THE APPLICATIONS OF SUPERCRITICAL FLUID AND SOLID-PHASE EXTRACTION TECHNIQUES FOR THE RECOVERY OF DRUGS OF ABUSE FROM BIOLOGICAL MATRICES

Thesis Submitted in Accordance with the Requirements of the University of Glasgow for the Degree of Doctor of Philosophy

By

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God Bless

THIS IS DEDICATED TO MY FAMILY

THE ALLENS

SUMMARY OF THESIS

The application of Solid-Phase Extraction (SPE) and Supercritical Fluid Extraction (SFE) were evaluated for the determination of various drugs of abuse analyzed in the field of forensic toxicology. Three biological matrices (blood, urine and hair) were investigated for the recovery of various commonly abused drugs.

The current SPE method used in the routine laboratory was optimized for the extraction of benzodiazepines in whole blood and applied to authentic forensic case samples. The optimized method and the routine method were then compared using authentic forensic samples. Successful methodology developed provided an efficient, less time consuming, reproducible and more robust alternative to the current routine method.

The use of SFE was applied to blood and urine samples for the extraction of cocaine and its metabolites, benzoylecgonine and ecgonine methyl ester. The method developed showed that the drugs can be successfully extracted from various biological matrices such as blood and urine with comparable results to SPE methods.

Forensic case samples positive for morphine were extracted using SFE and SPE techniques and the results compared. A good correlation was observed between the two methods rendering them compatible for the detection of morphine in whole blood.

Hair analysis was investigated using both SPE and SFE techniques. A study was carried out to determine whether a correlation existed between self-report data and hair analysis for five amphetamines (metamphetamine, amphetamine methylene-

dioxyamphetamine, methylenedioxyethylamphetamine and methylenedioxymeth-amphetamine). SPE methods developed proved that all five amphetamines could be extracted from human hair. However, a correlation did not exist between self-report and hair analysis data. Inter-laboratory studies carried out between two laboratories showed that both produced similar results for case samples containing amphetamines.

For the extraction of amphetamines (MDA, MDMA and MDEA) and cocaine and its metabolite from hair, developed SFE methods were applied. Amphetamines in hair were successfully extracted from spiked and authentic hair samples thought to contain amphetamines using developed SFE methods. Cocaine and ecgonine methyl ester were also successfully extracted from spiked human hair.

5. BENZODIAZEPINES

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GLOSSARY OF ABBREVIATIONS

AP	Amphetamine
atm	Atmosphere
BEC	Bond Elute Certify
BZE	Benzoylecgonine
BSTFA	N, O-bis (Trimethylsilyl) trifluoroacetamide
CO_2	Carbon dioxide
Cpt	Critical point
Cst	Critical state
C_1	Component one
C_2	Component two
Chlor	Chloroform
Cal	Calories
CV	Coefficient of Variants
DI	Distilled
DMD	Desmethyldiazepam
DF	Dilution Factor
DCM	Dichloromethane
d_3	tri-deuterated
Etoac	Ethyl acetate
GABA	Gamma amino butyric acid
GC	Gas Chromatography
GC-FID	Gas Chromatography-Flame Ionization Detector
GC-ECD	Gas Chromatography-Electron Capture Detector
GC-MS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography

IPA	Isopropyl alcohol
I	Ingestion
LLE	Liquid-liquid Extraction
L	Liquid
LRC	Low reservoir capacity
LLV	Liquid -liquid Vapor
LOD	Limit of detection
LOQ	Limit of quantitation
MA	Methamphetamine
MDA	Methylenedioxyamphetamine
MDMA	Methylenedioxymethamphetamine
MDEA	Methylenedioxyethylamphetamine
MeOH	Methanol
m/z	Mass to charge ratio
NR	No result
NA	Not available
O	Oral
P	Pressure
PAR	Peak Area Ratio
P_c	Critical Pressure
ρ_{rg}	Density of a gas
ρ_{rl}	Density of a liquid
ρ_c	Critical Density
δ	Solubility Parameter
RSD	Relative Standard Deviation

SDS	Sodium Dodecyl Sulfate
SF	Supercritical Fluid
SFE	Supercritical Fluid Extraction
SPE	Solid-Phase Extraction
SR	Slow releasing
StDev	Standard Deviation
TLC	Thin-layer Chromatography
TMCS	Trimethylchlorosilane
TEA	Triethylamine
T 1/2	Half-life
UCST	Upper Critical-Solution Temperature
V	Volume
x	Composition of heavy component
x*	Overall fixed composition
X	Mean

1. SUPERCRITICAL FLUIDS

1.1 LITERATURE REVIEW

1.1.1 INTRODUCTION

Over the past decade, there has been a growing interest in supercritical fluid extraction (SFE) as an alternative to conventional sample preparation techniques. SFE is a relatively new analytical technique based on the use of supercritical fluids (SF) for the isolation of analytes from various matrices. It is attracting great interest because of the increasing need for a simple, rapid, environmentally friendly, automated and selective extraction method.[1]

The principal goal of analytical chromatography is to provide precise, accurate, rapid and inexpensive measurements of unknown and target chemical species in complex samples.[2] Although research to improve the sensitivity, selectivity, speed, and separating capabilities of analytical techniques has increased, less attention has been focused on the preparation of the sample before introduction into the chromatographic system.

The identity or concentration of a compound is determined through chromatographic analysis and extraction of the target analytes from the matrix is necessary for preparation of the sample prior to the chromatographic process. Ideally, the extraction and preparation should yield quantitative and high recoveries of the target analyte, at low cost and risk to the environment.

The above criteria for ideal extraction and sample preparation resulted in the implementation of SF as a replacement for liquid solvents in the extraction process. Its combined gas-like mass transfer and liquid-like solvating characteristics first led analytical chemists to use them as chromatographic mobile phases and later as fluids capable of removing compounds from solid samples.

1.1.2 HISTORY

Supercritical fluid technology essentially involves the exploitation of the unusual variations in solubility with pressure and temperature immediately above the critical point of a volatile solvent. Supercritical fluids (SF) were first researched in 1822 by Baron Charles Cagniard de la Tour.[3] He discovered the critical point of a substance by sealing a liquid and a flint ball into a cannon barrel, heating and rocking it, while listening for changes in the sound of the rolling ball or sound changes made when the barrel was tapped. Discontinuities in the sound led him to describe the point known then as the "Cagniard de la Tour" point, which today is known as the critical point.

By the mid to late 1800s, Dr Thomas Andrews[4] carried out a series of investigations on the phase behaviour of CO_2. Later in his famous Bakerian Lecture in 1809 he described his experimental apparatus and observations of the critical properties of CO_2. Andrews reported that when carbonic acid is partially liquefied by pressure, while simultaneously raising the temperature gradually to 88 °F, the line of the liquid-gas demarcation surface fades, loses its curvature and then disappears to exist as a homogenous fluid.

Decreasing the pressure or slightly lowering the temperature of the extraction apparatus, cause the appearance of flicking striae throughout the entire mass of the fluid. Temperatures above 88°F, showed no apparent liquefaction of the carbonic acid and no separation of the two distinct forms of matter, could be effected, even when a pressure of 300 or 400 atmospheres was applied. The critical point of CO_2 was reported by Andrews as 30.92°C and 73.0 atm, which is in close agreement to presently accepted values of 31.1°C and 72.8 atm.

The ability of a SF to dissolve low vapour pressure solid materials using methyl halides in supercritical ethanol while increasing the pressure, was first reported by Hannay and Hogarth in 1879.[5] They modified the experimental apparatus used by Andrews [7] for an experiment carried out in a small-diameter glass tube, to monitor the effect of pressure changes which resulted in inorganic salts dissolving in or precipitating from ethanol at a temperature above its critical temperature.

In the same year, Amagat described a method of compressing gases to 400 atm using mercury columns extending to the bottom of a mine shaft.[6] This was followed by Cailletet in 1891, who described the generation of high pressure with a mercury column reaching to the top of the Eiffel Tower.[7]

The earliest published work on liquid CO_2 as a solvent was by Gore in 1861.[8] He found naphthalene and camphor to be soluble in liquid CO_2, but conclude that CO_2 was a very feeble solvent of substances in general, after testing a small number of organic compounds. In 1896, Villard described SF ability of methane, ethylene, CO_2 and nitrous oxide to dissolve numerous liquid and solid hydrocarbons.[9] Several years

later, Buchner carried out qualitative studies on SFs over a wide temperature range to observe cloud points, melting points, freezing points and the number of phases present for solubility determination.[10]

Researchers of the late 1800s and early 1900s met and overcame many obstacles found in carrying out their studies. By the early 1960s, the solubility of naphthalene and a variety of other gases were studied. Studies of SFs have continued over the decades to the present day, with numerous researchers contributing to this new application.

In 1962, Kelps *et al* first reported the use of a SF as a chromatographic mobile phase by using difluoromethane and monochlorodifluoromethane to separate nickel porphyrin isomers.[11] Sie *et al* investigated the use of carbon dioxide isopropanol, n-pentane and other materials as mobile phases with packed columns.[12-14] Giddings *et al* demonstrated the migration of carotenoids, sugars, amino acids, nucleotides, various polymers and other solutes using high pressure gas chromatography with CO_2 or ammonia as the mobile phase.[15-16] Their work showed great potential for this application of SFs.

Numerous applications of the use of SFs have been developed which include food, oils, coffee, tobacco and spices. This led to the building of the first large-scale production plant in 1979 by Hag, using SFE. It was used for the removal of caffeine from green coffee beans.[17] By the 1980s, SFE as an independent sample preparation technique became attractive to the analytical chemist and is still at the forefront of technology today.

1.1.3 DEFINITION OF A SUPERCRITICAL FLUID

By definition, a SF is a substance that at a temperature and pressure greater than its critical temperature (Tc) and pressure (Pc), is a gas-like, compressible fluid that takes the shape of and fills its container. It is not a liquid but has liquid-like densities (0.1 to 1g/mL) and solvating power.[18]

1.1.3.1 Supercritical Fluids

SFs are usually used at temperatures and pressures significantly higher than their critical values. Therefore, choosing an ideal SF, depends on the polarity and thermal stability of the analyte(s), the solvent strength and selectivity of extracted components at adequate operating temperatures and the limitations of the analysis instrumentation associated with high critical pressures required for some SFs.[19] The critical pressure determines the importance of the solvent power of the fluid, hence fluids with high critical pressures are more likely to dissolve polar components than fluids with lower critical pressures. Hence, critical temperature of a SF is influenced by the stability of the component to be extracted.

A wide range of SFs have been used in SFE covering a range of critical temperatures and pressures, molecular sizes and polarities. Among these, carbon dioxide seems to be the most widely used because of its moderate critical pressure (73.8 atm) and low critical temperature (31.1°C).

Table 1.1 Critical Parameters of commonly used SFs.[20]

Fluid	Critical Temp (°C)	Critical Pressure (atm)	Solubility Parameters
CO_2	31	73	10.7
N_2O	36	72	10.6
NH_3	132	112	13.2
MEOH	240	78	14.4
$CCIF_3$	29	38	7.8
Ethane	32	48	6.6
Ethylene	10	51	6.6

1.1.3.2 Carbon Dioxide

An SF such as CO_2 has a hydrocarbon like solvent strength at typical conditions, so it is an appropriate solvent for lipophillic substances.[21] Among the fluids shown in Table 1.1, CO_2 is the most frequently used for SFE for the following reasons.[1]

- Easy temperature and pressure manipulation (31 °C and 73.8 atm)
- Good solvent strength and miscibility with most solvents
- Compatible temperature with the thermal stability of solutes
- Low surface tension and viscosity
- Odourless, non-toxic, non-flammable and non-corrosive
- Readily available in high purity
- High diffusivity
- Economical (inexpensive)

1.1.3.3 SF Modifiers

Supercritical CO_2 has been the choice for most SFE studies, because of these attractive practical characteristics. It is an excellent extraction medium for nonpolar species and is reasonably good for moderately polar species. However, one of the disadvantages is that it is less useful for more polar compounds. One way to increase the polarity of a SF is to add small amounts of polar liquid solvents (e.g. methanol, acetonitrile or water) to optimise analyte recovery during SFE. These polar liquid solvents are known as cosolvent, moderators, modifiers or entrainers.[17,22]

SFE modifier can have dramatic effects on the analytes and matrices. They are a rapidly developing area of study in SFE technology in which new permutations and combinations are virtually unlimited. Most modifiers have solvent parameters greater than CO_2 so they are used to enhance the solubility properties and increase the yield or decrease the pressure and solvent requirements in the extraction of polar molecules.

The modifier exerts its effect by interacting with the analyte or matrix complex to promote rapid desorption into the SF.[23] The addition of minute amounts of modifier to a SF, can dramatically increase the solubility and critical properties and increase the solvent strength while maintaining its adjustably.[17] This increase can only be observed with the use of solvents with polar chemical functions. Yang *et al*, demonstrated that the combination of modifier and high temperature is highly effective.[24]

The use of different extraction pressures, entrainers and fluids with varying polarity is valuable in allowing class selective extraction methods to be developed for various compounds.[20,25] The modifier can be either added to the sample in the extraction cell prior to SFE, so that the modifier is swept directly from the cell when the SF starts to circulate through the sample or mixed with the CO_2 where it is continuously passed through the matrix.

1.1.3.4 SF Properties

SFs have numerous advantages necessary to meet the criteria for adequate extraction and preparation techniques.[15, 26-28] Some of these are listed below:

- Solvent strengths approaching those of liquid solvents, with low viscosity and high solute diffusivity, thus better mass transfer characteristics than liquids and faster extractions.
- Increase in pressure, results in an increased density which optimises the extraction conditions for the target analyte by changing the extraction pressure or temperature.
- Class-selective extractions of particular compound classes are possible with the extraction of the same sample at different pressures
- Relatively inert, pure and non-toxic
- Inexpensive per extraction
- Selective by adjusting pressure, temperature and degree of modifier
- Diffusion properties permit rapid penetration and extraction from the sample
- CO_2 has a relatively low critical temperature that allows good stability of thermally unstable components

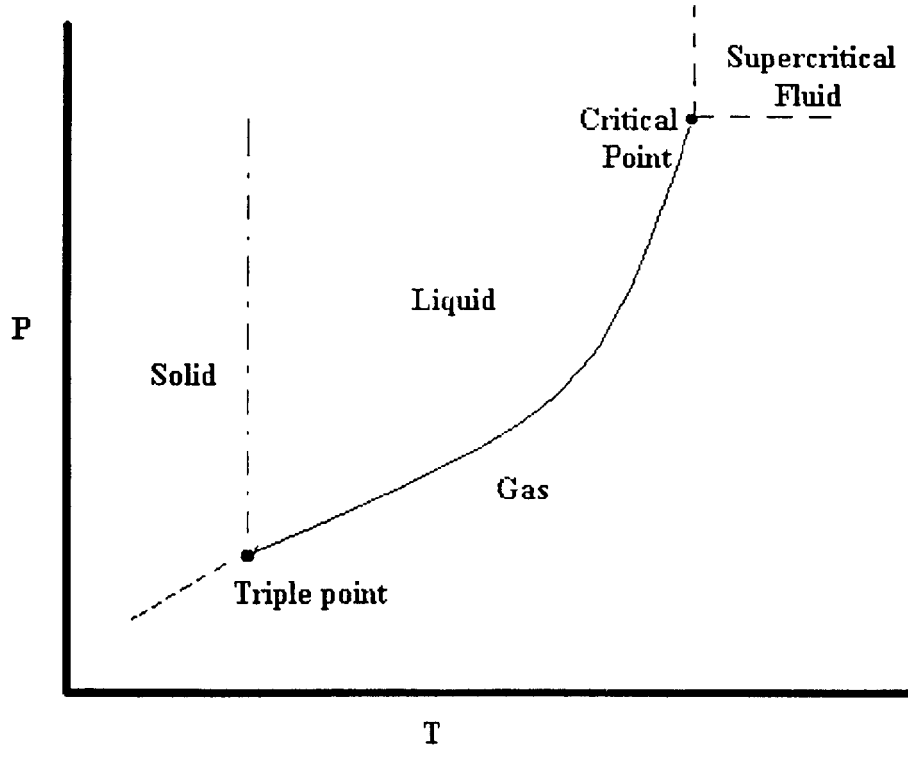
- Reduction or elimination of liquid solvents waste being generated and toxic solvent exposure in the laboratory

1.1.4 THERMODYNAMIC PROPERTIES

The thermodynamic state of a pure component is determined by the pressure (P), volume (V) and temperature (T). [17, 29, 30] This combination of variables is known as the state equation represented by a surface in the three dimensional plotting of P,V,T. Depending on the value of these parameters, a pure component will be either a solid, liquid or gas.

The phase diagram in Figure 1.1 shows the three phase transitions of a solid, liquid and gas boundaries. These transitions are accompanied by physical properties of increased pressure and temperature that move along the gas liquid boundary. Due to thermal expansion, the liquid becomes less dense and the gas becomes denser because of increased pressure.[31] When the critical point (Cpt) of a substance is reached, the densities of the two phases are identical and the distinction between the gas and liquid phase is absent. The substance now is no longer a gas or a liquid but described as a fluid.

Figure 1.1 Schematic phase diagram of a single substance.



A supercritical state is obtained at temperatures and pressures superior to those of critical temperature (T_c) and pressure (P_c). The Cpt or Cst corresponding to the T_c, P_c and critical density (ρ_c) is represented as C, which acts as an intermediate state between a liquid and a gas. Another important parameter is the critical volume (Vc) for one mole of substance. With an increase of temperature and pressure above a substances critical parameters, it becomes known as the supercritical state. If one of the parameters falls under its critical value while the other remains superior to its critical value, the substance is in a subcritical state. The critical parameters of various substances used as SF during analysis is listed in Table 1.2. The most commonly used substance is Carbon dioxide (CO_2), due to its convenient supercritical properties.[29]

1.1.5 PHYSICOCHEMICAL PROPERTIES

SFs possess physical properties intermediate between those having gas-like transfer and liquid-like solvating characteristics. These properties demonstrate favourable behaviour for the transportation of a solute and are influenced by diffusion, viscosity, density and solubility.[19] Table 1.2 shows physical parameters were SFs have densities comparable to liquids, viscosities comparable to gases and diffusivities comparable between liquid and gases.

Table 1.2 Physical parameters for Gas, SFs and a liquid.[32]

PHASE	DENSITY (g/ml)	VISCOSITY	DIFFUSION (m2 . s)
Gas	0.001	0.00005-0.00035	0.01-1.0
Supercritical fluid	0.2-0.9	0.0002-0.001	0.00033-0.00001
Liquid	0.8-1.0	0.003-0.24	0.000005-0.00002

Having these properties have led analytical chemists to use SF as a chromatographic mobile phase that is demonstrated in SFC and its use as an extractant which is demonstrated in SFE.[33] The most important factor to consider in both techniques is the solubility of the analytes of interest. Regardless of the extraction conditions, the solvent strength of a liquid is essentially constant. However, the solvent strength of a SF is dependent on the temperature and pressure used for the extraction.[34]

1.1.5.1 Viscosity

SFs are slightly viscous with viscosities higher than those of gases despite of their high densities. This property causes a pronounced viscosity during SFE, which causes an obvious pressure drop in an extraction cell.[1] The viscosity of a SF increases with pressure, but approaches that of a liquid less rapidly than the density.

Fluids in a supercritical state have viscosities inferior to that of a liquid even if very high pressures are used.

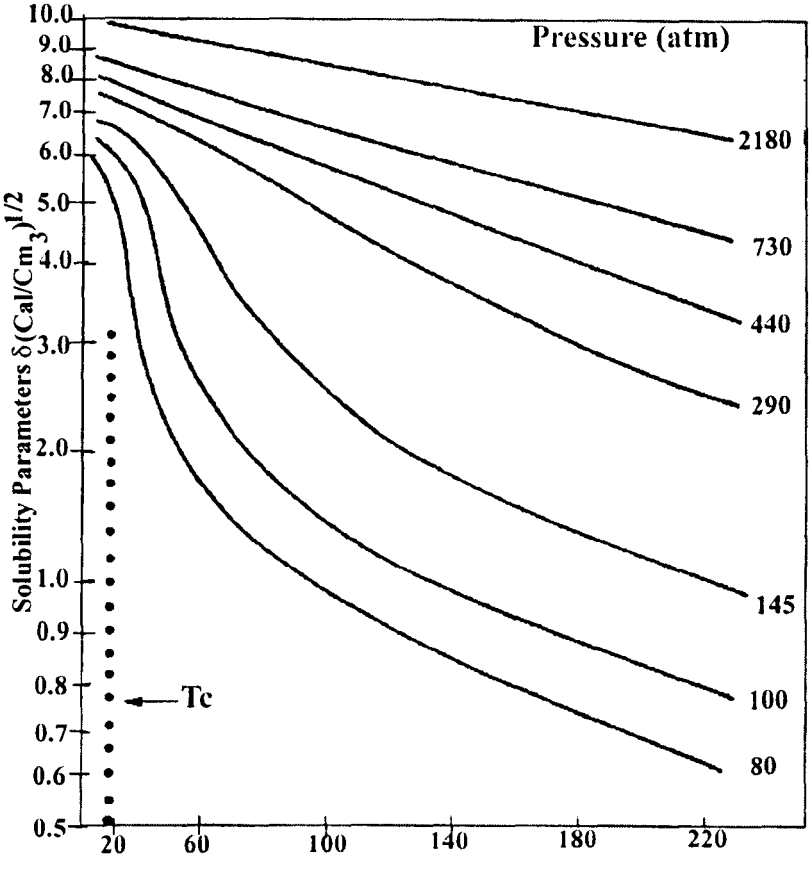
1.1.5.2 Density

Solvating power, diffusion and viscosity are all functions of density. Therefore, altering the density of the fluid, alters the properties of the fluid from gas-like to liquid-like form without crossing the gas/liquid equilibrium line. In addition, large changes in SF densities and solvating power effect small changes in pressure because the compressibility of a SF is largely above the Tc.[13,21] The density is directly related to the solvent strength of a SF hence, the SF solvating ability can easily be modified for a particular species by changing the extraction pressure and temperature.[17, 22, 26]

1.1.5.3 Solubility

The solvent strength of a liquid is essentially constant regardless of the extraction conditions, but the solvent strength of a SF depends on the pressure and temperature used for the extraction.[34] At constant temperatures, extraction at lower pressures will favour less polar analytes, while extraction at higher pressures will favour more polar and higher molecular weight analytes.[35] This allows the extraction to be optimised for a particular compound class by changing the pressure and to a lesser extent, the temperature, of the extraction. This solvent strength variation is demonstrated by the Hildebrand solubility parameter on temperature and pressure for carbon dioxide in Figure 1.2.

Figure 1.2 Dependence of the Hildebrand solubility parameter on temperature and pressure for supercritical CO_2.[35]



The choice of SFE conditions are pressure and temperature based, so that the target analytes have the highest solubility in SF.[35] The optimum conditions can be approximated if the solubility parameter of the analytes and the correlations are known; like the one proposed by Giddings[15]:

$$\delta = 1.25\ p_c^{1/2}\ (\rho_{rg}/\rho_{rl})$$

Where: the Hildebrand solubility parameter is δ = (cal cm^{-3}) $^{1/2}$, p_c is the critical pressure of the fluid (atm), ρ_{rg} is the density of the gas and ρ_{rl} is the density of the liquid. This equation also indicates that the polarity of the SF depends on its state by the relationship ρ_{rg}/ρ_{rl} and on its nature (chemical effect from the term 1.25 $Pc^{1/2}$).[1]

By using the above equation, the choice of which supercritical fluid is suitable and at what density for a given solute can be determined. Therefore, solubility parameters of the fluid and the solute should be identical at maximum solubility.

1.1.5.4 Solvent Strength

As mentioned earlier, the solvent strength of a SF is directly related to its density and may be changed using the pressure and or the temperature to adjust the reaction rates involved in the extraction process. This can be done by small changes in pressure and temperature producing relatively large changes in the density at the critical point. As pressure increases, the solvent power or fluid density increases at a given temperature. At a constant pressure, temperature increases lead to a decrease in solvent density. This strong pressure dependency of the SF solvating power is a key factor in SFE and is exhibited by all solid and many liquid solutes as long as the solute is not infinitely miscible with the solvent.[36]

In a SF, the effect of temperature on the solubility of a substance changes with pressure. Increases in temperature at a pressure close to the critical point (Cpt) lead to a decrease in the solute concentration in the supercritical phase. However, at a high pressure, an increase in temperature causes an increase in solubility leads to a simultaneous decrease in gas density thus increasing the vapour pressure of the solute.[30] This gas density reduction caused by the rise in temperature, is less pronounced at a higher pressure than a lower pressure. Therefore, the increase in the solute's vapour pressure overcomes the decrease in the gas density and leads to a higher concentration in the supercritical region.[36]

1.1.6 CLASSIFICATION OF FLUID PHASE BEHAVIOUR[17]

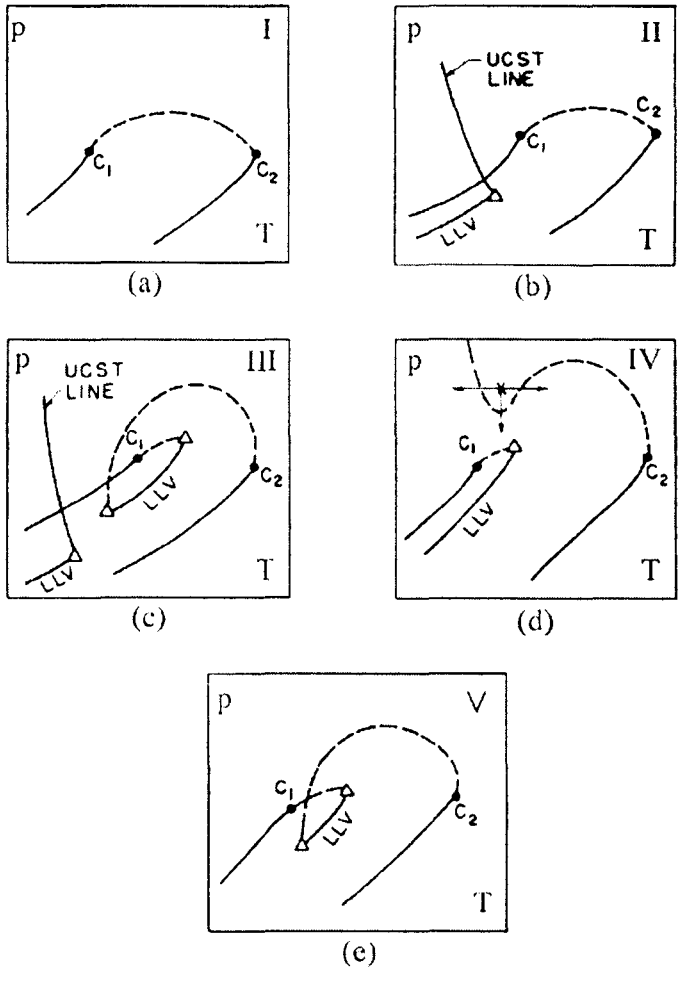
Interpreting high pressure phase behaviour data can be very difficult for a given practical application of SF technology. This can be explained by the mixture components varying in molecular size, shape, structure and even polarity. The diversity of a mixture's components and the amount can also contribute to the complexity of the phase behaviour.

The binary phase behaviour system is greatly varied and more complex than a single component system, but these too can be very complicated. The addition of polar modifiers to the SF can enhance SF-CO_2 solubility properties and influence the analyte or matrix complex causing rapid desorption into the SF.

This section is a review of vapour-liquid equilibria of binary mixtures. Generally of the different systems, the simplest is with a single solvent SF binary mixture with a single solute.[37] Although process mixtures are generally quite complex, it is instructive to study the phase diagrams of binary mixtures.[30] Useful detailed discussions of the thermodynamic behaviour of the various types of mixtures, with specific examples are provided by Rowlinson and Swinton [38], Streett [37] and McHugh and Krukonis.[17]

The currently accepted scheme for classification of binary mixture phase diagrams was described in 1970 by van Konynenberg and Scott.[39] They observed five classes of binary phase diagrams that could be qualitatively described using the van der Waals equation of state. There are five distinct phases that were derived according to this scheme, as shown in Figure 1.3.

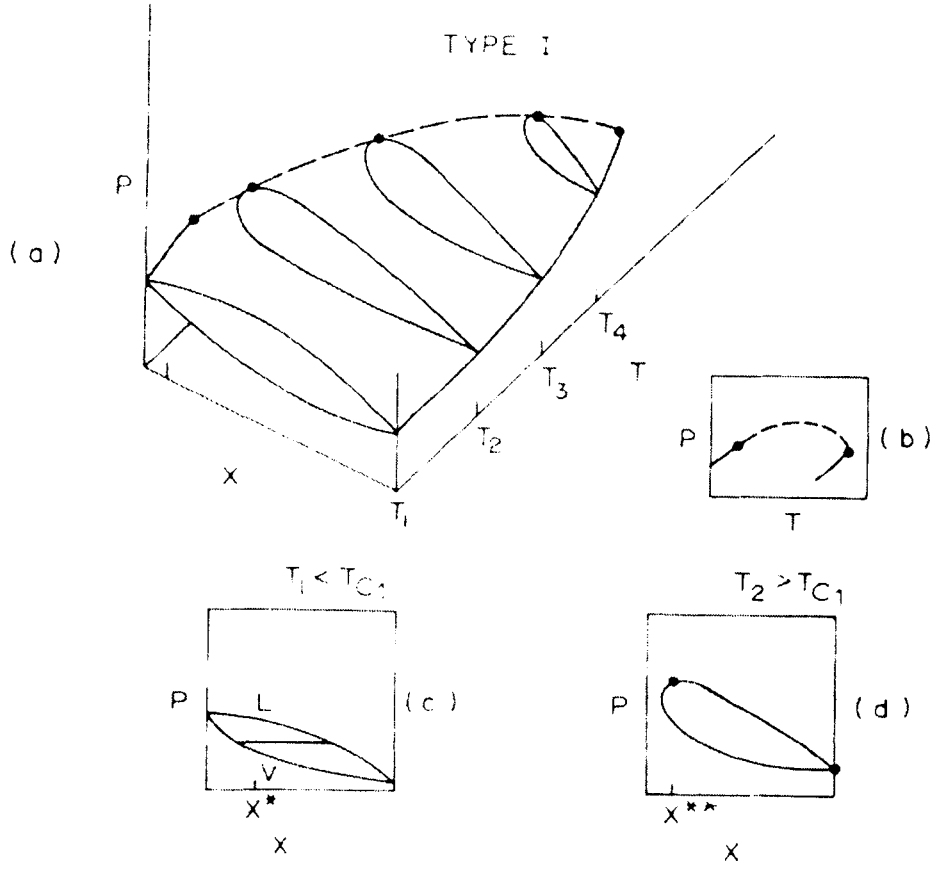
Figure 1.3 The five classifications for binary mixture phase diagrams.[17]



An understanding of the phase equilibrium principles of the phase diagrams above is crucial during SF solvent extraction of mixtures. Demonstrated in two dimensional p-T plots for simplicity, Figure 1.3 a is the simplest of the binary phase diagrams. The critical points are represented by component one (C1) and component two (C2), where mixture at its critical state runs continuously from the critical point of the heavier component to the lighter component forming the critical-mixture curve. In Figures 1.3 b-e, like Figure 1.3 a, the critical-curve of the binary mixture is represented by dashed lines connecting C1 to C2, with open triangles depicting the critical end points.

In general, the three-dimensional P-T-x diagram for type 1 binary mixtures, as shown in Figure 1.4, show that they can be constructed by compiling numerous isothermal P-x plots, as shown in Figures 1.4 c and d. In the P-T-x, P denotes pressure, T temperature and x the composition of the heavy component.

Figure 1.4 Diagrams for a type-1 Binary Mixture P-T-x (a), P-T (b), P-x (c).[17]



Temperature, which is below critical temperature of both components, is demonstrated as P-x at temperature T_1 in Figure 1.4 c. A familiar vapour-liquid envelope is observed which intercepts the pressure axis at the vapour pressure of pure component 1 and 2. The overall fixed composition denoted by x* in this figure, consists of a single vapour phase existing at low pressures. By isothermally increasing these pressures, the two-phase vapour-liquid envelope is intersected and a

dew or liquid phase now appears. The two phase vapour liquid is separated from the one phase vapour region by a locus of points called the "dew point curve". Within the two-phase boundary of the vapour-liquid envelope, the equilibrium vapour and liquid phase concentration is determined by a horizontal tie line as depicted in this figure.[17]

Further increasing the pressure increases the liquid phase mixture while decreasing the vapour phase, leaving only a small bubble of vapour. Increasing the pressure further, as the vapour liquid envelope is finally crossed the bubble of vapour disappears leaving a single liquid phase. The "bubble-point curve" is the name depicted for the locus of points that separate the two-phase vapour-liquid region for the one phase liquid region described in Figure 1.4 a.

Demonstrated in Figure 1.4 d, is the behaviour caused by increasing the temperature above the critical temperature of the component 1 (T_{c1}). Although the vapour-liquid envelope is similar to that in Figure 1.4 c, the envelope intersects the pressure axis at the vapour pressure of component 2 only. Since the temperature is greater than (T_{c1}), the vapour-liquid envelope on the left-hand side does not touch the pressure axis, because the vapour-liquid line for component 1 is never crossed.

Experimentally, at an overall composition equal to x** (shown in Figure 1.4 d), the vapour-liquid envelope is first intercepted along the dew-point curve at low pressures. It is then intercepted at its highest pressure, which corresponds to the critical mixture point for this temperature and composition. If the overall composition of the mixture is to the right of x** and the pressure is increased, a

bubble-point is observed; if it is slightly to the left of x**, a dew point is observed if the pressure is increased. This vapour-liquid envelope at T_2 is shown in the P-T-x diagram in Figure 1.4 a.

As a function of pressure and composition, critical mixture points are determined and P-x diagrams are determined at higher and higher temperatures to create a critical curve. Assembling these P-x diagrams to form three dimensional P-T-x representation (shown in Figure 1.4a), the critical mixture curve is obtained, shown by a dashed line though the locus of critical mixture points. Finally, if this P-T-x diagram is represented as a two dimensional P-T diagram, a continuous critical-mixture curve, (shown in Figure 1.4 b) is observed.

1.1.7 SUPERCRITICAL FLUID EXTRACTION

Supercritical fluid extraction (SFE) is an independent sample preparation method, whose extracts can be analysed using various appropriate techniques. The widespread interest paid to the process of SFE is due to its unique solvent characteristics. It is especially useful for separating substances with low volatilities that decompose before reaching normal boiling points.[21] The phenomenon of SFE is based on the process where the dissolving power of a solvent; as a first approximation, changes greatly with its density.[21]

The solvent power of the SF is controlled by its density in the Cpt vicinity where slight pressure or temperature changes can produce large density changes. The SF density is typically 100 to 1000 times greater than that of the gas and solvating characteristics approaching those of a liquid are imparted.[36] However, the diffusion

coefficient and viscosity of the fluid remain intermediate between the gas and liquid phases at moderate densities, allowing rapid mass transfer of the solute compared with the liquid.

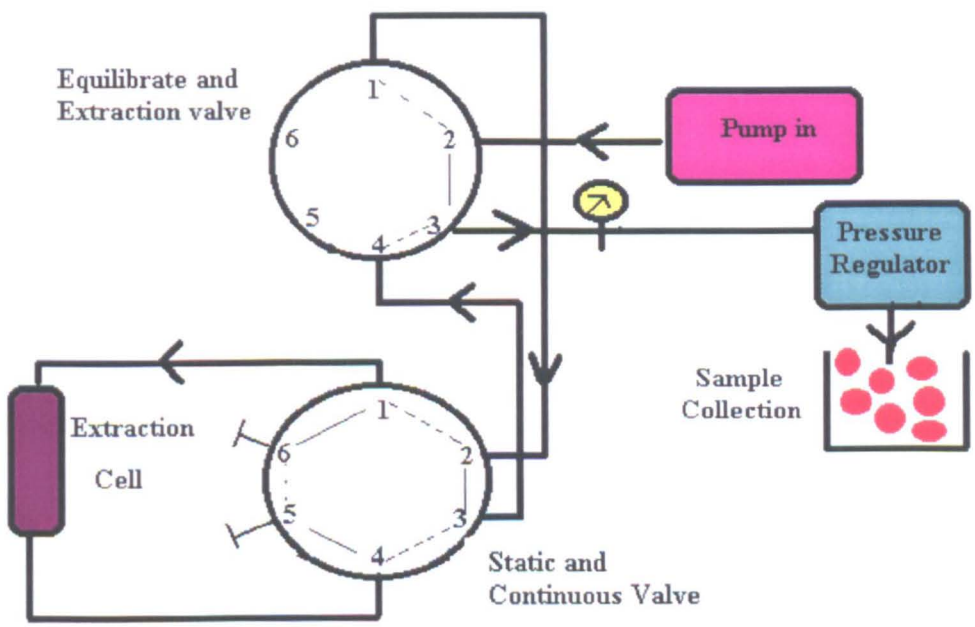
These liquid-like solvent powers and rapid mass transfer properties provide the potential for a more rapid extraction rate and a more efficient extraction than would be obtained with liquid due to better penetration into the matrix. Another advantage of using a SF as a solvent is its high diffusivity and low viscosity. This allows the SF to be able to penetrate into a polymer phase more effectively than a liquid solvent. In addition, the separation of the solute from the solvent can be achieved by the reduction in pressure and/ or cooling, thus making a very selective and efficient fractionation possible.

1.1.7.1 A Typical SFE Process

At the start of a SFE, the extraction fluid that is at or above its Pc is supplied by a pump to the extraction cell, which is placed in an oven at maintained temperatures at or above Tc. During the extraction, a preheat zone in front of the extraction cell helps to maintain the Tc, Pc and density conditions for soluble analytes to be partitioned from the sample matrix and incorporated into the SF. The analytes are then swept from the extraction cell at a flow rate and a time period empirically determined during the method development stage.[40] After passing through the cell, the SF containing extracted analytes flow through a restrictor and are depressurised into a collection vial containing an appropriate collection liquid.

The extraction can be carried out in two extraction modes, dynamic and static.[35] The dynamic mode (continuous mode), uses a continuous flow of SF through the extraction cell for continuous recovery of the analyte. The valve is opened allowing pressure to be maintained through the flow restrictor and cell allowing depressurisation into a collecting device. The static mode is carried out by closing the outlet flow of the SF allowing the cell to pressurise for a while, then the valve is opened (dynamic mode) allowing the analytes to be swept into the collection device. Figure 1.5 shows the schematics for Rheodyne dynamic and static extraction modes.

Figure 1.5 Rheodyne schematic of dynamic and static extraction modes.



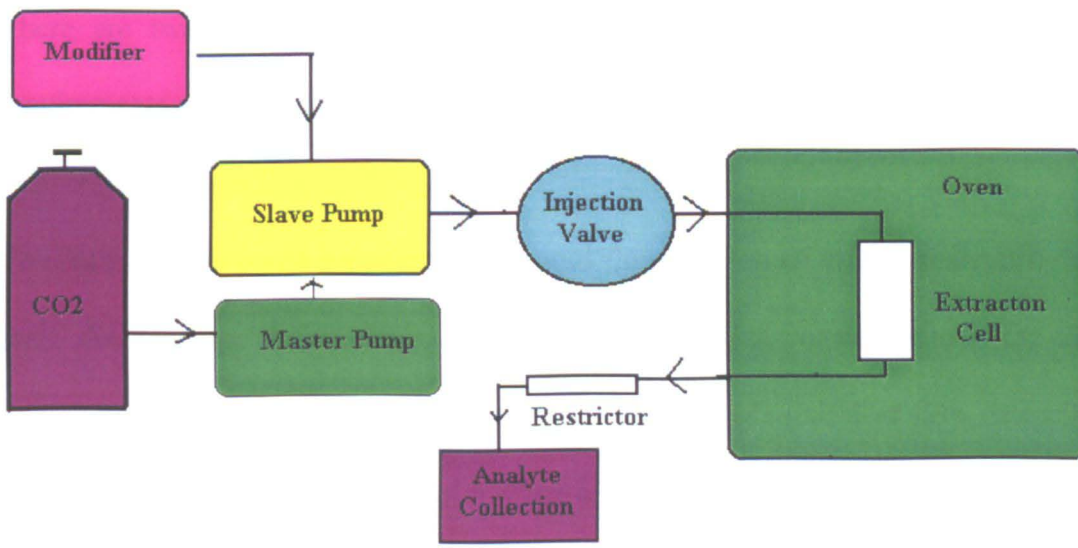
As demonstrated in Figure 1.5, when the first (top) Rheodyne valve is at the load (equilibrate) position, the flow is passed from position 2 to position 3, bypassing the second Rheodyne valve which is set in inject (continuous) mode. By setting the first valve to the inject (extract) position, the flow is diverted from position 2 to position 1, down to position 2 on the bottom valve. To perform a static extraction, the bottom valve is set to the inject position until the extraction cell is pressurised.

Once the required pressure is reached, the bottom valve in switched from the inject position to the load (static) position, diverting flow back to position 4 in the first valve via position 3 and out through the pressure regulator. Once the required time has elapsed during static extraction, the second valve is switched to the inject position and the analytes of interest along with the supercritical fluid are flushed out and depressurised through the restictor into the sample collection vial.

1.1.8 SFE INSTRUMENTATION

The main components in a SFE instrumentation are, the CO_2 source, the pump, the extraction cell, the restrictor, the oven and the analyte collection device. Generally, commercial extraction units will vary depending on the distributor, but all will consist of these major parts. Figure 1.6 demonstrates the schematics of the components consisted in a typical SFE system.

Figure 1.6 Schematics of a typical SFE instrumentation.



1.1.8.1 SF-Carbon Dioxide

High purity CO_2 is generally required for most SFE applications, so the analytes and their impurities, especially those with high boiling points, are collected during extraction. It is provided as a liquid-gas mixture in a 25-kg tank and at room temperature has a substantial vapour pressure that helps to displace the liquid to the pump. CO_2 is very dense, therefore a full length dip tube is installed which makes it more accessible and can be drained from the bottom of the tank easily. In addition, high purity CO_2 is better for certain detectors that can not tolerate impurities.

1.1.8.2 The Pump

The pump is used to pump liquid CO_2 at high pressure, to transport reproducible volumes, pump the modifier through with the SF and supply a constant fluid flow throughout the extraction. The constant flow rate provides a measure of the retention time of the separated analytes being collected for a finite time before further analysis. Therefore, the total SF passed through the extraction cell is of great importance. There are two types of pumps used in SFE that meet the flow and pressure requirements are the syringe pump and the reciprocating pump.[18]

The reciprocating pump supplies a continuous flow and has an infinite reservoir. Its main disadvantage is that the pump head must be cooled constantly to allow the pumping of the liquid CO_2. The syringe pump can provide pulseless flow, is easily filled with CO_2 and does not require to be cooled because the CO_2 is liquefied by pressure not temperature.[18] However, because of their limited volume, syringe pumps need to be filled and repressurised when the pump cylinder is emptied and thoroughly flushed when changing modifiers to prevent carryover. Fluid can be

pumped at flow rates of 0.5 to 4mL/min that enable faster flow rates to reduce extraction time.

1.1.8.3 The Extraction Cell

The extraction cell is generally a simple stainless steel tube, 100µl to 50ml in capacity with auto-sealing or compressed end fittings. They are placed inside the oven that controls the surrounding temperatures and must be able to withstand temperatures generated by the oven as well as the pressures generated by the pump.

1.1.8.4 Restrictor

In order for the system to reach a supercritical state during the extraction, the extraction cell is under pressure, which is incurred by using a fixed or variable restrictor. Fixed restrictors are commonly used during an extraction and consist of a piece of capillary tubing with an initial diameter and length inductive to produce appropriate backpressure. Varying the flow rate through the restrictor may change the SF density. Thus, fixed restictors must be changed between extractions because of the complex temperature, pressure, density and flow rate relationship, to vary the system pressure and change the density at a constant flow rate.[18]

Variable restrictors are more complex than fixed restrictors, but do not have to be changed during extractions. They regulate pressure independent of the flow rate by mechanically regulating the size of a small opening, allowing the de-coupling of pressure and flow rate.

1.1.8.5 Analyte Collection

After SFE has been completed, the trapping/collection efficiencies of the product analyte(s) are important for further analysis. Three ways of recuperating the analyte after depressurisation of the SF are by thermal, sorbent or solvent trapping.[1] Thermal trapping is where the SF is depressurised into a cooled vial. However, the high gas flows at the SFE outlet can cause volatile components to be lost, only retaining non-volatile components. Sorbent trapping is where the SF is depressurised, absorbing volatile and non-volatile components onto a solid support, that are eluted with a solvent after SFE is finished.

The simplest and quickest of the three is solvent trapping, where the SF is depressurised into a liquid solvent. It does not offer the selectivity of sorbent trapping, but is efficient for SFE, with optional collection solvent selectivity. Two major concerns in SFE for optimal analyte recoveries are, that the analyte of interest must be extractable from the matrix and the system used must be able to efficiently collect the analyte of interest.

1.1.9 SFE METHOD DEVELOPMENT

Analytical sample preparation techniques such as SPE have been widely used for a variety of matrices, compared to SFE, which is relatively new. During SFE method development, the appropriate conditions are often chosen by trial and error. The analytes that are contained in a solid matrix must be solubilzed in a SF in order to be extracted. Therefore, the relative affinities of the analyte for the SF and to the solid matrix have to be understood.

1.1.9.1 Analyte and Matrix Properties

Under SF conditions there are numerous analyte and matrix parameters that need to be considered to be adequate for sample preparation. The chemical properties of an analyte must be considered when selecting the starting conditions. The molecular weight, functional groups, polarity, solubility, volatility, pKa, thermal stability and concentration are all considerable parameters. Equally, matrix characteristics such as, the particle size, homogeneity, volume, porosity, composition, solubility, density and immobility are important for SFE. These parameters are all taken into consideration for SFE preparation to prevent the matrix from dissolving in the SF or the analyte being strongly retained during the extraction.

The morphology and physical form of the matrix can have an enormous influence on the transport of the analyte into the SF and this can effect quantitative recovery during SFE. If the matrix is in bulk form (e.g. solid polymeric pellets or hard soils), preliminary sample preparations such as grinding, sieving, drying or wetting may be used. The porosity and relative size of the matrix, smaller being better, effects the speed and efficiency of the extraction. This is due to the short internal distance the solute has to cover to attain the core of the SF solution.

In some cases, the geometrical form of the matrix used can influence the rate and efficiency of the extraction in the same way as the porosity of a matrix effects a solid-liquid extractions.[42,43] The use of solid supports such as diatomaceous earth, filter paper or a drying agent can be used for the application of a liquid sample to facilitate the extraction and at the same time preventing the matrix from being swept through the extraction cell.

The adjustment of pH or addition of solvent to the extraction cell can also aid in the SFE process for optimal recovery of the analyte of interest. Other parameters such as controlling the density, temperature, sample composition, flow rate and time can affect the reproducibility of SFE. Increased density increases solvent strength, thus, more polar analytes are removed. Controlling these parameters during SFE aids the selective fractionation of analytes into one or more fractions while leaving behind impurities in another fraction.[35] This results in fewer interferences due to impurities in the final analytical analysis.

The flow rate of the SF can also effect the extraction process. An increase in flow rate can accelerate analyte elution when optimum conditions are obtained. The other important parameter mentioned is the length of time needed to complete an extraction. Modifiers, as mentioned earlier, can be used to decrease the extraction time where most SFE can take 20 to 30 minutes depending on the type of sample.

1.1.10 COLLECTION TECHNIQUES

1.1.10.1 Off-line and On-line SFE Techniques

Sample preparation and extraction is often more difficult and time consuming than the actual analysis procedure and can lead to relatively inefficient analyte recoveries. Two types of extraction collection techniques that provide an alternative to conventional extraction techniques are by off-line and on-line SFE collection methods.

Off-line SFE is relatively simple to carry out. The analytes are collected into a collection device as described earlier, for subsequent analysis after extraction is

completed. The collected extracts may be analysed by a variety of appropriate analysis techniques (e.g. SFC[44-47], GC[2,26,48], HPLC[49], TLC[50], GC/MS[51]).

During off-line SFE, the analytes are depressurised and usually collected into a few millilitres of a liquid solvent. The vial is cooled by expansion of the SF that prevents rapid evaporation of the collection solvent due to the high gas flows. SFE eluents have been collected from numerous sorbents that aid analyte collection. Silica, bonded-phase packing and various forms of diatomaceous earth can be used to aid the analyte to be eluted successfully with a liquid solvent for subsequent analysis without interference.

1.1.10.2 On-line SFE

On-line SFE techniques can be directly coupled with an analysis technique such as SFC, GC, GC/MS, HPLC and TLC. The advantages of on-line SFE are that it eliminates sample handling and its associated errors and in most cases maximises sensitivity to yield high analyte recovery. A disadvantage of direct coupling of SFE is that the sample extracts are not viable for analysis using another method. On-line SFE coupled systems must be selected based on chromatographic techniques suitable to the analyte being extracted.[35]

1.1.11 SFE COUPLED TECHNIQUES

The direct coupling of SFE with various analysis techniques has been investigated and reported over the years. Stahl and Schilz in 1976, reported the use of a microextractor with TLC.[50] The coupling of SFE to HPLC was described by Unger and Roumeliotis with more recent developments of SFE coupled with SFC and GC.[22]

1.11.1 SFE-TLC

The coupling of on-line SFE to TLC has lead to the development of an extraction apparatus for the purpose of extracting a wide range of naturally occurring materials (e.g. coffee, dye-mix leaves etc.).[22] The extracts from this coupled technique are directly deposited onto the TLC plate, resulting in a static process being carried out. The components can be isolated from or detected on the support material in one or two dimensional chromatograms that can aid further analysis.

Although on-line SFE-TLC provides a rapid and simple insight into the extraction performance, it its not without its limitations. Quantification is difficult and there are problems with calculation of concentration levels, low resolution and high pressures that can cause instability of the support medium.

1.11.2 SFE-HPLC

There are numerous investigations reported for the application of off-line SFE-HPLC. For example, the technique was applied for the extraction of caffeine from roasted coffee beans using supercritical CO_2, the extraction of vitamin K_1 from commercial soy and milk based infant formulas, extracting polymer additives from polyethylene and polypropylene and for the isolation of morphine and quinine from various plant materials.[22]

The first coupled on-line SFE-HPLC device was described by Unger and Roumeliotis in 1983.[22] This system consisted of a connected series of two high-pressure sample-injection valves. The first valve serves as a switching valve to the

loop and pressure controller over a packed microbore column, while the second valve is operated as an injector for the normal-phase HPLC column.

1.1.11.3 SFE-GC

Selection of a chromatographic technique coupled with on-line SFE is based on choosing one that best fits the analyte of interest. The most commonly used SFs are gases under certain conditions, which make the coupling of SFE with capillary GC an appropriate analytical technique. On-line SFE-GC can be coupled either by direct depressurisation of the SF inside a conventional split or splitless injection port or via a capillary column with an extraction cell restrictor inserted through the on-column injection port.[35]

During SFE the analytes are cryogenically focused in the GC column stationary phase and the analysis is carried out as normal. On-line SFE-GC yields maximum sensitivity using smaller samples than off-line SFE-GC, because of the direct coupling of the extracted analytes onto capillary GC. For larger samples and a broader range of matrices, the use of split GC/MS can be used.[18]

1.1.11.4 SFE-SFC

The direct coupling of SFE with SFC has greater advantages for drug analysis than with the other coupled techniques, simply because both methods use SFs. Successful coupling can be attributed to the injection solvent being the same as the mobile phase and interfering components insoluble in the mobile phase will not be introduced onto the column following extraction.

During the coupling of on-line SFE-SFC, the analyte extracted is swept from the extraction cell and collected either into an accumulator trap containing a solvent material or thermally trapped at the head of the SFE column. After SFE, the extracted analytes are swept from the accumulation device by the mobile phase onto the SFE column. The analysis is then carried out under the appropriate conditions and chromatographic parameters.

On-line SFE-SFC involves three types of SFC techniques.[19]

- Conventional packed columns
- Capillary columns
- Packed capillary columns

Packed column SFC has a few advantages in comparison with capillary and conventional packed columns. It has a higher loading option and shorter analysis times, while capillary columns have the advantage of a lower pressure drop, higher efficiency and lower flow rates resulting in easy FID or MS interfacing.

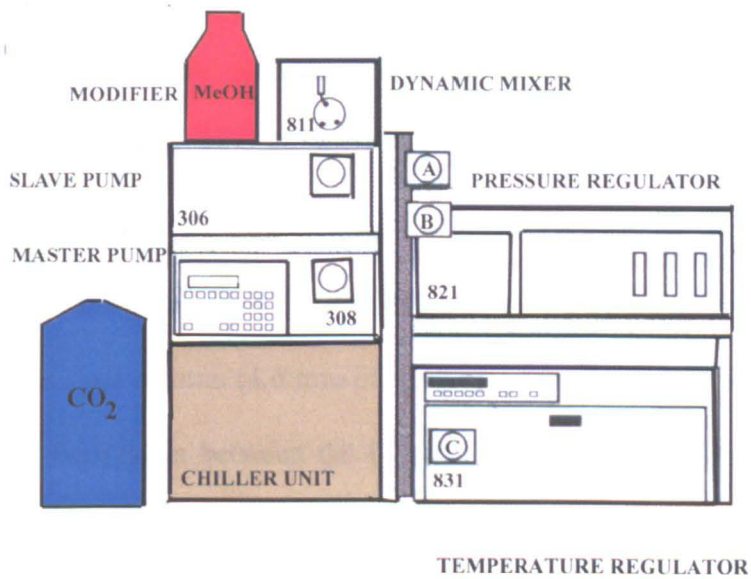
1.1.12 SFE INSTRUMENTION SET-UP

The SFE system used was manufactured by Gilson and consisted of all the necessary components. The system consisted of the following:

- CO_2 cylinder fitted with a full length dip-tube (7.2 kg of CO_2)
- A SFEC3 refrigeration chiller unit
- Two Gilson 10SC pumps (306 and 308) with built-in an automated programming system supplied via the GSIOC (Gilson Serial Input Output Channel) 308 pump
- A Gilson 811B dynamic mixer

- A 7125 Rheodyne (equilibrate/ extract valve (A))
- A second valve for static and dynamic extraction (B)
- Open/ close valve for extractions (C)
- A Gilson 821 pressure regulator
- A Rheodyne 7037 back-pressure regulator (inside oven)
- Gilson 831 temperature regulator (oven)

Figure 1.7 SFE instrumentation described above used in research laboratory



Polyvinyldifluoride (PVDF) check valves were placed in the CO_2-10SC 308 pumphead giving it more resistance for hot and cold temperatures. This pumphead was chilled using the CFC3 chiller unit. The second pumphead (306) contained polytetrafluoroethylene (PTFE) check valves and was used to pump the modifier.

The pressure, CO_2 amount, modifier and flow rate were all measured using the Gilson 308 "master" pump, which controls the 306 "slave" pump. The slave pump delivers organic modifier solvent to the 811B which is mixed with CO_2. The 821 pressure regulator is controlled by the 308 pump that controls the operation of the

pressure regulation valve indicated by P2 and P1 on the program. P2 maintains the required pressure levels at the outlet and P1 is used to implement the flow rate accuracy corrections and ensures that the system pressure is not above or below the limits entered in the program.

The HPLC 7125 Rheodyne valve (A), was placed in-line between the 811B mixer and the extraction cell, which was housed inside the oven, for the application of the gas and modifier to the cell. It would have been more ideal to have the Rheodyne inside the oven so that supercritical conditions are applied directly to the matrix containing the analytes, but with the initial set up used, this was not mechanically feasible.

To prevent deposits from the CO_2 cylinder that would contaminate the system, an HPLC stainless steel column (4.6 mm id. x 10 cm) loosely packed with lichoprep 60 was placed in the system between the CO_2 cylinder and the CO_2 pump head. The extraction cells were made from stainless steel empty HPLC columns. The dimensions were 3.4 cm x 4.6mm id and 7 cm in length.

This system described was used for method development throughout the course of research. Modifications that were made to the system will be discussed further through the course of this study.

1.1.13 CONCLUSION

Supercritical fluids as a medium for selectively separating components from a complex mixture via extraction and or coupling with chromatographic techniques are

currently being actively studied world wide. In an effort to effectively deal with thermally liable and non-volatile compounds (e.g. drugs, pharmaceuticals, foods, pesticides) analytical chemists have facilitated the use of SFs (e.g. CO_2) for adequate extraction and sample preparation techniques. This is mainly because of the advantages that SFs have over conventional liquid solvent techniques in the areas of efficiency, method development, cost and environmental consequences.

SFE utilises the properties of SFs for selective extractions of specific classes of solutes from complex matrices and can be used as an alternative to time- consuming solvent extractions for sample preparation. In addition, the incorporation of SF technology with analytical chromatography presents great potential towards quantitative or qualitative determinations of specific solutes in complex matrices.

SFs are finding wide acceptance in a number of analytical disciplines as unique solvating media, with the attributes of SFE well documented.[52] There are many areas in which the technology can and must improve, therefore, with the continued addition of new research, these improvements will be made.

2. COCAINE

2.1 LITERATURE REVIEW

2.1.1 INTRODUCTION

Over the past few decades, there has been rapid growth of illicit, medical and non-medical abuse of psychoactive drugs, one of which is cocaine. Cocaine is by no means a new drug. For over 3000 years, cocaine and cocaine-containing substances have been used in a custom known as coca chewing by the Native Indians in South America. [53]

Coca chewing can be done in two principle ways. First, toasted coca leaves are combined with an alkaline material and held in the mouth between the gum and cheek, this is known as a quid or wad.[54] This quid is re-chewed and the juice swallowed. It is kept in the cheeks for continuous low concentrations of cocaine to be released, because the effects of coca chewing are not long lasting.

A second method of coca chewing contains a fine powder made from ground dried coca leaves mixed with ashes. Small amounts of the quid are held between the cheek and gum as in the first method, with the juice and powder swallowed over several hours. The lime and ash combined with the coca leaves is used to facilitate drug absorption, as some of the cocaine is inadvertently swallowed while also being absorbed though membranes of the mouth.[53-54]

2.1.2 HISTORY

Controlled under the Misuse of Drugs Act (1971) Schedule II, cocaine is a highly lipophillic class A drug. It has been highly abused for decades as a topical anesthesia because of its properties. It is a natural alkaloid found in the leaves Erythoxylon coca and Erythroxylum Norgranatense, a plant indigenous to South America.[55] There are at least eighteen alkaloids contained in the leaves with the principle component cocaine, which represents 0.2-1% by dry weight of the leaf.[53, 56]

This pure alkaloid was first isolated in 1860 by Albert Niemann.[57] In 1884, Koller introduced cocaine into clinical practice as a topical anesthetic for ophthalmologic surgery, that later extended to dental, oral and urethral applications.[58] This stimulant of the central nervous system, had the potential to act as a local anesthetic and a sympathomimetic, increasing blood pressure, heart rate and body temperature. The earliest case of cocaine poisoning was reported in 1888 where a pharmacist made a deliberate suicide attempt by taking an overdose of the drug.[57, 58]

In addition, up until 1894 all other fatalities were accidental consequences because of cocaine usage for surgical applications.[58] This predominate accidental poisoning from cocaine usage, prompted the synthesis of synthetic substitutes such as procaine (Novocaine) by Einhorn in 1905.[57] This substance was less toxic and lacked the central effects caused by cocaine, because of its block anesthesia by hypodermic infiltration. However, in spite the dangers associated with cocaine hydrochloride, it was still used as an effective local anesthetic, mostly onto direct mucous membrane surfaces.

The use of cocaine has been available since the 1850s, when its addictive properties were appreciated.[54] The most widely used agents today are procaine, lidocaine, bupivacaine and tetracaine. Cocaine is now a commonly used addictive drug because of its psychoactive properties.

2.1.3 PHARMACOLOGY

The reinforcing effects of cocaine and its analogs correlate best with their effectiveness in blocking the dopamine transporter, leading to increased dopaminergic stimulation at critical brain sites. However, cocaine also blocks norepinephrine (NE) and serotonin (5-HT) reuptake. The clinical use of cocaine has steadily declined, mainly because of its toxic effects and abuse potential. The blockage of catecholamine uptake in the central and peripheral nervous systems is due to the high toxicity of cocaine, while euphoric properties are due to the inhibition of catecholamine uptake (primarily dopamine) at the central nervous system synapses. Other local anesthetics do not block the uptake of NE or produce the stimulus to catecholamines, vasoconstriction or mydriasis characteristics of cocaine.

2.1.4 PHARMACOKINETICS

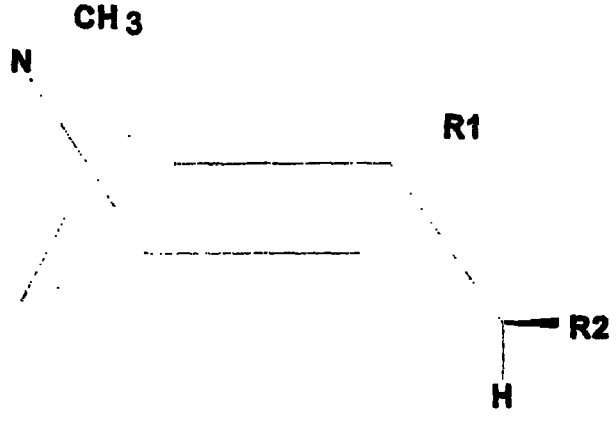
The conversion of cocaine to metabolites begins to occur soon after absorption.[46] Cocaine absorption is very variable, resulting in a wide spread of values for the time to peak concentration, peak concentration and half-life. Half-life varies with the individual with estimates ranging from 16-90 minutes (intranasal mean t1/2 75 minutes; intravenous, mean t1/2 54 minutes; oral, mean t1/2 48 minutes).[59, 60] In urine, excretion of cocaine depends on the pH[61] with approximately 1-9% of cocaine unchanged and detectable for only a few hours.[62] Therefore, for the detection of the

presence of cocaine in bodily fluids, more persistent urinary metabolites are looked at, however, cocaine itself is relatively stable in hair.

2.1.5 CHEMICAL STRUCTURE

The chemical structure of cocaine below in Figure 2.1 is referred to as a boat shaped structure that contains a methyl group attached to a nitrogen group, R1 which is a methyl ester group, R2 which is benzoyl group and a hydrogen.

