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A STUDY INVESTIGATING THE SUITABILITY OF GCMS AND GC FID TECHNIQUES FOR THE PURPOSE OF CREATING A ROBUST AND REPRODUCIBLE HEROIN PROFILING DATABASE

by

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

ABSTRACT

The continual growth in the manufacture, trafficking and abuse of illicit heroin are major areas of concern and heroin drug profiling is an important scientific tool for deriving intelligence to successfully counter ever-growing heroin abuse problems. The project aimed to build a database of heroin profiles amenable to rapid and accurate searching to aid the identification of heroin distribution and/or trafficking networks.

The majority of clandestine laboratories extract morphine from the seed pods of opium poppy plants, acetylation of crude morphine affords heroin. The final heroin composition incorporating the acetylated opium alkaloids plus added adulterants/diluents provides a unique chemical fingerprint for heroin profiling. The project initially aimed to develop a robust and reproducible heroin profiling method to quantify the major components found in heroin samples using the gas chromatography mass spectrometry (GCMS) instrument provided.

Validation of a non derivatised heroin GCMS method highlighted the impact of transesterification on method accuracy and reproducibility. Validation of a derivatised heroin GCMS revealed that the heroin components afforded quadratic calibration lines resulting in the inaccurate and non reproducible quantitation of low concentration heroin components. A series of experiments conducted varying the sample preparation method, GCMS inlet, column and detector parameters identified the MS detector as the source of the non reproducible quadratic behaviour.

Preliminary derivatised heroin studies carried out using a GC instrument equipped with a flame ionisation detector (FID) afforded linear calibration lines with the accurate quantitation of heroin components.

Ultimately GCMS was deemed not viable for heroin profiling and GC FID was established as a suitable technique for the purpose of creating a robust and reproducible heroin profiling database.

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ABBREVIATIONS

AAS	Atomic absorption spectroscopy
AMU	Atomic mass units
BSA	*N*,*O*-(bistrimethylsilyl)acetamide
BSTFA	*N*,*O*-bis(trimethylsilyl)trifluoroacetamide
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CE-MS	Capillary electrophoresis-mass spectrometry
CI	Chemical ionisation
DEA	Drug Enforcement Agency
DMF	*N*,*N*-dimethylformamide
EA	Elemental analysis
EA-IRMS	Elemental analysis-isotope ratio mass spectrometry
EI	Electron impact
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
FID	Flame ionisation detector
FSS	Forensic Science Service
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC-IRMS	Gas chromatography-isotope ratio mass spectrometry
GCMS	Gas chromatography mass spectrometry
HPLC	High pressure liquid chromatography
HPTLC	High performance thin layer chromatography
HSGC	Headspace gas chromatography
ICP-AES	Inductively coupled plasma atomic emission spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IP	Intermediate polarity
LC	Liquid chromatography
LCMS	Liquid chromatography mass spectrometry
LIF	Laser-induced fluorescence
LOD	Limit of detection

LOQ	Limit of quantitation
MAM	Monoacetylmorphines
3-MAM	3-Monoacetylmorphine
6-MAM	6-Monoacetylmorphine
MEX	Mexico
MS	Mass spectrometry
MSTFA	*N*-methyl-*N*-trimethylsilyltrifluouroacetamide
MTBSTFA	*N*-methyl-*N*-tertbutyldimethylsilyltrifluouroacetamide
MW	Molecular weight
m/z	Mass-to-charge ratio
PFTBA	Perfluorotributylamine
PTV	Programmed temperature vaporising injector
r	Correlation coefficient
r^2	Coefficient of determination
R	Resolution
RRT	Relative retention time
RSD	Relative standard deviation
RT	Retention time
S	Standard deviation
SA	South America
SEA	Southeast Asia
SIM	Selected ion monitoring
SKF_{525A}	2-Diethylaminoethyl-2,2-diphenylvalerate
S/N	Signal to noise ratio
SPSA	Scottish Police Services Authority
SWA	Southwest Asia
TEA	Triethylamine
TIC	Total ion chromatogram
TLC	Thin layer chromatography
TMCS	Trimethylchlorosilane
TMS	Trimethylsilane
TXRF	Total x-ray fluorescence spectroscopy

UNIDCP	United Nations International Drug Control Programme
UNODC	United Nations Office on Drugs and Crime
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry
% w/w	Mass percentage
$\bar{x}$	Mean

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Chapter 4

OUTLINE OF THESIS CHAPTERS

Chapter 1 introduces heroin including the historical origin of the drug, current heroin legislation and how heroin is administered with the corresponding effects. The extraction of opium from poppy seed pods and subsequent heroin synthesis is discussed in relation to the resulting heroin compositions. Heroin producers are outlined alongside the trends in heroin composition by geographical origin.

Chapter 2 introduces heroin profiling explaining how strategic and tactical intelligence is used to identifying the geographic origin of illicit heroin samples and heroin distribution networks. A review of the current literature identifies the numerous techniques currently amenable to heroin profiling. An in depth literature review of GCMS and GC FID heroin profiling methods evaluates major and minor heroin component GC profiling studies, heroin profiling GC quantification methodology, internal standard used in GC heroin profiling studies, the advantages and disadvantages of derivatising major heroin components prior to GC analysis, sample preparation methods for both non derivatised and derivatised major heroin components prior to GC analysis and both non derivatised and derivatised major heroin component literature GC methods.

Chapter 3 describes the development of a non derivatised heroin GCMS method incorporating sample preparation and optimisation of the GCMS method parameters. A transacetylation study however highlights the impact of transesterification on the accuracy and reproducibility of the non derivatised method. Chapter 3 further evaluates a similarly developed derivatised heroin GCMS method with method validation incorporating compound resolutions, retention times, relative retention times, GCMS reproducibility (intraday variation), derivatised sample stability (interday variation) and sensitivity measurements. A calibration study however reveals that the derivatised compounds afford quadratic calibration lines resulting in the inaccurate and non reproducible quantitation of heroin components.

Chapter 4 investigates the source of the quadratic calibration behaviour aiming to improve GCMS linearity and reproducibility by varying the sample preparation method, GCMS inlet, column and detector parameters. Experiments however define the MS detector as the source of the non reproducible quadratic behaviour and the GCMS system is deemed not suitable for heroin profiling purposes. Chapter 4 further evaluates the use of a GC FID instrument which affords linear calibration lines with the accurate quantitation of derivatised heroin components. Ultimately GC FID is established as a suitable technique for the purpose of creating a robust and reproducible heroin profiling database.

Chapter 5 summarises the conclusions reached from the thesis and considers both immediate and long term future work.

The experimental parameters employed throughout this thesis are documented in chapters 3 and 4 in parallel with the relevant discussion topics. The final optimised experimental methods are also detailed in the appendices.

CHAPTER 1 – INTRODUCTION TO HEROIN

Diacetylmorphine (diamorphine) is one of the most highly addictive, commonly abused fast acting opiates. Opiates belong to a family of compounds known as narcotic analgesics, narcotic meaning 'tending to induce sleep' and analgesic meaning without pain. The natural opiate morphine is extracted from the opium poppy, acetylation produces the semi-synthetic opiate diamorphine (figure 1). For the purpose of this thesis, heroin indicates the crude mixture of opium alkaloids obtained by acetylation of opium and diamorphine indicates the pure drug.

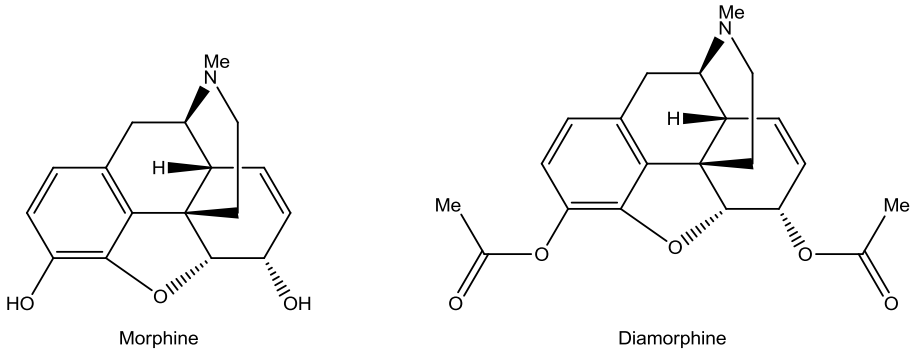


Figure 1 – Chemical structures of morphine and diamorphine

1.1 – HEROIN HISTORY

The psychological effects of opium have been known since 4000 B.C. and morphine was first isolated in 1805 followed by other opium alkaloids codeine and papaverine in 1832 and 1848. The pure alkaloids were prescribed for the relief of pain, cough and diarrhoea. In the 1860s morphine was extensively used as a pain killer for wounded soldiers during the American Civil War resulting in morphine addiction. Diamorphine was first synthesised in 1874 by acetylating morphine in an attempt to produce a new non-addictive painkiller, however, diamorphine was found to have narcotic and addictive properties far exceeding those of morphine. In 1914 the United States Congress called for control of each phase of the preparation and distribution of opium making it illegal to possess or supply these controlled substances. This in turn led to the start of illegal smuggling trades with the large scale smuggling of heroin into the United States in 1967.[7] Heroin is one of the

most frequently seized class A drugs in Scotland with an estimated 1.5% of the adult population injecting or smoking opiates in 2010.[8]

1.2 – HEROIN CONTROL

Currently in the United Kingdom, diamorphine is a class A drug controlled by the Misuse of Drugs Act 1971 which covers the production, supply or possession of heroin. Part 1 of Schedule 2 controls three natural products of the opium poppy: opium (whether raw, prepared or medicinal), poppy straw and concentrate of poppy straw. Part IV of Schedule 2 includes powdered or granulated opium within this definition. The stereoisomers, esters (monoacetylmorphines), ethers and salts are controlled in separate paragraphs of the schedule.[9]

Schedule 4 of the same act specifies the maximum punishments for the illegal possession or supply of heroin without a diamorphine prescription. Summary prosecution tried by a judge alone can result in 12 months imprisonment and/or a £400 fine whereas indictment before a judge and jury is punishable by up to 14 years imprisonment and/or an unspecified fine.[10]

1.3 – HEROIN EFFECTS

The activity of diamorphine is due to its metabolic hydrolysis to 6-monoacetylmorphine (6-MAM) and morphine which are the active opiate forms. The opiate chemical structures are very similar to the naturally produced endorphins and enkephalins and they act by engaging the same nerve-receptor sites in the brain to bring about similar narcotic analgesic effects. Heroin administration produces an intense euphoric rush lasting for a few minutes, feelings of tranquillity last for up to an hour before subsiding to leave a sense of dreamy contentment. At higher doses the user falls into a semi-conscious state as the central nervous system is suppressed, the effects of overdose include slow and shallow breathing, clammy skin, nausea, convulsions, coma and possible death. Physical and psychological dependence results from regular use which in turn increases tolerance and the need for greater quantities of the drug. Methadone is currently used as a substitution therapy for

heroin detoxification, though it is also an opioid causing dependence in its own right.[11]

1.4 – HEROIN ADMINISTRATION

Diamorphine is formulated in the form of either the free base or the hydrochloride salt as later discussed in chapter 1.6.

The more volatile heroin base is smoked by placing the powder on a piece of foil, heat is applied under the foil and the fumes are inhaled through a tube. This is often called 'chasing the dragon'.[12]

The water soluble heroin hydrochloride salt is injected by heating the powder in a spoon with water and an organic acid (commonly vitamin C, ascorbic acid, citric acid or tartaric acid) to aid uptake in the body. The heated mixture is injected using a syringe either intravenously (mainlining), subcutaneously (into fatty tissue under skin) or intramuscularly (into muscle).[12]

Heroin is snorted by inhaling the heroin powder.

Heroin is also eaten but this route is ineffective and therefore unpopular as the stomach metabolises heroin to morphine which is in turn broken down by the liver before reaching other parts of the body.

1.5 – OPIUM

Papaver somniferum var. *album* and *Papaver somniferum* var. *glabrum* are the two varieties of opium poppy plants cultivated for the illicit production of heroin due to their high morphine content (*Papaver setigerum* plants also contain morphine). Opium is the name for the latex produced from the seed pods of the opium poppy (figure 2).

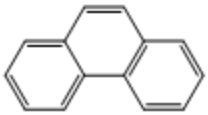
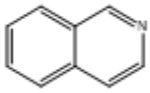
Opium poppy plants

Opium latex from seed pod

Figure 2 – Pictures of opium poppy plants and opium latex seed pod [2]

The raw latex of 1414 poppy seed pods were analysed by the United Nations International Drug Control Programme (UNIDCP) and the major alkaloid compositions are given in table 1 together with their structures in figure 3.[13] In total *Papaver somniferum* crude opium contains 25-30 alkaloids.

Table 1 – Major alkaloid compositions found in opium

Alkaloid			
Class	**Structure**	**Name**	**Composition (%)**
Phenanthrene		Morphine	3.1-19.2
		Codeine	0.7-6.6
		Thebaine	0.2-10.6
Isoquinoline		Papaverine	< 0.1-9.0
		Noscapine	1.4-15.8

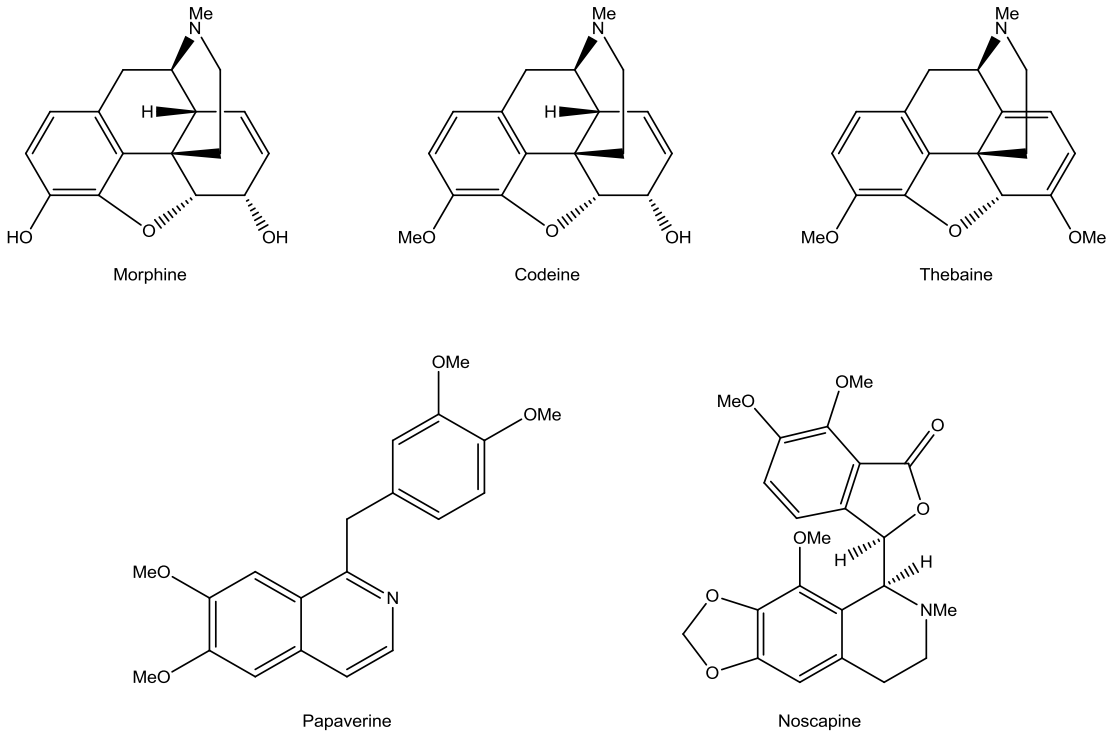


Figure 3 – Chemical structures of major alkaloids found in opium

1.6 – HEROIN SYNTHESIS

The majority of clandestine laboratories extract morphine from opium using the 'lime method'. The raw latex from the opium poppy seeds is dissolved in boiling water to remove the insoluble plant material. Lime (calcium hydroxide) is added to the opium solution converting water insoluble morphine into the water soluble calcium morphenate, other insoluble alkaloids precipitate on cooling and are removed. The calcium morphenate solution is heated and the pH adjusted to 8-9 by addition of ammonium chloride, upon cooling the precipitated morphine base is collected by filtration. Laboratories producing morphine as an end product perform further purification to remove traces of codeine, thebaine, papaverine and noscapine.[7]

Heroin clandestine manufactures typically acetylate the crude morphine base by addition of acetic anhydride followed by heating to generate diamorphine via the intermediate 3-monoacetylmorphine (3-MAM).[14] The cooled reaction mixture is typically treated with sodium carbonate to precipitate the heroin base. Dissolving the

heroin base in acetone with the addition of hydrochloric acid generates the heroin hydrochloride salt.

The range of morphine acetylating agents employed is given in figure 4. Use of acetyl chloride is documented in New Zealand for the 'homebake' preparation of heroin which involves the initial production of morphine by demethylation of codeine with pyridine hydrochloride.[15] Use of a mixture of trifluoroacetic anhydride and acetic acid or alternatively ethylene diacetate are reported, each of the different acetylation routes afford route specific markers.[16]

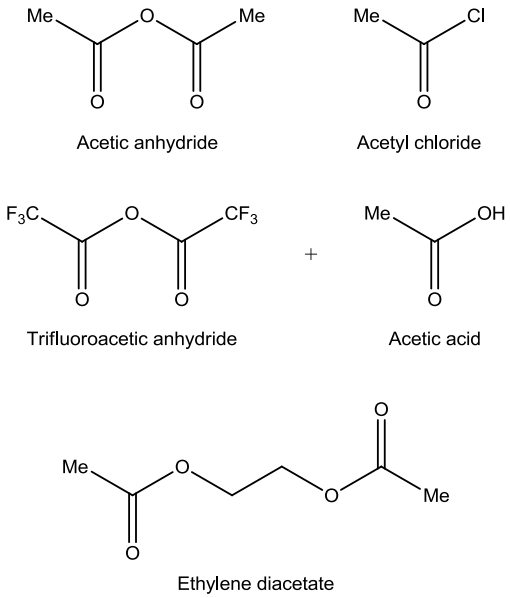


Figure 4 – Morphine acetylating agents

1.7 – HEROIN COMPOSITION

The raw opium natural product and subsequent synthetic steps involved in the illegal manufacture of heroin leave unique chemical fingerprints in the heroin. Chapters 1.7.1 to 1.7.4 discuss the various parameters which affect the chemical composition of the final heroin product.

