The development of appropriate methods for drug analysis at the Forensic Chemistry Laboratory, Cape Town

A dissertation submitted in fulfilment of the requirements for the degree of

Master of Science

at the University of Cape Town

by

Deidré Ilana Adams

Supervisor: Associate Professor Neil Ravenscroft
Co-supervisor: Dr Denise Lourens
Co-supervisor: André Baard

October 2008

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to everyone whose assistance and advice contributed to the creation of this dissertation, and in particular the following:

Associate Professor Neil Ravenscroft, my supervisor, for his continued interest, advice and guidance;

Dr Denise Lourens, now a Principal Specialist at the Forensic Pathology Services in Kimberley, Northern Cape, for her key role in coordinating the participation of the Salt River and Tygerberg Forensic Pathology Laboratories and for her subsequent guidance;

André Baard, my supervisor at the Forensic Chemistry Laboratory, Cape Town, for his enthusiasm, editorial input and invaluable assistance with all aspects of the project;

Ierefaan Batchelor, for his assistance with the extraction of the samples that were analysed to determine the extent of drug use in Cape Town;

the National Department of Health, for the use of the Laboratory's facilities to carry out this research;

my colleagues at the Laboratory, for their support;

Michael Rennie of PM Separations in Brisbane, Australia, for his assistance with the solid-phase extraction work.

Appendix 3

Working (Linear) Range (Measured Response)

	[Conc]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Cocaine Average Response	Cocaine Std. Dev
Concentration Level 1 (LoQ)	3	0.79	0.87	0.82	0.81	0.86	0.83	0.05753
Conc Level 2	5	1.07	0.85	0.97	0.97	0.94	0.96	0.15768
Conc Level 3	10	2.66	2.80	2.62	2.76	2.81	2.73	0.10390
Conc Level 4	15	4.39	4.46	4.52	4.39	4.39	4.43	0.04879
Conc Level 5	20	5.97	5.99	6.04	6.10	5.80	5.98	0.01414
Conc Level 6	25	6.35	6.96	6.89	7.09	7.66	6.99	0.43134
Conc Level 7	30	8.79	8.71	8.79	8.82	8.64	8.75	0.05657

Regression Statistics (Based on the average)	Slope, m	0.29365
	Intercept, b	-0.14
	Corr. Coeff.	0.99

TABLE OF CONTENTS

		page
ACKNOWLEDGEMENTS		i
TABLE OF CONTENTS		ii
ABBREVIATIONS		vi
INDEX OF FIGURES		viii
INDEX OF TABLES		x
ABSTRACT		xi
CHAPTER 1	Introduction	1
1.1	Forensic science	1
1.2	Forensic toxicology	2
1.3	Background	5
1.4	Extent of drug and alcohol use in the greater Cape Town area	7
1.5	Forensic Pathology Laboratory procedures	8
1.6	Current Forensic Chemistry Laboratory procedures	8
1.7	Extraction methods	10
1.8	New methods	12
1.9	Review of extraction methods	13
1.10	Aims and objectives	16
CHAPTER 2	Materials and methods	17
2.1	Blood ethanol analysis	17
2.2	Blood ethanol sample preparation	17
2.3	Reagents for blood ethanol analysis	18
2.4	Standards for blood ethanol analysis	18
2.5	Instruments and conditions for blood ethanol analysis	19

		page
2.6	Analytical methods used in toxicology	20
	2.6.1 Celite extraction method	20
	2.6.2 Butyl acetate extraction method	21
2.7	Reagents for toxicological analysis	22
2.8	Standards for toxicological analysis	22
2.9	Instruments and conditions for toxicological analysis	23
2.10	Drugs used in method development	24
CHAPTER 3	Extent of drug and ethanol use	32
3.1	Introduction	32
3.2	Experimental protocol	32
3.3	Results and discussion	33
3.4	Conclusions	35
CHAPTER 4	Development of solid-phase extraction method	37
4.1	What is solid-phase extraction?	37
4.2	Extraction mechanisms	37
	4.2.1 Reversed phase	37
	4.2.2 Normal phase	39
	4.2.3 Ion exchange	40
	4.2.4 Copolymeric bonding	42
4.3	Solid-phase sorbents	43
	4.3.1 Sorbent properties	43
	4.3.2 Sorbent specificity	43
	4.3.3 Silica-based sorbents	43
	4.3.4 Polymeric sorbents	44
	4.3.5 Copolymeric sorbents	44
4.4	Steps in solid-phase extraction protocols	46

		page
4.5	Method development and optimization	47
4.5.1	Effect of sample application pH on compound retention	48
4.5.2	Effect of elution solvent on compound retention	49
4.6	Experimental	51
4.6.1	Drug standards and chemicals	51
4.6.2	Preparation of spiked urine samples	52
4.7	Extractions	52
4.7.1	Investigation of Clean Screen® DAU column (200 mg, 3 mℓ capacity)	52
4.7.2	Investigation of XtrackT® column (200 mg, 3 mℓ capacity)	53
4.7.3	Investigation of dimethyl formamide as "keeper" solvent	53
4.7.4	Investigation of the extraction efficiency of the FCL CTN in-house methods compared to the XtrackT® column SPE method	54
4.8	Results and discussion	57
4.8.1	Clean Screen® DAU column (200 mg, 3 mℓ capacity)	57
4.8.2	XtrackT® column (200 mg, 3 mℓ capacity)	58
4.8.3	Dimethyl formamide as "keeper" solvent	60
4.8.4	The extraction efficiency of the FCL CTN in-house methods compared to the XtrackT® column using the SPE method	61
4.9	Conclusions	63

CHAPTER 5	Validation of the SPE method	65
5.1	Validation procedure	65
5.2	Specificity	66
5.3	Accuracy	67
5.4	Precision	69
5.4.1	Repeatability	70
5.4.2	Intermediate precision	71
5.5	Linearity of method	73

		page
5.6	Limit of detection	76
5.7	Limit of quantitation	77
5.8	Conclusions	78
CHAPTER 6	Conclusions and future work	81
6.1	Conclusions	81
6.2	Future work	83
REFERENCES		85
APPENDICES		93
Appendix 1	Ethanol and tertiary butanol chromatogram	93
Appendix 2	Settings for RapidTrace analysis of acidic, basic and neutral drugs in urine and whole blood: automated method for GC or GC-MS using a 200 mg XtrackT® extraction column	94
Appendix 3	Measured responses and calculation of the sum of squares for regression statistics for cocaine	96

ABBREVIATIONS

AAFS	:	American Academy of Forensic Sciences
CSF	:	cerebrospinal fluid
DMF	:	dimethyl formamide
DNA	:	deoxyribonucleic acid
EI	:	electron impact
EM	:	electron multiplier
FCL CTN	:	Forensic Chemistry Laboratory, Cape Town
GC	:	gas chromatography
GC-MS	:	gas chromatography coupled to mass spectrometry
HPLC	:	high-performance liquid chromatography
HSRC	:	Human Sciences Research Council of South Africa
IPA	:	isopropyl alcohol
IS	:	internal standard
LC	:	liquid chromatography
LC-MS/MS	:	liquid chromatograph with tandem mass-selective detectors
LD_{50}	:	lethal dose (where 50 % of a population will die after administration of the dose)
LIMS	:	Laboratory Information Management System
LLE	:	liquid-liquid extraction
LOD	:	limit of detection
LOQ	:	limit of quantitation
MDMA	:	methylenedioxymethamphetamine
MRC	:	Medical Research Council
MSD	:	mass-selective detector
m/z	:	mass-to-charge ratio
NET	:	norepinephrine transporter
NHLS	:	National Health Laboratory Service
NPD	:	nitrogen-phosphorus detector
NSAID	:	non-steroidal anti-inflammatory drug
PM	:	post-mortem
PSDVB	:	polystyrenedivinylbenzene

rpm	:	revolutions per minute
RSD	:	relative standard deviation
SACENDU	:	South African Community Epidemiological Network on Drug Use
SIM	:	single-ion monitoring
SOFT	:	Society of Forensic Toxicologists
SPE	:	solid-phase extraction
TIAFT	:	The International Association of Forensic Toxicologists

INDEX OF FIGURES

page

CHAPTER 1

1.1	Chemical grouping of poisons	4
1.2	A typical SPE column	13

CHAPTER 3

3.1	Manner of death of the 601 cases tested	33
3.2	Drugs related to ethanol level, based on the 164 drug-positive cases for which ethanol results were obtained	34
3.3	Number of occurrences of drugs in ethanol-negative victims, based on 218 cases	34
3.4	Number of occurrences of drugs in ethanol-positive victims, based on 372 cases	35

CHAPTER 4

4.1	Example of hydrophobic retention mechanism as a result of the preferred distribution of the less polar analytes to the similarly polar stationary phase	38
4.2	Example of normal-phase retention mechanism	39
4.3	Example of cation exchange retention	40
4.4	Example of anion exchange retention	41
4.5	Example of a copolymeric sorbent (anion exchange phase)	45
4.6	Example of a strong cation exchange bonding mechanism	45
4.7	Solvent polarity and elution strength	50
4.8	GC-MS chromatogram (of selected ions) of the cocaine standard (200 µg/mℓ), with the base peak at 82 m/z	54
4.9	GC-MS chromatogram (of selected ions) of the imipramine standard (200 µg/mℓ), with the base peak at 58 m/z	55
4.10	GC-MS chromatogram (of selected ions) of the lorazepam standard (30 µg/mℓ), with the base peak at 239 m/z	55

page

4.11	GC-MS chromatogram (of selected ions) of the methamphetamine standard (150 µg/mℓ), with the base peak at 58 m/z	55
4.12	GC-MS chromatogram (of selected ions) of the oxazepam standard (200 µg/mℓ), with the base peak at 205 m/z	56
4.13	GC-MS chromatogram (of selected ions) of the phenobarbital standard (200 µg/mℓ), with the base peak at 204 m/z	56
4.14	GC-MS chromatogram (of selected ions) of the secobarbital standard (250 µg/mℓ), with the base peak at 168 m/z	56
4.15	GC-NPD chromatogram of the acidic/neutral fraction from urine spiked with 10 µg/mℓ of the test mixture, and extracted from the CLEAN SCREEN® DAU column (2: imipramine, 3: lorazepam, 5: oxazepam, 6: prazepam (internal standard), 7: phenobarbital, 8: secobarbital)	57
4.16	GC-NPD chromatogram of the basic fraction from urine spiked with 10 µg/mℓ of the test mixture, and extracted from the CLEAN SCREEN® DAU column (1: cocaine, 2: imipramine, 6: prazepam (internal standard) 7: phenobarbital, 8: secobarbital)	58
4.17	GC-NPD chromatogram of the acidic/neutral fraction from urine spiked with 10 µg/mℓ of the test mixture, and extracted from the XtrackT® column (3: lorazepam, 5: oxazepam, 6: prazepam, 7: phenobarbital, 8: secobarbital)	59
4.18	GC-NPD chromatogram of the basic fraction from urine spiked with 10 µg/mℓ of the test mixture and extracted from the XtrackT® column (1: cocaine, 2: imipramine, 4: methamphetamine, 5: oxazepam, 6: prazepam (internal standard), 7: phenobarbital, 8: secobarbital)	59
4.19	GC-NPD chromatogram of the basic fraction from urine spiked with 10 µg/mℓ of the test mixture and extracted from the CLEAN SCREEN® DAU column with DMF added (1: cocaine, 2: imipramine, 4: methamphetamine, 6: prazepam (internal standard), 7: phenobarbital, 8: secobarbital)	60
4.20	GC-NPD chromatogram of the basic fraction from urine spiked with 10 µg/mℓ of the test mixture and extracted from the XtrackT® column with DMF added (1: cocaine, 2: imipramine, 4: methamphetamine, 6: prazepam (internal standard), 7: phenobarbital, 8: secobarbital)	61
4.21	Results of the comparison of the extraction efficiencies of the SPE, Celite and butyl acetate procedures (1: cocaine, 2: imipramine, 3: lorazepam, 4: methamphetamine, 5: oxazepam, 6: phenobarbital, 7: secobarbital) with error bars indicating the standard deviation of the measured recoveries	62

CHAPTER 5 **page**

5.1 Plot of regression residuals of cocaine over the linear range 75

INDEX OF TABLES

page

CHAPTER 4

4.1	Hydrophilic sorbents and structures	39
4.2	The four basic steps of an SPE procedure	47
4.3	Comparison of FCL CTN and SPE methods using extracted base peak ions analysed on GC-MS SIM	62

CHAPTER 5

5.1	Average percentage recovery and relative standard deviation (RSD; n = 3) for the seven drugs spiked at three concentration levels	68
5.2	Repeatability in terms of the RSD (n = 2) of the average percentage recoveries for the seven drugs spiked at three concentration levels and analysed on two different days	70
5.3	Intermediate precision in terms of the RSD (n = 2) of the average percentage recoveries for the seven drugs spiked at three concentration levels and analysed on two different instruments; ND = not detected	72
5.4	Sum of squares of regression statistics (slope, intercept and correlation coefficient) as well as residual regression analysis (linearity of method and linearity range)	74
5.5	Calculation of sum of squares for regression statistics for cocaine	75

ABSTRACT

The Forensic Chemistry Laboratory, Cape Town, analyses samples submitted by forensic pathologists in order to assist with determining the cause of death in cases of unnatural death. Many of these samples test positive for the presence of drugs and other toxic substances. Because of resource constraints, pathologists submit samples at their discretion and not on a routine basis. In this study, forensic and chemical aspects were combined and used as the motivation for the development of an improved extraction procedure for systematic toxicological analysis.

The scope of the study was therefore twofold. Firstly, a study was undertaken of unnatural deaths in the greater Cape Town area for which samples would not normally have been submitted. The results of this study indicated that cases of unnatural death should be submitted on a more routine basis, as 30 % of the samples tested positive for the presence of drugs. Secondly, solid-phase extraction, using spiked urine samples, was investigated as an alternative extraction procedure after the results indicated a need for a drug extraction procedure that would be able to isolate various classes of drugs during a single procedure with only one extraction cartridge.

Solid-phase extraction was compared with methods currently being used at the Forensic Chemistry Laboratory, Cape Town. The research focused on drugs that had previously been detected and included various classes of drugs with different properties. The selection comprised cocaine, imipramine, lorazepam, methamphetamine, oxazepam, phenobarbital and secobarbital, with prazepam being used as an internal standard. Clean Screen® DAU and XtrackT® columns from United Chemical Technologies were investigated for their extraction efficiency, and the use of dimethyl formamide as a "keeper" solvent, which is a solvent that prevents the evaporation of volatile components, was also studied. It was found that solid-phase extraction had a higher extraction efficiency than the current methods employed and that the XtrackT® cartridge used together with dimethyl formamide gave the highest recovery for the drugs that were spiked in urine. This column was also successfully used for the extraction of drugs from post-mortem blood, stomach contents and cerebrospinal fluid. The solid-phase extraction method was successfully validated for the extraction of drugs from urine, using an XtrackT® column, and a gas chromatograph with nitrogen-phosphorus detection was used for the determination of selectivity, accuracy, intra-assay precision, intermediate precision, linearity of method, limit of detection and limit of quantitation.

Some of the results of the study to determine the extent of drug and ethanol use, which are given in Chapter 3, have been published previously:

The International Association of Forensic Toxicologists (TIAFT) 43rd International Meeting, Seoul, Korea. 29 August – 2 September 2005. Poster presentation. The pattern of drug and alcohol use in victims of unnatural death in Cape Town, South Africa. Adams D, Baard A, Batchelor I, Lourens D, Wadee S

CHAPTER 1

INTRODUCTION

1.1 FORENSIC SCIENCE

Forensic science is the field in which scientific techniques are used to obtain evidence relevant to legal proceedings. In 1910 Edmond Locard formulated the "contact trace theory", which simply states that if two objects meet, evidence of that meeting can later be found and verified. This forms the foundation of forensic science [1].

Forensic science has come a long way since its early beginnings in the sixteenth century in Europe, when medical practitioners in army and university settings began to gather information on cause and manner of death. Writings on these topics began to appear in the late 1700s and included *A Treatise on Forensic Medicine and Public Health*, by the French physician Fodéré, and *The Complete System of Police Medicine*, by the German medical expert Johann Peter Franck [2]. One of the first uses of physical matching occurred in 1816 when a farm labourer was convicted of the homicide of a young maidservant. The police found an impression of a corduroy cloth with a sewn patch in the damp earth on the scene. This evidence corresponded exactly to the breeches of a farm labourer who had been threshing wheat nearby [2].

The basis of the investigation of a crime is the assembling of a sequence of facts that will assist the judicial process to be carried to a satisfactory conclusion. This process requires the application of various sciences to answer questions relating to biological evidence (by means of the DNA analysis of physiological fluids, for example), trace evidence, impressions such as fingerprints, footwear impressions

and tyre tracks, controlled substances, ballistics, and other evidence such as exhibits found at the scene of the crime. Such evidence is ultimately delivered to a forensic science laboratory, where skilled personnel perform analyses using analytical instrumentation.

1.2 FORENSIC TOXICOLOGY

Toxicology is the scientific study of toxins or poisons. A poison can be defined as a chemical substance that may be harmful to living organisms. It was Paracelsus who observed that anything can be a poison if it exceeds the threshold limit of an organism's ability to deal with it [3].

Toxicity is a biological concept and is usually determined by some sort of bioassay. Chemical analysis, however, is required to detect the presence of a toxin, identify it and measure its concentration, which must then be related to its known toxicity. Forensic toxicology is essentially the analysis of samples to determine the presence and concentration of drugs, alcohol and poisons in the body, followed by the interpretation of those results, which are typically used in a court of law. The use of forensic toxicology is crucial to the outcome of many legal cases, as it enables hard scientific evidence to be presented in court.

The first person to suggest a chemical method for the detection of poisons, according to Thorvald Jurgen in *The Century of the Detective*, was Dr Hermann Boerhaave [4]. His method, which was relatively unsophisticated, consisted of placing substances suspected of containing poison on red-hot coals and then testing the subsequent odours. In the early stages of forensic toxicology, arsenic was the most common poison of choice. It was known as the "poison of poisons" or as "inheritance powder", since many relatives used it to kill some aging patriarch [4].

Forensic toxicology became a scientific discipline in the nineteenth century. The first internationally acclaimed work on this subject was *Traité des poisons*, by Matieiv J B Orfila, which was published in 1813 [3]. It was the first systematic approach to the study of the chemical and physiological nature of poisons, and won its author the title of "Father of Toxicology" [3].

Another major step in the history of forensic toxicology was the development of methods for detecting the presence of plant alkaloids such as caffeine, quinine, morphine, strychnine, atropine and opium. Alkaloids leave no easily demonstrable traces in the human body and therefore require relatively complicated methods of extraction before an analysis can be performed. These poisons affect the central nervous system. Even Orfila had no success and he thought that the isolation of alkaloids from human tissues might be altogether impossible [3].

Orfila's student, Jean Servois Stas, had a different idea. In a homicide trial in 1850, a male victim showed clear chemical burns in his mouth, tongue and throat. Stas searched for three months for the agent and eventually managed to isolate nicotine from the body tissues. Using ether as a solvent, which he then evaporated to isolate the drug, he found the potent drug [5]. Several years later the extraction method was modified by F J Otto and became known as the Stas-Otto method [4].

Societies of forensics and toxicology such as the American Academy of Forensic Sciences (AAFS), The International Association of Forensic Toxicologists (TIAFT) and the Society of Forensic Toxicologists (SOFT) have been established to promote the cause of forensic toxicology, advance education in the field, and ensure knowledgeable input into standards governing the profession. Since 1991 the profession has matured in many ways. In 1996 the American Board of Forensic Toxicology launched a Forensic Toxicology Accreditation program based primarily on the SOFT/AAFS Guidelines. In 1997 New York

State passed legislation requiring the accreditation of all forensic laboratories in the public sector [6].

Compared with toxicologists in academic research or industry, forensic toxicologists face a task that is made more difficult by limited analytical material, severe time constraints, and generally limited resources at government laboratories. Forensic toxicology demands an overall analytical system designed to indicate or exclude the presence of any poison in each of the chemical groups shown in Figure 1.1. Most of the numerous screening procedures reported in the literature are too limited to permit a negative result to be reported with confidence. They are often oriented in classes or groups of drugs, despite the fact that not all known criminal poisonings involve drugs.

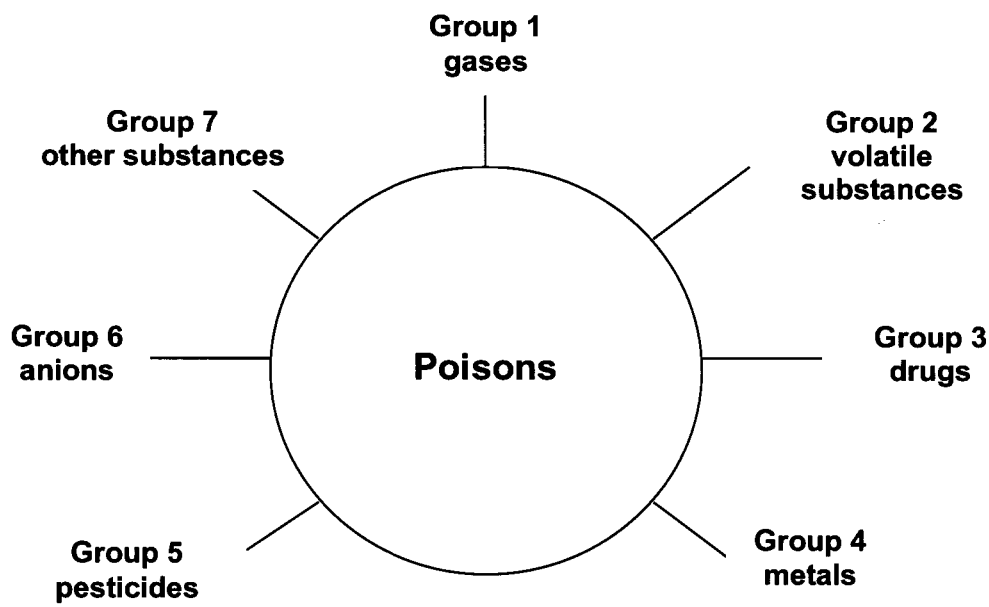


Figure 1.1 **Chemical grouping of poisons [3]**

Apart from analytical problems, the legal aspect of the work demands scrupulous attention to detail. Failure to make full descriptive notes on the items received, a simple error in the date an analysis was

performed, or neglecting to check reagent purity, can become evidence of careless work in the hands of an astute lawyer. The identification and quantification of the poison may be faultless and the conclusions correct, but, if the court's confidence in the forensic toxicologist as an unbiased scientific expert is destroyed, the case may be lost [3].

Guiding principles for forensic toxicology established by Orfila over 100 years ago are summarized as follows [5]:

- All chemists undertaking this work must have forensic experience.
- The analyst must be given a complete case history containing all the information available.
- All the evidential material, suitably sealed and labelled, must be submitted and examined.
- All the known identification tests should be applied and adequate notes made at the time.
- All the necessary test reagents should be pure, and blank tests should be performed to establish this fact.
- All tests should be repeated and compared to control samples to which the suspected toxin has been added.

