamine and methamphetamine are presented in Figure 1.11.

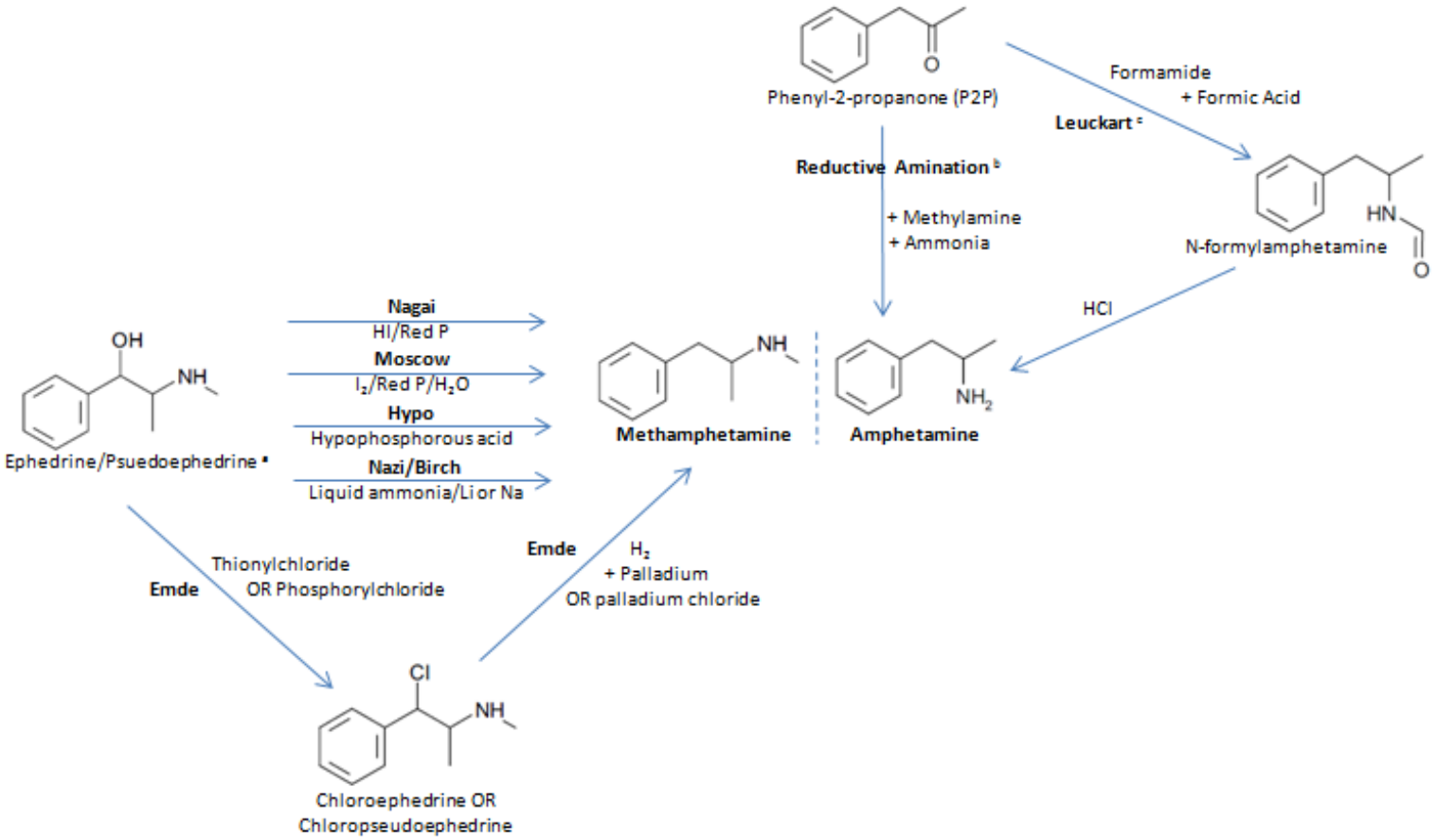


Figure 1.11 The predominant synthetic routes employed in the illicit manufacture of amphetamines. To synthesise amphetamine: [a] use norpseudoephedrine or norephedrine; to synthesise methamphetamine [b] use Al and $HgCl_2$ in the place of ammonia, [c] use N-methylformamide in the place of formamide. Adapted from [112]

Whilst methamphetamine is very easy to synthesise, the production process is very dangerous and harmful to the environment. Highly flammable and poisonous substances are used and some poisonous gases, or by-products, such as ammonia, phosphine and hydrochloride are often generated.[13] Manufacturing methods starting with P2P take longer and produce a racemic mixture of methamphetamine which is less potent. Thus, methods starting with ephedrine or pseudoephedrine are preferred as they produce the most active dextro isomer of methamphetamine.[113]

The reactive benzyl alcohol group of pseudoephedrine or ephedrine can be reduced via a range of different methods to produce methamphetamine.[111] The Birch/Nazi reduction is the most common in Australia and uses lithium or sodium metal dissolved in anhydrous ammonia. This method is simple and quick, however makes use of very hazardous chemicals. Any release of ammonia creates toxic environmental hazards and lithium and sodium react violently with water.[6] Lithium strips are obtained from batteries and anhydrous ammonia can be obtained from agricultural sources such as fertilisers.[111] Explosions or fires are not uncommon and this is often how clandestine laboratories are discovered.[13] The Birch/Nazi route is typically suited to small-scale production and can be carried out in one step. This method of one-step methamphetamine synthesis, referred to as *one pot shake and bake*, became popular in the US. Ammonia is generated *in situ* from ammonium nitrate fertilizer in a small amount of water with periodic addition of sodium hydroxide. All the ingredients i.e. pseudoephedrine, ammonia, starting fluid (used as a solvent) and lithium are mixed in a 2-litre bottle and the mixture is left for several hours. After conversion to methamphetamine the mixture is filtered and hydrogen chloride gas is introduced to form the hydrochloride salt of methamphetamine.[13,111]

Methods employing phosphorus species (Nagai, Moscow and Hypo) are the most common in New Zealand and are frequent in Australia.[7] In the Nagai method, hydriodic acid and red phosphorus are heated with pseudoephedrine/ephedrine to produce methamphetamine. This method is also simple and is often used for large scale production.[111] Red phosphorus can be obtained from matchbox strikers in small amounts. Hydriodic acid can be generated *in situ* from red phosphorus, iodine and

water (Moscow) or from hypophosphorus acid (Hypo). The former method produces a by-product of phosphorus acid and when the reaction mixture is overheated it generates the extremely toxic phosphine gas. Further, in the Hypo method the hypophosphorus acid also generates phosphine gas through the breakdown at a lower temperature.[114]

The Emde route of synthesis is less popular because it involves a more complex two-step procedure. The conversion of ephedrine/pseudoephedrine to a chloro-intermediate is facilitated by thionyl chloride or phosphorus pentachloride in chloroform. The subsequent conversion to methamphetamine is accomplished using hydrogen and a palladium catalyst.[111]

1.1.6.3 Amphetamine

The same synthetic pathways summarised in Figure 1.11 can be followed to synthesise amphetamine. Norephedrine or norpseudoephedrine can be used as starting products in the place of ephedrine or pseudoephedrine. However, amphetamine is more often synthesised from P2P via the Leuckart synthetic pathway or via reductive amination. The Leuckart reaction has been the most popular synthetic pathway for the clandestine manufacture of amphetamine in the US, UK and the Netherlands.[115] Formamide and P2P are heated in the presence of formic acid to form an intermediate of N-formylamphetamine. Hydrochloric acid is used to hydrolyse the intermediate product to form amphetamine.[13] This method is analogous to the Leuckart reaction of methamphetamine.

The reductive amination of P2P is popular.[51] Ammonia can be condensed with P2P to form an imine which is then reduced to amphetamine. A dissolving metal reduction is commonly employed using Al, Zn or Mg amalgams, for example Aluminium and Mercuric Chloride. Analogous methods used in the manufacture of methamphetamine and MDMA are illustrated in Figures Figure 1.11 and Figure 1.12, respectively. An alternative method involves the catalytic reduction of P2P in which the ammonia gas is charged with H_2 and a catalyst is used, such as palladium on

charcoal (Pd/C), platinum oxide (PtO) or Raney-nickel. Often a low pressure and temperature are used.[13]

1.1.6.4 MDMA and analogues

Most pre-cursors used in the manufacture of MDMA, and its analogues, contain the preformed methylenedioxy substitution pattern on the aromatic ring. Popular synthetic routes proceed via the 3,4-MDP-2-P intermediate which is an analogue of P2P.[116] The subsequent reduction of 3,4-MDP-2-P can be achieved using Leuckart's reaction or reductive amination.[117,118] The reductive amination of 3,4-MDP-2-P to form MDMA is the most direct: either via catalytic metal reduction (methylamine + hydrogen gas + platinum catalyst), aluminium amalgam ($Al/HgCl_2$) or metal hydride reduction ($NaCNBH_4$). Analogues such as MDA and MDEA can be produced using ammonia gas or ethylamine in the place of methylamine.[115] The Leuckart synthetic pathway is analogous to amphetamine manufacture. 3,4-MDP-2-P and N-methylformamide are reduced in the presence of formic acid to produce a formamide intermediate. The final product, MDMA, is obtained following hydrolysis with a strong acid or base. An extensive review of the reductive amination and Leuckart routes for the manufacture of MDMA-related substances was published by Carson in 1990.[119]

3,4-MDP-2-P can be made from piperonal, safrole or isosafrole which are all under international control.[120] Safrole can be extracted from the naturally occurring sassafras oil which typically contains between 80 and 90% safrole.[121] The synthesis of 3,4-MDP-2-P is achieved from safrole via Wacker oxidation and from piperonal via the Peracid or Wacker oxidation methods.[112] Piperonal can be produced via the nitropropene route.[121] These synthetic pathways are summarised in Figure 1.12.[121]

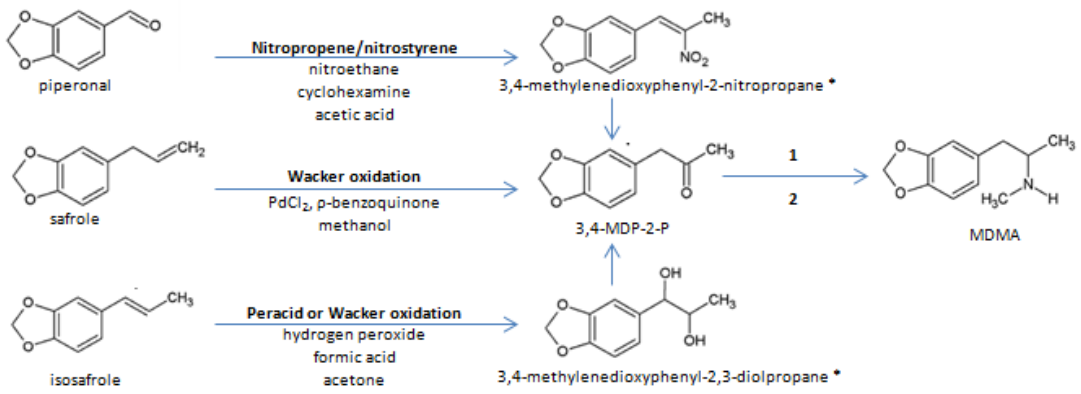


Figure 1.12 Synthetic pathways involved in the manufacture of 3,4-MDP-2-P from piperonal, safrole and isosafrole. * Intermediate products formed. To subsequently reduce 3,4-MDP-2-P to MDMA the: (**1**) Leuckart reaction and (**2**) reductive amination routes can be used. Adapted from [111,112]

MDMA and its analogues can be synthesised from the appropriately substituted benzaldehyde, for example piperonal. Piperonal and nitroethane react in the presence of acid or base to form a nitropropane/nitrostyrene intermediate which is converted to 3,4-MDP-2-P.[121,122] The appropriate allylbenzene i.e. safrole can be oxidised via the Wacker oxidation process using palladium chloride and p-benzoquinone. The oxidation of isosafrole is commonly achieved using hydrogen peroxide and formic acid, though the use of peracetic acid has also been documented. The intermediate of 3,4-methylenedioxyphenyl-2,3-diolpropane is then converted to 3,4-MDP-2-P.[123,124]

1.1.6.5 Cathinones

Similar methods used in the manufacture of amphetamines and MDMA-related substances can be used for manufacturing cathinones and depend on the desired ring substitution pattern. For example, methcathinone can also be manufactured from ephedrine/pseudoephedrine via oxidation of the ketone group, rather than reduction involved in amphetamine synthesis (Figure 1.13). The oxidation process is facilitated by sodium dichromate, potassium permanganate or chromium trioxide. A strong acid, such as concentrated sulphuric acid, is used and hydrogen chloride gas is employed to form the final salt form of methcathinone.[121,125,126]

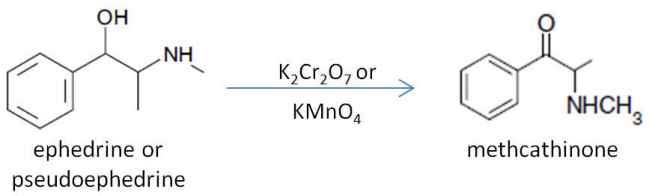


Figure 1.13 Preparation of methcathinone from ephedrine/pseudoephedrine. Adapted from [127]

Although less common, the racemic form of cathinones can be synthesised from the appropriately substituted propiophenone pre-cursor. The synthetic pathway used to manufacture methcathinone involves the bromination of propiophenone and subsequent treatment of the bromoketone intermediate with methylamine (Figure 1.14).[127,128]

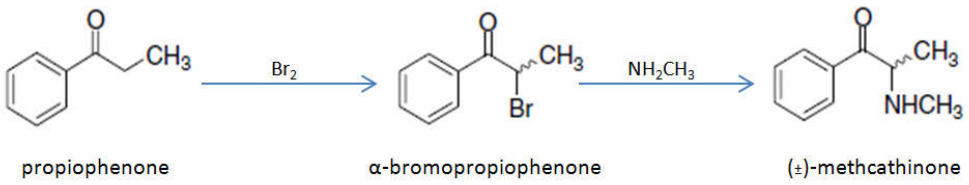


Figure 1.14 Preparation of racemic methcathinone from propiophenone. Adapted from [127]

1.1.6.6 Summary

The manufacturing methods for the ATS presented here are largely interchangeable given their structural similarities. The prominent synthetic routes used in the clandestine manufacture of ATS are summarised in Table 1.6.

ATS	Route of manufacture	Pre-cursor
Amphetamine	Reductive amination Leuckart	P2P P2P
Methamphetamine	Nagai Moscow Hypo Nazi/Birch Emde	PSE/EPH PSE/EPH PSE/EPH PSE/EPH PSE/EPH
MDMA and analogues	Reductive amination Leuckart Nitropropene/nitrostyrene Wacker oxidation Peracid/Wacker oxidation	3,4-MDP-2-P 3,4-MDP-2-P Piperonal Safrole Isosafrole
Cathinones	Oxidation Bromination and methylamination	PSE/EPH Propiophenone

Table 1.6 Summary of the predominant synthetic routes of clandestine ATS manufacture.

1.1.7 Analysis

The forensic analysis of ATS is performed using a range of analytical techniques. The choice of analytical technique and sample preparation method are based on the following factors:

(1) The questions or hypotheses to be addressed
(2) Laboratory resources
(3) Quantity and exhibit type

The legislative framework typically dictates which questions or hypotheses are to be addressed. Different levels of information may be required which include: identification of the drug, quantification and drug profiling. Identification confirms the presence, or absence, of controlled substances in the sample. Quantification is performed to determine the actual amount present which is required in some jurisdictions. The profiling of illicit drug samples involves the characterisation of traces of impurities or by-products (chemical profile) and physical attributes such as the colour, logo and packaging (physical profile). As summarised by Morelato *et al.* *'drug profiling can be used to link specific drug specimens, identify synthetic and trafficking routes, sources of supply and common origins between seizures* [12]*'*. The

comparison between illicit drug samples from two different cases, using profiling data, can suggest links and give valuable information for investigations.[14] From a drug intelligence perspective, this process can also be used to provide information about other cases that might be linked through chemical data, but were previously unsuspected. Numerous reviews and journal articles have been published which outline this concept. Further information can be found in a recent publication by Morelato *et al.*[12] To remain within the scope of this project, only the techniques involved in the identification of ATS will be discussed in detail.

The examination of powders, tablets or liquids suspected to contain illicit or controlled substances is performed sequentially. The general sequence employed in the laboratory is illustrated in Figure 1.15. The seized drug exhibit is visually examined and its physical characteristics are recorded. For example, the packaging, colour or characteristic marks or logos on tablets. The sample is then screened, if enough is present, to indicate if a certain class of illicit substances is likely to be present. Finally, a sample preparation procedure is followed and the sample is subjected to definitive analysis by a given technique.

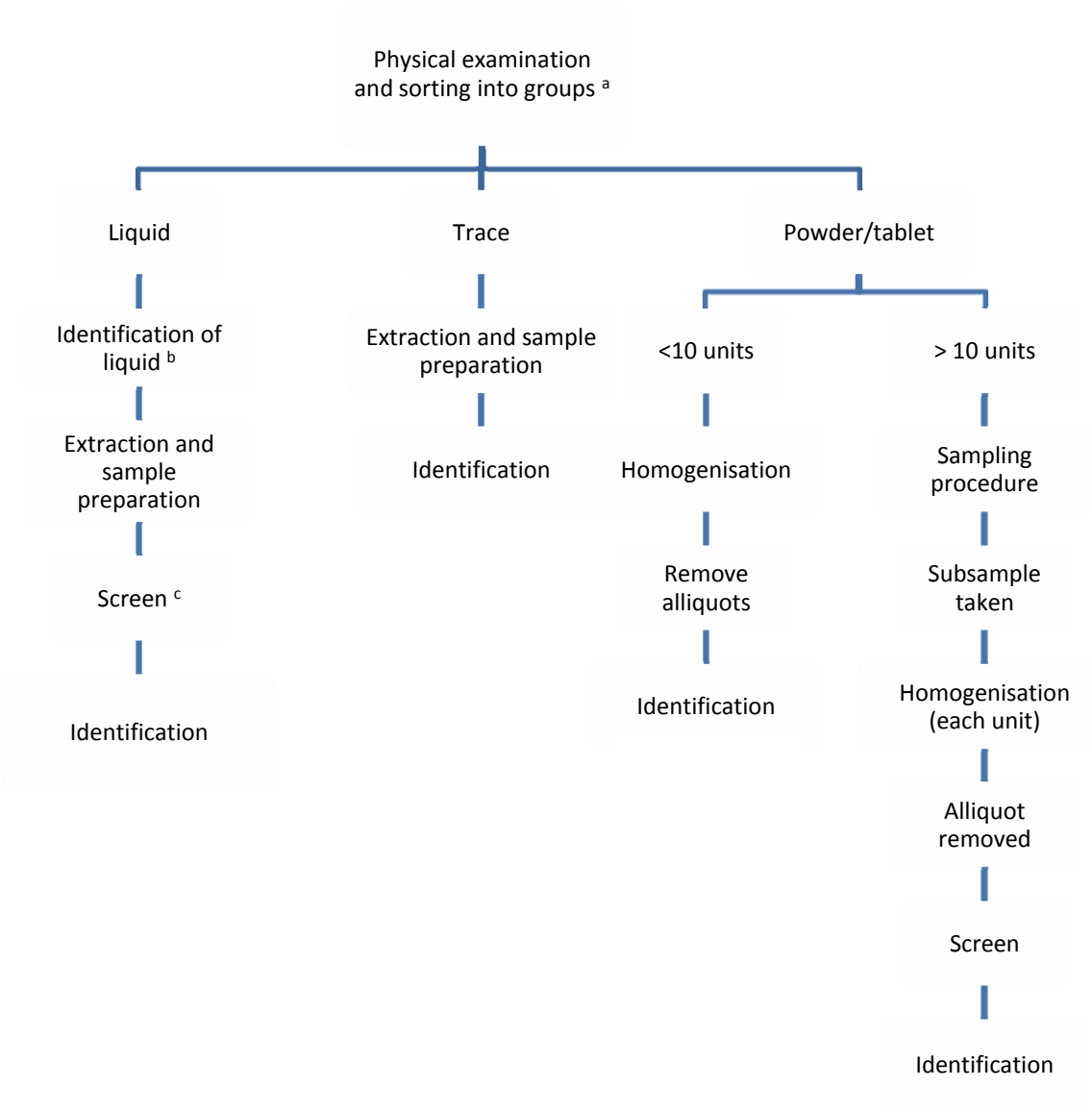


Figure 1.15 Flowchart illustrating the analysis scheme followed for the examination of drug seizures: [a] Sorting is based on visual similarity, [b] the procedure followed for identifying a liquid and processing liquids from clandestine laboratories is discussed further in chapter 3, [c] screen is performed if sample permits. Trace is defined as a sample that is barely visible to the naked eye, whilst bulk can be defined as more than 10 units or a surplus of one unit (≤ 10 kg). Adapted from [16]

The nature and quantity of the sample dictates which sequence to follow. For example, if a bulk quantity is seized then sampling and analysis will follow a screening procedure and confirmation. However, for trace samples there is only enough present for identification and the screening step is eliminated. Examples of traces related to drug seizures include residues inside reaction vessels, on bench tops or items suspected to have been in contact with illicit drugs.

A notable exception to the analysis scheme, presented in Figure 1.15, is when there is a visual or other identifier present. Pharmaceutical preparations, from which pseudoephedrine can be extracted, often have identifying features such as size, shape, markings and colour. In addition, the packaging is sometimes present at the site of illicit manufacture. This information can be combined with a quick presumptive test to be used for identification purposes.[129]

The analysis of liquids from clandestine laboratory sites is generally more involved. Additional sampling and analysis procedures are required in addition to the methods set out for seized drugs. A wide range of aqueous and non-aqueous liquids used in the manufacturing process of ATS are present at clandestine laboratory scenes and careful processing is required. Identifying the constituents of the sample matrix is required in addition to identifying the presence or absence of controlled substances.[130,131] The analysis of clandestine laboratory liquids is discussed in more detail in Chapter 3.

The sampling procedure, methods and analytical tools employed for the screening and identification of ATS depend on local jurisdictional requirements. Whilst these requirements are unique to a country, or even state, they are often based on internationally accepted guidelines. The United States scientific working group for the analysis of seized drugs (SWGDRUG) published a document outlining their recommendations for the analysis of seized drugs which was revised in July 2011. This document includes an excellent summary of the techniques available for identification purposes (Table 1.7).[130]

The techniques are grouped into three categories, A, B and C according to their maximum discriminating power. Category A includes the techniques with the most discriminating power which are predominantly based on mass or elemental analyses. Category B includes screening tools with enhanced discrimination e.g. chromatographic separation techniques. Whereas category C tests include colour tests and other physical tests which have the least discrimination.

The identification of a specific drug for use as evidence is very important and must be defendable in court. Guidelines have been designed for the analysis of drug exhibits for this purpose. According to SWGDRUG, the conclusive identification of seized drugs can be achieved by using: (1) one category A technique in combination with an additional technique from either category or (2) three techniques from categories B and C of which two should be category B. Gas chromatography (GC) or liquid chromatography (LC) when coupled with mass spectrometry (MS) are considered as separate techniques and satisfy the requirements for identification.

Further, SWGDRUG state that *'the classification of a technique may be lower if the sample, analyte or mode of operation diminishes its discriminating power* [130]*'*.

Category A	Category B	Category C
Infrared Spectroscopy	Capillary Electrophoresis	Colour tests
Mass Spectrometry	Gas Chromatography	Fluorescence Spectroscopy
Nuclear Magnetic Resonance Spectroscopy	Ion Mobility Spectrometry	Immunoassay
Raman Spectroscopy	Liquid Chromatography	Melting point
X-ray diffractometry	Microcrystalline Tests	Ultraviolet Spectroscopy
	Pharmaceutical Identifiers *	
	Thin Layer Chromatography	

Table 1.7 Categories of analytical techniques from SWGDRUG recommendations. *Examples of pharmaceutical identifiers include physical characteristics of tablets, capsules or packaging indicating the identity, manufacturer or quantity of the substances present.[130]

1.1.7.1 Screening

Various screening techniques are routinely employed, in-field and in the laboratory, to presumptively identify unknown powders. Screening tests are used to establish the possible presence of controlled substances and to classify these controlled substances into general categories.[132] They are often used to suggest which test to perform next and can be used to locate compounds of interest not visible to the naked eye.[133] These can be simple techniques based on colour reactions or crystal formations. Alternatively, separation techniques can be used to presumptively identify mixtures. The technique employed for presumptive in-field testing heavily depends on the nature and location of the drugs seized.

Colour or spot tests are routinely employed for the rapid screening of ATS and their pre-cursors. They were one of the earliest developed techniques for presumptive drug testing and are still popular due to their simplicity and inexpensive nature.[134] They were traditionally performed on porcelain spot tiles and often employed in a sequence using multiple reagents. They are now also available as presumptive field drug test kits.[14] Colour tests are often based on the reaction of specific functional groups with reagents. The result ranges from no change, one colour change to a

series of colour changes.[135] The most common reagents employed for ATS and their pre-cursors are summarised in Table 1.8. The most popular reagent used for the presumptive screening of ATS is Marquis.[16] Marquis tests distinguish between amphetamine and ring-substituted ATS.[13] Simon's reagent is used to presumptively identify secondary amines, for example when distinguishing between methamphetamine and amphetamine. However, piperidine also reacts with Simon's reagent. Chen's test reacts with phenethylamines possessing vicinal amino and hydroxyl groups.[136] This reagent is often utilised to distinguish pseudoephedrine, ephedrine, norephedrine, cathinone and methcathinone from amphetamine and methamphetamine.