1.7.1 – Opium poppy

The variety and age of the *Papaver somniferum* plant and the climate, altitude, soil fertility and moisture levels encountered during growth affect the level and number

of alkaloids found in the opium poppy. In 2008 the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) estimated a mean diamorphine content of 40% for heroin in the United Kingdom.[17]

Major opiate impurities originating from the opium include morphine, codeine, papaverine and noscapine. Thebaine is rarely observed in illicit heroin as it decomposes during acetylation generating acetylthebaol (figure 5). Minor opiate impurities from the opium include benzylisoquinolines (laudosine, narceine), tetrahydroisoquinolines, cryptopine plus alkaloids of unknown structure. Non-opiate derived impurities from the opium include meconin (figure 5).

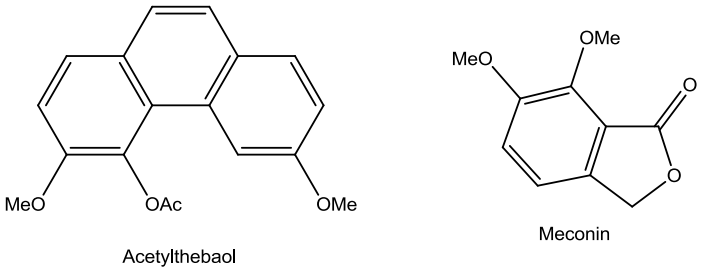


Figure 5 – Chemical structures of acetylthebaol and meconin

1.7.2 – Synthetic impurities

Impurities generated from the acetylation of codeine and thebaine are acetylcodeine (figure 6) and acetylthebaol (figure 5) respectively.

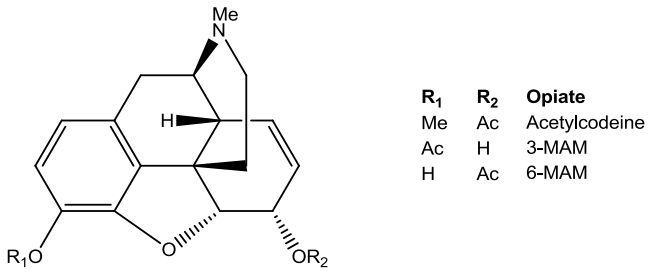


Figure 6 – Chemical structures of acetylcodeine, 3-MAM and 6-MAM

In addition, the incomplete acetylation of morphine using acetic anhydride affords 3-MAM whereas non quantitative acetylation of morphine with acetyl chloride affords both 3-MAM and 6-MAM (figures 6 and 7).[15, 18]

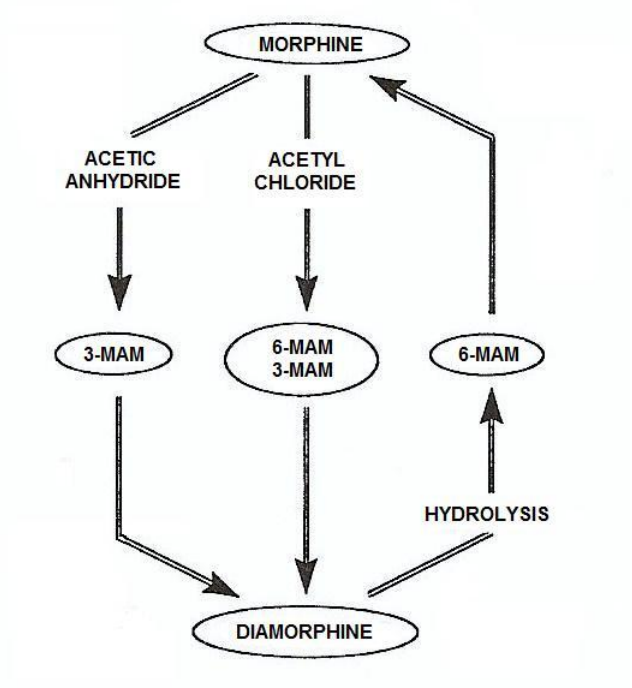


Figure 7 – Morphine acetylation and diamorphine hydrolysis routes [3]

The degree of skill of the illicit heroin manufacturer also determines the extent of diamorphine hydrolysis to generate predominantly 6-MAM (figure 7).[15] Huizer demonstrated that the reaction conditions used to convert diamorphine base to diamorphine hydrochloride also afford 3-MAM but further deacetylation to morphine occurs at a much faster rate than for 6-MAM.[18] Diamorphine is often present as the base or less frequently as the hydrochloride salt where the free base is less stable to hydrolysis than the salt.

Occluded solvents trapped in the heroin matrix may include acetone used in the conversion of diamorphine base to diamorphine hydrochloride or acetic acid generated during diamorphine deacetylation.

1.7.3 – Cutting agents

Pharmacologically active adulterants are added to heroin. Caffeine and paracetamol adulterants are most common in Western European countries where paracetamol increases heroin base volatility thereby increasing the effects from smoking heroin. Other adulterants include phenobarbitone, diphenyhydramine, procaine (to locally

relieve the pain of intravenous injection), quinine (to treat malaria amongst New York intravenous heroin users infected through use of shared syringes [19, 20]). Klemenc reported cases in Slovenia where seized heroin samples were found to contain very high levels of noscapine (up to 61%) supporting use of noscapine as an adulterant.[21]

Inert diluents are also added to expand the heroin bulk and increase profit with the most frequently encountered diluents being sugars (mannitol, lactose and glucose).[3]

Typically within the United Kingdom heroin samples imported directly into Southern England have lower levels of cutting agents than those found in Scotland following further adulterant/diluent opportunities.[22]

1.7.4 – Decomposition products

Heroin samples stored in the dark gave no changes in acetylcodeine, papaverine and noscapine content indicating their stability, however, storage for more than five years often resulted in diamorphine decomposition with increased 6-MAM and morphine content.[23] Post-processing diamorphine hydrolysis readily occurs if the sample contains non-bound water or excess acid.[24] Diamorphine decomposition studies varying pH and temperature have been conducted determining the ratio of 3-MAM and 6-MAM isomers obtained.[25]

1.8 – HEROIN PRODUCERS

In 1997 Southeast Asia (SEA) accounted for over half of the world's opium production, specifically Burma, Laos and Thailand as well as the adjacent areas of Southern China and Northwestern Vietnam, this area is known as the 'Golden Triangle'. Three other major areas for sources of illegal opium and heroin are Southwest Asia (SWA) including Pakistan, Iran and Afghanistan, known as the 'Golden Crescent', Mexico (MEX) and South America (SA) (figure 8).

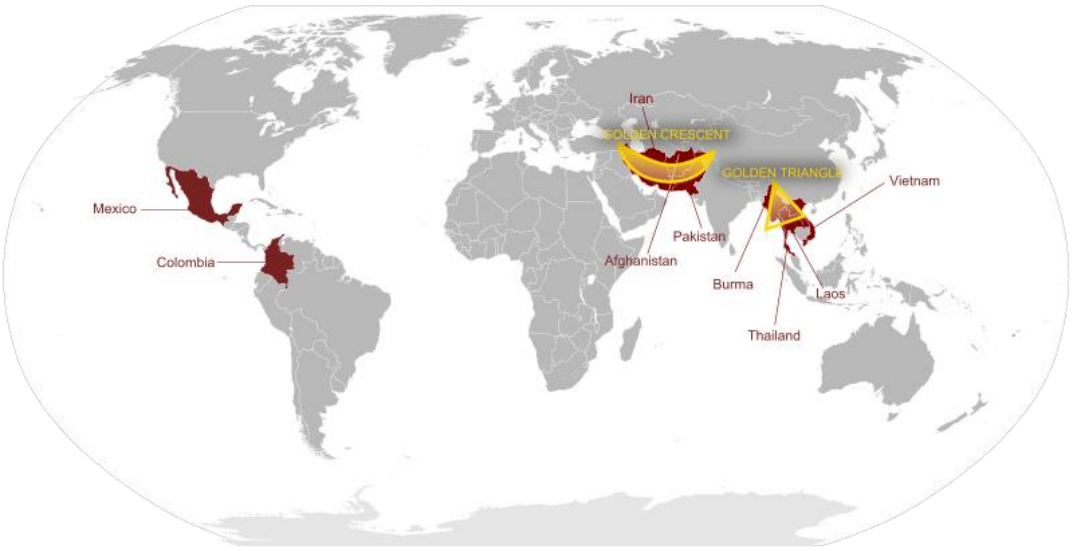


Figure 8 – World map of heroin producers [4]

In 1997 King reported that over 90% of heroin seized in the United Kingdom originated from Southwest Asia with the remainder mostly sourced from Southeast Asia.

1.9 – TRENDS IN HEROIN COMPOSITION BY GEOGRAPHICAL ORIGIN

There have been a large number of scientific investigations aiming to relate the relative and/or absolute alkaloid content of opium to the geographical source of the opium. Each major geographic source area produces heroin that can usually be recognised as a chemically distinct type.

1.9.1 – Southeast Asia heroin

SEA heroin is typically a white powder with high diamorphine purity (80%) as the hydrochloride salt with few other alkaloids or adulterants. Papaverine and noscapine are seldom present indicating effective purification of the intermediate morphine. The high similarity between chromatograms obtained from very pure samples results in poor resolution between subgroups (different areas of manufacture or processing techniques) in the SEA group.

1.9.2 – Southwest Asia heroin

In contrast, SWA heroin samples are far more variable than those from SEA. The most common form is medium brown and of lower diamorphine purity (40-60%) as the base with noscapine (20-30%), papaverine (2-6%), acetylcodeine (5-9%) plus many trace level alkaloid related impurities. The second most common SWA heroin is light brown with a higher diamorphine purity (60-85%) with proportional decreases in the remaining alkaloids.[13] Huizer similarly reported that heroin from SWA characteristically has high levels of both noscapine and papaverine with further discrimination possible based on the levels of acetylthebaol present.[26]

1.9.3 – Mexican heroin

MEX heroin is unique by its appearance as a sticky black tar (30-60% diamorphine purity) or less commonly as a dark brown powder.

1.9.4 – South American heroin

SA heroin is characteristically of high purity (> 90%) with low acetyl codeine (< 3.5%) and a low total content of summed 3-MAM and 6-MAM isomers (< 5%).[13] Other reports indicate low thebaine content with high papaverine levels.[16]

Heroin from India contains less acetylcodeine than heroin from other countries suggesting a lower proportion of codeine to morphine.[27]

The United Nations determined the alkaloid ratios for heroin samples of known provenance and applied the ratios to identify the geographical origin of unknown samples as SEA, SWA, MEX or SA.[13] The variance between the ratios was found to be quite significant and there was a large overlap for each data set across the different heroin producing regions.

Johnston and King determined the concentration of alkaloids and adulterants present in seized heroin samples and successfully classified their origin as the individual producer countries of Turkey, Pakistan, India or Southeast Asia in 83% of cases using a multivariate statistical analysis method.[28]

CHAPTER 2 – INTRODUCTION TO HEROIN PROFILING

Drug profiling is the extraction of a drug sample's chemical and/or physical profile, to be used in the application of policies against the illegal use of drugs (law enforcement, legislation, public health etc.).

The profile of a drug sample is a subset of the sample's characteristics specifically chosen with respect to the purpose of the process.

A class is a group of samples having similar profiles. It is a result of statistical methods applied to the output of the analytical process.

Profile and class can be chemical, physical or both, depending on the nature of the characteristics considered.[29]

2.1 – HEROIN PROFILING STRATEGY

Modern spectroscopic and chromatographic techniques provide the experimental tools to collect large amounts of data related to heroin sample compositions (discussed in chapter 2.4). This project focussed primarily on the development and validation of a suitable GC method to characterise heroin with the acquisition of heroin profiles.

Future work encompasses interpretation of the resulting GC data where the quantified heroin components are classified as either useful or non useful characteristics. The minimum number of components should be quantified to enable heroin sample discrimination without overcomplicating the data handling. Components with very low frequency of occurrence are typically omitted whereas those with a large concentration distribution over the whole heroin population are key where a number of characteristics may be highly correlated.[30]

Data interpretation techniques are essential to analyse and interpret the large volume of collected data as manual interpretation is not feasible. Objective and accurate evaluation of the data without the loss of information is achieved by chemometric

methods using statistical computer programmes such as Minitab or SPSS. Several authors have utilised chemometric methods to provide information about links between illicit heroin seizures and to identify the origin of heroin samples.[5, 30]

Ratle *et al*., utilised machine learning and chemometrics to highlight possible useful patterns in the chemical composition of illicit drug seizures to guide the investigation process.[31] Terrettaz-Zufferey *et al.,* applied pattern recognition techniques to establish possible relationships between the location of heroin seizures and the co-occurrence of particular heroin cutting agents. Graph theory helped develop hypotheses explaining the local cutting process to aid intelligence led policing.[32]

2.2 – HEROIN PROFILING INTELLIGENCE

2.2.1 – Strategic intelligence

Strategic intelligence gives information on the processing and/or geographic origin of illicit samples. This includes the identification of chemicals, reagents and/or solvents employed by laboratories and a scientific determination of the geographical origin of the sample. The monitoring of common methods used in illicit drug manufacture may provide information to help precursor monitoring programmes or support to differentiate between illicitly manufactured drugs and drugs from licit sources.

In order to establish a heroin programme capable of determining sample origin sufficient samples of known origin must first be acquired and analysed to compile an authentic database. The criteria for characterising and determining the origin of heroin samples is based on the different amounts of opiate alkaloids and related compounds carried over from the raw opium material and the acetylation process.[33, 34] The identification of adulterants and diluents provides little evidence in determining heroin origin.

2.2.2 – Tactical intelligence

Tactical intelligence indicates whether two or more samples came from the same source by comparing batches. This may aid the establishment of distribution and/or

trafficking links between multiple seized samples that have been obtained at different locations or in the possession of different individuals. A database can be used to confirm a hypothesis proposed during an investigation or can be searched to locate samples with similar profiles thereby directing cases that were otherwise unconnected.

2.2.3 – Public health intelligence

In addition, drug profiling studies can also generate information, which is essential to health authorities. Identification of adulterants in street heroin samples plays a key role in early warning systems concerning unexpected adverse health consequences. Several heroin users in Scotland and England died in 2009/2010 as heroin was found to be contaminated with anthrax.[35]

2.3 – HEROIN PROFILING CONSIDERATIONS

2.3.1 – Heroin supply chain

The heroin supply chain is long and complex, consisting of producer, trafficker, distributor, supplier and user. Cutting agents may be added at each stage of the chain thereby progressively increasing the complexity of the heroin profile (figure 9).

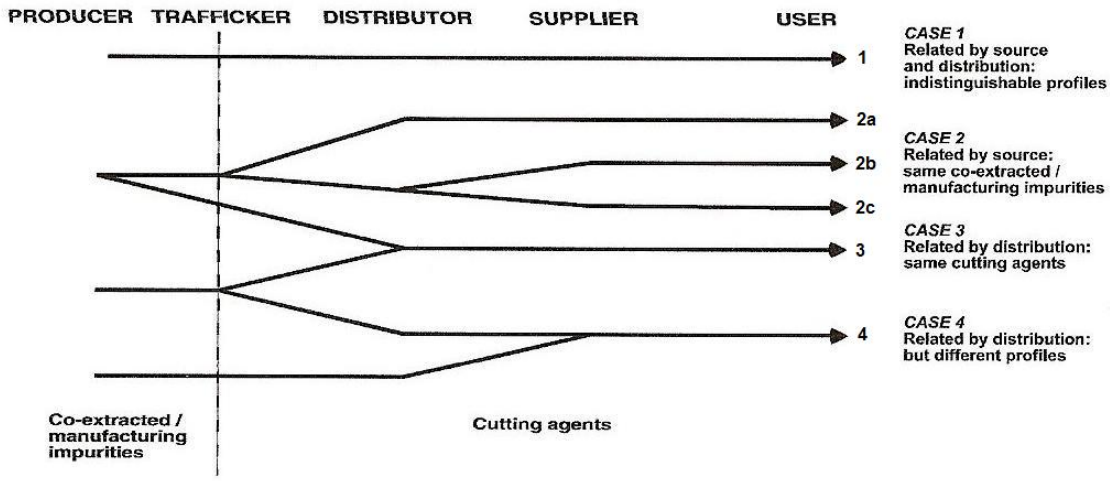


Figure 9 – The drug supply chain and its impact on chemical profiles [5]

Case 1 – Samples produced by the same producer and distributed by the same chain of supply. Chemical profiles contain the same relative proportions of alkaloids and cutting agents being indistinguishably linked by a common history.
Case 2 – Samples produced by the same producer but distributed by different supply chains. Chemical profiles contain the same relative proportions of alkaloids but different cutting agents being linked by a common source.
Cases 3 and 4 – Samples produced by different producers but are related by the chain of supply.

A further case occurs if samples are produced by different producers and are distributed by different chains of supply. This would generate samples with unequal quantitative contents of alkaloids, adulterants and diluents with different impurity profiles concluding that the investigated samples have different origins.[36]

2.3.2 – Heroin storage conditions

In depth chemical analyses of heroin samples of known geographic production are reported including the variations that take place with different storage conditions over time enabling the importance of each component for comparative analysis to be evaluated.[23, 37, 38]

Zhang *et al.*, profiled 500 illicit heroin samples by GCMS also studying their decomposition patterns. Increasing the storage period decreased diamorphine concentrations while 6-MAM contents increased and acetylcodeine levels remained almost unchanged. The decomposition profile led to the development of two source identification routes. Samples stored for less than 3 months were characterised by the ratios of acetylcodeine to diamorphine and acetylcodeine to 6-MAM, whereas, after storage of 3 months the content ratio of acetylcodeine to the sum of diamorphine and 6-MAM were employed. When the various ratios of different heroin samples were respectively similar, they were considered to be from the same source. The results using this approach were in good agreement with the information derived from the investigation process.[39]

2.3.3 – Batch variation

The process of drug profiling is further complicated by inconsistencies in the manufacturing process leading to inter-batch variation which may be as large as differences between different producers. If the heroin batch is non-homogeneous there may also be intra-batch variation with differences in impurity profile within a single batch. The changing chemical composition of seized heroin samples over time may reflect changes in origin but also changes in chemical practise so any classification of new samples using an old data set may not reflect the current situation thereby introducing errors. In addition a number of batches of heroin may be present in street samples resulting from batch mixing at various stages in the production or trade channels.[40]

2.4 – TECHNIQUES FOR HEROIN PROFILING

The continual growth in the manufacture, trafficking and abuse of illicit heroin are major areas of concern and heroin drug profiling is an important scientific tool for deriving intelligence to successfully counter ever-growing drug abuse problems.