Adherence to these principles makes forensic toxicology one of the slowest and most expensive forms of analysis; this must, however, be accepted to ensure justice for the victim and the accused [5].

1.3 BACKGROUND

With the change of government in South Africa in 1994, social, political and economic transitions occurred that resulted in rapid urbanization and greater unemployment. Because of these transitions, increased incidences of crime and violence were recorded [7].

South Africa's re-entry into the global market system created conditions that were conducive to the flow of illegal drugs into the country through drug syndicates. The country's ideal geographical position and solid infrastructure have made South Africa particularly vulnerable to drug trafficking [8, 9]. Research indicates that substance abuse has been a problem in Cape Town for many years, with alcohol being the most widely abused legal substance [9].

The influx of illegal drugs into the county led to a drop in prices [8, 9]. Drugs (such as cocaine) that were formerly affordable only by wealthy South Africans, suddenly became accessible even to the previously disadvantaged section of the population. Cocaine prices, for example, fell by 53 % between 1992 and 1998, and formerly "elite" drugs became more than a third cheaper compared to North America [9]. This price reduction was even more marked for drugs such as Mandrax (the active ingredient of which is methaqualone) and cannabis (a major component of which is Δ^9-tetrahydrocannabinol (Δ^9-THC)) [3].

The influx of drugs into South Africa and more specifically the port city of Cape Town, has created a climate in which crime and violence can flourish. Drugs such as methaqualone are generally associated with crime, violence and gang-related activities. Although methaqualone can be used orally, this mode of use is rare in South Africa, with users preferring to smoke it in a mixture with cannabis and tobacco in a unique South African drug combination called the "white pipe" [10].

Worldwide, drug and alcohol abuse are generally associated with the incidence of violence and crime [11]. Risky behaviour as a result of drug and alcohol abuse often leads to death, which in such cases would be classified as unnatural death or death due to unnatural causes. This is also the trend in South Africa [11]. Data on drugs present in unnatural death cases are provided to the mortuaries by the Forensic Chemistry Laboratory, Cape Town (FCL CTN). In South Africa post-mortem blood samples are sent for routine alcohol

screening, but, because of limited laboratory resources, mortuaries send cases for toxicological screening only at the discretion of the pathologist. This means that not all cases of unnatural death are toxicologically analysed for drugs and, as a result, the data reported by surveillance centres such as the Medical Research Council (MRC) are inaccurate.

1.4 EXTENT OF DRUG AND ALCOHOL USE IN THE GREATER CAPE TOWN AREA

Organizations such as the MRC, the South African Community Epidemiological Network on Drug Use (SACENDU) and the Human Sciences Research Council of South Africa (HSRC) have done extensive research into the alcohol and drug abuse problem in Africa and South Africa [10-14]. Charles Parry and associates carry out projects that monitor the trends of drug and alcohol use and the associated consequences on a six-monthly basis [10, 15]. Their "surveillance system" has done substantial work in highlighting the prevalence of drugs of abuse such as cannabis, methaqualone, cocaine and heroin, as well as alcohol, in South Africa. Their research has indicated that, since the inception of this program, all sentinel sites in South Africa except Cape Town have shown a drop in treatment demand [10]. The HSRC has confirmed these reports and has found that the Western Cape in particular exhibits a definite relationship between the level of drug and alcohol use, and crime [9, 13]. According to reports by the National Injury and Violence Surveillance Initiative, as well as numerous others [10-12], the risk of injury is increased by the intake of alcohol, both internationally and locally. Much less, however, is known about other drugs and their association with unnatural death, particularly in South Africa, where their availability and use appear to have increased since 1994 [11].

1.5 FORENSIC PATHOLOGY LABORATORY PROCEDURES

In terms of the Inquests Act (No 58 of 1959) and the Births and Deaths Registration Act (No 52 of 1992), all unnatural deaths and deaths due to undetermined causes must be subjected to medico-legal investigation. Victims are presented to the forensic pathology laboratories on a 24 hour basis. The purpose of the medico-legal autopsy is to determine the cause of death and make a medical determination of all the other factors that may be involved in the death. The autopsy provides the pathologist with an opportunity to examine the body both externally and internally to determine what wounds and injuries were sustained and to determine the cause of death. In some instances the cause of death cannot be determined, because of the condition of the body or the circumstances surrounding its discovery. In such a case, if the pathologist suspects that, deliberately or accidentally, poisoning, an overdose of medication, drugs of abuse, inhalation of volatile compounds, or alcohol might be involved, it is referred to a Forensic Chemistry Laboratory. Body fluids are sampled from the body and, if necessary, organs are removed. The samples are placed in toxicology and blood alcohol kits and sealed with unique seal numbers to preserve the "chain of custody", a term that refers to the actions taken to maintain the integrity of samples. The samples are then transported to the Forensic Chemistry Laboratory, where they are received, registered and bar coded.

1.6 CURRENT FORENSIC CHEMISTRY LABORATORY PROCEDURES

The FCL CTN is one of three Laboratories within the Directorate Forensic Pathology Services in the Cluster Non-Communicable Diseases of the National Department of Health. At the FCLs, biological and foodstuffs samples are analysed in terms of the Inquests Act (No 58 of 1959), the Road Traffic Act (No 93 of 1996), the Drugs and Drug Trafficking Act (No 140 of 1992), the Criminal Procedure Act (No 51 of 1977), and the Foodstuffs, Cosmetics and Disinfectants Act (No 54 of

1972). The FCL CTN delivers an analytical service to clients in the Western, Eastern and Northern Cape provinces. The FCLs in Johannesburg and Pretoria deliver a similar service to the other six provinces. The FCL CTN has three operational sections, which deal with the analysis of samples in the areas of toxicology, blood alcohol and food respectively.

The toxicology section receives on average 2 000 cases per annum to determine the presence and quantity of toxic substances such as drugs, medicine, heavy metals, volatiles and pesticides. The samples are received in sealed containers at reception after which they are registered and bar coded on a Laboratory Information Management System (LIMS). Two toxicology kits are available for use by pathologists: one is used for body fluids and the other one for organs. Only body fluids are usually analysed unless a request is received from the pathologist for the analysis of organs. The analysis requested, together with other relevant information, is indicated on the toxicology request form accompanying the samples. The kits are stored in a cold room at 5 °C until analysis. The samples are extracted using liquid-liquid extraction methods and the extracts are analysed on analytical instruments such as gas chromatographs (GC), GCs with mass selective detectors (GC-MS), high-performance liquid chromatographs (HPLC) and liquid chromatographs with mass-selective detectors (LC-MS/MS).

The blood alcohol section receives on average 13 000 ante-mortem (drunken driving) and 8 000 post-mortem samples for alcohol analysis per annum. Samples for alleged drunken driving are analysed in terms of section 212 of the Criminal Procedure Act. While keeping the chain of custody, the samples are opened, diluted with an internal standard and analysed using two GCs with flame-ionization detectors (GC-FID) under different operating conditions. The sodium fluoride concentrations of the samples are measured using a fluoride electrode.

1.7 EXTRACTION METHODS

The toxicology samples are extracted using liquid-liquid extraction (LLE) procedures. One of the methods consists of a two-step procedure in which ammonia is added to the biological fluid to ensure an alkaline medium, followed by extraction with butyl acetate. This method is referred to as the butyl acetate extraction method [16]. It enables reliable screening and quantification of basic drugs at therapeutic and toxic concentration levels in post-mortem biological fluids to be done.

The cause of acidity and basicity was explained in 1884 by the Swedish chemist Svante Arrhenius, who said that acids are substances that increase the concentration of hydrogen ions in aqueous solution, and bases are substances that increase the concentration of hydroxide ions in solution. More specific to molecules and ions, however, is the Brønsted-Lowry concept (formulated by the Danish chemist Johannes Brønsted and the British chemist Thomas Lowry in 1923), which says that the acid in a proton-transfer reaction is the species donating the proton, and the base is the species accepting the proton.

Acid-base chemistry is a vital aspect of drug chemistry and analysis, and drugs can be classified as acidic, basic or neutral. The functional groups that define their classes are amine groups for bases, and typically phenolic and carboxyl groups for acids. A drug molecule can have more than one acid or base group, each one of which is called an ionizable centre.

Amines are a major class of organic compounds and constitute most of the drug types encountered in forensic work. These are mainly basic drugs in that they act as proton acceptors in aqueous solutions. Amphetamine is a typical example of an amine-containing drug: when it is placed in water, the amine group is protonated, resulting in an alkaline solution:

$$\text{amphetamine-NH}_2 + \text{H}_2\text{O} \rightarrow \text{amphetamine-NH}_3^+ + \text{OH}^-$$

Blood and urine are also aqueous systems and follow similar considerations. In the literature, acidic and basic drugs are referred to as acids and bases, and this terminology will be used from here on [17-29].

The FCL CTN uses an LLE method in which basic drugs are converted back to their neutral form by the addition of ammonia solution to the blood or urine sample. An organic solvent, butyl acetate, is then used to extract the non-ionized form of the drug.

Given the widespread abuse of drugs in the greater Cape Town area and because of requests from pathologists based on drugs found at the death scene and observations during autopsies, this procedure has been used at the FCL CTN for all cases where drugs of abuse were suspected [16]. This extraction method, however, extracts mainly drugs that contain amine groups, that is, basic drugs. Common drugs of abuse such as lysergic acid diethylamide (LSD), and ibuprofen, are not extracted by this method and would therefore go undetected. This is also the case with morphine, an amphoteric drug whose charge depends on the pH of the matrix. As a result, it is not always detected using the butyl acetate extraction method and its percentage recovery is variable.

The other extraction procedure used at the FCL CTN, called the Celite extraction method, is based on the principles described by Missen [30] and Wood [31]. Celite is a calcined diatomaceous earth. In this method, the biological fluid is mixed with internal standard, potassium iodide, borate buffer and Celite powder and packed into a column. The Celite/fluid mixture is then eluted using a suitable solvent.

1.8 NEW METHODS

Over the past decade solid-phase extraction (SPE) has emerged as a powerful tool for selective chemical extraction and purification [17, 18]. From trace level sample preparation to industrial-scale chemical extraction, it plays an increasingly prominent role in a broad range of applications: forensic, pharmaceutical, fine chemical, biomedical, food analysis, organic synthesis, environmental and many others. Bonded silica sorbents, which consist of silica gel particles that have been reacted with organosilane reagents, are widely used as solid stationary phases in SPE work. The silica gel particles comprise a core of silicon atoms joined with oxygen atoms by siloxane (silicon-oxygen-silicon) bonds. Organosilane reagents containing various types of functional groups react with the silica gel substrate to form the bonded silica sorbent. The functional groups give the sorbent its analyte-retaining properties. These sorbents are rigid and do not shrink or expand in different solvents, unlike many polystyrene-based resins. This stability means that equilibrium is rapidly attained when solvent conditions are changed, enabling extraction procedures involving many solvents to be carried out in a short period of time [20-34].

Although in the past SPE was preferred for the selective extraction of specific drugs or drug groups, it has recently been proposed for screening purposes [17, 18]. Traditional LLE procedures are rapidly being replaced by SPE procedures, which use less solvent and are therefore safer and more cost-effective. The main reason is, however, that during the SPE process, the sample is selectively extracted, concentrated and purified on the extraction column. The analytes of interest in biological material are normally at very low concentrations and it is therefore useful to have a concentration step in the extraction procedure.

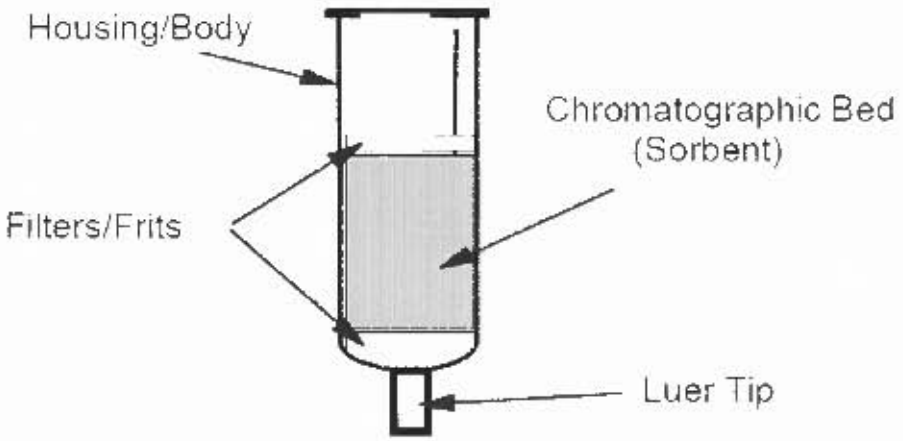


Figure 1.2 **A typical SPE column**

(modified from [20])

SPE is a method used for rapid sample preparation in which a solid stationary phase is packed into a syringe barrel, as shown in Figure 1.2.

1.9 REVIEW OF EXTRACTION METHODS

The extraction of drugs from biological matrices during a routine toxicological analysis is necessary in order to determine the cause of death. The procedure should be capable of extracting a wide range of compounds and at the same time should be able to remove concentrated interferences such as fatty acids and cholesterol in the case of samples such as whole blood. Liquid-liquid extraction is one of the most useful tools that a chemist has for extracting a desired component from a mixture. Selective partitioning of the compound of interest into one of the two immiscible (or partially miscible) phases occurs by the proper choice of extraction solvents. Often, however, it is not possible to find the optimum conditions that provide both high recovery and purity of the product in one extraction step. Low

recoveries may necessitate further extractions to achieve acceptable yields. In order to obtain an extract that contains as small an amount of impurities as possible, a second extraction procedure with a different solvent or pH may be required. Each successive step dilutes the sample further and also increases the extraction time.

In recent years, interest in SPE has grown because it is rapid and efficient, thus permitting the use of small quantities of sample. It is also versatile as can be seen when back-extractions are required, where the analyst is able to switch from an organic to an aqueous solvent and then back to an organic solvent within minutes. This is not the case with LLE.

The first reported use of SPE occurred in 1974 when Adams, Good and Telepchak accidentally dropped C18 packing material into a urine sample while working on high-performance liquid chromatography (HPLC) applications for clinical laboratories [21]. C18 packing material is composed of a silica backbone bonded with octadecane hydrocarbon chains. On testing the sample it was discovered that the steroids of interest were missing. Further testing revealed that the steroids were attached to the packing material, and only filtering, washing and re-suspending the packing material in methanol caused the steroids to be released for analysis [21].

The separation capability of gas chromatography (GC) combined with mass-selective (GC-MS) identification constitutes a powerful technique for doing routine toxicological analysis [21]. The sensitivity of these instruments, however, requires that the biological samples analysed be free from any matrix interferences. The challenge lies in developing a sample preparation method that provides sufficiently clean extracts from biological matrices such as whole blood and urine, and has the ability to extract a broad range of compounds. The need for a systematic approach to screening for drugs and poisons in biological samples has been emphasized by de Zeeuw [22], and the great

influence of the sample matrices on the recovery and detectability of analytes has been pointed out by Bogusz et al [23]. SPE has been shown to be a powerful technique in this field of application [17, 18]. Most publications, however, focus on the extraction of single compounds or groups of related compounds. The development of mixed-mode solid-phase extraction allowed for the extraction of acidic, neutral and basic compounds in a single procedure using one column, thus enabling SPE to be used for screening purposes with good reproducibility [17-27]. Mixed-mode solid-phase extraction uses sorbents that consist of two or more bonding mechanisms in the same column. In order to obtain reproducible results, the process of SPE must be carried out carefully, especially when controlling the flow rates of the sample and the eluent during the extraction process. With the development of fully automated SPE procedures [17, 18, 29], reproducible and faster extractions became possible as operator interventions were no longer required during the process.

Copolymeric bonded phases consisting of an ion-exchanger and a hydrophobic carbon chain attached to a silica backbone have been successfully used for the extraction of acidic, neutral and basic drugs [19-29]. Mixed-mode sorbents can be made by combining sorbents of each functional type, or they can be true copolymers in which different functional silanes are polymerized to the substrate. Copolymers typically give greater reproducibility, as no physical blending of phases is required [21]. Extraction columns containing these polymerized copolymeric phases have been successfully used to extract drugs from biological matrices [35]. Copolymeric sorbents have also been shown to give excellent recovery, reproducibility and selectivity and to be effective for the extraction of analytes from biological matrices [20, 36, 37]. Inconsistent eluent flow rates, and hence inconsistent recoveries, are caused by the presence of smaller than average particles, or fines, in the mixture of silica-containing hydrophilic particles and ion-exchange particles that comprise copolymeric sorbents.

1.10 AIMS AND OBJECTIVES

The objectives of this study can be stated as follows:

- To determine, through a study of the extent of drug and ethanol use, whether cases of unnatural death should be sent for drug screening on a routine basis.

- To develop an efficient SPE method that is able to extract acidic, neutral and basic compounds from blood and urine, in a single, automated procedure.

- To compare this SPE method with extraction methods currently being used at the FCL CTN.

- To validate this method on the basis of its specificity, accuracy, repeatability, intermediate precision, linearity of method, limit of detection, and limit of quantification.

CHAPTER 2

MATERIALS AND METHODS

2.1 BLOOD ETHANOL ANALYSIS

This method involves the quantitative determination of the ethanol concentration of blood samples. Public pressure for the strict enforcement of laws that restrict driving under the influence of ethanol has resulted in a high demand for blood ethanol analysis. The current South African limit is 0.05 % (g/100 mℓ of blood). Headspace gas chromatography is used internationally for the determination of blood ethanol and is the method used at the FCL CTN [32, 33]. The quantitative determination of ethanol in blood is performed using tertiary butyl alcohol (TBA) as internal standard [40, 41]. TBA is used, primarily because it is not a naturally occurring component of the blood samples analysed and the TBA and ethanol peaks are fully separated from each other in the chromatogram. Using an internal standard considerably decreases the requirements regarding the stability of temperature and eliminates the necessity of introducing equal volumes of the equilibrium gas into the gas chromatograph. The calculation of ethanol content is dependent on the internal standard response, which strengthens the validity of the result reported, provided the amount of TBA added is known and is determined reproducibly.

2.2 BLOOD ETHANOL SAMPLE PREPARATION

20 mℓ glass vials are prepared by labelling them for blank samples (water and internal standard), standards and samples, and by adding ~ 1 g of sodium fluoride to each vial to facilitate the migration of volatile components into the vapour phase according to Henry's Law [42]. The standards and blood samples are mixed with the internal standard

using a Hamilton Microlab 500 series dilutor, and dispensed into the marked vials [38, 43]. The vials are then capped with a rubber septum and crimped with an aluminium cap. Each sample is analysed in duplicate and the results are compared on a spreadsheet.

2.3 REAGENTS FOR BLOOD ETHANOL ANALYSIS

Standard ethanol in water solutions (10 mg/100 g, 20 mg/100 g, 50 mg/100 g, 100 mg/100 g, 300 mg/100 g and 500 mg/100 g) were supplied by the National Metrology Laboratory (NML) of the Council for Scientific and Industrial Research (CSIR). The sodium fluoride certified reference material (analytical grade) was also purchased from the NML. Tertiary butanol (99.5 % purity) and sodium sulphite were supplied by Merck (Darmstadt, Germany) and B & M Scientific respectively. Ultrapure water was obtained using the Milli-Q purification system at the FCL CTN.

2.4 STANDARDS FOR BLOOD ETHANOL ANALYSIS

The internal standard, 0.015 % TBA, was prepared by pipetting 150 µℓ of TBA into a 1 ℓ volumetric flask and diluting to the mark with ultrapure water. About 10 g of sodium sulphite was also dissolved in this water to prevent traces of acetaldehyde being formed by the oxidation of ethanol. Standard solutions of ethanol in water at concentrations of 10 mg/100 g, 20 mg/100 g, 50 mg/100 g, 100 mg/100 g, 300 mg/100 g and 500 mg/100 g were used to draw up calibration curves. These curves were drawn by plotting the ethanol and internal standard peak area ratio against ethanol concentration. 100 mg/100 g ethanol in water was used as a quality control standard and was run after every tenth sample during a run, to monitor the operation of the gas chromatograph. The sodium fluoride (NaF) standards were prepared in concentrations of 0.3 %, 1.0 % and 3.0 %, using water as solvent, and were used to calibrate and monitor the fluoride meter.

2.5 INSTRUMENTS AND CONDITIONS FOR BLOOD ETHANOL ANALYSIS

Hamilton Microlab 500 Series diluter

The diluter that was used is controlled manually by the pressing of a button. It was programmed to perform each of the following steps: firstly, draw up 2 250 µℓ of internal standard, then 10 µℓ of air, followed by 250 µℓ of the sample to be analysed; dispense 1 010 µℓ into a 20 mℓ headspace vial and dispense the remaining 1 500 µℓ to waste, to rinse the dispenser's tubing. Before a set of samples is pipetted, the analyst verifies that 1 mℓ of the blood sample and internal standard mixture is being dispensed consistently by the diluter. This is performed by weighing five empty headspace vials and then dispensing ultrapure water into each of the vials. These vials are then weighed and the actual mass of the dispensed water is determined. By using the density of water, it can be established that the instrument is dispensing the correct volumes as programmed. In this study, the diluter was successfully verified by means of this procedure and then it was used to prepare the samples for analysis.

Gas chromatographs with flame-ionization detectors, coupled to headspace autosamplers (headspace GC)

Two gas chromatographs (GC1 and GC2) with headspace autosamplers, each fitted with a column of different polarity, were used; the results obtained by the two instruments were not allowed to differ by more than 10 %.

The gas chromatographs used were both HP 5890 Series 2 instruments. GC1 was fitted with a ZB Wax column (30 m x 0.53 mm x 1.0 µm), and the temperature program was set at 35 °C isothermal. The temperatures of the injector port and the detector were set at 250 °C and 255 °C respectively. GC2 was fitted with a DBALC-1 Chromosorb column (30 m x 0.53 mm x 3.0 µm), and the temperature program was set at 40 °C isothermal. The temperatures of the injector

port and the detector were set at 250 °C and 320 °C respectively. Nitrogen was used as the carrier gas at a pressure of 100 kPa for both instruments. Both autosamplers had the platen (a heated rack) temperature set at 65 °C. The sample loop and transfer line temperatures were set at 85 °C and 100 °C respectively, and the sample equilibration time was set at 30 minutes. Once the vials are taken into the platens, the headspace autosampler needles are used to take samples from above the sample liquids (the headspace), which are then injected onto the GC columns.

Fluoride meter

The fluoride meter used was a Eutech Cyberscan pH5500 dual-channel ion meter. The tubes used for the collection of blood samples, contain sodium fluoride. It has been experimentally established that a sodium fluoride concentration of at least 1 % in blood samples inhibits the bacterial conversion of blood glucose to ethanol [41]. The sodium fluoride concentration of each blood sample was determined in order to verify that it was sufficient. The meter was calibrated using sodium fluoride standards with concentrations of 0.3 %, 1.0 % and 3.0 % respectively, after which the fluoride concentration of each sample was measured by placing the ion-selective electrode directly into the sample and recording the reading displayed on the screen.