Colour tests are quick and relatively sensitive. The National Institute of Justice reports detection limits of between 10 and 100 μg for selected ATS in a report evaluating some colour test reagents/kits for the preliminary identification of drugs of abuse.[137]

They are simple and easily employed by untrained personnel. They are often used by first responders in-field due to their inherent portability.[134] The results are non-specific and are influenced by the presence of other similar compounds and depend on drug concentration, salt form and pH.[13,134,135] The results depend on the interpretation of the analyst even with the aid of colour reference charts.[16] Further, many ATS, particularly MDMA-related stimulants, are often coloured which can mask the colour result of the test. In this case the procedure should be adapted to include a dye extraction step prior to performing the colour test.[13]

Gallic acid, not included in Table 1.8, can also be used to distinguish between ring-substituted ATS (e.g. MDMA, MDA and MDEA) and amphetamines. It reacts specifically with ring-substituted aromatic compounds.[138] A bright to dark green colour indicates the presence of MDA, MDMA and MDE. Pre-cursors of MDMA-related stimulants, 3,4-MDP-2-P and safrole, also react with Chen's reagent. 3,4-MDP-2-P turns brown and safrole is indicated by a brown colour turning to dark brown/red. The colour test solution is a mixture of gallic acid, concentrated sulfuric acid and aqueous acetic acid.[139]

	Marquis reagent	Simon reagent	Chen-kao reagent
ATS			
Amphetamine	Orange, slowly turning brown	No reaction *	No reaction *
Methamphetamine	Orange, slowly turning brown	Deep blue	No reaction *
MDMA	Dark blue/black	Deep blue	No reaction *
MDA	Dark blue/black	No reaction *	No reaction *
MDEA	Dark blue/black	(Deep) blue → brown	No reaction *
Cathinone	NR	No reaction *	Turns
Methcathinone	NR	Slightly blue, spot-or-ring-like precipitate	Yellow/orange Yellow/orange
Pre-cursors			
Pseudoephedrine	NR	No reaction *	Purple
Ephedrine	NR	No reaction *	Purple
Norephedrine	NR	No reaction	Purple
Safrole	Deep blue → dark purple	-	-
Isosafrole	Dark purple	-	-
Piperonal	Lemon yellow	-	-
P2P	Orange yellow	-	-
3,4-MDP-2-P	Orange-brown	-	-
Phenylacetic acid	Yellow → olive green	-	-
Other			
Piperidine	-	Deep blue	-

Reagents: ***Marquis****: formaldehyde/acetic acid/concentrated sulfuric acid,* ***Simons:*** *sodium carbonate/acetaldehyde/sodium nitroprusside,* ***Chen's****: aqueous acetic acid/copper(II) sulfate/sodium hydroxide. NR = No reaction. * The colour of the reaction should be considered as negative. Note: similar or other colours may occur in the presence of other controlled drugs or pre-cursors.*

Table 1.8 Colour test results observed with common reagents for ATS and their pre-cursors. Adapted from [13]

Chromatographic separation techniques can be used to screen mixtures to presumptively identify individual components. They are based on the interaction of compounds between two phases, the mobile phase and stationary phase. Traditionally thin layer chromatography (TLC) was used as a cost-effective screening tool with enhanced specificity to colour tests. Although TLC is often replaced with more sophisticated tools such as GC it is still utilised in some laboratories.

The sample, dissolved in a solvent such as methanol, is spotted onto the bottom surface of the chromatographic plate along with positive and negative controls. The plate is suspended in a solvent saturated tank which is left to travel up the plate. Individual components are separated on the basis of size, shape and their interaction with the solvent (mobile phase). After the solvent has travelled the length of the plate it is dried and examined using a visualisation technique such as ultra violet light. Often a developing reagent, such as the Marquis reagent, is used to enhance the

visualisation of components. Retention factor (R_f) values are calculated which are a measure of the distance each component travels along the plate relative to the distance moved by the solvent. A comparison is made to the values of reference standards run on the same plate. TLC is a fast and cost-effective separation technique with inherent flexibility. However, R_f values can fluctuate with temperature, sample concentration and adsorbent uniformity on the TLC plate. Other limitations include the lack of resolution, sensitivity and specificity of developing agents.[140,141]

GC and LC are more commonly used during the examination of liquids from clandestine laboratories. The ability to determine relative amounts of each constituent is useful in cases where sample concentration/dilution may be required prior to confirmatory testing.

Screening at airports, where typically trace amounts are present, is performed using ion mobility spectrometry (IMS). IMS achieves rapid responses, can screen for 40 compounds in 8 seconds and offers sensitivity down to the nanogram or picogram level.[142] Minimal sample preparation is required and the ability to screen on almost any surface is ideal for border security environments.[143] Its ruggedness and low power consumption enhances its degree of portability.[144,145] IMS has been used to help identify contaminated sites during the remediation of abandoned clandestine laboratory sites. It screens for the presence of traces of illicit drugs and other related hazardous chemicals.[146] However, the limited resolution attributed to IMS analyses can mask the positive identification of the drug in question as a result of interferences from other compounds present.[145,147]

1.1.7.2 Identification

The conclusive identification of seized drugs is achieved using a combination of general and more specific techniques, as outlined in the guidelines published by SWGDRUG (Table 1.7). Techniques with the highest discriminating power are favoured. Therefore, most laboratories employ gas chromatography - mass spectrometry (GC-MS) or liquid chromatography - mass spectrometry (LC-MS) due to their versatility, sensitivity and specificity.[148] Compounds are separated in the gas or

liquid chromatogram and the molecular fragment ion patterns produce a chemical fingerprint of the unknown substance.[140] Searchable libraries are used to compare the mass spectra of the drug exhibit with those of known standards.[129] Co-elution or poor peak resolution of structurally similar compounds can be problematic. A range of chemical derivatising agents have been designed to improve the specificity of these techniques which modify specific functional groups of ATS.[14,149] The use of GC-MS and LC-MS for forensic drug identification has been extensively reviewed in the literature. However, limited research has been conducted into the evaluation of these techniques from an intelligence perspective.[12] Some of the main advantages and disadvantages of these techniques are outlined in Table 1.9.

Fourier transform infrared (FTIR) spectroscopy is a rapid and non-destructive technique that offers excellent discrimination between pure drugs. A unique spectrum is obtained for each individual chemical compound (except for some isomers and homologues).[129] However, clandestinely manufactured drugs are typically impure and exist as mixtures and thus GC-MS is generally preferred. It is often difficult to isolate the spectra of individual constituents in a mixture.[130]

LC-MS (or HPLC-MS) often requires less sample manipulation and is frequently used for the analysis of highly polar, involatile and thermolabile compounds.[150] GC-MS and LC-MS are also the most utilised instrumental methods to accomplish quantification using an internal standard.[151] There are some cases where the presence of by-products or impurities cause interferences when using LC-MS.[151]

Technique	Advantages	Disadvantages
GC-MS	- Highly sensitive - Excellent resolving power - Impurities generally easily identified	- Sample must be volatile and thermally stable - Derivatisation sometimes required - Destructive
LC-MS	- Non-destructive - Volatility not required - Pre-treatment not usually required - Analysis is easily automated - Quantification without internal standard - Good resolving power	- Detection capabilities in liquid stream - Sample solubility in a wide range of solvents - Quantification can be slow - Large volume of solvents used - Impurities can interfere with identification
FTIR	- Powerful identification of pure compounds - Rapid - No derivatisation required - Non-destructive - Minimal sample preparation	- Often cannot resolve mixtures - Impurities can interfere with identification

Table 1.9 The advantages and disadvantages of some commonly employed for the conclusive identification of seized drugs. Adapted from [14]

1.1.7.3 Emerging techniques

Traditionally, the approach in forensic casework has involved the collection of samples at the scene for subsequent analysis in the laboratory.[152] However, the evolution of the contemporary drug problem has put increasing demands on law enforcement for expediting their investigations. Further, the increasing complexity of drug trafficking networks and dispersion of illicit manufacture to fuel local demands has created unique challenges for clandestine laboratory investigators.[7] Recent technological advancements have focused on the miniaturisation of traditional laboratory instruments. The need for more efficient and effective methods of processing drug seizures has moved away from the laboratory and towards in-field analysis methods. The development of field portable tools has been a major research focus. In 2001, this need was recognised by the Australian Federal Police (AFP):

> the ability to deploy more sophisticated analytical equipment into the field for screening and analytical purposes can greatly assist in the appropriate targeting of physical evidence at the scene.[153]

This is particularly relevant for clandestine laboratory investigations where the efficient collection and processing of seized materials is crucial. The immediate

identification of chemicals would provide valuable information for the assessment of hazards and to assist in scene processing. The United States National Forensic Science Technology Centre (NFSTC) compiles a list of reports on such emerging technologies and up to date information is available from their website.[154] In a recent report, the NFSTC summarised that:

> field-purposing technology puts the ability for timely collection and processing of forensic intelligence in the hands of deployed field personnel to enhance their ability to develop and disseminate actionable intelligence.[155]

This ultimately decreases the burden on forensic laboratories and truncates the time involved.[153]

The miniaturisation of existing analytical techniques has been a major focus over the past decade. Rapid technological advancements have driven the development of portable or hand-held devices, predominantly based on spectroscopic analysis techniques such as Raman or infra-red.[156] These devices are aimed at filling the technology gap between in-field and laboratory. A large number of portable devices have been developed, however this thesis will be confined to include only those developed for drug analysis. While additional models are available those that have been independently reviewed were chosen as examples. The unique advantages offered by some common portable tools are summarised in Table 1.10.

Technique	Advantages	Disadvantages
Raman **e.g. Ahura Scientific first defender** **From $35,000**	- Hand-held - Rugged - Easy to operate No sample preparation	- Software requirements - Difficulties with mixtures - Interference from fluorescence
Near InfraRed **e.g. Polychromix microPHAZIR**	- Non-destructive - Fast (8-10 seconds) - Hand-held - Rugged - Easy to operate - Hand-held (1.25 kg)	- Costly - Suffers with mixtures - Inferior specificity to FTIR
FTIR **e.g. Hazmat ID** **Approximately $55,000**	- Non-destructive - Searchable library - Accurate for pure drugs - Hand-held - Easy to operate - Heat and humidity resistant	- Difficulties with mixtures - Warm-up time of 20 minutes - Costly - Interference from water
Portable GC-MS **e.g. Griffin™ 450** **$85,000 – 88,000**	- Identification of unknown through spectral library comparison - No external pump required - Minimal sample preparation - User adaptable software - High sensitivity (pg - ng) - High specificity	- Relatively long analysis times - Warm-up time of 30 minutes and 4 minutes between analyses - Requires power generator - 40 kg - Requires manual injection - Requires training - Analysis time of 12 minutes
Lab-on-a-chip * **e.g. Bioanalyzer** **Approximately $20,000**	- High specificity - High sensitivity (µg) - Fast (60 second analysis) - No warm-up time required - Approximately $20,000 - Able to separate mixtures - Automated sample analyses - Software easy to use	- Requires power generator - 10 kg - Requires some training - Derivatisation required - Consumables required

Table 1.10 A summary of some commercially available portable drug analysis devices. * Although commercially available, lab-on-a-chip is not marketed for drug screening. This technique is presented as an alternative to existing commercial techniques.

Raman, near infra-red (NIR) and FTIR are spectroscopic techniques that detect molecular vibrations and are ideal for the analysis of organic molecules such as ATS and their pre-cursors. Three commercial devices of each type were evaluated by the NFSTC to compare the performance characteristics against specific in-field requirements.

The Hazmat ID employs a non-destructive sampling technique using attenuated total reflection – fourier transform infra-red (ATR-FTIR) spectroscopy. An accurate

identification of pure drugs can be achieved. However, results are not as reliable for mixtures as they depend on the IR characteristics of the primary component and compound(s) of interest within the sample. This technique allows for the qualitative identification of powders, liquids, pastes, gels and solids.[157] However, the strong absorbance bands of water can interfere with the IR spectra from wet ATS seizure samples.[158]

Portable Raman analysers (Ahura Scientific first defender) are non-destructive and able to screen liquids and solids without the need to transfer or contact the sample.[159,160] This is particularly useful in a clandestine laboratory setting for minimising exposure to hazardous materials.[161] They do not perform well with trace, fluorescent or pigmented samples and accuracy can be limiting. In this particular study by the NFSTC, only 50 percent of mixtures were identified correctly.[162] Raman spectrometers provide a chemical signature of the unknown material by illuminating it with a laser. The spectrum can be compared to a reference library.[159] The NFSTC comment that *'the use of Raman spectrometry is not inherently safe. Both eye hazards and explosion hazards exist if the unit is used incorrectly [163]'*.

The micro-PHAZAR, based on near-infrared technology, although not developed for illicit drugs was also tested by NFSTC. This technique was found to provide a quicker response than FTIR and Raman-based techniques but offers lower specificity. The scan range is narrow in comparison and therefore the NIR band differences are more subtle.[164] However, NIR does not suffer from interference from fluorescent, dark-coloured compounds, or water. As with Raman, NIR is non-destructive, easy to use and requires no sample preparation. Samples may be screened through containers. However, this technique is not as robust and can produce inaccurate results if any slight movement is made. Mixtures are also a problem with NIR spectroscopy.[155]

Whilst GC-MS provides excellent confirmatory analysis of drugs it is not well suited to miniaturisation. Despite this, research has been conducted into the development of robust MS detectors for in-field applications. The Griffin™ 450, a commercially available portable GC-MS device, was also evaluated by NFSTC. This device is more

expensive than spectroscopic-based models and not as portable with a weight of 40 kg. However, it has the potential to provide conclusive identifications. The limitations of this model include the required bench space, costs associated with maintenance and replacement columns, the need for manual injection and some safety hazards associated with attached compressed gas cylinders. In addition, it has a limited mass scan range of 40-425 m/z.[165]

The Bioanalyzer, based on lab-on-a-chip technologies, was designed for the analysis of biological molecules such as DNA. This technique is relatively inexpensive and simple to operate.[166] The Bioanalyzer is portable, with a weight of 10 kg, and has been investigated as a rapid screening tool for the in-field analysis of DNA at mass disaster sites.[167] Although some scientific training is required, enhanced specificity and sensitivity is often achieved in comparison with handheld spectroscopic techniques. Reproducible and accurate sizing of DNA fragments can be obtained with good resolution.[168] Although this device has not been evaluated for the analysis of illicit drugs its potential as a generic platform has been demonstrated.[169,170]

In summary, the available portable techniques have been extensively evaluated by NFSTC for forensic applications. Some of the spectroscopic-based tools are currently used by investigative personnel to aid in clandestine laboratory scene processing. They are useful for identifying pure pre-cursors and final products and have been evaluated for screening shipments at the border to replace IMS or as complementary tools.[160] A common limitation of spectroscopic techniques which restricts their use at clandestine laboratories is the inability to resolve mixtures or impure samples. The presence of fillers and binders saturate the spectra making it difficult to differentiate the drug from the background. This is particularly problematic when the compounds of interest are minor constituents. However, future developments are ongoing to address some of the issues with rapid advances in portable technology. In contrast, the available portable GC-MS models are expensive and are currently only suitable within the confinements of a mobile laboratory. The limitations of the presented techniques need to be overcome before they are widely employed at clandestine laboratory sites.

There still appears to be a technology gap and a need for the development of more specific and robust instrumentation to bridge this gap. A balance must be obtained between size, operating requirements and cost in order for these techniques to be routinely employed. Furthermore, there is often confusion regarding the use of portable and emerging techniques. The distinction between their screening and identification capacity is frequently not recognised and needs to be considered as the end-use or analytical focus of these techniques may be different. This should be further explored through research.

The end-use or focus of these techniques may be different and this should be clarified through research.

The UNODC identified that *'one key development to monitor will be the ongoing shift away from developed to developing countries, which would mean a heavier burden for countries relatively less equipped to tackle it* [7]*'*. Hence the development of low cost portable devices is crucial.

The use of microchip electrophoresis (MCE) for the analysis of illicit drugs is a viable option. The inherent portability and versatility of MCE could contribute to bridging the gap between the laboratory and in-field. Further, the ability to perform analyses using disposable microchips is also valuable.[171] Attractive features include short analysis times, minimal sample and reagent requirements, and ease of use. This technique has gained increasing popularity in analytical chemistry. In recent years, promising applications have been identified for the rapid on-site analysis of explosives for homeland security.[172]

1.2 Microchip capillary electrophoresis (MCE)

MCE is a miniaturised format of capillary electrophoresis (CE). The conventional capillary is replaced by finely etched micro-channels and electrophoretic separations are performed within these micro-channels on the planar surface of a microchip.

Prior to discussing MCE, the fundamental principles of CE are outlined to provide a basis for understanding.

1.2.1 Principles of capillary electrophoresis

CE is a rapid and versatile analytical separation technique. The multitude of separation modes and detection systems enable the analysis of a wide range of compounds from small inorganic ions to large deoxyribonucleic acid (DNA) fragments.[173] The ability to resolve complex mixtures of analytes efficiently, with minimal sample requirements, has contributed to its widespread application in forensic science. Applications include gunshot residues, low and high explosives, inks, soils, illicit drugs and DNA fingerprinting/DNA typing.[174-179]

Electrophoresis was traditionally performed in a slab gel format using a gel composed of agarose, polyacrylamide or starch.[180] Modern CE instrumentation is considered by many to have been initiated by the influential work of Jorgenson and Lukacs in the early 1980's. They demonstrated the high-speed electrophoretic separation of biomolecules in narrow-bore capillaries (75 µm internal diameter) made of glass.[181] The high surface-to-volume ratios of capillaries allow effective heat dissipation and thus separations can be performed at up to 30 kV offering high resolution separations.[173]

1.2.1.1 Instrumentation

Electrophoresis can be defined as the movement or migration of ions under the influence of an electric field.[182] Ions migrate toward the electrode of opposite charge i.e. positively charged ions migrate towards the cathode and negatively charged ions migrate towards the anode.

The instrumental set-up of a CE system is simple and consists of a fused-silica (SiO_2) capillary, two electrodes, two buffer reservoirs, a high voltage power supply and a detection system (Figure 1.16). The capillary is immersed in a buffer solution at both ends and the sample is introduced at the inlet (the anode in normal polarity mode). A

high voltage, typically between 20 and 30 kV, is applied and ionic species in the sample plug migrate towards the detector.[173] The detector signals are collected and a data acquisition system produces an electropherogram which plots the detector response, for example UV-visible absorbance, against time. The location of the detector is close to the cathode, as under normal polarity the flow of liquid is from the anode to cathode.

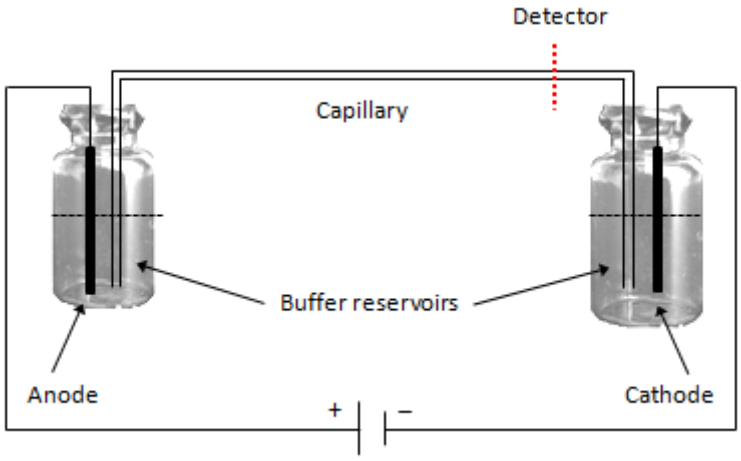


Figure 1.16 The instrumental set-up of a CE system. V_{inj} is the applied voltage. Adapted from [183]

The migration rate (electrophoretic mobility) of a solute ion is largely dependent on its size-to-charge ratio. The electrophoretic mobility (μ_e) of a charged species can be estimated using the Debye-Huckel-Henry theory:

$$\mu_e = \frac{q}{6\pi\eta r} \tag{1.1}$$

where q is the ion charge, η is the solution viscosity and r is the Stokes radius. Therefore, smaller, highly charged ions have a greater μ_e and thus migrate faster in the capillary. Individual sample ions separate according to their different mobilities.[173]

The velocity experienced by a charged ion is related to the electrophoretic mobility and the magnitude of the generated electric field. The electric field is directly proportional to the applied voltage and inversely proportional to the capillary length, illustrated in Equation 1.2:

$$v = \mu_e E = \mu_e \frac{V}{L} \quad (1.2)$$

where v is the migration velocity, μ_e is the electrophoretic mobility of the ion, E is the applied electric field, V is the applied voltage and L is the capillary length.[184]

1.2.1.2 Electroosmotic flow

A phenomenon known as electroosmotic flow (EOF) is the driving force behind the movement of liquid within the capillary.[173] This force, in addition to the electrophoretic mobilities of ions, enables the separation of anions and cations in a single run.[173] The EOF arises as a result of the surface charge at the capillary wall and the applied voltage. The capillary surface is made up of silanol groups that readily ionise to form a negative charge at the wall. At a pH of above 3, the negatively charged silica wall attracts an excess of positive ions from the buffer to form an electric double layer (Figure 1.17) and potential difference (zeta potential, ζ) close to the wall.

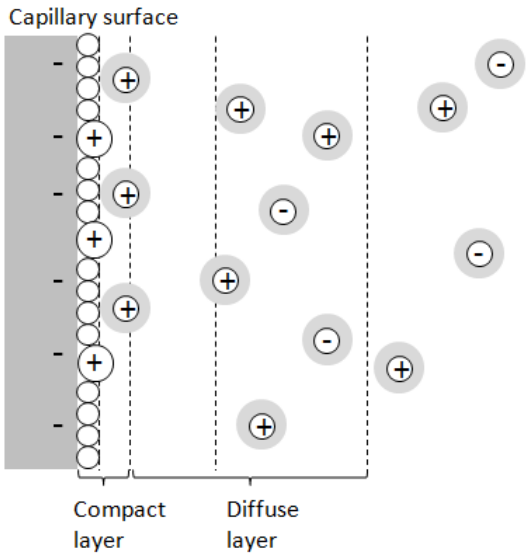


Figure 1.17 Schematic representation of the interface between a glass surface and an aqueous solution.[185]

The positive charge density closest to the wall forms a compact or Stern layer which is essentially static. Under the influence of an electric field the cations in the diffuse layer migrate towards the cathode carrying water molecules with them. The EOF acts

as an electric field-driven pump to propel all molecules (positive, neutral and negative) towards the detector. When the EOF is sufficient the difference in the electrophoretic mobility of each analyte leads to the formation of analyte zones by the time they reach the detector. If the EOF is slow, the analyte zones disperse causing band broadening and under extreme conditions some analytes may not reach the detector in a practical time frame. Above pH 3, the EOF drags all analytes contained in the injected sample, excluding those with an electrophoretic counter migration greater than the EOF.

Factors influencing the magnitude of the EOF include viscosity, zeta potential and the dielectric constant (Equation 1.3).

$$\mu_{eof} = \frac{\varepsilon\zeta}{\eta} \tag{1.3}$$

where μ_{eof} is the EOF mobility, ε is the dielectric constant, ζ is the zeta potential and η is the buffer viscosity.

1.2.1.3 Injection

The total capillary volume is small, in the order of microlitres, due to the narrow capillaries employed. Therefore, the sample volume (1-5 % of total capillary volume) is in the nanolitre range to avoid overloading and consequent band broadening.[173]

Sample injection is achieved by temporarily replacing the buffer reservoir with the sample reservoir. The most common injection modes involve the application of pressure, a vacuum (hydrodynamic) or an electric field (electrokinetic) to facilitate sample loading onto the capillary.[184] The buffer reservoir is then replaced and a potential is applied across the capillary to undergo separation.

The former pressure-driven technique is applied while the injection (inlet) end of the capillary is immersed in the sample. It is non-selective i.e. the injected sample plug has the same composition as the sample itself. The injection volume is directly proportional to the pressure difference between the sample vial and outlet buffer.

The amount injected also depends on the capillary dimensions, injection time and viscosity which can be described by Poiseuille law:

$$Amount = \frac{\Delta P r^4 \pi C t}{8 \eta L} \quad (1.4)$$

where ΔP is the pressure difference, r is the inner radius, C is the concentration of the sample, t is the injection time, η is the solution viscosity and L is the capillary length.[186]

Electrokinetic injection is induced by the application of a potential (1-10 kV) with the injection end dipped in the sample (inlet) and the other end in the buffer (outlet). This mode is selective as both ion mobility and the EOF mobility affect the amount of sample injected leading to preferential injection of components with the highest mobilites.[184] Electrokinetic injection is particularly useful for enhancing detectability when the analyte of interest represents a small percentage of the sample and has a higher electrophoretic mobility than the other components. For analytes such as DNA, which are dissolved in a gel matrix, electrokinetic injection is used to prevent damaging the gel with the application of pressure. The electrokinetic injection mode is summarised by Equation 1.5:

$$Amount = \frac{(\mu_e + \mu_{eof}) \pi r^2 V t}{L} C \quad (1.5)$$

where μ_e is the electrophoretic mobility of the analyte, μ_{eof} is the EOF mobility, r is the inner radius, V is the voltage, t is the injection time, C is the sample concentration and L is the capillary length.[186]

1.2.1.4 Separation modes

Capillary zone electrophoresis (CZE) is the simplest form of CE. To perform a separation, the capillary is filled with a suitable separation buffer adjusted to the desired pH and the sample is introduced at the inlet. When an electric field is applied, sample components migrate with the EOF and separate according to their electrophoretic mobilities. Under the right conditions, the sample components will

separate from each other and pass the detector as analyte zones. Neutral molecules migrate at the velocity of the EOF and cannot be separated using CZE.

Micellar electrokinetic chromatography (MEKC) was initially developed to facilitate the separation of neutral analytes. MEKC is a hybrid between electrophoresis and chromatography and involves the addition of micelles to the separation buffer that can interact with neutral molecules. This mode is also commonly used to separate charged compounds with similar electrophoretic mobilities.

