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DEVELOPMENT OF METHODS TO DETERMINE ANALYTES IN A VARIETY OF MATRICES WITH APPLICATION TO FORENSIC SCIENCE

Doctor of Philosophy

By

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This thesis is dedicated to my parents

Ali Breidi and

Hiba Badra Breidi

for their love, endless support

and encouragement.

I love you.

Abstract

This thesis comprises of studies based on the broad field of forensic analysis and the development of methods which can be applied to different matrices. The initial studies focus on the forensic determination of psychoactive drugs in hair matrix using newly developed gas chromatography–mass spectrometry analytical methods. The second study focuses on the discrimination and matching of skid marks and rubber tyre analysis by using novel chemical analysis methods. This thesis reports the development and application of a series of innovative analytical methods: gas chromatography–mass spectrometry, inductively coupled plasma mass spectrometry, pyrolysis–gas chromatography–mass spectrometry and attenuated total reflection Fourier transform infrared spectroscopy.

Gas Chromatography-Mass Spectrometric (GC-MS) methods for drug analysis routinely employ derivatising reagents. The aim of the first study was to develop a method for the analysis of two recreational drugs, delta-9-tetrahydrocannabinol (Δ^9-THC) and cocaine in hair samples using GC–MS, without prior derivatisation, thus allowing the sample to be analysed in its original form. Ten hair samples, that were positive to ELISA analysis for either Δ^9-THC and/or cocaine, were enzymatically digested, extracted and then analysed by gas chromatography–mass spectrometry. All samples measured contained Δ^9-THC and one sample contained cocaine. The limits of detection (LOD) and quantification (LOQ) were 0.02 ng/mg & 0.05 ng/mg, respectively for cocaine and 0.015 ng/mg & 0.02 ng/mg, respectively for Δ^9-THC. The wide detection window, ease of direct analysis by GC-MS, lower detection limits of un-derivatised samples and the stability of drugs using this technique offers an improved method of analysis.

This experiment has been designed to develop an immunological screening test followed by a GC–MS confirmation method for the simultaneous analysis of Δ^9-THC, THC-COOH, , OH-THC, cocaine, Benzoylecgonine (BZ), amphetamine (AP), methamphetamine (MA), in human hair, thus avoiding the significant factors responsible for drug degradation by acid and alkali hydrolysis and to obtain optimal recovery conditions. Enzymatic hair digestion was used to hydrolyse 18 Turkish

samples using Proteinase K, Dithiothreitol and Tris HCl buffer. At the beginning, all 18 samples tested screened positive on ELISA, though analysis by GC-MS indicated that only 2 samples were positive for Δ^9-THC and THC-COOH. Cross reaction lead to false positive results in the pre-screening step as a result of the degradation of the antibodies in the pre-coated ELISA microplate.

Tyre rubber analysis

Owing to an increase in the number of hit and run accidents, it is quite common for rubber traces to be left at the crime scene. The Forensic Scientist will have the task of analysing the tyre striation traces in order to identify the type of tyre involved in the accident. However, the tyre striations alone do not provide enough detail to show a high level of discrimination between different tyre manufacturers and individual models. In this study, Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) and pyrolysis GC-MS methods were developed to enable greater discrimination between different tyre rubber samples.

Seventy elements were screened for each sample by ICP-MS in both collision cell mode and reaction cell mode. ATR-FTIR analysis indicated a low intra-variability (analysis of similar tyres) which demonstrated high precision of the technique, and also showed a large inter-variability between different manufacturers and models, which supports their high potential as indicators to be used for discrimination between different tyres manufacturers and models. Principal Component Analysis (PCA) was utilised to distinguish between the different tyres.

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DECLARATION

This is to declare that this doctoral thesis and the work presented in it is based upon original research conducted by the author in the School of Pharmacy and Chemistry at Kingston University London between July 2010 and July 2014. The work done has not been submitted anywhere for any award other than part of it for publication in scientific literature. Other contributions made to the thesis have been acknowledged as text or by references.

LIST OF PUBLICATIONS

1- **Breidi, S.E.,** Barker, J., Petróczi, A. and Naughton, Declan, P., "Enzymatic digestion and selective quantification of underivatised Delta-9-tetrahydrocannabinol and cocaine in human hair using gas chromatography-mass spectrometry", Journal of Methods in Analytical Chemistry, (2012), doi:10.1155/2012/907893. 7 pages, ISSN 20908865.

2- **Breidi, S.E.**, Barker, J., Barton, S., Ghatora, B., a talk entitled "Tyre rubber analysis from car skid marks using multiple analytical techniques", 9th National FORREST (FORensic RESearch & Teaching) Conference 25-26th June 2013, Cambridge, Book of Abstracts, pg. 23.

3- Barker, James, Deshmukh, Nawed I.K., Shah, Syeda A.B., Archer, Roland, **Breidi, Salah E.**, Petroczi, Andrea, Cross, Paul and Naughton, Declan (2013) Development and application of hyphenated mass spectrometry techniques for drug analysis in human hair. In: BIT's 11th Annual Congress of International Drug Discovery Science & Technology 2013; 13-16 Nov 2013, Haikou, China.

4- **Salah Eddine Breidi**, James Barker, Steve Barton, Baljit Ghatora, Discrimination of car tyre rubber samples using ICP-MS, ATR-IR and Pyrolysis GC-MS and the subsequent matching to skid marks, World Forensic Festival 2014 (WFF 2014), Seoul, South Korea.

5- **Salah Eddine Breidi**, James Barker, Steve Barton, Baljit Ghatora, Chemical tyre rubbers analysis with analytical techniques for the discrimination of car's tyre samples, World Forensic Festival 2014 (WFF 2014), Seoul, South Korea.

6- **Salah Eddine Breidi**, James Barker, Steve Barton, Baljit Ghatora, Laboratory analysis for tyre rubber samples using various analytical chemical techniques for discrimination and the subsequent matching to tyre skid marks, forensic Science International Journal. In Press (2015).

CONFERENCES AND COURSES ATTENDED

• A talk titled: "Discrimination of car tyre rubber samples using ICP-MS, ATR-IR and Pyrolysis GC-MS and the subsequent matching to skid marks", World Forensic Festival 2014 (WFF 2014), Seoul, South Korea 2014.

• A talk titled: "Tyre rubber analysis from car skid marks using multiple analytical techniques", Forensic Research and Teaching conference, June 2013.

• A presentation at Kingston University research day 2012 and 2013.

• A poster presentation at the International Clarke Conference on 25 May 2011 at the Royal Pharmaceutical Society (RPS).

• Poster presentation at St. George's Research Day "Analysis of Psychotropic Drugs in Human Hair Using Gas Chromatography-Mass Spectrometry" Dec, 2010.

• Attended a Forensic instrumentation conference for Thermo Fisher on the 7th of June 2010.

• Thermo Scientific analytical conference on May, 2010.

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LIST OF ABBREVIATIONS

CNS	Central Nervous System
GC-MS	Gas chromatography mass spectrometry
mg/kg	Milligram per kilogram
LC-MS/MS	Liquid chromatography tandem mass spectrometry
DNA	Deoxyribonucleic acid
WADA	World anti-doping agency
mg	Milligram
GC	Gas chromatography
LC	Liquid chromatography
HPLC	High performance liquid chromatogrpahy
UV	Ultra violet
MS	Mass spectrometry
m/z	Mass to charge ratio
TIC	Total ion current
ESI	Electrospray ionisation
eV	Electron voltage
SIM	Selected ion monitoring
µl/min	Microlitre per minute
LLE	Liquid-liquid extraction
SPE	Solid-phase extraction
FDA	Food and drug administration
IS	Internal standard
LLOQ	Lower limit of quantification
LLOD	Lower limit of detection
RSD	Relative standard deviation
QC	Quality control
µl	Microlitre
mL	Millilitre
S/N	Signal to noise ratio
LC-MS	Liquid chromatography - mass spectrometry
µm	Micrometre

RT	Retention time
mg/mL	Milligram per millilitre
pg/mg	Pictogram per milligram
r^2	Regression coefficient
psi	Pressure per square inch
ng/mg	Nanogram per milligram
mM	Millimolar
µM	Micromolar
ELISA	Enzyme-linked immunosorbent assay
TfL	Transport for London
SRM	Selective reaction monitoring
V	Voltage
ICP-MS	Inductively coupled plasma mass spectrometry
Pyrolysis GC-MS	Pyrolysis–gas chromatography–mass spectrometry
ATR-FTIR	Attentuated total reflectance Fourier transform infrared spectroscopy
Pro K	Proteinase K
PCA	Principle component analysis
Δ^9-THC	Δ9-tetrahydrocannabinol
THC-COOH	11-nor-9-carboxy-delta-9-tetrahydrocannabinol
OH-THC	11-Hydroxy-Δ9-tetrahydrocannabinol
DTT	Dithiothreitol
AP	Amphetamine
BZ	Benzoylecgonine
MA	Methamphetamine
Be	Beryllium
B	Boron
Na	Sodium
Mg	Magnesium
Al	Aluminum
Ca	Calcium
Sc	Scandium
Ti	Titanium
V	Vanadium

Cr	Chromium
Mn	Manganese
Fe	Iron
Co	Cobalt
Ni	Nickel
Cu	Copper
Zn	Zinc
Ga	Gallium
Ge	Germanium
As	Arsenic
Se	Selenium
Rb	Rubidium
Sr	Strontium
Y	Yttrium
Zr	Zirconium
Nb	Niobium
Mo	Molybdenum
Ru	Ruthenium
Rh	Rhodium
Pd	Palladium
Ag	Silver
Cd	Cadmium
In	Indium
Sn	Tin
Sb	Antimony
Te	Tellurium
Cs	Cesium
Ba	Barium
La	Lanthanum
Ce	Cerium
Pr	Praseodymium
Nd	Neodymium
Sm	Samarium

Eu	Europium
Gd	Gadolinium
Tb	Terbium
Dy	Dysprosium
Ho	Holmium
Er	Erbium
Tm	Thulium
Yb	Ytterbium
Lu	Lutetium
Hf	Hafnium
Ta	Tantalum
W	Tungsten
Re	Rhenium
Os	Osmium
Ir	Iridium
Pt	Platinum
Au	Gold
Hg	Mercury
Tl	Thallium
Pb	Lead
Bi	Bismuth
Th	Thorium
U	Uranium

Chapter 1

Introduction

1.0 Introduction

1.1 Forensic toxicology

Forensic analysis is the use of toxicology and other techniques such as analytical chemistry, pharmacology and clinical chemistry to help the investigation of any crime including murder, poisoning, accidents and the use of drugs of abuse. This information is useful not only in the legal context, but also to obtain an explanation as to what the results dictate, whether negative or positive, how can it be statistically studied and its effect on society. The analytical forensic investigation can be applied to different types of samples depending on the type of the matrix. The forensic toxicologist must take into consideration the circumstances of any investigation and also which matrices are used. The matrices can depend upon the nature of any legal investigation, and can vary from very small and easy to collect matrices, to very niche ones such as photos, oral fluid, bullet, paint, dried blood spots, DNA, hair, documents, powder, tyres skid marks etc, which have their own particular idiosyncrasies (1). The type of sample collected for the analytical use depends on the nature of the source. Samples can be categorised based on origin and composition (2).

The analysis process will be determined based on the nature of the collected sample matrix. e.g. for the analysis of heroin in urine or blood the sample has to be collected and analysed (as frozen) within hours of the ingestion, while if it were hair samples it can be analysed differently within a longer analysis period, since the drugs will be metabolised differently in each matrix (hair, blood, etc.) (1,3).

A major aspect of forensic chemistry is the analytical concept that results in connecting the evidence to the criminal and thus a crime. Forensic testing for crime samples has become a useful diagnostic tool in determining the relationships and sometimes sequence of the crime case through a detailed analysis. For any analytical case, forensic analysts are often called to determine whether or not the evidence collected has an effect on the crime scene investigation. In order to make such evaluations, the Scenes of Crime Officer (SOCO) takes specimens to be submitted as evidence which can include blood, DNA, paint and anything else that can be associated with the scene. Nevertheless, analysis is dependent upon the type of evidence e.g. hair, blood, and the accessibility or availability of the evidence to be analysed and the sample size, which will determine the process of analysis and the technique to be used (4,5).

1.2 Analytical techniques

Forensic analysis is becoming increasingly sophisticated with the integration of the latest techniques in analytical chemistry and biology (2). Analytical methodologies face challenges in development and validation prior to the analysis of the pre-collected analytical and forensic samples. Moreover, the nature of the sample matrix and the technique used in the analysis presents a great challenge to analysts (6). The components of the matrices can have a big effect on the method sensitivity and reproducibility and thus can influence the analytical results (7,8). Careful methods of

sample preparation and choice of analytical technique coupled with the appropriate detector are the three important steps of method development.

1.3 Instrumentation

There are a number of techniques used for the separation and detection of the psychoactive drugs and other stimulants from different matrices for analytical purposes. The most widely used techniques are chromatography and spectroscopy. A chromatographic method that utilises separation, identification, and quantitation of one or more closely-related components of mixtures for unknown analytes is gas chromatography-mass spectrometry (GC-MS). GC-MS is essentially two techniques coupled together, which comprise the so-called "tandem" or "hyphenated" technique. Chromatography is a separation method which relies on partition between two phases for separation (mobile and stationary phases), after which detection occurs in the mass spectrometer (MS). The stationary phase is chosen based on the affinity of the sample being analysed to each phase. Along with the gaseous mobile phase, the sample passes through a column containing a stationary phase. Separation is based on the vapour pressure and polarity of the components. The boiling point of a particular compound is related to its volatility, where the compound with the lowest boiling point (vaporise first) and lowest polarity will have the shortest retention time and vice versa. The greater the forces of attraction the higher the boiling point, or greater the polarity the higher the boiling point. Mass Spectrometry is a technique used to determine the identity of compounds (9). MS has a high capability and sensitivity for accurate and precise identification of the separated unknown samples eluting through the GC column (10).

1.4 Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry is a technique to identify different substances within a test sample. Gas chromatography is a technique used in analytical chemistry for separating and analysing compounds. Mass spectrometry (MS) analysis can provide fragmentation of compounds thereby giving rise to various peaks. The highest mass peak usually corresponds to the atomic weight of the fragmented ion. In the mass spectrum, the peak corresponding to the compound's molecule is referred to as the molecular ion peak (M+) after the loss of electron and the most abundant peak in the spectrum is referred to the base peak (B+) (11). The results of interest are provided as GC chromatograms and mass spectra. GC can provide separation of a mixture of compounds based upon boiling points of components in the sample, so lower boiling points have a shorter elution time. The peaks presented in the GC spectra correlate with the elution of the compound from the column. The time taken for this compound to elute after entering the column is referred as the retention time (R_T). Comparing the R_t of the compound of interest with R_t of a known compound as a reference can allow us to determine the unknown drug present in a hair sample (12).

1.5 Ionisation

The mass to charge ratio m/z of the ions is the determinant for identification. Ions are created in the GC- interface or ionisation chamber, after which the quadrupole magnetic mass analyser guides the analyte ions to the detector (10). For the mode of

detection, the MS can be set at single or multiple ions monitoring for the selection of the charged ions. In the interface, the gaseous volatile molecules are ionised under vacuum to ease this transition to the ionised phase. Electron impact, also known as Electron ionization (EI), is an ionization technique whereby electrons interact with the gaseous organic molecules to produce ions (13). Electron ionisation ionises the analytes either positively or negatively. The ionisation mode allows the loss of an electron or protonation (positive ion mode, the addition of a proton) or deprotonation (negative ion mode, subtraction of a proton) to the molecular ion to be created and detected (14). The majority of interfaces use the EI interface for high ionisation and simplicity reasons (15).

In an EI ion source, ions are produced by electrons obtained from thermionic emission of a heated filament. The gaseous mobile phase containing the separated analyte atoms hits the electron filament in a perpendicular direction with energy of at least 70 eV. The high electron beam collides with neutral molecules causing a fluctuation in the electric field around the atoms leading to ionization and fragmentation (16). The ionized analytes are then focused and accelerated towards the MS quadropole, which analyses the charge to mass ratio of the selected ions that are going to be directed to the detector giving structural and molecular mass information of the sample.

1.6 Spectroscopy

Mass spectrometry measures the charged samples with respect to their mass to charge ratio (*m/z*), where the samples are ionised by the loss or gain of an electron(s)

(14) . The quadrupole mass analyser is the mostly widely used mass analyser due to its efficiency, rigidity, and relative cost. The analyser is made up of four parallel cylindrical rods which are connected in parallel electrically. Due to the direct current and the radio frequency (RF) applied, the selected ions pass between the rods Fig. 1.1. These ions are focussed and passed along the quadrupole rods to the detector. Other ions that have an unstable trajectory will collide with the quadrupole rods. The control of the RF permits the selection of particular ions with a specific *m/z* and also allows the instrument to scan for a range of samples at different *m/z* values by continuously varying the applied voltage along the analysis run (17).

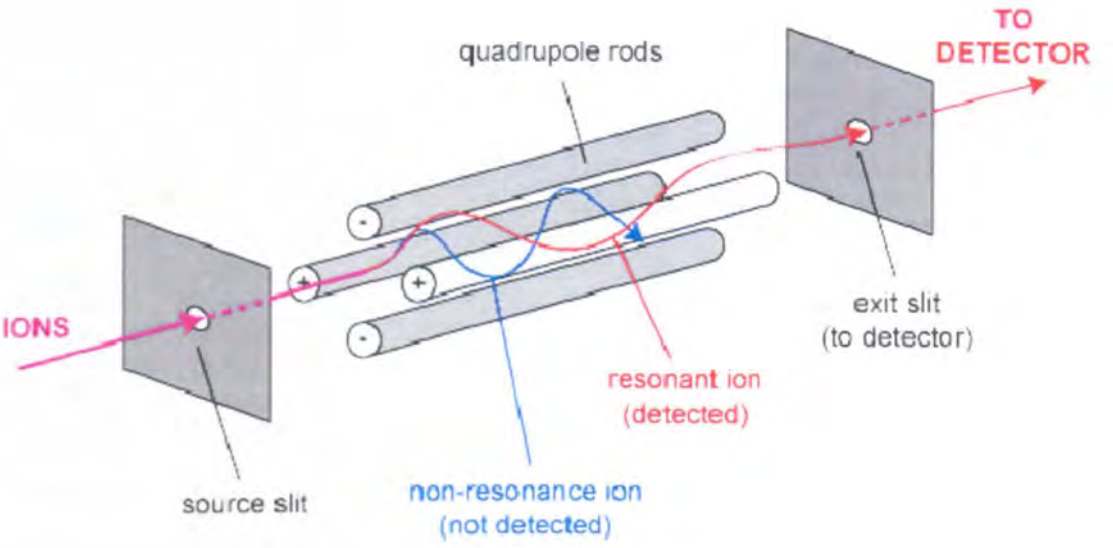


Figure 1.0.1: A schematic of a quadrupole analyser (18).

Every technique always has its own disadvantages. In-accurate quantification and analysis of the biological samples, when more than one analyte co-elutes at the same retention time can lead to a misinterpretation of the results. Also, the quality of analysis will always depend highly on the separation. Many components in matrices

themselves can influence the result of the analysis, affecting assay sensitivity and reproducibility.

Mass spectrometry analysis can be accomplished either by using the full mass scan mode or by selective ion monitoring (SIM), where a chosen number of masses are measured. The usual scan can last anywhere between 1 and 10 millisecond (ms) depending on the type of scan. These measurements and scans are plotted as the total ion current (TIC) Fig. 1.2. The TIC is a plot of the total number of ions in each MS scan plotted as intensity against time (19). Selected ion monitoring allows dwell times of masses to be increased and so precision and limits of detection are improved.

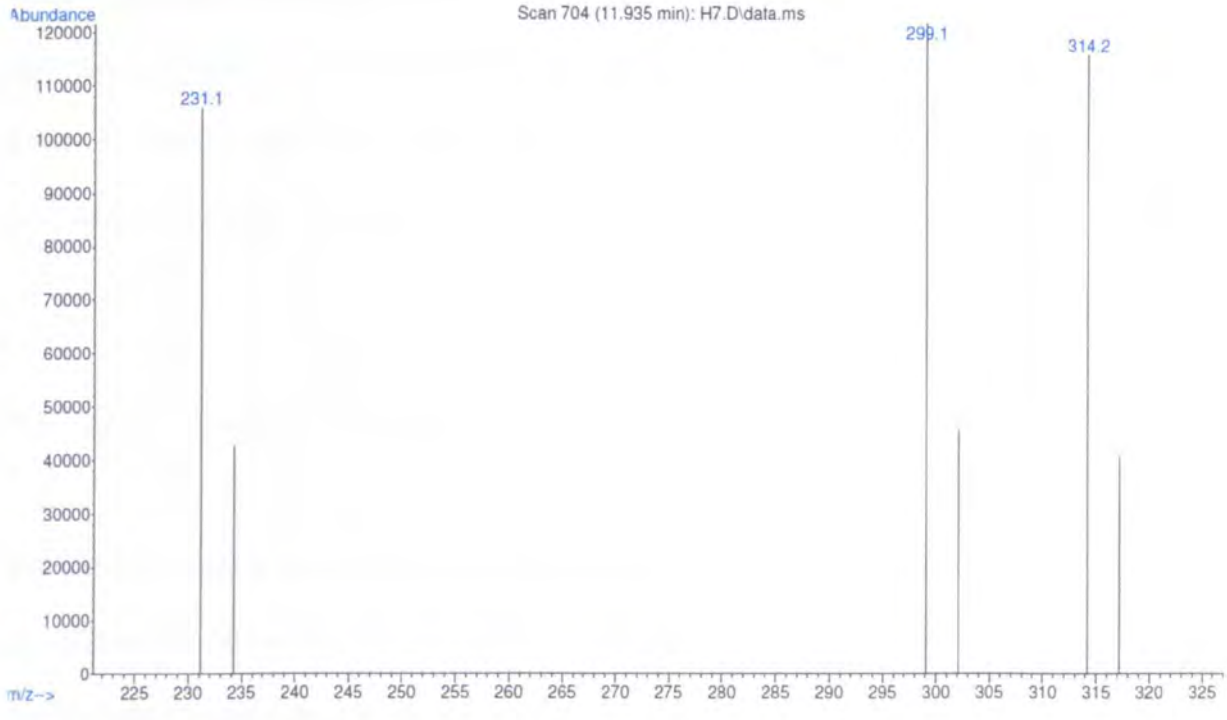


Figure 1.0.2: Mass spectra showing the base peak (314.2) and the fragment ions.

1.7 Pyrolysis GC-MS

Rubber samples cannot be analysed in their normal form using a conventional GC-MS, because of their cross-linked structure and lack of volatility. Pyrolysis gas chromatography mass spectrometry (Py-GC-MS) is an analytical technique whereby the samples are flash heated to high temperatures until they decompose and are then separated by gas chromatography and detected using mass spectrometry (20). This method differs from the regular GC-MS technique in the way the samples are introduced onto the GC column and the type of mass spectral ions produced. Samples are simply prepared by choosing the right minimal amount (> 1g to avoid overfilling the liner), inserting it in a quartz chamber (which is surrounded by a platinum coil) and placing it into the pyrolysis unit, where the coil heats it. The samples are heated (600-1000 °C) in an almost oxygen-free chamber at a pre-determined rate; this allows the breakage of the bonds between the macromolecule structures resulting in smaller chemical molecules. The cleaved molecules are then swept to the GC-MS by the gas stream to be processed and analysed as for the normal GC-M sample (21), (22).

1.8 Inductively coupled mass spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry (ICP-MS) was developed in 1971 at the University of Surrey, UK (23). It is an analytical technique that is widely used for elemental analysis of metals and other non-metals at concentrations as low as one part per trillion. This is achieved by the ionisation of the sample in the argon plasma torch and the separation and detection of the specific elements by mass spectrometry

(24). This technique has several advantages over the other spectroscopic techniques e.g. ICP-MS has a higher throughput than atomic absorption and optical emission spectrometry, including graphite furnace atomic absorption spectrometry (GFAAS) and ICP Atomic Emission Spectroscopy (ICP-AES); with the ability to handle different matrices due to its high plasma temperature.

The plasma source is energised by heating the gas stream with a coil using an electrical source. The ICP plasma source used in the analysis has a neutral charge, with each positive charge on an ion balanced by a free electron. The middle RF coil is surrounded by a gas stream passing through two outermost tubes made up of quartz. When a spark hits the gas (which is usually argon), it produces a negative charge leading to the formation of the argon ions. The plasma flame temperature may range between 6000-10000 K. The argon ions are trapped in the oscillating RF fields and collide with other argon atoms forming an argon discharge or plasma. The liquid sample is them injected into the nebulizer where the spray is formed. It is then vaporized by the high temperature and it gets ionised at the cone of the plasma source Fig. 1.3 (24).

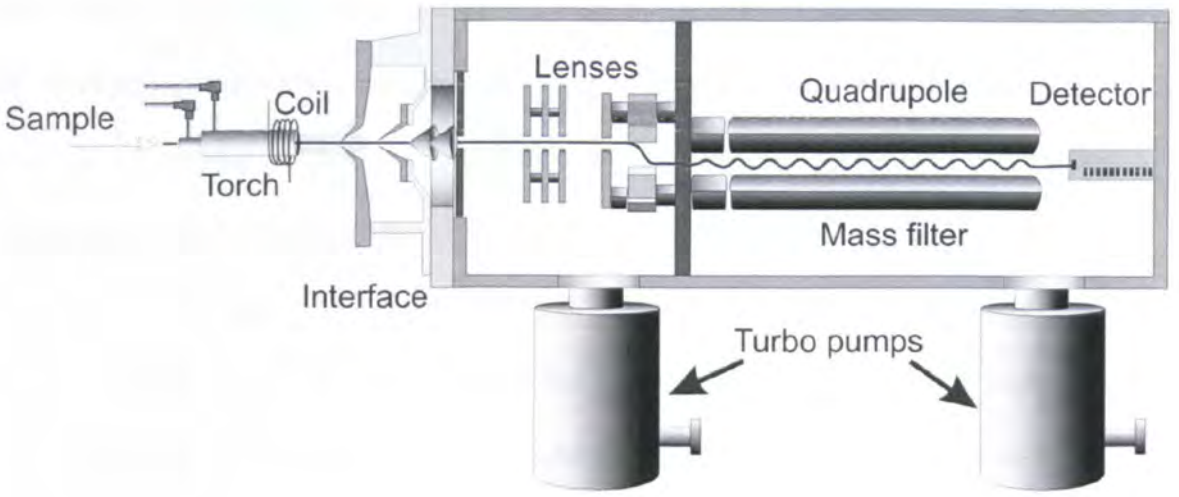


Figure 1.0.3: ICP-MS instrumentation analysis (25).

Once the ions of the specific elements are generated, they are then transferred to the mass spectrometer to be selected and then detected. The positively-charged electrostatic lens serves to align the positively-charged ion beam and focuses it into the entrance aperture of the mass spectrometer. The selected ions are separated on the basis of their mass-to-charge ratio and the detector gives a signal proportional to the concentration of the injected sample (26).

1.9 Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Attenuated total reflectance (ATR) is an analytical sampling technique which is combined with infrared (IR) spectroscopy for the direct quantitative and qualitative analysis of samples with little or no sample preparation. FTIR Fourier transform infrared spectroscopy (FTIR) is an infrared technique that instantaneously collects data from the sample over a wide spectral range, and this gives it the advantage over other narrow wavelength range continuous wave spectrometers. In contrast to regular IR (infrared) spectroscopy, samples don't need to be pressed into KBr discs prior to analysis to prevent then absorbing bands in the infrared spectrum. The most important advantage which can help in forensic investigation is that it's a non-destructive technique. ATR-FTIR spectral imaging has significant advantages compared to many other imaging methods for its ability to perform multiple analyses in a short time, its simplicity to operate and its ability to analyse a wide range of solids, liquids and gases (27). Applying pressure to the sample above the diamond slot is more than enough to achieve satisfactory results, which later can be identified

with the library search. The application of FTIR imaging to different samples has been presented in a number of publications (28)(29)(30).

ATR-FTIR measures the changes in a totally internally reflected infrared beam when it comes into contact with a sample. This beam is directed onto the sample through a higher refractive index crystal than the sample itself. In the crystal, the reflectance of the rays creates an evanescent wave that reaches the applied sample surface. Some of the created evanescent wave is absorbed by the examined surface and the attenuated evanescent wave is passed back as a weaker reflected IR beam, which is detected and an IR spectrum is generated Fig. 1.4.

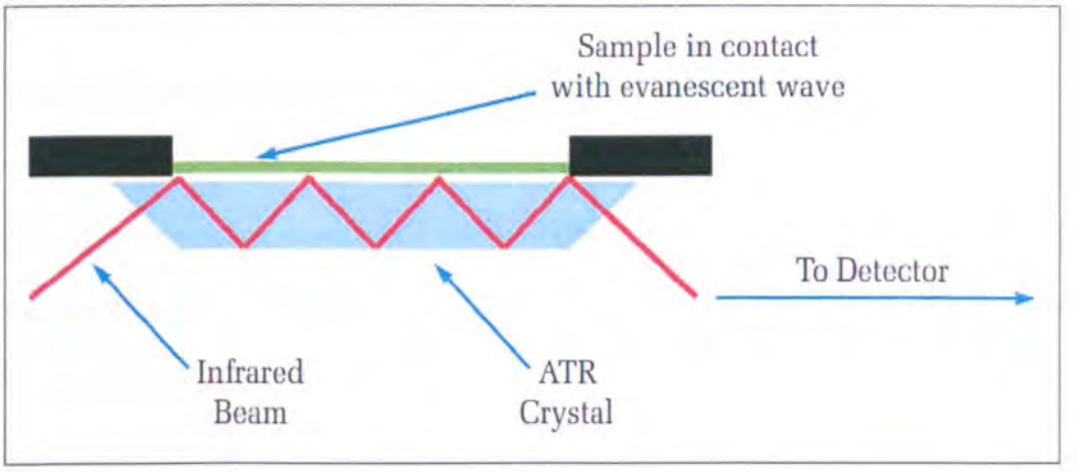


Figure 1.0.4: ATR-FTIR spectroscopy analysis (31).

1.10 Background: Drugs of abuse

Drug abuse is a serious burden to society contributing to detrimental public health and safety implications. The World Health Organisation (WHO) estimated that there were tens of millions of global consumers of illicit drugs in 2000, having greatest impact in developed countries (Europe, North America Australasia) in comparison to central and southern Africa (31). According to the World Drug Report 2008 by International Drug Policy Consortium (UNODC), there were an estimated 190,750,000 million cannabis, 19,380,000 million cocaine and 52,900,000 million amphetamine abusers globally aged between 15-64 years (32). The annual prevalence of illicit drug abuse had risen by 69% for cannabis, 16% for amphetamines (AM) and methamphetamines (MA) and 6% for cocaine over the period of 1998 to 2000. Globally, cannabis remains the most widely consumed drug worldwide. North America is the largest cocaine market with close to 40% of the global cocaine usage population. The proliferation of MA abuse still continues to be a major concern in USA, Australasia, East Asia and South-East Asia. The ability of drugs to promote compulsive repetitive abuse is referred to as "drug dependence". Reinforcement and neuronal adaptation can contribute to the individual's inability to control drug intake and continuing drug abuse despite the negative consequences. For the majority of individuals, the abuse of psychoactive substances may be to experience pleasure or to avoid pain. For many, gaining psychoactive effects, intoxication or altered body image outweighs the known risks (33).

Until this date, most of the drugs are analysed by urine testing. However, due to a small detection window, it is not possible to detect many drugs in urine after 48-72 hours, since the majority drugs are excreted by then. This makes it difficult to detect

drugs by random testing or monitoring the improvement of any clinical study or even prove the use or non-use historically. Hair analysis can solve these problems by having the ability to record the history of drug usage. Just as important, it can be used as a confirmatory test, when the initial screening was carried out by enzyme-linked immunosorbent assay (ELISA), for example. Hair analysis with its large detection window helps to determine the history of the individual's drug usage prior to sampling. Hair can be sampled months after any usage, as long as the hair length covers the tested period of time. Hair samples are far less likely to be replaced by bogus hair samples and cannot be easily diluted. Since hair testing can have a wide detection window, it does not needs to be examined as frequently as urine or oral fluid analysis, which may prove to be a cost-effective method of detection and more ethical method. Lawyers can use hair analysis evidence in court cases to prove drug abuse or abstinence. Hair samples are easier to store, transport and safer to collect due to the non-invasiveness properties of sampling (Table 1.1). Hair analysis can be used to differentiate between a one-off use and any addictive behaviour (34)(35)(36).

Table 1.1: Comparison between different matrices drugs analysis[26,37,42].

Matrices	Urine	Hair	Oral fluid	Blood
Detection window	1-4 (days)	1 week to 3 months	Approx. 24 hrs	Approx. 1-5 days
Collection	Tube collection (presence of the GP personnel)	Non-invasive (cut from posterior cortex)	Easy to collect by a syringe	Invasive (GP phlebotomist)
Storage	-Fridge/freezer -Special containers	-Room temp. -Paper envelope/bindle	-Fridge/freezer -Fluid collection tubes	-Freezer - Blood collection tubes
Transportation	Controlled temp (cold /ice)	Easy transportation (envelope)	Controlled temp (cold /ice)	Controlled temp (cold /ice)
Cost of storage and Transportation	Medium cost	Low cost	Medium cost	High cost
Substitution and Samples faking (exchange)	Very easy to substitute with another sample and dilute with any liquid	Controlled hair sampling	Easy to substitute	Controlled invasive sampling

1.11 Detection of drugs in hair

Drug users can substitute and sometimes dilute the urine samples during sample collection under supervision or when supervision during collection is absent (43). One hair sample is able to provide the information of up to 6 combined urine samples due to the long detection period that hair strands can hold; also 4 yearly hair samples can give absolute confidence of total drug abstention (44). Studying the drug history, which hair analysis is able to provide can give numerous benefits and information about the abuser (39,44).

1.12 Cocaine: background, chemistry, pharmacology and pharmacokinetics

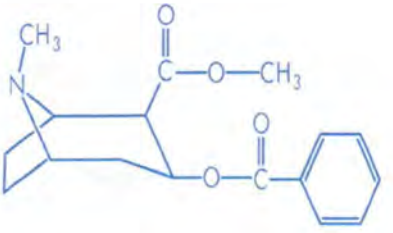
Cocaine (benzoylmethylecgonine) is a crystalline alkaloid derived from the leaves of the shrub Erythroxylon coca, native to South America. The molecule of cocaine consists of a nitrogen (N) containing ring layered hydroxy salt (Rhs) and a six carbon phenyl ring, both vital for the drug's biologic activity (see Figure 1.5A) (45). Other features of the molecule include the ecgonine (ester-type molecule), a local anaesthetic which adds to cocaine's numbing effects. Coca leaves consist of 0.6-1.8% w/w cocaine, containing 80% pure cocaine when initially extracted. The alkaloid is then converted to cocaine hydrochloride (HCl) and the sulfate (SO_4) salt. Cocaine HCl is polar and soluble in water and is therefore easily administered orally,

intranasally or via intravenous (IV) injection. However, its ability to transform back into the cocaine free base has increased health implications when smoked i.e. 'free base smoking' (34,45,46). Cocaine is an addictive central nervous system (CNS) stimulant that can be snorted, injected or smoked. Regardless of the different routes of administration, once absorbed and entered into blood circulation, cocaine is broken down via enzymes and rapidly eliminated with a half-life of 0.5-1.5 hours. The symptoms of euphoria, elation, mood elevation and fatigue suppression are exhibited through interactions with biogenic amine transporters (34,46)(31).

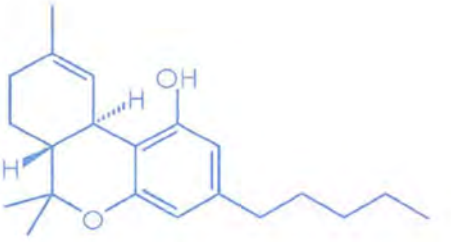
1.13 Cocaine: Mechanism of action

The majority of behavioural and physiological actions of cocaine are thought to be due to its ability to inhibit the re-uptake of dopamine (DA), norephrine (NE) and serotonin (5-HT). The stimulation of dopamine transmission in the limbic system is a fundamental property in the action of psycho stimulants such as cocaine. The system involves dopamine entering vesicles via amino acids present in our food (47). These amino acids are then converted to tyrosine via enzyme tyrosine hydroxylase. The tyrosine is then finally hydroxylated to L-Dopa forming dopamine via dopamine decarboxylase. The dopamine is synthesized into vesicles to allow appropriate release into the synapse (38). Dopamine is pumped out of the synapse and stored into the vesicles via the dopamine transporter. Cocaine acts by binding to specific dopamine rich sites of the brain (VTA) and nucleus accumbens (38,48). Cocaine binds tightly to DAT to prevent its re-uptake function, thereby causing dopamine to accumulate in the synaptic cleft. As a consequence of cocaine's action in the *nucleus accumbens,* there are elevated impulses activating the reward pathway. As a result of

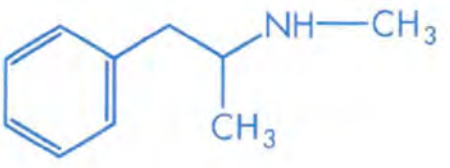
this inhibition, there is a prolonged and enhanced postsynaptic effect of dopaminergic signalling at dopamine receptors (38,48). Cocaine's action on the *nucleus accumbens* causes increased impulses and activation of the reward system and hence produces and reinforces pleasurable and natural rewards (See Figure 1.5C). The acute reinforcing effects of cocaine can lead to repetitive patterns of drug use and eventually addiction. The underlying explanation for addiction lies between compensatory adaptations in neural systems regulating autonomic and somatic functions, leading to physical dependence and withdrawal.



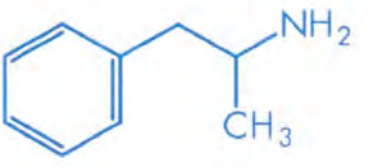
A: Cocaine



B: THC



C: Methamphetamine



D: Amphetamine

Figure 1.0.5: Molecular structure of A) Cocaine, B) THC, C) Amphetamine and D) Methamphetamine

1.14 Background on (Δ^9 THC) Delta-9 tetrahydrocannabinol

Cannabis is derived from cannabis sativa and originates from central Asia. It contains more than 60 unique compounds that are referred to as cannabinoids. The psychoactive properties of these compounds, particularly the principle active ingredient Δ^{-9} tetrahydrocannabinol (THC) account for repetitive cannabis use (34,45)(31). Cannabis exists in three preparations; marijuana a crude dried herbaceous preparation of cannabis sativa hashish, a resinous form and hash oil, a potent variation from hashish via distillation (31).

The molecule of THC is extensively metabolized in the liver by cytochrome P450 enzymes (CYP2C9, CYP2C19, and CYP3A4); the hydroxylation of THC leads to the primary production of active metabolites 11-hydroxy-Δ^9 – tetrahydrocannabinol (11-hydroxy-THC) and inactive metabolites 8 hydroxy-Δ^9 – tetrahydrocannabinol (45). Subsequent oxidation results in the production of the main inactive metabolite (9-carboxy-THC). The inhaled route of administration may result in rising levels in blood plasma which later decline due to drug metabolism and accumulation. In contrast, oral consumption has a prolonged, but poorer absorption, thereby resulting in lower variable concentrations and reduced bioavailability of THC. The psychoactive effects of THC include enhanced sensory perceptions, elevated appetite, memory impairment, anxiety and paranoia. Besides the major risk to public health, cannabis has its therapeutic benefits in providing pain relief (49). There is

increasing interest in the use of cannabis for treatment of medical conditions including chemotherapy-induced nausea and vomiting in cancer patients (50). In fact, the widely available antiemetic Dronabinol (Marinol®); a synthetic form of active constituent Δ^{-9} tetrahydrocannabinol is currently used in cancer patients (50).

1.15 THC: Mechanism of action

Cannabinoids, such as THC have the ability to exhibit such effects via mimicking the action of endogenous endocannabinoids (anandamide and 2-arachidonoylglycerol)(45). They exhibit their physiological effect by acting as agonists at the CB1 cannabinoid receptors expressed in the brain ventral tegmental area (VTA, *nucleus accumbens*) or CB2 cannabinoid receptors expressed in peripheral tissues, principally the immune system. In the CNS, endocannabinoids have a role as retrograde messengers that inhibit neurotransmitters (DA, NA, 5HT, GABA) through stimulation of pre-synaptic CB1 receptors on release from postsynaptic neurons (34). This raises intracellular Ca^{+} levels causing the release of endocannabinoids and activation of pre-synaptic CB1 receptors on the nerve terminal (See Figure 1.5A)(45).