Various literature reviews have summarised the many different methods employed in the comparative analysis of heroin samples.[3, 9, 41-43]

2.4.1 – Visual inspection

Visually recording the colour and form of heroin samples is a subjective process as colour shades must be recorded, consequently visual observations are only used to supplement more objective techniques.

2.4.2 – Colour tests

Presumptive colour tests provide an indication that heroin or other opiate alkaloids may be present but are not specific as many other compounds also give similar colours with the test reagents necessitating an additional confirmatory technique. The Marquis reagent (8-10 drops of 40% formaldehyde added to 10mL concentrated sulphuric acid [13]) is widely used as a heroin field test giving a characteristic purple

colour with diamorphine, morphine, codeine, 6-MAM or acetylcodeine, papaverine gives no colour and noscapine gives a bright yellow colour.[13]

2.4.3 – Thin layer chromatography

Thin layer chromatography (TLC) is often used as a simple and rapid screening technique to identify the opiate alkaloids and other components which may be present in heroin samples prior to examination by other methods.[44] The in-house solvent systems used by SPSA Forensic Services Edinburgh are cyclohexane/toluene/diethylamine (75:15:10) and toluene/acetone/ethanol/ammonia (45:45:7:3). Developed TLC plates can be visualised using UV light at 254nm or various spray reagents (Iodoplatinate, Dragendorff and Marquis).

2.4.4 – Infrared spectroscopy

Infrared spectroscopy is a useful screening technique that is more appropriate for the analysis of pure samples rather than multiple component mixtures such as heroin. However, Fourier transform infrared spectroscopy (FTIR) has successfully been utilised for heroin profiling.[36, 45-47]

Powdered heroin samples can be prepared as a halide disk using potassium bromide or potassium chloride or as a nujol mull using liquid paraffin. Major IR peaks are listed in order of magnitude of wavenumber absorbance (cm^{-1}) for the heroin base and heroin hydrochloride, the unique IR spectra enable their differentiation.[13, 48]

Heroin base:	1243	1196	1727	1214	1444	1757	1054	1370
Heroin hydrochloride:	1245	1736	1177	1194	1448	1765	1157	1386

However, determination of the heroin form within a sample is often not practical if the sample contains mixtures of the heroin salt and base forms or if the heroin base is adulterated with different salts.

2.4.5 – Gas Chromatography

A report by Gough and Baker in 1981 reviewed the gas chromatography (GC) analytical methods used for the quantitation of the major heroin constituents [49] and publications since 1999 indicate that GC remains the analytical technique of choice for drug profiling.[24] GC techniques routinely employed in heroin profiling afford high sensitivity and reproducibility providing greater resolution in the separation of complex heroin mixtures over liquid chromatography (LC) techniques (discussed in chapter 2.4.6). However, GC heroin sample derivatisation is often required eliminating the GC problems associated with compound adsorption, heat instability and transesterification (discussed in chapter 2.6.4).

GC coupled with mass spectrometry (MS), gas chromatography mass spectrometry (GCMS), combines the separating power of GC with the specificity and sensitivity of MS making GCMS a very useful tool for drug profiling. The MS detector generates highly specific mass spectral fragmentation data enabling the definitive identification of both known and unknown components within heroin samples, whereas a FID only provides 'visual pattern' information. Brenneisen *et al.,* used GCMS to study heroin pyrolysis obtaining 72 pyrolysis products. Heating the heroin street samples from 250 to 400°C gave substantial to complete diamorphine degradation, whereas morphine, codeine, acetylcodeine, papaverine and caffeine were heat stable.[50].

Two-dimensional GC has also been utilised for heroin profiling.[51] Headspace GC (HSGC) has been used to examine occluded solvents in heroin samples resulting from the heroin purification steps plus acetic acid produced by heroin deacetylation.[52-56] Traces of solvent residues trapped in the crystal lattice of the heroin samples are often unique to specific heroin producing regions. This technique offers the major advantage of being non destructive as the heroin remains intact and available for further analyses following HSGC analysis.

An in depth literature review of GCMS and GC FID heroin profiling methods is given in chapter 2.6.

2.4.6 – Liquid Chromatography

Liquid Chromatography (LC) techniques employed to study and/or profile major and minor heroin components [57] include densitometric determination using high performance thin layer chromatography (HPTLC) [58, 59], high performance liquid chromatography (HPLC) [16, 26, 34, 52, 60-70], sonic spray ionisation for liquid chromatography mass spectrometry (LCMS) [71] and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [72]. LC techniques eliminate the adsorption, heat instability and transesterification problems often associated with GC heroin analysis and sample derivatisation is not required. LC limitations include the need for component solubility, poorer resolution, high solvent consumption and associated solvent waste disposal.

2.4.7 – Capillary electrophoresis

Capillary electrophoresis (CE) [52, 73], capillary electrochromatography (CEC) with laser-induced fluorescence (LIF) [74] and capillary electrophoresis-mass spectrometry (CE-MS) [75] have been widely used to analyse heroin samples, a review is published by Anastos *et al.*,[76] The separation obtained by CE in narrow bore capillaries under the influence of an electric field is highly efficient, selective, rapid, and may be applied to both charged and neutral species. In addition CE has been used to determine the concentration of carbohydrates (glucose, sucrose, lactose, mannitol and mannose) found in heroin samples.[77, 78]

2.4.8 – Elemental analysis

A range of techniques have been studied to examine the trace inorganic impurities found in heroin samples originating from the elements present in the original opium poppy plus those introduced during the manufacturing process. Methods include elemental analysis (EA) to determine major metal (Ca, Mg, Al, Fe, Zn, Ba) and trace metal (Mn, Cu, Pb, Cd) concentrations.[79] Atomic absorption spectroscopy (AAS) [65], inductively coupled plasma atomic emission spectrometry using ultrasonic nebulisation (ICP-AES) [80] and inductively coupled plasma mass spectrometry (ICP-MS) [81] techniques have also been employed. The estimation of arsenic and strychnine adulterants found in heroin samples were carried out using the Gutzeit and

HPLC methods respectively [82] and trace elemental heroin profiling of iodine, bromine, calcium and zinc were investigated using total x-ray fluorescence spectroscopy (TXRF) [83].

2.4.9 – Isotope analysis

Isotopic analysis of heroin samples to determine isotope ratios of $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{18}O/^{16}O$ as markers for their geographical origin has been extensively studied using gas chromatography-isotope ratio mass spectrometry (GC-IRMS).[1, 84-92] Carbon isotope ratios of opiate alkaloids from opium natural products are influenced by photosynthesis and differences in plant location, climate or harvest time. Nitrogen isotope ratios are affected by the nitrogen composition of the soil and oxygen isotope ratios relate to precipitation and humidity.[3] The isotopic signature is further modified by ^{13}C enrichment during morphine acetylation to diamorphine providing additional discrimination depending on the acetylating agent used by the drug trafficker.[85] Desage *et al.*, measured the mean ^{13}C enrichment of diamorphine using GC-IRMS to distinguish heroin samples originating from Turkey but they could not discriminate between samples from Thailand, India, Pakistan and Nigeria.[91]

2.4.10 – Other analysis

The cling film samples used as wraps in heroin seizures have been analysed by elemental analysis-isotope ratio mass spectrometry (EA-IRMS) [87] and DNA profiles recovered from heroin packages have also been examined.[93]

2.5 – INTER-LABORATORY DATABASE COMPARISONS

A United Nations Office on Drugs and Crime (UNODC) report stated that experiences gained in the 1980s in the United States by the Drug Enforcement Agency (DEA) have shown that inter-laboratory comparisons of data can be problematic. In particular, the variance in the data generated using only major component analyses is too large to allow for successful inter-laboratory database searches hence nearly all retrospective database searches are performed as an intra-laboratory operation.[24]

More recently a collaborative heroin harmonisation study carried out between laboratories in Sweden, Germany and the Netherlands reached the same conclusion.[94] The European minor heroin component GC profiling method was proven to be robust and reproducible within one laboratory but despite a long collaboration between the three experienced laboratories, the inter-laboratory results were significantly less reproducible than the intra-laboratory results. This was attributed to the limits of the GC technique even though the GC instruments, columns and method parameters were identical. They concluded that the local determination at a large number of laboratories and the use of a common database was not realistic and the approach of conducting analysis in a central laboratory was recommended for international heroin comparisons.[94]

The above literature strongly suggests that heroin samples seized in Scotland could only be compared and potentially linked with samples seized in England if one laboratory is provided with samples from both seizures of interest. As such, the project aimed to develop a standalone Scottish heroin profiling database.

2.6 – REVIEW OF GCMS AND GC FID HEROIN PROFILING METHODS FROM THE SCIENTIFIC LITERATURE

2.6.1 – Major and minor heroin component GC profiling studies

Major components are defined as those present above 1% relative to diamorphine and include the most abundant alkaloids, adulterants and diluents. Characterisation of the major components enables screening of similar samples for possible linkages (tactical intelligence) as well as providing indications of the region of sample origin.

In contrast, minor components in heroin are present below 1% relative to diamorphine and include acidic and neutral by-products arising from acetylation of the opium alkaloids. Characterisation of heroin trace impurities generates signature spectra characteristic of geographical origin and also indicative of the processing or synthetic method utilised providing useful information for strategic intelligence.[95]

A number of research groups have profiled minor heroin components using GC techniques [1, 16, 36, 37, 51, 52, 94, 96-107] and studies have shown that 16 marker compounds are sufficient and robust for the purposes of impurity profiling.[37, 94] Examples of minor heroin component profiling by GC include:

- Structural characterisation of opium by-products related to noscapine and norlaudanosine.[106]
- Odell *et al.,* isolated oripavine, an alkaloid from the Tasmanian poppy, subsequent acetylation generated unique marker compounds enabling the identification of illicit heroin of Tasmanian origin.[96] Currently there is no evidence to suggest that heroin has ever been produced from Tasmanian poppies due to the low concentration of morphine.[108]
- Toske *et al.*, studied four tetrahydobenzylisoquinolines (laudanosine, reticuline, codamine and laudanine) present in the opium poppy, subsequent acetylation generated a series of 18 neutral minor heroin components enhancing the characterisation and classification of illicit heroin samples.[97]
- Morello *et al.*, described the isolation of 36 acidic and neutral manufacturing impurities found in illicit heroin samples. Over 500 authentic heroin samples of known origin were analysed enabling the geographical classification of illicit heroin based on the presence or absence of trace impurities.[107]

Minor heroin component analysis requires physically larger heroin samples than major component analysis. Typically a sample size equivalent to 15mg of pure diamorphine is required [94] which correlates to 150mg for a 10% diamorphine by weight heroin sample, and such sample sizes are not always available from street samples.

In addition, minor heroin component analysis often involves more complex and lengthy isolation procedures than major component analysis. The isolation process typically involves dissolving the heroin sample in dilute acid before extraction into an organic solvent using sonication, vortexing and centrifugation to isolate the

organic layer. Final evaporation is carried out before reconstitution in a suitable solvent for analysis or further derivatisation.

Odell *et al.,* compared the synthesis of heroin by acetylation of morphine with trifluoroacetic anhydride and acetic acid versus the traditional acetic anhydride route. The former route provided heroin with a lower diamorphine content and greater quantities of 3-MAM and 6-MAM. As the concentration ratios of the major alkaloids were found to be both production method as well as morphine extraction methodology dependant, they concluded that the examination of the major heroin alkaloid ratios was unsuitable for the development of a new heroin signature programme.[16] However, acetic anhydride remains the acetylating agent of choice being produced in large quantities for industry and easily diverted for illegitimate use.

A study by Gue´niat used both minor and major heroin impurity profiles to link different drug seizures. The similarities in profiles found by minor impurities were confirmed in 98% of cases by major impurities.[109]

Similarly a study by Dufey *et al.,* comparing the analytical results of 30 heroin samples showed that 95% of the links performed by their minor constituents method were confirmed by their major constituents method [104] which was in agreement with the conclusions of Esseiva *et al.,* [110]

2.6.2 – Heroin profiling GC quantification methodology

Profiling of major heroin components by GC frequently incorporates quantitation of diamorphine with semi-quantitation of the remaining components by normalising their peak areas relative to diamorphine [111] or an internal standard. If two or more heroin samples provide similar normalised peak area ratios, they are considered as possible matches and are selected for additional minor component analysis.

This approximate approach is reliable for the purposes of comparing heroin samples within one laboratory as both quantification errors and biases from the introduction

of adulterants and/or diluents are greatly reduced.[24] Furthermore, component data can be obtained and compared without the need for reliable drug standards.[111]

Besacier *et al.,* used normalised data measuring the alkaloid ratios of diamorphine and 6-MAM to acetylcodeine, diamorphine and 6-MAM to papaverine, and noscapine to papaverine enabling the differentiation of groups of samples.[1]

Morphine, 3-MAM, 6-MAM and diamorphine peak areas are often summed allowing the positive comparison of two samples that are identical except for the extent of hydrolysis. As acetylcodeine also hydrolyses to codeine their peak areas are similarly summed in samples where hydrolysis may have occurred.

An interesting normalised peak area application was developed by Narayanaswami. A characteristic increase of 1:3.8 was calculated when comparing the ratio of morphine/codeine in raw opium to the final ratio of diamorphine/acetylcodeine in clandestine heroin samples. Therefore applying a conversion factor of 3.8 for opium samples of known geographical origin provided the respective acetylated product ratios within heroin samples, the resulting diamorphine/acetylcodeine ratios were shown to be origin specific.[112]

2.6.3 – Internal standards used in GC heroin profiling studies

An internal standard is required to quantify major heroin components using GC techniques effectively removing response variations resulting from injection volume differences and MS detector instability. The internal standard must be commercially available and stable as the stock solution in the chosen injection solvent. When analysed they should provide good peak shape with a linear detector response and be fully resolved from the major heroin components commonly found in case samples.

A review of the literature regarding GC based heroin profiling methods showed that several compounds have been used as internal standards. These include benzopinacolone [16], 2-diethylaminoethyl-2,2-diphenylvalerate (SKF_{525A}) [39], amitriptyline hydrochloride [113] and numerous *n*-alkanes ranging from C_{16}

n-hexadecane [12], C_{22} *n*-docosane [40, 63], C_{24} *n*-tetracosane [24, 87, 89, 114, 115], C_{28} *n*-octacosane [10, 70] to C_{30} *n*-triacontane. [116] As none of the internal standards are chemically related to the opiates being quantified their analysis provides no indication of the efficiency of the heroin sample extraction, derivatisation or GC analysis.

Codeine has been utilised as a chemically related opiate internal standard for heroin profiling but as codeine is found in illicit heroin samples this represents a poor choice. [112, 117] Codeine and similar opiates are also not recommended as they undergo GC transesterification with diamorphine if heroin samples are not derivatised (discussed in chapter 2.6.4).

Use of analogous deuterated opiate internal standards is not feasible for GC FID analysis due to coelution with the opiates being quantified. The use of deuterated opiates as internal standards for heroin profiling by GCMS is possible if operating in selected ion monitoring (SIM).

2.6.4 – Advantages and disadvantage of derivatising major heroin components prior to GC analysis

Derivatisation is a process used to transform a chemical compound into another similar compound by altering one or more of its functional groups. Derivatisation is generally performed to alter compound reactivity or to change a physical property such as solubility, boiling point, melting point, thermal stability.

Several components present in heroin samples (morphine, 3-MAM, 6-MAM, codeine, paracetamol and phenobarbitone) possess polar groups (-OH, -NH) which form hydrogen bonds and sorb strongly onto various components of the GC system. Peak shapes in chromatographic systems are poor as hydrogen bonds cause peak tailing and components can be lost by adsorption onto the chromatographic system.

Derivatisation effectively caps the polar functional groups reducing their polarity, increasing volatility and stability to effectively enhance their overall

chromatographic behaviour. Figure 10 depicts the derivatisation of morphine by silylation using *N*-methyl-*N*-trimethylsilyltrifluouroacetamide (MSTFA) to afford morphine di-trimethylsilane (di-TMS). Specific methods employed to derivatise major heroin components are further discussed in chapter 2.6.6.1.