Micelles are amphiphilic organic compounds that possess a non-polar chain and polar head. Depending on the charge present on the head they can be anionic, cationic, zwitteronic or non-ionic. In low concentrations, surfactant molecules exist as single entities called monomers. When added to the buffer in sufficient molarity, above a threshold termed critical micellar concentration (CMC), the surfactant monomers aggregate to form micelles. The hydrophobic tails of the surfactant monomers orient towards the centre of the micelle excluding water and exposing the hydrophilic heads toward the surface of the aggregate. The most widely used anionic surfactant is sodium dodecyl sulfate (SDS) which has a CMC of 8.1 mM in water. The monomer and micelle of SDS are presented in Figure 1.18.

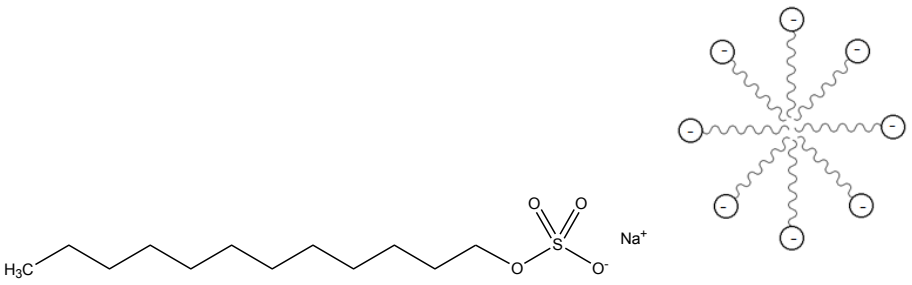


Figure 1.18 Sodium dodecyl sulphate; Left: monomer, Right: micelle.

The addition of micelles provides a pseudostationary phase with which sample components can partition based on such interactions as hydrophobicity, ionic attraction and hydrogen bonding. Thus the separation of sample components is dependent on their electrophoretic mobilities and their differential partitioning with the micelles.

Anionic SDS micelles, being negatively charged, are attracted to the positively charged anode and migrate in the opposite direction to the EOF. Generally their electrophoretic mobility is less than the EOF and the micelles move slowly towards the cathode retarding the migration of analytes that interact. Neutral analytes partition between the aqueous buffer phase and the pseudostationary micelle phase. They separate based on their affinity for the hydrophobic core of the micelle and electrostatic interactions with the hydrophilic surface (Figure 1.19).

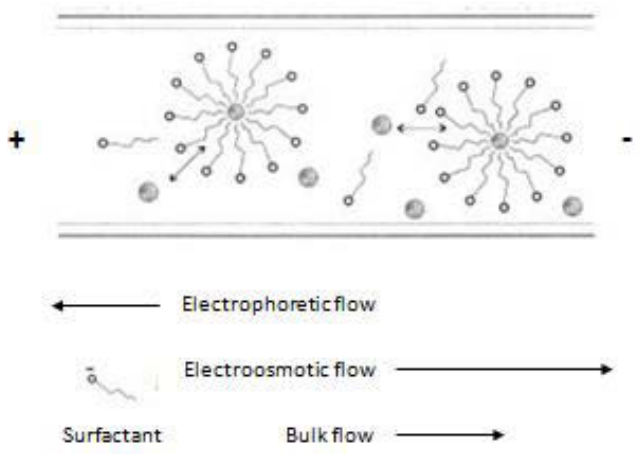


Figure 1.19 Micellar electrokinetic chromatography using an anionic surfactant beyond the CMC (normal polarity mode).

1.2.1.5 Detection

UV-visible absorbance is the most universally adopted detection mode in CE. The vast majority of compounds absorb within the UV region and thus chemical derivatisation is typically not required.[173] Detection can be observed down to a wavelength of 190 nM due to the high transparency of fused silica. Multi wavelength monitoring can be performed using diode array or fast scanning detection systems. Moderate sensitivities of the micromolar range are typically achieved using UV absorbance due to the small dimensions of the capillary and resulting short pathlength.[186] According to the Beer-Lambert law, the absorbance of UV-visible light depends on the optical pathlength (b), analyte concentration (C) and molar absorptivity (ε) of the analyte (Equation 1.6).

$$A = bC\varepsilon \quad (1.6)$$

Fluorescence is the most sensitive detection mode in CE. Unlike UV absorbance, the detection sensitivity does not depend solely on the pathlength and laser-induced fluorescence (LIF) can be miniaturised to the smallest diameter capillary.[173] Detection limits down to the nanomolar level can be achieved using fluorescence.[187] An inherent disadvantage of this technique is the need for derivatisation due to the lack of native fluorescence of most analytes (discussed in section 1.2.4.3). LIF has been introduced into many commercial CE systems. The most common laser sources are Ar-ion (488 nm), HeNe (543, 632 nm) and HeCd (325, 442 nm) mainly due to their wavelength compatibility with commonly available fluorescent reagents.[184]

Electrochemical detection (ED) is a popular alternative offering good sensitivity and versatility. Detection using this technique can be selective (amperometry) or universal (conductivity).[188] Amperometry detection is the most widely used and low limits of detection are typically achieved. Electroactive species can be detected as a result of the loss or gain of electrons on an electrode surface by measuring the electrical current output. The main challenge is the potential interference from the high voltage electric field. Conductivity is a universal mode of ED often employed for the detection of small, high mobility inorganic ions. It involves the measurement of conductivity/resistance of the solution between two electrodes when a small current is applied. Contactless conductivity is the most common form in which no direct contact is made between sensing electrodes and the electrolyte solution. This eliminates potential interference from the high voltage electric field.

For analytes that do not absorb UV, fluoresce or are not electrochemically active, an indirect detection scheme can be employed. A detectable reagent of the same charges as the analytes of interest is added to the separation buffer. The large background signal produced by the additive is displaced by the inactive analytes as they pass the detector. This decrease in signal is observed for each analyte as the concentration is lower in the analyte bands than the stable concentration of the

additive. Indirect modes offer universal detection for analytes exhibiting the same displacement mode.[189-191]

MS has also been coupled to CE. Electrospray ionisation (ESI) is the most common ionisation interface employed. Whilst CE-MS offers superior sensitivity to UV absorbance, lower sensitivity is achieved in comparison to LC-MS and GC-MS.[188] The coupling of CE to MS is more involved than for LC and GC. The poor compatibility of the flow rate and buffer species employed in CE is limiting.[192] However, the greatest challenge is maintaining stable electrical contact at the ESI-end of the capillary which is exposed to air. The most widely used configuration in commercial CE-ESI-MS instrumentation involves a co-axial sheath liquid.[192] Other configurations include liquid junction and sheathless/nanospray.[188]

1.2.1.6 Influencing factors

The main parameters influencing analytical performance in CE include the buffer composition, additives, temperature, applied voltage and capillary dimensions. These parameters should be optimised to maximise the selectivity and resolution of target analyte separation.

The composition of the buffer in CE separations is crucial. Buffers typically have an operating range of plus or minus one pH unit. The pH is important to monitor as it directly affects the EOF and hence has a large influence on the relative solute migration times. At a low pH (≈2) the EOF is almost eliminated and at a high pH (≈12) is very fast. The buffer concentration highly influences the zeta potential (section 1.2.1.2). An increase in ionic strength results in double layer compression and a consequent decrease in zeta potential and EOF. Often decreasing the ionic strength causes the separation to decelerate and peak resolutions and reproducibility improve.[186]

Shorter analysis times, but lower buffer concentrations, can also result in poor separations with broadened and asymmetric peaks. Concentrations of between 10 and 100 mM are typically employed.[193]

The EOF and electrophoretic mobility are proportional to the field strength therefore increasing the applied voltage will decrease the separation time. Theoretically, short separation times give higher efficiencies

Organic modifiers, such as methanol and acetonitrile, are often added to enhance the separation of analytes. The properties of the buffer are altered (e.g. conductivity or viscosity) which effects the separation. For MEKC analyses the EOF is often reduced which alters the selectivity.[194]

The standard flow of the EOF is from anode to cathode. The polarity can be reversed by using a coated capillary or a buffer with a cationic surfactant such as cetyltrimethylammonium bromide (CTAB). Cationic surfactants when of sufficient concentration reverse the net charge on the capillary wall and subsequently reverse the EOF. Reverse polarity is typically employed for the analysis negatively charged molecules such as DNA.

CE separations are typically employed at ambient temperature. Operating at elevated temperatures of up to 60 °C will not cause a significant increase in current. The EOF and electrophoretic mobility of analytes depend on viscosity which decreases with increasing temperature.

Capillary lengths of between 10 and 80 cm are typically employed. The shortest capillary should be used to achieve short analysis times. Longer capillaries can be used to improve resolution of closely migrating species, with a subsequent increase in applied voltage if the same field strength is desired. Internal diameters of between 50 and 75 µm are used – larger diameters can enhance detection sensitivity due to increased pathlength. However, surface-to-volume ratio is decreased and the dissipation of heat is less efficient resulting in a temperature gradient and band broadening.

Samples are dissolved in background electrolyte (BGE) which is made up predominantly of a buffering species. It provides a conductive liquid medium through which current can pass upon application of a high voltage. The BGE maintains a

constant pH and its composition significantly influences the migration and resolution of sample ions. Criteria for selection of buffers include buffering capacity in the range of choice, low absorbance at the detection wavelength and low mobility (minimises the generation of current). A wide range of buffers are available. Some popular choices for high pH separations are sodium tetraborate, phosphate and tris-phosphate. Other species are often added to the BGE to alter the separation selectivity.

1.2.2 Microchip design and function

1.2.2.1 Introduction

Miniaturisation has been a focal point of analytical science research since the early 1990's. The first micro fabricated device for chemical analysis was reported in 1979. Terry *et al.* constructed a GC column from silicon which was fitted with a thermal conductivity detection system.[195] The first miniaturised high performance liquid chromatography (HPLC) system was fabricated from silicon-Pyrex in 1990.[196] Initial adoption of these techniques, however, was minimal due to complexity and lack of application.[197]

The concept of μ-total analytical systems (μ-TAS), also known as lab-on-a-chip (LOC), was introduced by Manz *et al.* in 1992.[198] This research group constructed the first liquid-based miniaturised analytical system based on electrophoresis. The initial focus was to enhance analytical performance of electrophoresis rather than size reduction. The exponential growth of LOC is largely attributed to this work. Electrophoresis is well suited to LOC due to the ease of manipulating fluid at the nano and micro-litre scale, lack of moving parts and the resulting rapid, high-resolution separations.[197] More efficient electrophoretic separations can be obtained on chip format due to more effective heat dissipation, the capability of applying higher electric fields across a short separation channel and ability to inject a shorter plug size of sample.

Given this concept is multidisciplinary, numerous terms have been used to describe miniaturised analytical systems. The term LOC encompasses all devices that integrate

several laboratory functions on the platform of a small chip. Whilst MCE is a more specific term used to define microfluidic devices designed to perform electrophoresis.[197]

Modern microfluidic chips are typically constructed from glass or polymer substrates. Glass is often used due to its similar surface chemistry to the fused-silica capillaries used for CE.[199] Some ideal properties of glass include chemical resistance, optical transparence, dielectric, hardness and high thermal stability. Glass-based chips have been fabricated out of inexpensive soda lime glass through to high quality Quartz.[200] More recently, polymer microchips have become a popular cost-effective alternative to glass particularly for the manufacture of disposable microchips.[200] However, the surface chemistry of polymers is poorly understood.[197]

The design of microfluidic chips has evolved from a single cross channel design to a complex network of interconnected micro-channels that serve multiple sample wells. Figure 1.20 presents a 3-D representation of a planar microfluidic chip with a single cross channel design. The cross section of the microfluidic separation channel is illustrated in the top right (A-A). A series of voltages and currents are used to manipulate liquids in the micro-channels and drag the sample to the junction for injection. Following injection, the individual components in the sample are separated electrophoretically.

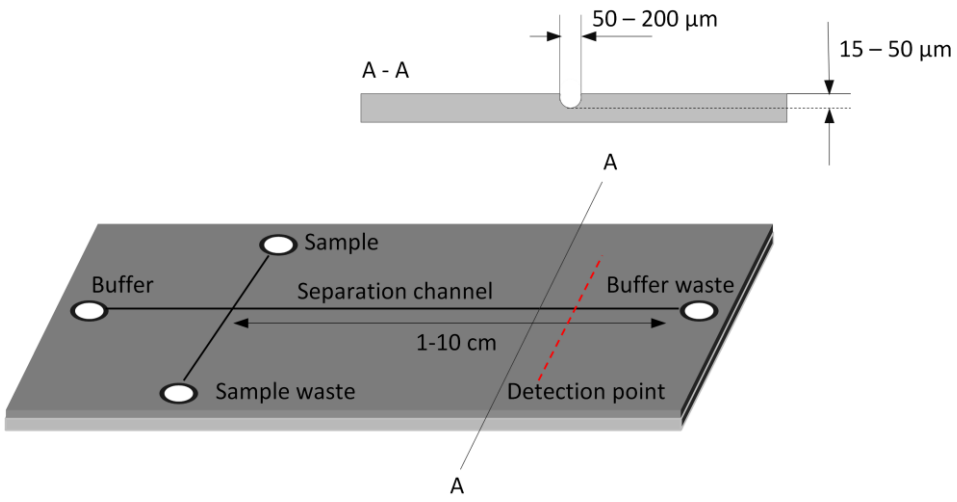


Figure 1.20 Three dimensional representation of a planar microfluidic chip. A-A = cross section of microchip. Adapted from [201]

Techniques used in the fabrication of microchips were adapted from the electrical engineering field.[197] Techniques typically used for glass substrates include classical photolithography and chemical wet etching procedures. Whilst popular methods for constructing polymer micro-channels include laser ablation, injection molding, silicon rubber casting and hot embossing.[200] Microchip fabrication from polymers is easier and faster which ultimately allows for high-throughput manufacture.[199]

In Forensic Science MCE has become an attractive platform for rapid separation and identification as outlined in 1.2.3. The analysis of illicit drugs, particularly ATS, will be discussed in detail to remain within the scope of this project.

1.2.2.2 Microchip design

Most microchips are relatively simple in their design, consisting of intersecting micro-channels that form an injection junction and four fluid reservoirs situated at each end. A separation channel, where the separation takes place, is elongated out from the injection junction and the detection system is positioned close to the end (Figure 1.21). Two typical layouts (geometries) of a simple planar microchip are illustrated: orthogonal and offset. The former cross-channel design is the most common, where the horizontal separation channel orthogonally intersects the vertical channel,

connecting the sample to waste. In the offset channel design, also known as double-t, the vertical sample channel is offset to form a larger injection region.[197,200,201]

The dimensions of micro-channels are in the order of microns, with typical depths and widths of tens of microns. In comparison to CE capillaries, however, the separation channels are significantly shorter of between 1 and 10 cm.[202,203]

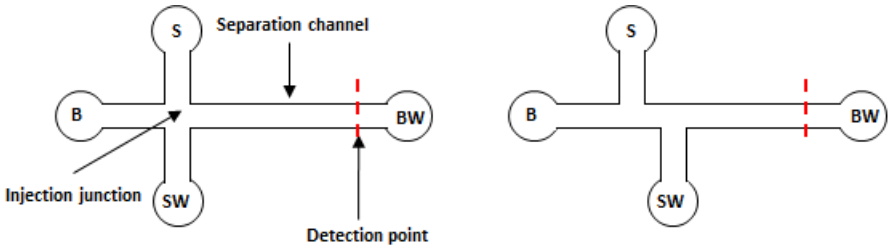


Figure 1.21 Cross-channel microchip designs. Left: orthogonal, Right: t-cross channel. Reservoirs refer to: S = sample, B = buffer, SW = sample waste and BW = buffer waste. An electrode is situated at each reservoir and connected to a high voltage power supply.

More sophisticated microchip designs have been created as a result of advances in fabrication technologies. These microchips incorporate on-chip processes and multiple sample reservoirs to enable high throughput and efficiency. The channel length can be increased without compromising on size by using a serpentine geometry. The turns on the microchip must be carefully designed to minimise dispersion.[202]

1.2.2.3 Injection

Unlike capillaries, microfluidic channels cannot be physically manipulated hence the development of new injection schemes.[197] Sample injection on a microchip is typically achieved using electrokinetic modes. They are simple to implement and do not require the integration of pumps or actuators (valves).

Several electrokinetic injection schemes exist of which the most common are pinched and gated (Figure 1.22).

The former involves two steps involving the precise control of potentials applied at the fluid reservoirs. A voltage gradient is applied across the sample channel (S to SW) allowing the flow of a sample solution to load the channel. After a specified time, the voltage gradient is switched to direct the flow down the separation channel. The sample contained in the injection junction is introduced into the separation channel. To prevent leakage into the separation channel small *pinching* voltages are applied in the sample channel towards the S and SW reservoirs during separation. This method of injection in the absence of *pinching* is referred to as floating injection.[197,202]

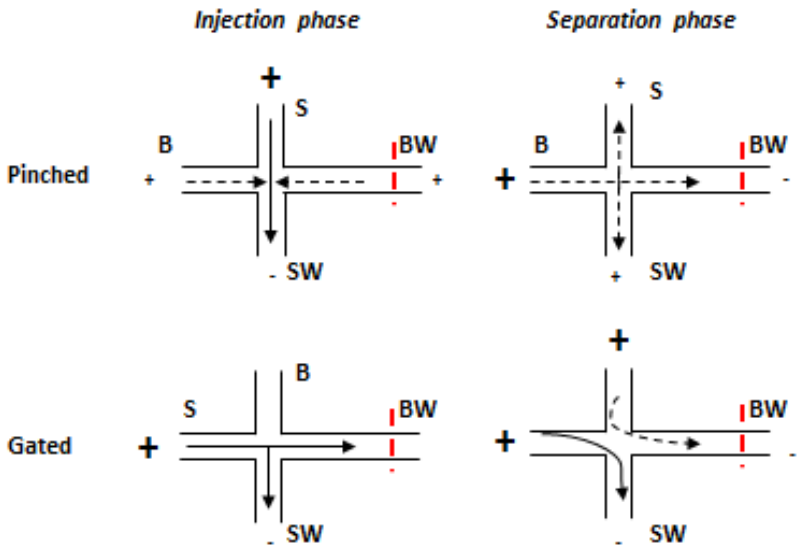


Figure 1.22 Gated and pinched injection modes. S = sample, B = buffer, SW = sample waste and BW = buffer waste. The dotted line represents BGE flow and the solid line represents sample flow. Adapted from [201]

In contrast, the gated injection method involves continuous sample flow. The buffer solution and sample solution flow continuously in parallel. The buffer flow is interrupted for a specified time by switching off the voltage gradient from B to BW, during which the sample is introduced into the separation channel. The buffer flow is then re-established to define the sample plug. This method is simple and popular when continuous injection is desired, however, it is subject to inherent electrokinetic bias (described in section 1.2.1.3). The plug length increases with injection time and for faster migrating species.[202] In the pinched injection scheme bias is avoided by defining the injection plug length and applying the voltage for a sufficient length of

time to ensure the injection volumes are representative of actual sample concentrations.[204]

Hydrodynamic injection (section 1.2.1.3) has also been integrated to avoid sample bias. This mode is particularly advantageous for the separation of analytes of a wide mobility range.[204] Hydrodynamic pressure can be implemented after electrokinetic loading. However, the use of hydrodynamic injection schemes often increases the complexity of the microfluidic device.[202] Pressure-facilitated injections require a transport or pumping mechanism for dissolved analytes. Flow control can be achieved using adjustable valves which direct the flow of solution.[200] The development of microvacuum pumps, syringe pumps and integrated peristaltic pumps for microchip devices has received much attention.[205]

1.2.2.4 Separation

The same separation modes employed in CE (section 1.2.1.4) are applicable to MCE.

1.2.2.5 Detection modes

The small size of the detection cell on microchips due to reduced micro-channel depths precludes the use of UV-visible absorbance.[206] The optical pathlength is shortened, resulting in low detection sensitivity; therefore it is not widely employed. A further limitation of UV absorbance is the required optical transparency of the detection cell which limits the material for microchip fabrication.

LIF is frequently interfaced to microchips due to its high sensitivity and compatibility with the flat chip surfaces. The laser beam is easily focused onto the micro-channel.[197] LIF was the first detection method to be coupled to MCE by Manz *et al.* in 1992.[207] However, the complex optical arrangement required for fluorescence detection limits the miniaturisation capabilities of the microchip device.

Electrochemical detection is principally compatible with miniaturisation and has been explored as an alternative to LIF. High sensitivities are attributed as the technique is easily miniaturised without loss of performance. Excellent compatibility with

microfabrication technologies allows electrodes and channels to be incorporated on the same substrate material and the fabrication of more compact microchip devices.[202] Amperometry is a proven highly sensitive detection system for MCE. Careful positioning of electrodes is required to reduce or adjust for interferences from the separation voltage. Figure 1.23 illustrates the typical set-up of this detection system for LOC devices. Isolation from the separation voltage is typically achieved by positioning the sensing electrode close to the end of the capillary or employing an electric field decoupler for off-column detection.[208] Contactless conductivity (C4D) has also been employed as a versatile detection method. The indirect electrode contact allows the use of inexpensive materials (aluminium, carbon and copper) for manufacturing electrodes. Therefore, the fabrication of low-cost, disposable microchips fitted with C4D detectors is possible.

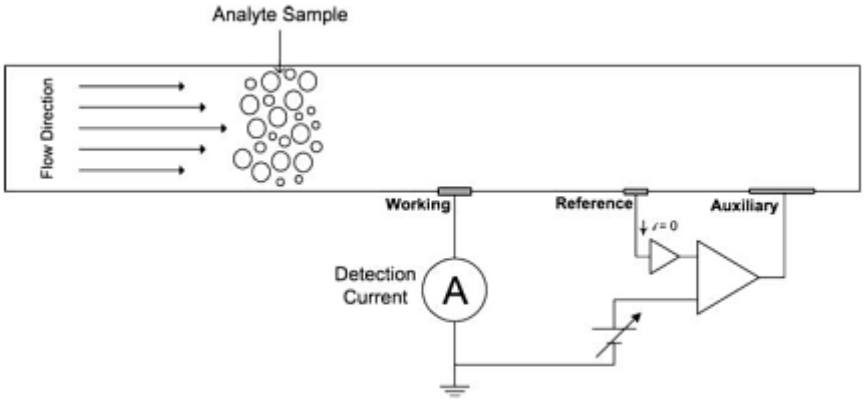


Figure 1.23 Schematic of the general set-up of an amperometric detection cell for LOC applications.[209]

MS has also been coupled to MCE predominantly using an electrospray ionisation interface and a range of different mass analysers.[210] Although MS is a powerful analytical technique, providing molecular weight information and fragmentation patterns, the interface between MCE and MS is complex and still in its early stages. The commercially available systems are large, expensive and have inferior sensitivity in comparison to LIF. The majority of MCE-MS applications to date have focused on protein sequencing (proteomics).[197]

Fluorescence achieves high detection sensitivities, typically down to the nanomolar level.[187] The sensitivity depends on the intensity of the laser or light-emitting diode (LED) source and can be enhanced by increasing the power. The most common optics configuration involves a confocal detection system.[200] An inherent disadvantage of LIF is the need for native fluorescence or the attachment of a fluorophore for detection.[197] However, sensitive and selective derivatisation schemes have been developed for many analytes to overcome this problem.[211]

The emission spectra of the fluorophore must overlap with the excitation spectra of the laser or LED source. In addition, the fluorophore must have a sufficiently high quantum yield and long lifetime. Other factors, such as the required reaction conditions and stability of the fluorescent derivatives, should be considered when choosing an appropriate fluorophore.

The high cost and bulky nature of laser sources and optical arrangements reduces the benefits of miniaturisation. The use of LEDs as alternate excitation sources has become popular in fluorescent detection schemes. LEDs are small, inexpensive and available in a wide range of wavelengths. They have been used in many applications due to their excellent stable output, long lifetimes and high emission intensities.[212,213]

The fluorescence signal results from the absorbance of a photon and subsequent emission. A laser or LED beam impinges upon the detection zone of the microchip separation channel and the resulting fluorescence is collected. The basic elements of a fluorescence detection system, illustrated in Figure 1.24, are the excitation source, optics to focus the excitation source onto the detection cell (capillary or micro-channel), collection optics and the detection system (photomultiplier tube).[184]

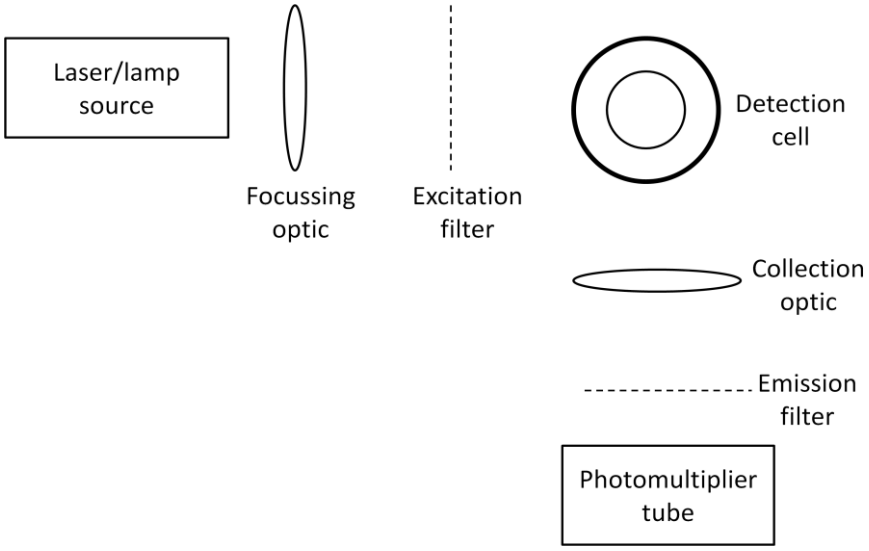


Figure 1.24 Basic elements of a fluorescence detection system. Adapted from [184]

The fundamental equation governing fluorescence is:

$$I_f = \emptyset_f \, I_o abc E_x E_c E_m E_{pmt} \tag{1.7}$$

where I_f is the measured fluorescence intensity, $\emptyset_f$is the quantum yield (photons emitted/photons absorbed), I_o is the excitation power of the light source, abc are Beer's law terms, $E_x E_c E_m E_{pmt}$ are the efficiencies of the excitation monochromator or filter, the optical portion of the micro-channel, the emission monochromator and detector (photomultiplier or charge-coupled device), respectively.[189]

The use of indirect fluorescence is also a popular means for avoiding analyte derivatisation when high sensitivity is not essential. A fluorescent species is added to the BGE to produce background fluorescence and non-fluorescent analytes quench the fluorescence and are detected due to the observed decrease in signal intensity. The modes LIF[214,215], LED-IF[212] and Indirect LIF[216,217] have all been applied for the detection of amphetamines or amine-related compounds on a chip format.