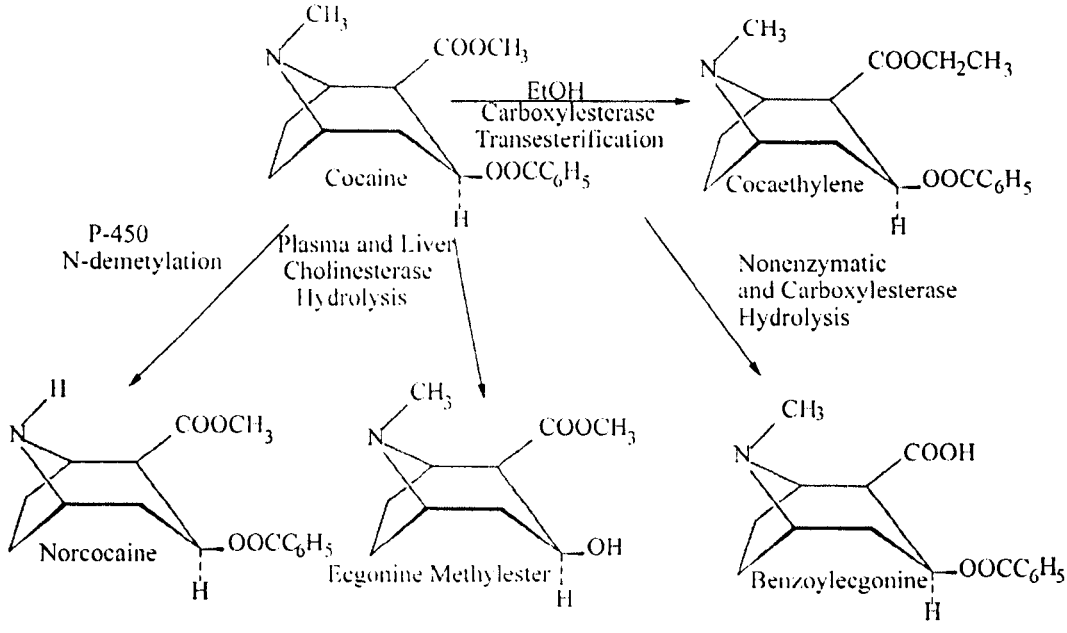
Figure 2.1 Chemical structure of cocaine



2.1.6 METABOLISIM

Metabolism of cocaine begins almost immediately after absorption with hydrolysis of the ester to give the two major metabolites of cocaine, benzoylecgonine [61-63], that is detected in most immunoassays and ecgonine methyl ester.[64-66] By the chemical hydrolysis of cocaine, benzoylecgonine is rapidly formed in biological fluids, while cholinesterases in serum or plasma and liver are responsible for the hydrolysis to ecgonine methyl ester. The plasma enzyme is less abundant, with a higher affinity than the liver, but the contribution of the two are similar except in users with cholinesterase.[63] Further hydrolysis of the two products leads to ecgonine.

Figure 2.2 Metabolic route of cocaine



Eleven metabolites of cocaine have be identified in urine including ecgonine[66], ecgonidine methyl ester[67], benzoylnorecgonine[61] and norcocaine[68], with the latter being found in only trace amounts. Arylhydroxy[69] and arylhydroxymethoxy[70, 71] metabolites and their ethyl ester analogues have also been found[71], with the latter formed with the simultaneous combination of ethyl alcohol. Norcocaine is the only active metabolite found in small concentration in urine up to 4 hours after a 100mg intravenous dose of cocaine.[67]

With the simultaneous combination of alcohol and cocaine, trans-esterification of cocaine occurs changing it to ethyl benzoylecgonine (cocaethylene).[72, 73] Coca-ethylene is an analyte with pharmacological activity similar to cocaine. The detection of cocaethylene is toxicologically important because of its frequent use with alcohol, its longer half-life than cocaine and prolongs the action of cocaine and is often present in similar or greater concentration than cocaine.[73] It can also be

hydrolyzed to benzoylecgonine and ecgonine ethyl ester through similar pathways as cocaine.[56] In addition, for the detection of cocaine in hair, the detection of cocaethylene can confirm the use of cocaine rather than accidental exogenous contamination.

2.1.7 COCAINE PREPARATION

Cocaine (benzomethylecgonine) being an aromatic residue, an intermediate chain and an amino, share common characteristics with other local anaesthetics.[58] It is an extract from the leaves of the plant with the use of an organic solvent such as paraffin and or petrol and sulfuric acid creating a coca paste (cocaine sulfate).[54] The paste is then treated with hydrochloric acid to refine and remove impurities, becoming a hydrochloric salt. The result is a pure white crystalline powder known as cocaine hydrochloride.

Cocaine hydrochloride can be administered intravenously, intranasally (orally) or by smoking.[54, 60, 74, 75] It has a melting point of 195°C and decomposes on heating, therefore, it can not be smoked and the hydrochloride must be removed. It is frequently reconverted into cocaine freebase or "crack" by a process known as freebasing. This process occurs when cocaine hydrochloride is mixed with sodium bicarbonate (baking soda), or water and ammonium hydroxide. Ether or a fast drying solvent can then be added to free the cocaine from the water, becoming an insoluble freebase form of cocaine.[54] It is then more volatile than cocaine hydrochloride and amenable to be ingested by smoking because the low melting point (98°C).

2.1.8 ROUTES OF ADMINISTRATION

Following administration, cocaine rapidly penetrates all body fluids and tissue. It is readily absorbed into the bloodstream and mucous areas depending on whether it is intravenously, orally or intranasally administered. Acute administration of cocaine through these routes, can cause mood elevation, euphoria, increased energy and self-esteem, alertness and can lead to a decrease in appetite and sexual interest.[56] Tolerance can be built up to the euphoria by chronic use of cocaine. Frequency of use increases anxiety, paranoia, hallucination and perceptual changes.[54] Respiratory arrest and convulsions can be experienced by an overdose and may be followed by death.

2.1.8.1 Intranasal administration

Intranasal administration or "snorting" is the most commonly used route of administering cocaine. Cocaine hydrochloride is ground finely and snorted into the nasal cavity using a small piece of tubing. Within seconds of snorting the cocaine, a numbing sensation is experienced which lasts up to five minutes. As the high commences, euphoria and an increase in energy is experienced. A peak high is reached within 10-20 minutes, subsiding within an hour.[54]

2.1.8.2 Intravenous administration

Cocaine is converted into a solution for Intravenous administration and then a tourniquet or belt is used to help swell the veins for easier access when injected. This produces an instant high. The same euphoric high is experience with intranasal usage. However, there is more of an intense and faster rush within minutes of intravenous administration. This usually wears off within about thirty minutes.[54] In

addition, intravenous use can cause hazardous problems such as overdose and transfer of infectious diseases such as AIDS or hepatitis.

2.1.8.3 Smoking

The most expensive and common form of cocaine is cocaine-freebase.[58] When cocaine hydrochloride is converted into cocaine-freebase the freebase is smoked through a water pipe to produce an intense rush and a euphoric like high. During this process, much of the cocaine is lost through condensation and escaped smoke, therefore, only 5-6 % of the drug gets through the pipe.[56]

One reason for freebasing being a common use of drug abuse today is because it produces increased craving for another dose, energy, and pleasure than the other routes of administration. In addition, because of the flammable solvents incorporated in cocaine freebase, it can damage the lungs.

2.1.9 DRUG INTERACTION

Sometimes drug combinations can be ingested inadvertently, because the user is not knowledgeable or concerned about drug interactions. The ingestion of two or more drugs, can produce various unpredictable effects greater or less than the effects of one of the consumed drugs or an effect unrelated to any of the drugs alone. Cocaine users tend to take other drugs such as benzodiazepines, barbiturates, marijuana or alcohol in combination with cocaine, thus having toxic effects. A common combination is with the use of heroin with cocaine known as a "speedball", that can have toxic effects resulting in death.

2.1.10 EFFECTS OF COCAINE

The psychological effects of cocaine are complex and influenced by the environment, the dose, the route of administration and the characteristics and experience of the user. The general transition of nerve impulses are blocked by cocaine in various nerve fibers. It has a profound prominent stimulatory effect on the central nervous system at the cortical level. This results in a heighten euphoric, physical and mental effect and the feeling of increased energy and in most cases hallucinogenic and paranoia effects. Doses of 25 mg produce euphoria within 2 minutes by intravenous injection or with in 3-5 minutes when taken intranasally.[74, 75] This initial euphoria is followed by a marked depression described by abusers as a "crash". This marked depression often leads to a prompt to experience the high over again every 10 to 30 minutes.[58]

2.1.11 STABILITY OF COCAINE

Cocaine is hygroscopic and benzoylecgonine forms a tetrahydrate, therefore, the stability and storage of cocaine and its metabolites is important to ensure proper preparation and preservation of the drug when in biological specimens and in solid or liquid forms.[76] Cocaine is most stable in aqueous solutions at pH 3-4, with stability of pH < 4 for over 24 days at 24 °C.[76] The base if stored in methanol at –15 °C, is stable indefinitely. Benzoylecgonine is most stable at pH 1.8 and in urine at pH 5-9 at 4°C up to 200 days.[77, 78] However, leaving benzoylecgonine in methanol for long storage periods leads to the formation of its methyl ester. At room temperature, vial to vial transfer, during transport or at pH 9, the loss of cocaine and its metabolites can be up to 50% over a 20-30 day period.[79]

To minimize the loss of the drug, a number of options may be taken. Evaporating the organic extracts to dryness can minimize the loss. Silanization of glassware or pre-treat auto-sample vials with 0.1% ethylene diamine tetra acetic acid (EDTA) can increase recovery and prevent tertiary amines forming complexes with metal ions in the glass.[80, 81]

2.1.12 CHROMATOGRAPHIC ANALYSIS

2.1.12.1 TLC ANALYSIS

For the detection of drugs of abuse, TLC can be used as a presumptive test. Cocaine has a relatively high Rf value on most solvent systems, while benzoylecgonine often remains at the origin.[76] For good separation of cocaine and its metabolites, suitable solvents are chloroform, methanol and ammonia. Dragendorff reagent is the most sensitive spray reagent used with TLC. For improved sensitivity this may be followed by over spraying with 20 %v/v sulfuric acid or by exposure to iodine vapors for 60-90 sec.[80]

2.1.12.2 GC and GC/MS ANALYSIS

The use of GC and GC/MS are more confirmatory methods for analysis. The specimen requires evaporation before being analyzed because of their volatility followed by derivatisation of its carboxyl groups to improve chromatography. Most methods in GC/MS use EI at 70-75 eV for the analysis of cocaine[82] alone or with nor-cocaine[83] with benzoylecgonine[84], or with benzoylecgonine and ecgonine methyl ester.[62]

2.1.12.3 HPLC ANALYSIS

The high water solubility of the amphoteric benzoylecgonine makes this a good candidate for estimation by HPLC without the need to derivatise. A loss of benzoic acid to form ecgonine methyl ester and ecgonine can occur and these compounds have poor UV absorbance. However, electrochemical detection at 1-2V over the limited pH range of 6-8 will detect cocaine, benzoylecgonine and ecgonine.[85]

UV absorbance for cocaine and its metabolites is monitored at wavelengths of 232-235 nm and occasionally at 200nm to increase sensitivty.[86] Reverse phase C_{18} columns are predominately used during HPLC analysis, however, phenyl and C_8 columns have also be used occasionally. Generally, methanol and or acetonitrile or acid buffer (pH 2-3) mobile phases combined with an amine modifier[87], sodium chloride[88], EDTA[89] or an ion-pair agent[90], are used during HPLC analysis of cocaine and its metabolites.

2.1.12.4 IMMUNOASSAY ANALYSIS

Relatively specific immunoassay techniques such as RIA (radioimmunoassay) and EMIT (enzyme multiplied immunoassay technique) are designed to detect benzoylecgonine. However, relative cross reactivity of cocaine can be observed, but not much research has been recorded for cross reactivity for ecgonine methyl ester. Low, medium and high known concentration level calibrators are supplied by manufactures, with cut-off levels of 300μg/L for benzoylecgonine, with levels lower than this considered negative.[76]

2.1.13 SFE ANALYSIS

Although the extraction of cocaine and its metabolites using SPE and LLE are well documented for biological fluids, there is no literature on the use of SFE for cocaine and metabolites from biological fluids. More recently, SFE has been applied for the extraction of cocaine from hair, but there has not been literature published on the use of SFE for cocaine and its metabolites in blood or urine.

2.1.14 CONCLUSION

The late twentieth century has seen the rapid growth of both the legitimate medical use and the illicit, non-medical abuse of an increasing number of drugs which alter ones state of mind. The widespread use and abuse of cocaine has led to a search for rapid and easy solutions to the problems of addiction. Cocaine continues to be one of the most popular and common drugs of abuse on the streets today.

Although treatment for the use of cocaine is on an increase, it can take a long time before a real cure is found. Even then it is unlikely that a single approach will be successful for all drug users seeking treatment, because the seriousness of the individual differs tremendously. Therefore, it is important that the specific needs of a patient are carefully evaluated, before a particular treatment is decided on.

2.2 CURRENT METHODOLOGY

Initially, a literature review on SPE methods was carried out to find out what types of solvent combination cocaine and its metabolites were extracted with, because there was no literature published for SFE.

The worldwide monitoring SPE method used currently in routine work was used as a reference for the extraction of cocaine and its metabolites.

- *Blood sample pre-treatment*: To a vial 1 ml of blood, 4 ml of de-ionized water and 100 µl of internal standard (d_3-cocaine) were added. pH was adjusted to 6 +/- 0.5 with mono or di-basic phosphate, mixed or vortexed and left for 5 minutes.
- *Urine sample pre-treatment*: To a vial 1 ml of urine, 2 ml of 0.1M phosphate buffer pH 6 and 100 µl of internal standard were added.
- *Column conditioning:* The column was conditioned with 3 ml of MeOH, 3 ml of de-ionized and 1 ml buffer. The sample was added to the column and pulled through
- *Column wash:* The column was washed with 2 ml de-ionized water, 2 ml 0.1M HCL and 3 ml MeOH and dried (5 min at ≥ 10inches Hg).
- *Elution:* The analytes were eluted with 3 ml of dichloromethane: isopropyl alcohol: ammonium hydroxide (78:20:2 v/v) which was prepared daily.

The eluent containing the drugs was evaporated to dryness at 40 °C. The vial was sealed and derivatise with 50 µl of BSTFA for 20 min at 70 °C. It was removed from heat source until cool and 1 µl was injected into the GC/MS for analysis.

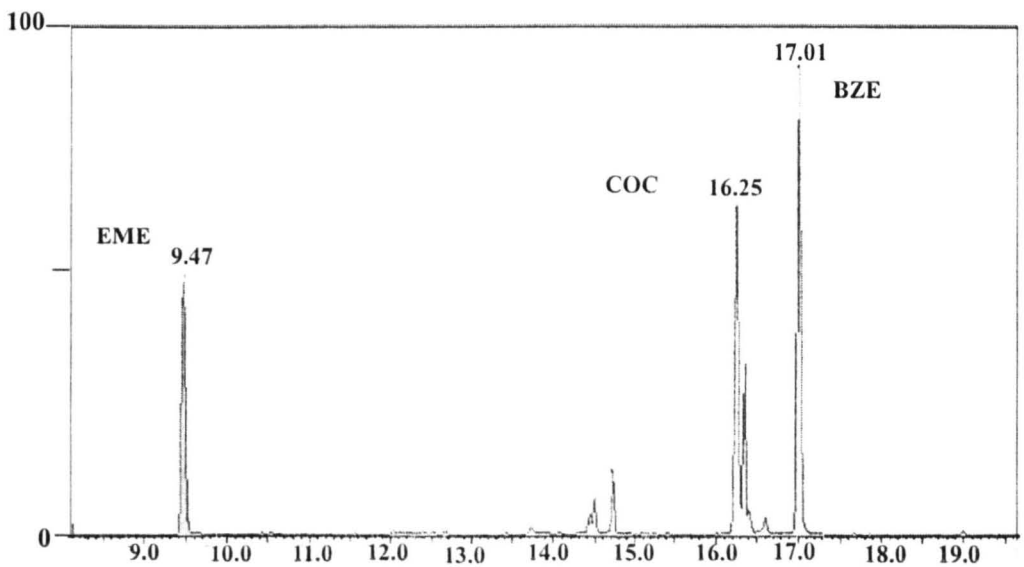
2.2.1 GC/MS Parameters

GC/MS for cocaine, benzoylecgonine and ecgonine methyl ester was carried out using a Fisons GC 8000 series gas chromatography with a mass detector MD 800. The GC was fitted with an HP1 capillary column (30 m x 0.25 mm x 0.25 µm film thickness). The GC/MS was operated in single ion monitoring mode (SIR) with an

injector temperature set at 250 ° C. The initial temperature was 100 ° C with an oven rise of 10 °C /minute and the final temperature was 300 ° C which was held for 2 minutes.

The mass to charge ratios (m/z) detected were 303, 182* for cocaine, 240* and 361 for benzoylecgonine, 182 and 82* for ecgonine methyl ester and for the internal standard deuterated cocaine (d_3-cocaine) the 185* ion was used for quantification. (* indicates the ion used for quantification).

Figure 2.3 GC/MS trace of cocaine, benzoylecgonine and ecgonine methyl ester.



2.3 SFE SYSTEM

The initial SFE set-up is described in section one of supercritical fluids. There are several startup checks to follow for turning on the system. Below is a list of steps:

Initial set-up:

- The Chiller unit is switched on first and left to cool the pump head
- Turn on the oven, 821 pressure regulator, both pumps and the mixer if needed

- Open the CO_2 cylinder so the gas can flow through the system
- Once the chiller unit is cold, the flow rate program can be initiated and the oven temperature set
- When the pressure is above 1.070 psi the selected pressure can be programmed
- Edit the method development parameter for the initial run
- Pack the sample into the extraction cell and connect it onto the on-line tubing which is housed in the oven
- Switch the valve from equilibrate to extract and immediately start the program
- Collect the sample analytes which will depressurize into a vial containing a solvent
- Analyze using GC/MS

At the end of an extraction:

- Switch the valve from extract to equilibrate
- Remove collection vial
- Dry and derivatise for analysis

Switching off the system:

- Go to the program and edit a program to depressurize the system
- If done manually, set the flow to 1500 psi which is the pressure limit for the back pressure regulator
- Once at this pressure, set it again to zero and set the flow rate to zero
- Turn off the CO_2, the oven, pressure regulator, both pumps and unplug the chiller unit.

2.4 METHOD DEVELOPMENT

Cocaine, benzoylecgonine and ecgonine methyl ester were all investigated by SFE from whole blood and urine matrices. Currently there is not a method for the SFE of cocaine and its metabolites from blood or urine. Therefore, an initial outline for a method had to be devised and then optimized.

2.4.1 CLEANING GLASSWARE

Glassware (e.g. Hypovials™) used throughout laboratory research were washed by hand using Decon 90. Decon 90 is a surface active cleaning agent and or radioactive decontaminant for laboratory use. It is biodegradable, phosphate-free, non-flammable and rinsable. The formation is comprised of an emulsion of highest quality, ionic and non-ionic surface-active agents, stabilizing agents, alkalis, nonphosphorus detergent builders and sequestering agents in an aqueous base. For a sufficient wash, 2-5 % solution mixture in water was used. Glassware was soaked for 24 hours, rinsed twice with water, twice with MeOH, then placed in a drying oven till dry.

2.4.2 EFFECTS OF TEMPERATURE

2.4.2.1 Experimental

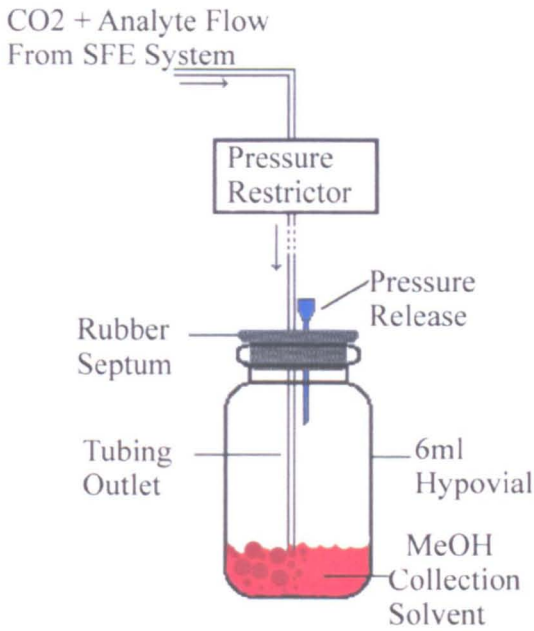
For the extraction of cocaine and its metabolites, approximately 0.13 g of plain Extrelut® was used as a sample matrix and extracted at temperatures ranging from 40-100 °C without the presence of blood or urine.

100 µl of each drug with internal standard (d_3-cocaine) was loaded onto the matrix and left to dry. Once dry the sample was loosely packed into the extraction cell. The

elution solvent DCM: IPA: NH_3 (78:20:2 v/v) was prepared and 300 µl was added to the extraction cell containing the sample. The initial conditions used were 1.5 ml/min flow rate, 2700 psi, 100 % CO_2 and the temperature set at 40 °C for the first four sets of matrices and then raised in increments of 10°C under the same conditions to 100 °C.

The extraction was under static conditions for 10 minutes and dynamic for 20 minutes for a total of 30 minute extraction time. The extracts were collected in 3 ml of MeOH at the flow outlet, evaporated to dryness under N_2 at 40 °C and derivatised with 50 µl of BSTFA at 70 °C for 20 minutes. Figure 2.4 shows the sample vial containing MeOH used for the collection of the eluted analytes. The vial was sealed using a butyl rubber septa with a disposable needle inserted into the top. The needle was used as a pressure release during depressurization of the aerosol containing the analytes into the vial. Unextracted standards containing all three drugs and the internal standard were prepared and used as references. Samples were run under cocaine parameters using GC/MS.

Figure 2.4 Collection vial used for SFE of cocaine and its metabolites



2.4.2.2 GC/MS Parameters

Analysis parameters for SFE were the same as set for SPE parameter for the analysis of cocaine and its metabolites discussed previously. The analysis parameters provided good results for this SFE methodology.

2.4.2.3 Results and Discussion

Out of all the samples extracted under these conditions, it was shown that only 40° C recovered cocaine and its metabolites but in low quantities using plain Extrelut®. However, 80° C did recover cocaine, but not the two metabolites. The temperature that was selected was 40° C and optimization to improve the method is discussed further.

2.4.3 EFFECTS OF SUPPORT MATRIX

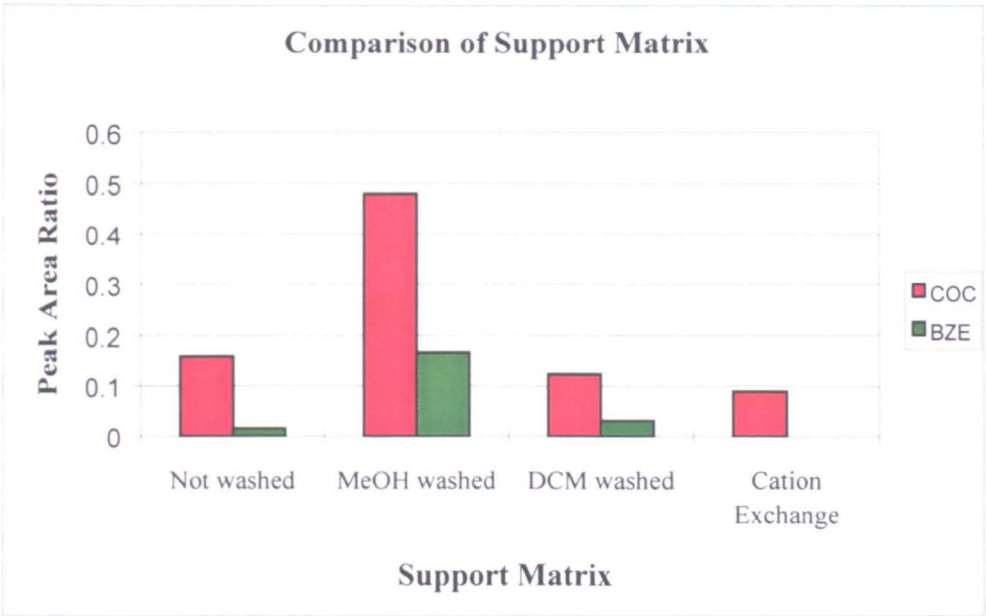
2.4.3.1 Experimental

A collection of four matrices, cation exchange silica packing, Extrelut® washed with DCM, MeOH washed, and Extrelut® without being washed in solvent were investigated. It was thought that by washing the Extrelut® in a polar solvent, might decrease any possibility of the drug binding to the sample matrix and increase elution of the drug of interest.

2.4.3.2 Results and Discussion

The four support matrices gave positive results for presence of the drugs at 40° C under parameters discussed earlier. However, out of the four, MeOH washed Extrelut® led to better recoveries of cocaine and benzoylecgonine more than the others. Shown in Figure 2.5 is a graph comparing the four support mediums determined by the peak area ratio. Extrelut® washed with MeOH, (a polar organic solvent), is thought to potentially enhance the extraction along with the modifier and makes it more sufficient.

Figure 2.5 Comparison of the four different sample matrices at 40 °C.



2.4.4 EFFECTS OF PRESSURE

2.4.4.1 Experimental

The pressure used in SFE operates in combination with temperature that controls the density of the SF. As the pressure and density increase, the solvating power of the fluid increases. Thus, a chemical change occurs from adjusting the pressure and or temperature and the solvating power also adjusts. Now that the temperature and extraction matrix were chosen, the pressure and flow rate had to be optimized. The pressure was increased to 3500 psi and the flow rate to 2 ml /min to see if this would make a difference in the quality of the extraction.

2.4.4.2 Results and Discussion

It was noted that by increasing the pressure at a constant temperature was more favorable for analytes that are more polar with higher analyte molecular weights. This with the inclusion of the modifier produced bigger peaks for all three drugs making them easier to quantify and elute the drugs at higher concentrations. The

making them easier to quantify and elute the drugs at higher concentrations. The flow rate was adjusted to 2 ml /min because the higher the flow rate, the better and quicker the analytes will be extracted from the matrix and less time is needed during extraction.

2.4.5 EFFECT OF THE MODIFIER

2.4.5.1 Experimental

The addition of a modifier incorporated directly to the sample enhances not only the polarity of the extraction, but increases the recovery of the drug of interest from the matrix while decreasing the extraction time. DCM: IPA: NH_3 was chosen in a 78:20:2 v/v ratio because of its polarity and its use in SPE as a potential eluent for cocaine and its metabolites.

2.4.5.2 Results and Discussion

This combination was chosen because of its characteristics and proved to be able to sufficiently elute cocaine, benzoylecgonine and ecognine methyl ester. Small amounts of concentrated ammonia was used because of its high polarity and strong solvating power to give the solvent combination added enhancement. Recoveries were estimated to be relatively high for cocaine and ecgonine methyl ester, however relatively low recoveries were found for benzoylecgonine.

2.4.6 EFFECTS OF DERIVATISATION TIME

2.4.6.1 Experimental

The effects of derivatisation time on cocaine, d_3- cocaine, benzoylecgonine and ecgonine methyl ester were investigated. Unextracted standards were prepared containing 50 µl of a 10 µg/ ml solution pipetted into a vial, evaporated to dryness and reconstituted with 50 µl of BSTFA (1%TMCS). Each standard was derivatised at various times from 20 minutes to 1 hour in intervals of 10 minutes at 70 °C.

2.4.6.2 Results and Discussion

The results of the effects of derivatisation times is shown in Table 2.1 with peak areas of cocaine (m/z 182, 303), d_3-cocaine (m/z 185), benzoylecgonine (m/z 240, 361) and ecgonine methylester (m/z 82, 182).

Table 2.1 Effects of derivatisation time

Time	m/z 185	m/z 182	m/z 303	m/z 240	m/z 361	m/z 82	m/z 182
20	**987642**	**2204723**	**1630202**	**224430704**	**99591216**	**5765876**	**723918**
30	287883	762050	287907	48802172	41070144	829800	46160
40	111123	314443	111154	4037605	6360242	361745	23710
50	92961	304923	108933	2414641	3550462	1134521	78350
60	203194	502818	196056	7612161	10698116	456558	20953

Derivatisation at 20 minutes proved to be the best time to derivatise cocaine and metabolites and therefore was the starting time for derivatisation to take place. From these results, 20 minutes is confirmed to be the best time for derivatisation and produced higher peak areas for each drug compared to the other times. By derivatising for 30 minutes was seen to still be acceptable, however more than this

time lead to a decrease in the peak areas for cocaine, d_3-cocaine, benzoylecgonine and ecgonine methyl ester

2.4.7 EXTRACTION OF DRUGS INDIVDUALLY

2.4.7.1 Experimental

Since the method was found to elute the drugs of interest under supercritical temperatures, before it could be fully optimized each drug was extracted individually under the same conditions discussed to rule out possible hydrolysis. 100 µl of each drug with 100 µl of internal standard was pipetted onto MeOH washed Extrelut® and dried. 300 µl of modifier was added to the extraction cell and the extraction started. Blanks samples containing modifier were also run before and after extraction just to eliminate the possibly of carry over.

2.4.7.2 Results and Discussion

Results showed that each drug was successfully extracted fine individually and there were no indications of cocaine hydrolyzing to form benzoylecgoine and ecgonine methyl ester. They were also individually extracted without the problem of hydrolysis. Overall there was no carry over detected in the system.

2.5 CONCLUSIONS FROM METHOD DEVELOPMENT

The final extraction conditions developed for cocaine, benzoylecgoine and ecgonine methyl ester are as follows:

- Flow Rate 2 ml /min
- Temperature 40 °C
- Pressure 3500 psi

- Support medium MeOH washed Extrelut®
- 100 % CO_2
- Run time 10 min static, 20 min dynamic
- Modifier 100 µl DCM: IPA: NH_3 (78:20:2 v/v)

Under these conditions, the SFE system was left to equilibrate while the sample was prepared. Approximately 0.13 g of MeOH washed Extrelut® was placed in a weighing boat with 100 µl aliquot of d_{-3} internal standard (1 µg /ml) and a 200 µl aliquot of sample blood or urine. The sample was left to dry at room temperature overnight or until a friable consistency was observed. The sample was loosely packed into the extraction cell with 100 µl of modifier, DCM: IPA: NH_3 and placed inside the oven The Rheodyne valve was switched from the equilibrate to the extract position with the valve closed for static extraction and opened for 20 minutes for dynamic extraction. This allows the flow of the SF to pass through the extraction cell. The analytes are depressurized at the flow outlet into a glass Hypovial™ containing 3 ml of MeOH.

2.6 METHOD VALIDATION

In order to investigate whether or not the optimized method was suitable for sample extraction, it was applied to blood and urine samples which were pipetted onto the MeOH washed Extrelut® matrix. The extraction was under optimized conditions with pressures of 3500 psi, flow rate 2 ml/ min, temperature 40 °C, 100 % CO_2 and 300 µl of modifier added to the extraction cell for 10 minutes static and 20 minutes dynamic.

2.6.1 RECOVERY

2.6.1.1 Experimental

To determine the percentage recovery of each drug, several spiked blood and urine samples were prepared without the internal standard $d\text{-}_3$ cocaine. The internal standard was added at the end of the extraction to the eluent as a reference ion to calculate how much of each drug was being recovered from the extraction.

2.6.1.2 Results and Discussion

Along with the addition of the internal standard after elution, an unextracted standard of the same concentration was analyzed and used to calculate the recovery. Recoveries for cocaine in blood were better than 70 %, benzoylecgonine 40 % and ecgonine methyl ester 85 %. Recoveries for cocaine in urine were better than 75 %, benzoylecgonine 49 % and ecgonine methyl ester 90 %. It was observed that out of the three drugs, benzoylecgonine produced very low recoveries for blood and urine.

Low recoveries of Benzoylecgonine may be due to poor analyte solubility and or a binding mechanism to the sample matrix that differs from cocaine and ecgonine methyl ester rendering the metabolite semi-extractable under these SFE conditions. Recoveries in urine were found to be a little higher than for blood. This could be explained partly because of the composition of the biological fluids and its affinity to adhere to the matrix. Urine is more lipophobic and is easier to extract than whole blood that forms a lipophilic bond with the proteins and drugs that can interfere with the extraction of the drug of interest.

2.6.2 REPRODUCIBILITY

2.6.2.1 Experimental

Several blood and urine blank samples were applied individually to several MeOH washed Extrelut® mediums. They were spiked with 100 µl (1 µg /ml) for each drug, cocaine, benzoylecgoine and ecgonine methyl ester and d_3-cocaine, which was pipetted onto the matrix, mixed and left to dry. Blanks were included during the run to observe any trace of carry over during the extractions. The extractions were carried out five times for blood and for urine at the selected parameters discussed earlier.

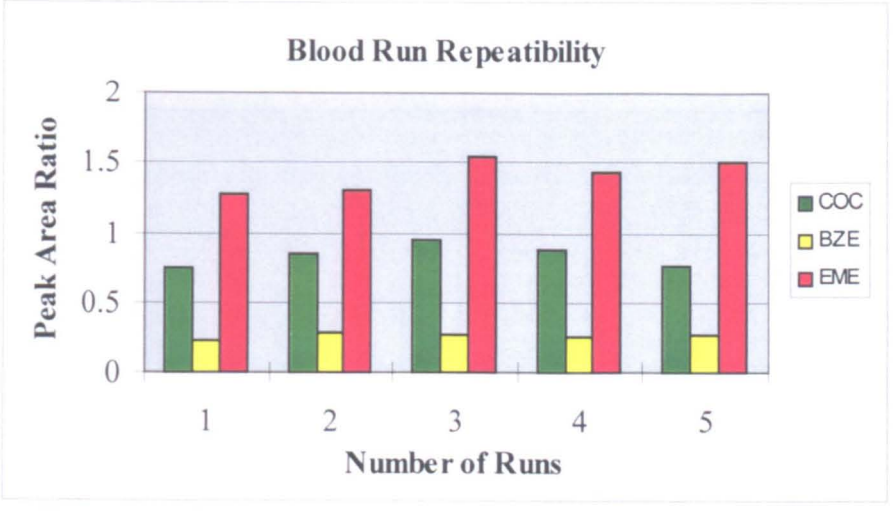
2.6.2.2 Results and Discussion

The following Figures 2.6 and 2.7 indicate the results obtained for the extraction of cocaine, benzoylecgonine, and ecgonine methyl ester at optimized conditions for urine and blood. As can be seen from these results, there was adequate run to run repeatability between each extraction (n = 5) indicating the accuracy of the method from run to run.

Table 2.2 Method repeatability of cocaine, benzoylecgonine and ecgonine methyl ester for blood

Blood	COC m/z 182/185	BZE m/z 240/185	EME m/z 82/185
1	0.746	0.22	1.28
2	0.849	0.278	1.30
3	0.949	0.27	1.55
4	0.876	0.251	1.43
5	0.769	0.267	1.51

Figure 2.6 Chart showing run to run repeatability of cocaine and its metabolites in blood.

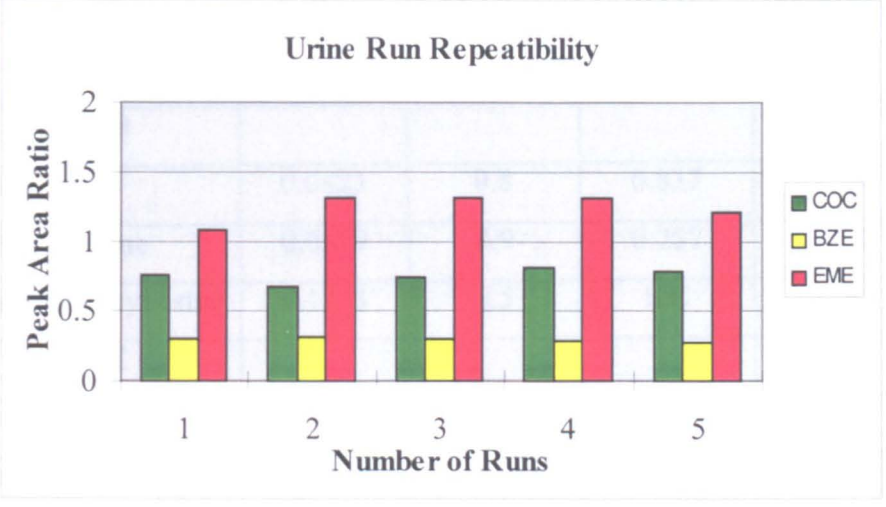


As shown in Figure 2.6 above, each spiked sample contained the same amount of drug concentration that was reproducible from run to run for each drug. The recovery varied for each drug with benzoylecgonine being the least recovered, however consistent within each run.

Table 2.3 Method repeatability of cocaine, benzoylecgonine and ecgonine methylester for urine.

Urine	COC m/z 182/185	BZE m/z 240/185	EME m/z 82/185
1	0.758	0.298	1.09
2	0.675	0.316	1.31
3	0.75	0.30	1.31
4	0.81	0.28	1.32
5	0.79	0.27	1.22

Figure 2.7 Chart showing run to run repeatability of cocaine and its metabolites in urine.



As demonstrated in Figures 2.6, Figure 2.7 also produced similar findings from run to run for urine samples with benzoylecgonine being the least recovered of the three drugs.

Table 2.4 summarises the method reproducibility of cocaine and its metabolites from both blood and urine. Overall, reproducibility from run to run for cocaine, benzoylecgonine and ecgonine methyl ester in blood and urine produced good standard deviations and relative standard deviations (% CV). Methods producing RSDs of less than 10 % are reported to be acceptable for method validation of biological specimens.[39]

Table 2.4 Method validation of cocaine, benzoylecgonine and ecgonine methyl ester in blood and urine (n = 5)

DRUG	StdDev	% RSD	PAR Mean	%Recovery
BLOOD				
Cocaine	0.0823	9.8	0.837	70
Benzoylecgonine	0.0229	8.9	0.257	40
Ecgonine methyl ester	0.1213	8.5	1.41	85
URINE				
Cocaine	0.0518	6.8	0.756	75
Benzoylecgonine	0.0180	6.1	0.292	49
Ecgonine methyl ester	0.098	7.8	1.25	90

2.6.3 LINEAR CALIBRATION

2.6.3.1 Experimental

Taken at various concentration ranges, aliquots from a 1 μg /ml solution standard of cocaine, benzoylecgonine, ecgonine methyl ester and $d\text{-}_3$ cocaine were pipetted onto a support matrix containing either 200 μl of blood or urine. For a calibration curve, the concentration range was from 5 ng to 100 ng /200 μl for both blood and urine. The sample was treated, extracted and derivatised using the developed method. The derivatised samples were analyzed by GC/MS.

2.6.3.2 Results and Discussion

Three separate calibration curves were created for each drug for blood and urine listed below in Figures 2.8-2.10. The results for the various concentrations cocaine,

benzoylecgonine and ecgonine methyl ester are shown in Tables 2.5 and 2.6 listed with the m/z ratios for each concentration for linear calibration.

Table 2.5 Linearity of cocaine, benzoylecgonine and ecgonine methyl ester in blood over a range from 5-100ng/200µl

Conc^n ng/µl	COC m/z 182/185	BZE m/z 240/185	EME m/z 82/185
5	0.008	0.001	0.76
10	0.032	0.006	0.97
20	0.07	0.02	1.21
40	0.19	0.03	1.52
60	0.27	0.44	1.83
80	0.42	0.068	1.97
100	0.58	0.10	2.21

Regression and slope were calculated for blood for all three drugs. Table 2.6 summarizes concentration range for the calibration curves and the limit of detection (LOD) and quantitation (LOQ) for each drug.

The LOD for cocaine, benzoylecgonine and ecgonine methyl ester was determined by using 200 µl of blank blood spiked from a 1µg/ ml solution. For the three drugs, the LOD was 1 ng with a LOQ of 10ng in 200 µl of blood. Figures 2.8-2.10 demonstrate the calibration curve for each of the three drugs along a concentration of 5 - 100ng as listed in Table 2.4 for blood.

Figure 2.8 Linearity of cocaine in blood

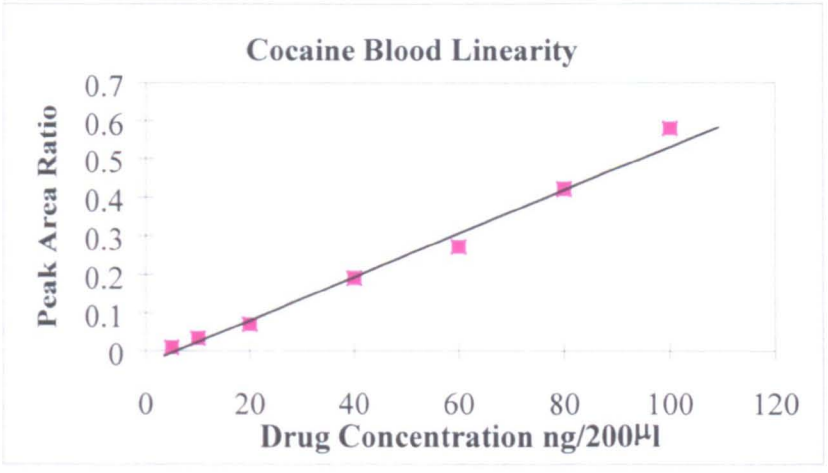


Figure 2.9 Linearity of benzoylecgonine in blood

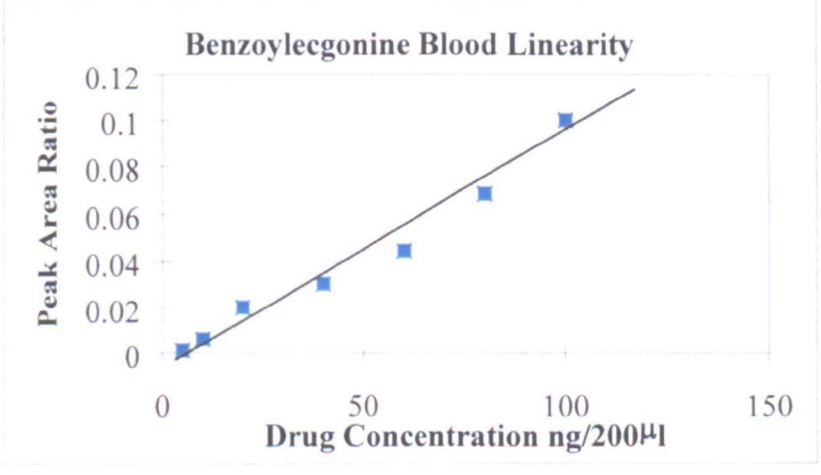


Figure 2.10 Linearity of ecgonine methyl ester in blood

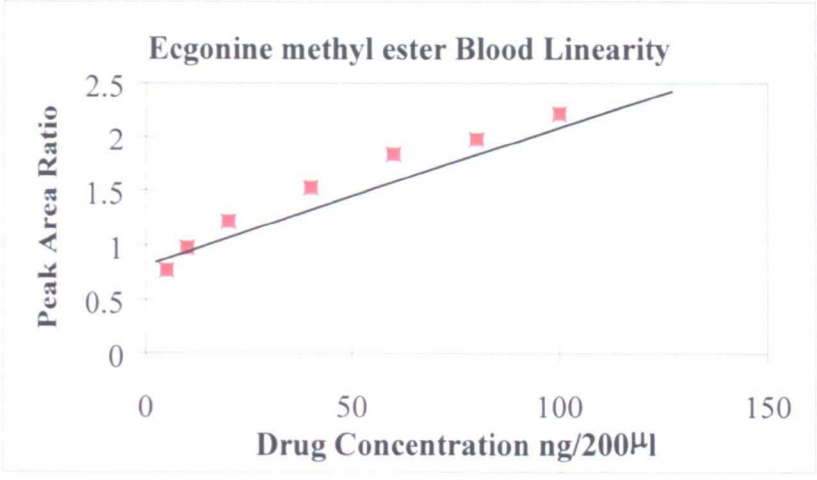


Table 2.6 Linearity of cocaine, benzoylecgonine and ecgonine methyl ester in urine over a range from 5 - 100ng/ 200µl

Conc^{n} ng	COC m/z 182/185	BZE m/z 240/185	EME m/z 82/185
5	0.0423	0.003	0.21
10	0.10	0.01	0.26
20	0.16	0.05	0.54
40	0.35	0.11	1.03
60	0.59	0.19	1.51
80	0.73	0.25	2.07
100	0.92	0.35	2.71

Figure 2.11 Linearity of cocaine in urine

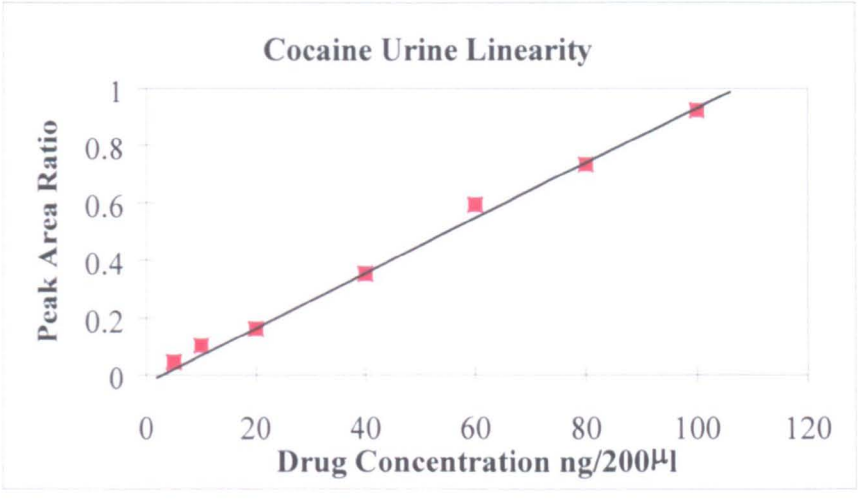


Figure 2.12 Linearity of Benzoylecgonine in urine

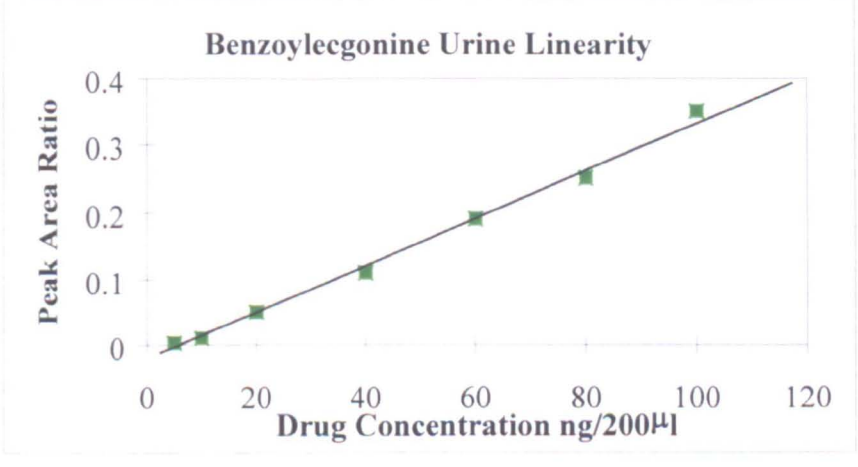


Figure 2.13 Linearity of ecgonine methyl ester in urine

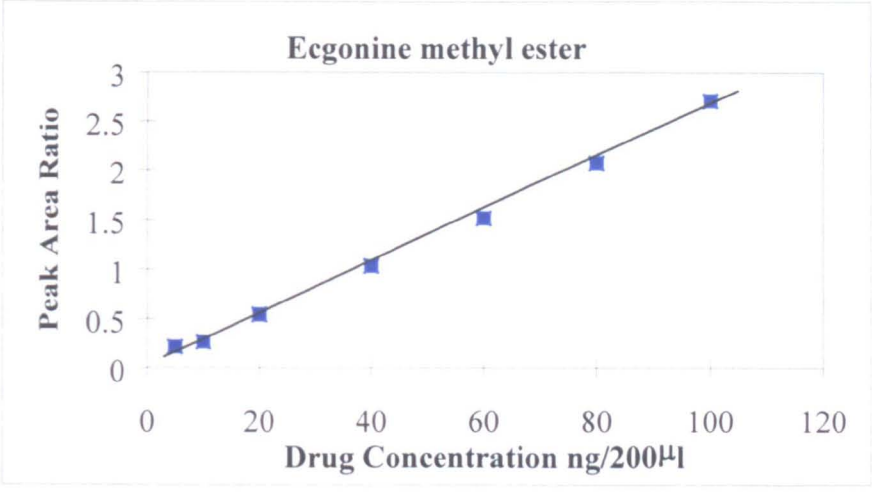


Table 2.7 summarizes concentration range for the calibration curves and the limit of detection (LOD) and quantitation (LOQ) for each drug for both blood and urine.

Table 2.7 Linear calibration and concentration range of cocaine, benzoylecgonine and ecgonine methyl ester.

DRUG	r^2	Slope	LOD/LOQ	Conc. Range
BLOOD			**ng / 200µl**	**ng / 200µl**
Cocaine	0.987	0.0058	1 ng / 10 ng	0 - 100
Benzoylecgonine	0.971	0.0009	1 ng / 10 ng	0 - 100
Ecgonine methyl ester	0.964	0.0145	1 ng / 10 ng	0 - 100
URINE				
Cocaine	0.997	0.0092	1 ng /10 ng	0 - 100
Benzoylecgonine	0.991	0.0034	1 ng / 10 ng	0 - 100
Ecgonine methyl ester	0.997	0.0262	1 ng / 10 ng	0 - 100

2.7 COMPARISON OF SFE AND SPE

2.7.1 Experimental

A comparison between SFE and SPE was made to investigate if the two methods correlate for the extraction of blood and urine for cocaine, benzoylecgonine and ecgonine methyl ester. Due to the lack of cocaine positive blood and urine forensic case specimens received into the laboratory, spiked samples were used for comparison between the two methods. From a stock solution of a 10 μg /ml, 50 μl of each drug was pipetted into three separate test tubes containing 1 ml of pretreated blood or urine for SPE and 200 μl of blood or urine for SFE. Samples were extracted and evaporated to dryness under a stream of nitrogen and derivatised.

2.7.2 Results and Discussion

Results from both methods for blood and urine are listed in Table 2.8 below.

Table 2.8 Comparison of SPE and SFE results of cocaine, benzoylecgonine and ecgonine methyl ester in urine and blood.

	Cocaine mg / L	Benzoylecgonine mg / L	Ecgonine methyl ester mg / L
SPE bld 1	0.207	0.16	0.103
SPE bld 2	0.21	0.11	0.084
SPE bld 3	0.18	0.17	0.093
SFE bld 1	0.25	0.13	0.17
SFE bld 2	0.20	0.11	0.11
SFE bld 3	0.26	0.12	0.12
SPE urn 1	0.19	0.10	0.08
SPE urn 2	0.17	0.07	0.081
SPE urn 3	0.20	0.08	0.09
SFE urn 1	0.29	0.14	0.27
SFE urn 2	0.23	0.13	0.26
SFE urn 3	0.22	0.13	0.24

The results show that a correlation existed between the two methods with small differences observed. The percent differences between the two methods for cocaine in blood were 0.04, 0.09, 0.08, for benzoylecgonine 0.03, 0, 0.05 and ecgonine methyl ester 0.07, 0.03, 0.03. For urine, the percent difference for cocaine was 0.10, 0.06, 0.02, benzoylecgonine 0.04, 0.06, 0.05 and ecgonine methyl ester 0.09, 0.07, and 0.06. The samples results for blood using SFE ranged from 0.084 – 0.21mg/l and SPE 0.11 – 0.26 mg/l. For SFE in urine the result range was 0.13 – 0.29mg/l and SPE 0.07 – 0.20mg/l. Figures 2.14 and 2.15 show graphs based on Table 2.6 for both methods for blood and urine, which clearly show the variation between the two methods.

Figure 2.14 Graph showing the comparison between the two methods for blood.

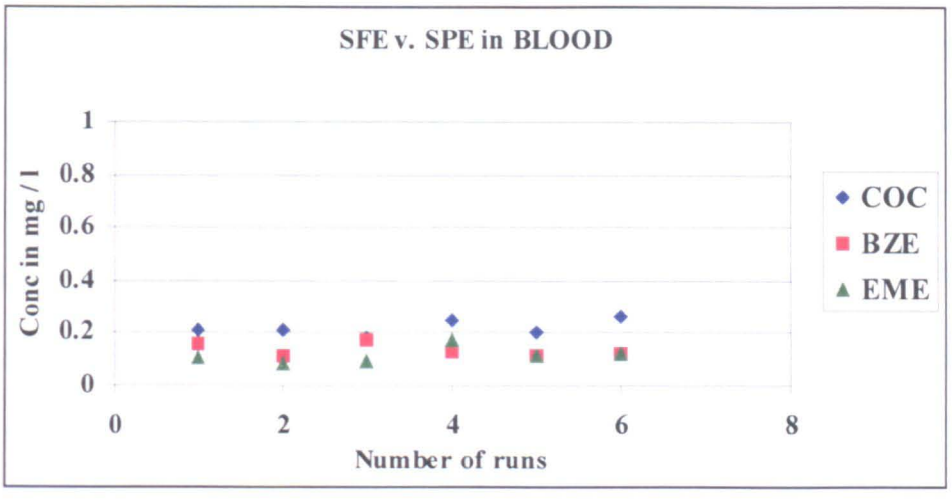
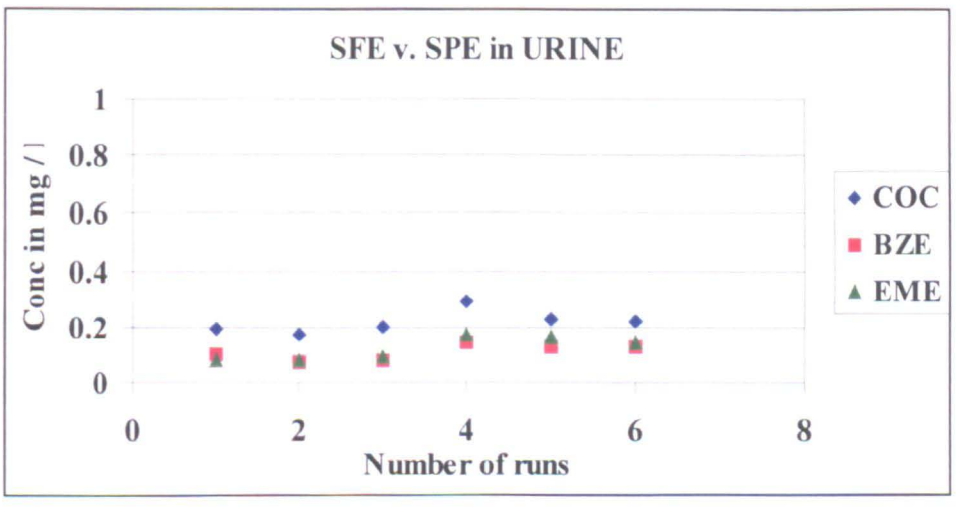


Figure 2.15 Graph showing the comparison between the two methods for urine.



2.8 CONCLUSION

At present, cocaine analysis from biological matrices involves a SPE method. The current study demonstrates that SFE can be used as a possible alternative to conventional methods for the isolation of drugs of abuse such as cocaine, benzoylecgonine and ecgonine methyl ester from blood and urine.

Various temperatures, pressures, flow rates and modifiers were investigated during method development. However, the described chosen conditions proved to give the best results for cocaine and its metabolites. Recoveries greater than 70% were reported for cocaine and greater than 85% for ecgonine methyl ester. However, benzoylecgonine recoveries were reported to be greater than 40%, which was lower than cocaine and ecgonine methyl ester. A possible explanation would be because the benzoylecgonine analyte is a zwitterion, a closer control of the extraction pH would have probably increased the yields. However since the developed procedures produced a reproducible extraction and was optimised to maximise the recovery of the major metabolite (benzoylecgonine), further development was deemed unnecessary.

SPE techniques were compared to the developed SFE techniques to find if a correlation existed between the two. A correlation was observed between SPE and SFE for the extraction of cocaine and its metabolites. Small percent differences were observed between the two methods, however both methods were comparable. This developed SFE method for the recovery of cocaine and its metabolites has demonstrated its potential usage for the extraction from spiked whole blood and urine.

3. OPIATES

3.1 LITERATURE REVIEW

3.1.1 INTRODUCTION

Opiates are comprised of as many as 25 different alkaloids, including pharmacologically active morphine, codeine and papaverine which are known abused drugs. In its pure form, weight for weight, heroin is several times more potent in its drug action as morphine.[91] For many years, purely synthetic narcotic analgesics have been sought for the use as pain-killers. Two compounds of this nature now generally used are pethidine (meperidine) and methadone. Although they lack some of the undesirable depressant effects of morphine, these potent analgesics still produce addictive dependency.

Heroin, morphine, opium, methadone and pethidine are all controlled under the Misuse of Drugs Act as Class A drugs. This makes them illegal to supply or possess without a prescription. Codeine and dihydrocodeine are Class B drugs, however prepared for injection they become Class A. Opium itself can be eaten or smoked and opiate powder can be dissolved in water and injected, sniffed or swallowed. Heroin is rarely eaten, as it is relatively ineffective this way. However it can be sniffed or smoked and the fumes inhaled, a term referred to as "chasing the dragon".

The desire to experience some altered state of consciousness is an intrinsic part of the human condition. The indulgence in something too much can be referred to as addictive or a drug. The same or similar drugs may be sold under various trade names for medical use, but still give the same effects. Morphine is just one of these

drugs that is commonly misused/ abused today and will be discussed further in this chapter.

3.1.2 HISTORY

The name opium is derived from the Greek name for the dried latex (juice) extracted from the unripe capsules of the opium poppy (Papaver somniferum).[92] The medicinal use of opium extends from the third century BC to the next three centuries with the cultivation of the opium poppy spread to the Mediterranean countries, Persia, India and China by Arab traders.

Clinically, opiates are used for the relief of pain with various potencies and pharmacological effects for the production of analgesia, sedation, diahorrea, respiratory depression and cough suppression.[92] Heroin (di-acetyl morphine) is produced by acetylation of morphine and in some areas of the UK, it is a serious problem where prescribed substitutes are not available. It was first isolated in 1874 at St Mary's Hospital London and then rediscovered in Germany in the 1890s.

After the First World War, an international agreement was made by Britain prohibiting the non-medical use of opium and opiates. By the early 1960s, heroin was on the rise being prescribed by some doctors for the relief of pain, which resulted in the spread of addiction. As a result, in 1968 the prescribing of heroin was prohibited which caused the beginning of an illicit black market for heroin production.

3.1.3 PHARMACOLOGY

Generally used as prescriptive pain-killers, opiates elicit their primary pharmacological effects on the central nervous, respiratory, cardiovascular and gastrointestinal systems. Several common central nervous system effects caused by opiates include euphoria, anesthesia, analgesia, increased tolerance to pain, stimulation of chemotrigger zone and production of miosis. The effects of opiates on the respiratory system include depression rate, volume and exchange of respiration and decreased responsiveness. Opiates also cause decreased motility and delayed gastric emptying in the gastrointestinal tract.[93] They are mainly abused for the production of their euphoric and pain relief effects.

3.1.4 PHARMACOKENETICS

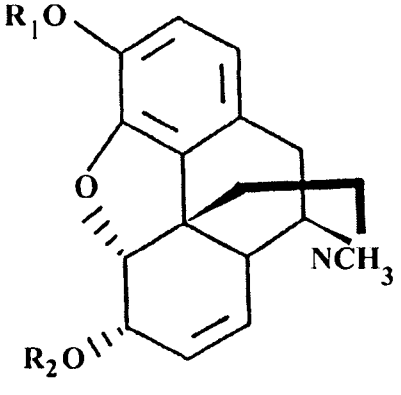
Morphine levels in the body fluids can be used as an indicator to detect therapeutic or harmful levels. Stead *et al* and Moffat reported therapeutic ranges between 0.1 to 1μg /ml.[94, 95] This indicates the tolerance range the body can develop to morphine over constant abuse requiring increased does for the same effect. The minimum lethal dose in man is approximately 200 mg, which can be exceeded by addicts due to tolerance.

The approximate half-lives of heroin and 6-MAM are 3 to 30 minutes respectively, while morphine has a half-life of 2-3 hours depending on the dose. Due to the rapid hydrolysis of diamorphine to 6-MAM, it is difficult to detect it in plasma.

3.1.5 CHEMICAL STUCTURE

Opiates are not naturally present in the body, but have structural similarities to a group of endogenous peptides called endorphins and enkephalins, discovered in 1978, that play an important role in the bodies own control of pain relief.[96] Figure 1shows the chemical structure of opiates. Only morphine will be discussed in this study, however the main related opiates have the same basic chemical structure differing only in the R_1 and R_2 groups as indicated in Figure 3.1. Diamorphine (R_1 $COCH_3$, R_2 $COCH_3$), 6-MAM (R_1 H, R_2 $COCH_3$) and Morphine (R_1 H, R_2 H)

Figure 3.1 The chemical structure of opiates[76]



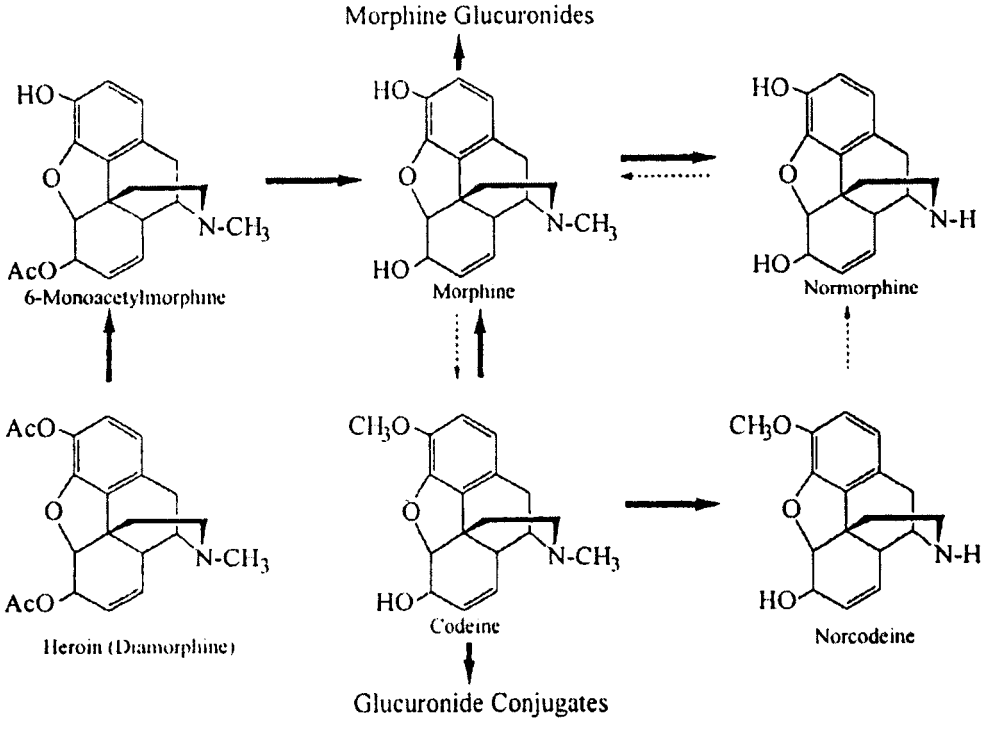
3.1.6 METABOLISM

Heroin is usually self-administered intravenously, by intra-muscular injection, snorted or smoked. Following the administration of heroin, it is rapidly metabolized by deacetylation into 6-MAM, that is then hydrolyzed into morphine. Morphine is then metabolized by conjugation to morhine-glucuronide and by N-demethylation to normorphine. Normorphine is further conjugated into normorphine-glucuronide. It

is also possible that codeine is produced as a metabolite of morphine (3-O-methylation).[97]

Primarily, morphine and conjugated morphine are metabolites of heroin commonly found in urine. However, morphine presence alone is not a reliable indicator of heroin use and 6-MAM is commonly used as a specific marker. The most common analgesic for the relief of severe pain is morphine. It is readily absorbed from the gastrointestinal tract, with high first-pass elimination on passage through the liver. Figure 3.2 shows the major metabolic pathway of diacetylmorphine.

