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Immunological Analysis of Drugs of Abuse with Reference to Anhydroecgonine Methyl Ester

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A thesis submitted in Partial Fulfilment of the requirement of Sheffield Hallam University for the Degree of Doctor of Philosophy

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‘To achieve your goals is to be successful, however, to achieve your dreams is the ultimate success’

Ricky Lankford

Abstract

The field of forensic drug testing is continually changing with analytical methodology being developed for an increasing number of drugs in a variety of biological matrices. The aim of this thesis was to develop a novel enzyme immunoassay (EIA) for screening oral fluid specimens for the presence of anhydroecgonine methyl ester (AEME), a pyrolysis product of cocaine. A confirmatory method was also to be developed to accurately quantify the levels of cocaine, its metabolites and pyrolysis products in oral fluid samples. The immunoassay development was started by synthesising an immunogen using anhydroecgonine (AE) and thyroglobulin. Following immunisation the antisera were screened by enzyme linked immunosorbent assay (ELISA) to enable selection of the antibody with the highest specificity and sensitivity. An enzyme labelled drug was synthesised and the titres of antibody and enzyme were optimised. A series of validation experiments were carried out which concluded that the EIA was sensitive, highly specific, and precise.

Gas chromatography-mass spectrometry (GC-MS) was investigated for the quantitation of cocaine and its metabolites. A temperature program was selected which allowed for the simultaneous analysis of all the analytes. A solid phase extraction (SPE) method was developed to extract cocaine and its metabolites from oral fluid. The SPE method provided high recovery for all analytes apart from the highly polar AE. Degradation of the GC column had a detrimental effect on the analysis of AEME, and so the confirmation method was switched to liquid chromatography-tandem mass spectrometry (LC-MS/MS). A series of LC columns and mobile phases were tested for optimum separation and ionisation. The instrument parameters such as capillary voltage, drying gas temperature, shield voltage, and needle voltage, were optimised. Following a number of validation experiments the method was found to be highly sensitive, precise, accurate and robust.

Both the EIA and LC-MS/MS methods were applied to the analysis of clinical samples from self declared crack cocaine users. The EIA showed good correlation to LC-MS/MS. It was evident that the presence of AEME can positively identify smoking as the route of cocaine administration however its absence does not necessarily mean the individual has not smoked cocaine.

Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
AE	Anhydroecgonine
AEEE	Anhydroecgonine Ethyl Ester
AEME	Anhydroecgonine Methyl Ester
AMP	Amphetamine
APCI	Atmospheric Pressure Chemical Ionisation
BCS	British Crime Survey
BSA	Bovine Serum Albumin
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
BZE	Benzoylecgonine
BZO	Benzodiazepines
CE	Cocaethylene
CEDIA	Cloned Enzyme Donor Immunoassay
Cl	Chemical Ionisation
CID	Collision Induced Dissociation
CNS	Central Nervous System
COC	Cocaine
CV	Coefficient of Variation
DCM	Dichloromethane
DIP	Drugs Intervention Programme
DMF	Dimethylformamide
DTTO	Drug Treatment and Testing Order
ECG	Ecgonine
EDC	1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide Hydrochloride
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrolidine
EEE	Ecgonine Ethyl Ester
El	Electron Ionisation
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
EME	Ecgonine Methyl Ester
EMIT	Enzyme Multiplied Immunosorbent Technology
ESI	Electrospray Ionisation

FN	False Negative
FP	False Positive
FPIA	Fluorescence Polarisation Immunoassay
G6PDH	bacterial glucose-6-phosphate dehydrogenase
GAR-HRP	Goat Anti Rabbit Horse Radish Peroxidase
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GC-MS/MS	Gas Chromatography-Tandem Mass Spectrometry
GHB	Gamma Hydroxy Buturate
HCl	Hydrochloric Acid
HPLC	High performance liquid chromatography
HRP	Horse Radish Peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPOL	Isopropanol
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS-MS	Liquid Chromatography-Tandem Mass Spectrometry
LLE	Liquid Liquid Extraction
LLOQ	Lower Limit of Quantitation
LOD	Limit of Detection
LOQ	Limit of Quantitation
LSD	Lysergic Acid Diethylamide
MAMP	Methamphetamine
MBDB	3,4-methylenedioxyphenyl-2-butanamine
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxy-N-ethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MeOH	Methanol
MES	Morpholinoethanesulfonic acid
MRM	Multiple Reaction Monitoring
MTD	Methadone
NBEZ	Norbenzoylecgonine

NCE	Norcocaethylene
NCOC	Norcocaine
NH3	Ammonia
OPI	Opiate
PBS	Phosphate Buffered Saline
PCP	Phencylclidine
PFP	2,2,3,3,3-pentafluro-l-propanol
PFPA	Pentafluoropropionic Anhydride
POCT	Point of Care Test
RIA	Radioimmunoassay
ROC	Receiver Operator Characteristic
RP	Reversed Phase
SAMHSA	Substance Abuse and Mental Health Services Administration
SD	Standard Deviation
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
SRM	Selected Reaction Monitoring
Sulfo-NHS	N-hydroxysulfosuccinimide
TFAA	Trifluoroacetic Anhydride
Tg	Thyroglobulin
THC	Tetrahydrocannabinol
TMB	Tetramethylbenzidine
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TN	True Negative
TOF	Time of Flight
TP	True Positive

1 Introduction

1.1 Overview of Drug use and Abuse

The term 'drug of abuse' can be defined as any drug that is used for a non-medicinal purpose, due to the desirable effect that particular substance may have.

Drug abuse is a growing problem in the UK and worldwide and places an immense burden on the health, social welfare and criminal justice systems.

The document 'tackling drugs to build a better Britain' was introduced in 1998 and forms the UK governments 10 year strategy for tackling drug misuse. The strategy comprises four key areas i.e. drug prevention, reducing supply, increasing availability of treatment, and reduction of drug related crime. The main focus is on the more dangerous class A drugs, in particular cocaine and heroin [1].

1.1.1 Regulation and Classification of Drugs

In the UK the primary drug legislation is the Misuse of Drugs Act 1971. The Act covers criminal offences such as possession, possession with intent to supply and production of controlled drugs without a licence. The advisory council on the misuse of drugs was established under the Misuse of Drugs Act 1971 to keep under review the drug situation in the UK, to advise the government on the appropriate classification of substances, facilitate treatment and educate the public [2]. Drugs are categorised as Class A, B or C. Class A drugs are considered the most harmful and carry the greatest penalty for offences related to them. Hundreds of drugs are categorised under the Misuse of Drugs Act, Table 1:1 summarises the categories under which the major abused drugs are placed.

Drug Classification	Compound
A	Cocaine
	Diacetylmorphine (Heroin)
	Ecstasy
	LSD
	Magic Mushrooms (if prepared for use)
	Metamfetamine
B	Amfetamine
	Barbiturates
C	Benzodiazepines
	Cannabis
	GHB

Table 1:1 Drug Classifications

The definition of the term drug includes natural, semi-synthetic, synthetic, and designer drugs.

- Natural drugs are the active ingredients or the secondary metabolic products of plants or other living systems.
- Semi-synthetic drugs are derived from natural sources that have undergone a chemical process.
- Synthetic drugs are artificially produced substances which are almost wholly manufactured from chemical compounds.
- Designer drugs are substances whose molecular structure has been modified in order to optimise their effect [3].

Plant derived substances such as cannabis and cocaine, and semi-synthetic substances such as heroin are the most prevalent illegal drugs abused in todays society. However the United Nations drug control programme believes that synthetic drugs such as

amfetamine, metamfetamine, 3,4-methylenedioxymethamfetamine (MDMA) and other related designer drugs are likely to pose more of a social problem in the future [4].

1.1.2 Drug Use Statistics

The British crime survey is a large nationally representative survey of adults living in private households in England and Wales. Since 1996 the survey has included a self completion module of questions related to illicit drug use [5]. According to the 2000 British crime survey; a third of 16-59 year olds in England and Wales have tried illicit drugs in their lifetime whilst the proportion of those using such drugs in the last year and month were 11% and 6% respectively [6], these figures remained stable for 2004/05 [5]. Cannabis has consistently been the most commonly consumed drug across all age groups. In 2000 over 20% of 16-29 year olds reported its use within the last year, followed by cocaine at 5% and heroin at 1% [6],

Younger age groups reported higher levels of drug use, and men were far more likely than women to have used any illicit and Class A drugs in the last year [5]. In general the age of first use of drugs is decreasing and individuals are experimenting with a wider range of drugs [2].

There were 1,623 drug related deaths reported in England and Wales in 2001, as well as related deaths and substantial ill health arising from blood borne viruses such as hepatitis and HIV [7].

1.2 Forensic Toxicology

Forensic toxicology is defined as the study of the effects of drugs and poisons on human beings and the investigation of fatal intoxications for the purpose of a medico-legal

enquiry [8]. Forensic toxicology can be separated into three separate areas, postmortem forensic toxicology, human performance toxicology, and forensic drug testing.

1.2.1 Postmortem Toxicology

Postmortem toxicology is used to determine whether alcohol, drugs or other poisons may have caused or contributed to the death of a person [9]. While the analytical techniques employed are the same for all forms of forensic toxicology, the time spent on sample preparation is greater in postmortem toxicology due to the nature of the samples. The specimens available for analysis in postmortem cases can be extensive. In cases of recent death they can include blood, urine, hair, vitreous humor, bile, liver, and on occasions lung, stomach contents, or spleen [10]. Alternatively with cases involving a decomposed body the only specimens available are likely to be hair, muscle and bone [9]-

Interpretation of results is more difficult in postmortem toxicology, the measured drug concentration may not accurately reflect the concentration at death as some drugs have been known to redistribute after death [11], in addition, some drugs are unstable and are metabolised postmortem [12].

1.2.2 Human Performance Toxicology

This area of forensic toxicology is concerned with the role of drugs in the modification of human behaviour. The behavioural toxicologist not only focuses on the effects of illicit drugs but also on the effects of correctly or incorrectly administered licit drugs. The metabolic profile of the drug is important in the evaluation of behavioural effects, the presence of particular metabolites may provide some information on the time of drug consumption [10]. The specimen most commonly used in human performance toxicology is blood, however, other specimens such as urine, sweat and oral fluid are

becoming increasingly popular. In general, human performance toxicology is applied to transport services and road safety [13].

1.2.2.1 Drugs and Driving

In the UK, The Road Traffic Act 1991 has a number of provisions relating to the use of alcohol or drugs whilst in charge of a motor vehicle [2]. Driving under the influence of drugs or alcohol is responsible for thousands of accidents every year and up to 25% of accidents involve drivers who test positive for the presence of drugs [14].

Legislating for driving under the influence of drugs is more difficult than legislating against alcohol as less is understood about exactly how drugs impair driving. Unlike alcohol there is no well established correlation between drug concentration in the blood and performance impairment [15].

Many roadside testing studies have been carried out on individuals suspected of being under the influence [16-20]. These studies have shown that cannabinoids and the amfetamine group are the most frequently detected drugs at the roadside. Single drug consumption was the most common with 61% of those tested confirming positive for at least one drug and 18% confirming positive for at least two drugs [16]. A recent roadside study in Glasgow where drivers were stopped on a random basis found that 16.8% of the 1396 specimens tested were positive, with MDMA being the most frequently encountered drug [21].

Questionnaires completed following voluntary roadside testing of 1000 people showed that 2.8 % of respondents admitted to having driven despite being under the influence of an illegal drug [18]. One study found that 95% of drug addicts questioned reported to have driven at least once within an hour of consuming heroin and other illicit drugs, while 65% admit driving daily while under the influence [22].

1.2.3 Forensic Drug Testing

Forensic drug testing is carried out to demonstrate the use or abuse of selected drugs in many areas such as the criminal justice system, military, public sector and private sector. Other areas include sport, the insurance industry, and hospitals. The use of drug testing in the workplace and criminal justice sector will be discussed further.

1.2.3.1 Workplace Drug Testing

Workplace drug testing is a well established procedure in the United States, beginning in the 1970's to prevent the spread of drugs amongst the US military forces during the Vietnam War. Drug screening was initiated following an accident on the naval carrier Nimitz which revealed that a number of military personnel were taking mind-altering drugs [23]. In 1986 an executive order was issued which stated that those federal employees in safety and security conscious positions were to be tested for drugs [24]. This resulted in the production of the mandatory guidelines for drug testing of federal employees now referred to as the Substance Abuse and Mental Heath Services Administration (SAMHSA) guidelines [25]. Testing can be undertaken on a random basis, following an accident, prior to promotion and 'for cause'.

The United Kingdom established similar workplace drug testing programmes within the armed forces and the transport industry approximately 15 years ago and workplace drug testing now has an estimated turnover of £12 million [24]. Workplace drug testing appears to be more widespread in the UK than in any other European country with the highest proportion of drug tests being carried out for the military [26].

1.2.3.2 Criminal Justice

The link between substance misuse and crime is well established, however what is less clear is whether the crime is always as a direct result of drug use or if the individual would have been an offender even without drugs [2].

In response to the 2000 British Crime Survey over 30% of people in England and Wales viewed drugs as a serious problem in their area [27]. This included instances of anti-social behaviour associated with drug dealing and also the activities of those individuals under the influence of drugs, such as robbery and organised crime, as well as the health risks of discarded syringes.

A strong link has been identified between drug misuse and crimes such as shoplifting, burglary, vehicle crime, and theft. Approximately 50% of these crimes can be linked to the use of heroin, cocaine and crack cocaine. Around 75% of crack cocaine and heroin users claim to be committing crime to maintain their habit [27].

Males are more likely to commit crimes such as supplying drugs, burglary, robbery and theft whilst females are more likely to shoplift, undertake fraud or solicit [2].

In 2003 the UK Home Office launched the drug interventions programme (DIP) which recognises that drug using offenders need to be monitored throughout every point in the criminal process from arrest to release from custody [28], as well as aftercare and resettlement [29]. As a result of the DIP a large proportion of police stations across England and Wales carry out a drugs test on individuals charged with the offences associated with illegal drug use. The testing is currently performed on-site using an immunoassay based point of care device for the presence of cocaine and heroin in oral fluid. If a positive result is obtained and contested the sample is confirmed by gas chromatography mass spectrometry (GC-MS). A positive result will not bring about a prosecution for unlawful possession or consumption, or even to support further police

investigation into the offence, it will however be used in court to aid sentencing. The individual may be referred into treatment and the courts can chose to pass the sentence of a drug treatment and testing order (DTTO). Between 2001 and 2003 a total of 17,596 tests were carried out of which 24% were positive for opiates, 12% for cocaine and 18% were positive for both [30].

Enrolment in treatment programmes produces a recognised reduction in acquisitive crime, with levels dropping by half after one year [31]. Treatment appears to be cost effective, the Home Office estimates that for every £1 spent on drug treatment at least £9.50 is saved in crime and health costs [27].

1.3 Analytical Methodology

Drugs of abuse testing usually includes a two step procedure, a preliminary screening procedure for groups of substances and a confirmation procedure for the identification of specific substances. The analytical strategy adopted is to a certain extent dependent on the potential consequences of the results obtained. Generally in the therapeutic care of drug addicts the analytical result is only one factor in the decision making process and is often a complement to self reported use. In this setting screening may be performed without subsequent confirmation of the positive samples. By contrast in a forensic setting (e.g. workplace, driving, criminal justice, and sports) the analytical result can have significant repercussions for the individual providing the specimen. In this situation the analytical approach is often screening of all samples followed by confirmation of the positives [32, 33].

Forensic identification of an analyte requires the use of two techniques that employ different physical and chemical principles [9]. Immunoassays are recommended for screening purposes and chromatographic methods coupled to mass spectrometry are required for identification [34].

1.3.1 Screening

Screening for drugs of abuse is primarily performed using immunoassays [32]. Immunoassays are sensitive analytical methods based on signal responses generated as a consequence of antibody-antigen reactions, the signal is generated from a label attached to either the antigen or the antibody, or in some assays from a secondary antibody [35]. Immunoassays offer a quick and easy method to screen large numbers of samples in a whole range of biological specimens. The sample does not require extraction of the drug prior to analysis [36], and only small sample volumes are required [37].

Immunoassays may be used as a point of care test for the analysis of a single specimen, or as a laboratory based test where it can be partially or fully automated enabling the analysis of thousands of samples per day.

1.3.1.1 Laboratory Based Immunoassay

A number of different laboratory based immunoassay methods are available for drugs of abuse including radioimmunoassay (RIA) [38-40], enzyme multiplied immunoassay technique (EMIT®) [41], fluorescence polarisation immunoassay (FPIA), cloned enzyme donor immunoassay (CEDIA) [42], and enzyme immunoassay (EIA) [43-45]. The differences between the assays relate to the type of labelled compound employed or the method of detection. All the immunoassays mentioned are used routinely in laboratories worldwide, the different techniques show reasonably comparable results [38, 41, 42, 44, 46]. The choice of immunoassay is therefore dependent on the requirements of each laboratory.

EIA has been reported as being the main immunological technique employed across Europe for drug testing [32]. Its increasing popularity is due to the ease of use, rapid turnaround of results, low sample volume, and the adaptability for use with various

biological matrices [47]. The technique has been used to measure qualitatively and semi-quantitatively for dmgs of abuse in hair [48-50], oral fluid [51-53], urine [54], meconium [55], blood [47] and sweat [56].

1.3.1.2 Point of Care Testing

Point of care testing (POCT) is defined as testing that is performed close to the patient. Important considerations for POCT are the fact that the test can be carried out virtually anywhere [57], there are no issues with chain of custody [58], and the patient can engage in immediate discussion of the results [33]. POCT is not suitable for high throughput screening as the devices lack automation.

POCT devices for drugs of abuse are generally based on lateral flow technology which use particles, such as colloidal gold, as the means of signal generation [59]. The devices can be in the form of cards, cassettes, dipsticks or cup devices. Often multiple drug lines are employed in the test making drug testing cheaper and quicker than assaying one drug at a time. Some manufacturers have developed an instrument to interpret the test result, taking away the individual subjectivity from visual interpretation.

This technique is well established in urine drug testing and a great number of devices are commercially available. In recent years the interest in the use of alternative biological matrices has seen the emergence of such devices for the application of oral fluid and sweat. As the technology is in its relatively early stages new devices are continuously appearing on the market, with some existing devices evolving or even becoming discontinued. Table 1:2 lists the point of care tests currently commercially available for the analysis of drugs of abuse in oral fluid.

Table 1:2 Summary of oral fluid point of care testing devices currently available for drugs of abuse

1.3.2 Confirmatory Analysis

The gold standard in forensic substance identification is considered to be GC-MS [1]. A study of the different analytical methodologies employed in drug of abuse testing laboratories across Europe showed that GC-MS was the main analytical method used for specific identification and quantification of drugs [32].

Mass spectral information of many compounds analysed by GC-MS have been compiled into mass spectral libraries, this allows for identification of unknown compounds in a sample following full scan analysis. However since GC-MS is limited to the analysis of volatile and thermally stable compounds, there is also a requirement for many polar compounds to be derivatised which extends the sample preparation time [60,61],

Liquid chromatography coupled to single stage or tandem mass spectrometry (LC-MS, LC-MS/MS) is becoming increasingly used in routine analysis [80, 81]. LC-MS is a powerful technique which has high sensitivity and specificity. It requires less sample volume which is an advantage when dealing with alternative biological samples, and does not require derivatisation of the sample prior to analysis thereby reducing the sample preparation time. In the areas where LC-MS has been used, the limits of detection or quantitation obtained are generally compared favourably with the other chromatographic techniques [82],

Limitations in the use of LC-MS(/MS) are that there are only certain volatile buffers and mobile phase additives which can be used. In addition the mass spectral libraries for electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are limited compared to electron ionisation (El) making identification of unknown compounds more difficult [36].

1.3.3 Sample Preparation

The main aims of sample preparation prior to analysis by GC-MS and LC-MS are to isolate and concentrate the relevant compounds from a complex sample matrix while removing any unwanted substances. Interfering compounds within biological samples are often a source of imprecision and inaccuracy [83].

A number of sample preparation techniques exist but the ones more commonly used in the analysis of drugs of abuse include liquid-liquid extraction (LLE), solid phase extraction (SPE) and solid phase microextraction (SPME).

SPE is becoming more popular for sample pre-treatment due to the possibility of high throughput automation, and also due to the increased commercial availability of innovative SPE sorbents. It offers the advantage of cleaner extracts, speed and reproducibility, and ease of operation [84]. Whole selections are marketed from polar to non-polar, mixed mode, ion exchange, polymeric and combinations of ion exchange and polymeric [85].

Mixed mode columns utilise a packed bed containing a mixture of non polar short alkyl chains (Cg) and strong cation exchange resin. These types of column are good for drugs of abuse as the acidic and neutral drugs are retained by hydrophobic interactions with the alkyl chains and the basic drugs by interactions with the cation exchange groups [83, 86, 87].

1.4 Analytical Cutoff Concentrations

It is common practice in drugs of abuse analysis to apply a cutoff value at which all samples with a concentration below the cutoff will be assigned a negative result and all those above will be classified as presumptive positives [88].

The UK guidelines for legally defensible workplace drug testing outline proposed cut off concentrations for screening and confirmation of drugs of abuse in urine, these are

displayed in Table 1:3 and 1:4 [89]. There are however currently no guidelines in the UK, or the rest of Europe, for the assignment of cutoff values in the analysis of drugs of abuse in alternative biological matrices.

The US mandatory guidelines for federal workplace drug testing programmes established cutoff concentrations for specific drugs of abuse in urine, oral fluid, hair and sweat (Tables 1:3 and 1:4) [25, 90]. These guidelines have been adopted by many manufacturers and laboratories worldwide, and have provided some uniformity in drug testing procedures [32]. However as they are not mandatory in Europe there are significant differences in cutoff concentrations applied to screening and confirmatory methods [62].

Drug	**Screening Cutoff in Urine (ng/mL)**		**Screening Cutoff in Oral Fluid (ng/mL)**
	UK	**US**	**US**
Amfetamines	300	1000	50
Cannabis	50	50	4
Cocaine	300	300	20
Opiates	300	2000	40
Phencyclidine	25	25	10
Methadone	300	None	None
Benzodiazepines	200	None	None
Barbiturates	200	None	None
LSD	1	None	None
Buprenorphine	5	None	None

Table 1:3 Proposed screening cutoff concentrations for urine and oral fluid in the UK and US

Drug	Confirmation Cutoff in Urine (ng/mL)		Confirmation Cutoff in Oral Fluid (ng/mL)
	UK	US	US
Amfetamines	200	500	50
Cannabis	15	15	4
Cocaine	150	150	8
Morphine	300	2000	40
6-Acetlymorphine	10	10	4
Phencyclidine	25	25	10
Methadone	250	None	None
Benzodiazepines	100	None	None
Barbiturates	150	None	None
LSD	1	None	None
Buprenorphine	5	None	None

Table 1:4 Proposed Confirmation Cutoff Concentrations for Urine and Oral Fluid in the UK and US

1.5 Drug Detection Times

The length of time a drug can be measured in a sample since the drug was last consumed is known as the detection time. There are a large number of factors which influence the detection time of drugs in a biological matrix such as, the rate of absorption, metabolism, and excretion of a drug. Other factors to consider are the administered dose, whether it is single or repeated dosing, the time of drug consumption in relation to the time the test was performed, and the route of drug administration. No one rule applies for all when it comes to drug detection times, it can, however, be generalised that the higher and more frequent the dose of drug the longer the drug will

be detected. Detection times can also vary depending on the biological matrix being tested, as shown in Figure 1:1.

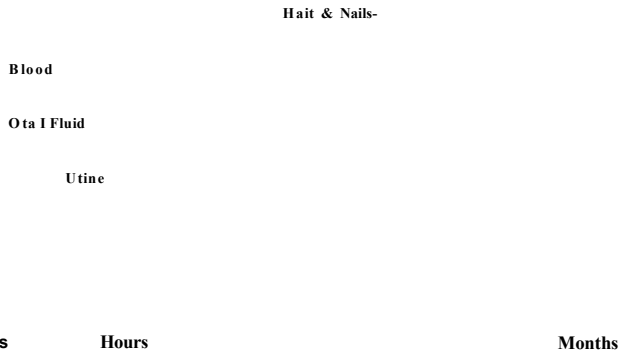

Figure 1:1 Drug detection times in different biological matrices [91]

1.6 Drug Testing Matrices

Traditionally the biological samples used for the qualitative and quantitative measurement of drugs in living subjects are blood and urine. Recently there has been a growing interest in the use of alternative matrices, such as saliva (oral fluid), hair, nails, and sweat. Meconium has also been used for the identification of foetal exposure to drugs [55, 92-95], The use of each matrix has its own advantages and disadvantages. No single technique or specimen can provide answers to all toxicological questions. It may be necessary to use more than one of these matrices in combination to give a better profile of an individual's drug exposure.

1.6.1 Urine

Mandatory workplace drug testing guidelines in the UK use urine as the chosen specimen for analysis of drugs of abuse [89], Urine has the advantage of being easy to collect and test, is cost effective, and also provides a more than adequate volume of sample for repeat analysis and confirmation if required.

Higher concentrations of the parent drug or metabolites are present compared to some other matrices. This is, however, dependant on the volume of liquid consumed by the individual, their degree of hydration at the time of drug ingestion and the amount of drug consumed.

A positive urine test does not necessarily mean that the subject was actually under the influence of the drug at the time of testing, it is merely a measure of the accumulated concentration of analytes since the last void of the bladder.

Disadvantages to using urine are the ease in which samples can be tampered with or adulterated. A donor may substitute his or her sample for a sample from a drug free individual, or they may try diluting their own sample with water or even adding substances to alter the test results [96]. It is for these reasons that it may be necessary to observe a urine specimen being given. This is not a dignified experience for the person observing or the person being tested, and is seen as an invasion of the individuals privacy.

1.6.2 Sweat

The mechanism for drug entry into sweat is unclear but it is most likely due to passive diffusion from the blood to the sweat gland [97]. Sweat consists of 99% water and can be collected by applying an absorbent patch to the surface of the skin. The patch can be left for several hours to give evidence of relatively recent use, or weeks to give an accumulative result. The disadvantages include high intersubject variability, and the potential for false positive results due to external contamination of the skin.

There is a variable time delay of 1-12 hours after drug administration before the drug is excreted in sweat, this makes sweat less of an appropriate matrix in detecting recent drug exposure [98].

1.6.3 Nails

Drugs remain in nails for an extended period of time which is advantageous in determining individuals' past drug exposure, but does not allow for determining recent drug use. There is a limited amount known about this aspect of drug testing [99, 100], with different mechanisms proposed for the incorporation of drugs into nails. Drug concentrations have shown to be higher in fingernails compared to toenails, the possible reasons could be the differences in blood supply to the nail bed, the rate of nail growth, or possible external contamination [93].

1.6.4 Hair

Hair has been widely evaluated as a drug testing matrix [48, 101-103] and offers a unique perspective on drug use. Hair gives a retrospective indication of drug use in the weeks or months preceding sampling. Sample collection is non invasive, unless head hair is unavailable, and there is the possibility of collecting a second almost identical specimen on another day for further testing. Given that it is not too long after collection of the first sample the two specimens will give almost identical profiles.

There are various theories as to how drugs become incorporated into the hair matrix, which include via the blood, sweat and sebaceous gland secretion and/or from the external environment [49, 102, 104]. It is generally assumed that scalp hair grows at approximately 1cm per month, but a wide range of growth rates have been reported [105].

There are many concerns and questions regarding the use and interpretation of hair testing. There is the question of drug stability once incorporated, problems with suitable specimen preparation for analysis, a lack of acceptable standards for reference material, bias due to hair colour or ethnic origin, value and validity of segmental analysis, dose

versus concentration relationship and acceptable analytical cut off concentrations [104, 106].

However one of the most controversial topics with respect to the validity of hair testing is the risk of false positive results due to external contamination [107-109].

1.6.5 Oral Fluid

Compared to urine or hair testing, oral fluid testing has lower refusal rates and is in general a more accepted sampling matrix [110]. Oral fluid has many advantages over other testing matrices as a diagnostic specimen. It is readily available, and multiple specimens may be collected from the same individual at the optimum time for diagnostic information. The sample can be collected non-invasively and, in contrast to collecting a blood sample, allows a specimen to be obtained from populations for whom it would normally be unethical to collect a blood sample, such as certain elderly patients, specific religious groups, and individuals with a mental or physical handicap. There is also the reduced risk of infection when collecting an oral fluid sample as there is no use of sharps and therefore no concerns over needle stick injuries [111]. Observed collection does not give rise to the privacy issues associated with urine collection.
Oral fluid is difficult to adulterate, the oral fluid in the mouth is rapidly turned over and a wait of approximately 10 minutes prior to collecting a sample allows re-equilibration of the oral cavity [68].

Disadvantages associated with oral fluid sampling are the limited sample size compared with urine [112]. Dry mouth syndrome is a common problem and can be caused by anxiety, lack of proper hydration, or the use of drugs known to reduce secretion of oral fluid such as the amfetamine group [113].

In general drug concentrations are lower in oral fluid than in urine and therefore more sensitive assays are required for their detection. However the oral cavity can become contaminated with drugs administered orally or through inhalation or insufflation, which can result in artificially elevated drug concentrations [114].

Many drugs are bound to plasma proteins, but only the free, lipid soluble, non ionised fraction is able to cross cell membranes and is physiologically active. Drug concentrations in oral fluid therefore generally reflect the free unbound parent drug and lipophilic metabolites circulating in the blood [115]. Studies involving paired oral fluid and blood samples have shown good correlation in analytical findings [20]. This means that oral fluid is a good specimen for detecting drug involvement in driving behaviour, or impairment of performance as there is a greater chance that the subject is experiencing pharmacological effects of the drug at that time [20].

In recent years there has been considerable interest shown in the use of oral fluid as a matrix for drug monitoring in treatment, criminal justice and driving under the influence testing programmes [68, 98, 116]. Oral fluid is being considered by SAMHSA as an alternative to urine for federal workplace drug testing programmes [25].

1.7 Physiology of Oral Fluid

The term oral fluid is preferred over saliva due to the definition of saliva being the secretion of the salivary glands, oral fluid therefore refers to the fluid contained within the oral cavity which consists of saliva and a mixture of other components such as mucosal transudate and crevicular fluid [80].

Oral fluid contains water (99%), mineral salts, proteins such as mucins and enzymes for digestion. Cell debris from the epithelial cells of the mouth and food residues are also

present. The composition of oral fluid can be affected by the time of day, food ingested, age, gender, health, and drug consumption. Between 0.5 and 1.5 L of saliva is produced daily in adults, this production being mainly from the secretions of the submandibular (65%), parotid (23%), and sublingual (4%) glands. The remaining 8% is produced from minor glands [115].

Mechanical and or chemical stimulation provokes a reflex response which in turn is controlled by the parasympathetic and sympathetic nerves resulting in the secretion of oral fluid. Drugs that stimulate the parasympathetic nerve increase the flow rate of oral fluid and therefore produce quite an aqueous solution. However stimulation of the sympathetic nerve results in the production of viscous and less abundant oral fluid, for example in the case of amfetamine use [115]. Flow rates may also depend on a person's age and also seasonal influences.

1.8 Collection of Oral Fluid

Oral fluid can be collected with or without stimulation. Unstimulated oral fluid is best collected by expectoration into an empty tube [117]. Sour candy and citric acid crystals have been used to stimulate salivation, alternatively a number of products exist to stimulate and collect oral fluid. Table 1:5 lists the oral fluid collection devices currently commercially available. These devices provide a relatively clean specimen in comparison to expectoration which may contain cell debris and/or food particles.

The most commonly used method to collect oral fluid involves the use of absorptive materials. Some collectors contain a sample adequacy indicator to show sufficient sample has been collected. Some devices also incorporate a proprietary buffer to which the collector is added resulting in a dilution of the sample.

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The recovery of drugs from some of the collection devices may limit their usability. A study which assessed the overall performance of a selection of oral fluid sampling devices showed that recovery of THC was poor for the Salivette® but good for the Cozart® collector [118].

Stimulation of oral fluid production by citric acid, chewing gum or other agents changes the pH of the oral fluid. This has been shown to lower analyte concentrations in the oral fluid compared to unstimulated collection methods [113, 119]. A possible explanation is a pH change associated with a change in flow rate, the mechanism of which is discussed later.

1.9 Transfer of Drugs into Oral Fluid

Drugs circulating in the blood are believed to pass into the salivary ducts by various mechanisms such as passive diffusion, active transport against a concentration gradient, ultrafiltration through pores in the membrane, or by pinocytosis [130]. Drugs also enter oral fluid directly from contamination of the oral cavity from smoking, nasal insufflation, sublingual tablets, or oral dosing [131].

The presence of drug in oral fluid is influenced by the physiochemical characteristics of the drug molecule such as molecular size, degree of protein binding, degree of ionisation, and lipid solubility [117, 132], Other factors include the plasma and saliva pH, and the saliva flow rate [133].

Passive diffusion is believed to be the major mechanism of drug transport, therefore smaller, non ionised and lipophilic molecules will diffuse more rapidly [117]. Due to their size, serum binding proteins do not cross the cell membrane therefore only the unbound fraction of the drug diffuses across into oral fluid.

The effect of pH has a significant influence on the concentrations of drug and metabolites in oral fluid. With regards to the degree of ionisation there are two drug groups. The first group comprises compounds which are largely non ionised under physiological conditions. These drugs are either neutral, have a pKa value greater than 8.5 or a pKa value less than 5.5, for this group the pH of the saliva will have little effect on the drug concentration. The second group consists of drugs which are largely ionised under physiological pH conditions, and will therefore be influenced by the pH of the saliva [134].

The pH of blood is approximately 7.4 and the pH of oral fluid is between 6 and 7 [135]. Drugs accumulate on the membrane side whose pH value favours a greater ionisation of the compound [136]. Acidic drugs tend to be lower in concentration and basic drugs tend to be higher in concentration in oral fluid than in blood. However if the salivary pH is higher than the blood pH then the reverse is true [130].

These rules are only representative of drugs entering saliva from plasma and do not take into account drug present due to contamination of the oral cavity from smoking or insufflation etc.

An increase in saliva flow from either chemical or mechanical stimulation increases the bicarbonate electrolyte concentration resulting in a higher pH value for saliva [137]. The pH of stimulated saliva can be as high as 8.0 [133].

Some oral fluid collection products use citric acid impregnated onto the collector to stimulate oral fluid, this can substantially decrease the pH of oral fluid to less than pH 3.0 [138]. The concentrations of certain drugs such as cocaine and its metabolites are subsequently reduced in the oral fluid compared to samples collected by expectoration [114]. It has been reported that the mean pH of oral fluid specimens collected using the citric acid Salivette® (pH 2.8) was lower than that observed after citric acid candy

expectoration (pH 4.3) while the mean pH of specimens collected with the neutral Salivette® was pH 6.0. Substantial inter-subject variability in pH (2.79 – 7.18) was observed in drug free oral fluid specimens collected using the citric acid treated Salivette® [128].

1.10 References

1. Bunn, S., *Drug Tests.* 2004, Parliamentary Office of Science and Technology, p. 1-4.
2. Payne-James, J., *Substance Misuse and the Legal System in England and Wales.* Journal of Clinical and Forensic Medicine, 2001. 8: p. 7-16.
3. Interpol, *Synthetic Drugs.* 2005, www.interpol.int/public/drugs/synthetic.
4. King, L.A., *The Misuse of Drugs Act - A Guide for Forensic Scientists.* RSC Paperbacks. 2003: The Royal Society of Chemistry.
5. Roe, S., *Drug Misuse Declared: Findings From the British Crime Survey 2004/05.* Home Office Statistical Bulletin, 2005. 16/05.
6. Ramsay, M., et al., *Drug Misuse Declared in 2000: Results from the British Crime Survey.* Home Office Research Study, 2001. 224.
7. Statistics, N., *Report: Deaths Related to Drug Poisoning: Reults for England and Wales, 1997-2001.* 2003. p. 65-71.
8. Anderson, R., *Forensic Toxicology,* in *Crime Scene to Court: The Essentials of Forensic Science,* P. White, Editor. 1998, Royal Society of Chemistry, p. 232-262.
9. Jones, G., *Postmortem Toxicology,* in *Clarkes Analysis of Drugs and Poisons,* A.C. Moffat, M.D. Osselton, and B. Widdop, Editors. 2004, Pharmaceutical Press.
10. Levine, B., *Principles of Forensic Toxicology.* 1999: American Association for Clinical Chemistry Inc.
11. Elliott, S.P., *MDMA and MDA Concentrations in Antemortem and Postmortem Specimens in Fatalities Following Hospital Admission.* Journal of Analytical Toxicology, 2005. 29: p. 296-300.
12. Drummer, O.H. and J. Gerostamoulos, *Postmortem Drug Analysis: Analytical and Toxicological Aspects.* Therapeutic Drug Monitoring, 2002. 24: p. 199-209.
13. Goldberger, B.A. and A. Polettini, *Forensic Toxicology: Web Resources.* Toxicology, 2002. 173: p. 97-102.
14. Kelly, E., S. Darke, and J. Ross, *A Review of Drug Use and Driving: Epidemiology, Impairment, Risk Factors and Risk Perceptions.* Drug and Alcohol Review, 2004. 23: p. 319-44.

15. Logan, B.K. and F.J. Couper, *3,4-Methylenedioxymethamphetamine (MDMA, Ecstasy) and Driving Impairment.* Journal of Forensic Sciences, 2001. 46: p. 1426-1433.

16. Steinmeyer, S., et al., *Practical Aspects of Roadside Tests for Administrative Traffic Offences in Germany.* Forensic Science International, 2001. **121:** p. 33-36.

17. Biermann, T., et al., *On-Site Testing of Illicit Drugs: The Use of the Drug-Testing Device 'Toxiauick'.* Forensic Science International, 2004. 143: p. 21-25.

18. Behrensdorff, I. and A. Steentoft, *Medicinal and Illegal Drugs Among Danish Car Drivers.* Accident Analysis and Prevention, 2003. **35:** p. 851-860.

19. Verstraete, A.G., *Oral Fluid Testing for Driving Under the Influence of Drugs: History, Recent Progress and Remaining Challenges.* Forensic Science International, 2005. **150:** p. 143-150.

20. Toennes, S.W., et al., *Screening for Drugs of Abuse in Oral Fluid - Correlation of Analysis Results with Serum in Forensic Cases.* Journal of Analytical Toxicology, 2005. 29: p. *22-21.*

21. Wylie, F.M., et al., *Drugs in Oral Fluid Part II: Investigation of Drugs in Drivers.* Forensic Science International, 2005. **150:** p. 199-204.

22. Albery, I.P., et al., *Illicit Drugs and Driving: Prevalence, Beliefs and Accident Involvement Among a Cohort or Current Out-of Treatment Drug Users.* Drug and Alcohol Dependence, 2000. 58: p. 197-204.

23. Spiehler, V., *Drug Screening in the USA.* Bandolier, 1994. **5:** p. 42-44.

24. Peat, M., *Workplace Drug Testing,* in *Clarke's Analysis of Drugs and Poisons,* A.C. Moffat, M.D. Osselton, and B. Widdop, Editors. 2004, Pharmaceutical Press, p. 68-79.

25. SAMHSA, *Mandatory Guidelines for Federal Workplace Drug Testing Programs.* 2001. Draft 4.

26. Verstraete, A.G. and A. Pierce, *Workplace Drug Testing in Europe.* Forensic Science International, 2001.**121:** p. 2-6.

27. Home Office, *Updated Drug Strategy 2002.* 2002, Home Office Drugs Strategy Directorate.

28. GLADA, *The GLADA Crack Cocaine Strategy 2005-08,* GLADA, Editor. 2004, Greater London Authority.

29. Hunter, G., T. McSweeney, and P.J. Turnbull, *The Introduction of Drug Arrest Referral Schemes in London: A Partnership Between Drug Services and the Police.* The International Journal of Drug Policy, 2005. 16: p. 343-352.

30. Cooper, G., et al., *Evaluation of the Cozart RapiScan Drug Test System for Opiates and Cocaine in Oral Fluid.* Forensic Science International, 2005. 150: p. 239-243.

31. Gossop, M., et al., *Reductions in Acquisitive Crime and Drug Use after Treatment of Addiction Problems: 1-Year Follow Up Outcomes.* Drug and Alcohol Dependence, 2000. 58: p. 165-172.

32. Badia, R., et al., *Analytical Approaches of European Union Laboratories to Drugs of Abuse Analysis.* Clinical Chemistry, 1998. 44: p. 790-799.

33. Phillips, J.E., T.M. Ambrose, and C. Hammett-Stabler, *Ligand Assay Technology in Drugs of Abuse Screening Point of Care Devices.* Journal of Clinical Ligand Assay, 2002. 25: p. 342-347.

34. Torre, R.D.L., et al., *Recommendations for the Reliable Detection of Illicit Drugs in Urine in the European Union, with Special Attention to the Workplace.* Ann Clin Biochem, 1997. 34: p. 339-344.

35. Wild, D., *The Immunoassay Handbook.* Third ed. 2005: Elsevier.

36. Langman, L.J. and B.M. Kapur, *Toxicology: Then and Now.* Clinical Biochemistry, 2006. 39: p. 498-510.

37. Dankwardt, A., *Immunochemical Assays in Pesticide Analysis,* in *Encylopedia of Analytical Chemistry.* 1998.

38. Armbruster, D.A., et al., *Enzyme Immunoassay, Kinetic Microparticle Immunoassay, Radioimmunoassay, and Fluorescence Polarization Immunoassay Compared for Drugs-of-Abuse Screening.* Clinical Chemistry, 1993.39: p. 2137-2146.

39. Swartz, M.S., J.W. Swanson, and M.J. Hannon, *Detection of Illicit Substance Use Among Persons with Schizophrenia by Radioimmunoassay of Hair.* Psychiatric Services, 2003. 54: p. 891-895.

40. Moody, D.E., et al., *Comparative Analysis of Sweat Patches for Cocaine (and Metabolites) by Radioimmunoassay and Gas Chromatography-Positive Ion Chemical Ionisation-Mass Spectrometry.* Journal of Analytical Toxicology, 2004. 28: p. 86-93.

41. Lu, N.T. and B.G. Taylor, *Drug Screening and Confirmation by GC-MS: Comparison of EMIT II and Online KIMS Against 10 Drugs Between US and England Laboratories.* Forensic Science International, 2006. 157: p. 106-116.

42. Armbruster, D.A., et al., *Cloned Enzyme Donor Immunoassay (CEDIA) for Drugs-of-Abuse Screening.* Clinical Chemistry, 1995. 41: p. 92-98.

43. Kupiec, T., et al., *Choice of an ELISA for Screening Postmortem Blood for Amphetamine and/or Methamphetamine.* Journal of Analytical Toxicology, 2002. 26: p. 513-518.

44. Kerrigan, S. and W.H. Phillips, *Comparison of ELISAs for Opiates, Methamphetamine, Cocaine Metabolite, Benzodiazepines, Phencyclidine, and Cannabinoids in Whole Blood and Urine.* Clinical Chemistry, 2001. 47: p. 540-547.

45. Spiehler, V., et al., *Performance of a Microtiter Plate ELISA for Screening of Postmortem Blood for Cocaine and Metabolites.* Journal of Analytical Toxicology, 2003. 27: p. 587-591.

46. Moore, K.A., et al., *Screening Postmortem Blood and Tissues for Nine Classes of Drugs of Abuse Using Automated Microplate Immunoassay.* Forensic Science International, 1999. 106: p. 93-102.

47. Laloup, M., et al., *Validation of an ELISA-based Screening Assay for the Detection of Amphetamine, MDMA and MDA in Blood and Oral Fluid.* Forensic Science International, 2005.153: p. 29-37.

48. Cooper, G., et al., *Validation of the Cozart Microplate ELISA for Detection of Opiates in Hair.* Journal of Analytical Toxicology, 2003. 27: p. 581-586.

49. Spiehler, V., *Hair Analysis by Immunological Methods from the Beginning to 2000.* Forensic Science International, 2000. 107: p. 249-259.

50. Lachenmeier, K., F. Musshoff, and B. Madea, *Determination of Opiates and Cocaine in Hair Using Automated Enzyme Immunoassay Screening Methodologies Followed by Gas Chromatographic-Mass Spectrometric (GC-MS) Confirmation.* Forensic Science International, 2006. 159: p. 189-199.

51. Barnes, A.J., et al., *Sensitivity, Specificity, and Efficiency in Detecting Opiates in Oral Fluid with the Cozart Opiate Microplate EIA and GC-MS Following Controlled Codeine Administration.* Journal of Analytical Toxicology, 2003. 27: p. 402-406.

52. Niedbala, R.S., et al., *Immunoassay for Detection of Cocaine/Metabolites in Oral Fluids.* Journal of Analytical Toxicology, 2001. 25: p. 62-68.

53. Cone, E.J., et al., *Oral Fluid Testing for Drugs of Abuse: Positive Prevalence Rates by Intercept Immunoassay Screening and GC-MS-MS Confirmation and Suggested Cut Off Concentrations.* Journal of Analytical Toxicology, 2002. 26: p. 541-546.

54. Huestis, M.A., J.M. Mitchell, and E.J. Cone, *Detection Times of Marijuana Metabolites in Urine by Immunoassay and GC-MS.* Journal of Analytical Toxicology, 1995. **19:** p. 443-449.

55. Moore, C., A. Negrusz, and D. Lewis, *Determination of Drugs of Abuse in Meconium.* Journal of Chromatography B, 1998. **713:** p. 137-146.

56. Spiehler, V., et al., *Enzyme Immunoassay Validation for Qualitative Detection of Cocaine in Sweat.* Clinical Chemistry, 1996. **42:** p. 34-38.

57. Hicks, J.M., et al., *Recommendations and Opinions for the Use of Point-of-Care Testing for Hospitals and Primary Care: Summary of a 1999 Symposium.* Clinica Chimica Acta, 2001. **303:** p. 1-17.

58. George, S. and R.A. Braithwaite, *Use of On-Site Testing for Drugs of Abuse.* Clinical Chemistry, 2002. **48:** p. 1639-1646.

59. Hand, C. and D. Baldwin, *Immunoassays,* in *Clarkes Analysis of Drugs and Poisons,* A.C. Moffat, M.D. Osselton, and B. Widdop, Editors. 2004, Pharmaceutical Press, p. 301-312.

60. Watson, *Introduction to Mass Spectrometry.* Third ed. 1997: Lippincott-Raven.

61. Wood, M., et al., *Recent Applications in Liquid Chromatography - Mass Spectrometry in Forensic Science.* Journal of Chromatography A, 2006. **1130:** p. 3-15.

62. Walsh, J.M., et al., *An Evaluation of Rapid Point-of-Collection Oral Fluid Drug-Testing Devices.* Journal of Analytical Toxicology, 2003. 27: p. 429-439.

63. Fucci, N., et al., *An Evaluation of the Cozart RapiScan System as an On-Site Screening Tool for Drugs of Abuse in a Non-Conventional Biological Matrix: Vitreous Humor.* Forensic Science International, 2005. 156: p. 102-105.

64. Giovanni, N.D., et al., *Cozart RapiScan System: Our Experience with Saliva Tests.* Journal of Chromatography B, 2002. 773: p. 1-6.

65. Jehanli, A., et al., *Blind Trials of an Opiate Saliva Drug Test for Marijuana and Opiates.* Journal of Forensic Sciences, 2001. 46: p. 1214-1220.

66. Kacinko, S.L., et al., *Performance Characteristics of the Cozart RapiScan Oral Fluid Drug Testing System for Opiates in Comparison to ELISA and GC/MS*

Following Controlled Codeine Administration. Forensic Science International, 2004. 141: p. 41-48.

67. Kolbrich, E.A., et al., *Cozart RapiScan Oral Fluid Drug Testing System: An Evaluation of Sensitivity, Specificity, and Efficiency for Cocaine Detection Compared with ELISA and GC-MS Following Controlled Cocaine Administration.* Journal of Analytical Toxicology, 2003. 27: p. 407-411.
68. Moore, L., et al., *Gas Chromatography-Mass Spectrometry Confirmation of Cozart RapiScan Saliva Methadone And Opiates Tests.* Journal of Analytical Toxicology, 2001. 25: p. 520-524.
69. Verstraete, A.G. and E. Raes, *Rosita-2 Project.* 2006.
70. Crouch, D.J., et al., *An Evaluation of Selected Oral Fluid Point-of-Collection Drug-Testing Devices,* journal of Analytical Toxicology, 2005. 29: p. 244-248.
71. Kintz, P., V. Cirimele, and B. Ludes, *Codeine Testing in Sweat and Saliva with the Drugwipe.* International Journal of Legal Medicine, 1998. 111: p. 82-84.
72. Pichini, S., et al., *On-Site Testing of 3,4-Methylenedioxymethamphetamine (Ecstasy) in Saliva with Drugwipe and Drugread: A Controlled Study in Recreational Users.* Clinical Chemistry, 2002. 48: p. 174-176.
73. Samyn, N., G.D. Boeck, and A.G. Verstraete, *The Use of Oral Fluid and Sweat Wipes for the Detection of Drugs of Abuse in Drivers.* Journal of Forensic Sciences, 2002. 47: p. 1380-1387.
74. Samyn, N. and C.V. Haeren, *On-Site Testing of Saliva and Sweat with Drugwipe and Determination of Concentrations of Drugs of Abuse in Saliva, Plasma and Urine of Suspected Users.* International Journal of Legal Medicine, 2000. 113: p. 150-154.
75. Corporation, A.B.M., *OralStat.* 2004, Marketing Information.
76. Wong, R.C., M. Tran, and J.K. Tung, *Oral Fluid Drug Tests: Effects of Adulterants and Foodstuffs.* Forensic Science International, 2005. 150: p. 175-180.
77. Barrett, C., C. Good, and C. Moore, *Comparison of Point of Collection Screening of Drugs of Abuse in Oral Fluid with a Laboratory Based Urine Screen.* Forensic Science International, 2001. 122: p. 163-166.
78. Yacoubian, G.S. and E.D. Wish, *A Comparison Between Instant and Laboratory Oral Fluid Analysis Among Arrestees.* Journal of Psychoactive Drugs, 2006. 38: p. 207-210.

79. Miki, A., et al., *Application of ORAL Screen Saliva Drug Test for the Screening of Methamphetamine, MDMA, and MDEA Incorporated in Hair.* Journal of Analytical Toxicology, 2004. 28: p.132-134

80. Maurer, H.H., *Advances in Analytical Toxicology: The Current Role of Liquid Chromatography-Mass Spectrometry in Drug Quantification in Blood and Oral Fluid.* Anal Bioanalytical Chemistry, 2005. 381: p. 110-118.

81. Jeanville, P.M., et al., *Rapid Confirmation/Quantitation of Cocaine and Benzoylecgonine in Urine Utilizing High Performance Liquid Chromatography and Tandem Mass Spectrometry.* American Society for Mass Spectrometry, 2000. 11: p. 257-263.

82. Marquet, P. and G. Lachatre, *Liquid Chromatography-Mass Spectrometry: Potential in Forensic and Clinical Toxicology.* Journal of Chromatography B, 1999. 733: p. 93-118.

83. Krishnan, T.R. and I. Ibraham, *Solid Phase Extraction for the Analysis of Biological samples.* Journal of Pharmaceutucal and Biomedical Analysis, 1994. 12: p. 287-294.

84. Zeeuw, R.A.d., *Drug Screening in Biological Fluids. The Need for a Systematic Approach.* Journal of Chromatography B, 1997. 689: p. 71-79.

85. Decaestecker, T.N., et al., *Suitability Testing of Commercial Solid-Phase Extraction Sorbents for Sample Clean-Up in Systematic Toxicological Analysis using Liquid Chromatography-(Tandem) Mass Spectrometry.* Journal of Chromatography B, 2003. 789: p. 19-25.

86. Franke, J.P. and R.A.D. Zeeuw, *Solid-Phase Extraction Procedures in Systematic Toxicological Analysis.* Journal of Chromatography B, 1998. 713: p. 51-59.

87. Gareri, J., J. Klein, and G. Koren, *Drugs of Abuse Testing in Meconium.* Clinica ChimicaActa, 2006. 366: p. 101-111.

88. Sunshine, I., *Preliminary Tests for Drugs of Abuse.* Clinical Chemistry, 1988. 34: p. 331-334.

89. Group, U.S., *United Kingdom Laboratory Guidelines for Legally Defensible Workplace Drug Testing.* 2001. Version 1.

90. SAMHSA, *Mandatory Guidelines and Proposed Revisions to Mandatory Guidelines for Federal Workplace Drug Testing Programs.* Federal Register, 2004. 69.

91. Caplan, Y.H. and B.A. Goldberger, *Alternative Specimens for Workplace Drug Testing.* Journal of Analytical Toxicology, 2001. **25:** p. 396-399.

92. Smith, F.P. and D.A. Kidwell, *Cocaine in Hair, Saliva, Skin Swabs, and Urine of Cocaine Users Children.* Forensic Science International, 1996. **83:** p. 179-189.

93. Engelhart, D.A. and A.J. Jenkins, *Detection of Cocaine Analytes and Opiates in Nails form Postmortem Cases.* Journal of Analytical Toxicology, 2002. **26:** p. 489-492.

94. Staub, C., *Chromatographic Procedures for Determination of Cannabinoids in Biological Samples, with Special Attention to Blood and Alternative Matrices like Hair, Saliva, Sweat and Meconium.* Journal of Chromatography B, 1999. 733: p. 119-126.

95. Kintz, P. and N. Samyn, *Use of Alternative Specimens: Drugs of Abuse in Saliva and Doping Agents in Hair.* Therapeutic Drug Monitoring, 2002. 24: p. 239-246.

96. Paul, B.D. and A. Jacobs, *Effects of Oxidizing Adulterants on Detection of 11-Nor-A9-THC-9-Carboxylic Acid in Urine.* Journal of Analytical Toxicology, 2002. **26:** p. 460-463.

97. Cone, E.J., *New Developments in Biological Measures of Drug Prevelance.* NIDA Research Monographs, 1997.167: p. 108-129.

98. Kintz, P., V. Cirimele, and B. Ludes, *Detection of Cannabis in Oral Fluid (Saliva) and Forehead Wipes (Sweat) from Impaired Drivers.* Journal of Analytical Toxicology, 2000. 24: p. 557-561.

99. Garside, D., et al., *Identification of Cocaine Analytes in Fingernail and Toenail Specimens.* Journal of Forensic Sciences, 1998. **43:** p. 974-979.

100. Valente-Campos, S., et al., *Validation of a Method to Detect Cocaine and its Metabolites in Nails by Gas Chromatography-Mass Spectrometry.* Forensic Science International, 2006.159: p. 218-222.

101. Sachs, H., *History of Hair Analysis.* Forensic Science International, 1997. **84:** p. 7-16.

102. DuPont, R.L. and W.A. Baumgartner, *Drug Testing by Urine and Hair Analysis: Complementary Features and Scientific Issues.* Forensic Science International, 1995. 70: p. 63-76.

103. Koren, G., *Measurement of Drugs in Neonatal Hair; a Window to Fetal Exposure.* Forensic Science International, 1995. 70: p. 77-82.

104. Kintz, P., *Drug Testing in Hair.* 1996: CRC Press Inc.

105. Charles, B.K., et al., *Opiate Recidivism in a Drug-Treatment Program: Comparison of Hair and Urine Data.* Journal of Analytical Toxicology, 2003. 27: p. 412-428.

106. Polettini, A., et al., *Determination of Opiates in Hair. Effects of Extraction Methods on Recovery and on Stability of Analytes.* Forensic Science International, 1997. 84: p. 259-269.

107. Schaffer, M.I., W.-L. Wang, and J. Irving, *An Evaluation of Two Wash Proceduresfor the Differentiation of External Contamination versus Ingestion in the Analysis of Human Hair Samplesfor Cocaine.* Journal of Analytical Toxicology, 2002. 26: p. 485-488.

108. Romano, G., N. Barbera, and I. Lombardo, *Hair Testingfor Drugs of Abuse: Evaluation of External Cocaine Contamination and Risk of False Positives.* Forensic Science International, 2001. 123: p. 119-129.

109. Romano, G., et al., *Determination of Drugs of Abuse in Hair: Evaluation of External Heroin Contamination and Risk of False Positives.* Forensic Science International, 2003. 131: p. 98-102.

110. Fendrich, M., et al., *Drug Testing Feasibility in a General Population Household Survey.* Drug and Alcohol Dependence, 2004. 73: p. 237-250.

111. Hofman, L.F., *Human Saliva as a Diagnostic Specimen.* American Society for Nutritional Sciences, 2001: p. 1621-1625.

112. Clauwaert, K., et al., *The Determination of Cocaine, Benzoylecgonine, and Cocaethylene in Small-Volume Oral Fluid Samples by Liquid Chromatography-Quadrupole-Time-of-Flight Mass Spectrometry.* Journal of Analytical Toxicology, 2004. 28: p. 655-659.

113. Drummer, O.H., *Drug Testing in Oral Fluid.* The Clinical Biochemist Reviews, 2006. 27: p. 147-159.

114. Kato, K., et al., *Cocaine and Metabolite Excretion in Saliva Under Stimulated and Nonstimulated Conditions.* Journal of Analytical Toxicology, 1993. 17: p. 338-341.

115. Samyn, N., et al., *Analysis of Drugs of Abuse in Saliva.* Forensic Science Review, 1999. 11: p. 1-19.

116. Neale, J. and M. Robertson, *Comparisons of Self-Report Data and Oral Fluid Testing in Detecting Drug Use Amongst New Treatment Clients.* Drug and Alcohol Dependence, 2003. 71: p. 57-64.

117. Kaufman, E. and I.B. Lamster, *The Diagnostic Applications of Saliva - A Review.* Critical Reviews in Oral Biology and Medicine, 2002. 13: p. 197-212.

118. Dickson, S., et al., *The Recovery of Illicit Drugs from Oral Fluid Sampling Devices.* Forensic Science International, 2007. 165: p. 78-84.

119. Schneider, N.G., et al., *Saliva Cotinine Levels as a Function of Collection Method.* Addiction, 1997. 92: p. 347-351.

120. Cooper, G., et al., *Validation of the Cozart Microplate EIA for Cocaine and Metabolites in Oral Fluid.* Journal of Analytical Toxicology, 2004. 28: p. 498-503.

121. Cooper, G., et al., *Validation of the Cozart Microplate EIA for the Analysis of Opiates in Oral Fluid.* Forensic Science International, 2005. 154: p. 240-246.

122. Cooper, G., et al., *Validation of the Cozart Amphetamine Microplate EIA for the Analysis of Amphetamines in Oral Fluid.* Forensic Science International, 2006. 159: p. 104-12.

123. Spiehler, V., D. Baldwin, and C. Hand, *Analysis of Drugs in Saliva,* in *Forensic Science: On Site Drug Testing,* A.J. Jenkins and B.A. Goldberger, Editors, Humana Press, p. 95-109.

124. Bennett, G.A., E. Davies, and P. Thomas, *Is Oral Fluid Analysis as Accurate as Urinalysis in detecting Drug Use in a Treatment Setting?* Drug and Alcohol Dependence, 2003. 72: p. 265-269.

125. Niedbala, R.S., et al., *Detection of Marijuana Use by Oral Fluid and Urine Analysis Following Single-Dose Administration of Smoked and Oral Marijuana.* Journal of Analytical Toxicology, 2001. 25: p. 289-303.

126. Clarke, J. and J.F. Wilson, *Proficiency Testing (External Quality Assessment) of Drug Detection in Oral Fluid.* Forensic Science International, 2005. 150: p. 161-164.

127. Dams, R., et al., *Oral Fluid as an Alternative Matrix to Monitor Opiate and Cocaine Use in Substance-Abuse Treatment Patients.* Drug and Alcohol Dependence, 2007. 87: p. 258-267.

128. Kim, I., et al., *Sensitivity and Specificity of the Cozart Microplate EIA Cocaine Oral Fluid at Proposed Screening and Confirmation Cutoffs.* Clinical Chemistry, 2003. 49: p. 1498-1503.

129. Quintela, O., D. Crouch, and D.M. Andrenyak, *Recovery of Drugs of Abuse from the Immunalysis Quantisal Oral Fluid Collection Device.* Journal of Analytical Toxicology, 2006. 30: p. 614-616.

130. Hold, K.M., et al., *Saliva as an Analytical Tool in Toxicology.* International Journal of Drug Test, 1996.1: p. 1-36.

131. Cone, E.J., J. Oyler, and W.D. Darwin, *Cocaine Disposition in Saliva Following Intravenous, Intranasal, and Smoked Administration.* Journal of Analytical Toxicology, 1997. 21: p. 465-475.