1.2.2.6 Influencing factors

As in CE, analytes migrate under the influence of an electric field following electrophoretic mobilities and the EOF. The buffer composition affects the mobility of the analyte of which molarity and pH are dominant factors. The length of the micro-channel also influences the separation of analytes (section 1.2.1.6). Operational parameters such as separation voltage, injection time and injection voltage/pinching current should be optimised to enhance the analytical performance of the method.

High voltages and longer micro-channels can enhance the separation, sometimes at the expense of time. The applied pinching current controls the amount of sample being injected and can improve detection limits.

1.2.3 Forensic applications

One of the leading applications of MCE technologies in forensic science is DNA analysis.[218] Chip-based techniques are efficient, reduce sample requirements, integrate on-chip sample preparation steps and typically offer higher resolution.[197] DNA profiling is used extensively for the forensic identification of humans. More recently the development of field portable devices for crime scene DNA analysis has been a research focus, reviewed by Horsman *et al*. in 2007.[219] Other areas of forensic DNA testing include paternity testing of children and establishing the geographical origin of unknown human DNA samples.[220-222]

MCE devices have been employed for homeland defense largely due to their small footprint, rapid response time and versatility.[223] Field deployable platforms for the rapid separation of pre- and post-blast explosives have been reviewed by a number of authors.[171,172,224] The detection of pre-blast explosives is frequently achieved using amperometry with either a carbon or gold electrode.[225-227] Nitroaromatic explosives have good redox properties therefore electrochemical detection methods are commonly coupled to MCE.[228] The inorganic ions present in post-blast residues are commonly detected via C4D.[229,230]

The analysis of illicit drugs on-chip has not been a major focus given the small number of suitable detection systems. The analysis of ATS using CE is commonly via a UV-detection mode.[231] In contrast, fluorescence is popular in MCE due to the ease of transferring this mode to a chip format and availability of fluorophores for labelling primary and secondary amines.[215] Basic amines, such as amphetamine, have high pKa values of around 10. They can be easily labeled with FITC to form highly fluorescent derivatives. The derivatives fluoresce at 488 nm and can be separated using CZE or MEKC. A high pH is often employed due to the negative charge on FITC-derivatives at this pH.

The first on-chip separation of FITC derivatives of ATS was reported by Ramseier *et al.* in 1998. Although the baseline separation of amphetamine and methamphetamine was achieved in less than 25 seconds, the derivatisation procedure took 24 hours at room temperature.[215] Using similar derivatisation conditions and detection by LIF, Belder *et al.* separated ephedrine and pseudoephedrine in less than 1 minute on a glass chip with MEKC.[232]

Using an alternative derivatisation agent, 4-fluoro-7-nitrobenzofurazan (NBD-F), Wallenborg *et al.* separated 7 ATS in 7 minutes using a longer S-folded separation channel of 160 mm. The NBD-F fluorescent products are weakly ionised or neutral at a high pH therefore MEKC was employed.[214]

More recently, electrochemical detectors have been applied to the analysis of small molecules like ATS. Schwarz and Hausier separated pseudoephedrine and ephedrine using MEKC with a 85 mm separation channel. Detection was by amperometry.[233] The use of acidic buffers for detection by amperometric modes is particularly favourable for resolution of basic compounds. In their unmodified form they are present as cations and at a low pH the EOF is minimised.[234] Tanyanyiwa and Hauser report the separation of some basic drugs using CZE with a 10 mM citric acid buffer.[235]

A literature summary of the analysis of ATS using MCE devices is presented in Table 1.11.

Analytes	Separation	Detection	Chip/setup	Ref
AMP, METH	CZE – 50 mM $Na_2B_4O_7$, pH 9.2	LIF – Argon ion laser 488 nm *Derivatisation:* FITC , 24 hours, 25 °C	Cyclic planar glass chip Laboratory made - confocal microscopy	215
EPH, PSE	MEKC – 10 mM Borate, pH 9.5, 35 mM SDS 40 seconds	LIF – Argon ion laser 488 nm *Derivatisation:* FITC, 2 hours, stirred, dark	Cross-channel glass planar chip, separation channel 85 mm Laboratory made -epifluorescence microscopy	232
Nor-EPH, EPH, CAT, AMP, PSE, MCAT, METH	MEKC – 50 mM phosphate, pH 7.35, 10 mM HS-γ-CD, 1.5 mM SDS 7 minutes	LIF – Argon ion laser 488 nm *Derivatisation:* NBD-F, 10 minutes, 60-80 °C	Schott glass wafers with S-folded separation channel 160 mm Laboratory made epifluorescence detection	214
DA, OA, NA, A, IP	CZE – 10 mM citric acid	C^4D – pair of identical tubular electrodes *Derivatisation:* none	PMMA microchips, separation channel 80 mm	235
PSE, EPH	100 mM NaOH, pH 12.9, 16 mg/mL CM-β-CD Under 160 seconds	Amperometry – detection potential 1300 mV	Glass microchips, separation channel 85 mm Laboratory built detector	233

Table 1.11 Summary of MCE analysis of ATS. AMP = amphetamine, METH = methamphetamine, EPH = ephedrine, PSE = pseudoephedrine, nor-EPH = nor-ephedrine, nor-PSE = nor-pseudoephedrine, CAT = cathinone, MCAT = methcathinone, DA = doxylamine, OA = octopamine, NA = noradrenaline, A = adrenaline, IP = isoprotenol.

1.2.4 Agilent Bioanalyzer

The Bioanalyzer 2100 was launched by Agilent Technologies in 1999 as the first commercial lab-on-a-chip device for biochemical assays. It was marketed as an automated platform capable of integrating multiple functions: sample handling, separation, detection and data analysis. The Bioanalyzer operates in two modes: electrophoresis and pressure-driven flow. Exchangeable cartridges facilitate switching between electrophoresis for the analysis of DNA, RNA and proteins and pressure-driven flow for fluorescent cell cytometric analyses. A range of pre-packaged reagent kits are available providing a streamlined approach for selected analytical methods. An application compendium published by Agilent Technologies lists approximately 100 life-science applications of the Bioanalyzer using these modes.[236]

The Bioanalyzer is compact, lightweight (10 kg) and can be laptop operated, thus is inherently portable (Figure 1.25).

Figure 1.25 Agilent Bioanalyzer 2100.[237]

The electrode cartridge employed for electrophoretic assays contains 16 electrodes which are connected to individual high-voltage sources (Figure 1.26). When the microchip is positioned on the platform and the lid is closed the fluid in each well

comes into contact with an electrode. Independent voltage control at each electrode allows sample transport within the micro-channels.

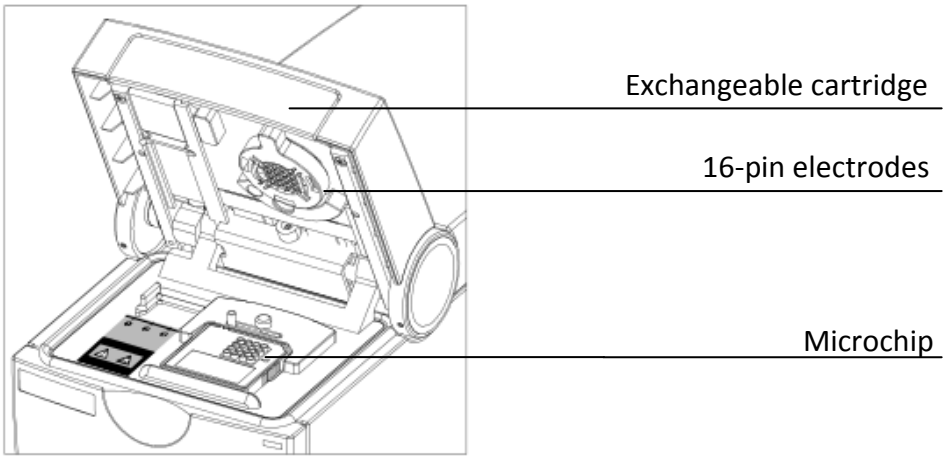


Figure 1.26 Bioanalyzer platform illustrating the point of contact between the microchip and the electrode cartridge.[238]

1.2.4.1 Chip design

The microchips consist of a soda lime glass sandwich structure with a network of channels micro machined onto a glass substrate. The micro-channels were created using photolithography and chemical wet etching techniques (Figure 1.27). They are sealed by thermal bonding with a top glass plate containing access holes exposing the ends of the micro-channels for reagent and sample introduction into the chip.[239]

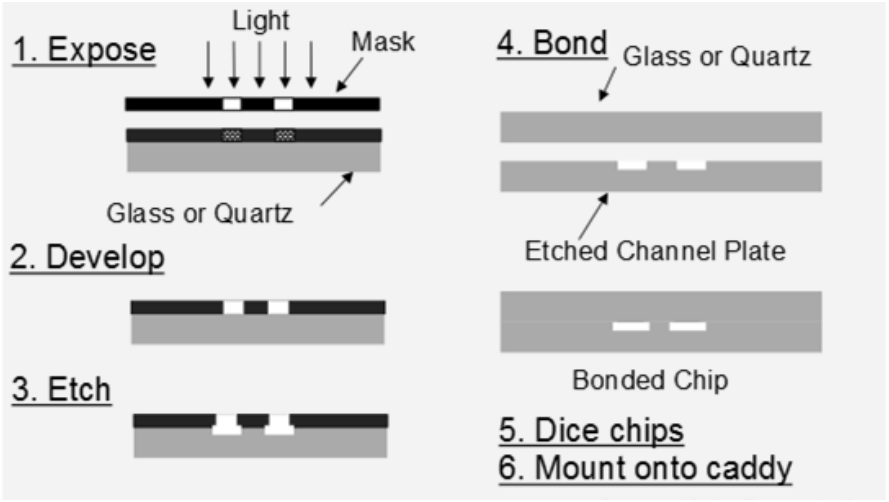


Figure 1.27 Microchip fabrication procedure.[240]

The extensive network of micro-channels inter-connect the multiple sample and waste wells to the separation channel. A simple schematic of the channel design is shown in Figure 1.28. Altogether, there are 16 wells labeled by row (A-D) and column (1-4). The wells located in column 4 (A4-D4) are reserved as buffer or waste reservoirs and the remaining 12 hold samples. The micro-channels which interconnect 12 sample wells have a depth of 10 µm and width of 50 µm. The separation channel length is 15 mm with an effective length of 12 mm.

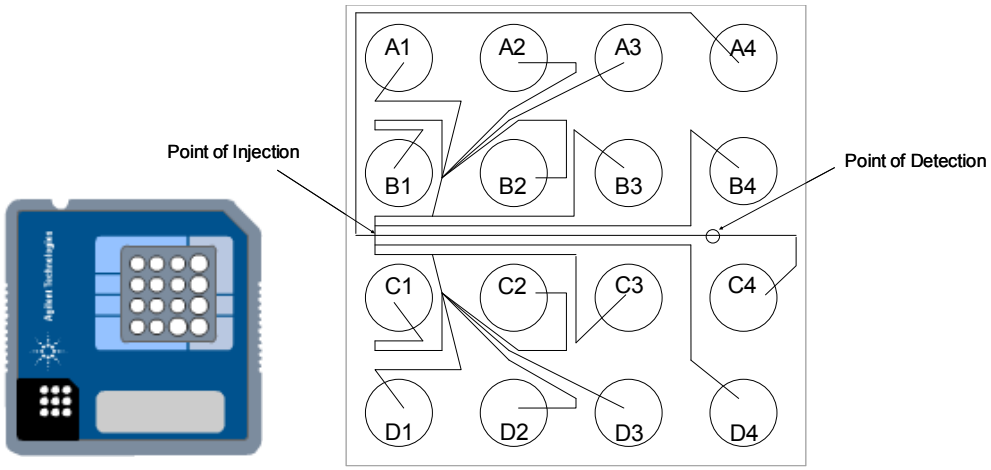


Figure 1.28 Chip design. Left: Microchip (Caliper). Right: schematic of the microchip design (actual size 17 mm square).[237]

1.2.4.2 Sample movement

Sample movement is achieved by the application of voltage gradients across the micro-channels. The basic principle is the movement of fluid from an area of high voltage to low voltage. The injection field strength must be sufficient to facilitate sample movement, typically between 500 and 2000 V.[211]

Figure 1.29 outlines the sequence involved with sample injection and separation. In step 1, a voltage gradient is applied across the sample channel which initiates sample movement towards the injection junction. In step 2, a pinching current of 0.5 – 1 µA is applied across the separation channel. This prevents the sample from entering the separation channel at this point. The sample plug volume can be reduced by applying a *sample pinch* low voltage across the separation channel simultaneously. Step 3 involves the application of a voltage gradient across the separation channel to accomplish injection. Following injection, a voltage gradient is applied across the

separation channel to initiate sample movement towards the detector. Individual sample components undergo separation via electrophoresis upon entering the separation channel. Simultaneously, a low voltage is applied across the sample channel to prevent sample diffusion during separation.

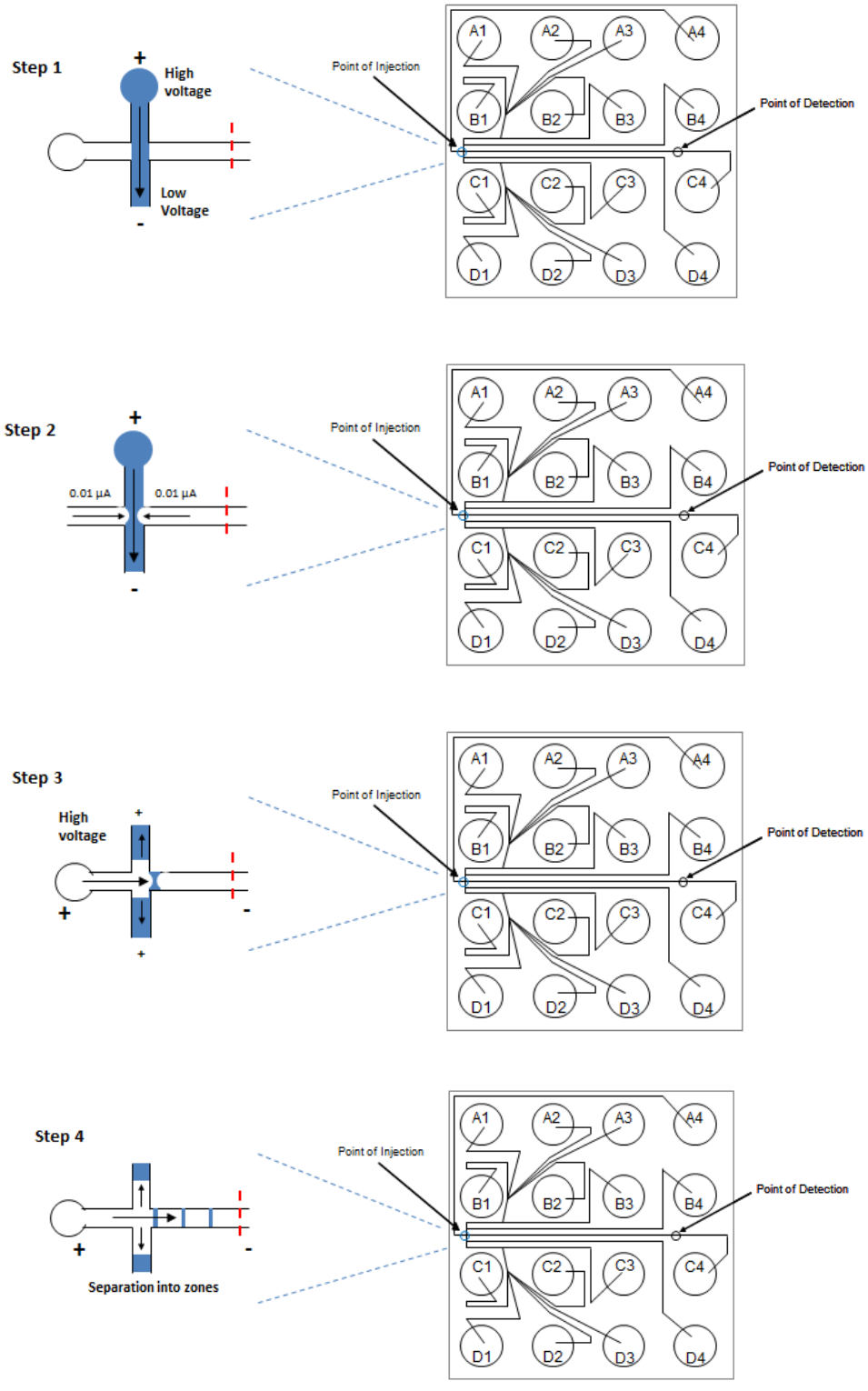


Figure 1.29 The steps involved in sample injection, separation and detection (not to scale). Adapted from [241]

1.2.4.3 Detection

The Bioanalyzer is fitted with two fluorescence detection systems: LED-IF (λ_{ex}= 510-540 nm, λ_{em} = 470 nm) and LIF (λ_{ex}= 674-696 nm, λ_{em} = 635 nm).[242] The LED-IF optics configuration incorporates a 475 nm blue LED source of 2 mW optical power (Figure 1.30). Using this mode, emission can be collected between 510 and 540 nm. The 635 nm red laser diode has 10 mW optical power and emission is collected above 685 nm.[243]

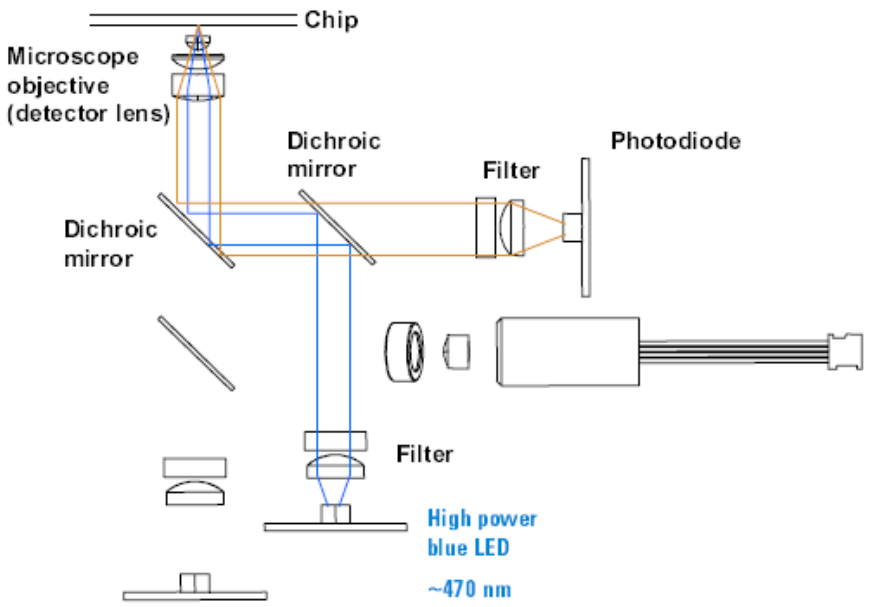


Figure 1.30 The optics configuration of the LED-IF detector in the Agilent Bioanalyzer 2100. The LED alignment and collection of the fluorescent signal is illustrated.[244]

As mentioned in section 1.2.2.5, derivatisation is required for the detection of non-fluorescent analytes. This is often seen as a downfall due to the resulting increase in sample preparation time. However, labelling reactions of between 1 and 10 minutes have been reported.[214,245,246] Furthermore, given the labelling procedure specifically targets the presence of certain functional groups, very high-fluorescing derivatives can be produced.

The primary factor involved in the selection of an appropriate reagent for fluorescent derivatisation is the selectivity for the analyte(s) of interest. Other important factors

include: the analyte stability under the reaction conditions, sample solubility in the required solvent, any occurrence of side reactions and the fluorescence sensitivity of the derivatisation. The purity, stability, commercial availability and cost of the label are also important to consider.[207]

Fluorescein-based dyes, such as fluorescein isothiocyanate isomer I (FITC), are compatible with the detector excitation and emission wavelengths of the LED source. The excitation and emission spectra of FITC (Figure 1.31) overlap with the excitation and emission wavelengths of the LED source in the Bioanalyzer.

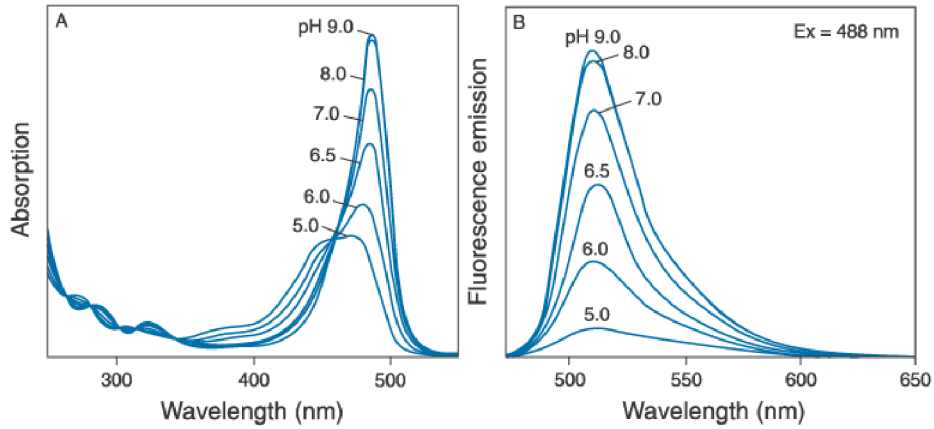


Figure 1.31 Excitation and emission spectra of FITC.[247]

The reaction of FITC with primary or secondary amines generates stable fluorescent products. This reagent also reacts with sulfhydryls and the phenolate ion, however unstable products are formed. Thus excellent selectivity is achieved for amines. The nucleophilic amine moiety attacks the central electrophilic carbon of FITC. The resulting electron shift and thiourea bond formed between FITC and the analyte forms a fluorescent derivative that is stable in aqueous solutions.[248] This scheme is illustrated in Figure 1.32.

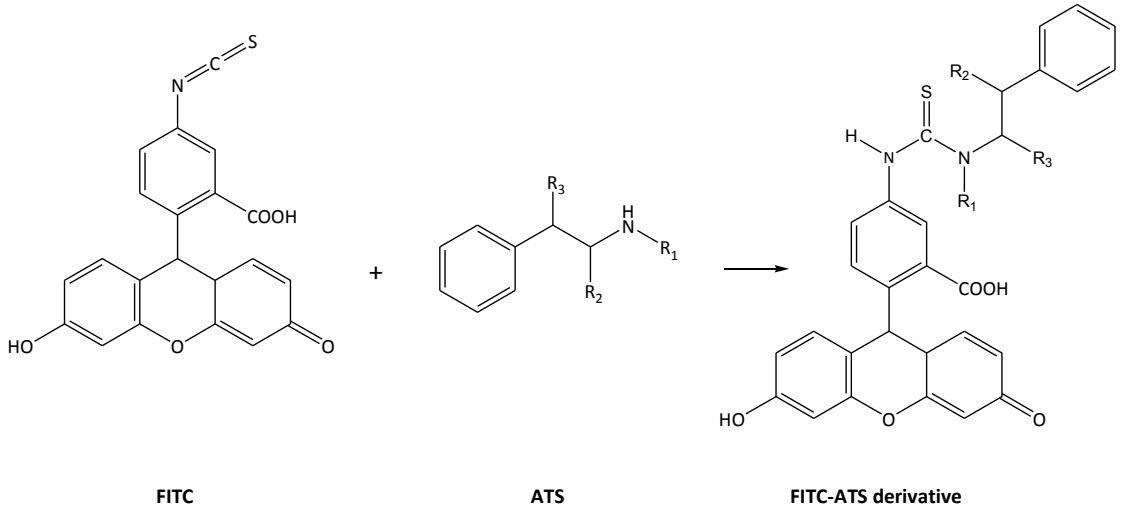


Figure 1.32 Thiocarbamylation reaction of FITC with amphetamine-type stimulants.

The pH of the background electrolyte is important as FITC and its conjugates only fluoresce at a basic pH. The sensitivity is typically enhanced with increasing pH.[207]

1.3 Aims of research

The primary aim of this project was to investigate the potential use of the Agilent Bioanalyzer platform for the analysis of amphetamine-type stimulants.

The growing demand for rapid, portable tools capable of screening samples from clandestine laboratory sites has been highlighted in section 1.1.7. Current methods employed in Australia and New Zealand lack the right combination of speed and selectivity to enable the collection of immediate data from trafficking. Further, these techniques have limited applications for handling the complex sample matrices encountered at typical clandestine laboratory sites. It is recognised that the ultimate integration of rapid in-field drug analysis into a broader crime analysis framework has the potential to deliver outstanding outcomes in the fight against illicit drugs and organised crime. Therefore, it is important to address these issues.

Efficient and effective sample processing is essential. In order to address the present technology gap, this research focused on developing a rapid analytical method for

substances found at clandestine laboratories. Of particular interest within this setting are ATS and their pre-cursors.