2.6 ANALYTICAL METHODS USED IN TOXICOLOGY

2.6.1 CELITE EXTRACTION METHOD

The Celite extraction method is used for cases in which no background information is supplied or a toxicology "screen" is requested. It is used to extract both drugs and pesticides from biological fluids. A piece of glass wool is placed in the tip of a plastic column, which is then placed in a vacuum manifold with a tube to collect the liquid beneath it. 750 µℓ (or a different, known volume) of blood or urine is pipetted into a glass dish, to which 5 drops of 0.5 M potassium iodide solution are added,

and then this mixture is stirred with a glass rod. Three drops of borate buffer (pH 9.2) are added and the mixture is stirred again. Two scoops of Celite powder are added and, after thorough mixing, the mixture is packed into the plastic column using the glass rod. Five to 10 mℓ of 1-chlorobutane is added to the sample dish, to rinse the contents, and then added to the plastic column, which is eluted under vacuum. The eluent is evaporated to dryness under a stream of nitrogen and the residue reconstituted in 200 µℓ of methanol (for drugs) or a hexane - acetone - ethyl acetate (6:4:1) mixture (for pesticides). Once the residues have been reconstituted, they are ready to be analysed by gas or liquid chromatography. Analytical results based on this extraction are calculated as follows:

$$\frac{\text{inj vol std}}{\text{inj vol sample}} \times \frac{\text{area sample}}{\text{area std}} \times \text{dil factor} \times \text{conc std} \times \frac{\text{final vol sample}}{\text{vol used}} \times 100 = \text{mg}/100\,\text{m}\ell$$

inj vol std = injection volume of standard
inj vol samp = injection volume of sample
area sample = area of sample peak
area std = area of standard peak

2.6.2 BUTYL ACETATE EXTRACTION METHOD

At the FCL CTN, this LLE procedure is used for the extraction of certain drugs of abuse from biological fluids. Liquid-liquid extraction (LLE), also known as solvent extraction, is a method used to separate compounds based on their solution preferences for two different immiscible liquids, usually water and an organic solvent.

600 µℓ of sample is placed in a plastic Eppendorf centrifuge tube. The sample is made alkaline by adding 50 µℓ of ammonia and then mixed on a Clifton vortex for approximately 10 seconds. This step in the procedure ensures that basic drugs, such as most drugs of abuse, are extracted. 150 µℓ of butyl acetate is then added and the sample is

vortexed for a further 60 seconds. The sample is placed in an Eppendorf centrifuge 5415 D and centrifuged for 15 minutes at 13 000 rpm. The top organic layer is isolated and used in the analysis. The concentration of each drug found and quantified using this procedure is multiplied by a factor of 0.25 to correct for the concentration step.

2.7 REAGENTS FOR TOXICOLOGICAL ANALYIS

Preparation of iodide solution for Celite extractions

A 0.5 M solution was prepared by weighing out 4.1 g of potassium iodide and making it up to a volume of 50 mℓ with distilled water. 1 mg of sodium thiosulphate was added per millilitre of iodide solution. The iodide solution was shaken with n-hexane in a separating funnel (ratio 1:1) and allowed to separate. The aqueous phase was stored in a dark bottle.

2.8 STANDARDS FOR TOXICOLOGICAL ANALYIS

Standards used for the quantification of compounds found positive during the study to investigate the extent of drug and ethanol use, were supplied by Industrial Analytical (Midrand, South Africa). Butyl acetate, ammonia and n-hexane (analytical grade) were supplied by Merck. The methanol was HPLC grade and was also supplied by Merck. Potassium iodide and sodium thiosulphate were supplied by Sigma Aldrich. Ultrapure water was obtained using the Milli-Q purification system at the FCL CTN. Separate stock solutions (1 mg/mℓ) of all compounds studied were prepared using methanol as solvent. The working standards were prepared by dilution of the stock solution with methanol. Solutions were stored at - 4 °C in the dark. A calibration curve based on five concentrations (each one of which was injected in triplicate) was obtained for each of the compounds identified on GC-MS. The peak areas were plotted against the concentrations, which ranged from 1.0 to 100 µg/mℓ.

2.9 INSTRUMENTS AND CONDITIONS FOR TOXICOLOGICAL ANALYSIS

Gas Chromatography – Mass-Selective Spectrometry (GC-MS)

The GC-MS system consisted of an Agilent 6890N GC System, a 7683 Series autosampler, a 7683B Series injector and a 5973 mass-selective detector with electron-impact (EI) capabilities and a diffusion pump. An Agilent HP-1MS capillary column (30 m x 250 µm x 0.25 µm) was used for separation. The temperature program used was as follows: the oven was set at 60 °C and increased to 320 °C at a rate of 25 °C/min, and held for a further 10 minutes. The detector was a mass-selective detector (MSD) and a splitless-injection mode was used with an injection volume of 1 µℓ. For single-ion monitoring (SIM) the dwell time per ion was 100 ms and the base peak area of each analyte was measured. A solvent delay of 3.8 minutes was used before the detector was switched on, to allow for the solvent used in the extraction process to pass the detector. Helium 6.0 was used as the carrier gas at a flow rate of 1 mℓ/min in the constant-flow mode and a pressure of 56.7 kPa at an average velocity of 37 cm/s.

The performance of the EI source of the instrument was monitored by performing an autotune, which is an automated source-optimization process of GC-MS systems, before each sequence. This was done to make sure that the source was free of water, that there were no leaks in the system and that the electron multiplier (EM) volts were at acceptable levels (< 3000 EM volts).

The column performance was monitored by analysing a known standard at the beginning and the end of each sample sequence, and checking for correct retention time and constant absorbance intensity.

Gas Chromatography – Nitrogen-Phosphorus Detection (GC-NPD)

The GC-NPD used was an Agilent 6890N instrument, which was set up in such a way that one volume of sample injected was split between

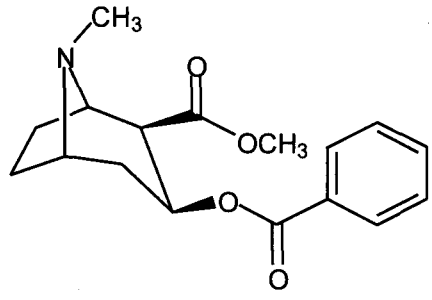
two different columns that each went to a separate nitrogen-phosphorus detector. In this way each compound detected on one column would be confirmed on another column with a different polarity while doing a single injection in one run. The columns used were a J&W DB-5MS column (30 m x 0.32 mm internal diameter) with a film thickness of 0.25 µm, and an Agilent Hewlett Packard 1MS column (30 m x 0.32 mm internal diameter), also with a film thickness of 0.25 µm. The injection mode was splitless with an injection volume of 3 µℓ. The temperature program used for the detection and quantification of the drugs was the following: the initial temperature of 60 °C was maintained for 1 minute and then raised at a rate of 20 °C/min to a final temperature of 280 °C, which was maintained for 15 minutes. The injector was held at 250 °C and the detector at 280 °C.

2.10 DRUGS USED IN METHOD DEVELOPMENT

The drugs chosen for the development of the SPE method in this study were: cocaine, imipramine, lorazepam, methamphetamine, oxazepam, phenobarbital, prazepam (internal standard) and secobarbital. These eight drugs, all of which can be detected on instrumentation currently available at the FCL CTN, were selected on the basis of positive identifications made during the study to determine the extent of drug use in Cape Town, and also because their pK_a values cover a large range (1.3 to 11.6). The decision to take pK_a values into consideration was necessary in order to ensure that the developed extraction method would be able to extract drugs of acidic, neutral and basic character.

The structure, properties, uses and pharmacological effects of these drugs are given below. Following the convention of the Merck Index, the pK_a refers to the protonated species of the drug [43].

Compound 1: COCAINE

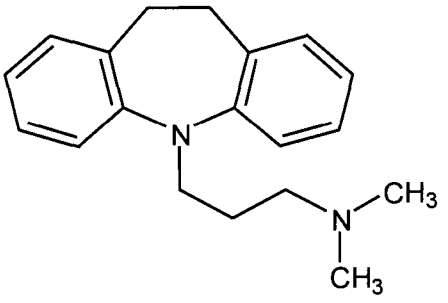


pK_a: 8.4; its principal mass spectral peaks are at *m/z*: 82, 182, 83, 105, 303, 77, 94 and 96 [44].

Cocaine is considered to be the most potent of the naturally occurring central nervous system stimulants. It is found in the leaves of *Erythroxylon coca,* a plant that occurs in South America [45]. Benzoylecgonine is the major metabolite of cocaine and is present in urine specimens two to four days after use and may be found without detection of the parent drug if it is present in low concentrations.

It is used therapeutically as a local anaesthetic, but is also used as a drug of abuse for its stimulant properties. It is normally used only as a surface anaesthetic in the eye, ear, nose and throat, because of the possibility of systemic toxic effects when given by other routes. Addicts may inject it or use it as a snuff; it is less toxic when taken orally, because of hydrolysis in the gastro-intestinal tract [45]. The estimated minimum lethal dose is 1.2 g, but susceptible persons have died from doses as small as 30 mg when applied to mucous membrane; addicts may be able to tolerate up to 5 g a day [3].

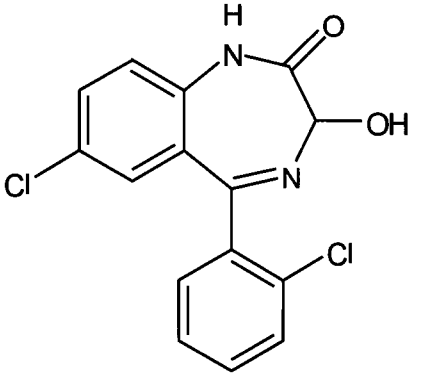
Compound 2: IMIPRAMINE



pK_a: 9.5; its principal mass spectral peaks are at *m/z*: 58, 235, 85, 234, 236, 195, 193 and 208 [44].

Imipramine is the prototype of the tricyclic antidepressant drugs and has been among the most frequently used agents for the treatment of depression [47]. It has as its primary active metabolite desipramine, which accumulates in plasma and may account for much of the therapeutic effectiveness of the drug. Numerous instances of imipramine intoxication have occurred and at least 40 fatalities are recorded in the literature [45]. In adults the estimated minimum lethal dose is 1 g, although fatalities have occurred with less than this and patients have survived the ingestion of as much as 5 g [3].

Compound 3: LORAZEPAM

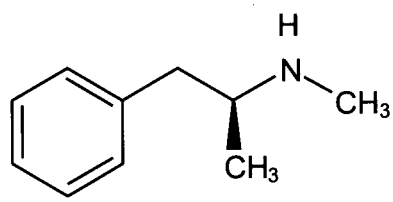


pK_a: 1.3 and 11.5; its principal mass spectral peaks are at *m/z*: 291, 239, 274, 293, 75, 302, 276 and 138 [44].

Lorazepam is a 3-hydroxy benzodiazepine, a group that includes oxazepam and temazepam [45]. Like many benzodiazepines that are administered in low doses, this drug is available as the free base in 0.5 to 2 mg tablets, which necessitates its detection at very low concentrations [3, 21].

Lorazepam, like most benzodiazepines, is frequently prescribed for the symptomatic treatment of anxiety and sleep disorders [45]. Benzodiazepines were introduced in the 1960s and have replaced barbiturates for many indications. This drug has been classified as a date-rape drug along with γ-hydroxybutyric acid (GHB), ketamine and flunitrazepam [3]. Lorazepam is rapidly conjugated with glucuronic acid, forming an inactive metabolite [45].

Compound 4: d-METHAMPHETAMINE



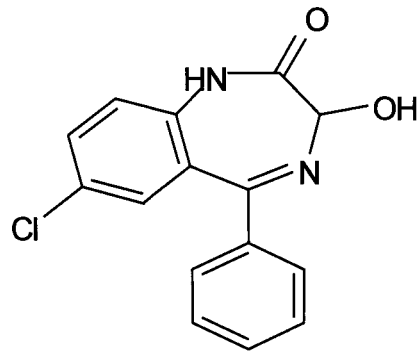
pK_a: 9.9; its principal mass spectral peaks are at *m/z*: 58, 91, 59, 134, 65, 56, 42 and 57 [44, 45].

d-Methamphetamine (hereinafter referred to as methamphetamine), the N-methyl derivative of amphetamine, was first prepared in 1919 [45]. As the hydrochloride salt, it is used in the treatment of obesity.

The l-isomer is used as a decongestant in certain non-prescription inhalers such as Vicks inhaler.

Methamphetamine is a potent central nervous system stimulant and is categorized as a phenylethylamine derivative. It has received a great deal of attention as a drug of abuse. Ilicit methamphetamine is readily synthesized from phenylacetone and N-methylformamide (dl-mixture), or from ephedrine using acid reduction (d-isomer) [46]. Methamphetamine is also formed as a metabolite of benzphetamine and famprofazone [46].

Compound 5: OXAZEPAM



pK_a: 1.7 and 11.6; its principal mass spectral peaks are at *m/z*: 257, 77, 268, 239, 205, 267, 233 and 259 [44].

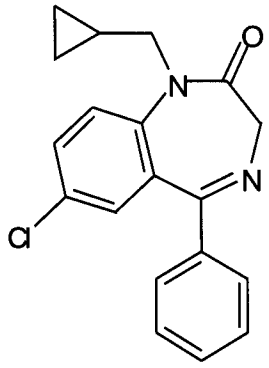
Oxazepam is a metabolite of several benzodiazepines including chlordiazepoxide, clorazepate, demoxepam, nordiazepam, diazepam, ketazolam, medazepam, prazepam and temazepam. Since many benzodiazepines can yield similar metabolic products, identification of the parent compound is difficult.

Oxazepam is the 3-hydroxy metabolite of nordiazepam and has been used since 1965 in the treatment of anxiety [45]. It is available as

tablets or capsules containing 10 to 30 mg of the free base and is administered orally [44]. Because of the low doses in which benzodiazepines are prescribed, nanogram-per-millilitre concentrations must be detected.

Oxazepam is rapidly conjugated with glucuronic acid and excreted in the urine, and only trace amounts of the free drug are found in the urine [45]. Glucuronic formation is a major metabolic route for benzodiazepines [21, 45].

Compound 6: PRAZEPAM

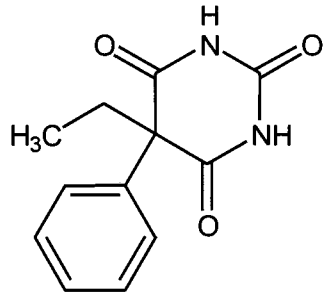


pK_a: 2.7; its principal mass spectral peaks are at *m/z*: 91, 269, 324, 55, 296, 295, 323 and 297 [44].

Prazepam is the N-cyclopropylmethyl analogue of diazepam. It was first synthesized in 1965 and is used in the treatment of anxiety [21, 45].

Prazepam is either dealkylated to nordiazepam, the major plasma species, or oxidized to 3-hydroxyprazepam. This latter compound may be either conjugated directly or first dealkylated to oxazepam, which is then conjugated [45]. Adverse reactions to prazepam administration include dizziness, fatigue, drowsiness, weakness, confusion and slurred speech [3, 45].

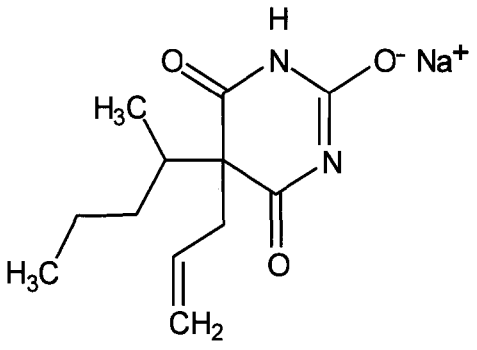
Compound 7: PHENOBARBITAL



pK_a: 7.5; its principal mass spectral peaks are at *m/z*: 204, 117, 146, 161, 77, 103, 115 and118 [44].

Phenobarbital is a barbiturate derivative and has been used as a daytime sedative and very extensively as an anticonvulsant since 1912 [45]. Barbiturates are central nervous system depressants and are potential drugs of abuse because of their hypnotic and sedative effects. The screening of urine for barbiturates is therefore widely practised. Toxic reactions to phenobarbital in chronically dosed subjects generally occur when plasma concentrations exceed 40 mg/ℓ [45]. The classical measurement of phenobarbital in biological specimens involves ultraviolet spectrophotometry [45]; gas chromatography, however, provides a more specific result and is usually done with flame-ionization or nitrogen-selective detection of the free drug.

Compound 8: SECOBARBITAL



pK_a: 8.0; its principal mass spectral peaks are at *m/z*: 167, 168, 41, 43, 97, 124, 39 and 55 [44].

Secobarbital is a barbiturate derivative of short duration of action and was first prepared in 1934 [45]. Like many barbiturates, secobarbital is a potential drug of abuse because of its hypnotic and sedative effects [45]. During its metabolism and excretion, secobarbital undergoes extensive biotransformation by oxidation of both side-chains to a series of more polar and pharmacologically inactive compounds [21, 45].

CHAPTER 3

EXTENT OF DRUG AND ETHANOL USE

3.1 INTRODUCTION

In this study the extent of drug and ethanol use in victims of unnatural death was investigated based on 601 deaths that occurred in Cape Town during the period from February to May 2005. The study population consisted of victims of unnatural death who presented to the Salt River and Tygerberg Forensic Pathology Laboratories, the two mortuaries in Cape Town. Blood and, if possible, urine were sampled from each victim fitting the inclusion criteria. These were that the victims had to be ten years or older and had to have died of unnatural causes; furthermore, there had to have been no attempted resuscitation of the patient, so that no drugs other than those taken by the victims themselves would be detected.

3.2 EXPERIMENTAL PROTOCOL

The samples were taken out of storage at 5 °C and allowed to reach room temperature at about 25 °C.

The blood samples were analysed quantitatively for their ethanol content according to the procedure described in Sections 2.1 to 2.5.

The blood and urine samples were extracted using the butyl acetate method described in Section 2.6.2; the extracts were then analysed by means of the procedures set out in the rest of Chapter 2.

Urine from each case was sent to the National Health Laboratory Service (NHLS), Green Point, to be analysed for Δ^9-

tetrahydrocannabinol, a component of cannabis. (This analysis was not performed at the FCL CTN at the time of the study.)

3.3 RESULTS AND DISCUSSION

The pathologists at the Salt River and Tygerberg Forensic Pathology Laboratories investigated 601 cases of unnatural death from February to May 2005. The manner of death was established (see Figure 3.1) and samples were provided for analysis.

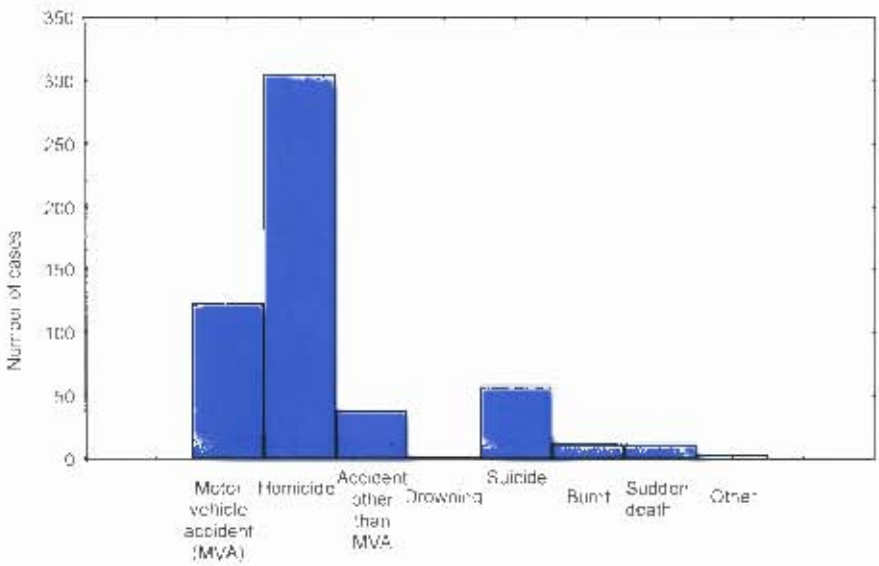


Figure 3.1 **Manner of death of the 601 cases tested**

Homicide was found to be the leading manner of death, with 306 victims of whom 106, or 35 %, tested positive for the presence of drugs. When the entire study population of 601 is taken into account, the number of drug-positive cases was 172, which was 29 % of the total; 97 of these cases were positive for more than one drug, giving a total of 163 occurrences of drugs for the ethanol-negative cases and 135 for the ethanol-positive cases.

It can be seen from Figure 3.2 that, when the ethanol levels are grouped into intervals, the victims of unnatural death who were negative for ethanol constitute the largest group of drug-positive cases.

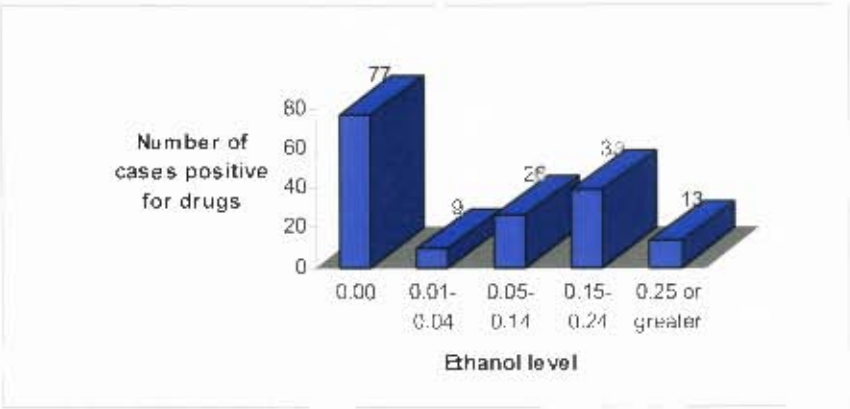


Figure 3.2 **Drugs related to ethanol level, based on the 164 drug-positive cases for which ethanol results were obtained**

It is possible that many of these drugs would have remained undetected if the usual procedure had been followed. This procedure involves routine testing for ethanol, but not for drugs, which are tested for only at the discretion of the pathologist. The most common drugs were found to be cannabis, diphenhydramine and methaqualone, which are frequently detected at the FCL CTN when drug analysis is performed. The extraction method used for the isolation of diphenhydramine and methaqualone (but not cannabis) has been the butyl acetate procedure and, as a result, only basic drugs have been detected.

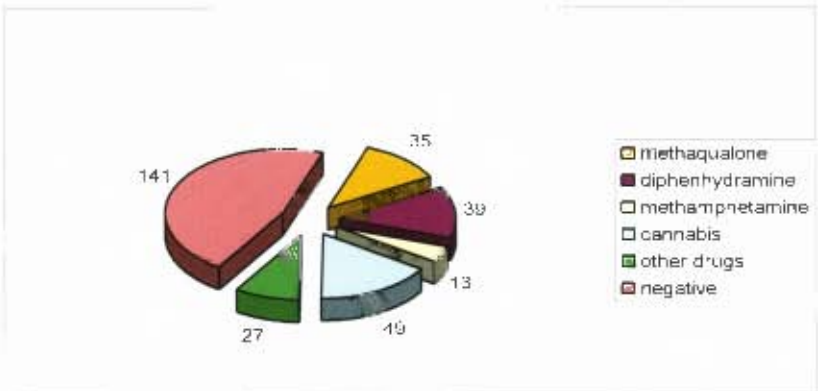


Figure 3.3 **Number of occurrences of drugs in ethanol-negative victims, based on 218 cases**

The number of occurrences of the drugs mentioned in the pie charts of Figures 3.3 and 3.4 are based on 77 and 87 cases respectively. The negative sectors, on the other hand, represent 141 and 285 cases respectively. (Of the 601 cases, eleven could not be analysed for ethanol, because there was either insufficient or no blood sample.) It is clear that a significant proportion of the cases was negative for the presence of drugs, this trend being more noticeable in the ethanol-positive cases. Whether this is a true reflection of these victims' use of drugs or is a consequence of the limitations of the butyl acetate extraction procedure used, is not known.