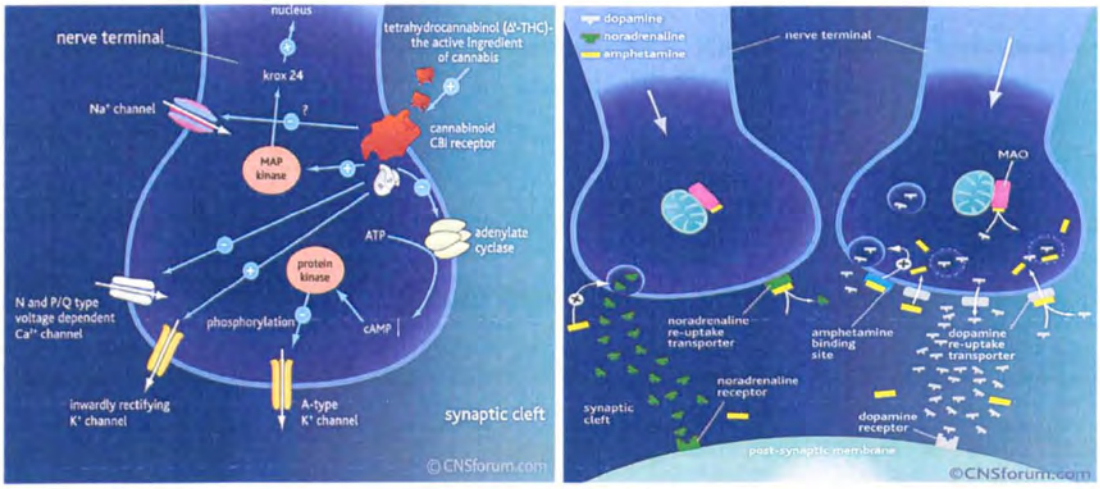
1.16 Background: chemistry, pharmacology and pharmacokinetics of amphetamines (AM) and methamphetamines (MA)

Amphetamine (N, α-methylbenzeneethanamine) is a parent compound of a family of synthetic psycho stimulants commonly produced in clandestine laboratories. The

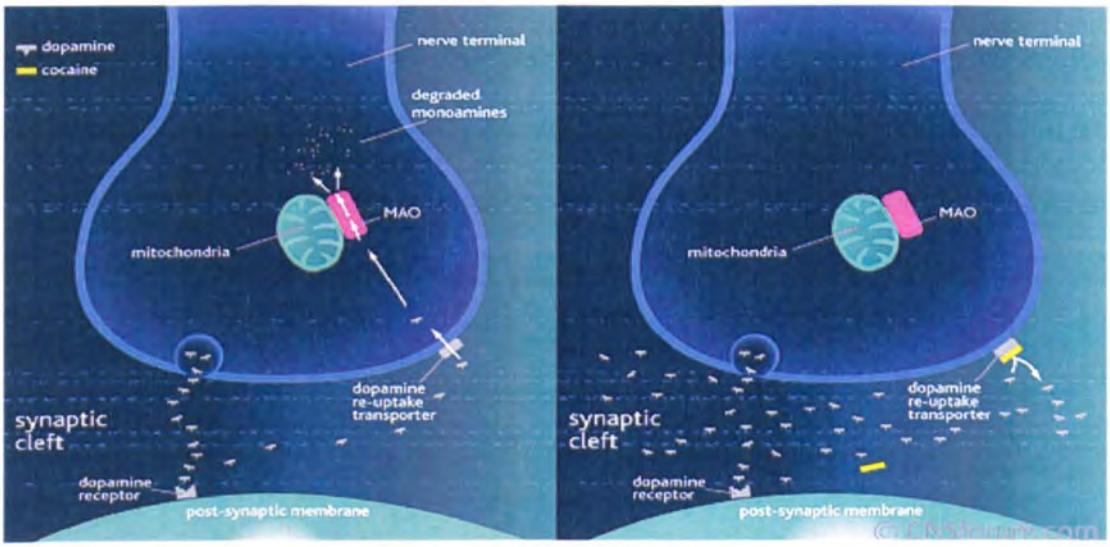
molecule of AM consists of an asymmetric α-carbon atom giving rise to two enantiomers (- l-stereoisomer or + d-stereoisomer) (see Figure 1.5B). Being the parent compound, it can produce an extensive range of psychoactive derivatives [MA, MDA (3,4-methylenedioxyamphetamine) and MDMA (3,4-methylenedioxy-N-methylamphetamine)]. The phenyl ethylamine structure of AM is related to the neurotransmitters dopamine and serotonin, which may demonstrate the CNS stimulant effects. Oral administration may be associated with an approximately 1 hour time lag, as a result of slow GI absorption, in contrast to the rapid and intense effects of the 'high' when inhaled or administered intravenously (31). The elimination half-life can range between 7 to 30 hours consequently leading to longer lasting effects from a single dose. Like cocaine, AM can provide feelings of heightened alertness, confidence, exhilaration and reduced fatigue.

1.17 Mechanism of action of AM

Amphetamine acts as indirect dopaminergic and nor-adrenergic agonist causing an increase in synaptic concentrations of dopamine (DA) like cocaine. However, it is unlike cocaine, which has the ability to simply block the action of dopamine active transporter (DAT), AM and MA enter the DA nerve terminal via DAT uptake. Once incorporated inside the nerve terminal, AM or MA enhance the release of DA from synaptic vesicles into the cytoplasm, leading to substantial increase in synaptic DA levels (Fig. 1.6) (51).



A) THC **B) AM**



C) Cocaine

Figure 1.0.6: Mechanism of action of drugs of abuse. A) Δ^9-THC B) AM C) Cocaine (51).

A) Δ^9-THC (Fig. 1.6A) exerts its effects by binding to CB1 receptors present on pre-synaptic nerve terminals in the brain. This activates G-proteins and inhibits signal transduction pathways. G-proteins inhibit N and P/Q-type voltage dependent Ca+ and Na+ channels and rectify K+ channels/ MAP Kinase signalling pathways to produce euphoric feelings.

B) AM (Fig 1.6B) has the ability to modify the action of (DA) and (NA) in the brain by promoting the release of dopamine from nerve terminals by either binding to pre-synaptic membrane dopaminergic neurons or interaction with synaptic vesicles (51). AM can also exert its effects by inhibiting the degradation of dopamine via binding to MAO in dopaminergic nerves or by binding to DAT to transport free dopamine out of the nerve terminal.

C) Cocaine (Fig 1.6C) acts on the 'brain's reward pathway' to modify dopamine levels by binding to DAT. This prevents the elimination of dopamine from the synaptic cleft and consequent degradation by MAO. Dopamine therefore remains in the synaptic cleft and free to bind to post-synaptic receptors, hence continuing the nerve impulses (51).

1.18 Traditional methods vs. hair analysis

Over the last 20 years, hair testing has increasingly gained recognition in the investigation of chronic drug exposure. For decades, the estimation of drug use has relied upon urine analysis, although it has been well been accepted that its main

limitations include high evasion potential, reduced retrospective capability of 1-3 days and lack of comparability with repeat samples. The limitations of urine can be improved through the use of hair as the testing medium. Hair analysis reduces the opportunity to evade or tamper with testing, unlike traditional methods (urine dilutions or abstention from drug prior to screening), Its advantages of providing an extensive drug history, larger surveillance window (weeks-months) and distinction between single or chronic exposures provides reliable and accurate results in comparison to traditional methods such as urine and blood (41,52,53) (37,54).

In fact, hair analysis has shown beneficial gains in several forensic applications such as chronic poisoning, surveillance of addicts, post mortem examination and differentiation between drug consumers and dealers (54). The patterns, colours, textures, diameters and growth rates of hair can vary between gender and age. The cycle of growth can initiate with the active hair growth period (anagen phase) during which the follicle develops and hair is produced. The follicle enters a short transition stage (catagen), a phase of regression where follicle activity discontinues and dermal papilla contracts (55). The follicle finally enters the resting phase (telogen) where the hair shaft growth ends and hair can be easily pulled for analysis prior to

commencement of the next growth cycle (56).

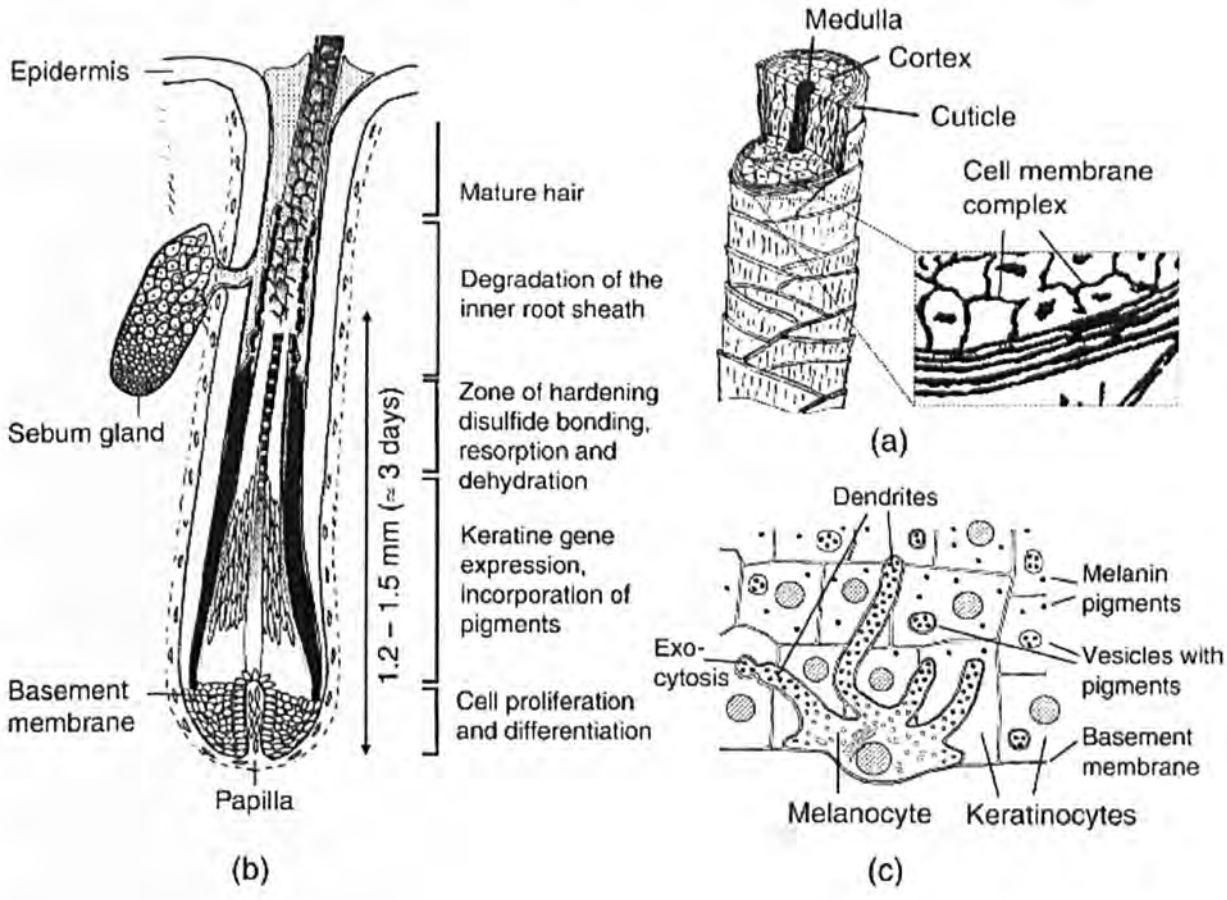


Figure 1.0.7: Structure of hair (1).

The affinity for sequestration in hair can vary among analytes; it appears certain drugs such as cocaine are tightly bound and stable in the hair for longer time periods in comparison to drugs such as cannabinoids (THC), which are weakly incorporated or retained in the hair (54). Drugs and their metabolites enter the follicles via the circulatory system and are then absorbed into the keratinaceous matrix of the hair shaft during hair protein synthesis (Figure 1.0.7). The period of time in which drugs remain embedded in the hair may vary with different drugs.

1.19 Mechanisms of drug incorporation

The precise mechanisms involved in incorporation of drugs into the hair are unknown, although several theories propose that the potential drug can enter hair from multiple sites via different mechanisms and periods of hair growth. This can be during formation of the shaft, (Fig.1.8) diffusion from blood to actively growing follicle, through external contaminations and via secretions of nearby apocrine or sebaceous glands (41). The incorporation of drugs into the hair matrix can be either through endogenous pathways, where the drug molecule passively diffuses from the systemic circulation into growing hair or through endogenous-exogenous pathway involving the absorption and transfer of drug molecules into the hair shaft via sweat, sebum or transdermal excretion (41). An alternative mechanism was proposed by Henderson et al, 1998 who suggested that drugs may bind with sulphydryl-containing amino acid (cysteine) abundant in the hair, forming cross-linking S-S bonds (57)(40). The incorporation into the hair can be limited by the lipophilicity and basicity of the drug. Lipophilic molecules have the ability to penetrate membranes and diffuse through the concentration gradient in hair matrix cells, whereas hydrophilic molecules form an impermeable barrier (40). The pKa of the drug compound and pH of matrix cells are important factors for drug incorporation. Basic drugs accumulate in pigmented hair as a consequence of the lower pH in melanocytes and binding to melanin. Acidic drugs such as THC are found at very low concentrations in the hair due to their distribution equilibrium that is favoured towards acidic matrix cells (40). This theory was confirmed by Nakhara et al, 1998 who had investigated into the effects of drug incorporation in the hair of Dark-Agouti rats. The results of the study revealed a greater incorporation rate (ICR: ratio

of drug concentration in rat hair) compared to THC, which was 3,600 fold lower than cocaine (12).

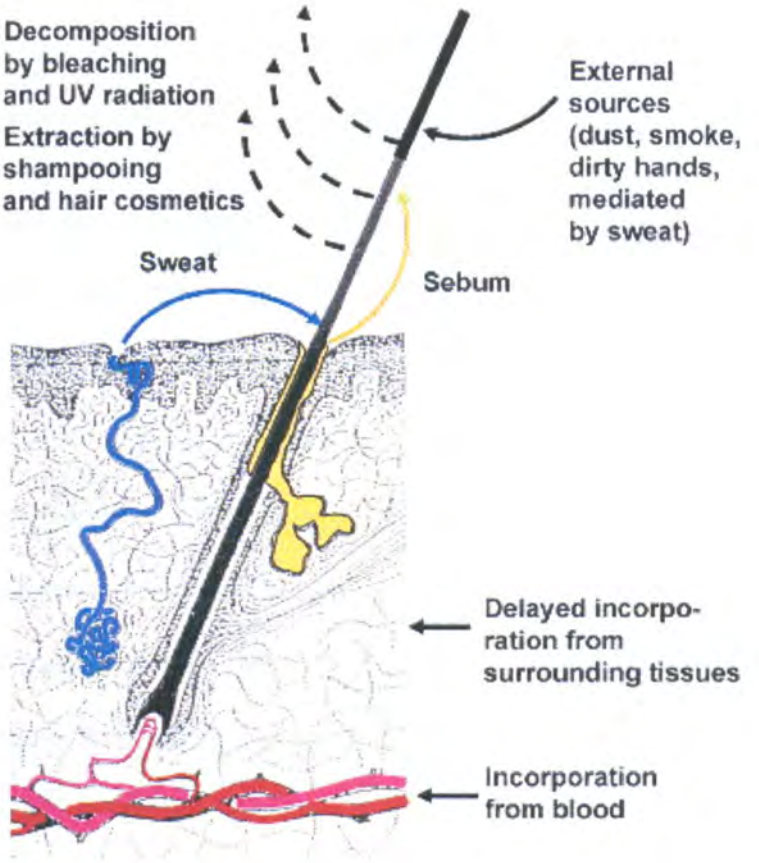


Figure 1.0.8: Pathways by which drugs can get incorporated into hair (14).

1.19.1 Influence of melanin content on drug incorporation

Human hair grows at an average rate of 1 cm per month, and every hair stem has its own blood supply through different capillaries that support the hair with the necessary nutrients for its growth. Once any type of drug is ingested; the drug is absorbed into the blood system through the epithelial cells lining up the intestinal

wall and starts its circulation in the human body. Through the process of blood feeding the hair for growth Fig.1.9, the hair takes the drug from the blood stream and incorporates it into the hair shaft. The hair remains for different periods in the hair shaft based on how often the hair is exposed to natural conditions and chemical reagents. But normally, 4 cm of hair should be enough to give the history of 4 months drug abuse or absence (56)(41)(48,53).

The structural and chemical properties of the drug along with the physical/physiological characteristics of an individual can influence drug incorporation (58). Hair colour is related to the melanin content and may vary between ethnic groups. Several studies have suggested variations in drug binding or differential incorporations of drugs in darker hair (black) compared to lighter hair (brown or blonde). Members of particular ethnic backgrounds with dominant phenotypes of dark hair (Africans, Asians and Hispanics) can have greater drug incorporation than those from the reference group (Caucasian) (58). A study by Henderson et al, 1998 has confirmed this theory by revealing an increased incorporation rate (2.9 times) of cocaine into hair samples of non-Caucasian subjects in comparison to Caucasian subjects under identical experimental conditions (57). Later, in 2000, Kelly et al. investigated the effects of various hair colours (red, grey, blond, light brown, medium brown, dark brown and black) on drug (cocaine, AM, THC) distribution (59). The results of the controlled study revealed a greater percentage of cocaine in black hair samples in comparison to red, blonde and brown hair. The concentration of cocaine had significantly decreased in grey hair samples suggesting a strong influence of melanin content. However, contrary patterns were observed for AM and THC with relatively high concentration values in medium to dark brown hair opposed to black hair. Contributing factors i.e. drug type and

intensity of use had influenced the variation in assay values. This implies a potential for bias in drug testing among particular racial groups, thereby suggesting inequity during doping control (59).

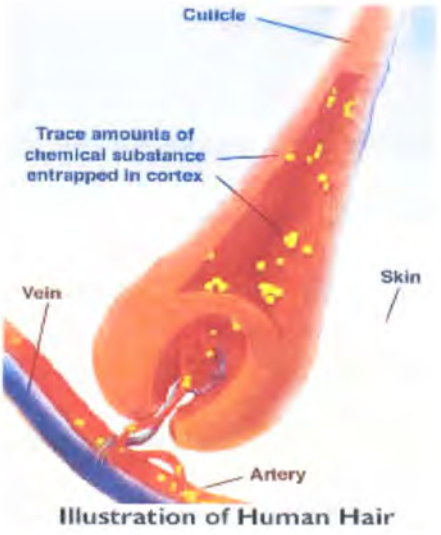


Figure 1.0.9: Incorporation and elimination of drugs in hair via various mechanisms, locations and sources (60).

1.19.2 External influences on drug incorporation

The stability of drugs in hair can be affected by ultraviolet (UV) exposure and chemical composition of various cosmetic treatments i.e. hair bleaching and permanent waving. Comparative studies on bleached and dyed hair revealed a 30-80% difference in drug concentrations for opiates and cocaine, implying there is a greater decrease in drug detection for bleached hair samples (54). The extent of hair damage also influenced the stability i.e. the greater the hair is damaged, the larger the variations in drug concentration levels. Research reveals the influence of cosmetic treatments on hair analysis to be biassed towards false-negative results as

opposed to false positive results of darker hair (58). Jurado et al, 1997 found a decrease in drug concentration subsequent to hair bleaching and waving. The hydrogen peroxide present in bleaching treatment had removed the hair colour thereby causing a loss of melanin granules. The reduced melanin content in bleached hair compared to unbleached hair consequently incorporates lower quantities of drugs (61).

Although hair analysis has the advantages of distinguishing repeated drug use, several cases of tampering with tests are reported each year. In fact, several AM and MA abusers attempt to purposely reduce the AM and MA concentrations present in hair either through repeated hair washing (> 10 times) or hair dyeing. A recent study in 2010 by SK Baeck et al. had confirmed this by investigating the effects of in-vitro repeated hair washing using shampoo and single hair dyeing in MA addicts in Korea (62). The quantitative results of the study showed MA and AM concentration ranges between (1.50-30.0 ng/mg) for non-treated hair samples and ranges of (0.41-12.90 ng/mg) for hair samples repeatedly washed with liquid soaps or dyed using black, brown or yellow hair dye. There was a (16.7-32.8%) decline in drug concentration in hair samples repeatedly washed in comparison to a (22.2-41.9%) decline in single dyed hair samples (62).

The differentiation between systemic and external contamination (Figure 1.0.10) for certain drugs such as cocaine, cannabis and heroin has been a frequent limitation for hair testing. Environmental contamination through passive smoking or drug handling has shown to contribute to the misinterpretation of results. Appropriate decontamination methods, detecting relevant metabolites and by placing threshold

values can potentially minimize misinterpretation. The concern of passive contamination of cannabis preparations containing THC still remains a problem. The diffusion of cannabis deposits into hair reduces the ability to distinguish between passive exposure and willing ingestion (63). A study was carried out to confirm whether cannabis smoke exposure had an influence in hair samples positive for cannabinoids (64). Natural hair strands has exposed to water, external contaminants (grease, sebum) or bleached/permed. The treated and untreated hair was exposed to cannabis smoke for 60 minutes and then decontaminated. GC-MS/MS analysis revealed elevated deposits of cannabinoids from cannabis smoke on untreated hair fibre samples in comparison to pre-treated hair samples. As a result, there was an increased risk of false positive test results when exposed to environmental cannabis smoke (64).

The influence of sunlight and humidity on hair samples of cannabis users has been investigated by Skopp et al, 2000. The results revealed a rapid decline in concentrations of cannabis components (THC) after 8 weeks of exposure to sunlight and humidity (65-67).

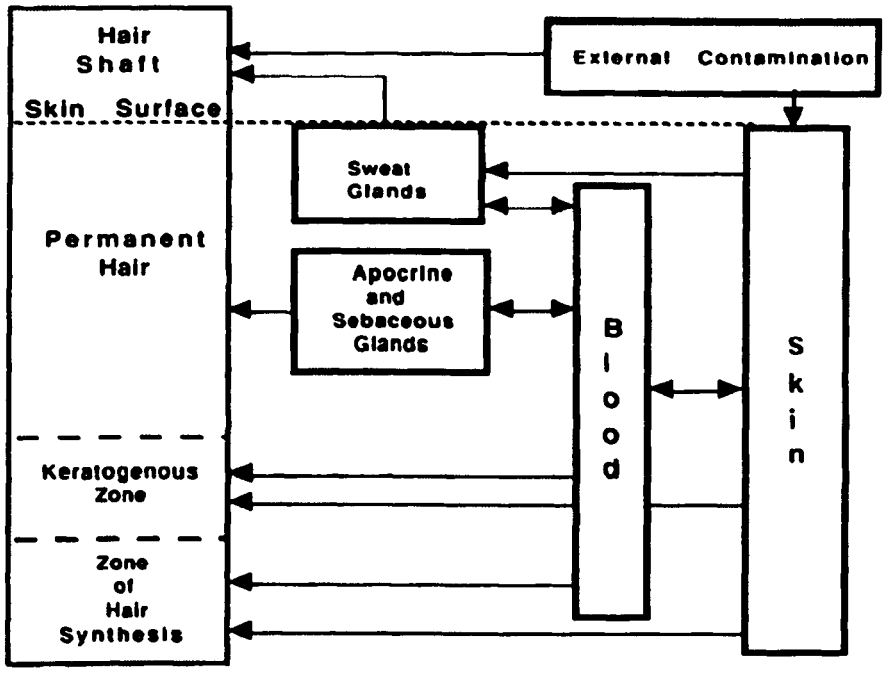


Figure 1.0.10 Proposed multi-compartment model for drug incorporation into hair [55]

1.19.3 Influence of Genetic Polymorphisms

Addiction to drug substances may arise from a combination of environmental and genetic factors. The increased genetic vulnerability to drug addiction has been supported by family, twin and adoption studies (68). The majority of clinical studies have revealed increased addiction and drug related disorders in first degree relatives of drugs addicts. Studies have also revealed significant correlations between drug disorders in adoptees and biological parents. These risks were significantly reduced in adoptees that had adoptive parents involved in substance abuse. Although several genetic studies have revealed and supported the role of genetic components in drug abuse vulnerability; data from monozygotic twin studies demonstrate the role of environmental factors in addictive disorders (68). Several molecular genetic studies have shown positive associations between polymorphisms of dopaminergic receptor

genes (DRD1, DRD2, DRD3 and DRD4). The involvement of A1 allele of DRD2 TaqI polymorphisms in prolonged cocaine abusers and drug disorders has been reported in numerous studies (68). A study by Ebstein et al, 1997 investigated the influence of dopaminergic system and D4 receptor expression in human brain regions involved in reward and mood; through examining drug seeking behaviour in 124 volunteers. The study found 7-repeat allele of DRD4 exonic polymorphisms associated with drug abuse (68,69). Another study carried by Dlugos A, 2010 revealed novel evidence of genetic variations in the fatty acid amide hydrolase (FAAH) gene associated with mood responses post AM administration (70). The findings of the study showed single nucleotide polymorphisms (SNP) (rs3766246) and (rs2295633) with higher reported stimulation in response to AM. The *post-hoc* analysis into the subcomponents of stimulation later revealed a significant greater decrease in fatigue after administration of AM in subjects possessing rs3766246 and rs2295633 SNP's in comparison to other genotypes (71). There was no significant association of any SNPs in the cocaine and AM regulated transcript (CARTPT) gene and susceptibility to cocaine dependence. The study did however manage to provide to scope for future studies with larger study groups of different populations with more comprehensive SNP coverage (71).

1.20 Immunological and analytical techniques

Methods of hair analysis usually involve some preparatory techniques of decontamination, acid or base extraction and digestion. Traditional approaches involve screening the hair samples by performing immunoassays using commercially available kits (i.e. ELISA kits) (12). The efficacy of immunological screening

techniques allow rapid analysis of drug negative hair prior to using expensive and time consuming confirmatory analytical techniques. The ability of ELISA kits to wash away the background matrix before colour generation can make the method extremely sensitive. The kits are designed to provide estimated drug concentrations before confirmation. Decisions on the positive or negative status of assays are determined through recommended cut-off concentrations. Positive samples from ELISA screening are subsequently confirmed via conventional analytical techniques. The most accepted technique for this purpose is gas chromatography mass spectrometry (GC-MS) due to the high specificity, sensitivity and selectivity that is necessary to detect analytes in keratin matrices (54). By meeting all the appropriate requirements, its use has increased for forensic, doping and clinical toxicological analyses. GC-MS is the most widely-used analytical technique for drugs of abuse, as it provides unambiguous identification of compounds (55). In most cases of analysis, electron impact or positive and negative chemical ionization mass detection is adopted; but due to the high sensitivity required in hair analysis, this may not necessarily be achievable by using full scan mode. For simultaneous analysis of multiple drugs, GC-MS EI-SIM (selected ion monitoring) can be employed.

1.21 Tyres rubber analysis

In recent years, 5,844 collisions were attributed to "hit and run" incidents on London roads (72). Presently a forensic site investigation of any road traffic accident relies mostly on the visual and physical observation of the street tyre striation that is left behind at crime scenes. These skid marks are produced due to the friction between

the road surface and the rubber tread of the car tyres of the vehicle responsible or related to the accident.

The tread rubber of the tyre is the major factor in this study, because it's the part which is in contact with the road surface. The purpose of this study was to look at the skid marks collected from the crime scene left by the tread friction with the tarmac surface after sudden vehicle braking e.g. by the suspected car responsible for the hit and run accident. The tyre's skid marks are left on the solid road surface and do not usually shows the tyre tread pattern that could identify the suspected vehicle. However, the tyre mark will contain traces of the tyre. A method that allows successful chemical identification and matching of the tread marks of the car tyre rubber will increase the likelihood of identifying the car responsible for the accident.

1.22 The components of tyre

Tyres are not completely made of natural rubber, although rubber is the most important ingredient. The tread of a tyre has a variety of components that are used to manufacture the final product. The choice of ingredients is reliant on the balance between the engineering conditions, cost, and the physical properties of the final product (73). The main five substances are: the natural rubber, synthetic rubber (Styrene-butadiene co-polymer), silica, black carbon and textile along with many other compounds that may be added depending on the type of the "compound" that is desired. Natural rubber is the basic elastomer that is used in tyre manufacturing, followed by the synthetic rubber styrene-butadiene co-polymer, which is mixed with the natural rubber and used to lower the cost of using the natural rubber alone. Also

another type of polymer that is used in the fabrication of the tyre is polybutadiene, which is used as a synthetic rubber too. Silica is also added to high performance car tyre rubber to reduce the heat build-up properties of the tyre. Carbon black, which acts as a binder also forms a main component in the tyre manufacturing process, because it adds abrasion resistance and reinforcement characteristics to the tyre rubber. Reinforcement of the tyre shape and rigidity is through the textile (fabric) properties (74,75).

The remaining constituents are present at much lower levels, these include different plasticisers, peptisers (rubber additive), and anti-oxidants that can be used in the tyre preparation. The variation in the formula used to manufacture the tyre rubber can vary between tyre producers, but can also differ between the same manufacturers. These differences can be used to discriminate between tyres from different manufacturers and tread rubber from the same maker. It is also likely that tyres from different manufacturers have very comparable formulations (76) (73).

1.23 The different layers of car tyres

1.24 Tread

The tread is the thickest part of the tyre which comes into contact with the road surface. It covers the first outer layer to provide the traction and protect the inner sides of the tyre. This layer needs to be more stable than the first layer to give it the needed protection. Grooves are a necessity to allow water to escape to the edges in an effort to avoid hydroplaning as seen in Fig. 1.11. The way the tread shape is

designed is based on different criteria; either to minimize tyre road surface friction noise levels and to meet specific product market positions, or it is based on the environment of the road that the car will be driven on. Tread void and softer rubber are for better traction but may wear quickly, while wintery weather needs a higher void ratio to channel away the mud and water thus providing a better gripping performance (77).

Figure 1.0.11: Tyre components (74)

The vast majority (80%) of the vehicles which were reported for causing the collision in hit and run accidents were cars and out of these 55% were vehicles driving straight ahead before being engaged in the accident (72). This means that there is an increased chance of leaving a skid as the driver could see the obstacle in his/her path before collision and may have tried to brake hard.

Hit and run accidents are not the only location for the presence of tyre marks (78). Under sudden braking circumstances, the tyre may not rotate correctly, as it scrubs against the surface of the road. The quantity of skid tyre rubber that is left on the road surface is usually determined by many factors; the speed of the vehicle, the characteristic and condition of the tyre, the condition of the road surface (tarmac, cement, etc), the circumstances around the car tyre and the temperature of the tyres. The car rubber skid mark residue will take the shape of a black striation presenting the width of each tyre of the vehicle and the direction that it was travelling towards and also the pace of the vehicle. These traces, either at a car collision or a crime scene determine its source and can importantly assist the forensic scientist to connect the vehicle to the scene. Physical features, the figurative measurement of the traces and the distances between the striations left by the voids and grooves of the tread can also be used to support the determination of the trace e.g. the distances between the tyres traces can be used to help determine its origin by linking the actions to the track or the wheelbase of the alleged vehicle (79). A system was developed to avoid the bias of subjective measurements of the grooves, divisions and measurement of the characteristics of the tyre skid mark, taking into account the difficulties linked to the roadway surface (80). In addition, it can be difficult or impossible to distinguish by measurement, the pattern of the tread of the selected tyres when not all the traces of the car tyres are visible or the quality of the skid marks are completely lost during the abrasion process. Moreover, the striation shape will be deformed if the tyre friction is perpendicular to the standard travel direction, resulting in unusable physical features (78)(81).

In Greater London, in 2004, there were a total of 4,898 victims of which 19 were fatality cases, 499 seriously injured and 4,380 slightly injured Fig. 1.12. These incidents represented 14% of all road traffic casualties in Greater London in 2004 (72). Due to these statistics, it's very important not only to visually identify the previous skid mark characteristics, but also to identify the car responsible for the collision. In situations where the tyre striation cannot be distinguished and matched with the specific tyre, the patterned rubber that has been scrubbed from the tyre to the road may provide evidence as to the real identity of the vehicle. The rubber skid marks that are left on the road surfaces are not all rubber polymers and are also made up of the raw components used to manufacture the tyre tread (73)(81).

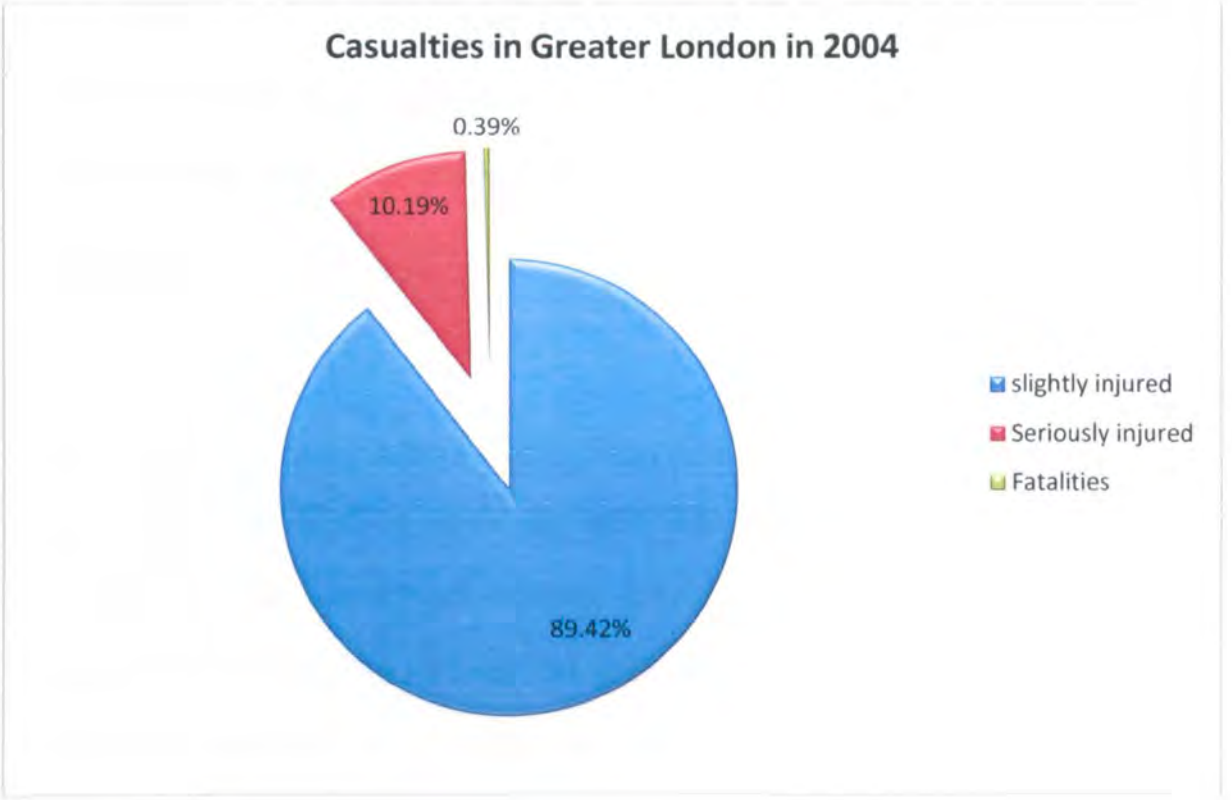


Figure 1.0.12: Year 2004 hit and run victims in Greater London (72).

The raw constituents of the car tyre debris on the road surface can be used for physical testing and can play a role in confirmatory chemical analysis. The physical properties of the tyre have no effects on the chemical approach which can be processed independently. There are many types of chemical testing which can be used in the analysis of the trace samples. These samples are collected from the suspected tyre tread and also from trace skid mark on the road surface for later possible matching and/or discrimination. The stored samples are analysed chemically based on their chemical formulation (78-83).

The comparison between the chemical components (concentration, ratio, presence etc) of the skid mark and the suspected car tyres can help to link the automobile to the crime scene. It has been proposed here that chemical analysis may not be an alternative method to visual physical testing at the crime scene, but could be used in a complementary fashion to physical examination to help confirm the results proposed.

Several papers have investigated forensic chemical analysis of vehicle tyres using different techniques; mainly pyrolysis GC-MS (73,82,83). This study developed a validated ATR-FTIR and ICP-MS methods to be used in the identification and matching of tyre analysis. For the latter, the results of the analysis were based on the calculated percentage and contents of the chemical compounds and elements of the rubber and the trace mark. Line Gueissaz, stated in his study "Tire traces– Discrimination and classification of pyrolysis-GC/MS profiles", that the previous methods were optimised without bearing in mind the repeatability of the results.

Also, assessment of the intra and inter-variation regarding the variables used as discriminant features was absent from some studies. Inconsistencies were detected in further studies between the tyres and the trace chromatograms (83). These variations were then described "by the fact that the braking process burns the tyre". In this study, principle component analysis (PCA) technique was used to compare the results, leading to grouping of tyres and trace samples. The pyrolysis process is not new; it has been applied in the past too, however, this research has revealed the advantages and weaknesses of Py GC-MS not only to distinguish but also match between tyre samples.

The present study of the chemical analysis of trace components in tyres offers a reasonable and robust framework to help to statistically relate a tyre trace to its source on the basis of chemical profiles obtained by many chemical analysis techniques. Most of the previous studies (including this study) focused on the tread analysis and its homogeneity across the whole tread area and the differentiation between tyres of similar and different manufacturers. Intravariability (variation within the tyre components) and intervariability (variation between the analysed tyres) are the statistical data that needs to be assessed and compared to reach the highest differentiation and/or matching levels (73,76,80,80,81).

The Department for Transport *Highways Economics* report found that the average cost of all road user casualties and the cost to the public of the losses resulting from

hit and run accidents in Greater London in 2004 was estimated to be around £0.5 million per day. This study demonstrates the potential, of the combined analysis using ATR-FTIR, Py GC-MS and ICP-MS with physical properties measurements for forensic purposes and evaluation of tyre rubbers and associated skid marks.

Chapter 2

Materials and Methods

1.0 Introduction

The following chapter describes in detail the steps that were followed in each method and the materials used for all the studies. The consumables and chemicals substances were purchased from VWR (Leicestershire, England), Fisher Scientific (Loughborough, England), Sigma Aldrich (Poole, England), LGC Standards (Teddington, England), Agilent technologies (Stockport, England), and Millipore (Watford, England).

1.1 Instruments

Table 0.1: instruments used.

Instrument	Source	Model
GC-MS	Agilent	5890 series II gas chromatograph (GC) 5971 Mass spectrometer (MSD)
GC-MS	Agilent	7890A (GC), 5975C XL (MSD)
Pyrolyser	CDS Analytical	Pyroprobe Series 5200
ICP-MS	AGILENT Technology	Agilent 7700
Centrifuge machine	Thermo Scientific	Labofuge 400 R
ATR-FTIR	Thermo Scientific	Nicolet ID5
Microplate reader	Varian	Cary 50
pH meter	Mettler Toledo	Seven Easy S20
Ball mill	Fritsch	Mini-mill Pulverisette 23
Sample concentrator	Techne	Dri-block DB-3D
Vortex	Fisher Brand	ZX Wizard

1.2 Consumables used

Table 0.2: Consumables used.

Type	Details	Source
BP-X5	30 m × 0.25 mm × 0.25 µm l,w,ID	SGE Forte Capillary column
Liner		SGE
Quartz tubes	Flame with quartz wool	SGE
GC Liner	Split / Splitless with double taper	SGE
Salanised glass inserts	200 µL	Capital Analytical
Salanised Amder glassware	4 mL with PTFE lines screw caps	Sigma Aldrich
Glass Bottle	300 mL	Wheaton
Glass centrifuge tubes	10 mL with PTFE lined screw caps	Fisher Scientific
Plastic centrifuge tubes	15 mL Polyethylene teraphthalate	Fisher Scientific
Plastic centrifuge tubes	50 mL Polyethylene teraphthalate	Fisher Scientific
Plastic centrifuge tubes	50 mL PP	VWR
Glass vials	10 mL	Fisher Scientific

The following chemicals were used in the experiments. All standard mixtures were prepared using high grade and pure solvents. The purity of the standards and their manufacturers are listed in Table 0.3.

Table 0.3: Solvents used.

Solvent	Grade	Source
Nitric acid	Trace metal	VWR
Nitric acid	69% lab grade	Fisher Scientific
Acetone	Lab grade	Fisher Scientific
Water	HPLC	Sigma Aldrich
Acetonitrile	HPLC	Sigma Aldrich
Pentane	Chromasolv for HPLC	Sigma Aldrich
Methanol	Chromasolv for HPLC	Sigma Aldrich
Chloroform	Chromasolv for HPLC	Sigma Aldrich
Ethyl acetate	Chromasolv for HPLC	Sigma Aldrich
Hexane	Chromasolv for GC	Sigma Aldrich
Dichloromethane	Chromasolv for HPLC	Sigma Aldrich

Table 0.4: General chemicals.

Chemicals	Details	Grade	Supplier
Proteinase K	Enzyme from Tritirachium album	Lyophilized powder, BioUltra, ≥30 units/mg protein	Sigma Aldrich
Tris Buffer	Tris HCl buffer	Laboratory	Sigma Aldrich
Dithiothreitol (DTT)	Cleland's reagent	Laboratory	VWR
Sodium hydroxide	1401534/ 1 kg	Analytical	Sigma Aldrich
Hydrochloric acid	1397813/ 500 mL	Analytical	Sigma Aldrich
Sodium hydrogen phosphate heptahydrate	1445623/ 250 mg	Analytical	Sigma Aldrich
Sodium phosphate monobasic dihydrate	BCBB7743/ 1 kg	Analytical	Sigma Aldrich
Melanin	109K1414V/ 100 mg	Bioreagant	Sigma Aldrich

Table 0.5: Standard drugs

Standard	Details	Source
Delta-9-tetrahydrocannabinol (Δ^9-THC)	1 mg/ mL (1mL)	LGC Standards
Cocaine	1 mg/ mL (1mL)	LGC Standards
Methamphetamine (MA)	100 µg/ mL (1mL)	LGC Standards
Amphetamine (AM)	100 µg/ mL (1mL)	LGC Standards
3,4-methylenedioxy-N-methylamphetamine (MDMA)	1 mg/ mL (1mL)	LGC Standards
3,4-methylenedioxyamphetamin (MDA)	1 mg/ mL (1mL)	LGC Standards
Ketamine	1 mg/ mL (1mL)	LGC Standards
Be, B, Na, Mg, Al, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Y, Zr, Nb, Mo, Ru, Rh, Pd, Ag, Cd, In, Sn, Sb, Te, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Hf, Ta, W, Re, Os, Ir, Pt, Au, Hg, Tl, Pb, Bi, Th, U	1 mg/ mL (100mL) in nitric acid	VWR

1.3 Human hair samples

The aim of this study was to develop a specific, sensitive and reproducible method to detect and quantify the presence of delta-9-tetrahydrocannabinol (Δ^9-THC), cocaine, amphetamine (AM) and methamphetamine (MA) in human hair from European participants. The hair samples were initially screened using ELISA; all samples shown to be positive were then further quantified and confirmed using GC-MS/MS. This robust quantitative method has the ability to detect low concentrations of Δ^9-THC, cocaine, AM and MA. In this study, the objective was to establish and validate a GC-MS/MS method for the determination of AM and MA in human hair using an appropriate derivatisation technique.

For the ELISA screening process of this study, hair samples were obtained from 180 European participants (108 males, 72 females, 62% athletes) aged 18- 53 years, the process of collection and identification was conducted by a previous study carried at Kingston University, School of Pharmacy and Chemistry by Nawed Deshmukh, Iltaf Hussain, James Barker, Andrea Petroczi, Declan P. Naughton (41, 42). The positive results from this screening process were further analysed for Δ^9-THC detection using GC-MS/MS. Blank hair samples were obtained from healthy, non-abusing volunteers from the UK.

The participants answered a questionnaire (39) regarding their exposure to psychoactive drugs during their career. Samples were collected from the posterior vertex (41). This was used because variation during growth of hair is less likely within the posterior cortex. The hair number in this area is more constant and is less affected by differences in age and/or sex. A minimum of 50 mg of hair was collected of each hair sample. They were fresh, untreated or processed with any chemical

treatment. The samples were individually sealed in paper envelopes, labelled and stored in a dry place prior to analysis (39).

Initially in the first hair analysis study, a batch of 10 hair samples was selected from athletes from a large sample group. Bigger batches of hair were pre-screened for a wider range of drugs using competitive binding ELISA to be able to narrow the following GC-MS confirmatory chemical analysis. As a result of the GC-MS confirmation test, 10 hair samples were chosen based on the presence of any traces of psychoactive drugs after the ELISA screening. The positive ten hair samples that were known positives for either Δ^9-THC and/or cocaine, were decontaminated, enzymatically digested, extracted and then analysed by GC-MS (see method in section 2.6).