MCE devices have shown great promise for the rapid analysis of biological molecules. In the field of forensic illicit drug analysis MCE is an emerging technique. Although this technique has been employed for the analysis of some ATS in tablet or powder form, liquids originating from illicit drug laboratories remain unexplored.

In order to investigate the use of MCE for this application this research focused on:

- developing and optimising a separation method applicable for in-field applications i.e. rapid, simple and cost-effective
- field testing using casework samples to evaluate the use of the developed method in real world scenarios

Chapter 2 Outlines the optimisation of the separation of amphetamine analogues. Initial method development focused on the rate-limiting fluorescent derivatisation procedure. Following this, the sequential optimisation of various factors affecting the separation of ATS was performed to obtain the best separation of the target analytes.

Chapter 3 Explores the development of a sample preparation procedure for clandestine laboratory samples. Preliminary research involved optimisation of the liquid-liquid extraction procedure. Following this optimisation process the method was applied to a range of previous casework samples.

Chapter 4 Investigates the derivatisation and separation of popular synthetic cathinones. The potential use of separation profiles for the comparative analysis of tablets is explored. The rapid use of these separation profiles to indicate the inter-variability of seized tablets/powders is also illustrated.

Chapter 5 Draws concluding remarks and indicates possible areas of future work.

Chapter 2

Optimisation of the analysis of ATS using the Bioanalyzer

Chapter 2: Optimisation of the analysis of ATS using the Bioanalyzer

This chapter summarises the method development carried out to optimise the analysis of ATS using the Bioanalyzer, beyond its design specifications. The ATS were rapidly derivatised with fluorescein isothiocyanate isomer I (FITC) by a temperature-accelerated procedure using a dry heating block. The sequential optimisation of the derivatisation procedure and parameters influencing the separation of selected analogues using MCE are discussed. The optimised separation and detection of amphetamine (AMP), methamphetamine (MA), pseudoephedrine (PSE), and selected analogues along with the performance characteristics of the developed method, are presented.

2.1 Introduction

The analysis of ATS using CE and MCE, as discussed in section 1.2.4.3, has been previously reported in the literature. Fluorescence is the most common method of choice for analyte detection in LOC applications. FITC is a common fluorescent labelling reagent for amines with excellent quantum efficiency and high stability of its derivatives.[249] The reaction of FITC with proteins and amino acids was first reported in 1969 by Maeda *et al*.[249] and has been used to label amphetamines and other amine-containing compounds.[215,250] Typical derivatisation times with FITC have been up to 12 hours, with recent attempts to shorten the reaction time. Dominguez-Vega *et al*.[251] described an accelerated FITC derivatisation of the amino acid ornithine. The reaction time was reduced from 16 hours to 10 minutes by the use of an ultrasonic probe. Zhou *et al*.[252] reported a microwave-assisted derivatisation procedure for amino acids with FITC, which took approximately 3 minutes. The signal intensity associated with the microwave procedure was improved in comparison to the procedures at room temperature and in a water-bath set at 100 °C.[252]

Ramseier *et al*.[215] reported the separation and detection of amphetamine analogues in human urine. The separation run time was 10 minutes, involving a 24 hour labelling process with FITC. This study utilised a laboratory constructed device and an argon ion laser with excitation at 488 nm. Alternative amino-reactive fluorophores such as o-phthalaldehyde (OPA), 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF) and nitro benzofurazan derivatives have also been used to label amphetamines.[214,253-255]

To facilitate analyses beyond the Bioanalyzer's design specifications, the software was set to *assay developer* mode. In this mode, operating parameters such as the voltage and current can be altered in order to optimise the separation of target analytes. Initial method development was performed using selected analogues of amphetamine due to their unrestricted nature (Table 2.1). As discussed in section 1.2.1.6, various parameters influence the degree of separation and efficiency. The separation channel length of the microchip is fixed, however, instrumental parameters and the background electrolyte composition can be optimised.

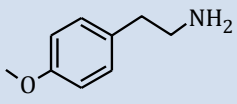
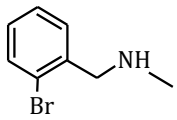
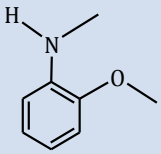
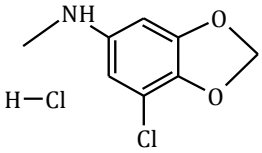
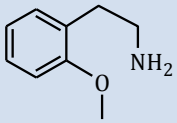
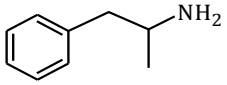
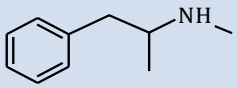
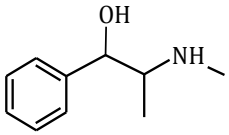
Name	Abbreviation	Structure
2-(4-Methoxyphenyl)ethylamine	*2-4-MPEA*	
2-Bromo-*N*-methylbenzenemethanamine	*BMBA*	
2-Methoxy-N-methylaniline	*2-MMA*	
5-Aminomethyl-7-chloro-1,3-benzodioxol hydrochloride	*AM-C-BD*	
2-Methoxyphenethylamine	*MPEA*	
Amphetamine	*AMP*	
Methamphetamine	*METH*	
Pseudoephedrine	*PSE*	

Table 2.1 Structures of the target amphetamines and amphetamine analogues.

2.2 Materials and methods

2.2.1 Apparatus

All experiments were performed on an Agilent 2100 Bioanalyzer using the Agilent 2100 Expert software (Agilent technologies, Waldbronn, Germany). Detection was by LED-IF (λ_{ex} 525 nm, λ_{em} 470 nm). All separations were performed using standard DNA 1000 microchips obtained from Agilent Technologies (Forest Hill, Australia). The chips were fabricated from soda lime glass and have a separation channel length of 15 mm, micro-channel depth of 10 µm and width 50 µm. Injection was performed at 1.5 kV for 2 seconds with a separation voltage of 1.5 kV.

2.2.2 Chemicals

Sodium hydroxide, sodium dodecyl sulfate, nile blue chloride, sodium tetraborate and FITC (>90%) were purchased from Sigma Aldrich (Sydney, Australia). Individual standards of 2-(4-methoxyphenyl)ethylamine, 2-bromo-N-methylbenzene-methanamine, 2-methoxy-N-methylaniline, 5-aminomethyl-7-chloro-1,3-benzodioxol-hydrochloride and 2-methoxyphenethylamine were also purchased from Sigma Aldrich (Sydney, Australia) as concentrated solids or solutions (in methanol). Pseudoephedrine hydrochloride, dexamphetamine sulfate and methamphetamine hydrochloride were obtained from the National Measurement Institute (Sydney, Australia). Table 2.1 lists the chemical structures of the target compounds.

2.2.3 Electrolyte preparation

Electrolytes and sample stock solutions were prepared in ultra-pure water (Arium 611, Sartorius AG, Goettingen, Germany). The separation electrolyte consisted of 50 mM sodium tetraborate buffer at a pH of 9.66 with the addition of 50 mM SDS. The electrolyte was mixed, sonicated for 5 minutes and filtered through a 0.20 mm syringe filter prior to injection (Millipore, Billerica, MA, USA). All electrolyte solutions and stock solutions of amphetamine analogues were kept in the refrigerator at 4 °C

and fresh solutions were prepared daily. A concentration of 1 mM nile blue dye was diluted in the running buffer and primed through the micro-channels prior to analysis for laser focusing.

2.2.4 Analyte preparation

2.2.4.1 FITC stock solution

A 10 mM stock solution of FITC was prepared in analytical reagent grade acetone and stored in a plastic bottle wrapped in aluminium foil at -18 °C.

2.2.4.2 Amphetamine analogues and ATS standards

Individual stock solutions of all analogues and ATS standards were prepared via dilution in ultra-pure water to a final concentration of 1000 mg/mL. Derivatisation of stock solutions was performed with FITC; 100 µL of analogue was diluted in 100 µL of 10 mM sodium tetraborate buffer (pH 9.66) and labelled for 3 minutes at 90 °C after the addition of 100 µL of 10 mM FITC. A dry heater block (Ratek-DBH30D)[256] was employed. Labelling was carried out in 1.7mL graduated microtubes wrapped in aluminium foil with the lid closed. Following derivatisation, the labelled analogues were diluted to 10 mg/mL in the running buffer for analysis. The set-up used for sample preparation and analysis is presented in Figure 2.1.

Figure 2.1 Sample preparation and analysis workflow. Left to right: exhibit or standard, sample vial containing FITC, dry heating block, Agilent DNA chip, Agilent 2100 Bioanalyzer platform.

2.3 Results and discussion

2.3.1 Labelling with FITC

FITC has been widely used as a derivatisation reagent due to its excellent quantum efficiency, high molar absorptivity and stability of products.[215,242] Experiments were performed to simplify the FITC derivatisation procedure using a dry heating block. Initially, experiments were conducted using a water-bath as performed by Zhou *et al*.,[252] however a dry heating block was chosen due to its reduced complexity. Whilst the heating block used is portable (*W* 283 x *D* 265 x *H* 100 mm, 4.5 kg and 60 samples capacity), a wide range of more compact heating blocks are also available which would further enhance the portability.

Figure 2.2 summarises the influence of temperature on the peak height for derivatised PSE with a 5 minute reaction time. Error bars were estimated from the standard deviation for the PSE fluorescence intensity from triplicate derivatisation of PSE at each temperature and triplicate injections on a single chip; i.e. a total of 3 chips were used for each temperature experiment. The response increased linearly with increasing temperature. As expected there was also a concomitant decrease in the signal of FITC. Temperatures beyond 100 °C were not investigated as the reaction mixture boiled. The optimal temperature was chosen as 90 °C; a compromise between sensitivity, high PSE and low FITC fluorescence intensities and reproducibility.

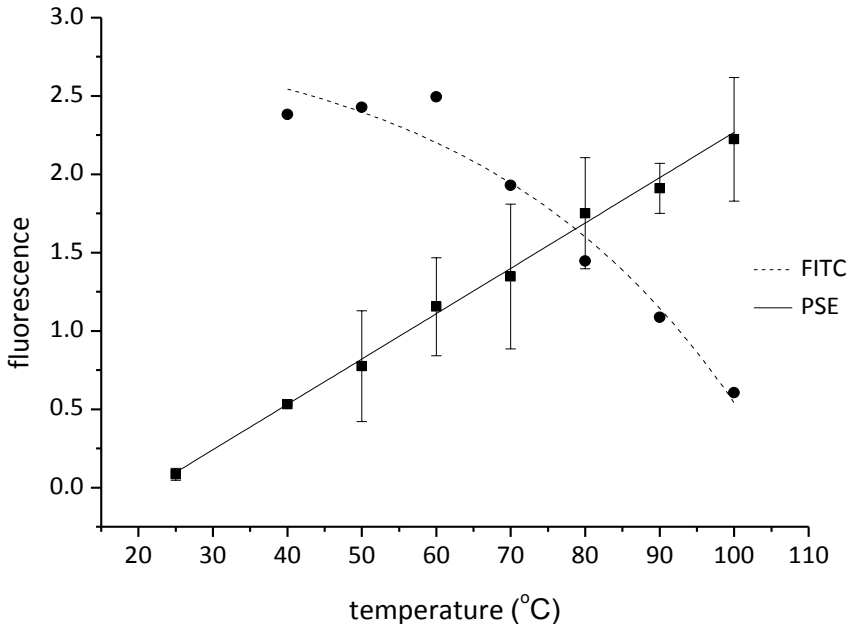


Figure 2.2 The influence of temperature on the fluorescence intensity of derivatised PSE (5 minute reaction time). Error bars represent the corresponding standard deviations of the peak heights for each temperature experiment (n = 3), and triplicate injection of derivatised PSE. Each experiment was performed on a separate chip.

Figure 2.3 illustrates the influence of reaction time upon the signal intensity at the optimum temperature of 90 °C. As before, error bars were calculated from the standard deviations of triplicate time experiments and triplicate injections on separate chips. The results show a sharp increase in the signal intensity of PSE up until 180 seconds where it reached a plateau. As expected, the FITC signal intensity gradually decreased over time, again confirming the consumption of FITC. The optimum time was 180 seconds determined by maximum PSE signal intensity, minimum FITC signal intensity and lowest fluorescence signal variability. The dry heating block procedure was compared against microwave and temperature assisted derivatisation methods described by Dominguez-Vega and Zhou *et al*.[251,252]

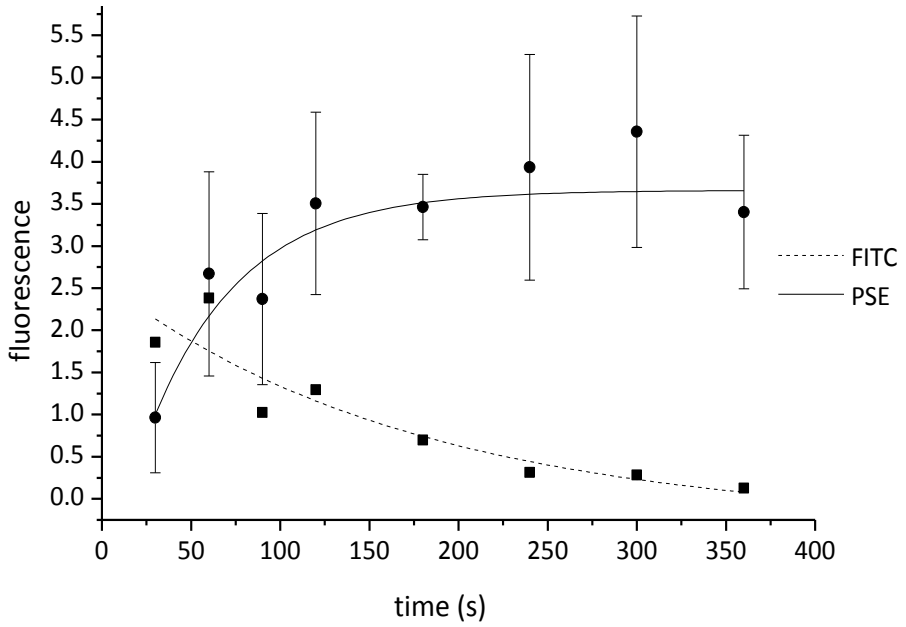


Figure 2.3 The influence of time on the fluorescence intensity of derivatised PSE for a reaction performed at 90 °C. Error bars represent the corresponding standard deviations of the derivatisation time periods for each temperature experiment (n = 3), and triplicate injection of derivatised PSE. Each experiment was performed on a separate chip.

The separation profile of a 24 hour standard procedure at room temperature is shown in Figure 2.4 (a). The 3 minute microwave programme consisted of alternating 30 second periods of microwave irradiation.

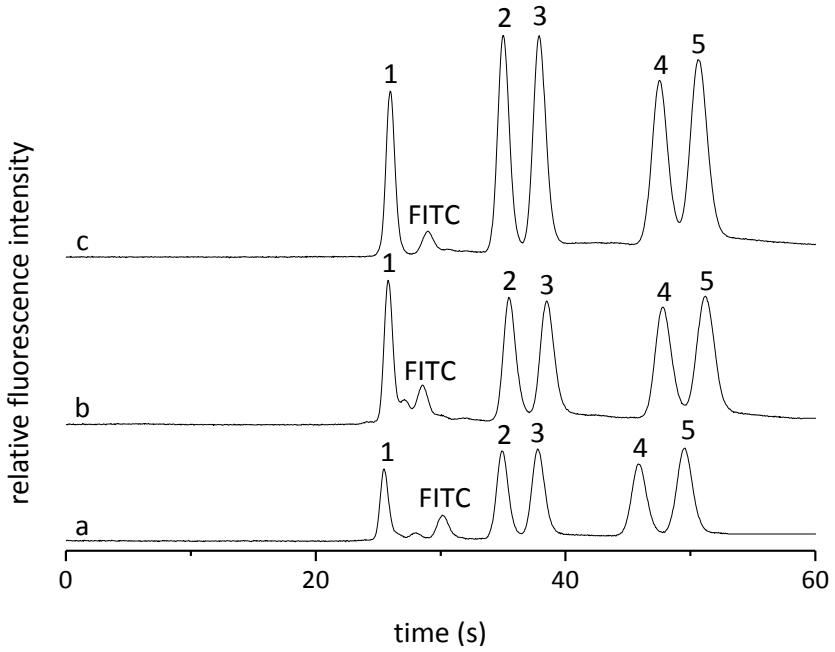


Figure 2.4 Separation profiles of the fluorescence intensity for amphetamine (a) 24 hours at room temperature, (b) 3 minute microwave program – 30 second periods alternating between microwave application and resting, (c) 3 minutes at 90 °C. Separation conditions as in Figure 2.5. (1) 2-MMA, (2) 2-4-MPEA, (3) MPEA, (4) AM-C-BD, (5) BMBA.

A microwave irradiation power of 250 watts was applied to a 300 µL mixture contained in a closed 1.7 mL centrifuge vial. With the application of microwave irradiation for 3 minutes (Figure 2.4(b)), similar fluorescence intensity was observed. When the reaction was performed using a dry heating block set at 90 °C, the fluorescence signal intensity was approximately double (Figure 2.4(c)). Microwave irradiation can accelerate the derivatisation process in comparison to conventional heating and can increase the yield. However, the temperature-assisted method was chosen for optimisation due to its simplicity and greater suitability for in-field analysis. A heating block was chosen due to its increased feasibility for laboratory and in-field analysis.

2.3.2 Electrolyte optimisation

Sodium tetraborate was chosen as a suitable buffer given the average pKa for the amphetamine analogues was 8.7. Optimisation experiments considered electrolyte pH, and the concentration of borate and SDS. At lower pH, the peaks broadened, decreasing the efficiency of the separation. The EOF is reduced due to the dispersed

double layer at the micro-channel surface (section 1.2.1.6). The separation efficiency increased with increasing borate and SDS concentration up to a maximum concentration of 50 mM of each. In the absence of SDS not all components were separated. The inclusion of SDS, which migrates in the opposite direction of the EOF, introduces an additional partitioning phase for the target analytes (section 1.2.1.4). Therefore the best separation buffer comprised 50 mM sodium tetraborate and 50 mM SDS at pH 9.66. The influence of hydroxyethyl-cellulose, methanol and acetonitrile were also investigated with the objective of improving the separation by slowing down EOF. However, there was no significant enhancement of separation and the baseline noise increased.

2.3.3 Instrumental parameters

The separation and injection voltages were optimised using a systematic approach. The optimal separation voltage of 1500 V was selected as a compromise between separation time and efficiency.

2.3.4 Microchip cleaning procedure

The microchips used throughout this research were designed to be disposed after analysis. This is advantageous for preventing contamination, however, relatively expensive for in-field analyses. A cleaning procedure was developed to enhance the cost-effectiveness of the method. This procedure, employing sodium hydroxide and deionised water, was similar to the method used to clean capillaries in CE. Using a pipette each well was filled with solution and flushed throughout the micro-channels by applying a vacuum at well C4 (refer to Figure 1.28). Multiple flushes, alternating between sodium hydroxide and water, were performed. In-between each flush the chip was dried thoroughly with a flow of compressed nitrogen (using a nitrogen gun). The use of a similar cleaning procedure has been reported by Chim and Lee.[257] Using this method the DNA sizing reproducibility for 10 repeats was within 5% RSD.

2.3.5 Separation of labelled analogues and ATS

Figure 2.5 shows the separation of five amphetamine analogues employing 50 mM sodium tetraborate and 50 mM SDS. These analogues were chosen due to their physical and chemical similarity to AMP and MA, as well as to highlight the potential of the Bioanalyzer lab-on-a-chip device for the analysis ATS and pre-cursors.

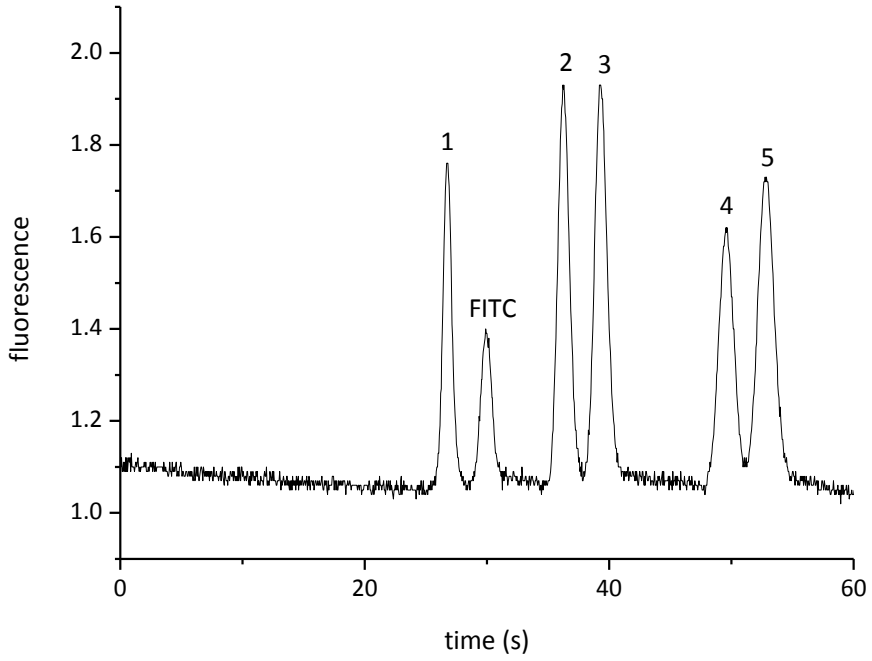


Figure 2.5 Electropherogram of 5 amphetamine analogues (5 µg/mL) using LED-IF (λem 470, λem 525). Conditions: 50 mM SDS + 50 mM sodium tetraborate, pH 9.66; 25°C; injection time 2 seconds; injection voltage 1.5 kV; separation voltage 1.5 kV; (1) 2-MMA, (2) 2-4-MPEA, (3) MPEA, (4) AM-C-BD, (5) BMBA.

Figure 2.6 shows the separation of pseudoephedrine, amphetamine, and methamphetamine. These compounds were treated separately to simulate a typical clandestine laboratory analysis such as the rapid identification of the presence of amphetamine or methamphetamine and its potential synthesis route, i.e. the manufacture of methamphetamine from pseudoephedrine as a starting material.

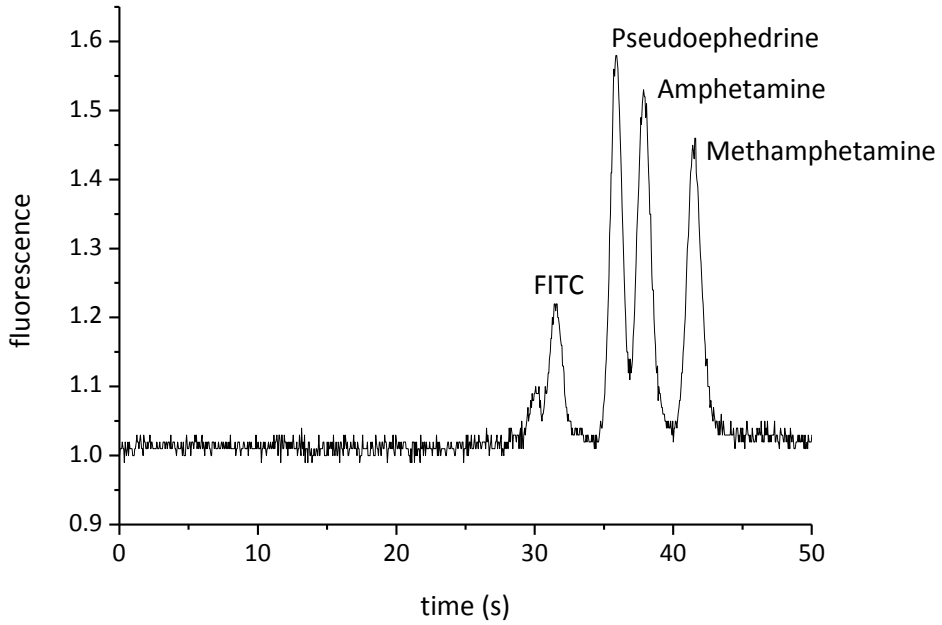


Figure 2.6 Electropherogram showing the optimised separation of 3 ATS standards (5 µg/mL) using LED-IF (λ_{em} 470, λ_{em} 525). Conditions: 50 mM SDS + 50 mM sodium tetraborate, pH 9.66; 25°C; injection time 2 seconds; injection voltage 1.5 kV; separation voltage 1.5 kV; (1) PSE, (2) AMP, (3) MA.

In both cases all compounds were resolved in less than 1 minute, with sufficient resolution for identification. The analytical performance of this method was evaluated in terms of sensitivity, repeatability and linearity. These results are summarised in Table 2.2.

A calibration curve was constructed for each analyte to determine the linear relationship between the analytical response (fluorescence signal) and the known analyte concentration for repeat measurements (n=6). Correlation co-efficients, measuring the strength of the linear relationship, ranged from 0.960 to 0.95. The linear range of the method was studied by analysing five concentrations over the range 1 to 50 mg/mL (in duplicate).

The ability of the method to detect and determine trace quantities of each compound was evaluated using the limit of detection (LOD) and limit of quantification (LOQ). The LOD is the lowest analyte concentration that is statistically different from the

background signal for qualitative analysis. The LOQ can be defined as the level above which quantitative results may be obtained with a specified degree of confidence. The LOD and LOQ were calculated for each compound (n=6) based on 3 times and 10 times the signal-to-noise ratio (S/N), respectively. This ratio measures the strength of the fluorescent signal (S) relative to the average strength of the background noise level (N). Average LOD and LOQ values were 0.6 mg/mL and 2.2 mg/mL, respectively. These values are consistent with detection limits of 0.5 mg/mL for 3,4-MDMA for a CE-LIF method reported by Fang *et al*.[258].