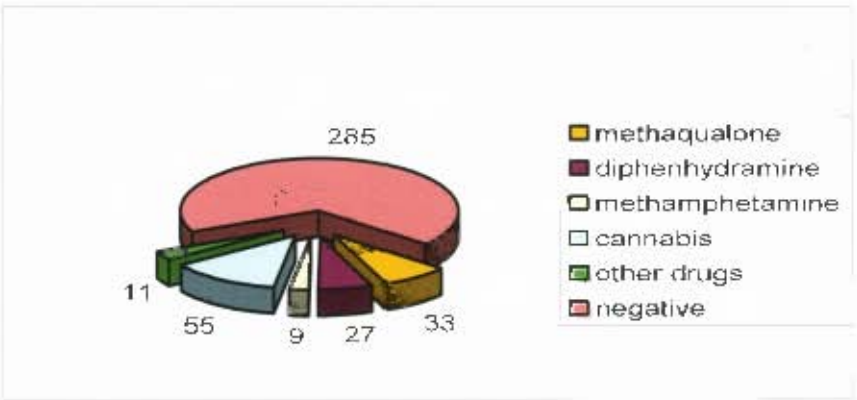


Figure 3.4 **Number of occurrences of drugs in ethanol-positive victims, based on 372 cases**

3.4 CONCLUSIONS

Overall, the results of this study indicate that the use of drugs remains a problem in Cape Town, and, more specifically, that there is a high prevalence of drug use among victims of unnatural death. Although cannabis and Mandrax (the active ingredients of which are methaqualone and, in most cases, diphenhydramine) were the two most common drugs found, the use of methamphetamine appears to be a growing problem in Cape Town: the FCL CTN detected this drug for the first time in December 2004, whereas in this study, which covers a period of only three months in the first half of 2005, there were 21 positive methamphetamine cases.

In order to ensure that as many of these drug-positive post-mortem cases as possible are detected, it will be necessary for the pathologists to adopt a routine approach to drug screening, as is presently the case with ethanol. They must be supported by the FCL CTN with the use of effective and comprehensive extraction and detection methods. The results of this study provided the motivation to investigate, specifically, solid-phase extraction procedures, which are known to be capable of extracting a wide range of drugs, even when they are present at therapeutic levels, but which were not in use at the FCL CTN at the time of this study. The purpose of this work was, therefore, to make a contribution to the improvement of the service offered by the FCL CTN in the light of the high incidence of drug use revealed by this study.

CHAPTER 4

DEVELOPMENT OF SOLID-PHASE EXTRACTION METHOD

4.1 WHAT IS SOLID-PHASE EXTRACTION?

Solid-phase extraction (SPE), which is also known as sorbent extraction, is a physical process involving a liquid and a solid: the compound to be extracted is attracted more strongly to the solid phase than to the solvent in which it is dissolved. As the solution moves through the sorbent bed, the compound of interest is concentrated by the sorbent, whereas other compounds in the solvent pass through the bed. Highly selective extractions that produce very pure and concentrated extracts can be performed by choosing sorbents to which the desired compound, but not others in the solution, is attracted. The extraction efficiency and the quality of the separation are dependent on the physical properties of the sorbent, such as pore and particle size, as well as its chemical properties [19, 21].

4.2 EXTRACTION MECHANISMS

Sorbents are most commonly categorized by the nature of their primary interaction or retention mechanism with the analyte of interest. The three most common extraction mechanisms used in SPE are reversed phase, normal phase and ion exchange.

4.2.1 REVERSED PHASE

Reversed-phase bonding, also called hydrophobic or non-polar bonding, occurs via attraction of non-polar groups to the sorbent by means of van der Waals forces (Figure 4.1).

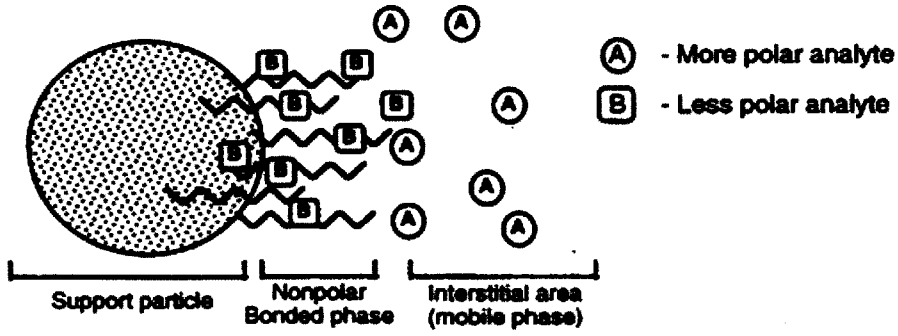


Figure 4.1 **Example of hydrophobic retention mechanism as a result of the preferred distribution of the less polar analytes to the similarly less polar stationary phase [36]**

Hydrophobic phases are alkyl chains and/or rings, with chain lengths from C2 to C18. Compounds are retained by non-polar interactions in the presence of polar solvents or matrix environments. Effective bonding also requires that the target analytes be in a neutral state. Elution, or disruption of the non-polar interactions, is achieved by solvents or solvent mixtures with a sufficiently non-polar character. Some polar solvents such as acetonitrile have enough non-polar character to disrupt non-polar bonding and to cause elution of a compound from the sorbent. Methanol can be used as well, but it should be noted that it will elute both polar and non-polar analytes of interest as well as interferences [21].

The retention of an analyte on a reversed-phase sorbent is dependent on the equilibrium distribution between the solvent and the sorbent. For a particular analyte, it is the combination of sorbent and eluent properties that determines how efficiently the analyte is removed from the sorbent.

Compounds that exhibit non-polar or neutral characteristics are extracted from biological matrices onto the sorbent by partition or adsorption, or by a combination of these two mechanisms. In the

partition mechanism, the analyte is transferred from the matrix to the sorbent, whereas in the adsorption mechanism, the analyte is not fully embedded in the sorbent, but is at the interface of the matrix and the sorbent.

4.2.2 NORMAL PHASE

Normal-phase bonding, also called hydrophilic or polar bonding, occurs by means of hydrogen bonding and/or dipole interactions. Compounds that are hydrogen donors, such as hydroxyls, carboxylic acids and amines, are characteristic of polar bonding (Table 4.1).

Table 4.1 **Hydrophilic sorbents and structures [21]**

sorbent	structure
silica	-SiOH
diol	-Si-$(CH_2)_3OCH_2CHOHCH_2OH$
cyanopropyl	-Si-$(CH_2)_3CN$

Normal-phase retention mechanisms are commonly used to extract polar analytes from non-polar organic solvents. These interactions are therefore between polar analytes and polar stationary phases (Figure 4.2).

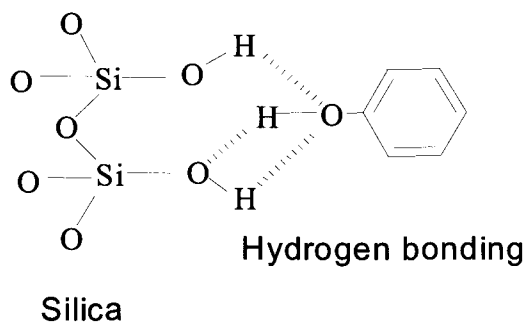


Figure 4.2 **Example of normal-phase retention mechanism**

These types of interactions occur when the distribution of electrons between individual atoms in functional groups is unequal, causing positive and negative polarity. Compounds typically extracted on a

hydrophilic column include analytes that have polar groups. Analytes can be eluted by the use of relatively low concentrations of polar organic solvents such as methanol or isopropanol, in combination with non-polar organic solvents.

4.2.3 ION EXCHANGE

For an ion-exchange mechanism to occur, the sorbent must be composed of a silica backbone bonded with a carbon chain that is terminated by a positively or negatively charged functional group. Ion-exchange bonding mechanisms are electrostatic interactions between oppositely charged sorbents and analytes. Types of ion-exchange bonding mechanisms include cation-exchange, anion-exchange and copolymeric-exchange mechanisms.

Charged sorbents are used to retain analytes of the opposite charge. For example, positively charged analytes containing amines are retained on negatively charged cation-exchange sorbents such as sulfonic acid or carboxylic acids. In contrast, negatively charged analytes containing sulfonic acid or carboxylic acid groups are retained on positively charged anion-exchange sorbents containing any one of a variety of different amine groups. This is illustrated in Figures 4.3 and 4.4.

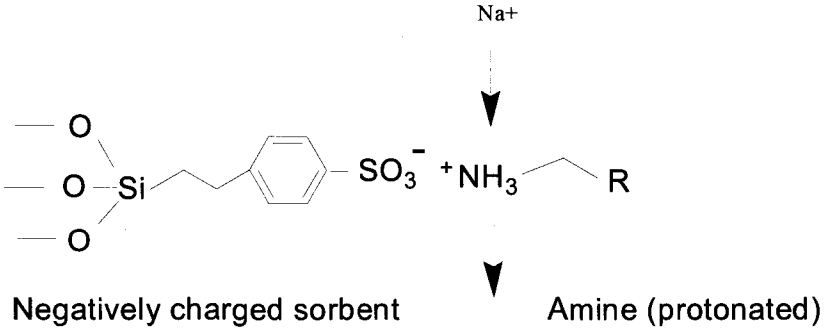


Figure 4.3 **Example of cation-exchange retention**

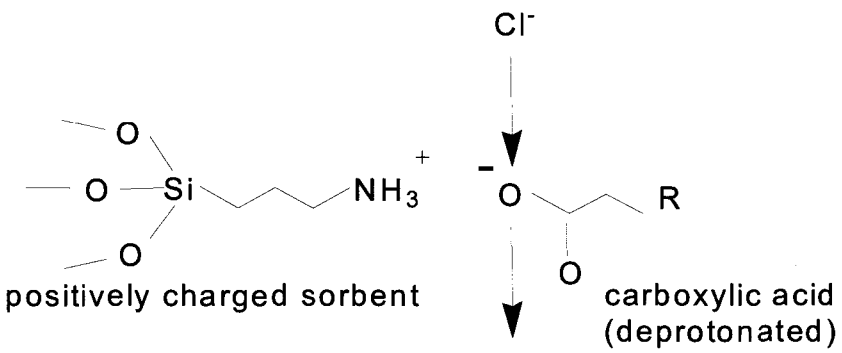


Figure 4.4 **Example of anion-exchange retention**

Only species of the proper charge are retained by the column, so most matrix contaminants are simply rinsed away to waste during the loading and washing steps. For this reason, cation-exchange SPE is commonly used for the extraction of basic compounds (drugs and other amine-containing compounds) from complex biological samples. The bonding can be reversed by changing the pH conditions or by introducing competing ions with greater sorbent affinity than the compounds of interest, which results in displacement of the analyte.

pH and pK_a play an important role in ion-exchange extraction procedures. The most significant factors in ion-exchange chromatography are the pK_a of the analyte, the sorbent, the pH, the ionic strength of the mobile phase, and the acid-base properties of the typical functional groups on the sorbent as well as the target analyte. The pK_a is the pH at which 50 % of the molecules are protonated and the other 50 % are deprotonated. The quantitative ionization or neutralization (99.5 %) of a functional group occurs at a pH value 2.0 units above or below the pK_a [20]. These relationships are described by the following equations:

$pK_a = -\log_{10} K_a$, where $K_a = \frac{[H^+][A^-]}{[HA]}$ with HA being an acid and A^- the conjugate base. The relationship between pK_a and pH is given by the equation $pH = pK_a + \log_{10}\left(\frac{[base]}{[acid]}\right)$. When the pH is equal to the pK_a, 50 % of the acid is protonated.

For example: the pK_a of a typical carboxylic acid is 4, and so the weak acids are charged at pH 6 and non-ionized (neutralized) at pH 2 [21]. At a pH of 4, 50 % of the molecules are protonated and non-ionized, and the other 50 % are ionized (charged). Consequently, low recovery values would be expected for carboxylic acid analytes on anion-exchange sorbents at pH 4 or below. In contrast, at pH 6, approximately 99.5 % of the analytes would be ionized and retained. Similarly, weak amines, such as most drugs of abuse, all tend to have fairly similar acid-base properties, even though minor differences do exist between secondary (2°) amines relative to primary (1°) and tertiary (3°) amines [21]. In general, weak amines have pK_a values of approximately 10, and are charged (and retained on cation-exchange sorbents) at pH 8 and non-ionized (and eluted) at pH 12. Elution occurs by using a solvent to raise the pH above the pK_a of the cationic group or to lower the pH below the pK_a of the anion to disrupt retention. At this point, the sorbent or compound will be non-ionized (neutralized).

4.2.4 COPOLYMERIC BONDING

Copolymeric sorbents have both hydrophobic and ionic-retention mechanisms. They consist of silica backbones with both reversed-phase and ion-exchange functionality and can therefore be used for the extraction of acidic, neutral and basic drugs.

4.3 SOLID-PHASE SORBENTS

4.3.1 SORBENT PROPERTIES

Solid-phase sorbents are available in a wide range of surface chemistries, pore sizes (60, 120, 200 and 300 Å), particle sizes (10, 40, 100 and 150 µm), and base supports (silica, alumina and polymers) [20, 21].

The optimal sorbent for any given extraction problem is dependent on the properties of the target analyte, the sample/matrix composition and other factors, which are discussed below [21].

4.3.2 SORBENT SPECIFICITY

Theoretically an "ideal" solid-phase sorbent binds only the target analyte(s), resulting in a contaminant-free eluent. In practice, however, the analysis is influenced by the retention of the target analytes as well as many contaminants, but the effect of contamination may be counteracted by the concentration of analytes on the sorbent and by properly optimized extraction conditions [20, 22]. It is therefore vital for the sorbent chemistry to be selected with care and for the extraction conditions to be optimal. Sorbent specificity is a function of the extraction conditions as much as of the surface chemistry, and the sample-loading conditions can even determine the chromatographic technique that will be used to analyse the extract [20, 33, 36].

4.3.3 SILICA-BASED SORBENTS

Silica-based sorbents are rigid, stable and relatively inexpensive, and they do not shrink or swell in most aqueous and organic solvents. Furthermore, it is easy to derivatize and manufacture them reproducibly. Those that are most widely used consist of chemically modified silica particles with covalently attached functional groups on the surface. Chromatographic selectivity can by significantly altered by modifying the silica surface. Unfunctionalized silica, for example, changes from being highly polar, with analytes being retained by

means of normal-phase and cation-exchange mechanisms, to being non-polar when a C18 saturated hydrocarbon is attached to the surface. Sorbents possessing various separation selectivities can be achieved by covalently attaching appropriate functional groups [19, 20, 36].

Silica-based sorbents may, after the initial bonding reaction, still contain a residual amount of unreacted –Si(OH) groups. These "free" groups constitute polar and acidic areas on the sorbent surface that can bind amines by means of hydrogen bonding and cation-exchange mechanisms. Interaction with these groups can lead to unwanted retention and low recoveries, because some analytes are ionized during extraction. The –Si(OH) groups are therefore methylated, usually with trimethylsilyl (TMS) groups, in a reaction known as endcapping [19, 20, 36].

4.3.4 POLYMERIC SORBENTS

Polymer-based sorbents typically consist of highly cross-linked polystyrenedivinylbenzene (PSDVB). Unlike unfunctionalized silica, PSDVB is non-polar and is capable of strong hydrophobic and π-π interactions with non-polar and electron-rich analyte molecules [19-21].

4.3.5 COPOLYMERIC SORBENTS

This sorbent is composed of a silica backbone with two types of functional chains attached: an ion-exchanger (or polar) chain and a hydrophobic carbon chain. It is produced in a way that allows for equal parts of each functional group to attach to the silica [21] (Figure 4.5).

Figure 4.5 **Example of a copolymeric sorbent (anion-exchange phase) [47]**

Such sorbents are referred to as "mixed-mode" sorbents. This type of dual chemistry is beneficial, especially if one is extracting both a neutral and a charged compound. This is common when a neutral parent drug is metabolized and becomes charged [19, 20].

A sorbent containing both cation-exchange and non-polar functionalities is optimal for extracting small, alkaline compounds with a moderate degree of hydrophobicity, since such compounds can be retained by both moderately strong non-polar interactions and by cation exchange. Opiates, benzodiazepines and tricyclic antidepressants fall into this category [19-21]. One such sorbent exhibits hydrophobic properties (because of a non-polar carbon chain) and cation-exchange properties (because of a benzenesulfonic acid functional group, which is a charged anion) (Figure 4.6).

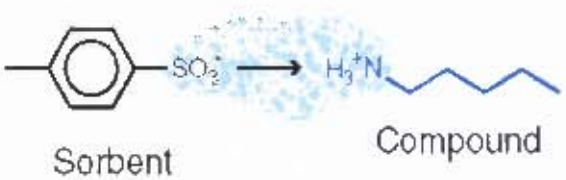


Figure 4.6 **Example of a strong cation-exchange bonding mechanism [47]**

Benzenesulfonic acid is considered to be a strong cation exchanger [19-21]. The drugs encountered in forensic samples are mostly weakly basic, thus easily forming anions dependent on the matrix pH. Benzenesulfonic acid, which is a strong acid, was therefore chosen as the ion exchanger on the sorbent.

This mixed-mode mechanism facilitates the isolation of acidic/neutral and basic drugs and can be performed in a single extraction using a single column, since the sorbent is composed of a silica backbone with an ion exchanger and a hydrophobic carbon chain. Depending on the pK_a of the metabolite, amine-containing groups may be positively charged, allowing the metabolite to bond to the negatively charged sorbent. Since the column also possesses a hydrophobic chain, the neutral parent drug is able to bond to the column.

4.4 STEPS IN SOLID-PHASE EXTRACTION PROTOCOLS

Sample preparation in SPE typically consists of four steps: conditioning, sample loading, washing and analyte elution. Because SPE takes advantage of the same physicochemical interactions used in HPLC, the recovery and purity of the target analyte(s) can be optimized by adjusting the composition of the solvents used during each of the steps (sample loading, washing and analyte elution) listed in Table 4.2.

Table 4.2 **The four basic steps of an SPE procedure**

(Modified from [21])

step	purpose
conditioning	To prepare the sorbent for effective and reproducible interaction with the analyte(s) by solvation. In effect, solvation is a wetting of the sorbent, creating an environment suitable for analyte retention. This is followed by equilibration in a solvent similar to the sample/matrix.
sample/matrix pre-treatment and loading	To vary the sample/matrix composition in a way that leads to retention of the analyte(s) while keeping impurities to a minimum.
washing	To remove impurities that are not as strongly bound to the sorbent as the analyte(s).
analyte elution	To disrupt functional bonding mechanisms and selectively release compounds for collection and analysis.

4.5 METHOD DEVELOPMENT AND OPTIMIZATION

An extraction method was developed using the CLEAN SCREEN® DAU and the larger-particle, XtrackT® columns, from United Chemical Technologies Inc (Pennsylvania, USA). These columns contain a copolymeric silica extraction support consisting of hydrophobic chains (octylsilane, *n*-C8) and strong cation-exchange moieties (benzenesulfonic acid) that have the ability to retain acidic, neutral, basic and amphoteric drugs on one column.

In this study, the method followed was based on several reported procedures in the literature for the extraction of drugs from biological materials [21, 23, 28, 34, 36, 37, 48]. It was then further modified to improve detection and recovery by the careful selection of solvents and by choosing the appropriate sorbent particle size. Capillary gas chromatography with fused-silica capillary columns and nitrogen-phosphorus detection (GC-NPD) has proved to be a powerful tool for

the analysis of underivatized drugs in biological extracts, and it was therefore used in this study. Since many drugs contain nitrogen, traces of them can be selectively detected with minimal interference from non-nitrogenous compounds, both endogenous and exogenous [49]. GC-MS with single-ion monitoring was employed for quantitative and qualitative analysis of the spiked compounds based on the diagnostic mass spectra of the analytes; the base peaks were used for quantification. Although this method was developed using urine, it was also intended to be used for the extraction of drugs from other body fluids as well as tissue [50-52].

The first step in developing an effective SPE method is the choice of the most appropriate retention mechanism and of the sorbent. The main considerations in the sorbent selection process are the physical nature of the sample/matrix, the chemical structure of the analyte, and the unique selectivity and specificity properties of each individual sorbent [53]. As the extraction method was required to extract a range of drugs with varying pK_a values during a single extraction procedure, a combination of separation strategies was needed. A copolymeric bonded phase was therefore selected for this purpose.

4.5.1 EFFECT OF SAMPLE APPLICATION pH ON COMPOUND RETENTION

There are three possible mechanisms of interaction between a sorbent and any compound, namely adsorption, hydrophobic and ion exchange. The application pH affects the retention and recovery of a drug. When a sample containing the drugs chosen for this study is prepared, phosphate buffer at pH 6.0 is added and loaded onto a copolymeric column. The basic drugs - cocaine, imipramine, methamphetamine and secobarbital (the pK_a's of which are 8.4, 9.5, 9.9 and 8.0 respectively) - are almost fully ionized at this pH. The average pK_a for these drugs is 9.0. Therefore, at the pH of 9, 50 % of the drugs are protonated (neutral) and the other 50 % are ionized. The pK_a of cocaine is 8.4 and therefore it is fully charged and retained on

the cation-exchange part of the sorbent at pH 6.4. At pH 10.4, the molecule is protonated/neutral and can be eluted. The pK_a of imipramine is 9.5 and it is therefore charged and retained at pH 7.5 and neutral at pH 11.5. The pK_a of methamphetamine is 9.9, which means that it is charged at pH 7.9 and can be eluted at pH 11.9, when it is protonated. At the application pH of 6, cocaine, imipramine, methamphetamine and secobarbital are all fully ionized and would be retained by the sulfonic acid moiety of the sorbent. The pK_a of secobarbital is 8.0 and it is therefore charged at pH 6.0 and neutral at pH 4.0, and will bind to the cation-exchange part of the sorbent through electrostatic bonding. The secobarbital molecule, however, also has a significant non-polar portion that would be attracted to the hydrophobic part of the sorbent.

At the same application pH of 6.0, of the acidic drugs in the sample - lorazepam, oxazepam and prazepam, the pK_a's of which are 1.3 and 11.5, 1.7 and 11.6, and 2.7 respectively - only prazepam is fully ionized. Since the sorbent does not contain any anion-exchange groups, these compounds cannot bind to the column via this mechanism and, in contrast to the binding mechanism of the basic compounds, the neutral or non-polar parts of the acidic drugs are attracted to the hydrophobic chains on the sorbent.