132. Crouch, D.J., *Oral Fluid Collection: The Neglected Variable in Oral Fluid Testing.* Forensic Science International, 2005. 150: p. 165-173.

133. Spiehler, V., *Drugs in Saliva,* in *Clarkes Analysis of Drugs and Poisons,* A.C. Moffat, M.D. Osselton, and B. Widdop, Editors. 2004, Pharmaceutical Press.

134. Kidwell, D.A., J.C. Holland, and S. Athanaselis, *Testing for Drugs of Abuse in Saliva and Sweat.* Journal of Chromatography B, 1998. 713: p. 111-135.

135. Schepers, R.J.F., et al., *Methamphetamine and Amphetamine Pharmacokinetics in Oral Fluid and Plasma after Controlled Oral Methamphetamine Administration to Human Volunteers.* Clinical Chemistry, 2003. 49: p. 121-132.

136. Haeckel, R. and P. Hanecke, *Application of Saliva for Drug Monitoring An In Vivo Model for Transmembrane Transport.* European Journal of Clinical Chemsitry and Clinical Biochemistry, 1996. 34: p. 171-191.

137. Gunnar, T., K. Ariniemi, and P. Lillsunde, *Validated Toxicological Detennination of 30 Drugs of Abuse as Optimized Derivatives in Oral Fluid by Long Column Fast Gas Chromatography/Electron Impact Mass Spectrometry.* Journal of Mass Spectrometry, 2005. 40: p. 739-753.

138. Shirtcliff, E.A., et al., *Use of Salivary Biomarkers in Biobehavioral Research: Cotton-Based Sample Collection Methods can Interfere with Salivary Immunoassay Results.* Psychoneuroendocrinology, 2001. 26: p. 165-173.

2 Preliminary Studies - Analysis of Amfetamines in Oral Fluid

2.1 Aims and Objectives

The aim of this chapter is to evaluate a laboratory based and a point of care immunoassay for the analysis of metamfetamine and MDMA in oral fluid samples. GC-MS will be used as the reference method and to provide data on drug concentrations in oral fluid from individuals self reporting the use of amfetamine or MDMA.

2.2 Introduction

The amfetamine group represents a class of phenylethylamine compounds that have varying degrees of sympathomimetic activity [1]. In the context of this chapter the amfetamines group includes synthetic drugs such as amfetamine and metamfetamine, and designer drugs such as 3,4-methylenedioxyamfetamine (MDA), 3,4-methylenedioxymethamfetamine (MDMA), 3,4-methylenedioxy-N-ethylamfetamine (MDEA), and 3,4-methylenedioxyphenyl-2-butanamine (MBDB). All these compounds are central nervous system (CNS) stimulants that are abused for their psychotropic effects including euphoria and increased energy and alertness.

Substitutions on the nitrogen and the ring system account for most of the structural variations of amfetamine (Figure 2:1).

The compounds in the amfetamine group all exist as 2 isomers, the dextro (d) and laevo (l) isomers. The different isomers exhibit different pharmacological properties, with the d-isomers producing greater CNS stimulant activity than the 1-isomers [2, 3].

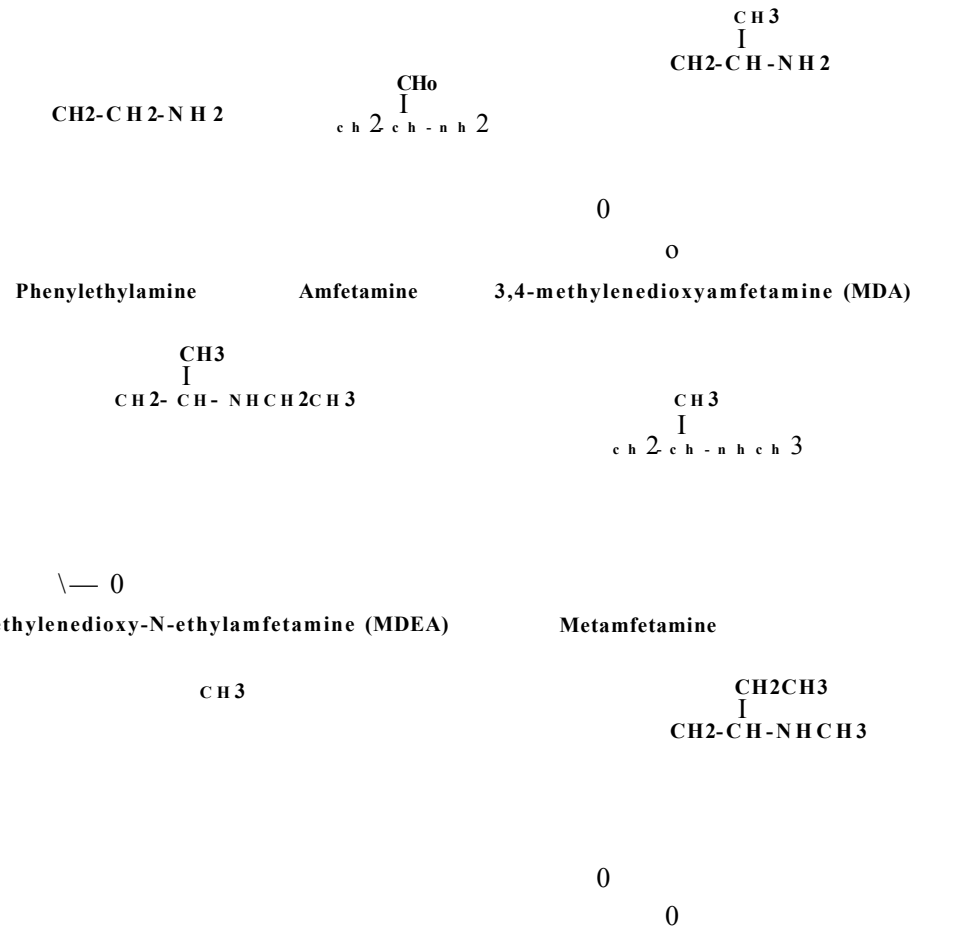

Figure 2:1 Structures of the amfetamine group

2.2.1 Use and Abuse

Amfetamine was first synthesised in 1877 [1]. In the late 1920's it was used as a replacement for ephedrine as a nasal decongestant [4]. During the Second World War amfetamine was used to maintain alertness and fight fatigue [5]. Currently amfetamine is available by prescription for narcolepsy, attention deficit hyperactivity disorder (ADHD) and as a dietary supplement for appetite suppression [1,6]. Single isomer forms of d-amfetamine are available as Dexedrine® [7].

Illicit amfetamine is more commonly seen in its powder form, with tablets representing approximately 10% of amfetamine available. Tablets have been shown to be poorly

made with drug content varying from 3mg to 50mg [8]. Amfetamine is generally abused via the oral or intravenous routes. Chronic abuse of amfetamine brings about sufficient tolerance such that it has been known for addicts to swallow or inject up to 2000mg daily [9].

Recent drug use statistics in the UK indicate that illicit use of amfetamine is declining [10].

Metamfetamine was first synthesised in Japan in 1919 and patented in 1920 [11]. In the US metamfetamine is prescribed in the d-isomer form, under the brand name Desoxyn®, for ADHD and narcolepsy [7], however due to its high abuse potential it is generally only prescribed if all other medications have failed. The l-isomer of metamfetamine exhibits vasoconstrictive effects and as a result is an ingredient of Vicks Inhaler and other nasal decongestants in the US [7].

Metamfetamine is a potent and highly addictive stimulant [12]. It is commonly found as a colourless crystalline solid, otherwise referred to as crystal meth, or alternatively in tablet or powder form. Metamfetamine is commonly abused via smoking, insufflation, intravenous and less commonly via the oral route [12]. Long term abusers have reported to use as much as 5000-15000mg/day [1].

Metamfetamine abuse became a social problem in the U.S in the 1960's [1], and it remains the most frequently encountered clandestinely produced controlled substance in the US [12]. In contrast it is relatively uncommon in the UK and has been quoted as representing only 0.1% of all amfetamine related cases [8]. There are currently fears that metamfetamine use could become increasingly popular within the next two to three years following the discovery of a small number of clandestine laboratories synthesising metamfetamine in Britain [13]. In general metamfetamine use in the UK has been confined to the gay community [14], however there are suggestions that a fall in the

price has led to its emergence into the clubbing scene. Following these recent concerns, recommendations from the Advisory Council on the Misuse of Drugs resulted in the government reclassifying metamfetamine from a class B drug to class A [13].

MDMA is one of the oldest designer drugs and is a derivative of metamfetamine [1]. MDMA was first synthesised in 1912 by two German chemists and was patented by the Merck Company two years later [15]. The first report that MDMA is psychoactive in humans appeared in a report by Alexander Shulgin in 1978 [16]. In the 1980's MDMA was being used in psychotherapy where it was said to increase patient self esteem and facilitate therapeutic communication [16].

The end of the 1980's saw a growth in the rave music scene in the UK and Europe and up to the present day MDMA has been used almost exclusively as a dance drug [9]. In the UK the use of MDMA has remained stable over the last year [10]. In contrast the availability of MDMA in the US has increased dramatically in recent years and it is now one of the most frequently used recreational drugs [17].

Ecstasy is the popular street name for MDMA but it has many other street names such as disco biscuits, love doves and Mitsibushies [18]. MDMA is available in tablet form and comes in a variety of colours, shapes and sizes and with different engraved pictures.

It has been reported that MDMA users often take a variety of other recreational drugs, with cannabis being the most popular [19-21]. Patterns of use vary considerably from half a tablet to the rare occasion where 10-25 have been consumed by experienced users possibly as a result of chronic pharmacodynamic tolerance [17], or as a result of low drug content [22]. On average the number of tablets consumed is between one and two [19].

Illicit manufacture of amfetamines produces street drugs containing variable concentrations of different amfetamines as well as other impurities, intermediate products and adulterants such as caffeine and/or ephedrine [8, 23, 24]. The purity of ecstasy tablets has shown many fluctuations over the years. Recent studies indicate that purity levels are between 90 and 100% [25, 26], Dosage is also highly variable. In a study whereby subjects purchased ecstasy tablets for their own use and sent one of them to the lab for analysis, the average consumed dose was found to be 57mg of MDMA [27]. The Laboratory of the Government Chemist between 1 January and 31 December 2002 found between 23 and 105mg of MDMA in ecstasy tablets, with an average content of 75mg [28]. Ecstasy tablets seized in Ireland in 2002 and 2003 were analysed and found to contain concentrations ranging from 7 to 79mg [29].

MDEA and MBDB have similar properties to MDMA [9], however their prevalence is low and the amount of information relating to these two designer drugs is limited.

2.2.2 Pharmacokinetic Effects

The amfetamines are generally well absorbed orally. They delay gastric emptying and decrease intestinal motility, thereby delaying their own absorption and that of other drugs taken at the same time [30]. A summary of the chemical properties of amfetamine, metamfetamine and MDMA can be seen in Table 2:1.

Amfetamine is metabolised in the liver and is excreted substantially unchanged in the urine [9, 30]. Following oral amfetamine administration the peak plasma concentrations are reported to be reached 2-3 hours later, with maximum subjective effects occurring at 2 hours. Subjective and behavioural effects then decline despite substantial amfetamine

concentrations [31]. The plasma elimination half life is 4-8 hours if the urine is acidic or 10-13 hours if urinary pH is uncontrolled [32].

Drug	Molecular Weight	Boiling Point (°C)	pKa	Protein Binding
Amfetamine	135	200	10.1	16%
Metamfetamine	149	214	10.1	0%
MDMA	193	100	9.0	65%

Table 2:1 Summary of chemical properties of amfetamine, metamfetamine and MDMA [30]

Following controlled administration of metamfetamine and amfetamine, the elimination half lives for oral fluid are comparable to plasma [12].

Metamfetamine has high lipid solubility allowing for rapid transfer across the blood brain barrier [33] which explains its greater CNS efficacy compared to amfetamine. Metamfetamine is excreted in the urine at concentrations approximately 37-45% of the dose [34]. Amfetamine is a metabolite of metamfetamine and represents approximately 10% of a metamfetamine dose [12]. However amfetamine is not always detected following metamfetamine administration [12, 34].

The bioavailability of metamfetamine is very dependant on the route of administration. When smoked the bioavailability was found to be 90% compared to 67% when administered orally [34]. Immediate euphoric effects are experienced when metamfetamine is smoked due to rapid absorption from the lungs into the blood [35]. The blood concentration versus time profile for smoked metamfetamine is very similar to intravenous administration.

The elimination half life of metamfetamine is significantly longer than any of the other amfetamines and is reported to be on average 10-12 hours regardless of the route of administration or biological matrix tested [12, 34, 36, 37]. The reported range is quite broad at between 6.4 and 15.1 hours [36].

Peak plasma concentrations are reached within minutes for the intravenous route and slightly later following smoking [35]. Oral dosing of metamfetamine produces maximum plasma concentrations at approximately 3 hours post dose [4].

Approximately 65% of MDMA is excreted unchanged in the urine [15]. MDA is one of the metabolites of MDMA and represents only approximately 8-9% of the total concentration of MDMA regardless of the dose administered [37] and the biological fluid tested [38]. 3,4-dihydroxymethamfetamine (HHMA) appears to be the major metabolite of MDMA in plasma and urine with concentrations showing to be equivalent to MDMA [37, 39], HHMA is however only present in its conjugated form in biological fluids.

The pharmacokinetics of MDMA has been observed to be non-linear [40, 41], this may have implications in cases of acute intoxication as small increases in MDMA dose may result in disproportionate rises in MDMA plasma concentration. Several mechanisms have been proposed for this observation such as saturation or inhibition of drug metabolism or the formation of a complex between a metabolite and endogenous enzymes [37].

The effects of MDMA have been reported to appear within 20-60 minutes following administration, with effects lasting 4-6 hours [15, 37, 42]. Peak plasma and oral fluid concentrations occur between 1.5-2 hours, [15, 21, 37, 38, 42].

The plasma elimination half life following lOOmg of MDMA has been reported as 8-9 hours [37].

2.2.3 Physiological and Pharmacodynamic Effects

The amfetamines exert their effects on the body by stimulating the release of the neurotransmitters serotonin, dopamine and noradrenaline, and blocking their presynaptic reuptake resulting in hyperstimulation of the CNS [43]. Amfetamine and metamfetamine exert the greatest effect on dopamine [1] while MDMA exhibits mainly serotonergic activity [16].

The acute and chronic subjective and toxic effects produced by the amfetamine group are difficult to distinguish clinically [43, 44]. The subjective effects include euphoria, increased alertness, intensified emotions, sensations of extreme physical and mental power, and in the case of MDMA enhanced sociability [1]. The euphoric effects of metamfetamine are longer lasting than those of the other amfetamines due to the longer elimination half life.

The acute effects closely resemble the physiological and psychological effects of the fight or flight response, including increased heart rate and blood pressure, vasoconstriction, bronchodilation and hyperglycaemia [37]. Other clinical symptoms include dilated pupils, hyperthermia, tremors, dry mouth and loss of appetite. Hyperthermia as a result of the amfetamines toxicity may lead to further fatal complications such as rhabdomyolysis, disseminated intravascular coagulation and acute renal failure [16, 44].

Undesirable psychological effects of the amfetamine group may be experienced up to 24-72 hours after drug consumption and have been recorded as aggression, lack of judgement, build up of fatigue, muscle aches, depression, anxiety, agitation, delirium,

seizures, hallucinations and paranoia. Long term and high dose use can induce an acute psychotic state [1, 45, 46],

Other effects recorded following the use of MDMA include nausea, chills, sweating, tremor, jaw clenching and insomnia [16], as well as more serious conditions such as metabolic acidosis and hyperkalemia. While relatively few cases of death involving MDMA use have been reported, the deaths have been attributed to cardiovascular, cerebrovascular and hyperthermic effects [47], while acidosis and electrolyte imbalance are sufficient to cause fatal cardiac arrythmias. Impaired thermoregulation resulting in hyperthermia is one of the major symptoms of acute MDMA induced toxicity and body temperatures >43 ℃ have been reported [16].

Different individuals may respond very differently to MDMA and so a lethal concentration can be as little as one tablet [42]. Adverse effects may be related to the hepatic enzyme CYP2D6 which is known to be deficient in 5-10% of the population, as a result poor metabolisers may be at greater risk of toxic responses to the drug even at low doses [26].

2.2.4 Amfetamines in Oral Fluid

Oral fluid concentrations of the amfetamines group have been reported to be higher than plasma concentrations [12, 27, 34, 36, 38]. This may be as a result of several factors including passive diffusion of drugs into the saliva across a concentration gradient, active transport, or ion trapping. Due to the low plasma binding of the majority of the amfetamines this may increase the diffusion of drug from plasma to saliva. Typically saliva is more acidic than blood, this provides a driving force for diffusion and partitioning of basic molecules into saliva, due to the basic properties of the amfetamines they will become ionised in the saliva which mean they can't diffuse back

into plasma resulting in their accumulation [48]. In addition the passage of drugs across cell membranes is favoured for low molecular weight molecules, another property of the amfetamines [38].

2.3 Experimental

2.3.1 Materials

Solutions of the reference standards (lmg/mL in methanol) amfetamine (AMP), metamfetamine (MAMP), MDMA, MDA, MDEA, MBDB and of the corresponding deuterated internal standards (O.lmg/mL in methanol) AMP-dn, MAMP-dn, MDMA-ds, MDA-ds, MDEA-d6 and MBDB-ds were obtained from Cerilliant (LGC Promochem, Teddington). The derivatising reagent pentafluoropropionic anhydride (PFPA) was purchased from Sigma Aldrich Company Ltd (Dorset, UK). Solid phase extraction (SPE) cartridges, Bond Elut™ Certify (50mg, 3mL), were purchased from Varian (Oxfordshire, UK). High recovery vials were purchased from Crawford Scientific (Lanarkshire, Scotland, UK). All other reagents and solvents were of analytical grade and were purchased from VWR International Ltd (Leicestershire, UK). The Cozart reagents including the oral fluid sample collector, drug test cartridge, RapiScan instrument, and metamfetamine EIA kits were supplied by Cozart Pic (Oxfordshire, UK).

2.3.2 Instrumentation

The GC-MS was an Agilent 5973N with 6890 GC purchased from Agilent Technologies (Cheshire, UK), equipped with Rtx-5Sil MS (30m x 0.25mm i.d x 0.25pm) capillary column, purchased from Crawford Scientific (Lanarkshire, Scotland, UK). The GC mobile phase was helium (99.999% purity) and was supplied by Air Products (Cheshire, UK).

SPE was performed on a vacuum manifold assembly purchased from Varian (Oxfordshire, UK). A Techne® Sample Concentrator with a DB-3 series Dri-Block® was purchased from LAB3 (Northampton, UK).

Microtitre plates were washed using a Dynex plate washer and the absorbance measurements were performed with a MRX microtitre plate reader in dual wavelength mode 450-650nm, both items were purchased from Dynex Techonologies (Worthing, UK).

2.3.3 Solution Preparation

2.3.3.1 Drug Standards

Combined analyte working solutions of lpg/mL and lOOng/mL were prepared in methanol from individual lmg/mL stock solutions of reference standards. A working solution of lpg/mL for the internal standards was prepared in methanol from the individual O.lmg/mL stock solutions. The standards were refrigerated at 4°C for up to 6 months.

2.3.3.2 Reagents and Buffers

- 0.1 mol/L Phosphate Buffer pH 7.4 (500mL).

  400mL of deionised water was added to 6.81 g of potassium dihydrogen orthophosphate (MW 139.09). The pH was adjusted to 7.4 using 10 mol/L potassium hydroxide. The volume was then made up to 500mL in a volumetric flask with deionised water and stored refrigerated (2-8°C) for up to 2 months.

- 10 mol/L Potassium Hydroxide (lOOmL).

  80mL of deionised water was added to 56.1 lg of potassium hydroxide (MW 56.11). The volume was made up to lOOmL in a volumetric flask with deionised water and stored at room temperature for up to six months.

- 0.01 mol/L Acetic Acid pH 3.3 (lOOmL).

  57.5pL of glacial acetic acid was added to 80mL of deionised water in a lOOmL volumetric flask. The volume was made up to lOOmL with deionised water and stored at room temperature for up to 2 months.

- lmg/mL (w/v) Tartaric Acid:Ethylacetate (lOOmL).

  lOOmL of ethylacetate was added to 0.1 g of tartaric acid and sonicated for 30 minutes. The solution was stored at room temperature for up to two months.

- 2% (v/v) Ammonium Hydroxide in Ethyl Acetate (50mL).

  lmL of ammonium hydroxide was added to 49mL of ethyl acetate. The solution was prepared daily and mixed well before use.

2.3.4 Methods

2.3.4.1 Oral Fluid Specimens

Three hundred and seventy oral fluid specimens were obtained from the analytical laboratory at Cozart Pic following their routine analysis for dmgs of abuse.

Prior to submission to the laboratory lmL of oral fluid was collected from each individual using the Cozart® RapiScan Collector, the oral fluid soaked sample pad was then inserted into the transport tube which contained 2mL of Cozart proprietary buffer. This collection method results in a 1:3 dilution of the sample, all concentrations noted are corrected for undiluted oral fluid. Detailed information regarding each individual was not available.

Samples were screened for MDMA and metamfetamine using the Cozart® RapiScan and/or the Cozart® EIA, and confirmed by GC-MS analysis. The time delay between receipt of samples into the laboratory for routine screening, and their subsequent testing was less than 4 months, during which time the samples were stored at -20°C.

An additional fifty six oral fluid samples were selected from the laboratory sample bank based on donor self reported use of amfetamine and/or MDMA up to 7 days prior to sample collection. Information on the gender of the donors was only available for 40 of the 56 samples, of which there was a ratio of females to males of 1:1.7.

The samples were quantified for amfetamines (AMP, MAMP, MDA, MDMA, MDEA, MBDB) by GC-MS, samples with a drug concentration greater than the top calibration point (180ng/mL) were serially diluted to give a concentration within the calibration curve.

2.3.4.2 Lateral Flow Point of Care Screening

The Cozart® RapiScan drug test cartridge is a competitive lateral flow immunoassay composed of a white nitrocellulose reaction strip with immobilised drug-binding sites. Gold-labelled polyclonal antibodies raised to d-metamfetamine are contained within a pad near the point of sample application. Addition of the sample rehydrates the gold-labelled antibodies and the mixture flows across the membrane, over the immobilised drug sites. If there is no drug present in the sample the antibodies will bind to the immobilised drug and a pink band will form, if drug is present then there is a decrease in band intensity.

The Cozart® RapiScan metamfetamine/MDMA test cartridge has a set cutoff of 45ng/mL in undiluted oral fluid. The manufacturer states that other designer amfetamines such as MBDB and MDEA show cross reactivity with the antibody, and that concentrations of amfetamine greater than 30,000ng/mL will give a positive screen result [49], details of the cross reactivity can be seen in Table 2:2.

Drug	Concentration (ng/mL)	Cozart® RapiScan Response
D-Metamfetamine	45	Positive
L-Metamfetamine	1500	Positive
MDMA	45	Positive
MBDB	45	Positive
MDEA	1500	Positive
D-Amfetamine	30,000	Positive
MDA	30,000	Negative

Table 2:2 Cross reactivity profile of Cozart® RapiScan drug test cartridge for MDMA and metamfetamine [49]

Samples were screened according to the manufacturers protocol [49]. In brief, four drops of oral fluid/buffer mixture were added to the cartridge using a disposable Pasteur pipette. As soon as the fluid appeared in the cartridge window the cartridge was inserted into the Cozart® RapiScan instrument and the timer was initiated by pressing the start button. Following five minutes incubation the cartridge was read and the result interpreted by the RapiScan instrument which then displayed either positive or negative on the screen, subsequently a print out of the results was produced from the attached printer.

2.3.4.3 EIA Screening

The components of the Cozart® EIA kit include a 96 well microtitre plate coated with mouse monoclonal antibodies raised to d-metamfetamine. The antibody also displays cross reactivity to additional compounds within the amfetamines group, see Table 2:3.

Drug	% Cross Reactivity
D-Amfetamine	0.7
MDA	0.4
MDMA	49
MBDB	154
MDEA	5

Table 2:3 Cross reactivity profile of the Cozart® Metamfetamine EIA [50]

The enzyme conjugate employed was metamfetamine labelled with horseradish peroxidase (HRP). The substrate solution used was hydrogen peroxide with 3,3,5,5-tetramethyl benzidine (TMB) as the chromogen, and the stop solution was 1 mol/L of sulphuric acid. The calibrators contained d-metamfetamine in oral fluid/buffer matrix.

Samples with sufficient volume following screening using the point of care device were screened using the Cozart® Metamfetamine EIA according to the manufacturers protocol [50]. 25pL of sample, calibrator or control was added in duplicate to the antibody coated microtitre plate, followed by lOOuL of enzyme conjugate. Following 30 minutes incubation at room temperature, the plate was washed four times with 350(iL of wash buffer. 100pL of substrate solution was added to each well and incubated for an additional 30 minutes. Finally lOOpL of stop solution was added and the absorbance read at 450nm, with a reference filter at 630nm.

2.3.4.4 Solid Phase Extraction (SPE)

Prior to analysis by GC-MS the samples were extracted by SPE according to a previously published procedure [51]. Specimens were allowed to equilibrate to room temperature, mixed and 0.5mL was taken for analysis. A seven point calibration curve, 0, 15, 30, 60, 90, 120, and 180ng/mL, was prepared using the working solutions of drug

standards, and negative oral fluid collected using the Cozart® collector. Using the l|ag/mL solution, deuterated internal standards at a concentration of 120ng/mL were added to each sample, blank, 60ng/mL control and standard followed by lmL of pH 7.4 phosphate buffer.

SPE was performed using Bond Elut Certify™ cartridges (50mg, 3mL). The columns were conditioned with lmL of methanol followed by lmL of phosphate buffer (pH 7.4, 0. lmol/L). Sample was loaded onto the column and then washed with lmL of deionised water. The column pH was adjusted with 0.5mL acetic acid (pH 3.3, 0.0lmol/L) and dried on full vacuum for 10 minutes. The column was then washed with 50pL of methanol and dried on full vacuum for 1 minute. 2mL acetone:chloroform (1:1) was added as the final wash step. The retained analytes were then eluted with lmL ethyl acetate containing 2% ammonium hydroxide (98:2, v/v). lOOpL tartaric acid was added to the eluates and evaporated to dryness at room temperature under nitrogen. The samples were then reconstituted in lOOpL PFPA:ethylacetate (1:1) and again evaporated to dryness at room temperature under nitrogen. The samples were reconstituted in lOOpL of ethylacetate and 2pi was injected into the GC-MS.

2.3.4.5 GC-MS Parameters

The GC-MS was run in electron ionisation mode with splitless injection. The mobile phase was helium at a flow rate of lmL/min and at a constant pressure of 8.7 psi. The injector port temperature was set at 290°C, with the transfer line and source temperatures set to 280°C and 230°C respectively.

The temperature programme consisted of an initial 2 minutes at 55°C, ramped at 20°C/min to 200°C, then ramped to 250°C at 10°C/min and finally to 300°C at

25°C/min where it was held for 2 minutes. The ions monitored and the individual retention times can be seen in Table 2:4.

Analyte	Retention Time	Ions Monitored (m/z)	Ions for Quantitation (m/z) !
Amfetamine	8.14	91, 118	190
Amfetamine-di i	8.10	98, 128	194
Metamfetamine	8.92	118, 160	204
Metamfetamine-di4	8.87	128, 163	211
MDA	10.32	162, 190	325
MDA-ds	10.31	167, 194	330
MDMA	11.16	162,339	204
MDMA-ds	11.13	165, 344	208
MDEA	11.42	162,353	218
MDEA-d6	11.39	165,359	224
MBDB	11.57	160,353	218
MBDB-ds	11.59	163,358	222

Table 2:4 Retention times and ions monitored for each analyte

All the analytes show good baseline separation of the peaks. Examples of the chromatograms for each analyte and their internal standards can be seen in Figures 2:2 and 2:5.

The method was linear (>0.9928) for all the amfetamines using seven data points ranging from Ong/mL to 180ng/mL, the CV was <0.5%. Inter-assay precision and intra-assay precision were <11% and <4% respectively. The limits of detection (LOD) for amfetamine, metamfetamine, MDA, MDMA, MDEA and MBDB were 2, 1,5, 1,2 and Ing/mL respectively, with the limits of quantitation (LOQ) as 5ng/mL for all compounds.

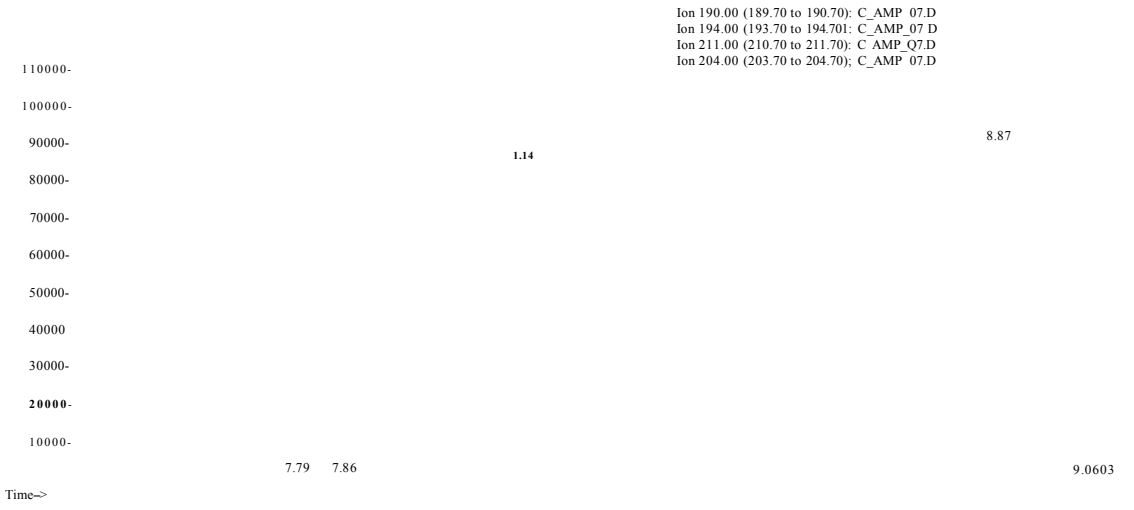

Figure 2:2 Amfetamine-dn, amfetamine, metamfetamine-dn and metamfetamine chromatogram

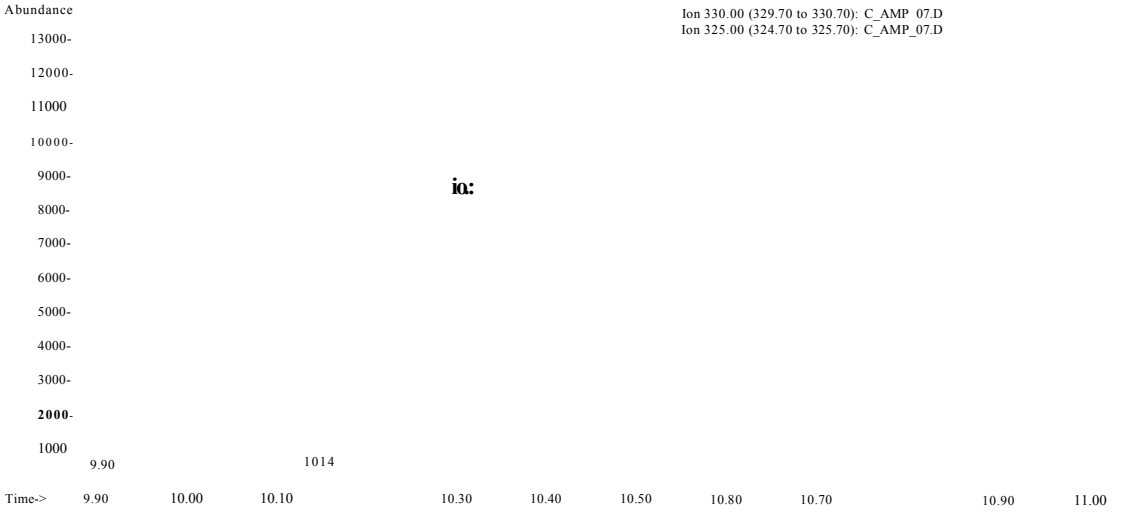

Figure 2:3 MDA-ds and MDA chromatogram

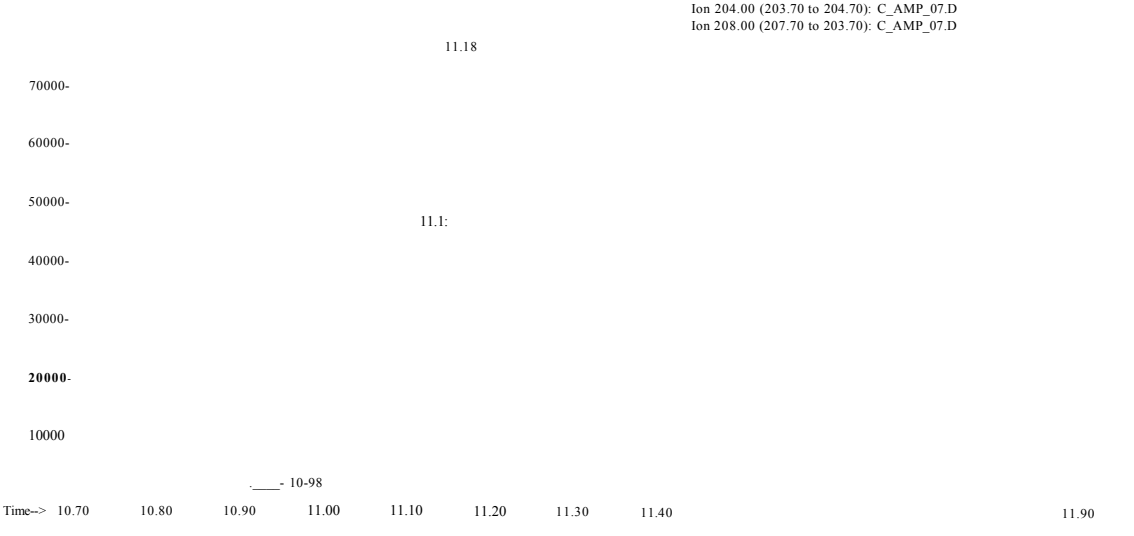

Figure 2:4 MDMA-ds and MDMA chromatogram

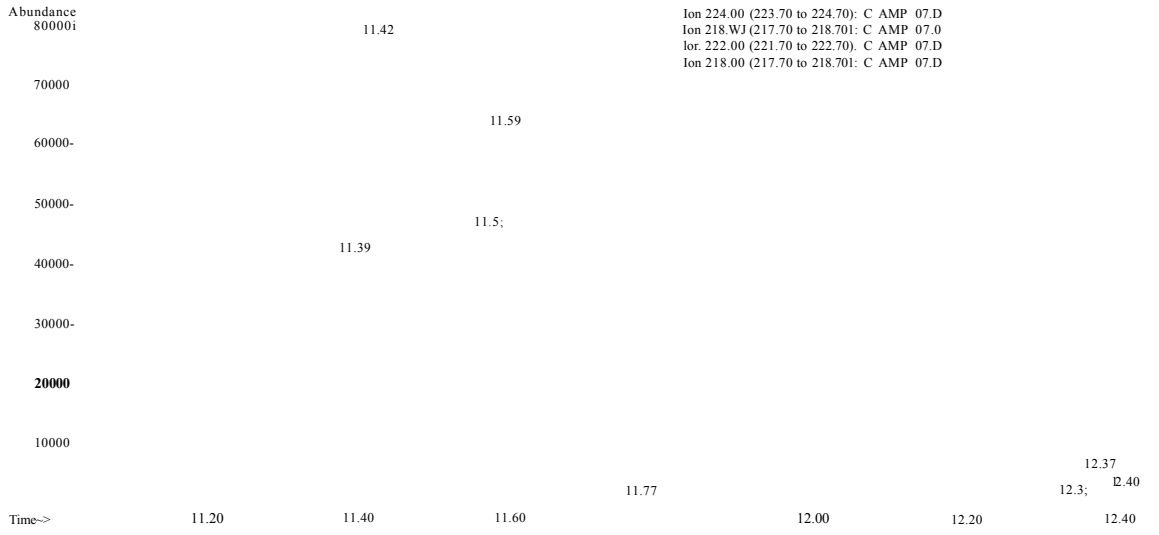

Figure 2:5 MDEA-d6, MDEA, MBDB-ds and MBDB chromatogram

2.3.4.6 Sensitivity, Specificity and Efficiency

In the laboratory the word sensitivity has two definitions, analytical sensitivity is the lowest concentration at which an analyte can be measured precisely, and clinical sensitivity is defined as the probability of a true positive test result as compared to a reference or gold standard [52], Clinical sensitivity is discussed here and is calculated from the tally of true positives (TP) and false negatives (FN): Sensitivity = [TP/(TP + FN)] x 100%.

Specificity is the probability that a test will produce a true negative result and is calculated from the tally of false positives (FP) and true negatives (TN): Specificity = [TN/(TN +FP)] x 100%.

Overall efficiency = [(TP + TN)/(TP + FP +TN + FN)] x 100%.

2.3.4.7 Receiver Operator Characteristic (ROC) Analysis

The efficiency of a diagnostic test is characterised by its sensitivity and specificity. As the cutoff point is varied over a spectrum of results the sensitivity and specificity will move in opposite directions, as one increases the other decreases. ROC plots are a

graphical representation of all the sensitivity/specificity pairs resulting from varying the cutoff point. A test with perfect discrimination has an ROC plot that passes through the upper left corner where the true positive fraction is 1.0 (perfect sensitivity), and the false positive fraction is 0 (perfect specificity). A test with no discrimination will produce a 45° diagonal line from the lower left hand corner to the upper right hand corner [53]. When results from multiple tests have been obtained the ROC plots can be graphed together. The relative positions of the plots indicate the efficiency of the tests. A plot lying above and to the left of another plot indicates greater efficiency, an illustration of this is shown in Figure 2:6.

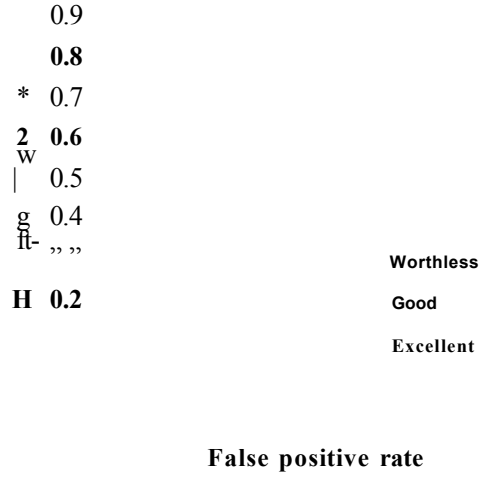

Figure 2:6 Illustration of ROC Curves

2.4 Results and Discussion

2.4.1 Lateral Flow Point of Care Screening

Of the three hundred and seventy oral fluid samples, one hundred and twenty one screened positive using the Cozart® RapiScan, and one hundred and twenty nine specimens were confirmed by GC-MS to contain detectable concentrations of MDMA. Two hundred and thirty specimens contained one or more of the amfetamines at

concentrations above the GC-MS LOQ of 5ng/mL. The Cozart® RapiScan and corresponding GC-MS results for these samples can be seen in Table 2:5.

Sample	Cozart® RapiScan Result	GC/MS (ng/mL) AMP	MDMA	MDA	MDEA	MAMP
139	N	13	0	0	0	0
140	N	14	0	0	0	0
141	N	21	0	0	0	0
142	N	23	0	0	0	0
143	N	31	0	0	0	0
144	N	35	0	0	0	0
145	N	53	0	0	0	0
146	N	57	0	0	0	0
147	N	58	0	0	0	0
148	N	60	0	0	0	0
149	N	60	0	0	0	0
150	N	67	0	0	0	0
151	N	79	0	0	0	0
152	N	87	0	0	0	0
153	N	94	0	0	0	0
154	N	104	0	0	0	0
155	N	110	0	0	0	0
156	N	125	0	0	0	0
157	N	128	0	0	0	0
158	N	128	0	0	0	0
159	N	133	0	0	0	0
160	N	136	0	0	0	0
161	N	141	0	0	0	0
162	N	150	0	0	0	0
163	N	167	0	0	0	0
164	N	173	0	0	0	0
165	N	176	0	0	0	0
166	N	177	0	0	0	0
167	N	>180	0	0	0	0
168	N	>180	0	0	0	0
169	N	>180	0	0	0	0
170	N	>180	0	0	0	0
171	N	>180	0	0	0	0
172	N	>180	0	0	0	0
173	N	>180	0	0	0	0
174	N	>180	0	0	0	0
175	N	>180	0	0	0	0
176	N	>180	0	0	0	0
177	N	>180	0	0	0	0
178	N	>180	0	0	0	0
179	N	>180	0	0	0	0
180	N	>180	0	0	0	0

Sample	Cozart® RapiScan Result	GC/MS (ng/mL) AMP	MDMA	MDA	MDEA	MAMP
181	N	>180	0	0	0	0
182	N	>180	0	0	0	0
183	N	>180	0	0	0	0
184	N	>180	0	0	0	0
185	N	>180	0	0	0	0
186	N	>180	0	0	0	0
187	N	>180	0	0	0	0
188	N	>180	0	0	0	0
189	N	>180	0	0	0	0
190	N	>180	0	0	0	0
191	N	>180	0	0	0	0
192	N	>180	0	0	0	0
193	N	>180	0	0	0	0
194	N	>180	0	0	0	0
195	N	>180	0	0	0	0
196	N	>180	0	0	0	0
197	N	>180	0	0	0	0
198	N	>180	0	0	0	0
199	N	>180	0	0	0	0
200	N	>180	0	0	0	0
201	N	>180	0	0	0	0
202	N	>180	0	0	0	0
203	N	>180	0	0	0	0
204	N	>180	0	0	0	0
205	N	>180	0	0	0	0
206	N	>180	0	0	0	0
207	N	>180	0	0	0	0
208	N	>180	0	0	0	0
209	N	>180	0	0	0	0
210	N	>180	0	0	0	0
211	N	>180	0	0	0	0
212	N	>180	0	0	0	0
213	N	>180	0	0	0	0
214	N	>180	0	0	0	0
215	N	>180	0	0	0	0
216	N	>180	0	0	0	0
217	N	>180	0	0	0	0
218	N	>180	0	0	0	0
219	N	>180	0	0	0	0
220	N	>180	0	0	0	0
221	N	>180	0	0	0	0
222	N	>180	0	0	0	0
223	N	>180	0	0	0	0
224	N	>180	0	0	0	0
225	N	>180	0	0	0	0
226	N	>180	0	0	0	0
227	N	>180	0	0	0	0
228	N	>180	0	0	0	0
229	N	>180	0	0	0	0
230	N	>180	0	0	0	0

Sample	Cozart® RapiScan Result	GC/MS (ng/mL) AMP	MDMA	MDA	MDEA	MAMP
231	N	>180	0	0	0	0
232	N	>180	0	0	0	0
233	N	>180	0	0	0	0
234	N	0	13	0	0	0
235	N	0	13	0	0	0
236	N	0	15	0	0	0
237	N	0	16	0	0	0
238	N	0	16	0	0	0
239	N	0	17	0	0	0
240	N	0	19	0	0	0
241	N	71	21	0	0	0
242	N	0	22	0	0	0
243	N	0	22	0	0	0
244	N	0	23	0	0	0
245	N	0	23	0	0	0
246	N	0	44	0	0	0
247	N	0	47	0	0	0
248	P	0	53	0	0	0
249	N	0	53	0	0	0
250	N	0	54	0	0	0
251	P	0	55	0	0	0
252	P	0	57	0	0	0
253	P	>180	58	0	0	0
254	P	0	65	0	0	0
255	P	0	69	0	0	0
256	P	0	70	15	0	0
257	P	0	81	0	0	0
258	P	>180	85	0	0	0
259	P	>180	88	0	0	0
260	P	0	95	0	0	0
261	P	0	97	0	0	0
262	P	>180	103	0	0	0
263	P	0	109	0	0	0
264	P	0	111	0	0	0
265	P	>180	112	0	0	0
266	P	>180	119	0	0	0
267	P	0	125	0	0	0
268	P	0	125	0	0	0
269	P	0	128	0	0	0
270	P	0	136	0	0	0
271	P	>180	146	0	0	0
272	P	0	149	0	0	0
273	P	0	153	0	0	0
274	P	0	161	0	0	0
275	P	0	174	0	0	0
276	P	0	179	0	0	0
277	P	>180	>180	0	0	0
278	P	0	>180	58	0	0
279	P	>180	>180	>180	0	0
280	P	>180	>180	>180	0	0

Sample	Cozart® RapiScan Result	GC/MS (ng/mL) AMP	MDMA	MDA	MDEA	MAMP
281	P	>180	>180	0	0	0
282	P	0	>180	0	0	0
283	P	0	>180	0	0	0
284	P	>180	>180	0	0	0
285	P	0	>180	0	0	0
286	P	>180	>180	85	0	0
287	P	>180	>180	>180	0	52
288	P	0	>180	0	0	0
289	P	0	>180	0	0	0
290	P	0	>180	113	0	0
291	P	147	>180	91	0	0
292	P	0	>180	0	0	0
293	P	0	>180	>180	0	0
294	P	>180	>180	>180	0	0
295	P	>180	>180	172	0	0
296	P	90	>180	175	0	0
297	P	>180	>180	>180	0	0
298	P	>180	>180	>180	0	0
299	P	0	>180	>180	0	0
300	P	>180	>180	>180	0	0
301	P	>180	>180	>180	0	0
302	P	>180	>180	>180	0	0
303	P	87	>180	>180	0	0
304	P	0	>180	>180	0	>180
305	P	0	>180	0	0	0
306	P	0	>180	145	0	0
307	P	>180	>180	107	0	0
308	P	0	>180	0	0	0
309	P	0	>180	0	0	0
310	P	0	>180	>180	0	0
311	P	0	>180	>180	0	0
312	P	0	>180	>180	0	0
313	P	0	>180	70	0	0
314	P	>180	>180	0	0	0
315	P	0	>180	0	0	0
316	P	0	>180	43	0	0
317	P	>180	>180	121	0	0
318	P	0	>180	23	0	0
319	P	0	>180	93	0	0
320	P	0	>180	0	0	0
321	P	>180	>180	43	0	0
322	P	>180	>180	>180	0	0
323	P	0	>180	>180	0	0
324	P	>180	>180	0	0	0
325	P	0	>180	136	0	0
326	P	84	>180	>180	0	0
327	P	0	>180	0	0	0
328	P	>180	>180	124	0	0
329	P	0	>180	20	0	0
330	P	>180	>180	0	0	0

Sample	Cozart® RapiScan Result	GC/MS (ng/mL) AMP	MDMA	MDA	MDEA	MAMP
331	P	>180	>180	0	0	0
332	P	0	>180	162	37	0
333	P	0	>180	0	0	0
334	P	>180	>180	35	0	0
335	P	>180	>180	>180	>180	0
336	P	>180	>180	0	0	0
337	P	>180	>180	46	0	0
338	P	0	>180	27	0	0
339	P	0	>180	57	0	0
340	P	>180	>180	26	0	0
341	P	>180	>180	>180	0	0
342	P	0	>180	133	0	0
343	P	0	>180	0	0	0
344	P	0	>180	176	0	0
345	P	0	>180	79	0	0
346	P	0	>180	36	0	0
347	P	0	>180	>180	0	108
348	P	0	>180	0	0	0
349	P	0	>180	34	0	0
350	P	0	>180	77	0	0
351	P	0	>180	147	0	0
352	P	0	>180	0	0	0
353	P	0	>180	0	0	0
354	P	0	>180	0	0	0
355	P	0	>180	0	0	0
356	P	0	>180	0	0	0
357	P	0	>180	0	0	0
358	P	>180	>180	0	0	0
359	P	0	>180	0	0	0
360	P	0	>180	28	0	0
361	P	0	>180	0	0	0
362	P	13	>180	29	0	0
363	P	>180	0	0	0	0
364	P	>180	0	0	0	0
365	P	>180	0	0	0	0
366	P	>180	0	0	0	0
367	P	>180	0	0	0	0
368	P	>180	0	0	0	0

Table 2:5 Cozart® RapiScan results for 230 samples containing amfetamine and its related compounds as quantified by GC-MS

The concentration range and frequency of amfetamines detected by GC-MS can be seen in Table 2:6. MDMA and metamfetamine were detected in 34.9% (N=129) and 0.8%

(N=3) respectively, of the samples tested. No specimens contained detectable quantities of MBDB.

Drug	Number	Range (ng/mL)
MDMA	129	13 ->180
Amfetamine	144	13 ->180
MDA	55	20->180
MDEA	2	37->180
Metamfetamine	3	52->180

Table 2:6 Range and frequency of amfetamine and related compounds confirmed by GC-MS following Cozart® RapiScan screening

Amfetamine was most frequently detected on its own, while MDMA was more often detected with its metabolite MDA, and/or amfetamine. On the few occasions where MDEA and metamfetamine were detected they were always present with MDMA. Table 2:7 displays the different combinations of amfetamine, MDMA and MDA encountered.

Analyte Combination	**Frequency**
Amfetamine	101
MDMA	57
Amfetamine + MDMA	18
MDMA + MDA	25
Amfetamine + MDMA + MDA	24

Table 2:7 Combinations of amfetamine and its related compounds detected in oral fluid

Six samples screened positive by the Cozart® RapiScan but did not contain detectable quantities of MDMA or metamfetamine by GC-MS. These samples contained concentrations of amfetamine which greatly exceeded the top calibration standard of

180ng/mL. The manufacturer's protocol states a positive response will be obtained with d-amfetamine concentrations of 30,000ng/mL and above, it is therefore suggested that these 6 samples contained concentrations in excess of this.

An additional sample which screened positive by the Cozart® RapiScan was subsequently identified as containing pseudoephedrine. Although the concentration could not be determined it is suggested that it may have exceeded 30,000ng/mL which would trigger a positive response according to the manufacturer's protocol.

The number of true positives (TP), false negatives (FN), false positives (FP), and true negatives (TN) were determined by comparing the Cozart® RapiScan test results to GC/MS using a cutoff of 30ng/mL, and the SAMSHA recommended cutoff of 50ng/mL of amfetamines in oral fluid (Table 2:8).

	GC-MS Cut-off Concentration	
	Cozart 30ng/mL	**SAMSHA 50ng/mL**
TP	113	113
FN	4	2
FP	8	8
TN	245	247
Sensitivity	96.6%	98.3%
Specificity	96.8%	96.9%
Efficiency	96.8%	97.3%

Table 2:8 Sensitivity, specificity and efficiency of the Cozart® RapiScan for metamfetamine/MDMA drug test cartridge versus the Cozart employed cutoff (30ng/mL) and the SAMSHA recommended GC-MS cut-off (50ng/mL)

When applying the 30ng/mL GC-MS cutoff there were a total of 4 false negatives, this decreased to 2 false negatives when the 50ng/mL SAMSHA cutoff was applied. The concentrations of MDMA in these samples were within 20% of the Cozart® RapiScan cutoff of 45ng/mL at 44, 47, 53 and 54ng/mL.

The sensitivity, specificity and efficiency of the Cozart® RapiScan versus a GC-MS cutoff of 30ng/mL were excellent at 96.6, 96.8, and 96.8% respectively. When the SAMSHA recommended cutoff of 50ng/mL was used these values increased slightly to 98.3, 96.9 and 97.3%.

A total of seven point of care devices have been identified in the literature as being designed to detect amfetamine and/or metamfetamine and MDMA in oral fluid [48, 54-57]. The cutoff points for these devices have ranged from 45ng/mL to 160ng/mL [58]. Previous studies have evaluated these devices at the roadside [55, 56], following controlled dose administration [21], or using spiked samples [57, 58].

The ORALscreen® assay for MDMA and metamfetamine was used to test nine samples from individuals declaring the use of amfetamines. Good correlation was shown when the oral fluid results were compared to the results of matched urine samples. However the number of samples analysed was small and only the urine samples were confirmed by GC-MS [54].

The Drugwipe® was used to test oral fluid from individuals administered lOOmg of MDMA. The device was initially tested onsite but performed poorly, samples were subsequently sent to the laboratory where the volume of sample applied to the device could be controlled [21].

Toxiquick® was evaluated during a roadside study, 263 oral fluid samples were tested using single amfetamine and metamfetamine test strips. Results of the study proved to be poor with only 77% correct results when compared to GC-MS [55], this result was

not surprising considering the original application of the device was for urine and as a result the cutoff was very high (500ng/mL).

A direct comparison of the Cozart® RapiScan with similar point of care devices is not possible as this is the first known study to provide sensitivity, specificity and efficiency data on an oral fluid point of care test for metamfetamine/MDMA using samples from a known drug using population.

2.4.2 EIA Screening

Following the point of care study a total of eighty-five samples contained sufficient volume to allow for further analysis using the Cozart® Metamfetamine EIA for oral fluid.

The calibrators supplied with the assay contain metamfetamine and the manufacturers' technical sheet states 50% cross reactivity of the assay to MDMA [50]. As previously shown in Table 2:6, MDMA was the second most frequently encountered analyte with only 3 samples containing metamfetamine. For this reason a set of calibrators were prepared using MDMA and were tested in parallel to the calibrators supplied with the kit.

The number of true positives (TP), false negatives (FN), false positives (FP), and true negatives (TN) were determined by comparing the Cozart® EIA test results, using both sets of calibrators, at various cutoff points, to the reference method GC/MS at a cutoff of 30ng/mL. From this data the sensitivity, specificity and efficiency were calculated for the assay against both the metamfetamine and MDMA calibrators (Table 2:9 and 2:10).

	Cutoff Concentration (ng/mL)									
	10	20	30	45	50	60	70	80	90	**100**
TP	**44**	**42**	**38**	**30**	**30**	**28**	**26**	**26**	**24**	**23**
FN	**1**	**3**	**7**	**15**	**15**	**17**	**19**	**19**	**21**	**22**
FP	**3**	**2**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
TN	**37**	**38**	**39**	**40**	**40**	**40**	**40**	**40**	**40**	**40**
Sensitivity	**97.8%**	**93.3%**	**84.4%**	**66.7%**	**66.7%**	**62.2%**	**57.8%**	**57.8%**	**53.3%**	**51.1%**
Specificity	**92.5%**	**95%**	**97.5%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**
Efficiency	**95.3%**	**94.1%**	**90.6%**	**82.4%**	**82.4%**	**80%**	**77.7%**	**77.7%**	**75.3%**	**74.1%**

Table 2:9 Metamfetamine calibrators: Sensitivity, specificity and efficiency of the Cozart Metamfetamine EIA for oral fluid at various cutoff concentrations versus a GC/MS cutoff of 30ng/mL

	Cutoff Concentration (ng/mL)									
	10	20	30	45	50	60	70	80	90	100
TP	**45**	**44**	**44**	**41**	**41**	**39**	**38**	**36**	**35**	**33**
FN	**0**	**1**	**1**	**4**	**4**	**6**	**7**	**9**	**10**	**12**
FP	**10**	**7**	**4**	**2**	**2**	**1**	**1**	**1**	**1**	**1**
TN	**30**	**33**	**36**	**38**	**38**	**39**	**39**	**39**	**39**	**39**
Sensitivity	**100%**	**97.8%**	**97.8%**	**91.1%**	**91.1%**	**86.7%**	**84.4%**	**80%**	**77.8%**	**73.3%**
Specificity	**75%**	**82.5%**	**90%**	**95%**	**95%**	**97.5%**	**97.5%**	**97.5%**	**97.5%**	**97.5%**
Efficiency	**88.2%**	**90.6%**	**94.1%**	**92.9%**	**92.9%**	**91.8%**	**90.6%**	**88.2%**	**87.1%**	**84.7%**

Table 2:10 MDMA calibrators: Sensitivity, specificity and efficiency of the Cozart® Metamfetamine EIA for oral fluid at various cutoff concentrations versus a GC/MS cutoff of 30ng/mL

Changing the calibrators so that the assay was calibrated to MDMA instead of metamfetamine increased the sensitivity, specificity and efficiency, at the manufacturers

recommended cutoff (45ng/mL), from 66.7%, 100%, and 82.4% respectively to 91.1%, 95%, and 92.9%, versus a GC-MS cutoff of 30ng/mL.

Using data from both sets of calibrators the sensitivity for each cut-off was plotted against 1-specificity as a Receiver Operator Characteristic (ROC) curve in Figure 2:7. From the ROC curve the optimum cutoff was 20ng/mL of metamfetamine in undiluted oral fluid and between 30 and 50ng/mL of MDMA.

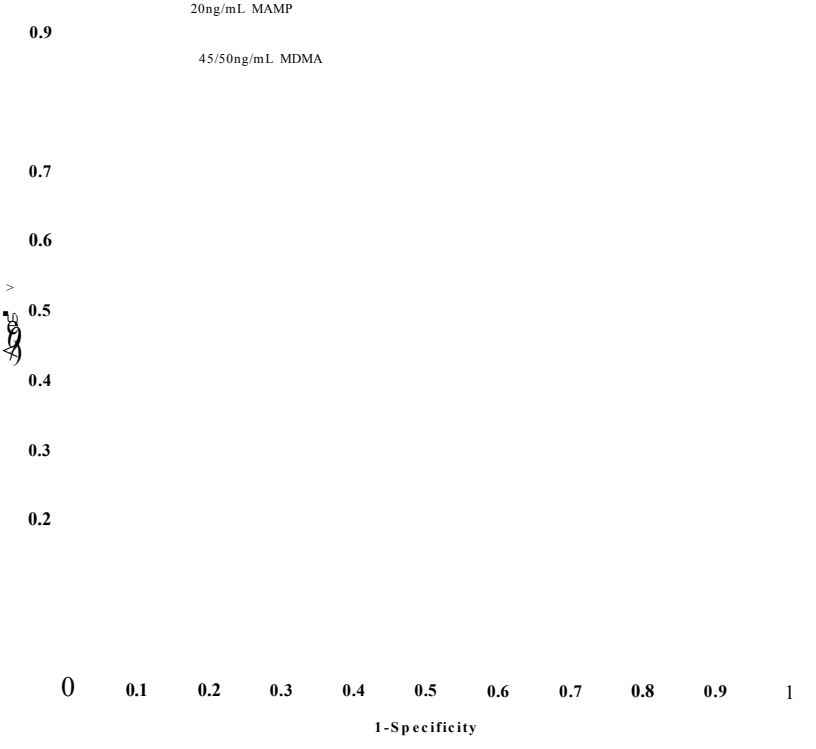

Figure 2:7 ROC Curve for the Cozart® Metamfetamine EIA for oral fluid comparing metamfetamine and MDMA calibrators. Data points from right to left are for cutoff points 10 to lOOng/mL

The population of samples encountered in these studies are consistent with reports that MDMA is more frequently abused in Europe while metamfetamine use is mainly found in the US [8], Therefore in European laboratories the assay can either be adapted by the

incorporation of MDMA calibrators, or applying the lower 20ng/mL cutoff. Countries with a higher incidence of metamfetamine abuse can use the kit as it is supplied by the manufacturer.

A large number of EIA kits specific to metamfetamine exist, the manufacturers of these assays state they can be used for various biological matrices including oral fluid. Despite their versatility there are a limited number of publications describing their evaluation. Where publications are available the assay has only been applied to blood [59], urine [60], or hair samples [61]. It is essential that every immunoassay is validated for the use of each biological fluid due to possible endogenous interferences from different matrices.

Previously the Cozart Amfetamine EIA was used to analyse oral fluid samples following controlled dose administration of MDMA. ROC analysis showed the optimum cutoff to be 51ng/mL versus an LC-MS/MS cutoff of lOng/mL, the sensitivity and specificity of the assay were 98.6% [62]. The cross reactivity of the EIA to MDMA was <0.1%, as a result the authors were relying on the presence of MDA which cross reacts at >140%. However, the data in Table 2:7 shows MDA is not always present with MDMA.

2.4.3 Self Reported Use

Information on the quantity and frequency of drug use is important when assessing an individual prior to and during a drug treatment programme [63]. Drug use can be monitored by self reported use and objectively measured by qualitative and quantitative analysis of biological specimens [64, 65].

In this study thirty-four individuals claimed to have used amfetamine within 6 days prior to sample collection, 18 people admitted to using MDMA, and 4 people admitted

using both amfetamine and MDMA either on the same day or within a day of each other. Full details of each sample can be found in Table 2:11.

GC-MS analysis confirmed the self report data in 87.5% of cases. On seven occasions the drugs declared were not consistent with the drugs detected. Three individuals claimed to have only used amfetamine, but their sample was found to also contain MDMA and MDA. Similarly three individuals who declared only MDMA use were found to contain detectable concentrations of amfetamine. One sample also contained metamfetamine when only MDMA had been reported. No detectable quantities of MDEA or MBDB were found in any of the samples.