Analyte	Detection limits (n = 6, µg/mL)		Calibration R^2 (n = 6, 1-50 µg/mL)
	LOD [a]	LOQ [a]	
2-4-MPEA	0.6	2.0	0.988
BMBA	0.7	2.3	0.991
2-MMA	0.7	2.3	0.989
AM-C-BD	0.9	3.0	0.983
MPEA	0.8	2.8	0.965
PSE	0.5	1.8	0.995
AMP	0.4	1.5	0.990
METH	0.4	1.5	0.960

Table 2.2 Analytical performance data for amphetamine analogues and ATS. [a] LOD and LOQ calculations were determined using 3 times and 10 times the signal-to-noise ratio, respectively (n = 6).

Repeatability is a measure of precision between replicate measurements. The repeatability of the migration times was evaluated for each analogue (30 mg/mL) from repeat injections both within-chip (n = 6) and between-chip (n = 12). The corresponding relative standard deviations (RSDs) for all analytes ranged from 1.8 to 4.4 and 2.7 to 4.9, respectively (Table 2.3).

Analyte	Average migration time ± Standard Deviation (s)	Adjusted migration times (% RSD) [a]	
		Within-chip	*Between-chip*
2-4-MPEA	26 ± 0.5	1.9	2.8
BMBA	34.9 ± 0.6	1.8	2.7
2-MMA	37.7 ± 0.8	2.1	3.5
AM-C-BD	47.6 ± 2.1	4.4	4.9
MPEA	50.5 ± 1.9	3.7	4.7
PSE	55.3 ± 2.3	2.3	4.1
AMP	58.4 ± 2.1	2.1	3.6
METH	67.2 ± 3.2	2.5	4.8

Table 2.3 Separation and detection reproducibility for amphetamine analogues and ATS. [a] Calculated from repeat injections of a 30 mg/mL standard mixture..

2.4 Conclusions

A fast and reliable method for the derivatisation of ATS with FITC and subsequent analysis via LED-IF using the Bioanalyzer was demonstrated. The rapid derivatisation reduced the typical 12 hour derivatisation of the analytes to 3 minutes. The procedure employed a portable dry heating block, set at 90 °C, for the labelling of amphetamines. The separation of AMP, MA and PSE was within 1 minute. Limits of detection and limits of quantification were similar to reported CE-LIF methods ranging from 0.4 to 0.9 mg/mL and 1.5 to 3.0 mg/mL, respectively. The derivatisation procedure with FITC was robust and reliable and was suitable for the in-field detection of amphetamines and other ATS.

Chapter 3

Screening of methamphetamine, ephedrine and pseudoephedrine

Chapter 3: Screening of methamphetamine, ephedrine and pseudoephedrine in samples from clandestine laboratories

The clandestine manufacture of methamphetamine in New Zealand predominantly involves the reduction of pseudoephedrine, extracted from pharmaceutical preparations, using hydrogen iodide. This method of illicit manufacture leaves a variety of materials at the scene that are a rich source of information. Efficient processing and preliminary identification of extraction and reaction mixtures, precursors and products is essential to minimise exposure to potential hazardous materials and to provide investigative and intelligence information. In this part of the project, the use of the Bioanalyzer was investigated for the rapid and cost effective screening of methamphetamine, ephedrine and pseudoephedrine in a variety of sample types found in typical clandestine laboratory scenarios.

3.1 Introduction

In 2010 and 2011, Police and Forensic Scientists in New Zealand attended approximately 400 clandestine laboratory related incidents.[259] The majority of these involved the manufacture of methamphetamine by reduction of pseudoephedrine extracted from pharmaceutical preparations. The manufacturing route via the reduction of pseudoephedrine/ephedrine using hydrogen iodide is most often employed.[260] Hydrogen iodide is sourced directly from hydriodic acid (Nagai method) or produced in situ using iodine in combination with either hypophosphorus acid or phosphorus acid (Hypo method), or, red phosphorus and water (Moscow method).[110,113,261] Figure 3.1 summarises the steps involved, as well as the likely clandestine product and matrix from each of these manufacturing methods.

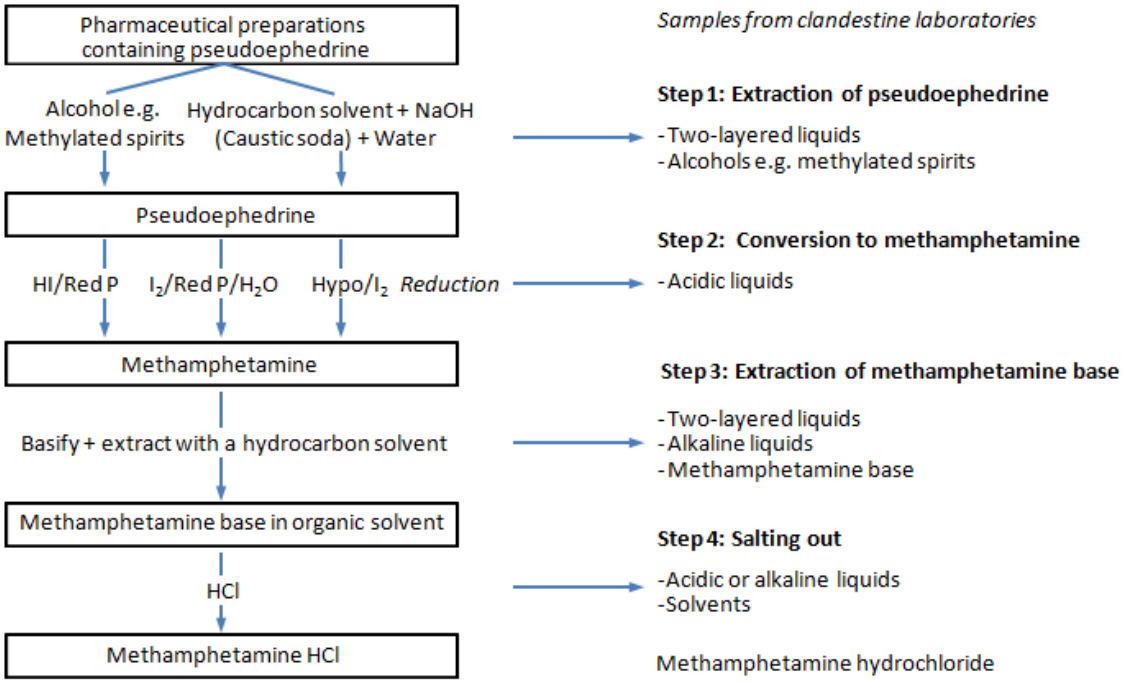


Figure 3.1 Left: Simplified outline of methamphetamine manufacture employing the HI reduction method; Right: samples resulting from each step.

The decision making process for first responders to the clandestine laboratory site involves consideration of efficient methods for exhibit recovery, preservation of traces and the minimisation of exposure to hazardous materials. Since all materials from which samples are collected are often sent immediately to a chemical contractor for destruction, the identification of reaction mixtures and waste liquids is crucial. Some of these materials may contain many, if not all, of the pre-cursors, final products and by-products for a given synthesis route.[10]

The presumptive testing and/or classification of compounds or mixtures are usually performed with pH tests, colour tests (spot tests) and the knowledge of relative physical and chemical properties of the sample. Whilst spot tests offer speed, simplicity, portability and cost-effective analyses, false positives and false negatives are frequent as they often lack specificity and/or sensitivity.[262]

An alternative to spot tests are portable devices that screen liquids present at the scene to identify samples likely to contain controlled substances, thereby prioritising

their collection, reducing costs and saving time both at the scene and at the laboratory.[10]

Portable devices available to first responders include hand-held Raman spectrometers and portable FTIR. These complementary tools are useful for identifying the major constituent(s) of powders, pure liquids and visible residues. However, they are of limited use when complex mixtures, certain liquid matrices and/or purities are sampled. Mobile laboratories, deployed for larger clandestine laboratories in some countries, are often fitted with a portable GC-MS. Difficulties experienced with rapid on-site analyses, mainly attributed to sample preparation requirements, limit the in-field use of these available tools.[140]

Minimal sample preparation requirements and fast analysis times contribute significantly to the Bioanalyzer's potential for screening.[228] Previous studies, outlined in Chapter 2, demonstrated the use of LOC for the analysis of ATS.[263] This chapter explores the suitability of this instrument for screening clandestine laboratory samples. Sample preparation methods were developed and its performance was tested with samples of different matrices representative of a typical clandestine laboratory. Unless stated otherwise, the reported samples were authentic, originating from previous clandestine laboratory seizures.

3.2 Materials and methods

3.2.1 Lab-on-a-chip apparatus

Refer to section 2.2.1.

3.2.2 Chemicals

Sodium dodecyl sulfate (SDS; ≥ 98.5 %), sodium tetraborate decahydrate (≥ 99.5 %) and fluorescein isothiocyanate isomer I (>90%) were purchased from Sigma Aldrich (St Louis, MO, USA). Analytical reagent grade methanol, heptane, toluene, acetone and chloroform and pH-indicator strips were obtained from Merck (Darmstadt,

Germany). Disposable skin cleansing 2-ply isopropyl alcohol wipes (70% saturated v/v) were purchased from Tyco healthcare (Mansfield, MA, USA). Individual primary drug standards of methamphetamine and ephedrine and pharmaceutical preparations were supplied by the Institute of Environmental Science and Research Ltd (ESR; Auckland, New Zealand). Pseudoephedrine hydrochloride (99+ %) was purchased from Acros Organics (New Jersey, USA). Nile blue chloride was purchased from Sigma Aldrich (Sydney, Australia). Concentrated hydrochloric acid and sodium hydroxide solutions were analytical grade.

3.2.3 Electrolyte preparation

Refer to section 2.2.3.

3.2.4 Preparation of stock solutions for analytical samples

FITC stock solution. A 50 µg/mL stock solution of FITC was prepared in analytical reagent grade acetone and stored in a 10 mL glass sample tube wrapped in aluminium foil at -18 °C. [1]

Buffer. A solution of 50 mM sodium tetraborate was prepared in distilled water and filtered through a 0.45 µm nylon membrane filter for use in extraction and derivatisation procedures.

ATS mixed standard. Individual stock solutions of ephedrine, pseudoephedrine and methamphetamine were prepared in distilled water to a final concentration of 2 mg/mL. Stock solutions were mixed equally (1:1) and 100 µL of the mixture was diluted 2-fold in 50 mM sodium tetraborate buffer, prior to derivatisation.

Sampling of simulated residues. A stock solution of methamphetamine, 10 mg/mL of the hydrochloride salt, was prepared in methanol. Solutions of 1, 0.1, 0.01 and 0.001 mg/mL [2] were prepared by further dilution and 100 µL was deposited on the surface

[1] *An excess of FITC was used in this part of the study to ensure all components would derivatise without using up the FITC.*
[2] *Corresponding masses deposited on the surface equate to 100, 10, 1 and 1µg, respectively*.

and left to evaporate prior to sampling. The surface was wiped, methanol (0.5 mL) was added to the sample wipe in a test tube and the liquid was drawn up using a glass pipette. The methanol extract was diluted 1:1 in buffer prior to derivatisation.

3.2.5 Sample preparation methods

3.2.5.1 Pharmaceutical preparations containing pseudoephedrine

Pharmaceutical preparations were selected at random from a collection at ESR and treated as unknowns to simulate a typical clandestine laboratory. The brand names are listed in Tables 2-4.

Solids. Tablets and caplets were crushed using a mortar and pestle; capsules were opened to remove the contents. A solution of approximately 3 mg/mL was prepared in distilled water and diluted (approximately 2-fold) in 50 mM sodium tetraborate buffer.

Liquids. The liquid/gel capsules were diluted 10-fold in buffer (v/v).

3.2.5.2 Clandestine laboratory samples

Preliminary investigations included the measurement of the pH of each liquid using pH indicator strips. For samples that were two-layered liquids, the pH of both layers was measured to identify the aqueous and organic layer as well as the appropriate workflow. A sodium tetraborate buffer (50 mM) was employed during sample preparation. Figure 3.2 outlines the initial clandestine laboratory assessment procedure that should be followed at the scene to identify the sample type. A different preparation procedure was followed for each sample type (a)-(f), prior to derivatisation with FITC. These methods are summarised in Table 3.1.

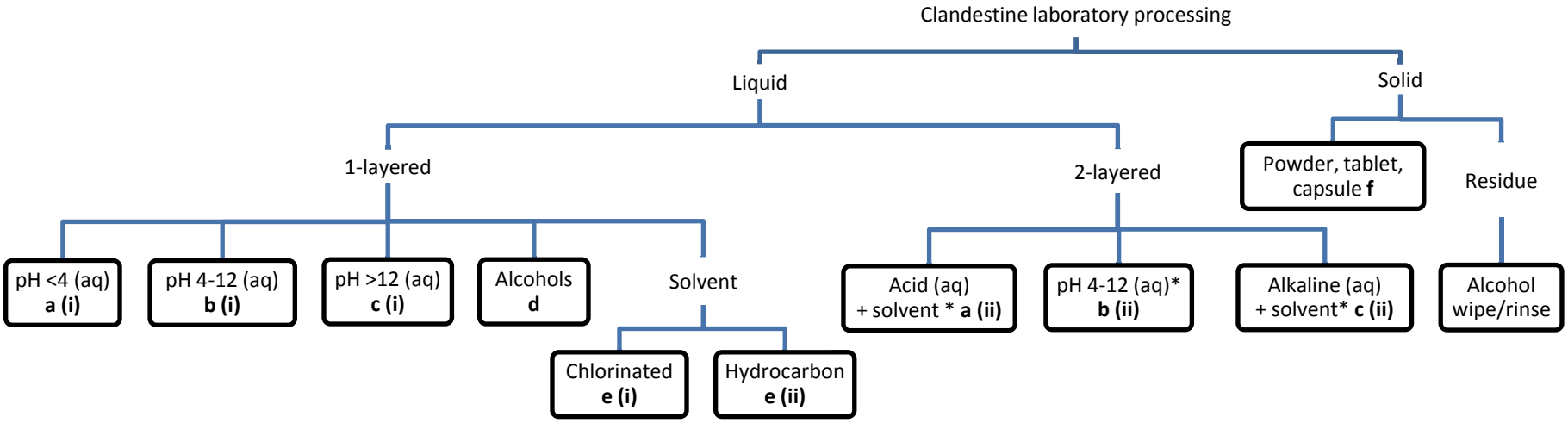


Figure 3.2 The clandestine laboratory processing sequence to determine which sample preparation method to follow. *These samples were two-layered liquids at the scene.

	Sample type	Sample amount	Step 1: Mix with toluene	Step 2	Step 3: Mix with buffer	Step 4: remove layer for analysis	Analyse
a (i)	Acid (aq) pH < 4	0.5 mL [a]	0.5 mL	Remove toluene	0.5 mL	Remove buffer (bottom) layer	Buffer layer [*a]
a (ii)	Acid (aq) + solvent	0.5 mL of acid (aq) layer	0.5 mL	Remove toluene	0.5 mL	Remove buffer (bottom) layer	Buffer layer [*a]
b (i)	Neutral (aq) pH 4 -12	0.5 mL	N/A	N/A	0.5 mL	N/A	Buffer mixture
b (ii)	Neutral (aq) + solvent	0.5 mL aq layer	N/A	N/A	0.5 mL	N/A	Buffer mixture
		0.5 mL solvent layer	N/A	N/A	0.5 mL	Remove buffer layer (refer to e)	Buffer layer
c (i)	Alkaline (aq) pH > 12	0.5 mL	0.5 mL	Remove toluene	0.5 mL	Remove buffer (bottom) layer	Buffer layer [*a]
c (ii)	Alkaline (aq) + solvent	0.5 mL of solvent layer	N/A	N/A	0.5 mL	Remove buffer layer (refer to e)	Buffer layer [*a]
d	Alcohol/alcohol rinse	0.5 mL	N/A	N/A	0.5 mL	N/A	Alcohol/buffer mixture [*a]
e (i)	Chlorinated solvent (e.g. chloroform)	0.5 mL	N/A	N/A	0.5 mL	Remove buffer (top) layer	Buffer layer
e (ii)	Hydrocarbon solvent (e.g. toluene, heptane)	0.5 mL	N/A	N/A	0.5 mL	Remove buffer (bottom) layer	Buffer layer
f	Solid	5 – 10 mg	N/A	Add 1 mL methanol	0.5 mL	N/A	Methanol/buffer

Table 3.1 Sample preparation procedures followed for each clandestine laboratory sample. * basify to pH 14 **adjust to pH 8-10, if required, prior to derivatisation.

3.2.5.3 Simulated alcohol wipes

A 100 cm^2 area of the fume hood was wiped. Methanol (0.5mL) was added to the wipe in a test tube. Using a glass pipette, the liquid was drawn through the wipe and added to an equal quantity of buffer solution for analysis.

3.2.6 Fluorescent derivatisation procedure

FITC (100 µL) was added to 200 µL of the prepared samples in a flat bottomed glass insert (placed inside a crimped 1.7 mL microtube). The microtube was sealed and wrapped in aluminium foil prior to being heated for 3 minutes in a Reacti-therm III heating module (Pierce) set at 90 °C. The derivative was cooled to room temperature and diluted 1:5 in electrolyte prior to analysis.

3.3 Results and discussion

3.3.1 Preparation of samples

Due to the wide range of liquid matrices typically found at clandestine laboratory sites, careful processing was required to determine the nature of the liquid prior to sample extraction. The miscibility of the sample with the 50 mM sodium tetraborate buffer solution was effective to distinguish between aqueous and non-aqueous liquids. An initial pH measurement dictated which sample type was present and thus which extraction procedure to follow. Figure 3.2 outlines the processing sequence followed to ascertain the sample type. For each type the sample amount, steps involved in extraction and the nature of the liquid prepared for analysis is provided in Table 3.1.

The relative solubilities of pseudoephedrine and methamphetamine in aqueous and non-aqueous solutions determined which layer to screen from two-layered liquids. In general, the order of solubility was as follows: acid > solvent > alkaline. Therefore, when a two-layered liquid containing an acidic layer (acid + solvent) was recovered and the acidic layer was extracted for analysis. In contrast, for a two-layered liquid

containing an alkaline layer (alkaline + solvent), the solvent layer was more desirable for screening. Where the pH of each layer was within a neutral range (e.g. water mixed with a solvent) both layers were screened.[10]

3.3.2 Migration time variation of the standard mixture

The electropherogram presented in Figure 3.3 illustrates the baseline separation of a mixed standard containing ephedrine, pseudoephedrine and methamphetamine. Intra-chip (within-chip) and inter-chip (between-chip) variations in migration times were calculated for a standard mixture of ephedrine, pseudoephedrine and methamphetamine from ten repeat injections. Corresponding RSDs of 3-5 % and 5-10 % were calculated for intra-chip and inter-chip, respectively. Therefore, a standard mixture was run on each chip to account for any chip-to-chip variations. For each unknown, a positive screening result was inferred when the migration time was within 4% RSD of the corresponding standard from the same chip.

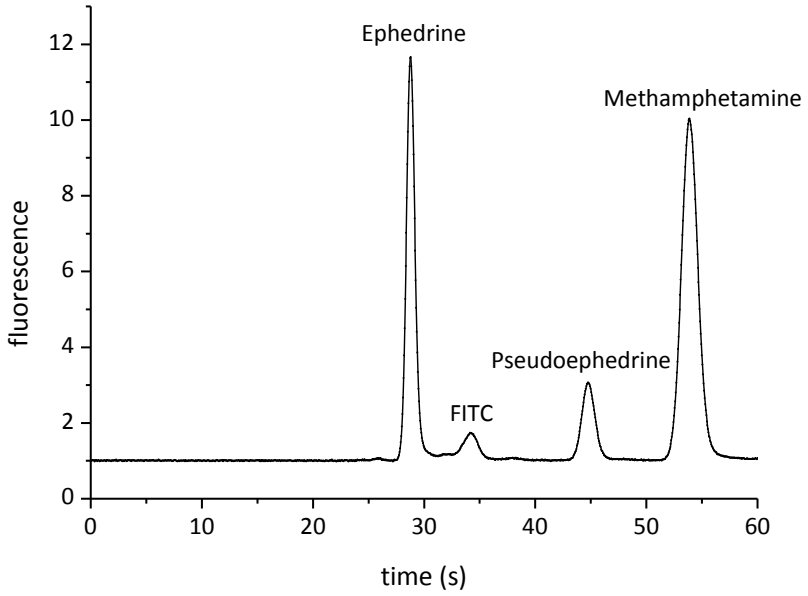


Figure 3.3 Separation of a standard mixture of ephedrine, pseudoephedrine and methamphetamine.

3.3.3 Analysis of clandestine laboratory samples

3.3.3.1 Part I: Pharmaceutical preparations containing pseudoephedrine

The analysis of unknown liquids, tablets, powders and gel capsules present at clandestine laboratory scenes is commonly performed to identify the presence of pseudoephedrine. Various discontinued pharmaceutical preparations, supplied by ESR, were analysed to determine if the method was suitable for the screening of pseudoephedrine in different matrices. The LOC method had high selectivity as most other constituents of pharmaceutical preparations are inert binders and fillers or tertiary amines, which did not derivatise with FITC. Paracetamol did form a derivative, however it was well separated from pseudoephedrine, co-migrating with underivatised FITC, and therefore easily identified.

Cold & flu liquids and gel capsules

The migration times for pseudoephedrine present in cough liquids and gel capsules, are summarised in Table 3.2 and Table 3.3. Relative standard deviations of 2.9 and 3.4 % were obtained for the range of cold & flu liquids and gel capsules, respectively, which were within the acceptable limits for screening purposes. The differences in viscosity and slight variations in the pH of sample solutions are likely to have contributed to the change in the overall migration time for each type of pharmaceutical preparation. The goal of this study was investigate if LOC is suitable for screening for the presence of pseudoephedrine in various matrices. Prior to assessing its suitability for routine analysis, inter- and intra- chip variations should be examined using a more challenging set of matrices.

	Pseudoephedrine concentration (mg/mL)	Average migration time (s) [a]
Robitussin	3	42.0
Sudomyl Elixir	6	44.0
Duro-tuss	4	44.2
Bisolvon sinus	4	43.8
Benadryl	6	44.3
Pseudoephedrine standard	2	45.9

Table 3.2 Data for the analysis of pseudoephedrine in 5 cold & flu liquids, reported concentrations were determined from the label on each bottle. * Analysed in duplicate.

	Pseudoephedrine % (w/w)	Average migration time (s) [a]
Actifed Dry	4.1	46.6
Dimetapp Cold & Flu	1.7	48.3
Actifed Chesty	3.7	49.1
Dimetapp Day & Night (Night)	1.8	49.9
Dimetapp Day & Night (Day)	1.7	51.0
Pseudoephedrine standard	-	49.3

Table 3.3 Data for the analysis of pseudoephedrine in 5 gel capsules. Concentrations were taken from the packaging of each gel capsule, w/w = average weight of pseudoephedrine present in each capsule. * Analysed in duplicate.

Tablets and capsules

Pseudoephedrine was identified in each of the representative tablets or capsules listed in Table 3.4. An overall RSD of 2.6 % was obtained across ten tablets. The relative peak areas are also listed.

	Pseudoephedrine concentration (mg/mL)	Normalised pseudoephedrine peak area [a]	Average migration time (s) [a]
Contac NT	2.4	6.2	36.8
Sinutab sinus & pain relief	0.3	3.2	35.6
Demazin night	1	2.0	37.0
Nurofen Cold & Flu	0.5	3.3	37.2
Sudafed sinus, pain & allergy relief	0.3	5.4	37.3
Coldrex Day & Night – Night	0.3	2.2	38.0
Telfast decongestant	0.9	5.3	38.0
Coldral Cold, Cough & Flu (Day & Night) – Day	0.3	2.4	38.4
Actifed	1.6	3.0	38.8
Coldral 4 Flu	0.3	2.9	38.7
Pseudoephedrine standard	1.0	1.0	37.1

Table 3.4 Migration time and peak area data for the analysis of pseudoephedrine in 10 pharmaceutical tablets for two aliquots. Concentrations of pseudoephedrine and paracetamol are reported for 3 mg/mL solutions of each tablet. Peak areas were normalised to a pseudoephedrine standard of 1 mg/mL.

3.3.3.2 Part II: Clandestine laboratory liquids

Samples from five different clandestine laboratory cases were selected. A range of samples (acidic, alkaline, solvents, alcohol rinses, two-layered liquids) were screened using LOC and subsequently cross checked by GC-MS. The LOC and the corresponding GC-MS results (data supplied by ESR) for each sample are summarised in Table 3.5. The results agree well for the presence/absence of the amines, indicating that the technique was suitable for screening prior to confirmatory analysis.

On each chip, a standard mixture of ephedrine, pseudoephedrine and methamphetamine was run to account for any inter-chip migration time variations. A positive identification of each was made if the migration time was within 5% RSD of the standard from the same chip.

As summarised in Table 3.5, the LOC results agree with the GC-MS data. There was only one case where screening of a two-layered liquid sample indicated the presence of pseudoephedrine and methamphetamine by LOC, however only pseudoephedrine was confirmed by GC-MS.