The second hair analysis study has been designed to develop an immunological screening test followed by a GC–MS confirmation method for the simultaneous analysis of Δ9-THC, THC-COOH, OH-THC, cocaine, Benzoylecgonine (BZ), amphetamine (AP), methamphetamine (MA), MDA, MDMA, Ketamine and Ephedrine in human hair. Samples were obtained from 18 male Turkish athletes. The samples, data and questionnaires were collected by Ihsan Sari and Andrea Petroczi at Kingston University. All the 18 samples were pre-screened by ELISA. The results are discussed within Chapter 4 as there was an unexpected high percentage of positive results for Δ^9-THC and cocaine.

The findings were then correlated with the participants' self-reported questionnaire, to verify them. The leftover samples were stored for further analytical studies. Experiments were carried according to the previous protocols established and

approved by the Kingston University Ethics Committee of the Faculty of Science, Engineering and Computing.

1.4 Methodology

The first two studies of this project mainly involved the development of optimised methods for the analysis of recreational drugs and other drugs in hair samples using GC–MS, without prior derivatisation, thus allowing the sample to be re-analysed in its original form. A decontamination and enzymatic digestion technique were also developed prior to the hair confirmatory step Figure 0.1.

Figure 0.1 Method summary for hair sample preparation.

1.5 Hair sample preparation

After each hair sample (50 mg) was removed from the paper envelope, it was then prepared for the analysis phase. The preparation stage started by decontaminating each hair strand through washing with dichloromethane (3 mL), then it was stirred using a rotatory vortex mixer for 2 minutes; this step was repeated 3 times. This cleanses the hair strands from contaminants such as colouring, sebum, shampoo, etc.,

which may interfere with the analysis and could cause false results. The advantage of DCM is that it is an aprotic solvent (a solvent that will dissolve many salts, but lack an acidic hydrogen) and will not cause the hair strands to swell (41). Any swelling that could occur will increase the possibility that drugs which are incorporated inside the hair shaft could become lost. After the decontamination step, the hair samples were neutralised with deionised water (DI) water (3 mL) and moved to glass vials where the digestion was carried out. The hair was left to dry at room temperature in a closed environment to avoid any contamination from the surrounding environment. After decontamination, the hair samples were pulverised to ca. 0.5 mm long segments by hand scissors or ball pulveriser. 50 mg of hair was weighed in separate 10 mL glass vials on a 5 decimal places calibrated balance. Two types of hair digestion method were used in this project, alkaline digestion and enzymatic digestion.

1.6 Hair digestion and extraction

Hair segments (50 mg) were accurately weighed and incubated with 1M sodium hydroxide (1 mL) at 95°C for no longer than 15 minutes (until a brown solution appeared) (Figure 0.2a). Incubation at temperatures greater than 95°C and longer than 15 minutes can potentially lead to drug degradation (14). Later, a more accurate enzymatic hair digestion method was used to lower the risk of drug degradation. The method involved weighing 20 mg of decontaminated hair samples which were then cut into even smaller segments (<1 mm). Subsequently, DTT (VWR, UK) (50 mg), Proteinase K (Pro K) (Sigma Aldrich, UK) (15 mg) and Tris buffer (pH 7.5) (Sigma

Aldrich) (1mL) were added to hair segments. The preparation was then incubated at 35-38°C for a duration of 1 hour 30 min. The rationale for these temperature ranges arises primarily due to the fact that temperatures of >38°C can destroy enzyme and temperatures <38°C may not be effective for digestion. The digested hair samples (homogenate) were then purified by liquid-liquid extraction using pentane (see Figure 0.2B). This was conducted by adding pentane (4 mL) to the homogenate, mixing the contents gently using a vortex mixer for 1 min and then centrifuging for 10 minutes at 3000 RPM (1750 x g) using Labofuge 400R centrifuge manufactured by Thermo Scientific, UK. Organic and aqueous layers were produced after separation; the organic layer of interest was then transferred into a new glass tube and the remaining aqueous layer was discarded (Figure 0.2). The organic layer was then dried through a stream of nitrogen gas at 50°C and finally reconstituted with 40 µl acetonitrile.

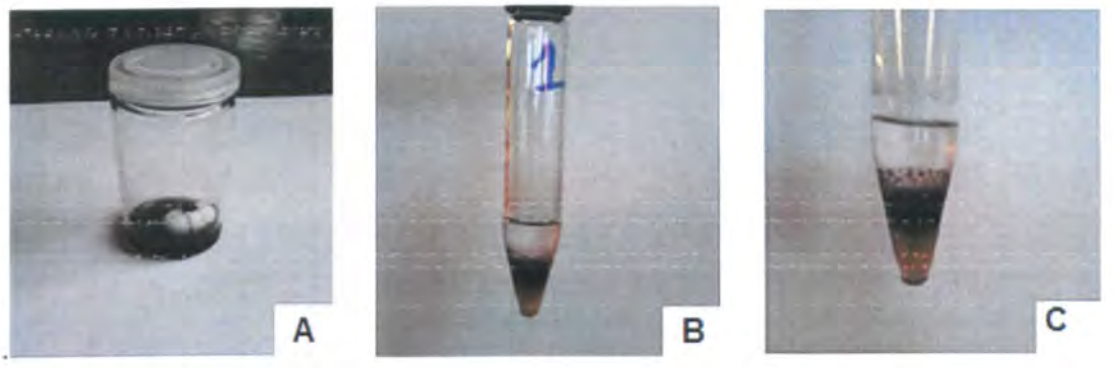


Figure 0.2: Photo images A) Hydrolysed hair samples post digestion using NaOH B) Extraction of drug using pentane. C) Post extraction displaying the separation with organic and aqueous layer.

1.7 Extraction method using (3:1:1) chloroform, ethanol and ethyl acetate

An alternative extraction method employed three reagents in ratio of 3:1:1 (chloroform: ethanol: ethyl acetate). The neutralized homogenate post digestion was transferred into a large centrifuge tube. Chloroform (150 mL), ethanol (50 mL) and ethyl acetate (50 mL) were transferred into the tube. The mixture was then vortex mixed for 1 minute and then centrifuged at 3000 RPM (1750 x g) for approximately 10 minutes. Once the mixture was centrifuged, the clear layer at the bottom of the tube (Figure 0.3) was removed using a syringe pipette and transferred into a fresh glass tube, the rest was discarded.

Figure 0.3: Photo image of the extraction procedure using (3:1:1) chloroform, ethanol and ethyl acetate.

1.8 Enzymatic digestion vs. digestion using NaOH

A comparative method to determine the effectiveness of the two digestion methods was carried out. The method involved preparing two sets of vials containing 50 mg of hair segments. Hair samples in Vial One were digested using 1M NaOH (1 mL). Hair samples in Vial Two were digested using the enzymatic digestion method using DTT (100 mg), Proteinase K enzyme from Tritirachium Album (lyophilized powder,

BioUltra, ≥30 units/mg protein) in a ratio of 1 mg hair: 1 mg enzyme; followed by (100 mg) Cleland's reagent (DTT) and Tris HCl buffer (1 mL) Figure 0.4. Both hair samples contained 25 µl of internal standard (THC-D3, cocaine-D3) and were spiked with 25 µl of drug (250 ng/mL). Two separate water baths were set; one at 95 °C for Hair Sample 1 and the other at 35-38 °C for Hair Sample 2. After the incubation, the digested samples were neutralized with phosphate buffer (1 mL) and HCl (1 mL). Both samples were centrifuged at 3000 RPM (1750 x g). The separated organic layer was then reconstituted with hexane (100 mL) for cocaine samples and ethyl acetate (100 mL) for Δ^9-THC and AM samples.

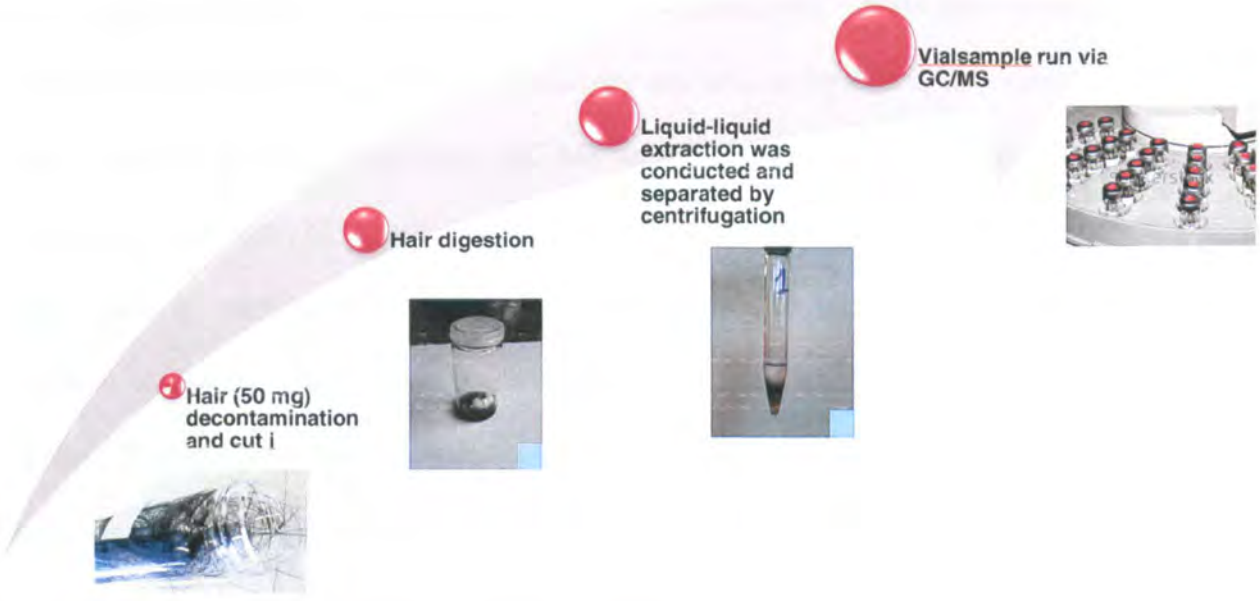


Figure 0.4: Figure 2.4: Enzymatic hair digestion, extraction and GC/MS analysis protocol.

1.9 Immunological and analytical techniques

Methods of hair analysis usually involve some preparatory techniques of decontamination, acid or base extraction and digestion. Traditional approaches involve screening the hair samples by performing immunoassays using commercially available kits (i.e. ELISA kits) (12,35). The efficacy of immunological screening techniques allow rapid analysis of drug negative hair prior to using expensive and time consuming analytical techniques. The ability of ELISA kits to wash away the background matrix before colour generation can make the method extremely sensitive. The kits were designed to provide estimated drug concentrations before confirmation. Decisions on the positive or negative status of assays were determined through recommended cut-off concentrations (84). Positive samples from ELISA screening were subsequently confirmed via conventional analytical techniques. The most popular technique for this purpose is gas chromatography – mass spectrometry (GC-MS) due to the high specificity, sensitivity and selectivity that is necessary to detect analytes in keratin matrices. GC-MS is the most widely used analytical technique for drugs of abuse, as it provides unambiguous identification of compounds. In most cases of analysis, electron impact or positive and negative chemical ionization mass detection is adopted; but due to the high sensitivity required, hair analysis may not necessarily be achievable using full scan mode. For simultaneous analysis of multiple drugs GC-MS EI-SIM (selected ion monitoring) can be employed (85-89). By meeting all the appropriate requirements, its use has increased for forensic and clinical toxicological analyses.

1.10 Enzyme linked Immunosorbent assay (ELISA) screening

For ELISA screening, an enzymatic digestion method was conducted. The homogenate was neutralized with 1 mL hydrochloric acid (HCL) and diluted with EIA buffer (1:1 v/v). ELISA screening works on the competition between the drug (Δ^9-THC, cocaine, AM and AM) and the Horseradish peroxidase (HRP) enzyme conjugate for the limited number of antibody sites present on a pre-coated microplate. Initially, hair samples were added to the wells followed by the HRP-enzyme conjugate and then the mixture incubated for 45 minutes. During incubation, competition between the drug (hair) and drug-enzyme conjugate for specific binding sites on a micro plate takes place. Unbound drug was thoroughly removed, by washing the plate (3 times) using a buffer supplied with the kit. 3,3',5,5'-Tetramethylbenzidine TMB substrate was added to detect bound drug-enzyme conjugate, allowing colour development after 30 minutes. The degree of colour development was directly proportional to amount of drug present in the hair sample (Figure 0.5) for qualitative analysis. The absorbance of each well was measured using a micro plate reader at 450 nm. The calibration curve was prepared by spiking blank hair samples with known concentrations of selected drug (Δ^9-THC, Cocaine, AM and MA). The calibration curve was used to determine the concentration of drug present in the hair samples. Hair samples that had shown positive results following the ELISA screening were further quantified using GC-MS/MS (90).

Figure 0.5: Schematic diagram displaying competitive ELISA principle [91]

Figure 0.5 represents the sample (red) which was added to each well followed by the enzyme conjugate (blue). During incubation, competition for the specific binding sites on the micro plate takes place. The solution was washed with buffer to remove the unbound drug. TMB substrate was added to detect bound drug-enzyme. Development takes place (yellow) after 30 minutes to determine the amount of drug present. Finally, absorbance readings of each well were taken at 450 nm.

1.11 Derivatisation method

Diluted hydrochloric acid ≈ 0.1 M (20 µl) (to prevent AP and MA from evaporating) was added to 5 mL of the extracted solution, and was evaporated at 500 °C under a nitrogen stream. Ethyl acetate (150 µL) was added with the derivatising reagent (TFAA) (150 µL) to the sample in a crimped vial, and then incubated to derivatise for 30 min at 70 °C; the reaction mixture was then evaporated and reconstituted with ethyl acetate (60 mL). 1 mL was injected into GC/MS system for the confirmation analysis of amphetamines and methamphetamines drug classes.

1.11.1 Derivatisation procedure: Identification of AM and MA

1.11.1.1 Method One

Samples were derivatised using two methods, firstly using BSTFA with 1% v/v Trimethylchlorosilicane (TMCS) and ethyl acetate. For comparison, the sample was then derivatised using MSTFA and catalysts (ammonia and merceptoethanol). Method One with BSTFA, 50 µL of sample was transferred into fresh glass vial and dried under nitrogen gas. The dried extract was then reconstituted with ethyl acetate (50 µL) and BSTFA (50 µL) with 1% v/v TMCS. The 100 µL solution was then heated at 80 °C for 30 minutes with a secured cap. The sample was cooled for GC-MS/MS analysis. In the second method with MSTFA, 50 µl of sample was dried under nitrogen gas and kept aside. Ammonium iodide (5 mg) was accurately weighed into a vial, MSTFA (1 mL) was added and the mixture was then heated at 80°C for 30 minutes (observation: yellow solution). After 30 minutes, merceptoethanol (5 µl) was added to the mixture (observation: transparent solution). Finally, the previously prepared dried extract was then reconstituted with MSTFA and heated at 80 °C for 30 minutes (92).

1.11.1.2 Method two

For the final method of derivatisation, ethyl acetate (100 mL) and derivatising agent (TFAA) (100 mL) was added to the glass vial of the dried sample. The mixture was then incubated for 30 minutes at 70°C. The sample was cooled, evaporated and then reconstituted with 60 µL ethyl acetate. Method Two was the final method used with

the addition of low concentration (10% volume of HCl) to prevent AM and MA evaporation.

1.11.2 Extraction

Two liquid-liquid extraction methods were used:

1- For hair that was obtained from the Turkish, Δ9-THC and Cocaine samples, extraction was achieved by the addition of a 6 ml pentane layer, followed by centrifugation for 10 min at 3,500 rpm. The supernatant was separated off to test tubes with the addition of 1% HCL (20 µl) to prevent evaporation of drug, then the solution was evaporated and the dried residue was reconstituted with hexane (60 µl) Figure 0.6. After this step, the sample was ready to be injected into the GC-MS without any derivatisation.

Figure 0.6: LLE of a hair sample using pentane

2- . A mixture of chloroform, ethanol and ethyl acetate (3:1:1 v/v) was used instead of pentane for the extraction of amphetamine and methamphetamine. But in this case, the supernatant had to be discarded and the lower layer had to be transferred into test tubes. 1% HCl in DI water (20 µl) was added to the

mixture to prevent the evaporation of the analytes and then was reconstituted with the derivatisation reagent for the derivatisation step Figure 0.7.

Figure 0.7: LLE of a hair sample using a mixture of solvents.

1.12 GC-MS analysis

1-3 µl of each sample were injected in the GC-MS (Figure 0.8) taking into consideration the volume of the injection liner and vapour volume of each solvent. Several steps were taken to improve the detection limits.

- Increasing the voltage of the multiplier made a big difference although it increased the noise level and the base line, but it was still more effective.
- Using pulsed splitless injection rather than split or just splitless injection allowed the injection system to put more pressure in the liner thus pushing more sample through the column, therefore more sample is being injected and detected by the MS (this step played a major role in the analysis).
- Increasing gas flow rate and pressure flow rate from 8 psi, which was mostly used in the other studies, to 24 psi in some cases to give a much

better peak shape and sensitivity, which gave the opportunity to have a higher signal to noise ratio and detection of lower concentrations.

Digestion, extraction and use of GC-MS in addition to the choice of suitable solvents and a temperature program helped in enhancing the sensitivity and achieving lower limits of detection.

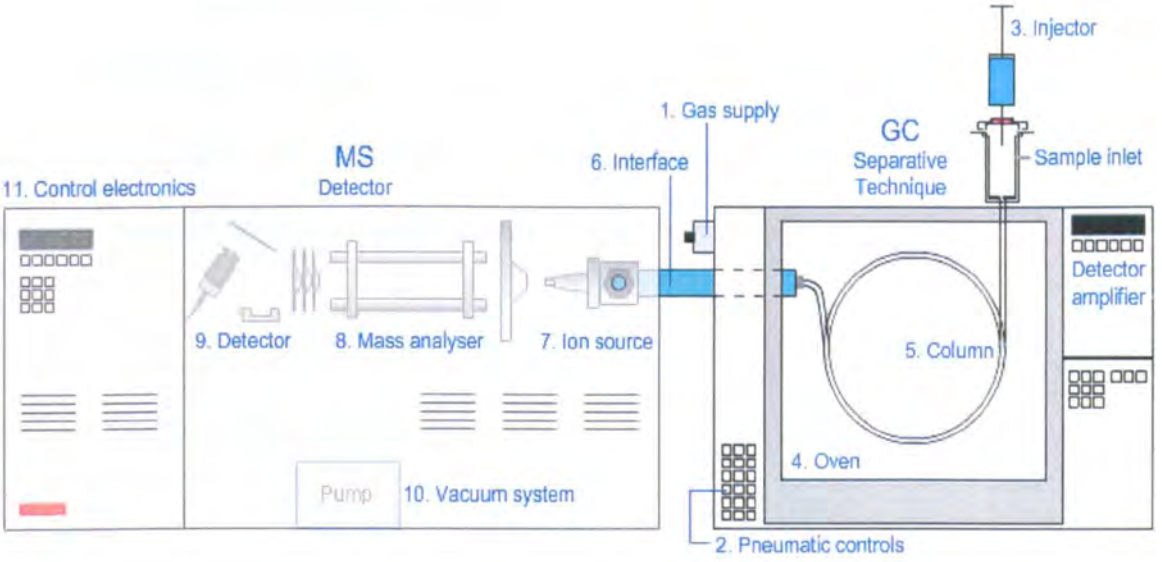


Figure 0.8: Components of GC-MS and how it is connected [68].

1.13 Validation

To be fit for the intended purpose, the method must meet certain validation characteristics. The performance of all the methods that were described in this project were validated according to the Food and Drug Administration (FDA) guidelines for different sets of parameters such as: lower limit of detection (LLOD), lower limit of quantification (LLOQ), linearity, accuracy, intravariability (intra-day precision), intervariability (inter-day precision), extraction recovery for each

substance and selectivity. For each method development and validation, a negative control sample representing the blank matrix was used. The calibration curves and quality control checks were prepared by spiking the blank matrix of each sample with a known concentration of the target analyte and internal standard (IS). Plotting of the calibration curves were obtained by calculation of the ratio of the IS against the known spiked concentration of the analyte in each standard. This ratio was attained by dividing the area under the peak of each of the analytes by the area of the peak of the IS. Some of the data were processed by MS Excel™ and others using the HP GC-MS bespoke analysis software. Linear regression was the method used to determine whether the numerical results quantifying hypothesized relationships between variables were acceptable as descriptions of the data.

1.13.1.1 LLOD, LLOQ and HLOQ

By definition, the limit of detection (LOD) is the lowest quantity of a substance that can be distinguished in a sample from the blank sample, but not necessarily quantified, with a signal to noise ratio of three. The lower limit of quantitation (LLOQ) and upper limit of detection (ULOQ) are the lowest and the highest points of analyte concentration of the calibration curve that that can be quantified with acceptable precision and accuracy (94).

1.13.1.2 Accuracy and precision

Although the two words precision and accuracy can be synonymous in colloquial use, they are deliberately contrasted in the context of the scientific method. The FDA regulations of accuracy validation stated that the results of the validated samples should be within the limit of 15% difference, and up to 20% when it comes to LLOQ. The level of accuracy and precision was determined based on the calculations of the analysis results of the quality controls which was formed by assessing the results from 18 successive samples divided into 3 groups of six standard points. For the determination of accuracy, the spiked samples were compared to known samples. The accuracy level was monitored by how close was the level of the known samples to the analysed spiked samples (95).

Precision, or as it can be called reproducibility or repeatability, by definition is the degree to which repetitive quantities under same conditions show unchanged results (95). Precision was divided into future interday variability (repeatability in 3 consecutive days) and intraday variability (repeatability in the same run day). The inter and intraday precision was evaluated by the calculation of the SD and the RSD of the repeated results in comparison with the mean of the repeated runs. The results according to the FDA guidelines should not be exceeding 15% difference when it comes to high limit of detection (HLOD) and medium limit of detection (MLOD), but it was accepted to be extended to 20% for the lower limit of detection (LLOQ) (96).

1.13.1.3 Selectivity

The International Union of Pure and Applied Chemistry (IUPAC) has expressed the view that "Specificity is the ultimate of Selectivity' and defined selectivity of a method by the degree to which the analytical of the determination of a particular analyte(s) in a composite mixture can be achieved without intervention from other components in the matrix. If the response level is distinguishable from the component of the matrix, this method can be identified as selective (97)(98).

The selectivity of this analytical method was established by providing data to prove the absence of interference peaks at the same retention time of the analyte and IS(s) with regard to degradation analytes, impurities and the matrix interference.

The selectivity of chromatographic and spectroscopic methods may be assessed by inspection of peak homogeneity. To avoid any interference, the analyte peak(s) was not overlapping to more than one component; thus leading to suppressing or enhancing of the intensity of the analyte and/or IS. The determination of the level of interference was evaluated by extracting the matrix and then spiking it with a known amount of the analyte and then comparing it with the analytical results of the same neat sample.

$$\textbf{\% interference} = (\textbf{Con}_n - \textbf{Con}_m) / \textbf{Con}_n \times \textbf{100} \qquad (99)$$

Equation 1: percent interference of the hair matrix on the analysis.

Where Con_n is the concentration response of the neat sample, and Con_m is the response of the matrix spiked sample.

1.13.1.4 Percentages extraction recovery

Percentage extraction recovery was determined by comparing a spiked matrix to a standard of the same concentration. The spiked negative control sample matrix with the analytes and IS are extracted with the validated extraction method and followed by comparing the peak area ratio of spiked matrix analyte to IS with the neat standards solutions of analyte and IS of the same known concentration (96).

1.14 Tyre study

1.15 Tyre rubber samples

Tyre rubber samples for this project were obtained from different global tyre manufacturers for analysis using three different analytical techniques (ICP-MS, ATR-FTIR, and Pyrolysis GC-MS). Different tyres sizes for different vehicles were used to replicate the widest range of cars on Britain's roads. All the collected samples were sampled from second hand tyres which had been fitted to a car and had been on British roads previously. Each tyre rubber sample consisted of approximately three fragments of the tyre tread Figure 0.9. These samples were collected from different areas of the tyre tread. The skid sample masses were approximately between 6-20 mg, the samples when collected were untreated with any type of solvent and decontaminated afterwards, based on the methodology of the technique used. Tyre rubber was cut into smaller samples to be weighed afterwards on an accurate five digits balance (≈ 100 mg each). The rubber fragment, in a similar

way to the skid mark residues sampling, was chosen from the inner end of the tread area, which is in direct friction with the road. To replicate the scenario of a real skid mark undertaken by a car on the road, the friction that occurs whilst a vehicle is braking was carried out by rotating a rig at a constant speed and an abrasive sheet was brought into contact with the tyre during the rotation period. This method was repeated for each tyre taking in consideration the swapping of the plastic sleeve. This process resulted in a tiny amount of residue which is usually produced by any tyre skidding. The tyre's rubber and the skid mark samples were then stored in plastic tubes screw capped with a lid in a dry place.

Figure 0.9: Tyres used in the study.

From the available 57 tyre samples, only 21 tyre samples of different sizes and manufacturers were chosen to be analysed for the determination of the chemical contents using different techniques as discussed on Chapter 5. The other samples were used in the experimentation and development of the methodology of each technique for the analysis of the 21 samples. Since the ICP-MS and Pyrolysis GC-MS techniques are destructive methods, ATR-FTIR was performed first.

1.16 Sample collection

For the tyre experiment, samples were collected from around 57 second-hand British car tyres. The tyres that were used were of different sizes in width and diameter and thickness. Second hand tyres were chosen to try to replicate everyday representative skid marks from a car suddenly braking. All the tyres details were recorded: name, model, date of manufacture, production location, size and part number.

All the sampled tyres were EU legal tyres; tyres of a good thread thickness (> 3 mm) were sampled to avoid the interference of the metal layer under the thread level and the other tyre components. Several samples were collected from each tread for validation purposes. A minimum of 3 grams of each tread was cut using a decontaminated razor. The blade was changed between the collection of each sample to avoid cross contamination. All the collected samples were untreated with any type of chemicals. Samples were then decontaminated with 5 mL DCM and 200 mL DI water to get rid of the road surface contaminant (such as oils, tarmac, dust etc...) and then dried under a gentle stream of nitrogen.

The size and the shape of the samples analysed by pyrolysis may have an impact on the repeatability of the results (100-103).The size effect factors cannot be controlled for the tyre traces and were expected to present variation in shape and thickness. This required an accurate weighing of the samples prior to the analysis. The overall size of the trace particles was expected to be around (4 mm (L) x 2 mm (W) x 1 mm (D)) for the ATR-FTIR analysis. To ensure an optimal comparison in pyrolysis analysis, the tyre samples were cut into fragments of approximately the same size, but of different shape and thickness weighing between 100-200 µg. For ICP-MS analysis, all the rubber samples were weighed on a 5 digits balance to the same accurate mass (100 mg).

1.16.1 Reproduction of tyre skid marks

The tyres (as described previously) were mounted on a rotating rig system. Skid marks were collected by reproducing a replica of a car braking under friction between the tyre and the road surface; these were performed on dry abrasive sheets.

The tyre trace samples were collected during the rotation of the rig at the same speed for all the tyre samples. The collection of the fragments was performed by applying a plate of plastic just under the rotating wheel. Plastic sheets were used throughout the study to reduce background contamination. For each test, the collection plastic plate was changed to avoid cross contamination between different tyres Figure 0.10.

To ensure that the abrasive sheet had no effect on the chemical analysis, a triplicate control sample was analysed with the same conditions as the tyre samples without showing any interference when compared to the pure control analysis results.

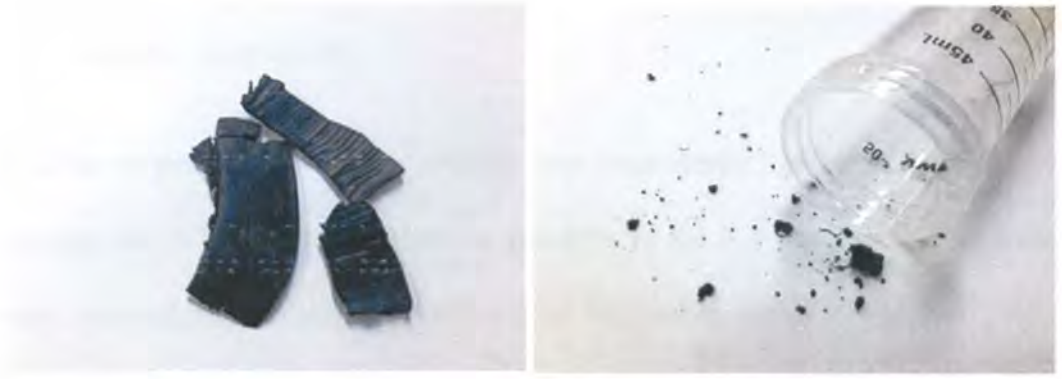


Figure 0.10: Rubber and skid marks samples.

1.16.2 Storage

All the collected samples were stored in plastic tubes screw capped with a lid in a dry place to avoid contamination and oxidation with the surrounding lab chemicals. Approximately 3-4 grams of each raw tyre rubber sample were collected before simulating friction, whereas due to the small amount of skid marks removable from the surface, only 10 mg for each skid mark produced were collected Figure 0.11.

Figure 0.11: Stored rubber samples.

1.17 Rubber digestion

In order to prepare the rubber samples and skid marks for the ICP-MS analysis, samples had to be digested before the injection in the ICP system. Rubber samples were decontaminated, and then 100 mg of the rubber samples were weighed and stored in glass vials (vials were pre-treated treated overnight in 50% nitric acid (HNO_3) in DI water to avoid cross contamination). Samples were then digested and metal trace nitric acid (5 mL) was added to the vessel (CEM Buckingham, UK). Vessels were then secured with the relief valve and fitted with a lid. The lid needed to be completely tight to stop the loss of any of the sample by evaporation. Advanced sensor technology for vessel recognition, a temperature control and MARS microwave was used in the digestion process (Buckingham, UK). The digestion method was optimised to get the best degradation results (104). The heating cycle, which lasted in total for 27 minutes, was altered to fit the samples' physical and chemical characteristics. Heating started at room temperature and then

increased to 400 W for 10 minutes at the rate of 100% and held there for 2 min and then increased to 800 W for another 10 minutes at the rate of 80% and then stopped to cool down for 5 min. The starting rate was lower than the second rate to try to avoid pressure and temperature building up, which can affect the continuation of the second heating program step. Samples were then allowed to cool for an extra 5 min at room temperature, resulting in a very light green colour Figure 0.12. Opening the microwave vessels was performed in a fume ventilation system to avoid toxic nitrogen oxide fumes that were usually produced during digestion. After digestion, the solution was made up to 50.00 mL with de-ionized water (tubes were soaked overnight with 20% nitric acid in DI water). Samples and skid marks were centrifuged at 3,500 rpm for 10 min to avoid blocking the ICP system tubes, then the top layer of liquid was moved after the precipitate has settled to a new sampling tube by decantation, to be analysed on ICP-MS Figure 0.13. The total time required for the solution ICP-MS analysis, including sample preparation, microwave digestion and ICP-MS analysis for 1 sample in 5 replicates was ~2.5 h.

Figure 0.12: MARS X microwave used in the rubber digestion and the digested sample

Figure 0.13: Digested tyre rubber sample.

1.18 Instrumentation

Three different instruments were used for the tyre rubber analysis. Pyrolysis GC-MS, ATR-FTIR and ICP-MS. Figure 0.14 represent the photographs of pyrolyser used in the project.

1.19 Pyrolysis gas chromatography mass spectrometry (GC-MS)

Pyrolysis analysis was achieved with a resistively-heated filament (platinum coil). The pyrolyser interface temperature was at a constant 60°C Figure 0.14. The quartz tubes that were used in this study were baked at 1250°C to avoid cross contamination. The pyrolysis was carried out using a Pyroprobe Series 5200 embedded with software (5000 DCI) version 1.62 from CDS Analytical, Inc. An AGILENT Technology 7560 gas chromatograph, in combination with an AGILENT 5860 XL EI/CI MSD Triple Axis Detector mass spectrometer operating in electron

impact ionisation (EI) mode using helium carrier gas with a flow rate of 1.4 mL/min was used for detection. The analytical column for the GC was a BP-X5 SGE Forte capillary column (Victoria, Australia) (30 m × 0.25 mm × 0.25 µm l,w,ID) (5% phenyl polysilphenylene-siloxane). The injector was at a stable temperature of 280°C. Split injection was performed for a purge time of 0.5 min with a split ratio of 53.6, a split flow rate of 75 mL/min and an initial pulse pressure of 20.4 PSI.

The GC oven temperature for the analysis of the rubber samples was programmed to start at 45°C, held for 6.5 min, then increased to 150°C at 5°C/min, held for 5 min, to a final step of 335°C at 70°C/min, held for 3 min to clean the column. Full scan mode was used in the MS method to detect all separated compounds. Analysis was performed using an Enhanced Data Analysis MSD enhanced Chemstation version E.02.00.493 from Agilent Technologies and then identified with the NIST 2008 mass spectra library.

Figure 0.14: Pyrolyser with Pyroprobe Series 5200, CDS analytical Inc.

1.20 Inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS system manufactured by AGILENT Technology (Agilent 7700) in combination with Agilent's Octopole Reaction System (ORS3) cell was used. Its high-temperature plasma was connected to a ASX-500 random access X,Y type autosampler (Agilent Ltd., California, USA) operating in a plasma of argon gas in both collision mode and reaction mode. The injection needle between samples was rinsed with DI water and 2% nitric acid in DI water. The standard analytes were measured in increasing calibration concentrations; multiple replicates (n=5) of digested spiked samples Figure 0.15.

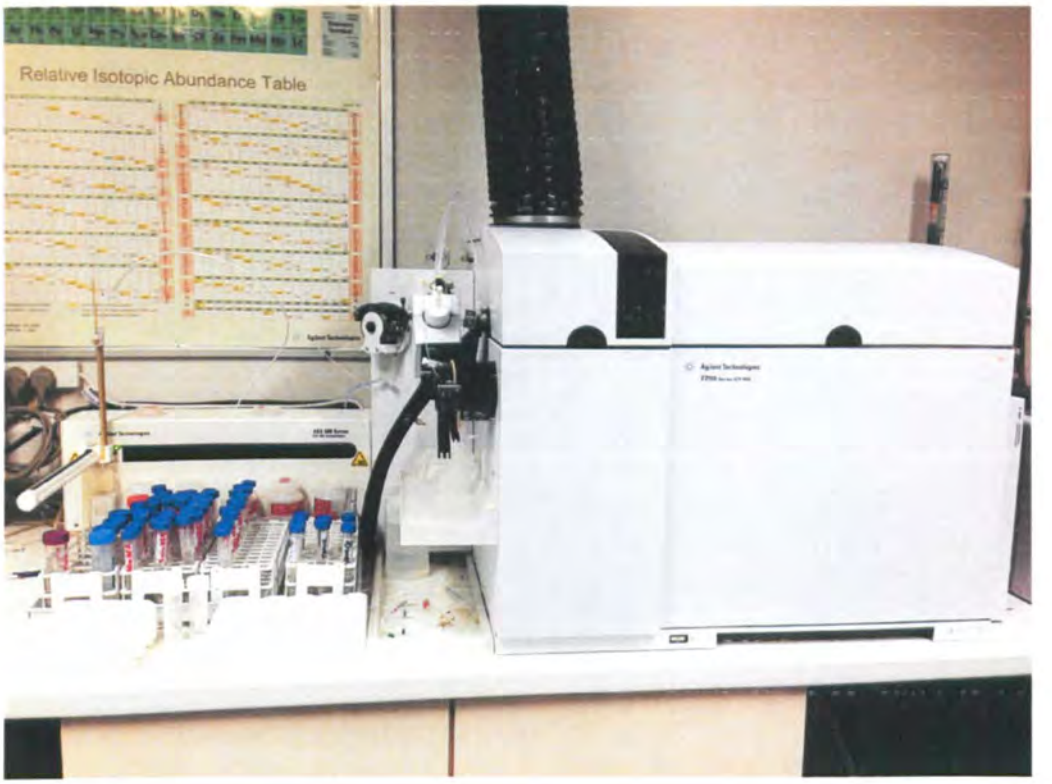


Figure 0.15: AGILENT Technology (Agilent 7700) inductively coupled plasma mass spectrometry (ICP-MS)

1.21 Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Thermo Scientific Nicolet ID5 ATR-FTIR spectrometer was equipped with deuterated triglycine sulfate (DTGS) KBr detector, and diamond single-reflection crystal. The number of scans was 8 and the resolution was 8 in % transmittance mode, scan ranged between 600-4000 cm^{-1}, and the optical velocity was of 0.4747 cm/sec.

Tyre samples were analysed under a reflective beam of infrared, which was then absorbed by the detector and the processed by OMNIC analysis software. The background was subtracted from each analysis to decrease the interference levels.

Chapter 3

Quantification of Underivatised Δ9-THC and Cocaine in Human hair

3.0 Introduction

The United Nation's Office on Drugs and Crimes (UNODC) and the World Health Organisation (WHO), in 2009, estimated that 149-272 million people used psychoactive substances at least once in the past 12 months (32)(105). The most commonly used substance was cannabis (between 125 and 203 million people) Figure 3.1, followed by amphetamine type stimulants, opioids and cocaine (32). Cannabis and cocaine analyses by GC-MS are two of the most frequently used drug assays (106,107). Research in analytical sciences has shown a sustained effort to develop methods with improved sensitivity and to facilitate fast, reliable and cost effective methods to identify users. The specific reasons behind the need for detection range from current risk to self and others, to future non-compliance (108). These individually lead to different detection windows. For example, authorities often require evidence of abstinence from drugs before re-granting driving licence (109,110), allowing child custody (111), returning to workplace (112,113) or licensing to practice (114). In these cases, the detection window stretches beyond the most recent consumption. The drug detection window is one of the main analytical challenges, since most drugs can only stay in the body system for shorts periods *e.g.* the plasma elimination half-life of tetrahydrocannabinol (THC) and THCCOOH is ca. 4.1 days and 5.2 ± 0.8 days respectively for frequent users (115). Other considerations include the accuracy, reproducibility, sample quantity required and limits of detection/quantification for a developed method.

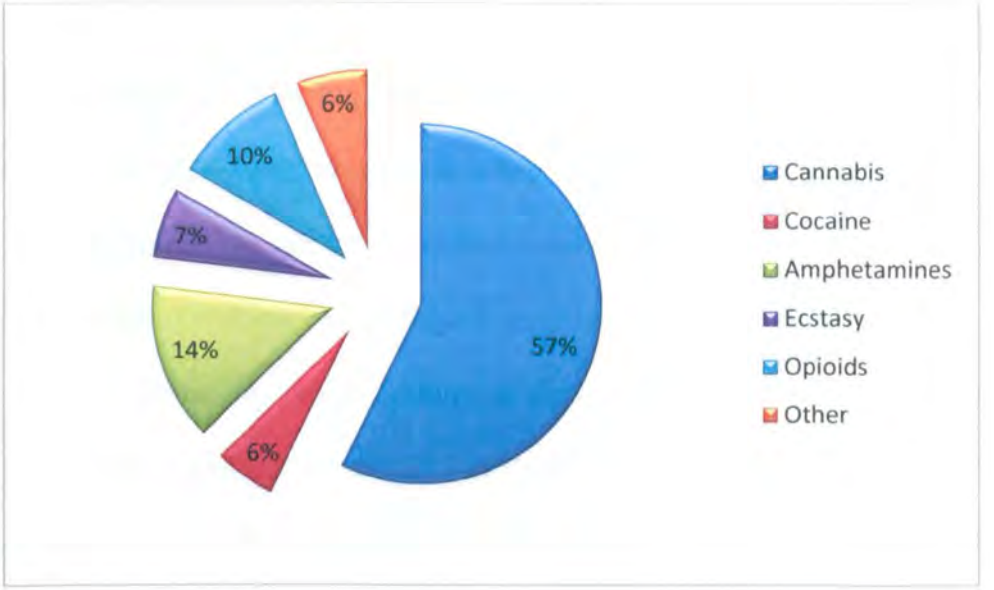


Figure 3.1: Annual prevalence of drug use at the global level. The picture varies between regions [32,105].

Drug analysis in hair has become a very common part of forensic and clinical toxicology (doping, work-place drug testing, rehabilitation programs and treatment centres) (12,106,116). Testing hair for drugs of abuse offers the possibility of a longer detection window than is commonly obtained from urine or blood analysis (41,117) and thus distinguishes between long term use and short term single exposure (118). Hair testing improves drug analysis by being non-invasive; samples are easy to store at room temperature and there is a negligible risk of infection. Hair grows at *ca.* 1-2 cm each month (106). Drugs can incorporate into hair through several mechanisms, either endogenously, by ingestion (via the blood during hair formation or through sweat and sebum), or exogenously, through external contamination (deposition of drugs on the surface of the hair and passive inhalation) Figure 3.2 (57). Due to the extremely low incorporation rate of the psychoactive ingredient of cannabis, (Δ^9-THC), cocaine and other drugs in hair [especially in blonde, brown and thin hair (119)], the development of sensitive techniques is essential for quantitative analysis (116).

The standard method of detecting abused psychoactive drugs is through urinalysis and blood analysis (120-122), however these methods are vulnerable to confounding results. For example, once the drugs are in the body, they are rapidly metabolised and have a detection window of 1-2 days in the blood, and 1.5 - 6 days (7 days in chronic users) in urine. In oral fluid, drugs of abuse can be detected for 5-48 hours at low nanogram per millilitre level (123). A study of Δ^9-THC demonstrated that the detection of Δ^9-THC in blood (serum) is not possible 6 hours after consumption (124). Psychoactive drugs are characterized as enhancers that allegedly improve mental functions such as cognition, memory, intelligence, motivation, attention, and concentration (125). Urinalysis, blood and oral fluid analysis are often unable in determining long term histories of drug use; they generally fail to identify long term usage of these types of drugs or the identification of any traces of pre-use. Genetic and metabolic variations are known to influence urinalysis based drug testing (126,127).

The concentration of the drug in hair may reflect the amount of drug used; *i.e.* if the rate of consumption is high or low. In addition, when a sample shows the absence of drug, it does not always mean that the sample is free from the drug, only that the concentration may be too low to be detected. The interpretation of hair analysis involves finding the correct concentrations to distinguish between the common or occasional consumer. Most methods include decontamination, digestion, drug extraction, reconstitution and derivatisation for sample preparation preceding hair analysis by GC-MS (117).

In this work, for the first time, lower limits of detection for Δ^9-THC and cocaine, have been achieved *without* the need for derivatisation. The limit of detection (LOD) is substantial in an analytical method where the detection limits are the backbone of the study (analytically significant). For forensic studies the LOD can be less significant whereby the presence or absence of the drug can give an enough evidence to determine the conclusion (forensically significant). In this analytical study, it is an improvement (LLOD 0.015ng/mg) on previously reported methods that achieved a lower limit of detection (LLOD) of 2.5 ng/mg for Δ^9-THC but *with* derivatisation (128). Further method developments in hair digestion and extraction have also been made, thus permitting direct measurement of the drug without issues such as contamination from reagents, formation of by-products, and reduction in recovery, chromatographic resolution and ionisation efficiency that may arise from derivatisation.