4.5.2 EFFECT OF ELUTION SOLVENT ON COMPOUND RETENTION

The column properties make provision for the effective retention of acidic, neutral and basic compounds, but it is also necessary to elute the compounds from the column selectively. The polarity of the solvent used to elute each compound from the column becomes critical (Figure 4.7). The elution solvent strength should be the weakest solvent that completely disrupts all binding mechanisms of the analyte as needed (hydrophobic, polar, and/or ion exchange). For each drug, the energy of elution of the specific solvent should be higher than the energy of adsorption.

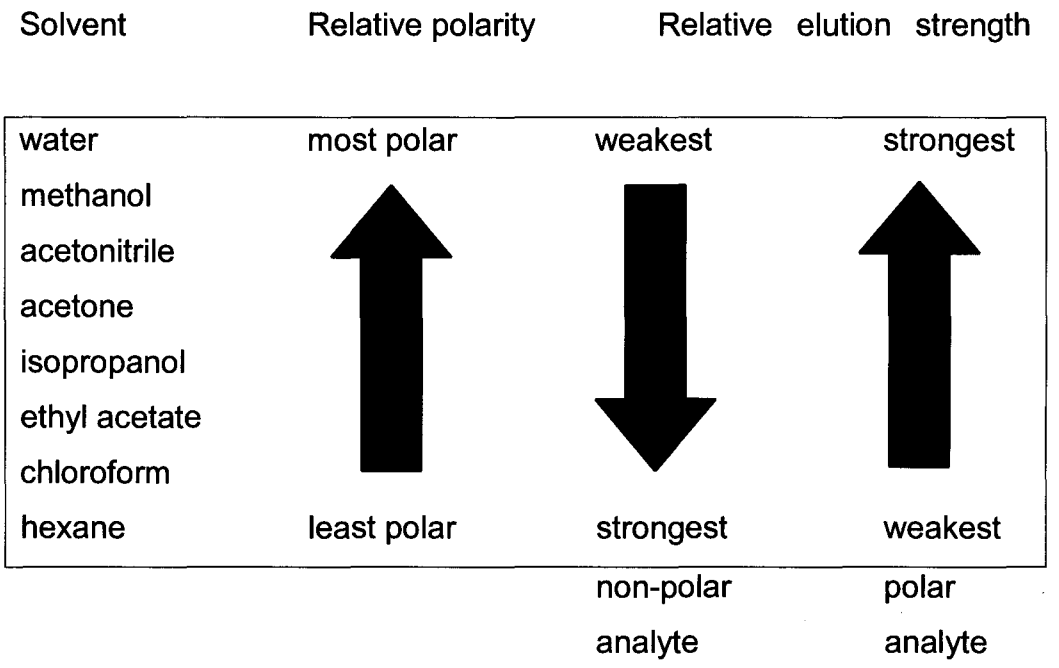


Figure 4.7 **Solvent polarity and elution strength [21]**

Acidic/neutral compounds and analytes that are hydrophobically bound can be removed using organic solvents of minimal polarity such as the hexane and ethyl acetate mixture (50:50 v/v), which can elute the hydrophobically bound analytes. On the other hand, cationic compounds and many others with intermediate polarity, including potential interferences, will remain bound to the column. Most of the potential interferences that are not ionically bound can be removed by means of a methanol wash. The effective elution of cationic analytes is achieved by applying an organic solution with a high pH, such as the methylene chloride - isopropanol - ammonium hydroxide mixture (78:20:2 v/v). The eluting strength and the pH of this solvent mixture are appropriate for these compounds. The methylene chloride - isopropanol - ammonium hydroxide mixture simultaneously disrupts the ionic interactions and successfully elutes the cationic compounds. The key principle that applies here is that "like dissolves like", which is another factor required for the elution of drugs from an SPE column.

Drying is performed before each elution step, under full vacuum or positive pressure (in the case of positive-pressure manifold or automated extraction systems), in order to remove all traces of residual sample/matrix and wash solvent, especially those that are immiscible with water (for example, hexane, ethyl acetate and methylene chloride), from the sorbent. This produces a more concentrated final extract with a constant volume and composition, and ensures better reproducibility and higher recoveries. See Appendix 2 for the details of the extraction method.

4.6 EXPERIMENTAL

4.6.1 DRUG STANDARDS AND CHEMICALS

A series of acidic/neutral and basic drugs was used to investigate the effect of the pH of sample application on isolate retention and subsequent recovery. Cocaine, imipramine, lorazepam, methamphetamine, oxazepam, prazepam, phenobarbital and secobarbital were obtained as drug standards from Industrial Analytical (Midrand, South Africa).

CLEAN SCREEN® DAU (200 mg, 3 mℓ capacity) and XtrackT® (200 mg, 3 mℓ capacity) copolymeric extraction columns were provided by PM Separations (Brisbane, Australia). The Caliper RapidTrace automated SPE system and the TurboVap evaporator were purchased from Microsep. HPLC grade solvents and reagent grade chemicals were obtained from Merck.

The analytes were purchased at a concentration of 1 mg/mℓ. Individual stock solutions of 100 µg/mℓ were prepared by mixing 100 µℓ of each pure compound with 900 µℓ of methanol. These stock solutions were stored in glass vials at 4 °C. Appropriate dilutions with methanol yielded the working solutions of the compounds used in the study. Prazepam was used as an internal standard to provide a reference to

which concentrations and responses could be compared. The presence of prazepam in urine has never been reported and only trace amounts of nordiazepam, its major metabolite, have been found [45].

4.6.2 PREPARATION OF SPIKED URINE SAMPLES

Blank urine samples collected from two subjects were combined and analysed for the presence of interferences. A mixture of drug standards was prepared by combining 100 µℓ of cocaine, imipramine, lorazepam, methamphetamine, oxazepam, phenobarbital and secobarbital respectively (each at 1 mg/mℓ) in a vial and diluting to 1 mℓ with methanol, giving a concentration of 100 µg/mℓ for each standard. 100 µℓ of this solution was added to 900 µℓ of the blank urine sample, which yielded a solution with a concentration of 10 µg/mℓ for each of the seven drugs. 500 µℓ of the 1 mg/mℓ prazepam solution (internal standard) was added to 49.5 mℓ of a phosphate buffer solution, giving a prazepam concentration of 10 µg/mℓ.

4.7 EXTRACTIONS

4.7.1 INVESTIGATION OF CLEAN SCREEN® DAU COLUMN (200 mg, 3 mℓ capacity)

Clean Screen® DAU columns were conditioned with methanol (3 mℓ), deionized water (3 mℓ) and 0.1 M phosphate buffer (pH 6; 1 mℓ). The sample (1 mℓ) was buffered to pH 6 and added to the sorbent at a rate of 1 to 2 mℓ/min. The column was washed with deionized water (3 mℓ) and 0.1 M acetic acid (1 mℓ). After drying the column for 5 minutes, the sorbent was rinsed with hexane (2 mℓ). The acidic/neutral drugs were then eluted with hexane - ethyl acetate (50:50 v/v; 3 mℓ) after which the column was washed with methanol (3 mℓ) and again dried for 5 minutes. The basic drugs were eluted with dichloromethane - isopropyl alcohol - ammonium hydroxide (78:20:2 v/v; 3 mℓ). The extracts were evaporated to dryness at < 40 °C under a stream of nitrogen, and

reconstituted in ethyl acetate (100 µℓ, acidic/neutral fraction) and methanol (100 µℓ, basic fraction) prior to analysis by GC-NPD and GC-MS.

4.7.2 INVESTIGATION OF XtrackT® COLUMN (200 mg, 3 mℓ capacity)

The extraction procedure described above was carried out, with the spiked mixture also being applied at pH 6.0, but using the XtrackT® column, which has a larger particle size. The extracts were evaporated to dryness at < 40 °C under a stream of nitrogen, and reconstituted in ethyl acetate (100 µℓ, acidic/neutral fraction) and methanol (100 µℓ, basic fraction) prior to analysis by GC-NPD and GC-MS.

4.7.3 INVESTIGATION OF DIMETHYL FORMAMIDE AS "KEEPER" SOLVENT

Certain compounds such as amphetamines and phencyclidine are heat labile, and care should be taken not to overheat or over-evaporate the samples when such drugs are present. In forensic samples, however, it is not always known what drugs are present in a sample and derivatization is therefore required. 40 µℓ of dimethyl formamide (DMF), a "keeper" solvent [45], was added to the elute of the basic fraction of the spiked urine to reduce the loss of the heat-labile compounds caused by their volatile nature. Such loss can occur during the evaporation stage or in the injection port of the GC-NPD or GC-MS when the extract is injected [54]. DMF solubilizes the analyte and promotes conditions that improve the reaction yield. It is not a very volatile organic solvent and acts to dissolve the drugs in an environment that has a higher boiling point than that of the drugs themselves; this reduces their volatility and hence increases their recovery.

4.7.4 INVESTIGATION OF THE EXTRACTION EFFICIENCY OF THE FCL CTN IN-HOUSE METHODS COMPARED TO THE XtrackT® COLUMN SPE METHOD

The butyl acetate and Celite extraction methods were compared to the XtrackT® column SPE method. The same sample, namely urine spiked with the eight drugs, including the internal standard, each at a concentration of 10 µg/mℓ, was used for each extraction procedure. DMF was added to the elute of the basic fraction of the SPE sample. The extracts were analysed on GC-MS with single-ion monitoring, and the ions for each compound were extracted (a term used to describe selecting specific ions out of a chromatogram) and the areas under the base peaks were compared with those from the other extraction techniques. The base peak of a compound refers to the most intense ion, which is assigned an abundance of 100. Figures 4.8 to 4.14 show the fragmentation patterns obtained for the respective compounds, with the base peak showing the highest abundance.

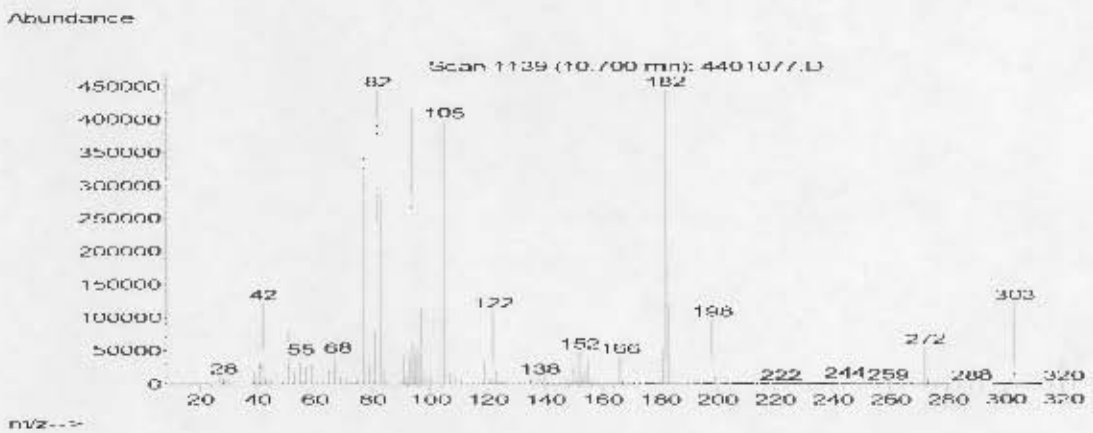


Figure 4.8 **GC-MS chromatogram (of selected ions) of the cocaine standard (200 µg/mℓ), with the base peak at 82 m/z**

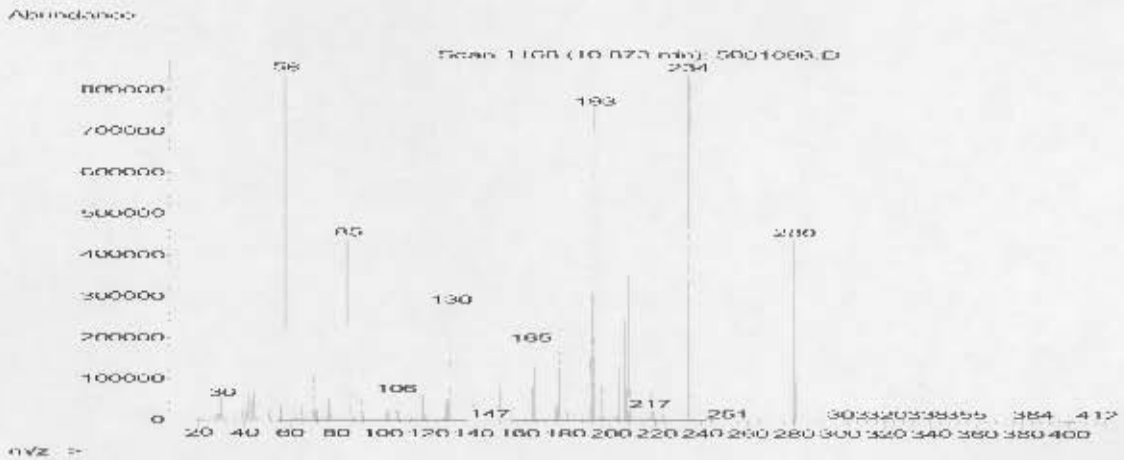


Figure 4.9 **GC-MS chromatogram (of selected ions) of the imipramine standard (200 µg/mℓ), with the base peak at 58 m/z**

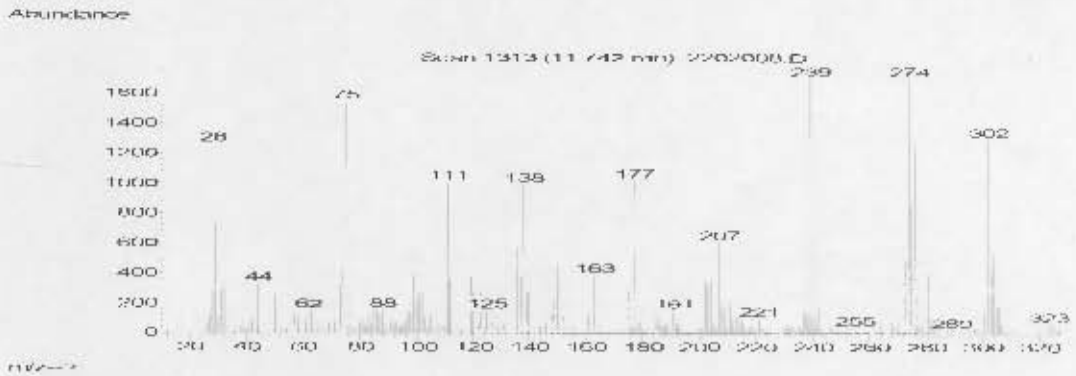


Figure 4.10 **GC-MS chromatogram (of selected ions) of the lorazepam standard (30 µg/mℓ), with the base peak at 239 m/z**

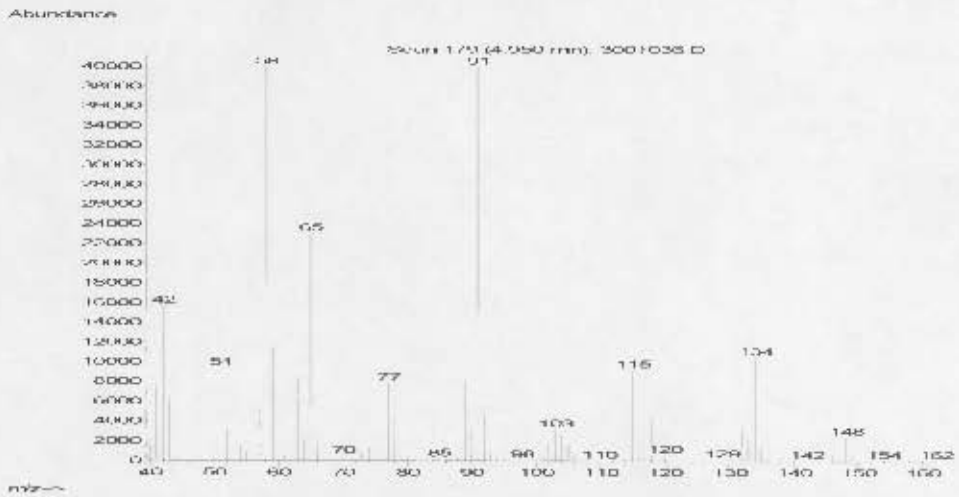


Figure 4.11 **GC-MS chromatogram (of selected ions) of the methamphetamine standard (150 µg/mℓ), with the base peak at 58 m/z**

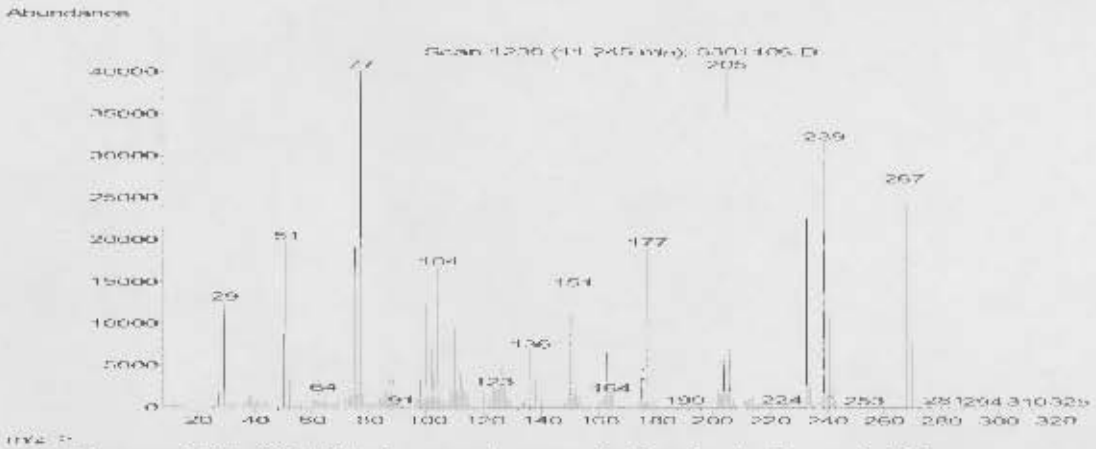


Figure 4.12 **GC-MS chromatogram (of selected ions) of the oxazepam standard (200 µg/mℓ), with the base peak at 205 m/z**

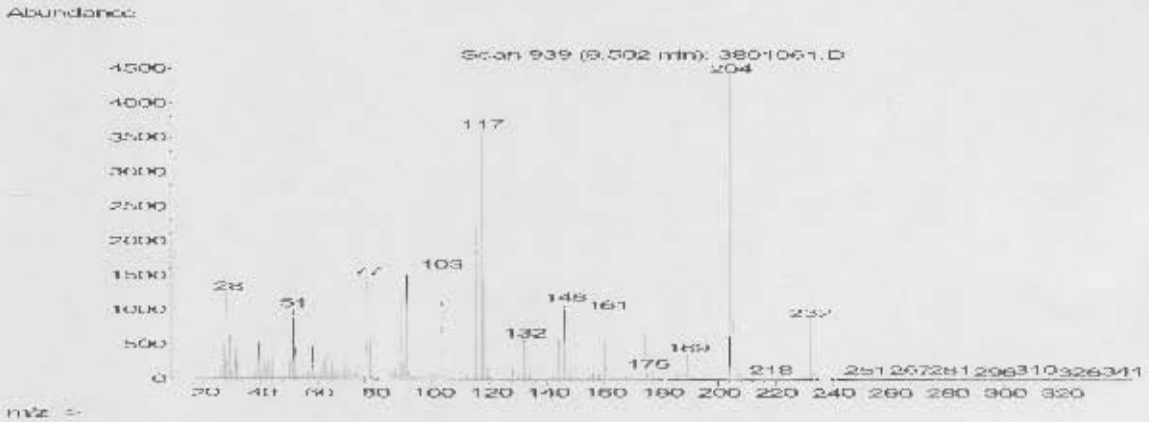


Figure 4.13 **GC-MS chromatogram (of selected ions) of the phenobarbital standard (200 µg/mℓ), with the base peak at 200 m/z**

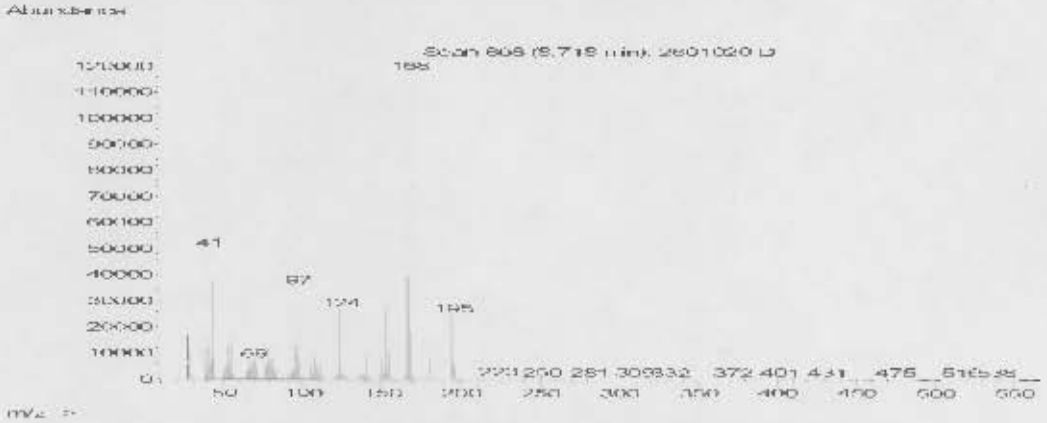


Figure 4.14 **GC-MS chromatogram (of selected ions) of the secobarbital standard (250 µg/mℓ), with the base peak at 168 m/z**

4.8 RESULTS AND DISCUSSION

4.8.1 CLEAN SCREEN® DAU COLUMN (200 mg, 3 mℓ capacity)

The analytes retained by the hydrophobic groups of the sorbent were eluted using the moderately polar solvent combination of hexane and ethyl acetate. The chromatogram is shown in Figure 4.15.

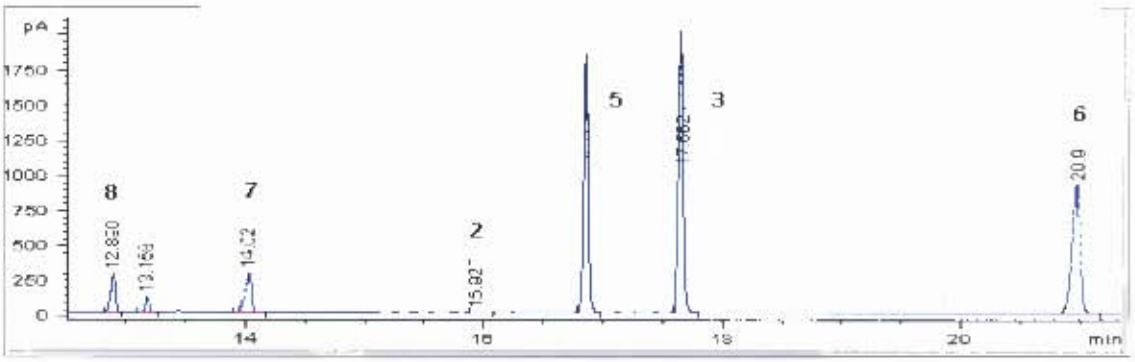


Figure 4.15 **GC-NPD chromatogram of the acidic/neutral fraction from urine spiked with 10 µg/mℓ of the test mixture, and extracted from the CLEAN SCREEN® DAU column (2: imipramine, 3: lorazepam, 5: oxazepam, 6: prazepam (internal standard), 7: phenobarbital, 8: secobarbital)**

The drugs with pK_a values of 8.0 or less – lorazepam, oxazepam, phenobarbital, prazepam and secobarbital – were all extracted predominantly in the acidic fraction. Imipramine (Compound 2) was, however, only partially extracted with this elution solvent.