Figure 3.2 The major metabolic pathway of diacetylmorphine. [76]



3.1.7 CHROMATOGRAPHIC ANALYSIS

There are various analytical methods for the analysis of morphine from biological fluids. The preferred tissue of choice has been plasma where morphine is present in

its unconjugated form and it is likely that interference will be minimal with the isolation of that drug. However, in post-mortem cases plasma is seldom available and the tissue most commonly supplied is haemolysed, often putrefied blood. Therefore, a suitable method should be applied capable of detecting morphine concentrations down to ng/ml levels. Typically, a 1ml sample volume is used incorporated with a pre-treatment or clean-up step to insure adequate chromatographic analysis.

Radioimmunoassay (RIA) has been used as a screening procedure before analysis, however for confirmation the most common analytical methods used are liquid-liquid extraction (LLE)[98-101], solid-phase extraction (SPE)[102-105] and more recently supercritical fluid extraction (SFE).[106,107] For chromatographic analysis gas chromatography - mass spectrometry (GC/MS) has been the preferred choice for opiates due to its sensitivity and selectivity.

3.2 SPE AND SFE COMPARISON OF MORPHINE

The principle of solid-phase extraction is well known and commonly used for the isolation of analytes from liquid matrices. Although SPE is easy and popular in terms of extraction techniques, supercritical fluid extraction (SFE), a relatively new sample preparation technique is becoming of increasing interest. Several authors have reported the super or sub critical analysis of opiates using various techniques.[106] This has lead to further research, applying SFE to biological fluids for the extraction of drugs of abuse.[107] A comparative study for the quantitative determination of morphine in whole blood using SPE and SFE has been investigated and discussed in

this chapter. This was to determine whether the two methods have any correlation when applied to authentic forensic case samples.

3. 2.1 CURRENT METHODOLGY

The current SPE methodology for morphine used in the routine laboratory is World Wide Monitoring, however, at the time of this study, the current methodology used is described below. The results obtained from this method were compared to the results obtained using SFE techniques to check that the same results were achieved for both methods for the extraction of blood.

3.2.1.1 SPE of Morphine

SPE columns were prepared by packing methanol: ethanol (1:1 v/v) washed Extrelut® into 10 ml syringes plugged with cotton glass wool. Samples were pre-treated in vials with 1 ml of 0.1M ammonium hydroxide solution and 50 µl of internal standard (d_3-morphine). The contents of each vial were pipetted onto corresponding Extrelut® columns, then conditioned with 5 ml of hexane and allowed to stand for 10 minutes. Labeled SCX columns (1cc) were placed on a VacElut and conditioned with 2 ml of methanol, 2 ml of de-ionized water and 1ml of ethyl acetate : isopropanol (9:1 v/v).

The Extrelut® columns were attached to the top of the SCX columns via adapters. The analytes of interest were transferred from the Extrelut® columns to the SCX columns using three 4 ml aliquots of ethyl acetate:isopropanol (9:1 v/v). The Extrelut® columns were discarded. The SCX columns were then washed with 5 ml

of acetonitrile: methanol (1:1 v/v) and the analytes of interest eluted using 2 ml of 10 % ammonia in acetonitrile: methanol (1:1v/v). The collected eluent was evaporated to dryness under nitrogen at 40 °C and derivatised with 50 µl of BSTFA (1% TMCS) at 90 °C for 10 minutes.

3.2.1.2 SFE of Morphine[108]

SFE pressure conditions were set at 3500 psi with the flow rate at set 2ml/min and temperature 100°C. A 10 % modifier of MeOH: Et_3N 85:15 was used with 90 % CO_2 for an extraction time of 30 minutes. The dried sample was derivatised using 50 µl of BSTFA and analyzed using GC/MS.

The samples were prepared by loading 100 µl of internal standard (d_3-morphine) onto the extraction media Extrelut® that was then mixed and dried. The Rheodyne is set in the load position and the instrument is left to equilibrate. The sample is then loosely packed into a stainless steel extraction cell that was placed over the loop position of the Rheodyne. The extraction is started by switching the Rheodyne from the load to the inject position and left for 30 minutes to elute the analytes though the outlet, which are collected by expansion in MeOH. The sample was then evaporated under nitrogen at 40 °C and derivatised with 50 µl of BSTFA at 90 °C for 10 minutes.[108, 109]

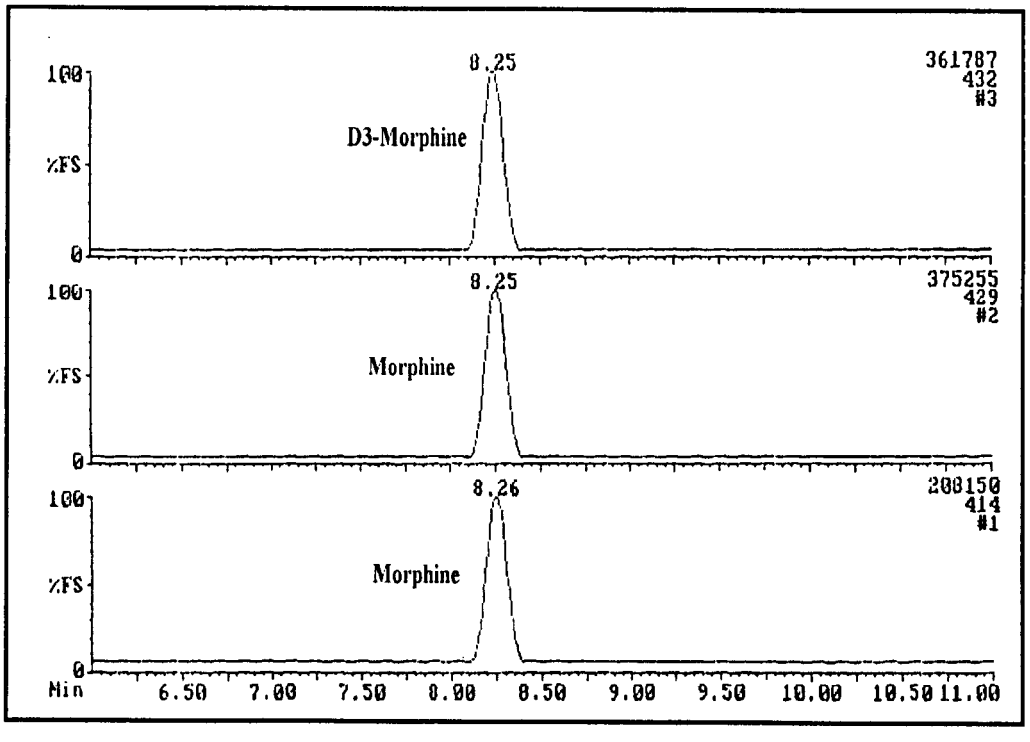
3.2.1.3 GC-MS Parameters

A Fisons model GC 8000 Series (MD 800 in the EI Mode (70 ev) gas chromatography-mass spectrometry was used for analysis, this was equipped with a

HP-1 (Cross linked Methyl Siloxane), capillary column, (30m x 0.53μm i.d, film thickness 0.88μm).

The temperature was set at an initial 200 °C for 1 min and a final 300 °C for 5 min with an oven rise of 10 °C /min. The injector temperature was 250°C. Figure 3.3 shows a GC/MS trace of morphine produced using analysis by GC/MS. The internal standard d_3-morphine m/z 432 ion, was used as a reference to calculate the concentration of morphine and ions m/z 414 and 429 for each sample.

Figure 3.3 Chromatograph of unextracted morphine standard



3.2.1.4 Results and discussion

Authentic forensic blood samples known to be morphine positives were chosen for this investigation. SPE is an efficient and reproducible extraction method that was compared to SFE to determine if the two yield corresponding results for blood.

Fourteen blood samples were extracted using both SPE and SFE with final analysis by GC-MS. For the extraction of morphine using SPE, the sample concentration of morphine ranged from 0.056 to 2.42 mg/ml in blood, compared to SFE results which ranged from 0.050 to 2.20 mg /ml in blood, shown in Table 3.1. The differences are also listed in Table 3.1. Small differences were shown between the two methods with a mean of 0.065. In addition, recoveries for SFE were found to be > 90% for morphine compared to that of SPE which was > 85%.

Table 3.1 Comparison between SPE and SFE of morphine from authentic blood case samples

Case #	SPE mg/l	SFE mg/l	Difference
T96-A	1.25	1.18	0.07
T96-B	0.59	0.67	0.08
T96-C	1.02	0.91	0.11
T96-D	**0.90**	**0.85**	**0.05**
T96-E	1.86	1.97	0.11
T96-F	2.42	2.20	0.22
T96-G	0.19	0.17	0.02
T96-H	0.35	0.39	0.04
T96-I	0.86	0.79	0.07
T97-J	0.45	0.45	0
T97-K	0.056	0.05	0.006
T97-L	0.85	0.74	0.11
T97-M	0.28	0.28	0
T97-N	0.27	0.29	0.02

Below in Figures 3.4 and 3.5 are traces showing the comparison between the same authentic blood samples (T98-D) run using SPE and SFE techniques. Both

techniques proved to be extractable for morphine in whole blood, with similar retention times and a percent difference of 5.9 %.

Figure 3.4 Chromatograph of an authentic case sample extracted by SPE

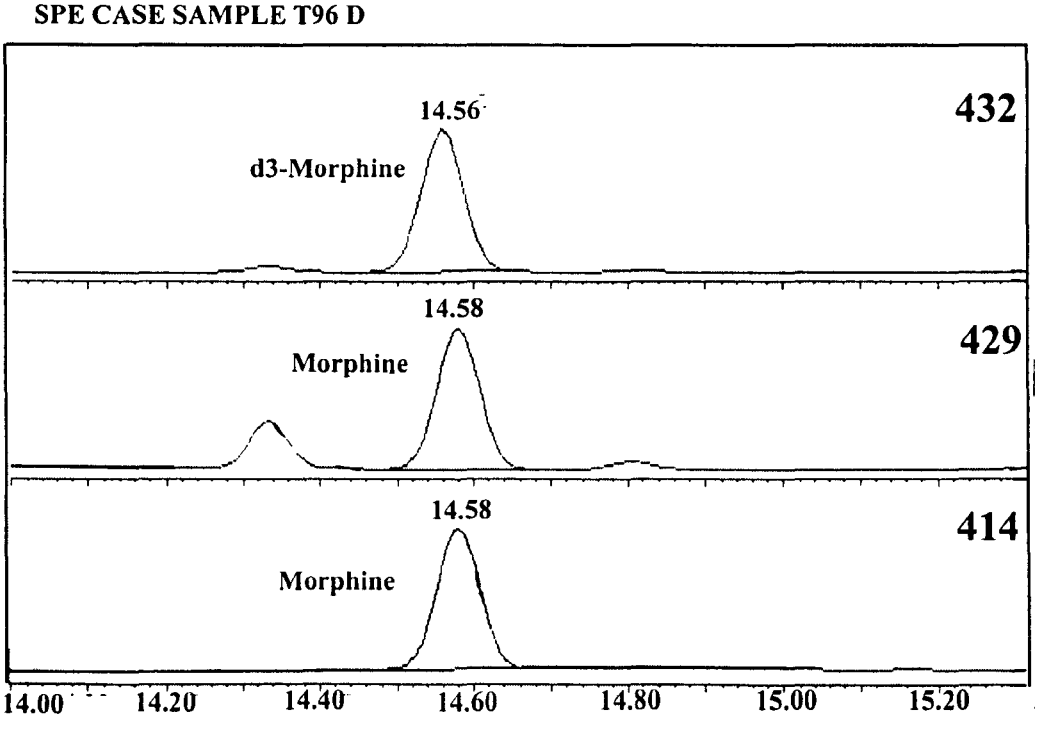
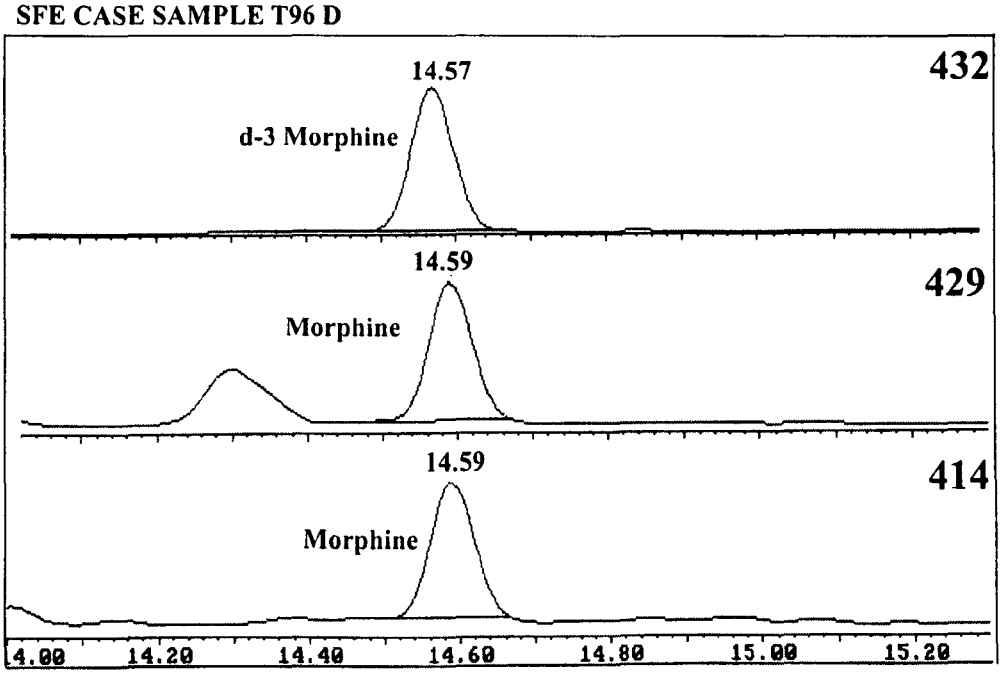
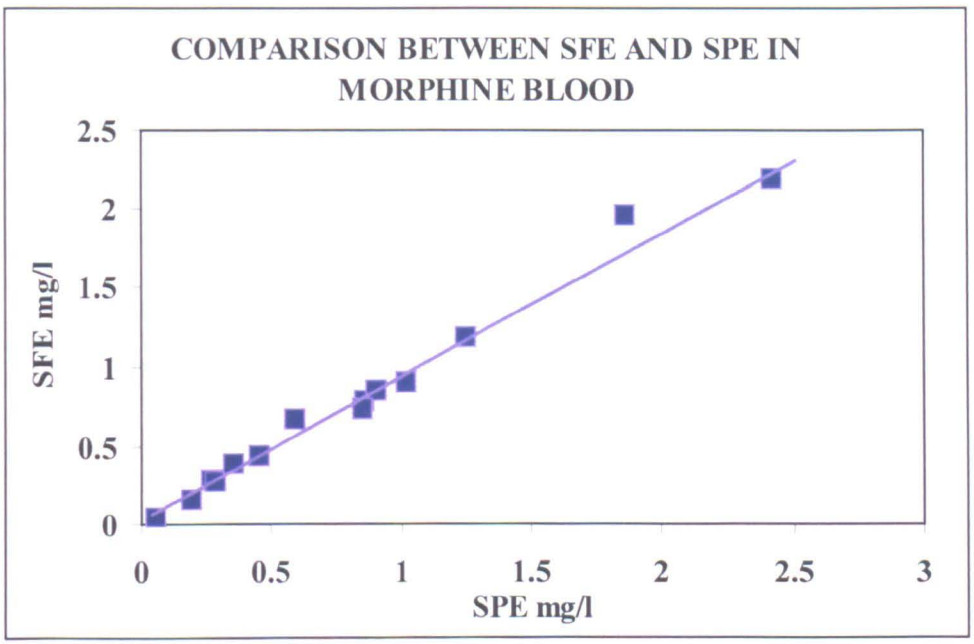


Figure 3.5 Chromatograph of an authentic case sample extracted by SFE



The standard deviation and mean for comparison of both methods was 0.633 and 0.782 with a linear relation ship of $r^2 = 0.986$ and a gradient of m = 0.94 seen in Figure 3.6. This shows that good correlation for morphine extracted from whole blood exists between the SPE and SFE methods. In a perfect world a straight line would be equal to 1.

Figure 3.6 Linear relationship between SPE and SFE for 14 authentic case samples.



3.2.2 CONCLUSION

Solid-phase extraction techniques have been the preferred technique for the extraction of drugs of abuse from various matrices. Although widely used as a sample preparation method, the technology is still advancing and exploiting more parameters to improve sample preparation, including SFE techniques.

SFE, a relatively new technique for sample preparation has until recently generally been confined to chemical processing applications. Now, it is emerging as a valuable analytical procedure for the forensic toxicologist.

The investigations carried out using SPE and SFE methods, show that the two analytical techniques correlate well for the analysis of morphine in whole blood. Both techniques are fast, yield reproducible results and give good recoveries for morphine extracted from whole blood.

4. SOLID-PHASE EXTRACTION

4.1 LITERATURE REVIEW

4.1.1 INTRODUCTION

In the last few years, the use of solid-phase extraction (SPE) as a tool for the isolation of drugs from biological matrices has become more acceptable in both clinical and forensic laboratories for routine analysis.[110-111] In analytical toxicology, the extraction or isolation of a compound of interest from biological specimens, is the most important process for the determination of a sample. A complete analytical process from sample collection to the final analysis of the results, has been developed for SPE.

The steps necessary for SPE include, sample collection and storage, sample preparation, the isolation and identification of the analyte and quantification. All of these will be discussed later on in this chapter. The principle objectives of sample preparation for chromatographic analysis are dissolution of the analytes into a suitable solvent and removal of as many interfering compounds as possible.[112]

With the use of a selection of commercially available sorbent columns, SPE can be suitably adapted to extract analytes from various samples (e.g. blood, urine, saliva, vitreous humor and more recently hair) with high percentage recovery and purity. Selective adsorption, adjustment of pH and partitioning of both the sample and the sorbent are very important during the extraction. The design of the extraction procedure is meant to selectively partition and exploit the analytes physical properties from interfering components in the sample.

As a result of the efficiency and less time consuming characteristics of SPE techniques, it is now the preferred technique over conventional extraction techniques such as liquid-liquid exaction (LLE) and has revolutionized sample preparation for the analysis of a wide variety of biological and non-biological samples.

4.1.2 HISTORY

The definition of SPE is "The separation or removal of an analyte or analytes from a mixture of compounds by selective partitioning of the compounds between a solid phase (sorbent) and a liquid phase (solvent)". This extraction technique has been used for the extraction of drugs in biological fluids and more recently hair samples in forensic laboratories. Due to its ability to provide clean, efficient extracts in a single step, it has recently gained popularity for many clinical and biochemical applications.

Whitehorn, in 1923 was recognized as one of the first researches to use solid-phase techniques to isolate drugs from biological samples.[113] The success of the technique continued in the 1950s with researchers like Lund[114] and his colleagues, who used alumina columns to extract adrenaline and noradrenaline from blood and Bergstron and Hanson[115], who used amberlite IRC-50 columns, to extract adrenaline, histamine and catechol bases from crude extracts of glands.

Research continued to improve extraction techniques with progress developing by the 1960s, with the introduction of cation exchange paper chromatography. Dole *et al*, used this qualitative technique to detect numerous tranquilizers, narcotics, amphetamines and barbiturates from urine samples.[116]

By the 1970s, the need for increased sensitivity made it necessary to develop more efficient techniques. The ion exchange principle was introduced to separate relevant drugs from biological samples using various columns. Fujimoto and Wang used amberlit XAD 2 resin columns to quantify narcotic analgesics at concentrations of 0.6 mg/ml^{-1}.[117] Adsorption techniques with average detection limits of 1 mg/ml^{-1} using charcoal columns were used by Meola and Vanko, to concentrate numerous drugs (e.g. quinine, barbiturates, morphine and cocaine and its metabolites) from urine samples.[118]

With the progress of HPLC technology, came the development of silica and bonded silica as the stationary phase by the late 1970s. This development lead to the concept first developed by Water Associates and Analytical Chemistry International, of using sorbents packed in miniature columns. These sorbent columns were capable of isolating drugs or chemicals of interest from interfering impurities in samples during extraction.

With the introduction of silica, bonded silica and other phases, small disposable cartridges termed SPE columns were commercially developed. They produced high recovery and purity of the analyte and reduced time, labour and solvent usage during extraction.

4.1.3 LLE AND SPE TECHNIQUES

The principles of SPE are very similar to LLE, in that they both involve a partitioning of compounds between two phases. Before the use of SPE was developed, LLE was without a doubt, one of the most frequently employed extraction

techniques. In LLE, the analytes to be extracted are partitioned between two immiscible liquids, whereas in SPE the analytes extracted are partitioned between a solid and a liquid.

Although widely employed, LLE techniques have a number of potential disadvantages which SPE techniques avoid or minimize.[119-120] These disadvantages of LLE include, the formation of emulsions, serial processing of samples, the need for ultra-pure solvents, the use of large volumes of toxic organic solvents, time and labour required for extraction, and the relatively large sample volumes required for extraction (e.g. a minimum of 0.5-1ml).

With the introduction of SPE methods, came the advantages for sample preparation and analyte recovery which were preferred over LLE techniques. Some advantages of SPE include, high recovery and purity of the analytes improving analysis precision, reduction of extraction time and labour, especially if automated methods are used, small sample volumes (e.g. 50-100 μl), small volumes of less pure solvent and the lack of emulsion formation that occurs in LLE.

4.1.3.1 Recovery

The comparison of SPE techniques with LLE techniques has been reported in literature showing a greater recovery range (from 80 % to 100 %) for SPE techniques.[121-122] Using both techniques, recovery studies on the extraction of the compound lidocaine, cocaine, benzolecgonine and norcocaine were researched by Sandberg.[123] For SPE procedures, the percent recoveries were lidocaine 92 %, cocaine 86 %, benzoylecogonine 91 % and norcocaine 85 %. In contrast, using LLE

procedures for the same drugs, the percentage recoveries were lidocaine 59 %, cocaine 57 %, benzolecogonine 66 % and norcocaine 21 %.[123]

In another comparative study, Okazaki *et al* reported recoveries of 39 % for Oflaxacin, 54.7 % for its dimethyl and < 5 % for its N-oxide metabolites using LLE methods respectively, as compared with 98 % recoveries using SPE C_8 columns for all three analyte types.[124] Numerous studies reported on the comparision of the two techniques have preferred SPE to LLE techniques.[125-126]

4.1.3.2 Sample Size

Successfully quantifying smaller sample sizes depends on the sensitivity of the procedure and high recovery of the analyte. The usage of various SPE columns, increases the sensitivity and decreases the amount of sample needed for extraction. SPE techniques are used in many applications because of its small sample size requirements.

The extraction of minute quantities of nicotine from human samples was reported by Sioufi *et al* as having achieved detection sensitivities of 1ng and 20 ng/ml^{-1} in plasma and urine, after the treatment with transdermal patches of nicotine.[127] With the use of SPE C_{18} columns to pre-concentrate mycotoxin form river waste, Hoke *et al* increased the sensitivity of the artemia bioassay by a factor >5.[128] Using SPE C_8 columns, Huang *et al* successfully quantified etintidine using plasma samples as small as 0.2 ml.[129]

Conventional methods made it unsuitable for neonates and infant clinical studies to be investigated because of the large sample amounts needed. Since SPE developments, studies such as pharmacokenetics of bumetamide by Wells *et al* using urine and plasma from human neonates has been reported.[130] By using SPE techniques in such studies, samples as small as 0.2 ml were used proving adequate.

4.1.3.3 Time

Employing rapid and less laborious methods is just another advantage that SPE techniques have over conventional LLE techniques. Extraction manifolds handling up to 24 samples simultaneously, which are commercially available, take less time for sample preparations than traditional procedures. The possibility of treating samples containing volatile analytes such as chlormethiazole[121] and 3-methylindole[131], are much more likely to be used because of these rapid procedures available.

In addition to reduction of time and labor, the use of organic solvents for elution are reduced with only a few milliliters needed for the extraction procedure to take place. This reduction minimizes solvent costs, waste-disposal and sample handling, reduces potential fire hazards which promotes safer laboratory conditions and produces an environmentally friendly environment.

Safer laboratory procedures using SPE methods have been applied and reported by Kupferschmidt *et al.*[132] They adapted a method to extract 3'-azido-3'-deoxythymidine from the blood of AIDs patients, which minimized sample handling and promoted a hazard free working environment.

4.1.4 OBJECTIVES OF SPE

There have been several objectives quoted on SPE techniques that are important for the extraction process to be successful.[112]

1. The use of appropriate solvents in the washing steps to remove all interfering compounds.

2. The principle objective in trace analysis starts with the pre-treatment of the sample for clean high recovery and purity of the analyte prior to the extraction process.

3. The ability to fractionate the sample into different compounds or groups of compounds using different liquid phases for elution.

4. Being able to store relatively highly volatile analytes that are unstable in liquid mediums.[112]

5. Derivitisation of the analyte(s) on the adsorbent surface and between relative groups, which can be directly eluted or retained and eluted later.

4.1.5 SPE COLUMNS

The principle of SPE is well known for its applications for the removal of compounds from body fluids and can fall into two categories, adsorption and absorption. Adsorption extraction relies on the reversible binding of the compound of interest to a soild-phase material. Absorption extraction relies on the absorption of one phase (e.g. the aqueous phase) onto an absorbent support usually diatomaceous earth (e.g. Extrelut®) and is designed to mimic liquid-liquid extraction techniques. Both extraction techniques are discussed further in this chapter.

4.1..5.1 Silica and Bonded Silica Columns

Silica or bonded silica gels, are the most popular SPE materials. Numerous functional groups with or without alkyl/chains that can be bonded to silica include groups such as -OH, $-C_6H_6$, NH_2, -CN, $-SO_3H$ and -COOH. As a result of the wide range of functional groups for SPE materials, the extraction can be non-polar, ion-exchange or polar, based on the interaction mechanism. Some commercially bonded silica SPE columns are noted to have all the functional groups, which cause multiple and secondary interactions during extraction.[133]

Residual silanol groups in SPE C_{18} sorbent material have been reported to cause secondary interactions in the analytes.[134] It has been reported that it is more advantageous in SPE applications, to make use of these groups rather than avoid or mask them. Utilization of residual silanol groups has been reported in the extraction of basic drugs from plasma.[135]

SPE columns are especially important for clean, efficient extracts, which are important in drug screening for the forensic toxicologist. They are generally disposable, polypropylene, cylindrical or conical shaped columns, with a male luer hub end used to fit into an extraction device (vacuum manifold). These SPE columns can hold up to 15 ml of sample and if necessary, can be adapted to have an attachment added at the top for increased volume. A solid sorbent approximately 40 μm thick is sandwiched between two polyethylene discs located just above the luer end.[136]

Solvation of a sorbent is necessary before the sorbet will interact reproducibly with isolates. Solvation is a wetting process of the sorbent bed with a solvent such as MeOH, creating a suitable environment for isolate retention. Once solvated, the excess solvent is removed by an additional solvent that prepares the sorbent to receive the sample. However, small amounts of the first solvent will remain associated with the sorbent. When the column is solvated, excess drying should be avoided, specifically before the sample application. Complete drying of the column bed requires usually at least 30 seconds of continuous airflow. Once the analytes are retained on the sorbent bed, drying is usually not a problem and sometimes is recommended between solvent steps.

The main columns used for SPE are the non polar, polar and ion exchange columns. The drugs of interest interact chemically based on the polarity and ion exchange properties within the column. The interactions are activated by pH adjustments from various solvents or buffers.

4.1.5.2 NonPolar or Reversed Phase

The principle of the nonpolar phase used in SPE is based the exploitation of hydrophobic interactions to extract non-polar compounds from a polar sample matrix, retaining them on the column sorbent for elution. Most matrices used are nonpolar, therefore, using nonpolar sorbents tends to be less selective during the extraction and alternative optimisation is necessary to elute specific compounds. Adjusting the pH of the sorbent and the sample are important for hydrophobic interactions between the functional groups of the analyte and the sorbent to take place.

Optimal elution conditions for nonpolar compounds will result in high solvent solubility and low solvent polarity of the eluted compounds. This means that because nonpolar interactions between nonpolar sorbents and nonpolar analytes are facilitated by polar solvents, the polar environment are generally not capable of disrupting the dispersion forces of the nonpolar interactions. However, nonpolar interactions between the analytes and the sorbent are best disrupted by solvents having nonpolar characteristics (e.g. MeOH, chloroform, hexane). Even though MeOH is polar, it has enough nonpolar character to disrupt the nonpolar interaction between the analyte and the sorbent causing elution of the analyte.

4.1..5.3 Polar or Normal Phase

In polar phase SPE hydrogen bonding or polar and hydrogen bonding of the polar compounds from nonpolar matrices, allow retention on the sorbent columns. Hydrogen bonding is one of the more significant polar interacts, were one hydrogen group bonds to an electo-negative atom such as oxygen or nitrogen and so on. High solubility and high solvent polarity give superior extraction and elution of the analyte.

Nonpolar solvents are used to condition the column prior to application of the sample and then used to elute interfering compounds from the sorbent bed. This is because strong electronic interaction between the sorbent and the analyte can not be easily disrupted by nonpolar solvents. Conversely, polar interactions are disrupted more by polar solvents because polar analytes are more soluble in polar solvents and they compete more effectively with the analytes for the sorbent than nonpolar sovlents.

At the end of the extraction, polar solvents are then used to elute the retained compounds of interest.

4.1.5.4 Ion Exchange (Anion or Cation Exchange)

The ionic character of the compound is based on its affinity for the ion exchange column. The ionic charge on the compound of interest should be opposite to the functional groups on the column for retention of the analyte. Low counter ion concentrations in a sample give superior extraction and elution of the analyte. The ion exchange columns can be cation or anion exchangers. Cation exchange columns attract analytes which have positive charged functional groups and anion exchange columns interact with the negatively charged analytes.

For retention to occur, the solvent or matrix must be at a pH where both the analyte and the sorbent are charged. Therefore, the pH is below the pk_a of the cation by two units and above the pKa of the anion by two units. At this pH approximately 99 % of the groups will be charged.

Ionic strength is important in ion exchange. It is a measure of the total ionic species concentration present in the solvent or matrix environment. Ion exchange is a competitive mechanism that cause the retention of analytes in the solvent or matrix that are capable of competing for available ionic groups on the sorbent. Low ionic strength promotes the retention of the analytes and high ionic strength disrupts it facilitating elution.

Table 4.1 The various phases present for SPE columns.

Adsorption Phase	silica gel (SiOH), florisil (MgSiO) alumina (Al_2O_3), Kleselguhr (SiO)
Normal Phase	amino (NH_2), cyano (CN), diol (COHCOH), silica (SiOH)
Reverse Phase	methyl (C_1), ethyl (C_2), hexyl (C_6), octyl (C_8) , octadecyl (C_{18}), cyano (CN), cyclohexyl (C_6H_{11}), phenyl (C_6H_5)
Anion Exchange	quaternary amine (N^+), amino (NH_2), diamino (NH_2NH)
Cation Exchange	aromatic sulphonic ($C_6H_5SO_3H$), carboxylic acid (COOH)

4.1.5.5 Diatomaceous Earth

Another type of substance which acts as a support for the absorption of an aqueous sample is called diatomaceous earth or Extrelut®. This Extrelut® is a porous material which is washed in solvent(s) such as MeOH or EtOH to clean the diatomaceous earth of contaminants before application of the sample.

Diatomaceous earth material used in absorption extraction, can provide clean and efficient extracts of sufficient quality for analysis by GC/MS or HPLC without an abundance of interfering compounds which are present in some biological matrices. Anderson and Fuller described good results and high recovery of the analyte of interest in post-mortem blood.[137] They used diatomaceous earth for the extraction of acid and neutral drugs prior to GC analysis.

There is not much literature on the use of diatomaceous earth. However, some applications using diatomaceous earth to elute sample compounds, have reported good results, but the use of prepacked SPE columns still proves to be the most widely used extraction method for elution of sample compounds. This is because of

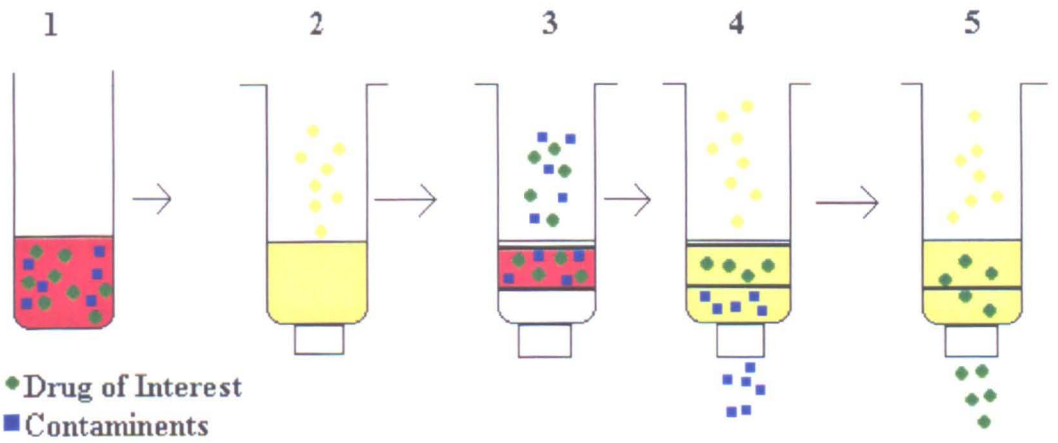
the numerous column phases that are commercially available and the ability to use various biological matrices on such columns.

4.1.6 PRINCIPLE OF SPE

The governing principle of SPE is to concentrate and isolate from a sample matrix, the analytes of interest. It is a physical extraction process involving a solid phase and a liquid phase.[138]

There are five principle steps and a derivatisation step required for SPE use, demonstrated in Figure 4.1: sample pre-treatment, column preconditioning, sample application, column wash and drying and elution of analytes.

Figure 4.1 Five steps for the elution of a sample using SPE columns



4.1.6.1 Sample Pretreatment

The first step in toxicological analysis is the pre-treatment of the sample prior to the extraction process. Depending on the type of sample used, biological or environmental, the sample pre-treatment will vary. The sample under goes a pre-

treatment application normally with a buffer with adjusted pH and internal standard used as a marker for detection of the ions during analysis.

The purpose of the sample pre-treatment step is to release the drugs from the matrix, remove interfering proteins and other endogenous irrelevant materials (e.g. salts) which may affect the analysis and extraction by adjusting the pH, ionic strength and concentration of the sample compound(s). Protein precipitation with organic solvents, dilution with buffers, water or sonication, may be carried out on the sample for extraction preparation.

4.1.6.1.1 Adjustment of pH and Ionic Strength

For optimal conditions, the adjustment of pH and ionic strength plays an import role in SPE and conventional techniques resulting in major difference in the final analysis. The extraction and pH of the sample depends on two factors: the physical and chemical properties of the relevant drug and the sorbent, and the drug interactions with functional groups of the sorbent. For example, for van der Waal forces and hydrogen bonding from non-polar sorbents (e.g. C_{18} octadecyl-bonded silica), the sample and sorbent column pH values should be adjusted so that the drugs to be extracted are in their uncharged forms.

With the principle of ion exchange, because there are opposite charges of the functional groups on the sorbent, the sample pH values must be adjusted to make sure the relevant drugs on the column are in their charged form and will be retained by the functional groups. Changing the pH of the extraction system, either neutralizes the charged sorbent or the compound to be extracted.

Compounds present in neutral form should have their pH adjusted at least 2 pH units higher than the pKa for basic compounds and at least 2 pH units lower than the pKa for acidic compounds.[139] This results in a yield of approximately 99% of the compounds of interest being eluted in their non-ionized form. For ion exchange SPE it is advisable to use buffers of < 0.1M or water as this helps to reduce ionic drug retention caused by highly concentrated samples which can act as counter ions.

4.1.6.1.2 Internal Standard

The internal standard is used in extraction procedures to correct for the recovery and the analytical variability during analysis. It must be added in the early process of the extraction procedure for accurate reflection of the recovery of the unknowns, errors produced from sample dilution and samples are correlated using internal standards.

The internal standard should be chemically similar to the compound of interest, chemically stable, have similar detection characteristics as the unknown compound, elute in a blank portion of the chromatogram and should be well resolved from adjacent peaks. Under normal conditions, 80 % well resolved full-scale peaks should be noted in a diluted sample with the internal standard clearly identified for sample identification on the chromatogram.

4.1.6.2 COLUMN PRE-CONDITIONING

Prior to sample application, it is necessary to precondition the sorbent column using suitable solvents for conditioning to obtain high reproducible recovery of the analyte. Suitable organic solvents (e.g. methanol) may be used on sorbents for nonpolar and multiple-interaction phases to solvate bonded functional groups. These functional

groups in turn help to open hydrocarbon chains and remove interfering organic residues which may affect analyte interactions or interfere in the detection stage.

Organic solvents are usually followed by the addition of water or buffers which have had their pH, ionic strength and polarity adjusted, to remove excess organic solvent and prepare the columns to receive an aqueous sample. For polar phases, the treatment with a nonpolar solvent (e.g. hexane) is necessary to activate the surface.

4.1.6.3 SAMPLE APPLICATION

Once the column has been pre-conditioned, the pretreated sample is applied onto the top of the pre-conditioned SPE column. Adsorption onto the sorbent bed is induced by applying a light vacuum or positive pressure at a flow rate of 1.5 ml /minute.

4.1.6.4 COLUMN WASH

After sample application, the columns must be washed again with water, buffer or an appropriate solvent, to selectively remove any endogenous compounds that may interfere with the analysis, without substantial loss or removal of the relevant drugs. The flow rate of the sample passing through should be kept at 1.5 ml /minute to retain the drug of interest or compound obtained from the biological sample onto the column.

4.1.6.5 COLUMN DRYING

Precautions must be taken in this step which is used to dry interfering solvents out of the sorbent bed before the elution step. If a water miscible organic solvent (e.g. methanol, buffer) is used as an eluent and the analysis is carried out on reversed-

phase HPLC, column drying is unnecessary, as residual water does not pose a problem during the analysis. However, analysis carried out on GC or GC/MS using a water-immiscible eluent, can interfere with the analysis and elution of relevant drugs if the column is not completely dried prior to the elution step.

4.1.6.6 ELUTION OF ANALYTES

A suitable eluent for the extraction of drugs from SPE columns should be strong enough to elute the relevant drugs completely using small amounts, while leaving behind any interfering components. Elution solvent selectivity is based on its polarity (P'), solvent selectivity and eluotropic strength (ε^{o}).[140] Solvent strength and selectivity are based on the solubility of a compound in a solvent. Solvent strength is the polarity or preferential ability to dissolve more polar compounds, while dissolving particular compounds where the polarity is not very different as the selectivity. Snyder, has reported 71 P' values and selectivity of solvents used in SPE techniques.[141]

The binary mixture for A and B solvents can be calculated for desired P' values using the following equation.[140]

$$P' = \Phi_a P_a + \Phi_b P_b$$

Φ_a / Φ_b = Volume Fractions of Solvents

A / B = in the solvent mix

P_a / P_b = P' values of pure solvents A/ B

E.g. P' of a 1:1 mix of chloroform (P'= 4.4) & Acetone (P' = 5.4) is 4.9

The eluotropic strength depends on the elution strength that is helpful for choosing a suitable eluent for compounds from a particular sorbent. ε° can be used to develop additional SPE methods and is useful in instances where the usage of an eluent system highly recovers not only the drug of interest, but interfering impurities as well. The use of another eluent with a similar ε° value can overcome this problem without greatly affecting the extraction efficiency.

Control of the rate of eluent flow through the column should be adequate enough for the mobile phase and the stationary phase to react with the relevant drug in the sample. The investigation of flow rates by Logan and Stafford using diatomaceous earth columns found that the best flow for the recovery of drugs was 2 ml /minute.[141] In the case of nonpolar, polar and ion exchange columns, a slower flow rate was found to be more suitable for ion exchange columns than polar and nonpolar columns, because of slower ion exchange interactions.[139]

4.1.6.7 DERIVATISATION

Converting sample compounds into a derivative form before or after extraction is useful in some instances. It can be used to reduce the polarity of the analyte for the use of partition columns rather than adsorption or ion exchange columns or to increase and selectively enhance the detector response to relevant compounds in the sample.

One of the derivatives used in forensic toxicology laboratories for the derivatisation of relevant drugs is the N,O - bis (Trimethylsilyl) trifluoroacetamide with a catalyst trimethylchlorosilane (BSTFA +1% TMCS). BSTFA is a colourless, clear and

moisture sensitive liquid that replaces labile hydrogens with a Si $(CH_3)_3$ group when reacted with polar compounds.[142] The addition of such groups helps to prepare volatile and thermally stable derivatives for GC/MS. It is an effective trimethylsilyl donor with donor strength similar to its unfluroinated analogue BSA (N,O - bis (trimethlysilyl) acetamide.

BSTFA is preferred over many other silylating reagents because good chromatographic separation can be obtained. This is because its volatile by-products mono-(trimethylsilyl)trifluoro-acetamide and trifluoroacetamide elute with the solvent front. TMCS is a catalyst which is normally added to react with BSTFA providing stronger donor strength. With the addition of this catalyst, many amides, secondary amines and hindered hydroxyls will be successfully derivatised, which BSTFA could not have done alone.

4.1.7 CHROMATOGRAPHIC ANALYSIS

4.1.7.1 SPE GC/MS

Gas chromatography- mass spectrometry (GC/MS) instruments have been widely used for environmental, biological and chemical sample analysis. This is mainly because of their sensitivity, selectivity and high resolution for the analyte from the compound mixture. Although good, these characteristics would not be possible if adequate sample preparation and extraction techniques where not carried out prior to analysis.

SPE techniques using GC/MS have resulted in clean and pure extracts, which elute the sample compound with high recovery and resolution. Both the extraction and

instrumental techniques are relatively quick, less laborious, can be automated and are easy to use. It has become the most preferred amongst analytical procedures for drug extractions in whole blood[143], serum[144], plasma[145], vitreous humor[146], urine[141], saliva[147], tissue[148] and more recently hair[149].

4.1.7.2 SPE HPLC

Many analytical methods employ high-performance liquid chromatography (HPLC) for the analysis of the sample extracts obtained from SPE techniques. The properties of the adsorbents in SPE columns, are very similar to HPLC adsorbents typically silica or bonded silica (e.g. C_{18}, C_8, C_5, C_2, cyano), so the same principle applys for retention and desorption of the analtye.

Efforts to automate sample preparation can lead to greater throughput of the samples prepared for analysis, more accurate methods, reducing time, labour and cost. Instrumentation and data process is far more easier to automate than sample preparation for SPE. This is because sample preparations are application specific and depend on the matrix and analyte properties, therefore manual procedures are preferred.

The success of SPE automation (e.g. ASPEC automated separation preparation systems by Gilson), allow samples to be applied to the cartridge and can be linked on-line or off-line to HPLC. Over the past few years, this automated process has been employed in numerous applications for the analysis of aqueous and biological matrices.

4.1.7.3 On-Line-SPE HPLC

SPE process can be performed either on-line as a trace enrichment technique or off-line, to prepare cleaner samples prior to analysis. The on-line SPE technique is a pre-column concentration technique that involves column-switching (e.g. automated valves) or coupled column process where the final eluent from the SPE column is injected into an HPLC system for partitioning and detection.[150]

By directly eluting SPE extracts onto the analytical HPLC system, improves the sensitively is enhanced and the evaporation and reconstitution steps normally used after the elution are eliminated thus increasing the recovery. For the confirmation of peak identity during analysis, a diode array detection system is employed in HPLC, which measures the complete UV/vis spectrum and expands the rule of HPLC from its traditional uses in quantitative procedures.

4.1.7.4 Off-Line SPE HPLC

Off-line SPE techniques were designed to produce cleaner samples prior to analysis. There are automatic and semi automatic off-line SPE instruments available that can carry out SPE procedures exclusively in automated systems, with the transfer of the eluents to HPLC done manually for semi-automatic systems. Multiple extraction devices have been introduced for the extraction process of numerous samples, which are performed manually, but can be done using off-line analysis.[112] An advantage of the off-line methods, is its ability to use a variety of solvents and multiple columns that can not be used with on-line techniques.

4.1.7.5 SPE SFE/SFC

SPE sorbents have been used in combination with SFE/SFC (supercritical fluid extraction and chromatography) techniques. As a result the sorbent acts as a chromatographic column with selective elution of the analyte of interest. Although SFE seems to have proven itself as a popular alternative to conventional liquid extraction methods, SPE will continue to be a popular sample preparation technique widely used in clinical and environmental laboratories.

4.1.8 CONCLUSION

SPE has been successfully established as the preferred sample preparation technique in a variety of chemical, biological and environmental applications. With numerous advantages over conventional methods that include; ease of automation, cost reduction and high sample recovery.

There is not doubt that SPE will continue to be used as the preferred extraction technique over traditional methods. However, with the introduction of SFE techniques have come many advantages over SPE techniques, with the major advantage being reduced contamination.

Using SPE techniques, impurities, antioxidants, contaminants etc., can often be observed causing interference during sample analysis and reduce the lifetime of chromatographic columns. By cleaning cartridges with solvents and running blanks to minimise interferences by contaminants, reduces sample throughput and increases solvent consumption and processing costs.

In general, it has been shown that SPE techniques are the method of choice over LLE techniques today. LLE methods offers the option of multiple extraction steps to provide a clean, selective and efficient extract. However, SPE adds further selectivity to the extraction procedure which makes it ideal for the elution of various compounds.

5. BENZODIAZEPINES

5.1 LITERATURE REVIEW

5.1.1 INTRODUCTION

The use of psychoactive drugs dates back to prehistory. Opium has been used to alleviate pain in Ancient Egypt, alcohol in religious ceremonies in the Near and Middle East, the chewing of coca leave by the Andean Indians to lessen fatigue and cocaine as a local anesthetic. Over the past decade, the rapid growth of both legitimate medical use and illicit non-medical abuse of numerous drugs that affect the mind have been observed.

A number of psychoactive drugs have been synthesized and introduced into medicine to treat psychological distress and psychiatric illness. Until recently, these tranquilizers have been thought to present little danger of abuse or dependence. However, long-term use can become addictive, causing dependency and withdrawals even under medical supervision.

5.1.2 HISTORY

In 1955 Dr Leo Sternbach synthesized a drug that he thought to be in the chemical family quinazoline N-oxide, but was later found to be a minor tranquillizer.[151] The drug was rediscovered later and released as chordiazepoxide, a minor sedative marketed as Librium® in 1961.[151] Some attributes that influenced the popularity of chlordiazepoxide were its calming effects, muscle relaxation and prevention of convulsions.

5.1.3 PHARMACOLOGY

Benzodiazepines are minor tranquilizers with anxiolytic, sedative, anti-convulsive and muscle-relaxing effects and are widely used as anti-anxiety drugs.[151] They are absorbed in the body, with peak concentrations occurring within 0.5-8hrs after administration. They are given in a wide range of dosages from less than 0.1 mg to 100 mg or more each day.[151] Researchers soon began to synthesis other drugs in the benzodiazepine family to determine the relation of its chemical structure to the drugs effects. Most benzodiazepines are extensively metabolized and many members of the group are in fact metabolites of the others.

5.1.3.1 System Reaction

The focus of benzodiazepines in the central nervous system has found to be located in specific receptors (dendrites with small areas) termed benzodiazepine receptors. These receptors cause the nerves to respond as if they were activated, resulting in release of chemical compounds called neurotransmitters. This reaction is also attributed to the receptors sensitivity to react to the neurotransmitter. The stronger the binding to a receptor, the stronger the drug will be to relieve anxiety thus, the two are directly related.

Researchers have found that the neurotransmitter responsible for the reduction of nerve activity and increasing the receptor-binding activity of benzodiazepine is called GABA (gamma-aminobutyric acid).[151] It was theorise that the GABA receptor is structurally similar to the benzodiazepine receptors, because GABA specifically bind only to the benzodiazepine receptor which in turn increases GABA binding to their own receptors.

5.1.3.2 Side Effects

Despite the success of benzodiazepines for the treatment of depression and anxiety, it does have dangerous side effects. When the problems associated with benzodiazepines became apparent, the initial enthusiasm and frequent uncritical distribution of prescription was replaced by restriction on the ease by which they were dispensed. They can be abused to the point where their dependability can be life threatening.

Acute poisoning due to an overdose of benzodiazepines is becoming a common problem and is indicated by drowsiness, confusion, slurred speech, lack of co-ordination and sometimes coma. More serious side effects that can be experienced are motor and memory impairment, sedation, behavior disinhibition and paradoxical agitation have been occasionally observed.[152]

5.1.4 PHARMACOKINETICS

The physicochemical and pharmacokinetic properties of the benzodiazepines greatly affect their clinical utility.[152] Lipophilicity is an important variable in the duration of action of benzodiazepines. The extent of binding correlates strongly with lipid solubility. The benzodiazepines are completely absorbed with the exception of clorazepate and some (e.g. prazepam and flurazepam) reach the systemic circulation in the form of active metabolites.[153]

Based on elimination half-life, benzodiazepine receptors can be divided into four categories: (1) ultra-short acting agents, (2) short-acting agents with t1/2 < 10 hrs, (3) intermediate-acting agents with t1/2 10-24 hrs and (4) long-acting agents with

t1/2 > 24 hrs.[154] Often, the longer the half-life of benzodiazepines can cause pronounced withdrawal symptoms on discontinuation of use, which may develop days after cessation of the drug taken. The duration of action of a certain benzodiazepine depends not only on the half-life of the drug itself, but on the possible formation of active metabolites.

5.1.5 PRESCRIPTION

The development of clordiazepoxide has led to the synthesis of hundreds of benzodiazepines and at present 16 available by prescription in the United Kingdom.[153,154] Diazepam, which has the trade name Valium®, is just one of the many synthesized benzodiazepines on the market as an effective sedative. It is much more potent than Librium® that results in less dosage needed to produce small but effective sedative effects.

Approximately 25 benzodiazepines are available by prescription for various needs. The generic names for some of them are lorazepam, flurazepam, triazolam, chlordiazepoxide, temazepam, oxazepam, clorazepate, diazepam and alprazolam. They range from sleeping pills to antidepressants, with some under investigation for use as anesthetics.

There are many questions that should be asked before you are prescribed benzodiazepines. Not all cases of anxiety should be treated using tranquilizers or sedatives, only in extreme cases should they be considered for use. Both doctor and patient should be aware of the potential side effects, dangers and correct usage of benzodiazepines.

Table 5.1 Various prescribed benzodiazepines and their use in treatment.[155]

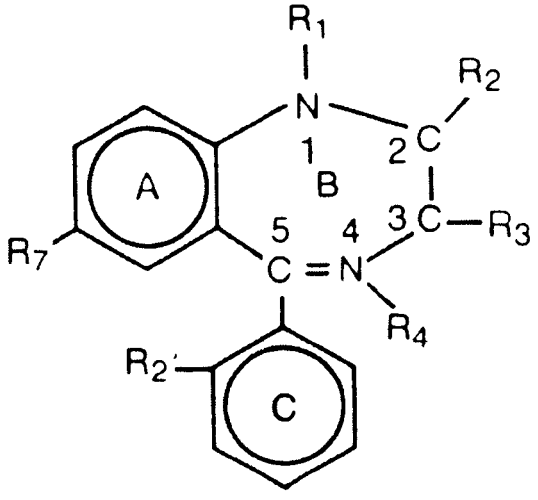
Nonproprietary Name(s)	Trade Name	Treatment	Daily Dose, mg	*Dosage form
Alprazolam	Xanax®	anxiolytic	0.75-1.5	O
Clorazepate	Tranxene®	anxiolytic	15-60	O, SR
Clonazepam	Rivotril®	anxiolytic, anti-seizure	1.5-10	O
Chlordiazepoxide	Librium®, others	anxiolytic	15-40	O, I
Diazepam	Valium®, others	anxiolytic, anti-seizure muscle relaxant	2-20; repeat in 3-4hrs	O, I ,L, SR
Flunitrazepam	Rohypnol®	hypnotic	NA	O, L
Lorazepam	Ativar®	anxiolytic, anti-seizure	2-4; 30-60	O, I
Midazolam	Hypnovel®	sedative	NA	I
Oxazepam	Seraz®, Zazopam®	anxiolytic	30-60	O

* Dosage forms O = Oral solid, L = Liquid, SR = Slow releasing, I= Injection

5.1.6 CHEMICAL STUCTURE [156]

Attempts to determine the relation of various aspects of the chemical structure of Librium®, have led to several other benzodiazepines being synthesized. However, because of the close chemical structures, most of them had very similar effects to Librium®, one being diazepam that is marketed as Valium®, being more potent. Below in Figure 5.1 is the basic structure of drugs belonging to the benzodiazepine class of molecules.

Figure 5.1 Basic Structure of 1,4-Benzodiazepine.[156]



The general structure consists of three rings denoted by the letters A, B and C in Figure 5.1, together with a number of locations on the drug molecule where specific atoms or groups of atoms are introduced at the sites labeled R1, R2, R3, R7 and R2'.[156] By substituting different molecule groups at the R sites results in new molecular structures which gives different drugs with different pharmacological activities.

Table 5.2 below lists the various common benzodiazepines and their structural groups. One example is diazepam, Valium®. By replacing R_1 with a methyl group CH_3, R_2 with a keto oxygen atom =O, R_3 and $R_{2'}$ with a hydrogen atom H and R_7 with a chlorine atom Cl, the structure becomes diazepam. From diazepam, changing R_1 from CH_3 to H results in nordiazepam, a second change in R_3 from H to OH, the hydroxy group, results in oxazepam and further change at $R_{2'}$ from H to Cl results in lorazepam. Going back to nordiazepam, change at the site R_7 from Cl to NO_2, the nitro group, gives nitrazepam and further change at $R_{2'}$ from H to Cl gives clonazepam, as listed in Table 5.2.

Table 5.2 Structural groups of various common benzodiazepines

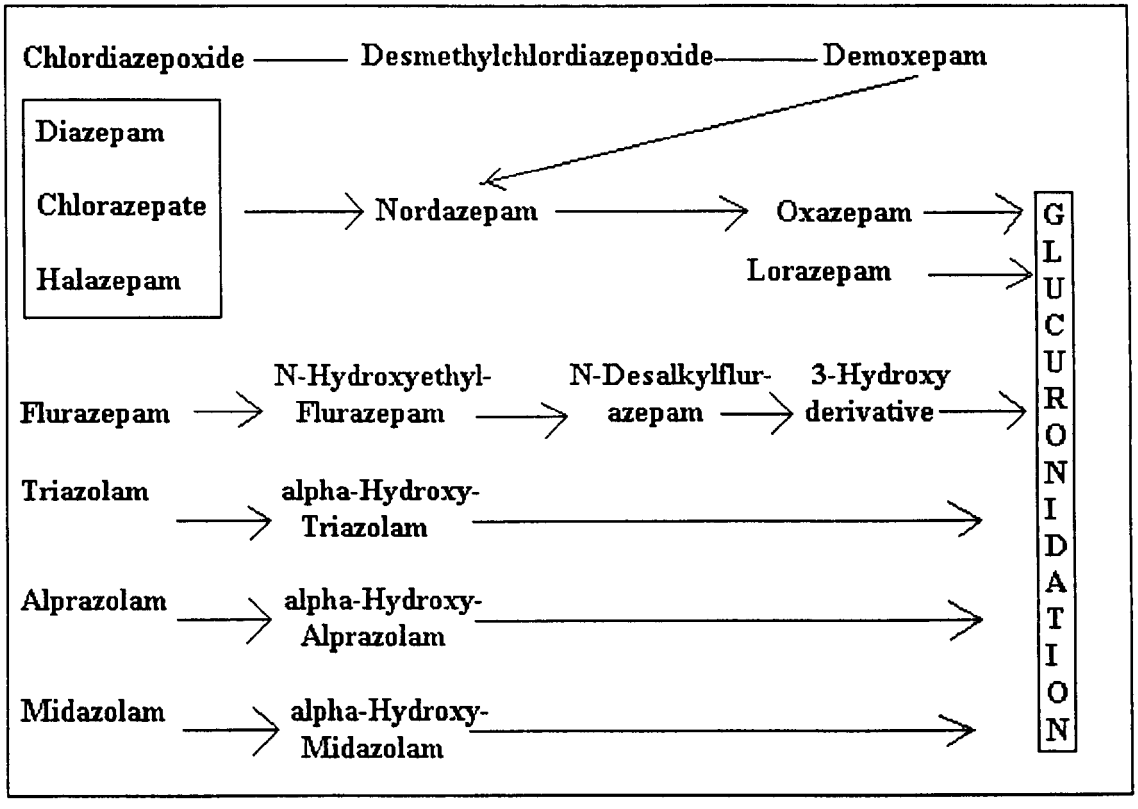
BENZODIAZEPINES	R1	R2	R3	R7	R2'
Chlordiazepoxide	(-)	$-NHCH_3$	-H	-Cl	-H
Diazepam	CH_3	=O	-H	-Cl	-H
Nordiazepam	-H	=O	-H	-Cl	-H
Nitrazepam	-H	=O	-H	$-NO_2$	-H
Clonazepam	-H	=O	-H	$-NO_2$	-Cl
Oxazepam	-H	=O	-OH	-Cl	-H
Lorazepam	-H	=O	-OH	-Cl	-Cl
Temazepam	$-CH_3$	=O	-OH	-Cl	-H

5.1.7 METABOLISM

Benzodiazepines are extensively metabolized by several different microsomal enzyme systems in the liver. Active metabolites are biotransformed more slowly than the parent compound. Hence, the duration of action of many benzodiazepines has no influence to the half-time of elimination of the drug that has been administered.[155]

The metabolism of benzodiazepines occur though hydroxylation (aliphatic and aromatic), desalkylation, reduction and acetylation reactions (phase I) followed by conjugation to glucuronic acid (Phase II) prior to excretion.[155] Biological activity is present in some Phase I metabolites which may be greater or less than that of the parent, whereas the conjugates possess no significant activity. Two of the most common metabolites of 1-4-benzodiazepine are oxazepam and nordiazepam. Nordiazepam has a half-life of 40-99 hours, therefore, all compounds metabolized to nordiazepam are considered long-acting.[155] Some parent drugs are rapidly metabolized into nordiazepam and are not detectable in blood or urine.

Figure 5.2 The Major Metabolic Route of some Benzodiazepine.[155]



5.1.8 CHROMATOGRAPHIC ANALYSIS

There are numerous publications of the analysis of benzodiazepines using methods such as SPE and LLE with analysis techniques using GC/MS, GC/FID and GC/ECD. Table 5.3 lists numerous recent literature on the types of extraction and analysis techniques used.

Table 5.3 Summary of extraction and analysis techniques used for detection benzodiazepines in various matrices.

Year	Extraction Technique	Analysis Technique
1989[157]	GC/MS	SPE
1991[158]	GC/MS/MS	SPE
1991[159]	GC/NPD, HPLC	LLE
1991[160]	FPIA, HPLC	LLE
1992[161]	HPLC	SPE
1994[162]	HPLC	LLE
1994[163]	/	IA
1995[164]	HPLC	LLE
1995[165]	GC/MS	LLE
1996[166]	GC/MS, HPLC	LLE
1996[167]	HPLC	SPE
1997[168]	HPLC	SPE

Recent literature published for the analysis of benzodiazepines, generally concerned with analysis by GC/MS. Both HPLC and GC/MS analysis can be used for benzodiazepine determination. However, it has been reported that HPLC has more advantage over GC/MS analysis for the recovery of some benzodiazepines.

5. 2 CURRENT METHODOLOGY

The current extraction and methodology for benzodiazepines in whole blood used in the routine toxicology laboratory is by SPE with analysis by HPLC with UV detection. The columns used for routine analysis are not sorbent columns and therefore, optimization was carried out using SPE columns and compared.

5.2.1 SPE DIATOMACEOUS EARTH

Label and fill a plastic 10 ml syringe with a small amount of glass wool and approximately ¾ full with dichloromethane washed Extrelut® (diatomaceous earth). Three vials blank, standard and sample were labeled.

- *Standard:* 0.9ml of blank blood was added to the vial with 1ml of pH 7.4 phosphate buffer, 25µl of 5% ammonia solution, 100µl of stock mix (triazolam, temazepam, chlordiazepoxide, desmethyldiazepam and diazepam 1 mg /ml) and 100 µl of internal standard (prazepam 2.61 mg /100 ml).
- *Blank:* 1ml of blank blood, 1ml of phosphate buffer, 100 µl of prazepam and 25 µl of 5% ammonia solution were added to a vial.
- *Sample:* 1ml of sample blood, 1ml of phosphate buffer, 100 µl of prazepam and 25 µl of 5% ammonia solution were added to a vial.

The contents of each vial were pippetted into their corresponding columns and left to stand for 5 minutes. Approximately 8 ml of diethyl ether was used for elution of benzodiazepines under gravity, then evaporated to dryness under nitrogen. The dried residue was reconstituted in 180 µl of mobile phase (0.01M Na_2HPO_4 + MeOH 30:70 v/v) and sonicated for 5 minutes.