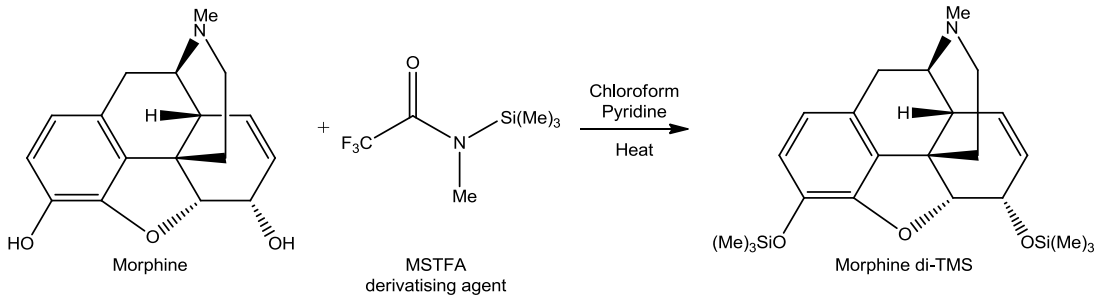


Figure 10 – Derivatisation of morphine using MSTFA

Heroin sample derivatisation is also beneficial in eliminating the GC problems associated with the transesterification of co-injected heroin sample components in the GC injection port. Transesterification involves the migration of acetyl groups from one component to another producing artefacts during GC analysis, this cannot occur if the polar 'acetyl accepting' groups (-OH, -NH) are effectively capped by derivatisation (figure 11).[118]

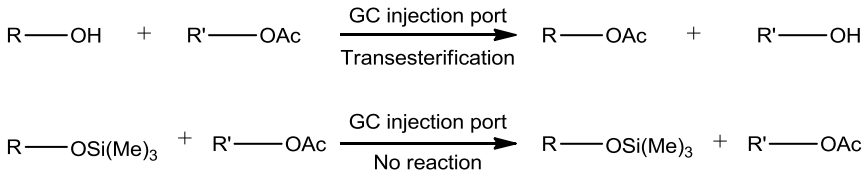


Figure 11 – Derivatisation prevents GC transesterification reactions

UNODC guidelines state that GC analysis of heroin without derivatisation results in the formation of three injection port artefacts, namely 6-MAM, 15,16-didehydroheroin and an unidentified component (MW = 381). The quantity of 6-MAM produced is a function of injection port temperature.[24]

Dautraix *et al.*, studied the specific GC transesterification of a 1:1 mixture of diamorphine:paracetamol. The amounts of diamorphine and paracetamol recovered

were not equal to those injected and 6-MAM and acetylparacetamol were also eluted (figure 12).[119]

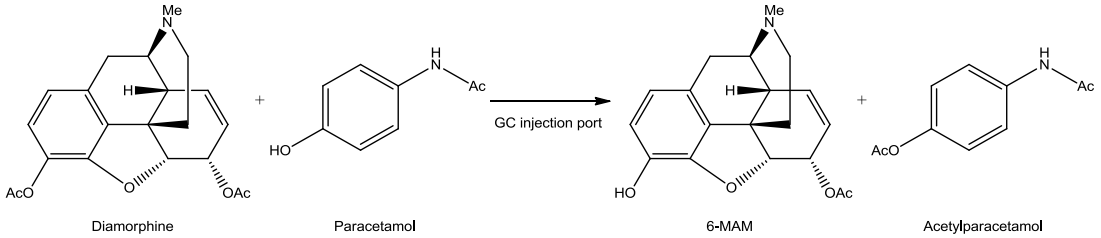


Figure 12 – GC injection port transesterification of diamorphine and paracetamol

A similar study by Huizer *et al.,* found that the levels of 6-MAM and acetylparacetamol produced were dependant on the amount of paracetamol adulterant added to the illicit heroin samples.[118]

Dybowski *et al.*, noted that increasing the GC injection port temperature increased thermal deacetylation of diamorphine to 6-MAM. In addition, they found that GC co-injection of a 1:1 mixture of morphine and diamorphine generated 6-MAM where the proportion of 6-MAM increased as the ratio of morphine to diamorphine increased. Increasing the injection port temperature also afforded trace 3-MAM.[120] Similar results were obtained when diamorphine and codeine were co-injected by GC affording trace 6-MAM and acetylcodeine. In each case artificially introducing a hold time after injection without carrier gas substantially increased the proportion of the 6-MAM and acetylcodeine artefacts.[120]

In the latter case of diamorphine and codeine co-injection, the heroin form was found to affect the degree of transesterification with methanolic heroin base solutions being more susceptible to codeine transesterification over methanolic heroin hydrochloric solutions which were more stable to GC deacetylation.[118]

In addition to improving GC chromatography and preventing transesterification, derivatising heroin samples enables the direct identification of sugars and

carbohydrates by GC analysis [121] and improves the resolution of components particularly isomers (3-MAM and 6-MAM).

A disadvantage of derivatisation results from the extra synthetic steps to derivatise the samples impacting on the reproducibility of the results via sample breakdown and/or contamination. A further associated problem is that heroin samples must be quantitatively derivatised to ensure no components amenable to derivatisation remain underivatised. As the derivatising reagents are moisture sensitive, a large excess is often employed to ensure complete derivatisation of all derivatisable components. In turn use of excess derivatisation reagents can be detrimental to the GC system and should be mopped up using triethylamine (TEA). Klemenc *et al.,* chose to omit derivatisation on these grounds.[21, 122]

Some guidelines also suggest that heroin transesterification reactions can be minimised if the injection port is properly cleaned and maintained as the reactions are enhanced/catalysed by contaminants in a dirty injection port serving as activation sites.[13, 24]

2.6.5 – Non derivatised major heroin component literature GC methods

2.6.5.1 – Sample preparation methods for non derivatised major heroin components prior to GC analysis

Major heroin component GC sample preparation omitting derivatisation involves extracting heroin samples using a suitable solvent with optional sonication and centrifugation to remove insoluble material.

UNIDCP guidelines state that use of an alcohol as an injection solvent can result in transesterification of certain components and advises choosing solvents which exclude sugars and salts other than organic halide salts. Numerous papers confirm this reporting transesterification combined with diamorphine and MAM hydrolysis when heroin samples are dissolved in methanol prior to injection [19, 58, 65, 88, 113, 115, 121, 123, 124] or ethanol.[39]

The alcohol solvent supports transesterification by extracting paracetamol present within the heroin sample which in turn promotes transesterification with diamorphine as discussed in chapter 2.6.4. As such many researchers recommend limiting the use of alcohol to dissolve the heroin sample thereby minimising paracetamol extraction. A 4:1 mixture of methanol:chloroform (or dichloromethane) dissolves most known adulterants or controlled substances found in heroin samples while excluding sugars although this approach may not be suitable for quantitation.[13]

Consequently researchers have qualitatively profiled heroin by dissolving the samples in a small volume of methanol followed by dilution with chloroform [63, 70, 114], 1:9 methanol:chloroform [10], 1:1:8 ethanol:isopropanol:chloroform [24] and 1:1 ethanol:chloroform.[10, 34, 119] Similarly, Fitsev *et al*., dissolved heroin samples in water and basified with ammonia to pH 9.0, before extracting the aqueous solutions three times into a 1:9 mixture of isopropanol:chloroform and concentrating the organic extracts for GCMS analysis.[125]

The extreme scenario of dissolving heroin samples in chloroform [1, 21, 67, 87, 89, 116, 122] or dichloromethane [16] excluding an alcohol solvent significantly reduces transesterification as paracetamol and other polar components are not extracted.

Barnfield *et al*., reported the use of a 1:9 *N*,*N*-dimethylformamide (DMF):ethanol mixture for heroin profiling where DMF was found to facilitate the dissolution of samples containing large proportions of caffeine, phenacetin and/or paracetamol.[111]

2.6.5.2 – Non derivatised major heroin component GC methods

The GC methods reported in the scientific literature for the analyses of non derivatised major heroin components from 1989 to the current date are summarised in table 2 (column packing 5% phenyl 95% dimethyl-polysiloxane, ZB-5 equivalent) and table 3 (column packing 100% dimethyl-polysiloxane, ZB-1 equivalent).

Table 2 – ZB-5 equivalent GC methods used to analyse major heroin components

Literature reference	Flow rate (mL/min)	Injection temperature (°C)	Oven programme
[58]	1.2	260	70°C to 280°C at 10°C/min
[125]	1.0	250	50°C to 300°C at 12°C/min
[123]	1.0	250	50°C for 2 min 50°C to 250°C at 5°C/min
[87, 89]	2.0	300 [a]	40°C for 2 min 40°C to 300°C at 10°C/min 300°C for 15 min
[88]	Not stated, 1.0 used	250	150°C for 1 min 150°C to 320°C at 10°C/min 320°C for 6 min
[39]	0.6	280	160°C for 1 min 160°C to 280°C at 10°C/min 280°C for 23 min
[12]	1.5	285	200°C for 1 min 200°C to 280°C at 12°C/min 280°C for 8 min
[50]	0.5	250	85°C for 1 min 85°C to 200°C at 8°C/min 200°C to 310°C at 6°C/min 310°C for 10 min
[16]	1.2	260	100°C for 1 min 100°C to 240°C at 6°C/min 240°C to 280°C at 2°C/min 280°C to 320°C at 6°C/min
[69]	Not stated, 1.0 used	225	60°C for 1.5 min 60°C to 200°C at 30°C/min 200°C to 300°C at 10°C/min 300°C to 310°C at 2°C/min

[a] Literature method ramped injection temperature from 50°C to 300°C at 200°C/min.

Table 3 – ZB-1 equivalent GC methods used to analyse major heroin components

Literature reference	Flow rate (mL/min)	Injection temperature (°C)	Oven programme
[65]	Not stated, 1.0 used	Not stated, 280 used	60°C to 280°C at 8°C/min
[125]	1.0	250	200°C to 280°C at 10°C/min
[15]	Not stated, 1.0 used	280	200°C to 280°C at 10°C/min
[91]	Not stated, 1.0 used	240	230°C for 1 min 230°C to 300°C at 8°C/min
[24] A3	1.0 used [a]	280	220°C to 300°C at 10°C/min 300°C for 3 min
[114]	Gas velocity 41cm/s	285 [b]	200°C for 1 min 200°C to 280°C at 12°C/min 280°C for 8 min
[1]	1.0	280	200°C for 1 min 200°C to 280°C at 4°C/min 280°C for 12 min
[24] A2	Not stated, 1.0 used	280	200°C to 260°C at 10°C/min 260°C to 310°C at 30°C/min 310°C for 5 min
[21, 122]	1.2	280	180°C for 1 min 180°C to 240°C at 10°C/min 240°C for 4.5 min 240°C to 290°C at 17°C/min 290°C for 4 min

[a] Literature method ramped flow rate from 0.9mL/min to 1.5mL/min at 0.1mL/min.

[b] Literature method injection temperature 75°C for 0.1 min then ramped to 285°C.

2.6.6 – Derivatised major heroin component literature GC methods

2.6.6.1 – Sample preparation methods for derivatising major heroin components prior to GC analysis

Major heroin component GC sample preparation incorporating derivatisation most commonly employs a silylation derivatisation method to trimethysilyl protect polar groups (-OH, -NH) as discussed in chapter 2.6.4.

From the list of proposed compounds for this study (table 5), codeine, morphine, 3-MAM, 6-MAM, paracetamol and phenobarbitone are all amenable to silylation affording the derivatives depicted in figure 13.

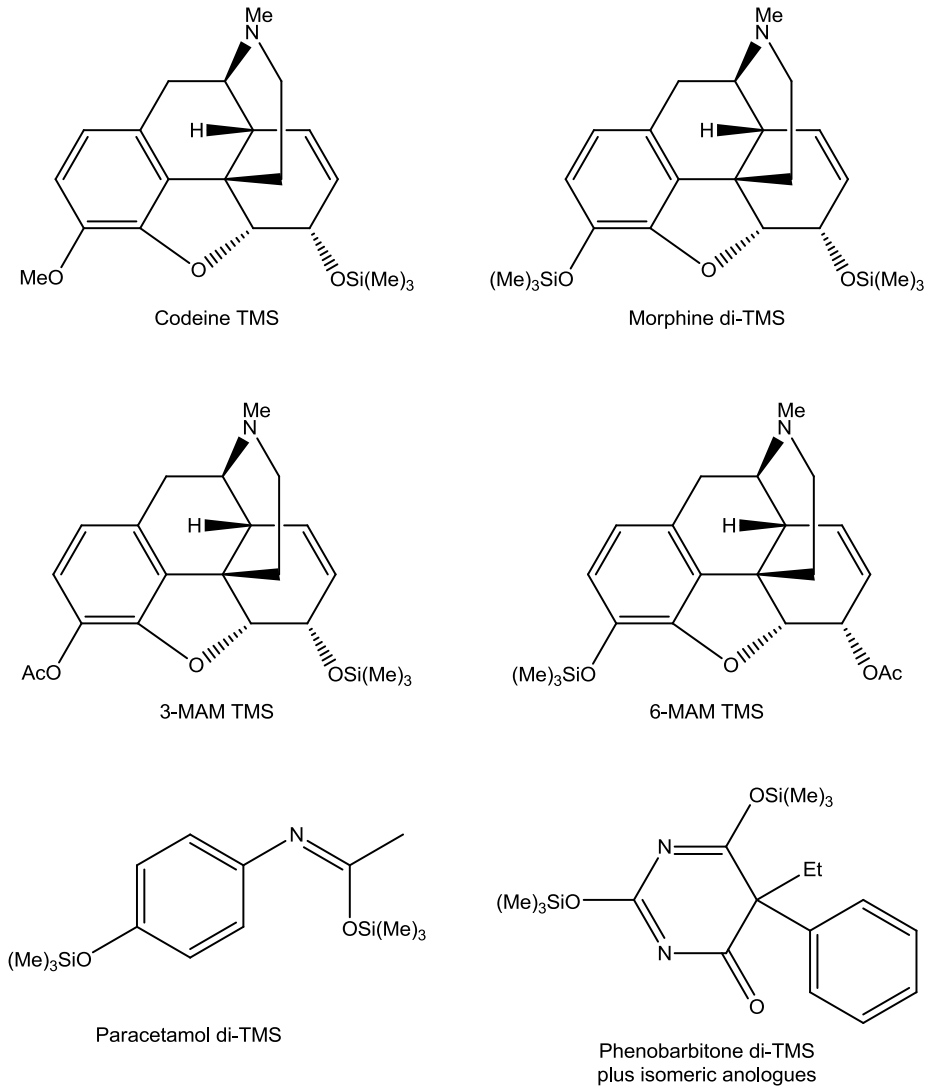


Figure 13 – Chemical structures of trimethylsilyl derivatised components

Ease of silylation follows 1° alcohol < 2° alcohol < 3° alcohol < phenol as steric hindrance increases derivatisation difficulty, amines and amides are harder to derivatise than alcohols.[121]

The most common derivatisation method used to silylate major heroin components for GC analysis dissolves the sample and internal standard in a 5:1

chloroform/pyridine mixture before adding MSTFA with heating at 70-80°C for 10-60 minutes and direct GC injection. Pyridine is added as an acid scavenger to drive the reaction forward.[13, 31, 104, 110, 126, 127]

Janhunen *et al*., extracted the major heroin soluble components into methanol with centrifugation to remove insoluble material before evaporating the extract. The residue was silylated with *N*,*O*-(bistrimethylsilyl)acetamide (BSA) in *n*-hexane heating at 60°C for 60 minutes prior to GC injection.[40] Other similar reports document heroin silylation using BSA in dichloromethane [13] or *N*,*O*-(bistrimethylsilyl)trifluoroacetamide (BSTFA) in chloroform [91] with heating at 60-70°C for 20-30 minutes prior to GC injection.

MSTFA has similar reaction properties to BSA and BSTFA as a trimethylsilyl donor for the silylation of all protic functional groups. At least 1-2 molar equivalents of silylating reagent are recommended per active hydrogen although unable to predict the proportion of alcohol and amine functional groups within heroin samples an excess is typically employed. In the case of BSA, silicon deposits from excess derivatising reagent form on the detector reducing its sensitivity. MSTFA and BSTFA trifluoroacetic acid based reagents are favoured volatilising the silicon as SiF_4 which acts as a cleaning agent to minimise build up and maintain sensitivity. MSTFA also benefits from elution of its *N*-methyltrifluoroacetamide by-product with the GC solvent front being more volatile than BSTFA and its by-product.

Researchers also suggest using MSTFA as the combined solvent and reagent thereby eliminating use of chloroform and pyridine. Heating at $\leq$ 80°C for $\leq$ 45 minutes is reported to prevent breakdown of the derivatised components with derivatisation complete on sample dissolution.[128]

A UNODC derivatisation method reports dissolving heroin samples in dilute hydrochloric acid with addition of solid sodium carbonate followed by acetylation with benzoyl chloride in chloroform and direct GC injection of the chloroform layer.

Care must be taken during the extraction procedure to avoid extremes of pH (< 3 and > 10) which increase the rate of diamorphine and MAM hydrolysis.[24]

Other researchers estimated the diamorphine content by direct GC analysis of heroin samples in methanol. Evaporation of the methanol extracts and acetylation using acetic anhydride/pyridine with GC analysis determining the original MAM content from the increased diamorphine content.[113, 117]

2.6.6.2 –Derivatised major heroin component GC methods

The GC methods reported in the scientific literature for the analyses of derivatised major heroin components from 1989 to the current date are summarised in table 4 (column packing 100% dimethyl-polysiloxane, ZB-1 equivalent).

Table 4 – GC methods used to analyse major derivatised heroin components

Literature reference	**Flow rate (mL/min)**	**Injection temperature (°C)**	**Oven programme**	**Resolution 6-MAM TMS/ 3-MAM TMS**
[13, 24] A4 [126, 129]	61 cm/sec	250	150°C to 300°C at 9°C/min 300°C for 2.4 min	0.37
[31, 110, 127]	1.43	290	150°C for 1 min 150°C to 250°C at 8°C/min 250°C to 320°C at 6°C/min 320°C for 1 min	0.52
[104]	1.4	290	150°C for 1 min 150°C to 250°C at 10°C/min 250°C to 300°C at 4°C/min	0.85

CHAPTER 3 – HEROIN PROFILING GCMS METHOD DEVELOPMENT

The project aim was to develop a robust and reproducible heroin profiling method and to build a database of heroin profiles amenable to rapid and accurate searching using the GCMS instrument provided. The literature reviewed from chapter 2.6.1 to 2.6.6 was considered prior to selecting the GCMS heroin profiling methodology appropriate for the study:

- Major heroin component impurity profiling was selected as the methodology for this study.
- Major heroin components were selected based on the most common heroin components and those used in corresponding profiling databases within the UK. The complete list of analytical standards used in this study together with the respective drug purities as sourced from the various suppliers is listed in table 5.
- Full quantification was chosen in this study to ensure long term reproducibility, quality control and a means of exchanging information between laboratories.
- Hexadecane was chosen as the internal standard based on commercial availability, cost, solubility, stability and GC peak shape, linearity and resolution.
- Overall no decision was made at the outset of the project whether or not to derivatise the major heroin components. Instead both non derivatised and derivatised sample preparation and GCMS methods were developed and assessed on their respective merits.

GCMS measurements were performed on a Shimadzu QP2010 Plus GCMS using helium carrier gas fitted with an automated split/splitless injector. The GC was operated in split mode with MS recording the full scan spectra in total ion chromatogram (TIC) mode unless otherwise stated. Initial studies used an available

ZB-5 capillary column (30m × 0.25mm internal diameter × 0.25µm film thickness, Phenomonex).

3.1 – DEVELOPMENT OF A NON DERIVATISED HEROIN GCMS METHOD

3.1.1 – GCMS analysis of non derivatised major heroin components

Based on the literature review in chapter 2.6.5.1, methanol was chosen as the most commonly utilised solvent for non derivatised major heroin component profiling and a chlorinated solvent was not added to enable quantitation. Each drug standard under study listed (table 5) and hexadecane internal standard were individually prepared at 1mg/mL in methanol and analysed.