Barcode	Declared Substance	Number of Days Since Use	GC-MS (ng/mL)			
			AMP	MDMA	MDA	MAMP
S336350	MDMA	0	0	8630	358	299
S206208	Amfetamine	0	136	0	0	0
S251329	Amfetamine	0	2357	0	0	0
SI06600	Amfetamine	0	2898	2370	244	0
S347050	Amfetamine	0	2352	0	0	0
S106390	Amfetamine	0	550	0	0	0
S328571	Amfetamine	0	2985	0	0	0
S328546	Amfetamine	0	35600	3132	196	0
S251651	Amfetamine	0	360	0	0	0
S347311	Amfetamine	0	617	0	0	0
S384891	Amfetamine	0	19725	900	442	0
S349402	Amfetamine	0	8970	0	0	0
S123026	Amfetamine/MDMA	0,1	14160	3932	192	0
S282143	MDMA	1	0	1650	0	0
S149811	MDMA	1	0	22	0	0
S311890	Amfetamine	1	2257	0	0	0
S250940	Amfetamine	1	167	0	0	0
S350710	Amfetamine	1	721	0	0	0
S336430	Amfetamine	1	413	0	0	0
S020829	Amfetamine	1	141	0	0	0
S351385	Amfetamine	1	938	0	0	0
S320379	Amfetamine, MDMA	1	246	505	0	0
S320066	Amfetamine	1	830	0	0	0
S351360	Amfetamine	1	1260	0	0	0
S359247	Amfetamine	1	9430	0	0	0
S352594	Amfetamine	1	9300	0	0	0
S347393	Amfetamine	1	10600	0	0	0
S334311	Amfetamine	1	382	0	0	0
S328582	Amfetamine	1	173	0	0	0
S334197	MDMA	1	0	44	0	0

Barcode	Declared Substance	Number of Days Since Use	GC-MS (ng/mL			
			AMP	MDMA	MDA	MAMP
S384595	Amfetamine	1	4675	0	0	0
S328028	MDMA	1	0	5232	457	0
S123095	Amfetamine	1	1070	0	0	0
S348304	Amfetamine	1	58	0	0	0
S049895	MDMA	2	0	75	0	0
S302216	MDMA	2	0	0	0	0
S365809	MDMA	2	0	0	0	0
S311324	MDMA	2	0	530	0	0
S351356	MDMA	2	16	32	0	0
S106166	Amfetamine	2	0	0	0	0
S383273	Amfetamine	2	946	0	0	0
S347235	Amfetamine	2	357	0	0	0
S311576	MDMA, Amfetamine	3	90	2868	175	0
S282229	MDMA	3	0	22	0	0
S332200	Amfetamine	3	294	0	0	0
S347038	MDMA	3	0	13	0	0
S401229	Amfetamine	3	0	0	0	0
S383259	Amfetamine	3	11505	0	0	0
S347219	Amfetamine	3	35	0	0	0
S206274	Amfetamine, MDMA	5	0	67	0	0
S250949	MDMA	5	0	0	0	0
S351450	MDMA	5	25	32	0	0
S366814	MDMA	5	0	0	0	0
S316506	MDMA	5	0	0	0	0
S316193	MDMA	6	13	0	0	0
S316838	MDMA	6	0	0	0	0

Table 2:11 Summary of self report data and corresponding GC-MS data

A total of 39% of MDMA positive samples also contained MDA at lower concentrations. Six of the samples contained MDA at concentrations 4.1-10.3% of the total concentration of MDMA, an average of 6.7% metabolic conversion. This data is consistent with previous studies where MDA was found in 37.8% of the MDMA positive samples [48], and where the average MDA concentration was 3.8% of the MDMA concentration [56, 66]. In this study one sample (barcode S384891) contained MDA at 49% of the MDMA concentration and also had a very high concentration of amfetamine (19,725ng/mL). The donor had only declared the use of amfetamine and so

it is possible that the presence of MDA, and maybe even MDMA, were as a result of drug impurity.

Twelve samples were classified as negative using the GC-MS cut-off of 30ng/mL, four of these samples contained a concentration of MDMA and/or amfetamine between the lower limit of quantitation (5ng/mL) and the cut-off (30ng/mL).

The concentration range of amfetamines measured in this population of samples can be seen in Table 2:12.

Drug	Frequency	Concentration Range (ng/mL)
Amfetamine	38	13-35600
MDMA	18	13-8630
MDA	7	175-457
Metamfetamine	1	299

Table 2:12 Frequency and concentration range of the amfetamine related compounds in oral fluid

Amfetamine was the most frequently reported drug and the highest concentration reached 35600ng/mL, from same day use. No published data has been found to determine if a concentration this high is common in this sample population. The highest measured concentration of MDMA in this study was 8630ng/mL, this is on average 21% higher than any other reported oral fluid concentration. The previously highest recorded concentrations of MDMA in oral fluid have been 6510ng/mL [38], 6871ng/mL [66], and 6982ng/mL [27]. These concentrations were achieved at 1.5 hours following controlled dose administration of either 75mg or lOOmg of MDMA. These doses are close to the average reported content of ecstasy tablets [27, 28] and therefore the oral

fluid concentrations quoted may be a realistic reflection of what should be expected from recreational users of MDMA.

The sample with the highest concentration of MDMA in this study was from an individual who reported consuming 4 tablets on the day the sample was collected, the purity and content of which cannot be ascertained. In a study representative of real life samples concentrations of MDMA in oral fluid ranged from 33 to 3533ng/mL when a single tablet was consumed and up to 7077ng/mL with multiple tablets [27].

Figure 2:8 displays the samples from donors declaring amfetamine use up to six days prior to sample collection, and categorises them into a series of concentration ranges. The same has been calculated for donors declaring MDMA use and is displayed in Figure 2:9.

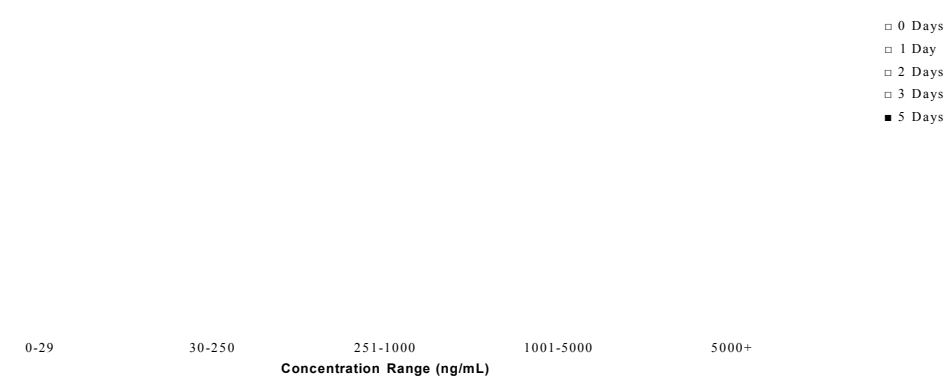

Figure 2:8 Concentrations of amfetamine in oral fluid up to 6 days following drug consumption

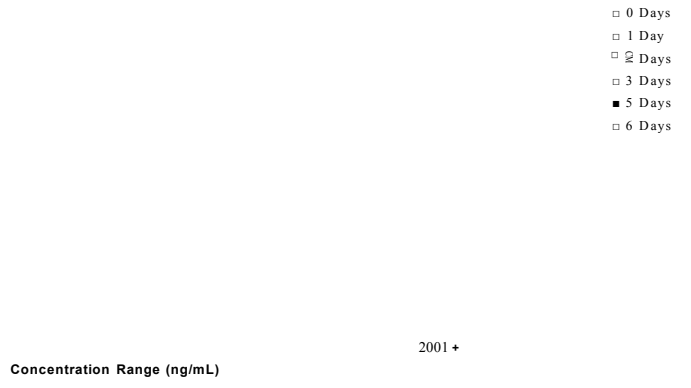

Figure 2:9 Concentrations of MDMA in oral fluid up to 6 days following drug consumption

Generally as the number of days since drug use increased, the concentrations of amfetamine and MDMA detected decreased. However on one occasion a concentration of amfetamine greater than 5000ng/mL was detected in a sample where the individual had claimed amfetamine use 3 days prior to sample collection.

Where drug use was declared on the same day as sample collection, concentrations were above 30ng/mL. No samples contained >30ng/mL of amfetamine or MDMA after 5 and 6 days respectively.

There are concerns about the reliability of self reported drug use [67], Although this study shows that the data is largely in accordance with the self reported drug use there are several inconsistencies in the data. It is suggested that either the purity or dose of the drug ingested may be variable, subjects may not know how much they have used, or they may be being deliberately untruthful. Alternatively self report histories may be inaccurate due to the memory deficits commonly encountered with use of the amfetamine compounds [68],

2.5 Conclusion

The work undertaken in this chapter has given an understanding of the fundamental processes of point of care and laboratory based immunoassay screening, GC-MS confirmatory analysis, and data interpretation. The knowledge gained can be applied to the development and validation of a novel EIA, and will help in the development of confirmatory techniques for the detection of smoked cocaine in oral fluid.

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2.6 References

1. Levine, B., *Principles of Forensic Toxicology.* 1999: American Association for Clinical Chemistry Inc.
2. Martins, L., et al., *Simultaneous Enantioselective Determination of Amphetamine and Congeners in Hair Specimens by Negative Chemical Ionisation Gas Chromatography-Mass Spectrometry.* Journal of Chromatography B, 2005. 825: p. 57-62.
3. Mendelson, J., et al., *Human Pharmacology of the Methamphetamine Stereoisomers.* Clinical Pharmacology and Therapeutics, 2006. 80: p. 403-420.
4. Drummer, O.H., *The Forensic Pharmacology of Drugs of Abuse.* 2001: Arnold Publisher.
5. Drummer, O.H., et al., *The Incidence of Drugs in Drivers Killed in Australian Road Traffic Crashes.* Forensic Science International, 2003. 134: p. 154-162.
6. Cody, J.T., S. Valtier, and S.L. Nelson, *Amphetamine Excretion Profile Following Multidose Administration of Mixed Salt Amphetamine Preparation.* Journal of Analytical Toxicology, 2004. 28: p. 563-574.
7. Holler, J.M., et al., *Quantitative and Isomeric Determination of Amphetamine and Methamphetamine from Urine using a Nonprotic Elution Solvent and R(-)-alpha-Methoxy-alpha-Trifluoromethylphenylacetic Acid Chloride Derivatisation.* Journal of Analytical Toxicology, 2005. 29: p. 652-657.
8. King, L.A., *Drug Content of Powders and Other Illicit Preparations in the UK.* Forensic Science International, 1997. 85: p. 135-147.
9. Braithwaite, R.A., et al., *Screening for Drugs of Abuse. I: Opiates, Amphetamines and Cocaine.* Ann Clin Biochem, 1995. 32: p. 123-153.
10. Roe, S. and L. Man, *Drug Misuse Declared: Findings From the 2005/06 British Crime Survey.* Home Office Statistical Bulletin, 2006. 15/06.
11. Logan, B.K., *Methamphetamine - Effects on Human Performance and Behaviour.* Forensic Science Review, 2002. 14: p. 133-148.
12. Schepers, R.J.F., et al., *Methamphetamine and Amphetamine Pharmacokinetics in Oral Fluid and Plasma after Controlled Oral Methamphetamine Administration to Human Volunteers.* Clinical Chemistry, 2003. 49: p. 121-132.
13. Travis, A., *Crystal Meth to Get Class A Listing in Bid to Limit Use,* in *The Guardian.* 2006.

14. Bolding, G., et al., *Use of Crystal Methamphetamine Among Gay Men in London.* Addiction, 2006. 101: p. 1622-1630.
15. Libiseller, K., et al., *Ecstasy - Deadly Risk Even Outside Rave Parties.* Forensic Science International, 2005.153: p. 227-230.
16. Green, A.R., et al., *The Pharmacology and Clinical Pharmacology of 3,4-Methylenedioxymethamphetamine (MDMA, "Ecstasy").* Pharmacological Reviews, 2003. 55: p. 463-508.
17. Scholey, A.B., et al., *Increased Intensity of Ecstasy and Polydrug Usage in the More Experienced Recreational Ecstasy/MDMA Users: A WWW Study.* Addictive Behaviours, 2004. 29: p. 743-752.
18. Drugscope, *Ecstasy.* 2004. www.drugscope.co.uk/st info.asp?file=Al l\l\l\ecstasy.html.
19. Sherlock, K. and M. Conner, *Patterns of Ecstasy Use Amongst Club-Goers on the UK Dance Scene'.* International Journal of Drug Policy, 1999. 10: p. 117-129.
20. McMillan, B., K. Sherlock, and M. Conner, *Expanding the Traditional User Versus Non-User Dichotomy Amongst Ecstasy Users.* Journal of Community and Applied Social Psychology, 2003. 13: p. 15-28.
21. Pichini, S., et al., *On-Site Testing of 3,4-Methylenedioxymethamphetamine (Ecstasy) in Saliva with Drugwipe and Drugread: A Controlled Study in Recreational Users.* Clinical Chemistry, 2002. 48: p. 174-176.
22. Cole, J.C., et al., *The Content of Ecstasy Tablets: Implications for the Study of Their Long-Term Effects.* Addiction, 2002. 97: p. 1531-1536.
23. Cox, D.E. and K.R. Williams, *'Adam' or 'EVE' - A Toxicological Conundrum.* Forensic Science International, 1996. 77: p. 101-108.
24. Villamor, J.L., et al., *A New GC-MS Method for the Determination of Five Amphetamines in Human Hair.* Journal of Analytical Toxicology, 2005. 29: p. 135-139.
25. Parrott, A.C., *Is Ecstasy MDMA? A Review of the Proportion of Ecstasy Tablets Containing MDMA, Their Dosage Levels, and the Changing Perceptions of Purity.* Psychopharmacology, 2004. 173: p. 234-241.
26. Schifano, F., et al., *Ecstasy (MDMA, MDA, MDEA, MBDB) Consumption, Seizures, Related Offences, Prices, Dosage Levels and Deaths in the UK (1994-2003).* Journal of Psychopharmacology, 2006. 20: p. 456-463.

27. Samyn, N., et al., *Plasma, Oral Fluid and Sweat Wipe Ecstasy Concentrations in Controlled and Real Life Conditions.* Forensic Science International, 2002. 128: p. 90-97.

28. LGC, *Ecstasy Drugs,* in *LGC Drugs News.* 2003. p. 4.

29. McFadden, K., et al., *Development and Application of a High-Performance Liquid Chromatography Method Using Monolithic Columns for the Analysis of Ecstasy Tablets.* Journal of Chromatography A, 2006. 1120: p. 54-60.

30. Samyn, N., et al., *Analysis of Drugs of Abuse in Saliva.* Forensic Science Review, 1999. 11: p. 1-19.

31. Angrist, B., et al., *Early Pharmacokinetics and Clinical Effects of Oral D-Amphetamine in Normal Subjects.* Biological Psychiatry, 1987. 22: p. 1357-1368.

32. King, L.A. and S.D. McDermott, *Drugs of Abuse,* in *Clarkes Analysis of Drugs and Poisons,* A.C. Moffat, M.D. Osselton, and B. Widdop, Editors. 2004, Pharmaceutical Press.

33. Nordahl, T.E., R. Salo, and M. Leamon, *Neuropsychological Effects of Methamphetamine Use on Neurotransmitters and Cognition: A Review.* The Journal of Neuropsychiatry and Clinical Neurosciences, 2003.15: p. 317-325.

34. Cook, C.E., et al., *Pharmacokinetics of Methamphetamine Self-Administered to Human Subjects by Smoking S-(+)-Methamphetamine Hydrochloride.* Drug Metabolism and Disposition, 1993. 21: p. 717-723.

35. Perez-Reyes, M., et al., *Clinical Effects of Methamphetamine Vapor Inhalation.* Life Sciences, 1991. 49: p. 953-959.

36. Cook, C.E., et al., *Pharmacokinetics of Oral Methamphetamine and Effects of Repeated Daily Dosing in Humans.* Drug Metabolism and Disposition, 1992. 20: p. 856-862.

37. Torre, R.D.L., et al., *Pharmacology of MDMA in Humans.* Annals of the New York Academy of Sciences, 2000. 914: p. 225-237.

38. Navarro, M., et al., *Usefulness of Saliva for Measurement of 3,4-Methylenedioxymethamphetamine and Its Metabolites: Correlation and Effect of Salivary pH.* Clinical Chemistry, 2001. 47: p. 1788-1795.

39. Segura, M., et al., *3,4-Dihydroxymethamphetamine (HHMA). A Major in Vivo 3,4-methylenedioxymethamphetamine (MDMA) Metabolite in Humans.* Chemical Research in Toxicology, 2001. 14: p. 1203-1208.

40. Farre, M., et al., *Repeated Doses Administration of MDMA in Humans: Pharmacological Effects and Pharmacokinetics.* Psychopharmacology, 2004. **173:** p. 364-375.

41. Torre, R.D.L., et al., *Non-Linear Pharmacokinetics of MDMA ('Ecstasy') in Humans.* British Journal of Clinical Pharmacology, 2000. 49: p. 104-109.

42. Karlovsek, M.Z., A. Alibegovic, and J. Balazic, *Our Experiences with Fatal Ecstasy Abuse (Two Case Reports).* Forensic Science International, 2005. 147S: p. S77-S80.

43. Albertson, T.E., R.W. Derlet, and B.E.V. Hoozen, *Methamphetamine and the Expanding Complications of Amphetamines.* Western Journal of Medicine, 1999. **170:** p. 214-219.

44. Derlet, R.W. and B. Heischober, *Methamphetamine Stimulant of the 1990s?* Western Journal of Medicine, 1990. **153:** p. 625-628.

45. Morgan, M.J., *Ecstasy (MDMA): A Review of its Possible Persistent Psychological Effects.* Psychopharmacology, 2000. 152: p. 230-248.

46. Huxter, J.K., A. Pirona, and M.J. Morgan, *The Sub-Acute Effects of Recreational Ecstasy (MDMA) Use: A Controlled Study in Humans.* Journal of Psychopharmacology, 2006. 20: p. 281-290.

47. Raikos, N., et al., *Amphetamine Derivative Related Deaths in Northern Greece.* Forensic Science International, 2002. **128:** p. 31-34.

48. Toennes, S.W., et al., *Screening for Drugs of Abuse in Oral Fluid - Correlation of Analysis Results with Serum in Forensic Cases.* Journal of Analytical Toxicology, 2005. **29:** p. 22-27.

49. Cozart, *Cozart RapiScan Single Panel Drug Test: Oral Fluid Methamphetamine/MDMA.* Technical Information, 2005. **01/05.**

50. Cozart, *Methamphetamine Microplate EIA - Oral Fluid Application.* 2003. p. 1-3.

51. Cooper, G., et al., *Validation of the Cozart Amphetamine Microplate EIA for the Analysis of Amphetamines in Oral Fluid.* Forensic Science International, 2006. **159:** p. 104-12.

52. www.rapid-diagnostics.org/accuracy.htm. *Accuracy of Diagnostic Tests.* 2005.

53. Zweig, M.H. and G. Campbell, *Receiver-Operating Characteristic (ROC) Plots: A Fundamental Evaluation Tool in Clinical Medicine.* Clinical Chemistry, 1993. **39:** p. 561-577.

54. Barrett, C., C. Good, and C. Moore, *Comparison of Point of Collection Screening of Drugs of Abuse in Oral Fluid with a Laboratory Based Urine Screen.* Forensic Science International, 2001. 122: p. 163-166.

55. Biermann, T., et al., *On-Site Testing of Illicit Drugs: The Use of the Drug-Testing Device 'Toxiquick'.* Forensic Science International, 2004. 143: p. 21-25.

56. Samyn, N., G.D. Boeck, and A.G. Verstraete, *The Use of Oral Fluid and Sweat Wipes for the Detection of Drugs of Abuse in Drivers.* Journal of Forensic Sciences, 2002. 47: p. 1380-1387.

57. Crouch, D.J., et al., *An Evaluation of Selected Oral Fluid Point-of-Collection Drug-Testing Devices,* journal of Analytical Toxicology, 2005. 29: p. 244-248.

58. Walsh, J.M., et al., *An Evaluation of Rapid Point-of-Collection Oral Fluid Drug-Testing Devices.* Journal of Analytical Toxicology, 2003. 27: p. 429-439.

59. Kupiec, T., et al., *Choice of an ELISA for Screening Postmortem Blood for Amphetamine and/or Methamphetamine.* Journal of Analytical Toxicology, 2002. 26: p. 513-518.

60. Kerrigan, S. and W.H. Phillips, *Comparison of ELISAs for Opiates, Methamphetamine, Cocaine Metabolite, Benzodiazepines, Phencyclidine, and Cannabinoids in Whole Blood and Urine.* Clinical Chemistry, 2001. 47: p. 540-547.

61. Han, E., et al., *Validation of the Immunalysis Microplate for the Detection of Methamphetamine in Hair.* Journal of Analytical Toxicology, 2006. 30: p. 380-385.

62. Laloup, M., et al., *Validation of an ELISA-based Screening Assay for the Detection of Amphetamine, MDMA and MDA in Blood and Oral Fluid.* Forensic Science International, 2005.153: p. 29-37.

63. Schumacher, J.E., et al., *Validity of Self-Reported Crack Cocaine Use Among Homeless Persons in Treatment.* Journal of Substance Abuse Treatment, 1995. 12: p. 335-339.

64. Preston, K.L., K. Silverman, and C.R. Schuster, *Comparison of Self-Reported Drug Use With Quantitative and Qualitative Urinalysis for Assessment of Drug Use in Treatment Studies.* NIDA Research Monographs, 1997. 167: p. 130-145.

65. Fendrich, M., et al., *Tobacco-Reporting Validity in an Epidemiological Drug-Use Survey.* Addictive Behaviours, 2005. 30: p. 175-181.

66. Wood, M., et al., *Development of a Rapid and Sensitive Method for the Quantitation of Amphetamines in Human Plasma and Oral Fluid by LC-MS-MS.* Journal of Analytical Toxicology, 2003. 27: p. 78-87.

67. Stark, M.M., et al., *The Validity of Self Reported Substance Misuse Amongst Detained Persons in Police Custody.* Journal of Clinical Forensic Medicine, 2002. 9: p. 25-25.

68. Bedi, G. and J. Redman, *Self-Reported Ecstasy Use: The Impact of Assessment Method on Dosage Estimates in Recreational Users.* Journal of Psychopharmacology, 2006. 20: p. 432-436.

3 Development of an Enzyme Immunoassay (EIA) for the Detection of Anhydroecgonine Methyl Ester

3.1 Aims and Objectives

The aim is to develop a novel application of EIA for the detection of the pyrolysis products of cocaine. The requirements are for the assay to be specific for anhydroecgonine methyl ester (AEME), one of the pyrolysis products of cocaine, and not to show cross reactivity to cocaine or any of its other metabolites. Following assay development a series of validation experiments are to be carried out to establish the performance characteristics of the assay.

3.2 Introduction

3.2.1 Cocaine and Crack Cocaine

Cocaine is an alkaloid derived from the plant *Erythroxylon coca.* Pure cocaine was first extracted from the leaf of the plant in the mid-19 century [1], and was used in various elixirs and tonics. In 1884 cocaine was recognised for its anaesthetic properties when Carl Koller became the first physician to use cocaine as a topical anaesthetic in ophthalmological surgery [1].

Under the Misuse of Drugs Act Cocaine is classified as a class A drug. It has a high potential for abuse but can be administered by a doctor for legitimate medical purposes, such as a local anaesthetic for some eye, ear and throat surgeries.

3.2.1.1 Types of Cocaine

There are two chemical forms of cocaine which are commonly abused, the hydrochloride salt and the cocaine base. The hydrochloride salt is generally a fine, white, crystalline powder known as 'coke', or 'blow'. Cocaine hydrochloride is extracted as a crude paste from the coca leaf, purified to form cocaine base, and then converted to cocaine hydrochloride [2]. In this form it is water soluble and is either taken intranasally or dissolved in water and taken intravenously. Smoked administration is less common as cocaine hydrochloride only volatilises at high temperatures and decomposes when it is burned [3].

Cocaine base, otherwise known as 'crack', is prepared by dissolving cocaine hydrochloride in water, mixing it with baking soda and heating. Cocaine base precipitates into a soft mass which becomes hard when dry [4]. The texture and colour of cocaine base ranges from a crumbly to hard crystalline solid and white to light brown. Cocaine base is insoluble in water which means it is not suitable for snorting or injecting. It is however suited to smoking as it vaporises at a low temperature, it is usually smoked in a pipe, glass tube, plastic bottle or in foil.

3.2.1.2 Use and Abuse

Cocaine is one of the most frequently identified drugs of abuse in the general population and in drug testing programmes [5-7]. In 2000 drug seizures in the UK involving cocaine accounted for 17% of all seizures of Class A drugs [8]. Cocaine was originally seen as a drug for the wealthy, but lower prices and less expensive forms, such as crack, have resulted in widespread use in all populations [9].

The results of the British Crime Survey (BCS) found that between 1998 and 2005/06 the use of cocaine hydrochloride has increased while the use of crack cocaine remained stable. The observed rise was mainly due to large increases in use between 1998 and

2000 [6]. However the overall use of crack cocaine may be underestimated due to the fact that the BCS is a household survey and therefore may not capture more problematic drug users. This is supported by findings that the use of crack cocaine by individuals attending a drug treatment centre was twice that of cocaine hydrochloride [5].

A decline in the use of some drugs such as amfetamines has been noted [10], although the overall use of stimulants has remained stable. This supports the idea that cocaine powder has replaced other substances as the drug of choice for stimulant users [6].

The reported number of deaths where cocaine was specifically mentioned on the death certificate has increased from 18 in 1996 to 147 in 2004 [11].

The use of crack cocaine is a concern to health care professionals due to the increased health risks associated with this form of cocaine. It is also of great concern to criminal justice officials due to its association with criminal activity [12].

3.2.1.3 Cocaine and Crime

Offenders who use crack cocaine and/or heroin commit significantly more crimes than those who don't use these particular Class A drugs. There are strong indicators that a significant proportion of the income used to pay for these drugs is obtained through low level crime, including property crime, benefit fraud, drug dealing and prostitution [5, 13-15]. This is more typical of crack users rather than cocaine hydrochloride use as it is suggested that the majority of cocaine hydrochloride users are in regular employment and can afford to fund their cocaine use through their legitimate income [13].

A recent study of 100 London crack cocaine users found that they spent on average £800 a week on their habit, and 9 out of 10 of these were committing crimes to fund their use [15]. This is further supported by a study of arrestees in the UK between October 2003 and September 2004 in which 38% reported the use of heroin or cocaine, 57% of which had been arrested for an acquisition crime [16].

In addition to the problem of acquisition crime there is also a link between the use of crack cocaine and violent crime. An increase in availability and use of crack cocaine in the UK and US over the years has seen a concomitant increase in the recording of violent crime, often involving firearms [13, 17].

3.2.1.4 Physiological and Phamiacodynamic Effects

Cocaine is a potent central nervous system stimulant, but also acts as a local anaesthetic and a sympathomimetic [18]. The local anaesthetic action is a result of its ability to block sodium channel conductance in neuronal cells thereby increasing the threshold required to generate an action potential [19].

Cocaine induces the release of the neurotransmitters nor adrenaline, dopamine and serotonin, the effect of this on the individual is a feeling of euphoria [20]. Cocaine also blocks the reuptake of the neurotransmitters at the synaptic junctions resulting in an increase in their concentrations, and therefore prolonging the euphoria. As nor-adrenaline is the primary neurotransmitter of the sympathetic nervous system its stimulation results in vasoconstriction, hypertension, tachycardia, mydriasis and hyperthermia [19, 21]. The behavioural effects however appear to be mediated by its dopaminergic actions, these effects are listed in Table 3:1. The euphoria of cocaine is followed by a 'crash' 20-60 minutes later, where the individual may experience anxiety, depression, fatigue and the desire for more cocaine [18]. This can be explained by the initial brief elevation of dopamine after an acute dose followed by a marked reduction of dopamine below normal concentrations [1].

The most common acute and chronic complications of cocaine use are related to the cardiovascular system. The highest risk of cardiovascular problems is within the first hour after cocaine use [22].

Dose	Physical Effects	Psychological Effects
Initial Low Doses	Tachycardia, tachypnoea, hypertension. Dilated pupils (& flattened lenses), sweating, reduced appetite, reduced need for sleep, reduced lung function, dry mouth, impaired motor control & performance of delicate skills and driving	Euphoria, sense of well being, impaired reaction time and attention span, impaired learning of new skills
Increased doses	Seizures, cardiac arrhythmias, myocardial infarction, ischemia, stroke, respiratory arrest	Anxiety, irritability, insomnia, depression, paranoia, aggressiveness, impulsivity, delusions, agitated/excited delirium, reduced psychomotor function
Chronic Use	Erosions, necrosis and perforation of nasal septum, anosmia, rhinorrhoea and nasal eczema, chest pains, muscle spasms, sexual impotence, weight loss, malnutrition, vascular disease, cardiomyopathy, myocarditis	Dependence, disturbed eating and sleeping patterns

Table 3:1 Physiological and psychological effects of cocaine use

The physiological, psychoactive and cardiovascular effects of cocaine hydrochloride and crack are similar. However smoking crack is the fastest route of cocaine entry to the brain at approximately 6-8 seconds. Smoked drugs enter the blood stream rapidly through the large surface area of the lungs producing rapid peak concentrations, the result of this is a much more rapid high which creates a higher potential for addiction [19, 23-25].

Crack cocaine users have reported more psychological health problems associated with their habit compared to users of other drugs [5].

Good correlations exist between peak cocaine oral fluid and plasma concentrations and peak subjective and physiologic effects, indicating that the presence of cocaine in oral fluid shows strong evidence that the subject is under the influence of the drug [23, 26-28].

3.2.1.5 Pharmacokinetics

The bioavailability of cocaine is variable depending on the route of administration. Intravenous administration consistently produces 100% drug bioavailability [4]. Orally administered cocaine takes approximately 30 minutes to enter the bloodstream and typically only 20-30% is absorbed, consequently the euphoric effects are reduced compared to other methods of administration and is therefore rarely the chosen route [1]. Intranasal bioavailability is variable and may be dose dependant, with increased bioavailability at higher doses due to the amount of drug available for absorption. Estimates have ranged from 25% to 94% [4, 23]. During insufflation there is a delay in absorption due to the barrier of the nasal mucosa and the vasoconstrictive properties of cocaine. In addition, a portion of the dose may be swallowed which leads to further delay in absorption [29].

The bioavailability of smoked cocaine is also highly variable and following a number of studies has been quoted to range from 25-110% [1, 19, 23]. The individual smoking technique, temperature, and the device used to smoke the cocaine can all influence the bioavailability of smoked cocaine. Approximately 26% of the original dose of cocaine has been recovered from the smoking device [23, 24].

3.2.1.5.1 Metabolism

The bicyclic structure of cocaine is characterised by functional groups including N-methyl, carboxyl methyl ester, and benzoyl ester, which are all susceptible to biotransformation (Figure 3:1) [30].

Cocaine is extensively metabolised, primarily in the liver, with less than 10% excreted unchanged in the urine [31, 32]. The main metabolites of cocaine are benzoylecgonine,

ecgonine methyl ester (EME) and ecgonine all of which are pharmacologically inactive. The excretion recovery of cocaine metabolites have ranged from 29 to 54% for benzoylecgonine to 26-60% for EME [18].

The metabolism of cocaine to benzoylecgonine occurs via spontaneous chemical hydrolysis of the methyl ester group [18, 30]. There is however some evidence to show that cocaine can also be enzymatically hydrolysed to benzoylecgonine by liver carboxylesterases [1,31]. Cocaine is metabolised to EME via enzymatic hydrolysis of the benzoyl group [30, 31]. Ecgonine is produced from the enzymatic hydrolysis of the benzoyl group of benzoylecgonine or the methyl ester group of EME [33, 34].

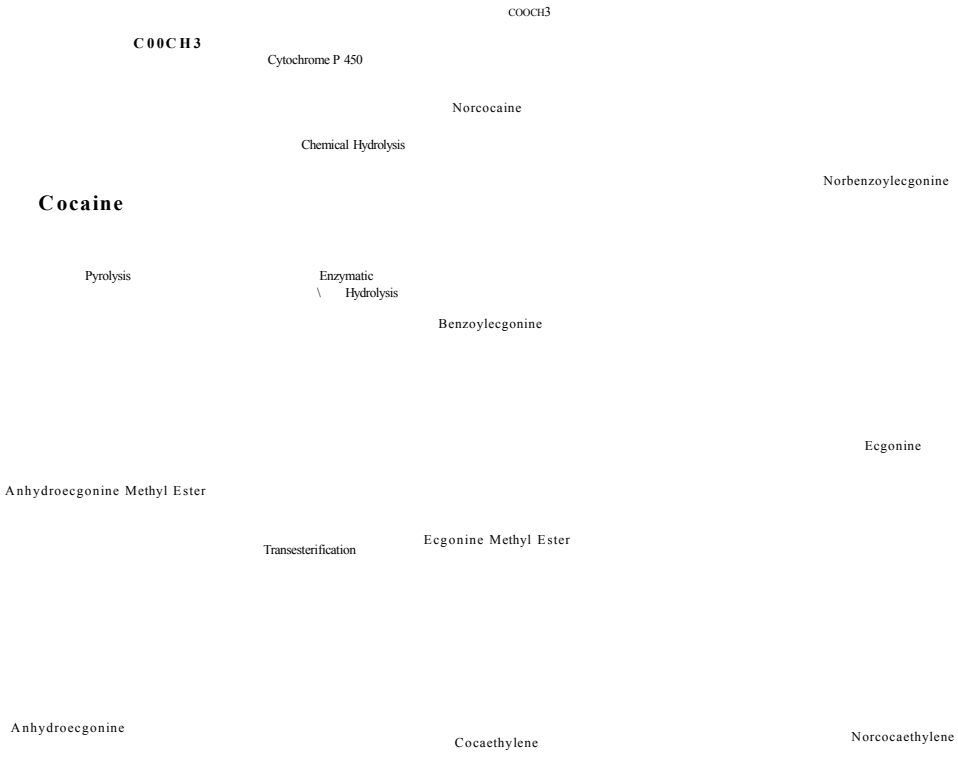


Figure 3:1 Metabolic and pyrolytic pathways of cocaine

Concurrent use of cocaine and alcohol results in the transesterification of cocaine to cocaethylene in the liver. Cocaethylene is biologically active and metabolises into two non active metabolites norcocaethylene and ecgonine ethyl ester [9].

Norcocaine is produced from the N-demethylation of cocaine mediated by microsomal cytochrome P-450. Norcocaine is pharmacologically active with similar actions to cocaine [30]. Norcocaine hydrolyses chemically to the pharmacologically inactive norbenzoylecgonine [33].

The minor metabolites such as norcocaine etc. have been shown to account for <2% of the administered dose of cocaine regardless of the route of administration [1, 29].

Research over the last two decades has identified a unique pyrolysis product of cocaine known as AEME [34-38]. AEME is not formed metabolically [39], it is solely produced by the elimination of benzoic acid during the thermal decomposition of cocaine at temperatures above 170°C [40, 41]. The pyrolysis of cocaine base to AEME has been studied and in general it has been found that the higher the temperature of the smoking device the greater pyrolysis of cocaine to AEME [24, 40, 42]. At 170°C the thermal degradation of cocaine base to AEME was found to be negligible, at temperatures up to 230°C the abundance of cocaine was 68-77%, and this decreased to 30% at temperatures over 255°C at which point AEME became the predominant compound. An increase in the temperature from 260°C to 400°C produced a linear increase in the degree of pyrolysis [40].

A similar pyrolysis experiment showed that at 350-400°C only 50% of cocaine was converted to AEME while at 650°C as much as 89% of cocaine was converted [42].

To prove the inefficiency of smoking cocaine hydrochloride it was subjected to pyrolysis, only 30% of cocaine was inhaled and there was no presence of AEME. When

the temperature exceeded 170°C there was no cocaine remaining but there was a small appearance of AEME (<10%) [40].

AEME is converted to anhydroecgonine (AE) via spontaneous chemical hydrolysis at basic pH [43, 44], and via enzymatic hydrolysis [37, 45]. The rate of metabolism of AEME to AE is unclear [45], and there is no evidence to suggest either are pharmacologically active [33]. AEME also undergoes transesteriflcation to anhydroecgonine ethyl ester (AEEE) in the presence of ethanol, as is seen with cocaine and cocaethylene [46].

Neither AEME nor AE have been found in street samples of cocaine [43], or following intravenous or intranasal administration of cocaine hydrochloride [29, 47], therefore the presence of AEME or its metabolite AE in biological matrices can positively identify the route of administration as being smoking.

3.2.1.5.2 Identification of Pyrolysis Products in Biological Fluids

It has been clearly established in the literature that the pyrolysis of cocaine produces a unique compound AEME. What is less clear is how useful AEME is for identifying the use of crack cocaine in a variety of biological matrices.

AEME has been detected in the urine [33, 34, 36, 38, 48], postmortem blood [9, 36, 49, 50], oral fluid [27, 34, 47, 51], sweat [34], nails [52, 53], and hair [34, 54, 55] of crack cocaine smokers.

In the analysis of postmortem and urine samples it appears that AE may be a more reliable maker for identification of crack cocaine use due to reports of significantly higher concentrations of AE compared to AEME. [9, 43, 45, 49, 56]. This is further

emphasised by the finding that AE was the predominant analyte in the urine of crack cocaine users [43, 56].

This is in contrast to sweat where a study of active cocaine and crack cocaine users showed that AEME was the predominant pyrolysis product [57].

The information on the presence of pyrolysis products in oral fluid is extremely limited. Due to the similarities between oral fluid and sweat with respect to parent cocaine being the predominant analyte [47, 58], it is possible that the identification of the pyrolysis products also follows a similar pattern to sweat with parent AEME being the predominant analyte over AE. Two controlled dose administration studies have been carried out, the results of which showed AEME to appear immediately in the oral fluid and clear within an hour. AE was not included in the analysis [27, 47]. AEME was detected in six oral fluid specimens from individuals with a history of drug use, the concentrations were reported to be low at <19ng/mL [34].

3.2.2 Screening for AEME

Typically biological samples are analysed for the presence of cocaine and or its metabolites, such as benzoylecgonine and ecgonine methyl ester, to identify cocaine use, [59-63], but determining the route of administration is not possible based on these analytes alone. It has been established that AEME is a pyrolysis product of cocaine [9, 35, 42], therefore its identification in biological matrices can establish the route of administration as smoking.

Although all forms of cocaine use are problematic, the consequences of crack cocaine use are greater compared to powder cocaine. This is reflected in the US by harsher penalties imposed for crack use and trafficking [45].

The use of crack presents a greater social problem, and has a high association with crime. Therefore the ability to distinguish between crack and powder cocaine would provide a valuable addition to drug treatment and criminal justice systems [57]. The identification and monitoring of crack cocaine use may also help to develop a better understanding of the prevalence and spread of crack use.

Although there are a number of studies published describing the application of EIA for the analysis of cocaine (see Table 3:2), there is currently no screening test available which will identify the presence of the pyrolysis products AEME or AE. Data regarding the prevalence of crack use has previously relied on self reports, the validity of which varies [12].

3.2.3 Immunoassay Development

3.2.3.1 Classification

In general immunoassays are categorised into two distinct groups, competitive and non-competitive. The general principle behind competitive immunoassay is that the drug antigen in the sample competes with a labelled antigen for a limited number of antibody molecules specific to that analyte. The amount of labelled analyte bound at equilibrium is inversely proportional to the number of unlabelled analyte molecules present [64]. The analysis of small molecules is largely limited to competitive immunoassay [65]. In non-competitive immunoassay the drug antigen reacts with an excess of antibody. The measured signal from the bound antibody increases with increasing antigen concentration [66].

Immunoassays which require separation of bound and free drug prior to signal measurement are known as heterogeneous and those which do not require separation are known as homogenous immunoassays. The advantage in using heterogeneous

immunoassay is that potential endogenous interferences from the biological matrix are removed during the separation step [66-68].

3.2.3.2 Preparation of Immunogen

Small molecular weight compounds, such as drug molecules, are not immunogenic alone. They require conjugation with a larger molecule, such as a protein, to create immunogens which are capable of inducing an immune response. Examples of carrier proteins include keyhole limpet hemocyanin (KLH), Bovine Serum Albumin (BSA) and thyroglobulin. Generally higher titre antisera are obtained when a rabbit is immunised with an immunogen conjugated to a large molecular weight protein such as thyroglobulin [72].

The presence of a reactive group on the drug molecule facilitates conjugation with the carrier protein, the common reactive groups include amino, carboxyl, sulfhydryl, and carbonyl. Drug molecules without one of these reactive groups can be derivatised to introduce one [65]. It is important that the functional group chosen is not fundamental in the specificity. If conjugation is performed using a spacer the drug molecule is better exposed to the immune system and therefore increases the specificity of the antibodies produced. However bridge antibodies may be produced which can decrease sensitivity [73].

Cross linkers are chemical reagents used to conjugate molecules together by a covalent bond [74]. A wide variety of crosslinking reagents with a choice of reactive groups is commercially available, the suitability of which depends on the type of reactive groups in the drug molecule and carrier protein [65].

Table 3:2 Validation data for cocaine and metabolites HA for various biological matrices

C2

3.2.3.3 Antibodies and Immunisation

Immunoassays can employ either monoclonal or polyclonal antibodies. Monoclonal antibodies are a single specific antibody population, produced by the clones of a single hybrid cell which is formed by the fusion of a B lymphocyte with a tumour cell [75]. As long as the cell line is maintained there will be a continuous supply of the specific antibodies [73]. While monoclonal antibodies show more specificity to the analyte than polyclonal antibodies they generally have lower affinity, this can result in less sensitive assays.

Polyclonal antibodies are a mixture of immunoglobulin molecules which have different affinity and specificity for the compound of interest. Rabbits are the principal animal used for the production of polyclonal antibodies, however other possibilities are goats and sheep. Avian antibodies harvested from egg yolks can also be used, they offer advantages of large quantities and exhibit less non specific binding than found with mammalian antibodies [68].

A blood sample is collected prior to immunisation to establish any pre-existing antibodies with specificity to the analyte of interest. The primary immunisation is made with the immunogen and an adjuvant. The adjuvant acts to release the antigen slowly by way of altering the physical state of the immunogen by forming depots thereby lowering the rate of elimination. This results in a prolonged persistence of the immunogen in tissues and a continuous stimulation of the immune response. A series of adequately spaced booster immunisations are then administered and blood samples are taken at various points during the immunisation process to assess the quality of the antiserum. Once the antiserum proves to be adequate the animal is bled [64]. The immune response to the third and subsequent booster injections mirrors that of the second injection. As

the immunisation schedule progresses the quality of the antibodies change due to the maturation of the immune response, therefore providing high affinity antibodies.

3.2.3.4 Antibody and Analyte Labelling

The antibody-antigen binding reaction itself does not produce a visible signal, therefore various labels are employed to produce a signal which is measurable [73]. Labels are conjugated to the drug molecule in a process similar to that of the preparation of the immunogen. A number of labels are used in immunoassay and the assays are often classified according to the label used. The more commonly used labels are radioisotopes (e.g RIA), active enzymes (e.g EMIT and ELISA), or fluorescent labels (e.g FPIA). Other labels exist such as chemi and bio-luminescent molecules, particles such as colloidal gold or latex beads.

3.2.3.5 Immunoassay Sensitivity and Specificity

While the general requirements of an immunoassay are speed, convenience, and robustness, the fundamental characteristics required are sensitivity and specificity. Analytical sensitivity relates to the degree to which an assay responds to small amounts of drug. High sensitivity is essential in any analytical technique as it allows smaller sample volumes to be analysed, and provides lower limits of detection which are often required in forensic analysis. The sensitivity of any immunoassay is a complex function of the physio-chemical basis of the technique, such as the type of label employed and the separation of bound and free drug, as well as the avidity of the antibody and the presence of non specific binding. Due to the dissociation of the antibody and antigen complex during the incubation and separation stages of the assay, it is the affinity of the

antibody in a competitive system which primarily determines the sensitivity of the assay [76].

Clinical sensitivity is often referred to when the performance of a screening test is being evaluated, it is defined as the probability of a true positive test result as compared to a reference or gold standard.

Typically immunoassays for drugs of abuse test for a class of compounds and cannot distinguish drugs from within its class due to the similarities in molecular structure, an example is the opiates class which consists of codeine, pholcodeine, morphine, diacetylmorphine and a few other derivatives [77]. Specificity is the degree to which an assay correctly identifies only the compound(s) of interest, the extent to which other endogenous molecules and drugs interfere is known as the cross reactivity. A good immunoassay should have high specificity with minimum cross reactivity to non related compounds.

3.3 Experimental

3.3.1 Materials

3.3.1.1 Chemicals and Reagents

Anhydroecgonine (AE), anhydroecgonine methyl ester (AEME) and all compounds used for cross reactivity testing were purchased from LGC Promochem (Teddington, UK), bovine thyroglobulin, bovine serum albumin (BSA), goat anti-rabbit immunoglobulin conjugated to HRP, morpholinoethanesulfonic acid (MES), bronidox, 1,2-propandiol, trizma base, sephadex G-50M dry beads, copper sulphate pentahydrate, folin reagent, and chromatography column (1.5 x 30cm) were purchased from Sigma Aldrich Company (Dorset, UK). Horseradish peroxidase (HRP) was purchased from

Biozyme Laboratories Ltd (South Wales, UK). Sodium hydroxide, 1M sulphuric acid, sodium carbonate, citric acid, sulphuric acid, EDTA, sodium chloride, sodium dihydrogen phosphate, sodium monohydrogen phosphate, sodium carbonate, and sodium bicarbonate were purchased from VWR International (Leicestershire, UK). Sodium azide was purchased from Molekula Ltd (Dorset, UK). 2mg/mL BSA solution, N-hydroxysulfosuccinimide (S-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Perbio Science Ltd (Cheshire, UK). Stabilzyme was purchased from Diarect AG (Freilburg, Germany).
Cozart Cocaine and Metabolites EIA for oral fluid, substrate solution (TMB), wash buffer and oral fluid collectors were supplied by Cozart Bioscience Limited (Oxfordshire, UK).

3.3.1.2 Buffers and Solutions

- Coating Buffer: 50mM Sodium Carbonate/Bicarbonate Buffer (30/70 ratio) pH 9.5 with 0.05% w/v sodium azide (500ml).
  0.795g of sodium carbonate (MW 106), 1.47g of sodium bicarbonate (MW 84) and 0.25g of sodium azide (MW 65) were dissolved in 450ml of distilled water. pH adjustment was not necessary. The solution was filled to 500ml with distilled water, poured into a pre-labelled bottle and stored at 4°C.
- Concentrated Coupling Buffer: 1M Sodium Bicarbonate (15ml).
  1.26g of sodium bicarbonate (MW 84) was dissolved in 14ml of distilled water, made up to 15ml and then stored at -20°C.
- Concentrated Activation Buffer: 0.5M Morpholinoethanesulfonic acid (MES)/Sodium Hydroxide Buffer pH 4.7 (25ml).

2.665g of MES (MW 213.2) was dissolved in 20ml of distilled water. The pH of the solution was adjusted to pH 4.7 with 1M sodium hydroxide before being made up to 25ml with deionised water. The solution was stored at -20°C.

- Phosphate Buffered Saline (PBS) (10X cone.): 0.2M Sodium dihydrogen/Sodium monohydrogen phosphate with 1.5M sodium chloride and 0.01% bronidox (w/v) (1000ml).

  A 5% bronidox solution was first prepared by dissolving 0.5g of bronidox in 10ml of 1,2-propandiol. 87.7g of sodium chloride (MW 58.44), 23g of sodium monohydrogen phosphate (MW 115g), and 4g of sodium dihydrogen phosphate (MW 20g) were dissolved in 800ml of deionised water. 2ml of the 5% bronidox solution was added and the final volume made up to 1L with deionised water. This solution was stored at room temperature.

- EIA Buffer: 50% Stabilzyme (Tris) (500mL).

  250mL of stabilzyme solution and 250mL of distilled water were measured out using a measuring cylinder. The solution was poured into a pre-labelled bottle and stored at 4°C.

- PBS (IX cone.) with 0.05% sodium azide (1L).

  100ml of 10X PBS was diluted with 900ml of distilled water. 0.5g of sodium azide was added. The solution was stored at room temperature.

- Affinity Chromatography Elution Buffer: 0.1M citric acid, 0.5M sodium chloride, 0.05% sodium azide and ImM EDTA (lOOmL).

  2.1g of citric acid (MW 210.14), 2.9g of sodium chloride and 0.05g of sodium azide were dissolved in 90mL of water. 250pL of a 400mM stock solution of EDTA was added and the volume made up to lOOmL. The solution was stored at 4°C.

- Neutralisation Buffer: 2M Trizma base/HCl buffer pH 9.5 (lOOmL).

24.2g of trizma (MW 121.14) was dissolved in 80mL of water. The pH was adjusted with hydrochloric acid to pH 9.5 and the volume made up to lOOmL with water. The solution was stored at 4°C.

- MES/NaOH Buffer pH 6.1 (25mL).

  2.665g of MES was dissolved in 20mL of water, the pH was adjusted to pH 6.1 with 1M sodium hydroxide and made up to 25ml with water. The solution was stored frozen at -20°C.

- Dialysis buffer: 0.05M Tris/HCl pH 7.5 with 0.15M sodium chloride (1L).

  6.06g of Trizma and 8.77g of sodium chloride were dissolved in 900mL of distilled water. The pH was adjusted to pH 7.5 using hydrochloric acid. The volume was then made up to 1L.

- Lowry Protein Assay Reagents

  Reagent A: 2% sodium carbonate, 0.4% sodium hydroxide, 0.16% potassium sodium tartarate and 1% SDS. This reagent can be stored in a plastic bottle at 37°C.

  Reagent B: 4% copper sulphate pentahydrate comes as ready to use.

  Reagent C: 100 parts of reagent A with 1 part reagent B, this must be prepared fresh.

  Reagent D: Folin reagent diluted 1:1 with water, must be prepared fresh.

3.3.1.3 Instrumentation

EIA experiments were performed on 96 well Costar flat bottomed polystyrene stripwell plates purchased from Appleton Woods (Birmingham, UK). Plates were washed with a Dynex plate washer and the absorbance measurements were performed with a MRX microtitre plate reader in dual wavelength mode 450-650nm, both items were purchased from Dynex Techonologies (Worthing, UK).

All buffers were made using filtered water from a Purite Select water purifier purchased from Purite Ltd (Thame, UK).

Absorbance measurements were carried out using a spectrophotometer purchased from Thermo Scientific (Basingstoke, UK).

3.4 Experimental Part 1 - Characterisation of Aatisera

This section describes the processes involved for the production of polyclonal antibodies to the pyrolysis product AEME, and the experiments performed to assess the sensitivity and specificity of the antibodies prior to development of the EIA.

3.4.1 Synthesis of Immunogen

Although the target analyte was AEME it was decided to prepare the immunogen using its metabolite AE. AEME and AE only differ by one functional group, by using this group for conjugation it will be hidden from the immune system and the antibodies produced should show specificity to both AE and AEME. The functional group in AE is a carboxyl group which can be directly used for conjugation, AEME however would require derivatisation to incorporate a carboxyl group into the molecule. Therefore the use of AE makes the process simpler while still retaining the immunogenicity required to detect AEME.

The carboxyl group of anhydroecgonine was conjugated to the amine group on the lysine residues of thyroglobulin using a carbodiimide mediated reaction. An illustration of the linkage is shown in Figure 3:2. Generally high titre antisera are achieved when a rabbit is immunised with an immunogen conjugated to a large molecular weight protein such as thyroglobulin [72]. Due to the large number of lysine residues on thyroglobulin the molecule will be highly substituted.

EDC is a water soluble derivative of the cross linker carbodiimide which reacts with carboxyl groups on the hapten to form an amine-reactive intermediate O-acylisourea [78]. This intermediate is unstable and can either be attacked by the amine group on the protein to form the conjugate, or undergo hydrolysis, which can severely limit the overall yields obtained.

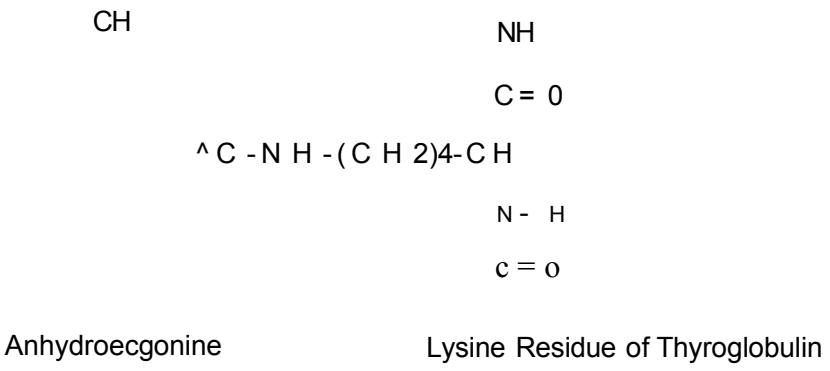


Figure 3:2 Illustration of the linkage between the carboxyl group of anhydroecgonine and the amine group on a lysine residue of thyroglobulin

A stabilisation step is therefore carried out by the addition of N-hydroxysulfosuccinimide (sulfo-NHS) which reacts with the intermediate to give stable active esters that hydrolyse very slowly, thereby enhancing the yield of EDC mediated coupling reactions [79], yields of up to 90% have been observed using this method [78]. The general conjugation process is illustrated in Figure 3:3.

One millilitre of a solution of AE (lmg/mL in methanol) was evaporated to dryness at 37°C. The drug was reconstituted in 0.5mL of 0.1M activation buffer, prepared as a dilution from the 0.5M stock solution. The solution was left stirring for 20 minutes. 1.5mg of Sulfo-NHS, dissolved in 50pL of 0.1M activation buffer, was added to the solution of AE, followed by 20mg of EDC, dissolved in 50pl of 0.1 M activation buffer. The mixture was left to stir for 30 minutes at room temperature.

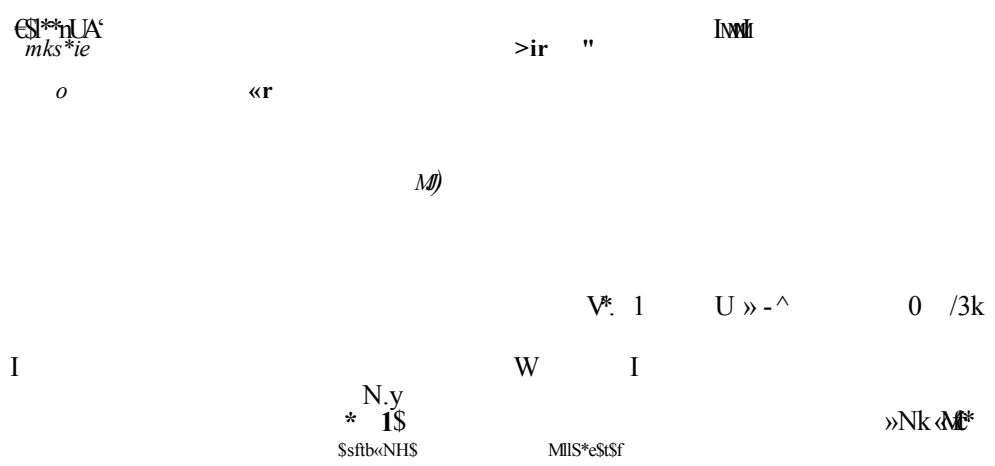


Figure 3:3 Illustration of conjugation reaction involving hapten (1), EDC, Sulfo-NHS, and carrier protein (2) [80]

In a separate vial 10.2mg of bovine thyroglobulin (Tg) was dissolved in 2mL of 0.1M coupling buffer, prepared as a dilution from the 1M stock solution. The solution was then left to stir for 20 minutes. The thyroglobulin solution and the AE/EDC/Sulfo-NHS solution were then combined and left to stir overnight at room temperature.

The Tg-AE conjugate solution was purified by dialysis to remove the unreacted small molecular weight substances. The solution was secured inside a dialysis membrane, immersed into 1L of dilute PBS, and dialysed at 4°C for 24 hours. The buffer was refreshed and dialysis resumed for a further 24 hours. The buffer was then replaced with 1L of 0.15M sodium chloride, containing 0.01% bronidox, and dialysed overnight at 4°C.

To determine the protein concentration, the purified Tg-AE conjugate was diluted 1:5, 1:10 and 1:20, and a Lowry assay was performed. Using the equation produced from the

Lowry assay standard curve (Figure 3:4), the protein concentration of each dilution was calculated. The results, corrected for the dilution factor, are displayed in Table 3:3.

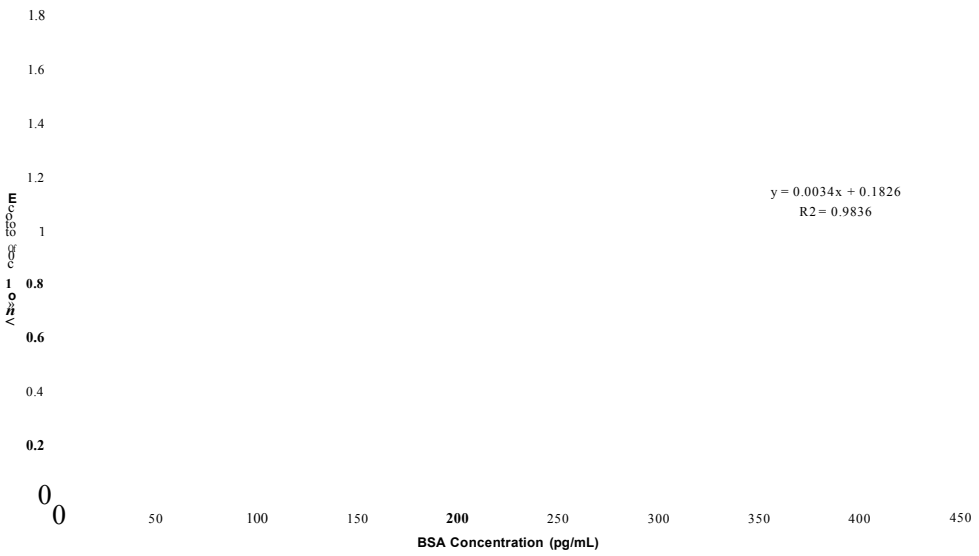


Figure 3:4 Lowry assay standard curve

Tg-AE Conjugate Dilution	Absorbance (660nm)	Final Protein Concentration (mg/mL)
1:5	1.168	1.45
1:10	0.724	1.59
1:20	0.436	1.49

Table 3:3 Protein concentrations of Tg-AE conjugate dilutions as determined by Lowry assay

The average concentration of the purified Tg-AE conjugate was calculated to be 1.51mg/mL.

3.4.2 Immunisation

Four female New Zealand white rabbits, 402717, 402719, 402734 and 402738, received lOOjag intramuscular inoculations of the purified Tg-AE conjugate. Immunisation was performed by CovalAb (Cambridge, UK), the protocol employed is outlined in Table 3:4.

Day	Protocol
0	Pre-Immune Test Bleed (4-5mL)
	1st Injection (0.5mL immunogen + 0.5mL Freund's Complete Adjuvant
	2nd Injection (0.5mL immunogen + 0.5mL Freund's Incomplete Adjuvant
21	
42	3rd Injection
53	Test Bleed (4-5mL)
63	4th Injection
74	Test Bleed (12-15mL)
88	Final Bleed (60mL)

Table 3:4 Immunisation protocol as performed by CovalAb

3.4.3 Synthesis of Coating Antigen

To evaluate the antisera produced following immunisation, a coating antigen was prepared using essentially the same carbodiimide mediated conjugation reaction as described in section 3.4.1 for the preparation of the immunogen. The carboxyl group of AE was conjugated to the amine group on the lysine residues of BSA. A different carrier protein was chosen for the coating antigen to avoid interference from antibodies specific to the carrier protein used for immunisation.

One millilitre of a solution of AE (lmg/mL in methanol) was evaporated to dryness at 37°C. The drug was reconstituted in 0.5mL of 0.1M activation buffer, prepared as a dilution from the 0.5M stock solution. The solution was left stirring for 20 minutes. 5mg

of Sulfo-NHS, dissolved in 50pL of 0.1M activation buffer, was added to the solution of AE followed by 30mg of EDC, dissolved in 50pL of 0.1M activation buffer. The solution was left to stir for 30 minutes at room temperature.