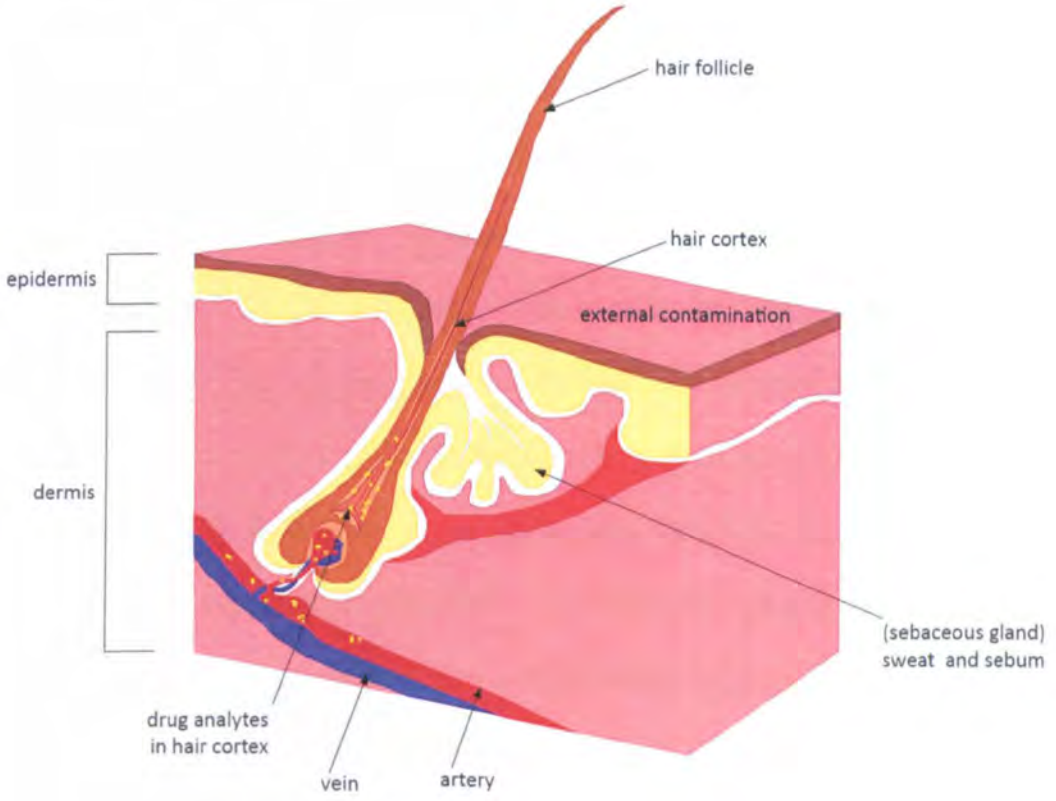


Figure 3.2: Drug incorporation into the human hair shaft

3.1 Materials and methods

3.2 Chemicals and reagents

Delta-9-tetrahydrocannabinol (Δ^9-THC) and cocaine (1 mg/mL) and their deuterated analogues Δ^9-THC-D3 and cocaine-D3 (100 µg/mL) were obtained from LGC standards (Teddington, UK). Proteinase K enzyme and HPLC grade pentane were obtained from Sigma Aldrich (Dorset, UK), DTT Cleland's reagent and TRIS HCl buffer were purchased from VWR, (Leicestershire, UK). Hexane, dichloromethane and all the other organic solvents were HPLC grade from Fisher Ltd. (Leicestershire, UK).

3.3 Preparation and standard solutions

Two stock solutions for cocaine and cocaine-D3; Δ^9-THC and Δ^9-THC-D3 were prepared in methanol at a concentration of 1 mg/mL Figure 3.3. These were mixed and diluted to prepare method development working solutions between 25-1000 ng/mL for each analyte. These underivatised prepared solutions were used for the development of the extracting method and gas chromatography method. Standards used to prepared standard curves were also prepared from stock solutions diluted in hexane. A similar method was used to prepare the quality control samples, but these were prepared from freshly made stock solutions. Real hair sample were used to optimise the developed method, and replicate real hair sample testing. All stock solutions were stored in crimped sealed glass vials under darkness in closed boxes at -20 °C and diluted solutions were stored in amber, silanised, glass vials under no more than 4 °C.

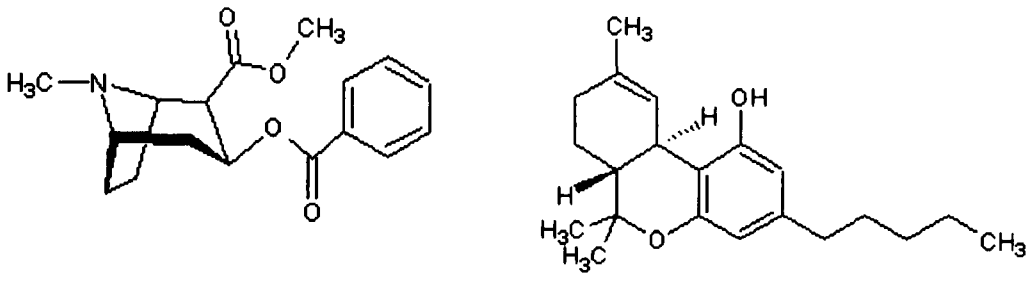


Figure 3.3: Structural formulae of cocaine and Δ9-THC

3.4 Hair Samples

All the hair samples were collected in envelopes, and were washed, labelled, and stored in 10 mL glass vials sealed with plastic caps. Hair samples analysed in this study were collected from the posterior cortex, by cutting with scissors, since the hair number in this area is more consistent and less affected by age and sex differences (129).

3.5 Samples preparation

3.5.1 Decontamination

Before laboratory analysis, the hair samples were washed to remove sebum, lipids, oils, cosmetics, and any adhering substances which may interfere with analysis. Each hair sample was decontaminated by washing with 3 mL dichloromethane. This step was repeated 3 times for 2 minutes with continuous stirring by micro-magnetic stirring bars on a stirring dock. After the last wash, the hair was dried completely under a very gentle stream of nitrogen.

3.5.2 Digestion

3.5.2.1 Pre-digestion

Washed hair samples were weighed accurately (50 mg) and cut to approximately 0.5 mm long segments using scissors.

3.5.2.2 Alkali digestion

Samples were spiked with the internal standard prepared deuterated (D) form of the analysed drug (prepared in 3.2.2.). 1M sodium hydroxide (NaOH) (1 mL) was added and incubated at 95°C for 10 minutes. Each vial was sealed during incubation to prevent any solvent evaporation (making some holes in the plastic cap prevented the cap from opening). The digested sample was then homogenised and neutralised (pH 7) with 2 M hydrochloric acid (HCl) (0.5 mL) and 0.2 M phosphate buffer (2 mL) to preserve it and stop it from degradation. Drug free hair was used as a negative control.

3.5.3 Enzymatic digestion

The cut hair was added to Proteinase K enzyme followed by DTT reagent (100 mg) and TRIS HCl buffer (1 mL). The digestion was continuously mixed using a magnetic stirring bar at low speed and incubated at 37.5°C for 50 min at pH 7.5. The hair was digested with the enzyme in the presence of Δ^9-THC-D3 and cocaine-D3; a spiked concentration of 1 ng/mg was used as internal standard. Drug free hair was used as a negative control.

3.5.4 Enzymatic digestion vs. alkaline digestion

In order to compare the efficiency of each of the two digestion methods the following experiment was undertaken.

a- Vials containing 1 M NaOH (1 mL) and 50 mg of blank hair containing 5 ng Δ^9-THC were incubated at 95^0C for 10 minutes. The alkaline solution was neutralised by adding 1 M HCl (1 mL) and of phosphate buffer (pH= 7, 0.2 M) (2 mL).

b- Vials containing 50 mg of blank hair sample was spiked with 5 ng Δ9-THC and incubated for 50 minutes in a solution containing Proteinase K enzyme (50 mg) and Cleland's reagent (DTT) (100 mg) and Tris HCl Buffer (1 mL).

Both samples were prepared on the same day, using similar procedure including laboratory equipment and consumables (pipette, hotplates, Vials Etc...) and solvents. To perform an accurate comparison experiment, a similar extraction procedure was followed for both tests.

3.5.5 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is one of the simplest techniques used in the separation of biological fluids such as urine, plasma, blood, and hair, based on the relative solubilities in two different immiscible solvents (130). The two liquid phases are mixed together in one container whereby the target analyte is preferentially soluble. After mixing, the layers are allowed to separate naturally by the

gravitational force or by centrifugation, and the extractant is either run out of the separating vessel via a valve or pipetted from the top layer. The effectiveness of LLE relies upon the difference in solubility of a compound in the chosen solvents.

In this method, the neutralised hair samples were subjected to liquid-liquid extraction using 2 different solvents mixtures. Analytes showed better extraction results using a mixture of chloroform: ethanol: ethyl acetate in 3:1:1 ratio compared with extraction using pentane (HPLC grade 6 mL) alone.

After vortex mixing and centrifugation (10 min at 2,383 x g), the supernatant organic layer was transferred into a fresh glass tube using a Pasteur pipette and the hair residue pellet was discarded. The organic layer was mixed with an aliquot of 2M HCl (10 µL) which had been diluted to 1% in phosphate buffer (pH= 7, 0.2 M) to prevent drug loss during evaporation.

The organic layer was evaporated under a gentle stream of nitrogen gas at 50 °C using a hotplate concentrator Techno DB-3 (Cambridge, UK).

Spiked samples within the range of 0.02-1.50 ng/mg were prepared for the Δ^9-THC and cocaine calibration plots. The extracted residue was reconstituted with hexane (60 µL), transferred to autosampler vials, and 3 µL was injected into the GC-MS system Figure 3.4.

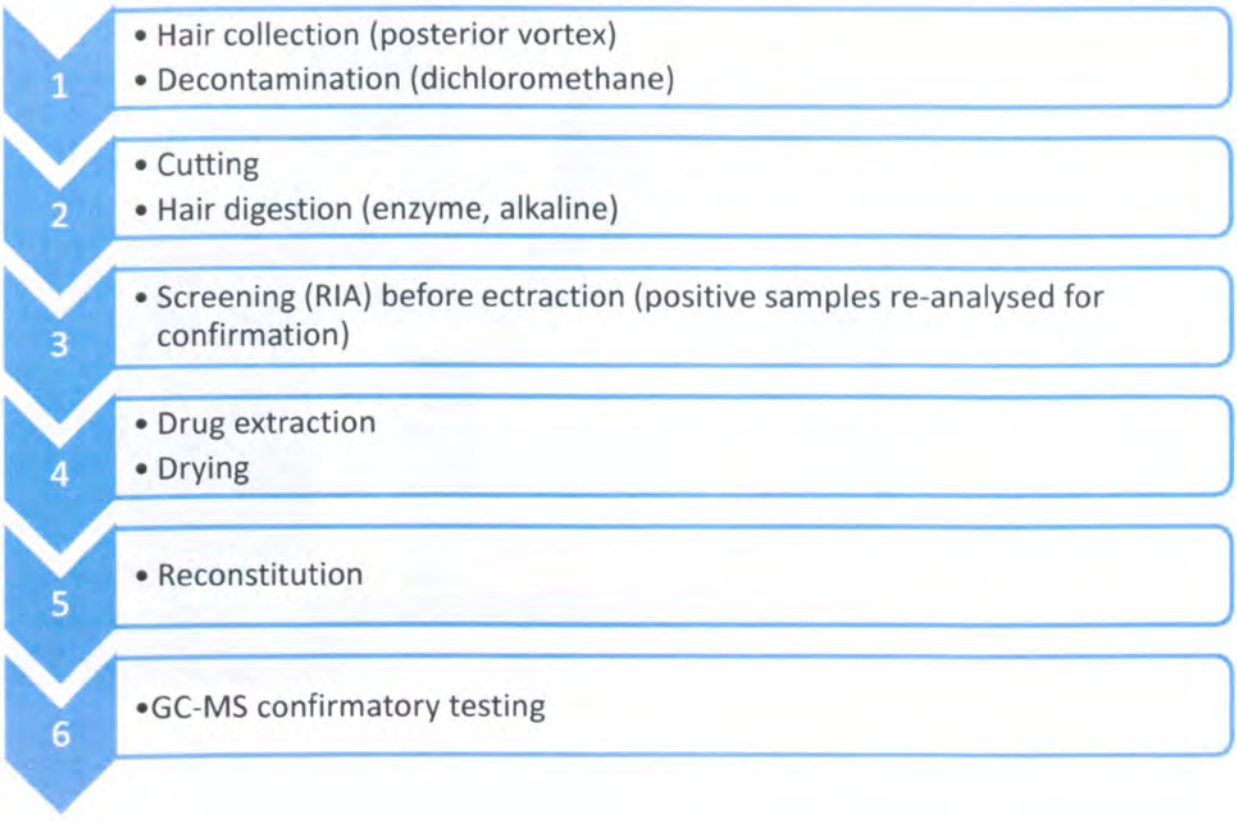


Figure 3.4: Hair analysis processing stages.

3.6 Gas chromatography-mass spectrometry analysis

The extracts were analysed using an AGILENT Technology 7890A gas chromatograph, in combination with an AGILENT 5975 XL EI/CI MSD Triple Axis Detector mass spectrometer connected to a 7683B autosampler (Agilent Ltd., California, USA) Figure 3.5.

Figure 3.5: GC-MS used in the analysis.

3.7 GC-MS conditions

The mass spectrometric parameters were optimised using the HP optimiser software (version 10.1.5). For method development, a 1 µL aliquot of 100 ng/mL solution of each of the analytes, plus its deuterated standard, were injected in the GC liner. The conditions which gave the highest sensitivity for each compound were saved in the method.

Pulsed, splitless injection was performed for a purge time of 1 min, a purge flow rate of 53 mL/min and an initial pulse pressure of 20 PSI reducing to 15 PSI during the run. This enhanced the peak shape and sensitivity by pushing most of the injected sample in the column. The construction of GC liners has great influence on the sample transfer during splitless GC process. Because of the long time the analytes spend in the liner during the splitless injection mode, a Double taper, deactivated

splitless inlet liner (Agilent, 5181-3315) was used to minimize the contact of the analytes with the bottom of the injection port. The gas chromatography separation was achieved on a BP-X5 SGE Forte Capillary column (Victoria, Australia) (30 m × 0.25 μm × 0.25 μm l,w,ID) (5% phenyl polysilphenylene-siloxane). The injection port temperature was set at 260°C. Better enhanced peak sensitivity was reported when the Helium carrier gas had a flow rate of 1.3 mL/min. The GC oven temperature for Δ^9-THC was programmed to start at 50°C, held for 1 min, then increased to 200°C at 40°C/min, held for 2 min, and increased to 280°C at 80°C/min, held for 3 min, to a final step of 310°C at 80°C/min, held for 4 min.

For cocaine analysis, the initial temperature was 50°C, held for 1 min, then increased to 200°C at 100°C/min, held for 2 min, then to 280°C at 80°C/min held for 3 min, to a final step of 310°C at 80°C/min, held for 4 min. (Table 3.1).

Table 3.1: GC oven temperature program for Δ9-THC and cocaine.

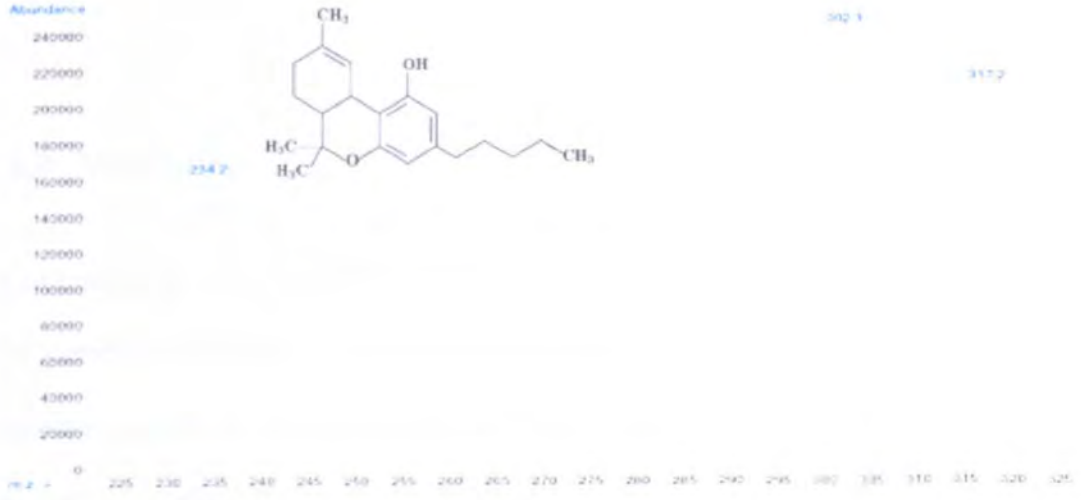
Δ^9-THC Analysis program	Temperature	Rate	Time
Colum temperature		50°C, held for 1 min	
Program 1	200°C	40°C/min	2 mins
Program 2	280ºC	80ºC/mins	3 mins
Final program	310°C	80°C/mins	4 mins.

Cocaine Analysis program	Temperature	Rate	Time
Colum temperature		50°C, held for 1 min	
Program 1	200°C	100°C/min	2 mins
Program 2	280ºC	80ºC/mins	3 mins
Final program	310°C	60°C/mins	4 mins.

The eluted peak of THC displayed in Figure 3.6a was confirmed by the MS fragmentation of unknown analyte provided in Figure 3.6b. The black, red and yellow peaks eluted at R_t (12.960 min) correlates to the ions 231.10, 299.10 and 314.20 m/z representing the THC drug. The dark green, light green and blue peaks eluted at R_t (12.929 min) correlates with the three ions 302.10, 317.20 and 234.20 m/z representing the internal standard THC-D3. The higher peak ions represent the internal standard and by subtracting 3 mass units from the m/z provided. This study was able to distinguish the ions representing the THC drug present in unknown hair

samples. So the SIM MS analysis was able to determine the molecular ion M+ peak at 314.20 (m/z corresponding to MW=314.45) of THC drug and fragmentation shown below.

a)



b)

Figure 3.6: a) GC chromatogram of ion extraction b) GC SIM mass spectra representing various peaks of THC structure and internal standard.

The mass spectrometric parameters were optimised using the Hewlett Packard optimiser software. Positive ionisation was used and operated in electron impact ionisation (EI) mode. Many MS parameters were tested in order to reach the optimum ionisation of different analytes, and the following MS parameters were applied. The transfer line was set at 280°C. The quadrupole temperature was set to 150°C. To choose the optimum voltage, the electron multiplier voltage (EMV) was examined to find the highest S/N ratio through the analysis of the standard of 0.5 ng/mg concentration at a range of 1300-2400 V. The analysis was performed in selected ion monitoring mode (SIM). The solvent delay time was 7 min; elution window 7-13 min. The precursor and product ions of Δ^9-THC, Δ^9-THC-D3 internal standard (IS), cocaine and cocaine-D3 (IS) were: - Δ^9-THC, *m/z* 314, 299; Δ^9-THC-D3, *m/z* 317, 302 cocaine, *m/z* 303, 182 and cocaine-D3, *m/z* 306, 185 (precursor ions, product ions) were used in the SIM mode. The retention times were 7.9 min and 12.9 min respectively for cocaine and Δ^9-THC. Between samples, at least one drug free sample was analysed to monitor cross-contamination.

3.8 Validation

Calibration plots were generated separately for Δ^9-THC and cocaine. The lower limit of detection (LLOD), lower limit of quantification (LLOQ), intraday precision, interday precision, accuracy and extraction recoveries for each analyte, of the analytical method was validated under the FDA guidelines (FDA, 2001 #37). Blank hair (50 mg) was spiked with solutions of the analytes to prepare the calibration curve. Calibration standard points were prepared by spiking the 50 mg hair with a specific concentration of the analytes and the IS dissolved in methanol.

The quality control (QC) samples were also prepared in the same way. The samples of calibration and QC were treated similarly to the hair samples. Calibration concentrations standards 0.02, 0.05, 0.1, 0.5, 1 and 1.5 ng/mg of Δ^9-THC and cocaine and were used to plot standard curves, using the ratio of analyte to internal standard, against standard known points of analyte of each sample. The analyte to internal standard ratio was calculated using the HP Data analysis software by dividing the peak area of the sample with the peak area of the internal standard. A blank hair sample was injected after the development of the method to confirm the selectivity of the analysis method by the lack of any interfering peaks at the illusion time of internal standard, cocaine and Δ^9-THC.

A full scan mode (m/z 50-500) was applied on all the analytes and the internal standard to acquire the full ion scan. Low intensity m/z ions were discarded due to their effect on the overall quality chromatogram baseline. More than one ion fragments resulted from the ionisation of each of the drugs. The most abundant fragment ions in the mass spectra of the deprotonated and protonated molecule of Δ^9-THC were 231.10, 299.10, and 314.20 and cocaine m/z 82.10, 105.00, 182.00, 303.10. The preferred selected ions: m/z Δ^9-THC 299.00, 314.00, cocaine 182.00, 303.00 for cocaine and of Δ^9-THC Δ^9-THC-D3 302.00, 317.00. The selection of the ion fragments for the analysis Δ^9-THC and cocaine in the quantification method was based on its intensity level, reproducibility and the noises levels from analysis of the real hair samples (with all the interfering hair matrix contaminants).For each analyte, the ratio of the two fragments ions were compared between the samples and the reference standards to enhance the method precision.

Peak area is proportional to the actual amount of solute in the injected sample; the detection of a constant flow of the solute by a sensitive detector and the volume of

mobile phase passing through the column. Accuracy, defined by calculation is the degree of confidence of analysed spiked real sample to the actual (true) standard concentration, which was obtained by calculation of the recovery concentration of the spiked real hair sample of cocaine and Δ^9-THC. The evaluated spiked samples were prepared with three different known concentrations (low, medium, high) of cocaine and Δ^9-THC in 50 mg of blank human hair samples. These solutions were prepared in replicates (n=6) and analysed using the same optimised GC-MS method. Precision was calculated by determining the reproducibility of the spiked real sample under the same conditions and method within the same day (intra-day precision), and three consecutive days (inter-day precision). Intra-day precision was calculated by analysing all 6 replicates of each measurement level in the same day. Inter-day precision was obtained by the measurements of the same 6 replicated of the three measurements within the 3 chosen consecutive days. LLOD is the lowest spiked concentration of the analytes that can be distinguished from the blank hair sample. In the spiked hair samples, detection was feasible for Δ^9-THC concentrations as low as 0.015 ng/mg and for cocaine at 0.02 ng/mg. To be able to calculate the LLOQ, the lowest concentration of the analyte, that can be reliably quantified must be reached, with a precision difference in RSD not exceeding 20% and having a signal to noise ratio S/N of 10 or better. LLOQ was possible at the concentrations of 0.02 ng/mg and 0.05 ng/mg for Δ^9-THC and cocaine respectively using a signal to noise ratio (S/N) of at least 3. The linearity of the method was measured by using linear regression analysis.

Extraction recovery was determined by comparing the area ratio of Δ^9-THC and cocaine extracted, from a 0.5 ng/mg spiked blank hair sample, with the area ratio of standard neat solutions prepared in pure hexane of the same concentration. The value

of the Δ^9-THC and cocaine peak area to IS peak areas ratio was then compared to the standard references peak areas (calibration curve points) to internal standard (IS) peak areas ratio. In order to assess the effect of matrix associated with real human hair on the analytes and analysed samples, 50 mg of drugs free hair (blank) was un-spiked sample and continued the extraction and analysis process in the same protocol as the previously analysed samples. The blank hair sample was spiked with a known concentration of the analytes and IS. The results of the analysis of the spiked hair sample were compared to the standard neat (no hair) solution GC-MS results of the same concentration. Cross contamination was tested by running different blank hair samples. No peaks were noted in the region of both drug's elution times. The assessment of the stability of the two analytes in the digested hair solution was done by spiking similar concentrations (100 ng/mL) of different hair digestions and then analysed at three different time periods, 48 hrs, 7 days and 28 days.

3.9 Results and discussion

3.10 Method development

3.11 GC-MS conditions optimisation

Full scan monitoring was used in the initial method development followed by selected ion monitoring (SIM), and retention times were used in the detection and quantification of Δ^9-THC and cocaine. Both analytes are reasonably different in their structures and their configuration; they have different melting points, 98°C for cocaine, and 157°C for Δ^9-THC. The difference in the chemical properties (volatility and polarity) of the two analytes and their relative affinities for the stationary phase

of the GC column promotes the separation of the compounds at different retention times as the sample travels the length of the column. The GC chromatograms (Figure 3.7) show peaks representing Δ^9-THC and cocaine eluting at R_t=12.9 ± 0.2 and 7.9 ± 0.2 minutes, respectively. The challenge was in the detection of both drugs without derivatisation. Different columns were tested to detect both drugs using the same method, since both can be important tests in drug confirmation after pre-screen testing.

The choice of fragment ions played a major role in measuring the reproducibility of the samples. The Δ^9-THC and cocaine that were detected, by assessing the effect of matrix associated with real human hair on the analytes. The interfering ions of the matrix had been avoided by using the specific SIM scanning mode selecting only the ions of the analytes. A chromatogram and mass spectrum and the predicted structures and fragmentation pathway of Δ^9-THC and cocaine from hair are represented in Figure 3.7 and

Figure 3.8.

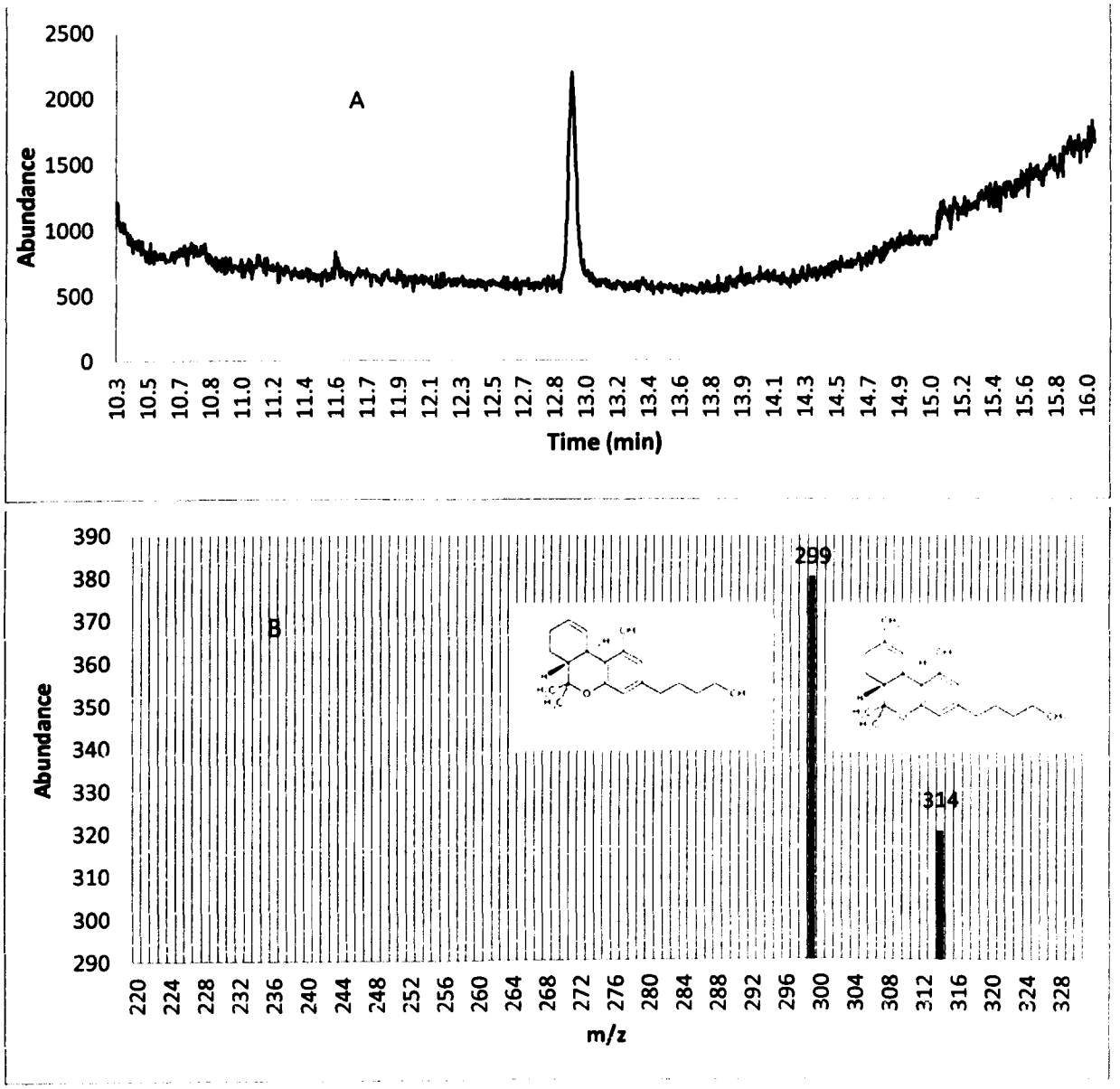
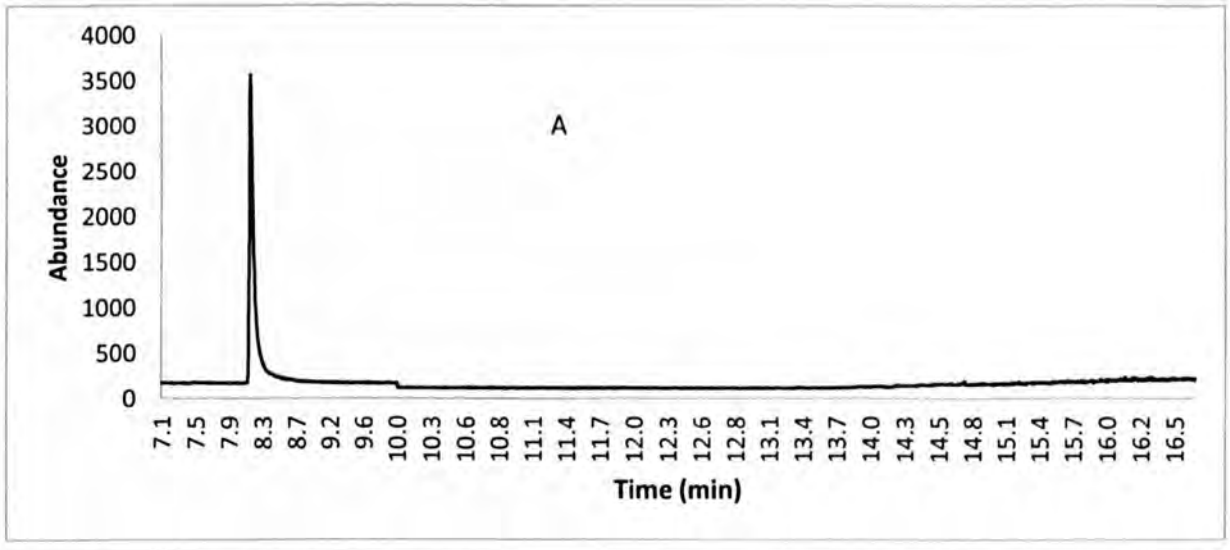


Figure 3.7 GC MS chromatogram A) and fragmentation pathway B) of Δ9 THC



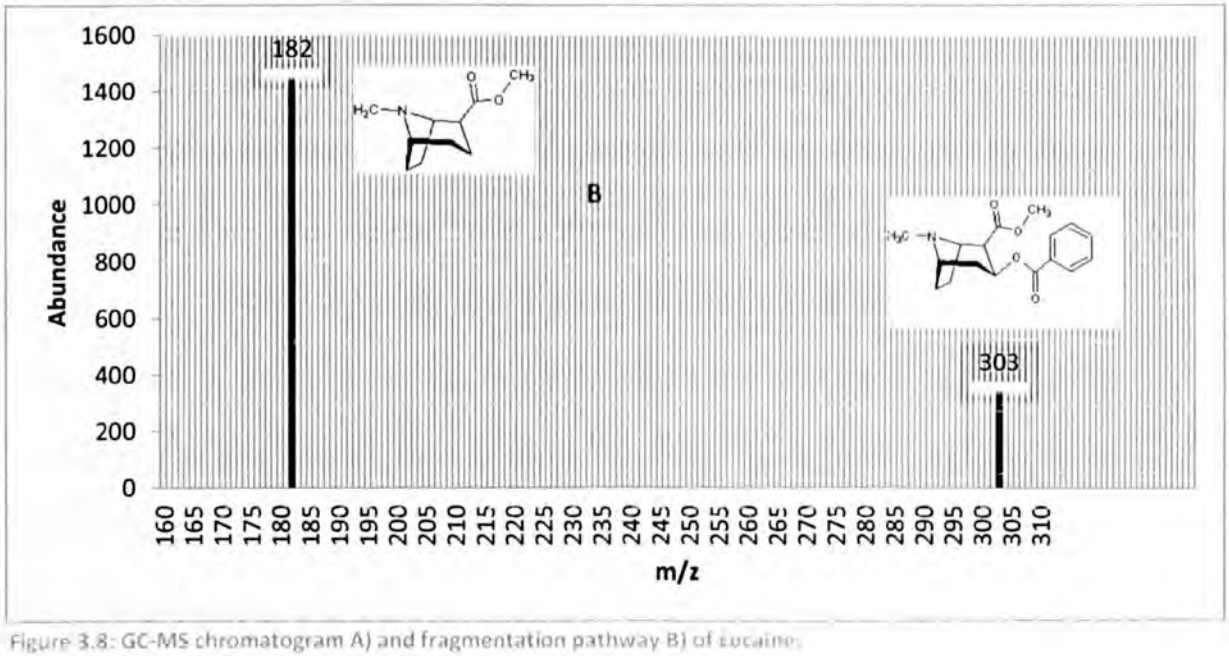


Figure 3.8: GC-MS chromatogram A) and fragmentation pathway B) of cocaine.

Increasing the injection volume of the analysed samples in the GC resulted in a better peak area. Different injected solvents can react in a different way to the column in the GC. To assess the solvents and their grade's influence on the column and thereby the peak shape, hexane, dichloromethane and methanol were tested. Hexane showed the highest peak area and best peak shape with a lower noise level. Most of the solvents used in GC-MS analysis (methanol, acetonitrile, ethyl acetate, DCM) have similar boiling points ranging between 60 and 80°C, so selecting the right solvent with the best sensitivity was challenging. The analyte must have a high

solubility level in the solvent. The standard 1 mg/mL solution of the drug, dissolved in methanol; hexane, was used instead of methanol for two reasons. Firstly, methanol had a lower S/N ratio due to its high boiling point which leads to it taking a longer time to be eluted completely from the GC column. Secondly, in order to increase the sensitivity of each run the injection volume was increased to 3 µL. The calculation of a single 3 µL methanol injection with the injector temperature between 260 and 280°C was ≈ 7 mL in volume. This volume was greater than the liner volume (800 µL). Hexane with a boiling point of 69°C and a 3µL injection vapour volume of 700 µL was used instead of methanol. A double tapered splitless inlet liner without glass wool was also used to avoid loss of the injected samples. To get a better peak shape, different gas flow rates ranging from 1.2-1.5 mL/min were investigated. A huge difference was observed possibly due to less sample loss from the liner and higher sample accumulation in the column. When the flow rate was changed, this also led to a change in the flow pressure from around 8 PSI to 30 PSI. However, the higher flow rates, which increased the pressure to almost 30 PSI, caused the system to shut down due to it exceeding the system and column optimum limits. A flow rate of 1.3 mL/min with a starting pressure of 20 PSI decreasing after injection to 10 PSI, and 2000 V for the ionisation source potential gave the best results, with optimal baseline and peak shape of the analytes and IS.

A full scan mode was applied to the MS electron multiplier voltage (EMV) at a voltage of 1,275 V when high concentrations (1 mg/mL) were used. Different ionisation voltages were also tested between the ranges of 1,300 V to 2,400 V of EMV during the SIM mode analysis of the samples and standards. By using low voltages, fewer analytes will be ionised, which indeed meant less signal. Also, using

high voltages such as 2,400 V resulted in an enhanced signal and also a higher noise level, which meant lower signal to noise ratio and thus poorer LLOD. The highest S/N ratio was reached at a transfer line temperature of 280°C and quadrupole temperature 150°C; electron multiplier voltage (EMV) 2,200 V.

The peak area is the integration of each specific solute, eluted at a specific time. For equal and accurate integration of each of the sample peaks, the auto integration Hewlett Packard software data analysis was performed. The peak area was calculated as the product of the height of the peak multiplied by its width at half height, taking into consideration the shape of the peak which should be typically Poisson, Gaussian shape.

3.12 Digestion and solvent selection

Hair samples were cut extra short to decrease the digestion time. Shorter hair requires shorter time for digestion, therefore a smaller amount of enzyme will be needed. Two digestion methods were used. A newly developed enzymatic digestion method was used in this experiment using Proteinase K from Tritirachium album for the digestion of the hair samples. Proteinase K is a stable and highly reactive serine protease (131). Although enzymatic digestion in contrast to alkaline digestion takes a longer period of incubation (up to 50 min), it's still performed under mild neutral conditions. Enzymatic digestion is superior to alkaline digestion in other areas too. The temperature which is used in alkaline and acidic digestion can reach up to 100°C, which therefore can easily denature most of the drugs and especially cocaine. Also, the strong basic media in which the digestion is carried out during alkaline

digestion may be one of the reasons for not detecting the drugs in some samples due to denaturation. Nevertheless, the proceeding steps that follow digestion can play a role in drug detection. In alkaline digestion, the samples are neutralised with buffer and strong acid which increase the dilution factor of the analytes; this may increase the risk of drug loss, and breakdown and/or affect the pre-screening ELISA test. The enzymatic digestion method was developed based on the chemical and physical properties of the enzyme Proteinase K. Hair samples were cut to fine strands, and then divided into separate vials. All the samples were collected from the same source to avoid the effect of the hair matrix of different humans. The enzyme (20 mg and 50 mg) were added to different vials of increasing gradient of Cleland's reagent (DTT) in 1 mL Tris HCl buffer (20 mg, 40 mg, 50 mg, 80 mg, 100 mg DTT). All of the hair samples were digested, but for varying periods of time. The shortest digestion time was 60 min in the vial that contained 50 mg of the enzyme with 100 mg of DTT at pH $\approx$ 7.6. Later on, slow continuous mixing with a magnetic stirring bar reduced the time to 50 min.

A study of the effectiveness of alkaline digestion in comparison with enzymatic digestion was completed. Alkaline digestion was employed for part of this study to hydrolyse hair so that the majority of the drugs in hair are released in the extracted solution. Alkaline digestion was used for real hair samples for ELISA screening for different drugs (129,132). Extraction of the hair samples was applied using LLE. This extraction method was sufficient to reduce all the contaminants and hair residues of the hair samples thus stopping it from affecting the GC-MS analysis. Recently, different solvents were used in many drug extraction methods. Ethanol, DCM and pentane were the most commonly used in extraction. Pentane for LLE was

found to be a very efficient method for the extraction of some analyte from hair samples in many methods previously tested before (133). Pentane has some disadvantages; it is harmful to human beings through inhalation or ingestion, also its high volatility increases the risk of losing a part of the extracted sample. Also, in some studies, other problems due to the formation of a gel-like substance (129) have been noted. This caused difficulty in separating the pentane layer and thus more drug sample was lost. The method developed here was to use a mixture of extraction solvents, in order to get the best extraction percentage.

It was discovered that a 3:1:1 ratio of chloroform: ethyl acetate: ethanol gave the best results after duplicates of 3 hair samples, spiked with 1 ng/mg of the analyte, alkali digested and then extracted using three different methods with pentane, ethyl acetate, and a mixture of the three (Table 3.2). Chloroform and ethyl acetate liquid solvents are insoluble in water, whereby they have a solubility of 0.056% at 20°C, and 3.3% at 20°C and in water respectively (119). Whereas, the hydrogen bonding of the hydroxyl group causes pure ethanol to be polar in nature and its nonpolar end make it capable of dissolving many ionic compounds, which make it perfect for the solubility of the analytes, especially cocaine (128,134). The combination of these factors gave a higher recovery in comparison with the other tested solvents regarding Δ^9-THC and cocaine. This LLE method provided a satisfactory precision, and requires low cost solvents and apparatus. Therefore, it is a more affordable method in comparison to other sample preparation techniques such as SPE. In the same way, the three solvent extraction efficiencies were compared by calculating the recovered percentage of a spiked hair sample to the same concentration of a neat standard analysis response. The solvent mixture achieved 95% mean recovery when the

samples were extracted at 3,500 x g relative centrifugal force for 15 minutes at 10 °C.

Table 3.2: Three extraction experiments to assess the recovery percentage

Extraction solvent	Mean recovery (%)
Ethyl acetate	70.4
Pentane	61.3
Mixture	95

This analytical procedure was able to detect Δ^9-THC and cocaine in human hair without any interference of any detectable contamination either from the hair matrix or from the accompanying procedures of digestion and/or extraction. Also, the hair decontamination technique that was used showed no interference in the mass spectrum of any of the usual contaminants of the human hair. To make sure that there is no variation in any of the method's sections (e.g. the instrumental response to the auto-injector of the GC system, interference between the drug and the matrix, effect of the digestion method and extraction or even loss of analytes during the sample preparation), the sample was spiked with a very similar deuterated internal standard to the compound being analysed. This step helps the variation to be corrected by the recalculation of the ratio of the analyte signal to the internal standard signal of each sample. Thus, it would be correcting for any matrix effect for all the samples. Although, under the given analytical standards that were used in

this study, no major interference was reported either regarding the hair sample itself or the analysis procedure.

To prevent the build-up effect of cross contamination from previous runs and to help regenerate column performance, two 3 μL blank injections of the sample's solvent (hexane) were injected through the auto-sampler in the liner in-between each sample run. In addition to overcoming any effect which may arise either from syringe contamination or inlet contamination due to the large number of injections, a baking method of the column was developed at an isothermal temperature, almost close to the column maximum operating temperature. (1 μL) of hexane was injected into the GC liner at 330°C resulting in a vapour volume of 750 μL, greater than the normal sample injection (700 μL) to decontaminate the liner. The column was baked at 340°C for 60 minutes with a continuous purge pressure of 15 PSI, and the MS was operated in a high ionisation voltage at a full scan mode between 50 and 500 m/z, to allow adequate time to flush out the residue of previous runs. After each baking run, a blank sample was injected using the normal analysis method to equilibrate the column; subsequently a standard was also injected to check the efficiency of the column. The septum and the liner were changed, and the column was trimmed at least twice during this study.