Table 5 – Analytical standard supplier specifications

Standard	Supplier	Purity (%)	Molecular weight
Acetylcodeine.HCl.H_2O	Kinesis	99.9	Salt 394.1, free 341.4
Acetylthebaol	LGC	98.2	Free 296.1
Caffeine	Sigma	99.0	Free 194.2
Codeine	Sigma	100.0	Salt 303.9, free 299.4
Diamorphine.HCl.H_2O	FSS	100.0	Salt 423.9, free 369.4
Diazepam	FSS	100.0	Free 284.7
Hexadecane	Sigma	99.0	Free 226.5
3-MAM amidosulphonate	Kinesis	99.5	Salt 424.5, free 327.4
6-MAM.HCl.$3H_2O$	Kinesis	99.6	Salt 417.9, free 327.4
Meconin	Thermofisher	90.0	Free 194.2
Morphine.HCl.$3H_2O$	Macfarlan Smith	100.0	Salt 375.8, free 285.3
Noscapine	Sigma	97.0	Free 413.4
Papaverine.HCl	Fluka	≥ 98.0	Salt 75.9, free 339.4
Paracetamol	Sigma	Minimum 99.0	Free 151.2
Phenobarbitone	Sigma	99.3	Free 232.2

GC method parameters documented by Sperling [114] (table 3) and subsequently optimised during a previous heroin GCMS profiling project [12] (table 2) provided an initial starting point for method development. Each of the compounds were defined by their relative elution order (hexadecane, paracetamol, meconin, caffeine,

phenobarbitone, codeine, morphine, diazepam, 3-MAM, acetylcodeine, 6-MAM, acetylthebaol, diamorphine, papaverine, noscapine) and characterised by their mass spectra [13] (supplied in appendices) before being added to the drug profiling GCMS library.

Thebaine was also analysed and the resulting poor chromatographic behaviour depicted in figure 14 was attributed to extensive thebaine rearrangement and decomposition in the GC injector.[13] As thebaine is also rarely found in illicit heroin samples decomposing during acetylation to afford acetylthebaol, thebaine was not included in the GCMS method.

Figure 14 – GCMS chromatogram of thebaine

3.1.2 – Optimisation of GC method parameters for non derivatised major heroin component analysis

The GC method was optimised to chromatographically separate each of the major heroin components by considering the GC method parameters:

- High plunger and syringe speeds were applied to ensure very short injection times. Heroin base and heroin hydrochloride provide different GC responses if evaporation from the needle occurs during slow injections.[118]

- Increasing the injection volume increases the sample amount applied to the column and the resulting peak areas, however, the risk of column overload must be minimised.
- Splitless injections were adopted as the high carrier gas flow rate ensured that the sample only resided in the injection port for a short time. This effectively minimised thermal decomposition and introduced the sample as a narrow band onto the column resulting in non tailing sharp peaks.
- Increasing the split ratio reduced the sample amount applied to the column but also increased the speed of analysis which may limit transesterification thereby reducing the formation of on column artefacts.[128]
- The injection temperature must be sufficiently high for instantaneous sample vaporisation providing well resolved sharp peaks without sample degradation and on column artefact formation. Strömberg *et al*., investigated heroin injection temperatures from 200-300°C and found that the later eluting heroin components required higher injection temperatures for volatilisation.[94]
- Increasing the GC oven temperature reduced retention times to afford sharp peaks but resolution was reduced, conversely decreasing the oven temperature improved resolution through increased retention times however broader peaks were obtained. Oven temperature ramps (isocratic systems) are often employed to balance the demands of retention time and peak shape versus resolution.
- Increasing/decreasing the flow rate similarly reduces/increases retention times causing retention time/peak shape/resolution issues as discussed above.
- The detector temperature must be greater than or equal to the injection temperature and the maximum oven temperature.

Each of the GC literature methods given in tables 2 and 3 (chapter 2.6.5.2) were replicated using the available ZB-5 capillary column to analyse a 1mg/mL mixed heroin standard solution in methanol. An injection volume of 1µL with a high 100:1 split ratio and a detector temperature of 310°C were applied throughout. Resolution (R) between two peaks was calculated according to the following formula where peaks were resolved to baseline if resolution ≥ 1.5.

$$R_{1/2} = 2 * \frac{t_{R2} - t_{R1}}{w_1 + w_2}$$

t_{Ri} = retention time of the compound *i*

w_i = width of the peak *i*

Only the methods by Odell *et al*., [16] (table 2) and Besacier *et al*., [1] (table 3) provided some resolution of all the components. The former method was discarded due to the prohibitively long run time (50 min) and the latter method (run time 33 min) was chosen for further method development. The oven programme was modified (changes highlighted in bold in table 6) to improve the problematic resolutions between 3-MAM/acetylcodeine, acetylcodeine/6-MAM and 6-MAM/ acetylthebaol whilst maintaining as short a run time as possible.

Table 6 – Optimisation of GC method to analyse major heroin components [1]

Oven programme (method run time)	**Resolution 3-MAM/ acetylcodeine**	**Resolution acetylcodeine/ 6-MAM**	**Resolution 6-MAM/ acetylthebaol**
200°C for 1 min 200°C to 280°C at 4°C/min 280°C for 12 min (33 min)	0.73	0.83	1.17
200°C for 1 min 200°C to 280°C at **3°C/min** 280°C for **8 min** (**36 min**)	0.84	0.88	1.54
200°C for 1 min 200°C to 280°C at **2°C/min** 280°C for **4 min** (**45 min**)	0.98	0.95	2.10
200°C for 1 min 200°C to **248°C** at 2°C/min **248°C** to **290°C** at **10°C/min** **290°C for 6 min** (**35 min**)	0.98	0.95	2.10

The final optimised GC method (full GCMS parameters detailed in appendices) afforded the non derivatised major heroin component chromatogram in figure 15.

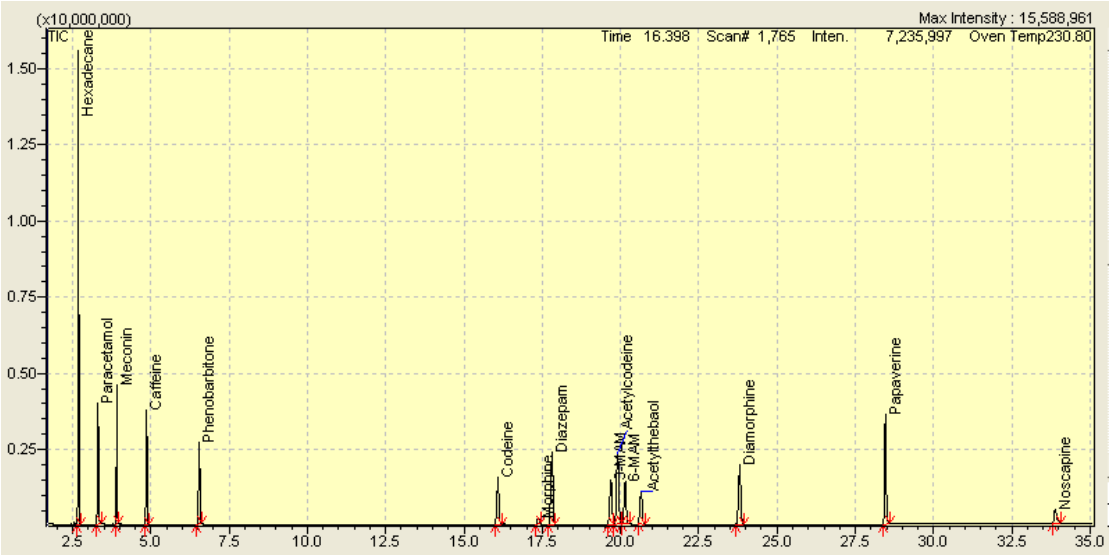


Figure 15 – Non derivatised major heroin component GCMS chromatogram

The compound resolutions are given in table 7.

Table 7 – Non derivatised major heroin component resolutions

Compound	Resolution
Hexadecane/paracetamol	4.32
Paracetamol/meconin	4.12
Meconin/caffeine	7.34
Caffeine/phenobarbitone	10.42
Phenobarbitone/codeine	46.77
Codeine/morphine	6.56
Morphine/diazepam	2.19
Diazepam/3-MAM	8.50
3-MAM/acetylcodeine	**0.98**
Acetylcodeine/6-MAM	**0.95**
6-MAM/acetylthebaol	2.10
Acetylthebaol/diamorphine	13.27
Diamorphine/papaverine	19.23
Papaverine/noscapine	20.62

The compound retention times (RT) and relative retention times (RRT) relative to diamorphine are given in table 8.

Table 8 – Non derivatised major heroin component RT and RRT

Compound	RT (min)	RRT diamorphine
Hexadecane	2.68	0.11
Paracetamol	3.31	0.14
Meconin	3.91	0.16
Caffeine	4.86	0.20
Phenobarbitone	6.55	0.28
Codeine	16.09	0.68
Morphine	17.38	0.73
Diazepam	17.81	0.75
3-MAM	19.69	0.83
Acetylcodeine	19.91	0.84
6-MAM	20.15	0.85
Acetylthebaol	20.66	0.87
Diamorphine	23.81	1.00
Papaverine	28.46	1.20
Noscapine	33.89	1.42

RRT calculated according to the formula:

$$RRT = \frac{t_{Ri}}{t_{R\ diamorphine}}$$

$t_{R\ diamorphine}$ = retention time of diamorphine

Finally the reproducibility of the GCMS system (intraday variation) was tested by carrying out six repeat injections of the 1mg/mL mixed heroin standard solution in methanol. The stability of the sample in methanol (interday variation) was similarly measured by injecting the sample six times over six consecutive days. As the chromatographic peaks were reasonably well resolved and symmetrical,

compound:hexadecane peak area ratios over peak height ratios [13] were used to determine the intraday and interday variation given in table 9.

Table 9 – Non derivatised major heroin component intraday and interday variations

Compound	**Intraday variation RSD (%)**	**Interday variation RSD (%)**
Paracetamol	2.4	2.6
Meconin	1.0	1.4
Caffeine	0.4	2.1
Phenobarbitone	0.7	2.2
Codeine	1.0	3.8
Morphine	2.4	**7.3**
Diazepam	1.0	1.3
3-MAM	1.4	3.5
Acetylcodeine	1.2	1.5
6-MAM	1.4	3.8
Acetylthebaol	1.2	1.2
Diamorphine	1.4	2.8
Papaverine	1.2	**5.1**
Noscapine	1.5	**7.8**

The compound relative standard deviations (RSD) in table 9 were calculated according to the formula:

$$RSD = 100 * \frac{S}{\bar{x}}$$

S (standard deviation) is calculated by: $S = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N-1}}$

N = number of measurements.

$\bar{x}$ (mean) is calculated by: $\bar{x} = \frac{\sum_{i=1}^{N} x_i}{N}$

Acceptable intraday variations with RSD values < 5% were obtained for all compounds demonstrating GCMS system reproducibility. However, interday variation RSD values > 5% were recorded for morphine and noscapine which could be ascribed to poor morphine chromatographic behaviour and large/comparatively non-volatile noscapine molecules providing inconsistent response factors. Interday variations were generally higher particularly for those compounds amenable to transesterification, highlighting reduced compound stability in methanol over time.

3.1.3 –Transesterification study of heroin components in methanol

A series of experiments were designed to establish whether the reduced stability of the non derivatised heroin components in methanol with time could be attributed to the internal transesterification reactions discussed in chapter 2.6.4.

Injection of diamorphine (1mg/mL) and hexadecane (1mg/mL) in methanol produced no 6-MAM demonstrating that diamorphine was stable and did not undergo thermal deacetylation with an injection temperature of 280°C (figure 16).[120]

Figure 16 – Co-injection of diamorphine (1mg/mL) and hexadecane (1mg/mL) in methanol

Injecting diamorphine (1mg/mL) and hexadecane (1mg/mL) with increasing amounts of paracetamol (0.2mg/mL to 9.0mg/mL) in methanol generated increasing levels of 6-MAM and acetylparacetamol (figure 17) via transacetylation.[118, 119]

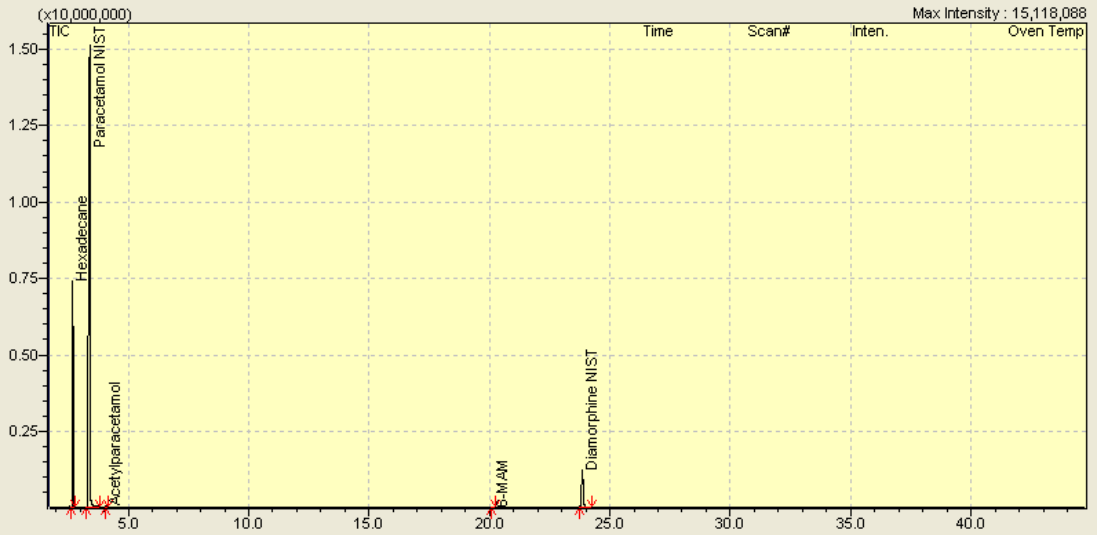


Figure 17 – Co-injection of diamorphine (1mg/mL), hexadecane (1mg/mL) and paracetamol (9.0mg/mL) in methanol

Injecting diamorphine (1mg/mL), morphine (1mg/mL) and hexadecane (1mg/mL) with increasing amounts of paracetamol (0.2mg/mL to 7.2mg/mL) in methanol afforded similar increasing levels 6-MAM and acetylparacetamol with further transacetylation between acetylparacetamol and morphine producing 3-MAM (figure 18).

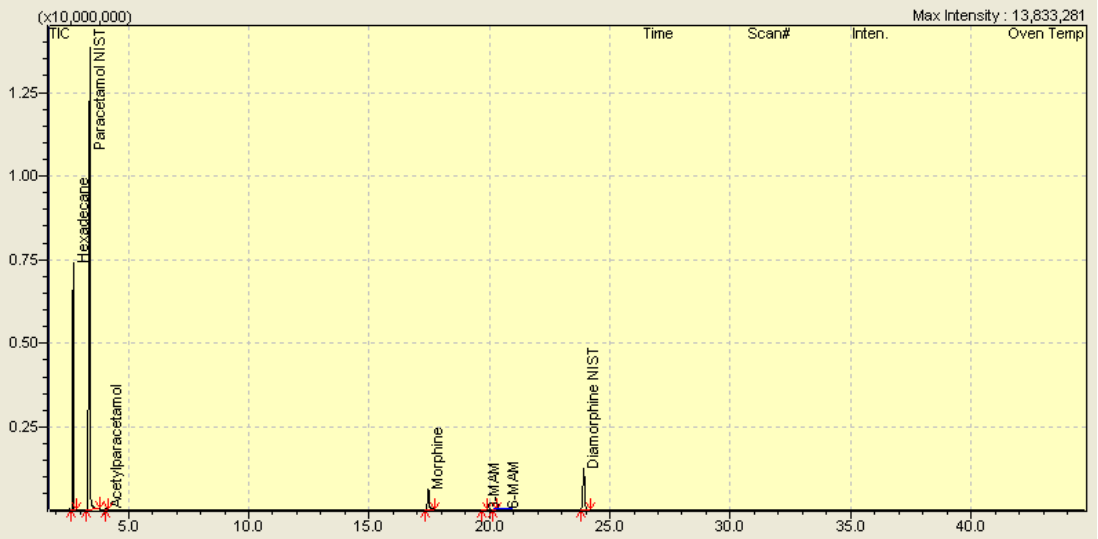


Figure 18 – Co-injection of diamorphine (1mg/mL), morphine (1mg/mL), hexadecane (1mg/mL) and paracetamol (7.2mg/mL) in methanol

Injecting diamorphine (1mg/mL), codeine (1mg/mL) and hexadecane (1mg/mL) with increasing amounts of paracetamol (0.2mg/mL to 7.2mg/mL) in methanol generated similar increasing levels of 6-MAM and acetylparacetamol with no further transacetylation of codeine to acetylcodeine (figure 19).