5.2.2 HPLC Parameters

A Gilson HPLC system was used which consists of a 307 Gilson pump with a 118 UV/vis detector and a wavelength set at 254 nm. The mobile phase which consisted of 0.01M Na_2HPO_4 : MeOH (30 : 70 v/v), was pumped through a 5 micron 25 cm x 4.6 mm i.d. Hypersil ODS Column at a flow rate of 1 ml /minute which gave a

pressure of approximately 2500 psi. Na_2HPO_4 buffer was prepared by dissolving 1.78 g of $Na_2HPO_4.2H_2O$ in 1 liter of de-ionized water. Add 120 ml of prepared buffer to 280 ml MeOH and de-gassed with helium for 10 minutes at room temperature. The injections were controlled via a 7125 Rheodyne fitted with a 20 µl loop and the data collected on a 3394A Hewlett Packard integrator.

5.3 CONCLUSION

Method development for the extraction of benzodiazepines using solid-phase extraction (SPE) was carried out for forensic purposes. SPE Bond elute certify LRC columns were used for method development, with results compared to routine drug screening using diatomaceous earth columns. Benzodiazepines used in method development were triazolam, temazepam, desmethyldiazepam, diazepam, chlordiazepoxide and prazepam which was used as the internal standard.

5.4 METHOD DEVELOPMENT

Alternative elution, various pH buffers and extraction methods were used maintaining constant apparatus parameters for consistent data. An unextracted sample was first analyzed for the calculation of the peak area ratio (PAR) and retention times for each drug. The standard mix used consisted of triazolam (1.04 mg /100 ml), temazepam (1.21 mg /100 ml), chlordiazepoxide (1.57 mg /100 ml), desmethyldiazepam (1.58 mg /100 ml), diazepam (1.31 mg /100 ml) and prazepam (internal standard 2.61 mg/100 ml) which were prepared as 10µg /ml standard solutions.

To find the percentage recovery for the unextracted sample, which is used as a reference, the PAR of the unextracted drug was divided by the PAR of the unextracted internal standard. The sample was then extracted and the internal standard added to the elution and analyzed.

This study involved the application of SPE Bond Elute Certify® (BEC) columns of benzodiazepines in whole blood. To find the best sample preparation and extraction technique, various preparation steps were investigated to determine the most robust method and then optimized. Eleven methods were investigated using Bond Elute Certify ® column to determine which extraction method eluted all five benzodiazepines. The column conditioning and wash steps were varied for initial determination.

5.4.1 SAMPLE PRE-PREPARATION

All methods consisted of the same sample preparation step for relatively consistent results. The preparations were as followed:

To a vial containing 1 ml of blank blood, 1 ml of 0.01M phosphate buffer (KH_2PO_4) pH 6 and 50 µl of 5 % ammonia prepared in de-ionized water were added. In addition, 100 µl of benzodiazepine spike solution, that consisted of triazolam, temazepam, chlordiazepoxide, desmethyldiazepam and diazepam were added to the vial and vortexed for 30 seconds. The internal standard prazepam (100 µl) was added to the final collected elution to establish the highest recovery from the various methods.

5.4.1.1 Experimental Method 1, 2 and 7

The column was conditioned with 2 ml of MeOH and 2ml of buffer pH 6 and then the sample was added. The columns were then washed with 1ml of water and dried for 2 min, 0.01M acetic acid pH 3.3 and dried for 1 minute and 50 µl of MeOH and dried for 2 min all under full vacuum. Method 1 was eluted with 4 ml of acetone : chloroform (1 : 1 v/v), method 2 with 4 ml of 2 % ammoniated ethyl acetate and method 7 with 4 ml of diethyl ether. Samples were blown down to dryness under nitrogen at 40 °C and reconstituted with 180 µl of mobile phase.

5.4.1.2 Experimental Method 3, 4, 5, 6 and 8

The column was conditioned with 2 ml of MeOH, 2 ml of buffer pH 6 and then the sample was added. The column was washed with 2 ml of buffer and dried for 2 min, 1ml of 0.01M acetic acid pH 3.3 and dried for 1 minute and 1 ml of hexane and dried for 2 min all under full vacuum. Method 3 was eluted with 4 ml of diethyl ether, method 4 with 4ml of acetonitrile : MeOH (1: 1v/v), method 5 with 4 ml of 100 % acetonitrile, method 6 with 4ml of hexane : ethyl acetate (3 : 1 v/v) and method 8 with 4 ml of 100 % chloroform. Samples were blown down to dryness under nitrogen at 40° C and reconstituted with 180 µl of mobile phase.

5.4.1.3 Experimental Method 9

The column was conditioned with 2 ml of MeOH and 2 ml of buffer pH 6 and then the sample was added. The column was washed with 1ml of MeOH and dried for 2 minutes, 1ml of 0.01M acetic acid pH 3.3 and dried for 1 minute and 1 ml of hexane and dried for 2 minutes all under full vacuum. Method 9 was eluted with 4 ml of

diethyl ether. Samples were blown down to dryness under nitrogen at 40 °C and reconstituted with 180 µl of mobile phase.

5.4.1.4 Experimental Method 10, 11

The column was conditioned with 2 ml MeOH and 2ml of buffer pH 6. The sample was added and the column washed with 2 ml of buffer and dried for 2 minutes, 1 ml of 0.01M acetic acid pH 3.3 and dried for 2 minutes and 2 ml of hexane and dried for 2 min all under full vacuum. Method 10 was eluted with diethyl ether and method 11 was eluted with 4 ml of 100 % chloroform. Samples were blown down to dryness under nitrogen at 40 °C and reconstituted with 180 µl of mobile phase.

5.4.1.5 Results and Discussion

Comparison of the various extraction methods described, established which of the solvent preparations methods produced the highest recovery for all five benzodiazepines. The selected method was then further optimized for improved recovery. Table 5.4 shows the percentage recovery for benzodiazepines using pH 6 phosphate buffer and various column conditionings, wash and elution methods.

Table 5.4 Percent recovery of benzodiazepine using the various methods.

DRUG %	1	2	3	4	5	6	7	8	9	10	11
Triazolam	81	NR	32	85	48	63	78	37	41	39	101
Temazepam	50	74	53	54	65	NR	51	40	50	50	68
Chlordiazepoxide	NR	NR	NR	22	NR	NR	9	NR	NR	NR	18
Desmethyldiazepam	37	24	14	34	11	NR	46	6	5	28	65
Diazepam	51	11	36	25	30	NR	58	18	18	45	60

***NR = the drug was not recovered**

From the table above, it could be seen that method 11 recovered all five drugs, but with very low recovery. Chlordiazepoxide was very difficult to recover, as benzodiazepines have different pH level which they will favor. Hence, to be able to recover all five of the drugs with good recovery, the pH level of the phosphate buffer, that was pH 6, was reduced.

5.4.2 VARYING THE BUFFER pH

5.4.2.1 Experimental

Five different pH levels were selected to investigate which had the most influence on the recovery of the benzodiazepines. Extractions using 0.01M phosphate buffer pH 4, 4.6, 5, 5.5, and 6 were carried out for comparison using the aforementioned sample preparation procedure and method 11 column conditioning, wash and elution steps. A blank and standard sample using pH 6 were also prepared.

5.4.2.2 Results and Discussion

Results showed that pH 5.5 phosphate buffer had the best and the highest recoveries for benzodiazepines. Most of the elution solvents investigated did not elute chlordiazepoxide, but 100 % chloroform gave the best results during elution depending on the pH of the buffer used. Below in Table 5.5 are the peak area heights of each drug when the buffer pH is varied. Buffers pH 6, 4 and 4.6 produced low recoveries. However, pH 5 gave good recoveries, but not as good as buffer pH 5.5.

Table 5.5 Peak area heights of each of the drugs using various pH buffers.

BUFFER	Triazolam	Temazepam	Chlordiazepoxide	DMD	Diazepam
Unextstd	114440	312970	576590	403670	395430
pH 4	129700	226230	188530	255930	270780
pH 4.6	140040	324540	64428	153100	314170
pH5	167620	313080	220950	317990	366110
pH5.5	178500	333240	276810	360360	405970
pH6	150030	312960	181660	19361	309418

5.4.3 INVESTIGATION OF OXAZEPAM

Oxazepam is one of the main metabolites of benzodiazepines. It was investigated during method development because sometimes if not properly stored or kept too long, benzodiazepines can be further broken-down into their metabolites. Oxazepam was prepared in a methanol solution of 1mg /100ml and injected several times for the detection of its retention time. It was found that oxazepam was not a factor during method development. Although it has a retention time very close to chlordiazepoxide and temazepam, it was not present during analysis.

5.4.4 CONSIDERATION OF EXTRACT QUALITY

The extracts obtained from the various eluent solvents used were considered on the basis of cleanliness. This was assessed by visual inspection and description of the extract (e.g. visible residue, oily/crystalline) and then by the appearance of co-extracted material in a chromatogram of an extract from a blank whole blood sample.

Visual inspection of the extracts prior to reconstitution showed that most of the solvents chosen (e.g. hexane combinations, acetonitrile, ethyl acetate, diethyl ether) gave good amounts of visible material apart from an oily residue or a brown film in

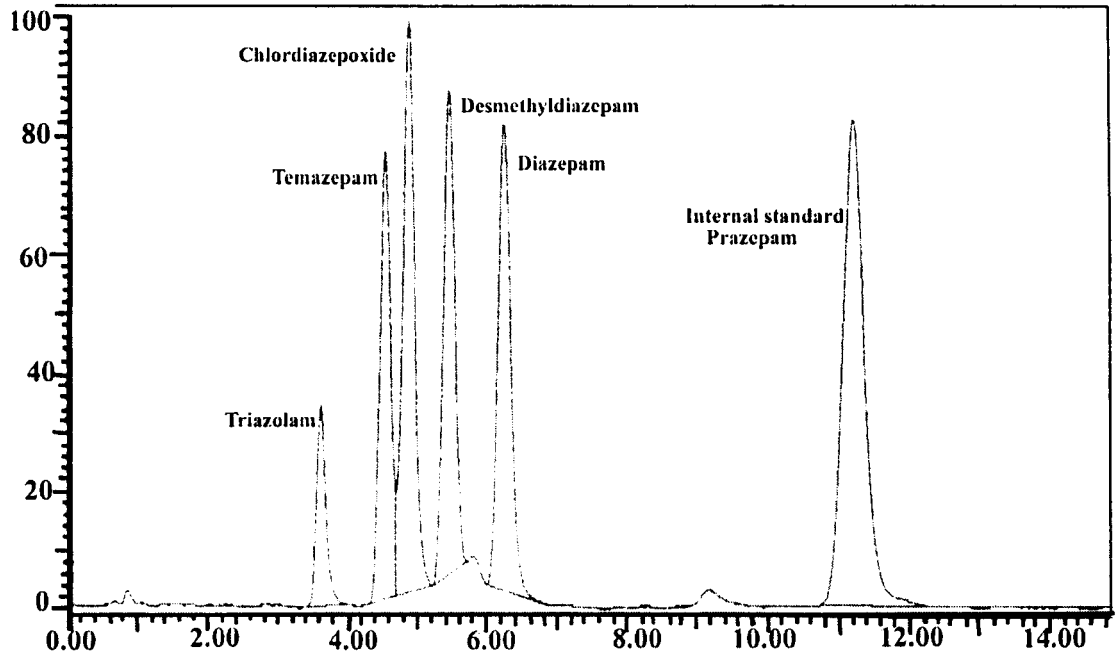
the extracts, but chloroform gave the most clean without these characteristics. This subjective visual assessment of extract quality was more significant than the subsequent interference assessment. This was because material that does not elute from the chromatographic column will have a deleterious effect on the chromatography and on the lifetime of the column and injection liner.

The second assessment of extract quality was based on the number of interference peaks in the extract from a drug-free or standard spiked blood sample. There were several contamination peaks within the first 4 minutes of chromatography caused by blood proteins. Oxazepam was investigated as a possible interference peak from breakdown of one of the drugs, but ruled out. Chloroform again proved to be the best elution solvent with the cleanest extracts and the least amount of interference peaks if any.

5.5 OPTIMIZED SPE METHOD

- *Sample pre-treatment:* To a vial 1 ml of sample blood, 50 µl of 5 % ammonia solution, 1 ml of 0.01M phosphate buffer pH 5.5 and 100 µl of internal standard were added and vortexed for 30 seconds.
- *Column conditioning:* BEC® LRC columns were conditioned with 2-3 ml of MeOH and 2 ml of 0.01M phosphate buffer pH 5.5 and pull through slowly not allowing the column to dry, then add sample (1 ml /minutes).
- *Column wash:* 2 ml of buffer was added and dry for 2 min, 1ml of 0.1M acetic acid pH 3.3 was added and dry 2 minutes and 2 ml of hexane was added and dry 2 minutes all under full vacuum (10 Hg).
- *Elution:* The sample was eluted into a clean vial with 4 ml 100 % chloroform.

Figure 5.3 Chromatograph of benzodiazepines extracted from whole blood using SPE BEC ® LRC columns with the optimized method.



5.6 METHOD VALIDATION

5.6.1 RECOVERY

5.6.1.1 Experimental

To determine the amount of drug being recovered by the method, several spiked samples were extracted. The internal standard, prazepam, was added at the end of the extraction to the eluent to determine the percent recovery.

5.6.1.2 Results and Discussion

Table 5.5 demonstrates the percent recoveries for each benzodiazepine at concentrations of 10μg/ml, ranged from 94-108% triazolam, 84-94% temazepam, 83-95% chlordiazepoxide, 85-95% for desmethyldiazepam and 88-101% diazepam,

respectively. Overall, recoveries were seen to be greater than 80% for each of the drugs.

Table 5.6 Percentage recovery and run to run reproducibility of benzodiazepines using the optimized method.

DRUG %	1	2	3	4	5	6	7	8	9	10	11	12
Triazolam	99	98	102	106	104	108	99	102	103	97	100	94
Temazepam	87	88	94	87	92	94	90	89	92	87	84	85
Chlordiazepoxide	95	92	95	91	91	91	87	86	85	85	85	83
DMD	91	91	95	89	96	95	91	91	93	88	85	85
Diazepam	94	96	97	93	100	101	94	95	96	91	88	91

5.6.2 REPRODUCIBILITY

5.6.2.1 Experimental

Samples were extracted several times and then analyzed several times by being introduced into HPLC. This was to find out if the method was reproducible with each extraction carried out separately.

5.6.2.2 Results and Discussion

Samples were prepared and injected several times to find out the recovery and reproducibility of the optimized method developed. Table 5.6 gives the Peak area ratios for each drug and unextracted standard. Comparison between the twelve runs show that this method is reproducible and the recovery is better than 80% for each of the drugs.

Table 5.7 Peak area ratio of each benzodiazepine resulting from multiple runs for recovery and reproducibility.

DRUG	Unextstd	1	2	3	4	5	6
Triazolam	0.1579	0.1566	0.1540	0.1622	0.1672	0.1651	0.1718
Temazepam	0.3939	0.3432	0.3475	0.3729	0.3461	0.3651	0.3715
Chlordiazepoxide	0.7133	0.6766	0.6585	0.6792	0.6542	0.6559	0.6558
DMD	0.5987	0.5420	0.5448	0.5737	0.5372	0.5766	0.5723
Diazepam	0.5449	0.5119	0.5228	0.5334	0.5096	0.5461	0.5506
		7	**8**	**9**	**10**	**11**	**12**
Triazolam		0.1571	0.1614	0.1633	0.1540	0.1592	0.1492
Temazepam		0.3577	0.3535	0.3639	0.3440	0.3346	0.3352
Chlordiazepoxide		0.6208	0.6165	0.6134	0.6088	0.6101	0.5991
DMD		0.5483	0.5493	0.5597	0.5301	0.5135	0.5102
Diazepam		0.5176	0.5199	0.5239	0.4971	0.4839	0.4971

The results of the unextracted standard were incorporated with the RSD for each of the drugs to find the overall percentage RSD. Table 5.8 gives the mean, median, range, RSD and recovery for each of the drugs based on table 5.7.

Table 5.8 Method validation for benzodiazepine in whole blood.

DRUG	MEAN	MEDIAN	RANGE	%RSD + STD	%Recovery
Triazolam	0.1604	0.1571	0.1492- 0.1718	0.6	> 94
Temazepam	0.3560	0.3475	0.3346- 0.3939	1.64	> 84
Chlordiazepoxide	0.6432	0.6558	0.5991- 0.7133	3.32	> 83
DMD	0.5504	0.5483	0.5102- 0.5987	2.45	> 85
Diazepam	0.5199	0.5176	0.4839- 0.5506	1.96	> 88

Figures 5.4-5.8 show the variation and reproducibility between each run of each drug when run at least three times. It was found that by using this method developed for benzodiazepines good run to run reproducibility could be seen.

Figures 5.4 Reproducibility of temazepam run in groups of three

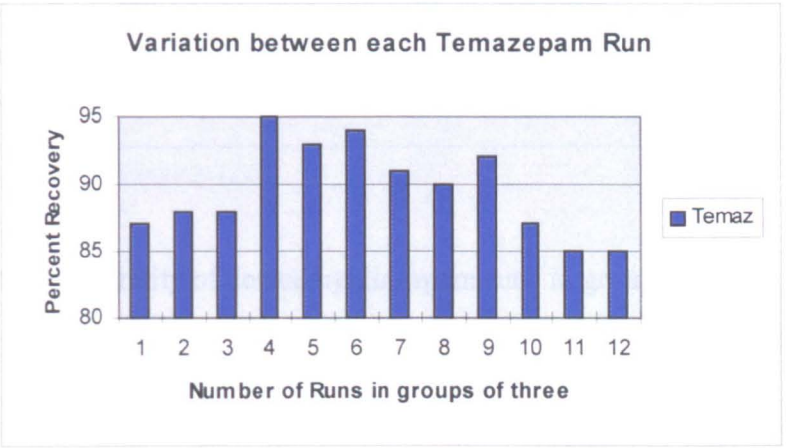


Figure 5.5 Reproducibility of triazolam run in groups of three

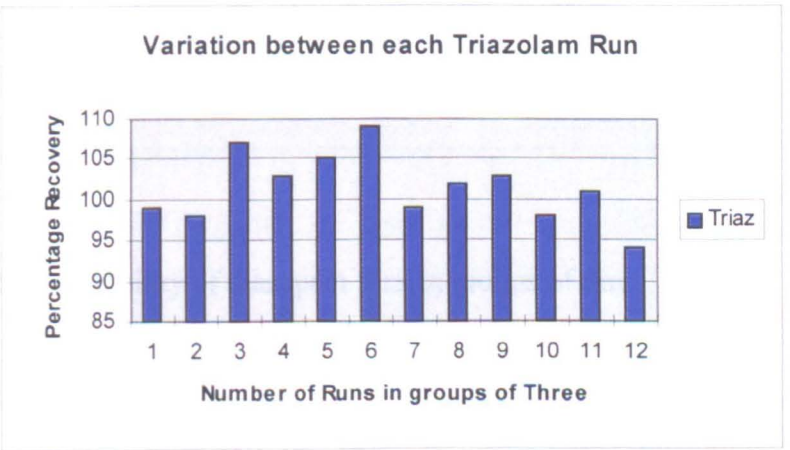


Figure 5.6 Reproducibility of chlordiazepoxide runs in groups of three

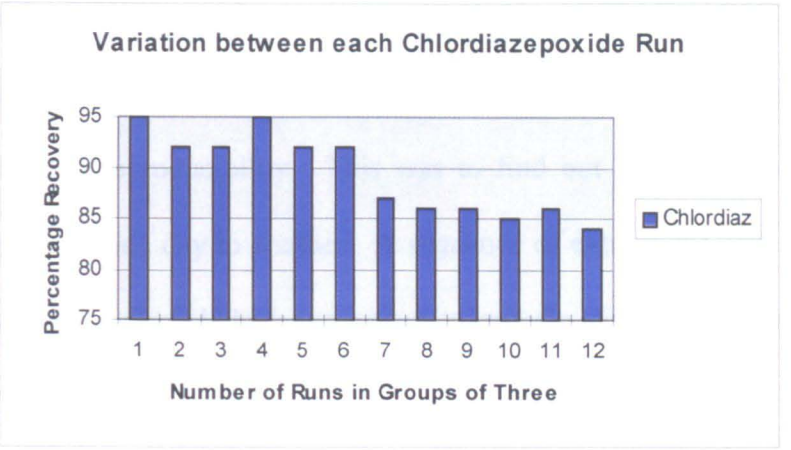


Figure 5.7 Reproducibility of desmethyldiazepam runs in groups of three

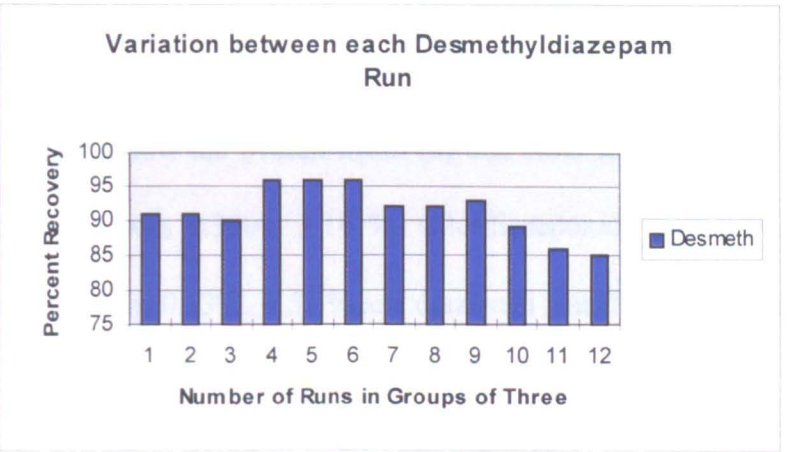
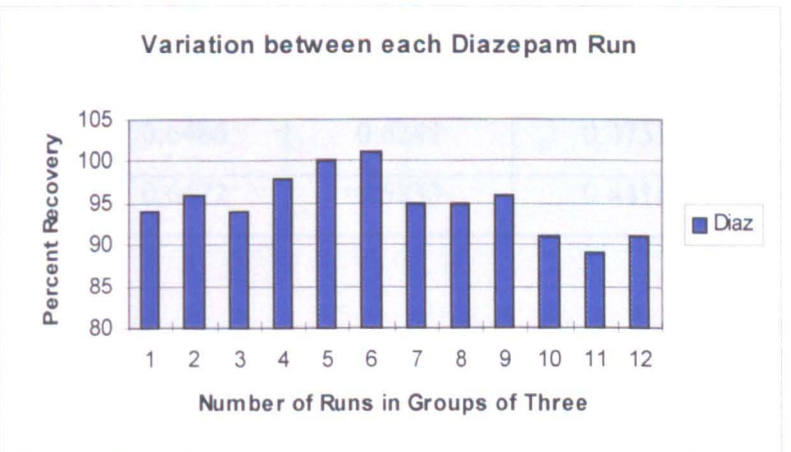


Figure 5.8 Reproducibility of diazepam runs in groups of three



5.6.3 INTRA AND INTER-DAY REPRODUCIBILITY

5.6.3.1 Experimental

The investigation of reproducibility of the method developed for benzodiazepines for intra and inter-day reproducibility. This was to find out if the method produced similar results from one day to another. A sequence of extractions were carried out on both days which included blank extractions to exclude any possible carry over.

5.6.3.2 Results and Discussion

To check the reproducibility of benzodiazepines from day to day several samples where prepared over the course of two days. It was found that, the intra and inter-day reproducibility was very good and correlated very well between both days. From Table 5.9 and 5.10 below the overall mean (x) and RSD for triazolam was 0.1624, 1.56 %, temazepam was 0.3564, 3.14 %, chlordiazepoxide was 0.5876, 4.19 %, desmethyldiazepam was 0.5392, 4.51 % and diazepam was 0.5045, 4.25 %.

Table 5.9 Intra-day reproducibility of benzodiazepines

DAY ONE	UNEXTSTD	RUN 1	RUN 2	RUN 3
Triazolam	0.1929	0.1930	0.1426	0.1685
Temazepam	0.4901	0.4186	0.3197	0.3638
Chlordiazepoxide	0.7119	0.6247	0.5123	0.5493
DMD	0.6486	0.6247	0.4753	0.5431
Diazepam	0.6672	0.5833	0.4414	0.5035

Table 5.10 Inter-day reproducibility of benzodiazepine

DAY TWO	UNEXTSTD	RUN 1	RUN 2	RUN 3
Triazolam	0.1649	0.1571	0.1592	0.1579
Temazepam	0.4115	0.3577	0.3346	0.3939
Chlordiazepoxide	0.7772	0.6208	0.6101	0.7133
DMD	0.5556	0.5483	0.5135	0.5987
Diazepam	0.5620	0.5176	0.4839	0.5449

5.6.4 LINEAR CALIBRATION

5.6.4.1 Experimental

The calibration curve obtained for each analyte, were constructed using linear regression analysis. For each of the drugs, linearity was achieved over a concentration range of 0.0039-1 μg /ml shown in Table 5.9.

Table 5.9 Linearity concentration range from 0.007-1μg /ml for each of the drugs

Conc. μg/ml	Triazolam	Temazepam	Chlordiazepoxide	DMD	Diazepam
1	0.369	0.388	0.805	0.650	0.778
0.5	0.215	0.223	0.445	0.367	0.457
0.25	0.111	0.116	0.234	0.191	0.239
0.125	0.041	0.043	0.084	0.072	0.089
0.0625	0.022	0.023	0.046	0.039	0.048
0.0325	0.013	0.016	0.028	0.023	0.029
0.0156	0.008	0.010	0.015	0.016	0.019
0.0078	0	0.005	0.008	0.007	0.010

5.6.4.2 Results and Discussion

Figures 5.9-5.13 demonstrate the linearity for each drug obtained in the selected concentration range. Analysis of the data from the regression lines (n = 9), exhibited

correlation coefficients for triazolam r^2=0.9917, temazepam r^2=0.9931, chlordiazepoxide r^2=0.9954, desmethyldiazepam r^2=0.9946 and diazepam r^2=0.9911 from whole blood.

Figure 5.9 Linearity of triazolam in whole blood

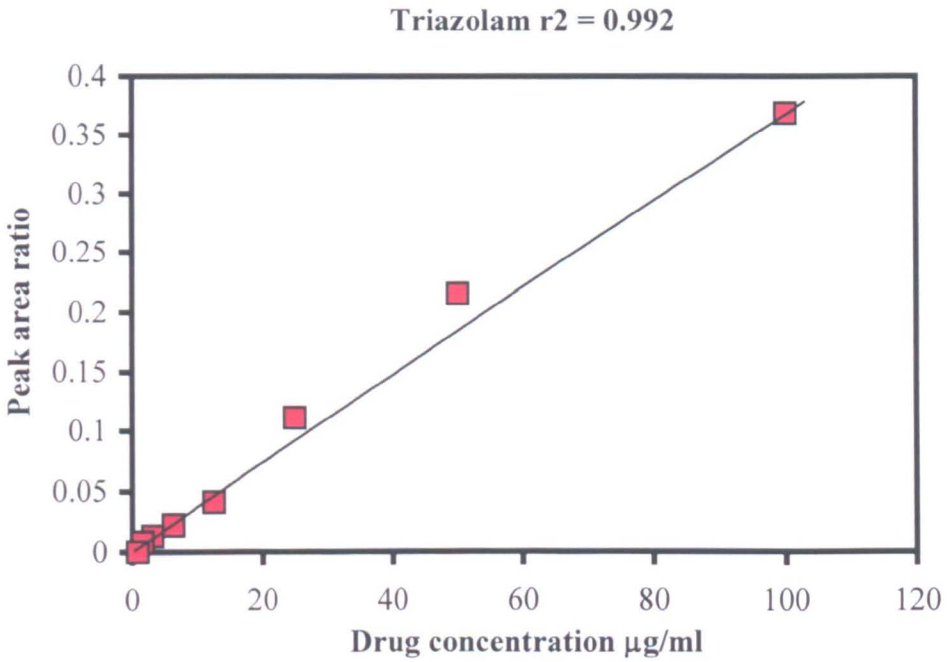


Figure 5.10 Linearity of temazepam in whole blood

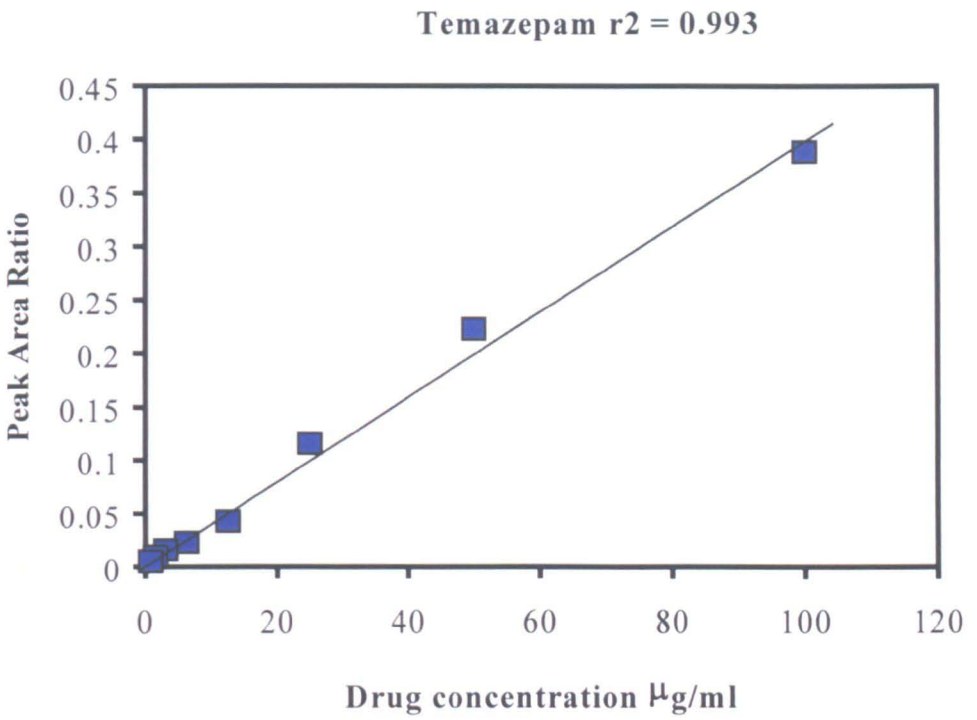


Figure 5.11 Linearity of chlordiazepoxide in whole blood

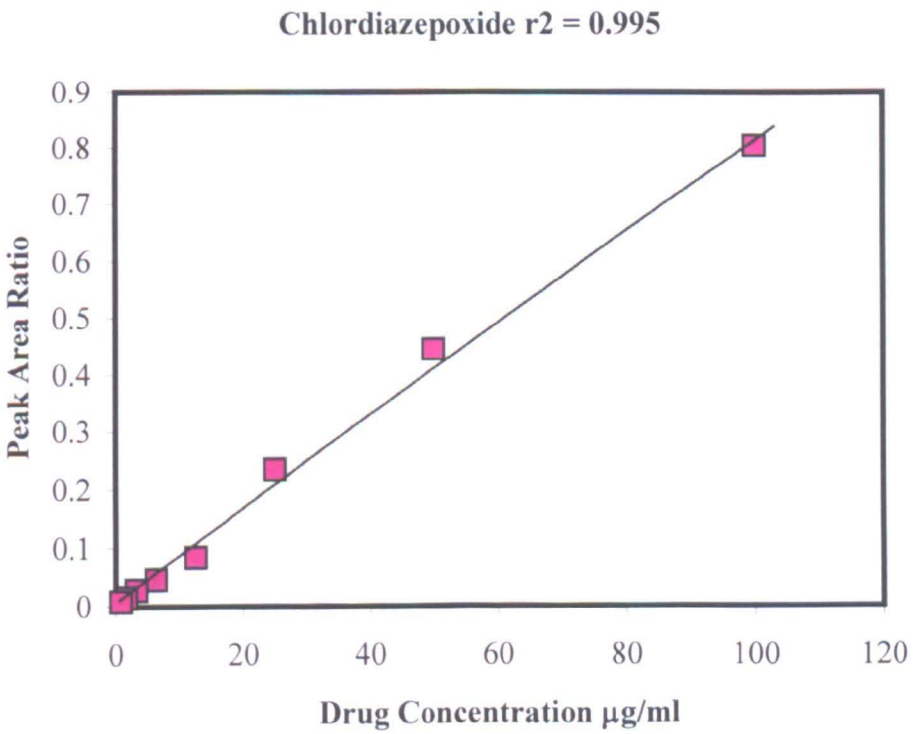


Figure 5.12 Linearity of desmethyldiazepam in whole blood

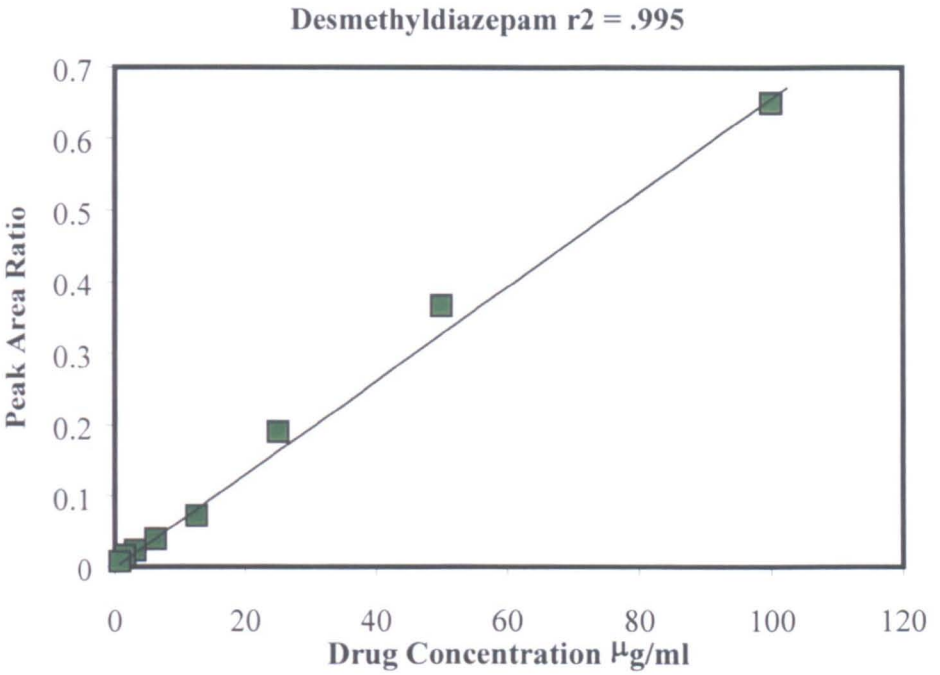
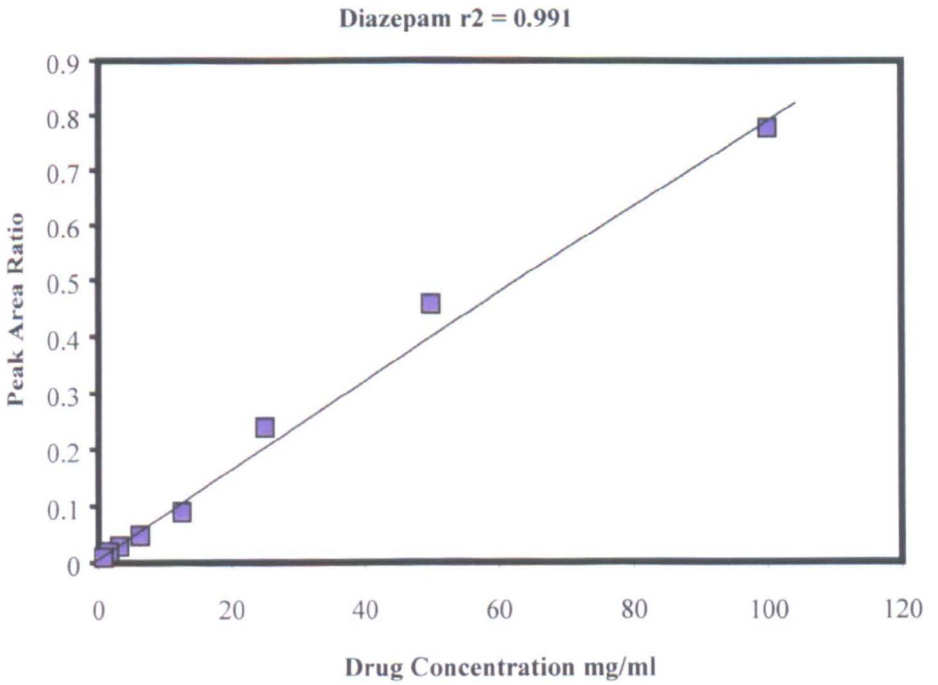


Figure 5.13: Linearity of diazepam in whole blood



5.6.5 LIMIT OF DETECTION AND QUANTIFICATION

5.6.5.1 Experimental

The limit of detection (LOD) and the limit of quantification (LOQ) was determined experimentally using blank extracted specimens with a range of 1 μg /ml to 7 ng /ml. The LOD is defined as the mean area ratio multiply three and the LOQ is defined as the mean area ratio multiply by ten.

5.6.5.2 Results and Discussion

The LOD was estimated to be 7 ng /ml and the LOQ estimated to be 60 ng /ml for this method. Triazolam was difficult to detect after 60ng /ml and the other benzodiazepines were difficult to observe and not able to quantitate very clearly because of the baseline noise.

5.7 COMPARISON OF EXPERIMENTAL AND ROUTINE METHOD

The solid-phase extraction procedure developed provides a reliable, rapid and reproducible technique for the isolation of benzodiazepines from whole blood. The Bond-Elute Certify® LRC columns selectively retains and elutes the analyte of interest using a mixed-mode interaction mechanism, constituting ion-exchange chromatography and hydrophobic and polar interactions. The high percentage recovery makes this method suitable for the analysis of benzodiazepines and detection is possible at low concentration levels.

5.7.1 Experimental

The developed SPE method was compared to the current routine method used in the laboratory. Blood case samples were analyzed using the two methods and compared.

Results produced between the two methods showed corresponding drug levels for each case sample.

5.7.2 Results and Discussion

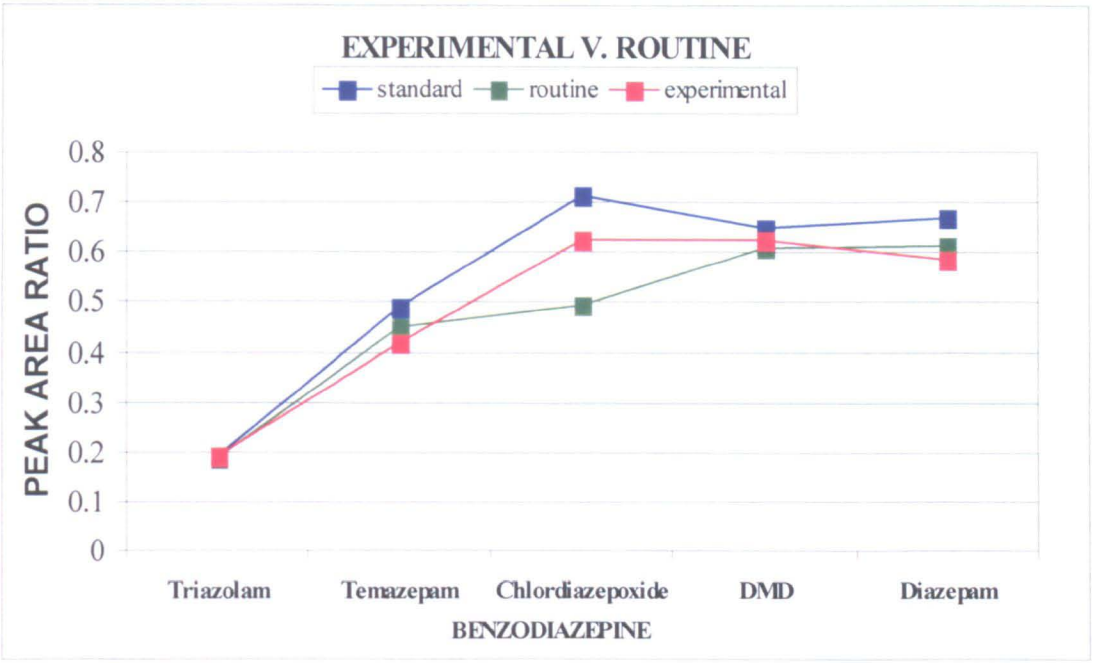
The routine and experimental methods were extracted and analyzed several times to find the average of the peak area ratios. The experimental method produced slightly better recover for all five drugs than the routine method. However, both methods are equally robust for the extraction of benzodiazepines from whole blood. Table 5.7 shows an average of the peak area ratios for the drugs.

Table 5.10 Comparison of the peak area ratios average between the two methods.

DRUG	UNEXTSTD	ROUTINE	EXPERIMENTAL
Triazolam	0.1929	0.1867	0.1930
Temazepam	0.4901	0.4485	0.4186
Chlordiazepoxide	0.7119	0.4934	0.6247
Desmethyldiazepam	0.6486	0.6101	0.6249
Diazepam	0.6672	0.6143	0.5833

The percent difference was calculated for each of the drugs from the results of both methods. The percent difference of triazolam between the two methods was 0.006, temazepam was 0.03, chlordiazepoxide was 1.3, desmehtyldiazepam was 0.014 and diazepam was 0.031. Figure 5.14 shows a graph based on Table 5.10 of the comparison between the routine and experimental method. The graph demonstrates that in comparison to the unextracted standard, the experimental method compares slightly better than the routine method, however, a correlation does exists between the two methods.

Figure 5.14 Comparison between the unextracted standard, routine and experimental method for the extraction of benzodiazepines in whole blood.



Several authentic forensic case samples were analyzed using both the routine and experimental method and the results compared. Table 5.11 shows eleven case samples that were analyzed using the two methods for experimental validation.

It can be seen that the results from the case samples extracted by the SPE experimental method developed for benzodiazepines, correlates well with those obtained from the routine SPE method currently used. Positive results for each drug were detected using the routine method and the experimental method with relatively close corresponding levels. Those that were listed as NA (not available) was because either the sample was negative or there was not enough sample for re-analysis.

Table 5.11 Comparison between the experimental SPE method and the routine SPE laboratory method using eleven whole blood case samples.

Case Samples	Routine Method mg/ml	Experimental Method mg/ml
T96-A	oxaz 0.13, dmd 0.22, diaz 0.25	oxaz 0.23, dmd 0.56, diaz 0.71
T96-B	dmd 0.44, diaz 0.25	dmd 0.56, diaz 0.73
T96-C	tem 0.35, dmd 0.55, diaz 0.26	tem 0.56, dmd 0.4, diaz0.66
T96-D	dmd 0.51, diaz 0.44	dmd 0.89, diaz 0.62
T96-E	dmd 1.1 diaz 1.58	dmd 1.35, diaz 2.45
T96-F	tem 2.79, dmd 1.29	tem 2.02 dmd 0.9, diaz 0.57
T96-G	NA	diaz 0.44
T96-H	tem 0.31, dmd 0.35, diaz 0.14	tem 0.20, dmd 0.24, diaz 0.10
T96-I	tem 0.87, dmd 0.18, diaz 0.08	tem 0.35, dmd 0.13, diaz 0.10
T96-J	tem 0.38, dmd 0.55, diaz 0.64	tem 0.29, dmd 0.58, diaz 0.71
T96-K	NA	tem 0.21

*NA = not available, tem = temazepam, dmd = desmethyldiazepam, diaz = diazepam, oxaz = oxazepam

Most of the samples were positive for temazepam, desmethyldiazepam and diazepam. An explanation for this is that temazepam and diazepam are the most widely abused benzodiazepines in the West of Scotland and are also mixed with other drugs of abuse, therefore more commonly found. Desmethyldiazepam is the major metabolite commonly identified where benzodiazepines have been abused, therefore encountered frequently. The overall average percent difference between the two methods for oxazepam was 0.1, temazepam was 0.34, desmethyldiazepam was 0.22, and diazepam was 0.30.

5.8 CONCLUSION

The aim of this study was to optimize and examine the applications of solid-phase extraction as a sample preparation technique for the screening of benzodiazepines in whole blood prior to HPLC analysis.

It was found that Bond Elute Certify® LRC SPE columns, were ideal for the elution of benzodiazepines. The method developed consists of suitable quality solvents in the sample preparation, column wash and conditioning steps to suit the group of compounds of interest. The effect of pH on the recovery of the benzodiazepine is very important during extraction, because basic drugs are recovered most efficiently at alkaline pH conditions. Visual inspection and description of the extract showed that the chosen eluent was the best elution solvent, which gave clean extracts and the least amount of interference peaks during analysis.

The Limit of detection and quantification was estimated to be 7 ng and 60 ng for the detection of benzodiazepines in whole blood. All of the benzodiazepines were difficult to accurately quantitative below 60ng, this was mostly because of the baseline noise. This method also produced good run to run reproducibility with overall recoveries better than 80% for each of the drugs.

This method provides a reliable rapid and reproducible technique for the isolation of benzodiazepines from whole blood. Comparison between the routine method and the optimized method using authentic forensic samples shows good correlation between the two. This indicated the potential usage for the developed method for real case samples. Bond Elute Certify® columns selectively retains and elutes the analyte of

interest using a mixed-mode interaction mechanism, constituting ion-exchange chromatography and hydrophobic and polar interactions. The high percentage recovery renders this method suitable for the detection of benzodiazepine, down to low concentration levels.

6. HAIR

6.1 LITERATURE REVIEW

6.1.1 INTRODUCTION

The analysis of human hair is purported to provide evidence of the use or lack of use of drugs of abuse and to some extent, estimation of the time of drug exposure. It has been used as a marker to toxicologists for more than a hundred years. Hoppe in 1857 published Caspers, "Practisches Handbuch der gerichtlichen Medicin", which reported the analysis of hair for the detection of poisons.[169] This translated means, Practical Guide to Legal Medicine. He investigated the presence of arsenic found in the hair from a body exhumed after 11 years. Nearly 100 years later, Goldblum *et al* in 1954, was the first to publish a report on the detection of barbiturates in guinea pig hair.[170] By 1979, Baumgartner published a report on opiates in hair analysis that has shaped the view of hair anaysis.[171]

Since then, over the last decade, there has been an accumulation of experimental data on the analysis of hair for trace elements and drugs. [172-176] This data demonstrated that drugs found in body fluids (e.g. blood or urine) can also be found in hair. Recent advances in drug testing have led to the development of new drug testing methodologies for the detection and measurement of drugs of abuse in hair. More than 400 clinical and forensic toxicology articles have been published on hair analysis. These methods show promise of providing drug exposure information over a wide window of time than is produced for traditional methods.

Hair analysis is a noninvasive technique, involving a relatively simple collection technique. It involves clipping strands of hair from the head or other regions of the body and extracting drugs from the hair matrix. The hair sample itself is stable indefinitely, alleviating many of the preservation storage and transportation problems associated other biological materials.[177] Due to the unique ability of hair to retain drugs for long periods of time, hair testing can provide a wealth of information of an individuals recent past.[178] A precise understanding of the mechanisms involved in the incorporation of drugs into hair is critical for forensic scientists to accurately interpret and understand the results of hair analysis in drug testing. There are basic structural similarities between hair of different color, ethnic origin and body region. The fundamentals of hair composition, anatomy and physiology a have been summarized in articles by Harkey[179] and Cone and Jospeh.[180]

Currently, hair testing is applied to various situations as a means of monitoring drug usage or exposure. This has been confirmed by several independent laboratories around the world.[181-185] The mechanisms of drug incorporation in hair are not fully known, with limited information on the physiochemical characteristics of how drugs bind to hair.[181] It has been suggested that drug incorporation into hair may depend on ethnic hair type, color and treatments. However, further research is necessary to clarify these issues which may cause any potential bias in hair testing.

6.1.2 ANATOMY OF HAIR

Hair is a complex fiber or tissue whose structure and biology are only partially understood. It is an epidermal outgrowth composed of cylindrical structures or shafts made up of matrix cells that grow from small sac-like organs called follicles. It can

also be described as an annex of skin originated from the hair follicle where the germination center is formed by matrix cells. Hence, these cells form different layers of the hair shaft for example the cuticle and medulla. The cells actively proliferate in the root, while residual metabolism is negligible in the hair shaft.

Hair consists of the definite morphological components; cuticle, cortex, medulla, melanin granules and cell membrane complex, each distinct in morphology and chemical composition. Sulfur rich proteins known as keratin form long fibrils in the shaft and are also a main component contained in hair. Keratin is tightly bound through the replacement of S-H bonds with S-S bridges that produce a tough, highly stable structure.[179, 180]

6.1.3 PHYSIOLOGY OF HAIR

During the growth of hair, moving in an upward direction, morphological and structural changes of the matrix cells occur to form different layers of the hair shaft. They may also acquire pigment in hair cells as they differentiate to form individual layers of hair that determine the color of the hair shaft. Differences in the type and quantity of proteins and pigments can be distinguished in individual layers of hair. These differences determine which structural components and layers of hair are responsible for binding drugs.

6.1.4 HAIR FOLLICLE

There are approximately 80,000-100,000 hair follicles on the human head with a decrease in the number with age.[186] The hair follicle is a highly complex skin appendage embedded 3-4mm below the epidermal epithelium of skin. They are

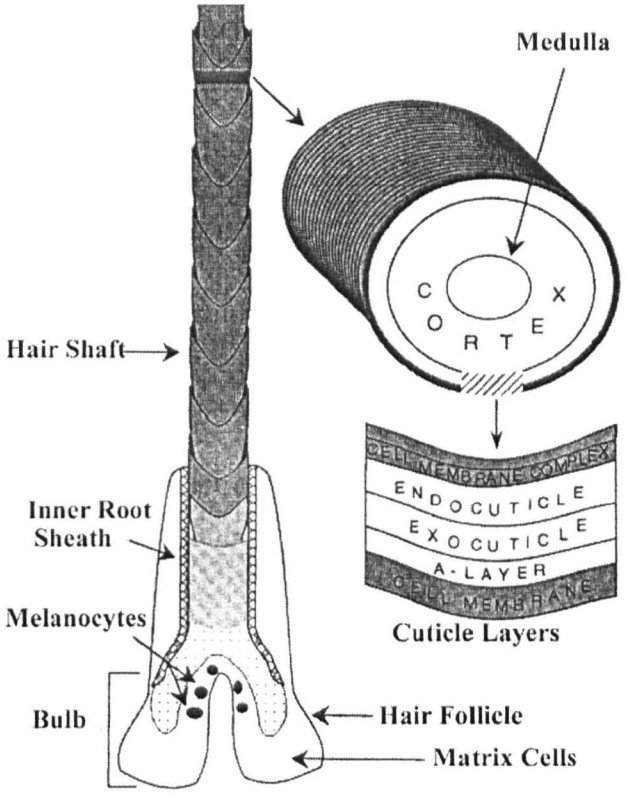
closely related to the apocrine and sebaceous glands that empty into the follicle and consist of epithelial (matrix and outer root sheath) and dermal (dermal papilla and connective tissue sheath) tissues.[187]

6.1.5 STUCTURE OF THE HAIR SHAFT

Regardless of hair color and ethnicity, there are basic structural similarities among all hair types. The diameter of individual hair shafts may range from 15-120 μm depending upon the type of hair a region of body the follicle is located.[179] The cuticle is the outermost layer of hair where the alpha (A) layer is located beneath the cell membrane and the exocuticle between the A-layer and the endocuticle. The cortex is located between the cuticle and innermost region, the medulla.[179, 186] The cortex forms the bulk of the hair shaft and is located immediately beneath the cuticle.[181] The medulla is small in relation to the cortex and consists of scattered cells and hollow spaces that may be continuous, discontinuous or absent in adult hair.[180, 184]

Melanin is among the most frequently occurring pigment in nature.[188, 189] It is synthesized in melanosomes that are discrete organelles located within melanocytes.[187] Melanin granules are the cell components of melanocytes situated in the upper portion of the hair bulb.[189] Melanosomes are transferred by the melanocytes to different cortical and medullary cells during growth as these cells move upward from the hair bulb to form the hair shaft.[190] Hair cells that form the hair shaft do not produce melanin.[189] Hair color depends on the number of melanin granules present, their size, arrangement and distribution in the hair shaft and their melanin composition.

Figure 6.1 Illustrates the anatomy of hair follicle and hair shaft.[191]



The hair shaft consists of three main functional zones, the innermost zone, the keratinogenous zone and the region of permanent hair. The innermost zone is the site of biological synthesis of hair cells in and around the bulb. Hardening and solidation of the hair and keratinization of the matrix cells occurs in the keratinogenous zone directly above the bulb. Fibrils fused by intercellular binding are composed of dehydrated cornified cells in the hair shaft, becoming the region of permanent hair.

6.1.6 CHEMICAL COMPOSTION

Hair is composed of approximately 1-9 % lipids, small quantities of trace elements, polysaccharides and water and 60-95 % protein, with the majority of sulfur-rich protein referred to as keratin.[179, 183] Although different in each layer, keratin is comprised in the cortex, cuticle and medulla in large amounts to form long fibers in

the hair shaft. High concentrations of cystine are contained in the keratin to form disulfide bonds that link the A-layer to the exocuticle. This makes the cuticle tightly bound together and protects the cortex from damage. This results in a very tough, highly stable structure.

In the cortex, keratin is comprised of 85 % or more of the mass of the hair shaft and two types of structural protein, matrix and fibrous proteins.[192-195] Matrix proteins are nonhelical structures with a high sulfur content and polypeptides, whereas fibrous proteins are helical structures that incorporate into matrix proteins with a low sulfur content. The structure, arrangement and ratio of matrix and fibrous proteins contribute to the physiochemical properties of keratinous tissues.[196-197] Fibrous proteins aid in the production of the cortex and the medulla in the keratogenous zone.

6.1.7 GROWTH CYCLE OF HAIR

Hair grows in cycles, alternating between periods of growth and quiescence. It has been stated that hair grows at a rate of 1cm per month or 0.5 inches per month. However, precise values for human hair growth rates are difficult to obtain. This is because the anatomical location and age of hair and growth does not occur all at once. Anatomical location and hair type are the most important factors determining growth rate, which is somewhat dependent on race, sex and age.

Literature reports scalp hair grows faster than pubic hair or axillary hair that grows faster than beard hair. Reported hair growth rates for scalp hair are between 0.2 mm/day and 1.12 mm per day.[179] There are three phases of hair growth, anagen (growing stage), catagen (transition stage) and telogen (resting stage).

6.1.7.1 Anagen Stage

The onset of the anagen stage starts the hair growth by increasing the metabolic activity of the matrix cells. The longer the stage, determines the length and type of hair.[187] The follicle becomes embedded into the dermis caused by continuous cell division that stimulates new hair growth as the cells elongate to form a thin filament. This activity causes the hair cells to form the cortex, cuticle and medulla region of the hair. At this time the keratinization process begins and this is where drugs and trace elements are incorporated into hair.[198]

6.1.7.2 Catagen Stage

During this transitional phase, the cell division taken place in the anagen stage is finished resulting in the base of the shaft becoming keratinized. A dry white node is formed followed by the degeneration of the bulb and the follicle shortens.[187]

6.1.7.3 Telogen Stage

This is known as the resting phase where the growth of the hair shaft stops making it easier for it to be pulled out or plucked retaining the white node. Depending on the area of the body, the percentage of hair that is growing varies compared to the percent resting.

The possible length of the hair and the density of a scalp depend on the duration of these stages and rate of growth. The duration of these stages varies from person to person, even in the scalp of one person. Each hair follicle has its own growth cycle, the longer the hair the longer the growing phase. Approximately 15% of scalp hair is at rest and the remaining 85% growing.[180, 199]

The duration of this process can be affected by a number of factors such as pregnancy, disease or nutritional deficiencies. Hair in the vertex region of the scalp is usually chosen as the best specimen, as it has the largest amount of hair in the telogen phase and the fastest growth rate.[179]

6.1.8 COMPARISON OF HAIR TYPES[179]

There are three main types of hair specimens that can be collected for analysis they are scalp, beard and pubic hair. Scalp hair has the highest growth rate of all the hair types. There are considerable variation in various regions and the growth rates in scalp hair. These variations make the choice area of sampling very important. Scalp hair is also exposed to external contamination, sweat and cosmetic treatments that can cause changes to its composition.

Beard hair has the slowest growth rate and can be exposed to external contamination and sweat like scalp hair. It is less likely to be contaminated through the sebaceous gland ducts because the hair shaft is in a separate channel of exit, where the hair shaft of scalp hair is directly exposed to the sebaceous ducts.

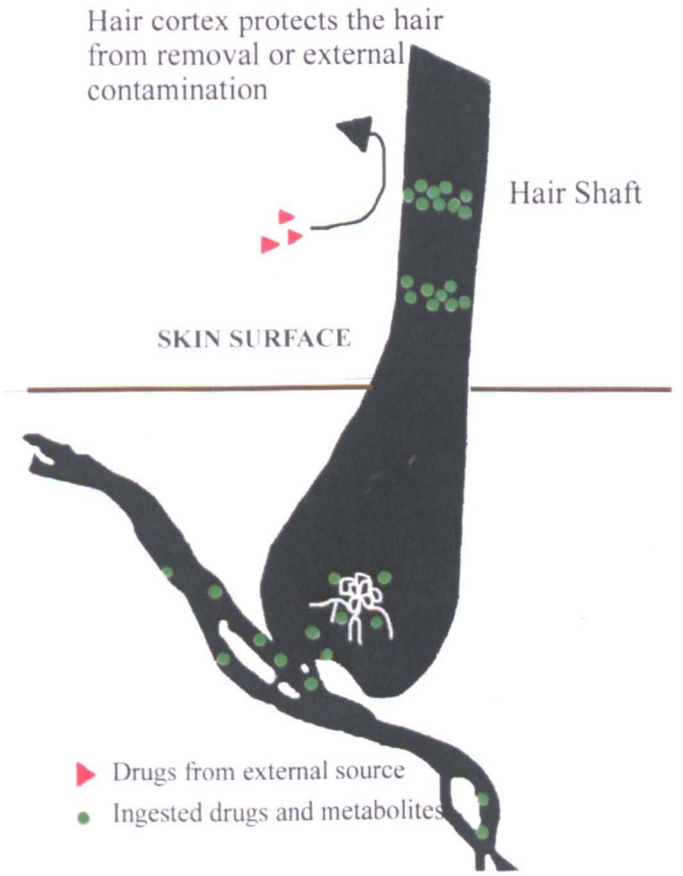
Pubic hair grows slower than scalp as well and has a longer resting period. It is less likely to be exposed to external contamination or chemical treatments, however it can be contaminated through sweat and urine. Segmental analysis can be very difficult with pubic hair as opposed to scalp hair because it is curled rather than straight.

6.1.9 MECHANISM OF DRUG INCORPORATION IN HAIR

The mechanism of drug entry into hair and the specific sites of drug sequestration is complicated and likely to involve a number of propertied processes. Several modes of entry suggested are: [200]

- Passive diffusion from blood
- Drug excretion in sweat with subsequent transfer to hair
- Drug excretion in sebum with subsequent transfer to hair (the oily secretion from sebaceous gland)
- Entry into hair from external environment

Figure 6.2 Drug entry into hair via the bloodstream[191]



By the ability of hair to directly absorb the drug, contamination by direct environmental exposure should be reasonably excluded if hair results are to be used. For example, nicotine is found in the hair of non-smokers and cocaine is found in the hair of children of cocaine users.[191, 201]

Baumgartner *et al*, have postulated that drugs enter the hair via the blood stream in direct proportion to their concentration.[202] However, recent studies have indicated that drug entry is likely to occur by passive diffusion from the blood stream into the growing hair cells at the base of the follicle. They then become bound in the interior of the hair shaft during subsequent keratogenesis (protein synthesis of the follicle as matrix cells move up the shaft).

The drug incorporation into hair is dependent on the drug concentration in blood that is dependent on the dose of drug ingested. Good correlation can sometimes be seen between the concentration of the ingested drug and the concentration of drugs in hair, while others correlate badly.[203, 204] This may be due to external contamination or inter-subject variability.

6.1.9.1 Sweat Transfer

There have been various studies to determine the contribution of drugs in sweat to drugs in hair. Drugs of abuse in concentrations often higher than in blood, have been found in sweat.[200] However, there are many factors that can influence the amount of drugs found in hair due to sweat contributions. Inter-individual differences of drug levels in subjects consuming known amounts of a drug may be an explanation. In addition, the fact that drugs are dispersed over a large area of the hair shaft can

conclude the excretion of sweat. For some individuals, sweat may be an important vehicle for the transfer of drugs into hair.

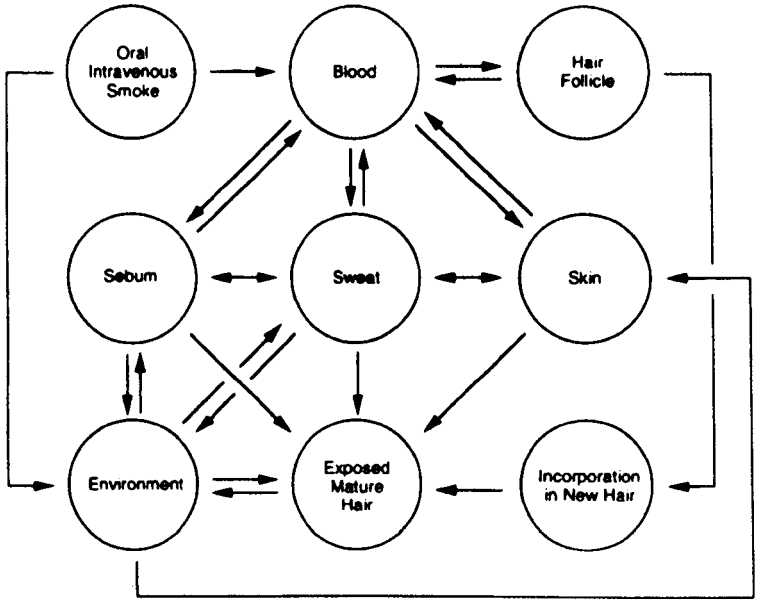
6.1.9.2 Skin Transfer

Another possible transfer of drugs into hair is through the skin. The skin can act as a reservoir for certain drugs, especially lipophilic drugs because of its two layers, epidermis and subcutaneous layer.[200] Studies have revealed that when drugs penetrate from the external environment across the skin, they are retained by the lipid layers of the outer layer of the epidermis and are released very slowly into the bloodstream. From the bloodstream, the drugs are retained primarily by adipose tissue of the subcutaneous layer and the outer layer of the epidermis where they surround the hair follicle and its associated glands.

6.1.9.3 Environmental Exposure

There are a variety of ways hair is exposed to environmental contaminates. Cosmetic treatments (e.g. bleaching), smoke (e.g. cigarette or drug exposure), or air (e.g. pollutants) are all possible contaminates which make it difficult to establish a concentration baseline for trace elements in hair.[200] External contamination could be a possible route of entry into hair for drugs that are smoked, therefore, various surface decontamination methods have been developed to eliminate such contamination before analysis.