The basic analytes retained by the cation-exchange groups of the sorbent were eluted by using the ammoniated dichloromethane and isopropylalcohol (IPA) solvent mixture. The chromatogram is shown in Figure 4.16.

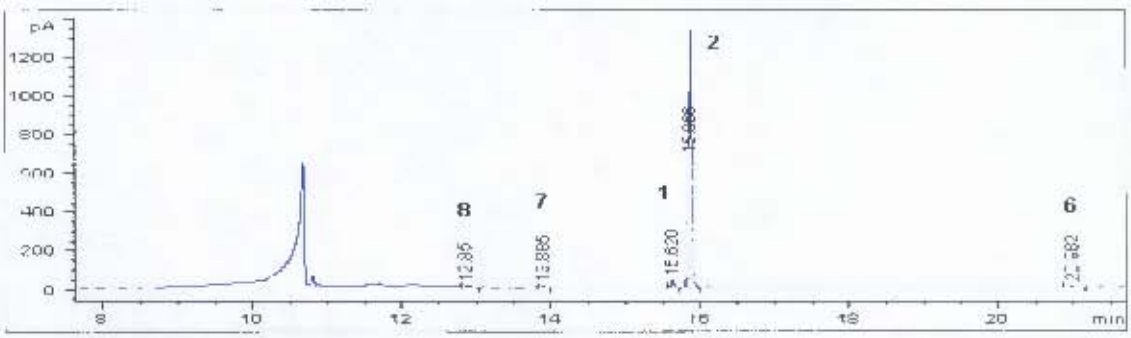


Figure 4.16 **GC-NPD chromatogram of the basic fraction from urine spiked with 10 µg/mℓ of the test mixture, and extracted from the CLEAN SCREEN® DAU column (1: cocaine, 2: imipramine, 6: prazepam (internal standard) 7: phenobarbital, 8: secobarbital)**

Only imipramine (pK_a 9.5) and cocaine (pK_a 8.4) were retained, with partial extractions of secobarbital, phenobarbital and prazepam occurring with this eluent. Methamphetamine was not, however, detected by GC-NPD in the basic fraction of the extraction procedure involving the CLEAN SCREEN® DAU column, as can be seen by the absence of a peak at the retention time of 9.2 minutes. The internal standard (prazepam), however, eluted only in the acidic/neutral fraction and not in the basic fraction during the extraction process. As a result, the ratios for analyte to internal standard response could not be calculated for all the compounds in both fractions. The calculated recoveries during the method development stage were therefore based on external standards only and are thus only a rough estimate of the recovery potential of the extraction columns.

4.8.2 XtrackT® COLUMN (200 mg, 3 mℓ capacity)

Figure 4.17 shows that lorazepam, oxazepam, phenobarbital, prazepam and secobarbital were, as with the CLEAN SCREEN® DAU column, extracted predominantly by the hexane and ethyl acetate solvent combination.

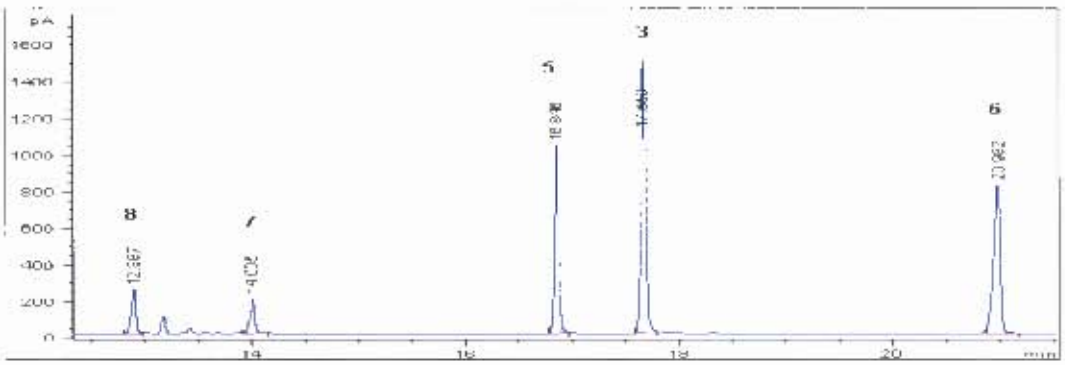


Figure 4.17 **GC-NPD chromatogram of the acidic/neutral fraction from urine spiked with 10 µg/mℓ of the test mixture, and extracted from the XtrackT® column (3: lorazepam, 5: oxazepam, 6: prazepam, 7: phenobarbital, 8: secobarbital)**

.No major differences were observed in the extraction capabilities between the XtrackT® and CLEAN SCREEN® DAU columns for the above-mentioned compounds.

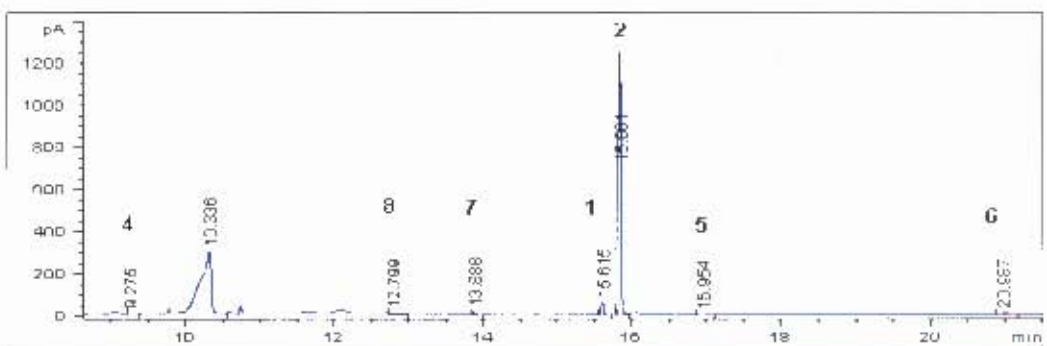


Figure 4.18 **GC-NPD chromatogram of the basic fraction from urine spiked with 10 µg/mℓ of the test mixture and extracted from the XtrackT® column (1: cocaine, 2: imipramine, 4: methamphetamine, 5: oxazepam, 6: prazepam (internal standard), 7: phenobarbital, 8: secobarbital)**

The elution fraction containing the basic drugs is shown in Figure 4.18. At the application pH of 6.0, cocaine, imipramine and methamphetamine were almost completely ionized and were

consequently well retained on the column. The ammoniated dichloromethane and IPA solvent mixture was able to deprotonate the drugs and thereby displace them. Methamphetamine (Compound 4) was, however, only partially detected in this elution fraction.

4.8.3 DIMETHYL FORMAMIDE AS "KEEPER" SOLVENT

The addition of the "keeper" solvent DMF to the elute of the basic fraction (Figure 4.19) of the extract prevented the volatile components in the sample, such as methamphetamine, from evaporating.

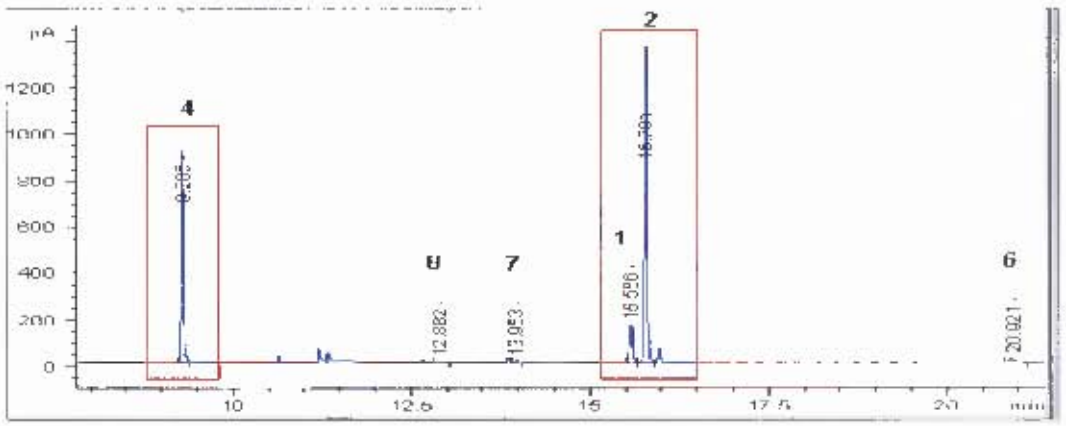


Figure 4.19 **GC-NPD chromatogram of the basic fraction from urine spiked with 10 µg/mℓ of the test mixture and extracted from the CLEAN SCREEN® DAU column with DMF added (1: cocaine, 2: imipramine, 4: methamphetamine, 6: prazepam (internal standard), 7: phenobarbital, 8: secobarbital)**

Following the addition of DMF to the basic fraction extracts, the methamphetamine was detected after use of the CLEAN SCREEN® DAU and the XtrackT® columns. The other basic components, cocaine and imipramine, also showed increased recoveries. These increases were, however, more noticeable with the XtrackT® column (Figures 4.19 and 4.20).

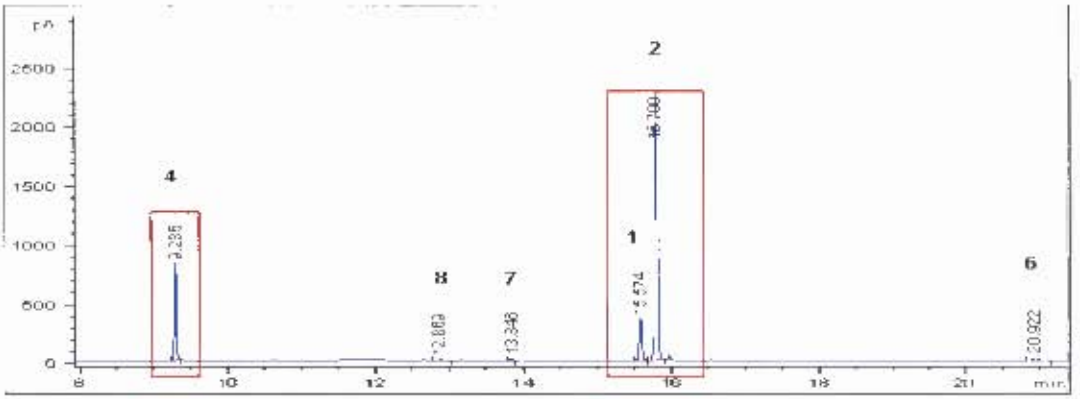


Figure 4.20 **GC-NPD chromatogram of the basic fraction from urine spiked with 10 µg/mℓ of the test mixture and extracted from the XtrackT® column with DMF added (1: cocaine, 2: imipramine, 4: methamphetamine, 6: prazepam (internal standard), 7: phenobarbital, 8: secobarbital)**

Although the urine sample was spiked with methamphetamine before the initial extractions, there was no indication of its presence when the CLEAN SCREEN® DAU column was used and only a small peak when the XtrackT® column was used. The addition of DMF improved the yield of methamphetamine and the other basic drugs in the extraction: the methamphetamine peak is now present in the CLEAN SCREEN® DAU chromatogram, and methamphetamine as well as cocaine and imipramine all show a higher abundance on the XtrackT® column (Figures 4.19 and 4.20).

4.8.4 THE EXTRACTION EFFICIENCY OF THE FCL CTN IN-HOUSE METHODS COMPARED TO THE XtrackT® COLUMN USING THE SPE METHOD

The XtrackT® SPE procedure was compared with the butyl acetate and Celite methods currently in use at the FCL CTN. Urine samples were spiked at 10 µg/mℓ, in triplicate, for each method, extracted and analysed on GC-MS using single-ion monitoring (SIM). For this experiment, the internal standard was added to the extracted fractions rather than to the urine sample before the extraction process. The

area under each of the base peaks for the spiked and extracted urine samples was measured, for the three extraction methods. The results of the comparative study are summarized in Table 4.3 and Figure 4.21.

Table 4.3 **Comparison of FCL CTN and SPE methods using extracted base-peak ions analysed on GC-MS SIM**

drug	retention time (min)	pK_a	base peak	SPE average % recovery (n = 3)	butyl acetate % average recovery (n = 3)	Celite average % recovery (n = 3)
1. cocaine	8.94	8.4	82	104.7	39.9	74.5
2. imipramine	9.06	9.5	58	102.6	39.5	82.5
3. lorazepam	9.67	1.3 / 11.5	239	23.0	19.3	ND
4. methamphetamine	4.27	9.5	58	99.9	65.5	ND
5. oxazepam	9.40	1.7 / 11.6	205	56.8	3.9	ND
6. phenobarbital	7.96	7.5	204	72.4	29.0	71.7
7. secobarbital	7.25	8.0	168	60.8	ND	58.9

ND = not detected

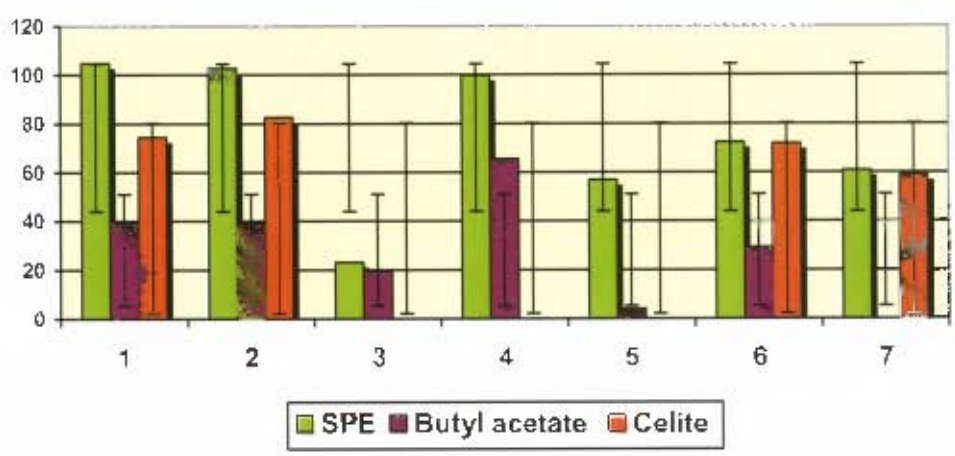


Figure 4.21 **Results of the comparison of the extraction efficiencies of the SPE, Celite and butyl acetate procedures (1: cocaine, 2: imipramine, 3: lorazepam, 4: methamphetamine, 5: oxazepam, 6: phenobarbital, 7: secobarbital) with error bars indicating the standard deviation of the measured recoveries**

The butyl acetate extraction method was able to extract all the drugs except secobarbital, its highest extraction recovery being that of methamphetamine at 65.5 %. The Celite method gave extraction recoveries of between 58 % and 82 %, but it was not able to extract lorazepam, methamphetamine or oxazepam. The SPE method was able to extract all the spiked drugs with recoveries of between 23 % and 104 %. The Celite and SPE methods yielded similar extraction recoveries for phenobarbital and secobarbital.

4.9 CONCLUSIONS

These experimental results show that factors such as the functional groups of the extracted compounds at given pH values, the pK_a values and the compound polarities need to be considered when determining the application pH and strength of eluents. The mixed-mode sorbents allowed the acidic/neutral compounds (lorazepam, oxazepam, phenobarbital and prazepam) to adsorb onto the column mostly through hydrophobic attractions. Similarly, the basic compounds (cocaine, imipramine, methamphetamine and secobarbital) were held predominantly through ionic but also some hydrophobic attractions at the application pH of 6.0. The CLEAN SCREEN® DAU and XtrackT® columns were equally effective at extracting all the spiked drugs, since the sorbent properties of both columns are similar, although the XtrackT® column has a slightly larger particle size. The low recoveries of oxazepam and lorazepam could be due to their limited solubility in water [36]. In real samples, however, a common route of biotransformation for benzodiazepines is conjugate formation in which the drug combines with a neutral sugar to form a glucuronide [36]. These samples must consequently be deconjugated before analysis, and chemical derivitization is required for better detection and confirmation results [36].

In order to elute the basic drugs, the ionic and hydrophobic interactions had to be suppressed. This was achieved by eluting with a relatively

non-polar organic solvent containing ammonium hydroxide so that the pH of the eluent was alkaline. The charges on the basic drugs were neutralized by the pH of the eluent, which allowed them to be removed from the sorbent easily. Methamphetamine was successfully recovered from the sorbent by the addition of IPA to the eluent, thereby increasing the polarity of this relatively polar drug and hence increasing its solubility.

The addition of the "keeper" solvent DMF to the basic fraction of the eluent to minimize the evaporative loss of the heat-labile compounds, improved the recovery not only of methamphetamine, but also of cocaine and imipramine. The only difference in the extraction of the spiked urine samples on the two columns resulted from the addition of DMF, which led to a greater recovery of the basic drugs using the XtrackT® column compared to the CLEAN SCREEN® DAU column. Overall, the SPE method using the XtrackT® column gave the best extraction recoveries and was also the only method to isolate all the spiked drugs, and this method was therefore chosen to be validated. Validation was necessary in order to determine the method's suitability for routine use. It was, however, observed during the method development process that the addition of prazepam as an internal standard to the urine sample prior to extraction, resulted in prazepam being eluted only in the acidic fractions of the extraction process. It was therefore not possible to compensate for variations in the extraction process for all the drugs in the SPE process. As a result of this observation, the comparative study was performed with prazepam being added to the eluted fractions of the SPE method, as well as for the butyl acetate and Celite methods, before drying and reconstitution. Prazepam was similarly applied during the validation process.

CHAPTER 5

VALIDATION OF THE SPE METHOD

5.1 VALIDATION PROCEDURE

Validation is the process by which it is established, by means of laboratory studies, that the performance characteristics of a test meet the requirements for the intended analytical application. The process also establishes the limitations of the test as well as any influences that may change these characteristics, and to what extent. The following criteria were used for the validation of the SPE method: specificity, accuracy, precision, linearity of method, limit of quantification and limit of detection. The validation process was performed using urine as the matrix and it was spiked with cocaine, imipramine, lorazepam, methamphetamine, oxazepam and phenobarbital. These drugs were extracted on XtrackT® columns. Prazepam was used as an internal standard and was added to the extracted acidic and basic fractions before the drying and reconstitution steps in the method. DMF was added to the basic fraction to prevent evaporation of the volatile analytes. Detection and quantification of the spiked compounds of both the acidic and basic fractions were performed by GC-MS with single-ion monitoring.

As the developed SPE method was based on recommended techniques and generic procedures in the literature on extraction from biological matrices, it had to be determined through the process of *validation whether the method was fit for its intended use.*

Stock solutions, each at a concentration of 1 mg/mℓ, were used for the preparation of the working solutions. A mixture of these calibration standards was then prepared by combining 600 µℓ of each of the seven

standards and diluting to 6 mℓ with methanol. An internal standard, prazepam, was added to each mixture to give a fixed concentration of 20 µg/mℓ. Dilutions were made from this mixture to obtain concentrations, for each drug, of 40 µg/mℓ, 35 µg/mℓ, 30 µg/mℓ, 25 µg/mℓ, 20 µg/mℓ, 15 µg/mℓ, 10 µg/mℓ, 5 µg/mℓ and 3 µg/mℓ respectively. A calibration curve was established for each drug. The internal standard, prazepam, was added to all the samples and standards in order to compensate for variations such as injection volume and detector performance during the analysis. The concentration of each analyte, calculated using the internal standard, is given by the formula:

$$C_{unknown} = \frac{A_{ISTD1}}{A_{ISTD2}} \times \frac{A_{unknown}}{A_{known}}$$

where $C_{unknown}$ is the concentration of the analyte,
A_{ISTD1} is the peak area of the internal standard in the standard,
A_{ISTD2} is the peak area of the internal standard in the sample,
$A_{unknown}$ is the area of the sample peak, and
A_{known} is the area of the standard peak.

5.2 SPECIFICITY

Specificity is the ability of a method to differentiate and measure the analyte accurately in the presence of interfering components in the matrix. The specificity was investigated by analysing blank urine samples obtained from four sources as well as two urine samples to which internal standard had been added. Specificity is established by ensuring that the sample matrix is free from interfering peaks and that the signal produced is due to the analyte only and not a chemically or physically similar or any other compound. No peaks that interfere with any of the compounds of interest were observed in the chromatograms of any of the urine samples, with or without the addition of internal standard.

5.3 ACCURACY

Accuracy expresses the closeness of agreement between the value that is accepted as a conventional true value, or an accepted reference value, and the value found [55]. In this study, the accuracy was investigated by analysing three separate urine aliquots that had been spiked at the three concentration levels of 50 %, 100 % and 150 % of the target concentration of 20 µg/mℓ (that is, 10 µg/mℓ, 20 µg/mℓ and 30 µg/mℓ respectively) in triplicate. The accuracy of the method was determined by calculating the percentage recovery of the spiked analyte based on the value of the certified reference material. It is given by the formula:

$$\text{Recovery} = \frac{(c_s - c_u)}{c_a} \times 100$$

where

c_s is the measured analyte concentration in the spiked sample,

c_u is the measured analyte concentration in the unspiked sample, and

c_a is the calculated analyte concentration in the spiked sample.

The accuracy of the analytical procedure was measured by calculating the average percentage recoveries, with three analyses by GC-MS SIM, and the relative standard deviation (RSD) in each case. RSD is calculated as follows:

$$\text{RSD} = \frac{s}{\bar{x}} \times 100 ,$$

where s is the standard deviation, with $s = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2}$,

$\bar{x}$ is the average of the observed values, with $\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$,

n is the number of observations, and

x_i are the observed values.

The results are given in Table 5.1.

Table 5.1 **Average percentage recovery and relative standard deviation (RSD; n = 3) for the seven drugs spiked at three concentration levels**

compound	average % recovery at 10 µg/mℓ	RSD (%)	average % recovery at 20 µg/mℓ	RSD (%)	average % recovery at 30 µg/mℓ	RSD (%)
cocaine	98.5	5.4	98.9	12.2	101.5	5.9
imipramine	94.3	14.0	106.7	6.1	96.5	3.0
lorazepam	20.9	13.8	15.3	10.9	13.7	14.8
methamphetamine	100.2	4.6	104.0	1.3	101.9	1.1
oxazepam	48.4	9.7	42.4	10.5	56.9	4.8
phenobarbital	67.6	7.3	63.0	10.4	65.8	4.2
secobarbital	54.2	8.8	57.3	8.8	50.6	11.7

The recoveries obtained for this procedure range from 13.7 % for lorazepam to 106.7 % for imipramine. The results obtained for cocaine, imipramine and methamphetamine compared well with the certified values of these analytes. Lower recoveries were, however, obtained for the other analytes extracted, with lorazepam yielding the lowest recovery. This indicated that the procedure was not optimal for the effective extraction of lorazepam, oxazepam, phenobarbital or secobarbital, or, by implication, of benzodiazepines or barbiturates in general. Variable analytical recoveries of benzodiazepines are often attributed to their limited solubility in water, and methods involving chemical derivatization to improve their detection have been reported [21]. It is also possible that the wash solvent, hexane, used during the extraction process, removed some of the hydrophobically bound compounds. While hexane is very effective in removing traces of water prior to analyte elution, it is also one of the least polar solvents and therefore a strong eluent for non-polar compounds. Nevertheless, the

recoveries for the barbiturates were, on average, acceptable and could possibly be improved by increasing the sample volume or decreasing the amount of phosphate buffer added. The RSDs were, in general, fairly consistent at the three concentration levels, with methamphetamine having the lowest RSD of all the compounds extracted.