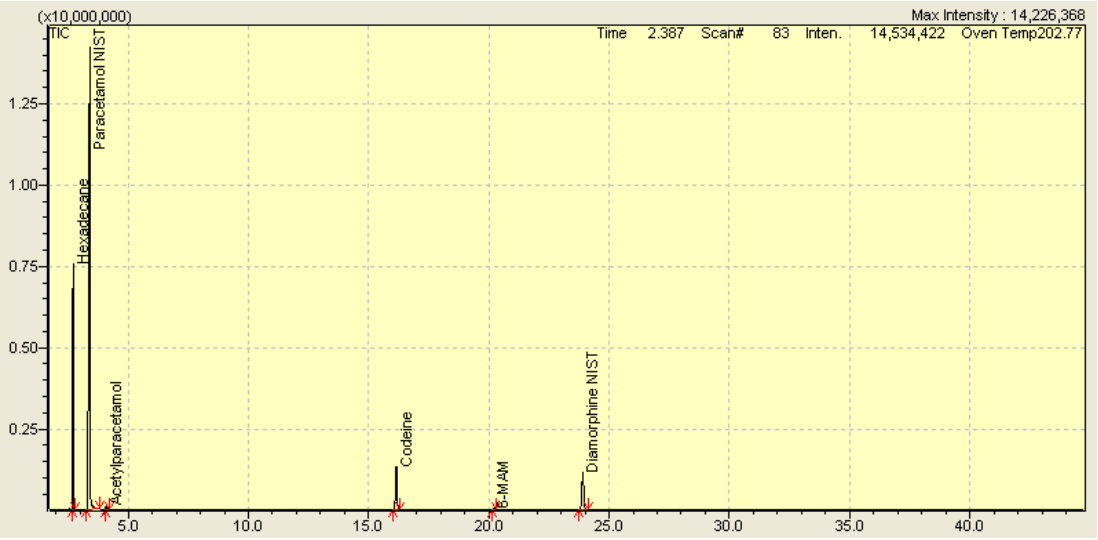


Figure 19 – Co-injection of diamorphine (1mg/mL), codeine (1mg/mL), hexadecane (1mg/mL) and paracetamol (7.2mg/mL) in methanol

Injecting diamorphine (1mg/mL) and hexadecane (1mg/mL) with increasing amounts of morphine (0.2mg/mL to 9.0mg/mL) in methanol afforded increasing levels of 6-MAM and 3-MAM (figure 20).[120]

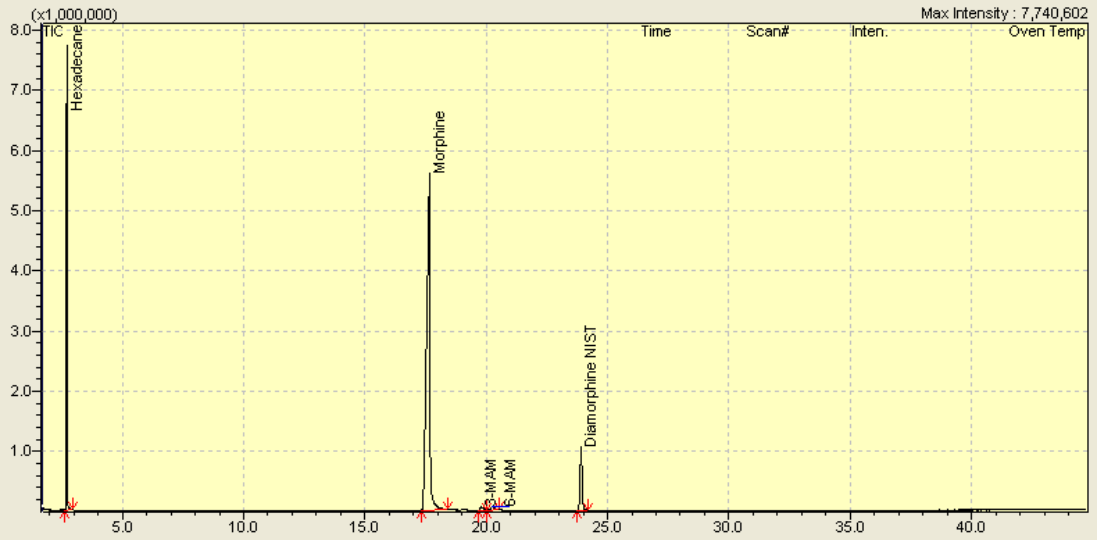


Figure 20 – Co-injection of diamorphine (1mg/mL), hexadecane (1mg/mL) and morphine (9.0mg/mL) in methanol

Attempts were made to correlate the production of 6-MAM, acetylparacetamol and 3-MAM as a function of increasing paracetamol and morphine concentrations but the GCMS instrument was too imprecise for a satisfactory transesterification kinetic study. Furthermore, minor variations in injection port temperature/number of active injection port sites/injection hold time could affect transesterification levels.[120]

In conclusion, GC transesterification of non derivatised heroin samples on column impacts both accuracy and reproducibility as the degree of artefact production is unique to the mixture of heroin components being analysed and affected by the injection port parameters. As such research began in parallel to develop a derivatised heroin GCMS method.

3.2 – DEVELOPMENT OF A DERIVATISED HEROIN GCMS METHOD

3.2.1 – GCMS analysis of derivatised major heroin components

Based on the literature review in chapter 2.6.6.1, silylation was chosen as the most common means of derivatising major heroin components for profiling. Amalgamating literature protocols [13, 24, 31, 104, 110, 126, 127], 1mg of each drug standard under study listed in table 5 was individually dissolved in chloroform (1mL) and pyridine (200µL) before adding MSTFA (150µL) and heating in a sealed vial at 80°C for 45 minutes. Similarly non derivatised drug standards were individually prepared at 1mg/mL in methanol.

GC method parameters documented by Esseiva *et al*.,[110] (table 4, chapter 2.6.6.2) provided an initial starting point for method development. Each of the derivatised and non derivatised compounds were analysed and defined by their relative elution order (hexadecane, paracetamol di-TMS, paracetamol TMS, meconin, caffeine, phenobarbitone di-TMS, diazepam, codeine TMS, morphine di-TMS, acetylcodeine, acetylthebaol, 6-MAM TMS, 3-MAM TMS, diamorphine, papaverine, noscapine). In addition the derivatised compounds were characterised by their mass spectra (supplied in appendices) before being added to the drug profiling GCMS library.

All of the paracetamol was consumed but quantitative conversion to paracetamol di-TMS failed and some intermediate paracetamol TMS remained. Further investigation of paracetamol derivatisation is discussed in chapter 3.2.3.

3.2.2 – Optimisation of GC method parameters for derivatised major heroin component analysis

The GC method was optimised to chromatographically separate each of the major derivatised heroin components by considering the GC method parameters outlined in chapter 3.1.2.

Each of the GC literature methods given in table 4 (chapter 2.6.6.2) were replicated using the available ZB-5 capillary column to analyse a 1mg/mL mixed derivatised heroin standard sample. An injection volume of 1µL with a high 100:1 split ratio

and a detector temperature of 325°C were applied throughout. The latter method by Dufey *et al*., [104] was chosen for further method development providing optimum resolution of the most problematic 6-MAM TMS and 3-MAM TMS derivatised compounds.

The oven programme was modified numerous times introducing additional complex oven temperature ramps to improve resolutions between paracetamol TMS/meconin, diazepam/codeine TMS, morphine di-TMS/acetylcodeine and 6-MAM TMS/3-MAM TMS whilst maintaining as short a run time as possible.

Oven programme	150°C for 1 min
	150°C to 224°C at 12°C/min
	224°C to 228°C at 0.25°C/min
	228°C to 275°C at 25°C/min
	275°C to 300°C at 5°C/min
	300°C for 2.95 min
Method run time	33 min

The final optimised derivatised GC method (full GCMS parameters detailed in appendices) afforded the derivatised major heroin component chromatogram in figure 21.

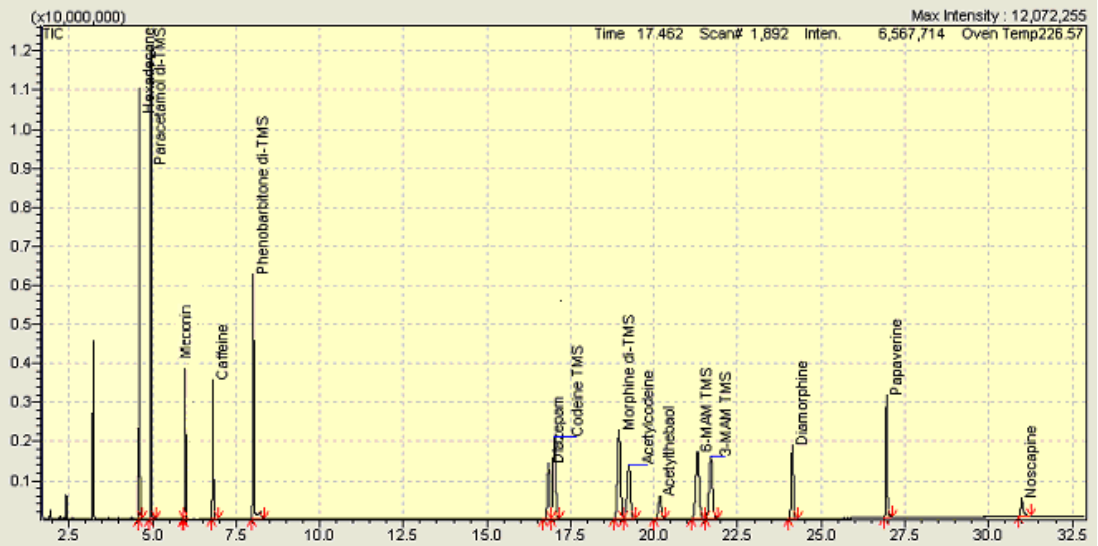


Figure 21 – Derivatised major heroin component GCMS chromatogram

Ultimately it was not possible to resolve all of the derivatised compounds to baseline requiring resolution ≥ 1.5 with the optimum compound resolutions given in table 10. The compound retention times and relative retention times relative to diamorphine are given in table 11.

Table 10 – Derivatised major heroin component resolutions

Compound	Resolution
Hexadecane/paracetamol di-TMS	2.94
Paracetamol di-TMS/paracetamol TMS	11.58
Paracetamol TMS/meconin	**0.80**
Meconin/caffeine	7.28
Caffeine/phenobarbitone di-TMS	9.73
Phenobarbitone di-TMS/diazepam	53.03
Diazepam/codeine TMS	**0.81**
Codeine TMS/morphine di-TMS	7.74
Morphine di-TMS/acetylcodeine	**1.03**
Acetylcodeine/acetylthebaol	3.74
Acetylthebaol/6-MAM TMS	4.39
6-MAM TMS/3-MAM TMS	**1.36**
3-MAM TMS/diamorphine	9.76
Diamorphine/papaverine	14.62
Papaverine/noscapine	23.70

Table 11 – Derivatised major heroin component RT and RRT

Compound	RT (min)	RRT diamorphine
Hexadecane	4.60	0.19
Paracetamol di-TMS	4.95	0.21
Paracetamol TMS	5.92	0.25
Meconin	5.98	0.25
Caffeine	6.80	0.28
Phenobarbitone di-TMS	8.02	0.33
Diazepam	16.85	0.70
Codeine TMS	17.02	0.71
Morphine di-TMS	18.96	0.79
Acetylcodeine	19.24	0.80
Acetylthebaol	20.18	0.84
6-MAM TMS	21.29	0.88
3-MAM TMS	21.69	0.90
Diamorphine	24.14	1.00
Papaverine	26.94	1.12
Noscapine	30.99	1.28

Finally the reproducibility of the GCMS system (intraday variation) was tested by carrying out six repeat injections of the 1mg/mL mixed derivatised heroin standard sample. The stability of the derivatised sample (interday variation) was similarly measured by injecting the sample six times over six consecutive days. Compound:hexadecane peak area ratios were again used over peak height ratios [13] to determine the intraday and interday variation. The compound relative standard deviations are given in table 12.

Table 12 – Derivatised major heroin component intraday and interday variations

Compound	**Intraday variation RSD (%)**	**Interday variation RSD (%)**
Paracetamol di-TMS	1.8	1.7
Paracetamol TMS	**62.1**	**45.6**
Sum paracetamol di-TMS + paracetamol TMS	0.9	1.2
Meconin	0.6	1.3
Caffeine	0.5	1.0
Phenobarbitone di-TMS	1.5	3.3
Diazepam	1.0	1.2
Codeine TMS	0.8	0.8
Morphine di-TMS	1.3	1.1
Acetylcodeine	0.8	0.9
Acetylthebaol	1.0	1.4
6-MAM TMS	1.1	1.5
3-MAM TMS	1.1	1.1
Diamorphine	0.8	1.3
Papaverine	0.6	2.6
Noscapine	2.7	3.4

Acceptable intraday and interday variations < 5% were obtained for all compounds excluding paracetamol TMS where the high RSD value was attributed to the observed conversion of paracetamol di-TMS to the intermediate paracetamol TMS. Satisfactory RSD values were calculated using the sum of both paracetamol derivatives:hexadecane peak area ratios as later discussed in chapter 3.2.3.

The chromatographic behaviour of phenobarbitone di-TMS deteriorated post derivatisation as depicted in figure 22 (black trace = phenobarbitone di-TMS day 1, pink trace = phenobarbitone di-TMS day 6). The MS data acquired throughout the phenobarbitone di-TMS time window matched that of the target compound and integrating both the main and shoulder peak in this region provided an acceptable interday variation RSD value of 3.3%.

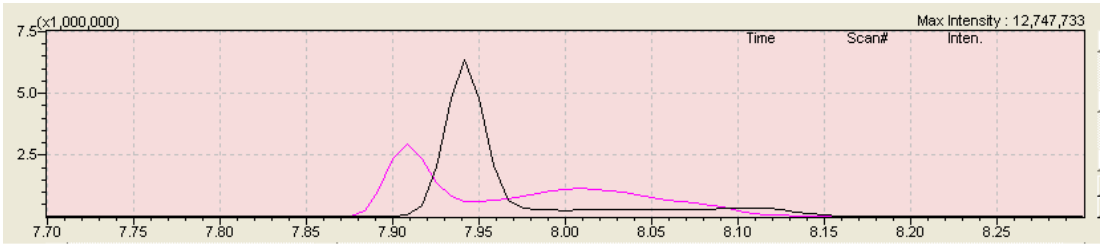


Figure 22 – Comparative GCMS chromatograms of phenobarbitone di-TMS

The cause of the phenobarbitone di-TMS peak degradation and tailing was unclear, although it may be attributed to formation of the phenobarbitone di-TMS isomeric analogous shown in figure 23.

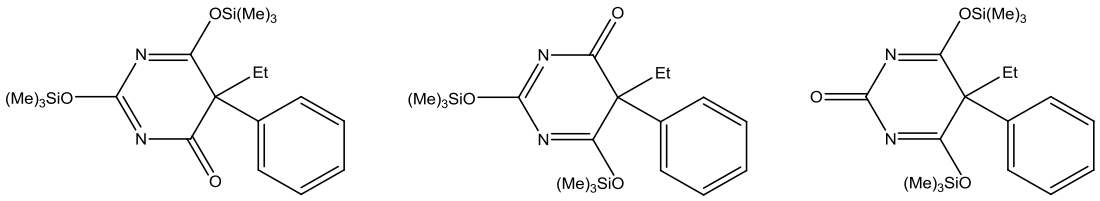


Figure 23 – Phenobarbitone di-TMS isomeric analogues

Overall, eliminating transesterification by derivatising the compounds afforded derivatised compounds which were more stable over time than the non derivatised compounds as demonstrated by the lower interday variation RSD values. As such research continued to develop a derivatised heroin GCMS method.

3.2.3 – Investigation of paracetamol derivatisation

As discussed in chapter 3.2.2, initially generated paracetamol di-TMS slowly reverts to the paracetamol TMS intermediate over time. The derivatising conditions were modified in an attempt to force and maintain the conversion of paracetamol to paracetamol di-TMS. Generating this single paracetamol derivative would simplify paracetamol quantitation and negate the paracetamol TMS/meconin resolution issue.

Using the derivatising conditions previously described in chapter 3.2.1, paracetamol (15mg) was dissolved in chloroform (1mL), pyridine (200µL) and MSTFA (150µL)

with heating at 80°C for 45 minutes affording a 20:80 ratio of paracetamol TMS:paracetamol di-TMS by peak area.

Incorporating all of the derivatising modifications below improved this ratio to 2:98 indicating that quantitative conversion to paracetamol di-TMS is not possible.

- Paracetamol amount reduced from 15mg to 10mg
- Anhydrous chloroform and anhydrous pyridine used
- Chloroform (0.5mL) and pyridine (100µL) volumes reduced
- MSTFA (250µL) volume increased
- MSTFA containing 1% trimethylchlorosilane (TMCS) used
- Derivatisation time increased to 1 hour
- Derivatisation temperature increased to 90°C

The paracetamol sample concentration was reduced to prevent intermittent MS detector saturation but the remaining above changes were not implemented due to the enhanced costs of anhydrous solvents and excess/alternative derivatising reagent plus the increased potential for derivatised component breakdown heating samples at ≥ 80°C for ≥ 45 minutes.[128]

An alternative approach was adopted whereby the paracetamol sample concentration was further reduced by dilution with 5:1 chloroform:pyridine and the ratio of paracetamol:MSTFA was increased by only derivatising an aliquot of the paracetamol solution.

Accordingly paracetamol (10mg) was dissolved in chloroform (5mL) containing hexadecane (0.5mg/mL), pyridine (1mL) was added and the solution was vortex mixed. A 500µL aliquot was then removed and derivatised using MSTFA (150µL) with heating at 80°C for 45 minutes. The derivatised sample was held at room temperature and repeatedly analysed over 24 hours monitoring the paracetamol TMS:paracetamol di-TMS peak area ratios as shown in table 13.

Table 13 – Changing paracetamol TMS:paracetamol di-TMS peak area ratios during paracetamol derivatisation

Hold time	Paracetamol TMS:paracetamol di-TMS peak area ratio
0 minutes	1.2:98.8
45 minutes	1.8:98.2
6 hours	2.2:97.8
24 hours	2.4:97.6

Heroin case samples (10mg) containing low, medium and high amounts of paracetamol (cases 1, 2 and 3) were similarly derivatised and each sample was analysed six times over a 24 hour period (chromatograms provided in appendices). In each case the paracetamol TMS:paracetamol di-TMS peak area ratios again remained constant within the range 1-2:99-98 indicating derivatised paracetamol sample stability. The RSD variations were calculated by peak area for paracetamol derivatives with respect to hexadecane as given in table 14.

Table 14 – Calculated RSD values for paracetamol TMS and paracetamol di-TMS

Sample	RSD paracetamol TMS:hexadecane (%)	RSD paracetamol di-TMS: hexadecane (%)	RSD sum paracetamol TMS and di-TMS: hexadecane (%)
Paracetamol	**26.2**	1.9	1.7
Case sample 1	**20.3**	1.5	1.2
Case sample 2	**19.2**	1.8	1.6
Case sample 3	**19.4**	1.8	1.6

The RSD values for paracetamol TMS were considerably larger than those for paracetamol di-TMS due to the relatively low amounts of the former derivative generated. Optimum RSD values were obtained by summing the peak areas of both paracetamol derivatives in relation to hexadecane peak areas. The GCMS Shimadzu software was unable to automatically sum the paracetamol derivatives within the method therefore all subsequent paracetamol quantitations were achieved manually using excel.

3.2.4 – Derivatised heroin GCMS method conversion from qualitative TIC to quantitative SIM/scan MS data acquisition

The derivatised heroin GCMS method was developed in TIC mode by recording the full scan spectra over the entire large mass range enabling the detection of all components present in the samples. Acquiring the MS data in TIC mode provided optimum selectivity but this was offset by reduced sensitivity.

For the purposes of reliable quantitation SIM mode is preferred recording more MS sampling data for each selected compound by targeting the specific compound ions.[130] Acquiring the MS data in SIM mode provides optimum sensitivity but selectivity is lost.[128]

The Shimadzu QP2010 Plus GCMS instrument provides an additional mode of MS data acquisition combining the advantages of both TIC and SIM, namely SIM/scan mode. This option was chosen offering the sensitivity to accurately quantify target compounds with the selectivity to identify any additional non target compounds present in the heroin samples.

Table 15 lists the quantitative and qualitative SIM target ions chosen for the derivatised major heroin components.