In addition, this method distinguished between ephedrine and pseudoephedrine, which is valuable information that the GC is only able to determine following a far lengthier derivatisation procedure.[264]

Sample type		LOC result	GC-MS result [3]
Acidic	1	PSE, METH	METH, PSE
	2	METH	METH
	3	NEG	NEG
	4	NEG	NEG
	5	METH	METH
Basic	1	PSE	PSE, chlorpheniramine
	2	NEG	NEG
Solvent	1	PSE, METH	METH, PSE/EPH, chlorpheniramine
	2	NEG	NEG
	3	NEG	NEG
	4	NEG	NEG
	5	NEG	NEG
Alcohol rinse	1	EPH, PSE	PSE/EPH, chlorpheniramine
	2	EPH, PSE	PSE/EPH, chlorpheniramine
	3	NEG	NEG
	4	NEG	NEG
	5	NEG	NEG
	6	NEG	NEG
	7	METH	METH
	8	NEG	NEG
Solid	1	PSE	PSE
	2	NEG	NEG
	3	NEG	NEG
2-Layered liquid	1	PSE	PSE
	2	PSE, METH	PSE
	3	EPH, PSE, METH	METH, PSE/EPH
	4	PSE	PSE/EPH, dextromethorphan, chlorpheniramine, codeine
	5	NEG	NEG
	6	METH	METH, P2P, N-dimethylamphetamine, chlorpheniramine
	7	PSE	TL = chlorpheniramine
	*	PSE	BL = PSE and chlorpheniramine
	8	METH	TL = METH
	9	PSE	METH, PSE/EPH

Table 3.5 Summary of the results obtained for clandestine laboratory samples using LOC with a comparison to GC-MS. NEG = no result, PSE/EPH – Either or both pseudoephedrine and ephedrine (i.e. – not confirmed by further analysis). * Two-layered liquid, both layers pH 6 therefore both layers analysed. TL = top layer, BL = bottom layer.

[3] *Agilent Technologies 6890N gas chromatograph coupled to a mass selective detector with capillary column BPX-5 (12 m x 0.2 mm ID x 0.25 µm film thickness). Samples analysed using a screening method [injector 250 °C, detector 280 °C, 60 °C (2 min) 20 °C/min 300 °C (7 min)].*

Figure 3.4 represents the fluorescent profiles for the solvent layer of a two-layered liquid sample that contained all target analytes, and an acidic sample containing methamphetamine. For each unknown, the migration times were measured relative to a standard mixture run on the same chip. The standard mixture is presented below each sample electropherogram. The migration times attributed to pseudoephedrine, ephedrine and methamphetamine from the solvent layer of two-layered liquid sample 3 were 30.85, 41.05 and 47.6 seconds, respectively. The corresponding standard migration times were 31.25, 41.45 and 48.05, respectively. Acidic liquid 3 contained methamphetamine with a migration time of 44.45, compared to 45.45 for the standard run on the same chip.

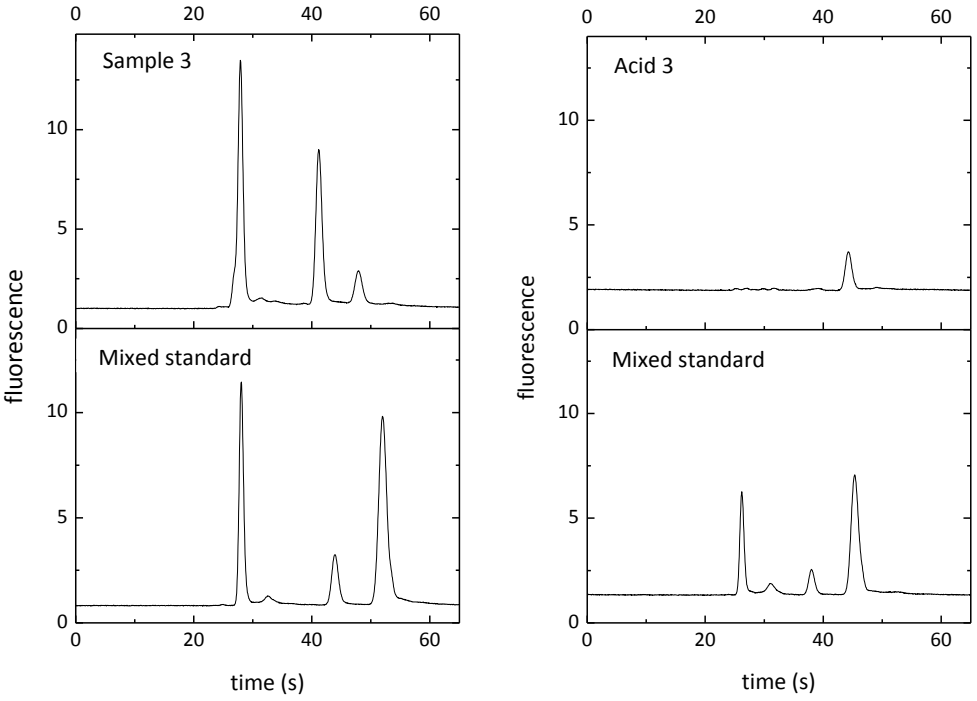


Figure 3.4 Fluorescent responses from LOC analyses of (a) the solvent layer of two-layered liquid sample 3, (b) acidic liquid 3. Numbers correspond to the results presented in Table 3.5.

Many by-products and intermediates generated during the methamphetamine manufacturing process do not contain a functional group for derivatisation with FITC.

They are often ketones or tertiary amines and therefore minimise the risk of false positives. A rate of 3% was calculated for these samples (refer to Table 3.5).[4] Some other impurities associated with this method of manufacture include amphetamine and methamphetamine-dimer, which have different migration times to the target analytes. Their presence in relatively trace amounts, in comparison to methamphetamine and pseudoephedrine minimises the risk of interference. In the case that a sample contains trace amounts using LOC, that particular sample is unlikely to be sent for further analysis if others generate results with higher levels.

Whilst the casework samples presented here are all from clandestine laboratories employing the hydriodic acid reduction of pseudoephedrine, the sampling procedure(s) and extraction methods are applicable to other manufacturing methods. Regardless of the starting products and the chosen route of synthesis, similar overall procedures (e.g. extraction, crystallisation) are involved in the synthesis. For example, the synthesis of methamphetamine from P2P employing the reductive amination or Leuckart routes.[110]

3.3.3.3 Part III: Simulated surface swabs

Swabs are commonly collected from various surfaces at clandestine laboratory sites to obtain evidence of manufacture. A screening tool that could identify contaminated surfaces would be useful for prioritising sample collection, particularly at larger sites. The extraction of methamphetamine with swabs from representative household surfaces at clandestine laboratories was evaluated for LOC analysis.[265] A recommended extraction method for alcohol swabs prior to GC-MS analysis involves a base/solvent extraction. A mixture of sodium hydroxide (0.5M) and chloroform are added to the swab in a glass sample tube and sonicated for 10-15 minutes. The chloroform layer is removed for analysis by GC-MS. The entire procedure takes at least 1 hour to generate a single result.

In comparison, a very simple and rapid extraction procedure was developed prior to analysis by LOC. A surface area of 100 cm^2 was wiped with an isopropyl alcohol wipe,

[4] *Calculation: # positive LOC results when negative GC-MS result/total # positives*

0.5 mL of methanol was added to the wipe in a test tube and the liquid was drawn up through a pipette and mixed with 50 mM sodium tetraborate buffer prior to derivatisation with FITC. The time from wipe to result was 15 minutes for one sample, or 1 hour for 12, which is 4 – 10 times faster than the currently employed method.

Methamphetamine standard

Methamphetamine was extracted from four surfaces including formica, ceramic tile, gloss painted wood and metal. The migration time of methamphetamine across all surfaces was within 2.4 % RSD and the average recovery was 24.1 % (Table 3.6). The lowest recovery (20.2 %) was obtained from a gloss painted wood surface, most likely due to interferences caused by that particular matrix.

Surface type	Methamphetamine concentration (mg/mL)	Average migration time (s) [a]	Average peak area [a]	Recovery (%)
Glossed wood	1.4	42.7	125	20.2
Ceramic tile	1.4	44.0	153	24.7
Formica	1.4	44.4	150	24.2
Metal	1.4	44.5	170	27.4
Standard	1.4	45.7	620	-

Table 3.6 Migration time data for the extraction of methamphetamine from different household surfaces. * Samples analysed in duplicate.

Simulated alcohol wipes

Two areas of a laboratory fume hood located at ESR, where the controlled manufacture of methamphetamine had previously been carried out, were sampled using alcohol wipes. Following analysis by LOC, one sample contained methamphetamine and the other produced a negative result. These results were confirmed by GC-MS.

During these experiments, the time from collection to a LOC result was recorded. A time of 15 minutes was recorded for one sample, whilst the screening of 12 samples

took less than 1 hour. This included sample extraction, derivatisation and microchip preparation and analysis procedures. In comparison to the current screening method, employing GC, where 1 hour is required per sample, LOC performs 4 or 10 times faster for an individual sample or multiple samples, respectively.

3.3.4 In-field applications

The liquid-liquid extraction methods outlined in the materials and methods section were simple, and the subsequent derivatisation and separation procedures were rapid. The Bioanalyzer is suitable for inclusion in mobile laboratories situated adjacent to the property/location for large or inter-city clandestine laboratory seizures to screen bulk samples. This method could also be applied at the border, particularly for large shipments of impure stimulants or dissolved drugs. Methods capable of screening liquids, such as LOC, would be valuable as liquids can be particularly difficult to deal with using the current instrumentation available to customs and border officers.

In order for it to be suitable for the *hot zone*, the confined space where the clandestine manufacture of methamphetamine was believed to be carried out, the degree of automation would need to be enhanced. Alternative extraction procedures, for example solid phase micro-extraction, could reduce the number of steps required, streamlining sample preparation methods. The modification of the instrument's hardware, to allow for on-chip derivatisation, would also enhance automation. Microchip devices incorporating a heating system have been designed,[266] which allow for on-chip derivatisation as reported by Yassine *et al*.[267] for a fluorescent derivatisation procedure employing naphthalene-2,3-dicarboxaldehyde.

3.4 Conclusions

The Bioanalyzer's versatility for the screening of ephedrine, pseudoephedrine and methamphetamine in a wide range of exhibits typically encountered at clandestine laboratories has been demonstrated, following simple sample preparation procedures. The instrument is portable and robust with good sensitivity, satisfying most requirements for in-field application. The Bioanalyzer offers a low cost, rapid alternative for the screening of clandestine laboratory liquids in mobile or office-based laboratories, to prioritise sampling procedures prior to GC-MS confirmation, where required. Further modifications to enhance the degree of automation and streamline preparation methods would improve its suitability for in-field analyses.

Chapter 4

Screening and comparative analysis of synthetic cathinone seizures

Chapter 4: Screening and comparative analysis of synthetic cathinone seizures

Variation in the chemical composition of illicit tablets and powders is common among samples from a given drug seizure population. Therefore, it is important to assess the homogeneity of samples from the same source to ensure that the conclusions being drawn are an accurate reflection of the entire seizure. Using MCE, multiple tablets can be screened in a cost-effective and timely manner. This method could be used in conjunction with reporting methods that focus solely on statistical sampling to infer homogeneity or otherwise of a larger subset of tablets. Some frequently observed synthetic cathinones, often present in illicit tablets seized in New Zealand, were chosen for analysis. The separation of synthetic cathinone mixtures is included in this chapter. A characteristic fluorescent profile was obtained for each tablet, in terms of the number of constituents, relative peak height ratios and migration times. The repeatability of the method was assessed for a wide range of tablets. The use of microchip tablet profiles in the forensic case comparison of illicit drug seizure samples in realistic scenarios is also discussed in this chapter.

4.1 Introduction

Due to the dynamic and resilient nature of the illicit drug market, an evolving number of synthetic stimulants are pressed into tablets.[120] Visually similar tablets from a given batch or seizure do not always contain the same tablet constituent(s) or relative proportions. This can be attributed to the lack of quality control at manufacturing or tabletting sites. Further, tablets from a given seizure do not always originate from the same source and/or manufacturing site. For instance, the tablets/powders may have been tabletted at different locations or distributed into various packets.[268]

The availability of MDMA has declined in recent years, a trend which has been accompanied by the increased use of other stimulants, such as synthetic cathinones: 4-methylethcathinone (4-MEC), 4-MMC, β-keto-3,4-methylbenzodioxylbutamine and

β-keto-3,4-methylenedioxymethamphetamine. These stimulants are often used as a substitute for *ecstasy* and typically sold via the internet.[8,25,83] Little is known of their detailed pharmacology; however they have no known medicinal uses. Synthetic cathinones are imported into New Zealand in powder form where they are often combined and distributed as *ecstasy*, of which 4-MEC is a predominant constituent. Given that these tablets normally contain 4-MEC, mixed with other stimulants, they are commonly referred to as MEC tablets.[76]

The forensic analysis of visually similar tablets is typically performed using different combinations of a confirmatory analytical technique (e.g. GC-MS), colour tests and statistical methods.

Various methods are used to determine the sample size for analysis. Classical, arbitrary sampling methods have been used i.e. the square root, 10 percent of the seizure population or n=1 (where 1 sample from the population is analysed).[16] Alternative methods based on frequentist or Bayesian statistical approaches are also employed. The frequentist approach makes use of a hypergeometric sampling population table whilst the Bayesian model makes use of prior knowledge and Baye's theorem to select a sample size for analysis.[269-271] In comparison to classical methods, the frequentist and Bayesian methods are more cost-effective and timely and still provide sufficient information for the purposes of law enforcement and judicial systems. Following the analysis of a representative sample of the seizure population, inferences are made regarding the rest of the population with an associated quantifiable degree of probability or likelihood. Although the presented statistical methods are based on sound mathematical principles, they are not easy to explain in court, thus are often misunderstood. Accordingly, the development of more simplistic approaches would be advantageous for the presentation of findings related to the forensic analysis of illicit drugs in court.

Currently GC-MS is the industry standard for the analysis of drug exhibits selected for analysis. Forensic analysis requirements vary between judicial systems, depending on resources and legal framework. However, most jurisdictions cannot justify the analysis of multiple tablets, beyond the requirements of statistical methods, using

confirmatory techniques such as GC-MS. In addition, some laboratories are unable to make use of GC-MS due to financial constraints. There is a need for fast and cost-effective techniques for the analysis of a larger sample size. This would improve accuracy, particularly for bulk seizures. The use of a screening tool capable of generating a profile for multiple tablets could be used to estimate the homogeneity within seizures. This would provide timely information to investigators and aid in courtroom visualisation of forensic drug analysis evidence.

Colour/spot tests are routinely employed in forensic drug laboratories during the examination of illicit drug exhibits. While they can be used to presumptively identify illicit drugs prior to GC-MS confirmation, they do not have the required specificity to distinguish between some synthetic cathinones and/or simultaneously identify those in a mixture. In addition, they can suffer from interference resulting in false positives/negatives and are dependent on the colour discrimination of the analyst.[134]

Other techniques available for screening multi-constituent tablets include HPLC. In comparison to HPLC, CE is fast, cost-effective and simple. Further, it is more suitable for miniaturisation and portability.[272] As a result, many CE-based microchip devices have been developed and applied for the portable analysis of DNA, proteins and small molecules.[248,273,274] This technique, a simple yet powerful separation tool, has also been reportedly applied for the analysis of illicit drugs in various matrices.[214,215,267,275]

These devices achieve rapid results, are cost-effective and require little maintenance. Recent growth in the area of microchip technologies has improved the functionality and robustness of portable systems, therefore enhancing their capability for high-throughput analyses.

This chapter evaluates the use of the Bioanalyzer for the analysis of MEC tablets. A simple and rapid sample preparation procedure is utilised. In this research, the sample preparation method described in chapter 2 was used. The method was applied to additional target analytes chosen to represent common synthetic cathinones seen in recent illicit tablet seizures in New Zealand. The sample preparation procedure was adapted and evaluated using MEC seizure tablets from

completed casework. Furthermore, the use of characteristic MCE profiles obtained for tablets containing different mixtures of synthetic cathinones was used to assess the homogeneity of visually similar tablets from the same seizure.

4.2 Materials and methods

4.2.1 Apparatus

Refer to section 2.2.1.

4.2.2 Chemicals and reagents

Sodium dodecyl sulfate (SDS; ≥ 98.5 %), sodium tetraborate decahydrate (≥ 99.5 %) and FITC (>90%) were purchased from Sigma Aldrich (St Louis, MO, USA). Analytical reagent grade methanol was obtained from Merck (Darmstadt, Germany). Nile blue chloride was purchased from Sigma Aldrich (Sydney, Australia). Individual primary drug standards of 4-methylethcathinone, 4-methylmethcathinone, N-ethylamphetamine and N-ethylcathinone were purchased from the National Measurement Institute (Sydney, Australia). Individual primary drug standards of β-keto-3,4-methylbenzodioxlylbutamine, β-keto-3,4-methylenedioxymeth-amphetamine, 3',4'-methylenedioxy-α-pyrrolidinobutiophenone and seizure tablets were supplied by the ESR (Auckland, New Zealand).

4.2.3 Electrolyte preparation

Refer to section 2.2.3.

4.2.4 Sample preparation

FITC stock solution. A 50 µg/mL stock solution of FITC was prepared in analytical reagent grade acetone and stored in a 10 mL glass sample tube wrapped in aluminium foil at -18 °C.

Buffer. A solution of 50 mM sodium tetraborate was prepared in distilled water and filtered through a 0.45 µm nylon membrane filter for use in the derivatisation procedure.

MEC tablets. Previous seizure tablets were provided by ESR. One tablet of each source and variety (i.e. visually similar) was available for analysis. Each tablet was homogenised using the method described below and confirmatory analysis was carried out using GC-MS.

Tablet homogenisation. Tablets were crushed into a fine powder using a mortar and pestle. Approximately 3-4 mg of powder was added to 1 mL methanol in a sample tube and thoroughly mixed by shaking. The solution was left to settle prior to derivatisation.

4.2.5 Fluorescent derivatisation procedure

To 100 µL of the homogenised tablet or target analyte solution, 100 µL each of FITC stock solution and buffer were added in a flat bottomed glass insert (placed inside a crimped 1.7 mL microtube). The microtube was sealed and wrapped in aluminium foil prior to being heated for 3 minutes in a Reacti-therm III heating module (Pierce) set at 90 °C. The derivative was cooled to room temperature and diluted 1:5 in electrolyte prior to analysis. The structures of the target analytes used in this study are presented in Figure 4.1.

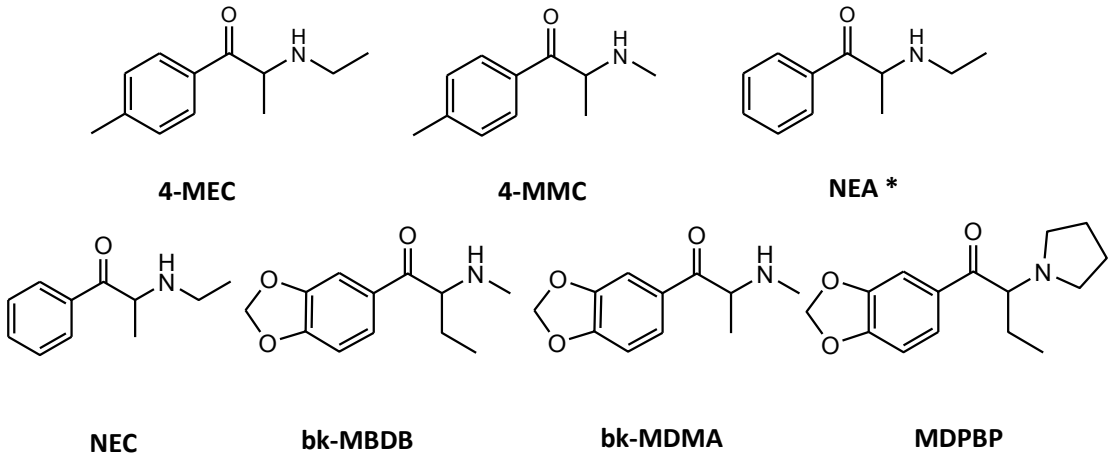


Figure 4.1 The structures of target analogues often present in MEC tablets**.** 4-MEC = 4-methylethcathinone, 4-MMC = 4-methylmethcathinone, NEA = N-ethylamphetamine, NEC = N-ethylcathinone, bk-MBDB = β-keto-3,4-methylbenzodioxlylbutamine, bk-MDMA= β-keto-3,4-methylenedioxymethamphetamine and MDPBP = 3',4'-methylenedioxy-α-pyrrolidinobutiophenone. * NEA, a derivative of amphetamine, is not part of the synthetic cathinone family.

4.3 Results and discussion

4.3.1 Derivatisation procedure

The derivatising agent, FITC, interacts with primary (NH_2) or secondary (NH) amino moieties of analytes to form fluorescent products for analysis by MCE. The generic structure of the synthetic cathinones analysed in this study is presented in Figure 4.2, where R^1-R^4 represent the different functional groups of each derivative.

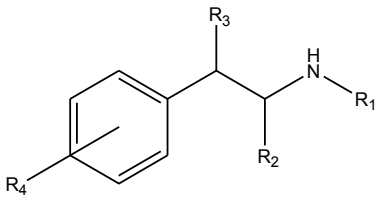


Figure 4.2 Generic ATS derivative chemical structure.

Table 4.1 outlines the structural classification of the target stimulants relative to the generic structure presented in Figure 4.2.

Name	R^1	R^2	R^3	R^4
4-MEC	Ethyl	Methyl	Carbonyl	Methyl
4-MMC	Methyl	Methyl	Carbonyl	Methyl
NEA	Ethyl	Methyl	H	H
NEC	Ethyl	Methyl	Carbonyl	H
β-keto-MBDB	Methyl	Ethyl	Carbonyl	3,4-methylenedioxy
β-keto-MDMA	Methyl	Methyl	Carbonyl	3,4-methylenedioxy
MDPBP	Pyrrolidino	Ethyl	Carbonyl	3,4-methylenedioxy

Table 4.1 Structural classification of the target stimulants. Refer to the ATS derivative structure presented in Figure 4.2.

Figure 4.3 illustrates the fluorescent response obtained for 4-MEC (a secondary amine) and MDPBP (a tertiary amine) relative to a blank response of FITC. The FITC derivative of 4-MEC is shown at approximately 50 seconds with some remaining FITC and by-products at around 30 seconds (labelled as FITC-related). In contrast, no fluorescent derivative is observed for MDPBP, a tertiary (N) amine, due to the absence of a site for FITC attachment and no reaction.[248] A strong fluorescent response can be seen at 29 seconds which corresponds to FITC. This negative response could also be indicative of the presence of another drug/product containing no amino group.

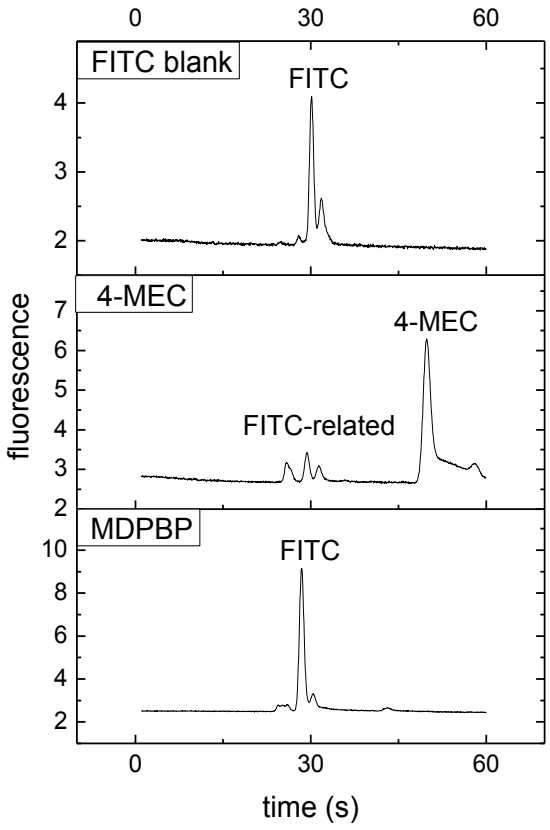


Figure 4.3 Comparison between MCE electropherograms for (a) FITC blank, (b) 4-MEC and (c) MDPBP.

The FITC-related peaks, seen at the beginning of each MCE profile, are most likely present due to hydrolysis, impurities and excess FITC from the reaction. The FITC-related peaks can also contribute to the MCE profile, as different ratios are present depending on the relative amount of constituents present in the tablet. A large response for FITC might indicate the presence of a tertiary amine due to the inactive nature of the nitrogen involved in the substitution reaction. All the target synthetic cathinones in this study are secondary amines due to the presence of the N-H group on the side chain.

4.3.2 Separation of visually different tablets

A previous publication shows the separation of amphetamine-type stimulants using this device.[263] The method was extended to include some frequently observed synthetic cathinones often present in tablets being marketed as *ecstasy*.

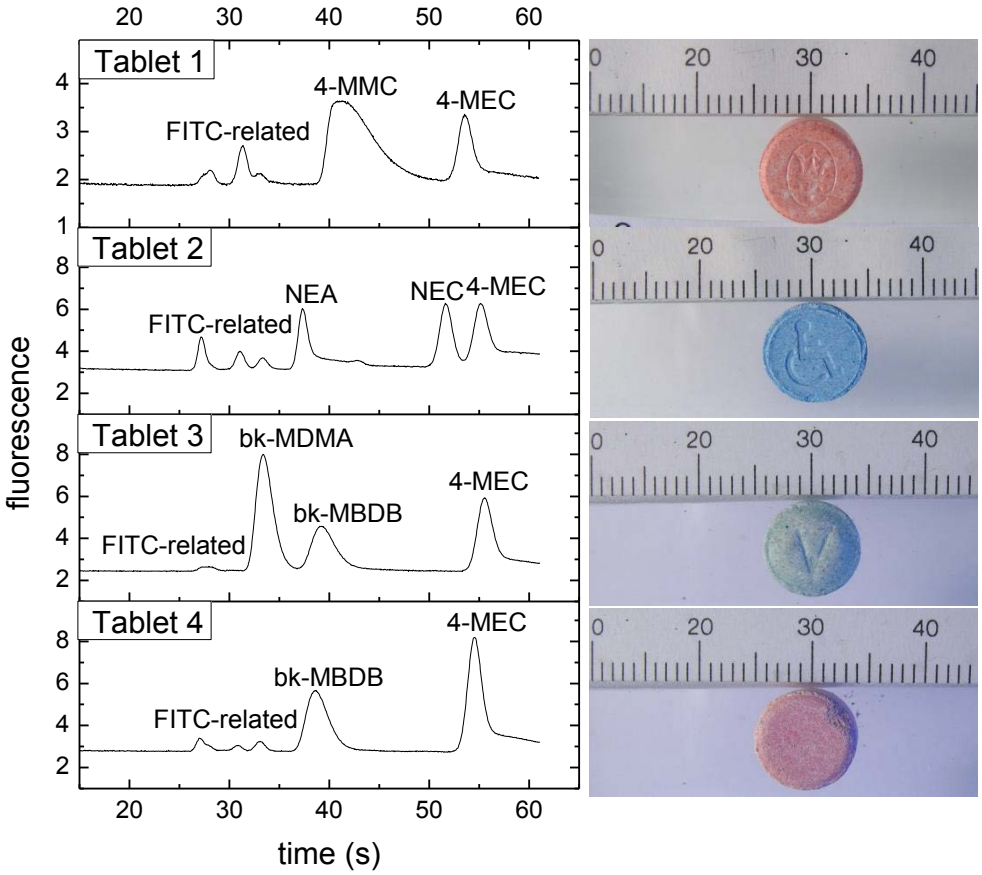


Figure 4.4 Comparison between the MCE profiles of four visually different MEC tablets found to contain different mixtures of synthetic cathinones.