Compared to the results obtained during the method development stage (Chapter 4), which was performed using the same extraction column but without an internal standard, similarly lower recoveries were observed for lorazepam, oxazepam, phenobarbital and secobarbital, but with higher RSD values. At a spiked concentration of 20 µg/mℓ, the average percentage recoveries, for three extractions, of lorazepam, oxazepam, phenobarbital and secobarbital were 30.5 %, 29.2 %, 17.2 % and 47.3 % respectively, whereas during the method validation stage, for the same spiked concentration, the average percentage recoveries, for three extractions, were 15.3 %, 42.4 %, 63.0 % and 57.3 % respectively. This shows the beneficial effect of the internal standard in producing more consistent results.

5.4 PRECISION

The precision of an analytical procedure is the closeness of agreement, or degree of scatter, among individual test results when the procedure is applied repeatedly to multiple samples under the same conditions. The two most common precision measures are repeatability and reproducibility. They represent the two extreme measures of precision that can be obtained. Repeatability, which is the smallest expected precision, indicates the variability to be expected when a method is performed in duplicate by a single analyst on one piece of equipment over a short period of time. For a sample that is to be analysed by several laboratories for comparative purposes, reproducibility, which is the largest type of precision usually found and which excludes variation with respect to time, is the preferred measure of precision. Precision between these two extremes can also be measured, for example the

variability between different analysts, over an extended period, within a single laboratory: this is called intermediate precision [55]. The precision data in this study covers repeatability and intermediate precision. Precision is expressed in terms of relative standard deviation [55, 56].

5.4.1 Repeatability

Repeatability expresses the degree of scatter within a series of measurements obtained under the same operating conditions over a short period of time. This precision was determined by analysing, by means of GC-MS SIM, three separate urine aliquots that had been spiked and extracted at the three concentration levels in triplicate, and by repeating this process two days later. The repeatability was evaluated by calculating the relative standard deviations of the recoveries obtained for each compound at the three concentration levels on the different days. The results are given in Table 5.2.

Table 5.2 **Repeatability in terms of the RSD (n = 2) of the average percentage recoveries for the seven drugs spiked at three concentration levels and analysed on two different days**

compound	average % recovery at 10 µg/mℓ		RSD (%)	average % recovery at 20 µg/mℓ		RSD (%)	average % recovery at 30 µg/mℓ		RSD (%)
	day 1	day 3		day 1	day 3		day 1	day 3	
cocaine	98.5	97.1	1.0	98.9	94.6	3.2	101.5	99.6	1.2
imipramine	94.3	91.8	1.9	106.7	88.7	13.0	96.5	98.8	1.7
lorazepam	20.9	19.7	4.2	15.3	14.7	2.8	12.7	13.4	3.8
methamphetamine	100.2	99.2	0.7	104.0	107.5	2.3	101.9	105.7	2.6
oxazepam	48.4	53.0	6.4	42.4	49.4	10.8	56.9	54.1	3.6
phenobarbital	67.6	70.7	3.2	63.0	68.1	5.5	65.8	65.2	0.6
secobarbital	54.2	49.1	7.0	57.3	58.5	1.5	50.6	53.7	4.2

The average percentage recoveries obtained on day 1 and on day 3 compare well with each other for all the compounds at all three concentration levels, and the relative standard deviations are all low. This indicates that no significant difference is expected between duplicate analyses when this method is used, thus meeting the criterion for good repeatability. The RSD values for imipramine, oxazepam, phenobarbital and secobarbital show more variation between the three concentration levels than do those for cocaine, lorazepam and methamphetamine.

5.4.2 Intermediate precision

Intermediate precision expresses the effect of variation within a laboratory such as different analysts over a period of time, or different instruments. Three separate urine aliquots were spiked and extracted at the three concentration levels in triplicate. Different instruments, GC-MS and GC-NPD, were used for the analysis in order to determine the intermediate precision, which was evaluated by calculating the relative standard deviations of the recoveries obtained for each compound on the two instruments. The results are given in Table 5.3.

Table 5.3 **Intermediate precision in terms of the RSD (n = 2) of the average percentage recoveries for the seven drugs spiked at three concentration levels and analysed on two different instruments; ND = not detected**

compound	average % recovery at 10 µg/mℓ		RSD (%)	average % recovery at 20 µg/mℓ		RSD (%)	average % recovery at 30 µg/mℓ		RSD (%)
	GC-MS	GC-NPD		GC-MS	GC-NPD		GC-MS	GC-NPD	
cocaine	98.5	69.8	24.1	98.9	90.8	5.8	101.5	84.0	13.2
imipramine	94.3	92.2	1.6	106.7	104.7	1.3	96.5	104.0	5.3
lorazepam	20.9	31.4	28.4	15.3	ND	-	12.7	56.0	89.1
methamphetamine	100.2	66.7	28.4	104.0	118.0	8.9	101.9	111.0	6.0
oxazepam	48.4	21.4	54.7	42.4	ND	-	56.9	43.0	19.7
phenobarbital	67.6	4.0	125.6	63.0	ND	-	65.8	ND	-
secobarbital	54.2	21.0	62.4	57.3	ND	-	50.6	ND	-

Even though all the fractions, both basic and acidic, were injected on the GC-NPD, not all the compounds were detected by the nitrogen-phosphorus detector [54]. The GC-NPD response was erratic throughout the experiment, as the bead voltage to the detector was fluctuating and at times it was zero. As a result, oxazepam, phenobarbital and secobarbital at 20 µg/mℓ, and phenobarbital and secobarbital at 30 µg/mℓ, were not detected by GC-NPD. Even though it could not be shown conclusively what the effect of variation between the two instruments was, the results obtained for cocaine at 20 µg/mℓ, for imipramine at all three concentration levels and for methamphetamine at 20 µg/mℓ and 30 µg/mℓ, indicated that the variation between the two instruments was minimal.

5.5 Linearity of method

The linearity of an analytical procedure (also referred to as range, calibration model or linear range) is its ability, within a given range, to

obtain tests results that are directly proportional to the concentration (or amount) of compound in the sample. For a quantitative method it is necessary to establish the range of compound concentration over which the method may be applied. The upper and lower end of the working linear range should also include the target concentration, which in this study was 20 µg/mℓ. Within the working linear range, the signal response will have a linear (first order) or higher order (quadratic, logarithmic or point-to-point fit) relationship with the compound concentration. For a linear (first order) relationship, calculations based on a linear least squares regression provide useful information on the goodness of fit or correlation coefficient, but this calculation does not determine the linearity of the method. Linearity is determined by the analysis of the regression residuals, which are the differences between the predicted and the actual values as functions of the known concentrations. For a method to be linear, the residuals, when plotted, should fall randomly on either side of the zero line over the linear range. If the plot of residuals shows a systematic, rather than random, pattern about the zero line, then the working range is not considered linear [57]. The standard error of the estimate for regression measures the amount of variability in the points around the regression line. It is the standard deviation of the data points as they are distributed around the regression line. The calculation involves first finding the sum of the squares of the errors (SS_E) and then using that value to find the standard error (SE). The standard error of estimate for regression is calculated as follows:

$$SS_E = SS_y - \frac{(SS_{xy})^2}{SS_x} \quad \text{and} \quad SE = \sqrt{\frac{SS_E}{n-2}}$$

where

SS_E is the sum of the squares of the errors,

SS_y is the sum of the squares of the dependent variable,

SS_x is the sum of the squares of the independent variable,

SS_{xy} is the sum of the squares of the cross-product,

SE is the standard error of the estimate for regression,

n is the number of samples [57].

The linearity of method was determined by preparing spiked samples at seven concentrations with equal intervals over the range 3 µg/mℓ to 30 µg/mℓ. Each sample was injected five times. Linearity was investigated by the calculation of the regression statistics and by calculating and plotting the residuals. The sum of the squares of regression statistics, and the calculation of these statistics for cocaine as an example, are given in Tables 5.4 and 5.5. The plot of the regression residuals over the linear range for cocaine is given in Figure 5.1.

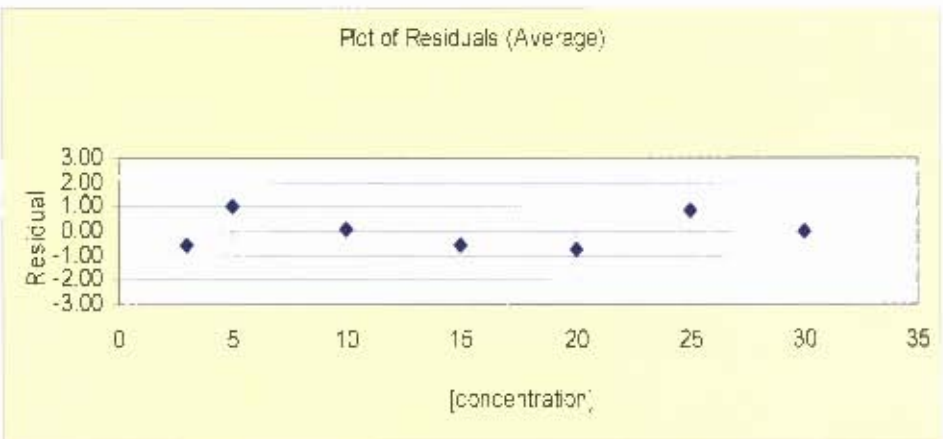
Table 5.4 **Sum of squares of regression statistics (slope, intercept and coefficient of determination) as well as residual regression analysis (linearity of method and linearity range)**

compound	slope (m)	intercept (b)	coefficient of determination (r^2)	*line equation: y = mx + c*	method linear? (Y/N)	linearity range
cocaine	0.29	-0.23	1.00	*y = 0.29x – 0.23*	Y	0 - 30 ppm
imipramine	0.19	0.62	0.97	*y = 0.19 + 0.62*	Y	0 - 30 ppm
lorazepam	0.03	-0.30	0.75	*y = 0.03x – 0.30*	N	10 - 30 ppm
methamphetamine	0.28	1.14	1.00	*y = 0.28x + 1.41*	Y	0 - 30 ppm
oxazepam	0.03	-0.11	0.79	*y = 0.02x – 0.11*	N	0 - 30 ppm
phenobarbital	0.03	0.16	0.96	*y = 0.03x + 0.16*	Y	0 - 30 ppm
secobarbital	0.05	-0.09	0.95	*y = 0.05x – 0.09*	Y	0 - 30 ppm

Table 5.5 Calculation **of sum of squares for regression statistics for cocaine**

Sum of squares for regression statistics					
	x	y	x^2	xy	y^2
	3.00	0.83	9.00	2.49	0.69
	5.00	0.96	25.00	4.80	0.92
	10.00	2.73	100.00	27.30	7.45
	15.00	4.43	225.00	66.45	19.62
	20.00	5.98	400.00	119.60	35.76
	25.00	6.99	625.00	174.75	48.86
	30.00	8.75	900.00	262.50	76.56
∑	108.00	30.67	2284.00	657.89	189.87
SS_{xy}	184.6957		Formula:		
SS_{xx}	617.7143		$y = mx + c$		
SS_{yy}	55.49289		y = 0.29x – 0.23		
SS_E	0.269123				
m	0.298999				
b	-0.23169				
R^2	0.99515				

Figure 5.1 **Plot of regression residuals of cocaine over the linear range**



The coefficient of determination was calculated to be 1.00 for cocaine and methamphetamine, indicating excellent linearity. When considering the plot of the regression residuals, imipramine, phenobarbital and secobarbital show linearity over the entire working

range, with the data points for these compounds falling randomly on either side of the zero line, even though their coefficients of determination range from 0.95 to 0.97. Lorazepam and oxazepam, however, demonstrate a slightly more systematic plot of regression residuals, with their coefficients of determination being 0.75 and 0.79 respectively, which indicates a non-linear response.

5.6 LIMIT OF DETECTION (LOD)

The LOD, expressed as the concentration c_L or the quantity $q_{L,}$ is derived from the smallest measure, $x_{L,}$ that can be detected with reasonable certainty for a given analytical procedure. The value of x_L is given by the equation:

$$x_L = x_{bl} + ks_{bl}$$

where x_{bl} is the mean of the blank measurements, s_{bl} is the standard deviation of the blank measurements, and k is a numerical factor chosen according to the confidence level desired [55].

When measurements are made at low concentration levels, for example in trace analysis, it is necessary to know what the lowest concentration of the compound is that can be confidently detected by the method. Several proposed approaches exist in the literature for the determination of LOD. In the "blank + 3s" approach, blank matrix samples are measured and the standard deviation, s, determined. The LOD is expressed as the analyte concentration corresponding to (a) the mean sample blank value + 3s or (b) 0 + 3s. This approach is, however, useful only when the sample blank gives a non-zero standard deviation. Furthermore, both the mean and the standard deviation of the sample blank are likely to be dependent on the matrix of the sample blank. The LOD will therefore be matrix dependent. A slightly more exact approach is to measure blank samples that have been spiked with the analyte(s) of interest at the lowest acceptable concentration. The standard deviation, s, of the spiked sample blank

values is calculated, and the LOD is expressed as the analyte concentration corresponding to the sample blank value + 4.65*s*, which derives from hypothesis testing [55-57].

Another approach is to determine the concentration below which specificity becomes unreliable [55]. The results may vary if the experiment is repeated at a different time with different reagents or spiking materials. Sample blanks are spiked with the compound at decreasing concentration levels and then measured. A response curve of results versus concentration is used to determine, by inspection, the concentration at which the test becomes unreliable. In this study, the LOD was determined in this way by injecting extracts of spiked samples, three times, at decreasing concentration levels. It was possible to differentiate the concentration of 2.5 µg/mℓ from background levels for all the drugs, except oxazepam and lorazepam, for which the threshold concentrations were 5 µg/mℓ and 10 µg/mℓ respectively.

5.7 LIMIT OF QUANTIFICATION (LOQ)

The LOQ of an analytical procedure is the lowest amount of compound in a sample that can be quantitatively determined with suitable accuracy and precision. It is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for the determination of impurities and/or degradation products. The ability to quantify is generally expressed in terms of the signal of analyte (true) value that will produce estimates having a specified relative standard deviation (RSD), commonly 10 %.

Thus:

$$L_Q = k_Q \sigma_Q$$

where L_Q is the LOQ, σ_Q is the standard deviation at that point, and k_Q is the multiplier whose reciprocal equals the selected quantifying RSD. The IUPAC default value for k_Q is 10 [55].

This value can also be determined experimentally by injecting standards of decreasing concentration and establishing the lowest concentration at which each drug can be measured. This approach was used in the present study and it showed that 3 µg/mℓ is the lowest concentration at which all the drugs can be measured with acceptable accuracy and precision, except oxazepam and lorazepam, which required concentrations of 5 µg/mℓ and 10 µg/mℓ respectively.

5.8 CONCLUSIONS

The SPE method is capable of selectively extracting cocaine, imipramine, lorazepam, methamphetamine, oxazepam, phenobarbital and secobarbital from a urine matrix, without extracting co-eluting substances. The matrix of the test material covered by this validation was only urine. The concentration range covered by the validation was 2.5 µg/mℓ to 30 µg/mℓ for the spiked compounds. Good resolution was obtained for all the compounds, including the internal standard.

In the accuracy studies, good recoveries were obtained for cocaine, imipramine and methamphetamine, with their average recoveries being 99.6 %, 99.2 % and 102.0 % respectively. The precision studies indicated good repeatability for these drugs. The test for intermediate precision was not conclusive for all the drugs spiked, because of the poor performance of the GC-NPD instrument. Nevertheless, the RSD for cocaine at 20 µg/mℓ was 5.8 %; for imipramine at the three concentration levels, the RSDs were 1.6 %, 1.3 % and 5.3 % respectively; and for methamphetamine at 20 µg/mℓ and 30 µg/mℓ, the RSDs were 8.9 % and 6.0 % respectively. These values indicate that there was minimal variation between the two instruments for these compounds.

The linearity studies gave excellent correlation for cocaine and methamphetamine, with both having a coefficient of determination of 1.00, and for imipramine, which had a coefficient of determination of

0.97. The regression residuals indicated linearity, with an equal distribution about the zero line for all three compounds within a range of 3 µg/mℓ to 30 µg/mℓ. For the determination of LOD, it was found that the threshold concentration at which the test became unreliable for these three drugs was 2.5 µg/mℓ. The LOQ formed part of the linearity of method determination, as it was determined individually by both these studies that the lowest analyte concentration that can be determined with an acceptable level of uncertainty is 3 µg/mℓ. For these three drugs, the SPE method has been successfully validated for linearity of method over the range 3 µg/mℓ to 30 µg/mℓ as well as for LOD and LOQ.

The accuracy studies for oxazepam, phenobarbital and secobarbital gave average recoveries of 49.2 %, 65.5 % and 54.0 % respectively. These lower recoveries could possibly be improved by applying more sample to the column than was used in this experiment. Another option is to change the wash solvent from hexane, which has a high relative eluting strength for non-polar compounds, to a more polar solvent like methanol. The precision studies showed repeatability for these drugs at an average RSD of 6.9 % for oxazepam, 5.9 % for phenobarbital and 9.7 % for secobarbital. Good linearity was obtained for phenobarbital and secobarbital with coefficients of determination of 0.96 and 0.95 respectively, and with their regression residuals distributed equally about the zero line within a linear range of 3 µg/mℓ to 30 µg/mℓ. For oxazepam, however, this could not be achieved: the coefficient of determination was only 0.79 and the regression residual showed a systematic trend rather that random distribution about the zero line. For the determination of LOD, it was found that the threshold concentration at which the test became unreliable was 2.5 µg/mℓ for these three drugs. The LOQ of phenobarbital and secobarbital was found to be 3.0 µg/mℓ, while that of oxazepam was 5.0 µg/mℓ.

The accuracy studies for lorazepam showed an average recovery of only 16.6 %. In the precision studies, the results obtained for the

repeatability test gave consistent results, with RSDs for the three concentration levels of 4.2 %, 2.8 % and 3.8 % respectively. The test for linearity of method produced a coefficient of determination of only 0.75 for this compound, for which the linear range was 10 µg/mℓ to 30 µg/mℓ. The LOD and LOQ were determined to be 5 µg/mℓ and 10 µg/mℓ respectively. The lack of linearity and the low coefficient of determination for lorazepam can be attributed to the degradation of this compound in the stock solution. This was investigated by reanalysing, over a period of three days, the stock solution from which the dilutions had been made, and it was confirmed that the lorazepam was decreasing in intensity and was then absent. Also, better linearity and correlation might have been obtained if the standards had been prepared individually and not by serial dilution, or if the standards had been checked against an independently prepared control standard from a different batch of material in order to rule out error as a result of the standard material and preparation.

This method was developed using mainly GC-NPD as an instrument for detection and quantification of the investigated compounds, but, because of the inconsistency of the results obtained from this instrument, GC-MS with single-ion monitoring was used for the validation procedure. It was able to detect and quantify all the spiked compounds with good accuracy and resolution and with very little variation between analyses.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 CONCLUSIONS

Liquid–liquid extraction procedures are still used for the systematic toxicological analysis of biological samples. SPE, however, allows the analyst to use smaller sample and solvent volumes; furthermore, it gives clean extracts, is capable of extracting a wide range of drugs, and lends itself to automation. The proposed solid-phase extraction procedure using a copolymeric column and followed by GC-MS and GC-NPD analysis, provides an accurate determination of acidic, neutral and basic drugs in urine. For a general screening procedure, suitable conditions must exist for all the drugs being extracted, but extraction optimization for certain drugs only would negatively affect the recovery of others.

The results of this study indicate that the developed methodology can be used for the isolation of various drug types from urine. By using a single, copolymeric CLEAN SCREEN® DAU COLUMN (200 mg, 3 mℓ capacity) during the method developments stage, drugs present in the pre-treated sample were effectively extracted and separated into two groups. The results obtained indicate that, in the screening of an unknown sample, the drug present in the first fraction should be acidic or neutral, and in the second fraction, the drug should be a basic one. When a drug is found in both fractions, this substance is likely to be a drug whose pK_a is close to the pH of the extraction system. The advantage of the two separate fractions is that two drugs that have similar retention times, but different physical properties, can be separated into two fractions. The method was further tested by using an XtrackT® (200 mg, 3 mℓ capacity) column, with a larger particle size,

to facilitate a better sample flow through the column, and with a view to potentially performing extractions from viscous matrices such as whole blood or tissue. Again, all the drugs were extracted from urine into two fractions. Better recoveries were obtained for the basic components with this column. The addition of DMF resulted in increased peak heights for cocaine, imipramine and methamphetamine with the use of the XtrackT® column. The validation process was based on the proposed SPE procedure using the XtrackT® column, with addition of the internal standard (prazepam) to the already extracted fractions prior to analysis. The ratios of the peak areas of the respective compounds and the peak area of prazepam were used for the recovery calculations.

The RSD for repeatability precision was less than 13 % for all the compounds; the recovery for the basic drugs was between 94.3 % and 106.7 %, and for the acidic drugs it was between 13.7 % and 67.6 %. This SPE method was fully validated for cocaine, imipramine and methamphetamine in terms of specificity, accuracy, repeatability precision, linearity of method, LOD and LOQ. The recoveries for oxazepam, phenobarbital and secobarbital ranged between 42.4 % and 67.6 %. The SPE method in this study can be used as a general screening procedure for drugs and, if positive for benzodiazepines or barbiturates, then more specific extraction procedures can be performed for quantification. For example, the amount of water added in the sample preparation step can be reduced to increase the amount of compound present, or the sample volume can be increased. Specifically for the benzodiazepines, glucuronide formation is a major metabolic route and therefore hydrolization or deconjugation can be performed before analysis. The poor results for lorazepam were confirmed to be due to the breakdown of this compound in the stock solution.

Although some drugs were not optimally extracted, the procedure used in this study showed potential as an effective means of doing systematic analysis of biological samples in a forensic laboratory.

6.2 FUTURE WORK

The success of the XtrackT® column in achieving clean extracts as well as acceptable recoveries for a wide range of drugs, could be extended to the extraction of post-mortem blood, serum, stomach contents, eye fluid and cerebrospinal fluid. These samples are considered to be more "difficult", as they are more viscous than urine and tend to form blockages in SPE columns, which would be obviated by the use of the XtrackT® column. The larger particle size of this column allows viscous samples to pass through the SPE cartridge evenly and uniformly without a significant amount of sample pre-treatment [54]. Because of the ability of the XtrackT® column to facilitate a uniform flow of more viscous samples, the FCL CTN is considering using these columns for the extraction of toxins from stomach and contents, liver and kidney samples. A few published applications that use this column for organ extraction, look promising [35], [53].