Figure 3.9: Analytes extraction using a mixture of solvents after centrifugation.

The major advantage of this method in comparison to the previously optimised methods (129,132,135) was that for the first time a Δ^9-THC and cocaine analytes were analysed without any derivatisation. This development made the method economical, rapid, precise, reproducible and cheaper to apply. During the analysis, the extracted hair sample was returned to the fridge after every run to prevent the reconstituted sample evaporating in the auto-sampler.

3.13 Optimisation of the procedure

Results shown in Figure 3.10 were obtained with the enzymatic and alkaline (NaOH) digestions. For these tests, a blank hair sample was used to reproduce true working conditions. Δ^9-THC (5 ng/mL) was spiked into two blank hair samples and digested with Proteinase K at 37°C or by NaOH at a temperature not greater than 95°C. This

process was repeated (n=6) times for each digestion method. Use of Proteinase K resulted in an average concentration of 4.85 ng/mL ± 0.23 with 95% recovery, whilst the samples digested by NaOH resulted in a mean of 3.15 ng/mL ± 0.1 and 62% recovery.

The reduced extraction recovery when using NaOH could be due to the drug degradation caused by the strongly basic NaOH conditions and high temperature. This enzymatic hydrolysis method is an improvement over other approaches, which could easily cause drug degradation in the presence of NaOH, HCl and high temperatures, and thus improve the stability of the method and therefore accuracy.

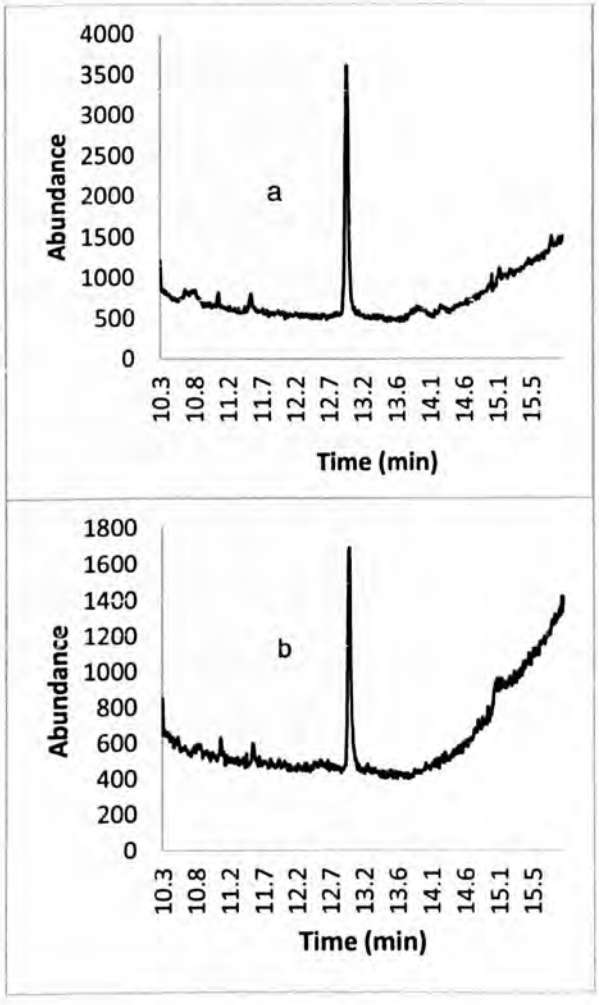


Figure 3.10: Chromatogram obtained after spiking hair with 5 ng Δ9-THC and digesting it with (a) Proteinase K and (b) NaOH.

3.14 Method validation

3.15 Chromatographic method

The calibration curves were prepared by spiking known concentrations of Δ^9-THC or cocaine to blank hair samples at 0.02, 0.05, 0.10, 0.50, 1.00 and 1.50 ng/mg, with a constant amount of Δ^9-THC-D3 and cocaine-D3 (1 ng/mg). Excellent peak shapes and peak resolution was achieved during the method validation. A linear regression analysis of Δ^9-THC and cocaine showed R^2 values of 0.995 and 0.997 respectively. Both were established at concentrations ranging from 0.02-1.00 ng/mg for Δ^9-THC and 0.05-1.00 ng/mg for cocaine. This analytical method demonstrated that it was capable of detecting as low as (LLOD) 0.015 ng Δ^9-THC and 0.02 ng/mg for cocaine per milligram of human hair with a S/N ratio greater than 3. The LLOQ 0.02 ng/mg and 0.05 ng/mg of hair respectively for Δ^9-THC and cocaine (Table 3.3). The S/N values of both analytes were found to be around 10. The extraction recoveries of both analytes were within the limits set by the FDA guidelines and summarised in Table 3.4. The calculated peak areas from GC chromatograms for spiked concentrations were divided by the area peak of the internal standard to determine the abundance ratio. To reach the optimum sensitiveness of the analytical method, sample testing concluded the solvent effect in determining a good peak shape and intensity and thereby reaching a lower LOD and LOQ.

Table 3.3: Summary of assay validation results

Compounds	Level	Concentration (ng/mg)	Precision RSD (%)		Accuracy (%)
			Intraday n=6 per each	Interday n=18 per each	
Δ^9-THC	Low	0.02	15.4	18.0	114
	Medium	0.10	15.6	7.0	110
	high	0.50	8.0	5.0	95
Cocaine	Low	0.05	12.1	10.6	98
	Medium	0.10	10.1	12.1	101
	high	0.50	5.1	2.8	110

Table 3.4: Extraction recovery results

Compounds	Level	Concentration (ng/mg N=6)	Extraction recovery (%)
Δ^9-THC	LLOQ	0.02	97.48
	Medium	0.10	100.92
	High	0.50	102.2
Cocaine	LLOQ	0.05	95.8
	Medium	0.10	99.5
	high	0.50	96.5

3.16 Real sample analysis

The method was applied to the analysis of 10 randomly selected hair samples out of 80 samples, which were qualitatively identified positive by ELISA [All the 10 samples in Table 3.5 were positively confirmed (by ELISA) for Δ^9-THC (10 samples) and 1 sample was found to be also positive for cocaine] and these were confirmed and quantified by GC-MS. Ten hair samples were positively identified for Δ^9-THC (H1-H10), and one also for cocaine. Using the abundance ratios, the actual drug concentrations (ng/mg) in the unknown hair samples ranged between 0.05-0.35 ng/mg hairs for Δ^9-THC. One sample (H10) tested positive for cocaine, and was measured to be 0.1 ng/mg. All other samples (H1-H9) detected negative for cocaine, i.e. it was below the ability of the method to detect it (lower than the LLOD). The results of the analysis are shown in Table 3.5:

Table 3.5 Hair analysis results of samples using GC-MS

Hair samples	Age	Gender	Δ^9-THC ng/mg	Cocaine ng/mg	SD	SEM
					n=3	
H1	22	F	0.08	ND	± 0.006	0.003
H2	22	F	0.05	ND	± 0.001	0.001
H3	18	F	0.35	ND	± 0.006	0.003
H4	21	M	0.20	ND	± 0.000	0.000
H5	24	M	0.18	ND	± 0.017	0.010
H6	22	M	0.09	ND	± 0.006	0.003
H7	20	M	0.08	ND	± 0.010	0.006
H8	27	M	0.14	ND	± 0.012	0.007
H9	18	M	0.13	ND	± 0.006	0.003
H10	23	M	0.15	0.1	± 0.00	0.000
					±0.015	0.009

ND = not detected, SD = standard deviation, SEM standard error of mean

The number of publications describing analytical proccdures relating to drug incorporation into hair, decontamination and analysis has increased in recent years (105,133,136), but in this research an improved digestion, and GC-MS method has been proposed which enhances drug analysis capabilities. The sensitivity achieved for Δ^9-THC (LOD 0.01 ng/mg ± 0.01 and LOQ 0.02 ng/mg ± 0.01) and for cocaine (LOD 0.02 ng/mg ± 0.015 & LOQ 0.05 ng/mg ± 0.01) is better than previous reports, which have been obtained from derivatised samples (LOD 0.025- 2.5 ng/mg, LOQ 0.05-7.5 ng/mg for Δ^9-THC and LOD 0.03-0.5 ng/mg, LOQ 0.05-1 ng/mg for cocaine) which is higher than what was achieved (132,137,138). However, although derivatisation is an important factor to improve sensitivity, it can be problematic in complex matrices. Derivatisation is sometimes time-consuming, can add possible

contamination to the sample mixture and could result in a decrease in the sensitivity of the method. Also, derivatisation can create new interfering degradation product ions of the drug itself (139). Negative results could also mean that the concentration of drug in hair is below the detection limit of the method. An additional advantage of the increased sensitivity is that the analysis required a reduced amount of hair, thus making the method more feasible for drug testing (140).

The performance in terms of reliability, feasibility and time for analysis can be improved by using enzymes. In this study, GC-MS has been performed on underivatised, informed positive hair samples for cocaine and Δ^9-THC.

3.17 Conclusions

This sensitive, specific, reliable, rapid, facile, and cheap detection method of hair analysis has proved to be a useful method of public health research in the case of Δ^9-THC and cocaine screening. An additional advantage of this method is that, unlike previously published work, it does not require derivatisation (118,132,136,141). The method that has been developed is capable of detecting exceptionally low levels of Δ^9-THC and cocaine in human hair when only 50 mg hair was processed. The chromatographic optimisation offered a maximal sensitivity for the analysis. Enzymatic digestion and the given chromatographic and mass spectrometric conditions were essential for reproducible and accurate analysis of these psychotropic drugs in hair without any interference. Thus, it is a convenient and potentially less problematic method which can be employed for routine drugs testing. This method can complement conventional blood and urine analysis with the advantages of non-invasiveness of sample collection, negligible risk of infection

(blood analysis), facile sample storage (small sample size, limited biohazard & adulteration/contamination risks) and negligible sample degradation. Also, hair analysis could help prevent false negative ELISA results that can be encountered from the higher limit of detection or even previously developed GC-MS methods by detecting ultra-low concentrations. The limit of detection for ELISA screening was found to be (0.5 ng/mg for the cocaine kit and 0.3 ng/mg for Δ^9-THC). These were used as cut-off levels for screening of the hair samples.

Chapter 4

False Positive Drug Results by ELISA Associated with Enzymatic Hair Digestion

5.0 Introduction

The most common definition of a drug is that it is a chemical, natural or developed substance and/or constituent used for the purpose of changing a person's mental state, and that may be used repeatedly by a person for that effect (142). The term 'drug' includes psychoactive substances and covers legal and illegal substances such as alcohol, caffeine, tobacco, kava, heroin, anabolic steroids, cannabis (marijuana), psychoactive pharmaceuticals and inhalants (143).

Throughout history, humans have always searched for ways to change they perceive and feel the world around e.g. music and spiritual meditation. Different cultures and societies developed ways to control these habitat changes and impact on their cultures (144). Drugs of abuse have serious consequences to public health and safety. Psychotropic drugs are usually divided into classification according to the effect they have on the individual. The most commonly accepted classifications of drugs include stimulants, depressants, narcotics, hallucinogens and cannabis. Stimulants include amphetamines and cocaine while cannabis includes delta-9-tetrahydrocannabinol, also known as Δ^9THC (145).

Globally, 185 million consumers of illicit drugs have been estimated in 2000 by the world health organisation (WHO). Cocaine, amphetamine (AM), methamphetamine (MA), 3,4-methylenedioxy-N-methylamphetamine (MDMA) and 3,4-

methylenedioxyamphetamine (MDA) are four of the most widely use stimulants whose primary action is to alter cognition and perception to produce distinctive emotional and social effects (146). Amphetamines are also used as a performance and cognitive enhancer, despite the significant health risks that are associated with their uncontrolled or high dosage use (147). Routes of administration are usually classified by application location e.g. by oral, inhalation or injection, which is the fastest route of administration (148,149). These drugs can easily and actively cross the blood-brain barrier and act on the brain cells to alter the normal brain function, which result in changes in awareness, mood, consciousness and behaviour (142,150). Amphetamines or empathogen-entactogens (including MA, MDA and MDMA) may produce an additional stimulant and/or euphoriant effect, thus increasing their use as addictive drugs. The detection of stimulant and Δ^9 THC in any matrix (blood, urine, hair, etc.) can result in severe consequences such as the loss of a job, and loss of driving license under German regulations. In a study carried out at San Francisco General Hospital from 1975-1987, approximately 25% of seizures were found to be caused by amphetamine use (151).

Despite these drug restrictions, drugs are still commonly abused by both athletes and the general public. The detection of psychotropic drugs is an increasing problem for the legal authorities, and hence there is a constant effort and need to develop new strategies for the detection, reduction, prevention and intervention of psychoactive drugs. Many social science research studies have focused on the statistical levels of amphetamine abuse around the world to try to understand the psychological effects (152-154)(155).

5.1 Hair detection

Ever since the early 90's, the potential of detecting some stimulants and cannabis in hair has been explored (116). Hair testing for drugs of abuse is a developing technique that offers the possibility of a longer detection window. Drugs can be incorporated into hair through several mechanisms and periods of hair growth. On the other hand, it is well known that hair analysis when compared to urinalysis, blood and saliva testing (depending on the hair length analysed) has the unique potential for determining the retrospective history of drug use. In addition, hair samples can be collected while being monitored. The average head has approximately 100,000 hairs. Keratin protein, in which also nails and outer layer of skin is made, also makes the hair. The visible part of the hair strand is called the hair shaft while the invisible one is called the hair follicle. The duration of the hair in the head is between two to six years. The hair grows at an average of 1 cm a month see Figure 5.1 (156). Hair analysis is not vulnerable to evasive manoeuvres such as temporary abstention, excessive fluid intake, and substitution or alteration of specimen and samples can be collected without any embarrassment e.g. swapping urine samples. Hair analysis is also not subject to false positives, which result from a single intake of any type of contaminant (e.g. food or drink); the washing processes should avoid false positive results. Furthermore, the samples are easy to store and free of biohazards (41). Generally, an appropriate strategy to prove cannabis consumption is done by an immunochemical pre-screening test followed by a confirmatory test using analytical technique such as GC/MS or LC/MS. Hair analysis is focused mostly on the identification of different analytes such as the primary psychoactive substance Δ9-tetrahydrocannabinol (Δ^9-THC), cannabinol

(CBN), cocaine etc. Laboratories follow different screening strategies, but GC-MS analysis is the most commonly used (157,158). Enzyme-linked immunosorbent assays (ELISA) are appropriate for pre-screening different drugs, but using this pre-test with a large number of different hair samples preparation steps can sometimes lead to misleading results, which are caused by the immunoassays (antibodies, protocol, etc.).

Figure 5.1: Hair growth cycle (23).

The results for this study reveal the method is suitable for testing a broad range of recreational drugs and provides scope for detecting therapeutic drugs and androgenic drugs. Thus hair analysis, either alone or in combination with urine, blood and saliva analysis can be an effective approach for the detection of psychoactive drugs through longer periods, without forgetting the side effects that can be caused by the interferences from different hair preparation steps.

5.2 Hair digestion and screening

Hair analysis techniques have been proposed for the detection of toxicities and an individual's nutritional status (159). Extraction of the analytes from hair is made by exposing of the hair to hot methanol (160) and overnight incubation of the hair strands in an alkaline or acidic medium (161). However, the solvent extraction has some disadvantages, including the wrong choice of the solvent, and temperature elevation, which can cause drug degradation. The solution provided in the hair analysis method should be able to solubilise the drug from the inner core of the hair shaft without causing damage to the analyte. This can be achieved by a mixture which is suitable for the dissolution of the keratinized source.

According to many researchers, hair analysis can be a promising technique for drugs of abuse screening (129,162-164). It is generally understood that drugs can penetrate through the hair shaft by several mechanisms, either endogenously, by ingestion (via the blood during hair formation or through sweat and sebum) in the course of passive diffusion from the circulatory system to growing hair follicles, which are then encapsulated in keratin fibres of the hair shaft, or exogenously, e.g. deposition of drugs through external contamination (57,162). In the past few years, many new biological extraction methods have been developed in addition to existing chemical and organic solvents hydrolysis methods (161,165). However, the chemical dissociation can cause drug degradation for chemically unstable compounds, such as cocaine, benzoylecgonine, heroine and other ester compounds (159,166). Solvent extraction cannot be guaranteed to give a complete recovery due to the variation in the physical properties of different types of hair (*e.g.* melanin content, thickness,

pores). As a result, the method of choice, which can help in overcoming most of the hair extraction and digestion method weaknesses, can be avoided by a more neutral, room temperature and analyte protective technique, which can be found in enzymatic digestion.

A biological digestion method works on destruction of the hair shaft structure and therefore releases the trapped analytes in hair (140). Enzymes such as proteinase K, protease E and protease type VIII have been previously used for hair hydrolysis (55,159) to hydrolyse the hair samples without degradation of the unstable drugs. Baumgartner and Hill (167) stated that under room temperature (37-40°C), rather than very high temperature, and at a neutral pH provides the typical medium for achieving best recoveries. A comparative extraction was conducted using NaOH digestion of spiked hair samples at 95°C for Δ^9-THC and cocaine to measure the drugs' degradation (60,166).

Early studies of hair analysis started with only qualitative analysis of hair samples (168). In current research, the digestion method is usually followed by radioimmunoassay of the samples before being confirmed by analytical methods *e.g.* GC-MS or HPLC-MS/MS. However ELISA has been used previously for the analysis of blood (12,123,169) and urinary samples; it is over the last few years that the method has been improved to meet the criteria of hair analysis (60,170,171). Unlike GC-MS, (due to impurities in the hair matrix), hair can be examined directly on ELISA after digestion. Radioimmunoassay (RIA) is considered as a screening test for a pre-analysis step which can be conducted by one of the spectroscopic or

chromatographic techniques. Poor agreement in analytical results has been reported between RIA and enzymatic digestion (159,172). It is reported that enzymatic hair digestion mixture can result in an undesired effect when used simultaneously with immunoassays kits.

5.3 Gas chromatography mass spectroscopy GC-MS

GC-MS is a sensitive technique that has the ability to combine gas chromatography (GC) and mass spectroscopy (MS) to determine the molecular weight and purity of compounds as seen in Figure 5.2.

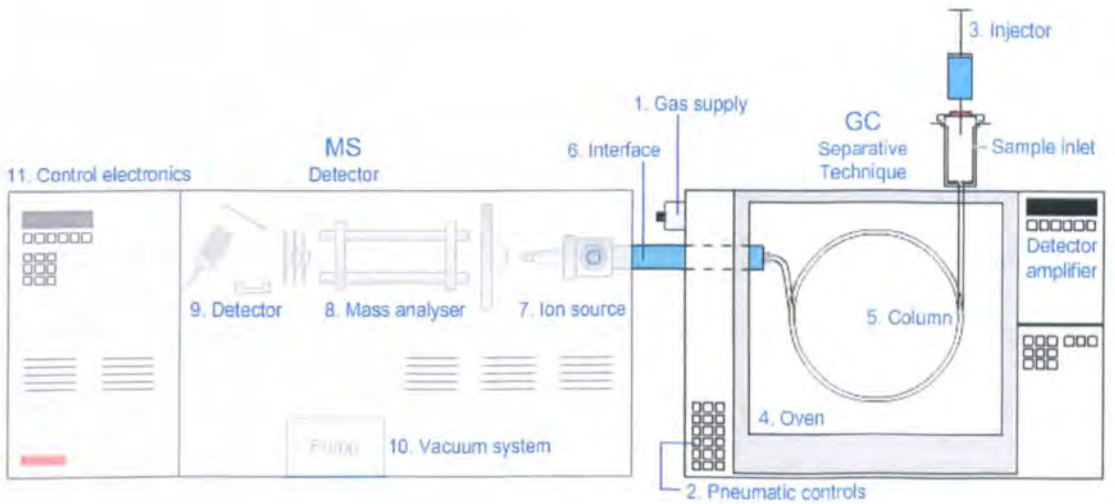


Figure 5.2: Schematic Diagram of a typical GC-MS/MS

(1) Gas supply fed through regulators to filter and obtain gas purity (2) Controls to regulate gas pressures (3) Sample is volatized and injected into the carrier stream via sample inlet entering GC column (4) Temperature programmed (5°C- 400°C) column oven. (5) GC Column (10-120 m) consisting of a mobile phase for appropriate transport of sample components via hollow capillary columns. Inner wall of column coated with a stationary phase (6) Interface to transport GC separated

analytes to the mass spectrometer for ionization, filtration and detection (7) Ion source to ionize prior to MS analysis (8) Mass analyser for separation of analytes on the basis of mass to charge ratio (9) Detection and transformation of ion beam from mass analyser into a usable peeks and data (10) Vacuum system to mass analysers for efficient operation (11) Control and selection of MS parameters via a computer-designed software (93).

5.4 Materials and method

5.4.1 Chemicals

The ELISA kits were obtained from Neogen Corporation (Lexington, KY 40511, USA). The kits consisted of a drug enzyme conjugate, wash buffer concentrate, specific enzyme immunoassay (EIA) buffer, 3,3', 5,5'-tetramethylbenzidine substrate, stop solution and antibody coated plate containing 96 wells. A Cary 50 MPR micro plate reader was provided by Varian Yarnton, UK. Proteinasc K was obtained from Tritirachium Album Bacteria, (highly pure ≥30 units/mg protein) in the form of lyophilized powder obtained from Sigma Aldrich (Poole, Dorset, UK). TRIS Buffer and DTT were obtained from MERK (Nottingham, UK). The following HPLC grade chemicals and reagents: Dichloromethane, pentane, sodium hydrogen phosphate, sodium hydroxide, hydrochloric acid, deionised water, formic acid and acetonitrile were obtained from Sigma Aldrich (Poole, Dorset, UK).

5.4.2 Specimen collection

Hair samples (50 mg) were collected anonymously from 18 male Turkish athletes. The hair collection process was approved by Kingston University Faculty Research Ethics Committee. The samples were decontaminated by washing 3 times with dichloromethane (3 mL) and vortex mixed for 20 seconds. This decontamination step should be enough to remove any hair contaminants such as colouring, sebum, shampoo, etc., which may interfere with analysis. The hair samples were cut to *ca.* 0.5 mm long segments with hand scissors. After the hair has been cleaned, the samples were enzymatically digested by Pro K enzyme.

For the ELISA screening process of this study, the positive results from this screening process were further analysed for amphetamines (same ELISA), cocaine and Δ^9-THC detection using GC-MS/MS. Blank hair samples were obtained from healthy, non-abusing volunteers living in the UK. All hair samples used for the study consisted of at least 48 x 2 cm chemically untreated hair strands cut directly at the skin surface (vertex posterior of the head) region, which exhibits a reduced variability in hair growth rate in comparison to other regions. All hair samples were then stored in individually labelled, sealable paper envelopes, according to approved protocols.

5.4.3 Specimen preparation

The samples were decontaminated by washing 3 times with dichloromethane (3 mL) and vortex mixed for 20 seconds. This decontamination step should be enough to

remove any hair contaminants such as colouring, sebum, shampoo, etc., which may interfere with analysis. The hair samples were cut to *ca.* 0.5 mm long segments with hand scissors. After the hair had been cleaned, the samples were enzymatically digested with Pro K enzyme.

5.5 Enzymatic digestion

The decontaminated hair (50 mg) was weighed for enzymatic digestion, to which DTT (Cleland's reagent) (50 mg) was added, and the mixture was conditioned with TRIS HCl buffer (1 mL) in a glass vial. Proteinase K "also called protease K" (Pro K) enzyme was added in a ratio of 1 mg hair: 1 mg enzyme to the pulverized hair. The mixture was incubated at 37.5°C for 50 min, whilst being continuously mixed. The drug free hair was used as a control.

5.6 Extraction of drugs

Hair samples spiked with 1 ng/mg of Δ^9-THC-D3 and amphetamine D3 (used as the internal standards), were digested with Pro K enzyme. Drug free hair was used as a control. The drugs were extracted by 2 different liquid–liquid extraction (LLE) methods using pentane (6 mL) and a solvent mixture of chloroform, hexane and ethanol 1:1:2 (6 mL). After vortex mixing and centrifugation (1 min at 2,383 x g), the supernatant organic layer was transferred into a fresh glass tube using a glass Pasteur pipette (to avoid plastic pipette degradation into the samples) and the hair residue pellet was discarded. The organic layer was mixed with an aliquot of 1M

HCl (25 μL) which had been diluted to 1% in methanol to minimise drug evaporation during the drying step. The organic layer was evaporated under a stream of nitrogen gas at 50°C using a sample concentrator Techno DB-3 (Cambridge, UK). Spiked samples of 0.02-1.50 ng/mg were prepared for the Δ^9-THC, cocaine, amphetamines, and their metabolites for GC-MS calibration plots. The extracted residue was reconstituted with 60 μL hexane and screened on ELISA. The remaining residue was transferred to autosampler vials, where 3 μL was injected into the GC-MS system for confirmation analysis.

5.7 ELISA

The hair samples were first screened by ELISA. All positive samples were then analysed on GC-MS for confirmation. The ELISA screening system consisted of a Cary 50 MPR microplate reader (Varian, UK). The enzymatically digested samples were screened by ELISA for the presence of Δ^9-THC, cocaine and amphetamines, using the manufacturer's procedure for analysis. The qualitative test kit is designed for screening and forensic detection of drugs and their metabolites. The ELISA kits operated on the basis of competition between the drug in the hair samples and the conjugate for the antibody binding sites on the pre-coated microplate. Enzymatically digested samples were loaded into the microplate wells followed by addition of the drug–enzyme conjugate and incubated at room temperature away from light, for 45 min. Unbound sample and drug-conjugate were used and the washing buffer was prepared with deionised water and the concentrated buffer supplied with the kit. K-blue substrate was added to detect the presence of the drug-enzyme conjugate. After 30 mins of incubation, the reaction was stopped by the addition of 1N H_2SO_4 (100

µL). The absorbance of each well was measured and read on the microplate reader at a wavelength of 450 nm. The concentration of the drug in the well is inversely proportional to the colour generated and hence the absorbance.

5.8 GC-MS calibration graph for AM, MA, Δ9-THC and cocaine

Calibration graphs were produced by initially weighing blank hair (50 mg) into 7 separate vials. Once the hair samples were cut into small segments; 50 ng of internal standard were added to each vial before the enzymatic digestion starts. The blank hair samples were then spiked with increasing known drug concentrations (0.04, 0.06, 0.08, 1, and 1.5 ng/mg for cocaine and benzoylecgonine, 0.02, 0.05, 0.10, 0.50, 1.00 and 1.50 ng/mg for Δ^9-THC, 11-Hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH), 0.05, 0.08, 0.1, 0.15, 0.2 ng/mg for amphetamine and its metabolites. Finally, the hair samples were digested for 50 minutes. The homogenate was extracted via liquid-liquid extraction using pentane and the solvent mixture (see above method).

The GC-MS chromatogram for each spiked sample was then used to produce a calibration graph. Individual peak areas and abundance for drug concentrations in the samples and the standards were determined using the software Enhanced Data Analysis MSD enhanced Chemstation version E.02.00.493 from Agilent Technologies and then identified with the NIST 2008 mass spectra library. The calculated abundance ratios of each analyte and IS against drug concentrations in ng/mL are then used to plot a calibration graph on MS Excel. Individual calibration

graphs for Δ^9 THC, cocaine, AM and MA allowed us to determine the drug concentrations of unknown hair samples.

5.9 Derivatisation of AM and MA

GC-MS analysis of AM and its metabolites require derivatisation prior to analysis. Due to the relatively high polarity of AM, chemical derivatisation is necessary to increase volatility and develop more characteristic mass spectral fragment ions (92). To achieve this, several derivatisation agents have been used such as trifluroacetic anhydride (TFAA), heptaflurobutyric anhydride (HFBA) N, methyl-bis-trifluroacetamide (MBFTA) or N,O-bis- trimethylsily- trifluoroacetamide (BSTFA). The most widely used and preferred reagent is BSTFA; it has the ability to react with a variety of polar organic compounds and replace active hydrogens with –Si $(CH_3)_3$ trimethylsilyl group (TMS). The increased volatility and reduced polarity of the derivative makes the molecule more thermally stable but in some cases more susceptible to disintegrate than the parent compound. The same concept can be applied to other derivatising agents. Derivatisation improves chromatographic properties (make the analytes more volatile) and to obtain higher abundance fragmentation patterns for mass spectrometry analysis Figure 5.3 (173)(174).

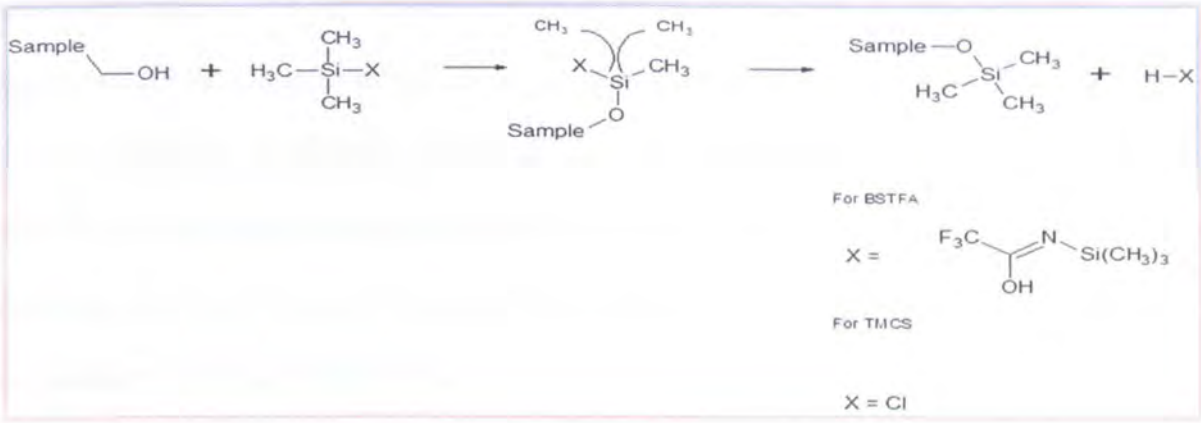


Figure 5.3: Silylation mechanism between sample and derivatising reagent BSTFA.

Figure 5.3, illustrates a nucleophilic attack upon the silicon atom of the silyl donor thereby forming a biomolecular transition state. After this point, the silyl compound leaving group is basic and able to stabilize a negative charge in the transition state, having no tendency to bond back to itself or the silicon atom (173).

The addition of TFAA to a sample containing AM can increase the molecular weight of the sample, thereby allowing better separation of AM and its derivative MA and improve detection during GC-MS analysis. TFAA achieves this by replacing the active hydrogen ions on the AM and MA structure with fluoro groups. The addition of three fluoro groups to the structure can then increase the mass of the molecule for improved separation (173,174).

For cocaine, and Δ^9-THC with its metabolites, BSTFA + 1% TMCS was the preferred choice, while for amphetamines, TFAA was the best choice. To find the best derivatising agent, 2 ng/mg spiked samples was reacted with different

derivatising agents. The derivatising agent was chosen based upon the sensitivity and precision (highest and most reproducible peak). The extract was dried under nitrogen gas at ≤ 50 °C and derivatised with bis(trimethylsilyl)trifluoroacetamide (BSTFA) (100 µL), 1% trimethylchlorosilane (TMCS) (100 µL) and ethyl acetate (50 µL) by heating at 55 °C for 2 hours. The best TFAA derivatisation results were achieved by the addition of 140 µL of the derivatising agent along with 70 µL of EA, incubated at 55 °C for 2 hours. All solutions were evaporated with a gentle stream of nitrogen at 55 °C, until near dryness, to prevent cross –contamination of the derivatising mixture with the sample. Samples were reconstituted with hexane (60 µL) and 3.0 µL of the sample was auto-injected into the GC column.

5.10 Detection and quantification using GC-MS/MS procedure

5.11 Cocaine and Δ^9-THC analysis

Electron impact (EI) ionisation mode was used with helium as the carrier gas at a flow rate of 1.3 mL/min. Pulsed splitless injection was performed at a purge time of 1 min and a purge flow of 53 mL/min with an initial pulse pressure of 30 PSI reduced to 10 PSI after injection, to enhance the peak shape and sensitivity. The oven temperature for the detection of Δ^9-THC was programmed to start from 50°C, held for 1 min, then increased to 200°C at 40°C/min, held for 2 min, and increased to 280°C at 80°C/min, held for 3 min, to a final step of 310°C at 80°C/min, and held for 4 min.

5.12 Amphetamine analysis

For amphetamines analysis, a 15 min run was programmed. The run started with an initial temperature of 50°C, was held for 1 min, then increased to 255°C at 20°C/min, which was then held for 2 min, and to 280°C at 50°C/min, to a final temperature of 300°C at 80°C/min, held for 1 min. The injection port temperature was set at 250°C. Pulsed splitless injection was performed at a purge time of 1 min and a purge flow of 54.2 mL/min. To enhance both the peak shape and sensitivity the initial pulse pressure was set at 20 PSI reduced to 10 PSI after the samples injection. The quadrupole temperature was kept at 150°C; electron multiplier voltage (EMV) 2,200 V.

The analysis of both methods was performed in selected ion monitoring mode (SIM) and between samples, at least one control sample was analysed to monitor cross-contamination. The solvent delay time was 7 min, where Δ^9-THC and cocaine metabolites exhibited retention times between 7.9 min and 12.9 min, while for amphetamines the solvent delay was 4 min Table 5.1. The precursor and product ions of the analytes and internal standard (IS) were: Δ^9-THC, *m/z* 371, 386 R_t 9.4 min; THC-COOH, *m/z* 371, 473 R_t 13.0 min; OH-THC, *m/z* 371, 474 R_t 11.8 min; Δ^9-THC-D3, *m/z* 413, 370 R_t 10.35 min, cocaine, *m/z* 303, 182 R_t 9.9 min benzoylecgonine, *m/z* 240, 261 R_t 10.9 min. For amphetamines, MDMA D5 was used as an internal standard with, *m/z* 158, 294 R_t 9.6 min, amphetamine (AP), *m/z* 140, 118 R_t 6.8 min; methamphetamine (MA), *m/z* 110, 118 R_t 7.6 min; Figure 5.4 and Figure 5.5.

Table 5.1: Parameters set on GC-MS/MS system for detecting Δ9-THC, Cocaine, MA, MA.

	Δ9-THC	Cocaine	Amphetamine
Inject Temperature	270°C	280°C	260°C
Injector Pressure	30 PSI for 1min	30 PSI for 1min	30 PSI for 1min
Injection Volume	3 µl hexane	3 µl hexane	3 µl hexane
Voltage	2000 V	2000 V	2300 V
MS temperature	280° C	280° C	280° C
RT	-Δ^9-THC ≈ 9.4min	-cocaine ≈ 9.9 min	-(AP) ≈ 6.8 min
	-THC-COOH ≈ 13.0 min	-Benzoylecgonine ≈	-(MA) ≈ 7.6 min
	-OH-THC ≈ 11.8 min	10.9 min	
	-Δ^9-THC D3 ≈ 10.35 min		

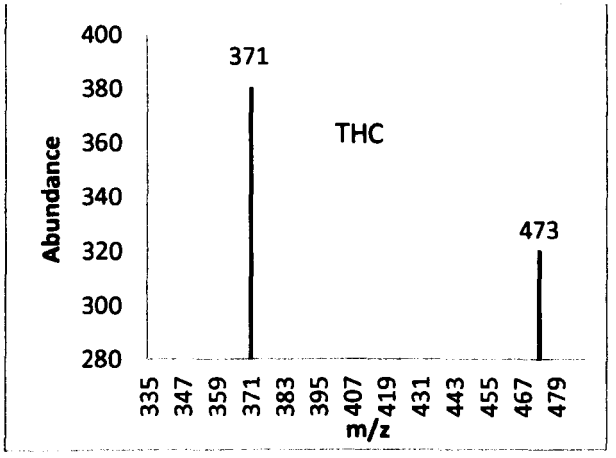
5.13 Data analysis & method validation

The limit of detection (LOD) was determined according to the signal-noise-ratio (S/N) equal to or greater than 3 (S/N≥3). Limit of quantification (LOQ) was determined depending on whether the S/N is equal to or greater than 10 (S/N≥10). Using cocaine-d3, THC-d3 and MD5 as internal standards, calibrator peaks were integrated to produce a linear calibration graph. This graph was used to determine the drug content (ng/mg hair) in the control and unknown samples. A full scan spectrum was used to determine the retention time of each drug and its respective fragment ions. These ions were later used in the SIM data selection. The drugs were finally verified by matching retention times and the fragment ions on the full scan spectra with a pure drug standard (control) on the same GC-MS machine and using the same derivatisation method in the case of AM Figure 5.6.

Samples for quantitative validation at low, medium and high drug concentration levels were prepared by spiking negative blank hair controls in a range of 0.01- 0.70 ng/mg of each drug to assess the inter (between different days), intraday (within day) variability, precision, % CV and accuracy (%Bias) for AM, MA, Δ^9-THC and cocaine. The % analytical recovery was assessed at three concentrations.

5.14 Method validation

GC-MS validation was achieved by spiking 50 mg of blank hair with solutions of the analytes in 60 µL hexane resulting in calibrator concentrations of 0.02, 0.05, 0.1, 0.5, 1 and 1.5 ng/mg for Δ^9-THC and cocaine Figure 5.5. Cross contamination was tested by running different blank hair samples. The results indicated there were no peaks in the region of both drug's elution times. The concentrations of the metabolites were calculated by comparison of peak/area ratio of the drug with IS to those from the calibration curve. The analytical measurement range was from 0.02 to 1.5 ng/mg.