In a separate vial 8.5g of BSA was dissolved in 2mL of 0.1M coupling buffer, prepared as a dilution from the 1M stock solution. The solution was then left to stir for 20 minutes. The BSA solution and the AE/EDC/Sulfo-NHS solution were combined and left to stir overnight at room temperature.

The BSA-AE conjugate was purified by gel chromatography to remove the unreacted small molecular weight substances. A glass chromatography column containing sephadex G50M beads was conditioned using 50mL of IX PBS. The BSA-AE conjugate was centrifuged at 3000rpm for 20 minutes and the supernatant was added to the sephadex column. The column eluant was collected immediately in 3mL fractions and the absorbance measured at 280nm using a spectrophotometer.

A total of 19 fractions were collected and the absorbance values plotted (Figure 3:5).

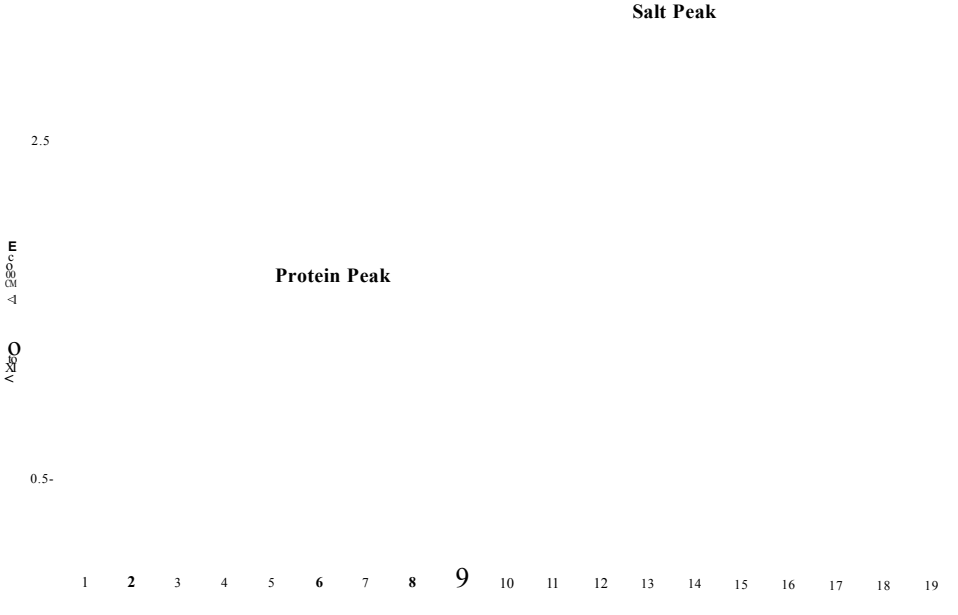


Figure 3:5 Absorbances of sephadex G50M eluted fractions

The BSA-AE conjugate eluted within the void volume and appears as the first peak on the graph. The second peak represents the unreacted free drug, EDC, and Sulfo-NHS. Fractions 5-7 which contained the BSA-AE conjugate were pooled, placed inside a dialysis membrane, and immersed into 1L of IX PBS for dialysis overnight at 4°C.

To determine the protein concentration, the purified BSA-AE conjugate was diluted 1:3, 1:5 and 1:10, and a Lowry assay was performed. Using the equation produced from the Lowry assay standard curve (Figure 3:6), the protein concentration of each dilution was calculated. The results, corrected for the dilution factor, are displayed in Table 3:5.

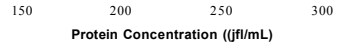


Figure 3:6 Lowry Assay Standard Curve

Coating Antigen Dilution	Absorbance (660nm)	Protein Concentration (mg/mL)
1:3	1.085	0.86
1:5	0.738	0.88
1:10	0.480	0.92

Table 3:5 Conjugate absorbance values and undiluted protein concentrations

The average protein concentration of the BSA-AE conjugate was calculated to be 0.89mg/mL. The approximate volume of the solution was 9ml which gives an approximate total amount of 8.0 lmg. As the starting amount was 8.5mg of BSA then the recovery was approximately 94%.

3.4.4 Screening Antisera

To monitor the progress of the immunisation a screening procedure was performed on the antisera. The sera of the pre-immune, and the antisera of the day 53, and final bleeds were titrated against lpg/mL BSA-AE coated microtitre plates. The sera from the pre-immune bleeds were also titrated against plates coated with lpg/mL BSA only. Three fold serial dilutions of the sera and antisera were performed and the ELISA procedure was carried out as described below, the microplate map in Figure 3:7 illustrates the titration.

	402717		402719		402734		402738			
	1	**2**	3	4	5	**6**			9	10
_A_	1/3000	1/3000	1/3000	1/3000	1/3000	1/3000	1/3000			
	i/9000	1/9000	1/9000	1/9000	1/9000	1/9000	1/9000	▪		
C_	1/27000	1/27000	1/27000	1/27000	1/27000	1/27000	1/27000	1/27000		
_D_	1/81000	1/81000	1/81000	1/81000	1/81000	1/81000	1/81000	1/81000		
_E_	1/243000	1/243000	1/243000	1/243000	1/243000	1/243000	1/243000	1/243000		
F	1/729000	1/729000	1/729000	1/729000	1/729000	1/729000	1/729000	1/729000		
J3_	1/2187000	1/2187000	1/2187000	1/2187000	1/2187000	1/2187000	1/2187000	1/2187000		
H	EIA Buffer	EIA Buffer	EIA Buffer	EIA Buffer	EIA Buffer	EIA Buffer	EIA Buffer	Buffer		

Figure 3:7 Microplate map for evaluation of sera and antisera

3.4.4.1 General Competitive ELISA Procedure

The BSA-AE conjugate was diluted in coating buffer. To coat the plate, lOOpL was added to the wells of a 96 well microtitre plate and incubated for 60 minutes on a shaker

at room temperature. The plate was washed 4 times with wash buffer, with the final wash left in the wells for 15-30 minutes before being removed. The antisera were diluted in EIA buffer and lOOpL was added to the wells in duplicate. The plate was incubated for 60 minutes and then washed three times with wash buffer. The horse radish peroxidase labelled goat anti-rabbit IgG (GAR-HRP) was diluted 1/6000 in EIA buffer and lOOpL was added to each well. The plates were incubated for 30 minutes before being washed three times in wash buffer. Colour was developed by adding 100pL of TMB solution to each well and incubating for 30 minutes. The reaction was stopped by the addition of lOOpL of 1M sulphuric acid. The absorbance was read using a microtitre plate reader at wavelength 450nm.

3.4.5 Titration of Coating Antigen

A two-dimensional chequerboard titration was used to assess the optimum coating concentration of the BSA-AE conjugate. Dilutions of the BSA-AE conjugate and the antiserum were prepared and the ELISA procedure followed as described in section 3.4.4.1. Figure 3:8 shows the microplate map.

	5|ig/mL BSA-AE		l^g/mL BSA-AE		0.2pg/mL BSA-AE		0.04ng/mL BSA-AE		5|ig/mL BSA Only			
	1	2	3	4	5	6	7	8	9	10	11	12
A	1/3000	1/3000	1/3000	1/3000	1/3000	1/3000	1/3000	1/3000	1/3000	1/3000		
B	1/9000	1/9000	1/9000	1/9000	1/9000	1/9000	1/9000	1/9000	1/9000	1/9000		
C	1/27000	1/27000	1/27000	1/27000	1/27000	1/27000	1/27000	1/27000	1/27000	1/27000		
D	1/81000	1/81000	1/81000	1/81000	1/81000	1/81000	1/81000	1/81000	1/81000	1/81000		
E	1/243000	1/243000	1/243000	1/243000	1/243000	1/243000	1/243000	1/243000	1/243000	1/243000		
F	1/729000	1/729000	1/729000	1/729000	1/729000	1/729000	1/729000	1/729000	1/729000	1/729000		
G	1/2187000	1/2187000	1/2187000	1/2187000	1/2187000	1/2187000	1/2187000	1/2187000	1/2187000	1/2187000		
H	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control		

Figure 3:8 Checkerboard titration of BSA-AE conjugate and antiserum

3.4.6 Competition Assay

The antibody affinity of each final bleed antiserum was investigated by calculating the percentage binding for a calibration curve containing AEME. Calibrators were prepared in EIA buffer, containing lOmg/mL BSA, at 0, 1, 10 and lOOng/mL AEME from a lmg/mL methanol stock solution. The absorbance of the Ong/mL standard represents 100% antibody binding.

The ELISA procedure was performed as described in section 3.4.4.1. Prior to the addition of the antisera, 50µL of each calibrator was added to the wells in quads as shown on the microplate map in Figure 3:9.

402717		402719		402734		402738	
100	100	100	100	100	100	100	100
100	100	100	100	100	100	100	100

Figure 3:9 Microplate map for AEME competition assay

3.4.7 Antiserum Specificity

Antiserum specificity was assessed by analysing 0, 10, 100, and lOOOng/mL solutions of AEME, AE, EME, benzoylecgonine, cocaethylene, and cocaine, prepared from lmg/mL stock solutions diluted in EIA buffer, containing lOmg/mL BSA.

The ELISA procedure was performed as described in section 3.4.4.1. Prior to the addition of the antisera, 50pL of each calibrator was added to the wells in quads.

3.5 Part 1 - Results and Discussion

3.5.1 Screening Antisera

The purpose of screening the pre-immune sera against the BSA-AE conjugate coated plates and the BSA coated plates was to eliminate the possibility of any pre-existing antibodies which may cross react with AE or with the carrier protein.

The binding profile was the same for each serum using both the BSA-AE and BSA only coated plates (see Figures 3:10 and 3:11).

The highest absorbance value was obtained at the first dilution point and is a result of non specific binding. The remainder of the dilutions produce absorbance values close to those provided by the control line. This establishes that there are no pre-existing antibodies present in the serum of the rabbits which may interfere with the assay and cause non specific binding.

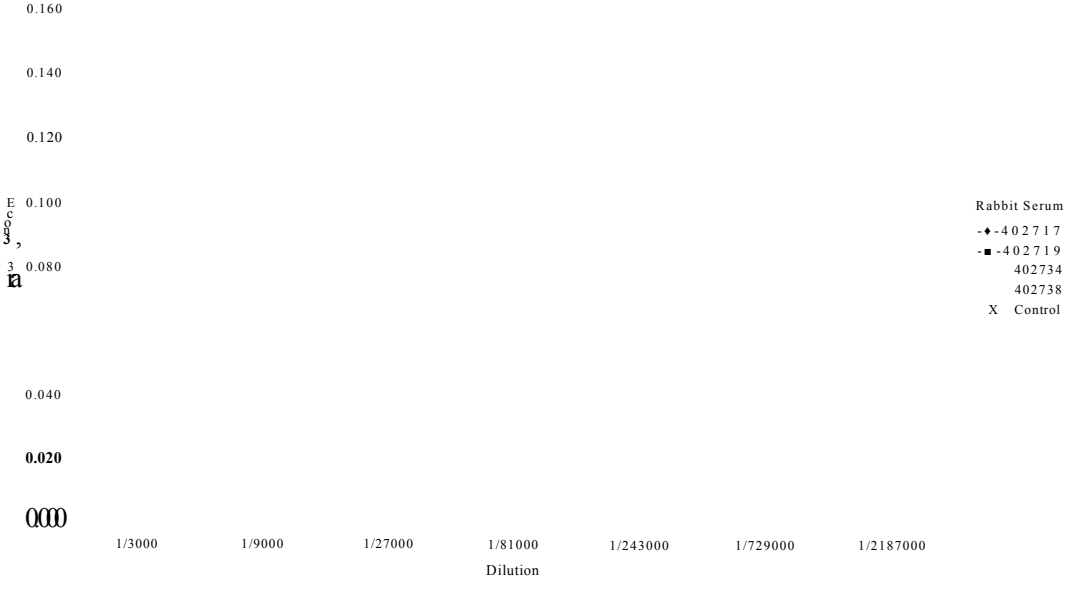


Figure 3:10 lpg/mL BSA-AE coated plate: Pre-immune sera. Results represent the mean of duplicate analyses, standard error of the mean is <5%.

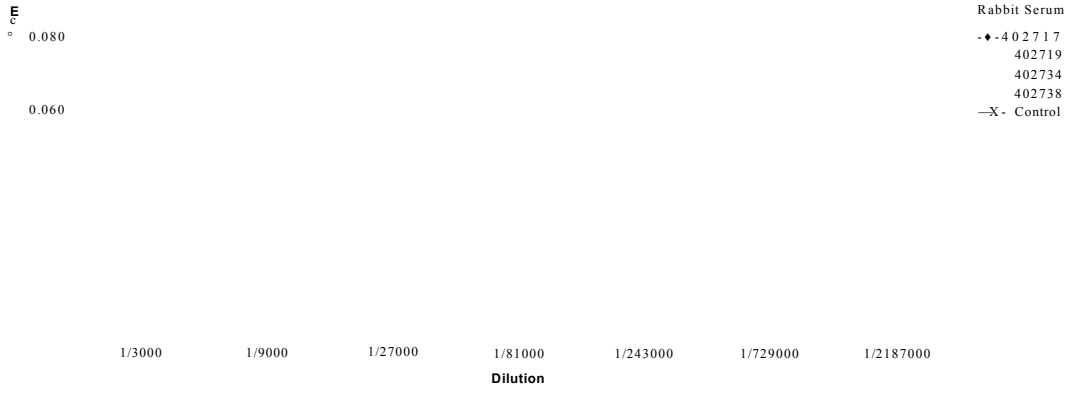


Figure 3:11 lpg/mL BSA coated plates: Pre-immune sera. Results represent the mean of duplicate analyses, standard error of the mean is <5%.

The ELISA screening of the antisera from day 53 of the immunisation shows all four rabbits have responded well to the immunisation protocol. The first few dilutions for most of the antisera produced absorbance values above the threshold of the plate reader, indicated by the arrows on Figure 3:12. Subsequent dilutions fell within the measurable absorbance range, with each antiserum showing a similar antibody titre.

The titration curves of the final bleed antisera were similar to those of the day 53 bleeds, however the titres were lower at equivalent dilutions. Overall the lowest antibody titre is provided by antiserum 402738, with the highest titre being provided by 402734 (Figure 3:13). However a high titre does not necessarily mean that the antibodies will have high affinity for the antigen.

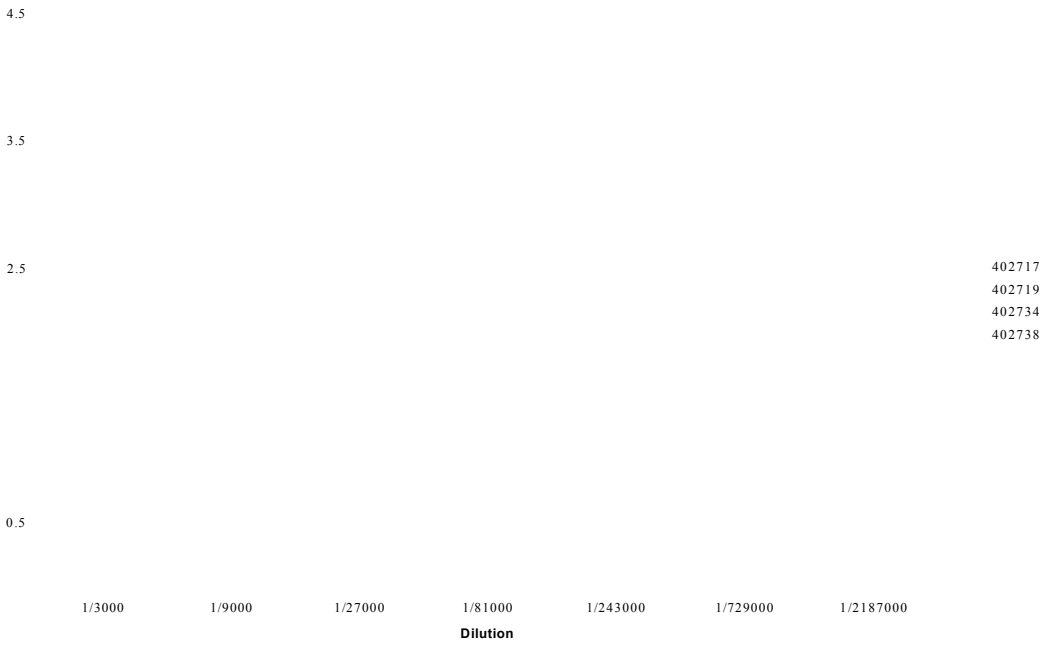


Figure 3:12 Titration of day 53 test bleed antisera. Results represent the mean of duplicate analyses, standard error of the mean is <5%.

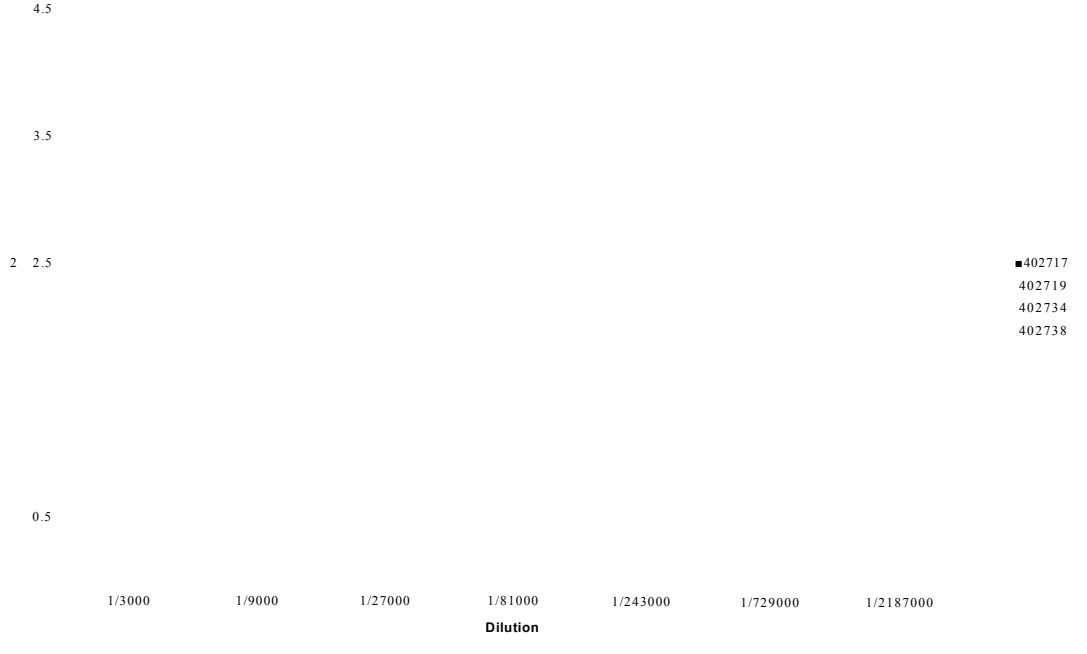


Figure 3:13 Titration of final bleed antisera. Results represent the mean of duplicate analyses, standard error of the mean is <5%.

Due to the limitations in microplate reader technology, optimum absorbance values should lie between 2.0 and 3.0. Dilution of the antisera to 1/300K should produce absorbance readings within this range for further studies.

Overall the immunisation proved successful with all antisera providing high titres.

3.5.2 Competition Assay

The competition assay was initially carried out using a BSA-AE coating concentration of lpg/mL and antisera dilutions of 1/300K. The percentage binding of each antiserum is plotted as a dose response curve in Figure 3:14. The degree of separation between the bindings at each concentration was small, therefore producing flat curves.

Figure 3:14 Competition assay: lpg/mL BSA-AE coating. Results represent the mean of four replicates, standard error of the mean is <5%.

The lack of inhibition suggested the concentration of free drug was not high enough to displace the antibody from the BSA-AE conjugate, or alternatively there was too much

immobilised drug. Reducing the concentration of BSA-AE conjugate would result in greater displacement of the antibody and steeper dose response curves.

To determine the optimum BSA-AE coating concentration a titration was carried out using antiserum 402717. The titration curves in Figure 3:15 show that there was no significant difference between antibody bindings at BSA-AE concentrations 0.2-5.0pg/mL.

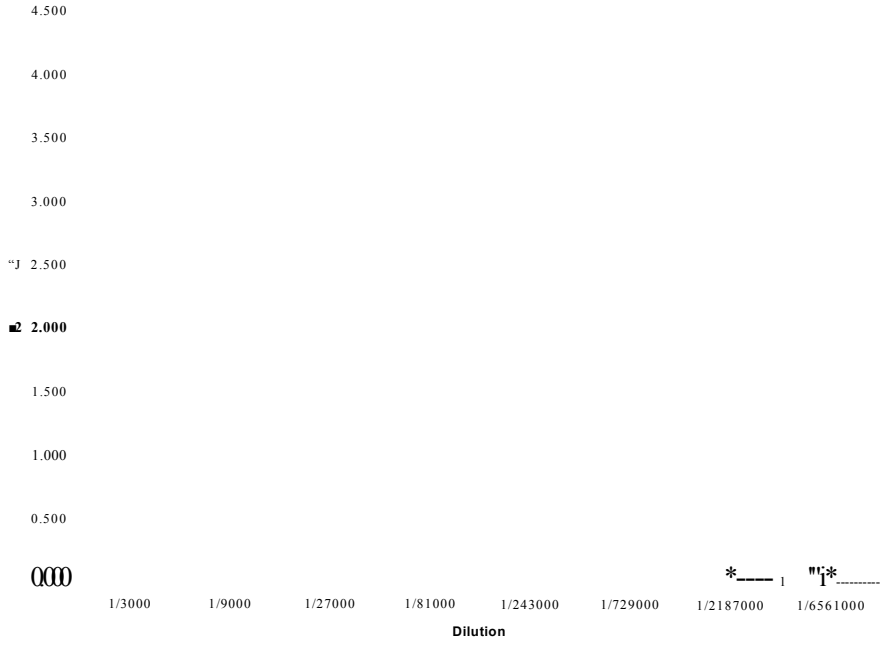


Figure 3:15 Titration of antiserum 402717 and BSA-AE conjugate. Results represent the mean of duplicate analyses, standard error of the mean is <5%.

Coating at 0.04pg/mL BSA-AE produced a drop in the absorbance indicating the concentration of coating antigen was no longer in excess. The competition assay was repeated for all four antisera using this concentration of BSA-AE. On this occasion the degree of separation between each drug concentration was greater, producing steeper dose response curves (Figure 3:16).

The concentration required to displace 50% of drug bound to the antibody is otherwise known as the IC50. This value can be used to establish the affinity of the antibody. Using this method the most sensitive antiserum was found to be 402717 with a value of lOng/mL, this was followed by 402734 at 35ng/mL, 402719 at 70ng/mL and 402738 at >100ng/mL.

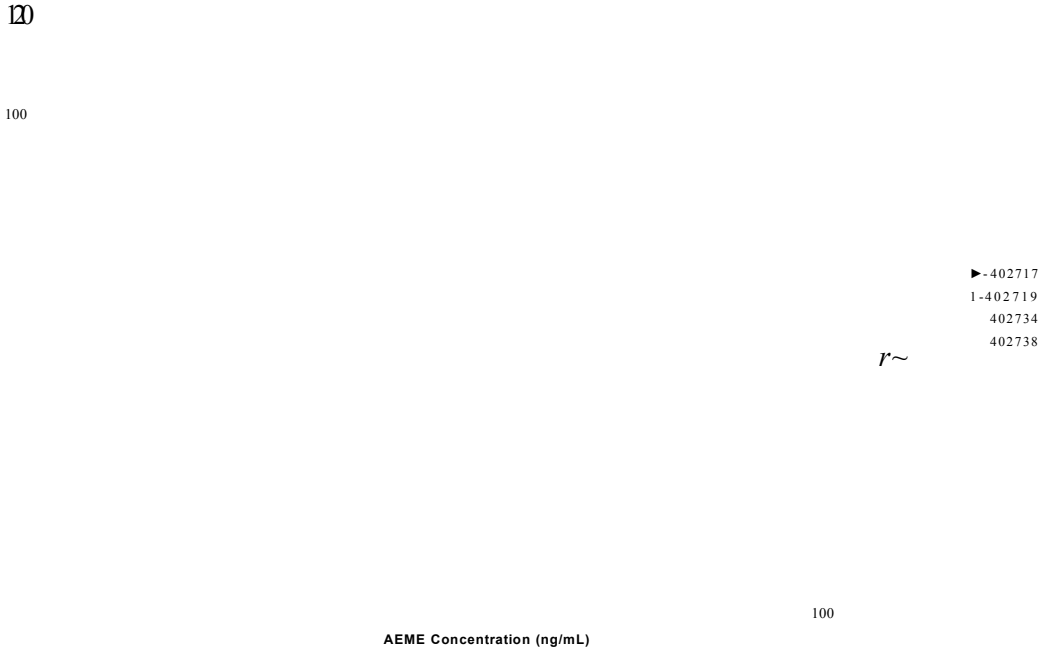


Figure 3:16 Competition assay: 0.04pg/mL BSA-AE coating. Results represent the mean of four replicates, standard error of the mean is <5%.

Antiserum 402717 was selected for further testing as a result of the higher sensitivity observed. Due to the low concentrations of drugs in oral fluid, and limited sample volume available, an antibody with high sensitivity is preferred.

3.5.3 Antiserum Specificity

The specificity of the antibody to AEME and AE alone is paramount in the development of the assay. Considering the choice of functional group used for the

synthesis of the immunogen it was expected that the antibodies produced would show equal specificity to both AEME and AE. This was not the case, AE only showed 0.6% cross reactivity at lOOOng/mL. The dose response curve for each analyte can be seen in Figure 3:17. A possible explanation for the unexpected lack of cross reactivity to AE may be a result of the negative charge on the AE molecule from the carboxyl group, at physiological pH. Conjugation of AE to the carrier protein involves the formation of a peptide bond using the carboxyl group of AE and therefore does not carry a charge. The antibody is subsequently raised to a neutral molecule and may not bind strongly with free negatively charged AE. The carboxyl group of AEME is esterified making the molecule neutral, it is also structurally related to AE. Therefore AEME resembles AE when bound to the carrier protein. The important fact is that the assay did not cross react with any of the other cocaine related compounds.

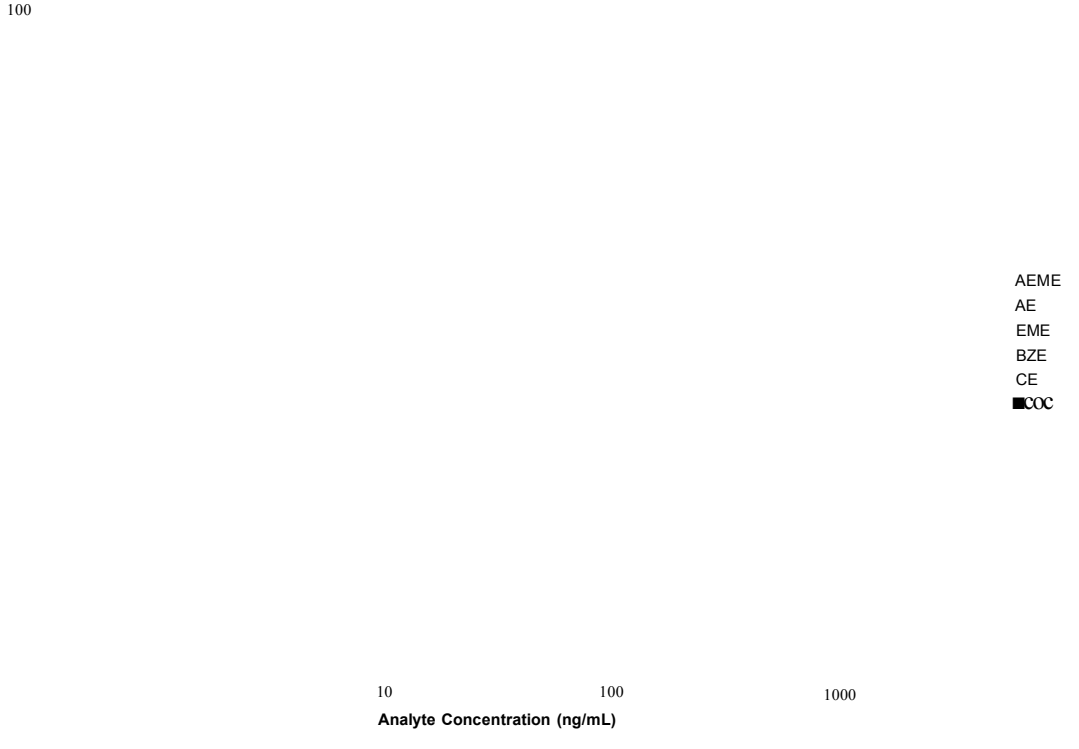


Figure 3:17 Specificity of Antiserum 402717. Results represent the mean of four replicates, standard error of the mean is <5%.

3.6 Experimental Part 2 - Development of EIA

This section details the purification of the chosen antiserum, the development and optimisation process of the EIA for AEME, and the necessary validation experiments to assess the overall performance.

3.6.1 Purification of IgG Fraction from Antiserum

The IgG fraction of antiserum 402717 was purified by affinity chromatography on a sepharose protein A column. Antibody purity is critical in providing a sensitive and reproducible assay [81]. Although the total immunoglobulin concentration in the serum of a polyclonal antibody preparation may be high, the desired antibody may only constitute 10% of the fraction [76].

lmL of antiserum was mixed with 4mL of PBS azide and filtered through a 0.45pm filter. The pre-packed protein A column was conditioned with lOmL of PBS azide. The antiserum was applied to the column followed by PBS azide, 3mL fractions were collected immediately and the absorbance measured by a spectrophotometer at 280nm. Once the absorbance values of the fractions fell below 0.1 the collections were stopped. To elute the bound IgG antibodies citric acid buffer was added to the column and 2ml fractions of the eluant were collected in test tubes containing 0.5mL of neutralisation buffer. The absorbance of each fraction was monitored at 280nm and collections were stopped once the absorbance value fell below 0.1. The column was then washed immediately with 20mL PBS azide so the acid pH did not damage the gel in the column. The eluted fractions produced two absorbance peaks as shown in Figure 3:18. The first peak represents the elution of the unbound material while the second peak represents the purified IgG.

Fractions 8-11 containing the purified IgG were pooled and dialysed overnight at 4°C in IX PBS. The solution was filtered through a 0.45pm filter, and its absorbance measured at 280nm. The concentration of IgG was calculated by dividing its absorbance at 280nm (0.623) by the absorbance of lmg/mL of IgG (1.4). Therefore the concentration of IgG was 0.445mg/mL which is a total concentration of 4.9mg in 11ml.

Fractions 1-5 containing the unbound material were also pooled for assaying.

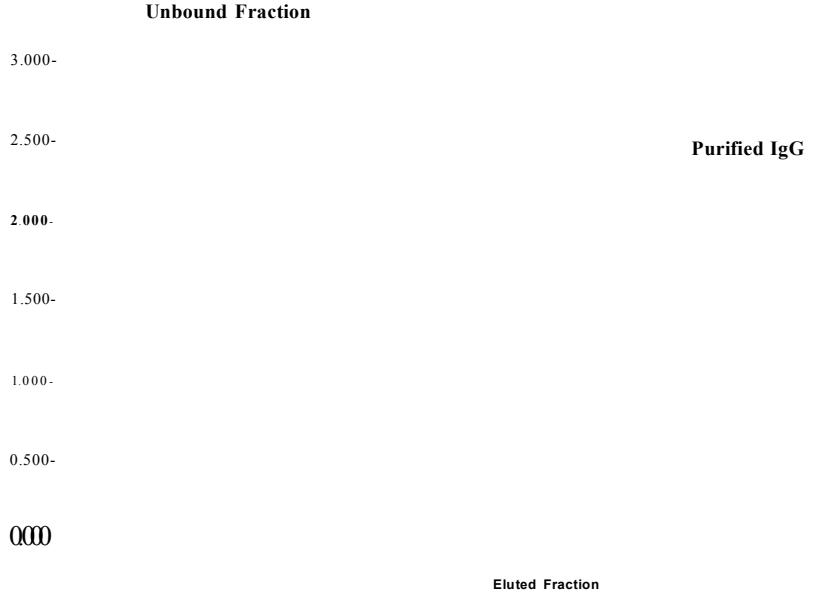


Figure 3:18 Absorbance of fractions eluted from protein A column

3.6.2 Assessment of Purified Antibody

The purified antibody, the unbound eluted fraction from the protein A column, and the original antiserum, were serially diluted in EIA buffer containing lOmg/mL BSA, and subsequently screened using the ELISA procedure described in section 3.4.4.1.

The microplate map is shown in Figure 3:19.

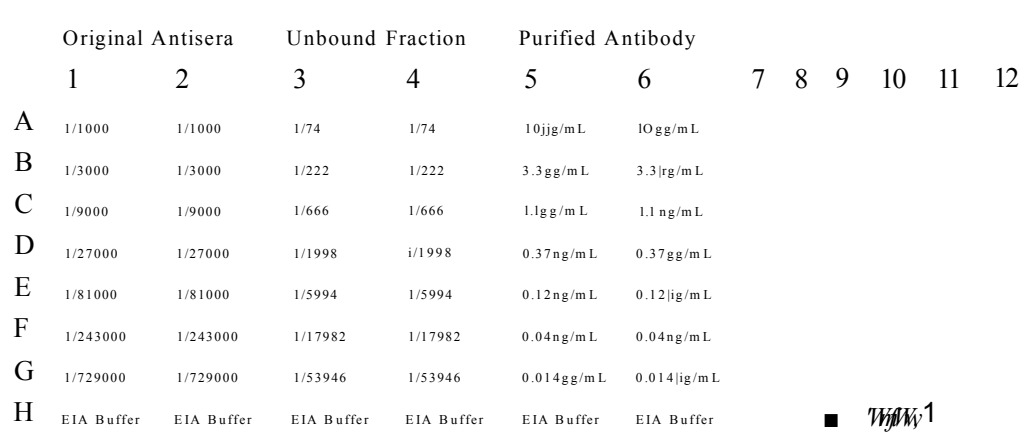


Figure 3:19 Assessment of purified antiserum

3.6.3 Synthesis of Enzyme Labelled Drug Conjugate

AE was conjugated to the enzyme label horseradish peroxidase (HRP).

0.3mL of a solution of AE (lmg/mL in methanol) was evaporated to dryness at 37°C. The drug was reconstituted in 0.5mL of 0.1M activation buffer, prepared as a dilution from the 0.5M stock solution. The drug solution was then added dropwise to lOmg of HRP, dissolved in ImL of activation buffer. 5mg of Sulfo-NHS, dissolved in 0.05mL of activation buffer, was slowly added to the HRP/AE solution. lOmg of EDC, dissolved in 0.5mL of activation buffer, was added in lOOpL aliquots every 10 minutes. The solution was left to stir for 2 hours at room temperature.

A further 5mg of EDC, dissolved in O.lmL of activation buffer, was added to the HRP/AE/Sulfo-NHS/EDC solution. The mixture was left to stir overnight at room temperature.

The HRP-AE conjugate was transferred to a dialysis membrane, immersed into 1L of dialysis buffer and left to stir overnight dialysis at 4°C.

The HRP-AE conjugate was desalted on a Sephadex G50M column using 50% stabilzyme. The column was conditioned with 50mL of EIA buffer. The HRP-AE

conjugate was added to the column. HRP produces a coloured liquid which is easy to track along the length of the column, as soon as the conjugate approached the lower part of the column the fractions were collected in 2ml aliquots and the absorbance measured at 403nm and 280nm.

Fractions 1-4 had an absorbance greater than 0.1 at 403nm and were pooled (see Figure 3:20).

Figure 3:20 Purification of HRP-AE conjugate

3.6.4 General Protocol for Drug Capture EIA

Plates were coated overnight with lOOpL of the purified antibody diluted in coating buffer. The plate was washed 4 times with wash buffer, with the final wash left in the wells for 15-30 minutes before being removed. The plate was incubated for 30 minutes with lOOpL of HRP-AE conjugate. The plate was washed three times in wash buffer and the colour developed by the addition of lOOpL of TMB substrate solution. The plate was incubated for 30 minutes and the reaction was stopped by the addition of lOOpL of

1M sulphuric acid. The plate was read using a microtitre plate reader at wavelength 450nm.

3.6.5 Titration of AE-HRP and Antibody

The optimal antibody coating concentration and enzyme dilution was established by performing a two-dimensional titration experiment.

The EIA procedure was followed as outlined in section 3.6.4 with lpg/mL, 5pg/mL and lOpg/mL antibody coating concentrations. The HRP-AE conjugate was serially diluted in 50% stabilzyme solution containing lOmg/mL BSA. The microplate map is shown in Figure 3:21.

	l|xg/mL			5[ig/mL		lOgg/mL						
	1	2	3	4	5	6	7	8	9	10	11	12
A	1/100	1/100	1/100	1/100	1/100	1/100						
B	1/200	1/200	1/200	1/200	1/200	1/200						
C	1/400	1/400	1/400	1/400	1/400	1/400						
D	1/800	1/800	1/800	1/800	1/800	1/800						UPr
E	1/1600	1/1600	1/1600	1/1600	1/1600	1/1600						B l
F	1/3200	1/3200	1/3200	1/3200	1/3200	1/3200						
G	1/6400	1/6400	1/6400	1/6400	1/6400	1/6400						
H	EIA Buffer	EIA Buffer	EIA Buffer	EIA Buffer	EIA Buffer	EIA Buffer						**fit**

Figure 3:21 Titration of HRP-AE and purified antibody

3.6.6 AEME Oral Fluid Calibration Curve

Pooled oral fluid samples from drug free volunteers were spiked with AEME at Ong/mL, Ing/mL, lOng/mL and lOOng/mL from a stock lmg/mL standard. 25pL of each calibrator was added in duplicate for each antibody concentration. Appropriate dilutions of the AE-HRP were carried out for each coating concentration. The EIA procedure in section 3.6.4 was followed.

A dose response curve was constructed based on percentage bindings versus drug concentration.

3.6.7 Assay Validation

Assay development should include a number of experiments to assess the suitability and validity of the assay. Evaluation of assay specificity and precision are the primary objectives followed by additional experiments such as sensitivity, selectivity, and storage stability of reagents [82].

3.6.7.1 Stability Testing of AEME Oral Fluid Calibrators

The stability of AEME in oral fluid calibrators was assessed at room temperature, refrigerated at 4°C, frozen at -20°C and incubated at 37°C. Time points of 0, 1, 7, 14, 21 and 36 days were tested. Fresh calibrators were prepared on day 0 and tested by the AEME EIA, lOOpL of the calibrators were then aliquoted into 0.5mL vials and stored at the various temperatures. At each subsequent time point a set of fresh calibrators were prepared and compared to each temperature point.

The stability of AEME in the calibrators was determined by the percentage binding values, an increase in the binding would be a result of degradation of AEME in the sample.

3.6.7.2 Matrix Effect

Assay interference is described as the effect of a substance present in an analytical system which causes a deviation of the measured value from the true value. Components in biological materials such as proteins, carbohydrates, lipids etc. can

affect analytical techniques. This is termed the matrix effect [83]. Immunoassays are prone to matrix effects due to the lack of sample extraction prior to analysis [82].

A total of 50 samples were collected from drug free volunteers using the Cozart® RapiScan collector. Each sample was screened using the newly developed EIA for AEME.

3.6.7.3 Cross Reactivity

The analytical specificity or cross reactivity of an immunoassay provides an indication of how the antibody responds to other drugs relative to the drug used to prepare the calibration curve [64]. Different methods are used to calculate cross reactivity, one way is the calculation of the ratio of the apparent concentration of analyte to the concentration of cross reactant. The accuracy of the immunoassay results will depend on minimising the cross reactive components [84].

A selection of commonly encountered over the counter and prescribed substances were tested for cross reactivity in both the Cocaine and Metabolites assay, and the AEME assay for comparison. The compounds were diluted 1:10 from the original stock concentration (lmg/mL or lOOpg/mL) using fresh oral fluid to give 100,000ng/mL and 10,000ng/mL respectively.

The cross reactivity of cocaine related compounds was also tested using the AEME EIA. Dilutions were carried out from the lmg/mL stock solutions to give 100,000ng/mL, 10,000ng/mL, and l,000ng/mL. If a measurable response was produced at lOOOng/mL the samples were diluted further and re-assayed until the concentration fell below the first calibration point.

3.6.7.4 Precision

The precision of an immunoassay is the extent to which replicate analyses of a sample agree with each other, while the reproducibility is the ability to yield the same results within analyses and between analyses [73].

Negative oral fluid/buffer samples were spiked at 0, 2.5, 5, 7.5, 10, 15, 30, and 50ng/mL using a lmg/mL stock solution of AEME. For inter-assay precision the samples were tested in duplicate, in two separate assays, every day for 20 days. Intra-assay precision was evaluated by testing each concentration 20 times in one assay. Both the absorbance values and concentrations were used to calculate the precision of the assay.

3.6.7.5 Limit of Detection

Sensitivity in immunoassay is otherwise known as the theoretical limit of detection or analytical sensitivity, and is determined as the concentration three standard deviations below the mean response for zero analyte concentration [65].

A standard set of calibrators plus an additional 0. Ing/mL calibrator were pipetted, and the zero calibrator was assayed 20 times. The average absorbance of the 20 replicates of the zero calibrator minus 3 SD was then read from the calibration curve, this value was the limit of detection for the assay.

3.7 Results and Discussion

3.7.1 Assessment of Purified Antibody

After establishing its high sensitivity and specificity, antiserum 402717 was purified by affinity chromatography. The purified antibody, original antiserum, and the eluted unbound fraction were assayed by ELISA. Figure 3:22 shows the binding of each of the fractions to the BSA-AE conjugate.

Figure 3:22 Assessment of purified antibody. Results represent the mean of duplicate analyses, standard error of the mean is <5%.

The unbound fraction showed considerable binding for the first two points on the curve but this was a result of non specific binding and the presence of IgM.

Both the original antisera and the purified antibody produced similar absorbance values suggesting that there had been no significant loss of antibody function during the purification process.

3.7.2 Titration of AE-HRP and Antibody

The absorbance readings from coating with lOpg/mL of antibody are above the threshold of the plate reader for all but the last enzyme dilution point (Figure 3:23). Coating at lpg/mL requires an enzyme dilution of approximately 1/800, while coating at 5pg/mL would require an enzyme dilution of approximately 1/4800.

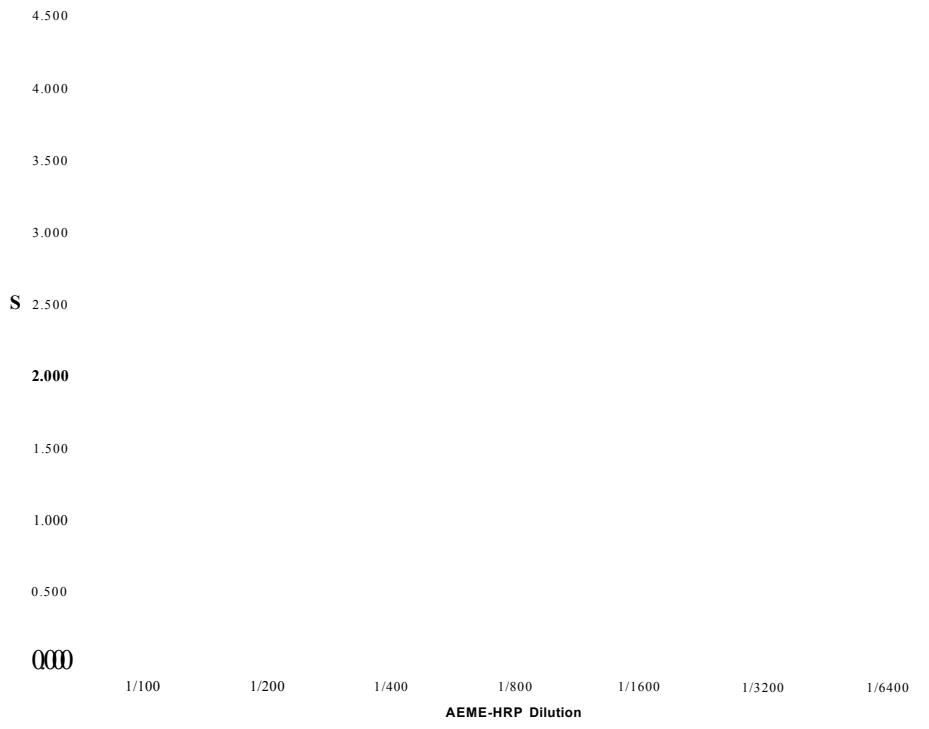


Figure 3:23 Antibody-enzyme titration. Results represent the mean of duplicate analyses, standard error of the mean is <5%.

Often in a commercial setting the antibody coating concentration is driven by cost. It is generally preferable to have a more dilute antibody and a less dilute enzyme conjugate than the other way round as the costs incurred in production of an antibody are far greater.

Both the lpg/mL and 5pg/mL antibody concentrations were used for further evaluation.

3.7.3 AEME Oral Fluid Calibration Curve

In general high antibody concentrations show poor slope sensitivity. More dilute antibodies will show good slope sensitivity at the lower drug concentrations but a flattened curve at higher concentrations [85],

The dose response curves for the lpg/mL and 5pg/mL antibody concentrations showed good separation between each calibration point (Figure 3:24). Using lpg/mL antibody

coating the steepest part of the curve was between Ong/mL and Ing/mL, and the IC50 value was calculated to be 0.85ng/mL. Using 5pg/mL antibody coating the steepest part of the curve was between Ing/mL and 1Ong/mL, and the IC50 value was calculated to be 2.75ng/mL. Therefore coating at lpg/mL produced the highest sensitivity at the lower drug concentrations.

Figure 3:24 AEME calibration curve in oral fluid. Results represent the mean of duplicate analyses, standard error of the mean is <5%.

3.7.4 Stability Testing of AEME Oral Fluid Calibrators

The oral fluid calibrators were stable for the 36 day duration of the experiment for the refrigerated and frozen samples. The room temperature samples were stable up until day 21 after which the percentage binding starts to increase slightly. The samples stored at 37°C were not stable, an increase in percentage binding was observed after day 1. Figure 3:25 presents the results for each storage temperature. Therefore the most appropriate storage of calibrators and samples would be at 4°C or at -20°C for longer term storage.

AEME Calibrator Stability at 4'C

AEME Calibrator Stability at -20C

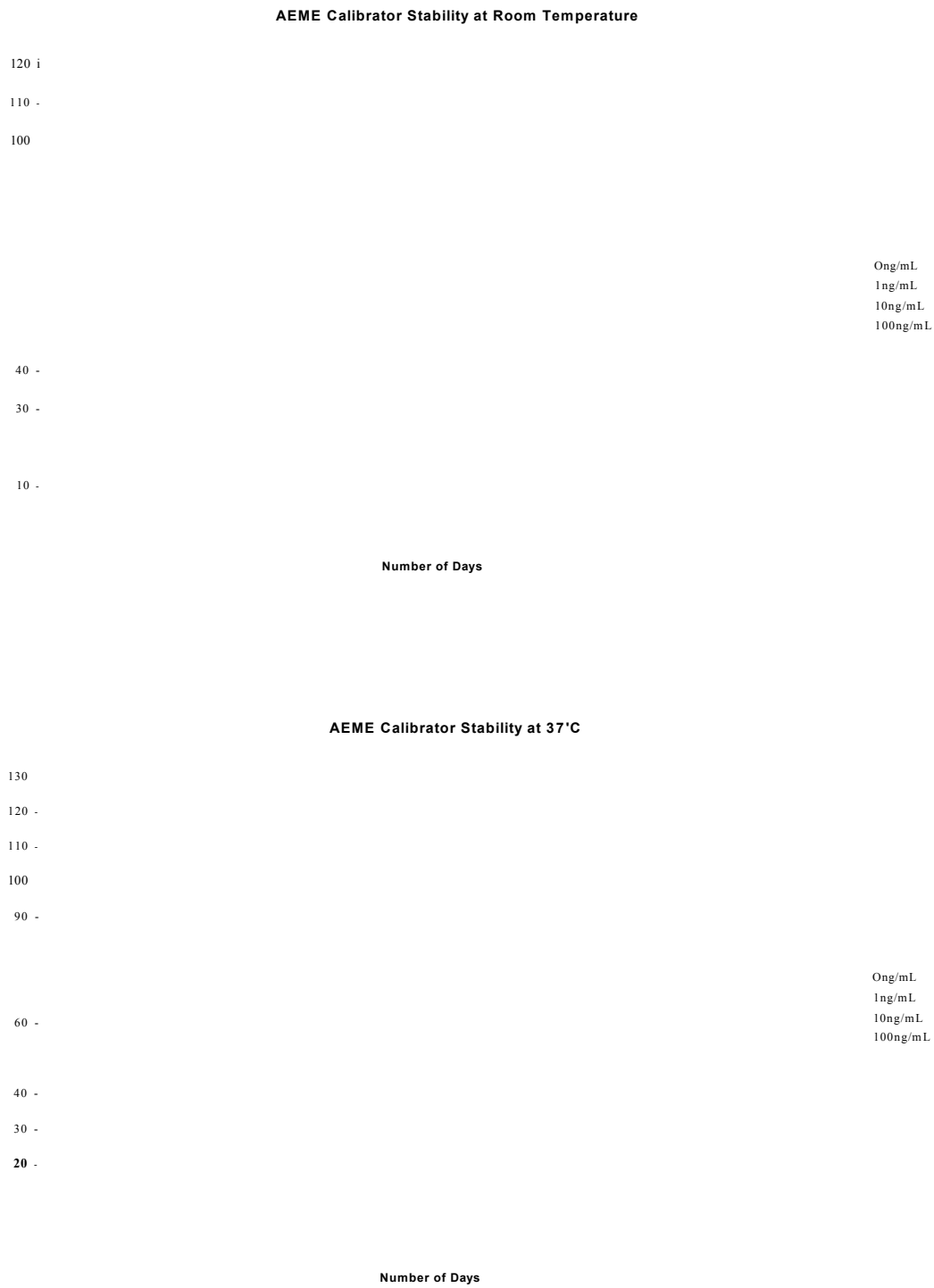


Figure 3:25 Stability testing of AEME oral fluid calibrators. Results represent the mean of duplicate analyses, standard error of the mean is <5%.

3.7.5 Matrix Effect

No significant matrix effect was observed as all samples were below the first calibration point of 1ng/mL for the AEME assay and therefore classified as negative. The average percent binding for the 50 negative samples was 88%.

3.7.6 Cross Reactivity

In this assay the antibody is required to be highly specific for AEME or its metabolite AE, without showing any cross reactivity to cocaine or any other structurally related compound. This is in contrast to a standard cocaine assay which requires the antibody to cross react to cocaine and its main metabolites benzoylecgonine and EME, but will not be negatively affected if it also cross reacted to other cocaine metabolites, as you are positively identifying the use of cocaine. The specificity of the antibody was fully evaluated with a series of cocaine related compounds as well as structurally unrelated compounds.

The cross reactivity data for cocaine and its structurally related compounds is shown in Table 3:6. None of the compounds produced significant cross reactivity in the AEME microplate. AE showed the highest cross reactivity at only 0.15-1.3%. Considering the choice of functional group for protein conjugation it was expected that the antibodies produced would show equal specificity to both AEME and AE. The important factor is that the assay does not cross react with any other compound and so is exclusive for the pyrolysis products of cocaine.

Analyte	Concentration (ng/mL)	Apparent AEME (ng/mL)	AEME % Cross Reactivity
Benzoylecgonine	100000	49	0.049
	10000	2.1	0.021
	1000	0.7	0.07
Cocaine	100000	118	0.118
	10000	4.6	0.046
	1000	0.95	0.095
AE	100000	146	0.146
	10000	77	0.766
	1000	9.4	0.94
	100	1.3	1.285
Cocaethylene	100000	111	0.111
	10000	6.4	0.064
	1000	0.9	0.09
EME	100000	37	0.037
	10000	2.2	0.022
	1000	0.8	o.o8 ;
Norcocaine	100000	0.6	0.001
	10000	0.6	0.006
	1000	0.6	o.o6 ;
Ecgonine	100000	16.2	0.016
	10000	1.4	0.014
	1000	0.6	0.06

Table 3:6 Cross reactivity of cocaine related compounds

The cross reactivity data for the non-related compounds is shown in Table 3:7. All compounds were tested at 100,000ng/mL in blank oral fluid except for propoxyphene, buprenorphine and 7-aminoflunitrazepam which were tested at 10,000ng/mL. The highest cross reactivity of any of the compounds was <0.3% and therefore none of the compounds were classed as having any significant cross reactivity.

Compound	Apparent AEME (ng/mL)	AEME % Cross Reactivity	
Pholcodeine	0.9	<0.001	
Codeine	0.6	<0.001	
6-Acetylmorphine	0.9	<0.001	
Heroin	0.5	<0.001	
Dihydrocodeine	0.4	<0.001	
Morphine	0.5	<0.001	
Tramadol	3	<0.01	
MDA	0.4	<0.001	
MDMA	5.9	<0.01	
MDEA	13.5	<0.02	!
MBDB	8.1	<0.01	
(+) Ephedrine	0.7	<0.001	
(+) Pseudoephedrine	0.4	<0.001	
(-) Pseudoephedrine	0.8	<0.001	
Tyramine	0.4	<0.001	I
Fenfluramine	6.3	<0.01	
Metamfetamine	2.4	<0.01	j
Diazepam	0.4	<0.001	
Lorazepam	0.4	<0.001	
Oxazepam	0.4	<0.001	
Nitrazepam	0.5	<0.001	
Nordiazepam	0.4	<0.001	
Fluoxetine	0.4	<0.001	
Temazepam	0.4	<0.001	
Lidocaine	12.4	<0.2	!
Nicotine	4.6	<0.01	
LSD	0.4	<0.001	
Phenobarbital	0.6	<0.001	
EDDP	1.7	<0.01	
PCP	0.8	<0.001	
Ibuprofen	3.4	<0.01	

Compound	Apparent AEME (ng/mL)	AEME % Cross Reactivity
Chloroquine	22.5	<0.3
Ketamine	1.2	<0.01
Fentanyl	0.9	<0.001
Warfarin	0.6	<0.001
Propranolol	0.8	<0.001
Dothiepin	0.5	<0.001
Chlordiazepoxide	0.5	<0.001
Naloxone	0.6	<0.001
Pheniramine	2.9	<0.01
Medperidine	0.9	<0.001
(-) Ephedrine	1.1	<0.01
Amfetamine	1.2	<0.01
Phentermine	1.3	<0.01
Cotinine	0.8	<0.001
Methadone	0.9	<0.001
11-Hydroxy-Delta-9-THC	0.7	<0.001
Cannabidiol	0.6	<0.001
Ranitidine	1.2	<0.01
Amitriptyline	0.9	<0.001
Phenylpropanolamine	0.6	<0.001
Paracetamol	0.8	<0.001
Caffiene	0.9	<0.001
Secobarbital	0.8	<0.001
*Propoxyphene	0.8	<0.01
*7-Aminoflunitrazepam	0.8	<0.01
*Buprenorphine	0.7	<0.01

Table 3:7 Cross reactivity of non-related compounds

3.7.7 Precision

The precision results are displayed in Tables 3:8 and 3:9. The inter-assay precision was 6.7-12.3% based on absorbance values, and 7.1-29.7% based on concentrations. In general the lower the drug concentrations the higher the %CV. The intra-assay precision was 2.9-7% based on absorbance and 6.6-27.1% based on concentrations.

Concentration	Absorbance			Concentration (ng/mL)		
(ng/mL)	Mean (n=40)	SD (n=40)	CV (%)	Mean (n=40)	SD (n=40)	CV (%)
0	2.391	0.25	10.4	0.45	0.12	26.2
2.5	1.567	0.19	12.3	2.4	0.72	29.7
5	1.147	0.11	9.8	6.1	1.05	17.3
7.5	0.954	0.08	8.8	9.97	1.45	14.5
15	0.626	0.05	8.4	30.4	5.00	16.5
30	0.470	0.04	9.2	59.7	7.58	12.7
50	0.389	0.03	6.7	86.8	6.19	7.1

Table 3:8 Inter-assay precision for AEME EIA using absorbance values and interpolated concentrations

Concentration	Absorbance			Concentration (ng/mL)		
(ng/mL)	Mean (n=20)	SD (n=20)	CV (%)	Mean (n=20)	SD (n=20)	CV (%)
0	1.917	0.07	3.9	0.4	0.08	21.5
2.5	1.221	0.08	6.2	2.9	0.77	27.1
5	1.028	0.05	4.5	5.1	0.69	13.5
7.5	0.858	0.04	5.0	9.1	1.30	14.3
15	0.640	0.03	5.4	21.4	3.08	14.4
30	0.489	0.01	2.9	41.3	2.72	6.6
50	0.375	0.03	7.0	70.6	9.30	13.2

Table 3:9 Intra-assay precision for AEME EIA using absorbance values and interpolated concentrations

Due to the greater inherent imprecision of immunoassays, acceptance criteria of 25% CV for precision in routine assays are proposed by some authors [86], while others suggest 20%, with 25% at the limits of quantitation [82], Using the absorbance values, inter and intra-assay precision were acceptable for this assay. Based on concentrations, inter and intra-assay precision were acceptable from 5ng/mL to 5Ong/mL.

3.7.8 Limit of Detection

The average absorbance value for the replicate analysis of 20 zero standards was calculated to be 2.635, and the SD was 0.12. The limit of detection was the concentration relating to the average absorbance minus 3 SD values, interpolated from the standard curve (Figure 3:26). The limit of detection was 0.5ng/mL.

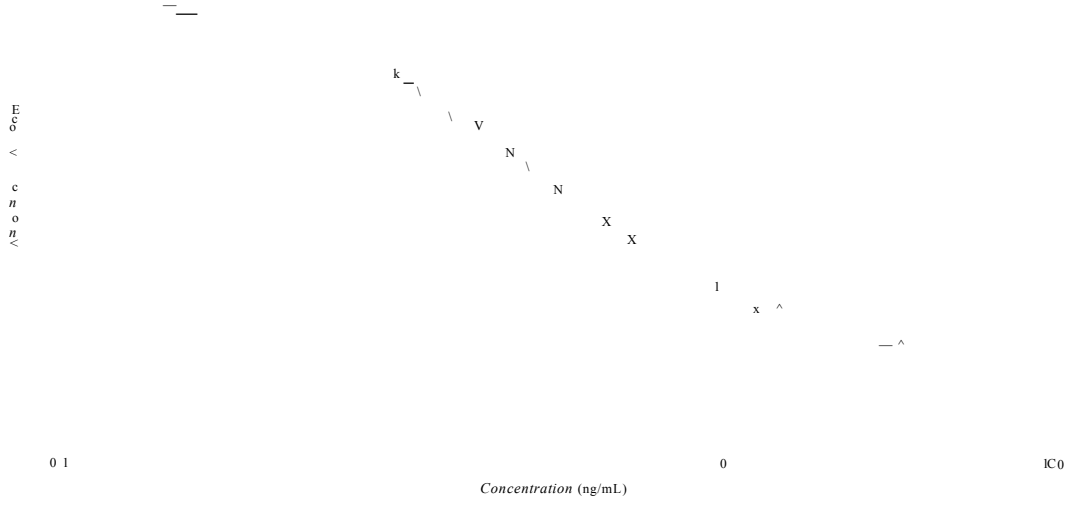


Figure 3:26 Log standard curve for estimation of limit of detection

3.8 Conclusion

The goals in developing an EIA are to have a sensitive, specific, robust and reproducible assay. The antibodies produced from immunisation with AE were of a high titre and were highly specific for AEME. The lack of cross reactivity to AE was surprising as the functional group used for conjugation should have provided an antibody with equal specificity to AEME and AE. The assay was successfully applied to oral fluid samples with acceptable precision, and no problems with matrix effect.

The assay, in combination with a confirmatory method, will be further evaluated using clinical samples to determine an appropriate cutoff concentration to positively identity the use of smoked cocaine.