The method using the XtrackT® column could be adapted for use on an automated extraction system, which would improve productivity as user intervention during the extraction process would be minimized. The present study showed the limitations of performing analysis using GC-NPD, but these were addressed by using GC-MS with SIM. Although sample decomposition at high temperature was reduced by the addition of DMF, this would not be required if the analysis was performed using LC-MS. A further improvement on this process would be the use of on-line SPE coupled with LC-MS/MS [58, 59]. Integration of sample extraction and LC into a single system permits direct injection of the sample. Instead of removing the eluted fractions to be dried and then reconstituted, the fractions are directly and automatically injected for analysis. This step prevents the loss of any

volatile components in the sample caused by evaporation and also reduces user intervention even further. The combination of online SPE with MS/MS would allow the development of a high-throughput method, resulting in higher and more reproducible recoveries of a more diverse range of drugs than what is identifiable on GC-MS [51, 58, 59].

REFERENCES

1. B Lane, *The Encyclopedia of Forensic Science: A guide to the science behind crime investigation,* Magpie Books, London, 2004, pp 267, 497

2. Forensic Science: The Early Years. http//www.troopers.state.ny.us/ Forensic_Science/Forensic_Science_History, accessed 7 June 2007

3. A C Moffat, J V Jackson, M S Moss, B Widdop, *Clarke's isolation and identification of drugs in pharmaceuticals, body fluids and post-mortem material*, Second Edition, The Pharmaceutical Press, London, 1996, Chapter 1, Figure 1

4. J C Brenner, *Forensic Science Glossary*, CRC Press, Boca Raton, Florida, 1999, p 174

5. G R Jones (Chair), W L Hearn, H H McCurdy, J R McCutcheon, SOFT/AAFS Laboratory Guidelines Committee, 1997/2002

6. B Levine, *Principles of Forensic Toxicology*, American Association of Clinical Chemistry, Washington DC, 2003, Chapter 1, pp 3-6

7. M Chopra, D Sanders, J Sundin, *Health and social transition: A case study from South Africa*, University of the Western Cape, South Africa, 2004

8. H Friedman, Win the drug war or lose a generation, *Cape Times*, Published on the web by IOL on 2001-04-04, 16:30:22

9. L Roch-Silva, E Mokoko, D Malaka, Drug use in South Africa, *HSRC Research Group Report,* Centre for Alcohol and Drug Related Research, Human Sciences Research Council, 21 May 1998

10. C Parrry, A Pluddemann, A Bhana, N Harker, H Potgieter, W Gerber, C Johnson, Alcohol and drug abuse trends: January – June 2004 (Phase 16), *MRC Research Group Report*, August 2004.

11. Fact Sheet – cannabis and mandrax use in South Africa, *Alcohol and Drug Abuse Research Group Report*, Medical Research Council, 2004

12. M E Foster, A comparative study of the incidences of ethanol, methaqualone and cannabis in homicide and pedestrian transport accident victims in the Western Cape Metropole area, *BSc(Med) (Hons) dissertation*, University of Cape Town, 1996

13. T Legget, What's up in the Cape? Crime rates in the Western and Northern Cape provinces, *SA Crime Quarterly No. 7*, Institute for Security Studies, March 2004.

14. K Ajam, *Cape Argus*, May 13, 2007, p 6, published on the Web by IOL on 2007-05-13 10:29:00

15. South African Community Epidemiology Network on Drug Use (SACENDU), Alcohol and drug abuse trends: January – June 2004 (Phase 16, 5e), *SACENDU Research Group Report*, 27 October 2004

16. S S Johansesn, J L Jenses, Liquid chromatography-tandem mass spectrometry determination of loperamide and its main metabolite desmethylloperamide in biological specimens and

application to forensic cases, *Journal of Chromatography B,* 2004, 811, pp 31-36

17. S Cardenas, M Gallego, M Valcacel, Rapid solid-phase extraction/ derivitisation system for sample preparation and gas chromatographic/mass spectrometric determination of drugs in human urine, *Rapid Communication in Mass Spectrometry*, 1997, 11, pp 298-306

18. S R Binder, M Regalia, M Biaggi-McEachern, M Mazhar, Automated liquid chromatographic analysis of drugs in urine by on-line sample cleanup and isocratic multicolumn separation, *Journal of Chromatography*, 1989, 473, pp 325-341

19. D D Blevins, M F Burke, T J Good, P A Harris, K C Van Horne, N Simpson, L S Yago, *Handbook of sorbent extraction technology*, Varian Inc, no date

20. *SPE Reference Manual & Users Guide*, Phenomenex, no date

21. M J Telepchak, T F August, G Chaney, *Forensic and Clinical Applications of Solid-phase Extraction*, Humana Press, Totowa, New Jersey, 2004

22. R A de Zeeuw, Drug screening in biological fluids: The need for a systematic approach, *Journal of Chromatography B*, 1997, 689, pp 71-79

23. M Bogusz, J Wijsbeek, J P Franke, R A de Zeeuw, J Gierz, Impact of biological matrix, drug concentration, and method of isolation on detectability and variability of retention index values in gas chromatography, *Journal of Analytical Toxicology*, 1985, 9, pp 49-54

24. X H Chen, J Wijsbeek, J P Franke, R A De Zeeuw, On-line coupling of sample pre-treatment with chromatography or mass spectrometry for high-throughput analysis of biological samples, *Journal of Forensic Science*, 1992, 37, pp 61-71

25. X H Chen, J P Franke, J Wijsbeek, R A de Zeeuw, Study of lot-to-lot reproducibilities of Bond Elut Certify and CLEAN SCREEN® DAU mixed-mode solid-phase extraction columns in the extraction of drugs from whole blood, *Journal of Chromatography*, 1993, 617, pp 147-151

26. X H Chen, J P Franke, J Wijsbeek, R A de Zeeuw, Isolation of acidic, neutral, and basic drugs from whole blood using a single mixed-mode solid-phase extraction column, *Journal of Analytical Toxicology,* 1992, 16, pp 351-355

27. X H Chen, J P Franke, K Ensing, J Wijsbeek, R A de Zeeuw, Semi-automated solid-phase extraction procedure for drug screening in biological fluids using the ASPEC system in combination with CLEAN SCREEN® DAU columns, *Journal of Chromatography,* 1993, 613, pp 289-294

28. Preparation of whole blood samples for use with Bond Elut Certify™, *Varian Sample Preparation Products application note*, no date

29. A Polettini, A Groppi, C Vignali, M Montagna, Fully-automated systematic toxicological analysis of drugs, poisons, and metabolites in whole blood, urine, and plasma by gas chromatography-full scan mass spectrometry, *Journal of Chromatography B, Biomedical Science Application*, 1998, 713, pp 265-279

30. A W Missen, A gas-liquid chromatographic procedure for the determination of drugs in small blood samples, *Report no. CD 2282*, Chemistry Division, Department of Science and Industrial Research, New Zealand, 1979

31. N F Wood, Extraction and cleanup of organochloride pesticide residues by column chromatography, *Analyst*, 1969, 94, pp 399-405

32. D Thieme, R K Muller, *Information theory and Systematic Toxicological Analysis in "general unknown" poisoning cases,* Fresenius Journal of Analytical Chemistry, 1997, 358, pp 785-792

33. D J Fox, E T Heebner, T L Phillips, L O'Dell, A comparative study of the extraction capabilities of various substituted silicas used in bonded phase extraction, *United Chemical Technologies, Inc*, Bristol, Pennsylvania, 1990

34. C Lora-Tamayo, M A Rams, J M R Chacon, Gas chromatographic data for 187 nitrogen or phosphorus containing drugs and metabolites of toxicological interest analysed on methyl silicone capillary columns, *Journal of Chromatography*, 1986, 374, pp 73-85

35. D J Fox, E T Heebner, C M Moore, G E Long, M J Telepchak, Principles of Solid-Phase Extraction on Multimodal Copolymeric Bonded Phases and Application for Novel High-Flow Sorbents, Electronic Catalogue, United Chemical Technologies Inc, Bristol, Pennsylvania, 2006

36. Microsep/Waters, The science of solid phase extraction, Presentation at an LC-MS/MS and SPE workshop, Sandton, 2006

37. J P Franke, R A de Zeeuw, Solid-phase extraction procedures in systematic toxicological analysis, *Journal of Chromatography B*, 1998, 713, pp 51-59

38. Drugs and traffic safety proceedings, Sixth International Conference on Alcohol, Toronto, 1994, 8

39. V J Emerson, *The measurement of breath alcohol: the laboratory evaluation of substantive breath test equipment and the report of an operational police trial*, First Edition, Forensic Science Society, Harrogate, North Yorkshire, England, 1980, pp 336-337

40. D V Canfield, M D Smith, H J Adam, E R Houston, Selection of an Internal Standard for Postmortem Ethanol Analysis, *Research Group Report*, Civil Aeromedical Institute, Federal Aviation Administration, February 2004

41. P S Lough, R Fehn, Efficacy of 1% sodium fluoride as a preservative in urine samples containing glucose and candida albicans, *Journal of Forensic Sciences*, 1993, 38, pp 266-271

42. D A Skoog, D M West, F J Holler, *Fundamentals of Analytical Chemistry*, Seventh Edition, Saunders College Publications, Fort Worth, 1996, p 870

43. A I Vogel, *A text-book of quantitative inorganic analysis: theory and practice,* Second Edition, Longmans Green, London, 1951, pp 336–337

44. The Merck Index on CD ROM, Version 13.0, 2001

45. R C Baselt, *Disposition of toxic drugs and chemical in man,* Fifth Edition, Chemical Toxicology Institute, Foster City, California, 2000

46. R Clouette, J Brendler, G Wimbush, J Garrdott, The determination of amphetamine, methamphetamine and other phenylalkylamines in blood and urine by a dual derivatization method, *SOFT Poster # 48*, 1997

47. *Solid Phase Extraction product catalogue*, United Chemical Technologies Inc, Bristol, Pennsylvania, Electronic version (version 2.0), no date

48. Y Hida, T Nagata, K Kudo, Elimination of interfering components in old blood for drug screening, *Japanese Journal of Forensic Toxicology*, 1991, 9

49. D W Newton, R B Kluza, *Principles of Medicinal Chemistry*, Second Edition, Lea & Febyer, 1981, pp 901-909

50. R C Baselt, Stability of cocaine in biological fluids, *Journal of Chromatography*, 1983, 268, pp 502-505

51. J P Franke, R A de Zeeuw, Solid-phase extraction procedures in systematic toxicological analysis, *Journal of Chromatography B: Biomedical Sciences and Applications,* 1998, 71, pp 51-59

52. X H Chen, J P Franke, J Wijsbeek, R A de Zeeuw, Determination of basic drugs extracted from biological matrices by means of solid-phase extraction and wide-bore capillary gas chromatography with nitrogen phosphorus detection, *Journal of Analytical Toxicology,* 1994, 18, pp 150-153

53. X H Chen, J P Franke, K Ensing, J Wijsbeek, R A de Zeeuw, Pitfalls and solutions in the development of a fully automated solid- phase extraction method for drug screening purposes in plasma and whole blood, *Journal of Analytical Toxicology*, 1993, 17, pp 421-426

54. S Zhou, H Wang, Improving Your NPD System Efficiency, Application, Agilent Technologies (Shanghai), 2004

55. The fitness for Purpose of Analytical Methods, EURACHEM Guide, document developed by a EURACHEM Working Group, LGC (Teddington) Ltd, Edition 1.0, 1998

56. HARMONIZED GUIDELINES FOR SINGLE LABORATORY VALIDATION OF METHODS OF ANALYSIS, IUPAC, Technical Report, Pure Appl. Chem., Vol. 74, No. 5, 2002, pp. 835–855

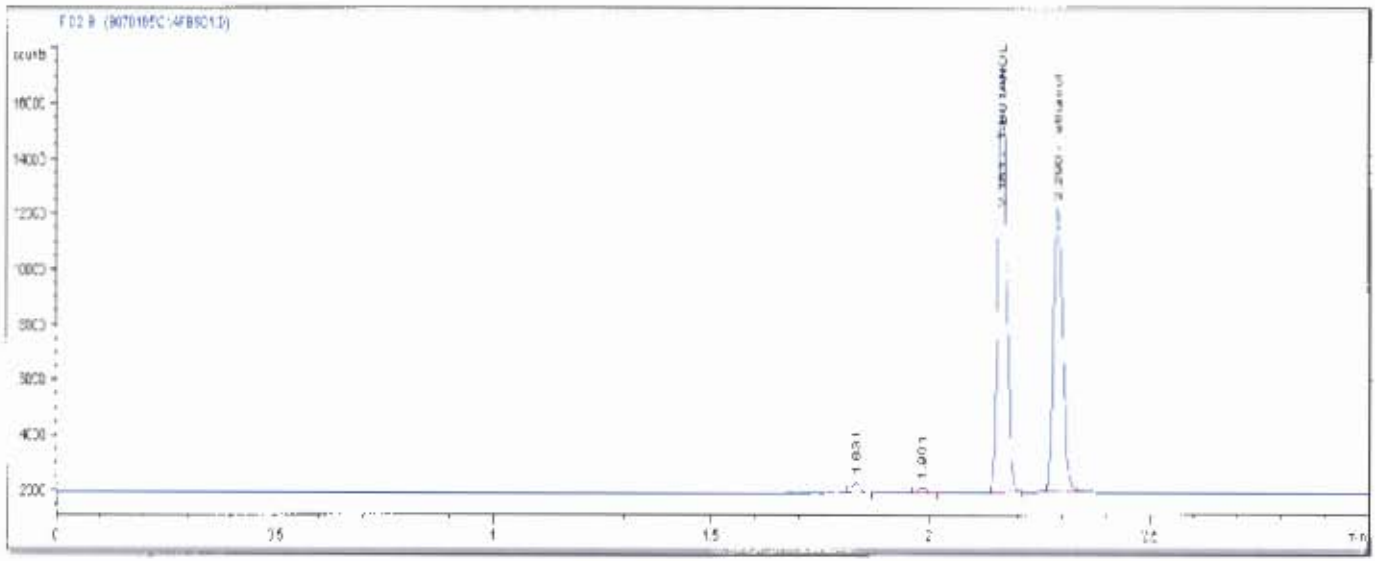
57. D.S Walonick, Survival Statistics, StatPac, Inc ,8609, Lyndale Ave. S, #209A, Bloomington, MN55420, 1997-2007, pp95-103

58. W M Mullet, Determination of drugs in biological fluids by direct injection of samples for liquid-chromatographic analysis, *Journal of Biochemical and Biophysical Methods,* 2007, 70, pp 263-273

59. M M Ariffin, R A Anderson, LC/MS/MS analysis of quaternary ammonium drugs and herbicides in whole blood, *Journal of Chromatography B*, 2006, 842, pp 91–97

APPENDICES

Appendix 1



Ethanol and tertiary butanol chromatogram

Appendix 2

ZYMARK RapidTrace Procedure

	step	source	destination	mℓ	mℓ/min	liquid check
1	Condition	MeOH	Aq.	3	12	No
2	Condition	H_2O	Aq.	3	12	No
3	Condition	pH 6.0	Aq.	1	12	No
4	Load	Sample	Bio.	5	1	No
5	Load	Sample	Bio.	2.2	1	No
6	Purge-Cannula	H_2O	Cannula	6	12	No
7	Purge-Cannula	MeOH	Cannula	6	12	No
8	Rinse	H_2O	Aq.	3	12	No
9	Rinse	HAc	Aq.	1	12	No
10	Dry	Time = 5 min				No
11	Rinse	Hexane	OrgSol	2	12	No
12	Collect	Hexane/Et Acetate (50:50)	Fract1	6	2	No
13	Rinse	MeOH	Org	3	12	No
14	Dry	Time = 5 min				No
15	Collect	CH_2CL_2/IPA/NH_4OH (78/20/2)	Fract2	3	2	No
16	Rinse	H_2O	Aq.	6	12	No
17	Rinse	MEOH	Aq.	6	12	No
18	Purge-Cannula	H_2O	Cannula	6	30	No
19	Purge-Cannula	MEOH	Cannula	6	30	No

Load Cannula Depth 0
Mix Cannula Depth 0
Mix Cycles 2
Mix Volume .5
Mix Speed 30
Reagent Mix Cycles 2

Reagent Setup

No.	Reagent Name	Abbreviation	Sip Speed
1.	Water	H_2O	30
2.	Methanol	MEOH	30
3.	Hexane	Hexane	30
4.	Acetic Acid 0.1 M	HAc	30
5.	CH_2CL_2/IPA/NH_4OH (78/20/2)	Elute 2	15
6.	Methylene Chloride	MeCl	30
7.	0.1 M pH 6 Phosphate Buffer	pH 6	30

8.	Hexane/ Et Acetate (50:50)	Elute 1	15
9.	Mixing Vessel	Mixer	30
10.	Sample	Sample	15

Column air push volume 2

Column air push volume speed multiplier 2

No.	Waste Name	Abbreviation
1.	Aqueous	Aq.
2.	Organic Solvent	Org.
3.	Biohazardous	Bio.

Settings for RapidTrace analysis of acidic, basic and neutral drugs in urine and whole blood: automated method for GC or GC-MS using a 200 mg XtrackT® extraction column

Appendix 3

	[Conc]	**Working (Linear) Range** (Measured Response) Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Cocaine Average Response	Cocaine Std. Dev
Concentration Level 1 (LoQ)	3	0.79	0.87	0.82	0.81	0.86	0.83	0.05753
Conc Level 2	5	1.07	0.85	0.97	0.97	0.94	0.96	0.15768
Conc Level 3	10	2.66	2.80	2.62	2.76	2.81	2.73	0.10390
Conc Level 4	15	4.39	4.46	4.52	4.39	4.39	4.43	0.04879
Conc Level 5	20	5.97	5.99	6.04	6.10	5.80	5.98	0.01414
Conc Level 6	25	6.35	6.96	6.89	7.09	7.66	6.99	0.43134
Conc Level 7	30	8.79	8.71	8.79	8.82	8.64	8.75	0.05657

Regression Statistics (Based on the average)	Slope, m	0.29355
	Intercept, b	-0.14
	Corr. Coeff.	0.99

Sum of Squares for Regression Statistics

x	y	x^2	xy	y^2
3.00	0.83	9.00	2.49	0.69
5.00	0.96	25.00	4.80	0.92
10.00	2.73	100.00	27.30	7.45
15.00	4.43	225.00	66.45	19.62
20.00	5.98	400.00	119.60	35.76
25.00	6.99	625.00	174.75	48.86
30.00	8.75	900.00	262.50	76.56
Σ	78	21.92	1384.00	395.39	113.31

SSxy	151.1386
SSxx	514.8571
SSyy	44.66789
SSE	0.300495
m	0.293554
b	-0.13961
R^2	0.993273

Formula:

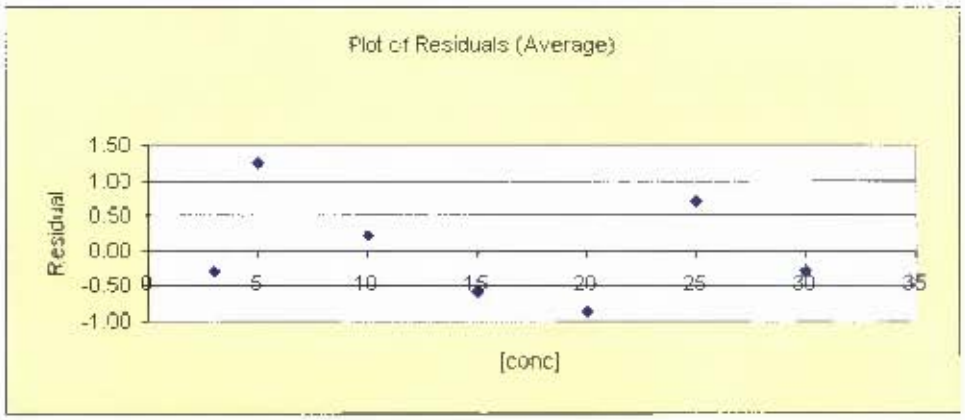
$y = mx + c$

$y = 0.29x - 0.14$

Regression Residuals Analysis

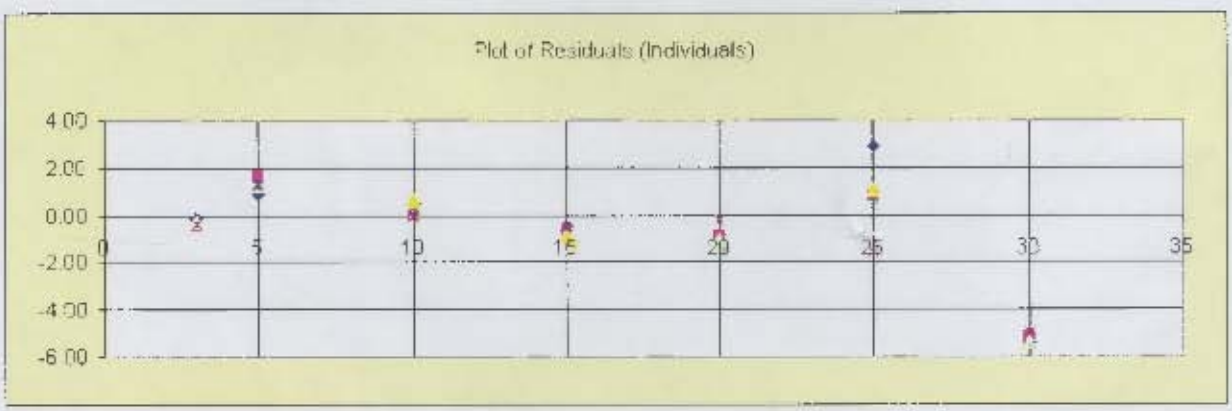
	[Conc]	Predicted Value	Residual (Difference)
Concentration Level 1 (LoQ)	3	3.30	-0.30
Concentration Level 2	5	3.75	1.25
Concentration Level 3	10	9.78	0.22
Concentration Level 4	15	15.57	-0.57
Concentration Level 5	20	20.85	-0.85
Concentration Level 6	25	24.29	0.71
Concentration Level 7	30	30.28	-0.28

[Conc]	Residual (Difference)
3	-0.30
5	1.25
10	0.22
15	-0.57
20	-0.85
25	0.71
30	-0.28



3	3.15	3.43	3.27	3.23	3.41
5	4.11	3.35	3.78	3.78	3.68
10	9.53	10.03	9.40	9.88	10.05
15	15.43	15.67	15.87	15.43	15.43
20	20.81	20.88	21.05	21.26	20.23
25	22.11	24.18	23.95	24.63	26.57
30	30.42	30.14	30.42	30.52	29.91

3	-0.15	-0.43	-0.27	-0.23	-0.41
5	0.89	1.65	1.22	1.22	1.32
10	0.47	-0.03	0.60	0.12	-0.05
15	-0.43	-0.67	-0.87	-0.43	-0.43
20	-0.81	-0.88	-1.05	-1.26	-0.23
25	2.89	0.82	1.05	0.37	-1.57
30	-5.42	-5.14	-5.42	-5.52	-4.91



Measured responses and calculation of the sum of squares for regression statistics for cocaine