Figure 6.3 Diagram of drug incorporation into hair showing drug transfer from potential pathways.[205]



6.1.10 FACTORS AFFECTING RETENTION OF DRUGS IN HAIR

There are a number of factors that affect retention of drugs into hair. From a chemical point of view, some factors for drug incorporation into hair are melanin affinity, lipophilicity, basicity[206] and pH[191].

6.1.10.1 Melanin

Scientific data show that melanin plays an important role from drug incorporation into hair.[205, 206] Studies have confirmed that in human hair, drug concentration in pigmented hair was higher than in nonpigmented hair.[207-210] Hair color is just one factor which affects binding of drugs. Studies have concluded that black hair from African and Asian people, tend to retain higher levels of drug than weakly pigmented Caucasian hair.[191, 210]

Melanin may also be considered to behave like weak cationic exchange polymers that form simple ionic bonds with metals and chemicals.[180] This may possibly explain why drugs with cationic properties such as cocaine, amphetamine and opiates appear to be bound and retained by hair to a greater degree in comparison to anionic compounds such as aspirin and tetrahydrocannabinol (THC).[180]

6.1.10.2 Lipophilicity

Lipophilicity of drugs is another key factor for drug incorporation into hair. An example is cocaine and 6-acetylmorphine being incorporated better than benzoylecgonine and morphine.[211, 212]

6.1.10.3 Basicity

Another factor that affects the uptake and/ or retention of drugs in hair is the basicity of the drug. As the pH of hair is slightly acidic, it is more likely for acidic drugs to have lower concentrations in hair than basic drugs.[191, 199] Nakahara 1995, found that the pKa of amphetamine analogues plays a significant role in its drug retention in hair.[206]

6.1.10.4 Cosmetic Treatment

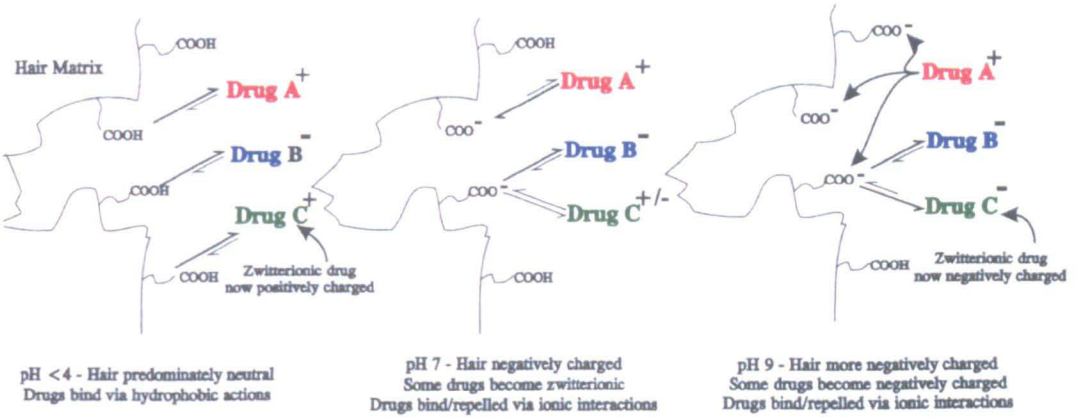
The everyday treatment of hair has a significant affect on the retention of drugs in hair. Controlled experiments have shown that with frequent use of shampoo, hydrogen peroxide and conditioners contributes to losses of some drugs in hair.[213-215] Bleaching hair also reduces the concentration of drugs such as heroin and cocaine metabolites in hair.[215]

6.1.10.5 Effects of pH in Hair[191]

Hair contains three major functions whose characteristics can vary as a function of pH (i.e. side chains of aspartic acid, glutamic acid and sulfate groups produced by oxygen degradation of the amino acid cystine). Chemically treated or damaged hair contains sulfate groups that have a lower isoelectric point than pH4. At pH 4 (acidic), drugs bind through weak hydrophobic interaction provided they have similar hydrophobicities and charge as hair. The pH of hair can produce a change in the charge of the hair, drug and hydrophobicity of both the drug and hair, which determine the relative uptake of drugs into hair.

Positively charged drugs at a low pH (pH 4) bind less well than neutral or negatively charged drugs. If the hair has a pH higher than its isoelectric point (pH 7), aspartic and glutamic acid side chains become deprotonated and hair becomes negatively charged and some drugs (i.e. benzoylecgonine) become zwitterionic, some bind and some repel via ionic interaction. Hence the more positively charged the drug, the more the binding. Increasing the pH further (pH 9), more side chain carboxyl groups are deprotonated and hair becomes more negatively charged. Hence, some drugs become negatively charged and are bound or repelled via ionic interaction.

Figure 6.4 A model of the drug incorruption into the hair based on pH.[191]



6.1.11 DRUG CORRELATION STUDIES

There are numerous studies on the correlation between drug intake and the concentrations found in hair. Positive correlations have been reported by Baumgartner and co-workers using self reported drug use history and concentrations of either heroin[214], marijuana[216], cocaine[217] or PCP[218] in their hair. However, these studies compared immunoassay data with self-reported drug intake. Other methods (e.g. GC-MS) have more recently been used to show positive correlation between dose and the amount of drug in hair.

In a five subject study, Nakahara *et al* found good correlation between the dose of methozyamphetamine and the concentration of drug in the hair.[219] Similarly Cone *et al* using two subjects, found positive correlation between the dose of opiates and the concentrations in beard hair.[220] In contrast, Puschel *et al* analyzed hair from carcinoma patients receiving morphine doses and found poor correlation between the dose and drug concentration in hair.[221]

Other studies for cocaine have suggested that increased cocaine dose leads to increase in the concentration found in hair. However, inter-subject variability is so great that to estimate the drug amount administered based on the hair drug concentration is very difficult. Further, this variability could not be explained by plasma pharmacokinetic difference because of relatively small differences in pharmacokinetic parameters not sufficient enough to explain the large differences in the hair drug concentrations.

6.1.12 PARENT DRUG-METABOLITE RATIOS

Drugs of abuse are metabolized by the liver to form polar metabolites readily eliminated from the body via urine. The chemically nonpolar parent drug is rapidly metabolized compared to urinary excretions. However, urinary excretion of the chemically polar metabolites is more rapid and extensive than the parent drugs.

Generally, finding drug metabolites in body fluids are used to prove the ingestion or injection of a drug into the body, since the bodys metabolism is responsible for the formation of the metabolite. The plasma drug metabolite concentration (or ratio between parent drug and metabolite) is also used to differentiate between acute and chronic drug administration. However, these drug distribution generalities do not always hold when hair is the matrix.

Current studies clearly show high parent drug to metabolite ratios found in hair. Goldberger *et al* have reported high metabolically labile drugs in hair like heroin and its metabolite 6-monoacetylmorphine.[222] Cocaine with its relatively short half-life has also been studied as the primary analyte in hair. It showed approximately 6-fold

and 10 fold greater concentrations in hair than its primary metabolites benzoylecgonine and ecgonine methyl ester.[223-225]

6.1.13 DECONTAMINATION OF HAIR

There are a number of published surface decontamination methods for hair. It is not clear which technique offers any real advantage over any other, but all are considered suitable for reducing surface contaminants. Several methods include the use of sodium dodecyl sulfate (SDS)[172], acetone[213], water and acetone[226], methanol[174], ethanol[175], and dichloromethane[176]. Wash procedures are used to remove external contamination and are based on the following properties of hair with respect to exogenous incorporated drugs. These properties have been established by earlier studies.

- Cocaine, opiates, amphetamines, marijuana and phencyclidine and their metabolites do not bind strongly to keratin proteins and can be removed by washing.
- Through contact with hair, metabolites can not be formed from the original drugs.
- Endogenous and exogenous deposition of drugs in hair involve three domains, surface, accessible interior and inaccessible interior domain.[227]

i. **Surface Domain:** Located at the surface of the hair, this domain is readily accessible to drug incorporated by external contamination. However, because they are loosely bound to the hair, they can be easily removed by washing solvents.

ii. **Accessible Interior Domain:** Located in the interior structure of the hair, this domain resists the penetration of exogenous drugs through hair swelling solvents such as water or possible perspiration. However, as a result the drugs can also be removed by swelling the hair using water. Since the accessible interior domain is smaller in comparison to the inaccessible interior domain and contains majority of the exogenous drugs, the loss of endogenous drugs from the accessible domain through washing is not very significant to the overall detection of drug in hair.

iii. **Inaccessible Interior Domain:** Located in the interior of the hair, this domain can not be contaminated by exogenous drugs or washed out using wash procedures. It is a relatively large domain and appears to depend on the type and condition of hair (e.g. thick or thin, porous or nonporous). Endogenous drugs are only released though digestion and extraction of the hair.

6.1.13.1 Wash Procedures

There are two main wash procedures used in the pre-treatment of hair, they are extended and truncated wash procedures. Continual washes carried out until a plateau drug concentration is reached is termed extended washes.[184] Fixed wash procedure over a fixed length of time is known as truncated wash procedures. This wash procedure can be used for mass sample production, where extended procedures are used for individual forensic samples.

General wash procedures consist of first a cleansing wash with a detergent (e.g. SDS), then a solvent wash with an alcohol (e.g. MeOH, EtOH), followed by an aqueous and final solvent wash. Washing with an alcohol ensures that all of the oils

incorporated in hair do not interfere analysis and to remove loosely adhering drugs from the surface. The aqueous wash causes the hair to swell and in turn allows penetration into the interior. A final solvent rinse step is advisable to help the hair dry. These steps can be repeated before the final rinse step to ensure full surface decontamination.

6.1.14 ANALYTICAL METHODS

The isolation of drugs in hair has become of increasing interest to the analytical chemist, with numerous extraction techniques being published. A typical analysis is usually in four stages, washing the hair of any exogenous contaminates, the pre-treatment (e.g. solvents, hydrolysis), extraction of the sample (e.g. SPE, LLE, SFE) and analysis (e.g. GC/MS, HPLC).

Treatment of the hair without solubilisation (e.g. methanol or enzymes) can lead to reduced extraction yield. A ball mill is commonly used to pulverize hair followed by extraction into methanol or dilute buffer that helps to minimize drug loss. Despite intentions, variability in drug hair concentrations are affected by the washing and extraction method chosen. Therefore, the method of choice should always be taken into consideration when evaluating hair drug concentration data.

An alternative extraction procedure to conventional techniques is the use of SFE for the extraction of drugs of abuse in hair. With the use of SFE, the aforementioned preparative wash, pre-treatment and extraction steps can be carried out using the same apparatus, minimizing potential problems associated with other techniques.[228]

6.1.15 HAIR ANALYSIS USING SFE

During SFE, the rate of extraction is due to the solubility of the analyte in the supercritical fluid and the transfer of the mass of the solute outside of the matrix. Diffusion of the analyte depends on the particle size, therefore in hair analysis, the pulverization of hair will increase the recovery of the drug in interest. With the addition of a more polar modifier subsequently increases the recovery even more.

The extraction of drugs of abuse in hair using SFE was first reported by Sachs and Uhl in 1992.[229] Sachs was also the first to quantitatively determine opiates in hair using SFE techniques and GC/MS analysis.[230] Further studies by Edder *et al* [231] and Morrison *et al*[232] also investigated the possible use of SFE for the selective recovery of drugs of abuse from hair. More recent investigations by Cirimele *et al* in 1995 reported a methodology for the extraction of opiates from hair with investigations on the effective parameters that influence recovery.[233] Staub and co-workers in 1996 modified the method by Edder for the extraction of opiates and methadone.[228]

6.1.16 CONCLUSION

Hair testing for drugs of abuse can provide useful information for questionable drug usage. It is a noninvasive technique that provides an extended analysis time window for drug detection and can not be adulterated, unlike urine or blood. Several publications have proposed various wash criterias to distinguish active from passive exposure. However, there is no set criteria as yet.

The extraction of drugs from the hair is another important variable besides decontamination. Not all extraction solutions can remove all the drugs from hair,

therefore, dissolution or pulverization is necessary to ensure removal for analytical purposes. Already hair analysis has proved useful in a variety of applications. However, further research in this field is necessary to establish definitive data regarding some of the unanswered issues for hair analysis.

7. AMPHETAMINES IN HAIR

7.1 LITERATURE REVIEW

7.1.1 INTRODUCTION

Most of the literature written dealing with the detection of amphetamine in hair has come from Japanese researchers. In the past, target drugs have been amphetamine (AP) and methamphetamine (MA), however, more recently methylene-dioxyamphetamine (MDA) derivatives like methylenedioxymethamphetamine (MDMA) are of particular interest.

Nakahara published a detailed review in 1995 on the detection of amphetamines in hair.[234] Techniques published before 1990 used acid or alkaline hydrolysis or a combination of hydrochloric acid and methanol followed by SPE or LLE and derivatised. Recently Rohrich and Kauert developed a screening procedure for the simultaneous detection of AP, MA, MDA, MDMA and MDEA based on methanol sonication with methaqualone used as the internal standard.[235] Kintz and Cirimele compared four different procedures for AP, MDA and MDMA (methanol sonication and acid, alkaline and enzymatic hydrolysis) where alkaline hydrolysis was determined to give greatest recoveries.[236]

The detection of amphetamine and ecstasy in hair is becoming of growing interest in the forensic laboratory. Since Moeller *et al* first identified MDMA in human hair, it has become one of the most frequently identified, especially in Europe.[237] Therefore, it is included in all screening procedures.

7.1.2 HISTORY

The Substance MDMA has been said to have first been synthesized as early as 1898[238], although, it was patented by the German pharmaceutical company E. Merck in 1914. It was allegedly for the use as an appetite suppressant for the German army, however, because of the side effects, its use was discontinued. Merck also synthesized MDA (3,4-methylenedioxyamphetamine) which is closely chemically related to MDMA. MDMA reappeared in 1977 in the pharmacopoeia when a Californian chemist, Alexander Shulgin synthesized MDMA in his laboratory. Shulgin is known as the father of ecstasy, but refers to himself as the stepfather as he only rediscovered MDMA. He has also synthesized and tested 179 different phenethylamines and may other psychoactive drugs.[239]

The substance known as "ecstasy", has become a prevalent hallucinogenic drug associated with the "rave scene" or described as the pre-eminent youth movement of the decade. However, ecstasy is not a new drug, it is also know as MDMA (3,4-methylenedioxymethamphetamine). MDMA belongs to a large family of hallucinogenic substances known as phenethylamines.[239]

Ecstasy has been incorrectly described as a "designer drug" because MDMA is a synthetic combination of hallucinogens and amphetamines.[240-242] Smith and Seymour contend that, MDMA is not a designer drug, quoting Donald R. Wesson's definition of a designer drug *as "where the defined psychoactive properties of a scheduled drug have been retained, but the molecular structure has been altered in order to avoid prosecution under the Controlled Substance Act".*[243]

Today drugs purported to be ecstasy, may not necessarily contain only MDMA, but adulterants or MDMA related drugs in combination to produce similar effects.[244-246] A wide variety of combined drugs known as cocktails of MDMA and other psychoactive substances exist, that produced similar effects.[243]

7.1.3 PHARMACOLOGY

As a group, amphetamines are relatively strong bases with pKa values ranging from 9.5 to 10. High pKa values account probably for the significant urinary elimination as unchanged drugs.[247] They are lipid soluble as free bases, but water soluble as the hydrochloride or sulfate salts, which are the common dosage forms. Following ingestion the compounds are transferred to the brain rapidly and appear to localize in this tissue relative to plasma. The short term and side effects of MDMA have been documented well, with consistent clinical, laboratory and self-reported findings.[248-253]

The initial effects common to MDMA include an increased pulse rate and blood pressure, peaking one half to three hours following ingestion.[248] These effects are greatest with the highest levels of dose to body weight. Blood pressure is eventually reduced to below pre-ingestion levels after approximately six hours.

MDMA is a monoaminergic drug which are a group of neurotransmitters, like serotonin (5-HT2 (5-hydroxytryptamine)), dopamine and noradrenaline.[254] The main function of serotonin is to control mood. Serotonin is pumped out of the neuron by transporter proteins into the synapse, then taken back into the same neuron by the transporter protein. This process helps to continuously replenish the neuron with serotonin, keeping the signal brief.[254] MDMA works by binding to these transporter

proteins and preventing the reuptake of serotonin. A full review of the neurotoxic effects of MDMA is given by McKenna and Peroutka.[255]

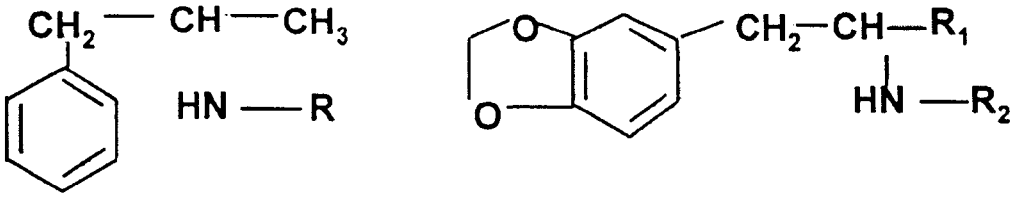
Peroutka, *et al* conducted a study using five recreational MDMA users for any abnormalities in serotonegic, dopaminergic and adrenergic activity.[256] Extraction and analysis of the lumbar cerebrospinal fluid taken from the subjects found no evidence suggesting long term neurotoxic effects in humans. However, it is possible the MDMA may cause neurotoxic effects to some extent similar to other amphetamine derivatives and drugs. If such damage occurs, it may be acute and reversible, with the possibility of long term damage.

The after effects of MDMA are very similar to those associated with amphetamines. Users have reported complaints of insomnia, muscle aches and jaw.[248] With more sever reactions, paranoia, weight loss, exhaustion, flash backs, jaundice, irritability and depression have been included.[257] Several other side effects of ecstasy use include loss of appetite and alertness, dry mouth, pupil dilation, tachycardia, nystagmus (eye wiggle), bruxism (teeth grinding) and trismus (jaw clench).

7.1.4 CHEMICAL STUCTURE

The basic chemical structure contained in speed and ecstasy tablets or powders are shown in Figure 7.1. The R in each structure represents where a substitute group should be place when synthesized. AP, R = H, MP, R = CH_3, MDA, $R_1 = CH_3$, $R_2 =$ H, MDMA $R_1 = R_2 = CH_3$, MDEA, $R_1 = CH_3$, $R_2 = C_2H_5$.

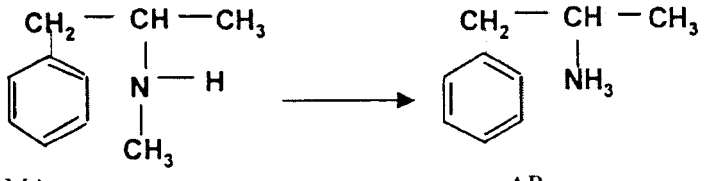
Figure 7.1 Structure of the drugs contained in "speed" and "ecstasy" tablets.



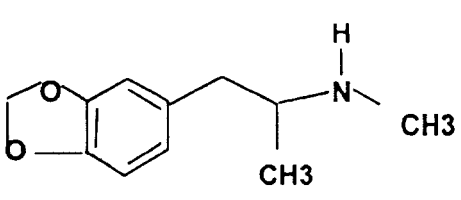
7.1.5 METABOLISM

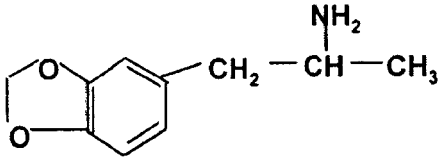
The methylenedioxy derivatives of amphetamine have received a great deal of attention because of the popularity of MDMA as an illicit recreational agent.[257] MDA and MDEA are both analogs of MDMA. MDMA is demethylated into MDA and then is demethylated into the catechol dihydromethylamphetamine (DHMA) by cytochrome P450.[247] MDMA being the most recognized metabolite of ecstasy, at single dose levels of 75-100mg is moderately effective, with effects experienced after 20-60 minutes after ingestion and lasting for several hours.[247]

Figure 7.2 The products of drugs formed during synthesis or when metabolized.

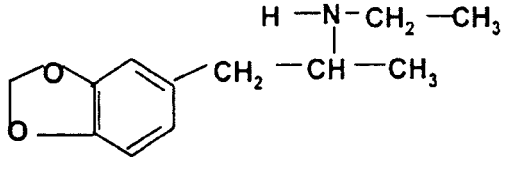


MA AP





MDMA MDA



MDEA

7.1.6 PHARMACOKINETICS

There are several reports on the pharmacokinetics available.[258-260] The half-life of amphetamine ranges from 7 +/- 1.2 to 11 +/- 2.1 hours. Approximately 30% of an amphetamine dose is excreted unchanged. Excretion in sensitive to urinary pH and by acidifying it (pH~5), the urinary excretion half-life becomes ~4.6.[258]

The half-life of methamphetamine in urine at pH 5 was determined to be ~4.9 hours. Under these conditions, 70 % of the dose is eliminated as methamphetamine therefore the condition of the urine determines the concentration of drug excreted. Studies involving the pharmacokinetics of MDMA enantiomers in rats, was

investigated by Cho *et al.*[261] The half-life of (+) isomers was found to be 74 minutes and that of the (-) isomer was 100 minutes.

7.1.7 ROUTES OF ADMINISTRATION

Amphetamines can be taken via various routes either orally, intranasal or intravenous. If the amphetamine is in its powdered form, then it can be sniffed, smoked or injected. If in capsule or tablet form, which most of the time MDMA is presented, then it can be taken by mouth. Illicitly manufactured amphetamine powders can be found on the illicit market and some capsules or tablets are produced for medical use. After cannabis, amphetamines are the most widely misused controlled drugs.

7.1.8 EFFECTS OF ECSTASY

7.1.8.1 Psychological Effects

Often feelings of euphoria and increased physical and emotional energy, empathy and personal insight have been reported by users.[246] MDMA does not produce hallucinogenic or disillusioned states, hence, if these symptoms are reported then it is likely that MDMA was not the only drug present.[238] Unlike most drugs, MDMA does not appear to produce psychological dependence. It has been reported that the more often MDMA is used, the less enthusiastic the user becomes about the drug.[262-263]

7.1.8.2 Social Effects

Experimental studies on the social effects that MDMA has on the users life, indicated varied response either positive or negative.[249] Some claimed that the drug improved their lives, enhancing personal communication skills even with strangers.[264] Ecstasy

has also been known to be an aphrodisiac, partly because of its association with MDA (the love drug) and its name "ecstasy". Buffum 1986 conducted a study of 76 users, which concluded that, the use of MDMA increased emotional closeness, but not sexual desire.[265]

7.1.9 CHROMATOGRAPHIC ANALYSIS

7.1.9.1 AMPHETAMINE HAIR ANALYSIS

Until recently, literature on the detection of amphetamines in hair has been published.[173, 184, 208, 234, 235] A large number of the literature published has been by Japanese researchers marking the prominence of amphetamine consumption in the Far East. In 1983 Ishiyama used GC/MS to identify methamphetamine, nicotine, amitriptyline and imipramine.[266] By 1984 Suzuki, published a method to detect amphetamine in a single hair.[267] In Genoa in 1993 standard screening procedures for the identification of amphetamine-like designer drugs were easily identified.[268] More recently Nakahara published an informative survey detailing the interpretation of amphetamine in hair.[269]

7.1.10 CONCLUSION

In the UK, ecstasy tablets have been found to contain a range of stimulants (e.g. amphetamine, caffeine) without containing the psychoactive ingredients or variants of the MDMA compound (e.g. MDEA). Some tablets have been found to contain the anesthetic ketamine and some variable amounts of the active ingredient. Therefore, it is unlikely that consumers will be aware what substance they are consuming.

In addition to this, consumers usually takes a combination of recreation drugs as adjuncts to the ecstasy tablets and therefore, it is not possible to attribute their behavior unequivocally to MDMA. Although studies have attempted to measure the extent of ecstasy-related harm and the prevalent use; there is still a need for more research on MDMA or ecstasy and its effects.

7.2 CURRENT METHODOLOGY

There are numerous methods for amphetamines and at present the method of extraction employed in the routine laboratory is solid-phase extraction for biological samples. There are several methods that have investigated the extraction of amphetamines in hair. However, not many include the investigation of a correlation between the self-reported use of speed and ecstasy consumed and levels found in hair.[173]

The worldwide monitoring method is used for the extraction of amphetamines from blood and urine in the routine Laboratory.

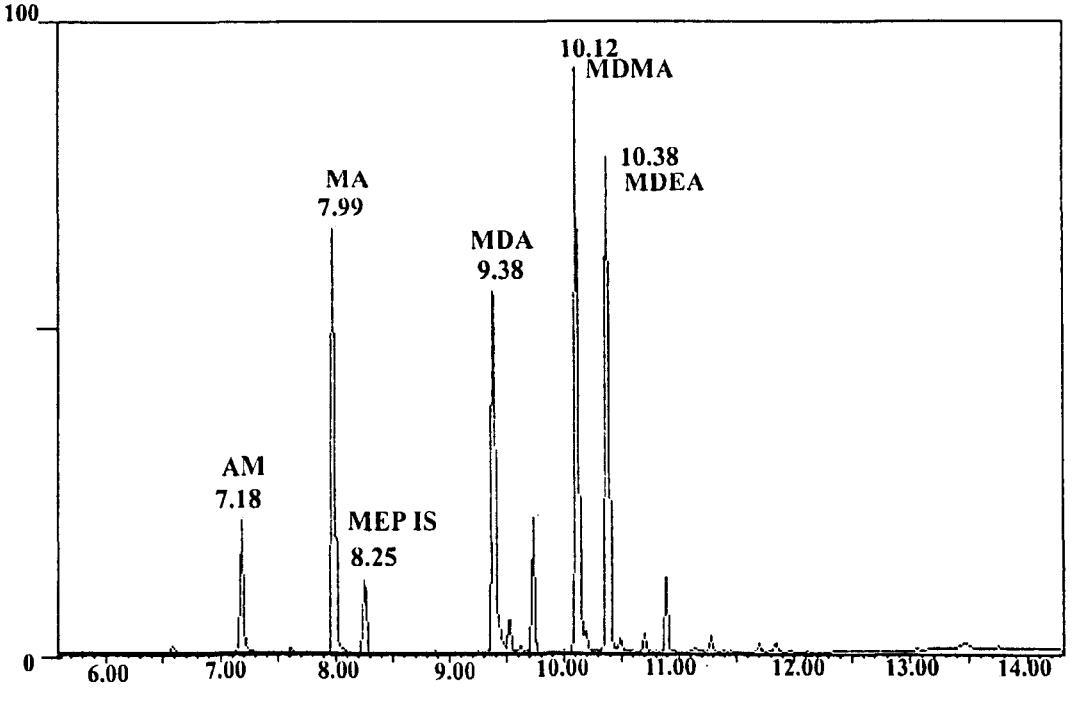
- *Blood sample pre-treatment*: To a vial 1 ml of blood or urine, 100µl of internal standard, (d_3-amphetamine) and 2 ml of 100mM phosphate buffer pH 6 were added.
- Column conditioning: The column was conditioned with 3 ml of MeOH, 3 ml of de-ionized water and 1 ml buffer. The sample was then added to the column
- Column wash: The column was washed with 3 ml de-ionized water, 2 ml 1.0 M acetic acid and 3 ml MeOH and dried for 5 min at ≥ 10inches Hg).
- Elution: The analytes were eluted with 3 ml of dichloromethane: isopropyl alcohol: ammonium hydroxide (78:20:2 v/v), which was prepare daily.

The eluent containing the drugs was evaporated to dryness without heat at room temperature or under nitrogen. The vial was sealed and derivatised with 50 µl of PFPA for 20 min at 70°C. It was evaporated to dryness at 40° C and reconstituted with 100 µl of Etoac. The sample was removed from the heat source, cooled and 1 µl was injected into the GC/MS for analysis.

7.2.1 GC/MS Parameters

A Fisons model GC 8000 MD800 series was fitted with a HP-1 capillary column. The initial temperature was 55°C held for 2 minutes and final temperature was 280°C held for 5 minutes. The oven rise was at a rate of 20 °C / min.

Figure 7.3 Chromatograph of an unextracted sample of the five amphetamines



7.3 AMPHETAMINES IN HAIR

The aim of this study was firstly to investigate and confirm the accuracy of self-reported use of speed and ecstasy using hair analysis procedures. Second, to optimize a method which would extract and identify amphetamine (AP), metamphetamine (MA), methylenedioxyamphetamine (MDA), methylenedioxy-methamphetamine (MDMA) and methylenedioxyethylamphetamine (MDEA) in hair. Third to determine the existence of a correlation between the self-reported amounts of drug consumed and drug levels found in hair.

7.3.1 Experimental Overview

This study took place in Glasgow Scotland jointly by the Faculty of Law at the University of Sheffield; the Scottish Centre for Criminology and the Department of Forensic Medicine and Science at the University of Glasgow.

Ecstasy was investigated because of its expanded use among adolescents, especially in the rave scenes or discotheques. Subjects were recruited through chain referral (e.g. subjects referring others) from several rave scenes and asked to honestly answer a detailed questionnaire on their drug used over the last 12 months. They were then asked to donate a hair sample for completion of this study, which was collected after completion of the questionnaire.

The most important information gained from the questionnaire was the subjects self-reported account of their past drug usage in the last 12 months. The amount of ecstasy used was recorded by the number of tabs consumed per month and speed was

recorded as ever consumed and how often (previous week, month or year) over the past 12 months.

7.4 SUBJECTS DRUG HISTORY

From the interview conducted, a report on drug use histories was recorded for over the past 12 months. Out of 100 subjects, 90 admitted consuming ecstasy, with the number of tabs consumed ranging from 1-144 (mean 19, median 10). Most reported consuming ecstasy regularly and 28 subjects reported sporadic or irregular consumption of the drug, where 6 - 72 tablets were consumed in a month, one case reported consuming 400 tabs.

Subjects were also asked to record to their best knowledge, their use of other drugs beside for amphetamine and ecstasy over the past year. Table 7.1 summarizes the various drugs consumed. Majority of the subjects consumed alcohol (96%), cannabis (95%). Speed (84%) and tobacco (84%).

Table 7.1 Drugs consumed by the 100 subjects over the past 12 months

DRUG	YES	NO	NO RESPONSE
ALCOHOL	96	4	-
CANNABIS	95	5	-
SPEED	84	16	-
TOBACCO	84	16	-
LSD	67	33	-
COCAINE	53	42	5
NITRILES	46	50	4
MUSHROOMS	30	67	3
TEMAZEPAM	29	68	3
OTHER BENZOS	19	75	6
OTHER OPIATES	13	82	5
GLUE	7	88	5
HEROIN	6	86	8
TEMGESIC	6	84	10
KETAMINE	5	86	9

7.5 HAIR SAMPLING TECHNIQUES

Collection procedures have not been standardized, but hair was often collected from the area at the back of the head (vertex posterior), cut as close as possible to the scalp and stored in dry tubes. In this case they were wrapping in foil and put in labeled plastic ziplock bags. This area is said to have less variability in hair growth rate, the number of hairs in the growing phase is more constant and the hair is less subject to age and sex-related influence.[270]

Hair samples were collected and separated into categories of male and female and the age, length and color was recorded. 100 subjects were interviewed, 51 were male and 49 female with the age range from 15 to 44 years (mean = 24yrs). The hair

samples taken had lengths that ranged from 1.5 to 12 cm for 100 samples. Although hair growth may vary from 0.6 to 3.36 cm, the rate of growth is approximately 1cm/ month, therefore 12 months is equivalent to 12cm worth of hair growth.

The hair samples were separated for segmental analysis by measuring 6 cm from the root (proximal) along the hair shaft and 6cm from that cut off point from the tip (distal). This gave a total of 12 cm or 12 months hair growth data. Some of the samples were either too small for analysis or had less than 6 months (6 cm) worth of hair growth, therefore they were analyzed as full samples. In total 139 hair segments were analyzed, with 46 of them analyzed in full for self-reported speed and ecstasy use. Table 7.2 shows details the data used for hair analysis.

Table 7.2 Data of the hair region, length and number of hair segments analyzed.

Hair Region	Length data (cm)	Number analyzed
Roots	< 3 cm	4
	3 cm < 6 cm	10
	6 cm	40
Tips	6 cm	29
	6 cm < 12 cm	10
Full	12 cm	46
Total Analyzed		**139**

7.6 SAMPLE PREPARATION

Stock standard solutions of AP, d_3-AP, MA, MDA, MDMA and MDEA (0.1mg/ ml) were prepared in methanol. From these, solutions of 1 and 10 μg/ ml were prepared as working standards.

7.6.1 Experimental

A detailed description of the wash procedures is described in the hair chapter. The sample preparation used for ecstasy hair samples is as follows:

- Hair samples were stored in glass vials and washed once with 0.1% SDS (sodium dodecyl sulfate), three times with de-ionized water and sonicated for 15 minutes. The hair was rinsed with MeOH and dried overnight in a dessicator.
- The washed hair was weighted, measured and cut into root and tip segments for analysis. Using liquid nitrogen and a pestle and mortar, the hair was cut and ground into a fine powder and weighed for further analysis.
- The powdered sample was pretreated using an enzymatic hydrolysis (2ml 0.1M phosphate buffer pH 7.4 and 50 µl of 0.1mg /ml β-glucuronidase/ arylsulphatase in prepared in buffer) with the addition of 100µl d_3-AP (IS) heated at 40 °C for 2 hrs.
- The sample was vortexed then centrifuged and the supernatant pipetted off into a clean vial. The addition of 2ml of buffer was repeated and centrifuged and the supernatant added to the first fraction.

7.6.2 Solid-Phase Extraction Methodology[271]

- *Column conditioning:* 2ml of MeOH was added to the column and dried under vacuum, then 2ml of phosphate buffer pH 7.4 was added to Isolute™ Confirm HCX-3 mixed mode column.
- *Sample application:* The pretreated sample containing the combined fractions was added to the conditioned column under slow vacuum.

- *Column wash:* The column was washed with 2ml of de-ionized water and the pH was adjusted using 1ml 0.01M acetic acid pH 3.3 and dried under full vacuum for 4 minutes. 2ml MeOH was added and dried under full vacuum for 1 minute.
- *Elution:* The acidic and neutral drugs (fraction A) were eluted first using 2ml acetone : chloroform (1:1) and discarded. The basic drugs (fraction B) were eluted using 2ml of 2% ammoniated ethyl acetate and blown down to dryness under nitrogen with no heat or left to evaporate at room temperature.
- *Derivatisation*: Fraction B was reconstituted with 50µl PFPA : Etac (2:1) and sonicated for 5minutes. Then derivatised for 15 minutes at 50° C, evaporated to dryness and reconstituted in 50 µl ethyl acetate. 1 µl was injected for analysis by GC/MS.

Various ions were chosen to identify the drug during chromatographic analysis. AP (m/z 118, 190*), MA (m/z 118, 160, 204*), MDA (m/z 135, 162, 190, 325*), MDMA (m/z 185, 162, 204, 339*) and MDEA (m/z 135, 162, 190, 218, 353*). The astrex (*) indicates the ions which were used for quantitation of each drug.

7.6.3 Results and Discussion

Blank hair was spiked (10 mg) were known amounts of standard mix were incorporated into the hair at levels of at 5, 10 and 20 ng/mg for method validation. Each drug had very good recoveries, which were all over 70 %. AP 71 %, MA 79 %, MDA 82 %, MDMA 87 % and MDEA 85 % relatively. The method proved linear over a concentration range of 0 to 100 ng/ mg. The LOD for AP and MA was 0.5 ng /mg and 0.1ng /mg MDA, MDMA and MDEA. These results are summarized in Table 7.3.

Table 7.3 Method validation for the confirmation of amphetamines from hair.

DRUG	%RECOVERY	% RSD	LOD ng/mg	LINEARITY r^2
AP	70.6	7.92	0.5	0.990
MA	79.4	8.68	0.5	0.993
MDA	82.2	6.26	0.1	0.993
MDMA	87.1	4.62	0.1	0.995
MDEA	85.4	2.12	0.1	0.996

7.7 DRUG CONCENTRATIONS IN HAIR

The amount of drug found in hair can vary depending on the amount consumed. The use of segmental analysis to determine drug usage patterns is based on the assumption that hair grows at a constant rate and drug diffusion is absent along the hair shaft.

7.7.1 Experimental

Table 7.4 lists the type of drug investigated the number of positives for each drug, the range of drug levels and the median and mean of the concentrations detected in hair.

Table 7.4 Drug concentrations detected in hair.

Drug	Sample Positives	Range ng/mg	Median ng/mg	Mean ng/mg
AP	11	0.7 - 97.7	2.5	12.5
MA	27	0.6 - 32.3	2.6	5.2
MDA	20	0.1 - 8.4	1.0	1.9
MDMA	56	0.1 - 82.9	0.7	4.6
MDEA	23	0.1 - 15.0	0.5	2.8

7.7.2 Results and Discussion

The amount of drug concentration in hair was determined for all five drugs for 139 samples. In total, 73 (52.5 %) tested positive for at least one of the amphetamines and the remaining 66 tested negative for all five drugs. Each drug had a relatively large range from very low levels to very high levels, which are consistent with other studies. [173, 235, 266, 267, 272] It is important to note that these hair samples were analyzed once due to the sample size and the reliability of this single analysis should be taken into account when interpreting the results summarized in Table 9.4.

7.8 DRUG - METABOLITE RATIOS

Goldberger *et al* had found that highly metabolically labile drugs like heroin and its short-lived metabolite 6-MAM are present in hair. Cocaine, with its short plasma half-life, has similarly been identified as the primary analyte in the hair of individuals that have ingested cocaine. Cocaine is present in high concentrations in hair even though it has a short-lived plasma half-life, 6-fold and 10-fold greater than its metabolites benzoylecgonine and ecgonine methyl ester respectively.[225, 273-276] The Society of Hair testing has set ratios for cocaine and heroin and their metabolites, but not as yet for amphetamines.[277]

7.8.1 Experimental

To determine whether a positive result is due to contamination or from ingestion of a drug, the metabolite to parent drug ratio is determined. The concentration of the parent drug is normally higher than that of the metabolite, but expected to be lower than 1.0 due to parent drug to metabolite incorporation. However, levels reported to

be greater than 1.0 are determined to be caused from contamination[277, 278] The metabolite to parent ratios are summarized in Table 9.5.

Table 7.5 Metabolite to parent drug ratio

Metabolite and Parent	Number and (Range)	Median	Mean
MA and AP	9 (0.15 -44.40)	0.85	7.26
MDA and MDMA	12 (0.01 - 6.28)	0.40	0.54
MDA and MDEA	11 (0.10 - 6.50)	0.67	1.59
MDA (MDMA + MDEA)	14 (0.03 - 6.50)	0.33	0.95

7.8.2 Results and Discussion

All of the ratios except for those of MDA and MDMA had greater ranges than in other studies. This can be explained by the various materials incorporated in some of the tabs where the contents are unknown. For example, although methamphetamine is a metabolite of amphetamine, speed can contain the parent drug and the metabolite, which can affect the metabolite to parent drug ratio.

Where a range of 0.03 to 6.5 was reported for MDA: (MDMA+MDEA) ratios, Rothe and co-workers reported a range of 0.03 to 0.2 (n = 67) with some cases having ratios > 1 like in this study.[173] Similarly, this can be explained as in the case of AP and MA, if unknown to the user, MDA was consumed as well as the parent drugs MDMA and MDEA.

7.9 ECSTASY PREPARATIONS

7.9.1 Experimental

At the time of the hair analysis study, 15 illicit ecstasy tablets were analyzed in the laboratory. A Home Office licence was obtained to be able to collect substances that were sold as ecstasy. This was investigated to find the percentage composition of the ecstasy tablets or powders that were in circulation at the time of sampling, which would identify the predominant parent drug(s). Table 4 details the percentage, actual amounts found and the type of drug found in various brands of ecstasy tablets or powders analyzed during the study.

7.9.2 GC-FID and GC-MS Parameters

Samples were analyzed using a HP 5890 gas chromatography fitted with flame ionization detection (GC-FID) and a CP-Sil 5 0.5id x 10 m column. The initial temperature was set at 80°C, with a final temperature of 200°C at a rate of 10 °C per minute held for 5 minutes. The injector and detector temperatures were set at 290 °C. Confirmation was carried out by GC-MS using a Fisons GC 8000 MD 800. The initial temperature was 60 °C and final temperature was 300 °C at 10°C/ minute held for 10 minutes.

7.9.3 Results and Discussion

Table 7.6 shows a list of the 15 substances thought to contain ecstasy, which were analyzed during this study. Overall, MDMA was the parent drug identified in the majority of the tablets ranging from trace levels to70 % (trace - 239 mg median 90 mg). Trace levels of MDMA were a result of AP and caffeine, which comprised of

87% of the tablet. MDEA was detected in several samples, but only from trace levels to 3.5 mg.

Although MDA was not detected in any of the substances analyzed, these are not representative samples and it does not eliminate the possibility that ecstasy was consumed by individuals in this study contained MDA. It has been reported that substances known to contain ecstasy seized in Britain have been identified as having MDA as one of its main constituents.[279, 280]

Table 7.6 List of 15 ecstasy samples analyzed at the time of hair study

ECSTASY NAME	FORM	MDMA %	OTHER DRUG
Emerald E	Capsule	MDMA trace	AP(46%)CAF(41%)
Tornado Powder	Powder	MDMA 13	-
Rhubarb+Custard	Capsule	MDMA 17	-
Lemon and Lime	Capsule	MDMA 19	MDEA (1%)
Clog	Tablet	MDMA 20	-
Pink Snowball	Tablet	MDMA 20	-
Madman	Tablet	MDMA 21	-
Turbo	Tablet	MDMA 21	-
Disco Biscuit	Tablet	MDMA 31	MDEA (trace)
Un-named	Tablet	MDMA43	-
Blaster	Tablet	MDMA 46	-
White/Love Dove	Tablet	MDMA 58	-
Madwoman I	Powder	MDMA 65	-
Super dove/robin	Powder	MDMA 68	MDEA (trace)
Madwoman II	Tablet	MDMA 70	-

7.10 SELF-REPORTED AND LABORATORY FINDING

7.10.1 Experimental

A direct comparison was made to find if a correlation exists between self-report data and the levels detected in hair for the 139 segments. Out of 73, 71 of the segments tested positive for at least of one of the amphetamines and agreed with the self-report data, where 2 were positive but disagreed. The remaining 66 tested negative for any amphetamine, however 6 out of the 66 agreed with having taken any amphetamine. There was an overall concordance of over 50% with a low number of false positives, which is consistent with other studies.

Figure 7.4 Summary of the overall concordance between self-report and hair analysis

		SELF-REPORT	
		YES	NO
LAB	YES	71 (51.1%)	2 (1.4%)
	NO	6 (4.3%)	60 (43.2%)

7.10.2 Results and Discussion

The relatively large number of false negatives reported can be caused by a number of possible problems. With the amount of any amphetamine present in tablets (e.g. very low contents, if any), the method may not be sensitive enough to recover low levels. The over-reporting of drug use and the influence of cosmetic treatments on the hair could also be an explanation.[281-283] However true this may be, information was not

available to assess this hypothesis. In addition, many of the hair samples collected were very small which could also influence the number of false negatives reported.

7.11 COMPARISON BETWEEN ROOTS, TIPS AND FULL

7.11.1 Experimental

By comparing root samples with tips, root samples are expected to be more accurate than tips because they are not greatly exposed to environmental or cosmetic influence, which can affect drug stability in hair. Figure 7.5 A, B and C compare the segments as well as show the concordance between self-report and laboratory recorded data.

Figure 7.5A Summary of self-report and hair analysis data for roots (n=54)

		SELF-REPORT	
		YES	NO
LAB	YES	29 (53.7%)	0 (0%)
	NO	22 (40.7%)	3 (5.6%)

Figure 7.5B Summary of self-report and hair analysis data for tips (n=29)

		SELF-REPORT	
		YES	NO
LAB	YES	13 (44.8%)	0 (0%)
	NO	14 (48.3%)	2 (6.9%)

Figure 7.5C Summary of self-report and hair analysis data for full (n=56)

		SELF-REPORT	
		YES	NO
LAB	YES	29 (51.8%)	2 (3.6%)
	NO	24 (42.9%)	1 (1.8%)

7.11.2 Results and Discussion

Root samples have a higher concordance (53.7%) than tips (44.8%) and full (51.8%), In addition, roots having a higher correlation is possibly due to the more accurate self-reporting over the last 3 months of the 12 month due to better recollection.

7.12 COMPARING SPEED AND ECSTASY SELF-REPORT

7.12.1 Experimental

When individually comparing speed or ecstasy self-report data to hair data, a low concordance for speed (28.3 %) is seen. This can be explained by the cut-off levels being higher compared to ecstasy, the speed data not being recorded for each month over 12 months and subjects were not aware of what they had consumed. A combination of these can affect the number of expected positives. The figures shown in figures 7.6A and B summarize the concordance for both speed and ecstasy only.

Figure 7.6A Speed data for self-report and hair analysis data.

SPEED ONLY*

		SELF-REPORT	
		YES	**NO**
LAB	**YES**	**23** (16.7%)	**6** (4.3%)
	NO	**93** (67.4%)	**16** (11.6%)

(*) Indicates that one subject did not answer question on speed use.

Figure 7.6B Ecstasy data for self-report and hair analysis data.

ECSTASY ONLY

		SELF-REPORT	
		YES	**NO**
LAB	**YES**	**58** (41.7%)	**7** (5%)
	NO	**52** (37.4%)	**22** (15.8%)

7.12.2 Results and Discussion

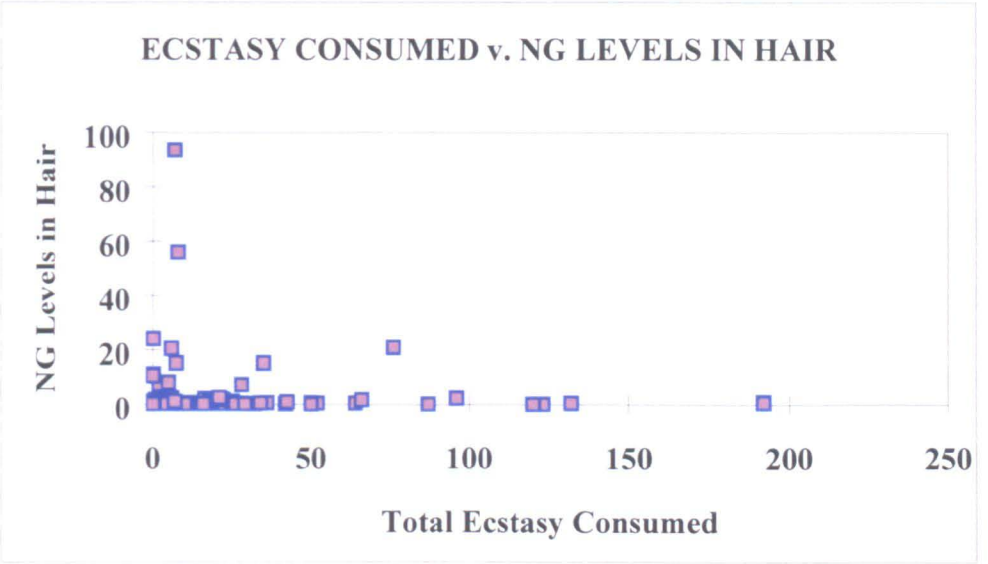
Looking at the speed and ecstasy data in Figure 7.6 A and 7.6 B, 6 subjects denied the use of speed, but their hair tested positive (false positive) and 5 were positive for ecstasy and admitted consuming ecstasy. In the ecstasy data, 6 out of the 7 false positives admitted consuming speed but denied ecstasy use with only one case positive for speed.

7.12 TOTAL ECSTASY v. DETECTED LEVELS

7.12.1 Experimental

The amount of ecstasy recorded being consumed was compared to the levels detected in hair analysis to find if a correlation existed. Figure 7.7 shows the correlation between the number of ecstasy consumed over the prior 12 months compared to the levels found in hair

Figure 7.7 Comparison between the amount of ecstasy consumed and the levels detected in hair for 139 samples.



7.12.2 Results and Discussion

The amount of ecstasy found in hair was based on a combination of MDMA and MDEA levels compared to the total amount of ecstasy tabs consumed by the subject over twelve months. A large degree of scattering of data was observed with the majority gathered along the axis. A linear dose response relationship was not observed ($r^2 = 0.0484$). If ecstasy was used sporadically, then this result would prove true with the results varying greatly. However if ecstasy had been consumed on a regular basis consistently over twelve months, then a higher correlation would possibly exist between ecstasy consumed and the amount detected in hair.

This correlation was expected because of assumed irregular consumption and the possibility that what was thought to be consumed as ecstasy was probably not or contained very low amounts. In addition, comparing the amount of ecstasy consumed month to month to corresponding hair data, it was found that a relationship

existed between the data. Similar results were produced comparing the amount of ecstasy consumed and MDMA or MDA+MDMA+MDEA data.

7.13 ECSTASY CONSUMED PER MONTH

7.13.1 Experimental

Hair segment data was separated into groups of five based on the frequency of ecstasy consumed each month. Each group consisted of approximately 20 % of the segments analyzed. Table 7.7 summarizes the number of ecstasy tabs consumed each month, the average consumption of each month and the mean, median and range of the levels detected in hair.

Table 7.7 Correlation between ecstasy consumed per month and ng levels detected for MDMA +MDEA

Ecstasy tablets consumed per month	Frequency	ng / mg Levels in Hair	
		mean/median	Range
None	29	0.086 / 0.00	0 - 1.00
< 0.5	29	1.493 / 0.10	0 - 20.3
0.5 - 1.25	28	4.425 / 0.00	0 - 93.4
1.25 - 3.5	27	1.615 / 0.00	0 - 23.9
> 3.5 +	26	4.285 / 0.55	0 - 55.8
TOTAL	**139**	**1.942 / 2.00**	**0 - 93.4**

7.13.2 Results and Discussion

A correlation between the number of ecstasy tablets consumed each month and MDMA + MDEA levels found in hair was investigated. The mean and median values increased from low to high, with the exception of ecstasy tabs consumed each month in the range of 0.5 - 1.25. The mean value is higher due to one case what has

a total concentration of 93.4 ng / mg, which can be considered to be an outlier, being the only high level found. In addition, the number of high false negatives recorded, were due to subjects claiming ecstasy use, which influenced the data.

7.14 INTERLABORATORY COMPARISON

The purpose of this study was to determine whether a concordance could be established between two laboratories using similar techniques for the recovery of amphetamines and its derivatives in hair samples. Work was carried out in Lab one; the Tricho-Tech Laboratory Ltd., located at the Cardiff Medicentre in Cardiff England and Lab two; the Department of Forensic Medicine and Science in Glasgow Scotland.

Hair samples were analyzed without prior knowledge of the data and procedures by either lab. Both laboratories used different extraction procedures with GC/MS for confirmation of AP, MA MDMA and MDA. In addition, MDEA was only screened for by Lab two and Lab one screened also for opiates using RIA techniques.

7.14.1 METHODOLOGY

Lab one analyzed 49 case hair samples (n = 93 segments analyzed total) which were divided into two segments of 3 cm for root and tip analysis. Lab two analyzed 100 case hair samples (n = 139 segments were analyzed total) which were also divided into two segments. However the segments where in 6 cm lengths for root and 6 cm for tip.

If the sample was deemed too small to be analyzed in separate segments, the whole sample was analyzed. Table 7.8 shows a summary of the number of hair sample lengths and the total amounts analyzed by both labs.

Table 7.8 Summary of the number of hair sample lengths analyzed by both labs.

Sample	Lab one	Lab two	Total
ROOT	**49**	**54**	**103**
< 3cm	4	4	8
3cm Root	45	0	45
3.1cm -5.9cm	0	10	10
6cm Root	0	40	40
TIP	**44**	**29**	**73**
(1cm/ 2cm)Tips	3	0	3
3cm Tip	41	0	41
6cm Tip	0	29	29
FULL	**0**	**56**	**56**
6.1cm - 11.9cm	0	10	10
12 cm	0	46	46
TOTAL	**93**	**139**	**232**

Limited information was available regarding the precise extraction and wash criteria used by Lab one due to commercial interests. The analysis of the hair samples was a four-phase process.

7.4.1.1 Experimental

7.14.1.1.1 Lab One: Pre-treatment and Analysis

The Hair sample was accurately measured and weighed. The sample was then fully digested to release all of the bound drugs from the hair and extracted with organic

solvents to remove potentially interfering material and concentrated extracted drugs. The analysis used was GC/MS for AP, MA, MDA and MDMA. The limit of detection was recorded as being 0.5 ng/mg for 10 mg of hair.

7.14.1.1.2 Lab Two: The pre treatment, extraction method and analysis carried out for Lab two has been listed throughout this chapter.

7.14.1.2 Results and Discussion

Despite several differences between the two laboratory techniques, analysis and the amounts of hair sample (e.g. 3 cm v. 6 cm) used for analysis, both labs produced similar results for the selected case samples. Table 7.9 below lists the drug, range, median and mean of the number of positives recorded from both labs.

Table 7.9 Drug concentrations detected in hair samples by both labs.

Drug/ Lab One	No. (Range)ng/mg	Median	Mean
AP	22 (0.3 - 48.4)	3.2	6.7
MA	2 (0.3 – 0.6)	0.5	0.5
MDA	17 (0.20 – 8.8)	0.4	1.1
MDMA	46 (0.30 – 44.6)	1.9	5.3
Drug /Lab Two			
AP	11 (0.7 – 97.7)	2.5	12.5
MA	27 (0.6 – 32.3)	2.6	5.2
MDA	20 (0.10 – 8.4)	1.0	1.9
MDMA	56 (0.10 – 82.9)	0.7	4.6
MDEA	23 (0.10 – 15.0)	0.5	2.8

Concordance between self-report and laboratory findings for the total amounts of amphetamines was compared for both lab one and lab two. Lab one had a

concordance of 61.1 % (n = 93) which was a slightly better concordance than Lab two which had a concordance of 56.8 % (n= 139). The false positives were low for both labs where lab one had one false positive and lab two had two.

For concordance between root and tip for both labs, Lab one which analyzed 3 cm of root hair, had a concordance of 71.5 % (n = 49) and Lab two which analyzed 6 cm of root hair had a concordance of 61.1 % (n = 54) both recorded no false positives. For concordance of tip hair samples, Lab one (3 cm) had a concordance of 50 % with 1 false positive (n = 44) and Lab two had a concordance of 55.2 % (n = 29) with no false positives. As expected, it can be seen that samples containing the 3 cm root samples were found to yield more accurate results than the 6 cm root samples. However, the 6 cm tip samples were slightly more accurate than the 3 cm tip samples.

7.15 CONCLUSION

Hair analysis is a very new technique in the field of the analytical chemist. Hair is an attractive matrix for the detection of drug exposure because of the accessibility of hair and its presumed ability to retain drugs longer than either plasma or urine. The method developed has proven successful for the extraction of five amphetamines (AP, MA, MDA, MDMA and MDEA) using enzymatic extraction followed by SPE with confirmation carried out using GC/MS. Authentic hair samples were used in this study taken from participants in the Glasgow dance scene to confirm speed and ecstasy use.

All five amphetamines could be detected in hair using this method with over 50% concordance between self-reported and lab findings. However a high number of false negatives (43.2 %) were recorded, which may highlight the methods possible lack of sensitivity in confirming low use of speed and or ecstasy. It is also possible that by asking participants to tick a check list of the possible drugs they may have consumed over the past year might be difficult to remember. The self-reported use can only be taken as an estimation of what that person felt they consumed. Another methodological consideration would be that the ecstasy or speed the subject thought they might be taking, may not in fact be pure MDMA, rather contain other active or non-psychoactive materials or stimulants.

No correlation was observed between the amount of ecstasy consumed and the levels detected in hair. However, there is evidence of a relationship existing between the level of use (measured in average ecstasy tabs consumed each month) and the levels detected in hair from low to high use. A combination of low concordance between self-report data and hair analysis results and the high number of false negatives raises questions regarding the sensitivity of the method and reliability of the self-report data.

The collection of hair samples is also an important factor in this study. Where possible, a sample should be analyzed in triplicate to decrease experimental errors and increase the validity of the analytical result. Some of the samples were too small to be analyzed more than once as mentioned earlier in this study. This led to the possibility of false negative results, as the sample size was too small to give a significant level by the method used with subsequent GC/MS analysis. However,

obtaining larger samples on a voluntary basis can prove to be problematic due to the area being sampled (vertex region).

It was very difficult to accurately correlate the amount of ecstasy tabs consumed with the levels detected in hair because of a lack of dose-response relationship for the segments analyzed. Large discrepancies observed in some cases could explain this correlation. One example of this was where one subject reported over a sixth month period, consuming 52 ecstasy tablets, however the hair analysis was negative for all amphetamines. Conversely, another subject denied ecstasy use, but 0.6 ng / mg was found in the corresponding 2 cm of hair segment.

Determining the smallest dose detectable in hair, in combination with dose- response studies, would explain the variation of levels consumed and levels detected. In one case, a subject consumed 5 ecstasy tabs and a level of 1.1ng / mg of MDMA was detected. However, when another subject consumed 50 tabs, 0.5 ng / mg of MDMA was detected. As mentioned earlier in the ecstasy preparation study, this can be due to the constituents in the tabs taken. Some may contain ecstasy in small quantities and the major constituent could be caffeine or speed.

There are many influences that can effect the levels detected in hair. Chemical treatments, an individuals metabolism, hair characteristics (i.e. damaged hair), sweat and other parameters must be taken into consideration during hair analysis. There are no set wash procedures or followed criteria for hair analysis yet, however there are quite a few methods investigating the extraction of amphetamines from hair.

In order to obtain more precise results for the comparison of self-report data and any amphetamine consumption, a controlled study must be carried out. This would include controlled dosages given to set amounts of subjects (with same hair colour) in a controlled environment what included controlled daily hair treatment procedures. Although possible, in the real world, this is not; therefore, the accuracy of the self-report data compared to the levels detected in hair will only prove useful as an indicator for drug usage.

From the results of both labs, it can be seen that even though different size samples and techniques were used, both laboratories reported similar results for the detection of amphetamines in hair. The data also reported that in general, root samples give more accurate concordance than tip and full samples. Tip samples were slightly more accurate than full but they did not differ significantly. In addition, by using smaller hair samples (3 cm or the first three months growth) yields a higher and possibly more accurate result, depending on the purported recent drug use.

8. SFE OF AMPHETAMINES IN HAIR

8.1 LITERATRUE REVIEW

8.1.1 INTRODUCTION

Amphetamine and methamphetamine are the two most abused drugs in the far East and were amongst the first drugs investigated in hair.[284] The recent spread of illicit drugs in the West has made these drugs and their analogues the third most prevalent in hair analysis. The determination of methamphetamine has also been detected in nails, sweat and saliva by Suzuki *et al* .[285]

Nakarhara[286], Kintz *et al*[236] and other researchers have published literature on the detection of amphetamine and their analogues in hair. It has been proven that these drugs can be extracted from hair following the addition of various extraction solutions, followed by GC/MS analysis to confirm their presence in hair. Table 8.1 lists several studies on the extraction of amphetamine and its analogs from hair.

Table 8.1Amphetamines in Hair

Drug	Hair Conc. ng /mg	Reference
Amphetamine	0.1 - 4.8	Rothe et al 1997[173]
	0.02 - 6.5	Rohrich & Kauert 1997[235]
Methamphetamine	0.05 – 0.89	Rothe et al 1997[173]
	1 – 39	Nakahara et al 1990[287]
MDMA	0.1 – 8.3	Rothe et al 1997[173]
	0.05 – 2.9	Rohrich & Kauert 1997[235]
MDA	0.05 – 0.89	Rothe et al 1997[173]
	0.04 – 1.2	Rohrich & Kauert 1997[235]
MDEA	0.8 – 3.07	Rohrich & Kauert 1997[235]

8.1.2 SFE HAIR ANALYSIS

Although, the analysis of amphetamine and its analogues (e.g. MDMA, MDEA and MDA) have been detected in hair using several extraction techniques, there is no present literature on the extraction of hair for amphetamines by SFE. However, there has been literature published on the applications of SFE for the extraction various other drugs from hair. [229, 230, 232, 233]

The purpose of this study was to investigate the use of SFE for the detection of MDA, MDMA and MDEA in human hair. Second, to optimize a method that would extract and identify the selected amphetamines. Also, to determine if this method can be used for the extraction of authentic case samples.

8.2 HAIR PREPARATION

8.2.1 Wash Procedure

It is necessary to wash the hair to remove any exogenous contaminates, prior cosmetic treatments and grease that can interfere with analysis or create false positives. The samples are first washed with a detergent sodium dodecyl sulfate followed by a wash with distilled water and then dichloromethane with a final wash with methanol. Each stage was repeated twice followed a 15 minute sonication at room temperature and dried at room temperature overnight.

8.2.2 Pulverization Procedure

Hair samples were ground to provide a large surface area to particle size ratio for better drug recovery.

8.2.3 Spiking Procedure

A measure of 1gm of blank hair sample was spiked with 1 ml (10 μg /ml) of each drug standard (MDA, MDMA, MDEA) in a glass vial. To ensure the hair was coated sufficiently, 3 ml of MeOH was added to the vial, sealed and sonicated for 15 minutes. The sample was then left dry at room temperature. The internal standard for all drugs was added to the hair during hair spiking preparation or before packing the hair into the extraction cell.

It is possible that this method of spiking is not the most ideal, as most drugs coat the surface of the hair and do not incorporate into the hair structure. However, it is accurate enough for estimation of exact concentration levels and is the only way to spike hair samples with known concentrations.

8.3 METHOD DEVELOPMENT

Hair samples were collected from persons known not to have taken any drugs for the use as blank hair samples. The hair samples were washed, ground and spiked with the appropriate prepared standards (10μg/ gm) as described above and in the hair chapter. All samples extracted were spiked samples based on 500ng/ 50mg (10ng/mg) of hair for each extraction.

The optimization of amphetamines by SFE can be achieved in three ways:

1. Increase or decrease in temperature
2. Increase or decrease in pressure
3. Increase or decrease in the % of modifier and CO_2

These three parameters will be investigated further to find the optimum conditions for the extraction of amphetamines from human hair.

8.3.1 EFFECTS OF TEMPERATURE

8.3.1.1 Experimental

The temperature is known to effect the extraction of a sample under supercritical condition. It is one of the parameters that determine whether a drug can be eluted under such conditions. By varying the temperature and pressure can increase or decrease the polarity and or mass transfer of the SF and density. To determine the optimum temperature for the extraction of amphetamines (MDA, MDMA and MDEA), temperatures at a range of 60° C to 100° C with a rise of 10° C increments were studied. Several samples were extracted for each temperature.