3.9 References

1. Isenschmid, D.S., *Cocaine - Effects on Human Performance and Behaviour.* Forensic Science Review, 2002.14: p. 62-100.
2. King, L.A. and S.D. McDermott, *Drugs of Abuse,* in *Clarkes Analysis of Drugs and Poisons,* A.C. Moffat, M.D. Osselton, and B. Widdop, Editors. 2004, Pharmaceutical Press.
3. Chiang, C.N. and R.L. Hawks, *Research Findings on Smoking of Abused Substances.* NIDA Research Monographs, 1990. 99: p. 1-152.
4. Levine, B., *Principles of Forensic Toxicology.* 1999: American Association for Clinical Chemistry Inc.
5. Gossop, M., et al., *Changes in Use of Crack Cocaine after Drug Misuse Treatment: 4-5 Year Follow-up Results from the National Treatment Outcome Research Study (NTORS).* Drug and Alcohol Dependence, 2002. 66: p. 21-28.
6. Roe, S. and L. Man, *Drug Misuse Declared: Findings From the 2005/06 British Crime Survey.* Home Office Statistical Bulletin, 2006. 15/06.
7. Cone, E.J., et al., *Urine Testing for Cocaine Abuse: Metabolic and Excretion Patterns Following Different Routes of Administration and Methods for Detection of False Negative Results.* Journal of Analytical Toxicology, 2003. 27: p. 386-401.
8. George, S., *Has the Cocaine Epidemic Arrived in th UK?* Forensic Science International, 2004. 143: p. 121-125.
9. Lewis, R.J., et al., *Determination of Cocaine, its Metabolites, Pyrolysis Products, and Ethanol Adducts in Postmortem Fluids and Tissues using Zymark Automated Solid-Phase Extraction and Gas Chromatography-Mass Spectrometry.* Journal of Chromatography B, 2004. 806: p. 141-150.
10. Ramsay, M., et al., *Drug Misuse Declared in 2000: Results from the British Crime Survey.* Home Office Research Study, 2001. 224.
11. Dale-Perera, A., *Drug-Related Deaths,* in *www.nta.nhs.uk/pro2ramme/drd3.* 2006, National Treatment Agency for Substance Misuse.
12. Lu, N.T., B.G. Taylor, and K.J. Riley, *The Validity of Adult Arrestee Self-Reports of Crack Cocaine Use.* American Journal of Drug and Alcohol Abuse, 2001.27: p. 399-419.
13. NCIS, *NCIS UK Threat Assessment 2003,* N.C.I. Service, Editor. 2003, Corporate Communications.

14. Abigail, N., *Tackling Crack - A National Plan.* 2002.
15. GLADA, *The GLADA Crack Cocaine Strategy 2005-08,* GLADA, Editor. 2004, Greater London Authority.
16. Boreham, R., et al., *The Arrestee Survey Annual Report: Oct 2003 - Sept 2004.* Home Office Statistical Bulletin, 2006. 04/06.
17. Degenhardt, L., et al., *Was an Increase in Cocaine Use Among Injecting Drug Users in New South Wales, Australia, Accompanied by an Increase in Violent Crime?* BMC Public Health, 2005. 5.
18. Braithwaite, R.A., et al., *Screening for Drugs of Abuse. I: Opiates, Amphetamines and Cocaine.* Ann Clin Biochem, 1995. 32: p. 123-153.
19. Warner, E.A., *Cocaine Abuse.* Annals of Internal Medicine, 1993. **119:** p. 226-235.
20. Srinivasan, K., et al., *Liquid Chromatography-Tandem Mass Spectrometry Analysis of Cocaine and its Metabolites from Blood, Amniotic Fluid, Placental and Fetal Tissues: Study of the Metabolism and Distribution of Cocaine in Pregnant Rats.* Journal of Chromatography B, 2000. 745: p. 287-303.
21. Stillman, R., et al., *Improved Performance 4 Hours After Cocaine.* Psychopharmacology, 1993. **110:** p. 415-420.
22. Egred, M. and G.K. Davis, *Cocaine and the Heart.* Postgraduate Medical Journal, 2005. **81:** p. 568-571.
23. Cone, E.J., *Pharmacokinetics and Pharmacodynamics of Cocaine.* Journal of Analytical Toxicology, 1995.**19:** p. 459-478.
24. Perez-Reyes, M., et al., *Free-Base Cocaine Smoking.* Clinical Pharmacology and Therapeutics, 1982. 32: p. 459-465.
25. Hatsukami, D.K. and M.W. Fischman, *Crack Cocaine and Cocaine Hydrochloride. Are the Differences Myth or Reality?* The Journal of the American Medical Association, 1996. **276:** p. 1580-1588.
26. Cone, E.J., et al., *Correlation of Saliva Cocaine Levels with Plasma Levels and with Pharmacologic Effects after Intravenous Cocaine Administration in Human Subjects.* Journal of Analytical Toxicology, 1988.**12:** p. 200-206.
27. Cone, E.J., J. Oyler, and W.D. Darwin, *Cocaine Disposition in Saliva Following Intravenous, Intranasal, and Smoked Administration.* Journal of Analytical Toxicology, 1997. **21:** p. 465-475.

28. Foltin, R.W. and M.W. Fischman, *Smoked and Intravenous Cocaine in Humans: Acute Tolerance, Cardiovascular and Subjective Effects.* Journal of Pharmacology and Experimental Therapeutics, 1991. 257: p. 247-261.

29. Cone, E.J., et al., *Cocaine Metabolism and Urinary Excretion After Different Route of Administration.* Therapeutic Drug Monitoring, 1998. 20: p. 556-560.

30. Inaba, T., *Cocaine: Pharmacokinetics and Biotransformation in Man.* Candian Journal of Physiology and Pharmacology, 1989. 67: p. 1154-1157.

31. Samyn, N., et al., *Analysis of Drugs of Abuse in Saliva.* Forensic Science Review, 1999. 11: p. 1-19.

32. Ambre, J., et al., *Urinary Excretion of Cocaine, Benzyolecgonine, and Ecgonine Methyl Ester in Humans.* Journal of Analytical Toxicology, 1988. 12: p. 301-306.

33. Peterson, K.L., *Detection of Cocaine and its Polar Transformation Products and Metabolites in Human Urine.* Forensic Science International, 1995. 73: p. 183-196.

34. Kintz, P., et al., *Evidence of Crack Use by Anhydroecgonine Methyl Ester Identification.* Human and Experimental Toxicology, 1997. 16: p. 123-127.

35. Jacob, P., et al., *Cocaine Smokers Excrete a Pyrolysis Product, Anhydroecgonine Methyl Ester.* Clinical Toxicology, 1990. 28: p. 121-125.

36. Jenkins, A.J. and B. Goldberger, *Identification of Unique Cocaine Metabolites and Smoking By-Products in Postmortem Blood and Urine specimens.* Journal of Forensic Sciences, 1997. 42: p. 824-827.

37. Jacob, P., et al., *A Pyrolysis Product, Anhydroecgonine Methyl Ester (Methylecgonidine), is in the Urine of Cocaine Smokers.* Journal of Analytical Toxicology, 1990. 14: p. 353-357.

38. Zhang, J.Y. and L. Foltz, *Cocaine Metabolism in Man: Identification of Four Previously Unreported Cocaine Metabolites in Human Urine.* Journal of Analytical Toxicology, 1990.14: p. 201-205.

39. Toennes, S.W., A.S. Fandino, and G. Kauert, *Gas Chromatographic-mass Spectrometric Detection of Anhydroecgonine Methyl Ester (Methylecgonidine) in Human Serum as Evidence of Recent Smoking of Crack.* Journal of Chromatography B, 1999. 735: p. 127-132.

40. Nakahara, Y. and A. Ishigami, *Inhalation Efficiency of Free-Base Cocaine by Pyrolysis of 'Crack' and Cocaine Hydrochloride.* Journal of Analytical Toxicology, 1991.15: p. 105-109.

41. Wood, R.W., et al., *Methylecgonidine Coats the Crack Particle.* Pharmacology Biochemistry and Behaviour, 1996. 53: p. 57-66.

42. Martin, B.R., L.P. Lue, and J.P. Boni, *Pyrolysis and Volatilization of Cocaine.* Journal of Analytical Toxicology, 1989. 13: p. 158-162.

43. Paul, B.D., L.K. McWhorter, and M.L. Smith, *Electron Ionisation Mass Fragmentometric Detection of Urinary Ecgonidine, a Hydrolytic Product of Methylecgonidine, as an Indicator of Smoking Cocaine.* Journal of Mass Spectrometry, 1999. 34: p. 651-660.

44. Fandino, A.S., S.W. Toennes, and G.F. Kauert, *Studies on In Vitro Degradation of Anhydroecgonine Methyl Ester (Methylecgonidine) in Human Plasma.* Journal of Analytical Toxicology, 2002. 26: p. 567-570.

45. Riley, K.J., et al., *Monitoring the Crack Epidemic Through Urine Testing: Establishment of Routine Detection Methods.* Addiction Biology, 2001. 6: p. 83-95.

46. Myers, A.L., et al., *Identification of Anhydroecgonine Ethyl Ester in the Urine of a Drug Overdose Victim.* Journal of Forensic Sciences, 2005. 50: p. 1481-1485.

47. Jenkins, A.J., J.M. Oyler, and E.J. Cone, *Comparison of Heroin and Cocaine Concentrations in Saliva with Concentrations in Blood and Plasma.* Journal of Analytical Toxicology, 1995. 19: p. 359-374.

48. Cone, E.J., M. Hillsgrove, and W.D. Darwin, *Simultaneous Measurement of Cocaine, Cocaethylene, Their Metabolites, and 'Crack' Pyrolysis Products by Gas Chromatography-Mass Spectrometry.* Clinical Chemistry, 1994. 40: p. 1299-1305.

49. Shimomura, E.T., G.D. Hodge, and B.D. Paul, *Examination of Postmortem Fluids and Tissues for the Presence of Methylecgonidine, Ecgonidine, Cocaine, and Benzoylecgonine Using Solid Phase Extraction and Gas Chromatography-Mass Spectrometry.* Clinical Chemistry, 2001. 47: p. 1040-1047.

50. Toennes, S.W., et al., *Artifact Production in the Assay of Anhydroecgonine Methyl Ester in Serum Using Gas Chromatography-Mass Spectrometry.* Journal of Chromatography B, 2003. 792: p.345-351

51. Dams, R., et al., *Oral Fluid as an Alternative Matrix to Monitor Opiate and Cocaine Use in Substance-Abuse Treatment Patients.* Drug and Alcohol Dependence, 2007. 87: p. 258-267.

52. Ragoucy-Sengler, C. and P. Kintz, *Detection of Smoked Cocaine Marker (Anhydroecgonine Methyl Ester) in Nails.* Journal of Analytical Toxicology, 2005. 29: p. 765-768.

53. Garside, D., et al., *Identification of Cocaine Analytes in Fingernail and Toenail Specimens.* Journal of Forensic Sciences, 1998. 43: p. 974-979.

54. Cognard, E., et al., *Analysis of Cocaine and Three of its Metabolites in Hair by Gas Chromatography-Mass Spectrometry Using Ion-Trap Detection for CI/MS/MS.* Journal of Chromatography B, 2005. 826: p. 17-25.

55. Kintz, P., et al., *Testing Human Hair and Urine for Anhydroecgonine Methyl Ester, a Pyrolysis Product of Cocaine.* Journal of Analytical Toxicology, 1995. 19: p. 479-482.

56. Paul, B.D., et al., *Concentration Profdes of Cocaine, Pyrolytic Methyl Ecgonidine and Thirteen Metabolites in Human Blood and Urine: Determination by Gas Chromatography-Mass Spectrometry.* Biomedical Chromatography, 2005.19: p. 677-88.

57. Liberty, H.J., et al., *Detecting Crack and other Cocaine Use with Fastpatches.* Addiction Biology, 2003. 8: p. 191-200.

58. Kato, K., et al., *Cocaine and Metabolite Excretion in Saliva Under Stimulated and Nonstimulated Conditions.* Journal of Analytical Toxicology, 1993. 17: p. 338-341.

59. Engelhart, D.A. and A.J. Jenkins, *Detection of Cocaine Analytes and Opiates in Nails form Postmortem Cases.* Journal of Analytical Toxicology, 2002. 26: p. 489-492.

60. Niedbala, R.S., et al., *Immunoassay for Detection of Cocaine/Metabolites in Oral Fluids.* Journal of Analytical Toxicology, 2001. 25: p. 62-68.

61. Spiehler, V., et al., *Enzyme Immunoassay Validation for Qualitative Detection of Cocaine in Sweat.* Clinical Chemistry, 1996. 42: p. 34-38.

62. Kim, I., et al., *Sensitivity and Specificity of the Cozart Microplate EIA Cocaine Oral Fluid at Proposed Screening and Confirmation Cutoffs.* Clinical Chemistry, 2003. 49: p. 1498-1503.

63. Cooper, G., et al., *Validation of the Cozart Microplate EIA for Cocaine and Metabolites in Oral Fluid.* Journal of Analytical Toxicology, 2004. 28: p. 498-503.

64. Hand, C. and D. Baldwin, *Immunoassays,* in *Clarkes Analysis of Drugs and Poisons,* A.C. Moffat, M.D. Osselton, and B. Widdop, Editors. 2004, Pharmaceutical Press, p. 301-312.

65. Gosling, J.P., *Immunoassays: Practical Approach.* 2000.

66. Wild, D., *The Immunoassay Handbook.* Third ed. 2005: Elsevier.

67. Spiehler, V., *Hair Analysis by Immunological Methods from the Beginning to 2000.* Forensic Science International, 2000. **107:** p. 249-259.

68. Gosling, J.P., *A Decade of Development in Immunoassay Methodology.* Clinical Chemistry, 1990. 36: p. 1408-1427.

69. Lachenmeier, K., F. Musshoff, and B. Madea, *Determination of Opiates and Cocaine in Hair Using Automated Enzyme Immunoassay Screening Methodologies Followed by Gas Chromatographic-Mass Spectrometric (GC-MS) Confirmation.* Forensic Science International, 2006. **159:** p. 189-199.

70. Spiehler, V., et al., *Performance of a Microtiter Plate ELISA for Screening of Postmortem Blood for Cocaine and Metabolites.* Journal of Analytical Toxicology, 2003. 27: p. 587-591.

71. Kerrigan, S. and W.H. Phillips, *Comparison of ELISAs for Opiates, Methamphetamine, Cocaine Metabolite, Benzodiazepines, Phencyclidine, and Cannabinoids in Whole Blood and Urine.* Clinical Chemistry, 2001. 47: p. 540-547.

72. Watanabe, T., et al., *Development of a Class-Specific Immunoassay for the Type IPyrethroid Insecticides.* Analytica Chimica Acta, 2001. **444:** p. 119-129.

73. Dankwardt, A., *Immunochemical Assays in Pesticide Analysis,* in *Encylopedia of Analytical Chemistry.* 1998.

74. Uptima, *EDAC (carbodiimide) Heterobifunctional cross-linker.* Uptima Technical Sheet, 2005.

75. Wu, A.H.B., *A Selected History and Future of Immunoassay Development and Applications in Clinical Chemistry.* Clinica Chimica Acta, 2006. 368: p. 119-124.

76. Deshpande, S.S., *Enzyme Immunoassays From Concept to Product Development.* 1996: Chapman & Hall.

77. Langman, L.J. and B.M. Kapur, *Toxicology: Then and Now.* Clinical Biochemistry, 2006. 39: p. 498-510.

78. Sehgal, D. and I.K. Vikay, *A Methodfor the High Efficiency of Water Soluble Carbodiimide-Mediated Amidation.* Analytical Biochemistry, 1994. 218: p. 87-91.

79. Staros, J.V., R.W. Wright, and D.M. Swingle, *Enhancement by N-Hydroxysulfosuccinimide of Water-Soluble Carbodiimide-Mediated Coupling Reactions.* Analytical Biochemistry, 1986. 156: p. 220-222.

80. Inc, P.B., *Crosslinking Reagents: Technical Handbook.* 2006.

81. Huse, K., H.-J. Bohme, and G.H. Scholz, *Purification of Antibodies by Affinity Chromatography.* Journal of Biochemical and Biophysical Methods, 2002. 51: p. 217-231.

82. Findlay, J.W.A., et al., *Validation of Immunoassays for Bioanalysis: A Pharmaceutical Industry Perspective.* Journal of Pharmaceutical and Biomedical Analysis, 2000. 21: p. 1249-1273.

83. Selby, C., *Interference in Immunoassay.* Ann Clin Biochem, 1999. 36: p. 704-721.

84. Miller, J.J. and R. Valdes, *Approaches to Minimising Interference by Cross-Reacting Molecules in Immunoassays.* Clinical Chemistry, 1991. 37: p. 144-153.

85. Osterloh, J. and G.P. Butrimovitz, *Interactions Among Enzyme, Antibody, Drug, and Time in Enzyme Immunoassays for Drugs of Abuse.* Clinical Chemistry, 1982. 28: p. 327-332.

86. Shah, V.P., et al., *Bioanalytical Method Validation - A Revisit with a Decade of Progress.* Pharmaceutical Research, 2000. 17: p. 1551-1557.

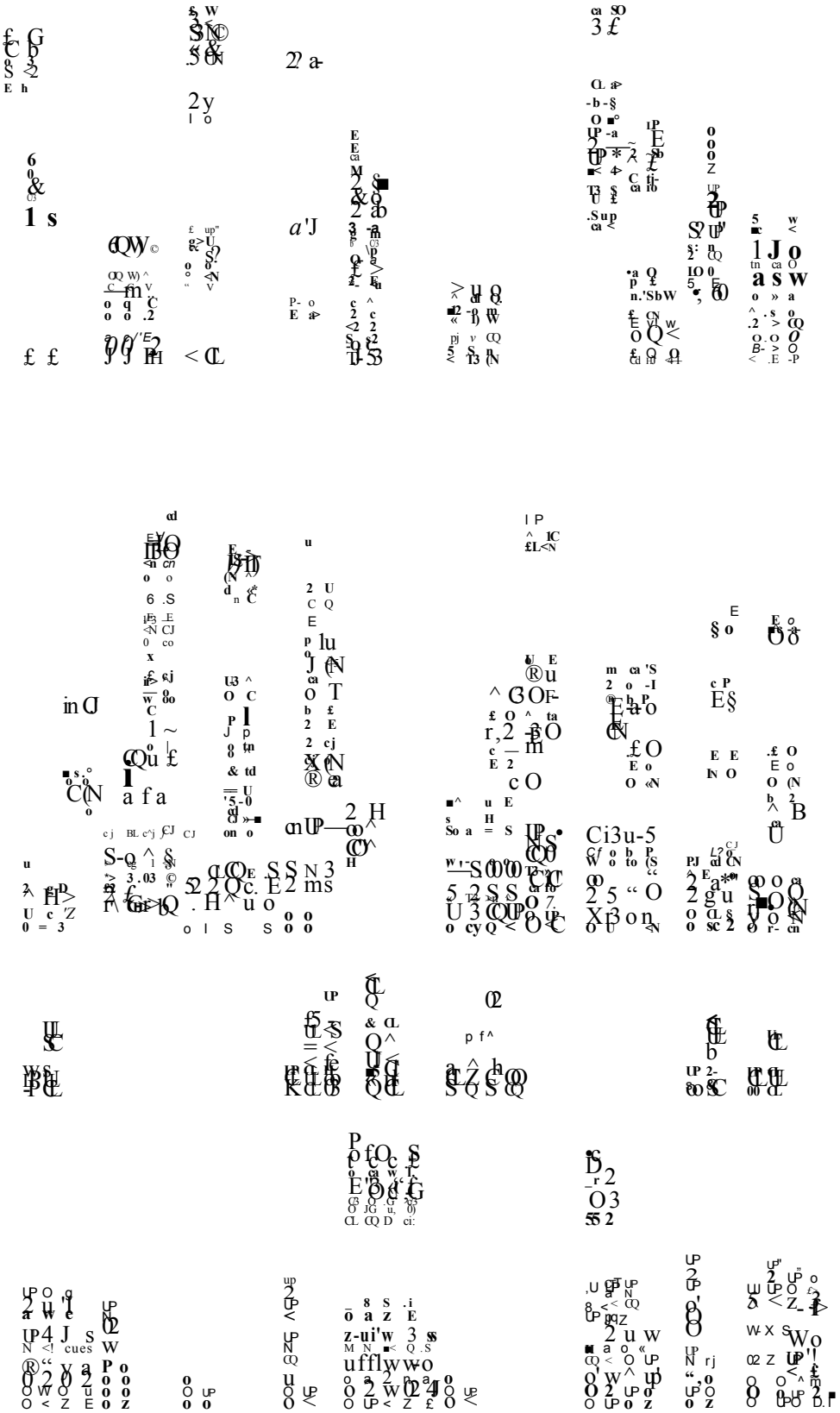
4 Gas Chromatography-Mass Spectrometry Method Development for the Quantitation of Cocaine, its Metabolites and Pyrolysis Products

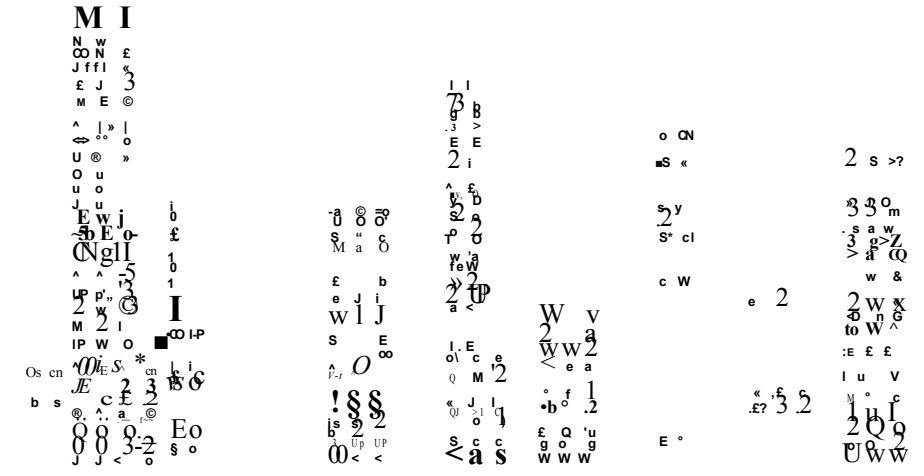
4.1 Introduction

The simultaneous analysis of cocaine and its metabolites presents many analytical challenges. Difficulties are encountered both at the sample preparation stage and during chromatographic separation due to the large polarity difference between the analytes [1]. Cocaine and cocaethylene are non-polar, benzoylecgonine is moderately polar, and AEME, EME and AE are highly polar.

The quantification of cocaine and its metabolites in biological fluids has been commonly performed using GC-MS. In previous years the focus has been on cocaine and its major metabolites benzoylecgonine and EME, however since the identification of the unique pyrolysis products of cocaine some methods have also included AEME. A selection of GC-MS methods published since 1990 detailing the identification of cocaine and its metabolites in a variety of biological matrices are listed in Table 4:1. Twenty one of the thirty five methods described, include AEME in the analysis and only four of these methods were applied to oral fluid samples [2-5]. None of the oral fluid methods included the metabolite AE in the analysis despite the indication that the half life of AEME is short [6].

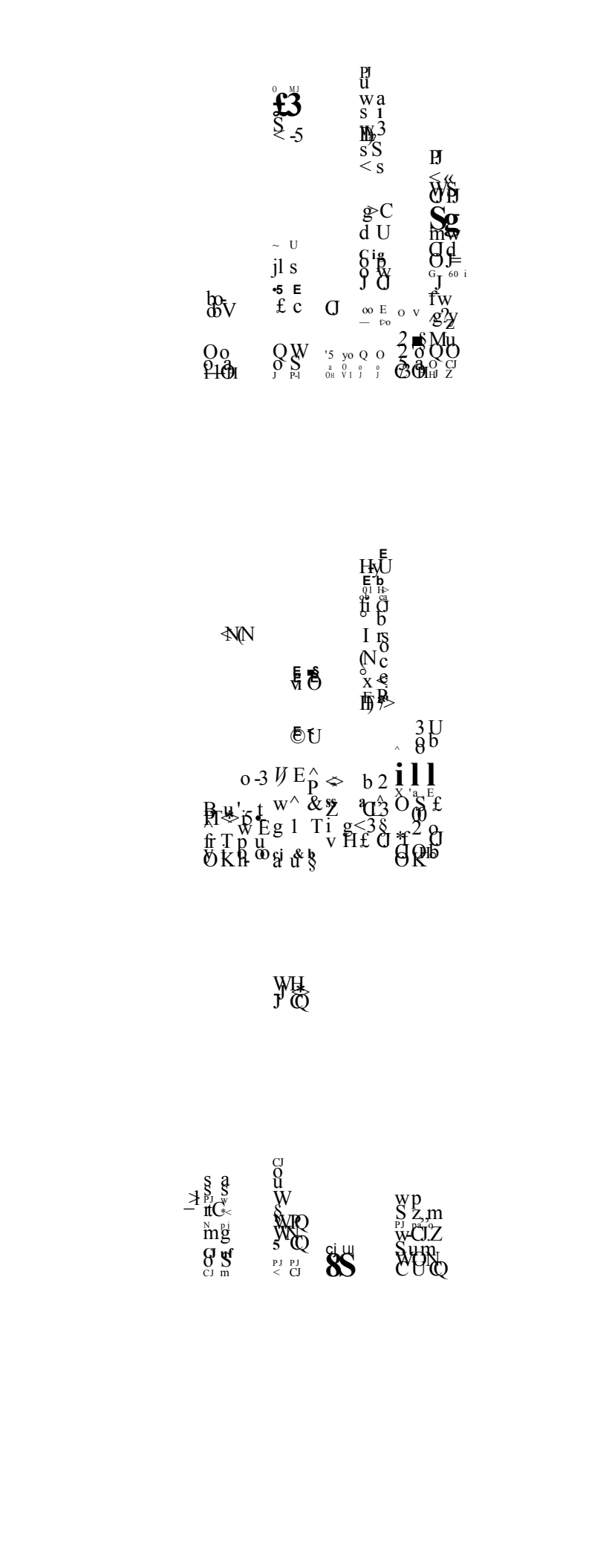
A wide range of temperature programmes have been used, starting as low as 55°C and increasing to a maximum of 320°C. The majority of these temperature programmes have employed a series of ramps in temperature.





3

Analytes	Matrix	Sample Workup	Chromatography/Mass Analysis	Validation Data and Comments
COC, EME, AEME, BZE	Serum	SPE MBDSTFA	GC-MS (EI) (SIM) OPTIMA-1-MS capillary column (30mm x 0.25mm, 0.25pm) 55°C (2min) —>170°C at 20°C/min —»310°Cat 12°C/min (5min)	Method applied to 21 samples, also investigation of artifact AEME
COC, AEME, BZE, EME, CE	Plasma, Oral Fluid, Urine, Sweat and Hair	LLE BSTFA	GC-MS (EI) (SIM) HP-5 MD capillary column (30m x 0.25mm) 60°C to 290°C at 30°C/min (5min)	Recovery: AEME 79-88% in various matrices AEME LOD: fluids Ing/mL, hair O.lng/mg, sweat 0.5ng/patch AEME Precision: 7-10%
COC, BZE, EME, CE, NCOC, EEE	Sweat	SPE BSTFA/MTBSTFA	GC-MS (EI) (SIM) HP-1MS capillary column (30m x 0.32mm, 0.25pm) 70°C (lmin) to 175°C at 30°C/min to 250°C at 23°C/min to 310°C at 18°C/min (5min)	Evaluated excretion of cocaine in sweat following controlled dose administration LOQ: 2.5ng/patch
COC, BZE	Blood, Skin, Interstitial Fluid, Sebum, Stratum Comeum	SPE MTBSTFA	GC-MS (CI) Ion Trap DB-5MS capillaiy column (J&W)	
COC, BZE, EME	Oral Fluid	SPE PFPA/PFP	GC-MS (EI) Quadrupole MS (SIM) J&W 0.25mm 3pm column	LOQ COC 4ng/mL, BZE 7ng/mL Precision COC 11%, BZE 2%, EME 10%
COC, BZE, EME	Sweat	SPE HFIP/PFPA	GC-MS (EI) (SIM) DB-1 capillary column (12m x 0.8mm) 90°C to 130°C at20°C/min to 270°C at 35°C/min (1.5min) to 300°C at 30°C/min (lmin)	LOQ 4ng/mL COC, 2ng/mL BZE, 3ng/mL EME. LOD 3ng/mL COC, 2ng/mL BZE, EME
COC, CE	Oral Fluid	SPME	GC-MS (EI) (SIM) Capillary column (12m x 0.2mm, 0.33pm) 40°C (lmin) to 280°C at 20°C/min (5min)	Precision: 4-10% COC, 6-28% CE LOD: 20ng/mL COC, 5ng/mL CE LOQ: 50ng/mL COC, 1Ong/mL CE
COC	Oral Fluid	SPME	GC-MS (EI) (SIM) HP5MS capillary column (30m x 0.25mm, 0.25pm) 150°C (2min) to 220°C at 15°C/min to 250°C at 20°C/min (5min)	Recovery: 73-93% LOD/LOQ: 5ng/mL Precision: 2-6%
COC, BZE, CE	Oral Fluid	SPE BSTFA	GC-MS (EI) Quadrupole MS (SIM) DB-5MS capillary column (30m x 0.25mm, 0.25pm) 150"C (1,5min) to 230"C at 60"C/min (8min) to 300°C at 60°C/min (4min)	217 clinical samples tested
COC, BZE, CE	Hair	SPE MSTFA	GC-MS (EI) (SIM) HP-5MS capillary column (30m x 0.25mm, 0.25pm) 180°C (lmin) to 190°C at 15°C (lOmin) to 250°C at 5°C/min to 290°C at 30°C/min (2min)	Precision <7%
AEME, EME, COC, CE, BZE	Hair, Urine	LLE BSTFA	GC-MS (EI) HP-5MS capillary column (30m x 0.25mm) 60°C to 290°C at 30°C/min (5min)	Precision: <18% LOD: 5ng/mLBZE, Ing/mL others



4.1.1 Artifact Production of AEME

AEME, as well as being produced from smoked cocaine, can be formed as an artifact of cocaine at high temperatures within injector ports in the GC. Similarly AE can be formed from the thermal decomposition of benzoylecgonine [13]. AEEE, a transesterification product of smoked cocaine co-abused with alcohol has also been established as an artifact of cocaethylene in the GC injector port [38].

Studies have shown that the amount of AEME formed as an artifact is <1.0% of the concentration of cocaine [4, 15, 20, 33], and that a linear relationship exists between AEME artifact production and the concentration of cocaine in the sample [24].

Artifact production can be monitored by injecting a known concentration of cocaine standard as a control and monitoring the peak corresponding to the 152 ion of AEME [15]. Alternatively the production of AEME-d_3 as an artifact of COC-d_3 can be measured [20].

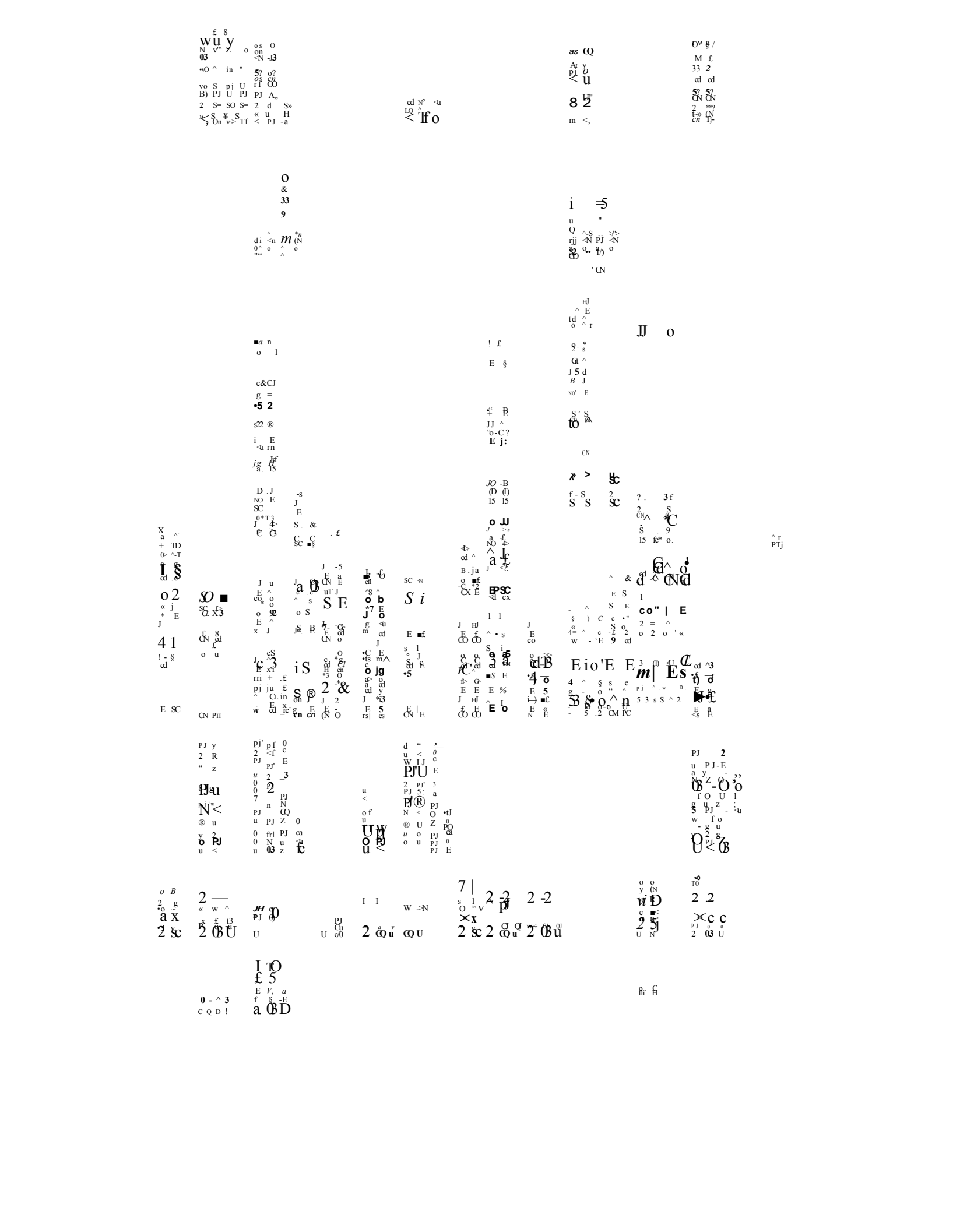
It is suggested that decreasing the injector port temperature will reduce artifact formation of AEME and AE [21]. Some authors report a decreased formation of artifact AEME when the injector port temperature was reduced to 210°C [13], others claim there was no significant difference observed [15]. A compromise may need to be met whereby the injector port temperature is high enough so as not to decrease the response for all the other analytes but low enough to reduce the formation of artifact AEME.

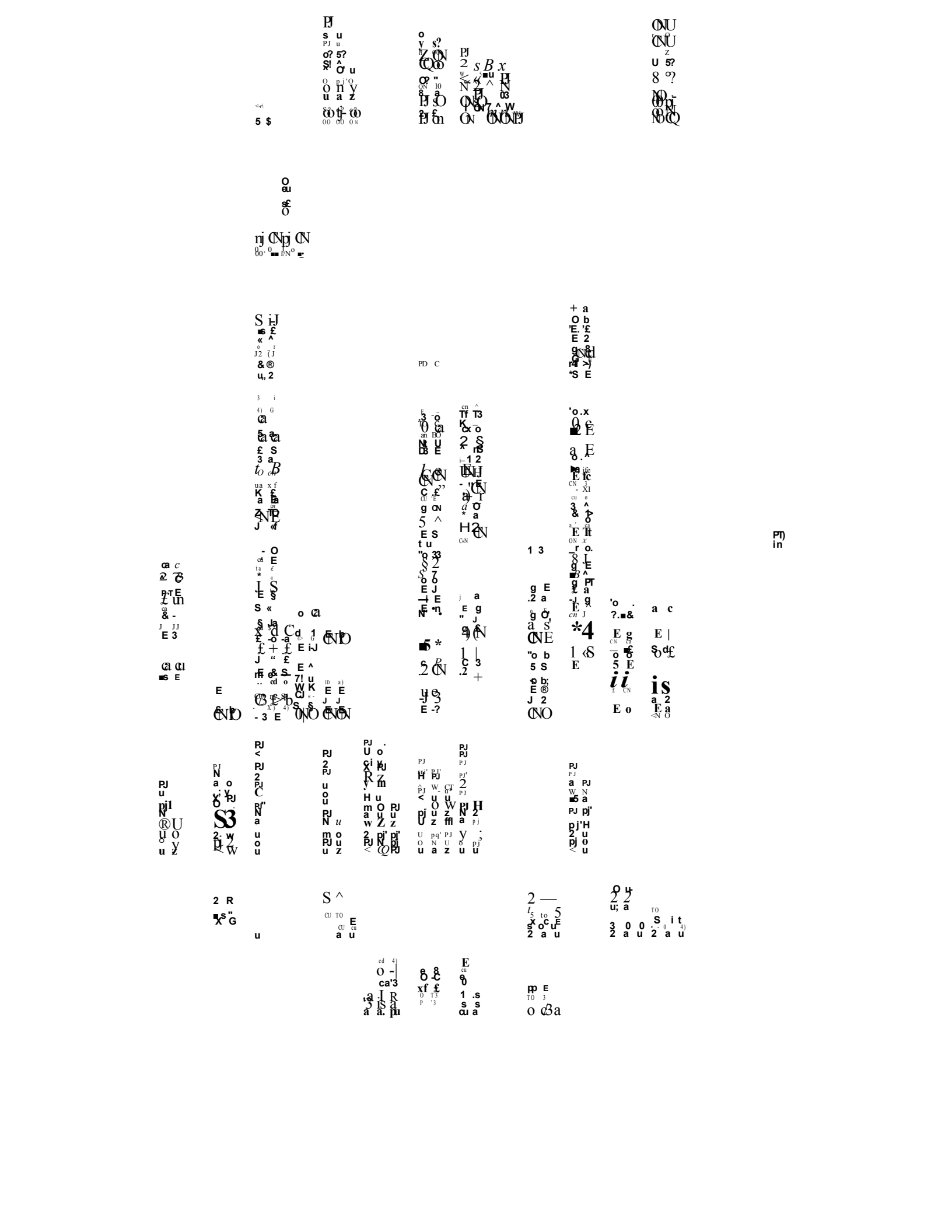
The state of the insert liner has also been suggested as a contributing factor to artifact formation of AEME, so recommendations are to use a clean liner when analysing for AEME [24, 38].

4.1.2 Sample Preparation

Sample preparation techniques which have proved successful in the extraction of cocaine and its metabolites from biological matrices include liquid-liquid extraction (LLE), solid phase microextraction (SPME), and more commonly, solid phase extraction (SPE). The different SPE methods employed for the analysis of cocaine and its metabolites are listed in Table 4:2. Mixed mode sorbents such as Bond Elut Certify™ are versatile and are frequently used for the analysis of drugs of abuse [39]. The extraction recoveries quoted vary considerably between different analytes and also between different extraction methods.

A large number of methods have utilised phosphate buffer at pH 6 for sample pre-treatment and column conditioning. In general at pH 6 a high percentage of cocaine, cocaethylene, benzoylecgonine, and AEME are retained on the SPE column, however the more polar compounds ecgonine, and AE, are not adsorbed under these conditions and so pass through the column into waste [18]. Very low recoveries (0.7-4.1%) have been reported for AE and ecgonine [15]. The difficulty experienced in extracting these compounds from biological matrices means they are not frequently analysed [40]. EME is another compound which has reasonably low recovery due to its polar functional group, most methods report recoveries of approximately 40-45% [3, 13, 14, 41]. The reasoning for the low reported recoveries using mixed mode sorbents may lie in the low ion exchange capacity of the products [42].





A number of different methods have been tested in order to overcome the poor recoveries of the highly polar metabolites. Reducing or removing the wash steps has proven successful in increasing recovery while not significantly decreasing the sensitivity of the analytical method [1, 19, 45]. However the highest reported recoveries (50-99%) have been achieved by performing a two step SPE method [11, 12, 18, 21]. The eluant from the first SPE, following addition of the sample, is saved and then subjected to a second SPE extraction under different experimental conditions.
Even though AE is highly water soluble and a zwitterion, like benzoylecgonine, it cannot be extracted under the same conditions. The dissociation of the carboxylic acid is expected to be more than that of the sterically hindered carboxylic acid group in benzoylecgonine [12]. The key to increased recovery of the highly polar compounds AE and ecgonine appears to lie in the pH of the solution. Adsorption of AE was studied in the pH range of 2.0-5.0. Recoveries of 99% were found at pH 2.0, compared to 9% at pH 5.0 [12]. Under acidic conditions the dissociation of the carboxylic acid is reduced and the lipophilic and cationic character is improved allowing for more efficient adsorption to the solid phase [11, 12].

Two solvents, methanol and acetonitrile have been evaluated in the wash step prior to elution. The use of methanol produced low recovery of benzoylecgonine (30%), this increased considerably to 91% when acetonitrile was used [49]. There was no data provided to establish what effect this has on the non polar compounds such as cocaine and cocaethylene.

The extraction of cocaine and cocaethylene from biological materials is relatively simple, their hydrophobic nature allows extraction into non polar liquid or stationary phases [50]. This approach however is not suitable for the more polar metabolic and

pyrolytic products of cocaine [51]. Mixtures of solvents such as dichloromethane or chloroform, plus ethanol or isopropanol have proved most efficient in extracting the majority of metabolites [39, 50]. Elution of substances retained via the cation exchange sites is achieved using an amine, in general 2-3% strong ammonia is used. The vast majority of methods listed in Table 4:2 have utilised dichloromethane and isopropanol with 2% ammonia solution.

4.1.3 Derivatisation

The presence of polar functional groups on some of the cocaine metabolites makes the analysis by GC-MS extremely difficult [51]. Derivatisation using different types of agent can be performed to change the structure of the molecule and mask the polar hydrogen bonding characteristics of these molecules [1].

Trimethylsilylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) is the main derivatising agent used as the hydroxyl and carboxylic groups present are readily derivatised. A disadvantage however is that the trimethylsilyl (TMS) matrix compounds have been found to elute in the same range as AEME, interfering considerably with sensitivity [20].

The use of pentafluoropropionic anhydride (PFPA) and 2,2,3,3,3-pentafluro-l-propanol (PFP) as derivatising agents has been reported as providing a much more stable derivative with a much higher molecular weight and less background noise than TMS [15]. PFPA derivatises hydroxyl groups and secondary amine functional groups, while PFP derivatises the carboxylic groups [13].

4.2 Experimental

4.2.1 Materials

Solutions of the reference standards (lmg/mL in methanol or acetonitrile) AEME, AE, EME, cocaine, benzoylecgonine and of the corresponding deuterated internal standards (O.lmg/mL) EME-d3, cocaine-d3, benzoylecgonine-d3 and were obtained from Cerilliant (LGC Promochem, Teddington, UK). The derivatising reagents BSTFA with 1% v/v TMS, PFPA, and PFP were purchased from Sigma Aldrich Company Ltd (Dorset, UK). All other reagents and solvents were of analytical grade and were purchased from VWR International Ltd (Leicestershire, UK). Solid phase extraction (SPE) cartridges, Bond Elut™ Certify (50mg, 3mL), were purchased from Varian (Surrey, UK). High recovery vials were purchased from Crawford Scientific (Lanarkshire, Scotland, UK).
Oral fluid collectors were provided by Cozart pic (Oxfordshire, UK).

4.2.2 Solution Preparation

4.2.2.1 Drug Standards

Combined analyte working solutions of lpg/mL and lOOng/mL were prepared in acetonitrile from individual lmg/mL stock solutions of the reference standards. A working solution of lpg/mL for the internal standards was prepared in acetonitrile from the individual O.lmg/mL stock solutions. Analyte matched internal standards do not exist for every compound of interest, it is important therefore to use an internal standard that matches as close as possible the polarity of the compound being analysed [1]. For the compounds used in this study internal standards were only available for cocaine, benzoylecgonine, and EME. EME-d3 was used as the internal standard for both AEME and AE. The working solutions were refrigerated at 4°C for up to 6 months.

4.2.2.2 Buffers and Reagents

- 0.1mol/L Phosphate Buffer pH 6 and 4. (500mL)

  400mL of deionised water was added to 6.81g of potassium dihydrogen orthophosphate (MW 139.09). The pH of the buffer was adjusted to the required pH using 10 mol/L potassium hydroxide. The buffer was made up to 500mL in a volumetric flask with deionised water and stored refrigerated (2-8°C) for up to 2 months.

- 2mol/L Hydrochloric Acid (100mL)

  19.2mL of a 32% hydrochloric acid solution was added to a 100mL volumetric flask made up with deionised water.

- 0.01 mol/L Hydrochloric Acid (100mL)

  500µL of the 2M hydrochloric acid solution was added to a 100mL volumetric flask made up with deionised water.

- 0.1 mol/L Hydrochloric Acid (100mL)

  5mL of the 2M hydrochloric acid solution was added to a 100mL volumetric flask made up with deionised water.

- Dichloromethane:Isopropanol (DCM/IPOL) (80:20) (1L)

  800mL of dichloromethane and 200mL of isopropanol were measured out individually using measuring cylinders and then combined. The solution was stored in a 1L glass bottle.

4.2.2.3 SPE Elution Solvents

- Dichloromethane/Isopropanol/Ammonium Hydroxide (80/20/2 v/v). Prepared daily as required.

  1ml of ammonium hydroxide was added to 49mL of DCM/IPOL, the solution was mixed vigorously before use.

- Methanol/Isopropanol/Ammonium Hydroxide (40/60/2.5 v/v). Prepared daily as required.

  20mL of methanol and 30mL of isopropanol were measured out using a 50mL measuring cylinder, 1.25mL of ammonium hydroxide was added and the solution was mixed vigorously before use.

- Methanol/Ammonium Hydroxide (98/2 v/v). Prepared daily as required.

  lml of ammonium hydroxide was added to 49mL of methanol, the solution was mixed vigorously before use.

4.2.3 Instrumentation

The GC-MS was an Agilent 5973N with 6890 GC purchased from Agilent Technologies (Cheshire, UK), equipped with Rtx-5Sil MS (30m x 0.25mm i.d x 0.25pm) capillary column, purchased from Crawford Scientific (Lanarkshire, Scotland, UK). The GC mobile phase was helium (99.999% purity) and was supplied by Air Products (Cheshire, UK).

SPE was performed on a vacuum manifold assembly purchased from Varian (Surrey, UK). A Techne® Sample Concentrator with a DB-3 series Dri-Block® was purchased from LAB3 (Northampton, UK).

4.2.4 GC-MS Parameters

The GC-MS was run in electron ionisation mode with splitless injection. The mobile phase was helium at a flow rate of lmL/min and at a constant pressure of 8.7 psi. The injector port temperature was set at 210°C, with the transfer line and source temperatures set to 280°C and 230°C respectively. Due to short retention times reported for AE and AEME the MS acquisition was set at 4 minutes.

4.2.5 Method

4.2.5.1 GC Temperature Programme

Seven temperature programmes were chosen from the literature and evaluated for the simultaneous detection of AEME, cocaine, EME and benzoylecgonine. A summary of these can be seen in Table 4:3. A cross section of methods published within the last 10 years was considered. The detection of AEME was an important factor and most of the methods chosen described its analysis. Two methods did not analyse AEME but did however have excellent reproducibility and low limits of detection for cocaine and benzoylecgonine.

Method	GC Oven Temperature Programme	Reference
CRK01	**150°C (1.5min) —»220oC at 5°C/min —>300°C at 30°C/min (3.83min)**	**Cozart in house method**
CRK02	**90°C (lmin) —>180°C at 20°C/min —>240°C at 5°C/min —>290°C at 30°C/min**	**[17]**
CRK03	**55°C (2min) —>170°C at 20°C/min —»310°Cat 12°C/min (5min)**	**[24]**
CRK04	**70°C —>250°C at 30°C/min (4min)**	**[21]**
CRK05	**80°C (0.5min) —>150°C at 20°C/min (2min)**	**[12]**
CRK06	**70°C —>135°C at 30°C/min —»140°C at 2°C/min —>230°C at 30°C/min —>234°C at 2°C/min —>250°C at 50°C/min**	**[13]**
CRK07	**70°C —>130°C at 30°C/min ->140°C at 5°C/min -+210°C at 35°C/min —>222°C at 4°C/min —»290°C at 45°C/min (0.49)**	**[15]**

Table 4:3 Summary of GC oven temperature programmes tested

4.2.5.2 Sample Derivatisation

Each temperature programme was assessed using an un-extracted standard which was prepared using the 1pg/mL working solution. Drug was added to a high recovery vial

and evaporated to dryness at 40°C under a stream of nitrogen. Two different derivatising techniques were tested, acylation using PFPA and PFP, and silylation using BSTFA. For silylation BSTFA (50pL) was added to the vial and heated to 70°C for 20 minutes. Acylation was performed by adding PFPA (50pL) and PFP (50pL) to the vial and heated to 70°C for 20 minutes. The solution was evaporated to dryness under a stream of nitrogen at room temperature. Ethylacetate (100pL) was added and the solution was evaporated to dryness again to remove any residual derivatising agent. The drug was reconstituted in 50pL of ethylacetate.

The derivatised sample (2pL) was injected onto the column using the HP autosampler. For each method described in Table 4.3, the sample was first analysed in fall scan mode, 50 to 550 AMU, to obtain the retention times of each analyte. After which the MS was set to selective ion monitoring (SIM) to increase the sensitivity and reduce the presence of background interference. The ions monitored for positive identification of the analytes are displayed in Table 4:4.

Analyte	**Ions Monitored**	
	BSTFA	**PFPA and PFP**
AE	N/A	270, 271,299
AEME	152, 166, 181	152, 166, 188
EME	82, 97, 271	182,314, 345
EME-d3	85, 99, 274	185,317, 348
Benzoylecgonine	82, 240, 361	300,316, 421
Benzoylecgonine-d3	85, 243, 364	303,319, 424
Cocaine	82, 182,303	182, 272, 303
Cocaine-d3	85, 185, 306	185,275,306

Table 4:4 Ions monitored for each analyte using BSTFA or PFPA/PFP as derivatising agents

4.2.5.3 Solid Phase Extraction

Following a literature review of solid phase extraction methods for cocaine and its metabolites, it was found that the main differences between each published method were in the solutions used for pH adjustment and column washing. Four different SPE methods were tested to encompass a variety of solutions for evaluation. The volumes used were kept consistent between methods. The summary of each SPE method used can be seen in Table 4:5.

Method	SPE Method	Elution Solvent
1 (Cozart)	**lmL Methanol, lmL pH6 phosphate buffer, sample + lmL buffer, lmL water, lmL 0.01M HC1, dry lOmins, 2mL methanol, dry 2mins.**	**DCM/IPOL/NH3**
2 (Adapted Cozart)	**lmL Methanol, lmL pH4 phosphate buffer, sample + lmL buffer, lmL water, lmL 0.01M HC1, dry lOmins, 2mL methanol, dry 2mins.**	**DCM/IPOL/NH3**
3 [15]	**lmL Methanol, lmL 0.1M pH4 sodium acetate buffer, sample + buffer, lmL water, lmL 0.1M HC1, lOmin dry, 2mL methanol, dry 2 min**	**DCM/IPOL/NH3**
4 [19]	**lmL methanol, lmL 0.1M HC1, sample + lmL 0.1M HC1, lmL water, lmL 0.25M acetic acid, dry lOmins, 2mL methanol, dry 2mins.**	**MeOH/IPOL/NH3 (4:6:0.25)**

Table 4:5 Summary of solid phase extraction methods used for the extraction of cocaine, its metabolites and pyrolysis products

Drug and internal standard were added to 500pL of negative oral fluid buffer mixture at a concentration of 180ng/mL. Samples were extracted in duplicate according to each method as outlined in Table 4:5. An un-extracted standard was also prepared for comparison. Following elution each sample was evaporated to dryness under nitrogen at 40°C and derivatised using PFPA and PFP as outlined in section 4.2.5.2.

Each method was compared based on absolute recovery for each analyte. The analyte peak areas of the un-extracted standard were taken as 100% recovery and the peak areas of the extracted samples were calculated against it.

The choice of elution solvent can have a significant impact on the recovery of analytes from the SPE column. Three different elution solvents were tested, they included dichloromethane/isopropanol/ammonium hydroxide (80:20:2), methanol/isopropanol/ammonium hydroxide (4:6:0.25), and methanol/ammonium hydroxide (98:2).

Recovery of AE using SPE has been reported to be very low due to the polar nature of the analyte [15]. Some authors have used a two step extraction technique for AE whereby eluates from the first SPE after sample loading were saved and subjected to a second SPE. Two methods from the literature were selected, the details are summarised in Table 4:6.

Method	SPE Method
AE Method A **[11]**	**lmL Methanol, lmL 0.1M HC1, sample + lmL 2M HC1, lmL water, lmL 0.1M HC1, 2mL Methanol, lOmin dry.**
AE Method B **[21]**	**lmL methanol, lmL 0.2M phosphoric acid, sample + lmL 0.2M phosphoric acid, lmL water, lmL 0.2M phosphoric acid, 2mL methanol, lOmin dry.**

Table 4:6 Summary of SPE methods for extraction of AE.

The SPE was started by conditioning the column with methanol, after which the waste tubes were removed and replaced with fresh pre-labelled tubes to collect the fraction containing AE. Figure 4:1 fully illustrates the process of the two step SPE method.

The sample was loaded onto the column which was subsequently washed with the addition of lmL of water, the tubes were removed and saved for the second extraction. The original waste tubes were replaced and the rest of the extraction was continued as previously described. The saved eluants were then extracted using each of the two methods outlined in Table 4:6.

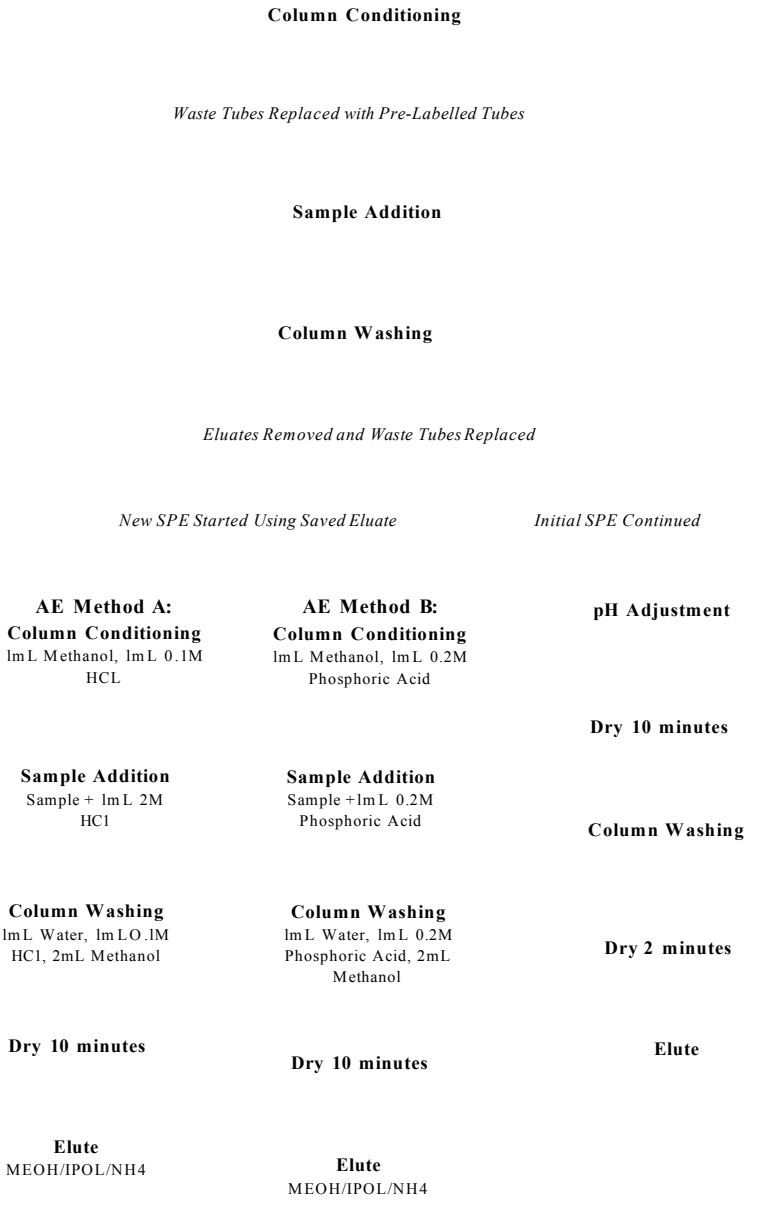


Figure 4:1 Overview of 2 step SPE methods

Drug was eluted with methanol/isopropanol/ammonia in a ratio of 4:6:0.25. They were each evaporated to dryness under nitrogen at 40°C and derivatised using PFPA and PFP at 70°C for 20 minutes. The drugs were reconstituted in ethylacetate (100µl), and the contents of the vial containing AE were added to the vial containing the other analytes to allow for simultaneous analysis. The samples were evaporated to dryness under nitrogen again and reconstituted in 50µL of ethylacetate.

4.2.6 Calibration Curve Linearity

A calibration curve prepared using drug free oral fluid was extracted. The calibration standards were prepared from the drug standard working solutions and consisted of eight points representing 0, 15, 30, 60, 90, 120, 180 and 360ng/mL of each analyte. Internal standards were added at a concentration of 120ng/mL.

To generate a calibration curve the peak area ratios between the standards and the internal standard were plotted against the concentration. The coefficients of determination (r^2) obtained for the calibration curves were calculated.

4.3 Results and Discussion

4.3.1 GC Temperature Programme

Seven GC oven temperature programmes and two different derivatising agents were tested to identify the method which provided identification of all the required analytes from a single injection, with good resolution and peak shape.

Due to instrument availability two identical GC-MS instruments were used to evaluate the temperature programmes. Instrument 1 was used initially to evaluate methods CRK01 to CRK06 for the identification of AEME, EME, cocaine and benzoylecgonine using BSFTA as the derivatising agent. Methods CRK02, CRK03, and CRK04 allowed

the simultaneous identification of all the analytes. AEME eluted from the column first followed by EME, cocaine, and benzoylecgonine. Chromatographic peaks typically take the shape of a normal Gaussian distribution curve. Method CRK02 provided better peak shapes compared to methods CRK03 and CRK04. The full scan chromatograms and mass spectra for method CRK02 can be seen in Figures 4:2 to 4:5.