Figure 4.4 shows the separation profiles observed for four visually different tablets (colour, logo etc.), containing MEC mixed with other constituents. The presence of each constituent was confirmed using GC-MS prior to analysis using MCE and spiked standards were used to identify each compound. The MCE profiles in Figure 4.4 represent some of the most frequent synthetic cathinones present in MEC tablets sent to ESR. The response for MEC can be seen at around 55 seconds which is well separated from other tablet constituents. Relative separations and peak heights vary according to the presence of other constituents which can be seen by comparing tablets 1 – 4, shown in Figure 4.4.

The characteristic profile observed for 4-MMC, which can be attributed to the instability of some cathinones, can also be used to identify its presence.[273]

The migration times of tablet constituents can vary chip-to-chip and day-to-day. This can be due to buffer degradation, pH or temperature changes. However, the appearance of the constituent peaks and the relative separation between them (i.e. the overall profile) remains similar among these repeats. Although not necessary, the injection of a standard mixture on the same chip can be used for comparison purposes.

4.3.3 Reproducibility of tablet profiles

As the chemical constituents of visually similar tablets within a given case may vary, the reproducibility of profiles for repeats between samples of a homogenised tablet was examined. The reproducibility of the method was determined by RSDs of peak heights and migration times of all target analytes.

Table 4.2 illustrates the analytical data for 10 tablets, where three aliquots of each tablet were derivatised in duplicate. Most tablets contained MEC, except for the *Wii* tablet. The tablets all contained a number of additional constituents; the peak height ratios illustrate the relative proportions of each constituent. The RSDs within repeats ranged from 1.9 and 5.5 % and 0.4 to 6.0 % for peak height ratios and migration times, respectively.

Tablets were analysed by GC-MS to confirm the tablet constituents prior to analysis by ME.

	Migration times*						Peak heights*			Peak ratios**	
Tablet	**MEC**	**MMC**	**NEA**	**NEC**	**βk-MBDB**	**βk-MDMA**	**1**	**2**	**3**	**1**	**2**
	49.0 (1.25)	38.0 (1.1)	-	-	-	-	4.5 (2.5)	3.8 (3.6)	-	0.84 (2.0)	-
	-	-	35.4 (1.6)	48.7 (1.8)	-	-	7.0 (14.4)	7.5 (15.4)	-	0.93 (3.9)	-
	50.6 (1.5)	-	-	-	36.4 (1.3)		5.1 (7.0)	8.5 (11.8)	-	0.6 (5.4)	-
	54.2 (1.3)	-	38.7 (1.0)	57.9 (1.3)	-	-	6.0 (4.7)	6.3 (5.5)	5.6 (4.4)	0.97 (5.0)	0.90 (1.6)
	50.2 (4.7)	38.7 (3.7)	-	-	-	-	4.7 (10.3)	2.7 (10.4)	-	0.59 (5.5)	-
	58.8 (0.4)	-	-	-	40.9 (0.4)	34.6 (0.2)	8.5 (5.2)	6.6 (4.0)	7.6 (4.8)	0.77 (3.2)	0.89 (4.5)
	55.6 (5.0)	-	37.5 (4.1)	52.3 (5.0)	-	-	6.3 (7.3)	6.6 (9.7)	6.6 (8.1)	0.97 (2.6)	0.95 (2.4)
	54.3 (5.2)	-	36.9 (3.8)	51.2 (5.2)	-	-	5.6 (9.5)	6.6 (9.5)	6.1 (10.2)	0.91 (3.4)	0.85 (1.9)
	53.7 (3.4)	-	-	-	-	-	14.5 (3.6)	-	-	-	-
	53.0 (0.9)	-	-	-	37.7 (0.4)	-	3.5 (3.5)	5.1 (4.6)	-	0.69 (4.9)	-

*Averages are presented with corresponding % RSDs in brackets.*One tablet was analysed from each seizure from which 3 aliquots were derivatised in duplicate, a total of 6 repeats. **Peak height ratio calculated relative to the largest constituent in the mixture.*

Table 4.2 Summary of the migration time and peak height data for 10 tablets.

In this study, the focus was not to calculate amounts of each constituent but determine relative proportions for comparison purposes. Therefore, the sample preparation method was designed to be simple and rapid, eliminating such time-consuming steps as accurate weighing. Approximate samples (3-4 mg) were taken of the homogenised tablets and as expected the % RSDs associated with each individual peak height are large. The peak heights were normalised to the peak height of the constituent with the highest intensity (largest fluorescence response) and the reproducibility achieved was within 5% RSD. For example, for the *Wii* tablet the percent RSD for the peak heights of NEA and NEC were 14.4 and 15.4, respectively. Taking a ratio of NEA/NEC reduces the percent RSD to 3.9. While the concentration of each constituent varies depending on the weight sampled, the relative concentrations are the same across replicates/aliquots, if homogenised.

Two visually different tablets from different seizures *Nike tick* and *New York Yankees* were found to contain the same constituents with similar MCE profiles. Average migration times of 38.0, 49.0 (MMC, MEC) and 38.7, 50.2 (MMC, MEC) were achieved for *Nike* and *New York Yankees* tablets, respectively. A more detailed look at the peak height ratios observed for each tablet indicated some differences. The peak height ratio for the *Nike* tablet was 0.84 and the *New York Yankee* tablet was 0.59. These peak height ratios were consistent across repeats with percent RSDs of 2 and 5.5 for the *Nike* and *New York Yankee* tablets, respectively.

Two visually similar tablets from the same police operation, *wheelchair 1* and *wheelchair 2*, can be compared. These tablets contained the same constituents; MEC, NEA and NEC. Peak ratios for each tablet were very similar being 0.97 and 0.90 compared with 0.97 and 0.95 for *wheelchair 1* and *wheelchair 2*, respectively. These tablets, although seized in different locations in fact belonged to the same case, indicated here by their similar MCE profiles.

4.3.4 Current methods

As mentioned in the introduction, statistical methods are often employed in forensic illicit drug analysis. Arbitrary methods of selecting a sample size from a seizure population are simple and require a manageable number of samples for small seizure populations. For large seizures, however, the sample size can become excessive.[16] In contrast, frequentist and Bayesian statistical approaches are preferred as they are more statistically sound. The frequentist paradigm is more widely accepted in court as the method of inference is based on simple probabilities. Bayesian inference makes better use of available information and effectively reduces analysis requirements. However, the visual assessment of the degree of seizure homogeneity depends on the analyst's interpretation. Background information is also used to determine the sample size based on the analyst's knowledge and experience e.g. the frequency of a sample with a particular visual appearance and what it was previously found to contain.

Some laboratories may opt to use a *bulking* method where one mixture composed of multiple samples is prepared for analysis. Since this method gives an average result for multiple samples it can give a more representative sample of the population but removes the possibility of measuring within-batch variability. This method does not perform well for relatively inhomogeneous seizure populations as individual samples are not characterised.[276]

4.3.5 Applications of MCE as a comparison tool

The use of a simple screening tool would provide a means for analysing a larger number of samples without increasing the burden on forensic laboratories. Analysing a larger number of samples could reduce the likelihood of missing crucial information; particularly for cases where tablets are visually similar but the seizure population is relatively inhomogeneous.

The aim of this method was to analyse one tablet using GC-MS, in which the tablet constituents would be confirmed. Then, as an alternative to current methods, analyse a further number of tablets to sufficiently represent a visually homogenous population of tablets.

Large seizures are made up of numerous individual packets/tablets suspected to contain illicit drugs. Colour tests can be used as a presumptive test prior to confirmatory analysis by GC-MS. MCE is more specific and responds to the presence of a wide range of synthetic cathinones. Furthermore, comparison with the corresponding standard surpasses the specificity of a colour test in determining the nature of tablet constituents.

The majority of drugs of interest include a substituted amine, either NH_2 (primary) or NH (secondary). FITC, the derivatising agent, reacts with the amino group to form a fluorescent derivative. A fluorescence response on MCE indicates the presence of a primary or secondary amine. The migration time of the unknown, previously identified using GC-MS, can be matched to that of a standard. A further number could be screened to indicate whether all tablets/packets respond similarly. A profile may

also be created showing a graphical similarity between tablets/packets based on the number of constituents showing a response, their migration times and peak height ratios with % RSD.

Two realistic scenarios were chosen to show how the discriminating power of ME, along with visual examination and the use of GC-MS as a primary identification technique, has the potential to be used for the more informative forensic analysis of multiple tablets/packets.

4.3.5.1 Scenario 1

A number of different tablets were seized from one location (i.e. same seizure). Three varieties of tablets were present, each visually different. As illustrated in Figure 4.5, tablet 1 was blue, with a wheelchair logo, tablet 2 was white, half-scored with no logo and tablet 3 was red, with a *Coca-Cola* bottle logo. One tablet from each variety was homogenised and analysed using GC-MS. Tablets 1 and 2 were found to contain the controlled drugs NEA, NEC and MEC, however the analysis of tablet 3 confirmed the presence of MEC only. The MCE profiles illustrated in Figure 4.5 were in agreement with GC-MS results. The corresponding analytical data is presented in Table 4.3. For the two tablets containing a mixture of compounds the highest response was obtained for NEC, however slightly different ratios for NEA and MEC were calculated relative to NEC. For tablet 1, the fluorescence response for NEA, NEC and MEC was similar, with ratios of 0.97 and 0.96. However, the ratios for tablet 2 were slightly lower at 0.91 and 0.85 for NEA and MEC, respectively.

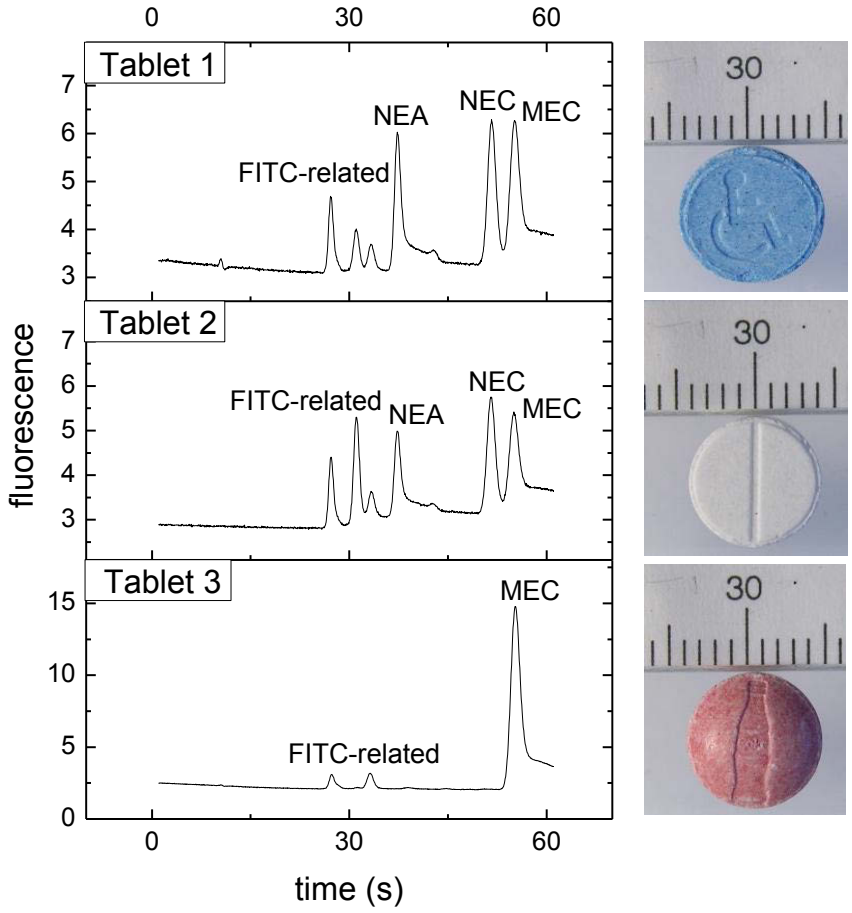


Figure 4.5 MCE separation profiles obtained for 3 tablets from the same seizure with corresponding tablet photos.

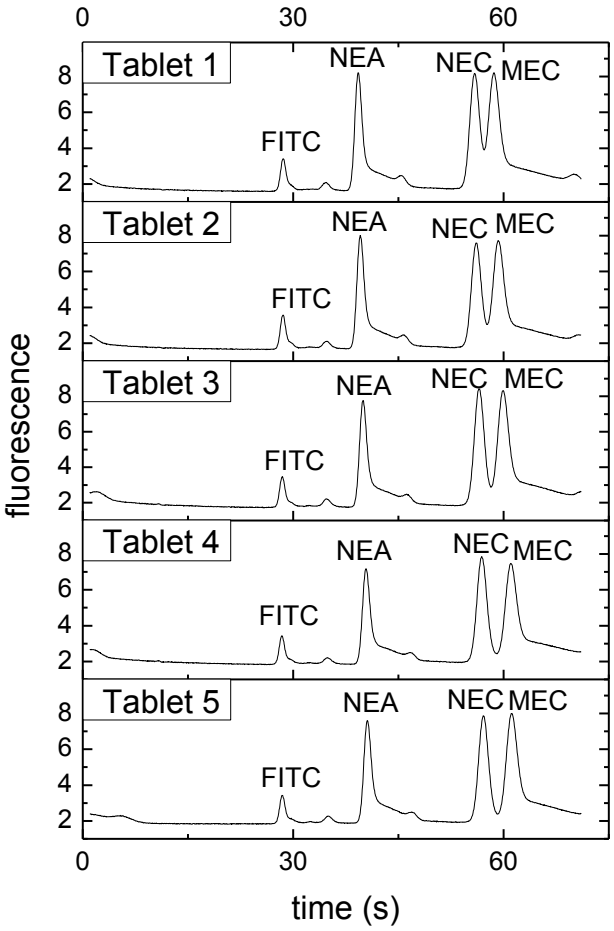
	Migration times			Peak heights			Peak height ratios	
Tablet #	NEA	NEC	MEC	NEA	NEC	MEC	1*[b]	2*[c]
1	37.5	52.3	55.6	6.6	6.6	6.3	0.97 (2.6)	0.95 (2.4)
2	36.9	51.2	54.3	6.6	6.1	5.6	0.91 (3.4)	0.85 (1.9)
3	-	-	53.7	-	-	14.5	-	-

**[a](NEA/NEC), *[b](MEC/NEC). *[c] Data presented as Av (%RSD)*

Table 4.3 Summary of analytical data obtained for 3 visually different tablets from one seizure.

4.3.5.2 Scenario 2

Multiple, visually similar, tablets were seized. As shown in Figure 4.6 (c) all tablets were red with a New York Yankees logo and were of a similar size and thickness. One tablet was homogenised and analysed by GC-MS, which confirmed the presence of MEC, mixed with NEA and NEC. This tablet and the four others from this seizure were analysed using MCE to assess the homogeneity of tablets within this particular seizure. A summary of the profiles obtained for each tablet is illustrated in Figure 4.6 (a). The migration time and peak ratio data is presented in Figure 4.6 (b). The tablets all contain 4-MEC, NEC and NEA in almost the same amounts. The ratios calculated relative to NEC (the constituent with the highest fluorescence response) were 0.98 and 1.01 for NEA and MEC, respectively. This demonstrates the rapid use of MCE to prepare and analyse five tablets in less than 1 ½ hours. Using GC-MS, the same results would have been obtained after 1 day and the cost of analysis would have been significantly higher. It appears that the tablets from this seizure have the same composition, with ratios of 0.98 and 1.01 relative to NEC (largest response) and corresponding % RSDs of 3.7 and 3.4 across the 10 repeats.



a

Migration times			
	NEA	NEC	MEC
Av	39.6	55.9	59.3
%RSD	2.1	2.9	2.9
Peak heights			
Av	7.8	8.05	8
%RSD	3.7	3.5	3.4
Ratio	0.98*	1	1.01*

** 3.6 % RSD*

b

c

Figure 4.6 Summary of the MCE analyses of 5 visually similar tablets from the same seizure (a) MCE profiles (b) Analytical data (c) Photo of the tablets analysed, showing their visual similarities.

4.3.6 Further enhancements for routine use

In order to adapt the Bioanalyzer or any alternative microchip device for routine use, the method could be further automated. Following the analysis of tablets/powders from the same seizure, the data could be fed into a macro, designed to calculate a similarity degree between multiple analyses. The integration of peaks using a software package could be used to improve the accuracy of peak comparison, an alternative to peak heights. The fluorescent derivatisation procedure could be adapted by the integration of a heating device for on-chip derivatisation, which has been previously reported in the literature.[266,267] The use of a portable device, such as the Bioanalyzer, could also be used to help screen bulk seizures at customs or international mail/cargo sites. Although confirmatory analysis is required at the

laboratory, the on-site screening of powders, tablets or liquids could reduce the need for bulk transport of seizure materials. Flexible access to drug exhibits would allow large scale field-testing and enable the evaluation of suggested improvements. The examination of a larger subset of tablets from a wide range of sources is required in order to perform a full method validation.

4.4 Conclusions

The rapid separation of synthetic cathinones present in MEC tablets was demonstrated using the Agilent Bioanalyzer. Reproducible profiles were obtained for each tablet. Using ME, the presence of tablet constituents and their relative amounts is indicated in a cost-effective and timely manner. The presumptive identification of samples from a given seizure population, using the presented method, offers enhanced specificity to colour tests.

Two realistic scenarios were presented to illustrate the use of these profiles in illicit drug case comparisons. The homogeneity of a seizure population using MCE profiles, in conjunction with GC-MS, could be more accurately assessed in comparison to current methods. This method allows for a larger number of illicit drug samples to be analysed without increasing the burden on forensic laboratories which would ultimately enhance the speed of analysis and reporting accuracy. Despite inherent compromises with miniaturisation, such as resolution, this commercial MCE device has proven to be a practical solution to a current problem in forensic drug analysis.

Chapter 5

Concluding remarks and future work

Chapter 5: Concluding remarks and future work

The analysis of forensic drug exhibits plays a significant role in the investigation of clandestine laboratories and drug-related cases. Drug analysis, in particular drug profiling, provides crucial information for investigations and a broader understanding of illicit drug activity. The nature of the activity and/or the identification of sample constituents also contribute to qualify the offence and determine the penalty and/or sentence to be imposed.

The development of portable tools for in-field applications endeavors to reduce the risk of contamination, the need for transportation and the chance of missing important information.

The principle aim of this research was to evaluate the use of the Agilent Bioanalyzer, one type of MCE, for the analysis of ATS. This research has highlighted MCE as a competitive platform for the screening of ATS. The potential use of LOC technology for screening drug-related exhibits was demonstrated for a wide range of sample types.

A timely and cost-effective method for the analysis of ATS was successfully developed using the Bioanalyzer. Following optimisation, the sample preparation procedure consisted of a 3 minute derivatisation with FITC and the separation of ATS was achieved within 1 minute employing MEKC. The use of the Bioanalyzer surpasses the specificity of colour tests as a screening method. The excellent specificity and sensitivity of FITC for primary and secondary amines was demonstrated for a wide range of clandestine laboratory samples. As a screening device it would assist with sample collection and/or the selection of exhibits for further analysis.

Its potential use as a comparison tool for bulk seizures of powder or tablets has also been identified. The Bioanalyzer separated multiple 4-MEC tablet components, which

were very similar in structure, in approximately 1 minute. In comparison to other techniques MCE is capable of detecting minor tablet components. In conjunction with statistical sampling methods of the given jurisdiction (simple random, stratified, multistage or composite)[277] LOC could be used to enhance reporting accuracy. This would be particularly beneficial for laboratories with limited resources.

This project has identified specific in-field applications of the Bioanalyzer for the analysis of ATS in a variety of matrices. It could be adapted as a ready tool for the analysis of ATS and be incorporated in mobile forensic laboratories. These laboratories, which are deployed for large crime scenes, are fitted with several analytical tools to assist in high-volume processing. The presented methods could be further developed into automated analysis schemes. A similar approach to the development of DNA and protein kits could be applied. For example, an ATS kit could be marketed for rapid screening of methamphetamine, ephedrine and pseudoephedrine in clandestine laboratory liquids. Although the Bioanalyzer requires some level of scientific training the software could be adapted for routine use. The automation of data analysis steps would facilitate rapid interpretation by non-scientific users. The cleaning procedure developed to enable the repeated use of microchips could be further enhanced by designing an automated cleaning tool. Research into alternative sample extraction methods, such as solid phase micro-extraction, would further streamline the sample preparation process.

There are specific requirements in terms of size, speed and portability for the use of analytical tools at clandestine laboratory sites. The Bioanalyzer is suitable for the rapid screening of ATS. Certain modifications, however, would accelerate its uptake in the field such as the inclusion of a portable power source and a reduction in sample/reagent handling requirements. Flexible access to drug exhibits would allow extensive testing of the in-field capabilities of the Bioanalyzer.

The fixed nature of the Bioanalyzer's detection system limited the scope of this research. The absorption and emission spectra of analytes must correspond with the wavelength ranges of one of the detectors. As most analytes are not naturally

fluorescent the number of possible derivatisation agents is restricted. FITC was identified as the most suitable derivatising agent for labeling the ATS using the Bioanalyzer. In order to achieve high sensitivity, a limited working pH range of between 7 and 10 was available for the optimisation of analyte separation. The absorption and emission spectra of FITC are pH dependent. At pH 9 complete ionisation is achieved and FITC is in its most fluorescent form, refer to Figure 1.31 (section 1.2.4.3).[247] Many other fluorescent derivatisation agents are commercially available for labeling amino acids which can subsequently be applied for ATS. These reagents often have emission and excitation wavelengths outside the range of the Bioanalyzer's detection system however could be used in the absence of a fixed wavelength detector. For example, 4-chloro-7-nitrobenzofurazan (λ_{ex}= 337 nm, λ_{em} = 512 nm), 3-(2-furoyl)-quinoline-2-carboxyaldehyde (λ_{ex}= 486 nm, λ_{em} = 600 nm) and dansyl chloride (λ_{ex}= 372 nm, λ_{em} = 557 nm).

The microchips employed in this research have a fixed channel design and separation channel length to allow for multiple analyses. A research avenue worth pursuing would be the design of microchips with longer separation channel lengths adaptable to Bioanalyzer platform. For example, microchips with an s-folded channel geometry. With the ability to lengthen the separation channel length, improvements in the resolution of current methods and chiral separations could be achieved. This information is particularly valuable from a forensic intelligence perspective as information regarding the chirality of the drug can be used to indicate the route of synthesis employed. This is one aspect of analysis that could be combined along with other chemical and physical characteristics of the sample to generate strategic or operational intelligence (outlined in section 1.1.7). The derivatisation procedure requires a heating block set at 90 °C which does not lend well to in-field analysis sites beyond mobile laboratories. The integration of a heating device would open up the possibility of performing on-chip derivatisations. On-chip derivatisation procedures have been reported in the literature for other derivatising agents and alternative microchip designs. This adaptation would further increase portability and ultimately its suitability for in-field applications.

Another possible research avenue is to extend the research scope to include other illicit drugs. An investigation into derivatisation reagents that target different functional groups could identify suitable reagents for labeling other drugs or pre-cursors. Although more expensive, the nitrobenzofurazan reagents are particularly useful for labeling amines, ketones and aldehydes. These have also been employed for chiral separations and could be more suitable for this application due to their low molecular weight in comparison to fluorescein reagents. Alternatively, indirect fluorescence should be investigated in order to develop screening methods for other components present in drug-related exhibits.

A unique challenge in designing analytical methods for the analysis of ATS is the burgeoning new designer stimulants. These substances, often analogues of existing controlled ATS, are not easily detected and identified by forensic laboratories. The present challenges that legislators face with the rapid introduction of novel designer stimulants onto the market include: their similarities in chemical structure and pharmacological properties, marketing under disguises such as *bath salts*, the vast number of existing and potential analogues and the lack of research into the harmful effects of each. A more proactive approach to legislative control is required in order to combat the rapidly expanding illicit drug market. In some countries recent measures have been taken including the NZ law change in September 2011 to put the onus of proof on the manufacturers.

The traditional approach involved in the identification of new substances is not feasible. The development of adaptable analytical tools that are able to respond quickly to these new potentially harmful drugs is an important area of research.

A rapid method for the separation of ATS was developed using the Agilent Bioanalyzer platform. Its practical use for screening casework exhibits has identified promising applications in forensic drug analysis. Future research, focused on streamlining the analysis procedure and enhancing the Bioanalyzer's portability, would extend its in-field capabilities. Furthermore, the complementary use of other MCE devices, capable of suggesting the route of manufacture, combined with the

development of a systematic screening approach for exhibits could enable the effective use of rapid information for intelligence purposes. This research has highlighted MCE as a viable platform for screening ATS and identified possible areas of future work that would enhance its capacity for routine use.

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