8.3.1.2 Results and Discussion

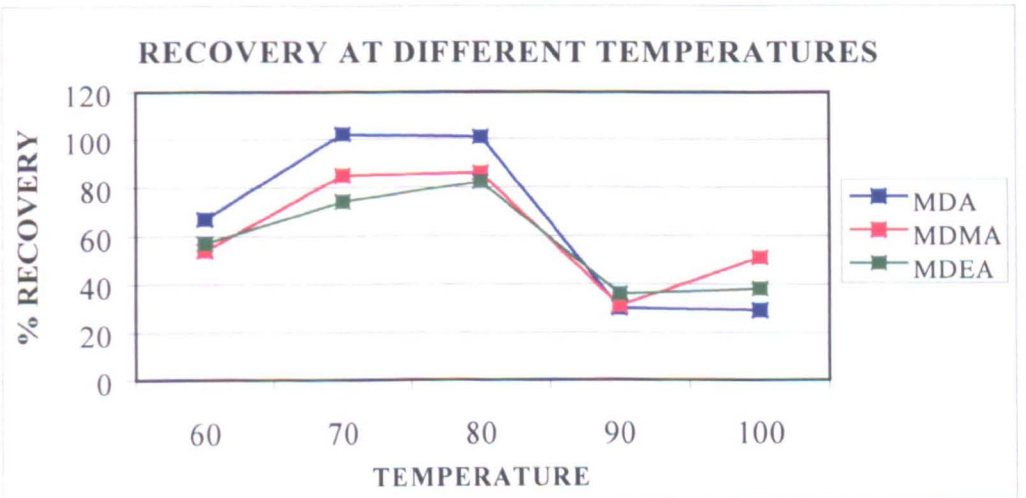
The optimum temperature was found to be 70 °C with the pressure set at 3500 psi and flow rate at 2 ml/ min. Various modifiers were also used to determine optimum conditions. The extraction was carried out in dynamic mode (continuous) for 30 minutes with the analytes collected in a vial containing chloroform. The solvent strength of the SF was dependent on the pressure and temperature used for the extraction. Constant temperatures at high pressures allowed the extraction to be optimized for a particular compound class. Table 8.2 lists the various temperatures investigated.

Table 8.2 Varying the temperatures of the extractions with chloroform: isopropyl alcohol (90:10 v/v)

DRUG	UNEXTSTD	60° C	70° C	80° C	90° C	100° C
MDA	1.662	1.1208	1.6955	1.6756	0.4895	0.4873
MDMA	0.890	0.4846	0.7593	0.7660	0.272	0.4538
MDEA	0.4878	0.2770	0.3582	0.3986	0.1719	0.1456

Table 8.2 showed that 70 and 80 °C were the best temperatures, however, it was shown that the higher the temperatures, the worse the recovery and chromatography for each drug. Figure 8.1 shows the percent recovery of each drug extracted at different temperatures.

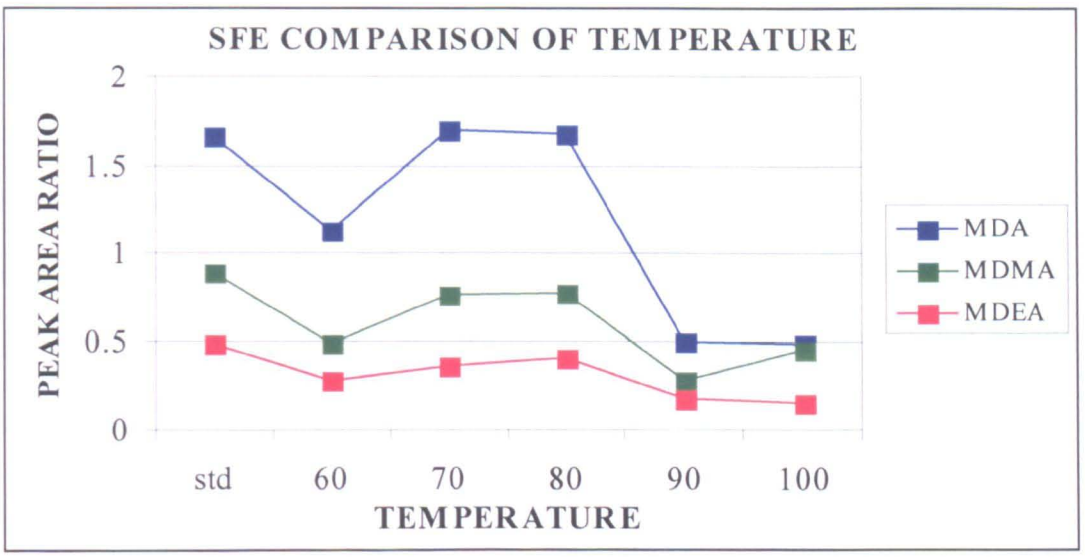
Figure 8.1 Percent recovery of MDA, MDMA and MDEA extracted at different temperatures



Recoveries at 70 °C were 110 % for MDA, 85 % for MDMA and 74 % for MDEA. Recoveries at 80° C were equally as high for each of the drugs as 70° C, however, 70 ° C was chosen as the best temperatures as amphetamines are relatively stable at

lower temperatures. Figure 8.1 compares the PAR of the temperatures from Table 8.2.

Figure 8.2 Comparison of the PAR of the temperatures used for the extraction of amphetamines from hair



With comparison to the PAR of the unextracted standard, 70° C and 80° C proved to be the best extraction temperatures for MDA, MDMA and MDEA.

8.3.2 EFFECTS OF PRESSURE

8.3.2.1 Experimental

As described in chapter one of supercritical fluids, pressure has a great effect on the SF and its solvent strength. An increase in pressure causes the solvent power or fluid density to increases at a given temperature. The pressure dependency of the SF solvating power is very important in SFE and is exhibited by all solids and many liquid solutes.

The pressure during SFE was investigated to determine the optimal pressure for the extraction of amphetamines. The temperature was set at the optimum 70° C, the flow at 2 ml/ min and the modifier at 10 % chloroform: isopropyl alcohol (90: 10 v/v) and 90 % CO_2.

8.3.2.2 Results and Discussion

There was fluctuation during each extraction that made it a little difficult to observe an accurate pressure. However, from the pressure study, it was determined that pressures between 3500 – 4000 psi produced the best extractions for amphetamines. Pressure ranges of 45- 5000 psi had just as good peak area ratios, but there seemed to be more fluctuation. Table 8.3 lists the various pressure ranges investigated for the extraction of MDA, MDMA and MDEA from hair.

Table 8.3 Pressure ranges of the peak area ratios

		PRESSURE RANGE PSI				
DRUG	**UNEXTSTD**	**28 -3000**	**32-3300**	**35-4000**	**45-5000**	**55-6000**
MDA	1.4299	0.1598	0.1548	1.212	1.2484	0.4318
MDMA	0.9485	0.2118	0.1205	0.4974	0.5470	0.1645
MDEA	0.4813	--	0.395	0.2727	0.2705	0.0918

Figure 8.3 shows a comparison of the pressure ranges during each extraction from MDA, MDMA and MDEA based on Table 8.3.

Figure 8.3 Comparison of the range of pressures used in the SFE of amphetamines.

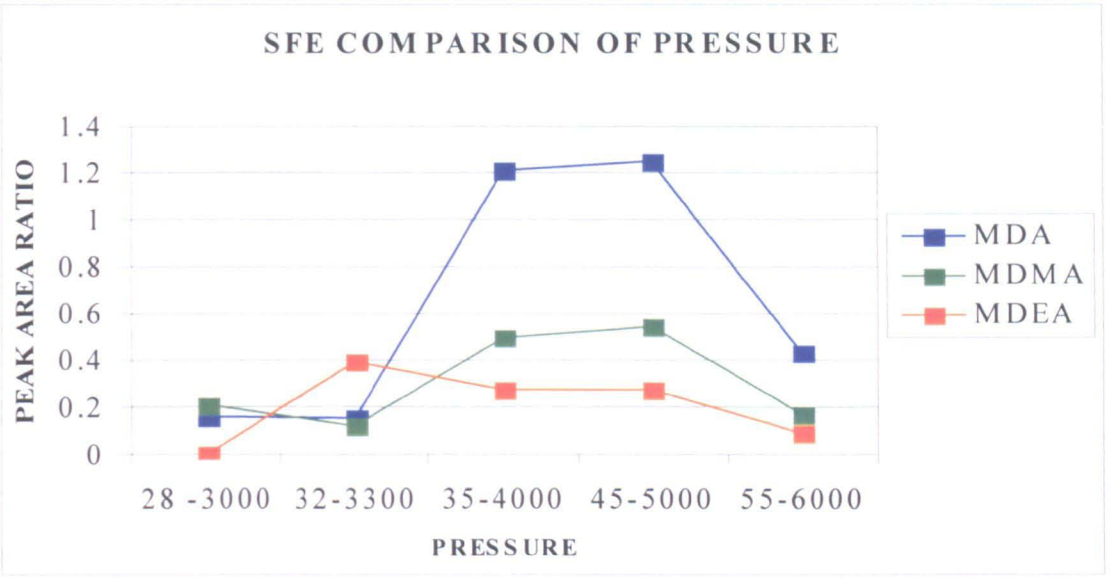


Figure 8.2 shows that temperatures ranging from 3500 –5000 psi produced greater peak area ratios compared to the unextracted standard. Temperature was selected at 3800 psi for extraction. Recoveries within that pressure range, ranged from 85-87 % for MDA, 52-58 % for MDMA and 56-57 % from MDEA.

8.3.3 EFFECTS OF MODIFIER

8.3.3.1 Experimental

The use of modifiers during extraction increases the polarity of the CO_2 during extraction to increase the recovery of the analytes. Various modifiers were used to investigate which gave the best recoveries for the selected amphetamines. At the selected temperature of 70° C and the selected set parameters, four different combinations of modifiers were used. Table 8.4 lists the extraction modifiers used for the extraction of amphetamines in hair.

Table 8.4 Extraction modifiers selected.

	EXTRACTION MODIFIERS
1	**Chloroform : isopropyl alcohol (90 :10 v/v),**
2	100 % chloroform
3	chloroform: isopropyl alcohol: isoamyl alcohol (90:9.5:0.5 v/v)
4	chloroform: n-heptane: isopropyl alcohol (50:17:33v/v)

8.3.3.2 Results and Discussion

In combination with CO_2, modifiers listed in Table 8.4 were introduced through the column at 10 %. The best recoveries were found with chloroform: isopropyl alcohol (90: 10 v/v) at the set parameters. Table 8.5 shows the concentration ratios for each drugs extracted and modifiers used in the extraction.

Table 8.5 The average concentration ratio for each drug produced by each modifier.

		EXTRACTION MODIFIERS			
DRUG	**UNEXTSTD**	**1**	**2**	**3**	**4**
MDA	1.384	1.6345	0.3819	1.5684	1.3353
MDMA	1.180	1.2941	0.4530	1.2661	1.3611
MDEA	0.967	0.593	0.2397	0.4677	0.045

The percent recovery was calculated for each of the extraction modifiers. Chloroform: isopropyl alcohol (90: 10 v/v) was found to produce the best recovery for each of the drugs. Modifier 4 produced good recoveries for MDA and MDMA, but recoveries were low for MDEA.

Table 8.6 The average percent recovery for each of the modifiers.

DRUG	% RECOVERY FOR EXTRACTION MODIFIERS			
	1	2	3	4
MDA	118	28	113	96
MDMA	109	38	107	115
MDEA	61	25	48	5

8.3.4 EFFECTS OF PERCENTAGE CO_2 AND MODIFIER

8.3.4.1 Experimental

By varying the percent modifier can establish the optimum conditions for the extraction. The modifier was varied from 5 % to 30 % at increments of 5 (e.g. 5, 10, 15, 20, 25, 30) and the CO_2 was varied in accordance to the percentage of modifier introduced into the system (e.g. 95, 90, 85, 80, 75, 70).

8.3.4.2 Results and Discussion

The percent modifier was introduced into the system from 5 – 30 %. An increase in the percentage of the modifier decreases the amount of CO_2 being pumped thought the system under supercritical conditions. This decrease in CO_2 leads to the decrease in the supercritical state the extraction is under. Therefore, it is recommended that if a modifier is used, it must be 15 % or less. Although Cleland *et al* reported the use of 20% methanol-modified CO_2 to recover arsenic from dogfish muscle.[288] Studies showed that using a 10 % gave good recoveries for MDA, MDMA and MDEA. Less than 10 % modifier recovered less drug and greater than 10 % recovered just as much as 10 %.

8.3.5 EFFECTS OF DERIVATISATION

8.3.5.1 Experimental

Derivatisation is an important step for the analysis of some samples using GC/MS. Amphetamines are known to be labile, therefore, the effects of derivatisation on MDA, MDEA and MDMA were investigated to find out how long amphetamines are to be derivatised

8.3.5.2 Results and Discussion

Samples containing 500 ng of MDA, MDMA and MDEA were left to derivatise over a period from 15 to 45 minutes. Table 5 lists the peak area ratios of each drug over the selected derivatisation times.

Table 8.7 Derivatisation of Amphetamines

	DRUG		
TIME	**MDA**	**MDEA**	**MDMA**
15 MIN	4.77^{E008}	2.64^{E008}	5.92^{E007}
30 MIN	4.60^{E008}	2.86^{E008}	5.89^{E007}
45 MIN	5.09^{E008}	2.91^{E008}	5.66^{E007}

*E = 10 to the power

It was found that derivatising MDA, MDMA and MDEA for 15 minutes gave similar peak areas as 30 and 45 minutes. Therefore it can be concluded that the length of derivatisation up to 45 minutes does not effect the recovery of MDA, MDEA and MDMA.

8.4 DEVELOPED SFE CONDITIONS

Pressure: 3800 psi

Temperature: 70° C

Flow rate: 2 ml/ min

Modifier : chloroform : isopropyl alcohol (90: 10 v/v) @ 10 %

CO_2 : 90 %

Collection solvent: 4 ml of 100 % chloroform

Dynamic extraction for 30 minutes

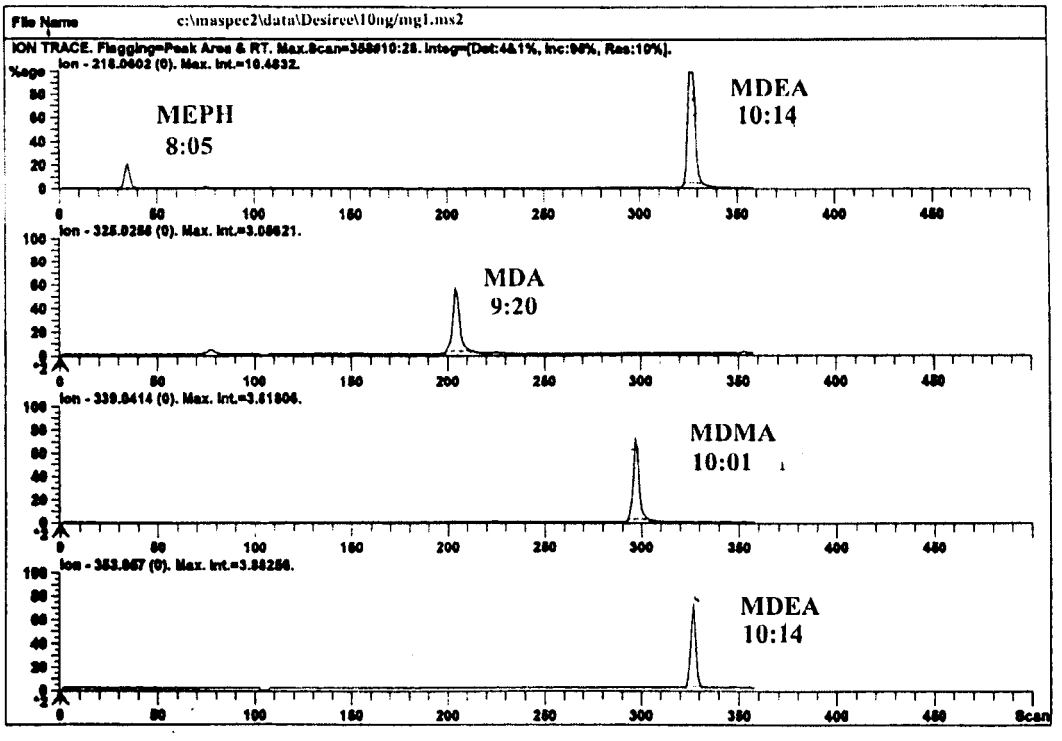
Extractions were carried out under the above conditions for the extraction of MDA, MDMA and MDEA in 10 ng /mg of hair. Figure 8.4 shows a chromatography of the drugs identified using SFE at the above set parameters.

8.4.1 GC/MS Parameters

A Hewlett-Packard gas chromatography model 5890 fitted with a VG Analytical VG70- 250S mass spectrometry was used for analysis. The MS is a magnetic sector instrument that was operated in EI and SIM mode. The GC was fitted with a HP-5 X- link 5 % PH Me silicone capillary column, 30 m x 0.25 μm, 0.88 μm film thickness.

The selected ions were monitored in the electron ionization mode at 70 eV and the trap current was set at 200 μA. The initial temperature was 55° C held for 2 minutes and final temperature was 280° C held for 5 minutes. The oven rise was at a rate of 20° C /min. The ions monitored were m/z 218 mephentermine, m/z 325 MDA, m/z 339 MDMA and m/z 353 MDEA.

Figure 8.4 Chromatography of MDA, MDMA, MDEA and internal standard mephentermine from a 10ng/ mg spiked hair sample.



8.5 METHOD VALIDATION

8.5.1 RECOVERY

8.5.1.1 Experimental

Hair samples at levels of 10 ng/ mg were extracted under the aforementioned SFE parameters to investigate the percent recovery of MDA, MDMA and MDEA. The internal standard (mephentermine) was made up to 10 μg /ml solution and 50 μl of that standard was added to each extracted eluent.

8.5.1.2 Results and Discussion

Under the developed SFE parameters from amphetamines, several hair samples were extracted and analyzed using GC/ MS. At 10ng /mg hair samples produced

recoveries at an average of 84 % for MDA, 71 % for MDMA and 77 % for MDEA. Table 8.8 shows the average recoveries and peak area ratio range of each drug.

Table 8.8 Peak area ratios and average recovery for MDA, MDMA and MDEA from hair.

DRUG	% AVG RECOVERY	UNEXTRACTED STD PAR	PAR RANGE
MDA	84.2	1.6722	1.0976 – 1.5090
MDMA	71.2	0.7352	0.4071 – 0.7345
MDEA	77	0.3785	0.2294 – 0.3690

8.5.2 REPRODUCIBILITY

8.5.2.1 Experimental

Hair sample individually extracted at a concentration of 20 ng/ mg five times were investigated for reproducibility from sample to sample.

8.5.2.2 Results and Discussion

Summarised in Table 8.9 below shows five different extractions of hair containing the same concentration of hair taken from the same spiked hair batch.

Table 8.9 Run to run reproducibility of MDA, MDMA and MDEA in hair

DRUG	1	2	3	4	5
MDA	12.309	9.263	9.058	9.169	11.214
MDMA	9.072	6.782	6.733	6.962	8.292
MDEA	6.900	5.593	5.208	5.558	6.214

The mean, range, StdDev and percent RSD was calculated for the reproducibility of each drug for n = 5 runs. The results are summarized in Table 8.10 below.

Table 8.10. Reproducibility of amphetamines in hair.

DRUG	MEAN	RANGE	StdDev	%RSD
MDA	10.202	9.058 - 12.309	1.47	14
MDMA	7.556	6.733 – 9.072	1.065	14
MDEA	5.899	5.208 – 6.900	0.665	11

8.5.3 INTRA-DAY AND INTER-DAY REPRODUCIBILITY

8.5.3.1 Experimental

Over a period of two days, a sequence of hair samples were extract at the same concentration under the developed SFE conditions. The investigation of intra and inter-day reproducibility was to show that the method can be reproduced on a day to day bases.

8.5.3.2 Results and Discussion

Five hair samples at 10ng/ mg were extracted. Table 8.11 and 8.12 list the peak areas of MDA, MDMA and MDEA extracted over period of two days.

Table 8.11 Day one reproducibility of each drug

DRUG	UNEXTSTD	1	2	3	4	5
MDA	2.119	1.913	1.1897	2.7397	2.2025	2.4690
MDMA	2.0746	2.068	1.788	1.939	1.6371	1.5929
MDEA	2.0549	0.456	0.677	0.855	0.7037	0.7699

Table 8.12 Day two reproducibility of each drug

DRUG	UNEXTSTD	1	2	3	4	5
MDA	3.3182	2.1934	2.1173	2.6670	3.3232	2.2103
MDMA	2.5970	1.7711	1.8597	1.9111	3.133	1.9840
MDEA	2.0833	1.1376	1.2789	1.1837	1.6681	1.2103

For MDA, MDMA and MDEA for day one, the standard deviation was 0.595, 0.200 and 0.148, the mean 2.102, 1.804 and 0.692 and the RSD 28 %, 11 % and 21 %. For MDA, MDMA and MDEA for day two, the standard deviation was 0.507, 0.565 and 0.214, the mean 2.5022, 2.1318, 1.2960 and RSD 20 %, 26 % and 16 %.

There was some variation between the two days but comparable. MDEA for day one is much lower than for day two. A possible explanation for this could be because of the spiking of the blank hair. Spiking blank hair with a drug does not incorporate into the hair as fortified hair samples, therefore not all the time will there be precise consistency from batch to batch. However, it is accurate enough for estimation of exact concentration levels and is the only way to spike hair samples with known concentrations. Also the sensitivity of the GC/MS from day to day can be a possible contributory factor.

8.5.4 LINEAR CALIBRATION

8.5.4.1 Experimental

Linearity of the developed method over a concentration range from 20ng /mg to 0.02ng /mg was investigated. Five individual samples (n = 5) at each of the six concentration levels were extracted for validity purposes.

8.5.4.2 Results and Discussions

Over the selected range of 20ng/mg to 0.02ng /mg of hair, the linearity of each drug was extracted five times and the average taken. Table 8.13 below shows the concentration range investigated detailing the average peak area ratios for each drug.

Table 8.13 Linear range of MDA, MDMA and MDEA over a range from 20 ng/mg to 0.1ng/mg

	CONCENTRATION OF HAIR ng/ mg				
DRUG	**20**	**10**	**2**	**1**	**0.1**
MDA	10.2024	2.9308	1.2761	0.4507	0.3934
MDMA	7.5682	2.4232	1.1079	0.3827	0.1683
MDEA	5.899	2.3561	0.6522	0.2006	0.1038

Figures 8.5-8.7 show the linear calibration range of the three drugs based on the average peak area ratios listed in Table 8.13.

Figure 8.5 Linearity of MDA in hair

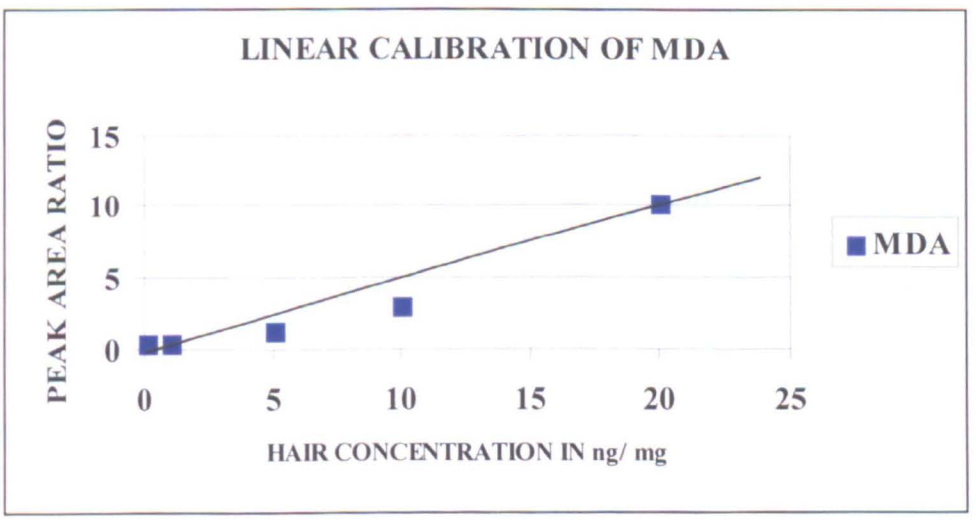


Figure 8.6 Linearity of MDMA in hair

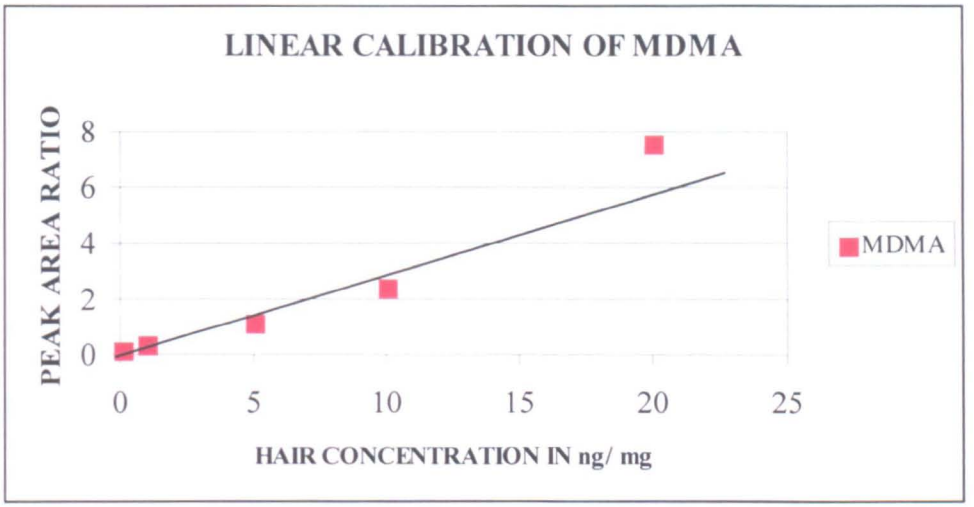
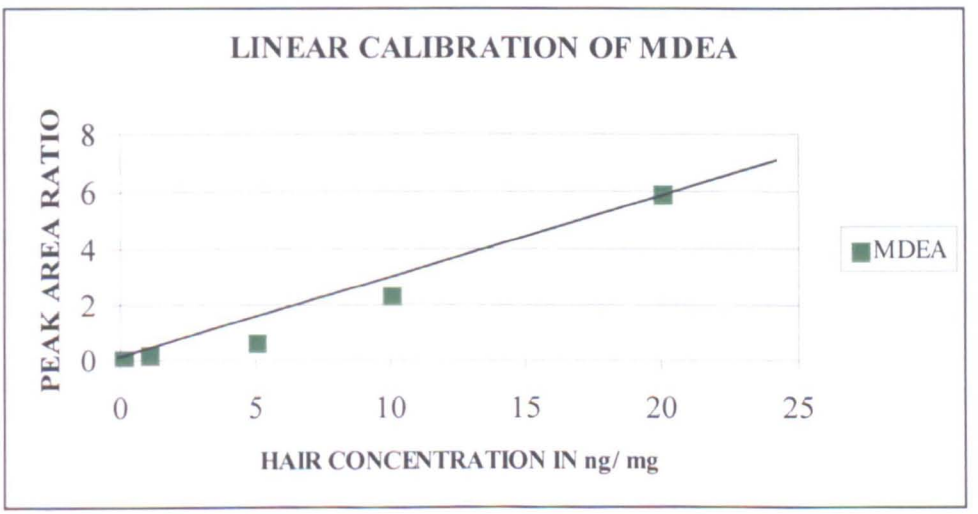


Figure 8.7 Linearity of MDEA in Hair



The correlation coefficient, range, LOD, intercept and slope were calculated for each of the drugs. The method proved linear over a concentration range of 0 to 20 ng/ mg. The LOD for MDA was 0.02 ng/mg and MDMA and MDEA was 0.1ng /mg. These results are summarized in Table 8.14.

Table 8.14 Method validation for the detection of MDA, MDMA and MDEA in hair

DRUG	Correlation coefficient (r^2)	Conc range ng /mg	LOD ng /mg	Intercept A	SLOPE B
MDA	0.9703	0.02 - 20	0.02	0.4736	0.085
MDEA	0.9775	0.02 - 20	0.1	0.3535	0.0106
MDMA	0.9938	0.02 - 20	0.1	0.2870	0.0581

8.6 AUTHENTIC CASE SAMPLES

8.6.1 Experimental

Hair samples taken from subjects participating in the self-report study for amphetamine use which was discussed in Chapter 7 were analyzed under SFE conditions. The samples were too small (too short or had only a few hairs) to be separated into segments of root or tip so they were analyzed in bulk or Full.

Before analysis, the hair samples were washed and dried under the aforementioned wash conditions and the colour, weight and length were applicable of each hair sample were recorded. Table 8.15 below lists twenty cases analyzed for MDA, MDMA and MDEA.

Table 8.15 Case samples analyzed for MDA, MDMA and MDEA

CASE #	MDA ng/mg	MDMA ng/mg	MDEA ng/mg	Length cm	Weight mg
A99-A	0.255	0.638	0.396	11	60
A99-B	0.125	0.288	0.067	4	30
A99-C	0.252	0.153	0.171	11	40
A99-D	**ND**	0.117	**ND**	11	70
A99-E	**ND**	0.659	0.286	11	19
A99-F	**ND**	**ND**	**ND**	12	78
A99-G	**ND**	ND	**ND**	11	60
A99-H	0.274	0.53	0.11	5	35
A99-I	0.061	0.336	0.082	4	20
A99-J	**ND**	**ND**	**ND**	11	53
A99-K	**ND**	3.88	**ND**	11	60
A99-L	**ND**	**ND**	**ND**	11	21
A99-M	0.059	1.15	**ND**	12	10
A99-N	**ND**	**ND**	**ND**	11	40
A99-O	**ND**	**ND**	**ND**	2	66.5
A99-P	**ND**	**ND**	**ND**	2	7
A99-Q	2.14	6.44	**ND**	8.5	10
A99-R	**ND**	0.71	**ND**	11	9
A99-S	**ND**	1.62	**ND**	11	10
A99-T	**ND**	**ND**	**ND**	11	52.5

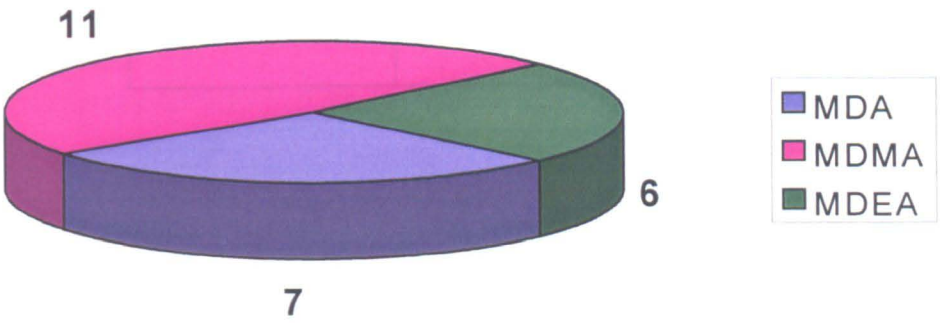
ND = Not detected

8.6.2 Results and Discussion

Authentic hair samples analyzed in Table 8.15 were taken from subjects claiming amphetamine use over the past 12 months. Out of the 20 samples analyzed, 5 were positive for MDA, MDMA and MDEA, 5 were positive for MDMA only, 2 were positive for MDA and MDMA only, 1 was positive for MDMA and MDEA only and 7 were not detected. Samples that were not detected may not have been negative but the levels were possibly to small to detect. Below in Figure 8.8 are the result

summarized for the case samples positive overall for all three drug. Samples that were listed as not detected are not included. There were 7 overall MDA positives, 11 MDMA positives and 6 MDEA positives.

Figure 8.8 Chart of overall positive cases



The self-report data was compared to the SFE findings from the hair samples to find if a correlation existed. It was found that some of the subjects that reported no use or little use (e.g. half an ecstasy tablet monthly) over the past 12 months were found to be negative and others found positive with low levels detected. Figure 8.9 below summarizes the results of the self-report data to the hair findings.

Figure 8.9 Self-report data compared to hair analysis for MDA, MDMA and MDEA

		SELF-REPORT	
		YES	NO
LAB	YES	11 (55%)	3 (15%)
	NO	6 (30%)	0 (0%)

Out of the 20 hair samples analyzed, 11 (55 %) were positive for all or either MDA, MDMA or MDEA and said yes to consuming ecstasy. 3 (15 %) also tested positive but disagreed with having taken any ecstasy and 6 (30 %) were negative for MDA, MDMA or MDEA, but agreed with having consumed ecstasy in the past 12 months. There was no one that claimed to be negative and was found to be negative.

8.7 CONCLUSION

Supercritical fluid extraction is a good technique that can be used as an alternative extraction method to conventional techniques. Among its many advantages, SFE is fast, inexpensive and produces less solvent waste. The use of SFE for the extraction of hair has been reported in various studies. Despite the controversial aspects of hair testing, the use of hair analysis is rapidly expanding.

The aim of this study was to determine if amphetamines, MDA, MDMA and MDEA, could be extracted from hair samples using SFE, optimize a method for extraction and its application to authentic hair samples. The results in this chapter demonstrate

that MDA, MDMA and MDEA can be extracted from hair under the developed SFE conditions. Recoveries better than 84 %, 71 % and 77% were produced for MDA, MDMA and MDEA. Based on spiked hair samples, the method was found to be linear of a concentration range of 0.02 to 20 ng/mg of hair. The correlation coefficient (r^2) for MDA, MDMA and MDEA was 0.9703, 0.9775 and 0.9938 showing the linearity of the method. The method was also found to be reproducible from sample to sample and on a day to day basis.

It has also been demonstrated that by the application of a polar modifier directly through the system providing better extraction conditions for the drugs of interest. By varying the temperature and pressure for optimal conditions increased the extraction potential. Temperature and pressure are the two main parameters that effect SFs. An increase in pressure at a constant temperature, increasing the density and mass transfer of the SF to allow more polar and higher molecular weight analytes of interest to be eluted.

Authentic hair samples taken from subjects participating in a self-report study were analyzed using developed SFE techniques. Out of the 20 samples analyzed, 7 overall were positive for MDA, 11 for MDMA and 6 for MDEA. Samples were positive for either MDA or MDMA or a combination of MDA, MDMA and MDEA. Samples that were not detected may not have been negative, but had levels too low to detect using this method. The results from the hair analysis were compared to the self-report data collected to find if a correlation existed between vhe two. It was found that with some of the subjects, a corrleation did exist between what they had reported to have consumed from month to month and hair findings and others it did not. The accuracy of the self-report data was questionable because of the possibility of under

or over reporting of consumption and whether or not what was taken contained MDA, MDMA or MDEA.

It can be concluded that, SFE is an appropriate method for the extraction of amphetamines in hair. It is fast (30 minutes), reproducible and can be applied to authentic hair samples with results comparable to those generally observed in hair analysis.

9. SFE OF COCAINE IN HAIR

9.1 LITERATURE REVIEW

9.1 .1 INTRODUCTION

Although during metabolism, cocaine rapidly degrades and benzoylecgonine is the predominant drug found in biological tissue, cocaine is the primary analyte found in hair. This is also observed to be true when hair is soaked in aqueous solutions containing equal amounts of cocaine and benzoylecgonine.[73] This can be attributed to the greater affinity of cocaine to bind to the binding sites than benzoylecgonine. Once cocaine is incorporated into the hair, it is very stable compared to biological fluids. Other metabolites found in hair include, ecgonine methyl ester, ecgonine, cocaethylene, norcocaine, anhydroecgonine methyl ester and norcocaethylene.

Hair texture differences, ones diseased state and damaged hair can all lead to varying amounts of cocaine incorporated into hair and subsequently detected. Harkey *et al* observed little correlation between self-reported and cocaine concentrations in hair of light users compared to heavy users.[275] The incorporation of cocaine in hair is still very unclear. Investigators have consistently reported higher cocaine concentrations in hair than any of its metabolites.[225, 211, 274, 289] This is surprising since benzoylecgonine and ecgonine methyl ester have longer plasma half-lives than cocaine and are present at higher concentrations in biological fluids.

Nakahara and kikura investigated the incorporation of cocaine and its metabolites in rat hair using trideuterated standards.[211] From this it was suggested that most of the benzoylecgonine detected during hair analysis arises from the hydrolysis of cocaine

incorporated into hair and the benzoylecgonine and ecgonine methyl ester incorporation rates are relatively low.

9.1.2 COCAINE IN HAIR

Early studies employing immunoassay indicated that hair analysis was effective for detecting cocaine and its metbolites.[290-292] As interest grew a variety of analytical methods and assays evolved for the analysis of hair samples for cocaine and benzolyecgonine. Studies of hair obtained from cocaine users have revealed that cocaine is the primary chemical species found in hair.[225]

Early publication concerning cocaine in hair have been investigated by Valente in 1980.[290] By 1987, Balabanova and Homoki[293] identified cocaine by GC/MS, followed by Kidwell in 1988[224] using pyrolysis and CI/MS/MS. By 1991, cocaine and selective metabolites were quantified from hair by Cone.[225]

9.2 METHOD DEVELOPMENT

Blank hair samples were collected for method development from persons known not to have used drugs. The hair samples were prepared by washing, grinding and then spiking with the appropriate prepared standards. For the method development of cocaine and its metabolites from hair, several conditions have to be investigated for optimum extraction. They are the effects of temperature, pressure and the amount of modifier introduced during the extraction. These three parameters will be described throughout this chapter.

9.2.1 EFFECTS OF TEMPERATURE AND MODIFIER

9.2.1.1 Experimental

The effects of temperature were investigated to determine the best temperature for the recovery of cocaine and its metabolites from hair. Temperatures from a range of 60 to 100° C at 10 °C increments were investigated. A combination of varying temperature and modifier were used to first find out if cocaine, benzoylecgonine and ecgonine methyl ester could be extracted from hair before a method could be developed.

Samples containing 50 ng (10ng/ mg) of spiked hair were packed individually in an extraction column that was set at varying test temperatures to find the best temperature for extraction. The flow rate was set at 2 ml /min, pressure 3500 psi and 90 % of CO_2 with a 10 % selected modifiers was applied to the system to assist with analyte elution during extraction. Some modifiers were introduced directly thought the system and others directly into the extraction column before extraction.

9.2.1.2 Results and Discussion

Two hair samples were extracted at each temperature range of 60, 70, 80, 90 and 100°C. Modifiers made up of 16 various solvent combinations were either added to extraction column or through the system to the extraction column. Hair samples were prepared and extracted for each modifier used. Table 9.1 lists the various modifiers used.

Cocaine was not elute out using most of the modifiers listed in the table however, modifiers 5, 6, 7 and 9 did eluted cocaine. Benzoylecgonine and ecgonine methyl ester were only eluted out with modifiers 6 and 7, but in very low concentrations.

Modifier 6 (H_2O: NH_3 85:15 v/v) gave better results than 7 and 9 gave the best results for cocaine only. However, during extraction modifier 6 caused a precipitant to develop that caused blockage in the Rheodyne value and tubing so its use was discontinued. The other modifier combinations (e.g. 1 – 4 and 8, 10 and 11) did not elute cocaine or its metabolites.

Table 9.1 Modifiers used during SFE extraction of spiked hair samples

No.	SFE MODIFIERS
1	DCM: IPA: TEA (78:20:2 v/v)
2	DCM: H_2O: TEA (80:17:3 v/v)
3	MeOH: IPA: TEA (80:17:3 v/v)
4	Chloroform :IPA: TEA (80:17:3 v/v)
5	H_2O: TEA (85:15 v/v)
6	DCM: IPA: NH_3 (78:20:2 v/v)
7	H_2O: NH_3 (85:15 v/v)
8	IPA:TEA (85:15 v/v)
9	H_2O: TEA (85:15 v/v)
10	MeOH: TEA (85:15 v/v)
11	MeOH:NH_3 (85:15 v/v)
12	100% Chloroform
13	Chloroform: IPA (90:10v/v)
14	Chloroform: n-Heptane: IPA (55:33:17 v/v)
15	Chloroform: IPA: Isoamyl alcohol (90:9.5:0.5 v/v)
16	**Toluene: IPA: Isoamyl alcohol (70:20:10v/v)**

Several other modifiers listed in Table 9.1 (12-16) were used to investigate if all three drugs would be eluted. These modifiers were pumped directly thought the system at 10 % with CO_2 at 90 % using the aforementioned temperature range. Studies showed that by using various combinations of all five modifiers, eluted cocaine and ecgonine methyl ester only at all temperature. However, only with

100% chloroform was benzoylecgonine eluted, but in small amounts along with cocaine and ecognine methyl ester at higher concentrations. Table 9.2 shows the peak area ratios of each drug extracted using various modifiers and temperature conditions.

Table 9.2 Effects of temperature and modifier under SFE conditions for cocaine and ecgonine methyl ester in hair

DRUG	**TEMPERATURE °C**				
Chloroform (Chlor)	**60**	**70**	**80**	**90**	**100**
Cocaine	0.6388	0.9607	0.8534	0.5638	0.2570
Benzoylecgonine	0.1566	0.2199	0.1255	0.0276	0.0921
Ecgonine methyl ester	0.2142	0.4453	0.3218	0.1577	0.0628
Chlor: IPA					
Cocaine	0.8342	0.620	1.5323	0.1496	0.8630
Benzoylecgonine	--	--	--	--	0.1520
Ecgonine methyl ester	0.3542	--	0.4670	0.5020	0.4360
Chlor: IPA:n-Heptane					
Cocaine	1.6319	1.5280	1.6677	1.5320	--
Benzoylecgonine	--	--	--	--	--
Ecgonine methyl ester	0.5730	0.6002	--	0.4686	--
Chlor: IPA:Isoamyl					
Cocaine	0.1460	1.5815	1.6670	1.5313	0.6268
Benzoylecgonine	--	--	--	--	0.0268
Ecgonine methyl ester	0.3125	0.5113	--	0.4886	0.1448
Tolulene:IPA:Isoamyl					
Cocaine	1.4899	**1.5008**	1.4732	1.4563	1.4466
Benzoylecgonine	--	--	--	--	--
Ecgonine methyl ester	0.4563	**0.5452**	0.4732	0.5908	0.5775

Toluene: IPA and isoamyl alcohol (70:20:10 v/v) at 70° C was chosen as the modifier because it produced good recovery and reproducibility overall for both cocaine and ecgonine methyl ester.

9.2.2 EFFECTS OF PRESSURE

9.2.2.1 Experimental

The pressure of the extraction was varied from 3500 psi to 5500psi, which was the limit that the pressure could reach in intervals of 500 psi (e.g. 3500, 4000, 4500, 5000, 5500). Parameters were set at temperatures of 70° C and flow rate of 2ml/ min with only the pressure varied. By increasing the pressure and keeping temperature constant should increase the solvating strength of the supercritical fluid (CO_2) for more polar and high molecular weight analytes to be eluted out better (e.g. benzoylecgonine).

9.2.2.2 Results and Discussion

For the set temperature (70 °C) it seem that between 3600 to 3800 psi was the best pressure to elute cocaine and ecgonine methyl ester from hair samples. However, benzoylecgonine was not eluted out at any of the pressures.

9.2.3 EFFECTS OF CO_2 AND MODIFIER

9.2.3.1 Experimental

Using modifiers during extraction, aid to enhance the extraction of polar analytes from the sample matrix to increase the recovery of the drug during extraction. Several extractions were carried out using spiked powdered hair samples containing 50 mg (20ng /mg) and various combinations of CO_2 and modifier (toluene: IPA:

isoamyl (70:20:10 v/v)). Combinations ranged in intervals of 5 %, where CO_2 was measured from 95%–80% and toluene: IPA: isoamyl from 5 %- 20 %.

9.2.3.2 Results and Discussion

Out of the combinations of CO_2 and modifier used, 90% CO_2 and 10 % toluene: IPA: isoamyl (70:20:10v/v) was preferred. This combination seemed to produce the best results for the extraction of cocaine and ecgonine methyl ester. Using the other combinations did not produce any of the drugs.

9.3 DEVELOPED SFE CONDITIONS

Pressure: 3800 psi

Temperature: 70° C

Flow rate: 2 ml/ min

Modifier: Toluene: IPA: Isoamyl alcohol (70:20:10 v/v)

CO_2: 90 %

Collection solvent: 2 ml of MeOH

Dynamic extraction for 30 minutes

Extractions were carried out under the above conditions for the extraction of cocaine and ecgonine methyl ester in 10 ng /mg of hair. Figure 9.1 shows a chromatography of the drugs identified using SFE at the above set parameters.

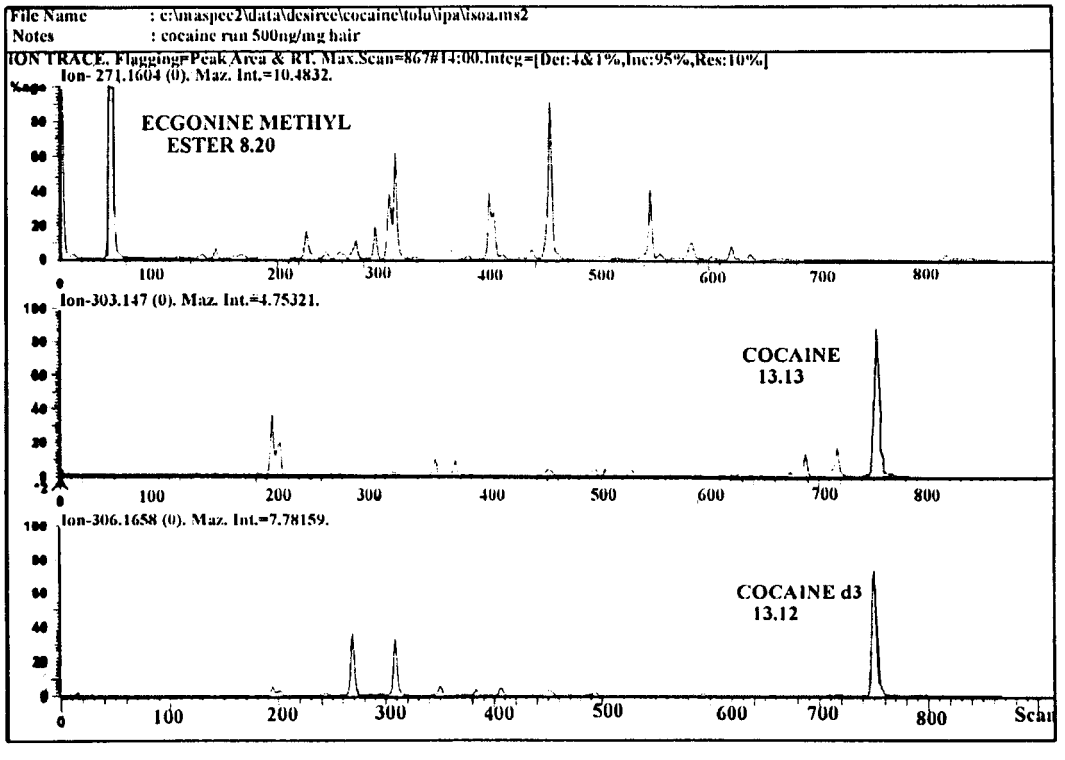
9.3.1 GC/MS Parameters

A Hewlett-Packard gas chromatography model 5890 fitted with a VG Analytical VG70- 250S mass spectrometry was used for analysis. The MS was a magnetic sector instrument operated in electron ionization and single ion monitoring (SIM)

mode. The GC was fitted with a HP-5 X- link 5 % PH Me silicone capillary column, 30 m x 0.25 μm, 0.25 μm film thickness.

The selected ions were monitored in the electron ionization mode at 70 eV and the trap current was set at 200 μA. The initial temperature was 100° C held for and final temperature was 280° C held for 2 minutes. The oven rise was at a rate of 10° C /min. The ions monitored were m/z 271 ecgonine methyl ester, m/z 303 cocaine, m/z 362 benzoylecgonine and m/z 306 d_3 cocaine.

Figure 9.1 Chromatography of the extraction of cocaine and ecgonine methyl ester from 10 ng/mg of hair



9.4 METHOD VALIDATION

9.4.1 RECOVERY

9.4.1.1 Experimental

Using 10ng /mg spiked hair samples, developed SFE conditions were applied to find the percent recovery for cocaine and ecgonine methyl ester.

9.4.1.2 Results and Discussion

From the 10ng/mg spiked hair samples, Six (n = 6) samples were extracted under the developed SFE conditions. Recovery for cocaine was 70 % and 100 % for ecgonine methyl ester.

9.4.2 REPRODUCIBILITY

9.4.2.1 Experimental

Samples were extracted five times (n = 5) using spiked hair samples containing 10ng/mg of cocaine, benzoylecgonine and ecgonine methyl ester.

9.4.2.2 Results and Discussion

Below in Table 9.2 are the results from the reproducibility of cocaine and ecgonine methyl ester from 10ng /mg of hair. The extraction of cocaine and ecgonine methyl ester under the developed SFE conditions has shown good reproducibility from sample to sample.

Table 9.3 Run to run reproducibility of cocaine and ecgonine methyl ester in hair

DRUG	Unextstd	1	2	3	4	5
Cocaine	2.351	1.6448	1.5995	1.669	1.5907	1.682
Ecgonine methyl ester	0.594	0.3406	0.4613	0.4370	0.4939	0.4427

The overall mean, StdDev and % RSD for cocaine and ecgonine methyl ester were calculated and the results summarized in Table 9.4.

Table 9.4 The reproducibility of cocaine and ecgonine methyl ester in hair

DRUG	MEAN	RANGE	StdDev	% RSD
Cocaine	1.6372	1.5905 – 1.682	0.0408	2.4
Ecgonine methyl ester	0.4351	0.3406 – 0.4613	0.0573	13.1

9.4.3 INTRA AND INTER-DAY

9.4.3.1 Experimental

Over a period of two days, the intra and inter-day reproducibility of cocaine and ecgonine methyl ester were investigated. Samples of 10ng/mg of hair were extracted five (n = 5) times under the same conditions each day.

9.4.3.2 Results and Discussion

Cocaine and ecgonine methyl ester were reproducible on a day to day basis and from sample to sample. Tables 9.5 and 9.6 summarize the reproducibility results from day to day.

Table 9.5 Day one intra-day reproducibility

DRUG	Unextstd	1	2	3	4	5
Cocaine	2.953	2.459	2.368	2.244	2.290	2.356
Ecgonine methyl ester	1.152	1.023	0.8057	0.871	0.8606	1.026

For day one, the mean for cocaine was 2.343, StdDev 0.0819 and % RSD 3.4%. For ecgonine methyl ester, the mean was 0.8900, StdDev 0.0931 and % RSD 10.4 %.

Table 9.6 Day two inter-day reproducibility

DRUG	Unextstd	1	2	3	4	5
Cocaine	3.045	1.973	2.538	2.547	2.133	2.329
Ecgonine methyl ester	1.061	0.8404	0.9716	0.8833	0.8003	0.8870

For day two, the mean for cocaine was 2.304, StdDev 0.251 and % RSD 10.9 %. The mean for ecgonine methyl ester was 0.876, StdDev 0.0638 and % RSD 7.2 %.

9.4.4 LINEAR CALIBRATION

9.4.4.1 Experimental

Linear calibration of the developed method was investigated from a concentration range on 10 ng /mg to 0.05 ng /mg. Five samples (n = 5) were extracted at each concentration level.

9.4.4.2 Results and Discussion

Over the selected concentration range, the average PAR of each sample was calculated for each concentration level. Table 9.7 shows average PARs for the linear range for cocaine and ecgonine methyl ester.

Table 9.7 Linearity of cocaine and ecgonine methyl ester in hair

	CONCENTRATION OF HAIR ng / mg					
DRUG	**10**	**5**	**1**	**0.5**	**0.1**	**0.05**
Cocaine	1.6372	0.9354	0.2589	0.1815	0.1032	0.0755
Ecgonine methyl ester	0.4351	0.2573	0.1351	0.102	0.0737	0.0582

Figures 9.3 and 9.4 show the linear calibration for cocaine and ecgonine methyl ester over a concentration range of 0.05 to 10ng /mg of hair.

Figure 9.5 Linearity of cocaine in hair

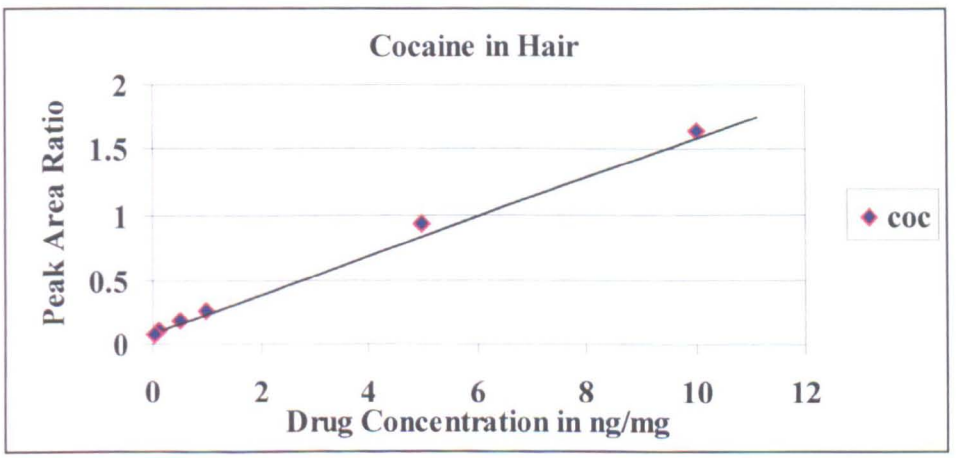
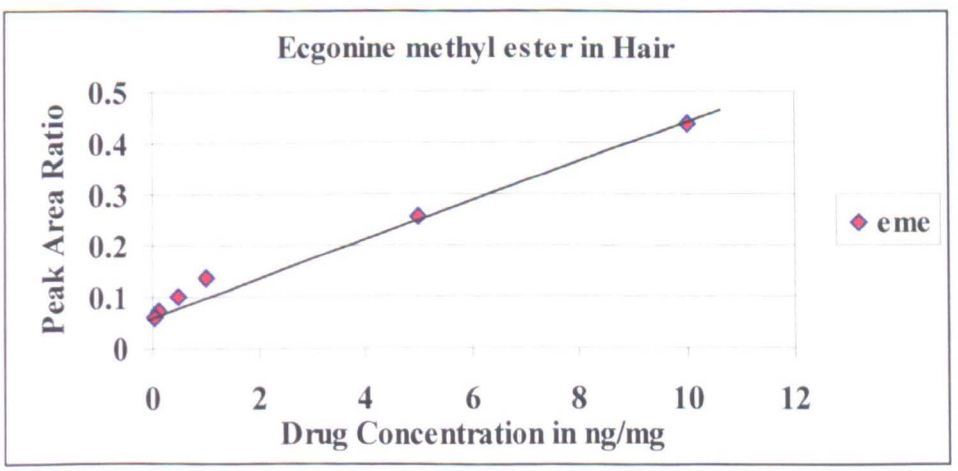


Figure 9.6 Linearity of ecgonine methyl ester in hair



The results for the method validation for the developed SFE method are summarized in Table 9.8.

Table 9.8 Method validation for cocaine and ecgonine methyl ester in hair

DRUG	Correlation Coefficient r^2	LOD ng/mg	Intercept A	Slope B
Cocaine	0.9987	0.05	0.0976	0.0911
Ecgonine methyl ester	0.9951	0.05	0.0767	0.0360

9.5 CONCLUSION

In recent years, a large number of drug abuse studies based on the analysis of human hair have been described and reported. With the epidemic spread of cocaine and crack use, attempts have been made to take advantage of this ostensibly longer detection time of hair analysis compared to urine testing for identifying cocaine users. SFE is just one of the techniques being explored as an alternative to conventional methods for the extraction of drugs of abuse from human hair.

The aim of this study was to develop a method which SFE could be applied for the extraction of cocaine and its metabolites from hair. Based on spiked hair samples, successful methods were developed for cocaine and ecgonine methyl ester with recoveries of 70 % and 100 %.

By altering the temperature and pressure during extraction aided to enhance the extraction potential for the recovery of both cocaine and ecgonine methyl ester. The method was found to work best at a temperature of 70° C with at a pressure of

3800psi. The introduction of the polar modifier, toluene:IPA:isoamyl (70:20:10 v/v) enhanced the solvating power of the supercritical fluid to extract cocaine and ecgonine methyl ester, however benzoylecgonine was not eluted, even with the use of various modifiers.

The method was found to be linear over a concentration range of 0.05 to 10 ng/mg of hair, with detection limits down to 0.05 ng/mg for cocaine and 0.01ng/mg for ecgonine methyl ester. It was also found to be reproducible on a day to day basis and from sample to sample.

CONCLUSIONS

The objective of this study was to develop new sample extraction methodology using solid-phase extraction and supercritical fluid extraction techniques for the determination of drugs of abuse from biological matrices. Benzodiazepines, morphine, amphetamines and cocaine and its metabolites were all investigated using either of these techniques and applied to authentic case samples.

The use of SPE was applied for the extraction of benzodiazepines from whole blood. At present, a routine method does exist, however, optimization was necessary to find a more robust method for routine analysis. Successful methodology was established for benzodiazepines in spiked whole blood and authentic forensic case samples.

SPE and developed SFE techniques were carried out for the extraction of morphine from whole blood on authentic forensic samples and compared to determine if a correlation existed between the two methods. A correlation was found between the two methods with comparable results for morphine from authentic whole blood samples.

For the extraction of cocaine and its metabolites from blood and urine, SFE techniques were developed. It was found that cocaine and its metabolites could be extracted under SFE conditions with relatively good recoveries with the exception of benzoylecgonine, which had low recoveries. Using a modifier combination of dichloromethane, isopropyl alcohol and ammonia at a temperature of 40 °C and pressure of 3500 psi, successfully extracted cocaine, benzoylecgonine and ecgonine methyl ester from blood and urine samples.

Human hair was another matrix investigated for drugs of abuse. The interest of hair for drug testing has grown as an alternative biological sample in the field of forensics. Its use has been investigated in a number of extraction techniques because of its wide window of drug analysis time and noninvasiveness compared to that of blood and urine.

SPE methods were successfully developed to extract amphetamines (AP, MA, MDA, MDMA and MDEA) from spiked and authentic human hair samples. This study determined and confirmed the accuracy of the self-reported use of amphetamines using hair analysis. It was found that a correlation did not exist between the self-reported consumed amounts of drug and the drug levels found in hair.

SFE techniques were also developed and applied to specific amphetamines (MDA, MDMA and MDEA) for the investigation in human hair. With the aid of chloroform as the polar modifier, recoveries better than 71 % were found overall for amphetamine use with reproducible results. The application of this method to fortified human hair samples proved successful for the recovery of MDA, MDMA and MDEA in hair. These findings render SFE as an appropriate method for the extraction of amphetamines in hair.

For the extraction of cocaine and its metabolites (benzoylecgonine and ecgonine methyl ester) from spiked hair samples, developed SFE techniques were applied. Results showed that cocaine and one of its metabolites, ecgonine methyl ester, were extracted under the developed SFE conditions. Reproducible results were produced with recoveries for cocaine and ecgonine methyl ester of 70 and 100 %.

It has been demonstrated that SFE is an appropriate method for the extraction of drugs of abuse from various biological matrices. It has proven to be rapid, reproducible and efficient for solid matrices such as human hair with excellent results for authentic hair samples. Comparing SFE to conventional techniques such as SPE has proven to give comparable results with good correlation from sample to sample.

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LIST OF APPENDICES

APPENDIX ONE: Calculations used during method development.

Blood and urine sample and standard ratio calculations:

$$\textbf{Sample Ratio} = \frac{\text{Response of Drug in Sample}}{\text{Response of Drug in Standard}}$$

$$\textbf{Standard Ratio} = \frac{\text{Response of Drug in Standard}}{\text{Response of Internal Standard}}$$

The Dilution Factor (DF) is used when the volume of the standard and the sample are not the same. (e.g. 1 ml of standard and 100µl of sample gives a DF of 10)

$$\textbf{Drug Concentration (mg/l)} = \frac{\text{Sample Ratio} \times \text{Concentration of Drug in Standard}}{\text{Standard Ratio Dilution Factor}}$$

The percent recovery was calculated to find out how much of the drug was being recovered from the sample matrix using the developed method.

$$\textbf{\% Recovery} = \frac{\text{Extracted Standard Ratio}}{\text{Unextracted Standard Ratio}} \times 100$$

APPENDIX TWO: Standard Deviation and Relative Standard Deviation

To assess the variability of the data, the Standard (StdDev) and Relative Standard Deviation (RSD) were calculated.

StdDev

$$\sigma n-1 = \sqrt{\frac{\Sigma^{n}(x_i - x)^2}{(n-1)}} = \sqrt{\frac{\Sigma x^2 - (\Sigma x)^2/n}{n-1}}$$

RSD (%) = StdDev divided by the X (mean) multiplied by 100

APPENDIX THREE: Linear Regression

To find the calibration curve produced by the data, linearity was calculated using slope where **B** = the gradient, **A** = the intercept and **X** and **Y** = the axis.

Linearity = $y = bx + a$

APPENDIX FOUR: Papers published in support of this thesis.

Desiree L. Allen, Karen S. Scott and John S. Oliver. *The Comparison between Solid-Phase Extraction and Supercritical Fluid Extraction for the Analysis of Morphine in Whole Blood.* Journal of Analytical Toxicology, May/June (1999), Vol. 23, 216-218

Desiree L. Allen and John S. Oliver. *The Application of Supercritical Fluid Extraction of Cocaine and its Metabolites from Various Biological Matrices.* (Accepted for publication) Journal of Analytical Toxicology 1999

Gail A. A. Cooper, Desiree L. Allen, Karen S. Scott, John S. Oliver, Jason Ditton and Iain D. Smith. *Hair Analysis: Self-Reported Use of "Speed" and "Ecstasy" Compared with Laboratory Findings.* (Accepted for publication) Journal of Forensic Science in press March 2000

Jason Ditton, Gail A. A. Cooper, Karen S. Scott, Desiree L. Allen, John S. Oliver and Iain D. Smith. *Forensic Hair Testing for "Ecstasy" in a Large Volunteer Sample of Scottish Drug Users.* Addiction Biology1999 in press

Desiree L. Allen and John S. Oliver. *The Use of Supercritical Fluid Extraction for the Determination of Amphetamines from Human Hair.* (Accepted for publication) Forensic Science International 1999 in press

Comparison of Solid-Phase Extraction and Supercritical Fluid Extraction for the Analysis of Morphine in Whole Blood

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Abstract

A comparative study of the quantitative determination of morphine in whole blood using solid-phase extraction (SPE) and supercritical fluid extraction (SFE) is described. Comparative studies were made of the two techniques for the extraction of morphine from authentic forensic blood specimens. Quantitative results indicate that morphine levels measured using SPE correspond well to morphine levels produced using SFE. The two techniques are therefore comparable, although SFE is faster and cleaner and extracts may be produced with higher analyte recoveries than with SPE. This paper presents a comparison of the two techniques and the morphine concentrations determined in blood.