Table 15 – Quantitative and qualitative SIM target ions selected for the derivatised major heroin components

Compound	Quantitative		Qualitative		Qualitative	
	Ion (m/z)	Ratio (%)	Ion (m/z)	Ratio (%)	Ion (m/z)	Ratio (%)
Hexadecane	57.1	100	71.1	74.26	85.1	50.26
Paracetamol di-TMS	206.1	100	280.1	74.31	295.1	50.79
Paracetamol TMS	181.0	100	223.0	73.60	208.0	17.92
Meconin	165.1	100	194.0	92.71	147.1	91.42
Caffeine	194.1	100	109.1	59.27	82.1	28.60
Phenobarbitone di-TMS	146.1	100	361.1	28.63	261.1	12.74
Diazepam [1]	283.0	100	256.0	127.09	284.0	89.13
Codeine TMS	371.1	100	178.1	55.01	234.1	50.54
Morphine di-TMS [2]	236.0	100	429.1	142.73	414.1	61.03
Acetylcodeine	341.1	100	282.1	67.06	229.1	37.68
Acetylthebaol	254.2	100	239.2	74.92	296.2	32.75
6-MAM TMS	399.1	100	340.1	60.39	287.1	39.37
3-MAM TMS [3]	357.1	100	399.1	111.92	234.1	88.73
Diamorphine	327.1	100	369.1	68.51	268.1	58.00
Papaverine	338.1	100	324.1	95.21	339.1	81.44
Noscapine	220.0	100	205.0	15.46	221.0	13.37

[1] 256.0 ion present in non-baseline resolved codeine TMS.

[2] 429.1 ion present in non-baseline resolved acetylcodeine.

[3] 399.1 ion quantitative ion for non-baseline resolved 6-MAM.

The specific target ions given in table 15 were selected for each derivatised compound requiring SIM quantitation adhering to the following guidelines:

- Selected target ions must be distinctive, high mass ions with good abundance.
- The target quantitative ion is characteristic of the target compound, preferably one that distinguishes the compound from any others with similar retention times.
- Two target qualifier ions are selected from the MS of the target compound. The presence of these ions in the correct ratios relative to the target ion gives evidence of correct target compound identification.

- Default ion allowance ratio required for compound identification set to 30%.
- The chosen quantitative ion corresponds to the most abundant ion unless this ion is also found in the MS of non-baseline resolved compounds.
- Target ions cannot include m/z 73 as this corresponds to the non unique TMS functional group common to the TMS derivatised heroin compounds.

3.2.5 – Derivatised method linear calibration study 200-1000µg/mL

A heroin profiling study conducted in Scotland in 2007 determined the following percentage composition ranges for heroin samples:

Paracetamol	1-62% w/w
Caffeine	0.5-31% w/w
Diamorphine	0.5-85% w/w
Papaverine	2-5% w/w
Noscapine	5-30% w/w

Mass percentages (% w/w) were used to express the concentration of each component within a heroin sample. For example, if a heroin sample is 50% diamorphine by mass (50% w/w), every 100g of heroin contains 50g of diamorphine. Mass percentages are calculated as 100% times the mass of a component divided by the mass of the heroin sample. A sum of the % w/w quantified components less than 100% provides an indication that unknown adulterants/diluents/salts may be present within the heroin sample.

A decision was made to generate calibration lines for each compound with linear ranges of 0-100% w/w to encompass the full scope of possible concentrations. The weighing balance was only accurate for masses ≥ 10mg, therefore 10mg free base equivalents of each compound were weighed into individual vials accounting for the drug purities and salt contributions listed in table 5:

Acetylcodeine.HCl.H_2O	11.6mg
Caffeine	10.1mg
Codeine	10.2mg
Diamorphine.HCl.H_2O	11.5mg
Diazepam	10.0mg
3-MAM amidosulphonate	13.0mg
6-MAM.HCl.$3H_2O$	12.8mg
Meconin	11.1mg
Morphine.HCl.$3H_2O$	13.2mg
Noscapine	10.3mg
Papaverine.HCl	11.3mg
Paracetamol	10.1mg
Phenobarbitone	10.0mg

Acetylthebaol (10.2mg) was omitted from the initial method development work due to delays in procuring the drug standard.

Initial attempts to follow the previously optimised derivatisation protocol (chapter 3.2.3) proved unsuccessful as the compounds failed to completely dissolve in 5:1 chloroform:pyridine (10mL) despite vortex mixing the solutes/solvents with subsequent sonication at 40°C.

An alternative approach of dissolving the compounds in methanol was evaluated as diamorphine free base and the hydrochloride salt are soluble and freely soluble in methanol respectively.[13] A 1mg/mL mixed standard solution in methanol was prepared by transferring the 10mg free base equivalents of each compound to a 10mL volumetric flask. The vials were rinsed with methanol (2 x 250μL), added to the flask and the flask sonicated for 5 minutes before making the total volume up to 10mL with methanol.

A 0.779mg/mL internal standard solution in chloroform was prepared by weighing hexadecane (78.7mg) into a 100mL volumetric flask, chloroform was added and the

flask sonicated for 5 minutes before making the total volume up to 100mL with chloroform. Hexadecane was not prepared as a 5:1 chloroform:pyridine solution as the addition of pyridine caused yellow discolouration of the solution on standing.

Calibration and control samples were prepared by transferring aliquots of the 1mg/mL mixed standard methanol solution to individual vials as detailed in table 16. The methanol was immediately removed by evaporation at 30°C under nitrogen to minimise hydrolysis of diamorphine to 6-MAM/morphine which has been shown to occur the day after diamorphine sample preparation in methanol.[131]. Care was also taken to ensure that all traces of methanol were removed as any residual solvent would react and quench the MSTFA affecting the derivatisation reproducibility.

Table 16 – Preparation of calibration and control heroin standard samples

Sample	**Volume 1mg/mL mixed standard solution (µL)**	**% w/w**	**Concentration (µg/mL)**
Standard 0	0 [a]	0	0
Standard 1	130	20	200
Standard 2	260	40	400
Standard 3	390	60	600
Standard 4	520	80	800
Standard 5	650	100	1000
Control 1 [b]	65	10	100
Control 2 [b]	325	50	500
Control 3 [b]	585	90	900

[a] 650µL methanol added

[b] Six samples prepared

Hexadecane 0.779mg/mL in chloroform (417µL), pyridine (83µL) and MSTFA (150µL) were added to each evaporated vial. A blank was also prepared by adding chloroform (417µL), pyridine (83µL) and MSTFA (150µL) to a separate vial. Final hexadecane sample concentrations of 500µg/mL were achieved. Each of the vials were capped, heated at 80°C for 45 minutes, cooled to room temperature and analysed.

The derivatised paracetamol calibration line was obtained by manually plotting the peak area ratio of the summed paracetamol derivatives to hexadecane against the paracetamol concentrations using excel. The calibration line was then used to manually calculate the control concentrations/control percentage errors/RSD values.

Correlation coefficients (r) given in table 17 indicate the measure of linear fit, r values ≥ 0.995 signify good linearity.

The maximum control percentage error obtained for each series of six control samples also given in table 17 provides a measure of the accuracy of the measured control concentrations in relation to the actual spiked control concentrations, percentage errors ≤ 15% indicate good accuracy. Percentage errors calculated according to the formula:

$$\%\ error = \frac{(measured\ concentration - actual\ concentration)}{actual\ concentration}\ x\ 100\%$$

Finally the RSD variations between each series of six control samples given in table 17 determined using compound:hexadecane peak area ratios denote sample preparation reproducibility.

Table 17 – Linear calibration results 200-1000µg/mL

Compound	**r**	**900µg/mL control**		**500µg/mL control**		**100µg/mL control**	
		% error	**RSD (%)**	**% error**	**RSD (%)**	**% error**	**RSD (%)**
Sum paracetamol derivatives	**0.985**	5.2	2.2	**15.4**	1.9	**27.2**	1.4
Meconin	0.999	2.5	1.5	6.6	1.5	13.2	1.4
Caffeine	0.998	3.1	1.3	6.2	1.6	**18.4**	1.8
Phenobarbitone di-TMS	0.997	6.7	0.9	11.7	2.8	**16.4**	1.5
Diazepam	0.997	4.0	1.1	13.3	4.5	9.6	3.6
Codeine TMS	0.998	2.8	1.8	13.3	4.7	**22.8**	4.3
Morphine di-TMS	0.998	3.5	1.7	2.9	1.6	**18.7**	1.5
Acetylcodeine	0.999	3.2	2.0	2.3	0.7	**21.0**	4.4
6-MAM TMS	0.998	4.0	2.4	6.2	1.1	**19.5**	**6.8**
3-MAM TMS	0.998	6.7	2.1	2.5	1.4	**16.8**	**5.0**
Diamorphine	0.996	3.6	0.7	3.9	1.0	13.5	**5.4**
Papaverine	0.996	7.2	3.4	9.4	4.0	**18.3**	**6.7**
Noscapine	**0.984**	4.4	1.2	**23.1**	3.4	**17.8**	**5.4**

Excellent linearity was seemingly observed for all compounds excluding derivatised paracetamol (attributed to MS detector saturation by the higher concentration standard samples) and noscapine (ascribed to the large non-volatile noscapine molecules providing inconsistent response factors). However, only meconin and caffeine calibration lines naturally incorporated the origin. The linearity of the remaining compound calibration lines significantly improved on discarding the origin calibration point as depicted for papaverine in figure 24, plot 1 incorporates the origin (r = 0.997), plot 2 omits the origin (r = 0.999).[12]

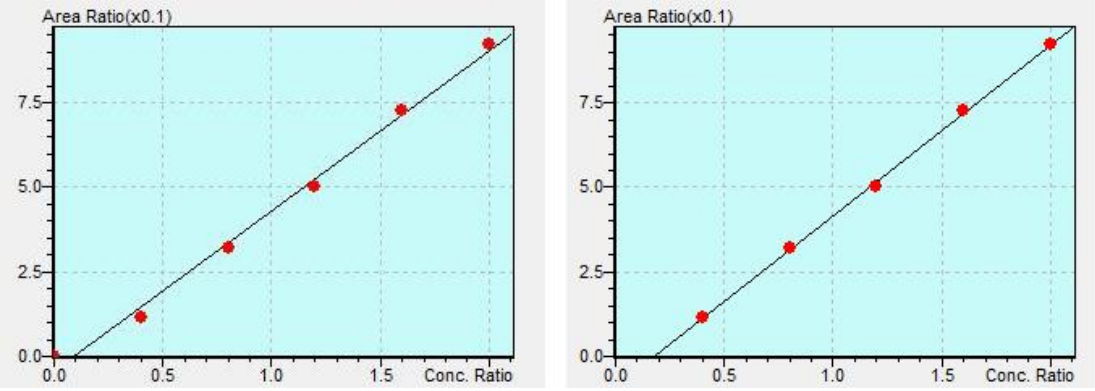


Figure 24 – Papaverine 200-1000µg/mL linear calibration with/without origin

The poor linearity at low concentrations was reflected by inaccurate quantitation and non reproducible sample preparation of the lowest concentration control samples (100µg/mL). On the assumption that the linear range may be too large (200-1000µg/mL) the calibration study was repeated effectively halving the linear range.

3.2.6 – Derivatised method linear calibration study 100-500µg/mL

Derivatised sample preparation mimicked that outlined in chapter 3.2.5, halving both the concentration of hexadecane in chloroform (0.39mg/mL) and the volumes of 1mg/mL mixed standard solution used. The results given in table 18 were compared with the analogous results obtained in chapter 3.2.5.

Table 18 – Linear calibration results 100-500µg/mL

Compound	**r**	**450µg/mL control**		**250µg/mL control**		**50µg/mL control**	
		% error	**RSD (%)**	**% error**	**RSD (%)**	**% error**	**RSD (%)**
Sum paracetamol derivatives	0.999	2.0	0.7	4.3	2.1	**22.8**	1.5
Meconin	0.999	3.3	1.2	5.0	0.7	**15.6**	1.0
Caffeine	0.999	2.7	0.8	6.3	1.0	**16.3**	1.6
Phenobarbitone di-TMS	0.996	8.0	1.4	10.6	3.3	10.1	1.3
Diazepam	**0.991**	3.2	0.8	**18.0**	4.2	3.5	2.7
Codeine TMS	**0.994**	4.1	2.0	11.4	1.9	9.7	2.1
Morphine di-TMS	**0.993**	3.5	0.5	12.1	1.5	8.6	1.8
Acetylcodeine	0.996	3.5	1.4	10.0	1.2	14.7	2.4
6-MAM TMS	**0.992**	4.7	1.8	13.1	1.7	4.3	2.1
3-MAM TMS	**0.994**	4.1	1.6	9.9	1.3	11.9	2.5
Diamorphine	**0.991**	3.4	1.4	**15.4**	2.0	3.0	2.4
Papaverine	**0.985**	6.5	1.3	**17.0**	2.5	8.8	3.7
Noscapine	**0.970**	11.4	3.5	**27.4**	3.3	**36.6**	1.7

Reducing the calibration range notably afforded lower correlation coefficients. The majority of calibration lines (excluding meconin and caffeine) again failed to pass through the origin and linearity improved on omitting the origin calibration point as depicted for papaverine in figure 25, plot 1 incorporates the origin (r = 0.985), plot 2 discards the origin (r = 0.996).[12] Control percentage errors remained high particularly for the 250µg/mL and 50µg/mL control samples with variable sample preparation RSD reproducibility values.

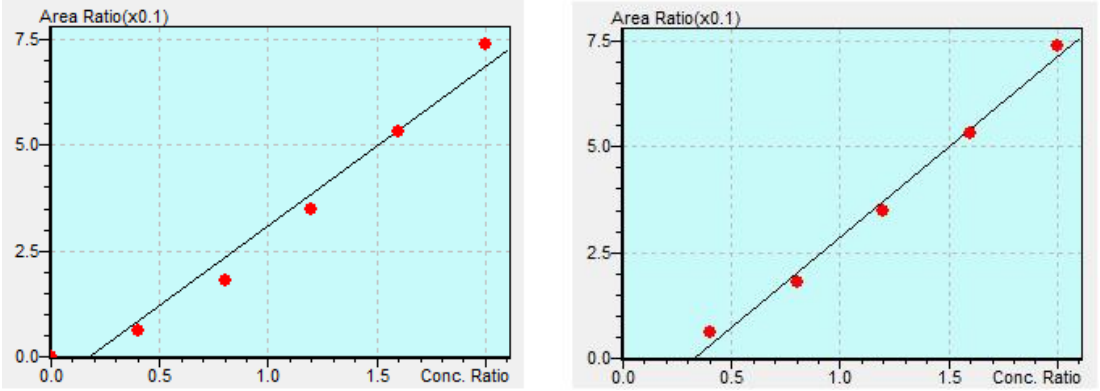


Figure 25 – Papaverine 100-500µg/mL linear calibration with/without origin

Clearly the main accuracy and reproducibility issues related to the lower concentration samples therefore the calibration study was extended incorporating more calibration samples at lower concentrations to further investigate the calibration model.

3.2.7 – Derivatised method calibration study 5-500µg/mL

Derivatised sample preparation replicated that outlined in chapter 3.2.6 and further lower concentration calibration/control samples were added as depicted in table 19.

1mg/mL mixed standard solution prepared in methanol (chapter 3.2.5, stock A).
Stock A (100µL) + methanol (900µL) → 0.1mg/mL mixed standard solution (stock B).
Hexadecane (0.39mg/mL) prepared in chloroform (chapter 3.2.5).

Aliquots of the mixed standard solution in methanol (table 19) were transferred to individual vials and evaporated at 30°C under nitrogen. Hexadecane 0.39mg/mL in chloroform (417µL), pyridine (83µL) and MSTFA (150µL) were added to each evaporated vial. Final hexadecane sample concentrations of 250µg/mL were achieved. Each of the vials were capped, heated at 80°C for 45 minutes, cooled to room temperature and analysed.

Table 19 – Preparation of additional calibration and control heroin standard samples

Sample	Volume mixed standard solution (μL)	% w/w	Concentration (μg/mL)
Standard 0	0 [a]	0	0
Standard 1	32.5 B	1	5
Standard 2	65.0 B	2	10
Standard 3	162.5 B	5	25
Standard 4 [b]	32.5 A	10	50
Standard 5	65.0 A	20	100
Standard 6	130.0 A	40	200
Standard 7	195.0 A	60	300
Standard 8	260.0 A	80	400
Standard 9 [b]	325.0 A	100	500
Control 1	97.5 B	3	15
Control 2	32.5 A	10	50
Control 3	55.25 A	17	85
Control 4	97.5 A	30	150
Control 5	195.0 A	60	300
Control 6	292.5 A	90	450

[a] 325μL methanol added

[b] Six samples prepared

It immediately became apparent that each of the derivatised compounds (excluding paracetamol) favoured quadratic over linear calibration lines achieving coefficients of determination (r^2) ≥ 0.998 signifying good measures of quadratic fit. However, even application of quadratic calibration lines failed to realise control percentage errors ≤ 15% for the control sample concentrations at 15μg/mL.

3.2.8 – Derivatised method quadratic calibration studies 5-100μg/mL and 100-500μg/mL

The calibration data obtained in chapter 3.2.7 was reprocessed introducing individual low (5-100μg/mL) and high (100-500μg/mL) quadratic calibration lines again attaining r^2 values ≥ 0.998 for all derivatised compounds. The control percentage errors in table 20 were calculated using the appropriate low or high quadratic

calibration lines (derivatised paracetamol calibration data discussed separately in chapter 3.2.9).

Table 20 – Quadratic calibration results 5-100µg/mL and 100-500µg/mL

Compound	**Control sample % error**					
	450 µg/mL	**300 µg/mL**	**150 µg/mL**	**85 µg/mL**	**50 µg/mL**	**15 µg/mL**
Meconin	2.0	1.3	3.3	2.5	2.5	1.7
Caffeine	1.6	0.8	2.0	1.8	5.6	1.3
Phenobarbitone di-TMS	2.8	3.3	9.3	5.5	4.8	1.3
Diazepam	1.0	5.8	5.9	7.5	4.8	7.3
Codeine TMS	0.5	5.2	5.4	5.9	7.0	1.7
Morphine di-TMS	0.4	3.6	5.9	5.2	5.5	4.7
Acetylcodeine	1.2	4.1	5.7	6.4	9.0	9.3
6-MAM TMS	1.1	4.9	5.8	6.9	7.2	4.3
3-MAM TMS	1.9	5.2	5.8	6.0	7.0	2.3
Diamorphine	0.9	4.0	6.2	4.7	3.0	9.7
Papaverine	1.7	4.2	6.5	3.6	3.2	9.8
Noscapine	3.9	4.1	7.4	2.0	6.6	9.7

Evidently adopting two quadratic calibration lines significantly improved accuracy as all control percentage errors were < 10%. Sample preparation also proved reproducible for both high (500µg/mL) and low (50µg/mL) samples with RSD values ≤ 2.3% obtained by injecting each series of six standard samples. RSD interday variation values measured from six repeat injections of standard 9 (500µg/mL) over six consecutive days are given in table 21.