400
380
360
340
320
300
280
Abundance
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THC
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395
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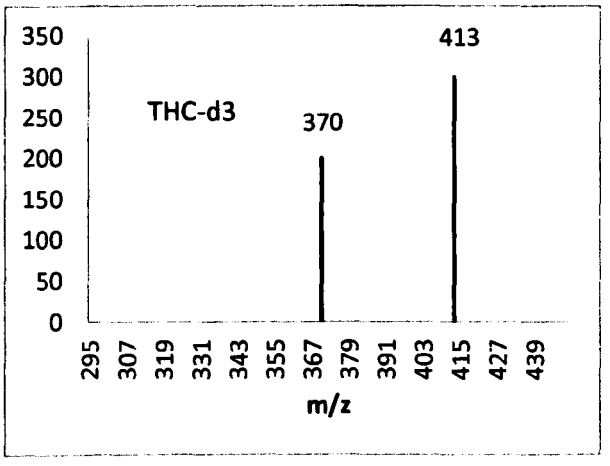
467
479
m/z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THC-d3
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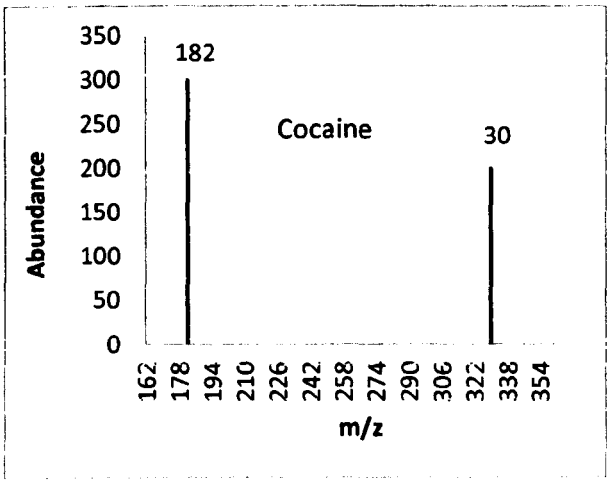
439
m/z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Cocaine
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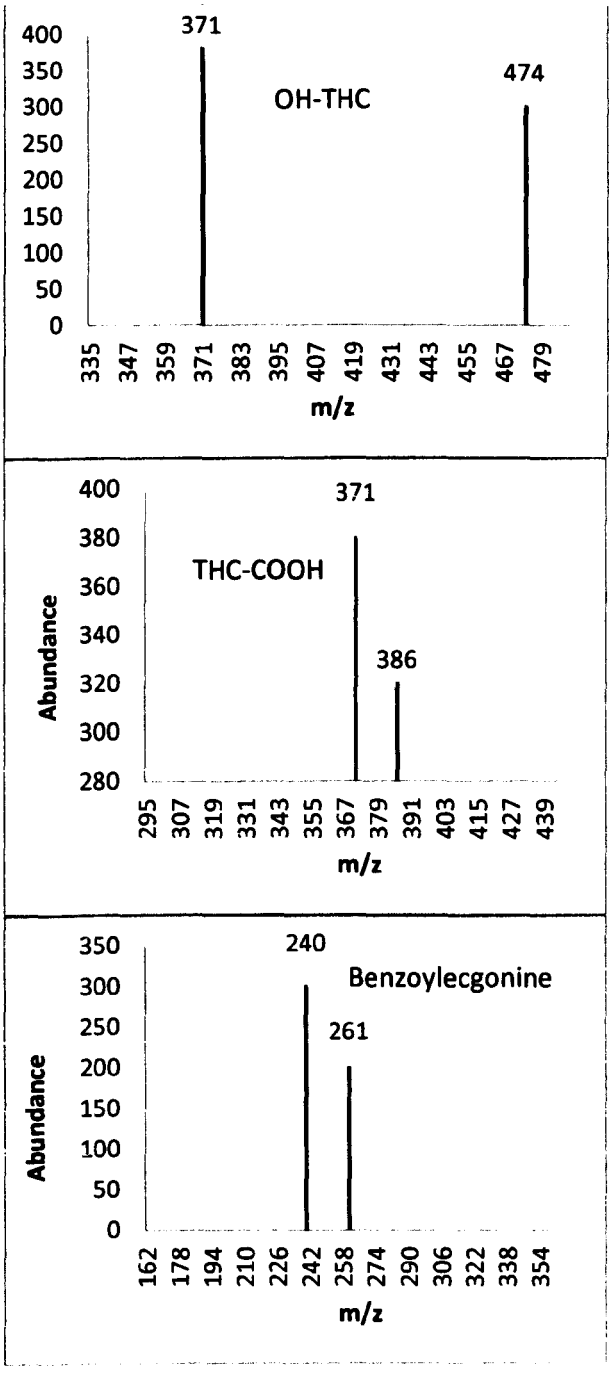
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OH-THC
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360
340
320
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280
Abundance
371
THC-COOH
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m/z
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Abundance
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261
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m/z

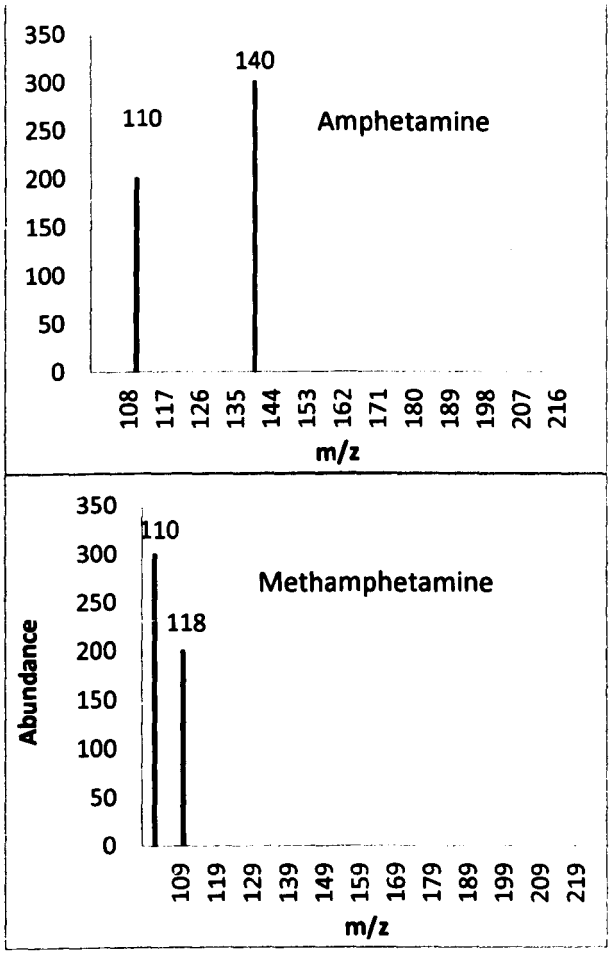


Figure 5.4: Mass spectrometer ion fragments of drugs and metabolites

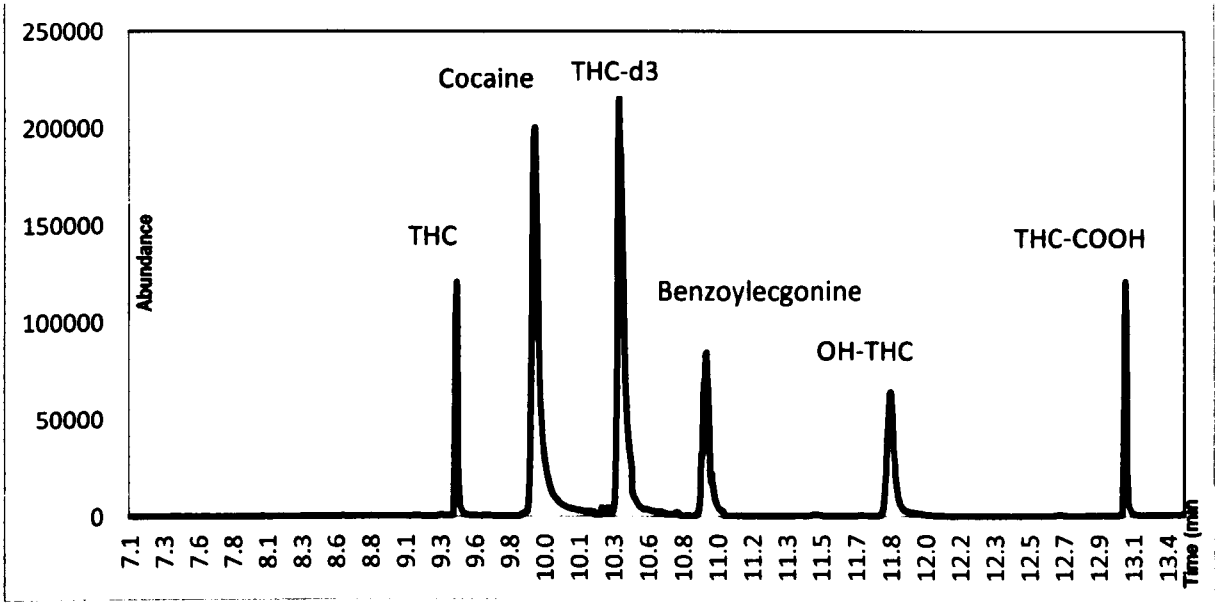


Figure 5.5: Chromatogram for Δ9-THC, cocaine and their metabolites

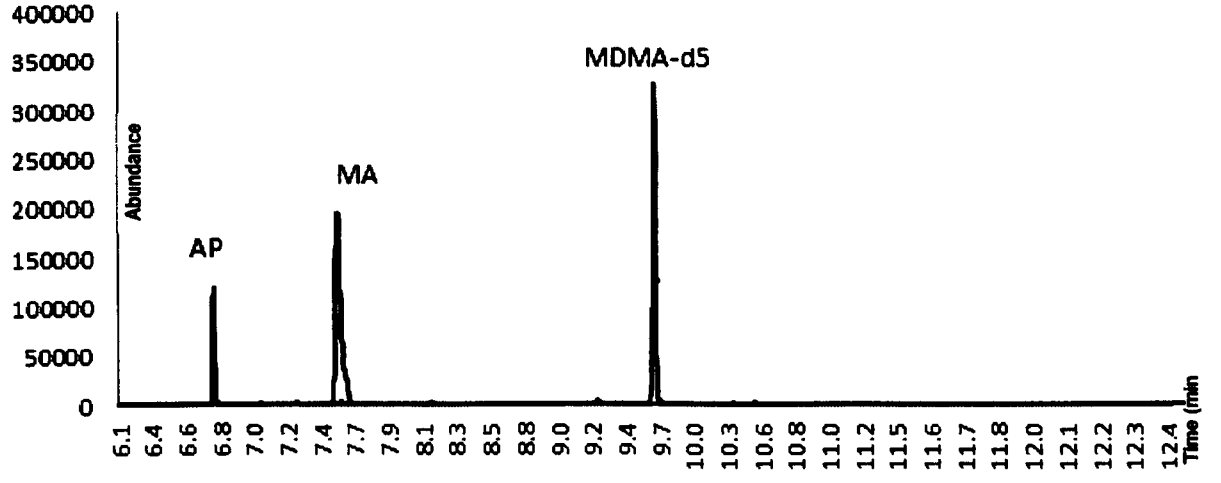


Figure 5.6: Chromatogram for amphetamines

5.15 Results & discussion

5.15.1 ELISA

The initial ELISA screening using the hair samples derived from Turkish participants was carried out. Among these hair samples, 18 had shown positive results. The positive 18 samples were further analysed to confirm for drug abuse using GC-MS/MS.[14]

5.15.2 Amphetamine and Methamphetamine

Linear calibration graphs were established at concentrations ranging from (0-3 ng/mg) for AM and MA. The calibration curves were prepared by spiking known concentrations of AM and MA to blank hair samples at (1, 1.5, 2.0, 2.5 and 3.0 ng/mL) or (0.05, 0.08, 0.1, 0.15, 0.2 ng/mg hair).

Figure 5.7 displays the GC chromatograms of spiked known concentrations at 2 ng/mg. The first smaller peak in

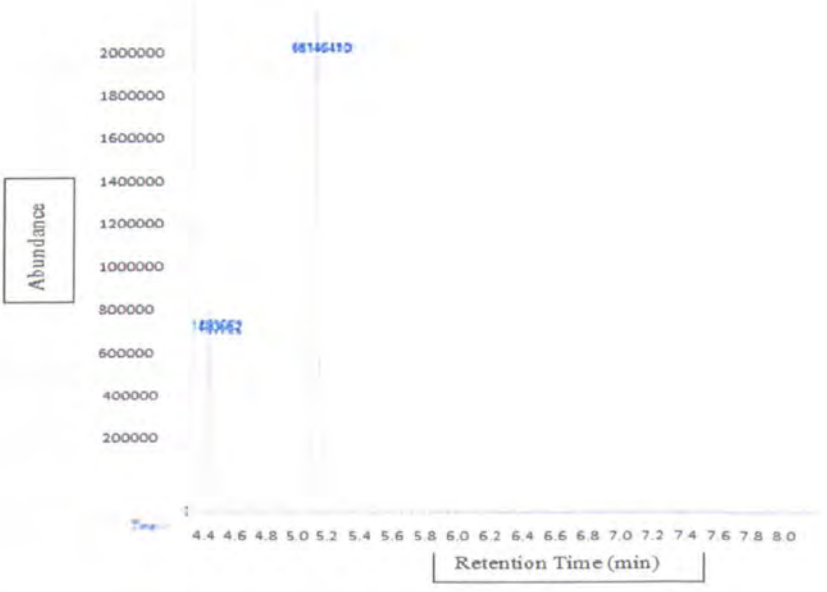


Figure 5.7 indicates the elution of AM and the larger peak indicates the later elution of MA. The shape of the peaks for AM and MA for each chromatogram also look comparable. The peak areas had shown to elevate with increasing concentrations.

The peak area for 1 ng/mg concentration exhibited the following; abundances were AM was (8986359) and for MA (23251130). The abundances then exhibited increases for 3 ng/mg, AM was (23041080) and MA was (75508116).

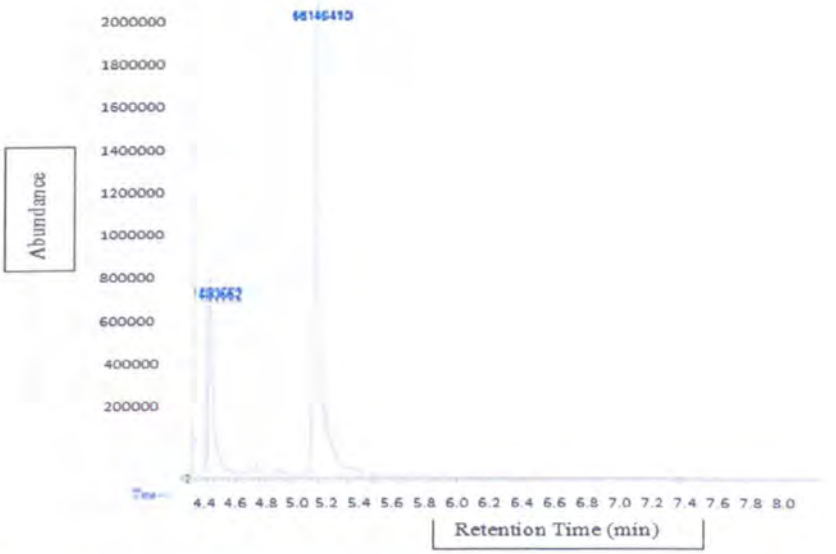


Figure 5.7: GC chromatograms displaying peak area for samples spiked with known concentrations (2 ng/mg) to produced calibration graphs for AM and MA.

5.15.3 Quantification

The peak areas of both AM and MA peaks were then divided by the peak area of the internal standard to determine the concentration using the abundance ratio. The abundance ratio against AM and MA known concentrations in (ng/mg) are used to produce a linear calibration curve. GC chromatograms provide adequate separated peaks to distinguish AM and MA analytes.

The peak areas of AM and MA were divided by the peak area of the internal standard to determine the abundance ratio and thereby the concentration. The

calculated abundance ratio was then cross-referenced with the calibration graph, Figure 5.8 and Figure 5.9 to determine the actual AM and MA concentrations (ng/mg) in unknown hair samples. All error values were determined by calculating the SD of each drug.

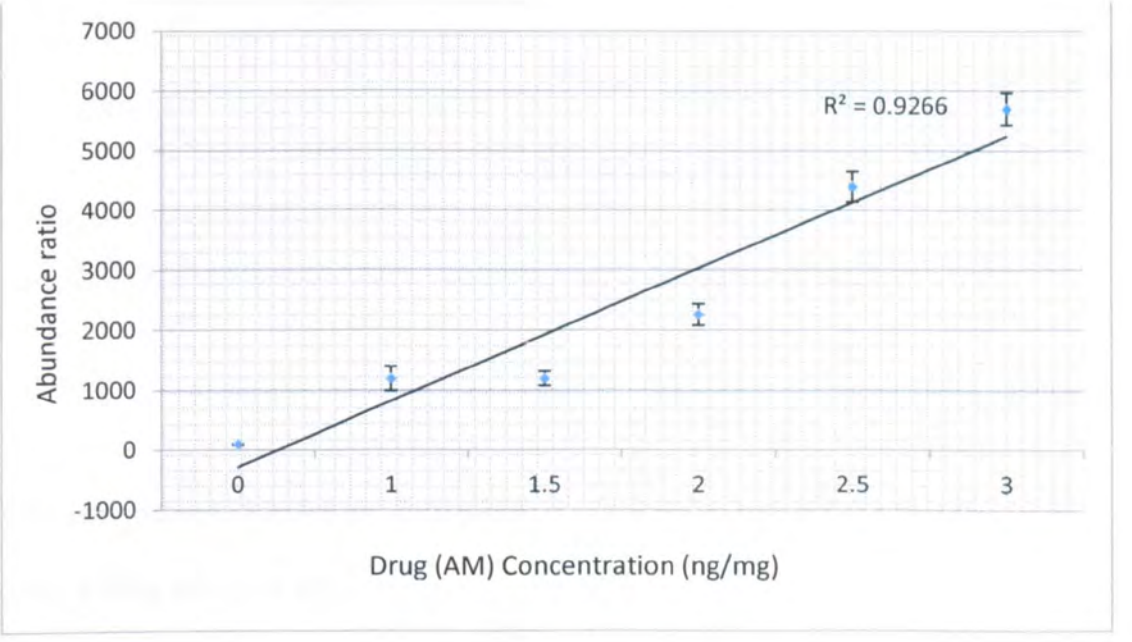


Figure 5.8: Calibration graph of known concentrations of AM against abundance ratio (n=6).

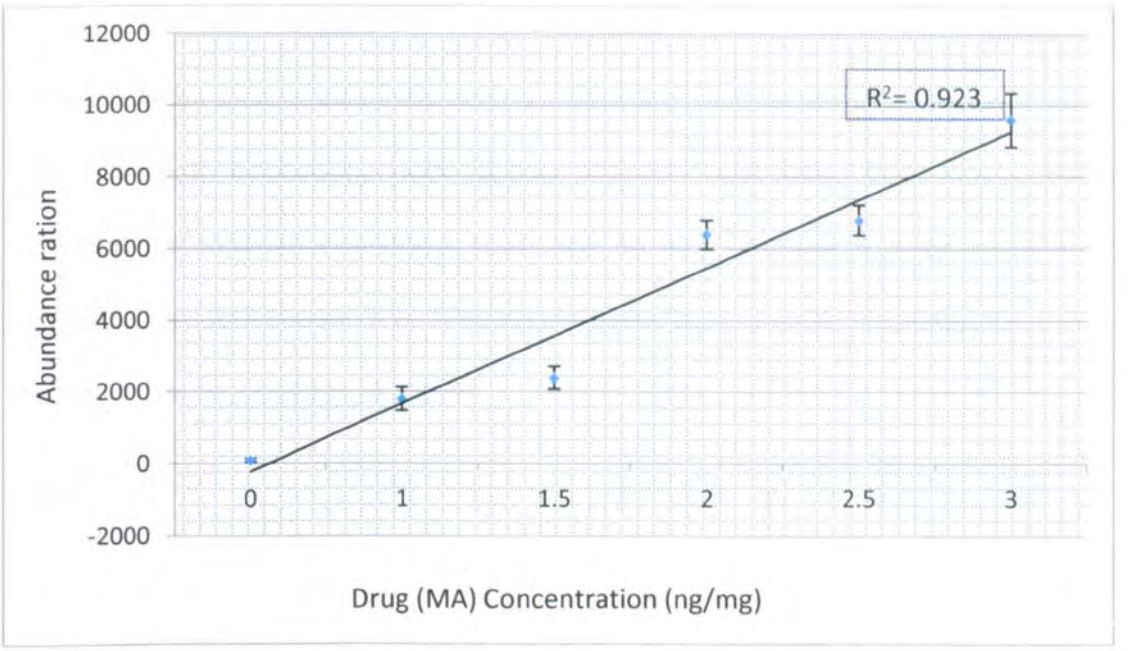


Figure 5.9: Calibration graph of known concentrations of MA against abundance ratio (n=6).

The chromatogram prior to derivatisation displays a reduced peak area, broader peak, tailing and poor separation as a result of the less volatile small molecular mass of AM (MW=135) and MA (MW=149) molecules. This is the reason why samples were derivatised using TFAA to provide improved chromatographic sensitivity and specificity, hence providing more informative fragmentation of the analyte of interest. Figure 5.10 and Figure 5.11display the difference in AM and MA separation through the eluted peaks before and after derivatisation of a control compound. Post derivatisation can provide a more sensitive and improved separation, therefore making it easier to differentiate between AM and MA The derivatised sample had eluted 2 tight peaks in comparison to almost a single peak with subsequent tailing by

the un-derivatised sample. The increased volatility and molecular weight, and altered polarity as a result of the derivatisation provided improved chromatographic selectivity and non-tailing peak shapes for AM and MA. Post derivatisation increased the MW of AM-TFAA (m/z 140) to m/z 231, and MA-TFAA MW of m/z154 to m/z 245 Figure 5.12. The eluted peaks from the GC chromatogram were confirmed by the MS fragmentation.

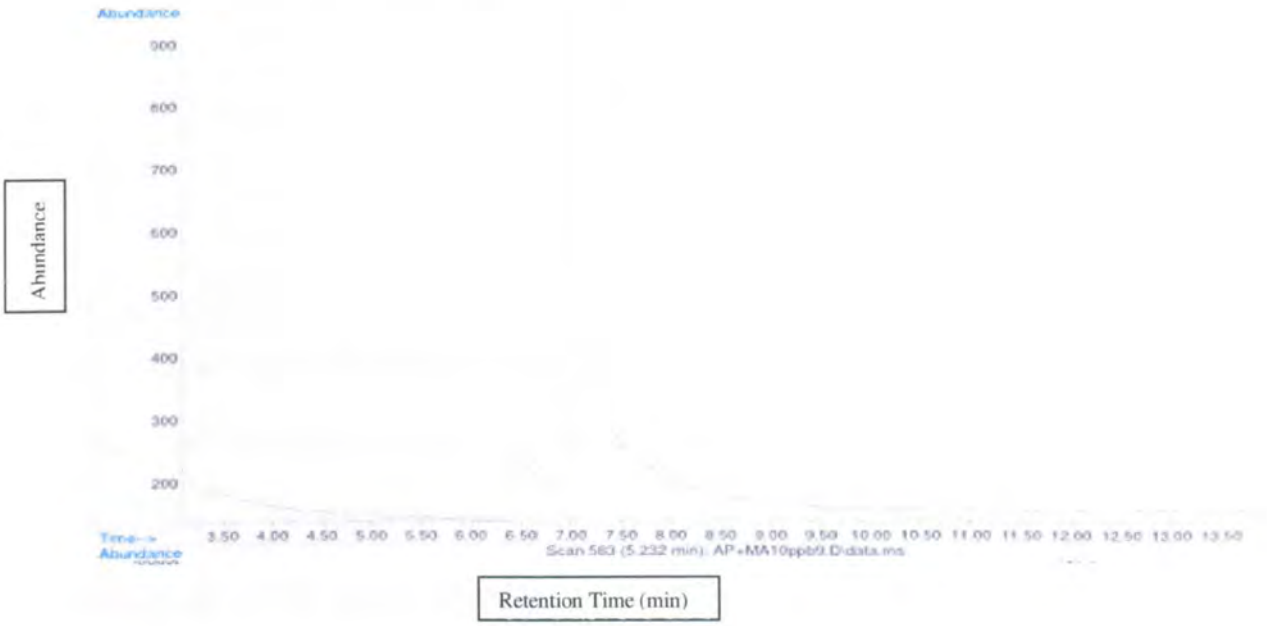


Figure 5.10: GC-MS/MS chromatogram prior to derivatisation. Small peak area and tailing of peak explains the reduced sensitivity and poor separation. Difficult to distinguish AM and its derivative MA.

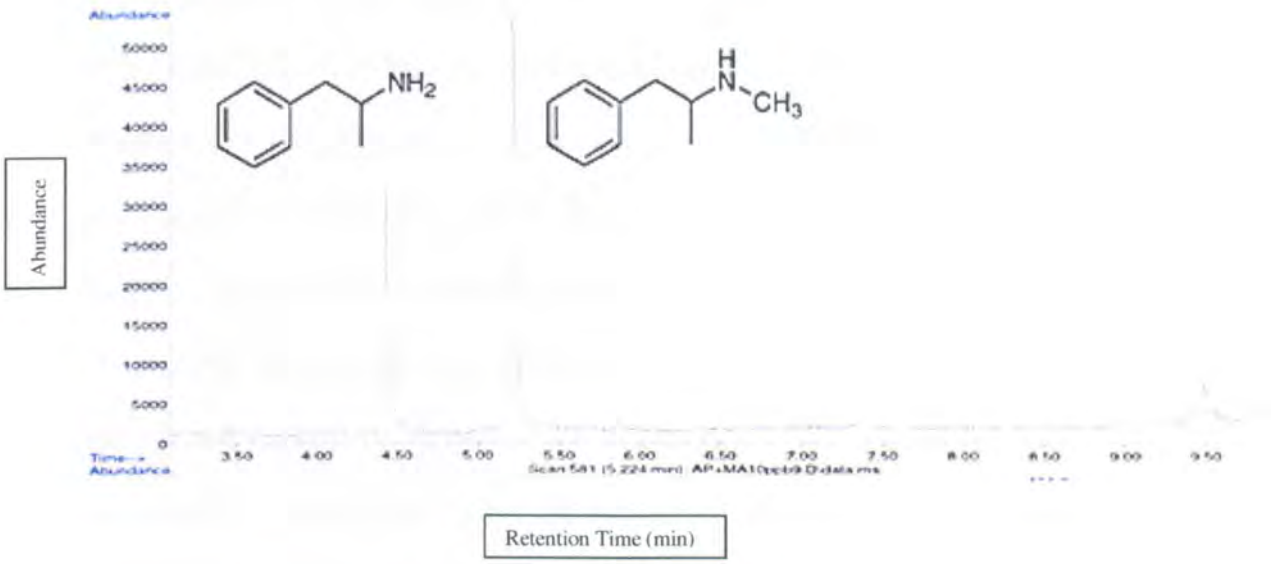
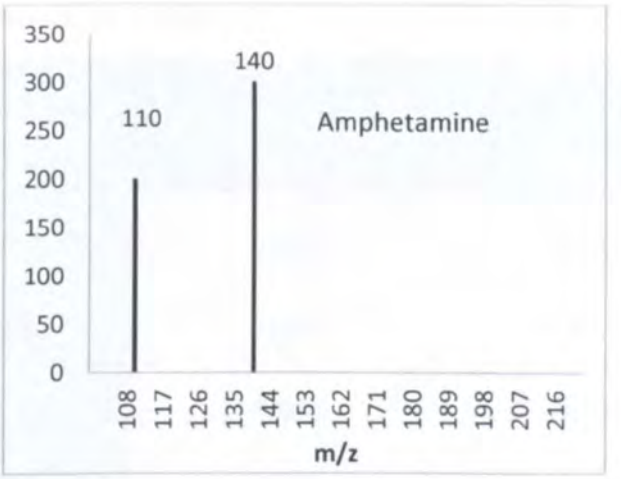


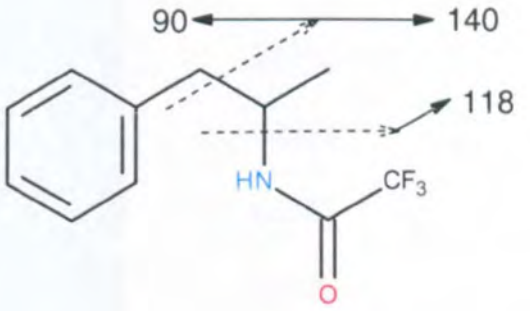
Figure 5.11: GC-MS/MS spectra post derivatisation using TFAA.

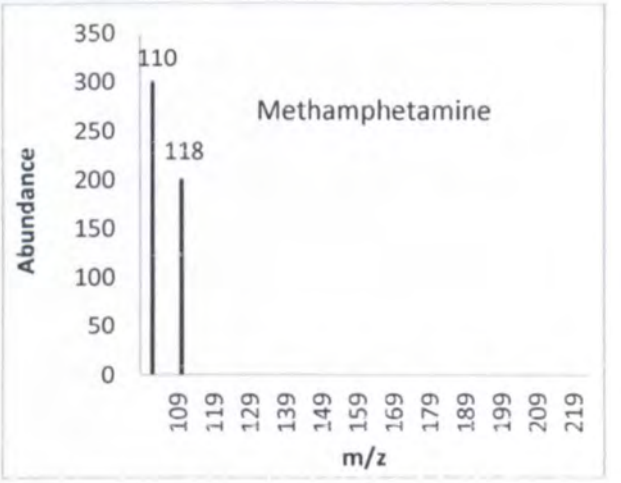
Figure 5.11: Wider peak area and presence of baseline between peaks can provide an improved detection limit.

The LOD for AM and MA was (0.02 ng/mg) and (0.01 ng/mg) respectively, according to the signal to noise ratio (S/N) (<3). The LLOQ for AM was (0.04 ng/mg) and (0.02 ng/mg) for MA. The intra and inter day accuracy (% Bias) and precision (%CV) were evaluated by spiking quality control (QC) blank samples (n=6) with three known AM and MA concentrations (AM= 0.16, 0.32, 0.70 ng/mg) and (MA= 0.1, 0.2, 0.5) in one day for the intra-day study. For the inter-day study, each concentration was analysed on three different days. The average concentrations and standard deviation (SD) for AM for intra and inter day ranged from (0.16-0.69 ± 0.01-0.05 ng/mg) and (0.16-0.73 ± 0.01-0.06 ng/mg) (Table 5.3). For MA, the average concentrations for intra-day and inter-day ranged from (0.11-0.52 ± 0.01-0.05 ng/mg).

The intra and inter day accuracy (% Bias) for AM ranged from (98.02-98.33%) and (101.04-103.8%). The % accuracy for MA had ranged from (96.67%-109.20%) and (96.67%-108.3%) for intra and inter day. These limits for both are still acceptable, since they are between the ±15 % different (see Validation section in material and methods chapter). The analytical recovery was determined by comparing the analysis of extracted and non-extracted spiked samples at low (0.16 ng/mg), medium (0.32 ng/mg) and high (0.70 ng/mg) of six replicates for AM and at low (0.01 ng/mg), medium (0.20 ng/mg) and high (0.50 ng/mg) for MA (Table 5.4). The recovery was over 90% and the regression coefficient (r^2) for AM was (0.92657) and (0.92362) for MA Figure 5.8 and Figure 5.9. Among the 8 unknown hair samples, AM and MA concentrations as low as (0.08 ng/mg) and (0.30 ng/mg) were able to be detected (Table 5.2).







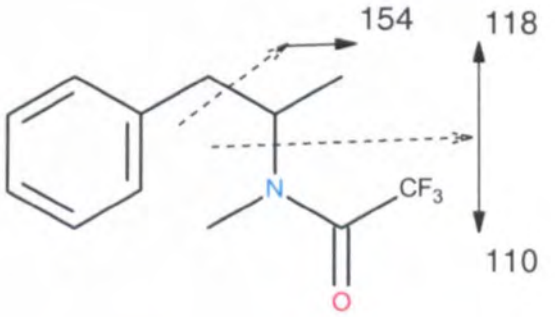


Figure 5.12: GC full scan spectra a) represents peak ions 140, 110 m/z and fragmented structure of AM b) represents peaks ions 154, 118 m/z and fragmented structure of MA

Table 5.2: AM and MA concentrations (ng/mg) for 7 positive unknown samples analysed by GC-MS (n=6).

Sample	Hair samples	Amphetamine (ng/mg)	Methamphetamine (ng/mg)
1	G004	0.1 ±0.06	0.30 ±0.035
2	G017	0.13 ±0.02	0.79 ±0.03
3	G018	0 ±0.00	0.55 ± 0.05
4	G029	0 ±0.00	0.50 ±0.06
5	G030	0 ±0.00	0.95 ±0.016
6	G033	0.2 ±0.025	1.10 ±0.05
7	G034	0 ±0.00	0 ±0.00
8	G037	0.08±0.003	1.50 ±0.2
	Mean	**0.13**	**0.81**

Table 5.3: AM intra-day and inter-day validation for GC-MS analysis (n=6).

Drug	Control level (ng/mg)	Average	Standard deviation (SD) ±	% CV	Accuracy (% Bias)	LOD ng/mg	Recovery (%)
Intra-Day	LLOQ(0.04)	0.04	0.005	12.55	90.00		
	QCL (0.16)	0.16	0.01	5.01	98.02	0.02	
	QCM (0.32)	0.33	0.01	4.40	103.44		0.16 ng/mg = 95.82%
	QHC (0.70)	0.69	0.05	7.49	98.33		0.32 ng/mg = 99.50%
Inter-Day	LLOQ (0.04)	0.04	0.01	14.08	91.67		0.70 ng/mg = 96.50%
	QCL (0.16)	0.16	0.01	7.23	101.04	0.02	
	QCM (0.32)	0.33	0.02	7.24	103.60		
	QHC (0.70)	0.73	0.06	7.91	103.8		

Table 5.4: MA intra-day and inter-day validation for GC-MS-MS analysis (n=6).

Drug	Control level (ng/mg)	Average	Standard deviation (SD) ±	% CV	Accuracy (% Bias)	LOD ng/mg	Recovery (%)
Intra-Day	LLOQ (0.02	0.02	0.005	23.14	112.50		
	QCL (0.1)	0.11	0.01	12.81	106.67	0.01	
	QCM (0.2)	0.21	0.02	7.97	105.00		0.01 ng/mg = 93.94%
	QHC (0.5)	0.52	0.05	8.69	104.00		
Inter-Day	LLOQ (0.02)	0.02	0.01	24.84	114.17		0.20 ng/mg = 101.61%
	QCL (0.01)	0.10	0.02	16.89	96.67	0.01	
	QCM (0.02)	0.22	0.01	3.45	109.20		0.50 ng/mg = 97.20%
	QHC (0.5)	0.54	0.01	1.39	108.3		

Linear calibration graphs were established at concentrations ranging from (0.1-1 ng/mg) for Δ^9-THC. The calibration curves were prepared by spiking known concentrations of THC-D_3 to blank hair samples at (0.1, 0.2, 0.4, 0.6, 0.8 and 1 ng/mg). The calculated peak area from GC chromatograms for spiked concentrations was divided by the peak of internal standard to determine the abundance ratio. The linear calibration curve was then produced via plotting abundance ratio against known concentrations (ng/mg hair) Figure 5.13. The standard calibration was used as a reference to determine the Δ^9-THC and cocaine concentrations (ng/mg) in unknown hair samples. The GC chromatogram (Figure 5.14) displayed a peak representing Δ^9-THC eluting at (RT=9.4 min). The elution time was confirmed by R_t of pure Δ^9-THC as a reference. Using the abundance ratio, the actual Δ^9-THC concentrations (ng/mg) in the unknown hair sample was determined.

The method was validated through verifying the method linearity, limit of detection (LOD), limit of quantification (LLOQ) intra-day, inter-day precision, and accuracy, and extraction recovery. The sensitivity of the method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) for the analyte. The LOD and LLOQ for the analytical method for Δ^9-THC detection was (LOD = 0.01 ng/mg) and (LLOQ = 0.02 ng/mg). The intra and inter day accuracy (% Bias) and precision (%CV) were evaluated by spiking QC blank samples with a known concentrations in one day for the intra-day study (same day) and in 3 days for inter-day (Table 5.5). The inter and intra-day percent accuracy ranged from (102-115.50%) and (95.4-110.1%). The analytical recovery of Δ^9-THC was determined by

comparing the analysis of extracted and non-extracted spiked samples at low (5 pg/mg hair), medium (20 pg/mg hair) and high (40 pg/mg hair). The recovery of Δ^9-THC was over 90% and the regression (R^2) of the calibration curve was 0.95303.

Table 5.5: Δ9-THC Intra-day and Inter-day Validation data for GC-MS analysis (n=6).

Drug	Control level (ng/mg)	Average	Standard deviation (SD) ±	Precision (% CV)	Accuracy (% bias)	LOD ng/mg	Recovery (%)
Intra-Day	LLOQ(0.02)	22.42	3.44	15.35	112.08		
		41.45	6.46	15.58	103.63	0.01	
	QCL (0.04)	115.50	9.20	7.97	115.50		0.005 ng/mg
	QCM	1020.0	56.57	5.55	102.00		=102.17%
	(0.10)	0					0.02 ng/mg =
	QHC (1.0)						100.92%
	LLOQ	0.6	0.1	18.1	114		0.04 ng/mg =
Inter-Day	(0.02)	2.8	0.2	7.0	110.1	0.01	97.48%
	QCL (0.04)	4.8	0.2	5.0	95.4		
	QCM	20	0.6	2.9	100.1		
	(0.10)						
	QHC (1.0)						

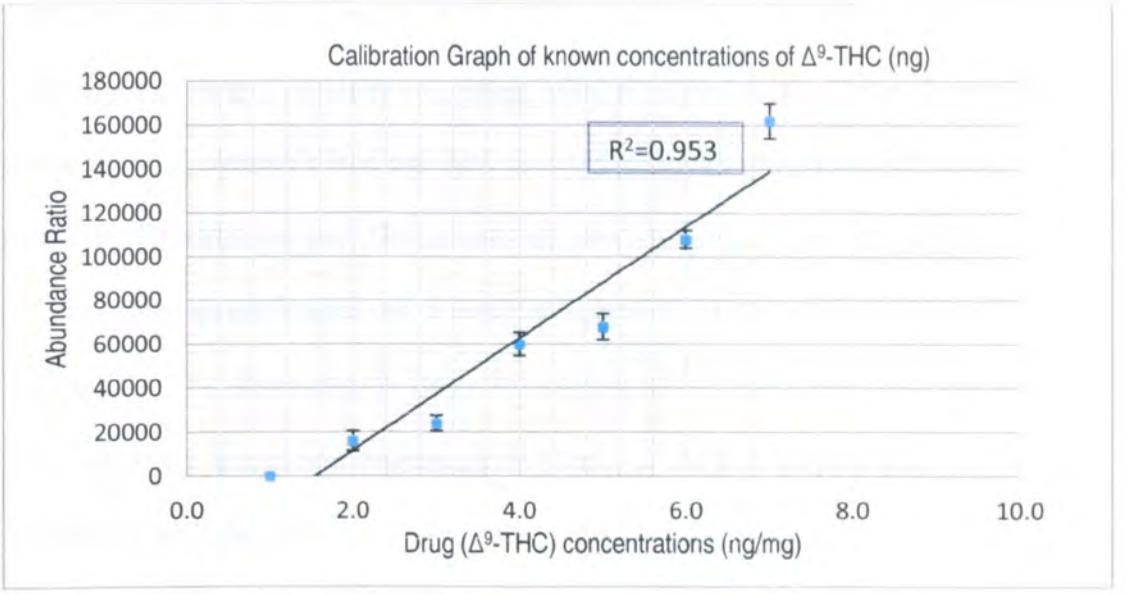


Figure 5.13: Calibration graph of known drug concentrations of Δ9-THC (n=6).

Figure 5.14: Chromatogram displaying the peaks of unknown hair sample 5. Peak eluted at 12.7 min represents Δ9-THC.

The results indicate the method is efficient in detecting AM, MA and Δ^9-THC at low levels when 20-50 mg of hair was used for analysis, particularly when working with

limited samples. The method was able to detect as low as (0.04- 0.5 ng/mg) for amphetamines, (0.015 ng/mg) for Δ^9-THC and (0.03 ng/mg) for cocaine. Similar ranges have been reported in other studies. Wu Y.H, 2007 were able to simultaneously quantify AM and MA at concentrations as low as (0.03-0.05 ng/mg) using GC-MS/MS analysis. The results of their study confirmed the suitability for a simultaneous quantification of a broader spectrum of drugs through a single hair specimen (175). Similarly, a study by Martin et al, 2005 had also been able to simultaneously determine enantiomeric ratios of AM, MA and other derivatives. Among 11 hair samples tested, the study had found 8 samples containing AM and MA. The ranges of AM were (0.17-0.59 ng/mg) and from (0.12-0.31 ng/mg) for MA. In fact, their method had also been able to detect AM and (MA) alone in two hair specimens; suggesting ingestion of optically pure substances (176). A different approach of quantifying AM, cocaine and other metabolites was taken by Paterson and Cordero, 2006. Through a two-step derivatistion method using MBTFA and MSTFA, + 1% TCMS agent and GC-MS analysis via full scan and SIM mode; the study was able to determine LOQ equivalent to 0.1 and 0.2 ng/mg for AM and cocaine.

The relatively small study group in comparison to the 115 participants in this study may have the largest impact on the results. There are also the considerations of high subject-to-subject variability of Δ^9-THC pharmacokinetics and various routes of administration taken by participants. The increased systemic bioavailability of inhaled Δ^9-THC in comparison to smoking may provide variations in the detection limits. The reported dosage levels, frequency and time frame of abuse (months to years) may also play a role. It was unable to detect cocaine concentrations in the hair samples of participants, however according to literature the expected cocaine

concentrations should ideally range between (0.1-28.9 ng/mg). These ranges were confirmed by a sensitive method developed by Cognard et al, 2005 to quantify cocaine and related metabolites in hair of long term cocaine abusers (who had to fill a questionnaire of the period of drug abusing). The study had found cocaine ranging between (0.5-5.0 ng/mg) (177,178). The limit of detection for AM, MA and cocaine in the urine had ranged between (12.5-50 ng/mL) (179). Later Adriaan A.S et al, 2009 had been able to accurately and precisely detect similar ranges for AM, of 1.5-6.25 ng/mL, and various analytes in the urine through extractive-derivatisation and rapid GC-MS analysis. Their analysis on urine samples from a large study group of 110 individuals enabled successful detection of AM and MA at levels as low as 11 and 19 ng/mL, with a limit of quantification of 20 ng/mL. These results clearly show the ability to detect higher levels of AM, MA and cocaine in urine samples in comparison to hair samples (180).

The reason for such higher concentrations detected in urine in comparison to hair is due to the pharmacokinetics of the drug via the different routes of administration. The rapid absorption into the blood stream, metabolism through appropriate enzymes and renal excretion of the drug via urine, can improve the detection time and reduce the opportunity for drug degradation. Although urine analysis has the ability to provide rapid drug detection, its drawbacks include limited drug history, increase risks of false positives and samples can be easily tampered with. The limits of drug detection are low with hair specimens in comparison to blood and urine, but these can be outweighed by the advantages of providing a larger surveillance window (weeks-months), long term drug histories and ability to determine frequency of use. If the drug is not metabolized into its metabolites, this allows us to detect the parent compound of the drug in the hair matrix. If however the drug is metabolized, this can

lead to partition of the parent compound into numerous metabolites such as THC-COOH, OH-THC, Benzoylecgonine. Phase 1 and 2 Metabolism can lead to biotransformation of the drug into more polar metabolites and facilitate the excretion and elimination of the drug. The consequences of such changes may decrease the half-life and accumulation of the drug, therefore increase the renal excretion (52).

The classes of drugs and concentration of these drugs detected may vary among different subject groups. This can depend on the sample size and the targeted population for the study. It is difficult to point out the exact explanation for variations in the results that have been presented, and in comparison to others, without knowing the subjects drug history. There are clear variations in the detectable levels between long and short exposure to drugs. Clearly evident from the results of the study by Skopp et al, 2007, where an increasing amount of cannabinoids including its derivatives had been detected with increasing cumulative dose over a period of months in comparison to no exposure by control subjects (66). As with increasing age, the deterioration in renal function can have a large impact on the excretion of drugs. The reduced excretion of both therapeutic and harmful substances can be detected at large concentrations or have a substantial risk of drug toxicity. The degree of drug incorporation into hair has a large influence on the quantification and detection of drugs. Several studies have confirmed the increasing incorporation of drugs in individuals with high melanin content in the hair. Although two subject groups may have consumed the same drug concentrations, the variability in the results may still lie among different ethnicities or racial groups. The different hair colours, textures, conditions and cosmetic treatments among participants may also have influenced the results obtained here and by other researchers in literature.

Lower detection limits of analytical methods have improved accuracy of hair testing methods therefore providing future scope for analysing particular psychoactive drug abuse, potential toxic therapeutic drugs or several other harmful substances of abuse. Hair analysis can provide several advantages over traditional methods of detection. Nevertheless, the limitations of hair analysis include the biological variations of hair growth, multiple mechanisms of drug incorporation, effect of external factors on stability of and retention of drugs and role of pigmentation; which may vary among different ages, genders, race and ethnicity. The frequency of drug of consumption and lag time between drug administration and appearance in the hair may vary among individuals.

The false positive results obtained from above study made it necessary to investigate this issue further in detail. The following part of the study examines the reasons leading to these false results and ways to overcome this obstacle.

5.16 False positive drug results by ELISA associated with enzymatic hair digestion

This experiment has been designed based on the previous study to develop an immunological screening test followed by a GC–MS confirmation method for the simultaneous analysis of Δ^9-THC, THC-COOH, OH-THC, cocaine, benzoylecgonine (BZ), amphetamine (AP), methamphetamine (MA) in human hair and avoiding the significant factors responsible for drug degradation by acid and alkali hydrolysis and to obtain optimal recovery conditions. Enzymatic hair digestion was used to hydrolyse 18 Turkish samples using Proteinase K, Dithiothreitol and Tris HCl buffer. Ail samples were positive when screened by ELISA for Δ^9-THC and cocaine.

5.17 Enzymatic digestion and analysis

Cross reaction lead to false positive results in the pre-screening step as a result of the degradation of the antibodies in the pre-coated ELISA microplate. In this study, the method investigated the pattern of drug screening and analysis. At the beginning, 18 out of 18 samples screened positive on ELISA, but later it was revealed that only 2 samples were positive for Δ^9-THC and THC-COOH. Hair (50 mg) was digested enzymatically with Proteinase K and the sample was incubated in a water bath at 37 °C for 1 h. Using a threshold of 0.1 ng/mg in 50 mg hair, two positive results were found in 18 cases. 16 out of 18 positive detections were not be confirmed by GC–MS. It was concluded that the use of hair analysis can reveal both unknown

drug use, as well as confirm a period of drug consumption. The proposed GC-MS method showed high sensitivity, was simple and easy to perform and showed appropriate feedback for screening purposes.

5.18 Desalting column

5.19 Protein desalting spin column preparation

Desalting Zeba column (Thermo Scientific, UK) is a size-exclusion chromatography resin that provided excellent protein performance to desalt the enzymatic buffer of protein samples.

Desalting was performed by unsealing the bottom through twisting and loosening the cap to prepare it for the run. The 5 mL columns were inserted in 15 mL tubes as collection tubes. The columns were then centrifuged at 1000 × g for 2 minutes to remove the stored solution and then a mark was placed on the side of the column where the compacted resin is slanted upward. The columns need to be conditioned by the addition of 2.5 mL of the buffer and centrifuged at the same speed for 2 minutes to remove the buffer. This step was repeated 3 times. New, 15 mL collection tubes were used for the samples' desalting. The 1 mL enzymatic digested samples were slowly added to the centre of the compact resin bed. At the final step, the Zeba columns were centrifuged at 1000 × g for 2 minutes to collect the sample. The columns were discarded after use without the option of reusing it. The collected samples were then screened by ELISA.