Introduction

Solid-phase extraction (SPE) techniques are widely used for the isolation of analytes from liquid matrices. Although SPE is easy and popular in terms of extraction techniques, supercritical fluid extraction (SFE), a relatively new sample preparation technique, is becoming of increasing interest.

SFE is based on the use of carbon dioxide above its critical temperature and pressure. The development of SFE as an alternative extraction method to SPE methods has aroused interest because of the limitations of conventional methods. Supercritical fluids are gases and liquids at temperatures and pressures above their critical points; in this state, they have the solvation powers of a liquid while retaining properties such as high diffusivity and low viscosity of a gas (1). The high diffusivity of the supercritical carbon dioxide allows for rapid and effective extractions of mainly nonpolar solutes without costly organic solvent use (2).

An ideal extraction method should be rapid, yield quantitative recovery of the target analyte without loss or degradation, be relatively simple and inexpensive, permit minimal sample handling, and generate no additional laboratory wastes (3).

The use of SFE as a sample preparation technique is rapidly increasing as an alternative for the analysis of compounds from a wide variety of matrices. Studies have been carried out using conventional SPE methods, and they have been compared with SFE methods to investigate the correlation between the two methods for the extraction of morphine from authentic forensic blood specimens.

Experimental

Apparatus

A Fisons model GC 8000 series (MD 800 in the EI Mode [70ev]) gas chromatograph–mass spectrometer (GC–MS) was used for analysis, and it was equipped with an HP-1 (cross-linked methyl siloxane) capillary column (30 m × 0.53-mm i.d, 0.88-µm film thickness).

Temperature was initially set at 200°C and held for 1 min; oven temperature increased 10°C/min to a final temperature of 300°C and was held for 5 min. The injector temperature was 250°C.

Materials

Tri-ethylamine (Merck G-P-R™ grade) and isopropanol (AnalaR grade) were supplied by Merck (Poole, U.K.). Chromatographic (HPLC)-grade ethyl acetate, methanol, and acetonitrile were obtained from Analytical Sciences Lab-Scan (Dublin, Ireland). CO_2 was from Air Products (Walton-on-Thames, U.K.); 10-mL plastic syringes were from Becton Dickinson (Oxford, U.K.); silanized glass wool was from Jones Chromatography (Mid Glamorgan, U.K.); and Extrelut® columns were from Merck. Six-milliliter Hypovial™ SFE vials were obtained from Pierce (Oud-Beyerland, The Netherlands); butyl rubber septa were from Pierce and Warner (Chester, U.K.). Columns for SFE (3.4 cm × 4.6-mm i.d. stainless steel tubing) and SPE (1-mL SCX columns) were from Varian.

Stock solutions

Drug and internal standard stock solutions were prepared in methanol (1 mg/100 mL).

SPE sample preparation

SPE columns were prepared by packing Extrelut washed with methanol/ethanol (1:1, v/v) into 10-mL syringes plugged with cotton glass wool. One-milliliter whole-blood samples were pretreated in vials with 1 mL of 0.1M phosphate buffer (pH 6), 50 µL

of ammonium hydroxide solution, and 50 µL of internal standard (morphine-d_3).

The contents of each sample vial were pipetted onto corresponding Extrelut columns, then conditioned with 5 mL of hexane and allowed to stand for 10 min. Labeled SCX columns (1 mL) were placed on a VacElut and conditioned with 2 mL of methanol, 2 mL of deionized water, and 1 mL of ethyl acetate/isopropanol (9:1, v/v).

The Extrelut columns were attached to the top of the SCX columns via adapters. The analytes of interest were transferred from the Extrelut columns to the SCX columns using three 4-mL aliquots of ethyl acetate/isopropanol (9:1, v/v). The Extrelut columns were discarded. The SCX columns were then washed with 5 mL of acetonitrile/methanol (1:1, v/v), and the analytes of interest were eluted using 2 mL of 10% ammonia in acetonitrile/methanol (1:1, v/v). The collected eluent was evaporated to dryness under nitrogen at 40°C and derivatized with 50 µL of *N,O-bis*(trimethylsilyl)trifluoroacetimide catalyzed with 1% trimethylchlorosilane (BSTFA + 1%TMCS) at 90°C for 10 min.

SFE set-up, sample conditions, and preparation

The pump pressure at the end of the system (i.e., before the extraction cell) was measured by a Gilson 805S manometric module and monitored using Gilson 715 software. The software controller allowed programming of up to four pumps. A Gilson 811B dynamic mixer was used to premix the CO_2 and modifier being pumped through the system. An HPLC 7125 rheodyne valve fitted with a 100-µL injection loop was used to apply the samples to the extraction vessel. This was placed in line between the dynamic mixer and the extraction cell that was housed in a GC oven.

SFE pressure conditions were set at 3500 psi with a 2-mL/min flow rate and 100°C temperature. Three-hundred microliters of MeOH/Et_3N (85:15, v/v) was used as a modifier, and 90% CO_2 was used as the supercritical fluid.

The samples were prepared by loading 100 µL of internal standard (morphine-d_3) onto the extraction media Extrelut that contained 100–300 µL of mixed and dried blood. The rheodyne was set in the load position, and the instrument was left to equilibrate. The sample was then loosely packed into a stainless steel extraction column, and 300 µL of modifier was added. The column was sealed and placed over the loop position of the rheodyne into an oven set at 100°C. The extraction was started by switching the rheodyne from the load (equilibrate) to the inject (extract) position. The instrument was left for 30 min to elute the analytes through the outlet; the analytes were collected by expansion in methanol. The sample was then evaporated under nitrogen at 40°C and derivatized with 50 µL of BSTFA at 90°C for 10 min (4).

Table I. Comparison of SPE and SFE Results for Morphine

Case #	SPE (mg/L)	SFE (mg/L)	%Difference
T96-A	1.25	1.18	0.07
T96-B	0.59	0.67	0.08
T96-C	1.02	0.91	0.11
T96-D	0.90	0.85	0.05
T96-E	1.86	1.97	0.11
T96-F	2.42	2.20	0.22
T96-G	0.19	0.17	0.02
T96-H	0.35	0.39	0.04
T96-I	0.86	0.79	0.07
T96-J	0.45	0.45	0
T96-K	0.056	0.05	0.006
T96-L	0.85	0.74	0.11
T96-M	0.28	0.28	0
T96-N	0.27	0.29	0.02

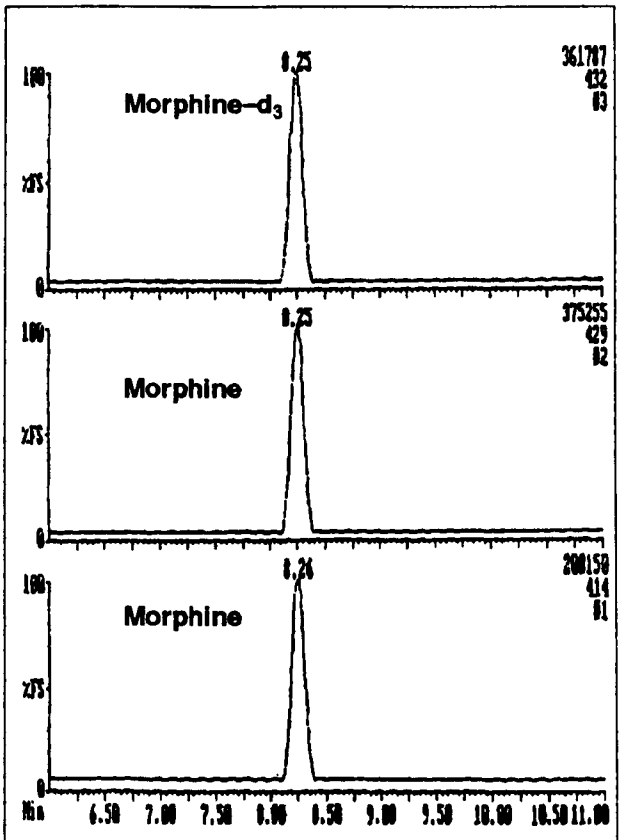

Figure 1. Chromatograph of morphine ions by GC–MS.

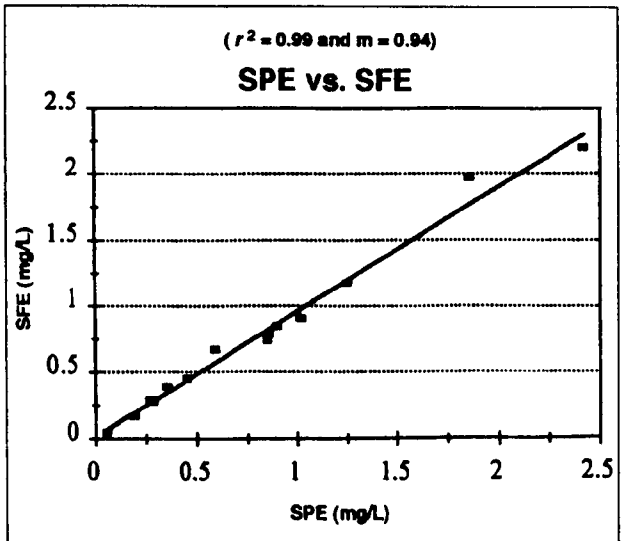

Figure 2. Comparison of SPE and SFE for morphine/blood case samples.

Results and Discussion

Authentic forensic blood samples known to be morphine positive were chosen for this investigation. SFE is an efficient and reproducible extraction method that was compared with SPE to determine if the two yield corresponding results.

Fourteen blood samples were extracted using both SPE and SFE with final analysis by GC–MS. The internal standard morphine-d_3 *m/z* 432 ion was used as a reference to calculate the concentration of morphine using ions *m/z* 414 and 429 for each sample, as shown in Figure 1. For the extraction of morphine using SPE, the sample concentration of morphine ranged from 0.056 to 2.42 mg/mL in blood compared with SFE results that ranged from 0.050 to 2.20 mg/mL in blood. Recoveries for SFE were found to be > 90% for morphine.

The standard deviation and mean for comparison of both methods were 0.633 and 0.782 with a linear relationship of r^2 = 0.990 and a gradient of m = 0.94, seen in Figure 2. Table I lists very small percent differences between the two methods (mean 0.0647). This indicates that good correlation for morphine extracted from whole blood exists between the SPE and SFE methods used.

Conclusions

SPE techniques have been the preferred technique for the extraction of drugs of abuse from various matrices. Although widely used as a sample-preparation method, the technology is still advancing and exploring more parameters to improve sample preparation, including SFE techniques.

SFE, a relatively new technique for sample preparation, has until recently generally been confined to chemical-processing applications. Now, it is emerging as a valuable analytical procedure for the forensic toxicologist.

The investigations carried out using SPE and SFE methods, show that the two analytical techniques correlate well for the analysis of morphine in whole blood. Both techniques are fast, yield reproducible results, and give good recoveries for morphine extracted from whole blood.

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THE APPLICATION OF SUPERCRITICAL FLUID EXTRACTION OF COCAINE AND ITS METABOLITES FROM BLOOD AND URINE

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Abstract

Supercritical fluid extraction (SFE) is emerging as a valuable analytical technique for the use as an alternative to conventional solid-phase and liquid-liquid extraction (SPE/ LLE) techniques. It is a relatively new technique based on the use of supercritical fluids (SF) for the isolation of analytes from various matrices. It is attracting great interest because of the increasing need for a simple, rapid, environmentally friendly, automated and selective extraction method.

A new method was developed using SFE procedures for the extraction of cocaine (COC) and its major metabolites, benzoylecgonine (BZE) and ecgonine methyl ester (EME) from whole blood and urine. This study has shown that cocaine and its metabolites can be successfully extracted from blood and urine using SFE techniques.

Levels measured using SFE have shown analyte recovery better than 70 % for cocaine, better than 40 % for benzoylecgonine and better than 85 % for ecognine methyl ester from whole blood and urine. Good run to run reproducibility was observed between each extraction with limits of detection and quantitation of 1 ng and 10 ng based on 200 µl of blood and urine. A comparison between solid-phase extraction (SPE) and developed SFE techniques was investigated to observe if a correlation existed between the two methods. Studies proved that a correlation did exist between the two methods for spiked blood and urine samples with comparative results. This paper details a procedure for the extraction of cocaine and its metabolites from blood and urine.

Introduction

A Supercritical fluid (SF) is a substance that is above its critical temperature and pressure.[1] They have unique physical properties, which make them suitable as alternative extraction solvents. An important feature of SFs as solvents is that the solvating strength of the fluid is directly related to density. The density can be varied easily as a function of pressure and temperature. However, in general, SFs have solvating properties similar to a liquid, while having viscosities closer to a gas.

The SF of choice is CO_2 because of its low critical pressure and temperature (73.8 bar and 31.1° C). It is a non-polar gas that exhibits great solvent strength towards polar molecules[2]. CO_2 is non-toxic, non-flammable, inexpensive, a good solvent for organics and can be used with small amounts of entrainers (e.g. MeOH). These entrainers or modifiers can vary the extraction power of the fluid to produce greater analyte recovery. In addition, for quantitative analysis, the selection of suitable conditions to extract analytes from complex matrices is relatively straightforward.

SFE is simple to carry out and can be analysed using any technique that is appropriate for the extracted analytes (e.g. GC/MS). Supplied by a pump, a known pressure of the extraction fluid (CO_2) is applied to the extraction column at ambient conditions. The extraction cell containing the sample is placed in an oven at a set temperature above the critical temperature of the supercritical fluid. During the extraction, soluble analytes are partitioned from the sample matrix into the supercritical fluid. The analytes are then swept through a restrictor at ambient pressures and depressurised into a collection vial containing a solvent (e.g. MeOH). The extraction fluids are vented from the collection vial while the analytes are retained for analysis.

There are numerous studies for the extraction of drugs of abuse from various biological matrices, such as serum[3] or plasma[4], urine[5], hair[6] and vitreous humor[7] using solid-phase or liquid-liquid extraction, immunoassay and other extraction techniques. However, most literature published on SFE relates to organic chemical substances, including organic chemical separation from water, caffeine from coffee beans, pesticide residue, polynuclear aromatic hydrocarbons and polychlorinated biphenyls[8] and oils from natural products.[9] More recently, SFE has been used for the

extraction of drugs of abuse from various matrices[10-14]. Several authors have reported the super or subcritical analysis of drugs using various techniques. Janicot *et al*[10] used CO_2 for the successful separation of opium alkaloids from poppy straw followed by Edder *et al*[11] who reported the use of SF-CO_2 for the extraction of opiates from biological matrices and Lawrence *et al* who used SFE to extract benodiazepines from their solid dosage forms.[15] More recently, other authors such as Sach and Uhl[16] and Sach and Raff[17] demonstrated for the first time the use of supercritical fluids in the extraction of drugs in hair.

There are various analytical methods for the analysis of cocaine and its metabolites from both blood and urine. However, in forensic analysis of post-mortem case samples, plasma is seldom available and the tissue most commonly supplied is haemolysed, often putrefied blood. Therefore, a suitable method should be applied capable of detecting concentration levels of cocaine and its metabolites down to nanogram levels. This study reports the development of an application of SFE to the recovery of cocaine and its metabolites (benzoylecgonine and ecgonine methyl ester) from both whole blood and urine.

Experimental

Materials

AnalaR grade isopropyl alcohol was supplied by Merck (Poole, UK) and HPLC grade methanol, dichloromethane and ammonia were supplied by Analytical Sciences Lab-Scan in Dublin, Ireland. CO_2-Air Products were provided by Walton-on-Thames in the UK and the diatomaceous earth (Extrelut®) was supplied by Merck. SFE 6mL Hypovial™ vials were purchased from Pierce in Oud-Beyerland, The Netherlands and the Butyl rubber septas from Pierce and Warner, Chester, UK. The columns for SFE were 3.4 cm x 4.6 mm i-d. stainless steel tubing.

Cocaine, d_3-cocaine, benzoylecgonine and ecgonine methyl ester stock standards were purchased in ampules of 1mg/ml and 100 μg/ ml , which were supplied by Sigma Chemicals, St. Louis MO. The derivatisation agent N,O - bis (Trimethylsilyl) trifluoroacetamide (BSTFA) was supplied by Pierce Chemical Co., USA. The SFE

instrument used was manufactured by Gilson Instruments and supplied by Anachem Instruments in Luton UK.

Stock solutions

Drug and internal standard stock solutions were prepared in methanol (1 μg/ ml and 10 μg/ ml).

Supercritical Fluid Extraction

The pressure, CO_2 amount, modifier and flow rate were all measured using a Gilson 308 "master" pump, that controlled a Gilson 306 "slave" pump. The slave pump delivers organic modifier solvent to a Gilson 811B dynamic mixer that mixes the solvent with the CO_2. The Gilson 821-pressure regulator was controlled by the 308 pump that controlled the operation of the pressure regulation valve indicated by P2 and P1 on the program. P2 was used to maintain the required pressure levels at the outlet. P1 was used to implement the flow rate accuracy corrections and ensures that the system pressure was not above or below the limits entered in the program.

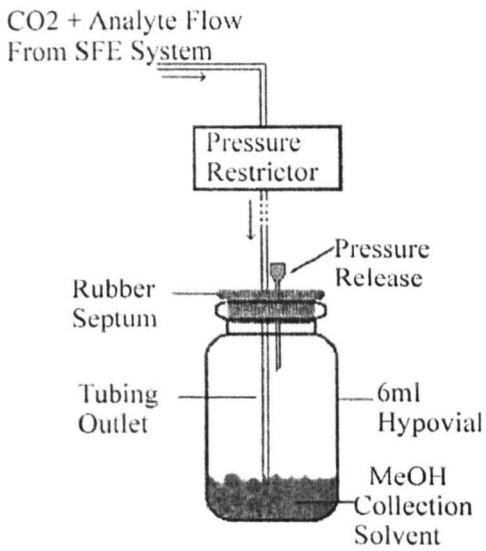
An HPLC 7125 Rheodyne valve, was placed in-line between the 811B mixer and the extraction cell, that was housed inside a Gilson 831 temperature regulator (oven), for the application of the gas and modifier to the cell. A second valve located below the 7125 valve was used to switch the extraction from static to dynamic (continuous) mode. SFE pressure conditions were set at 3500 psi with a flow rate of 2 ml/ min and temperature 40° C. 100-200μl DCM:IPA:NH_3 (78:20:2 v/v) was used as a modifier and added to the extraction cell with 100% CO_2 as the supercritical fluid.

Samples were prepared in weighing boats containing approximately 0.13g of the extraction media Extrelut®. 100 μl of internal standard (100 ng d_3-cocaine) and 100 μl from each stock standard (100 ng COC, BZE, and EME) were added to 200 μl of blood or urine, which was then pipetted onto the media. The contents of the boat were mixed and allowed to dry overnight in a drying chamber at room temperature or until a friable consistency was achieved.

The Rheodyne valve was set to the load position and the instrument left to equilibrate until ready for extraction. The sample was loosely packed into a stainless steel extraction cell and 100 - 200 µl of modifier was added directly to the sample cell. The extraction cell was sealed and placed inside the temperature regulator that was set at 40° C.

The extraction was started by switching the Rheodyne valve from the load (equilibrate) to the inject (extract) position and left for 10 minutes in static mode, then switched to dynamic mode for 20 minutes. The analytes were eluted though the outlet and were collected by expansion into a Hypovial containing 3 ml of MeOH. Figure 1 shows the depressurisation of the analytes into the Hypovial containing solvent. The sample was evaporated under nitrogen at 40° C and derivatised with 50 µl of BSTFA at 70° C for 20 minutes and 1 µl was injected into the GC/MS.

Figure 1: Depressurisation of analytes from the SFE system into a collection vial containing MeOH



Gas Chromatography-Mass Spectrometry Analysis

A Fisons model GC 8000 Series (MD 800 in the EI Mode (70ev) gas chromatography-mass spectrometry operated in single ion monitoring mode (SIM) was used for analysis. This was equipped with an HP-1 (Cross-linked Methyl Siloxane), capillary column, (30 m, 0.53 mm i.d., film thickness 0.88 µm). The carrier gas was Helium with gas flow rates of 1 ml / min. The temperature was set at

an initial 100° C and a final temperate of 300° C for 2 min with an oven rise of 10° C per min. The injector temperature was set at 250° C.

Comparison between SFE and SPE

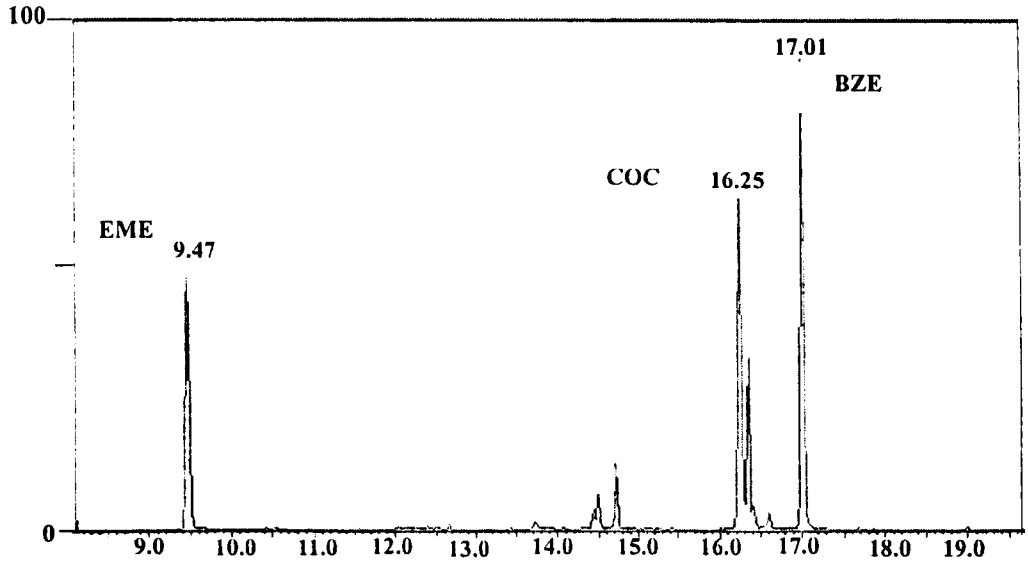
A comparison between SFE and SPE was made to investigate if the two methods correlate for the extraction of blood and urine for cocaine, benzoylecgonine and ecgonine methyl ester. Due to the lack of cocaine positive blood and urine forensic case specimens received into the laboratory, spiked samples were used for comparison between the two methods. From a stock solution of a 10 μg /ml, 50 μl of each drug was pipetted into three separate test tubes containing 1 ml of pre-treated blood or urine for SPE and 200 μl of blood or urine for SFE. Samples were extracted and evaporated to dryness under a stream of nitrogen and derivatised. Then 1 μl of sample was injected into the GC/MS for analysis.

Results and Discussions

SFE can be used as an alternative sample extraction technique for the extraction of cocaine, benzoylecgonine and ecgonine methyl ester from blood or urine. The favourable mass transport and variable solvating powers of supercritical fluids have encouraged researchers to investigate SFE as an alternative to conventional procedures for drug testing. However, there is very limited literature on the SFE of drugs of abuse from biological matrices, with the exception of matrix bound drugs from hair.

Several spiked blood and urine samples were prepared and extracted under the aforementioned conditions. Quantition by SFE method was achieved by the addition of d_3-COC m/z = 185 that was used as a reference ion to calculate the concentration ratios of COC m/z = 182, 303, BZE m/z = 361, 240 and EME m/z = 82 for both blood and urine. Figure 2 shows a chromatograph of a typical GC/MS trace for COC, EME and BZE.

Figure 2: Chromatograph of a COC, BZE and EME trace analysed by GC/MS.



Good run to run reproducibility was observed between each extraction (n = 5) indicating the accuracy of the method from run to run. Table 1 and 2 show comparison of the variability of each drug for each SFE run for blood and urine (n=5).

Table 1: Method repeatability of cocaine, benzoylecgonine and ecgonine methyl ester for blood.

Blood	COC m/z 182/185	BZE m/z 240/185	EME m/z 82/185
1	0.746	0.22	1.28
2	0.849	0.278	1.30
3	0.949	0.27	1.55
4	0.876	0.251	1.43
5	0.769	0.267	1.51

Table 2: Method repeatability of cocaine, benzoylecgonine and ecgonine methyl ester for urine.

Urine	COC m/z 182/185	BZE m/z 240/185	EME m/z 82/185
1	0.758	0.298	1.09
2	0.675	0.316	1.31
3	0.75	0.30	1.31
4	0.81	0.28	1.32
5	0.791	0.27	1.22

Recoveries for cocaine in blood were better than 70 %, benzoylecgonine 40 % and ecgonine methyl ester 85 %. Recoveries for cocaine in urine were better than 75 %, benzoylecgonine 49 % and ecgonine methyl ester 90 %. Because of the low recoveries of benzoylecgonine, each drug was individually spiked onto blood and urine Extrelut® samples and extracted under SFE conditions. This was to investigate possibility of hydrolysis of cocaine to benzoylecgonine or ecgonine methyl ester, which were not observed.

Overall, reproducibility from run to run for cocaine, benzoylecgonine and ecgonine methyl ester in blood and urine produced good standard deviations and relative standard deviations (% CV). Table 3 summarises the method validation of cocaine and its metabolites from both blood and urine. Methods producing RSDs of less than 10 % are reported to be acceptable for method validation of biological specimens.[18]

Table 3: Method Validation of cocaine, benzoylecgonine and ecgonine methyl ester in blood and urine

DRUG	StdDev	% RSD	Mean	%Recovery
BLOOD				
Cocaine	0.0823	9.8	0.837	70
Benzoylecgonine	0.0229	8.9	0.257	40
Ecgonine methyl ester	0.1213	8.5	1.41	85
URINE				
Cocaine	0.0518	6.8	0.756	75
Benzoylecgonine	0.0180	6.1	0.292	49
Ecgonine methyl ester	0.098	7.8	1.25	90

Three calibration curves shown in Figures 3 and 4 were produced for each drug from 200 µl of blood and urine over a range of 5 – 100ng. The results are summarised in Tables 4 and 5.

Table 4: Linearity of cocaine, benzoylecgonine and ecgonine methyl ester in blood

Conc^{n} ng/µl	COC m/z 182/185	BZE m/z 240/185	EME m/z 82/185
5	0.008	0.001	0.76
10	0.032	0.006	0.97
20	0.07	0.02	1.21
40	0.19	0.03	1.52
60	0.27	0.44	1.83
80	0.42	0.068	1.97
100	0.58	0.10	2.21

Figure 3: Linearity of cocaine, benzoylecgonine and ecgonine methyl ester in blood

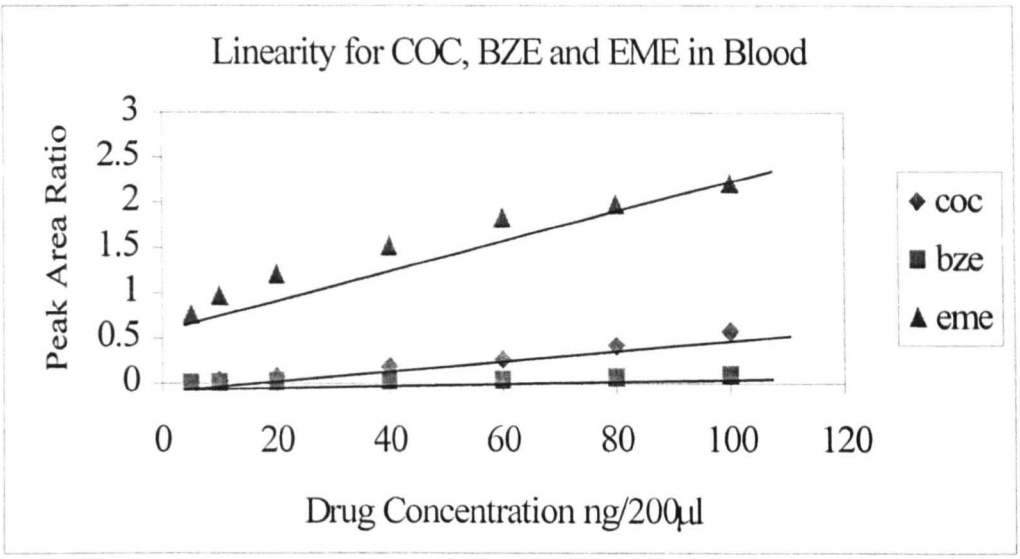


Table 5: Linearity of cocaine, benzoylecgonine and ecgonine methyl ester in urine

Conc^{n} ng/µl	COC m/z 182/185	BZE m/z 240/185	EME m/z 82/185
5	0.0423	0.003	0.21
10	0.10	0.01	0.26
20	0.16	0.05	0.54
40	0.35	0.11	1.03
60	0.59	0.19	1.51
80	0.73	0.25	2.07
100	0.92	0.35	2.71

Figure 4: Linearity of cocaine, benzoylecgonine and ecgonine methyl ester in urine

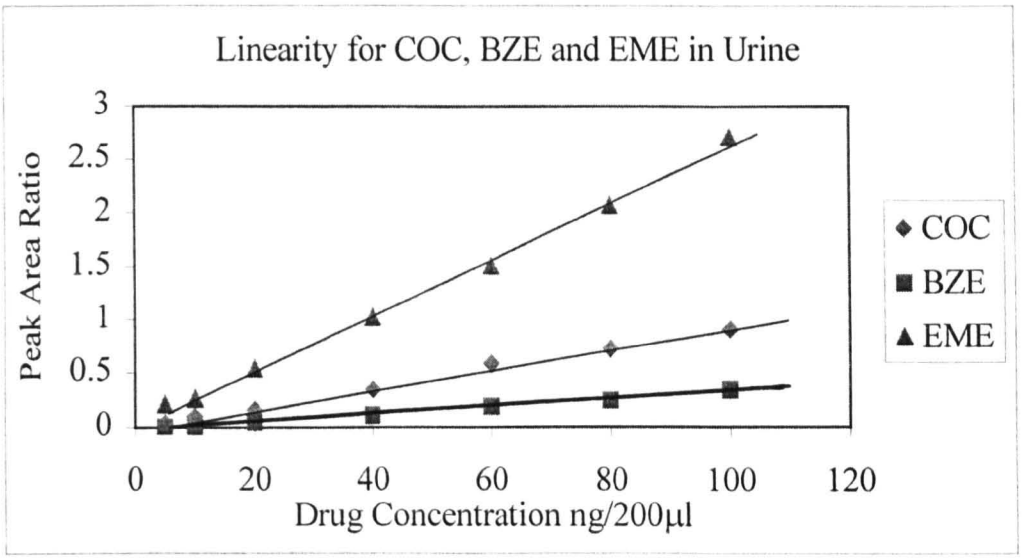


Table 6 summarises concentration range for the calibration curves and the limit of detection (LOD) and quantitation (LOQ) for each drug. The limit of detection (LOD) for cocaine, benzoylecgonine and ecgonine methyl ester was determined by using 200 µl of blank blood spiked from a 1µg/ ml solution. For the three drugs, the LOD was 1 ng with a limit of quantification (LOQ) of 10ng in blood and urine.

Table 6: Linear calibration and concentration range of cocaine, benzoylecgonine and ecgonine methyl ester.

DRUG	r^2	Slope	LOD/LOQ	Conc Range
BLOOD			**ng / 200µl**	**ng / 200µl**
Cocaine	0.987	0.0058	1 ng / 10 ng	0 - 100
Benzoylecgonine	0.971	0.0009	1 ng / 10 ng	0 - 100
Ecgonine methyl ester	0.964	0.0145	1 ng / 10 ng	0 - 100
URINE				
Cocaine	0.997	0.0092	1 ng /10 ng	0 - 100
Benzoylecgonine	0.991	0.0034	1 ng / 10 ng	0 - 100
Ecgonine methyl ester	0.997	0.0262	1 ng / 10 ng	0 - 100

SPE and the developed SFE methods were compared to find the existence of a correlation. Results showed that a correlation existed between the two methods with

small differences observed. The percent differences between the two methods for cocaine in blood were 0.04, 0.09, 0.08, for benzoylecgonine 0.03, 0, 0.05 and ecgonine methyl ester 0.07, 0.03, 0.03. For urine, the percent difference for cocaine was 0.10, 0.06, 0.02, benzoylecgonine 0.04, 0.06, 0.05 and ecgonine methyl ester 0.09, 0.07, and 0.06. Results from both methods for blood and urine are summarised in Table 7. The samples results for blood using SFE ranged from 0.084 – 0.21mg/l and SPE 0.11 – 0.26 mg/l. For SFE in urine the result range was 0.13 – 0.29mg/l and SPE 0.07 – 0.20mg/l. Figures 5 and 6 show graphs based on Table 5 for both methods for blood and urine, which clearly show the variation between the two methods.

Table 7: Comparison of SPE and SFE results of cocaine, benzoylecgonine and ecgonine methyl ester in urine and blood.

	Cocaine mg / L	**Benzoylecgonine mg / L**	**Ecgonine methyl ester mg / L**
SPE bld 1	**0.207**	**0.16**	**0.103**
SPE bld 2	**0.21**	**0.11**	**0.084**
SPE bld 3	**0.18**	**0.17**	**0.093**
SFE bld 1	0.25	0.13	0.17
SFE bld 2	0.20	0.11	0.11
SFE bld 3	0.26	0.12	0.12
SPE urn 1	**0.19**	**0.10**	**0.08**
SPE urn 2	**0.17**	**0.07**	**0.081**
SPE urn 3	**0.20**	**0.08**	**0.09**
SFE urn 1	0.29	0.14	0.17
SFE urn 2	0.23	0.13	0.16
SFE urn 3	0.22	0.13	0.14

Figure 5: Graph showing the comparison between the two methods for blood.

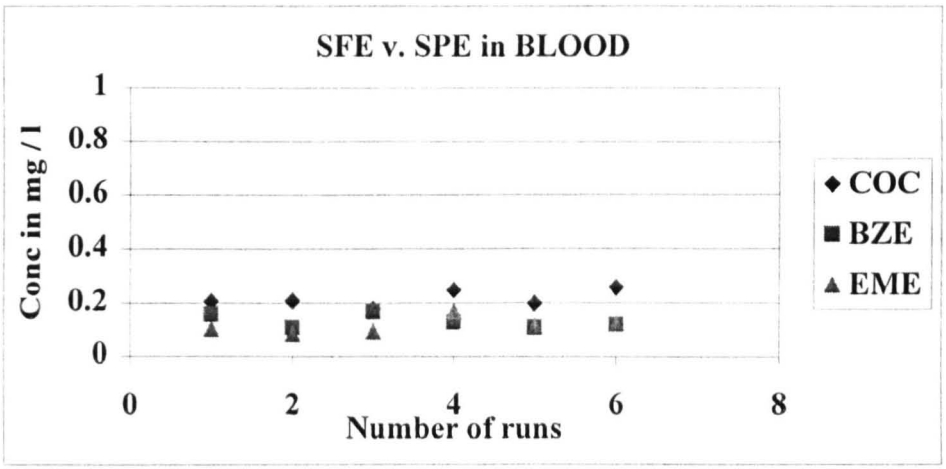
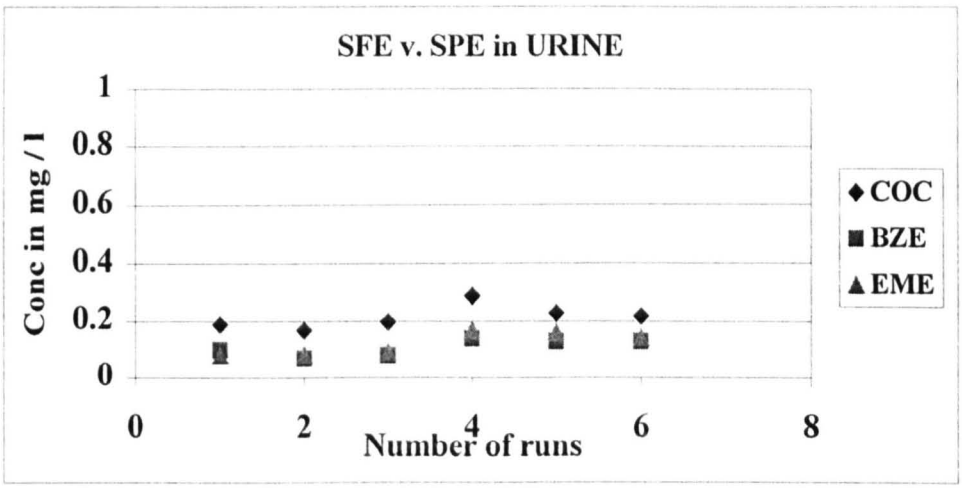


Figure 6: Graph showing the comparison between the two methods for urine.



Conclusion

At present, cocaine analysis from biological matrices involves a SPE method. The current study demonstrates that SFE can be used as a possible alternative to conventional methods for the isolation of drugs of abuse such as cocaine, benzoylecgonine and ecgonine methyl ester from blood and urine.

Various temperatures, pressures, flow rates and modifiers were investigated during method development. However, the described chosen conditions proved to give the best results for cocaine and its metabolites. Recoveries greater than 70% were

reported for cocaine and greater than 85% for ecgonine methyl ester. However, benzoylecgonine recoveries were reported to be greater than 40%, which was lower than cocaine and ecgonine methyl ester. A possible explanation would be because the benzoylecgonine analyte is a zwitterion, a closer control of the extraction pH would have probably increased the yields. However since the developed procedures produced a reproducible extraction and was optimised to maximise the recovery of the major metabolite (benzoylecgonine), further development was deemed unnecessary.

SPE techniques were compared to the developed SFE techniques to find if a correlation existed. A correlation was observed between SPE and SFE for the extraction of cocaine and its metabolites. Small percent differences were observed between the two methods, however both methods were comparable. This developed SFE method for the recovery of cocaine and its metabolites has demonstrated its potential usage for the extraction from spiked whole blood and urine.

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Hair Analysis: Self-Reported Use of "speed" and "ecstasy" Compared with Laboratory Findings*

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Abstract

Drug use histories were collected from 100 subjects recruited from the "dance scene" in and around Glasgow, Scotland. In addition, each subject donated a hair sample which was analysed by gas chromatography – mass spectrometry (GC/MS) for amphetamine (AP), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA).

The hair samples were analysed in two 6cm segments or in full, ranging from 1.5 - 12cm depending on the length of the hair. Approximately 10mg of hair was ground to a fine powder before treatment with β-glucuronidase/aryl sulphatase. A solid-phase extraction procedure was carried out followed by derivatisation with pentafluoropropionic anhydride (PFPA). All extracts were analysed by gas chromatography/mass spectrometry (GC/MS).

Of the 139 segments analysed, 77 (52.5%) were positive for at least one of the five amphetamines. The drug concentrations found in the hair were compared with the self-reported drug histories. A concordance of greater than 50% was found between the self-report data and levels detected in hair. However no correlation was found between the reported number of "ecstasy" tablets consumed and the drug levels detected in hair. An increase in the average drug levels measured was observed from low to high use (number of "ecstasy" tablets/month). A large number of false negatives and a low number of false positives were observed.

Keywords: forensic science, hair analysis, self-report, "speed", "ecstasy", solid-phase extraction

Introduction

The analysis of hair is now accepted as an alternative method for determining drug use. It has several possible advantages over other biological matrices (e.g. blood and urine), including collection of information regarding long-term drug use and determination of compliance with treatment programs. (1-3) Over the past few years there has been a substantial increase in the number of scientific papers detailing methods for the analysis of a variety of both prescription and non-prescription drugs in hair.

Several independent laboratories in Europe, Japan and the US have confirmed the accumulation of drugs such as cocaine (4-6), heroin and other opiates (4,7-9), amphetamines (10-13) and phencyclidine (14,15) in the hair of drug users. Other studies published have reported a dose-response relationship in hair for a variety of drugs in controlled animal studies (16-19). Most of the studies involving human subjects compared self-reported drug use to hair or urinalysis. A linear relationship was observed between hair cotinine levels and daily nicotine intake (20), however, this is an exception to what is generally reported. A relationship between low and high use and the levels of drugs detected in hair has been observed for cocaine (21), heroin (22) and buprenorphine (2). The lack of a linear dose-response relationship is not surprising due to the number of unknown variables, such as the exact dose or purity of drug consumed, the accuracy of the self-report data, individual metabolism variations and the types and frequency of hair treatments.

The work presented here is part of a larger study investigating the use of "speed" and "ecstasy" in Glasgow (23). Subjects claiming use of "speed" and "ecstasy" were

recruited from the "dance scene" by chain referral (i.e. subjects nominated other subjects). They were asked to complete a detailed questionnaire of their drug use over the previous 12 months and to donate a hair sample for analysis. Subjects were compensated for samples.

The aims of the project were to investigate the accuracy of hair analysis in confirming self-reported use of "speed" and "ecstasy" and to determine whether or not a correlation exists between the amount of drug ingested and the levels detected in hair. AP and MA were identified to confirm "speed" use and MDA, MDMA and MDEA for "ecstasy" use.

Materials and Methods:

Sample Collection

100 subjects were interviewed, 51 of whom were male. Ages ranged from 15-44 years, with a mean age of 24 years. 95% of the participants were Caucasian. "Ecstasy" use was recorded as the number of tablets consumed per month for each of the previous 12 months. "Speed" use was recorded as "ever consumed" and then broken down into how often they had consumed it in the previous week, month and year.

The hair samples were cut close to the scalp in the vertex region, wrapped in aluminium foil and sealed in labelled plastic bags. The self-report "ecstasy" data was recorded for each month prior to interview, therefore it was possible to do segmental analysis on a month by month or cm by cm basis. Ideally 10 mg of hair is required for analysis, however, some of the hair samples were very small and thus, had to be analysed in full. Where possible, two 6 cm segments were analysed. The proximal

6 cm was labelled the "root" sample and the distal 6cm the "tip" sample. In total, 139 hair segments were analysed without prior knowledge of the self-report data.

A summary of the segments analysed is shown in Table 1. The hair lengths ranged from 1.5-12 cm. A total of 56 samples were analysed in full.

Summary of Drug Use History

Of the 100 subjects questioned, 90 admitted taking "ecstasy" during the year prior to interview. Consumption of "ecstasy" was primarily at one of four locations (night-clubs, licensed raves, illegal parties and private parties). Not all users consumed "ecstasy" on a regular basis and were classed as stable or erratic users. In contrast to stable users (n = 43) who consistently used the same amount of "ecstasy" each month, erratic users (n = 48) tended to have an irregular pattern of use which varied from month to month.

The total number of "ecstasy" tablets consumed within these two groups ranged from 1 – 144 (mean = 19, median = 10) over the previous year. In this study, the heaviest user consumed 144 tablets in the previous year, which translates to more than weekly but less than daily consumption of "ecstasy". This is in sharp contrast to heavy heroin users, who use heroin at least once a day. The control group (n = 9) consisted of individuals who participated in the dance scene, but denied ever using "speed" or "ecstasy". This number is low due to the difficulty in recruiting individuals who satisfied this criteria.

Questions regarding use of other drugs were also answered by each subject. The drugs used by the respondents over the previous year are summarised in Table 2 in descending order of frequency of use. The majority of respondents consumed alcohol (96%), cannabis (95%), "speed" (84%) and tobacco (84%).

Experimental

Standards and Reagents

Amphetamine (AP), methamphetamine (MA), 3,4-methylene-dioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), d_3-amphetamine (d_3-AP), lauryl sulfate (sodium dodecyl sulphate (SDS)) and β-glucuronidase (activity = 300,000 – 400,000 units/g solid, sulphatase activity = 15,000 – 40,000 units/g solid) were obtained from Sigma® Chemical Co., (Dorset, U.K.). 3,4-methylenedioxy-ethylamphetamine (MDEA) was manufactured by Radian International and supplied by Promochem, (Herts., U.K.). Pentafluoropropionic anhydride (PFPA) was supplied by Fluka Chemicals (Dorset, U.K.). HPLC-grade methanol, acetone, chloroform, deionised water and ethyl acetate chromatographic (HPLC) grade were obtained from Lab-Scan Analytical Sciences (Dublin, Ireland). Analytical-grade ammonium hydroxide and glacial acetic acid and HiPerSolv grade potassium-dihydrogen phosphate (KH_2PO_4) were obtained from Merck (Poole, UK).

Stock solutions

A combined stock standard solution (0.1mg/ml) of AP, MA, MDA, MDMA and MDEA was prepared in methanol. Working solutions were also prepared in methanol (10, 1μg/ml) by appropriate dilution of the stock standard. The internal standard (I.S.), d3-AP, was prepared in methanol to give a stock standard solution of

0.1mg/ml. Working solutions of d_3-AP (10, 1µg/ml) were prepared by dilution of the stock standard with methanol. All stock and working solutions were stored in a freezer at -20°C.

Gas Chromatography - Mass Spectrometry

A Fisons model GC8000 series was used, fitted with a HP-1 capillary column. The initial column temperature of 55°C was held for 2 minutes and then increased to 280°C at a rate of 20°C/min. and held for a further 5 minutes. The carrier gas used was Helium. Injections were made in the splitless mode.

The GC8000 was interfaced to a Fisons MD800 mass spectrometer operated in the electron - ionisation mode at 70eV. The ions monitored were m/z 118, 190* (AP), m/z 118, 160, 204* (MA), m/z 135, 162, 190, 325* (MDA), m/z 135, 162, 204, 339* (MDMA), m/z 135, 162, 190, 218, 353* (MDEA) and m/z 193* (d_3-AP). The ions labelled "*" were used for quantitation.

Sample Pre-treatment

Hair samples were washed once with 0.1% SDS in deionised water and then in triplicate with deionised water. Each wash step was carried out by sonicating for 15 minutes. The hair was then rinsed with methanol and allowed to dry overnight in a dessicator.

The dried hair was measured and cut to the appropriate lengths, before being ground to a fine powder under liquid nitrogen using a mortar and pestle. The powdered hair was placed in a clean vial and weighed accurately.

Each hair sample was treated with 50 µl of β-glucuronidase [0.1mg/ml in phosphate buffer (pH7.4, 0.1M)] and 2 ml of phosphate buffer (pH7.4, 0.1M). 100 µl of the internal standard, d_3- AP (10ng/mg based on a 10mg hair sample) was added and the samples were incubated at 40 °C for two hours. The samples were allowed to cool to room temperature, and then the supernatant was removed following centrifugation (2000 rpm, 5 min.). A further 2 ml of phosphate buffer was then added, centrifuged as before and the two supernatants combined for further analysis.

Solid-Phase Extraction

To separate the drugs from the hair matrix, solid-phase extraction was carried out on Isolute® Confirm HCX-3 (130 mg) SPE columns [manufactured by International Sorbent Technology LTD (Hengoed, U.K.) and supplied by Crawford Scientific (Strathaven, U.K.)].

The method used was adapted from a method previously reported for the analysis of methadone[24] and differs only by the pH of the phosphate buffer used. The SPE columns were conditioned with methanol and phosphate buffer (pH7.4, 0.1M). The supernatant was applied onto the column which was then washed with deionised water. The pH was adjusted with 0.01M acetic acid pH 3.3 and the column subjected to two drying steps before elution of the drugs. The first fraction (A) was eluted with acetone:chloroform (1:1v/v) and was discarded as no quantifiable levels of the drugs were detected. The analytes of interest were eluted into the second fraction (B) using 2 ml of 2 % ammoniated ethyl acetate.

Derivatisation and GC/MS Analysis

Fraction B was evaporated to dryness under a stream of nitrogen at room temperature. The drugs were then reconstituted using 50µl of PFPA:Ethyl acetate (1:1) sealed and allowed to derivatise at 50°C for 15 minutes. After derivatisation was complete, the samples were evaporated to dryness as before and reconstituted in 50µl of ethyl acetate. 1µl was injected for analysis by GC/MS.

Method Validation

Hair samples were spiked by adding a set volume of the working solution to 100mg of ground blank hair (washed prior to milling). The methanol was allowed to evaporate to dryness overnight at room temperature. The hair was then mixed thoroughly to ensure homogeneity of the sample.

The developed method was validated using blank hair spiked with the five amphetamines at three different concentrations (5, 10 and 20 ng/mg), using approximately 10 mg of hair.

The recoveries from the spiked hair were greater than 70 % for all five amphetamines and the method was linear in the concentration range 0 to 100 ng/mg. The limit of detection (L.O.D.) for AP and MA was 0.5ng/mg and 0.1ng/mg for MDA, MDMA and MDEA. The results are summarised in Table 3.

Spiking hair results in surface bound drugs and does not accurately represent how drugs are bound in the inner layers of drug users hair. This should be taken into account when interpreting the validation results.

Analysis of "ecstasy" Preparations

Substances (n = 15) which were sold as "ecstasy" were obtained by the research group. A Home Office licence (Ref. No.: 93/MM/254) was obtained which authorised the possession of drugs specified in Schedule I and II to the Misuse of Drugs Regulation 1985 (As amended). The 15 "ecstasy" preparations were analyzed by gas chromatography - flame ionisation detection (GC-FID) and GC/MS. GC-FID analysis was carried out using an HP 5890 GC fitted with a CPSil 5 0.5 i.d. x 10 m column. The intial temperature was set at 80°C and then ramped to a final temperature of 200°C at a rate of 10°C/min. and held for five minutes. The injector and detector temperatures were set at 290°C. Confirmation was carried out by GC-MS using a Fisons GC8000/MD 800. The initial temperature of 60°C was ramped to 300°C at 10°C/min. and held for 10 minutes.

Results and Discussion

Due to the limited sample size, each hair segment (n = 139) could only be analysed once by the described method. The reliability of this single analysis should be taken into account when interpreting the results.

Of the 139 hair segments, 73 (52.5%) tested positive for at least one of the five amphetamines, the remaining 66 segments were negative for all five. In general, the levels of AP, MA, MDA, MDMA and MDEA detected in the hair samples are in the same range as those reported elsewhere (10,13,25-27).

Some exceptions to this were found where levels detected were higher than those previously reported. These cases were few and the median of the results was found to lie within the reported ranges. The results are summarised in Table 4.

The ratio of metabolite to parent drug levels in hair has been used to determine whether a positive result is due to contamination or from ingestion.(28,29) This ratio is expected to be lower than 1.0 due to the preferential incorporation of the parent drug to the metabolite. Therefore, a value greater than one is thought to result from contamination. The Society of Hair Testing recognises set ratios for cocaine and heroin and their metabolites, but not as yet for amphetamines (29).

The metabolite to parent drug ratios are summarised in Table 5. With the exception of the ratio MDA: MDMA, the ranges of the others were higher than those previously reported. This can be explained in the case of AP and MA. "Speed" can contain both AP and MA as the parent drug, which will effect the metabolite to parent drug ratio. Combining the levels of MDMA and MDEA found in individual hair samples was carried out to determine if a relationship exists between the combined levels of these parent drugs in hair and the amount of drug consumed. Results obtained for the ratio MDA: (MDMA+MDEA) ranged from 0.03 to 6.5 in contrast to those found by Rothe et al (25) (0.03 to 0.2, n=67). However, they too reported cases with ratios > 1. A possible explanation for this would be the consumption of MDA as a parent drug as well as its metabolic contribution from MDMA and MDEA.

To investigate this, the contents of 15 illegal "ecstasy" preparations collected at the approximate time of sampling were analysed. The results are summarised in Table 6. The amount of MDMA present was found to range from trace levels in "Emerald E" (which mainly consisted of AP and caffeine) to 70 % in "Madwoman" (n = 15, range: trace – 239mg, median = 90). MDEA was detected in only three samples (range:

trace – 3.5mg). MDA was not detected in any of the 15 preparations. However, this is not a representative sample and does not eliminate the possibility that "ecstasy" consumed by individuals in this study contained MDA. "Ecstasy" tablets seized within the U.K. have been reported to contain MDA (30-31).

Concordance between the self-reported data and the levels detected in hair was investigated for all 139 segments. A total of 73 segments tested positive for at least one of the five amphetamines with 71 of these agreeing with the self-report data. 66 were were found to be negative, with only six agreeing with the self-report data. Overall, agreement or concordance was greater than 50% with a low number of false positives, consistent with other studies. The results of which are summarised in Figure 1.

A large number of false negatives were found. There are a number of possible explanations for this including the method lacking sensitivity, over reporting of drug use by the individual and the influence of cosmetic treatment on the hair (32-34). Unfortunately, no information was available on previous hair treatments (e.g. perms, bleaching etc.) and so the contribution of this to the results could not be assessed.

Comparing the different types of sample analysed, it was expected that root samples would be more accurate than tip samples as they have been subject to fewer environmental and cosmetic influences, which have been shown to affect the drugs stability in hair. In addition, the self-report data would be expected to be more reliable due to better recollection of drug use in recent months as opposed to 12 months prior to interview.

The root samples have slightly better concordance (59.3%) than the tips (51.7%) and full (53.6%) samples. Figure 2 summarises the concordance between self-report and hair analysis for each of the segment groups. These consist of roots (proximal sample ≤ 6cm), tips (distal 6cm) and full samples (> 6cm).

When comparing the self-report data for the use of "speed" only or "ecstasy" only, the concordance is very low for "speed" (28.3%) but higher for "ecstasy" (57.5%). The results are illustrated in Figure 3. Unlike the "ecstasy" data, the "speed" data was not recorded for each month of the previous year and in addition, higher limits of detection were employed. The combination of these factors will decrease the number of potentially positive results.

Of the six false positive segments where individuals denied "speed" use but tested positive for amphetamine and/or methamphetamine, five admitted "ecstasy" use, and were also positive for MDA, MDMA or MDEA. Six of the seven false positives for "ecstasy" admitted taking "speed" but only one case was positive for amphetamine. It is certainly plausible that the subjects were unaware whether they were consuming "speed" or "ecstasy" and this would account for some of the false positive or negatives. For example, one of the 15 "ecstasy" preparations consisted of mainly amphetamine and caffeine, with only trace levels of MDMA.

Figure 4 illustrates the correlation between the total number of "ecstasy" tablets consumed and the total concentration of "ecstasy" (MDMA + MDEA) detected in each hair segment. There is a large scatter of results with the majority congregated along the axes. No linear dose-response relationship was observed ($r = 0.0484$, $p =$

0.572). This was also the case when comparing the amount of "ecstasy" consumed and the total concentrations of MDMA or (MDA + MDMA + MDEA) measured.

The results for each hair segment were separated into five groups depending on the average number of "ecstasy" tablets consumed each month. Each group consisted of approximately 20% of the segments analysed. The mean, median and range are noted for each group and summarised in Table 7.

An increase in the mean and the median values for the total concentration of MDMA + MDEA is observed from low to high frequency of use. The one exception to this is the group where between 0.5 to 1.25 tablets were consumed each month. The mean value is higher due to one case which had a total concentration of 93.4 ng/mg. Another important point here is the high number of false negatives which are prevalent throughout each of the four groups claiming "ecstasy" use. However, the number of false negatives did decrease from low to high use.

The data was evaluated with respect to sex and hair colour. There was no significant difference between the sexes. 95% of the participants were Caucasian with the vast majority with hair of various shades of brown. The lack of a relationship with hair colour is probably indicative of this.

Conclusion

The described method combining enzyme extraction, SPE, derivatisation and GC/MS, successfully detected all five amphetamines (AP, MA, MDA, MDMA and

MDEA) in hair samples donated by participants in the Glasgow dance scene, to confirm the use of "speed" and "ecstasy".

Although concordance between self-reported data and laboratory findings was approximately 50%, there were a high number of false negatives (43.2%) which may highlight the methods lack of sensitivity in confirming low use of "speed" and/or "ecstasy". No correlation was observed between the amount of "ecstasy" consumed and the levels detected in hair. However, there was evidence of a relationship existing between the level of use (measured in average "ecstasy" tablets consumed per month) and the levels detected in hair from low to high use.

The results of this study have given rise to a number of key questions which need to be answered to realise fully the potential of hair analysis, in particular, for the analysis of ecstasy in hair. A combination of low concordance between self-report data and hair analysis results, and the high number of false negatives, raises questions regarding both the method sensitivity and the reliability of the self-report data.

The method of collection of hair samples is also of utmost importance. As highlighted earlier in this paper, the samples obtained for this study were too small to be analysed more than once. In some cases, this one analysis yielded a false negative result as the sample size was too small to give a significant level by GC/MS. It is acknowledged that obtaining larger samples on a voluntary basis could be problematic due to the sampling area (vertex region). However it is practical to be able to analyse samples in triplicate in order to decrease experimental errors and thus increase the validity of analytical results.

The large number of false negatives and low drug levels detected is likely to be a reflection on low drug consumption, with the heaviest users consuming approximately 1 "ecstasy" tablet per week of unknown purity. Comparing this to heavy heroin or cocaine users (>1 per day), approximately 80% of "ecstasy" users in this study consumed less than 1 tablet per week.

The parent drug to metabolite ratio is unusual in comparison to the studies carried out with heroin and cocaine, where ratios of less than one are expected. The ecstasy preparations analysed during this project were not a representative sample. Tablets seized from within the UK prior to the hair samples being collected, had high quantities of MDA present. With this in mind the ratios are not unusual due to the unknown contribution of MDA as either a parent drug and/or a metabolite.

Large discrepancies between cases are reflected in the lack of a dose-response relationship for the segments analysed. Two examples are given which clearly illustrate this.

One subjected reported use of 52 "ecstasy" tablets over a six month period, however the hair was negative for all five amphetamines. Conversely, another subject denied use of "ecstasy" but 0.6 ng/mg of MDMA were found in the corresponding 2 cm hair segment.

Determining the smallest dose detectable in hair, in combination with dose-response studies, would certainly help explain why in one case, where only 5 "ecstasy" tablets

were consumed, a level of 1.1 ng/mg of MDMA was detected. However, when 50 tablets were consumed by another subject, this only gave rise to a level of 0.5 ng/mg of MDMA.

The influence of various factors, i.e. cosmetic treatments, individual metabolism variations, and the uncertainty of the content and dose of the "ecstasy" tablets, are currently being investigated further. A wealth of information can be gained from studies involving consumption of known doses and subsequent hair analysis. One additional factor, highlighted by Rothe et al (25), is the influence of drug incorporation via sweat. This is of particular relevance with "ecstasy" use, where participants in the dance scene are known to dance for several hours in hot, humid conditions.

The relationship observed between the average number of "ecstasy" tablets consumed per month and the total concentration of (MDMA + MDEA) detected in hair, gives some indication of the potential of hair analysis as an indicator of "ecstasy" use. This in addition to the previous points, will aid greatly with future interpretations of amphetamine levels in hair.

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TABLE 1 – Lengths of hair segments analysed

Sample Type	Length (cm)	Total
Roots	< 3cm	4
	3cm < 6cm	10
	6cm	40
Tips	6cm	29
Full	6.1cm < 12cm	10
	12cm	46
Total		**139**

TABLE 2 – Drugs consumed by 100 respondents over the year prior to interview

Drug	Yes	No	No Response
Alcohol	96	4	-
Cannabis	95	5	-
"speed"	84	16	-
Tobacco	84	16	-
LSD	67	33	-
Cocaine	53	42	5
***Poppers**	46	50	4
†Mushrooms	30	67	3
Temazepam	29	68	3
‡Other Benzos	19	75	6
§Other Opiates	13	82	5
Glue	7	88	5
Heroin	6	86	8
Temgesic	6	84	10
Ketamine	5	86	9

* Alkyl nitrites

†Hallucinogenic mushrooms

‡All benzodiazepines with the exception of temazepam

§All opiates with the exception of heroin

TABLE 3 – Recovery and linearity results for the extraction of amphetamines from hair

Analyte*	Recovery (%) ± Std. Dev. †	L.O.D. ‡ (ng/mg)	Linearity (r) §
AP	70.6 ± 7.92	0.5	0.990
MA	79.4 ± 8.68	0.5	0.993
MDA	82.2 ± 6.26	0.1	0.993
MDMA	87.1 ± 4.62	0.1	0.995
MDEA	85.4 ± 2.12	0.1	0.996

* AP: amphetamine, MA: methamphetamine, MDA: 3,4-methylenedioxyamphetamine, MDMA: 3,4-methylenedioxymethamphetamine, MDEA: 3,4-methylenedioxyethyl-amphetamine

†Standard Deviation

‡ Limit of Detection

§ Correlation Coefficient

TABLE 4 – Drug concentrations detected in hair segments

Drug	No. (Range) ng/mg	Median ng/mg	Mean ng/mg
AP	11 (0.7 - 97.7)	2.5	12.5
MA	27 (0.6 – 32.3)	2.6	5.2
MDA	20 (0.1 – 8.4)	1.0	1.9
MDMA	56 (0.1 – 82.9)	0.7	4.6
MDEA	23 (0.1 – 12.0)	0.5	2.8

TABLE 5 – Metabolite to parent drug ratios

Ratio	No. (Range)	Median	Mean
AP : MA	9 (0.15 – 44.40)	0.85	7.26
MDA : MDMA	12 (0.01 – 6.28)	0.40	0.54
MDA : MDEA	11 (0.10 – 6.50)	0.67	1.59
MDA : (MDMA + MDEA)	14 (0.03 – 6.50)	0.33	0.95

TABLE 6 – Content of "ecstasy" preparations analysed by GC-FID and GC/MS

Street Name	Form	MDMA	OTHER
Emerald E	Capsule	MDMA (tr.)*,	AP(46%), †Caff.(41%)
Tornado Powder	Powder	MDMA(13%)	
Rhubarb + Custard	Capsule	MDMA(17%)	
Lemon and Lime	Capsule	MDMA(19%)	MDEA (1%)
Clog	Tablet	MDMA(20%)	
Pink Snowball	Tablet	MDMA(20%)	
Madman	Tablet	MDMA(21%)	
Turbo	Tablet	MDMA(21%)	
Disco Biscuit	Tablet	MDMA(31%)	MDEA (tr.)
Un-named	Tablet	MDMA(43%)	
Blaster	Tablet	MDMA(46%)	
White/Love Dove	Tablet	MDMA(58%)	
Madwoman Powder	Powder	MDMA(65%)	
Super dove/robin	Powder	MDMA(68%)	MDEA (tr.)
Madwoman	Tablet	MDMA(70%)	

* Trace levels detected

† Caffeine

TABLE 7 – Correlation between the number of "ecstasy" tablets consumed/month and the total concentration of (MDMA + MDEA) detected in hair

No. E Tablets per month*	**Frequency**	**ng/mg in hair**		
		Mean	**Median**	**Range**
None	29	0.086	0.00	0 – 1.00
< 0.5	29	1.493	0.10	0 – 20.3
0.5 – 1.25	28	4.425	0.00	0 – 93.4
> 1.25 – 3.5	27	1.615	0.00	0 – 23.9
> 3.5	26	4.285	0.55	0 – 55.8
Total	**139**	**1.942**	**2.00**	**0 – 93.4**

* The average number of ecstasy tablets consumed each month.