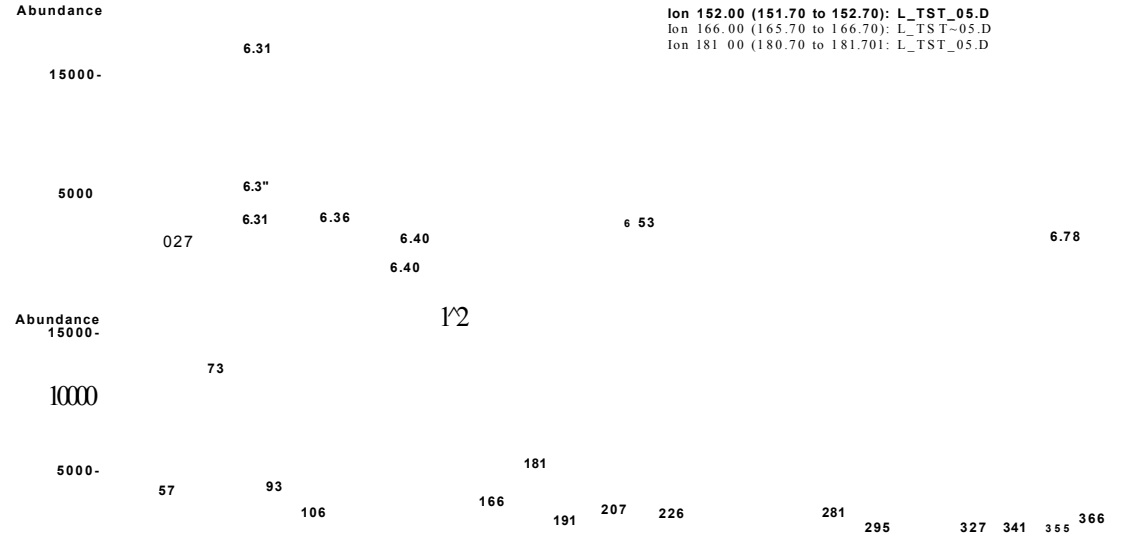


Figure 4:2 Full scan chromatogram and mass spectra for AEME using method CRK02

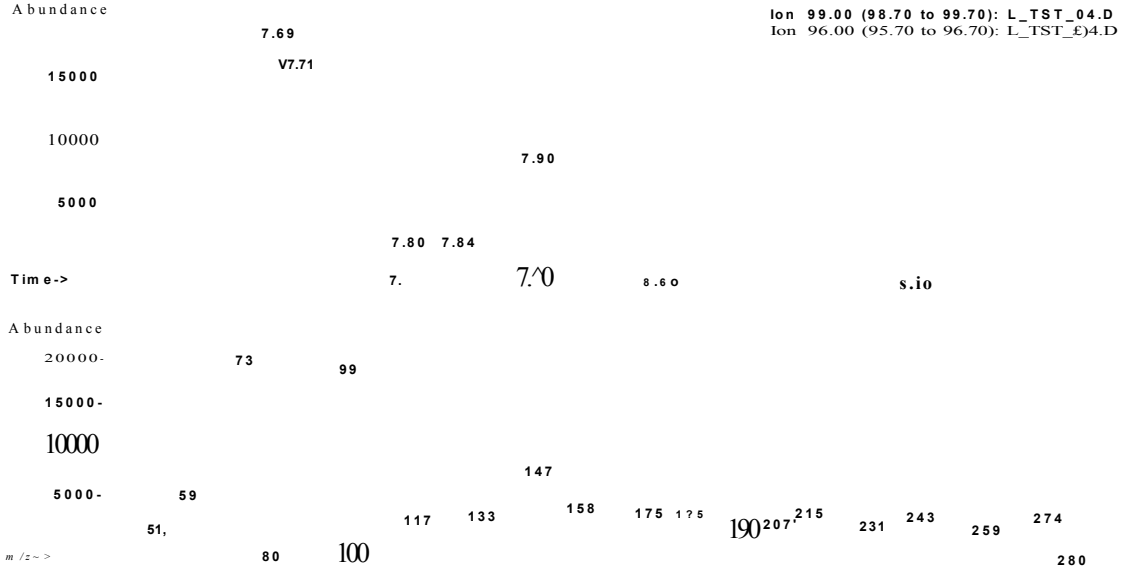


Figure 4:3 Full scan chromatogram and mass spectra for EME-d3 and EME using method CRK02

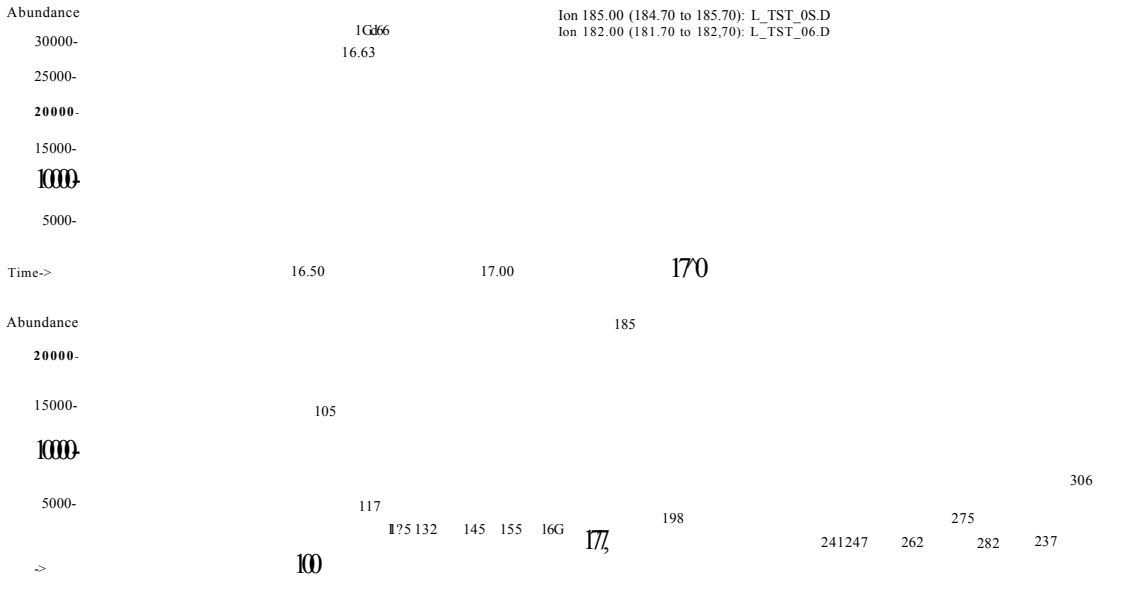


Figure 4:4 Full scan chromatogram and mass spectra for cocaine-d3 and cocaine using method CRK02

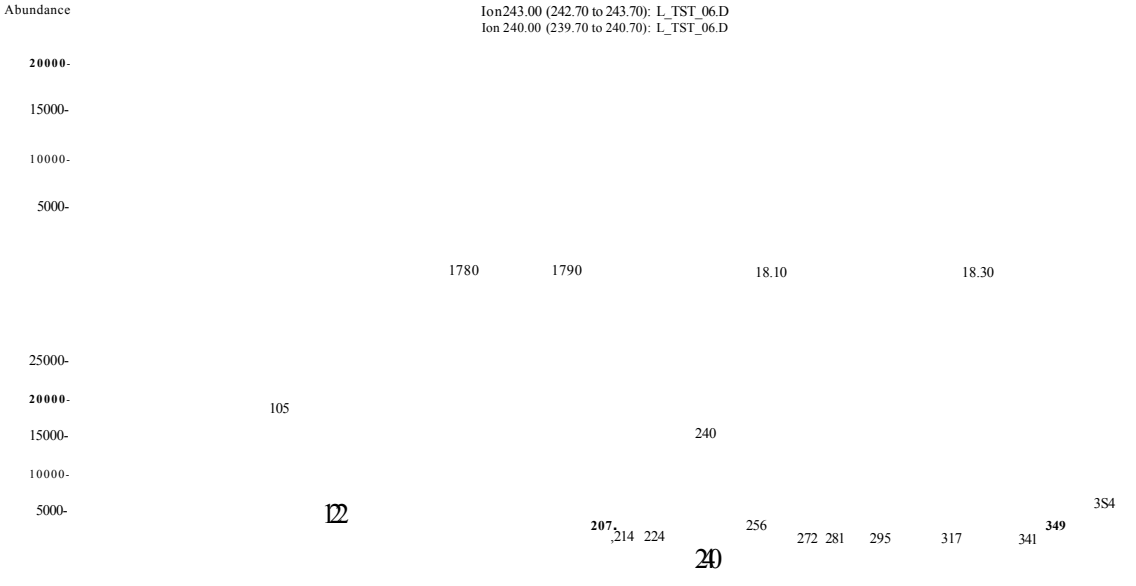


Figure 4:5 Full scan chromatogram and mass spectra for benzoylecgonine-d3 and benzoylecgonine using method CRK02

Method CRK03 and CRK04 produced very poor peak shapes for cocaine and benzoylecgonine, with either very poor selectivity or split peaks (Figure 4:6).

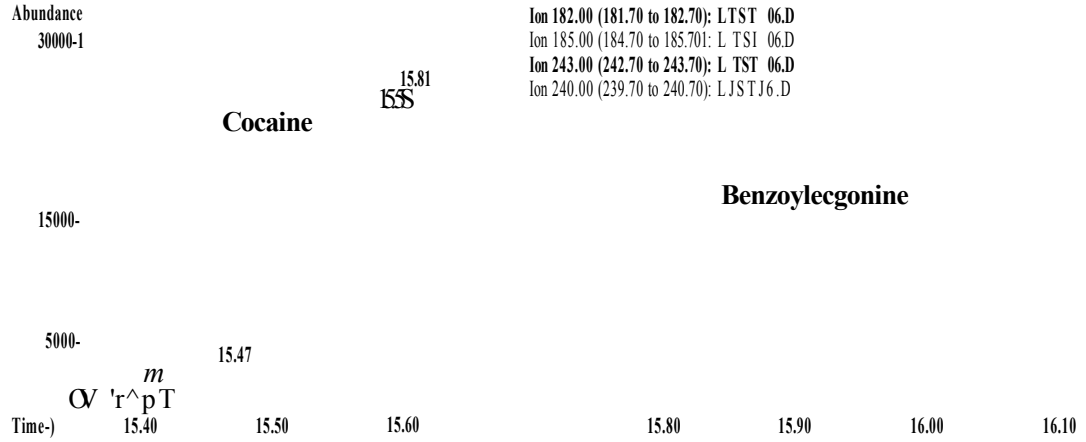


Figure 4:6 Chromatograms for cocaine, benzoylecgonine and their relative internal standards using method CRK03

Methods CRK02, CRK03, and CRK04 were subsequently tested using PFPA and PFP as the derivatising agents. AEME was not identified using PFPA and PFP in any of the methods, however significantly improved peak shapes for benzoylecgonine and cocaine were observed (Figure 4:7).

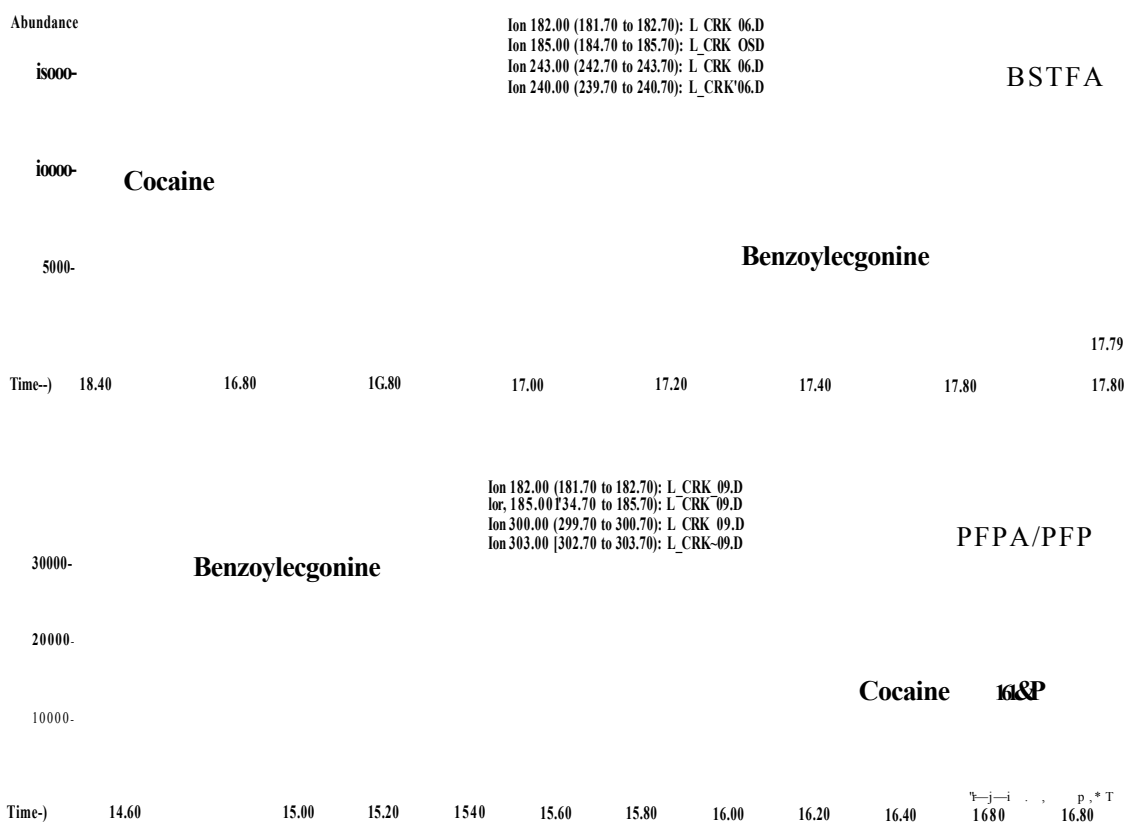


Figure 4:7 Effects of derivatising agents on analysis of cocaine and benzoylecgonine

Using PFPA and PFP the order of elution changed, with benzoylecgonine eluting before cocaine. Cocaine does not contain any hydroxyl or carboxyl groups and therefore is not derivatised by BSTFA, PFPA, or PFP, benzoylecgonine on the other hand contains a carboxyl group which is derivatised. The benzoylecgonine derivative formed using PFP is structurally and chemically different to the derivative formed using BSTFA. The PFP derivative has a higher molecular weight and a reduced affinity for the column which resulted in earlier elution of benzoylecgonine. As cocaine is not derivatised there was no change in its retention time.

The advantages of improved peak shape and less background noise observed using PFPA led to the selection of two GC-MS methods in the literature which used PFPA for derivatisation of the cocaine metabolites. One of the methods chosen was previously tested using BSTFA as the derivatising agent and was method CRK06. Both methods were very similar to one another with respect to the analytes identified and the pattern of oven temperature ramping, however the second method, CRK07, produced a slightly longer run time and a higher final oven temperature.

Due to equipment availability instrument 2 was used to perform the remainder of the method development. AE was also included in the chromatographic analysis at this stage of the investigation.

Method CRK06 enabled the successful identification of AE, AEME, and EME. However neither cocaine nor benzoylecgonine were identified using this temperature programme. Method CRK07 was successful in allowing for the identification of all analytes, with good separation between each compound. The analyte retention times for both methods are summarised in Table 4:7.

Analyte	Retention Time	
	CRK06	**CRK07**
AE	5.57	5.25
AEME	6.16	5.7
EME	6.54	5.99
Benzoylecgonine	ND	10.13
Cocaine	ND	10.73

Table 4:7 Summary of analyte retention times for methods CRK06 and CRK07

The chromatograms produced using method CRK07 are shown in Figures 4:8 and 4:9. The smallest peak area was provided by AEME while AE produced the largest. Benzoylecgonine and cocaine were the last two compounds to elute from the column for each temperature programme tested. The polarity and volatility of these two compounds is very different to AE, AEME and EME which all elute relatively early in the chromatographic run.

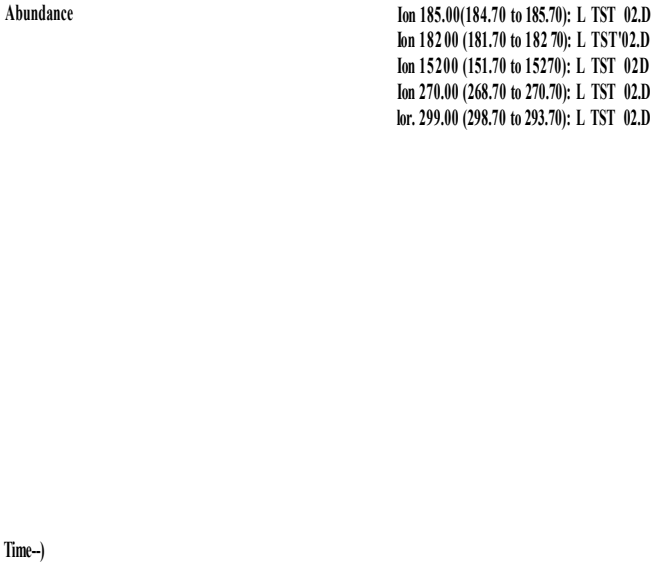


Figure 4:8 Chromatogram of AE, AEME, EME-d3 and EME produced using method CRK07

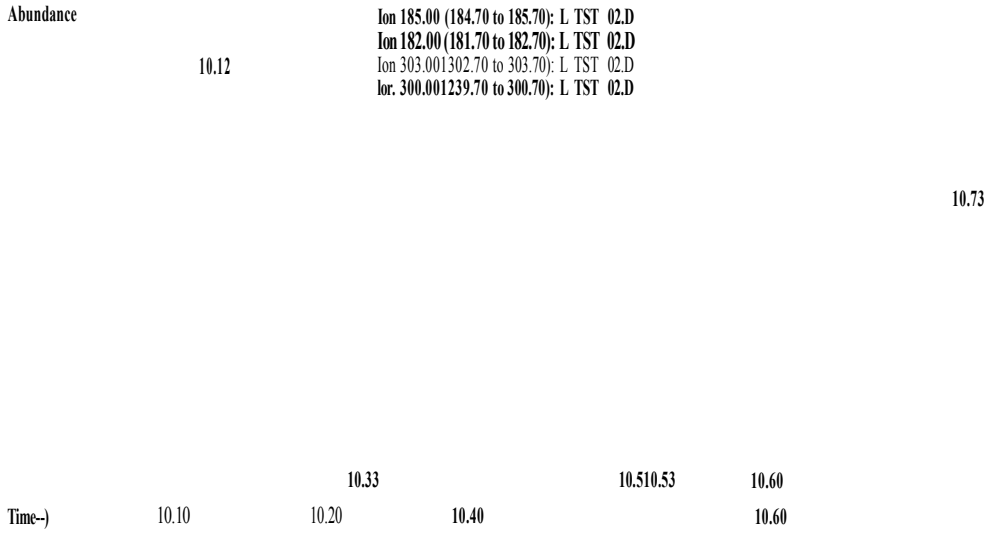


Figure 4:9 Chromatogram of COC-cb, COC, BZE-d3 and BZE produced using method CRK07

The retention times of AE, AEME and EME were slightly longer using method CRK06, the absence of benzoylecgonine and cocaine may be a result of the oven programme being too short. Method CRK06 has a total run time of 9.99 minutes, while method CRK07 has a total run time of 11 minutes. The column temperatures at the point where benzoylecgonine and cocaine were eluted, using method CRK07, were calculated to be 273 °C and 290°C respectively. The fact that the final column temperature for method CRK06 was 250°C may also be a reason for the failure in detection of benzoylecgonine and cocaine.

4.3.2 Solid Phase Extraction

An ideal SPE should provide analyte recovery close to 100%. Cocaine and its metabolites vary greatly in polarity and so SPE methods should be optimised to provide recoveries as high as possible, particularly for AEME as this is the compound of most interest. The recoveries of each analyte using the different SPE methods are summarised in Table 4:8.

Analyte	Un-extracted	SPE 1		SPE 2		SPE 3		SPE 4	
	Peak Area	Peak Area	% Recovery	Peak Area	% Recovery	Peak Area	% Recovery	Peak Area	% Recovery
AEME	21509	2934	13.6	25594	119	17702	82.3	22046	102.5
AE	39179	156	0.4	184	0.5	232	0.6	4292	11
EME	32132	9739	30.3	9822	30.6	8188	25.5	11992	37.3
COC	30281	30516	100.8	28797	95.1	26653	88	30343	100.2
BZE	39740	20218	50.9	19744	49.7	15010	37.8	23472	59.1

Table 4:8 Recovery of analytes following different solid phase extraction methods

The highest recoveries of AEME were produced using SPE 2 and 4 which gave >100%, SPE 1 performed poorly with only 14%. Cocaine recoveries were >95%, with the exception of SPE 3 which gave 88%. Recovery of benzoylecgonine was lower and ranged from 38-59%, while EME ranged from 26-37%. As expected the analyte which produced the lowest recovery was AE, which at its highest was only 11% using SPE 4. Using 0.1M HCl locks the analytes onto the sorbent using ion exchange mechanisms [52], and using acetic acid in the wash step helps remove a variety of impurities without affecting the recovery of the acidic substances [49]. This may be the reason for the increased recoveries shown for SPE 4.

Overall SPE 4 provided the highest recoveries and was selected to develop the extraction further.

4.3.3 Choice of Elution Solvent

The extraction recoveries using the different elution solvents are shown in Table 4:9.

The greatest variability in extraction recoveries was for AEME which ranged from 59-96%. AE recovery was still low using each elution solvent, with the least recovery provided by DCM/IPOL.

Analyte	Un-extracted	DCM/IPOL/NH4		MeOH/IPOL/NH4		MeOH/NH4	
	Peak Area	Peak Area	% Recovery	Peak Area	% Recoveiy	Peak Area	% Recovery
AEME	20845	17126	82.2	20029	96.1	12259	58.9
AE	48670	500	1	5300	11	4425	9.1
EME	26941	10876	40.4	12359	45.6	8753	32.5
COC	22106	26201	118.5	25142	113.7	27577	124.8
BZE	36444	22363	61.4	23393	64.2	28353	77.8

Table 4:9 Recovery of analytes using three different elution solvents

The elution solvent methanol/isopropanol/ammonium hydroxide consistently gave the highest recovery for AE, AEME and EME compared to the other two elution solvents and was selected for further experiments.

4.3.4 Two Step SPE

Recovery of AE using the SPE methods investigated so far has been very poor. Employing a two step SPE did not produce significant increases in recovery of AE, despite some authors previously reporting an increase [11, 12, 18, 21]. A 5% increase was shown for method B, however this may not be attributed to the method, and may be a result of variability between extractions. Table 4:10 summarises the extraction recoveries for each analyte using the two step SPE methods.

Analyte	Un-extracted	AE Method A		AE Method B	
	Peak Area	Peak Area	% Recovery	Peak Area	% Recovery
AEME	49521	48185	97	60062	121
AE	83143	11036	13	13376	16
EME	62911	28644	46	40288	64
COC	43014	50870	118	50722	118
BZE	68162	41770	61	46312	68

Table 4:10 Analyte recoveries using a two step extraction technique

The recovery of EME was increased considerably using method B, and was the only analyte to show a significant increase. No difference in recoveries was shown between SPE4 from section 4.3.2 and AE method A.

The simultaneous analysis of cocaine and its metabolites presents difficulties in the extraction from biological matrices because of the large polarity difference between them. High recovery of cocaine and its metabolites is dependent on the sorbent and solutions used. Bond Elut™ Certify was chosen as it is effective in retaining both non polar and cationic analytes under the right extraction conditions. Previous publications have shown excellent recoveries using this sorbent [14, 19, 44].

Cocaine and AE represent the two extremes when performing SPE. Cocaine is strongly adsorbed via the hydrophobic interactions with the alkyl chains of the sorbent, subsequent wash steps do not disturb these bonds and so optimal recovery is achieved. This is illustrated by the high recoveries shown for each SPE method tested.

AE is not adsorbed under the same optimal conditions as cocaine and subsequently passes directly through the column into waste. Decreasing the pH of the solution ionises AE which should allow for retention via the cation exchange groups. The recovery however was still very poor, possibly as a result of inadequate analyte retention

following sample addition or due to loss of AE in subsequent wash steps particularly when water is used.

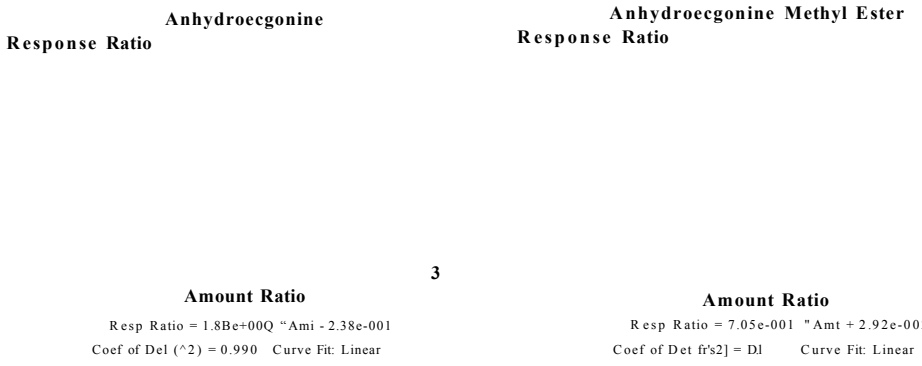
The reduced recovery of the other analytes may also be linked to analyte loss during column washing, alternatively losses may have occurred during sample evaporation under nitrogen, particularly for EME which is known to be very volatile.

When analysing a large number of compounds with differing polarities a SPE method may need to be chosen which provides a compromise on recovery for each analyte of interest. The compromise on this occasion is the poor recovery of AE, using AE method 4B achieved optimal recovery of AEME and cocaine and significant recovery of benzoylecgonine. These are the analytes which are most important for identifying cocaine hydrochloride and crack cocaine use.

4.3.5 Calibration Curve Linearity

Examples of the extracted calibration curves for each analyte are displayed in Figure 4:10. The coefficients of determination were >0.99 for all analytes except for AEME which gave 0.988.

The peak area for AEME was very low, so to rule out poor extraction recovery an un-extracted standard was tested. Similar results were obtained and significant peak tailing was also observed.



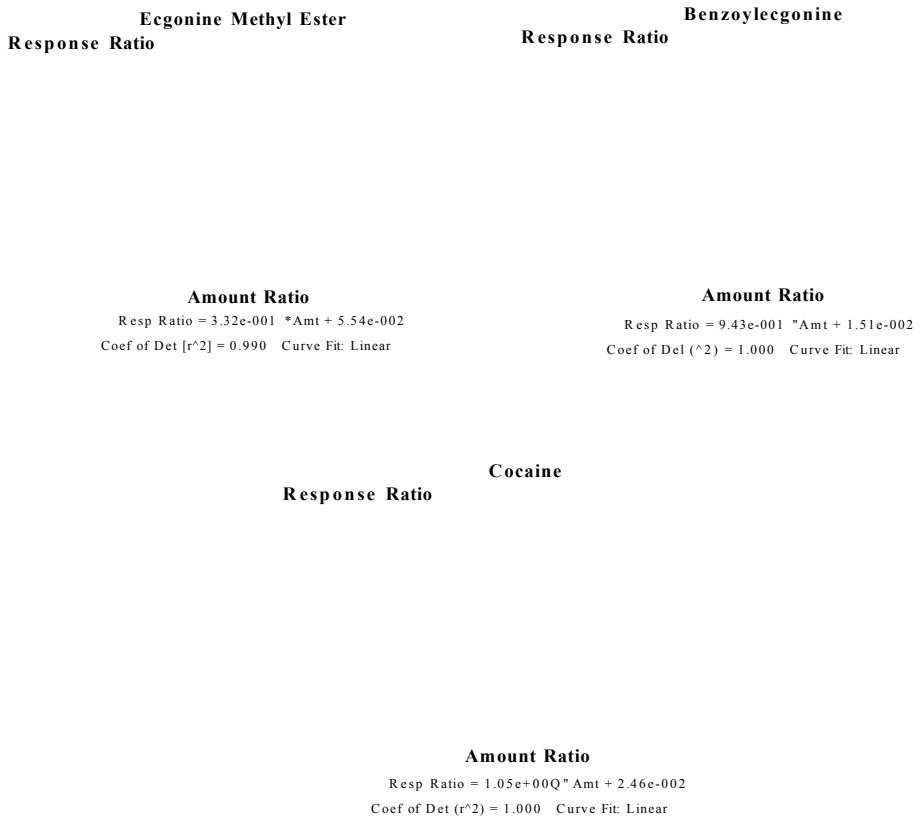


Figure 4:10 Extracted Calibration curves for AE, AEME, EME, cocaine and benzoylecgonine

Column degradation can be caused by many different factors and can result in poor quality peaks, peak tailing, decreased peak size, and loss of column efficiency. Column contamination is a common problem encountered in GC, the contaminants can be either non-volatile or semi-volatile. Non volatile components in a sample can collect on the head of the column, this can result in deterioration of column performance [53]. Certain compounds can cause chemical damage to the GC column. PFPA is an example of an organic compound which can cause column damage. More problems are experienced when using splitless injection due to the large volume of sample deposited at the front of the column. While every effort is made to remove PFPA from the sample after derivatisation there is the possibility that small amounts could still enter the column,

over time this may lead to a build up of the compound at the front of the column and subsequently cause column degradation.

Cutting approximately 10-20cm from the front end of the column removes the section of column with the greatest contamination and allows for improved chromatography. In this study the column was cut and a significant improvement in peak shape and peak area for AEME was observed. However the improvement was short lived, as deterioration of the chromatography occurred rapidly. Investigation into the maintenance log of the instrument showed that at the time of initiation of the experiments on instrument 2 a new column had been installed. It is speculated that the successful results observed early on in the method development was a result of a newly installed column, and that subsequent deterioration in the chromatography for AEME was most likely directly related to the deterioration of the GC column. The other analytes did not appear to be affected.

Active compounds such as carboxylic acids, amines, phenols and diols are particularly affected by contamination.

Column degradation also appeared to be a problem for another author, daily maintenance was performed on their GC-MS which included clipping the column and replacing the septum, liner and injector seal [3].

4.4 Conclusion

The work carried out in this chapter produced a sample preparation technique which was successful in providing good recoveries for the majority of the analytes. The GC-MS method enabled the simultaneous analysis of cocaine, its metabolites, and pyrolysis products. However further analysis using GC-MS was stopped due to frequent column degradation resulting in poor chromatography for AEME. To proceed with GC-MS it would have been necessary to continually cut the column or install a new column more

regularly. This would introduce a significant rise in the cost of analysis, and also increase the turnaround time of sample analysis as a result of increased instrument maintenance.

Other problems associated with the use of GC-MS in the analysis of AEME have been reported to be artifact formation of AEME from cocaine in the GC injector port leading to false identification of crack use [4, 15, 20, 33].

It was therefore decided to pursue the development of a LC-MS/MS method. There would be no issue with regards to artifact formation of AEME as the temperature of the system does not exceed 55°C, also sample preparation is quicker using LC-MS/MS as there is no requirement for sample derivatisation.

4.5 References

1. Srinivasan, K., et al., *Liquid Chromatography-Tandem Mass Spectrometry Analysis of Cocaine and its Metabolites from Blood, Amniotic Fluid, Placental and Fetal Tissues: Study of the Metabolism and Distribution of Cocaine in Pregnant Rats.* Journal of Chromatography B, 2000. 745: p. 287-303.
2. Wang, W.-L., W.D. Darwin, and E.J. Cone, *Simultaneous Assay of Cocaine, Heroin and Metabolites in Hair, Plasma, Saliva and Urine by GC/MS.* Journal of chromatography B, 1994. 660: p. 279-290.
3. Cone, E.J., M. Hillsgrove, and W.D. Darwin, *Simultaneous Measurement of Cocaine, Cocaethylene, Their Metabolites, and'Crack'Pyrolysis Products by Gas Chromatography-Mass Spectrometry.* Clinical Chemistry, 1994. 40: p. 1299-1305.
4. Kintz, P., et al., *Evidence of Crack Use by Anhydroecgonine Methyl Ester Identification.* Human and Experimental Toxicology, 1997. 16: p. 123-127.
5. Cognard, E., S. Bouchonnet, and C. Staub, *Validation of a Gas Chromatography - Ion Trap Tandem Mass Spectrometry for Simultaneous Analyse of Cocaine and its Metabolites in Saliva.* Journal of Pharmaceutical and Biomedical Analysis, 2006. 41: p. 925-934.
6. Jenkins, A.J., J.M. Oyler, and E.J. Cone, *Comparison of Heroin and Cocaine Concentrations in Saliva with Concentrations in Blood and Plasma.* Journal of Analytical Toxicology, 1995.19: p. 359-374.
7. Zhang, J.Y. and L. Foltz, *Cocaine Metabolism in Man: Identification of Four Previously Unreported Cocaine Metabolites in Human Urine.* Journal of Analytical Toxicology, 1990. 14: p. 201-205.
8. Bourland, J.A., et al., *Quantitation of Cocaine, Benzoylecgonine, Cocaethylene, Methylecgonine, and Norcocaine in Human Hair by Positive Ion Chemical Ionisation (PICI) Gas Chromatography-Tandem MAss Spectrometry.* Journal of Analytical Toxicology, 2000. 24: p. 489-495.
9. Cognard, E., et al., *Analysis of Cocaine and Three of its Metabolites in Hair by Gas Chromatography-Mass Spectrometry Using Ion-Trap Detection for CI/MS/MS.* Journal of Chromatography B, 2005. 826: p. 17-25.
10. Liberty, H.J., et al., *Detecting Crack and other Cocaine Use with Fastpatches.* Addiction Biology, 2003. 8: p. 191-200.

11. Paul, B.D., et al., *Concentration Profiles of Cocaine, Pyrolytic Methyl Ecgonidine and Thirteen Metabolites in Human Blood and Urine: Determination by Gas Chromatography-Mass Spectrometry.* Biomedical Chromatography, 2005. 19: p. 677-88.

12. Paul, B.D., L.K. McWhorter, and M.L. Smith, *Electron Ionisation Mass Fragmentometric Detection of Urinary Ecgonidine, a Hydrolytic Product of Methylecgonidine, as an Indicator of Smoking Cocaine.* Journal of Mass Spectrometry, 1999. 34: p. 651-660.

13. Cardona, P.S., et al., *Simultaneous Analyses of Cocaine, Cocaethylene, and their Possible Metabolic and Pyrolytic Products.* Forensic Science International, 2006. **157:** p. 46-56.

14. Torre, R.D.L., et al., *Determination of Cocaine and its Metabolites in Human Urine by Gas Chromatography/Mass Spectrometry After Simultaneous use of Cocaine and Ethanol.* Journal of Pharmaceutical and Biomedical Analysis, 1995.**13:** p. 305-312.

15. Lewis, R.J., et al., *Determination of Cocaine, its Metabolites, Pyrolysis Products, and Ethanol Adducts in Postmortem Fluids and Tissues using Zymark Automated Solid-Phase Extraction and Gas Chromatography-Mass Spectrometry.* Journal of Chromatography B, 2004. 806: p. 141-150.

16. Schramm, W., et al., *Cocaine and Benzoylecgonine in Saliva, Serum and Urine.* Clinical Chemistry, 1993. 39: p. 481-487.

17. Campora, P., et al., *Quantitation of Cocaine and its Major Metabolites in Human Saliva Using Gas Chromatography-Positive Chemical Ionisation-Mass Spectrometry (GC-PCI-MS).* Journal of Analytical Toxicology, 2003. 27: p. 270-274.

18. Shimomura, E.T., G.D. Hodge, and B.D. Paul, *Examination of Postmortem Fluids and Tissues for the Presence of Methylecgonidine, Ecgonidine, Cocaine, and Benzoylecgonine Using Solid Phase Extraction and Gas Chromatography-Mass Spectrometry.* Clinical Chemistry, 2001. 47: p. 1040-1047.

19. Fandino, A.S., S.W. Toennes, and G.F. Kauert, *Studies on In Vitro Degradation of Anhydroecgonine Methyl Ester (Methylecgonidine) in Human Plasma.* Journal of Analytical Toxicology, 2002. 26: p. 567-570.

20. Toennes, S.W., A.S. Fandino, and G. Kauert, *Gas Chromatographic-mass Spectrometric Detection of Anhydroecgonine Methyl Ester (Methylecgonidine)*

in Human Serum as Evidence of Recent Smoking of Crack. Journal of Chromatography B, 1999. 735: p. 127-132.

21. Scheidweiler, K.B., et al., *Stability of Methylecgonidine and Ecgonidine in Sheep Plasma in Vitro.* Clinical Chemistry, 2000. 46: p. 1787-1795.
22. Kato, K., et al., *Cocaine and Metabolite Excretion in Saliva Under Stimulated and Nonstimulated Conditions.* Journal of Analytical Toxicology, 1993. 17: p. 338-341.
23. Jacob, P., et al., *A Pyrolysis Product, Anhydroecgonine Methyl Ester (Methylecgonidine), is in the Urine of Cocaine Smokers.* Journal of Analytical Toxicology, 1990. 14: p. 353-357.
24. Toennes, S.W., et al., *Artifact Production in the Assay of Anhydroecgonine Methyl Ester in Serum Using Gas Chromatography-Mass Spectrometry.* Journal of Chromatography B, 2003. 792: p. 345-351.
25. Kacinko, S.L., et al., *Disposition of Cocaine and Its Metabolites in Human Sweat After Controlled Cocaine Administration.* Clinical Chemistry, 2005. 51: p. 2085-2094.
26. Lester, L., et al., *Disposition of Cocaine in Skin, Interstitial Fluid, Sebum, and Stratum Corneum.* Journal of Analytical Toxicology, 2002. 26: p. 547-553.
27. Niedbala, R.S., et al., *Immunoassay for Detection of Cocaine/Metabolites in Oral Fluids.* Journal of Analytical Toxicology, 2001. 25: p. 62-68.
28. Spiehler, V., et al., *Enzyme Immunoassay Validation for Qualitative Detection of Cocaine in Sweat.* Clinical Chemistry, 1996. 42: p. 34-38.
29. Fucci, N., N.D. Giovanni, and M. Chiarotti, *Simultaneous Detection of Some Drugs of Abuse in Saliva Samples by SPME Technique.* Forensic Science International, 2003.134: p. 40-45.
30. Yonamine, M., et al., *Solid Phase Micro-Extraction Gas Chromatography-Mass Spectrometry and Headspace-Gas Chromatography of Tetrahydrocannabinol, Amphetamine, Methamphetamine, Cocaine and Ethanol in Saliva Samples.* Journal of Chromatography B, 2003. 789: p. 73-78.
31. Cooper, G., et al., *Validation of the Cozart Microplate EIA for Cocaine and Metabolites in Oral Fluid.* Journal of Analytical Toxicology, 2004. 28: p. 498-503.
32. Lachenmeier, K., F. Musshoff, and B. Madea, *Determination of Opiates and Cocaine in Hair Using Automated Enzyme Immunoassay Screening*

Methodologies Followed by Gas Chromatographic-Mass Spectrometric (GC-MS) Confirmation. Forensic Science International, 2006. 159: p. 189-199.

33. Kintz, P., et al., *Testing Human Hair and Urine for Anhydroecgonine Methyl Ester, a Pyrolysis Product of Cocaine.* Journal of Analytical Toxicology, 1995. 19: p. 479-482.
34. Lowe, R.H., et al., *A Validated Positive Chemical Ionisation GC/MS Method for the Identification and Quantification of Amphetamine, Opiates, Cocaine and Metabolites in Human Postmortem Brain.* Journal of Mass Spectrometry, 2005. 41: p. 175-184.
35. Ragoucy-Sengler, C. and P. Kintz, *Detection of Smoked Cocaine Marker (Anhydroecgonine Methyl Ester) in Nails.* Journal of Analytical Toxicology, 2005. 29: p. 765-768.
36. Yang, W., et al., *Simultaneous Quantification of Methamphetamine, Cocaine, Codeine and Metabolites in Skin by Positive Chemical Ionisation Gas Chromatography-Mass Spectrometry.* Journal of Chromatography B, 2006. 833: p. 210-218.
37. Valente-Campos, S., et al., *Validation of a Method to Detect Cocaine and its Metabolites in Nails by Gas Chromatography-Mass Spectrometry.* Forensic Science International, 2006. 159: p. 218-222.
38. Myers, A.L., et al., *Identification of Anhydroecgonine Ethyl Ester in the Urine of a Drug Overdose Victim.* Journal of Forensic Sciences, 2005. 50: p. 1481-1485.
39. Franke, J.P. and R.A.D. Zeeuw, *Solid-Phase Extraction Procedures in Systematic Toxicological Analysis.* Journal of Chromatography B, 1998. 713: p. 51-59.
40. Isenschmid, D.S., *Cocaine - Effects on Human Performance and Behaviour.* Forensic Science Review, 2002. 14: p. 62-100.
41. Bogusz, M.J., *Liquid Chromatography-Mass Spectrometry as a Routine Method in Forensic Sciences: A Proof of Maturity.* Journal of Chromatography B, 2000. 748: p. 3-19.
42. Liu, Y., *Use of an Improved Version of C8 + SCX Mixed Mode Solid Phase Extraction Material for Clean Extraction and Recovery of Basic and Zwitterionic Compounds from Biological Fluids.* Journal of Liquid Chromatography and Related Technologies, 2006. 29: p. 1405-1431.
43. Clauwaert, K., et al., *The Determination of Cocaine, Benzoylecgonine, and Cocaethylene in Small- Volume Oral Fluid Samples by Liquid Chromatography-*

Quadrupole-Time-of-Flight Mass Spectrometry. Journal of Analytical Toxicology, 2004. 28: p. 655-659.

44. Pichini, S., et al., *Development and Validation of a Liquid Chromatography-Mass Spectrometry Assay for the Determination of Opiates and Cocaine in Meconium.* Journal of Chromatography B, 2003. 794: p. 281-292.

45. Xia, Y., et al., *An LC-MS-MS Method for the Comprehensive Analysis of Cocaine and Cocaine Metabolites in Meconium.* Analytical Chemistry, 2000. 72: p. 764-771.

46. Garside, D., et al., *Identification of Cocaine Analytes in Fingernail and Toenail Specimens.* Journal of Forensic Sciences, 1998. 43: p. 974-979.

47. Mortier, K.A., et al., *Simultaneous Quantitative Determination of Opiates, Amphetamines, Cocaine and Benzoylecgonine in Oral Fluid by Liquid Chromatography Quadrupole-Time-Of-Flight Mass Spectrometry.* Journal of Chromatography B, 2002. 779: p. 321-330.

48. Rook, E.J., et al., *The Quantitative Analysis of Heroin, Methadone and their Metabolites and the Simultaneous Detection of Cocaine, Acetylcodeine and their Metabolites in Human Plasma by High-Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry.* Journal of Chromatography B, 2005. 824: p. 213-221.

49. Soriano, T., et al., *Improved Solid Phase Extraction Method for Systematic Toxicological Analysis in Biological Fluids.* Journal of Analytical Toxicology, 2001. 25: p. 137-143.

50. Braithwaite, R.A., et al., *Screening for Drugs of Abuse. I: Opiates, Amphetamines and Cocaine.* Ann Clin Biochem, 1995. 32: p. 123-153.

51. Peterson, K.L., *Detection of Cocaine and its Polar Transformation Products and Metabolites in Human Urine.* Forensic Science International, 1995. 73: p. 183-196.

52. Wood, M., et al., *Quantitative Analysis of Multiple Illicit Drugs in Preserved Oral Fluid by Solid-Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry.* Forensic Science International, 2005. 150: p. 227-238.

53. Dawling, S., *Gas Chromatography,* in *Clarke's Analysis of Drugs and Poisons.* 2004, Pharaceutical Press, p. 425-499.

5 Liquid Chromatography-Tandem Mass Spectrometry Method Development for the Quantitation of Cocaine, its Metabolites and Pyrolysis Products

5.1 Aims and Objectives

The aim of this chapter is to develop and validate a sensitive LC-MS/MS method for the simultaneous analysis of cocaine, its metabolites and pyrolysis products in oral fluid.

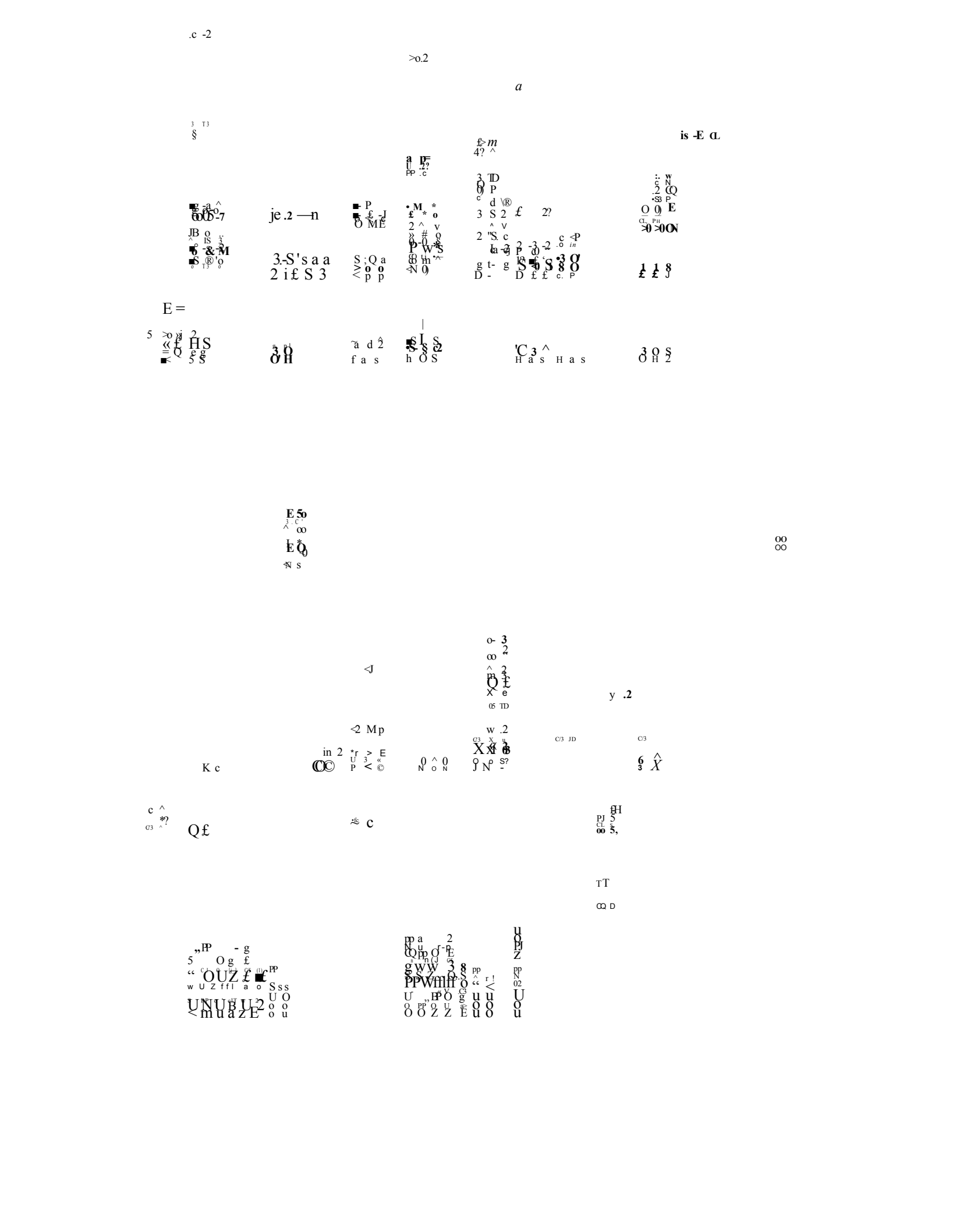
5.2 Introduction

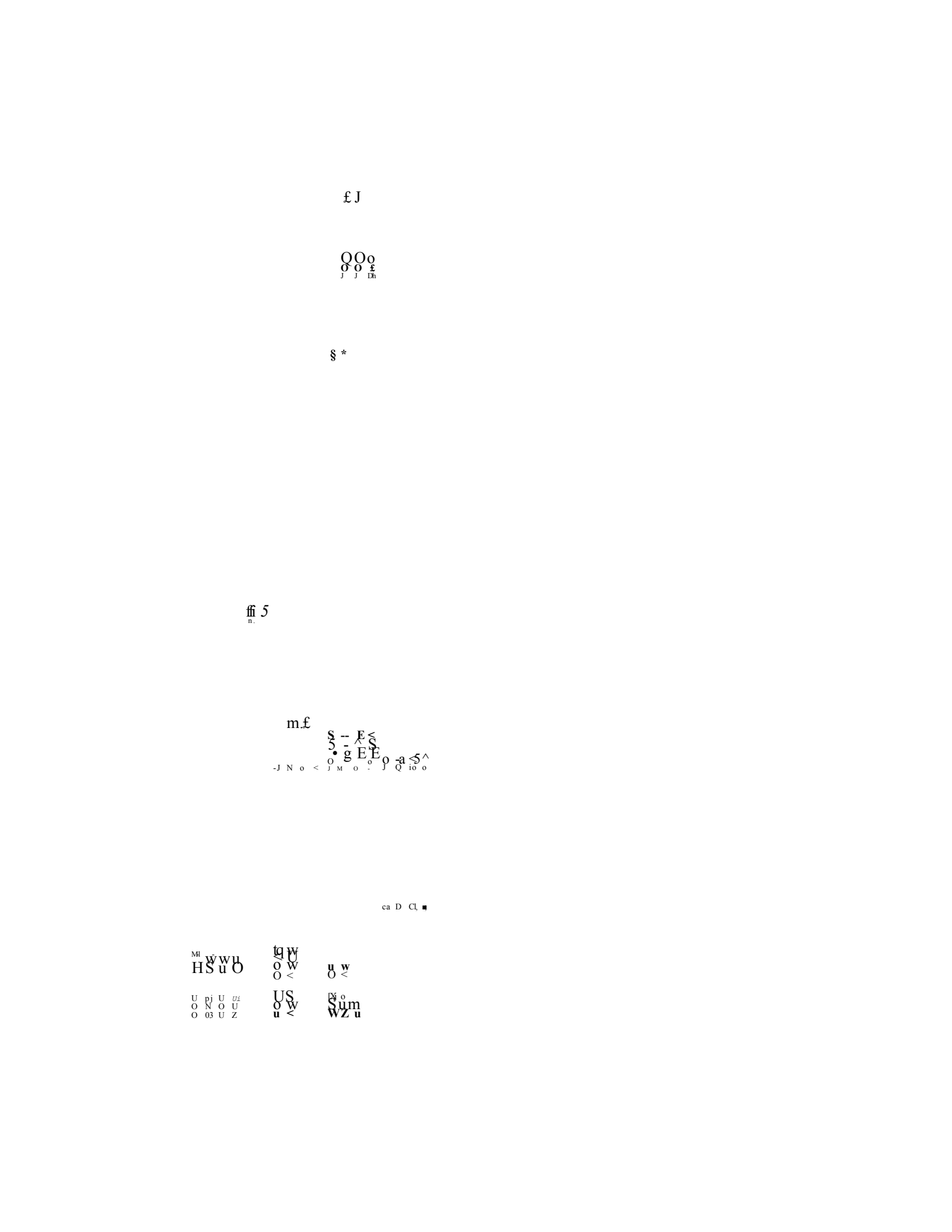
Traditionally GC-MS has been used to quantify cocaine and its metabolites in biological fluids. However the high sensitivity and selectivity provided by LC-MS/MS, and the reduced sample analysis time [1, 2], has led to an increase in its use and an increase in the number of methods published. Also the reports of artifact formation of AEME from cocaine in the injector port of the GC [3-6] highlights the importance of using LC-MS for the accurate analysis of the pyrolysis products of cocaine.

A literature search produced fourteen methods describing the use of LC-MS(MS) in the analysis of cocaine and its metabolites, the details of which are summarised in Table 5:1. Six of these included the analysis of the pyrolysis product AEME but none included its metabolite AE. Only one of these methods was used for the analysis of oral fluid samples [7].

Across the methods the chromatographic conditions have varied significantly with regards to the LC columns employed and the compositions of the mobile phases. The MS conditions were predominantly electrospray ionisation with tandem MS.

Methods of sample preparation employed in the analysis of cocaine and its metabolites have been previously described in chapter 4.





5.3 Experimental

5.3.1 Materials

Solutions of the reference standards (lmg/mL in methanol or acetonitrile) AEME, AE, EME, cocaine, cocaethylene, benzoylecgonine and of the corresponding deuterated internal standards (O.lmg/mL) EME-d_3, cocaine-d_3, cocaethylene-d_3 and benzoylecgonine-d_3 were obtained from Cerilliant (LGC Promochem, Teddington, UK). Water, methanol, acetonitrile, acetic acid, formic acid, and ammonium formate were purchased from VWR International Ltd (Leicestershire, UK). Solid phase extraction (SPE) cartridges, Bond Elut™ Certify (50mg, 3mL), were purchased from Varian (Surrey, UK). High recovery vials were purchased from Crawford Scientific (Lanarkshire, Scotland, UK).

Oral fluid collectors were provided by Cozart pic (Oxfordshire, UK).

5.3.2 Solution Preparation

5.3.2.1 Drug Standards

Individual and combined analyte working solutions of lpg/mL, lOOng/mL and lOng/mL were prepared in acetonitrile using individual lmg/mL stock solutions of the reference standards. A working solution of lOOng/mL for the internal standards was prepared in acetonitrile using the individual O.lmg/mL stock solutions. The working solutions were refrigerated at 4°C for up to 6 months.

5.3.2.2 Mobile Phase Solutions

- 0.1% Acetic Acid in Water/Methanol/Acetonitrile (500mL)

500pL of glacial acetic acid was added to 499.5mL of deionised water, methanol or acetonitrile. The solution was stored at room temperature for up to 2 months.

- 0.1% Formic Acid in Water/Methanol (500mL)

  500pL of formic acid was added to 499.5mL of deionised water, methanol or acetonitrile. The solution was stored at room temperature for up to 2 months.

- lOmM Ammonium Formate (pH 3/pH 4) (500mL)

  450mL of deionised water was added to 0.315g of ammonium formate in a volumetric flask. The pH was adjusted with formic acid to the required pH. The volume was made up to 500mL and stored at room temperature for 2 months.

- 20mM Ammonium Formate (pH 4) (500mL)

  450mL of deionised water was added to 0.631g of ammonium formate in a volumetric flask. The pH was adjusted to pH 4 with formic acid. The volume was made up to 500mL and stored at room temperature.

- 5mM Ammonium Formate (pH 3) (500mL)

  450mL of deionised water was added to 0.158g of ammonium formate in a volumetric flask. The pH was adjusted to pH 3 with formic acid. The volume was made up to 500mL and stored at room temperature.

5.3.3 Instrumentation

LC-MS/MS analysis was performed on a Varian 1200L triple quadrupole mass spectrometer equipped with an atmospheric pressure ionisation source via an electrospray interface, ProStar 410 autosampler, and ProStar 210 isocratic solvent delivery modules. The MS was operated in positive ion multiple reaction monitoring (MRM) mode. The collision gas was 99.999% pure argon, purchased from Argo International (Basildon, UK).

The HPLC columns were manufactured by Agilent and purchased from Crawford Scientific (Lanarkshire, Scotland, UK) and included the Eclipse XDB-Phenyl (150 x 2.1mm, 5pm), ZORBAX Eclipse Plus C18 (150 x 2.1mm) in 5pm and 3.5pm, and ZORBAX Eclipse Plus C8 (150 x 2.1mm, 3.5pm).

5.3.4 LC-MS/MS Parameters

The LC-MS/MS was initially set up according to the manufacturer's recommendations. The drying gas was nitrogen and was maintained at 300°C with a constant pressure of 18psi. The capillary, shield, and needle voltage were set at 70V, 600V, and 5000V respectively. The mobile phase flow rate was set at 0.25mL/min. The column oven temperature was set at 40°C.

5.3.5 Fragment Elucidation

A 1pg/mL standard solution of each analyte was directly infused into the MS ion source using a syringe infusion pump at a flow rate of 50pL/min. The detector was turned on and the ion with the greatest intensity was identified as the protonated molecular ion, this was confirmed by comparison to the known molecular weight of the analyte. The CID gas was turned on to generate the product ions and the collision cell was set to scan the collision energy range. The optimum collision energy for each product ion was established as the energy which produced the greatest ion abundance.

Two product ions for each analyte and one for each internal standard were selected based on their abundance. The product ions and the optimum collision energies for the ten compounds are displayed in Table 5:2.

Compound	Precursor Ion –> Product Ion (m/z)	Collision Energy (eV)
AEME	182-122	14.5
	182-118*	19.0
AE	168-122	15.0
	168-91*	20.5
EME	200- 182*	13.0
	200 - 82	21.0
EME-d3	203- 185*	13.0
Benzoylecgonine	290- 168*	14.5
	290- 105	24.5
Benzoylecgonine-d3	293- 171*	14.5
Cocaine	304- 105	26.0
	304- 182*	15.0
Cocaine-d3	307- 185*	15.0
Cocaethylene	318-196*	15.0
	318-150	20.5
Cocaethylene-d3	321 - 199*	14.5

Table 5:2 Product ions and optimum collision energies

Quadrupole 1 was set to select ion monitoring (SIM) mode to pass only the protonated molecular ions through to the collision cell. The specific collision energies for each fragment ion were set in the collision cell, and quadrupole 3 was set in SIM mode to pass only the selected product ions through to the detector.

The product ions used for quantitation are denoted by an asterisk in Table 5:2, the others were used as qualifier ions.

5.3.6 Mobile Phase and Column Selection

The objective of any chromatographic method is to achieve optimal separation of the analytes with the shortest possible run time. Four LC columns and eleven mobile phases were compared for selectivity and efficiency. The details of the columns used can be found in section 5.3.3, and the different mobile phases tested are described in section 5.3.2.2. The mobile phase flow rate was set to 0.25mL/min. The columns were selected based on those most commonly used for the analysis of cocaine. Two C18 columns with different particle sizes were compared.

Columns were equilibrated for 20 minutes prior to each new mobile phase followed by the injection of a solvent blank at the start of each run. Drug solutions were prepared in the appropriate mobile phase and injected onto the column. Comparisons were made based on peak area and retention time.

5.3.7 Optimising Analyte Separation

Gradient elution has proved to be useful in the separation of compounds which span a relatively wide polarity range, separations are achieved more quickly and with better efficiency [13]. Due to the unknown hydrophobicity of all the analytes a simple linear mobile phase gradient was first adopted, the gradient composition is detailed in Table 5.3. Manipulation of the gradient was subsequently performed to provide optimal separation and analysis time.

Time (minutes)	% Mobile Phase A	% Mobile Phase B
0	95%	5%
19	5%	95%
20	95%	5%
30	95%	5%

Table 5:3 Mobile phase gradient composition

5.3.8 Optimisation of Instrument Parameters

Instrument parameters were adjusted to establish settings which provided optimal ionisation of the analytes. Drying gas temperature was tested in 50°C increments from 100°C to 400°C. Needle voltage was tested in 500V increments from 3000V up to 6000V. Capillary voltage was increased from 50V to 90V in 5V increments and then from 90V to 100V. The shield voltage was increased from 100V to 700V in 100V increments.

Each parameter was assessed based on peak area for an un-extracted standard.

5.3.9 LC-MS/MS Method Validation

Method validation was carried out using oral fluid samples extracted using the optimised SPE method described in chapter 4.

5.3.9.1 Linearity

Very high concentrations of analyte can cause partial or total saturation of the stationary phase [21], therefore it is important to establish linearity. The linearity of the method over the working range of 0-360ng/mL was assessed using 4 separately prepared standard curves. Calibration curves were prepared using 200pL of negative oral fluid, drug standard, and 60ng/mL of internal standard. The method was deemed linear if the correlation of determination of the standard curve was greater than 0.99 [22].

5.3.9.2 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ of the assay were determined by the analysis of negative oral fluid spiked at concentrations 0, 0.5, 1, 2, 3, and 4ng/mL using the 10ng/mL working drug standard. The experiment was repeated on three different days. The LOD was determined as the lowest concentration at which the method could reliably differentiate the analyte peak from background noise, where a signal to noise ratio was equal to or greater than 3 [23]. The LOQ was determined as the lowest concentration of analyte which can be quantitatively determined with acceptable precision and accuracy (<20%). Quantification below the LOQ is by definition not acceptable and can only produce semiquantitative or qualitative data.

5.3.9.3 Precision and Accuracy

Three concentrations within the linear calibration range (21, 60 and 120ng/mL) were analysed five times within an analytical run to evaluate the intra-assay precision. The assay was repeated over three runs to assess the inter-assay precision. The precision of the method was expressed as the % CV of replicate analyses. Acceptable precision is <15% or 20% near the LLOQ [24].

Accuracy was assessed by calculating the percent deviations of the observed mean values from the respective true values for each concentration of spiked sample. Acceptable accuracy should be within 15% of the theoretical value, or within 20% near the LLOQ [24]. The calculation is as follows:

$$\text{Accuracy} = \frac{(\text{mean measured value} - \text{true value})}{\text{true value}} \times 100$$

5.3.9.4 Robustness

The robustness of the method was investigated by assessing variation of peak retention times, ion intensity ratios, and assay drift throughout the run.

The variation of the retention time should be within 4%. The ion ratios for an analyte, measured as the peak area of a qualifier ion divided by the peak area of the target ion, should be within 20% of the average [25]. The drift of the assay was assessed by placing the 21ng/mL spiked sample at the beginning and end of the run. This determines if an analytical run could be reanalysed in case of unexpected delay in the analysis, such as instrument failure [26]. The assay drift should not be greater than 10%.

5.3.9.5 Selectivity

Selectivity is the ability of a method to measure unequivocally and differentiate the analyte in the presence of components which may be expected to be present such as metabolites, impurities, degradants and matrix components. To prove lack of response in negative matrix, 48 samples collected from non cocaine users were analysed.

5.3.9.6 Recovery and Ion Suppression

Recovery was determined at three concentrations within the linear calibration range (21, 60 and 120ng/mL). The first set of samples were spiked prior to extraction, the second set were spiked after sample extraction, and the third set were un-extracted standards. Analysis was performed in triplicate on three different days. Absolute recovery was calculated from the average peak areas obtained from set one and set three.

Ion suppression from the use of oral fluid samples was assessed by comparing sets two and three.

5.4 Results and Discussion

5.4.1 Mobile Phase and Column Selection

The column and mobile phase combination chosen should ideally provide adequate retention of the more polar analytes, baseline separation of the peaks, and optimal ionisation. Faster elution times can be achieved by adjusting the column and mobile phase composition [10]. Assessment of each variable is based on the retention time and peak area response.

The first experiments were carried out using methanol or acetonitrile as the organic mobile phase, and water as the aqueous mobile phase. The organic and aqueous mobile phase pH was also modified with 0.1% acetic acid. The different combinations of organic and aqueous mobile phases used are listed in Table 5:4.