5.20 Discussion

Enzymatic digestion can provide a recovery of 80% for cocaine metabolite, while alkaline hydrolysis causes its chemical destruction (166). The verification of alkaline destruction of psychoactive drugs and their metabolite was achieved by a recovery study that was carried out on reference specimens, where it was reported a loss of drugs due to alkaline digestion with a lower recovery as compared to direct spiked hair extraction (60,166). In order to ensure the maximum amount of drugs were retained, enzymatic digestion was used. Cross-reactivity was observed for the enzymatically-digested metabolite.

The ELISA assay carried out on selected drugs of enzymatically hydrolysed samples was positive in 18 cases; higher readings with respect to the values expected were observed for enzymatically digested samples, where DTT and Pro K were used. Three experiments with different sample preparation methods were used to evaluate the optimum digestion method. A replicate of 30 ng/mg of spiked hair sample and a blank sample were screened on ELISA, the comparison of the enzymatically digested sample with the control showed very high concentration values. The results of these preliminary tests are illustrated in Table 5.6. After many replicates and different digestion methods, it appeared that the cross reactivity was the result of the enzymatic digestion. Instead, no significant interferences of Solutions B and C were observed when digested differently with the ELISA kit.

Table 5.6: Mean recovery of a spiked hair sample with different methods

	Info	ELISA Recovery (ng/ml)
Solution A	Hair + Proteinase K + DTT+ buffer	65
Solution B	Hair + NaOH + HCL + buffer	27
Solution C	Hair + Methanol + buffer	28

For composition of individual solutions used see ***Materials and methods***

The desalting columns were able to filter the samples of proteins which are bigger than 1000 daltons (Da). When samples were screened by ELISA it gave the same false positive values. The desalting columns were capable of desalting some of the proteins (digestion enzyme Pro K) without being able to avoid the negative effect of the 5% of the Pro K left behind after the filtration step (95% can only be desalted according to Thermo Scientific). Another factor that the desalting columns were able to avoid was DTT. The desalting column is not engineered to be able to trap DTT. The combined effect of the 5% of the enzyme left in the samples and the DTT led to the false positive results Figure 5.15.

According to the ELISA assay theory, the kit operates on the principle of competition between the enzyme conjugate and the drugs in the sample for a specific number of specific antibody sites on the pre-coated assay. Analytes are added at the beginning to the microplate wells, then followed by the enzyme conjugate and incubated at room temperature. A competition for the binding sites between the analytes and the ELISA conjugate takes place. After incubation, the wells are then washed, clearing any unbound extra materials. At the end, another incubation period takes place in the presence of the substrate which binds to the enzyme conjugate. As described in the materials and method section, the concentration of the drug in the competitive ELISA is inversely proportional to the colour intensity of the plate reader results. For example, the presence of the drug will show in a light colour or no

colour as the concentration of the analyte increases. The absence of the drug analytes in the microplate will result in a dark colour.

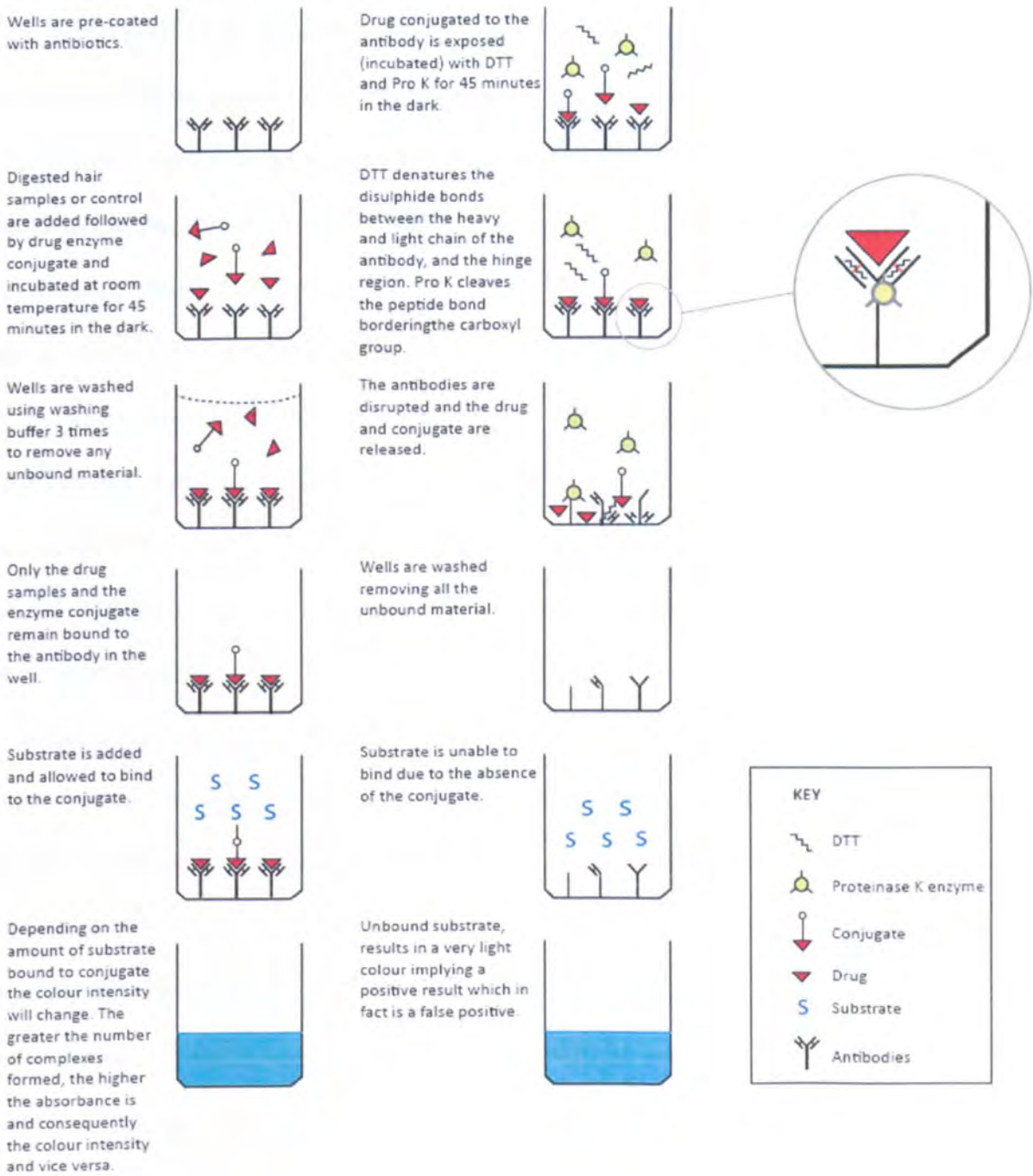


Figure 5.15: Normal and enzymatically digested samples procedure for competitive direct enzyme-linked immunosorbent assay (ELISA).

DTT is a strong reducing agent which has a redox potential of -0.33 V at pH 7 (181). The reduction of a typical disulfide bond proceeds by two sequential thiol-disulfide exchange reactions (181-183). The same principle apply to the antibodies in the plate and it proves that the oxidised DTT breaks the disulfide-bonded resulting in the denature of the disulfide bond-linked heavy and light chains and also at the hinge region of the ELISA's antibodies. Proteinase K extracted from fungus Tritirachium album is commonly used for its broad specificity and its ability to hydrolyse keratin (hair), hence, the name "Proteinase K". The main location of cleavage is the peptide bond bordering to the carboxyl group mostly on the fragment crystallisable region (Fc region) of aliphatic and aromatic amino acids with a blocked alpha amino group (131). DTT as a denaturing agent is used to unfold the protein substrates and make them more accessible to the enzyme to attack and break up the hair. After studying the function of both DTT and pro K, it appeared that both have an undesired effect on the particular antibodies. It was found that it has the same effect on the RIA assays. This was further proved when a couple of the microplate wells were washed with only a solution of Pro K and DTT followed by a blank sample and a spiked sample and all results were with a low absorbance value, strengthening the theory of the denatured antibodies. As shown in Figure 5.15, if the drug is not present more substrate-conjugate binding will occur and higher colour intensity should be seen under the microplate reader. On the other hand, when the antibodies are been destroyed by DTT and the enzyme, there will be no place for the conjugate or the antigen (drug in this case) to bind and therefore it results in no binding of the substrate- conjugate complex and showing no colour, but giving the assumption of low absorbance reading of the wells and therefore false positive results.

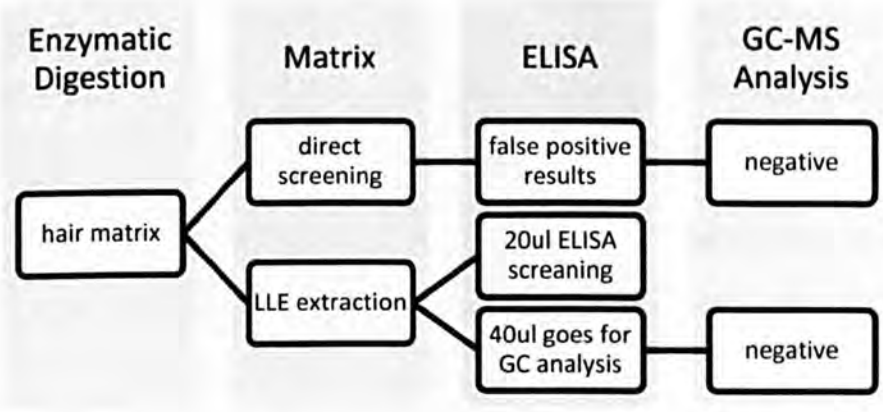


Figure 5.16: clearing of DTT and Pro K from the hair matrix.

Due to the false positive results that were appearing at the end of the ELISA screening, Liquid-Liquid Extraction was used to clear the DTT and deactivate Pro K enzyme inhibitor. Pentane was used in LLE due to its low polarity and since DTT is not soluble in pentane.

All the 18 hair samples were clear of the additives and extracted and screened again by ELISA Figure 5.16. The LOQ of ELISA using enzymatic digestion and extraction by pentane was 0.02ng/mg while the LOD of the GC-MS method was 0.015 ng/mg hair. The results this time were different, 2 samples appeared to contain Δ^9-THC on ELISA. GC-MS confirmed these samples contained Δ^9-THC and THC-COOH (Table 5.7).

5.21 ELISA validation

At the beginning of the validation process, several analytical parameters were first examined for ELISA. The optical absorbance of the calibration standards on the plate reader decreased as the concentration increased (0.04, 0.06, 0.08, 1, and 1.5 for

cocaine and metabolites and 0.02, 0.10, 0.50, 1.00 and 1.50 ng/mg for Δ^9-THC and metabolites. Positive control and the calibration curves were conducted for each new plate to ensure the kit was working properly. After this, negative quality controls (hair samples) were run for each series in the same kit (any reading on the plate reader was below 1.276 at 650 nm was considered positive and any reading higher than this value was considered negative) (Figure 5.17: Standard curve for ELISA cocaine results (n=3). The two positive samples detected on ELISA were not quantified, but were only qualitatively analysed. Repeatability of the assay was evaluated by screening spiked hair specimens with a known amount of drugs on the same day.

Table 5.7: Quantitative GC-MS hair results.

Samples	Δ^9-THC ng/mg	THC-COOH ng/mg	Cocaine and amphetamines and metabolites
Hair sample 1	ND	ND	ND
Hair sample 2	ND	ND	ND
Hair sample 3	ND	ND	ND
Hair sample 4	ND	ND	ND
Hair sample 5	ND	ND	ND
Hair sample 6	ND	ND	ND
Hair sample 7	ND	ND	ND
Hair sample 8	ND	ND	ND
Hair sample 9	**0.3**	**0.1**	**ND**
Hair sample 10	ND	ND	ND
Hair sample 11	ND	ND	ND
Hair sample 12	ND	ND	ND
Hair sample 13	ND	ND	ND
Hair sample 14	ND	ND	ND
Hair sample 15	**0.2**	**ND**	**ND**
Hair sample 16	ND	ND	ND
Hair sample 17	ND	ND	ND
Hair sample 18	ND	ND	ND

*ND: Not detected

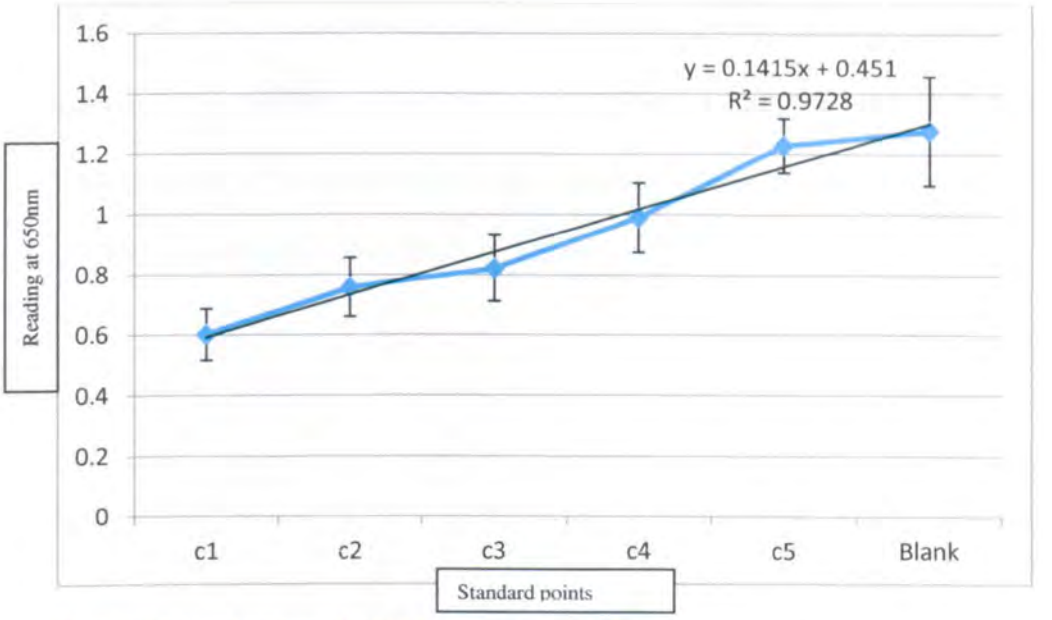


Figure 5.17: Standard curve for ELISA cocaine results (n=3).

5.22 Conclusion

There is an increasing interest in toxicological hair investigation as a marker of individual exposure to many toxicants such as illicit substances. This has made the analysis feasible by the extension of a simple, highly sensitive and reliable method of detection using gas chromatography mass spectrometry instead of many other techniques (184). Hair analysis for many substances can be difficult and expensive; also ELISA kits don't have a general acceptance due to their very narrow selectivity without being able to specify some drugs and metabolites. However, the rapid ELISA screening method can shorten the time and minimise the waste of resources on blank samples, so only positively screened samples continue their way through to confirmation analysis. The enzymatic hair digestion that was performed followed by

ELISA screening showed some interferences with ELISA and the enzymatic digestion. False positive samples appeared to be confirmed as negative on ELISA due to DTT and Pro K denaturing the antibodies inside the microplate wells. LLE and enzyme inhibitors were the key to overcome this problem. This removed 16 false positives lowering it down to only 2 users.

Chapter 5

Discrimination of car tyre rubber samples using ICP-MS, ATR-IR and Pyrolysis GC-MS and the subsequent matching to skid marks

7.0 Introduction

Road safety statistics showed that there were 4,379 road traffic hit and run collisions on the roads of Greater London in 2004, which makes up 15% of the total road traffic collisions (72). The inability to accept responsibility for the accident is not the only reason for hit and run accidents; in 2006 Transport for London estimated that approximately 4-5% of drivers in the UK are driving illegally, this could be as a result of drink driving conviction, driving without a legal licence, valid insurance, using a stolen vehicle, speeding, or driving under age (72).

Vehicle tyre skid marks are useful pieces of evidence following hit-and-run accidents. The tyre traces are produced due to the friction (braking or skidding) between the tyre and the tarmac or other road surface. The rubber particles which deposit on the road surface as skid marks can be collected for assessment and investigation purposes to determine linkage between tyre marks on the scene and the vehicle suspected to be involved in the accident (81,185).

A variety of visual physical measurements are made at the scene from the tyre striation marks. These include; the number of skid marks, the width of each striation left behind (deposited by the left and the right tyres of a vehicle) and the distance between each parallel and diagonal skid mark measurement (i.e. between left and right skid marks) compared to the wheelbase of a suspected vehicle. These measurements provide the forensic scientist with physical assessment of the features of the tyre, which can enable to identify the tyre that produced the striation, with the help of databases, to identify the tyres (i.e. brand and model of tyres) which could be at the source of the trace, for example, the Treadmate® database (186). However,

any defective features of tyre skid marks observed on the road surface could make the identification questionable. The physical measurements may not be satisfactory to link the vehicle to the crime scene due to poor weather or surface conditions which make it more difficult for the measurements to be completed. Furthermore, during the skidding process the shape and details of the tyre patterns and ribs become unreadable (81). In some cases, where the recognition of the tyre pattern is poor, it may affect the visibility of the number and width of striations, information which is vital to help determine the source of the trace. For example, in cases where only one trace is visible, this will make the calculation of the wheelbase measurements almost impossible, thus making the physical traces completely useless (80).

The disadvantages associated with the visual match tests that are carried out at the crime scene highlight that a controlled chemical method could help strengthen the integrity of the evidence within the case. Chemical tests may answer many questions related to tyre samples, independent to the features of the car tyre, pattern and physical properties of the skid mark on the crime scene.

The tyre black skid mark will be left by the abrasion of the tread; it is a result of the friction during the braking or skidding process between the tread areas of the tyre with the road surface. The rubber that the tread is made up of is a composite of different materials such as styrene-butadiene co-polymer (SBR), a synthetic rubber that is often substituted in part for natural rubber (187). Polybutadiene is also used in combination with other rubbers because of its high resistance to wear and its low heat-build-up properties (188). Carbon black supports the rubber with high reinforcement and abrasion resistance (189,190) along with natural rubber and other chemical additives (191-193). The ratio of each of these compounds varies with each tyre manufacturer and model, therefore these features could be used as a comparative

tool to differentiate between the types and models of tyres, as well as match the tyre with the skid mark at a crime scene (194).

Pyrolysis gas chromatography mass spectrometry (PY GC-MS) is an analytical technique where the tyre rubber samples are heated to breakdown, and the individual constituents of the decomposed materials are separated by gas chromatography, before being detected and identified using mass spectrometry (195). Several studies have investigated tyre trace analysis using PY GC-MS (73,78,82,83). These co-workers analysed between 12-59 different tyre samples; and based on the calculated relative percentage content of selected compounds that were used within each tyre, and the subsequent variations of specific chemical compounds across all samples (194), and showed that it was possible to discriminate between different tyre manufacturers.

Many research methods have been published on inductively coupled plasma mass spectrometry (ICP-MS) (196-199) and its importance in the analysis of metals in different matrices; however to the authors' knowledge, this study is the first of its kind that has analysed metals in rubber from tyres. ICP-MS is a reliable and accurate technique that is capable of detecting trace metal concentrations down to part per trillion levels (200) and could accompany Py GC-MS in tyre identification (198), along with attenuated total reflectance infrared spectroscopy (ATR-IR). These can be utilised in order to compare raw rubber tyres and the skid mark produced from them, in order to produce a match.

7.1 Materials and methods

7.2 Optimisation of the methods

Twenty-one tyres, which were previously used on Britain's roads, were donated by National Tyres Ltd, Kingston upon Thames. The brand, model, size, and country of manufacturer for each tyre are listed in Table 7.2. All twenty-one samples were used to simulate skid marks. The skid marks were also analysed using the same analysis methods and interpreting techniques which was used in the tyres analysis to compare it and discriminate it from the related tyres. Four different brands of tyres (N° 2, 3, 12, 17); Michelin, Avon, Firestone and Pirelli tyres (plus different models within each brand) were used for the development and optimisation of Pyrolysis GC/MS, ICP-MS and ATR-IR methods. Replicates (n=5) of different tyres brands and models Michelin Energy [1 Spanish (N° 21) and 4 French manufactured (N° 17, 18, 19, 20)], Michelin Agilis 87 (N° 9), Michelin Primacy (N° 5), (3 tyres) Firestone TZ300 [2 Spanish (N° 2, 4) and 1 French manufactured (N° 8)] Avon ZV5 (N° 3), Pirelli PZERO (N° 14), Pirelli Euforia (N° 16), and Pirelli P6000 (N° 12) were used to assess if the methods were replicable and accurate. Any variation that may exist within the manufacturing location was also taken into consideration.

Table 7.1 Tyre samples used in the method optimisation

Tyre brand/model	Sample ID
Michelin Energy(Spanish)	21
Michelin Energy(4 French)	17, 18, 19, 20
Michelin Agilis 87	9
Michelin Primacy	5
Firestone TZ300 (2 Spanish)	2, 4
Firestone TZ300 (1 French)	8
Avon ZV5	3
Pirelli PZERO	14
Pirelli Euforia	16
Pirelli P6000	12

7.3 Sample collection

Fifty seven tyres, which were previously used on Britain's roads, were used in the whole study. Out of the original number, only twenty-one tyres and their skid (trace) marks were used in the comparison and matching part of the study. The tyres were donated by National Tyres Ltd, Kingston upon Thames. All twenty-one samples were used to simulate skid marks. Four different brands of tyres; Michelin, Avon, Firestone and Pirelli tyres (plus different models within each brand) were used for the development and optimisation of Pyrolysis GC/MS, ICP-MS and ATR-IR methods. Replicates (n=5) of different tyres brands and models Michelin Energy (1 Spanish and 4 French manufactured), Michelin Agilis 87, Michelin Primacy, (3

tyres) Firestone TZ300 (2 Spanish and 1 French manufactured) Avon ZV5, Pirelli PZERO Pirelli Euforia, and Pirelli P600 were used to assess if the methods were replicable and accurate. Any variation that may exist within the manufacturing location was also taken into consideration.

7.4 Rotating rig

A wheel balancing rig was used for this purpose at a speed of ≈ 200 rpm.. Each wheel was fitted on the rotating rig, clipped with the safety nut to ensure the stability of the wheel during the rotation period. The wheel starts its rotation until the machines reaches the max stable speed, then an abrasive sheet was brought close to the rubber material of the tread area (avoiding any cross contamination from the different areas of the tyre e.g. side walls) of the tyre to replicate the friction that is occurring in the real-time sudden brake scenario. The rubber fragments, which were produced as a result of the friction between the tyre rubber and the abrasive sheet, are then collected on an uncontaminated plastic plate to be stored later in a plastic tube (with lids) for further analysis. The rig is used rather than a skid pan to prevent the exposure of tyre samples to any contamination from the road surface Figure 7.1.

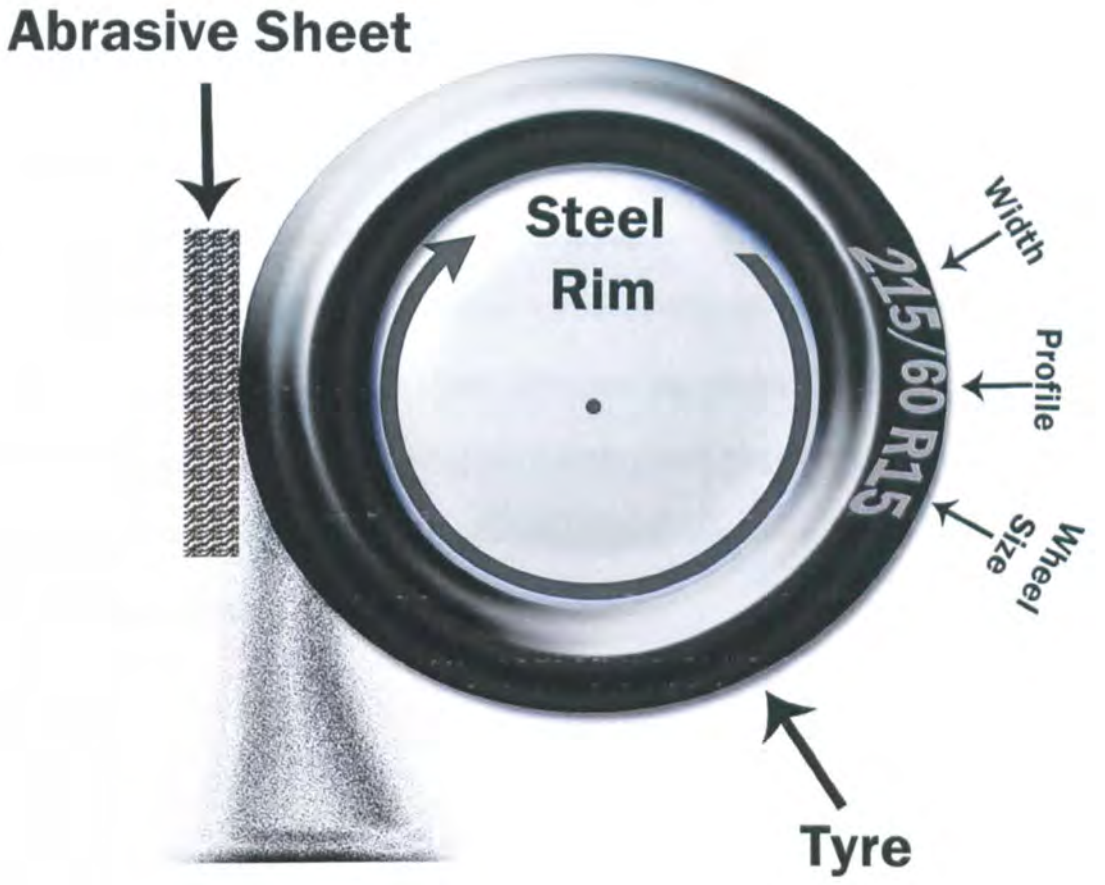


Figure 7.1: Trace rubber collection scheme.

7.5 Tyre trace sample collection

Samples were collected from a variety of tyre manufacturers using car tyres of different sizes Table 7.2. A replication/simulation of the friction that occurs whilst a vehicle is braking was carried out to expose the rubber to the same conditions that the tyre is exposed to during the braking process. The braking test was performed on a tyre mounted on a rotating rig. The rig rotated at a constant speed for all tyres, and an abrasive sheet was brought into contact with the tyre during the rotation period to

replicate the friction between the tyre and the road surface (abrasive sheets were changed with every tyre to avoid cross contamination). The tiny tyre rubber residue ($\approx$ 10 mg) traces were scratched off and collected on separate plastic plates.

The samples were then stored in a dry place using plastic screw cap tubes. Due to the small amount of skid marks removable from the surface, only 10 mg for each skid mark produced from the same collection run was collected for the analysis. To examine if the abrasive sheet had any effect on the chemical analysis, control samples of polypropylene fragments of (n=3) were tested using the same conditions as the tyre samples using. No compounds were found, apart from a trace amount of aluminium when tested with ICP-MS.

Table 7.2: Details of the tyres that were used in this research.

Samples	Manufacturer	Model	Size	DOM	Origin
1	Michelin	Pilote HX	205/50/R16	08/2004	France
2	Firestone	TZ300	185/65/R15	42/2010	Spain
3	Avon	ZV5	195/60/R15	23/2009	England
4	Firestone	TZ300	185/65/R15	05/2011	Spain
5	Michelin	Primacy hp	205/55/R16	30/2011	France
6	Bridgestone	Turanza ER300	205/55/R16	27/2009	Spain
7	Avon	Enviro CR322	175/70/R13	22/2002	china
8	Firestone	TZ300 A	205/55/R16	02/2010	France
9	Michelin	Agilis 87	205/65/R16C	12/2006	France
10	Continental	contipremium contact 2	195/60/R15	38/2006	France
11	Avon	ZV5	205/50/R16	12/2010	England
12	Pirelli	P6000	165/65/R14	10/2005	Italy
13	Michelin	Energy	205/55/R16	01/2005	Spain
14	Pirelli	PZERO Rosso	225/45/ZR17	10/2010	Romania
15	Michelin	Energy	175/65/R14	23/2000	Spain
16	Pirelli	Euforia	195/55/R16/87H	45/2008	Germany
17	Michelin	Energy	185/65/R15	32/2006	France
18	Michelin	Energy	185/65/R15	31/2006	France
19	Michelin	Energy	185/65/R15	31/2006	France
20	Michelin	Energy	185/65/R15	32/2006	France
21	Michelin	Energy	175/65/R14	23/2000	Spain
22	Dunlop	SP Sport Maxx	205/55/R16	42/2012	Germany
23	Michelin	pilot exalto	195/50/R15	50/2003	Spain
24	Bridgestone	b391	165/70/R14	34/200	Spain

				5	
25	**Michelin**	**energy**	**195/60/R15**	**48/2004**	**Germany**
26	Kleber	Nioxer	165/65/R13	17/2007	France
27	**Continental**	**contipremium contact 2**	**195/50/R15**	**09/2008**	**Germany**
28	Bridgestone	Turanza ER300	215/55/ZR16	05/2010	Poland
29	**Pirelli**	**P6000**	**205/50/R17**	**04/2007**	**Germany**
30	Barum	Brilliant 2	175/70/R14	24/2011	Brazil
31	**Michelin**	**energy**	**185/65/R15**	**48/2004**	**UK**
32	Yokohama	Advan A11A	205/50/R16	38/2008	Japan
33	**Bridgestone**	**b250**	**175/65/R15**	**10/2007**	**Italy**
34	Bridgestone	potenza	225/45/R18	48/2010	Poland
35	**Kumho**	**Sulus KH15**	**185/60/R14**	**13/2008**	**Korea**
36	Pirelli	P3000	165/65/R14	10/2005	Italy
37	**Bridgestone**	**potenza**	**225/45/R18**	**03/2010**	**Poland**
38	Firestone	F590	175/65/R13	04/2004	Spain
39	**Firestone**	**F590**	**175/65/R13**	**09/2005**	**Spain**
40	Belshina	GEA391	155/70/R13	08/2004	Belarus

DOM indicates the date of the tyre's fabrication; the first number shows the week of the year and the last number shows the year it was made.

7.6 Tyre sample collection

Approximately between 3-4 g of each raw tyre rubber sample was collected from the tread of each tyre before simulating friction. The rubber samples were then stored in a dry place using plastic screw cap tubes Figure 7.2.

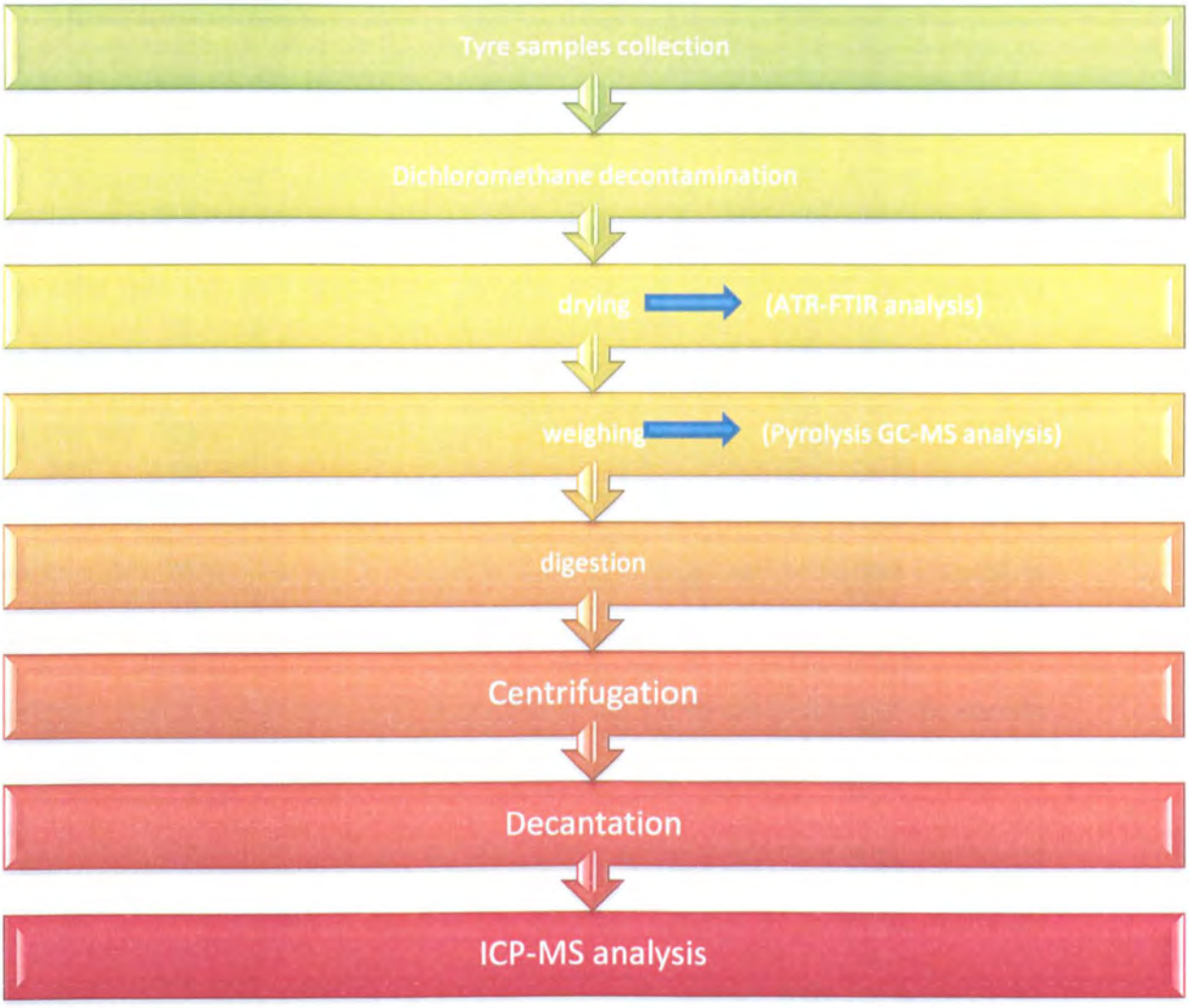


Figure 7.2: Rubber samples analysis porcess.

7.7 Methods

7.8 Chemicals and reagents

Nitric acid 67% NORMATOM® for trace metal analysis and metal standard reagents were obtained from VWR (Leicestershire, UK). Hexane, dichloromethane, nitric acid lab grade and all the other organic solvents were from Fisher Ltd. (Leicestershire, UK).

7.9 Instrumentation

7.9.1 Pyrolysis GC-MS sample preparation

Similar sample sizes and thicknesses were used to get the best replicable sensitivity. The solid tyre and skid mark debris samples were cut into pieces of approximately the same shape, thickness and mass before the analysis (200–300 µg), and inserted without any further preparation into the quartz tubes. The combustion was carried out in pure fused quartz tubes (99% SiO_2 deposited on a bed of similar quantities of quartz wool) filled to almost 10 % of the tube. The quartz tube was then inserted in the probe bore of the pyrolysis unit and placed with the plunger into the furnace pyrolyzer (CDS, Oxford USA). To minimise errors due to the position of the sample inside the pyrolyser filament, each piece of tyre was deposited on the surface of quartz wool, filling half of the quartz tube. Three analyses per trace were performed with the same Py-GC/MS method as for the tyres samples to check the method's repeatability. To achieve that in method development, samples were ran again by the pyrolysis method to check that no more compounds are still in the left carbon solid powder.

7.9.2 Pyrolysis GC-MS

The pyrolysis method was performed by introducing the samples into a heated chamber, where the sample is vaporised and pyrolysed with a heated platinum coil filament, installed in a Pyroprobe Series 5200, embedded with software (5000 DCI) version 1.62 from (CDS Analytical, USA). The helium gas flow was maintained at a specific pressure throughout the pyrolysis period until decomposition vapours

reached the GC column. The separation and detection of the resultant chemicals was carried out using an AGILENT Technology 7560 gas chromatograph, in combination with an AGILENT 5860 XL EI/CI MSD Triple Axis Detector mass spectrometer operating in electron impact ionisation (EI) mode using helium carrier gas with a flow rate of 1.4 mL/min. The analytical column for GC was a BP-X5 SGE Forte Capillary column (Victoria, Australia) (30 m × 0.25 mm × 0.25 μm l,w,ID) (5% phenyl polysilphenylene-siloxane). The injector was set at 280 °C. Split injection was performed for a purge time of 0.5 min with a split ratio of 53.6, a split flow rate of 75 mL/min and an initial pulse pressure of 20.4 PSI. This enhanced the peak shape and sensitivity. The GC oven temperature for the analysis of the rubber samples was programmed to start at 45°C, held for 6.5 min, then increased to 150°C at 5°C/min, held for 5 min, to a final step of 335°C at 70°C/min, held for 3 min to clean the column. Full scan mode was used to detect all separated compounds. The pyrolyzer interface temperature was set at 60°C. Quartz tubes were baked at 1,250°C to avoid cross contamination.

7.9.3 ATR-IR sample preparation

Using the same sample collection method, tyre samples were decontaminated prior to the IR screening. The tyre samples were sliced to a thickness 0.5-1 mm. In the same way, 5 mg of the skid marks were analysed on the ATR diamond crystal.

7.9.4 ATR-IR

Thermo Scientific Nicolet ID5 ATR-FTIR spectrometer was equipped with deuterated triglycine sulfate (DTGS) KBr detector, and diamond single-reflection crystal. The instrument was controlled with Thermo Scientific OMNIC software for data analysis. Eight scans were made between 600-4000 cm^{-1}. The resolution was 8 cm^{-1} and the optical velocity was 0.4747 cm/sec.

7.9.5 ICP-MS sample preparation

The collected tyre rubber samples and trace samples were washed with DI and then decontaminated to remove any road debris or road tar residue. Decontamination was carried out using a mixture of dichloromethane and ethanol 1:1 ratio. This solvent mixture (10 mL) was added to each sample and vortex mixed for 2 mins (repeated 3 times). The samples were rinsed with DI water to remove all the excess solvent and thus prevent rubber damage. The organic and water residues were evaporated under a gentle stream of nitrogen gas at 50°C using a hotplate concentrator Techno DB-3 (Cambridge, UK). The samples were then weighed and digested for ICP-MS analysis.

7.10 Rubber digestion

In order to prepare the rubber samples and skid marks for the ICP-MS analysis, samples had to be digested. Rubber and trace samples were decontaminated. 100 mg

in excess (≈ 2 mg were used) of the rubber samples and 10 mg of the trace were weighed and placed into glass vials (vials were pre-treated treated overnight in 50% nitric acid (HNO_3) in DI water to avoid cross contamination). Metal trace nitric acid (5 mL) was added to the vessel (taking in consideration the 10 fold dilution between the trace and the rubber sample). Vessels were then covered with a relief valve and outer lid. An advanced sensor technology MARS (MARSXpress by CEM, USA) was used in the digestion process (Buckingham, UK). The digestion method was optimised to get the best digestion (104). The heating cycle lasted in total for 27 minutes. Heating started at room temperature and then increased to 400 W for 10 minutes (at a rate of 100%) and held there for 2 min and then increased to 800 W for another 10 minutes (at a rate of 80%). After digestion, the solution was made up to 50.00 mL with de-ionized water (tubes were soaked overnight with 20% nitric acid in DI water). The solutions were centrifuged at 3,500 rpm for 10 min and decanted to fresh tubes prior to analysis by ICP-MS.

7.11 ICP-MS

Tyre and skid mark samples were analysed using an AGILENT Technology Agilent 7700 inductively coupled plasma mass spectrometry (ICP-MS) in combination with a 7700 Agilent Octopole Reaction System (ORS3) cell for effective removal of interferences in complex and variable samples. Its high-temperature plasma of argon gas operating at both collision mode and reaction mode was connected to an ASX-500 autosampler (Agilent Ltd., California, USA) Table 7.3. The injection needle was rinsed with deionised water (DI) and 2% nitric acid in deionised water between

samples. At least one metal-free sample was analysed to monitor cross-contamination between samples.

Table 7.3. ICP-MS system operating parameters.

Conditions	Value
Sample depth (mm)	8
RF power (W)	1550
RF matching (V)	2.2
Carrier gas flow (L/min)	1.01
Dilution flow (mL/min)	0.25
Nebulizer pump (rps)	0.1
Spray chamber temperature (°C)	2
Helium gas flow (mL/min)	4.3
Extraction lens 1 (V)	0
Extraction lens 2 (V)	-140

7.12 ICP-MS method development

An ascending standard solution containing 65 metals and their isotopes of concentration range 0-1000 ng/mL (Be, B, Na, Mg, Al, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Y, Zr, Nb, Mo, Ru, Rh, Pd, Ag, Cd, In, Sn, Sb, Te, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Hf, Ta, W, Re, Os, Ir, Pt, Au, Hg, Tl, Pb, Bi, Th, U) was prepared from a fresh 1000 ppm stock in 3% HNO_3 solution. Tyre rubber samples, in replicates (n=5), were analysed for

these 65 metals. For interpretation of the results, three criteria were set; the presence of the metal in the tyre rubber, the precision between each replicate [metals were chosen which have the lowest relative standard deviation (RSD) between replicate runs to improve precision], and how reproducible are concentrations of the metals. Six tyres (N° 2, 4, 3, 11, 17, 18), Michelin Energy tyres manufactured in France (x 2), Firestone TZ300 manufactured in Spain (x 2), and Avon ZV5 manufactured in England (x 2), were used in the validation process. Replicates of each of these samples were digested and analysed for all the 65 metal elements on both mass spectrometry collision and reaction modes.

7.13 ICP-MS method validation

ICP-MS validation was achieved by spiking blank samples with solutions of the analytes in 10% nitric acid producing 10 calibration standards for each the 10 metals. The levels found were 0-10 ng/mL (0, 0.1, 0.5, 1, 2, 5, 10 ng/mL standards) for V, Cr, Co, Sr & Sn and 0- 50 ng/mL (0, 0.5, 2.5, 5, 10, 25, 50 ng/mL standards) for Ni and Pb, whereas for Ti it ranged between 0-500 ng/mL (0, 5, 25, 50, 100, 250, 500 ng/mL standards) and for Al 0-1000 ng/mL (0, 10, 50, 100, 200, 500, 1000 ng/mL standards).

Negative quality controls of blank samples, of 1:5 trace nitric acid in DI water, under the same digestion and analysis conditions were run for each series in the same batch. Repeatability of the method was evaluated by screening spiked polypropylene fragments control specimens with a known amount of metal concentration in the same day and in 3 successive days. Standard curves were linear with R^2 values of 0.984, 0.982, 0.981, 0.983, 0.958, 0.986, 0.984, 0.982, 0.984, and 0.979 respectively.

A set of control checks of a specific concentration (100 ng/ml) were performed in-between each batch of analyses to monitor precision and accuracy.