		SELF	
		Yes	No
LAB	Yes	71 (51.1%)	2 (1.4%)
	No	60 (43.2%)	6 (4.3%)

SELF

LAB	Yes	No
Yes	29 (53.7%)	0 (0.0%)
No	22 (40.7%)	3 (5.6%)

A

SELF

LAB	Yes	No
Yes	13 (44.8%)	0 (0.0%)
No	14 (48.3%)	2 (6.9%)

B

SELF

LAB	Yes	No
Yes	29 (51.8%)	2 (3.6%)
No	24 (42.9%)	1 (1.8%)

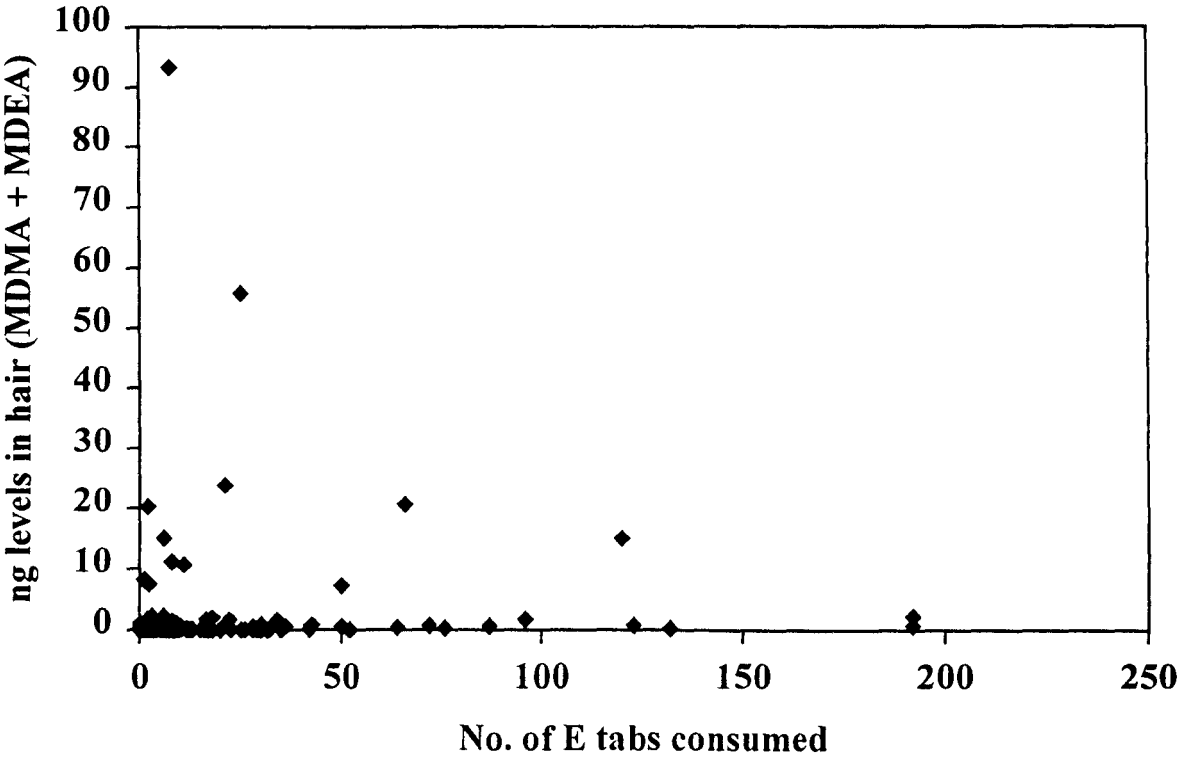
C

		SELF	
		Yes	No
LAB	Yes	23 (16.7%)	6 (4.3%)
	No	93 (67.4%)	16 (11.6%)

A*

		SELF	
		Yes	No
LAB	Yes	58 (41.7%)	7 (5.0%)
	No	52 (37.4%)	22 (15.8%)

B


ng levels in hair (MDMA + MDEA)
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
No. of E tabs consumed

List of Legends

Hair testing for "ecstasy" in a large volunteer sample of Scottish drug users

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Introduction

Although the results of testing hair for the presence of poisons have been accepted - at least in American courts - for over a century,[1] hair testing for evidence of the use of drugs has not yet reached that degree of full judicial acceptability,[2] although there are various reports of quasi-judicial use.[3,4,5,6]

Hair testing began slowly about 20 years ago, initiated, perhaps, by Baumgartner's pioneering article.[7] Sachs suggests an erratic growth thereafter, with a "gold rush" period between 1986 and 1992 typified by relatively uncritical use of hair testing, followed by a "hang over" period between 1992 and 1996 characterised by more critical reflection.[8] It seems that a renewed "gold rush" - at least of published papers if not of conclusive results - began thereafter.

Hair offers one crucial potential advantage when compared to, for example, urine as a medium for detection - the long time window. Whereas urine can only indicate use for 2-3 days for opiates, cocaine and the amphetamines, hair offers the possibility of retrospective use examination for at least 3 months, and possibly for much longer. Indeed, Baumgartner, *et al.* claim to have tested 5 hairs, 7.6cm in length, from Keats, and, 167 years later, discovered the presence of opiates therein;[9] and Kidwell & Blank report the discovery of cocaine in the hair of 1,000 year old Peruvian mummies.[10] Specifically, hair offers the opportunity to examine evidence of drug use for each of the months separately, rather than merely for all of the months together. A number of other advantages of hair are sometimes cited (difficult to falsify, easy to store, lengthy shelf life, low body invasion), but for others, these apparent benefits merely offer more opportunity to tramel with people's civil liberties for longer periods.[2,10,11]

Previous work on MDMA (3,4-methylenedioxymethamphetamine) in hair is obviously of some relevance here (although Mieczkowski considers that MDMA is only

incorporated in hair to a "moderate degree", p. 60),[1] and thus deserves a detailed review. The case reported in Moeller, *et al.* is almost entirely in Japanese, which we are unable to interpret.[12] Cirimele *et al.* discovered AP (amphetamine), MDA (3,4-methylenedioxyamphetamine) and MDMA in both the fingernails and head hair of a 22 year old male "known to be a drug user" (p. 68).[13] Kikura and colleagues analysed hair samples from 3 rats that had been given MDMA (amongst other drugs) and also analysed head hair from 7 human "MDMA abusers", in whose hair, MDMA was indeed discovered.[14]

Kintz & Cirimele report a methodological trial involving interlaboratory comparisons of the analysis of a hair sample containing MDMA, MDA and AP, and the laboratory comparison is discussed in a later section of this paper.[15] At this point, it is merely worth noting that the sample came from "a male subject, aged 24 years, known to be a stimulant abuser and deceased from fatal methylenedioxymethamphetamine overdose" (p. 152).

Rohrich & Kauert spiked drug-free hair samples with MDA, MDMA and MDEA (3,4-methylenedioxyethylamphetamine) for calibration purposes, and subsequently tested "authentic hair samples from [303] individuals with suspected drug abuse" (p. 180).[16] They discovered that "28 of these samples (9.3%) contained amphetamine and/or methylenedioxy-amphetamine-derivatives" (p. 187). AP was detected in 23 samples, MDMA in 9, MDA in 6 and MDEA in 5. They conclude "Considering the increasing use of amphetamine and particularly of 'Ecstasy', this number of 28 amphetamine-positive samples seems remarkably low" (p. 180).

Finally, Rothe, *et al.* examined (proximate 3 cm length) hair samples from "20 regular 'speed' or 'ecstasy' users of the techno scene...who anonymously reported their drug history in a drugs advice centre" (pp. 111, 113).[17] While these "abusers" could report the number of tablets consumed, they could not comment on their relative purity

(another issue to be taken up later). The 20 were classified as follows: 7 "insignificant abusers" (up to 1 tablet per month); 5 "moderate abusers" (1-3 tablets per month); 3 "strong abusers" (3-8 tablets per month); and 5 "very strong abusers" (over 8 tablets per month). The mean discovered ng/mg (nanograms per microgram) levels matched their rank order by tablets consumed, although there was considerable variation within each group. For example, in the very strong abuser group, concentrations ranged from none detected to over 16 ng/mg. Their ability to detect MDMA in the hair of subjects who had consumed less than 1 tablet per month persuades the authors of the sensitivity of hair analysis; indeed, they suggest "in cases of ecstasy abuse the intake of 1 tablet may lead to a hair concentration in the order of 1 ng/mg" (p. 126). However, they recognise considerable interindividual variation; and, separately, that participation in the "techno or rave" scene, and associated sweating, can lead to false positives through deposition, and chronological misinterpretation from sweat-assisted migration along the hair shaft.

Method

Recruitment

Subjects were recruited for interview using a strategy which combined the use of "key informants" using "chain referral",[18,19] as informed by a "theoretical sampling" strategy,[20] in which subjects themselves nominate another subject (individuals well known in the dance scene who could introduce interviewers to a large number of MDMA users) coupled to 'multi-site sampling' (attempting to initiate chains in many separate and diverse locations). The initial criterion for inclusion in the study was that subjects had taken MDMA at least once, although not necessarily a whole tablet of it.[21]

The first interview took place on the 1st December 1993, and the 229th on the 9th June 1995 - nearly 18 months later. The 100 subjects analysed here are a sub-set of the 229-strong main sample.

Sample

All respondents who reported ever taking "ecstasy" answered an array of questions about their lifetime use of it, and a very extensive set of questions relating to their use of it in the 12 months prior to interview. These included estimations of the number of days on which they had consumed "ecstasy" for each of the previous 12 months, together with an estimation of how many "ecstasy" tablets they had taken on each day of use.

Users are classified in Table 1 in terms of their "ecstasy" consumption in the past 12 months. One subject had not used in the past year, and is grouped with the 8 non users. The "Light" users had consumed between ¼ and 11 tablets in the previous year (broadly speaking, their averaged consumption was 'less than monthly but more than yearly'); the "Medium" users had consumed between 12 and 47 tablets in the previous year (similarly, their averaged consumption was 'more than monthly but less than weekly'); the "Heavy" users had consumed 48 or more tablets in the previous year (their averaged consumption was 'more than weekly but less than daily'). No users qualified as 'daily' users.

Previous research on substance use has tended to treat self-report measures of quantity/ frequency as sufficient, but this study also collected information on pattern of use. Data were obtained about "ecstasy" use during each of the twelve months preceding interview: specifically on how many days "ecstasy" was consumed during each month and for each day of use, and on how many tablets were consumed on

average. Measures used were mean reported number of tablets used per occasion per month and the mean reported number of days used per month. From these were derived additional measures: reported use for each month (being the number of tablets per occasion multiplied by the number of days of use); and reported variation in use per month (being the standard deviation calculated across use for each of the 12 months). These data theoretically made it possible to distinguish 'stable' users from 'erratic' users at each level of consumption. Essentially, a stable user was one who tended to use the same amount of "ecstasy" the same number of days each month, while an erratic user tended to have different patterns of use during different months.

Insert Table 1 about here

Hair donation

After completing the main structured interview and various self-completion scales, all subjects were asked to provide some hair for laboratory testing. From those that agreed to donate a sample, between 30 and 50 hairs were taken from the scalp vertex. Cut ends were aligned, wrapped in aluminium foil for ease of identification of the root end, and sealed in individual plastic bags, numbered with each subject's ID code.

Hair analysis

This paper reports the analysis of hair from 100 of the 229 subjects (44%). Hair from a further 49 subjects was analysed by a commercial laboratory, and is not reported here as the laboratory was unable to provide sufficient technical information relating to their analytic procedures. Of the 80 subjects for whom a sample was not available, 12 refused to (or could not) donate one, and 68 donated a sample which later proved to be of insufficient volume to permit reliable analysis. Of the 100 remaining hair samples,

11 were 6 cm long, and 68 were 12 cm long. The remaining 21 were odd lengths ranging from 2 cm to 10 cm. Donors were compensated for hair loss with reimbursement of expenses incurred. The 100 samples reported here were analysed at the Department of Forensic Medicine and Science at the University of Glasgow (Scotland).

The method is reported in full elsewhere.[22] In brief, each hair sample was washed with sodium dodecylsulphate and deionised water, rinsed with methanol and allowed to dry overnight. The methanol rinses were all analysed, and were found to be negative. The hair was cut and ground finely, treated with the enzyme glucuronidase in the presence of pH7.4 phosphate buffer, and the internal standard, amphetamine-d_3. The samples were then sealed and heated at 40°C for 2 hours. The resultant supernatant was separated from the hair matrix by centrifugation. The analytes of interest were separated from the supernatant by solid phase extraction, and analysed by a combination of derivatisation and GC/MS (gas chromatography/mass spectrometry). For MDA, MDMA and MDEA the Limit of Detection (LOD) was 0.1ng/mg and the Limit of Quantitation (LOQ) was 0.5ng/mg. For AP and MA, the LOD was 0.5 ng/mg, and the LOQ was 1 ng/mg.

Results

This section is divided in two. First, a discussion of the results relating to "gross concordance" between the self-report "ecstasy" consumption, and MDMA (or allied compound) ng/mg data; second, "net concordance", or degree of correlation between the number of "ecstasy" tablets reportedly consumed, and the ng/mg of MDMA (or related compounds) traced in the hair of each subject that self reported "ecstasy" use,

and in whose hair one of the five drugs tested for (MDMA, MDA, MDEA, MA, AP) was found.

Gross concordance

Self-report "ecstasy" consumption data was matched to ng/mg data using the rule of 1 cm of hair growth per calendar month. For example, with a subject donating 6 cm of hair, self-report data relating only to the previous 6 months was analysed. Both the self-report tablet consumption and ng/mg data were interval variables, but each was recoded as a nominal variable (i.e., as a simple yes/no response) to estimate gross concordance using cross-tabulation and tested using Cohen's Kappa.[23] Results are in Table 2.

Table 2 about here

Overall percentage gross concordance is weak, with no cell exceeding 59% concordance, and with the Cohen's Kappa for no cell exceeding 0.08. Magura & Kang indicate that only Kappa values that exceed 0.8 are of interest.[24] The fourth column ("anyxtc") is a result of a nominal version of summed data from the ng/mg output data for, separately, MDMA, MDA and MDEA, each of which was tested for separately. The seventh column ("anya") is similarly summed from the AP and MA (methamphetamine) ng/mg data. The eighth column ("any") is the sum of "anyxtc" and "anya". Neither amphetamine consumption separately, nor amphetamine consumption coupled to "ecstasy" consumption (the bottom two rows of Table 2) improve concordance. Accordingly, data from row one column eight ("ecstasy" consumption by "any") is used for the remainder of this part of the paper.

Figure 1 illustrates the breakdown of the gross concordant data, with 52% being lab and self positive and 7% being lab and self negative (summing to 59%).

Figure 1 about here

Net concordance

This section deals only with the 52 users that self-reported "ecstasy" use, and later hair-tested positive for MDMA (or related compound) use. The demographics of this sub-sample are in Table 3. The 52 subjects who self-confessed to "ecstasy" use and hair-tested positive for MDMA (or related compound) use could only be distinguished from the 48 who did not meet this definition on only three of 49 demographic and other related sociological variables. These three variables were:- whether or not they had taken cocaine last year, been ill in bed last year, and suffered from mood swings last year (all $p<0.05$), with those who tested positive for MDMA (or related compound) use being more likely to have experienced all three. Neither hair weight (expressed as weight per cm), nor hair colour (coded as a 9-point ordinal from white through black) correlated with any of the ng/mg data.

Table 3 about here

At this level (lab and self positives) correlations between interval versions of the variables are most appropriate, rather than the simple nominal versions used to assess levels of gross concordance. The results are in Table 4.

Table 4 about here

No correlation approaches even the lowest level of statistical significance, and no correlation is positive. In the latter sense, that between ng/mg of MDMA, MDEA and MDA combined ("threemng" in Table 4) and number of tablets consumed comes

closest to a positive correlation. The degree of scatter is enormous, however, and is illustrated in Figure 2, in a way suggested by Marques.[25]

Figure 2 about here

So far, when considering net concordance, the possible relationship between tablet consumption and ng/mg detection has been posed at the interval level, and has concluded that nanograms, as an "absolute dosimeter",[26] do not predict tablet consumption. At a weaker level, the relationship may be tested at an ordinal level (nanograms as a "relativistic dosimeter"[27]), which has proved successful elsewhere, and with other drugs.[28,29,30,31] Table 5 shows the results of such a test, when subjects are grouped by tablet consumption (*pro rata* by length of hair sample) using the same ordinal groupings as in Rothe, *et al.*,[17] and compared with interval nanogram data.

Table 5 about here

Interpretation

Gross concordance

From Figure 1, the 5 discrepant lab positives are within the range reported elsewhere.[32] The 36 false negatives are considerably proportionately higher than those found elsewhere, and are probably accounted for, at least in part, by the fact that this sample of users were volunteers with no apparent motive to under report "ecstasy" use.

A method of assessing the competence of hair analysis as a test for the presence of "ecstasy" (assuming the accuracy of self-report) is provided by Bernadt.[33] An effective

test must have sensitivity of at least 90% (i.e., it must pick up most cases with the condition), and a positive predictive value of at least 40% (i.e., at least this many of the positive tests must be cases of the condition). Here, the test results fail at both levels: sensitivity is only 64%, and the positive predictive value is only 23%.

Net concordance

The analysis of data at this level also fails standard tests. For none of the nanogram data (whether separately or in summated form) do the means progressively increase with grouped reported tablet consumption, and in every case, the lowest recorded nanogram reading in the greatest consumption category is considerably smaller than the highest recorded nanogram reading in the lowest consumption category. The bottom row of Table 5 attempts to duplicate Rothe *et al.*[17]

Overall concordance

These results do not inspire confidence in any credible individual or aggregate relationship between careful and unrestrained self-report on the one hand, and laboratory testing on the other. Analysis has relied on two sources of data which, in a perfect world, should have correlated. They were: self-report "ecstasy" consumption; and laboratory discovered nanograms of ecstasy in the consumer's head hair. In principle, there are a large number of reasons why a highly correlated match was not achieved. In practice, all or any of them might have been in play, to some or other degree, on this occasion. We consider these problems in the Discussion.

Discussion

Such problems are considered under two main headings, with those relating to the validity of self-report data considered before those relating to laboratory analysis.

Self report data

The most critical variable influencing putatively the quality of self report must be the circumstances of report itself. Confessions from those in constrained settings (arrestees at booking facilities, inmates of correctional establishments) may be required of persons in a relatively traumatised frame of mind and/or believing themselves to be at risk of sanction for admitting prior drug use. The first may affect memory, the second willingness to self-report fully. It is consequently believed that "self report may be the least reliable among criminal justice clients" (p. 27).[34]

Since the circumstances of self-report from subjects reported here was one of voluntary participation with credible guarantees of confidentiality and anonymity, the abnormally large number of discovered false negative gross non-concordant individual results begins to make some sort of sense. On the other hand, Cook *et al.* compared various types of self report with urine and hair analysis when investigating drug use among 928 employees at a US steel plant.[32] They discovered a low false positive rate of 4 per cent, and a low false negative rate of 5 per cent. Although theirs was not a criminal justice sample, it appears not to have been a volunteer one either.

However, unconstrained circumstances do not necessarily guarantee accurate self-report data. Experimentation has shown both that overreporting of drug consumption is more common than underreporting,[35] and *vice versa*.[36] This is sometimes explained by reference to bravado and/or reticence, although it may be more appropriate instead to consider what experimental and other evidence exists related to the ability of memory even for those with no wish to report levels of drug use higher or lower than those actually experienced.

Hammersley has recently published a detailed review of the literature on memory problems which casts considerable doubt on the reliability of self-report in research into drug use.[37] Nevertheless, complete disregard of self report (the stance adopted, *inter alia*, by Marques, *et al.* who comment: "the widespread use of self report data to validate technical measures of drug exposure is roughly analogous to having a patient self report temperature in order to validate a thermometer reading" - p. 172 [25]) probably goes too far.

Independent of these problems of truthful recall and memory difficulty, are problems of substance purity (only rarely referred to in the literature on hair analysis). Street purchased MDMA is known to vary greatly in terms of the proportions of MDMA and other inert components and/or psychoactive adulterants. Indeed, as part of the general research design of which hair analysis was a component, the research team obtained 15 substances bought as "ecstasy" (the first author held a Home Office licence to be in possession of Class A substances at the time). The analysis of the 15 substances recovered this way was conducted at the same laboratory. Resulting data is in Table 6.

Table 6 about here

MDMA content ranged from less than 1% to a high of 70% (with a mean of 34%). A frequent heavy user of "Emerald Es" would probably consume less actual MDMA than an occasional light user of "Madwomans". Far more extensive tablet analysis conducted in Amsterdam (2,689 tablets, analysed during the whole of 1997) indicated an MDMA average content of 41%, and a range from 24% to 61% per month. Interestingly, when MDMA content was low, amphetamine content was high, and *vice versa*.[38] Purity is clearly an issue deserving more sustained attention.

Laboratory analysis

Turning now to problems associated with laboratory analysis, perhaps the main one is an unswerving belief in the validity of laboratory data, which, when produced by GC/MS, is often "assumed to be virtually 100 percent accurate" (p. 20).[34] However, laboratory analysis of hair for the presence of drugs is not unproblematic. Relevant problems fall into one of two types: problems relating to hair, and problems relating to its analysis.

First, rates of hair growth. A norm currently is to assume a growth rate of 1 cm/month (the assumption on which the technical analysis in this paper was based). Not so long ago, however, the American assumption was half an inch/month. Twelve cm is the equivalent of four and three-quarter inches. On the current assumption, this would indicate 12 months of hair growth: on the earlier one, nine and a half months. In addition, rates of hair growth are believed to vary individually from, by one estimate, from 0.6-3.36 cm/month.[39] For an individual at the low end of this scale, 12 cm of hair would reflect 20 months of hair growth; for one at the high end, 12 cm of hair would imply just over three and a half months of hair growth. This is a major, yet typically unaddressed problem.

These latter are only averages: the situation for individuals is more complex as hair growth varies by the length of the individual's hair (with "in general, the longer the hair is, the longer the growing phase is", p. 306)[40]; by race (Causcasian hair grows faster than Asian hair); by sex (scalp hair in women grows faster than in men); age (hair growth generally decreases with age); by position on the scalp (hair grows faster in the vertex region than elsewhere); by general location (compared to scalp hair, pubic hair grows more slowly, and beard hair more slowly still); by hair colour (darker hair is more likely to reveal substances or their metabolites); and possibly by hair texture (whether "coarse" or "fine"). In addition, a certain proportion of head hair - usually

estimated at 15 per cent of it - is dormant at any one time, and could not, thus, be any sort of calendrical record of consumption.

It is further the case that "hair is a very complex part of the anatomy whose biology is only partially understood" (p. 9).[39] This leads to two more problems. First, rates of incorporation of substances into hair are believed to vary by many of the dimensions that affect rates of hair growth as noted above. Kidwell & Blank suggest that cocaine incorporation ratios for Africans:Whites are 2.9:1, and for Koreans:Whites, 6.8:1.[10] Second, here is the more immediately relevant difficulty: the problem of the possibility of external contamination of hair.[41] Harkey comments: "the hair shaft is exposed directly to sebaceous secretions before it emerges from the skin. Scalp hair is also exposed to sweat secretions as well as contaminants in air, water, or dust" (p. 16).[39]

However, most drugs can be delivered by a variety of different methods, and might be consumed differently by one individual on different occasions. Tablets, such as those of MDMA (which is also available in powdered - as well as capsule and tablet - form, and has been known to be injected) are generally handled by those not wearing surgical gloves, who may thereafter run their hands through their own, or somebody else's, hair. If MDMA is consumed in hot dance venues, which is typical in the UK, then the problem of sweat contamination is presumably greater than it may be in cooler drug consumption environments. On the other hand, handling (and subsequent hair-fondling) has been challenged by Mieczkowski who refers to his work with undercover narcotics officers "who are chronically exposed to cocaine", but who, even after "several thousand" exposures, have "near-zero levels of contamination [and] are readily differentiated from cocaine users" (p. 62).[1]

The effects of the differential regularity and frequency of individual non-experimental washing, dyeing, perming and otherwise cosmeticising hair are unknown, but cannot be dismissed. Jurado, *et al.* examined the hair of those with similar drug consumption

histories, and concluded that "in all cases the drug content in hair that had undergone cosmetic treatment decreased in comparison to untreated hair" (p. 159).[42] They also conclude that shampooing may not have much effect on drug retention, but that bleaching, dyeing and streaking all do: albeit with different individual variations. Skopp, *et al.* appear to disagree. Their analysis of bleaching indicates that: "in general, the drug concentrations in virgin hair were slightly lower compared to cosmetically treated hair" (p. 47).[43]

As for laboratory analysis, first, there are problems of comparison. Hair preparation procedures adopted prior to testing itself vary widely, and new ones are developed with a regularity unjustified by citation of conclusive evidence of superiority. Subsequent laboratory tests selected are from a variety of different options which are known to vary in their ability to detect the presence of various substances. Further, cut-off points, which crucially determine the ratio of false positives to false negatives, are not always reported, and when they are, they are not always similar.

Next, there are occasionally problems of credibility. Of the five English language reports of laboratory testing for MDMA use, one analysed hair from somebody "known to be a drug user", a second analysed hair posted from overseas where it had been harvested from alleged "MDMA abusers", and a third analysed a sample from somebody "known to be a stimulant abuser". Only the samples used by Rohrich & Kauert,[16] and Rothe, *et al.*[17] come anywhere near sizes or sampling techniques that would permit any acceptable generalisations.

Post-harvesting wash procedures are designed to eliminate traces on the external surface of the hair. The extent to which they do so is clearly related to the reagents and to thoroughness and frequency of washing, with Baumgartner, *et al.* indicating that washing has a variable effect with, in one case, removal of 88 per cent of cocaine from a hair sample, and in another, 41 per cent.[9] It is sometimes believed that tracing drug

metabolites (rather than the drugs themselves) in hair is indicative of consumption rather than of contamination, but Kidwell & Blank offer evidence that metabolites have been traced in street samples.[10]

The washing problem is akin to the cut-off problem noted above. Too much washing might lead to too many false negatives: too little, too many false positives. It has been noted, by Blank & Kidwell that, even after "substantial washing" by one method, two cocaine spiked samples still retained 211 ng of cocaine or its metabolites/10mg hair.[44] This is equivalent to amounts found in the hair of those classified as heavy cocaine users. Finally, the degree of external contamination will relate to the mechanism and context of original drug delivery. Drugs which are smoked or chased will, presumably, stand a greater chance of externally contaminating hair than those which are consumed orally or by injection.

In the introduction to this paper, reference was made to Sachs' distinction between a "gold rush" phase for hair analysis, followed by a "hang over" one.[8] One implication is that, in brief, during the "gold rush" era, hair was believed to possess the probable potential to be an "absolute dosimeter" for drug consumption. The period of retreat typifying the subsequent "hang over" era has forced a stocktaking, and a revision of expectations down to hoping that hair may offer what Baumgartner & Hill latterly have referred to as a chronometrically operating "relativistic dosimeter".[31]

That even this has not proved possible in the analysis presented above does not necessary mean a further retreat is called for. Instead, improved research protocols might well be more appropriate, and it is to this that the Recommendations attend.

Recommendations

Whilst the analysis of the materials presented here offers no obvious source of optimism for the future of hair analysis in the confirmation of MDMA (or related compound) use, the failure to generate impressively positive results may well be due to the failure of self report and/or laboratory analysis. How could faith in each be enhanced?

Self report would seem to be more reliable if samples are large, voluntary, and not derived from criminal justice populations. Self report could be more credible if it were contemporaneous (perhaps as diary record) rather than retrospective, and limited, perhaps, to a three month period. The issue of the consumption of variously adulterated complicates matters further. Until sampled users can be permitted to return samples of substances along with their self reported consumption details, little impact on this key issue will be possible. An alternative is for the sort of controlled dosage studies that Mieczkowski, *et al.* have called for, although these may be harder to organise.[45]

Laboratory analysis would, in principle, be surprisingly easy to check for reliability, and if a high degree can be demonstrated to exist, then the need for research into self report itself declines. There would appear to be room for improvement in laboratory analysis, and this would be facilitated if considerably larger hair samples were harvested in future. This would enable double testing of samples: something which should become the norm rather than merely an ideal. Another part of the problem is the proliferation of different washing procedures, assays, etc., on which, Mieczkowski comments (p. 68):[1]

> "There is not yet a universal standard for washing the specimens, conducting the assay or establishing quantitative outcomes which are readily extrapolated to consumption levels. Systematic comparison of hair analysis values will require a

standardised assay method, and be greatly facilitated by a standard system of reporting outcomes."

The establishment of a single method for washing, assaying and reporting could be the basis of a sound interlaboratory and intralaboratory research programme. This has been tried, in part, but only with small samples of rather dubious authenticity, or with possibly flawed sample spiking. Welsh, *et al.* conducted two exercises with "hair from suspected drug users" and "drug-free hair".[46] In the first exercise, 8 laboratories were sent 8 powdered samples, and 7 laboratories returned quantitative results. Ninety four per cent correctly identified positives, and all correctly identified negatives. However, "scatter in quantitative results was high" (p. 295). In a second exercise, ten laboratories were sent eight hair segments rather than powder. The qualitative results were not as good (78% correctly identified positives, 94% correctly identified negatives), and they comment "quantitatively, the results were more scattered for the second exercise when the hair was in form of short segments."

Mieczkowski, *et al.* report the despatch of cocaine positive hair samples from 141 individuals to two laboratories.[47] The two laboratories used different assay methods (one RIAH, the other GC/MS) and different cut-offs. None of those deemed negative by GC/MS were deemed positive by RIAH, but 16 of those deemed positive by GC/MS were deemed negative by RIAH. Finally, Kintz & Cirimele sent a hair sample spiked with AP, MDA and MDMA to 16 different laboratories.[15] The laboratories all used GC/MS (but used a variety of washing procedures) and were asked to test for AP, MA, MDA and MDMA. Two laboratories detected MA (with which the samples were not spiked), three failed to detect AP, two failed to detect MDA and three failed to detect MDMA. For the remainder, the scatter in the concordant set was: for AP (13 positive results) 3.3-17.5; for MA (2 positive results) 0.8-1.8; for MDA (14 positive results) 2.5-19.5; and for MDMA (14 positive results) 3.3-100.00.

Such interlaboratory testing for MDMA (and related compound) use as has been conducted so far does not inspire confidence, but divergent results may be because of insufficient sample size,[46] different assay methods,[47] different washing procedures,[15] and/or because some amphetamines are notoriously volatile in the testing process itself.[48]. Clearly, if the first can be boosted, the middle two standardised, and the latter overcome, then an appropriate level of faith in hair analysis might be calibrated.

Table 1: Sample demographics; and % reporting having used various drugs at least once in year prior to interview

n=	ex-users 9 %	light stable 20 %	light erratic 27 %	medium stable 12 %	medium erratic 15 %	heavy stable 11 %	heavy erratic 6 %	**ALL 100 %**
% female	67	40	48	50	67	36	33	**49**
% aged 14-19	11	15	15	8	53	18	16	**20**
% aged 20-24	56	40	44	42	40	46	83	**46**
% aged 25+	33	45	41	50	7	36	-	**34**
cannabis	67	100	100	92	100	100	83	**95**
alcohol	89	100	96	92	100	100	83	**96**
ecstasy	-	90	100	100	100	100	100	**90**
tobacco	67	90	89	75	80	82	100	**84**
amphetamine	56	80	82	100	93	91	83	**84**
LSD	22	70	70	83	73	55	83	**67**
cocaine	11	26	63	55	75	82	100	**56**
nitrites	44	45	39	64	64	55	20	**48**
mushrooms	11	26	37	33	43	20	33	**31**
temazepam	-	30	19	25	57	50	33	**30**
benzodiazepine	11	20	15	8	42	33	17	**20**
other opiate	-	10	11	25	27	10	17	**14**
heroin	-	11	4	17	9	-	-	**7**
temgesic	-	5	4	9	18	11	-	**7**
solvent	-	10	12	8	8	-	-	**7**
ketamine	-	-	8	9	-	22	-	**6**

Note: all rows do not sum to 100. None of the differences in sample demographics are statistically significant; neither are most of the prior year drug consumption differences, except for cannabis and cocaine (at $p<0.001$). Drugs listed in descending order by prevalence in whole sample. "Light" users report using between ¼ and 11 tablets in the previous year; "medium" users between 12 and 47 tablets in the previous year, and "heavy" users 48 or more tablets in the previous year. At each level of use, "stable" users tended to use the same amount of "ecstasy" the same number of days each month, and an "erratic" user tended to have different patterns of use during different months.

Table 2: Gross concordance between "ecstasy" (and/or related compounds) reportedly consumed, and ng/mg found (n=100).

% concordant p Cohen's K	mdma	mdea	mda	*anyxtc*	ap	ma	*anya*	***any***
"ecstasy"	43%	33%	27%	*55%*	22%	32%	*34%*	***59%***
consumption	-	-	-	-	-	-	-	-
	0.05	0.07	0.03	*0.09*	0.03	0.05	*0.05*	***0.08***
amphetamine	37%	31%	27%	*51%*	24%	32%	*34%*	***55%***
consumption	-	-	-	-	-	-	-	-
	-0.07	0.01	-0.01	*-0.01*	0.02	0.02	*0.02*	***0.01***
"ecstasy" and/or	41%	25%	21%	*55%*	14%	24%	*26%*	***59%***
amphetamine	-	-	-	-	-	-	-	-
consumption	0.05	0.02	0.02	*0.08*	-0.00	-0.00	*-0.00*	***0.06***

Figure 1: Gross concordance and non-concordance (% & n = 100)

		LAB	
		Yes	No
SELF	Yes	52 (+)	36 (+ive)
	No	5 (-ive)	7 (-)

Table 3: Demographics of those who confess to "ecstasy" use and who also test positive for at least one of the five methamphetamines or amphetamines; and % reporting using various drugs at least once in year prior to interview

	ex-users	light stable	light erratic	medium stable	medium erratic	heavy stable	heavy erratic	ALL
n=		13	13	6	7	9	4	52
	%	%	%	%	%	%	%	%
% female	-	46	46	67	71	33	50	**50**
% aged 14-19	-	15	8	-	4	2	-	**17**
% aged 20-24	-	46	46	33	29	44	100	**46**
% aged 25+	-	39	46	67	14	33	-	**37**
alcohol	-	100	100	100	100	100	75	**98**
ecstasy	-	92	100	100	100	100	100	**98**
cannabis	-	100	100	83	100	100	75	**96**
amphetamine	-	77	85	100	86	89	75	**85**
tobacco	-	85	77	83	86	78	100	**83**
LSD	-	85	62	100	86	67	100	**79**
cocaine	-	42	92	20	67	78	100	**67**
nitrites	-	46	31	80	83	67	25	**52**
temazepam	-	39	23	33	67	50	25	**38**
mushrooms	-	33	39	33	43	25	25	**34**
benzodiazepine	-	23	31	-	60	29	-	**25**
other opiate	-	15	8	33	60	13	-	**18**
heroin	-	17	8	17	-	-	-	**9**
solvent	-	15	15	-	-	-	-	**8**
temgesic	-	8	-	-	20	14	-	**7**
ketamine	-	-	15	-	-	14	-	**6**

Note: all rows do not sum to 52. None of the differences is statistically significant. Drugs listed in descending order by prevalence in whole sample. "Light" users report using between ¼ and 11 tablets in the previous year; "medium" users between 12 and 47 tablets in the previous year, and "heavy" users 48 or more tablets in the previous year. At each level of use, "stable" users tended to use the same amount of "ecstasy" the same number of days each month, and an "erratic" user tended to have different patterns of use during different months.

Table 4: Net concordance between "ecstasy" consumed, and ng/mg discovered (n=52) - all Pearson's' coefficients.

Pearson's r (n) p	mdma ng	mdea ng	mda ng	*three* *mng*	ap ng	ma ng	*two* *ang*	*five* *mng*
"ecstasy"	-.1022	-.1739	-.2744	*-.0518*	-.1236	-.2016	*-.1392*	*-.1008*
consumption	(36)	(20)	(16)	*(52)*	(8)	(19)	*(52)*	*(52)*
	.553	.463	.304	*.715*	.771	.408	*.325*	*.477*

Table 5: Net concordance between "ecstasy" reportedly consumed, and ng/mg found (n=52) - means and ranges for ordinal groups

Mean (*) range of ng	n=23 <1 tab	2=14 1-3 tabs	n=7 3-8 tabs	n=8 8+ tabs	N=52
mdmang	2.7 (9) 0.1-13.7	15.3 (6) 0.1-82.9	1.1 (1) 0.5-3.3	10.8 (-) 0.3-55.8	7.0 (16) 0.1-82.9
mdeang	3.8 (14) 0.2-15.0	3.1 (9) 0.1-10.5	2.4 (5) 0.9-3.9	2.4 (4) 0.1-8.9	3.2 (32) 0.1-15.0
mdang	3.2 (14) 0.1-8.6	0.9 (12) 0.8-1.0	0.2 (5) 0.1-0.2	1.0 (5) 0.5-1.4	2.1 (36) 0.1-8.6
threemng	*4.4 (-)* *0.0-30.4*	*9.9 (-)* *0.0-93.4*	*1.7 (-)* *0.1-7.2*	*12.4 (-)* *0.3-57.2*	*6.8 (-)* *0.0-93.4*
apng	5.0 (19) 1.2-13.3	51.5 (12) 5.2-97.7	- (7) - - -	6.4 (6) 2.4-10.4	16.9 (44) 1.2-97.7
mang	7.4 (12) 0.6-32.3	7.1 (8) 0.6-28.0	- (7) - - -	0.7 (6) 0.6-0.7	6.6 (33) 0.6-32.3
twoang	*4.4 (-)* *0.0-32.3*	*10.4 (-)* *0.0-99.9*	*- (7)* *- - -*	*1.8 (-)* *0.0-11.1*	*5.0 (-)* *0.0-99.9*
fivemng	***8.8 (-)*** ***0.0-59.5***	***20.3 (-)*** ***0.0-193.3***	***1.7 (-)*** ***0.1-7.2***	***14.2 (-)*** ***0.3-59.6***	***11.7 (-)*** ***0.0-193.3***
mdmang + mdeamg	4.3 (6) 0.1-21.8	17.2 (6) 0.1-93.4	2.7 (-) 0.5-7.5	12.0 (-) 0.3-55.8	8.1 (12) 0.1-93.4

* Figure in parenthesis = no. of negative cases

Table 6: Analysis of 15 substances, bought as "ecstasy"

street name	type	believed contents	actual % MDMA	other active substances
clog	tablet	MDMA	20	
madman	tablet	MDMA	21	
white dove	tablet	MDMD + heroin	58	
pink snowball	tablet	MDMA +	20	
rhubarb and custard	capsule	MDMA	17	
emerald E	capsule	MDMA, LSD Amphetamine,	trace*	46% Amphetamine; 41% Caffeine
turbo	tablet	MDMA	21	
blaster	tablet	MDMA	46	
madwoman	tablet	MDEA or MDA	70	
madman	powder	MDMA	68	
disco biscuit	tablet	MDMA	31	trace of MDEA
	tablet	MDMA	43	
super dove	tablet	MDMA	68	trace of MDEA
white tornado	powder	MDMA	13	
lemon and lime	capsule	MDMA	19	1% MDEA
Average			**34**	

* = less than 1%

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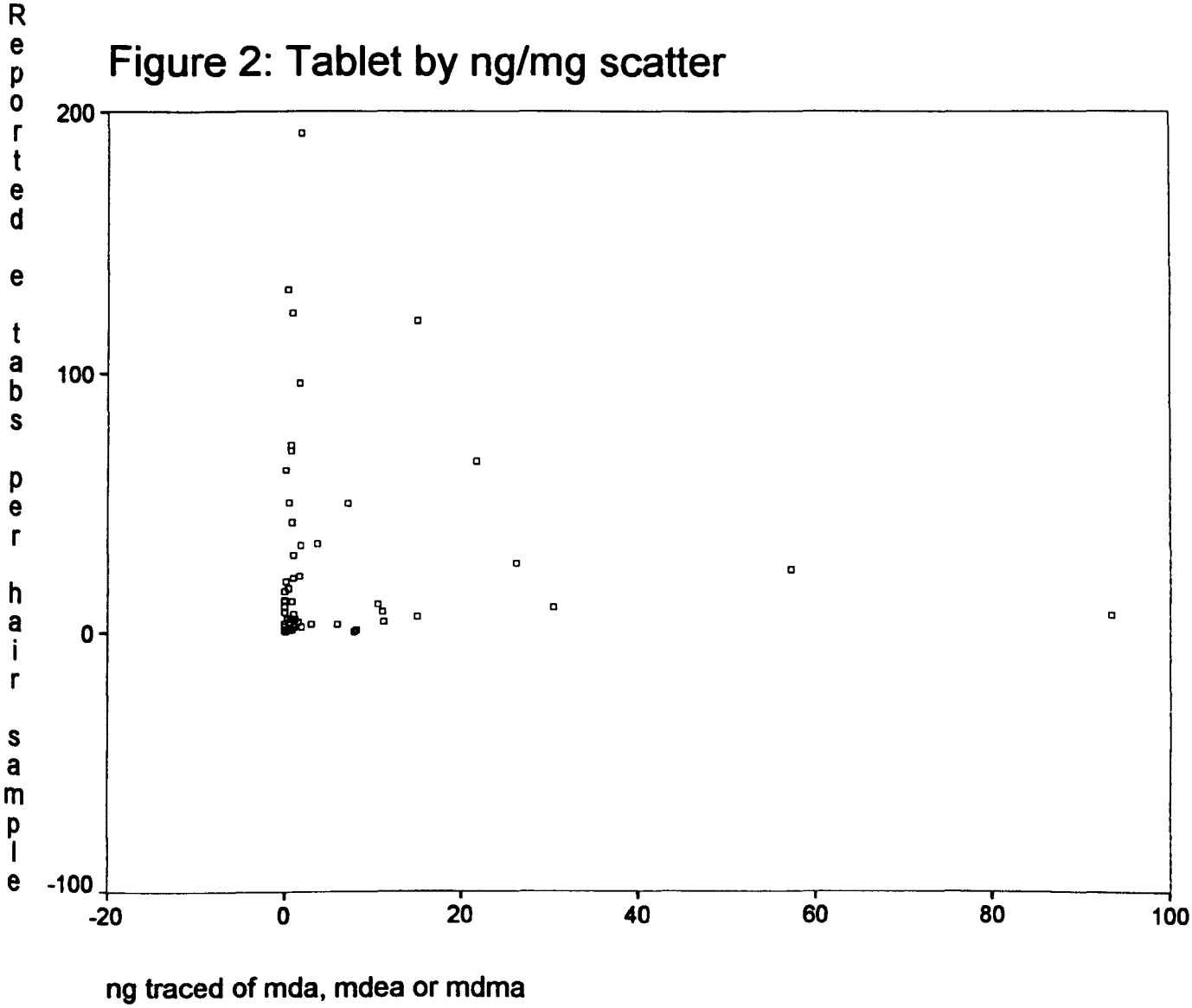
Figure 2: Tablet by ng/mg scatter



THE USE OF SUPERCRITICAL FLUID EXTRACTION FOR THE DETERMINATION OF AMPHETAMINES IN HAIR

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ABSTRACT

A laboratory study interested in the analysis of human hair for drugs of abuse was conducted to determine if drugs could be detected and quantified from hair. Supercritical fluid extraction (SFE) techniques followed by GC/MS analysis were applied to extract amphetamines from hair. The group of amphetamines included methylenedioxyamphetamine (MDA), methylenedioxymetamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA) and internal standard mephentermine (MP).

To validate information on amphetamine use in hair, powdered hair samples free from drugs were collected and soaked in a known amphetamine standard solution. Authentic fortified case hair samples taken from known drugs users known to have consumed amphetamines were also analyzed for amphetamine.

Results from this study show that amphetamine use can be detected in spiked and authentic fortified human hair using SFE techniques for qualitative and quantitative reproducible results.

Key Words: Hair analysis, Supercritical fluid extraction, methylenedioxy-amphetamine, methylenedioxyethylamphetamine, methylenedioxymethamphetamine

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INTRODUCTION

Most of the literature written dealing with the detection of amphetamine in hair has come from Japanese researchers. This is mainly because of the vast amphetamine consumption in the East that has prompt increase analytical research. In the past, target drugs have been amphetamine (AP) and methamphetamine (MA), however, more recently MDA derivatives like MDMA are of particular interest.

Nakahara published a detailed review in 1995 on the detection of amphetamines in hair.[1] Techniques published before 1990 used acid or alkaline hydrolysis or a combination of hydrochloric acid and methanol followed by SPE or LLE and derivatised. Recently Rohrich and Kauert developed a screening procedure for the simultaneous detection of AP, MA, MDA, MDMA and MDEA based on methanol sonication with methaqualone used as the internal standard.[2] Kintz and Cirimele compared four different procedures for AP, MDA and MDMA (methanol sonication and acid, alkaline and enzymatic hydrolysis) where alkaline hydrolysis was determined to give greatest recoveries.[3]

The detection of amphetamine and ecstasy in hair is becoming of growing interest in the forensic laboratory. Since Moeller *et al* first identified MDMA in human hair, it has become one of the most frequently identified, especially in Europe.[4] Therefore, it is included in all screening procedures.

There are various techniques used for the extraction of drugs from hair. Conventional techniques such as solid-phase extraction have been a popular choice, along with alkaline or acid hydrolysis and enzymatic digestion techniques. However, more recent techniques such as supercritical fluid extraction has been applied for the extraction of drugs of abuse from human hair.

Supercritical fluid (SF) is a substance that is above its critical temperature and pressure. Supercritical extraction depends on the ability of the SF to selectively dissolve various amounts of nonvolatile substances.[5] The favorable mass transport properties and ability to vary the temperature and pressure of the SF, changes the solvent properties to be able to extract substances from various matrices. For these reasons researchers have investigated the use of SFE as an alternative to conventional methods for drug analysis in hair.[6,7]

Although, the analysis of amphetamine and its analogues (e.g. MDMA, MDEA and MDA) have been detected in hair using several extraction techniques, there is no present literature on the extraction of hair for amphetamines by SFE. It was Sachs and Uhl [8] and Sachs and Raff [9] that demonstrated for the first time the use of supercritical fluids in the extraction of drugs in hair. Since then there has been studies for the extraction of opiates and cocaine in hair. [10, 11]

The purpose of this study was to investigate the use of SFE for the detection of MDA, MDMA and MDEA in human hair. Second, to optimize a method that would extract and identify the selected amphetamines. Also, to determine if this method can be used for the extraction of authentic case samples.

Experimental

Materials

Chloroform and Isopropyl alcohol AnalaR grade were supplied by Merck (Poole, UK). Ethyl Acetate chromatographic (HPLC) grade (Analytical Sciences Lab-Scan Dublin, Ireland). Pentafluoropropionic anhydride (PFPA) was supplied by Fluka Chemika. CO_2 Air Products (Walton-on-Thames, UK). SFE vials 6ml Hypovial™ (Pierce, Oud-Beyerland, The Netherlands), Butyl rubber septa (Pierce and Warner, Chester, UK) and columns for SFE were 3cm x 4.6 mm i-d. stainless steel tubing.

Stock solutions

Drug and internal standard stock solutions were prepared in methanol (1μg /ml and 10μg /ml).

Sample Conditions and Preparation

It is necessary to wash the hair to remove any exogenous contaminates, prior cosmetic treatments and grease that can interfere with analysis or create false positives. The samples are first washed with sodium dodecyl sulfate (detergent) followed by a dichloromethane wash, a methanol wash and a final wash with distilled water. Each stage was repeated twice followed a 15 minute sonication at room temperature and dried at room temperature.

After being washed and dried, blank hair samples were finely cut and weighed out into 50 mg samples. The blank sample was spiked with 10 ng/mg of drug standards (e.g.

MDA, MDMA and MDEA) in a glass vial. To ensure the hair was coated sufficiently, 3 ml of MeOH was added to the vial, sealed and sonicated for 15 minutes then opened and left overnight to dry at room temperature. The internal standard for all drugs was added to the hair during hair spiking preparation or after extraction for recovery purposes.

It is possible that this method of spiking is not the most ideal, as most drugs coat the surface of the hair and possibly do not incorporate into the hair structure. However, it is accurate enough for estimation of exact concentration levels and is the only way to spike hair samples with known concentrations.

SFE Parameters

A Gilson 308 "master" pump, which controlled a 306 "slave" pump and regulated the pressure, CO_2 amount, modifier and flow rate was used. A Gilson 811B dynamic mixer combined the solvent with CO_2 and a Gilson 821-pressure regulator controlled by the 308 pump regulated the pressure.

Placed in-line between the 811B mixer and the extraction cell, an HPLC 7125 Rheodyne valve was housed inside an 831 Gilson temperature regulator (oven), for the application of the gas and modifier to the cell. A second valve located below the 7125 valve, was used to switch the extraction from static to dynamic (continuous) mode.

SFE pressure conditions were set at 3800 psi with the flow rate at set 2ml /min and temperature 70° C. Modifier was Chloroform and Isopropyl alcohol (90:10 v/v) pumped at 10 % in 90 % CO_2 in dynamic extraction mode for 30 minutes. Derivatise by adding 50µl of PFPA: Ethyl acetate (1:1v/v) and analyzed using GC/MS.

GC/MS Parameters

A Hewlett-Packard gas chromatography model 5890 fitted with a VG Analytical VG70-250S mass spectrometry was used for analysis. The GC was fitted with a HP-5 X- link 5 % PH Me silicone capillary column 30 m x 0.25 µm, 0.88µm film thickness.

The selected ions were monitored in the electron ionization mode at 70 eV and the trap current was set at 200 µA. The initial temperature was 55° C held for 2 minutes and

final temperature was 280° C held for 5 minutes. The oven rise was at a rate of 20 Celsius ° per min. The ions monitored were m/z 218 mephentermine (internal standard), m/z 325 MDA, m/z 339 MDMA and m/z 353 MDEA.

Results and Discussions

Under the developed SFE parameters from amphetamines, hair samples were extracted and analyzed using GC/ MS. At 10ng /mg hair samples produced recoveries at an average of 84 % for MDA, 71 % for MDMA and 77 % for MDEA. These results are summarized in Table 1.

For the linear calibration of each drug, the method proved linear over a concentration range of 0 to 20ng/ mg of hair was investigated. The limit of detection for MDA was 0.02ng/mg and 0.1ng/mg for MDMA and MDEA. Table 2 shows the method validation for MDA, MDMA and MDEA.

The reproducibility of this method was investigated and was found to be relatively reproducible from sample to sample extraction. The results are summarized in Table 3.

Intra and inter-day reproducibility produced comparable results from sample to sample and day to day. Tables 4 and 5 shows the peak area ratios produced for day one and day two for MDA, MDMA and MDEA.

For 10ng/mg MDA, MDMA and MDEA for day one, the standard deviation was 0.595, 0.200 and 0.148, the mean 2.102, 1.804 and 0.692 and the RSD 28 %, 11 % and 21 %. For MDA, MDMA and MDEA for day two, the standard deviation was 0.507, 0.565 and 0.214, the mean 2.5022, 2.1318, 1.2960 and RSD 20 %, 26 % and 16 %.

There was some variation between the two days but comparable. MDEA for day one is much lower than for day two. A possible explanation for this could be because of the spiking of the blank hair. Spiking blank hair with a drug does not incorporate into the hair as fortified hair samples, therefore not all the time will there be precise consistency from batch to batch. However, it is accurate enough for estimation of exact concentration levels and is the only way to spike hair samples with known concentrations. Also the sensitivity of the GC/MS from day to day can be a possible contributory factor.

Hair samples taken from subjects participating in the self-report study for amphetamine use which were analyzed under SFE conditions. The samples were too small to be separated into segments of root or tip so they were analyzed in bulk or Full. Before analysis, the hair samples were washed and dried under the aforementioned wash conditions and the colour, weight and length were applicable of each hair sample were recorded. Table 6 lists twenty cases analyzed for MDA, MDMA and MDEA.

Out of the 20 samples analyzed, 5 were positive for MDA, MDMA and MDEA, 4 were positive for MDMA only, 2 were positive for MDA and MDMA only, 1 was positive for MDMA and MDEA only and 8 were not found to contain drugs. Samples that were not detected may have contained drugs below the limit of detection. Below in Figure 2 are the result summarized for the case samples positive overall for all three drug. Samples that were listed as not detected are not included. There were 7 overall MDA positives, 11 MDMA positives and 6 MDEA positives.

The self-report data was compared to the SFE findings from the hair samples to find if a correlation existed. It was found that some of the subjects that reported no use or little use (e.g. half an ecstasy tablet monthly) over the past 12 months were found to be negative and others found positive with low levels detected. Figure 3 summarizes the results of the self-report data to the hair findings. Out of the 20 hair samples analyzed, 11 (55 %) were positive for all or either MDA, MDMA or MDEA and said yes to consuming ecstasy. 3 (15 %) were also positive but disagreed with having taken any ecstasy and 6 (30 %) were negative for MDA, MDMA or MDEA, but agreed with having consumed ecstasy in the past 12 months. There was no one that claimed to be negative and was found to be negative.

CONCLUSION

Supercritical fluid extraction is a good technique that can be used as an alternative extraction method to conventional techniques. Among its many advantages, SFE is fast, inexpensive and produces less solvent waste. The use of SFE for the extraction of hair has been reported in various studies. Despite the controversial aspects of hair testing, the use of hair analysis is rapidly expanding.

The aim of this study was to determine if amphetamines, MDA, MDMA and MDEA, could be extracted from hair samples using SFE, optimize a method for extraction and

its application to authentic hair samples. The results demonstrate that MDA, MDMA and MDEA can be extracted from hair under the developed SFE conditions. Recoveries better than 84 %, 71 % and 77% were produced for MDA, MDMA and MDEA. Based on spiked hair samples, the method was found to be linear of a concentration range of 0.02 to 20 ng/mg of hair. The correlation coefficient (r^2) for MDA, MDMA and MDEA was 0.9703, 0.9775 and 0.9938 showing the linearity of the method. The method was also found to be reproducible from sample to sample and on a day to day basis.

It has also been demonstrated that by the application of a polar modifier directly through the system providing better extraction conditions for the drugs of interest. By varying the temperature and pressure for optimal conditions increased the extraction potential. Temperature and pressure are the two main parameters that effect SFs. An increase in pressure at a constant temperature, increasing the density and mass transfer of the SF to allow more polar and higher molecular weight analytes of interest to be eluted.

Authentic hair samples taken from subjects participating in a self-report study were analyzed using developed SFE techniques. Out of the 20 samples analyzed, 7 overall were positive for MDA, 11 for MDMA and 6 for MDEA. Samples were positive for either MDA or MDMA or a combination of MDA, MDMA and MDEA. Samples that were not detected may not have been negative, but had levels too low to detect using this method. The results from the hair analysis were compared to the self-report data collected to find if a correlation existed between the two. It was found that with some of the subjects, a correlation did exist between what they had reported consumption from month to month and hair findings and others it did not. The accuracy of the self-report data was questionable because of the possibility of under or over reporting of consumption and whether or not what was taken contained MDA, MDMA or MDEA.

It can be concluded that, SFE is an appropriate method for the extraction of amphetamines in hair. It is fast (30 minutes), reproducible and can be applied to authentic hair samples with results comparable to those generally observed in hair analysis.

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Figure 1 Chromatography of a 10 ng/ mg hair sample of MDA, MDMA and MDEA extracted using supercritical fluid extraction.

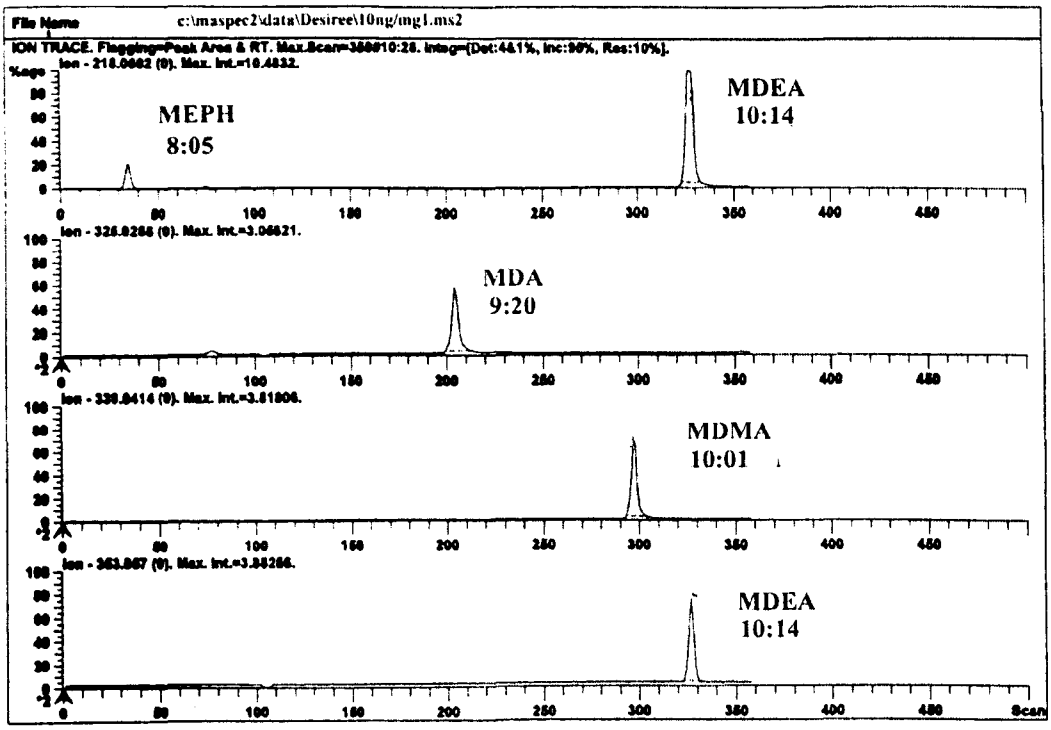

Table 1 The average recovery of amphetamines from hair

DRUG	% AVG RECOVERY	UNEXTRACTED STD PAR	PAR RANGE
MDA	84.2	1.6722	1.0976 – 1.5090
MDMA	71.2	0.7352	0.4071 – 0.7345
MDEA	77	0.3785	0.2294 – 0.3690

Table 2 Method validation for amphetamines in hair.

DRUG	Correlation coefficient (r^2)	Conc. range ng /mg	LOD ng /mg	Intercept B	SLOPE A
MDA	0.9703	0.02 - 20	0.02	0.4736	0.085
MDEA	0.9775	0.02 - 20	0.1	0.3535	0.0106
MDMA	0.9938	0.02 - 20	0.1	0.2870	0.0581

Table 3 Reproducibility of amphetamines in hair at 2ng/mg.

DRUG	MEAN	RANGE	StdDev	%RSD
MDA	1.2761	1.2309-1.3241	0.038	± 3
MDMA	1.1079	0.9470-1.2779	0.165	± 14
MDEA	0.6522	0.577-0.7230	0.059	± 9

Table 4 Day one reproducibility of each drug

DRUG	UNEXTSTD	1	2	3	4	5
MDA	2.119	1.913	1.1897	2.7397	2.2025	2.4690
MDMA	2.0746	2.068	1.788	1.939	1.6371	1.5929
MDEA	2.0549	0.456	0.677	0.855	0.7037	0.7699

Table 5 Day two reproducibility of each drug

DRUG	UNEXTSTD	1	2	3	4	5
MDA	3.3182	2.1934	2.1173	2.6670	3.3232	2.2103
MDMA	2.5970	1.7711	1.8597	1.9111	3.133	1.9840
MDEA	2.0833	1.1376	1.2789	1.1837	1.6681	1.2103

Table 6 Case samples analyzed for MDA, MDMA and MDEA

CASE #	MDA ng/mg	MDMA ng/mg	MDEA ng/mg	Length cm	Weight mg
A99-A	0.255	0.638	0.396	11	60
A99-B	0.125	0.288	0.067	4	30
A99-C	0.252	0.153	0.171	11	40
A99-D	**ND**	0.117	**ND**	11	70
A99-E	**ND**	0.659	0.286	11	19
A99-F	**ND**	**ND**	**ND**	12	78
A99-G	**ND**	**ND**	**ND**	11	60
A99-H	0.274	0.53	0.11	5	35
A99-I	0.061	0.336	0.082	4	20
A99-J	**ND**	**ND**	**ND**	11	53
A99-K	**ND**	3.88	**ND**	11	60
A99-L	**ND**	**ND**	**ND**	11	21
A99-M	0.059	1.15	**ND**	12	10
A99-N	**ND**	**ND**	**ND**	11	40
A99-O	**ND**	**ND**	**ND**	2	66.5
A99-P	**ND**	**ND**	**ND**	2	7
A99-Q	2.14	6.44	**ND**	8.5	10
A99-R	**ND**	0.71	**ND**	11	9
A99-S	**ND**	1.62	**ND**	11	10
A99-T	**ND**	**ND**	**ND**	11	52.5

ND = Not detected

Figure 2 Chart of overall positive cases

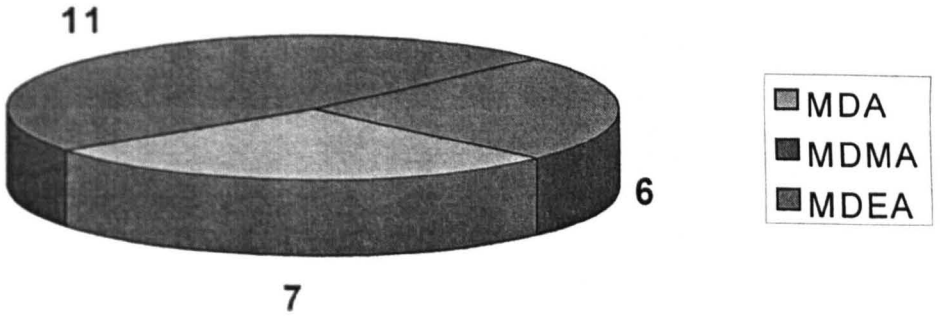

Figure 3 Self-report data compared to hair analysis for MDA, MDMA and MDEA

		SELF-REPORT	
		YES	NO
LAB	YES	11 (55%)	3 (15%)
	NO	6 (30%)	0 (0%)