	Aqueous Mobile Phase	**Organic Mobile Phase**
Combination 1	Water	Methanol
Combination 2	0.1% Acetic Acid in Water	Methanol
Combination 3	0.1% Acetic Acid in Water	0.1% Acetic Acid in Methanol
Combination 4	Water	Acetonitrile
Combination 5	0.1% Acetic Acid in Water	Acetonitrile
Combination 6	0.1% Acetic Acid in Water	0.1% Acetic Acid in Acetonitrile

Table 5:4 Mobile phase combinations applied

The highest analyte retention was achieved using the phenyl followed by the C_{18} and finally the Cg. There was no significant difference between the two different Cig columns. The differences in analyte retention can be attributed to the molecular interactions and non-polar properties of the columns. The Cs column has the shortest

hydrocarbon chain and as a result has fewer hydrophobic interactions compared to the Cis columns. The phenyl column however has additional molecular interactions which offer additional retention of the analytes [27, 28].

The first analyte to elute was either AE or EME and the retention times were greater than 2 minutes using the phenyl column compared to 1.5-1.7 minutes for the other columns. Similar studies have failed to retain EME using a cyano column or various types of C_{18} column [2]. It is important for increased sensitivity that analytes elute after 2 minutes. Ion suppression from interfering compounds is often observed with analytes which elute within the first two minutes.

In each case the last analyte to elute was cocaethylene. AEME was often the third analyte to elute, while the elution order of cocaine and benzoylecgonine changed depending on the type of column and the mobile phase combination used. Tables 5:5 to 5:8 show the analyte retention times for each column using the six different mobile phase combinations. The representative chromatograms for each column using mobile phase combinations 3 and 6 are shown in Figure 5:1.

The use of acetonitrile instead of methanol as the organic phase reduced the retention times of most of the analytes by 2-3 minutes. There was no significant difference shown for the early eluting analytes AE and EME.

Table 5:8 Phenyl Column

Table 5:7 C8 Column 3.5|im Diameter

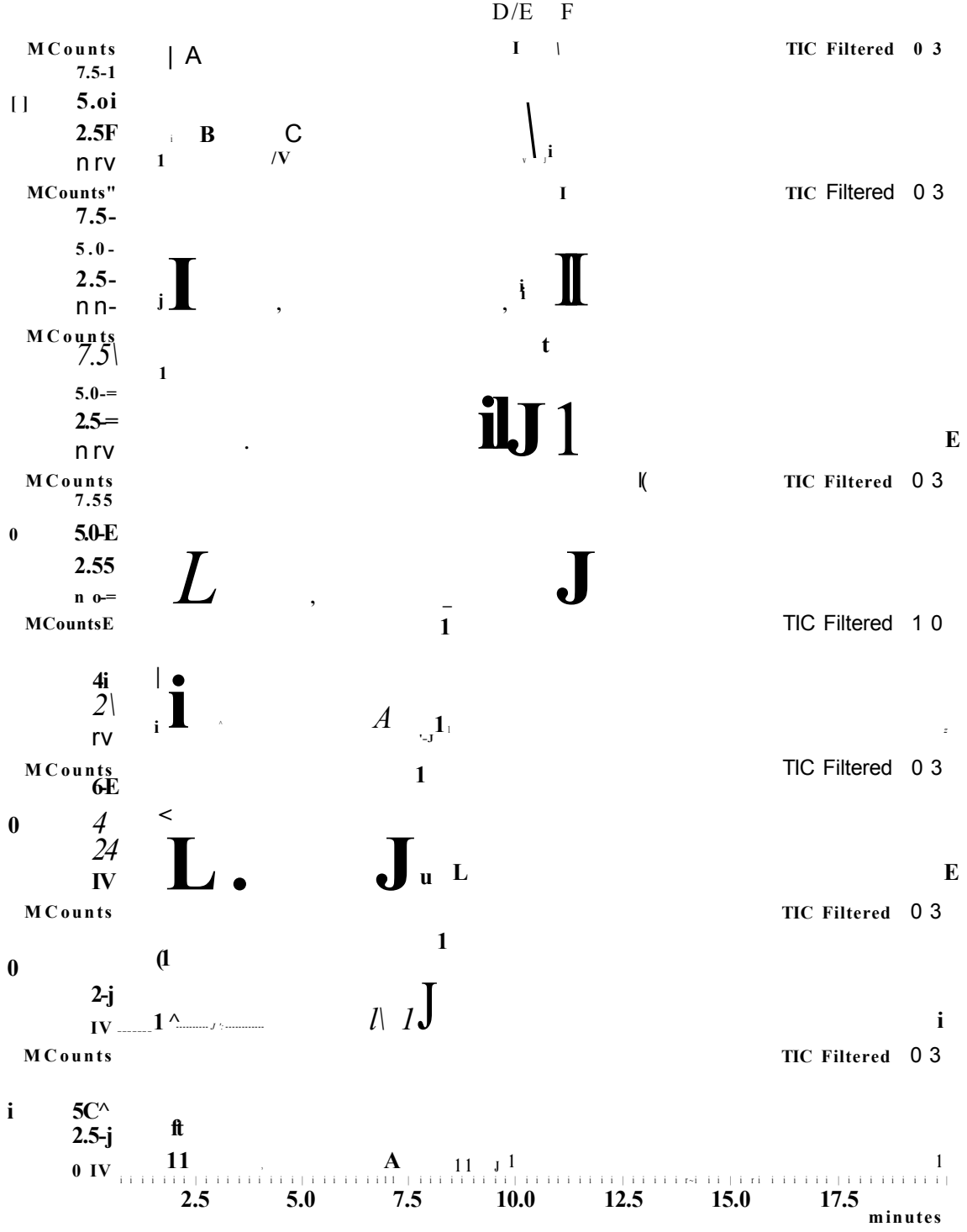


Figure 5:1 LC-MS/MS MRM chromatograms of (A) EME, (B) AE, (C) AEME, (D) benzoylecgonine, (E) cocaine, (F) cocaethylene using (1, 5) C 1₈ column 3.5 pm diameter, (2, 6) C18 column 5pm diameter, (3, 7) C₈ column, (4, 8) phenyl column. The mobile phase was 0.1% acetic acid in the aqueous and organic mobile phase with methanol (1-4) and acetonitrile (5-8).

Cocaine and cocaethylene consistently produced the largest peak area, and AE consistently produced the smallest. It is hypothesised that as the concentration of organic solvent in the mobile phase is increased the ESI-MS signal increases due to more efficient desolvation in the ESI process compared to water [1, 29]. As cocaine and cocaethylene are the last two analytes to elute from the column the high percentage of organic phase may be a reason for their large peak area. The peak areas produced with each mobile phase are presented in Figures 5:2 to 5:5, the graphs relate to each column type.

The addition of an organic acid controls the pH of the mobile phase, improves peak shape and resolution, and can change the hydrophobicity of the analyte [30], as well as increase ionisation of the analytes [1]. The acid can neutralise the charge on any residual exposed silica on the stationary phase and act as ion pairing agents to neutralise charge on the analyte. The use of acetic acid in the mobile phase produced marked increases in the analyte peak areas.

In general the analyte peak areas were greater when methanol was used as the organic mobile phase. This is contrary to the findings of Jeanville *et al* who showed approximately 50% reduction in sensitivity for EME and cocaine when switching from acetonitrile to methanol [1].

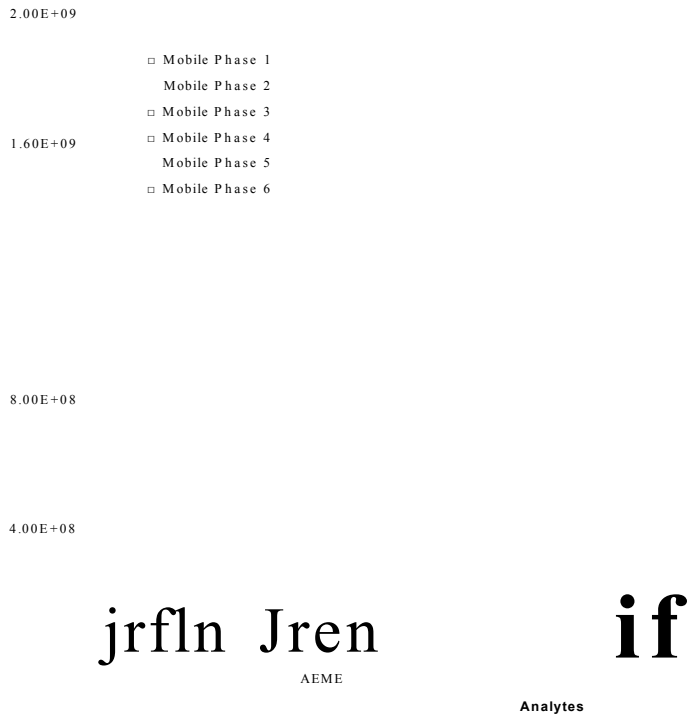


Figure 5:2 Comparison of analyte peak area with different mobile phase combinations using a C_{18} column with 3.5pm diameter

Figure 5:3 Comparison of analyte peak area with different mobile phase combinations using a Cig column with 5pm diameter

Figure 5:4 Comparison of analyte peak area with different mobile phase combinations using a Cg column

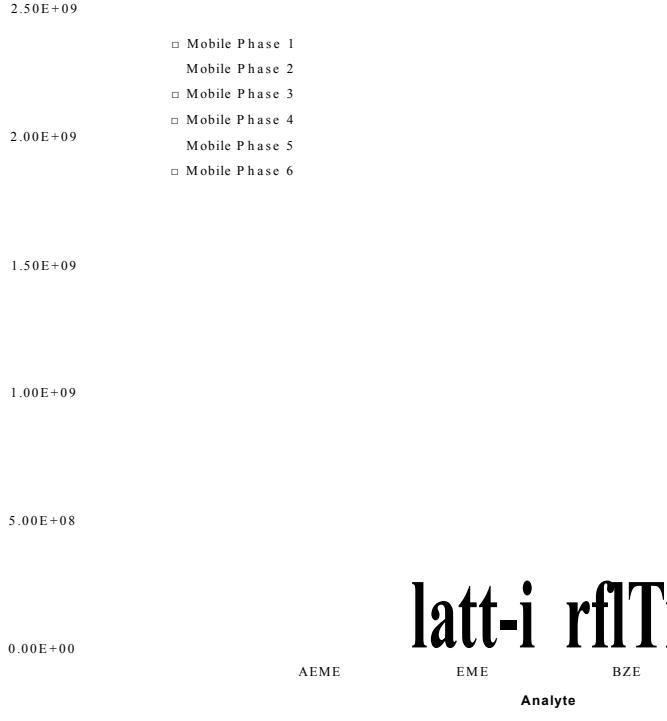


Figure 5:5 Comparison of analyte peak area with different mobile phase combinations using a phenyl column

Although the LC column simply acts to retain the analytes and has no effect on the ionisation, it was observed that the peak areas produced when using the phenyl column were significantly larger than the other columns particularly for cocaine and cocaethylene.

Resolution is the parameter that describes the separation power of the chromatographic system and is expressed as the ratio of the distance between two peak maxima to the mean value of the peak width at the base line. If the ratio is less than 1 the analyte peaks overlap, a ratio >1.0 indicates the two peaks overlap by approximately 2% and is usually considered acceptable, whereas a ratio >1.5 indicates the peaks are completely resolved otherwise known as baseline resolution [28].

There was always baseline resolution between cocaethylene and its neighbouring peak. This was the same for AEME on all but one occasion where it eluted around the same time as benzoylecgonine. AE and EME eluted close together as did benzoylecgonine and cocaine. The resolution ratios of these peaks are displayed in Figure 5:6.

When acetonitrile was used as the organic mobile phase, AE and EME were not resolved using any of the columns, however the benzoylecgonine and cocaine peaks were completely resolved when acetic acid was added. When methanol and acetic acid were used a resolution of >1 was achieved for AE and EME in all but the Cs column. Resolution of the benzoylecgonine and cocaine peaks was low using all the columns apart from the phenyl column where baseline separation was achieved.

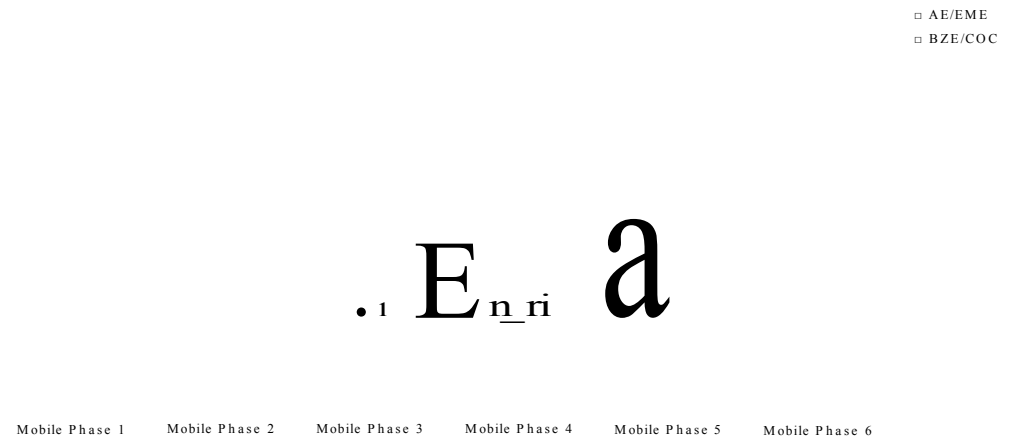


Figure 5:6 Resolution ratios of AE and EME, and benzoylecgonine and cocaine using different mobile phases and HPLC columns

A study attempting to separate two pharmaceutical analytes of similar polarities found that they were only successfully separated using a phenyl column compared to a Cig, this was attributed to the additional molecular interactions occurring with a phenyl column. The organic phase used was also found to be important as separation was only achieved using methanol and not acetonitrile. It is suggested that the use of acetonitrile suppresses the molecular interactions *[21].*

Phenyl columns are good for the separation of complex mixtures [28], and have shown higher retention and enhanced resolution in the analysis of drugs of abuse [31].

The phenyl column provided increased retention for the early eluting compounds and greater resolution between the closely eluting analytes, so was selected for assessment of additional mobile phases. Methanol was chosen as the organic mobile phase based on

the greater peak areas produced and improved resolution of all analytes when using the phenyl column.

Mobile phase combination 3 was used for comparison. The new organic phase used was methanol with 0.1% formic acid. The different aqueous phases contained either 0.1% of formic acid or various concentrations of ammonium formate adjusted to either pH 3 or 4 using formic acid. The different aqueous mobile phases used are listed in Table 5:9.

Aqueous Mobile Phase
0.1% Formic Acid in Water
lOmM Ammonium Formate pH4
20mM Ammonium Formate pH4
lOmM Ammonium Formate pH3
5mM Ammonium Formate pH3

Table 5:9 Aqueous mobile phase compositions

The chromatograms produced using each mobile phase are shown in Figure 5:7, and the representative peak areas are displayed in Figure 5:8. The use of formic acid improved ionisation compared to using acetic acid, however it was the use of ammonium formate which proved to be superior. Similar findings in the analysis of cocaine and its metabolites have been published [1]. There was no significant change in the analyte retention times using the different mobile phases.

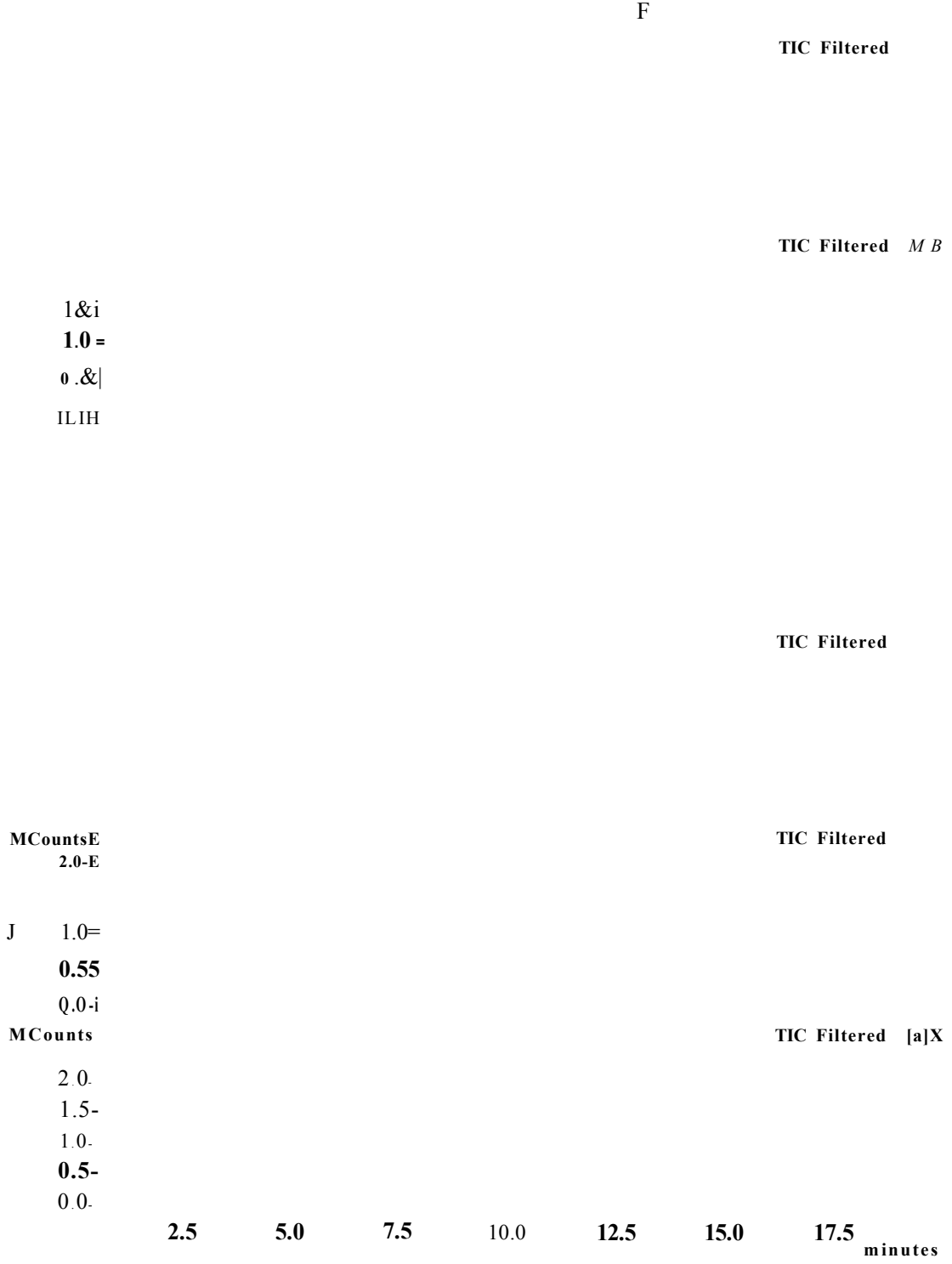


Figure 5:7 LC-MS/MS MRM chromatograms of (A) EME, (B) AE, (C) AEME, (D) benzoylecgonine, (E) cocaine, (F) cocaethylene using a phenyl column. The mobile phase was 0.1% acetic acid (1), 0.1% formic acid (2), ammonium formate lOmM pH 4 (3), 20mM pH 4 (4), lOmM pH 3 (5), and 5mM pH 3 (6)

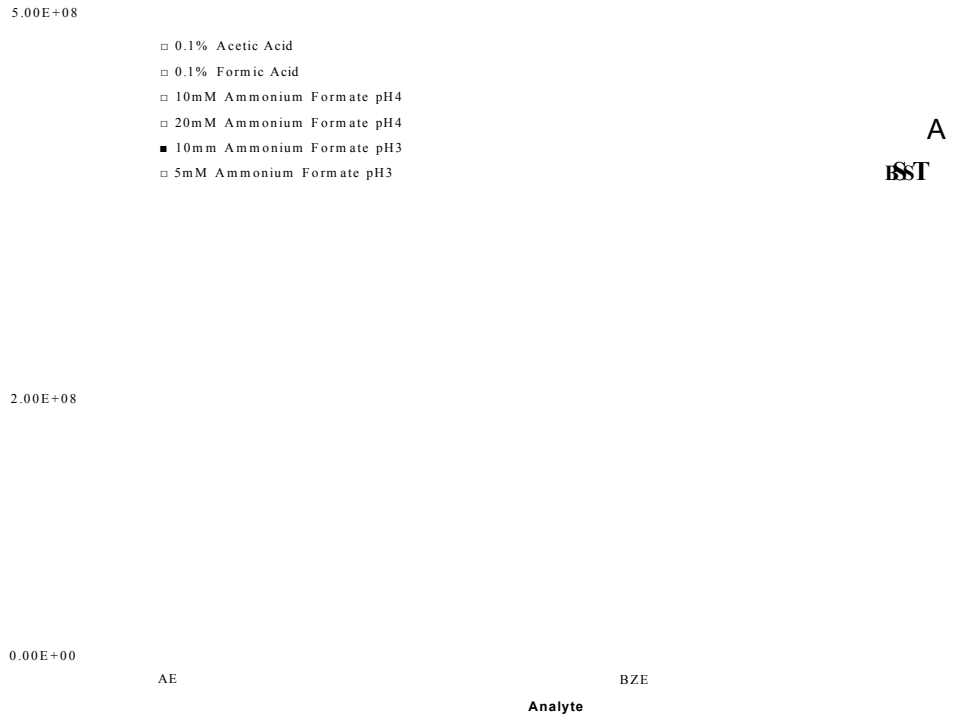


Figure 5:8 Comparison of analyte peak areas using different concentrations of ammonium formate and formic acid

The use of ammonium formate at lower molar concentrations provided improved ionisation. The use of 5mM ammonium formate produced the largest peak areas for AE, cocaine and cocaethylene however the SD of the replicate analyses were high for most of the analytes. The correlation between ionisation and molar concentrations of ammonium formate is unclear. Some authors found that lower concentrations increase ionisation [29], while others showed the opposite [1], or no significant effect [18]. Half of the analytes showed an improvement when pH 3 lOmM ammonium formate was used versus pH 4.

These experiments led to the selection of the phenyl column with an organic mobile phase of 0.1% formic acid and an aqueous mobile phase of lOmM ammonium formate at pH 3.

5.4.2 Gradient Elution Optimisation

Some of the metabolites of cocaine are highly polar, therefore low concentrations of organic solvent must be used to provide adequate retention of the analytes [29]. The gradient was started at 5% organic solvent so the hydrophobic hydrocarbon chains were exposed, at 0% they would be flattened to the silica particle and therefore not available for capturing analyte.

When first performing a reversed phase separation of a complex sample, a broad linear gradient should be used to determine where the analytes will elute. In this case the gradient was increased to 95% organic phase. The amount of organic phase required to elute each analyte was estimated and is shown in Table 5:10 along with the retention times.

Analyte	**Retention Time (minutes)**	**Organic Phase (%)**
EME	2.175	15.3
AE	2.583	17.2
AEME	5.33	30.3
Benzoylecgonine	11.054	57.4
Cocaine	11.772	60.8
Cocaethylene	12.823	65.8

Table 5:10 Percentage organic mobile phase required to elute each analyte

Cocaethylene was the last analyte to elute and required approximately 66% organic mobile phase. Stopping the gradient increase at 75% organic mobile phase allowed for the total analysis time to be reduced by 4 minutes. The time taken to return to 5% organic was reduced to 30 seconds and column equilibration was reduced to 7 minutes, this reduced the run time by an additional 3 and a half minutes making the total run time

22.5 minutes. The retention times, percentage of organic mobile phase, and resolution ratios for the new gradient are displayed in Table 5:11.

Analyte	Retention Time (minutes)	Organic Phase (%)	Resolution Ratio
EME	2.08	14.8	N/A
AE	2.482	16.7	1.2
AEME	5.128	29.1	8.0
Benzoylecgonine	11.003	56.7	16.8
Cocaine	11.448	58.8	1.2
Cocaethylene	12.486	63.7	2.7

Table 5:11 Summary of data for gradient elution with 75% final composition organic mobile phase

The final percentage of organic mobile phase was reduced further to 65% at 14 minutes. This reduced the total run time by an additional one minute. A summary of the retention times, percentage of organic mobile phase, and resolution ratios are displayed in Table 5:12.

Analyte	Retention Time (minutes)	Organic Phase (%)	Resolution Ratio
EME	2.022	12.9	N/A
AE	2.445	14.6	1.2
AEME	5.044	24.8	7.4
Benzoylecgonine	11.926	51.9	19.1
Cocaine	12.364	53.6	1.2
Cocaethylene	13.597	58.4	3.2

Table 5:12 Summary of data for gradient elution with 65% final composition organic mobile phase

Both gradients provided resolution ratios of >1, the second gradient was shorter and so was selected for method development. While baseline resolution (>1.5) of all peaks is ideal it is less necessary in LC-MS/MS as long as the unresolved peaks have different m/z values or produce fragment ions of different masses [18] which in this case they do

As the analytes eluted in three distinct time bands three segments were introduced into the MRM programme. This allowed the MS to scan for fewer ions at each time point thereby increasing the sensitivity of the method. AE and EME were grouped into segment 1, AEME was in segment 2, and benzoylecgonine, cocaine, and cocaethylene were in segment 3.

5.4.3 Optimisation of Instrument Parameters

The effect of needle voltage on ionisation of the analytes is shown in Figures 5:9 and 5:10. Increasing the needle voltage appears to have no significant effect on the peak areas of AE, EME, AEME or benzoylecgonine. Cocaine and cocaethylene peak areas show a gradual decline across the range.

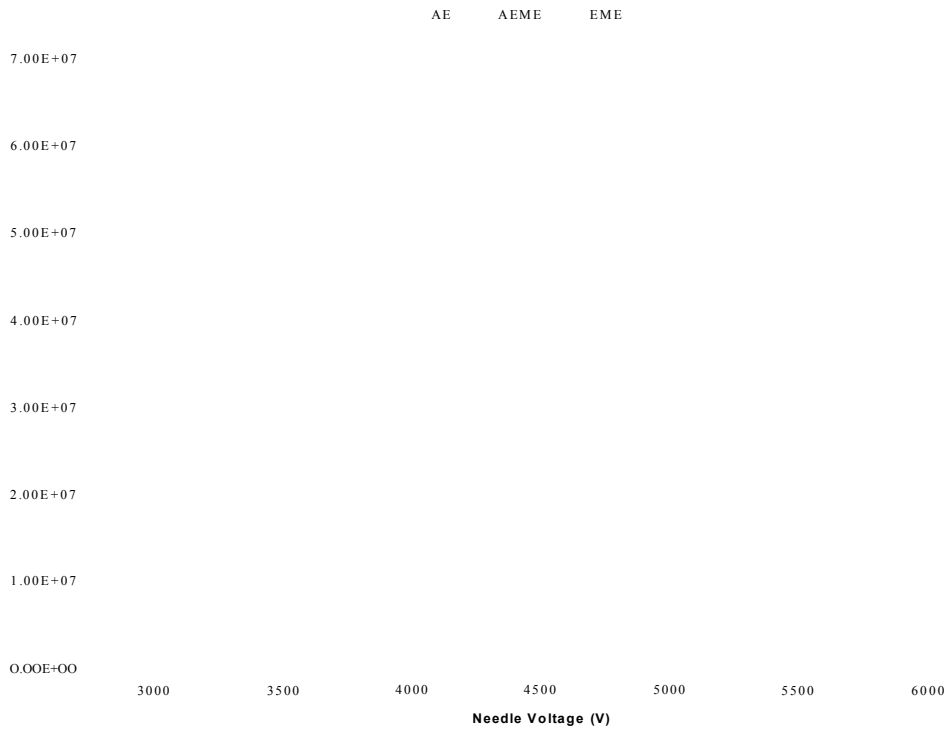


Figure 5:9 Optimisation of needle voltage for AE, AEME, and EME

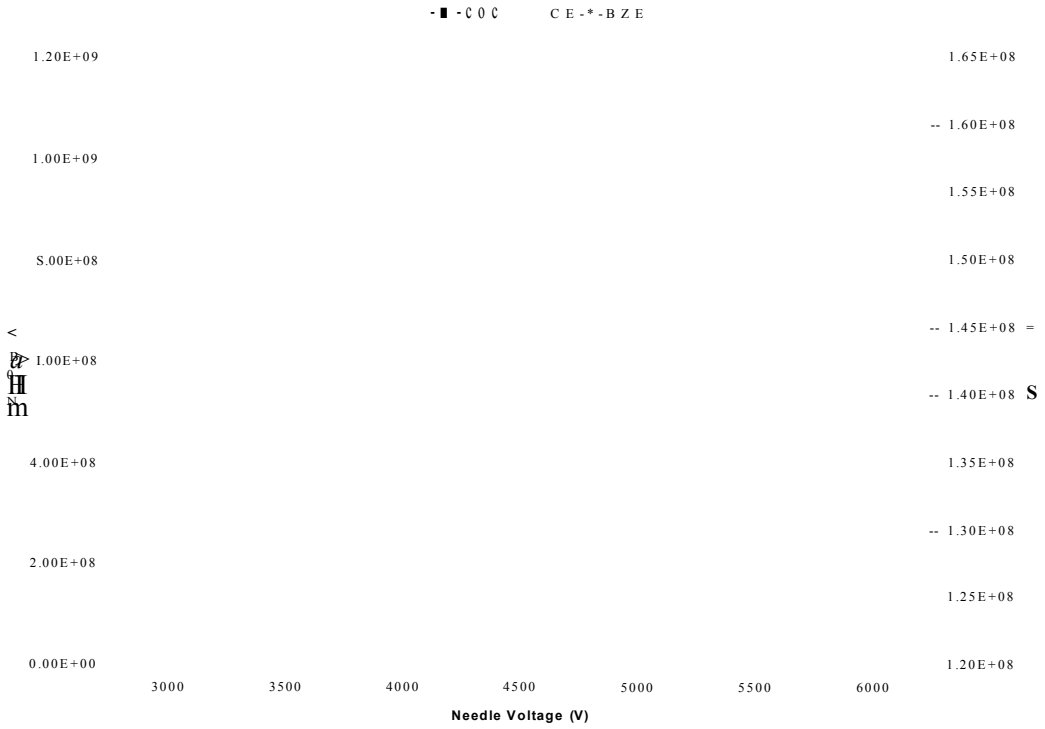


Figure 5:10 Optimisation of needle voltage for benzoylecgonine, cocaine, and cocaethylene

Due to the absence of clear optimum needle voltage for all of the analytes the instrument default setting of 5000V was selected.

The drying gas temperature was started at 100°C as temperatures below this do not allow efficient evaporation of the solvent from the column eluant and result in a build up of liquid in the ion source. The results displayed in Figure 5:11 show that increasing the drying gas temperature produced an increase in analyte peak areas up to 200-3 00 °C after which they gradually declined. A drying gas temperature of 250°C was selected as it provided a suitable compromise for all the analytes.

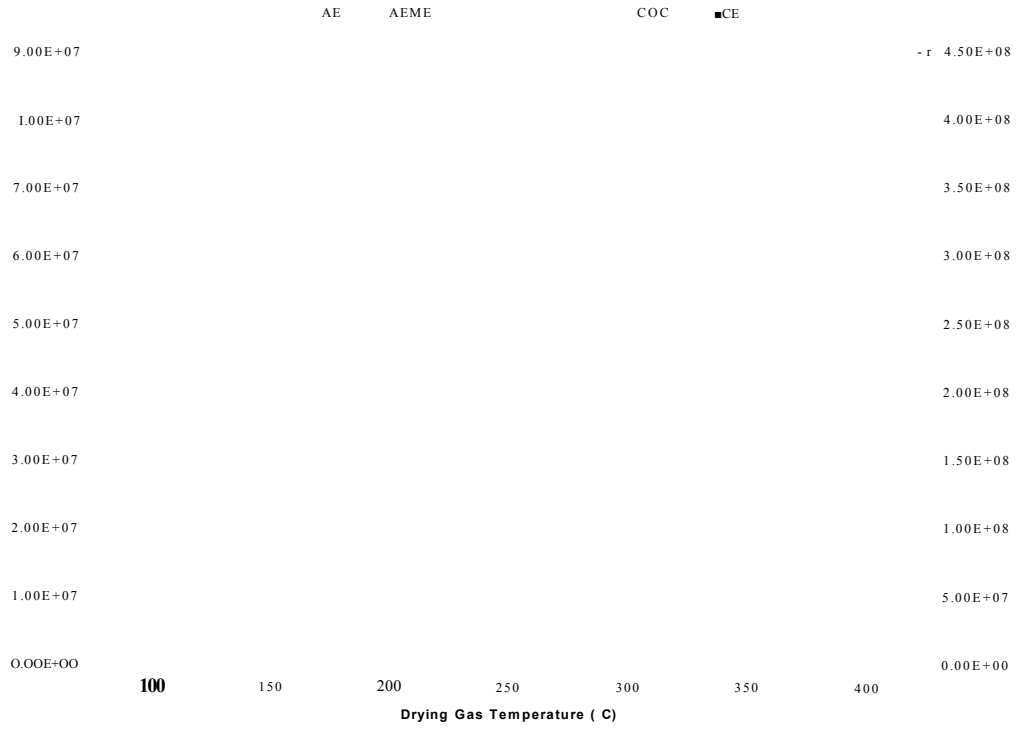


Figure 5:11 Effect of drying gas temperature on ionisation of AE, AEME, EME, cocaine, cocaethylene and benzoylecgonine

Each analyte may have an optimum capillary voltage at which maximum ionisation is achieved without fragmentation of the ion. An increase in the capillary voltage from

50V to 55V produced an increase in analyte peak area as shown in Figure 5:12. Further increases in capillary voltage produced gradual decreases in analyte peak area.

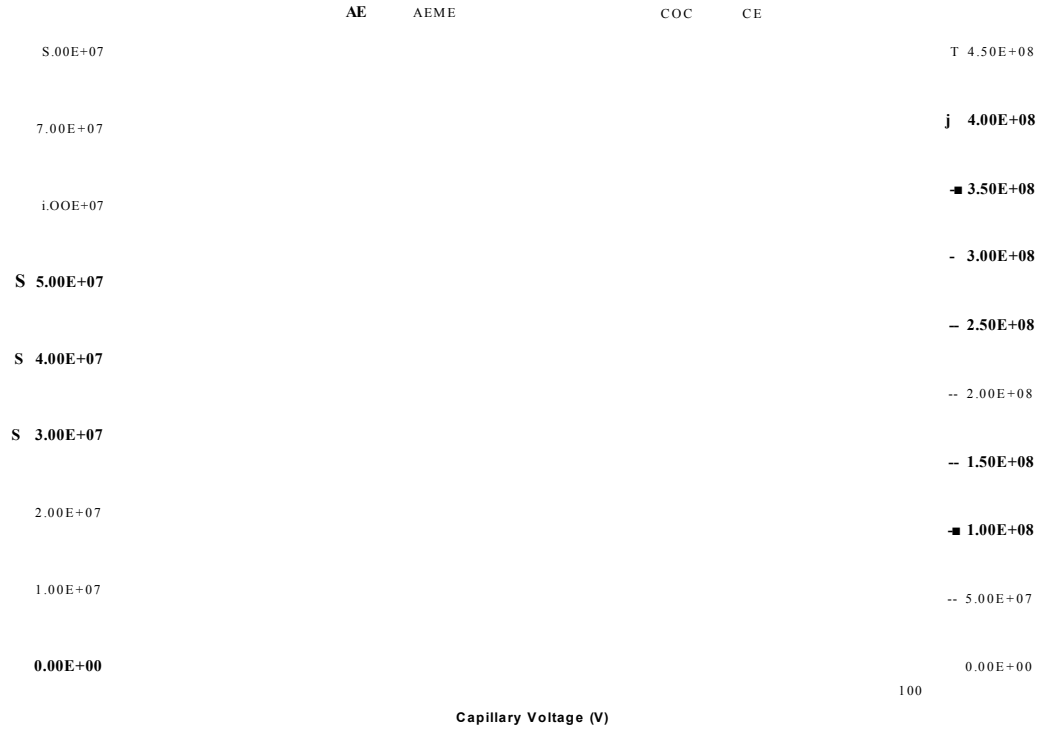


Figure 5:12 Effect of capillary voltage on ionisation of AE, AEME, EME, benzoylecgonine, cocaine, and cocaethylene

As the capillary voltage increases the energy applied to the analyte ions increases causing fragmentation in the ion source. As a result less molecular ions pass through to the collision cell for fragmentation causing a decrease in the ion signal.
The capillary voltage was therefore set to 55V.

The results displayed in Figure 5:13 and 5:14 show that increasing the shield voltage produced an increase in analyte peak areas up to 400-500V after which they gradually declined. Based on these results a shield voltage of 500V was chosen.

Figure 5:13 Effect of shield voltage on ionisation of cocaine, cocaethylene, benzoylecgonine and AEME

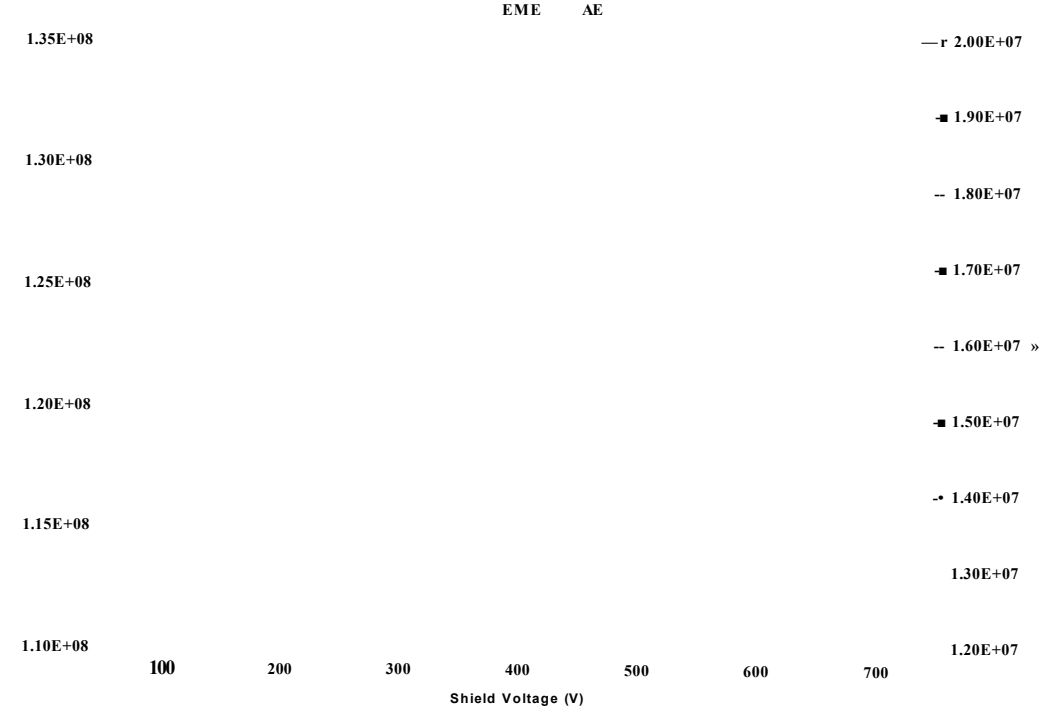


Figure 5:14 Effect of shield voltage on ionisation of EME and AE

5.4.4 Validation Data

Method validation is required to demonstrate the performance of a method and the reliability of analytical results [32].

The method was first assessed by establishing the linearity of extracted calibration curves over the range of 0-360ng/mL for each analyte, see Table 5:13. Calibration curves were produced from the ratios of analyte peak area to its internal standard. All the analytes produced good linearity with coefficient of determinations greater than 0.99, with the exception of AE which failed to produce a linear curve. At this point AE was excluded from further validation experiments as accurate quantitative results would not have been possible.

Analyte	**Range (ng/mL)**	**Coefficient of Determination**	
		Mean	**SD (n=4)**
EME	0-360	0.9980	0.0012
AEME	0-360	0.9968	0.0015
Benzoylecgonine	0-360	0.9983	0.0011
Cocaine	0-360	0.9988	0.0006
Cocaethylene	0-360	0.9976	0.0017

Table 5:13 Calibration curve linearity data

Three analytical runs were performed to establish the LOD, LOQ, precision, accuracy, and robustness of the method. The LOD and LOQ for each analyte are displayed in Table 5:14. The results are favourable compared to other oral fluid LC-MS methods for the analysis of cocaine which state 0.2-5ng/mL LOD and 0.9-1 Ing/mL LOQ [7, 9, 16, 17].

Analyte	LOD (ng/mL)	LOQ (ng/mL)
EME	1	2
AEME	0.5	1
Benzoylecgonine	1	2
Cocaine	1	2
Cocaethylene	1	2

Table 5:14 LOD and LOQ of LC-MS/MS method

The LOQ can be further separated into the upper limit of quantitation and the lower limit of quantitation, which represent the concentration of the highest and lowest calibrations standards which in this case is 5ng/mL and 360ng/mL.

The goal of quantitative analysis is to determine with accuracy and precision how much analyte is present in a sample. Precision is the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample. Accuracy describes the closeness of mean test results obtained to the true concentration of the analyte [24]. Very good inter-assay precision (1.9-7.1%), and intra-assay precision (1.4-4.9%) were obtained across the different concentrations. The results are an improvement of previously published methods which range from 4-23% [7, 9, 16]. The accuracy of the results was 0.1-16%, the highest % bias was produced by benzoylecgonine. The data is displayed in Table 5:15.

Analyte	Inter-assay Precision (n=15)			Intra-assay Precision (n=5)			Accuracy
	Mean Concentration (ng/mL)	SD (ng/mL)	CV (%)	Mean Concentration (ng/mL)	SD (ng/mL)	CV (%)	% Bias
EME	19.8	0.9	4.4	19.8	0.8	4.1	-5.9
	60.5	2.1	3.5	60.6	2.0	3.4	0.9
	117	3.4	2.9	117	3.2	2.7	-2.5
AEME	21.2	1.5	7.1	21.2	0.75	3.6	1.0
	60.6	3.8	6.3	60.6	2.3	3.8	0.1
	113.3	6.1	5.4	113.3	3.7	3.2	-5.6
Benzoylecgonine	24.4	1.3	5.2	24.4	1.2	4.9	16.0
	69.1	2.9	4.2	69.1	2.4	3.4	15.2
	135.7	4.0	2.9	135.7	2.9	2.1	13.1
Cocaine	20.0	0.9	4.7	20.1	0.9	4.5	-4.5
	61.4	1.4	2.3	61.4	1.2	2.0	2.4
	118.8	3.1	2.6	118.8	2.1	1.8	-1.0
Cocaethylene	21.3	1.0	4.7	21.3	0.97	4.6	1.3
	62.5	1.2	1.9	62.5	0.9	1.4	4.2
	121.4	3.4	2.8	121.4	2.8	2.3	1.2

Table 5:15 Intra-assay and inter-assay precision, and accuracy of cocaine and its metabolites at different concentrations in oral fluid

The retention times and ion intensities were assessed across each run and the results are shown in Tables 5:16 and 5:17. The retention times were very close with % CV within 0.6%, and the ion ratios were within 7.6%.

The results of the assay drift showed very little change in the drug concentration with good accuracy values of 1.9% (Table 5:18).

Analyte	Mean Retention Time (min)	SD	CV (%)
EME	2.45	0.014	0.59
AEME	5.94	0.02	0.34
Benzoylecgonine	11.43	0.019	0.17
Cocaine	12.36	0.015	0.12
Cocaethylene	13.41	0.01	0.07

Table 5:16 Retention time variation data

Analyte	Ion transition 1 (IT1)	Ion transition 2 (IT2)	Mean Ratio (IT2 as % of IT1)	SD (%)	CV (%)
EME	200—>182	200—>82	44.1	1.7	3.9
AEME	182—>118	182—>122	75.3	2.5	3.3
Benzoylecgonine	290—>168	290—>105	26.4	2.0	7.6
Cocaine	304—>182	304—>105	11.5	0.6	5.0
Cocaethylene	318—>196	318—>150	8.8	0.4	4.5

Table 5:17 Ion intensity variation data

Analyte	Drift (%)		
	21ng/mL	60ng/mL	120ng/mL
EME	-1.7	1.6	1.4
AEME	-0.1	1.9	1.4
Benzoylecgonine	2.7	3.1	-0.5
Cocaine	-1.8	1.6	1.6
Cocaethylene	0.7	0.2	1.9

Table 5:18 Assay drift data

The analysis of 48 oral fluid samples from non cocaine users produced negative results. The method showed good selectivity as there were no interfering peaks present in the chromatograms.

Extraction recoveries based on samples spiked prior to extraction and un-extracted samples were evaluated and the data is shown in Table 5:19. The average recoveries were as follows, EME 53.6%, AEME 88.3%, benzoylecgonine 99.1%, cocaine 72.9%, and cocaethylene 72.1%. The results are similar to those previously reported. EME has reasonably low recovery due to its polar functional group, recoveries of approximately 40-45% have been reported [20, 25, 33, 34], Although high recovery is desirable it is not needed to provide good accuracy and precision if adequate detection is attained [22].

Analyte	**Absolute Recovery**								
	21ng/mL			**60ng/mL**			**120ng/mL**		
	Average	SD (N=3)	CV (%)	Average	SD (N=3)	CV (%)	Average	SD (N=3)	CV
EME	50.4	3.3	6.5	59.9	5.4	9.0	50.5	4.6	9.2
AEME	90.5	4.3	4.8	96.8	8.4	8.7	77.7	6.4	8.2
Benzoylecgonine	106	4.5	4.2	101.5	5.5	5.4	89.9	10.3	11.4
Cocaine	71.2	2.1	2.9	78.6	4.3	5.5	68.9	3.9	5.77
Cocaethylene	68.2	7.2	10.5	81.9	4.3	5.2	66.1	4.9	7.4

Table 5:19 Absolute recovery of extracted samples

The presence of biological material can result in ion suppression at the ion source, this was investigated by comparing the results of samples spiked after extraction and un-extracted samples. The results are displayed in Table 5:20. The average recoveries were as follows, EME 100.3%, AEME 104%, benzoylecgonine 98.7%, cocaine 89%, and cocaethylene 79.8%. This indicates that there is no ion suppression for EME, AEME, or

benzoylecgonine using oral fluid specimens. The recoveries were lower for cocaine and cocaethylene suggesting a small degree of ion suppression towards the end of the analytical run.

The use of an isotopically labelled internal standard corrects any ion suppression effect experienced [23].

Analyte	Recovery - Ion Suppression								
	21ng/mL			60ng/mL			120ng/mL		
	Average	SD (N=3)	CV (%)	Average	SD (N=3)	CV (%)	Average	SD (N=3)	CV (%)
EME	93	4.4	4.8	112.3	7	6.2	96.4	4.5	1.0
AEME	102.8	7.1	6.9	116.1	7.6	6.5	93	1	4.7
Benzoylecgonine	96.8	8.5	8.7	108.2	15.5	14.3	91.1	6.5	7.1
Cocaine	83.3	4.1	4.9	102.1	13.3	13	81.7	4.5	5.5
Cocaethylene	73.7	7.1	9.7	91.7	9.1	9.9	74	3.2	4.3

Table 5:20 Assessment of ion suppression using spiked oral fluid samples

5.5 Conclusion

This chapter documents the development of a quantitative LC-MS/MS method for the analysis of cocaine, benzoylecgonine, cocaethylene, AEME, and EME in oral fluid. The column, mobile phase and instrument parameters were all optimised to give improved analyte ionisation and separation. The validation data summarised meets the required acceptance criteria and demonstrates that the method is sufficiently reproducible, robust and sensitive to carry out routine analysis of oral fluid samples.

The application of this method to clinical samples is discussed in the following chapter. Cocaine and its metabolites pose difficulties in their analysis due to their chemistry ranging from polar (acidic and basic) to non polar. A compromise is often met in order

to analyse all compounds within one analytical method. In this case the compromise was the decision to exclude AE from the method. The main reason was based on the lack of linearity for AE which would have meant that the method could only provide qualitative not quantitative results. The peak area for AE was considerably lower compared to the other analytes suggesting poor ionisation. This would reduce the sensitivity of the method for AE and would result in poor reproducibility. Other problems associated with the analysis of AE were the low extraction recoveries as detailed in chapter 4.

Further investigations are required to discover an appropriate chromatographic system which will allow AE to be accurately analysed either separately or simultaneously with other analytes.

5.6 References

1. Jeanville, P.M., E.S. Estape, and I.T.-N.d. Jeanville, *The Effects of Liquid Chromatography Eluents and Additives on the Positive Ion Responses of Cocaine, Benzoylecgonine, and Ecgonine Methyl Ester Using Electrospray Ionisation.* International Journal of Mass Spectrometry, 2003. 227: p. 247-258.
2. Johansen, S.S. and H.M. Bhatia, *Quantitative Analysis of Cocaine and its Metabolites in Whole Blood and Urine by High Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry.* Journal of Chromatography B, 2007. 2007. 852: p. 338-344.
3. Kintz, P., et al., *Evidence of Crack Use by Anhydroecgonine Methyl Ester Identification.* Human and Experimental Toxicology, 1997. 16: p. 123-127.
4. Lewis, R.J., et al., *Determination of Cocaine, its Metabolites, Pyrolysis Products, and Ethanol Adducts in Postmortem Fluids and Tissues using Zymark Automated Solid-Phase Extraction and Gas Chromatography-Mass Spectrometry.* Journal of Chromatography B, 2004. 806: p. 141-150.
5. Toennes, S.W., et al., *Artifact Production in the Assay of Anhydroecgonine Methyl Ester in Serum Using Gas Chromatography-Mass Spectrometry.* Journal of Chromatography B, 2003. 792: p. 345-351.
6. Toennes, S.W., A.S. Fandino, and G. Kauert, *Gas Chromatographic-mass Spectrometric Detection of Anhydroecgonine Methyl Ester (Methylecgonidine) in Human Serum as Evidence of Recent Smoking of Crack.* Journal of Chromatography B, 1999. 735: p. 127-132.
7. Dams, R., et al., *Oral Fluid as an Alternative Matrix to Monitor Opiate and Cocaine Use in Substance-Abuse Treatment Patients.* Drug and Alcohol Dependence, 2007. 87: p. 258-267.
8. Dams, R., et al., *Urine Drug Testing for Opioids, Cocaine and Metabolites by Direct Injection Liquid Chromatography/Tandem Mass Spectrometry.* Rapid Communications in Mass Spectrometry, 2003. 17: p. 1665-1670.
9. Clauwaert, K., et al., *The Determination of Cocaine, Benzoylecgonine, and Cocaethylene in Small-Volume Oral Fluid Samples by Liquid Chromatography-Quadrupole-Time-of-Flight Mass Spectrometry.* Journal of Analytical Toxicology, 2004. 28: p. 655-659.

10. Jeanville, P.M., et al., *Rapid Confirmation/Quantitation of Cocaine and Benzoylecgonine in Urine Utilizing High Performance Liquid Chromatography and Tandem Mass Spectrometry.* American Society for Mass Spectrometry, 2000.11: p. 257-263.

11. Xia, Y., et al., *An LC-MS-MS Method for the Comprehensive Analysis of Cocaine and Cocaine Metabolites in Meconium.* Analytical Chemistry, 2000. 72: p. 764-771.

12. Pichini, S., et al., *Development and Validation of a Liquid Chromatography-Mass Spectrometry Assay for the Determination of Opiates and Cocaine in Meconium.* Journal of Chromatography B, 2003. 794: p. 281-292.

13. Rook, E.J., et al., *The Quantitative Analysis of Heroin, Methadone and their Metabolites and the Simultaneous Detection of Cocaine, Acetylcodeine and their Metabolites in Human Plasma by High-Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry.* Journal of Chromatography B, 2005. 824: p. 213-221.

14. Needham, S.R., et al., *Performance of a Pentafluorophenylpropyl Stationary Phase for the Electrospray Ionisation High-Performance Liquid Chromatography-Mass Spectrometry-Mass Spectrometry assay of Cocaine and its Metabolite Ecgonine Methyl Ester in Human Urine.* Journal of Chromatography B, 2000. 748: p. 77-87.

15. Fandino, A.S., et al., *Identification of Anhydroecgonine Methyl Ester N-Oxide, a new metabolite of Anhydroecgonine Methyl Ester, using Electrospray Mass Spectrometry.* Journal of Mass Spectrometry, 2002. 37: p. 525-532.

16. Mortier, K.A., et al., *Simultaneous Quantitative Determination of Opiates, Amphetamines, Cocaine and Benzoylecgonine in Oral Fluid by Liquid Chromatography Quadrupole-Time-Of-Flight Mass Spectrometry.* Journal of Chromatography B, 2002. 779: p. 321-330.

17. Wylie, F.M., et al., *Drugs in Oral Fluid Part I: Validation of an Analytical Procedure for Licit and Illicit Drugs in Oral Fluid.* Forensic Science International, 2005. 150: p. 191-198.

18. Srinivasan, K., et al., *Liquid Chromatography-Tandem Mass Spectrometry Analysis of Cocaine and its Metabolites from Blood, Amniotic Fluid, Placental and Fetal Tissues: Study of the Metabolism and Distribution of Cocaine in Pregnant Rats.* Journal of Chromatography B, 2000. 745: p. 287-303.

19. Myers, A.L., et al., *Identification of Anhydroecgonine Ethyl Ester in the Urine of a Drug Overdose Victim.* Journal of Forensic Sciences, 2005. 50: p. 1481-1485.
20. Bogusz, M.J., *Liquid Chromatography-Mass Spectrometry as a Routine Method in Forensic Sciences: A Proof of Maturity.* Journal of Chromatography B, 2000. 748: p. 3-19.
21. Higson, S.P.J., *Analytical Chemistry.* 2003: Oxford Univeristy Press.
22. Bressolle, F., M. Bromet-Petit, and M. Audran, *Validation of Liquid Chromatographic and Gas Chromatographic Methods: Applications to Pharmacokinetics.* Journal of Chromatography B, 1996. 686: p. 3-10.
23. Peters, F.T., O.H. Drummer, and F. Musshoff, *Validation of New Methods.* Forensic Science International, 2007.165: p. 216-224.
24. CDER, *Guidance for Industry: Bioanalytical Method Validation.* 2001, U.S Department of Health and Human Services. www.fda.gov/cder/guidance/index.htm.
25. Cardona, P.S., et al., *Simultaneous Analyses of Cocaine, Cocaethylene, and their Possible Metabolic and Pyrolytic Products.* Forensic Science International, 2006. 157: p. 46-56.
26. Shah, V.P., et al., *Bioanalytical Method Validation - A Revisit with a Decade of Progress.* Pharmaceutical Research, 2000. 17: p. 1551-1557.
27. Yang, M., et al., *Impact of Methanol and Acetonitrile on Separations Based on π-π Interactions with Reversed Phase Phenyl Column.* Journal of Chromatography A, 2005. 1097: p. 124-129.
28. Kupiec, T., et al., *High Performance Liquid Chromatography*, in *Clarkes Analysis of Drugs and Poisons,* A.C. Moffat, M.D. Osselton, and B. Widdop, Editors. 2004, Pharmaceutical Press.
29. McMillan, B., K. Sherlock, and M. Conner, *Expanding the Traditional User Versus Non-User Dichotomy Amongst Ecstasy Users.* Journal of Community and Applied Social Psychology, 2003. 13: p. 15-28.
30. Maurer, H.H., *Liquid Chromatography-Mass Spectrometry in Forensic and Clinical Toxicology.* Journal of Chromatography B, 1998. 713: p. 3-25.
31. Dams, R., et al., *Comparison of Phenyl-Type Columns in the Development of a Fast Liquid Chromatographic System for Eighteen Opiates Commonly Found in Forensic Toxicology.* Journal of Chromatography A, 2000. 896: p. 311-319.

32. Causon, R., *Validation of Chromatographic Methods in Biomedical Analysis Viewpoint and Discussion.* Journal of Chromatography B, 1997. 689: p. 175-180.

33. Cone, E.J., M. Hillsgrove, and W.D. Darwin, *Simultaneous Measurement of Cocaine, Cocaethylene, Their Metabolites, and 'Crack' Pyrolysis Products by Gas Chromatography-Mass Spectrometry.* Clinical Chemistry, 1994. 40: p. 1299-1305.

34. Torre, R.D.L., et al., *Determination of Cocaine and its Metabolites in Human Urine by Gas Chromatography/Mass Spectrometry After Simultaneous use of Cocaine and Ethanol.* Journal of Pharmaceutical and Biomedical Analysis, 1995.13: p. 305-312.

6 Analysis of Clinical Samples for the Presence of Cocaine, its Metabolites and Pyrolysis Products

6.1 Aims and Objectives

The aim of this chapter is to analyse a large number of oral fluid samples from self declared cocaine hydrochloride and crack cocaine users. The samples will be screened using the newly developed AEME EIA and confirmed for the presence of cocaine, cocaethylene, benzoylecgonine, EME and AEME using liquid chromatography tandem mass spectrometry (LC-MS/MS).

The results will be used to establish an optimum cutoff concentration for the AEME EIA, and provide much needed information on the concentrations and relative detection times of AEME in oral fluid.

6.2 Introduction

The majority of published studies which report concentrations of abused drugs in biological fluids relate to controlled dose administration of drug. While this is a useful way of studying the pharmacokinetics and pharmacodynamics of a particular drug it does not accurately reflect real life cases where individuals may be using drugs frequently, which vary in purity and dose. In these situations the concentration of drug tends to be higher and have a longer window of detection than following controlled dose administration. For ethical reasons the frequent administration of high doses of drug in a controlled environment is not possible, therefore biological samples must be obtained from a drug using population. To enable a proper interpretation of data and estimation of detection times an accurate history of drug use is essential and therefore requires cooperation from the drug user to provide this information.

A disadvantage of relying on self reported use is the inaccuracy and bias associated with it. If it is perceived that negative consequences, such as cessation of treatment or legal consequences, will be associated with reporting certain activities then the respondents may feel compelled to conceal the truth [1]. A 32% discrepancy between urine sample positives for cocaine and self report data was observed from a population of homeless persons. Half of this study population were guaranteed access to therapy and housing subject to abstinence from cocaine [2].

Individuals with a prior history of drug treatment or those who have been in contact with the criminal justice system are more likely to report their drug use accurately [3], while individuals who lead more socially acceptable lifestyles are more likely to under-report their drug use [4].

Higher rates of agreement were found between self report data and urinalysis for patients upon entry to treatment, the rate of agreement declined after intake with underreporting more common [5]. When patients were categorised into groups relative to their frequency of attendance at a drug treatment clinic it was found that there was poorer concordance between self-report and urinalysis for patients attending the clinic more frequently. It was concluded that older subjects have a tendency to be more honest [6] as well as those who have spent more money on drugs [3].

Recollection of drug consumption may be impaired by the duration of time since use, and the possible concurrent use of other psychoactive drugs that may impair the memory [7].

Drug use is usually reported as the amount of drug used, for example in grams or number of pills, or in the amount of money spent [7]. This information can be problematic as the subject may be a poor judge of weight, but more importantly the purity of the drug purchased on the street is unknown and may change frequently. This

issue was highlighted in a study in the US where individuals self reported the use of ecstasy within two to three days prior to testing, only 20% tested positive for MDMA. However 65% of this population tested positive to metamfetamine [4]. Collecting information relating to monetary value has its disadvantages as prices change over time and may also differ significantly around the country.

Self report data with accompanying analytical results provides additional data to support that provided by controlled dose administration studies.

6.3 Experimental

6.3.1 Materials

The LC-MS/MS instrumentation, materials, and reagents used were as previously described in chapter 5. The AEME EIA used was as previously described in chapter 3.

6.3.2 Method

6.3.2.1 Clinical Specimens

Two hundred and seventy samples were obtained from the analytical laboratory at Cozart pic following their routine analysis for drugs of abuse. In general samples were selected on the basis of declared cocaine use from the donor, with the intention to identify two types of cocaine use, cocaine hydrochloride and crack. Of the 270 samples analysed a total of 229 were provided with self report information relating to the type of cocaine, quantity, and date consumed.

Prior to submission to the laboratory lmL of oral fluid was collected from each individual using the Cozart® RapiScan Collector, the oral fluid soaked sample pad was then inserted into the transport tube which contained 2mL of Cozart proprietary buffer.

This collection method results in a 1:3 dilution of the sample, all concentrations noted are corrected for undiluted oral fluid. The samples were stored frozen following receipt at the laboratory and were tested within 2 months.

The samples were screened using the AEME EIA and confirmed for the presence of cocaine, cocaethylene, benzoylecgonine, EME, and AEME by LC-MS/MS. All procedures for the immunoassay, sample preparation, and confirmation by LC-MS/MS were as previously described in chapters 3, 4 and 5 respectively.