7.14 Results and discussion

Pyrolysis is a sensitive technique that decomposes each rubber sample, with evolved gases detected by a conventional GC-MS; the size of the sample and the heat rate of the pyrolysis are the main factors that can affect sample analysis (100). Initial analysis indicated that these tyres were of very similar chemical composition when analysed using PY-GCMS, however, when these samples were analysed for metal content by ICP MS and chemical composition by ATR-IR, they showed noticeable differences in their composition. These differences and similarities were evaluated by principle component analysis (PCA) to determine if the techniques are successful in discriminating between different tyre samples

7.14.1 Principal Components Analysis (PCA)

PCA is powerful tool to identify high dimension data in patterns by using mathematical concepts such as standard deviation, covariance, eigenvectors and eigenvalues to analyse these data in such a way as to highlight their similarities and differences (201). This technique has been widely used in different fields and applications to match and discriminate between different data (81,202,203). In this study, PCA was carried out using the "The Unscrambler" software version 10.0.1

(CAMO, Norway) was used to discriminate and match between "different" and "similar" tyre samples based on the results obtained from ATR-IR, Pyrolysis GC-MS and ICP-MS.

PCA enables data produced from different tyre samples, including their replicates, to be displayed in clusters on a single graph. The ICP-MS results demonstrated clustering of the five replicates per sample for the tyre rubber sample sets and ATR-IR also illustrated the clustering of the five replicates per sample using all the 21 tyre samples. These clusters show the replicates of each of the tyre and skid marks samples after being analysed by ICP-MS and ATR-IR. Using the mathematical variation and similarities; replicates of tyres that produced similar data were plotted within the same area of the graph. Data from different tyre samples were positioned in different areas, allowing for discrimination between different tyre brands and models to be visually displayed. Using PCA, discrimination can be determined between "different" tyre brands when analysed by either one of the chemical analysis techniques, as well as allowing similar samples and skid marks to be matched up when necessary. Some samples were overlapping because there are two or more sets of the similar tyre rubber samples of the identical manufacturer and model (e.g. Michelin energy samples, tyres N° 17, 18, 19, 20 and Firestone TZ300 N° 2 and 4 etc.). Outliers were discarded from the PCA analysis prior to data plotting.

7.14.2 Outliers

One ICP-MS run of Zn and another of Cd analysis results were discarded. In order to discard errors from the large amount of data processed; statistical analysis was

used to remove any outliers. All sample data were compared to their subsequent replicates, and unknown samples were chosen to be randomly matched with the previously analysed tyre. Grubb's and Dixon's tests were used to check the suspected outliers in the data at 95% confidence intervals. Any unidentified sample, which does not match with the mean of parent samples, or different from the rest of the samples (with a large RSD value > 20%), were checked by using the Q test for identification and rejection of outliers.

Grubb's test

$$G = \frac{\text{Mean - suspected value/ SD}}{\bar{X} - X_{min}} = \frac{\bar{X} - X_{min}}{S}$$

Dixon's Q test

$$D = \frac{X_2 - X_1}{X_n - X_1}$$

X2= suspected value
X1= nearest value to suspected
Xn= largest value

7.14.3 Pyrolysis GC-MS

The pyrolysis method was chosen to suit the sizes and thicknesses of the weighed samples so that all the samples are decomposed. A number of preliminary experiments were conducted to ensure that the optimised pyrolysis GC-MS method is sufficiently robust in terms of high sensitivity and precision. As part of this process, previously analysed samples tubes were rerun using the same method to check that no more compounds remain in the pyrolysed carbon powder. Previous studies focused more on the design of temperature and time of pyrolysis

(73,78,81,83,186). The pyrolysis method started by studying the impact factors of different parameters of the analytical technique. The initial temperature and the ramp increase were major factors that affected the retention of the compounds and also the separation.

Method optimisation showed that maximum sensitivity was achieved under the following settings: interface transfer line was set at 300 °C, and the valve at 280 °C to avoid pyrolysis product condensation, and the valve set to release the pyrolysed samples at 275 °C with a filament rate of 20°C/msec increased to reach 750 °C for 15 seconds (run time 15.035).

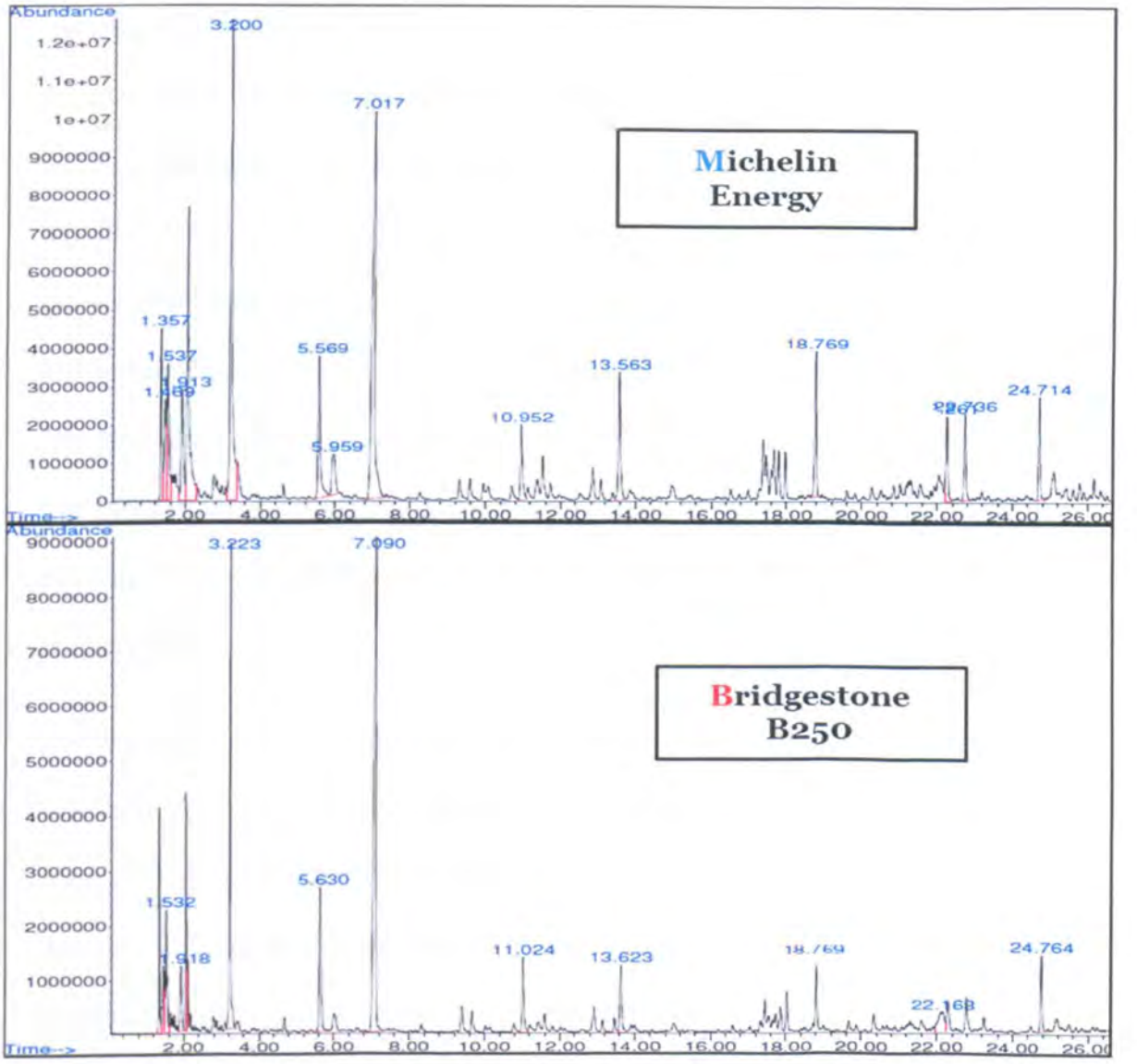


Figure 7.3: Pyrolysis GC-MS chromatograms of 2 tyres

Once the samples were analysed, differences were observed during the first 15 minutes, where 8 of the peaks (Toluene, ethylbenzene, styrene, α-methylstyrene, indene, azulene, Naphthalene-1-methyl, biphenyl) showed either much lower or much higher intensities than the other tyres Figure 7.3. This suggested that there is a variation in the amount of these decomposition products mixtures between each tyre's brand and model. The differences in the tread samples showed possibilities for differentiation in the trace samples spectra too.

For the entire set of analysed rubber samples, there are no peaks present in the skid mark spectra that had not been present in the tyre rubber chromatogram. All of the samples of the 21 tyres were analysed at least three times with the same pyrolysis GC-MS method parameters and conditions. The run for each sample lasted for 25 minutes plus 5 minutes in-between each sample to bake the column to avoid the cross contamination and the deposition of leftover samples from the previous run. The retention time and availability of the chemical compounds were replicable in the tyre and skid marks results for each sample. The peaks' area integration was performed using Chemstation version E.02.00.493 Enhanced Data Analysis MSD software from Agilent Technologies and then identified with the NIST 2008 mass spectra library.

The abundance data of each of the selected peaks was used in PCA analysis. PCA was used to find the critical variation in compounds between the pyrograms data Figure 7.4, from which toluene, Ethylbenzene, Styrene, α-Methylstyrene, Indene, Azulene and biphenyl were chosen to be the indicators of discrimination and matching between the tyre rubber samples and the skid marks. The area of each peak was normalised by the weight of the sample which ranged from 200–300 μg. The tyre rubber replicates showed a reasonable precision of intensities and retention times within all the different 8 compounds, whereas the skid marks showed smaller peak areas under the peak of the same compounds in comparison with the tyres' rubber. This is due to the friction and heat between the tyre and the adhesion sheet generated while the skid mark was created, decomposing the compounds. Previous literature reports didn't identify as many peaks, thus did not give comparison results of sufficient quality for the supplier to be identified (73,78). In this study, an updated

version of the mass spectra library (2008) was used resulting in more peaks being identified than has been found in previous studies.

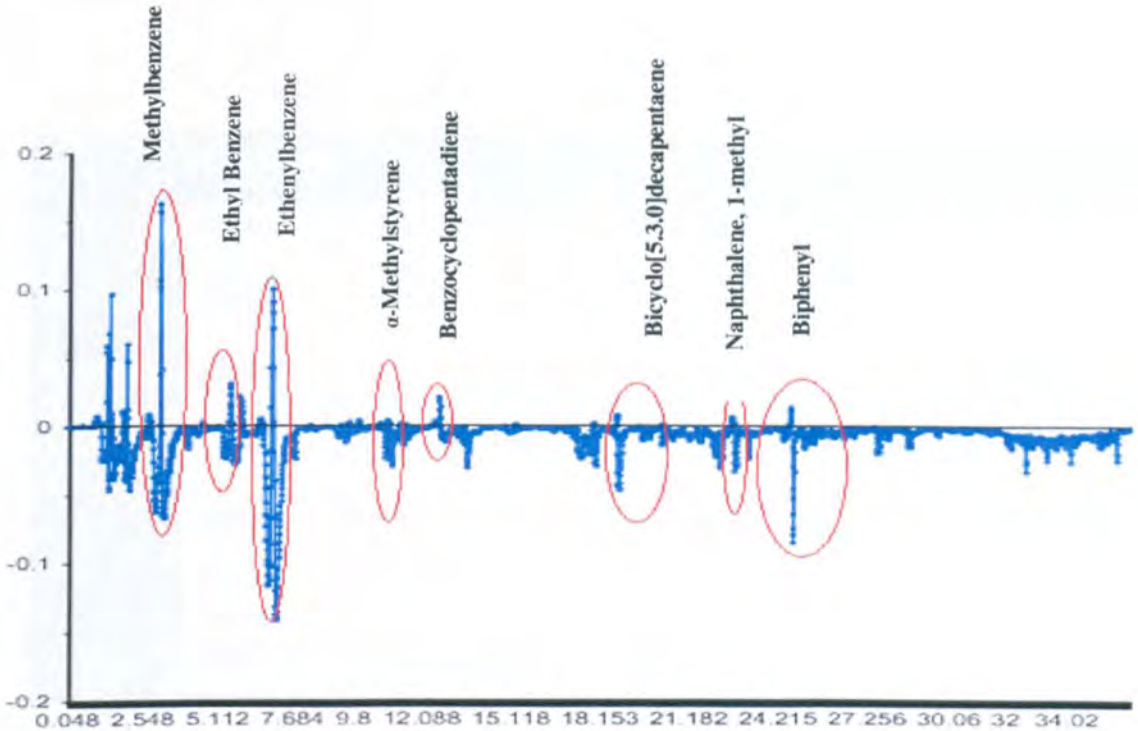


Figure 7.4: Graph showing the chosen compound according to its variation levels between different tyres' pyrograms.

7.14.4 ICP-MS

During the method development, samples were examined for consistency on each analytical technique. The levels of the 10 metals of choice were corrected based on the weight of each sample prior to analysis. Even though the weights used were within 1% of each other, in ICP –MS minor mass differences played a major role in the quantification analysis. The results indicated that the less concentrated metals (Cd and Cr) tended to result in less precision between replicates than the other meta

ls. Four tyres (5 replicates) of the same manufacturer and model were analysed for

the 10 metals to check the precision and reliability of the method. With the RSD below 6%, intra-variability analysis of these 4 (Michelin Energy) samples showed very precise results. Table 7.4 shows the repeatability and the effectiveness of this method on tyre analysis

Table 7.4: ICP metal element analysis of 4 Michelin Energy tyres (mean of 5 replicates)

	Conc(ppb)	Al	Ti	V	Cr	Co	Ni	Zn	Sr	Cd	Pb
Michelin 1	Mean	120.94	147.85	1.54	3.43	0.88	1.72	947.82	2.19	8.51	6.77
Michelin 2	Mean	124.98	141.38	1.58	3.01	0.92	1.67	926.06	2.24	8.83	6.15
Michelin 3	Mean	132.40	149.96	1.70	3.27	0.95	1.66	959.20	2.50	9.01	6.35
Michelin 4	Mean	129.73	149.48	1.63	3.15	0.96	1.57	922.88	2.33	8.60	6.02
	SD	5.08	3.96	0.07	0.18	0.03	0.06	17.44	0.14	0.23	0.33
	Pooled Mean	127.01	147.17	1.61	3.21	0.93	1.66	938.99	2.32	8.74	6.32
	RSD	4.00	2.69	4.11	5.52	3.75	3.78	1.86	5.99	2.61	5.16

The replicate control samples of the 3 different brands; Michelin Energy (4tyres) (N° 17, 18, 19, 20), Firestone TZ300 (3 tyres) (N° 2, 4, 8), Avon ZV5 (2 tyres) (N° 3, 11) etc Figure 7.5. showed an RSD (above 74%) between the different brands. The tyres analysed showed high precision for replicates of the same sample and large inter-variability between different tyre samples (manufacturer and model), which supports their high potential as indicators to be used for discrimination between different tyres brands and models.

The control check standards that were tested between each batch sample runs were made to evaluate signal stability of ICP-MS system; with less than 3% RSD. This supported the validity of the final results of the analysed samples.

All tyre and skid mark samples were analysed for the 10 metals. Based on the three selection criteria, only 10 metal elements (Al, Ti, V, Cr, Co, Ni, Zn, Sr, Sn, and Pb) out of 65 were chosen for use in distinguishing between different tyre brands/models. For interpretation of the results, three criteria were set; the presence of the metal in the tyre rubber, the precision between each replicate [metals were chosen which have the lowest relative standard deviation (RSD) between replicates runs to improve precision], and how reproducible are concentrations of the metals.

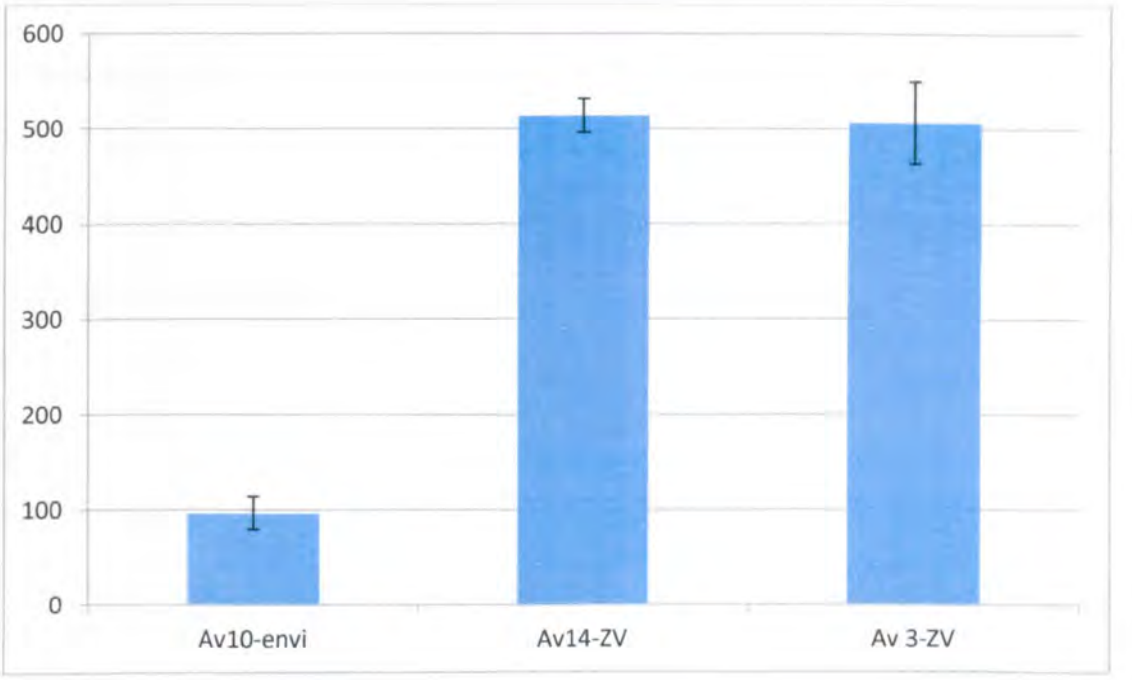


Figure 7.5: Aluminium levels in different Avon tyres models (2 x ZV5 & Enviro) (No 3, 11 & 7).

Twenty out of 21 tyres were discriminated except for tyre number 28 (Bridgestone Turanza) which couldn't be discriminated from the other 2 Bridgestone Putenza tyres. Different manufacturers showed different metal percentage content. By analysing the levels of the 10 different metals in all tyres, it became apparent that Zn, Al and Sn were the most useful as they could be used to differentiate between different tyres brands (manufacturers) and models Figure 7.6. For example, the

concentration of Al in Michelin Energy (N° 17) tyres was 1457.09 ng/mL ± 3.2% and for Zn were 11816.04 ng/mL ± 1.8% while that of Firestone TZ 300 (N° 3) was 577.03 ng/mL ± 5 % and 15547.71 ng/mL ± 4.4% respectively (n=5). The robustness of this method coefficient of variation (% CV) was assessed on consecutive days and demonstrated that the % CV was between 3.7 - 14.03 % ± 0.7 % for all 10 metals. After the method was validated, 10 blind match tests were carried using randomly chosen tyre rubber samples and comparing the ICP-MS metal content profile to the pre-analysed samples. The entire blind test was able to detect the different tyre manufacturers/brands/skid marks, which were statistically distinguishable from each other due to the metal content and also match the similar tyres brands (manufacturers) and models due to similar metal concentration between identical tyres brands and models.

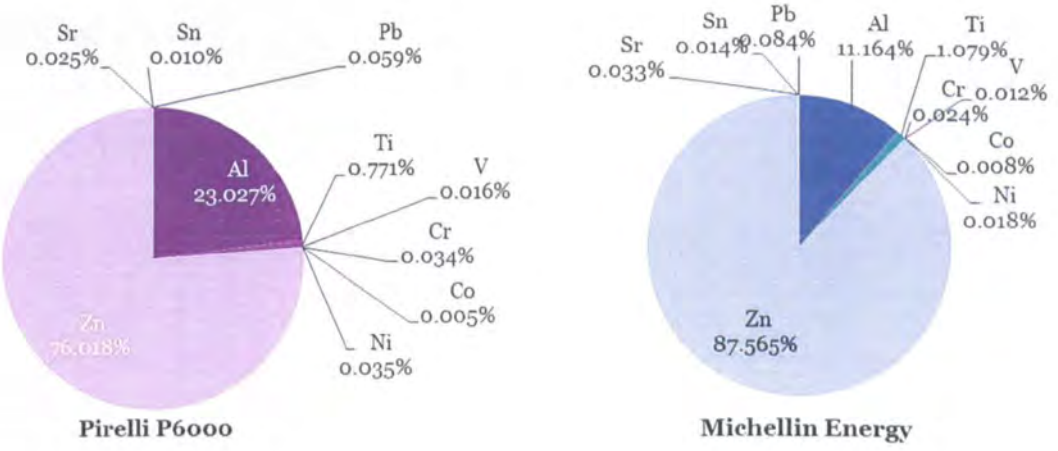


Figure 7.6: Percentage content of the 10 metals in tyre rubber

The PCA results of the samples run by ICP-MS showed a good discrimination between the manufacturers (brands) and tyre model samples. Almost 85% of the tyres studied were successfully grouped in different clusters based on the level of each metal measured by ICP-MS. The rest (15%) including tyre number 28, shared similarity in some of the rubber metal concentration levels in each tyre, which was later discriminated by using Py GC-MS and ATR-FTIR. In the same way that the percentages of each metal was a successful way to separate between different rubber manufacturers and models, ICP-MS analysed the differences and similarities between these rubber samples, and matched the similar tyre models together. Based on the variations between the levels of each metal concentration in the tyres skid mark samples, these were automatically matched with the applicable tyre models and type. Figure 7.7(A) represents a cluster of 5 replicates of Pirelli P3000 (N^o 36), (B) Michelin Energy (4 identical samples of 5 replicates each) (N^o 17, 18, 19, 20) and (C) Bridgestone (3 model samples of 5 replicates each) (N^o 28, 34, 37). These results proved the effectiveness of the method to group similar tyre samples and to discriminate from the rest of the samples.

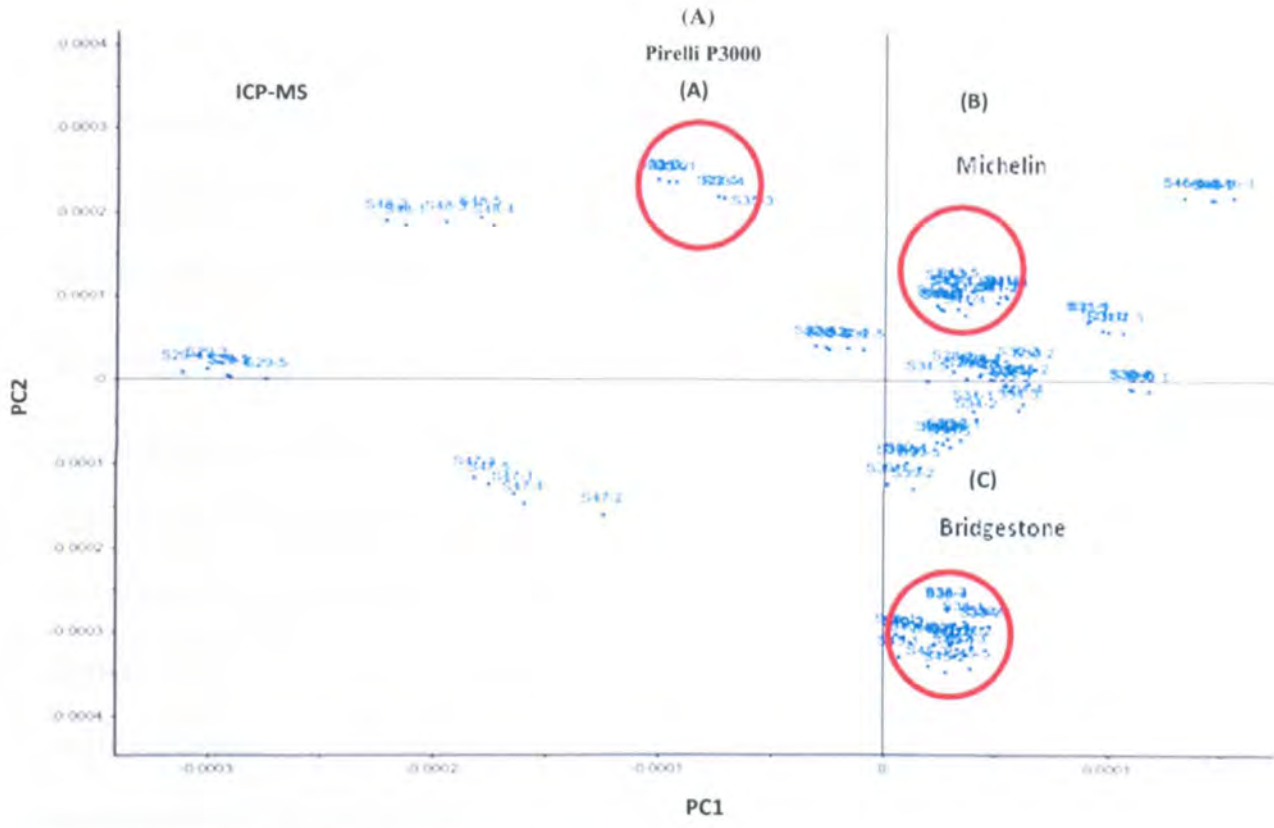


Figure 7.7: PCA of ICP-MS analysis of 21 tyre samples.

7.14.5 ATR- IR

Five replicates were analysed from each tyre sample and their accompanying trace skid marks that were reconstructed from each tyre.

Figure 7.8 shows the ATR-FTIR spectra of some of the analysed tyre rubber positioned at different angles and different areas. ATR-FTIR analysis of samples T1 and T21 confirms the presence of the various compounds used in tyre production. The figure indicates the identified peaks of 4 Michelin tyres, e.g. aromatic compounds, alkenes, alkyl, EPDM (ethylene propylene diene monomer- synthetic rubber), talc, carboxylic acid and esters (204). In addition, strong bands were observed for all samples at 1488– 1398 cm^{-1}, which are attributed to the C-H

bending bands. Natural rubber cis-isomers can be observed at 870–780 cm^{-1} due to the combination of the tyre synthetic rubber with the natural rubber; also a synthetic rubber consisting of hydrocarbons displayed a clear peak in the region of 2900–2800 cm^{-1} for the skid marks and tyres, attributed to stretching of the C-H bond (204,205).

ATR-FTIR repetitive spectra of the samples and analysis of the same samples on the opposite sides, showed an exceptional precision in the analysis. When comparing the means of n=5 replicates, they were within the satisfactory deviation values (mean ±0.7), and the RSD values calculated the absolute value of the coefficient of variation, between the sample readings and the mean of all the replicates (% relative variance) ranged between (0.3-2%) of the same five samples which also proved the precision and repeatability of the method. These validation results made the samples more identifiable when compared with the other samples. Variations in these compounds were observed in the analysis of the tyre rubber samples and its skid marks. Each compound may vary slightly in composition from sample to sample however this could be due to ageing of the rubber and from some unavoidable road contamination. The tyres can still be discriminated though due to the variation in the overall chemical profile of the rubber. ATR-IR data variations (similarities and differences) were processed by PCA to discriminate and match between different tyre manufacturers and models and sometimes even manufacturer location.

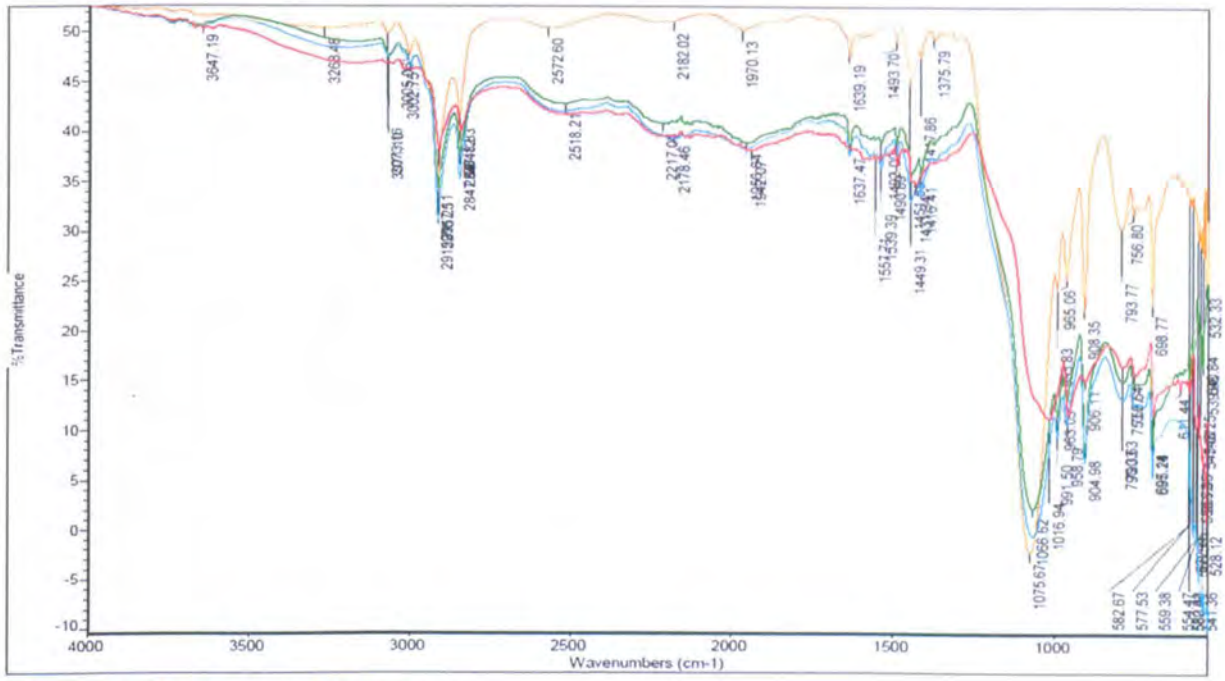


Figure 7.8: ATR-IR analysis of Michelin tyres.

Figure 7.9 shows the tyre samples plotted on a graph in clusters by PCA based on the variations in the spectrum values (Wavenumber and intensity) and hence the intensity of each chemical group resulting from the ATR-FTIR analysis. Group (A), (B) and (C) represents two Michelin Energy (N° 17, 18, 19, 20) tyres of 5 replicates, Bridgestone B391 (N° 24) (n=5), and 3 tyres of Firestone 590 (N° 38) (n=5), respectively. Different tyre brands (producers) and models were separated from the others and similar brands and models, thus tyres were grouped together due to the same chemical compounds contained within. Skid mark samples were also matched to the tyre rubber samples. In the previous ICP-MS PCA cluster results Bridgestone B391 was grouped with the other Bridgestone models, making discrimination difficult; however using ATR-FTIR differentiation between Bridgestone tyre models was possible. This demonstrates the efficiency of this technique in complimenting the other 2 chemical analysis methods.

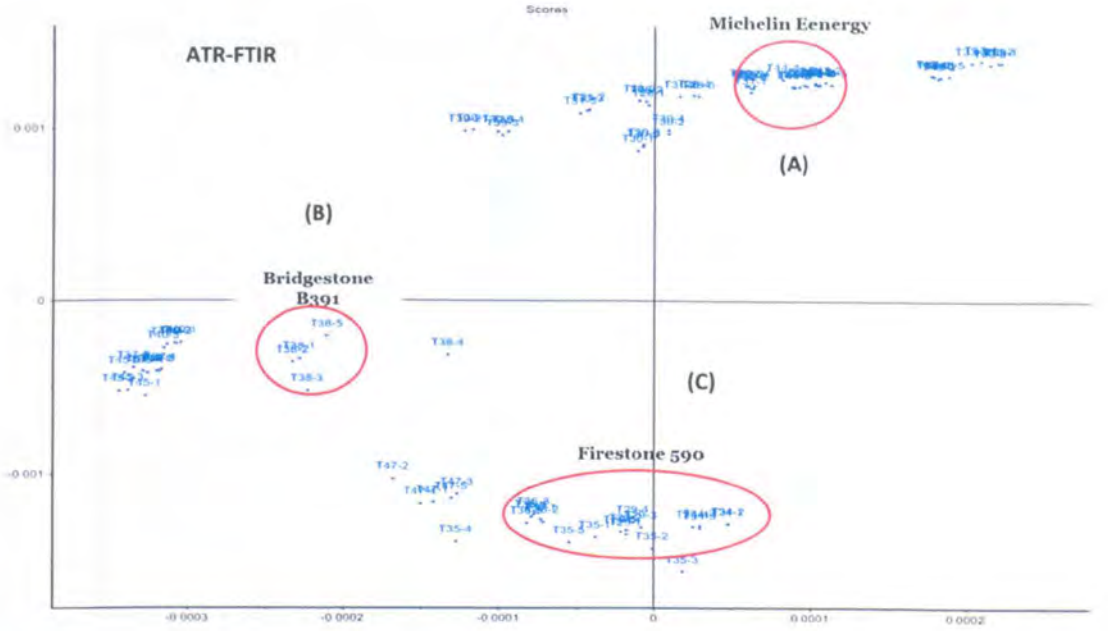


Figure 7.9: PCA plotting results of the ATR-FTIR analysis.

7.15 Heat as a result of friction effects study

During the ATR-FTIR analysis, it was found that tyres are homogeneous and that differences will be rarely seen in the repeat analysis of a sample. Although this technique showed very good repeatability, physical changes were observed between the tyre sample and the skid mark sample when the speed of the rotation increased. The observed differences were mainly in the 3000 – 2800 cm^{-1} region (Figure 7.10). The replicate analysis showed a decreasing peak sp^2 with the increase in the speed of the tyre rotation. Increasing friction and heat in the tyres lead to the reduction of the alkyl group peak (C-H). These properties might be used in future work to estimate the speed of the vehicle.

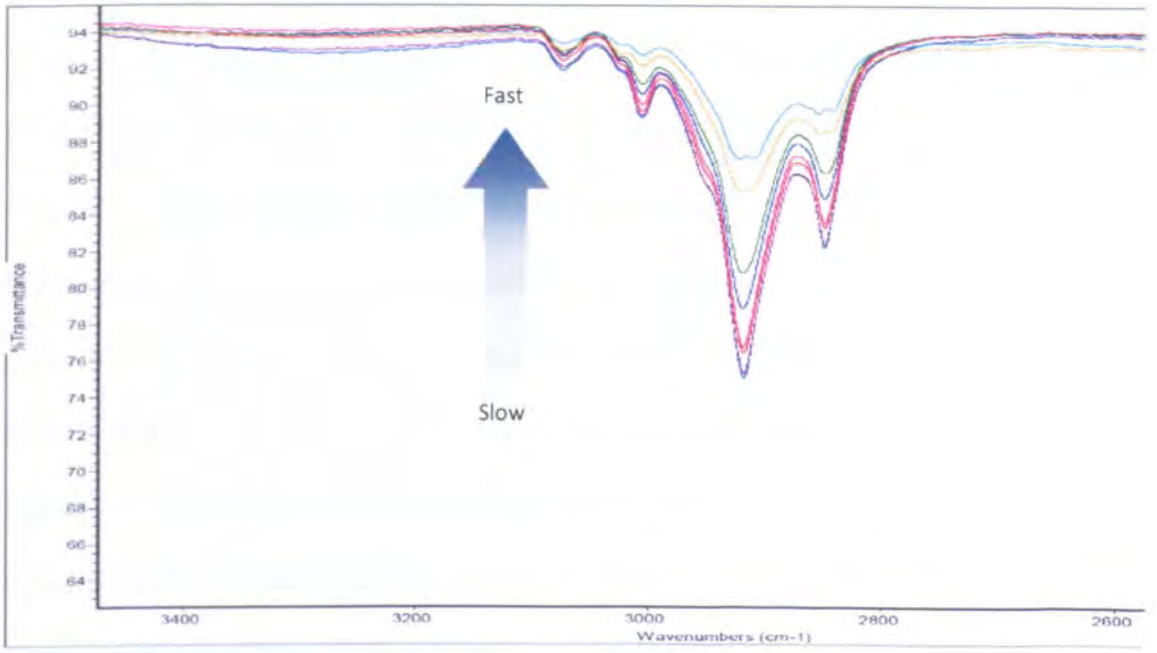


Figure 7.10: Replicates of tyre skid marks at different speeds.

7.16 Conclusion

There were 12 hit and run casualties per day in 2004 in UK, with a subsequent cost to the community of approximately £0.5 million per day and a 41% increase in the figure of hit and run accidents from the 1994-98 average to 2004 (206). The majority of hit and run collisions occurred in fine weather (86%) and on a dry road (81%) which increases the possibility of leaving a rubber trace from skidding (72). Decreasing this number should be a priority. Three different techniques were used to discriminate between tyres of different manufacturers and models and also to match between similar samples and trace skid marks. For the first time, ICP-MS was used to compare the metal element concentrations and this showed a high potential in discrimination and matching between tyres and skid marks. Twenty-one tyre rubber and twenty-one skid mark trace samples were analysed by using newly developed and optimised ICP-MS, ATR-IR and Pyrolysis GC-MS methods. In combination, the

three techniques resulted in possible distinguishing factors that allow all the 16 tyres producers and models (manufacturers and models) to be differentiated. Pyrolysis-GC-MS was not fully able to separate all the tyres alone; however in conjunction with the other chemical techniques discrimination was successful. Principal Component Analysis (PCA) was utilised to distinguish between the different (manufacturers and models) tyres and match with similar ones. The robust PCA technique not only looked at the levels of the compounds and elements in each tyre, but also looked at the level of variation between these factors and showed a high potential level of discrimination between different tyre manufacturers, different models and sometimes, different location of manufacturing. ATR-FTIR utilises a simple preparation technique and is also very efficient in obtaining very precise and replicable results, either in the discrimination between tyre samples or in matching skid marks and the raw rubber samples. All three techniques successfully passed the test of a blind analysis of replicates of random samples from different tyres and sample collection areas. This method was developed to accompany the visual skid mark measurement test to be able to have a more accurate and robust matching and discrimination between different tyres (cars) at the hit & run crime scene and strengthen an already established link on the basis of physical properties.

7.17 Concluding remarks and future work

I- To conclude the first section of this thesis describes a novel sensitive and reliable method for the detection of psychotropic drug Δ^9-THC and cocaine without any derivatisation, where only 50 mg of hair was used. The GC-MS method developed is accurate, reproducible and according to FDA guidelines. Hair analysis is a very useful tool for providing information on individual's drug use. Future work can involve method development for simultaneous detection of diuretics hormones in different matrices such as hair, blood and urine using LC-MS/MS. The assay can complement urinalysis to determine an individual's drug use. In addition, further research needs to be carried out on the metabolism of these drugs, in vitro. The method can also be extended to simultaneous detection of other psychoactive drugs of the same category.

Future work

- Again hair analysis can be used to complement urinalysis and blood analysis. Future work can include developing analytical methods in matrices with even better sensitivity to cover wider range of drugs, also to develop a new method for the detection of diuretic supplements in athletes' human hair using GC-MS and LC-MS/MS.
- To investigate the effect hair analysis investigation compared to blood and urinalysis.

II- The second project in this thesis reports new enzymatic hair digestion method for the quantification amphetamines, cocaine and THC and their metabolites in human hair by GC-MS. A novel enzymatic method was developed for detecting very low concentration of the drugs while avoiding the problems associated with the acidic or alkaline digestion methods that could lead to the degradation of the drug and thereby decrease the chance of the detection. The method developed is sensitive, reliable and reproducible. This first part of the study researched for the simultaneous detection of the amphetamines, cocaine and THC and their metabolites in human hair. The second part focused on identifying the complications that caused the false positive ELISA results. Hair analysis for many substances can be difficult and expensive; also ELISA kits don't enjoy a general acceptance due to their very narrow selectivity without being able to specify some drugs and metabolites. However, the rapid ELISA screening method can shorten the time and minimise the waste of resources on blank samples, so only positively screened samples continue their way through to confirmation analysis. The false positive ELISA results that contradicted the GC-MS confirmatory results were investigated to find the weak link in this method. Experiments were conducted and findings were gathered to overcome the false positive results.

- To develop new methods for the detection of other widely used drugs in different matrices (nails, sweat etc.).
- To study the effect of the washing and hair preparation steps on the levels of drugs in hair before and after.

III- The final project was based a novel, research study on the matching between the striations of the leftover tyres on the road surface tarmac with the suspected car tyres. This method was developed to conjoin the investigators physical car skid marks analysis and confirm it. Tyre rubber and residues were digested in nitric acid using an industrial microwave. Three techniques (ICP-MS, ATR-FTIR and Pyrolysis GC-MS) were developed, optimised and used in the chemical analysis of the tyre trace residue marks and the tyre rubber. The ICP-MS results showed the differences in the major elemental contents of different rubbers. The variation of these elements concentration was used to discriminate between different brands and models. The similarities of the specific elements concentrations were used to match the skid marks to the suspected tyres. ATR-FTIR and Pyrolysis GC-MS studied the chemical compounds in which different tyres rubbers and made up of. The collected data were analysed by the PCA technique which were able using the three techniques to discriminate and in most of the cases match between the skid marks residues and the tyre rubber.

- Study the effect of braking speed and friction between the road surface and the tyre on the compounds forming the tyre rubber to be able to estimate the speed of the car at the moment of the accident.
- Take the research to track level and build a bigger data base on the components of the most used tyres in UK to be able to cover as many brands and models as possible.

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7.19 Appendix

Table 1: ELISA False positive run

Positive Controls

Name	Description	Concentration g/L	Well 650.0 nm
POS-1		G6	0.1652
POS-2		H6	0.1417

Negative Controls

Name	Description	Concentration g/L	Well 650.0 nm
NEG-1		A7	0.8160
NEG-2		B7	0.7990

Samples

Name	Description	Concentration g/L	Well 650.0 nm
SML-1		A1	0.1169
SML-2		A2	0.1094
SML-3		A3	0.1148
SML-4		A4	0.1122
SML-1		A5	0.1201
SML-1		B1	0.1147
SML-2		B2	0.1166
SML-2		B3	0.1253

SML-3	B4	0.1219
SML-3	B5	0.1197
SML-4	C1	0.1463
SML-4	C2	0.1091
SML-5	C3	0.1412
SML-5	C4	0.1931
SML-6	C5	0.1260
SML-6	D1	0.1279
SML-7	D2	0.1684
SML-7	D3	0.1571
SML-8	D4	0.1806
SML-8	D5	0.1257
SML-9	E1	0.1240
SML-9	E2	0.1205
SML-10	E3	0.1766
SML-10	E4	0.1541
SML-11	E5	0.3753
SML-11	F1	0.1391
SML-12	F2	0.1422
SML-12	F3	0.1881
SML-13	F4	0.1132
SML-13	F5	0.3445
SML-14	G1	0.3138
SML-14	G2	0.7715
SML-15	G3	0.1229
SML-15	G4	0.1494
SML-16	G5	0.1318

SML-16	H1	0.3169
SML-17	H2	0.8522
SML-17	H3	0.1256
SML-18	H4	0.2333
SML-18	H5	0.1294

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