6.3.2.2 Statistics

A receiver operator characteristic (ROC) analysis was carried out on the data obtained from the AEME EIA and LC-MS/MS methods to determine the most appropriate cutoff concentrations for accurate analysis of clinical samples.

The number of true positives (TP), false negatives (FN), false positives (FP), and true negatives (TN) were determined by comparing the AEME EIA results at various cutoff concentrations, to the reference method LC-MS/MS at cutoffs of 5ng/mL and lOng/mL. These values were used to calculate the sensitivity and specificity as follows:

Sensitivity = [TP/(TP + FN)] x 100%

Specificity = [TN/(TN +FP)] x 100%

To create the ROC curve the sensitivity is plotted against 1-specifcity for each cut off value. The accuracy of the test is determined by the area under the curve, the greater the area under the curve the greater the accuracy.

Pearson's correlation coefficient is a measure of the linear relationship between two variables. The closer the value is to 1.0 the greater the correlation. The correlation coefficient value squared is otherwise known as the coefficient of determination, and

gives the proportion of variance between two data sets. It represents the percent of data that is closest to the line of best fit.

The concentrations of AEME measured by EIA and LC-MS/MS were compared to see if there was any correlation between the two different methods. This was achieved by creating a scatter plot with the data and calculating the coefficient of determination. This statistical analysis was also carried out on the other analytes quantified by LC-MS/MS to see if there is a relationship between concentrations of cocaine and its metabolites.

6.4 Results and Discussion

6.4.1 AEME EIA Data Analysis

The full set of data for the AEME EIA and LC-MS/MS analysis is provided in Table 6:3.

6.4.1.1 Correlation

Good correlation was found between the AEME EIA and the results provided by LC-MS/MS. The coefficient of determination for the immunoassay across its calibration range 3-300ng/mL was calculated to be 0.76 (n= 157). The scatter plot can be seen in Figure 6:1.

The typical dose response curve of an immunoassay is sigmoidal in shape, the linear range is obtained around the middle of the curve and consequently are where the most precise measurements are obtained. Experimental errors increase towards the upper and lower portion of the curve [8]. The non linear characteristics of an immunoassay calibration curve means that samples containing drug concentrations outside of the

calibration range are extrapolated from the curve and as a result the concentrations may vary considerably from the true value.

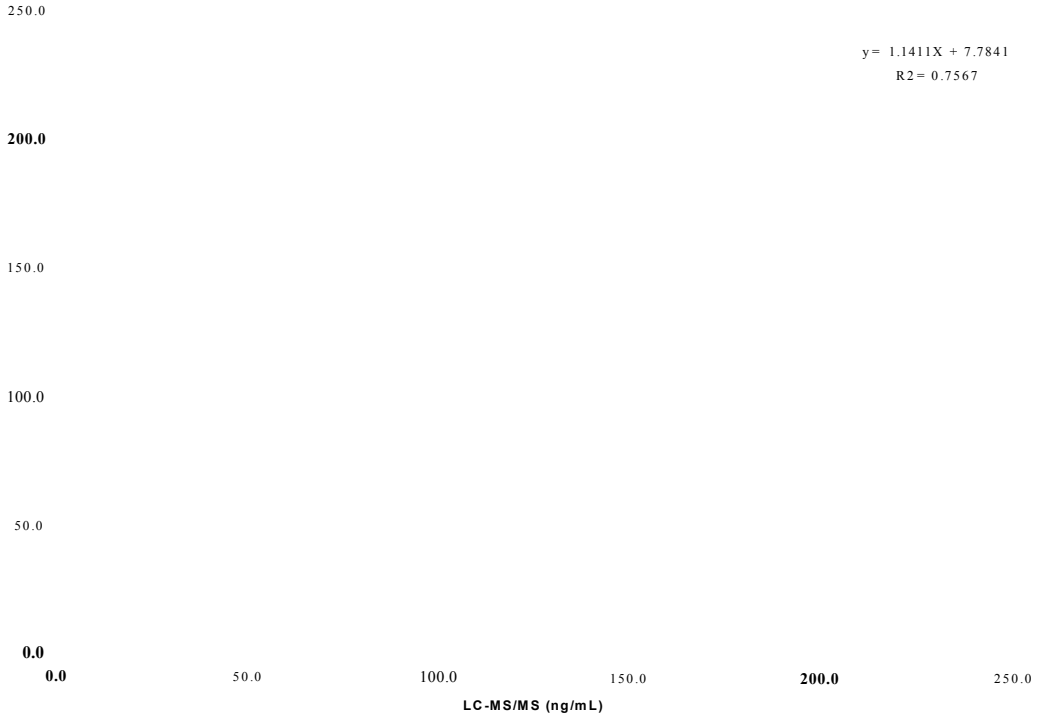


Figure 6:1 Correlation of AEME EIA and LC-MS/MS

Correlation studies between immunoassays and confirmation analyses have been performed by several authors and have provided varied results. A comparison of GC-MS results to the results of four different immunoassays, for drugs of abuse in whole blood, found that despite the occasional matching result, no correlation was found between the methods making quantitative analysis by immunoassay impossible [9], Many immunoassays are directed at classes of drugs rather than individual analytes, therefore good correlation would require the confirmation method to analyse for all the compounds detected by the immunoassay. However due to the nature of an immunoassay each analyte may have a different degree of cross reactivity which may produce an overestimated or underestimated quantitative result, and as a result give low correlation between the screen and confirmation methods. Overestimation of

quantitative results has been reported more often and as a result of this a semi-quantitative approach to the immunoassay has been adopted [10-12].

The AEME EIA has good correlation to LC-MS/MS and the reason may be that the assay is specific to AEME and does not cross react to cocaine or any of its other metabolites.

6.4.1.2 ROC Analysis

There are currently no mandatory guidelines which specify cutoff concentrations for immunoassay screening of drugs of abuse in oral fluid. For this reason, and the fact that this is a unique immunoassay, an ROC analysis was performed on the data to establish an appropriate cutoff.

The sensitivity, specificity, and efficiency data are presented in Tables 6:1 and 6:2.

LC/MS Cutoff	**AEME Cut-off Concentration (ng/mL)**								
5ng/mL	**3**	**5**	10	**15**	20	**25**	**30**	**40**	**50**
TP	84	82	**68**	63	56	50	46	38	37
FN	**0**	**0**	16	**21**	28	34	38	46	47
FP	**86**	45	14	**6**	**1**	**1**	**0**	**0**	**0**
TN	**100**	143	172	180	185	185	186	186	186
Sensitivity	**100**	**100**	81	75	66.7	59.5	54.8	45.2	44.1
Specificity	53.8	76.1	92.5	96.8	99.5	99.5	**100**	**100**	**100**
Efficiency	**68.1**	83.3	88.9	90.0	89.3	87	85.9	83	82.6

Table 6:1 Sensitivity, specificity, and efficiency data for AEME EIA at various cutoff concentrations versus an LC-MS/MS cutoff of 5ng/mL

LC/MS Cutoff	AEME Cut-off Concentration (ng/mL)								
lOng/mL	3	5	10	15	20	25	30	40	50
TP	65	65	63	60	55	49	46	39	38
FN	0	0	2	5	10	16	19	26	27
FP	105	62	19	9	2	2	1	0	0
TN	100	143	186	196	203	203	204	205	205
Sensitivity	100	100	96.9	92.3	84.6	75.4	70.8	60	58.5
Specificity	48.8	69.8	90.7	95.6	99	99	99.5	100	100
Efficiency	61.1	77	92.2	94.8	95.6	93.3	92.6	90.4	90

Table 6:2 Sensitivity, specificity, and efficiency data for AEME EIA at various cutoff concentrations versus an LC-MS/MS cut-off of lOng/mL

A ROC curve was constructed from the data in Tables 6:1 and 6:2, and is shown in Figure 6:2. The results suggest that the most efficient screening cutoff was between 10 and 15ng/mL of AEME in undiluted oral fluid using the lOng/mL LC-MS/MS cutoff, and lOng/mL EIA cutoff using the 5ng/mL LC-MS/MS cutoff.

Looking at the ROC curve it is clear that the greatest efficiency will be provided by employing the lOng/mL LC-MS/MS cutoff as this provided the greatest area under the curve.

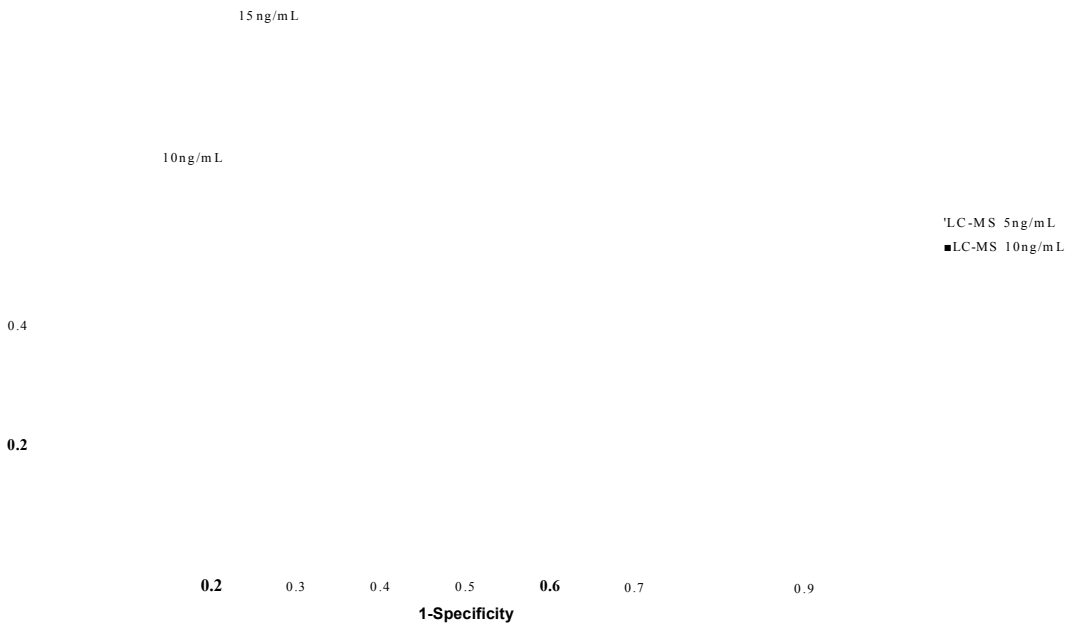


Figure 6:2 ROC curve for AEME EIA versus LC-MS/MS

6.4.2 Quantitative LC-MS/MS Analysis

The full set of results for both screening and confirmation can be seen in Table 6:3. The samples are organised in ascending concentration of apparent AEME from the EIA. A total of 270 samples were tested by both methods, the most predominant analyte was benzoylecgonine contrary to the findings of some studies [13, 14]. The reason for this may reside in the spontaneous hydrolysis of cocaine to benzoylecgonine in the collected oral fluid sample, or alternatively may be attributed to the longer half life of benzoylecgonine compared to cocaine [15]. On each occasion where EME was detected, benzoylecgonine was also present. This is similar to the findings of Kim et al [15].

	Barcode	Drug Declared	Quantity	Days Since Drug Use	Apparent AEME by EIA (ng/mL)	LC-MS/MS (ng/mL) AEME	EME	BZE	COC	CE
	S702462	Crack	£20	1	<3	0	3.3	41	1.2	0
	S702195	Crack	1 Pipe	1	<3	0	0	27	0	0
	S705540	Crack	£20	3	<3	0	0	5.6	0	0
	S697738	Crack		1	<3	0	4.5	11	6.7	0
	S735016	Cocaine	£20	2	<3	0	0	48	2.1	0
	S705544	Crack	£5	1	<3	0	33	154	7	0
	S735562	Crack	£5	1	<3	0	0	19	0	0
	S776242	Crack	1 Rock	1	<3	0	7.4	24	1.6	0
	S529421	Crack	£40	3	<3	0	0	41	1.3	0
	S644257	Cocaine	£5	2	<3	0	0	15	0	0
	S736170	Crack	£20	1	<3	0	43	172	4	1.3
	S705542	Crack	£20	1	<3	0	7.2	58	2.2	2.2
	S693937	Crack	£5	1	<3	0	0	3.3	0	0
	S737724	Cocaine	£50	5	<3	0	0	0	0	0
	S699482	Crack	0.2g	1	<3	1.5	29	133	4.4	0
	S768695	Crack	£20	1	<3	1.7	126	261	32	0
	S705900	Cocaine	1 Line	2	<3	0	0	8	0	0
j	S739097	Crack	lg	3	<3	0	1.9	4.3	0	0
	S705896	Cocaine	1 Line	3	<3	0	0	16	0	0
	S694817	Crack	4 Pipes	3	<3	0	1.1	14	1.7	0
	S736116	Crack	£10	1	<3	0	6	36	12	0
	S705756	Crack	2 Rocks	5	<3	0	0	1.5	0	0
J	S632493	Crack	1 Stone	1	<3	0	0	8.8	0	0
	S736433	Cocaine	£40	3	<3	0	5.3	109	4.3	0
	S644256	Cocaine	£5	3	<3	0	0	18	0	0
	S702190	Cocaine	2 Lines	2	<3	0	0	23	0	0
	S693928	Crack	1 Pipe	1	<3	0	17	126	3.7	0
	S706133	Crack	£10	4	<3	0	3.4	18	14.7	0
	S702118	Crack	£10	1	<3	0	6.8	67	0	0
	S693929	Crack	£10	2	<3	0	0	16	0	0
	S633268	Crack	£20	2	<3	0	0	16	0	0
	S698969	Crack	£10	1	<3	0	0	41	0	0
	S653859	Cocaine		2	<3	2.8	14.7	160	15	0
	S706697	Cocaine		1	<3	0	28	275	32	0
	S782767	Crack	£20	1	<3	0	2.9	24	0	0
	S641474	Crack	£5	2	<3	0	7.2	42	0	0
	S614055	Crack		0	<3	0	3	16	2.9	0
	COZ 0879/06				<3	0	89	461	12	0
	S696996	Crack	0.8g	1	<3	3.1	246	715	88	8.1
	S768688	Crack	£10	1	<3	0	18	104	3.6	0
,	S698620	Cocaine	1 Line	2	<3	0	0	3.3	0	0
	S693930	Crack	£10	1	<3	0	2.4	71	1	0
	S786617	Crack	£20	1	<3	0	9.2	60	2.6	0
	S645746	Crack	£20	1	<3	0	0	14	0	0
	S603058	Crack	£10	2	<3	0	0	14	3.7	0
	S706839	Crack	£20	0	<3	0	6.5	101	2.8	0
	S643631	Crack		2	<3	0	0	3.1	1.1	0
	S639076	Cocaine	3g	3	<3	0	0	0	0	0
	S754343	Crack	£5	1	<3	0	1.3	16	0	0
	S745629	Crack	1 Pipe	1	<3	1.9	70	261	56	17
	S707068	Crack	2 Rocks	2	<3	0	0	11	0	0
|	S610234	Crack	0.5g	2	<3	0	0	54	0	0
	S638760	Cocaine		1	<3	0	8.9	61	34	0
1	S616822	Cocaine	1 Line	3	<3	0	0	23	4	0
	COZ 0844/06				<3	0	0	0	0	0

	Barcode	Drug Declared	Quantity	Days Since Drug Use	Apparent AEME by EIA (ng/mL)	LC-MS/MS (ng/mL) AEME	EME	BZE	COC	CE
	S468000	Cocaine	£10	3	<3	0	0	60	9.1	0
i	S748357	Crack	£30	1	<3	0	0	11	13	0
!	S637267	Cocaine	0.2g	3	<3	0	2.5	41	0	0
	S501536	Crack	O.lg	3	<3	0	0	15	0	0
	S877191				<3	0	1.7	42	46	0
	S735320	Cocaine	£20	1	<3	0	3.9	61	24	0
	S653033	Cocaine	£2	3	<3	1.3	3	85	14	0
	S735316	Crack	£30	3	<3	0	11	73	22	0
	S696749	Crack	1 Pipe	3	<3	0	9.6	131	46	0
	S786368	Crack		4	<3	0	0	11	4.6	0
	S745626	Crack	£10	1	<3	1	138	177	16	0
	S639548	Crack	£20	1	<3	0	34	419	11	0
	COZ 0841/06				<3	0	0	0	0	0
:	S735551	Crack	£10	1	<3	0	13	216	3.2	0
	S754316	Crack	£30	1	<3	0	23	369	7.2	6.1
	S735484	Crack	£20	1	<3	0	74	208	38	1.4
	S706700	Crack		1	<3	0	9.2	70	2.9	0
	COZ 0827/06				<3	0	0	0	0	0
	S637387	Crack	£10	3	<3	0	0	2	0	0
	S768669	Cocaine	£50	1	<3	2.2	39.6	163	122	2.3
	S639547	Crack	£30	1	<3	0	4.9	219	0	0
	COZ 0914/06				<3	0	0	1.3	0	0
	COZ 0926/06				<3	0	1.2	40	1.2	0
	S653858	Cocaine	£2	2	<3	0	1.4	89	10	0
	1215261				<3	0	2.9	7.4	7.8	0
	S802542	Crack	£10	1	<3	0	17	75	7.4	0
	COZ 0821/06				<3	0	5.9	30	12	0
	COZ 0919/06				<3	0	2.8	54	14	0
	S707670	Crack	£10	2	<3	0	8.3	75	4.5	0
|	S647895	Cocaine		2	<3	3	33	292	5.3	0
I	S706127	Crack	£30	3	<3	3	27	196	25	0
	S640254	Cocaine	1 Pipe	7	<3	0	0	7.2	0	0
	COZ 0872/06				<3	0	0	0	0	0
	S706150	Cocaine	£10	4	<3	0	1.8	56	11	0
	S707027	Crack	£10	1	<3	1.8	4.2	33	24	0
	S705546	Crack	£20	1	<3	2.7	4.2	92	5.7	0
	S603929	Crack		1	<3	0	6	260	9	0
	COZ 0866/06				<3	0	0	0	0	0
	S737475	Crack		3	<3	0	0	2.9	0	0
	S754257	Crack	£40	5	<3	0	1.4	5.8	3.7	0
	S754253	Crack	1 Pipe	2	<3	0	1.7	14.7	8.8	0
	S782769	Crack	£20	3	<3	0	0	8.3	2.3	0
	S706554	Crack	£20	2	<3	4.4	560	951	60	0
	1218379				<3	0	3.7	33	16	0
	S786616	Crack	£10	2	<3	0	14	314	2.6	0
	S699404	Crack	0.75g	2	3.0	2.9	215	507	137	0
	S844287				3	1.3	0	14.6	101	0
	COZ 0874/06				3	0	0	19	6.4	0
	S706138	Crack	£10	2	3.0	0	0	27	1.1	0
	S708410	Crack		1	3.1	2.3	2.0	228	0	0
	S738251	Cocaine	£10	2	3.1	1.8	7.7	170	5.8	0
	COZ 0856/06				3.1	1.6	0	46	10	0
	S718765	Crack	1 Pipe	1	3.1	0	5.9	49	0	0
	1201162				3.2	0	36	116	48	1.6
	S705147	Cocaine	£20	1	3.4	0	77	276	26	0

Barcode	Drug Declared	Quantity	Days Since Drug Use	Apparent AEME by EIA (ng/mL)	LC-MS/MS (ng/mL)				
					AEME	EME	BZE	COC	CE
S768576	Crack	£20	0	3.4	2.1	26	257	21	0
COZ 0858/06				3.4	0	8.7	145	3.9	0
S768795	Crack	£25	1	3.5	0	1.6	31	1.1	0
S796760	Crack		1	3.5	4.5	53	329	94	0
S735019	Crack	£60	3	3.6	0	0	5.8	0	0
S745733	Cocaine	2 Lines	2	3.6	0	3.4	51	6	0
S646905	Crack	0.5 Rock	1	3.6	0	0	126	0	0
S776243	Crack	0.25 Rock	0	3.6	3.5	26	253	216	0
S644728	Crack	1 Pipe	2	3.6	0	13	107	11	0
1225034				3.6	0	0	0	0	0
S778018	Crack	£10	2	3.6	0	5.1	34	2.3	0
S844284				3.7	3.1	21	164	89	0
COZ 0842/06				3.7	0	0	0	8	0
S706141	Crack	£20	1	3.8	0	0	38	0	0
S776345	Crack		1	3.8	1.4	96	245	43	9.1
S735796	Cocaine	2 Lines	6	3.9	0	0	2.9	0	0
S707318	Crack	3 Rocks	1	3.9	5.5	12	211	28	0
S603646	Crack	1 Pipe	4	3.9	0	1.5	6.4	1	0
S772864	Crack		0	4.0	2.5	44	206	35	0
S782871	Crack	£20	1	4.0	1.1	8.2	128	22	0
S700608	Crack	£20	1	4.2	6.7	26	661	76	4.3
COZ 0917/06				4.2	0	0	1.7	0	0
S706140	Crack	£5	2	4.3	2	0	342	0	0
S718771	Crack	£10	1	4.3	0	78	172	7.6	0
S699476	Crack	0.2g	2	4.3	0	4.2	38	4.9	0
S467998	Crack		2	4.4	0	7.4	74	7.0	0
S529693	Cocaine	1 Line	5	4.4	0	0	5.5	1.9	0
S746908	Crack	£20	1	4.7	0	9	47	66	0
COZ 0833/06				4.7	0	0	0	0	0
S776349	Crack		4	4.8	0	0	3.8	1.3	0
S501310	Crack	0.8g	3	4.9	0	35	81	8.1	0
COZ 0836/06				4.9	0	0	0	0	0
S467977	Crack	2 Pipes	4	4.9	0	31	76	27	0
1225290				5	2.7	174	261	33	0
1202200				5	5.4	23	59	113	0
S472084	Crack	0.4g	1	5.1	0	6	44	3.6	0
S745874	Crack	£5	1	5.1	0	1.9	12	2.2	0
COZ 0922/06				5.2	0	0	3.9	4.4	0
S706143	Cocaine		0	5.3	4.7	286	763	36	46
S776117	Crack	2 Pipes	1	5.4	0	1.6	16	0	0
COZ 0918/06				5.4	0	0	36	27	0
S706147	Crack	£20	2	5.4	4.1	0	218	1.3	0
S647206	Crack		1	5.5	8.4	83	445	359	0
S706146	Crack	£5	1	5.5	8	47	412	35	0
S782481	Crack	0.5 Rock	3	5.8	0	7.3	65	28	0
S697493	Crack		1	5.8	5.3	9.1	191	15	0
S707306	Crack	6 Rocks	1	5.9	14	51	413	127	0
S735479	Crack		1	6.1	6.4	30	137	72	0
S768681	Crack	£10	2	6.2	1.5	11	53	18	1.3
S813044	Crack	0.5g	1	6.3	0	0	9	0	0
S796552	Cocaine	£10	1	6.4	0	4.5	21	1.3	2.7
S754322	Crack	£10	1	6.6	6	51	586	66	0
S776423	Crack		1	6.9	0	73	229	25	24
COZ 0840/06				7	5.8	6.7	51	38	0

	Barcode	Drug Declared	Quantity	Days Since Drug Use	Apparent AEME by EIA (ng/mL)	LC-MS/MS (ng/mL) AEME	EME	BZE	COC	CE
;	S735339	Crack	2 Pipes	2	7.2	0	1.2	8.1	0	0
!	S632489	Crack	0.7g	1	7.4	4.8	29	415	171	0
	S636051	Crack		1	7.5	5.3	102	660	55	0
	COZ 0849/06				7.5	5.9	4.9	107	322	0
	S772494	Crack	£40	1	7.8	0	1.0	16	3.2	0
	1219049				8.1	0	1.3	9.7	22	0
	S471160	Cocaine	4 Lines	3	8.2	0	1.3	46	7.7	0
	S812910	Crack	£30	1	8.3	0	19	208	36	1.4
	S796800	Crack		1	8.3	0	47	337	5.5	0
	1227037				8.4	6	76	221	88	0
	S861860				8.6	2.9	14	138	158	0
	S644530	Cocaine	£100	5	8.6	9.4	8.6	222	98	0
	S647220	Crack		1	[illegible]	4.7	26	82	41	0
	S787110	Crack	1 Rock	1	8.8	0	27	238	100	0
i	1218380				[illegible]	2.7	295	353	49	0
	S772727	Crack		1	8.8	7.8	90	307	154	0
	S775655	Crack		2	8.8	1.6	3.1	69	18	0
	S754354	Cocaine	1 Line	3	8.9	0	1.6	11	1.9	0
	S772285	Crack	£5	1	8.9	0	7.8	46	0	0
'	S472086	Crack	£20	1	9.4	0	3.2	85	0	0
i	S706129	Cocaine	£25	1	9.6	13	43	1008	205	2.6
	S812901	Crack	£20	1	9.9	0	22	105	20	0 I
	S749911	Cocaine	1 Line	6	9.9	0	0	8.2	5.2	0
,	S775545	Crack	£100	1	9.9	0	26	103	28	0
	S776394	Crack	£10	1	10	0	30	61	12	0
	S776083	Crack	3 Spliffs	2	11	5.7	52	201	73	0
	S715231	Crack	£40	3	11	11	22	94	58	0
	S813032	Crack	£20	2	11	0	0	9.9	2.1	0
	S768941	Crack	1 Rock	0	11	0	5.9	62	23	6.6
	S768874	Crack		2	11	0	79	302	11	0
	S692665	Cocaine	£20	1	11	14	212	666	325	0
	1202191				11	8.8	12	199	199	0
	S754319	Crack		4	12	0	1.6	8.3	2.7	0
	S802691	Crack	2 Pipes	1	12	0	22	113	23	0
	S775628	Crack		3	13	0	0	8.7	4.3	0
	S776388	Crack		1	14	0	71	131	19	0
	S736130	Crack	£30	1	14.8	14	101	761	60	63
	S802969	Cocaine	2 Lines	4	15	0	0	7.2	4.5	0
i	S736168	Crack	1 Pipe	1	16	13	15	70	44	0
	S735190	Cocaine	2 Lines	2	17	0	7	56	33	3.9
	1210049				17	12	12	238	288	0
	S768935	Crack	£20	4	17	1.3	127	458	42	0
	S702428	Crack	£10	0	17	21	52	390	236	0
	S706534	Crack	£10	2	18	6.4	55	509	42	0
	S736174	Crack	£10	1	18	10	216	812	457	0
	S816288	Cocaine	1 Line	4	18	0	1.5	23	4.3	0
	S812903	Crack	3 Rocks	1	19	8.4	34	195	63	0
	S813029	Crack	£50	2	19	0	0	3.6	1.3	0
	S754325	Crack	£10	2	19.8	10	7.2	274	70	0
	S707495	Crack	£10	1	21	26	139	1946	297	34
	S644729	Crack	1 Rock	3	21	23	230	649	192	0
	S782856	Crack	£10	1	22	21	203	1537	1406	0
	S702468	Crack	£20	2	22	17	190	1570	318	0
	S739046	Crack	£40	0	23	22	140	562	438	0
	S777302	Crack		1	24	23	118	632	289	0

	Barcode	Drug Declared	Quantity	Days Since Drug Use	Apparent AEME by EIA (ng/mL)	LC-MS/MS (ng/mL) AEME	EME	BZE	COC	CE
	S693944	Crack	£5	2	25	19.6	14	342	15	0
i	S618766	Crack	1 Pipe	2	26	3.2	45	233	20	0
	S645680	Crack	£30	1	26	14	69	791	85	0
	S644272	Crack	£10	1	26	22	102	518	417	0
	S736156	Crack	£10	1	29	28	111	799	676	0
'	S782870	Crack	£20	1	32	15	93	307	183	0
!	S715361	Crack		0	32	29	4.4	32	38	0
	S706536	Cocaine	£40	1	33	22	10	232	11	0
	S750051	Cocaine	1 Line	1	33	6.9	39	507	399	0
	S796863	Crack	£2.50	2	34	14.9	27	195	75	0
	S647885	Crack	£20	0	34	14.9	22	501	22	0
	S706013	Crack	4 Pipes	2	38	53	16	982	70	0
	S737507	Cocaine		1	39	69	446	3772	1479	553
	S746914	Crack	1 Pipe	1	43	19.5	37	348	467	0
	S739096	Crack		1	51	53	410	1278	695	71
	S735183	Cocaine	3 Lines	2	53	10	31	209	131	0
	S736110	Crack	£30	0	58	43	114	762	1998	2.3
	S812920	Crack	2 Rocks	1	58	10	65	381	65	0
	S736143	Crack	£45	1	60	64	1204	2751	316	0
	S754214	Crack	£20	1	67	64	128	903	1800	0
	S706139	Crack	£80	1	75	104	211	2076	799	0
	S647217	Crack		1	90	95	103	1340	992	o 1
	S772861	Crack		1	90	30	163	841	1127	25
|	S754305	Crack	£10	0	90	74	433	2207	1123	0
	S738567	Cocaine	2 Lines	1	94	58	36	2195	180	2.1
	S776291	Crack	0.5g	1	95	48	330	1629	656	0
	COZ 0915/06				101	40	61	1356	620	0
	S647864	Crack		3	104	61	35	407	65	0
	S707463	Crack	1 Pipe	1	105	69	221	2871	831	0
	S738284	Crack	£20	0	107	52	365	2381	614	0
	S746618	Crack	1 Rock	0	113	117	44	339	454	0
	S772482	Crack	£200	1	120	46	247	768	147	0
	S812923	Crack	1 Rock	0	121	38	68	731	543	0
	COZ 0878/06				139	62	343	1108	495	0
	S700668	Cocaine	£20	0	158	41	201	1577	243	116
	S781645	Crack	£5	1	160	80	232	1003	1603	0
	S746974	Crack	0.5g	1	178	123	135	1107	2075	0
	COZ 0813/06				212	221	213	2029	5292	0
	S746912	Crack	£20	1	>300	296	287	3057	710	0
j	S633326	Crack		1	>300	118	155	1471	2316	0
i	S636717	Crack	£2	2	>300	499	396	5037	1852	0
	S603071	Crack	£10	4	>300	825	909	3595	6593	0
	COZ 0925/06				>300	75	176	589	169	37
	111317	Crack	O.lg	2	>300	647	175	1878	895	45
	S707007	Crack	£60	1	>300	919	665	3481	889	0
	S775509	Crack		1	>300	989	1063	3668	3058	9.9
	S735330	Cocaine	£30	1	>300	718	3161	8045	6557	1
	S644258	Cocaine	£20	0	>300	357	354	2170	972	0
	S775612	Crack		1	>300	948	290	855	1747	0
i	S644928	Crack	£50	1	>300	406	956	4166	2308	3.2
	S778095	Crack	2 Rocks	1	>300	441	1834	3901	4275	0

Table 6:3 EIA and LC-MS/MS results of oral fluid clinical samples

A summary of the frequency of analyte detection, the average concentration, and the range of concentrations measured is presented in Table 6:4. Only concentrations above the LC-MS/MS LLOQ of 5ng/mL were taken into consideration.

Analyte	Frequency	Average Concentration (ng/mL)	Concentration Range (ng/mL)
Benzoylecgonine	246	441	0-8045
Cocaine	170	256	0-6593
EME	163	86	0-3161
AEME	84	36	0-989
Cocaethylene	16	4	0-553

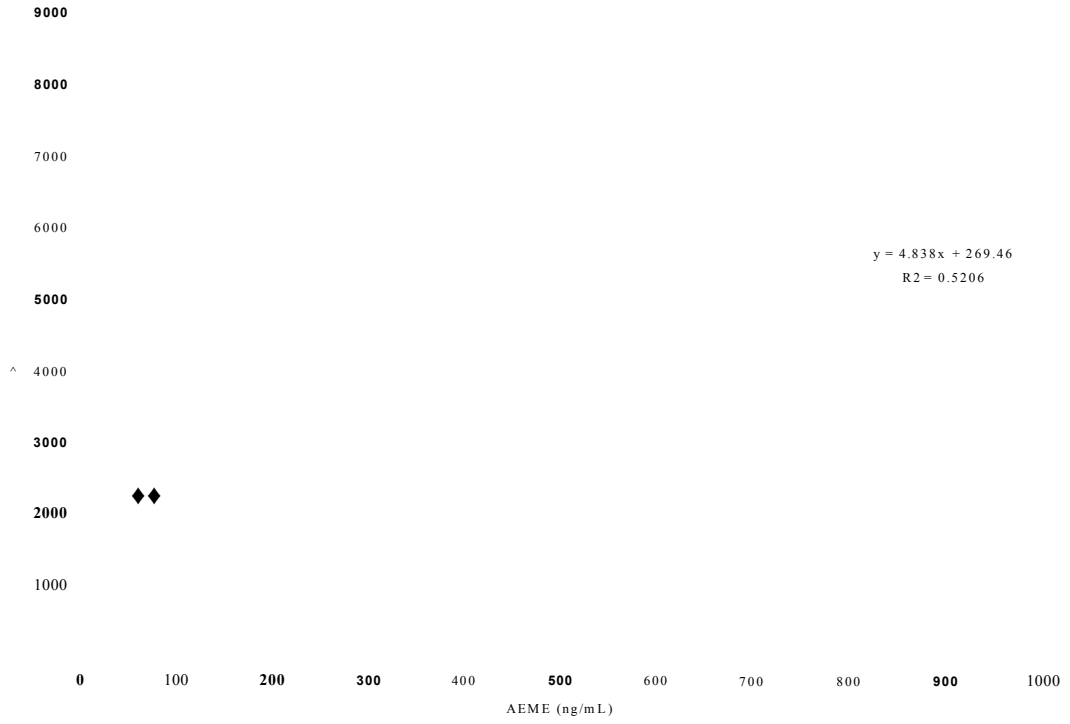
Table 6:4 Summary of LC-MS/MS data

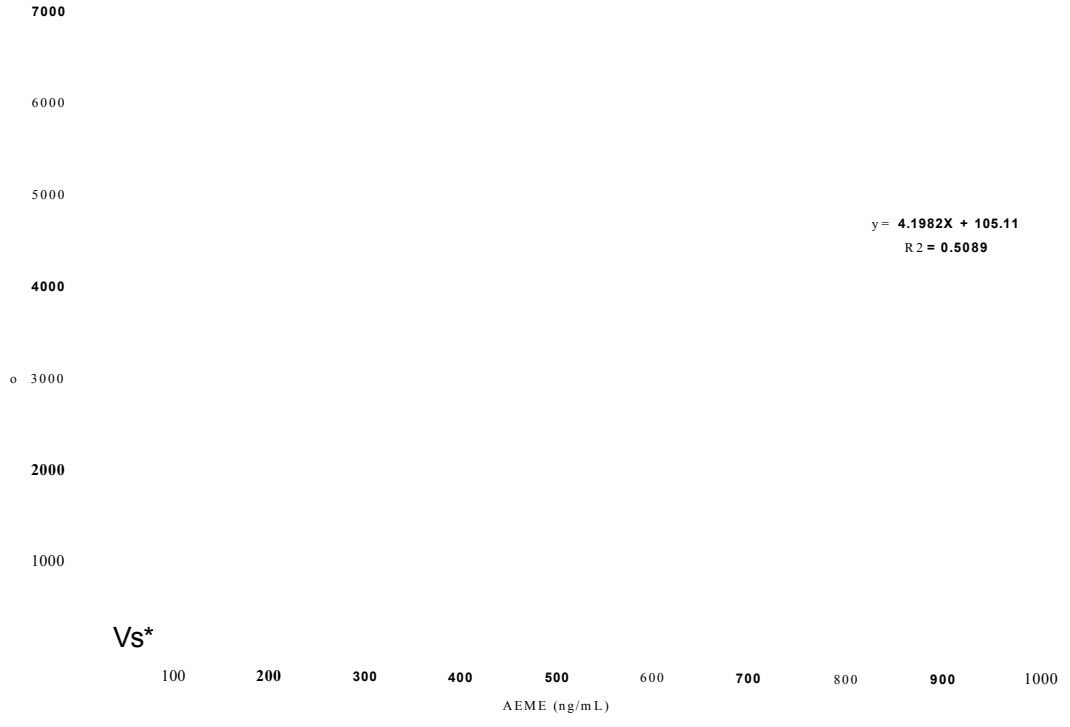
Out of 270 samples analysed the total number of samples positive for AEME by LC-MS/MS above the LLOQ of 5ng/mL was 84. The concentrations exceed any other reported concentrations in clinical studies from a drug using population. There are only two studies known which have quantified AEME in oral fluid specimens obtained from drug treatment clinics and forensic cases. A small study of only six oral fluid samples detected AEME by GC-MS at concentrations of 5-18ng/mL [16]. A larger study of 130 oral fluid samples from 16 cocaine users found that five samples contained measurable quantities of AEME by LC-MS/MS at 25-143ng/mL, with an average of 96ng/mL [17]. Other investigations into the concentrations of AEME in oral fluid have included controlled dose administration of cocaine base by smoking. One study administered 40mg of cocaine base to 7 subjects and found that the highest concentrations of AEME, 558-4374ng/mL, were achieved in the first sample which was collected after two minutes. Concentrations then declined below the LOD of the method between 10 and

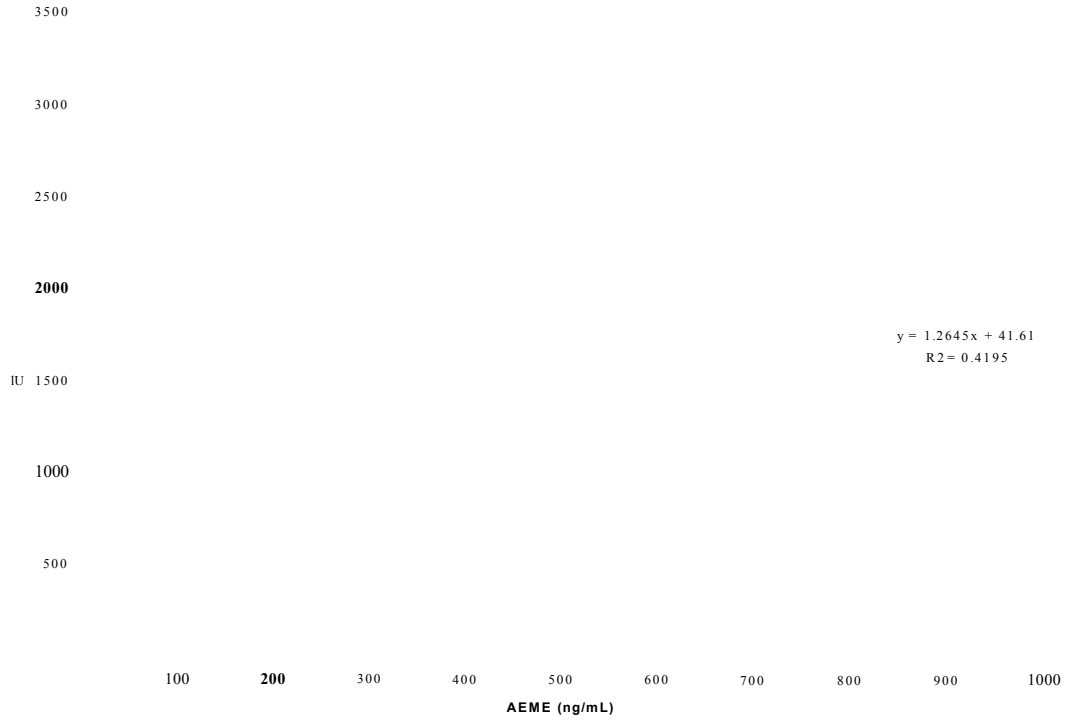
120 minutes [13]. A similar study administered 42mg of cocaine base by smoking to 6 subjects, peak concentrations of 51-775ng/mL were also achieved in the first sample which was collected after 5 minutes. Concentrations declined below the LOD of the assay between 10 and 60 minutes [18]. Despite the equivalent doses being administered in both studies, the peak concentrations of AEME were significantly lower in the second study. This is likely to be a result of the longer delay in collecting the first sample. When the 5 minute time point was compared for both studies the concentrations were similar.

In samples where AEME was present, above the LLOQ of 5ng/mL, EME was present (6.7-316 Ing/mL) in all but two of the samples, while cocaine and benzoylecgonine were always present at 11-6593 and 32-8045ng/mL respectively.
Cocaethylene was only detectable in a small number of samples, and was always accompanied by EME, benzoylecgonine and cocaine. EME was never found in isolation it was always accompanied by benzoylecgonine. A similar pattern was seen for cocaine where benzoylecgonine was also present in all but one sample.

The concentrations of analytes present in the samples were compared to one another using a scatter plot, their coefficient of determinations were calculated to identify any correlation between pairs of analytes. The plots are shown in Figure 6:3.


9000
8000
7000
6000
y = 4.838x + 269.46
R2 = 0.5206
5000
^ 4000
3000
2000
1000
0
100
200
300
400
500
600
700
800
900
1000
AEME (ng/mL)


7000
6000
5000
y= 4.1982X + 105.11
R2 = 0.5089
4000
o 3000
2000
1000
Vs*
100
200
300
400
500
600
700
800
900
1000
AEME (ng/mL)


3500
3000
2500
2000
lU 1500
1000
500
y = 1.2645x + 41.61
R2 = 0.4195
100
200
300
400
500
600
700
800
900
1000
AEME (ng/mL)


7000
6000
5000
4000
o 3000
2000
1000
0
y = 0.6836x - 47.249
R2 = 0.6065
1000
2000
3000
4000
5000
6000
7000
8000
9000
Benzoylecgonine (ng/mL)

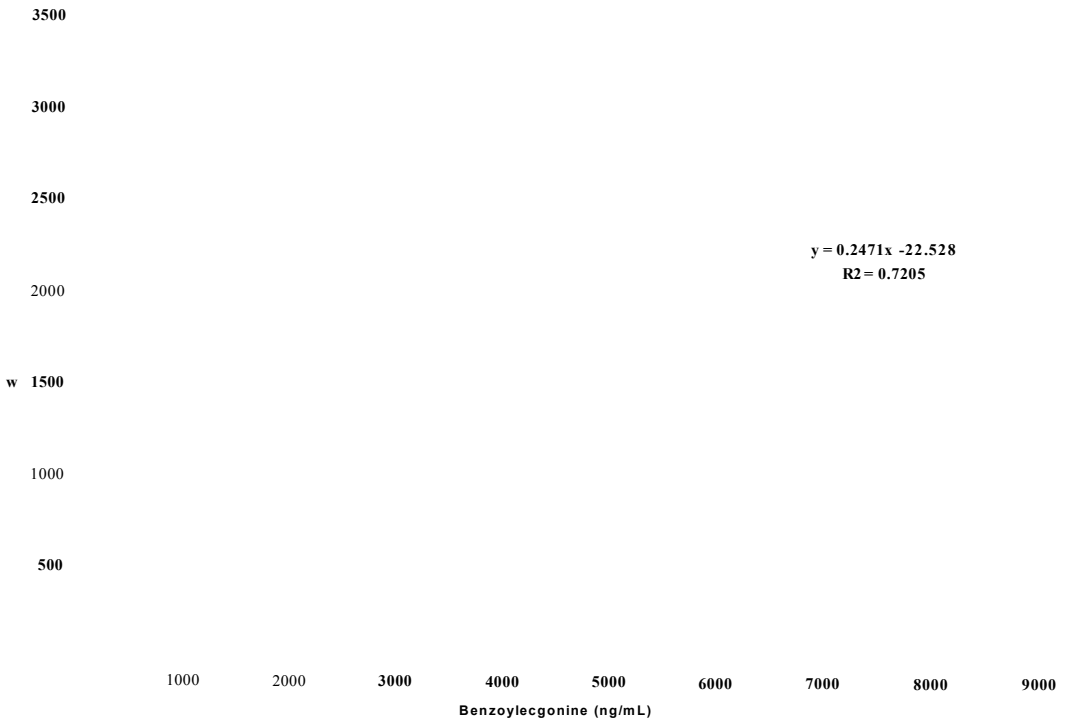


Figure 6:3 Scatter plots to establish correlation between analyte concentrations

No significant correlation was found, the highest coefficient of determination was 0.72 which was produced by the comparison of benzoylecgonine and EME. All the other

analytes showed only moderate correlation towards each other, with values between 0.42 and 0.61. These results are similar to those of Lewis et al who found no correlation between AEME and cocaine, benzoylecgonine or EME concentrations in a variety of biological matrices [19]

Looking at the raw data for AEME it was observed that in general its presence was accompanied by reasonably high concentrations of benzoylecgonine and cocaine.

6.4.3 Self Report Data Analysis

Two hundred and thirty samples were provided with self report information, from this it was established that 184 declared the used of crack cocaine specifically, 17 declared the use of powder cocaine only, and 29 of the cases simply declared 'cocaine' making the distinction between the type of cocaine impossible.

The number of samples from cocaine hydrochloride users was very low and is likely to be a result of the study population. Samples were obtained from drug rehabilitation clinics and the criminal justice sector, and so the incidence of cocaine hydrochloride use in this population is much lower in comparison to crack cocaine [20].

AEME was detected in three of the samples from donors declaring powder cocaine use, indicating an inconsistency in the reporting of the type of cocaine use.

It was not possible to compare the quantity of drug consumed with the concentrations of analytes present in the oral fluid sample due to the different ways in which the quantities were reported. Cost was the most common method of reporting quantity where an average £22 was spent (range £2-£200), however this can represent differing drug weights due to the variation in prices across the country as well as the purity of drug present.

Table 6:5 summaries the data in relation to the number of days following drug use. The range was 0-7 days with the average calculated to be 1.7 days. Cocaethylene was not detectable after 2 days, and AEME and EME were detectable for up to 5 days. Cocaine and benzoylecgonine were detectable up to 6 and 7 days respectively, however the concentrations were approaching the LLOQ.

Days Since Use	Number of Samples	Frequency					Peak Analyte Concentration (ng/mL)				
		AEME	EME	BZE	COC	CE	AEME	EME	BZE	COC	CE
0	18	11	16	18	16	3	357	433	2381	1998	116
1	118	48	92	117	84	11	989	3161	8045	6557	553
2	47	10	25	44	25	1	647	560	5037	1852	45
3	28	3	9	24	11	0	61	230	649	192	0
4	11	1	3	10	5	0	825	909	3595	6593	0
5	5	1	1	3	1	0	9	9	222	98	0
6	2	0	0	1	1	0	0	0	8	5	0
7	1	0	0	1	0	0	0	0	7	0	0

Table 6:5 Summary of analyte frequency and peak concentrations up to 7 days following drug use

Only general assumptions can be made from the data generated from the self report information. The analyte concentrations were at their highest at day 1 and from that point onwards the concentrations declined until day 4 where a significant rise in peak concentration was observed. This was a result of one particular sample which gave unusually high concentrations for each analyte.

The data produced from previous controlled dose studies indicates a short detection time for AEME of less than 120 minutes [13, 18]. However the data produced here provides evidence that AEME is detectable for a longer period of time, possibly up to

48 hours following smoked cocaine use. The detection times can only be rough estimates in this study due to many factors such as inconsistencies when dealing with self report information, frequency of an individual's drug use, and the different rate of metabolism of each person. Interpretation of concentrations and detection times of AEME is made more problematic due to the reported variability in the pyrolysis of cocaine to AEME at different smoking temperatures and using different smoking devices [21-23].

The self report data provided in this study proved to be reasonably reliable. Overall the comparison of the self reported declarations with the analytical results shows 95% concordance based on the presence of benzoylecgonine in the oral fluid samples up to 7 days after declared use. However the information becomes less consistent when looking specifically at the concentrations of analytes present compared to the number of days since drug use. An example of this is where crack cocaine use was declared four days prior to sample collection and the concentrations of the analytes present were more consistent with those likely to be found in samples collected the same day or the day after drug use.

Other inconsistencies were found with the type of cocaine reported, three of the samples in which only powder cocaine use had been reported were also found to contain AEME.

6.5 Conclusion

This is the largest reported study of clinical samples in which the concentration of AEME is measured in oral fluid. The AEME immunoassay was shown to be highly sensitive and specific in the detection of AEME, with good correlation to LC-MS/MS. The concentrations of AEME in oral fluid quantified by LC-MS/MS are the highest reported to date in real life clinical samples, and have provided an indication of the

possible detection times of AEME in oral fluid. Although the presence of AEME can be used to positively identify the route of cocaine administration as smoking, its absence cannot rule it out. The use of self report data has also shown to be a useful tool in establishing an individuals recent drug use, however it is not infallible and is best supported by the addition of analytical data to give a more reliable overall picture.

6.6 References

1. Darke, S., *Self-Report Among Injecting Drug Users: A Review.* Drug and Alcohol Dependence, 1998. 51: p. 253-263.
2. Schumacher, J.E., et al., *Validity of Self-Reported Crack Cocaine Use Among Homeless Persons in Treatment.* Journal of Substance Abuse Treatment, 1995. 12: p. 335-339.
3. Lu, N.T., B.G. Taylor, and K.J. Riley, *The Validity of Adult Arrestee Self-Reports of Crack Cocaine Use.* American Journal of Drug and Alcohol Abuse, 2001. 27: p. 399-419.
4. McGregor, K. and T. Makkai, *Self-Reported Drug Use: How Prevalent is Under-Reporting?* Trends and Issues in Crime and Criminal Justice, 2003. 260: p. 1-6.
5. Sherman, M.F. and G.E. Bigelow, *Validity of Patients' Self-Reported Drug Use as a Function of Treatment Status.* Drug and Alcohol Dependence, 1992. 30: p. 1-11.
6. Kilpatrick, B., et al., *Drug Use, SelfReport and Urinalysis.* Drug and Alcohol Dependence, 2000. 58: p. 111-116.
7. Preston, K.L., K. Silverman, and C.R. Schuster, *Comparison of Self-Reported Drug Use With Quantitative and Qualitative Urinalysis for Assessment of Drug Use in Treatment Studies.* NIDA Research Monographs, 1997.167: p. 130-145.
8. Dankwardt, A., *Immunochemical Assays in Pesticide Analysis,* in *Encylopedia of Analytical Chemistry.* 1998.
9. Kroener, L., F. Musshoff, and B. Madea, *Evaluation of Immunochemical Drug Screening of Whole Blood Samples. A Retrospective Optimization of Cutoff Levels after Confirmation-Analysis on GC-MS and HPLC-DAD.* Journal of Analytical Toxicology, 2003. 27: p. 205-212.
10. Lachenmeier, K., F. Musshoff, and B. Madea, *Determination of Opiates and Cocaine in Hair Using Automated Enzyme Immunoassay Screening Methodologies Followed by Gas Chromatographic-Mass Spectrometric (GC-MS) Confirmation.* Forensic Science International, 2006. 159: p. 189-199.
11. Segura, J., et al., *Immunological Screening of Drugs of Abuse and Gas Chromatographic-Mass Spectrometric Confirmation of Opiates and Cocaine in Hair.* Journal of Chromatography B, 1999. 724: p. 9-21.

12. Mao, C.-L., et al., *Development of an Enzyme Linked Immunosorbent Assay for Fentanyl and Applications of Fentanyl Antibody Coated Nanoparticles for Sample Preparation.* Journal of Pharmaceutical and Biomedical Analysis, 2006. 41: p. 1332-1341.

13. Jenkins, A.J., J.M. Oyler, and E.J. Cone, *Comparison of Heroin and Cocaine Concentrations in Saliva with Concentrations in Blood and Plasma.* Journal of Analytical Toxicology, 1995.19: p. 359-374.

14. Kato, K., et al., *Cocaine and Metabolite Excretion in Saliva Under Stimulated and Nonstimulated Conditions.* Journal of Analytical Toxicology, 1993. 17: p. 338-341.

15. Kim, I., et al., *Sensitivity and Specificity of the Cozart Microplate EIA Cocaine Oral Fluid at Proposed Screening and Confirmation Cutoffs.* Clinical Chemistry, 2003. 49: p. 1498-1503.

16. Kintz, P., et al., *Evidence of Crack Use by Anhydroecgonine Methyl Ester Identification.* Human and Experimental Toxicology, 1997. 16: p. 123-127.

17. Dams, R., et al., *Oral Fluid as an Alternative Matrix to Monitor Opiate and Cocaine Use in Substance-Abuse Treatment Patients.* Drug and Alcohol Dependence, 2007. 87: p. 258-267.

18. Cone, E.J., J. Oyler, and W.D. Darwin, *Cocaine Disposition in Saliva Following Intravenous, Intranasal, and Smoked Administration.* Journal of Analytical Toxicology, 1997. 21: p. 465-475.

19. Lewis, R.J., et al., *Determination of Cocaine, its Metabolites, Pyrolysis Products, and Ethanol Adducts in Postmortem Fluids and Tissues using Zymark Automated Solid-Phase Extraction and Gas Chromatography-Mass Spectrometry.* Journal of Chromatography B, 2004. 806: p. 141-150.

20. Gossop, M., et al., *Changes in Use of Crack Cocaine after Drug Misuse Treatment: 4-5 Year Follow-up Results from the National Treatment Outcome Research Study (NTORS).* Drug and Alcohol Dependence, 2002. **66**: p. 21-28.

21. Nakahara, Y. and A. Ishigami, *Inhalation Efficiency of Free-Base Cocaine by Pyrolysis of 'Crack' and Cocaine Hydrochloride.* Journal of Analytical Toxicology, 1991. 15: p. 105-109.

22. Perez-Reyes, M., et al., *Free-Base Cocaine Smoking.* Clinical Pharmacology and Therapeutics, 1982. 32: p. 459-465.

23. Martin, B.R., L.P. Lue, and J.P. Boni, *Pyrolysis and Volatilization of Cocaine.* Journal of Analytical Toxicology, 1989. 13: p. 158-162.

7 Conclusions and Further Studies

A number of advances have been made in the field of forensic toxicology in recent years. An increasing number of laboratories worldwide are now routinely analysing oral fluid samples from donors in different settings such as workplace, criminal justice and rehabilitation settings [1]. The use of crack cocaine presents a great social problem, and has a high association with crime. The ability to distinguish between crack and powder cocaine would provide a valuable addition to drug treatment and criminal justice systems [2]. The identification and monitoring of crack cocaine use may also help to develop a better understanding of the prevalence and spread of crack use.

Preliminary studies carried out in this thesis involved screening a large number of samples from a drug using population, for the presence of MDMA and metamfetamine using a laboratory based EIA and a POCT device. All samples were subsequently confirmed by GC-MS. Overall the clinical sensitivity and specificity of both assays were excellent.

There is a limited amount of information available regarding the concentrations of amfetamine and MDMA present in oral fluid samples from a drug using population. Therefore the clinical data obtained from the self report study provided a significant contribution to the field of drug testing. There was good concordance between the self report data and analytical findings, although some inconsistencies were found. Overall these studies provided an understanding of the fundamental processes of point of care and laboratory based immunoassay screening, GC-MS confirmatory analysis, and data interpretation.

The development of a novel EIA was described for the identification of AEME in oral fluid. It was found that immunisation with AE, linked via its carboxyl group to the carrier protein thyroglobulin, produces antibodies highly specific for AEME without exhibiting cross reactivity to any other cocaine related compound. This was the most important factor in the development of this assay as any cross reactivity, other than to AEME or its metabolite AE, would result in the false identification of crack cocaine use. The lack of significant cross reactivity to AE was surprising considering it was used for immunisation, and its structure is similar to that of AEME. Each of the four antisera contained a high titre of antibody, with 402717 providing increased sensitivity compared to the others.

The wide polarity range of cocaine and its related compounds presents many analytical challenges. Solid phase extraction (SPE) in this study provided good recovery for the majority of the analytes except for the highly polar AE. Attempts to significantly increase the recovery of AE by performing a two step SPE method were unsuccessful despite previous reports describing marked improvements [3-5]. Further research into different types of sorbent material is required to attempt to improve the recovery of AE.

Optimum chromatographic separation by LC and GC is difficult due to the large difference in elution times for the highly polar and non polar compounds. The use of GC-MS for quantification was ruled out due to the increased amount of column maintenance required during the early studies. As time progressed the chromatography of AEME deteriorated and as a result the column required regular cutting. Other concerns regarding the reported artifact formation of AEME from cocaine in the GC injector port confirmed the decision to switch to LC-MS/MS [6]. A number different column types and mobile phases were tested to identify the optimum chromatographic

conditions. Adequate retention of EME and AE was only achievable using the phenyl column, and peak resolution was improved in many instances. The use of acetonitrile instead of methanol shortened the elution time of the later eluting non polar compounds but it also reduced analyte ionisation. The greatest analyte ionisation was achieved using low concentrations of ammonium formate adjusted to pH 3 with formic acid. AE continuously provided low peak areas and the lack of a linear calibration curve meant that accurate quantitation of AE would not have been possible. While it may have been feasible to analyse AE using a different LC-MS/MS method this was not the criteria in this study, it was important to analyse all analytes simultaneously. Further investigations to enable AE to be incorporated into the LC-MS/MS method are required.

A large number of samples from a drug using population were screened using the EIA for AEME and then confirmed by LC-MS/MS. Overall the clinical sensitivity and specificity of the EIA were excellent, concluding that the EIA successfully identifies the use of crack cocaine. Following on from this success a point of care test could be developed using this antibody to enable screening to be performed virtually anywhere. There is a limited amount of information available regarding the presence of the pyrolysis products of cocaine, particularly in the oral fluid of a drug using population [7, 8]. The large amount of clinical data generated in this thesis provides information relating to the possible detection times of AEME, peak concentrations, and the correlation of AEME with cocaine and its main metabolites. The majority of samples were provided with declarations of drug use, the degree of concordance between self reported use of cocaine and the presence of cocaine, or any of its metabolites, was 89.6%, this reduced to 34.4% when looking specifically at declared crack cocaine use and the presence of AEME. This highlights two possible scenarios, either the self reported information regarding crack cocaine use was inaccurate, or the detection of

AEME is problematic. The detection of AEME in oral fluid can be affected by various factors such as the temperature and flow rate of the smoking device, the rate of metabolism of AEME in the body, and its stability in collected oral fluid. Therefore the presence of AEME in a clinical sample can positively identify the use of crack cocaine, however, its absence does not necessarily mean that crack cocaine has not been used.

A number of studies can be undertaken following on from this work. The detection times of AEME can be investigated further by obtaining specimens from crack cocaine users over a number of days, in a controlled environment following cessation of drug use. The contribution of AE in oral fluid samples needs to be established to assess the extent of metabolism of AEME. Similarly the stability of both AEME and its metabolite in collected oral fluid also needs to be evaluated across a range of temperatures and pH values. The pH of oral fluid is known to affect the concentration of cocaine and benzoylecgonine present [9], therefore similar studies may be carried out to determine the effect of oral fluid pH on the concentration of the pyrolysis products of cocaine.

The EIA and LC-MS/MS methods have both proved to be successful in the analysis of AEME in oral fluid, and so may be applied to the analysis of additional biological fluids such as hair, blood, and urine. Full validation will be required to establish adequate sensitivity and precision for each biological matrix.

7.1 References

1. Clarke, J. and J.F. Wilson, *Proficiency Testing (External Quality Assessment) of Drug Detection in Oral Fluid.* Forensic Science International, 2005. 150: p. 161-164.
2. Liberty, H.J., et al., *Detecting Crack and other Cocaine Use with Fastpatches.* Addiction Biology, 2003. 8: p. 191-200.
3. Paul, B.D., et al., *Concentration Profiles of Cocaine, Pyrolytic Methyl Ecgonidine and Thirteen Metabolites in Human Blood and Urine: Determination by Gas Chromatography-Mass Spectrometry.* Biomedical Chromatography, 2005.19: p. 677-88.
4. Shimomura, E.T., G.D. Hodge, and B.D. Paul, *Examination of Postmortem Fluids and Tissues for the Presence of Methylecgonidine, Ecgonidine, Cocaine, and Benzoylecgonine Using Solid Phase Extraction and Gas Chromatography-Mass Spectrometry.* Clinical Chemistry, 2001. 47: p. 1040-1047.
5. Scheidweiler, K.B., et al., *Stability of Methylecgonidine and Ecgonidine in Sheep Plasma in Vitro.* Clinical Chemistry, 2000. 46: p. 1787-1795.
6. Cardona, P.S., et al., *Simultaneous Analyses of Cocaine, Cocaethylene, and their Possible Metabolic and Pyrolytic Products.* Forensic Science International, 2006. 157: p. 46-56.
7. Kintz, P., et al., *Evidence of Crack Use by Anhydroecgonine Methyl Ester Identification.* Human and Experimental Toxicology, 1997. 16: p. 123-127.
8. Dams, R., et al., *Oral Fluid as an Alternative Matrix to Monitor Opiate and Cocaine Use in Substance-Abuse Treatment Patients.* Drug and Alcohol Dependence, 2007. 87: p. 258-267.
9. Kato, K., et al., *Cocaine and Metabolite Excretion in Saliva Under Stimulated and Nonstimulated Conditions.* Journal of Analytical Toxicology, 1993. 17: p. 338-341.