Table 21 – Effect of measurement parameter on interday variation RSD values

Compound	**Interday variation RSD (%) of 500µg/mL standard**	
	Calculated using compound:hexadecane peak area ratios	**Calculated using measured sample concentrations**
Meconin	**5.3**	5.0
Caffeine	3.8	3.6
Phenobarbitone di-TMS	4.5	2.6
Diazepam	4.2	2.9
Codeine TMS	**6.0**	4.5
Morphine di-TMS	2.9	2.1
Acetylcodeine	**6.3**	4.9
6-MAM TMS	3.9	2.7
3-MAM TMS	3.8	2.9
Diamorphine	4.0	2.8
Papaverine	3.0	1.9
Noscapine	**9.7**	**5.3**

For the 500µg/mL sample, RSD values decreased if calculations were based on the measured sample concentrations (RSD $\leq$ 5.3) rather than compound:hexadecane peak area ratios (RSD $\leq$ 9.7). This observation relates to the quadratic behaviour of the derivatised compounds and is discussed further in chapter 3.2.11.

As the derivatised components are ultimately quantified by concentration this parameter was chosen to determine all future RSD values.

3.2.9 – Derivatised method paracetamol calibration studies 5-100µg/mL

Previous derivatised paracetamol calibration data for the linear range 100-500µg/mL (chapter 3.2.6) provided excellent linearity (r = 0.999) and control percentage errors $\leq$ 4.3% with the exception of the lowest 50µg/mL control sample (table 18). As such attention was focussed on the low derivatised paracetamol calibration range (5-100µg/mL) preparing a series of standard samples within this concentration range following the procedure outlined in chapter 3.2.7.

The derivatised paracetamol calibration data obtained by plotting the peak area ratio of the summed paracetamol derivatives to hexadecane (x-axis) against paracetamol concentrations (y-axis) in figure 26 clearly favoured a power regression curve fit (blue curve, r = 0.999) over a linear curve fit (black line, r = 0.987).

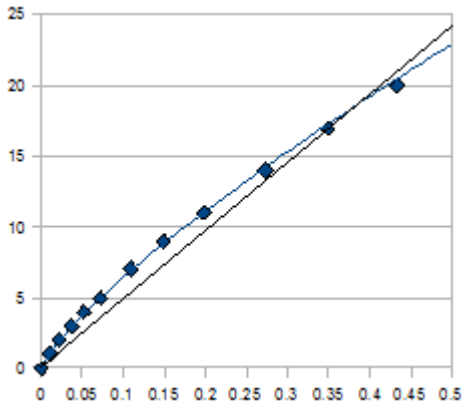


Figure 26 – Power regression and linear calibration lines for derivatised paracetamol 5-100µg/mL

Power regression curves are a transformation to a linear model where the exponential equation obtained in figure 26 was transformed according to the formula:

$$y = b * x^{a}$$
$$In\,(y) = In\,(b) + a * In\,(x)$$

The derivatised paracetamol calibration data acquired in chapter 3.2.8 was processed introducing individual low and high calibration lines.

5-100µg/mL standards generated a power regression curve with r = 0.999, equations:

$$y = 48.77 * x^{0.86}$$
$$In\,(y) = In\,(48.77) + 0.86 * In\,(x)$$

100-500µg/mL standards generated a linear line with r = 0.999, equation:

$$y = 40.71x + 2.75$$

The derivatised paracetamol control percentage errors in table 22 were calculated using the appropriate low or high calibration line.

Table 22 – Derivatised paracetamol calibration results 5-100µg/mL and 100-500µg/mL

Control sample % error					
450 µg/mL	**300 µg/mL**	**150 µg/mL**	**85 µg/mL**	**50 µg/mL**	**15 µg/mL**
1.7	2.9	6.8	7.7	8.5	3.2

Again adopting two independent calibration lines significantly improved accuracy as all control percentage errors were < 10%. Sample preparation proved reproducible for both high (500µg/mL) and low (50µg/mL) standard samples with RSD values of 0.7% and 1.1% respectively. An interday variation RSD value of 5.2 was obtained for the 500µg/mL standard sample.

3.2.10 – Sensitivity of derivatised heroin GCMS method

The sensitivity of the derivatised heroin GCMS method was assessed by analysing 15, 10 and 5µg/mL mixed derivatised heroin standard samples to determine the limit of quantitation (LOQ) and limit of detection (LOD) for each compound.

LOQ is defined as the lowest concentration of a sample that can still be quantified with acceptable accuracy and precision. Alternatively the LOQ can be based on the signal to noise ratio (S/N) where the ratio between the height of the analyte peak and the amplitude of the baseline noise is ≥ 10.[132] Compound LOQ are given in table 23.

LOD is defined as the lowest concentration of a sample that can still be detected but not necessarily quantified as an exact value. LOD is based on a S/N ratio ≥ 3.[132] LOD ≤ 5µg/mL for each compound.

Table 23 – Compound LOQ and similarity index/search values from 5µg/mL mixed derivatised heroin standard sample

Compound [a]	**LOQ (µg/mL)**	**Similarity index**	**Similarity search normal fit**	**Similarity search reverse fit**
Paracetamol di-TMS	5	92	93	97
Meconin	5	91	93	97
Caffeine	5	89	91	97
Phenobarbitone di-TMS	5	80	86	94
Diazepam	10	41	50	85
Codeine TMS	10	40	56	82
Morphine di-TMS	10	36	50	85
Acetylcodeine	10	27	31	75
Acetylthebaol	10	44	56	85
6-MAM TMS	10	26	30	75
3-MAM TMS	10	18	28	75
Diamorphine	10	20	33	74
Papaverine	10	32	41	76
Noscapine	15	19	19	No match

[a] Paracetamol TMS not detected in 5, 10 or 15µg/mL mixed derivatised heroin standard samples.

The mass spectrum of the 5µg/mL mixed derivatised heroin standard sample was matched against instrument defined similarity index/search parameters (table 23). Similarity index matches the quantitative and qualitative ion ratios of the unknown sample against ratios obtained from standard samples. Similarity searches match the acquired mass spectra against library standards. The normal library match fits the unknown mass spectrum into the library spectrum, whereas the reverse library match verifies that the peaks in the library mass spectrum are present in the unknown spectrum and any extra peaks in the unknown spectrum are ignored.[128] Typically similarity index < similarity search normal fit < similarity search reverse fit.

The derivatised major heroin component GCMS chromatogram (figure 21) demonstrates that compound responses decrease as compound retention times increase. This problem was exacerbated as the gradient of the oven temperature programme was reduced to achieve resolution of the derivatised components.

Typically the peak height for paracetamol di-TMS (RT 4.95min) is 5x that of morphine di-TMS (RT 18.96 min) and 28x that of noscapine (RT 30.99 min) even though the compounds are present in equimolar amounts. Increased amounts of derivatised compounds can be added to the GC column by increasing the sample concentration/injection volume or decreasing the split ratio. However, increasing the sensitivity for all compounds effectively decreases the LOQ for later eluting compounds but early eluting compounds then trigger MS detector saturation.

3.2.11 – Summary of developed derivatised heroin GCMS method

Validation of the developed derivatised heroin GCMS method appeared complete:

- Derivatised components sufficiently resolved ($R \geq 0.80$).
- GCMS system reproducible (RSD intraday variations $\leq 2.7\%$ for 1mg/mL mixed derivatised heroin sample).
- Derivatised compounds stable (RSD interday variations $\leq 3.4\%$ for 1mg/mL mixed derivatised heroin sample).
- Two calibration ranges required to accurately quantify each compound (5-100µg/mL and 100-500µg/mL).
- Summed paracetamol derivatives favoured a power regression fit for the low calibration range with a high linear calibration range ($r = 0.999$).
- Remaining derivatised compounds favoured quadratic fits for both low and high calibration lines ($r^2 \geq 0.998$).
- Quantitation of control samples accurate for concentrations ranging from 15-450µg/mL (% errors $\leq 9.8\%$).
- Sample preparation reproducible for 50µg/mL and 500µg/mL (RSD variations $\leq 2.3\%$).
- Derivatised compound LOQ range from 5µg/mL to 15µg/mL.
- Derivatised compound LOD $\leq$ 5µg/mL.

The non linear quadratic behaviour presented by the majority of derivatised compounds was unexpected and unusual. Figure 27 represents a typical 0-500µg/mL

quadratic calibration plot comparing the differences between peak area ratio and measured sample concentration measurements at low and high sample concentration.

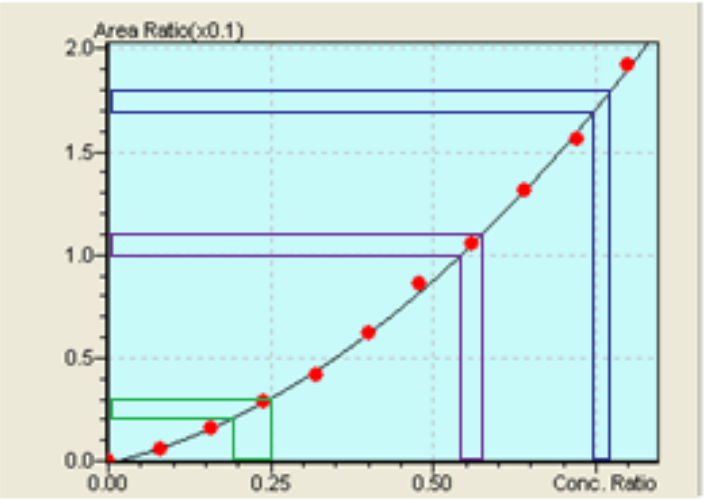


Figure 27 – Quadratic calibration model comparing peak area ratios versus measured sample concentrations

For any given point of high sample concentration on the calibration curve, variations in peak area ratios are slightly more significant than changes in concentration as indicated by the blue band (figure 27). This calibration model explains an observation made in chapter 3.2.8, where the interday RSD values for a high concentration (500µg/mL) mixed derivatised sample were higher basing calculations on the compound:hexadecane peak area ratios (RSD ≤ 9.7) rather than the measured sample concentrations (RSD ≤ 5.3).

Only sample concentrations within the mid range of the quadratic calibration curve differ equally by peak area ratio and concentration as shown by the purple band (figure 27). Conversely points of low sample concentration on the curve experience more variation by concentration than by peak area ratio as signified by the green band (figure 27). As samples are quantified by concentration, the quadratic calibration curve automatically disfavours the accurate and reproducible quantitation of low concentration samples.

Further investigation was carried out in chapter 4 to try and explain the source of the quadratic calibration behaviour and to determine the overall suitability of the GCMS system for quantifying both high and low concentration derivatised heroin components.

CHAPTER 4 – SUITABILITY OF GCMS SYSTEM FOR QUANTIFYING HEROIN SAMPLES

The following criteria were used to select a model test compound for the GCMS feasibility study:

- Compound must display typical non reproducible quadratic calibration lines ruling out meconin and caffeine which favour linear calibration lines.
- Compound must be stable in methanol eliminating use of diamorphine, 3-MAM and 6-MAM.
- Compound must not undergo derivatisation enabling simple sample preparation discounting paracetamol, phenobarbitone, codeine, 3-MAM, 6-MAM and morphine.
- Compound must exhibit good chromatographic behaviour negating noscapine.
- Compound must ideally posses a short retention time enabling rapid analysis with a reduced GCMS method run time disfavouring papaverine.
- Compound must be commercially available at reasonable cost excluding acetylcodeine and acetylthebaol.

Diazepam was chosen as the only test drug which satisfies all of the above criteria where methanol stability was established from in-house methanolic diazepam solutions which displayed no degradation over six months as determined by LCMS.

Initially two series of diazepam samples in methanol (50µg/mL and 500µg/mL) were each injected six times consistently affording RSD values by peak area < 2%. These results met the Shimadzu GCMS specification criteria requiring RSD values < 6% for six repeat injections of the same sample.

Hexadecane internal standard was incorporated to standardise the data eliminating any possible discrepancies from sample evaporation (via the punctured vial cap) or variations in injection volume. A quadratic diazepam calibration line (r^2 = 0.998)

was generated by analysing diazepam samples (40-400µg/mL) with hexadecane (200µg/mL) in methanol to enable diazepam quantitation by concentration. Diazepam control samples also containing hexadecane were then analysed six times over six consecutive days measuring the diazepam concentrations, control percentage errors and interday variation RSD values by concentration (table 24).

Table 24 – Effect of diazepam control sample concentration on interday RSD values

Control concentration (µg/mL)	**Measured concentration (µg/mL)**	**Maximum % error**	**RSD %**
40	36-76	**90.0**	**34.2**
200	195-233	**16.5**	**7.2**
360	348-368	3.3	**5.1**

The results in table 24 clearly confirm that the quadratic calibration lines cannot be used to accurately and reproducibly quantify low concentration diazepam samples over the period of a week. All of the control sample percentage errors only remained < 10% for one day after sample preparation despite the known stability of diazepam in methanol. After four, five and six days the 40, 200 and 360µg/mL control samples respectively reached control percentage errors > 10%.

These findings are consistent with the previous accurate quantitation of low concentration mixed derivatised samples immediately following calibration with acceptable intraday variation and sample preparation RSD values (chapter 3.2.8). However, former interday variation studies were only modelled using high concentration mixed derivatised samples (500µg/mL-1mg/mL).

The heroin profiling method requires calibration of the instrument and subsequent analysis of heroin samples with the intermittent accurate quantitation of freshly prepared control samples proving the validity of the calibration lines. Given the high cost of drug standards and the time required to generate two derivatised quadratic calibration lines per heroin component, the ability to only accurately quantify low concentration samples within a few days of calibration is not viable.

4.1 – INVESTIGATION INTO QUADRATIC BEHAVIOUR OF GCMS SYSTEM

The observed GCMS quadratic behaviour could be attributed to compound instability or to adsorption of active compounds by the inlet, column, detector or contaminants. The high heat and surface area needed to uniformly vaporise samples can cause compounds to break down or be adsorbed. Although GCMS analysis of the mixed derivatised heroin samples in SIM/scan mode afforded no detectable degradation products, they may not be amenable to analysis.

Sample thermal degradation can occur if the GC injection port, oven or detector temperatures are too high. Sample adsorption of active compounds with -OH, -NH or -SH functionality typically occurs by GC. The derivatised heroin method effectively caps all of the protic functional groups but the basic compounds also contain nitrogen and/or oxygen electron pair donors. The pathway surfaces of the GC system contain active silanol groups (Si-OH) that act as electron pair acceptors adsorbing the basic compounds.

Considering the quantitation of diazepam with hexadecane internal standard, both compounds are stable in methanol and the predicted relationship between diazepam:hexadecane peak area ratio and diazepam concentration is linear regardless of the diazepam concentration. However, if diazepam undergoes thermal degradation/adsorption, the measured diazepam concentrations would be lower than the true diazepam concentrations. These discrepancies would be more significant for lower diazepam concentration samples as the degraded/adsorbed molecules correlate to a larger relative proportion of the sample. Sample degradation/adsorption causes peak tailing, reduced responses and poor reproducibility and for low level analyses such losses can be significant affording poor linearity.[133]

A systematic series of experiments were conducted to monitor the effects of varying the sample preparation, GCMS inlet, column and detector parameters (chapters 4.1.1 to 4.1.16). Diazepam was again chosen as the test compound for the reasons outlined in chapter 4. Unless otherwise stated, each modification was tested by analysing

diazepam samples (40-400µg/mL) with hexadecane (200µg/mL) in methanol to generate a diazepam calibration line. Diazepam control samples containing hexadecane were then analysed sufficient times to determine diazepam quantitation accuracy, control percentage errors and RSD values by concentration.

4.1.1 – Sample preparation

Trials were conducted preparing and storing the diazepam samples in glassware silanised by rinsing with BSA followed by evaporation to minimise diazepam adsorption. However, using deactivated glassware failed to recover additional diazepam and responses remained unchanged. This was despite also preparing the diazepam samples in methanol enabling the alcohol to compete for the adsorptive sites on the surface pathways of the GCMS to minimise diazepam adsorption losses.[133]

4.1.2 – GC liner and solvent vapour volumes

Backflash occurs when the volume of the vaporised sample exceeds the volume of the GC liner. Most of the excess vaporised sample escapes out of the top of the GC liner and some is swept down the septum purge line. The remaining portion can back up into the carrier gas supply line being reintroduced into the injection port causing poor peak area reproducibility and sample carryover.

An online backflash calculator provided by Thames Restek was used to determine the internal vapour volume of the GC liner and the injection solvent vapour volume.
The GCMS method currently uses intermediate polarity deactivated Shimadzu straight focus liners packed with wool as they are suitable for most common split injection analyses.

Liner length = 95mm
Liner internal diameter = 3.4mm
Liner internal volume = 862µL
(Presence of carrier gas in the liner diminishes available liner volume by up to 25%).
Liner vapour volume = 646µL

Injection temperature = 290°C
Injection volume = 1µL
Head pressure = 18.5 psi
Methanol vapour volume = 505µL
(Chloroform and pyridine afford lower vapour volumes than methanol as less polar).

As the methanol vapour volume (505µL) does not exceed the liner vapour volume (646µL) using the defined method parameters, the possibility of backflash was ruled out and the GC liner dimensions remained unchanged.

4.1.3 – Non packed GC liners

Non packed liners are typically used for splitless methods however they were assessed as some reports indicate that adsorption may take place in the injection port when packing material is present in the GC liner. Huizer *et al.*, noted that deviations in the linearity of diamorphine calibration curves, particularly near the origin, may be indicative of adsorption by wool in the injection liner.[118]

In order to effectively evaluate a splitless liner without packing while maintaining the high split ratio, three injection techniques were individually incorporated to minimise needle discrimination during split injections.

Needle discrimination occurs when the needle begins to heat in the injection port causing low molecular weight analytes to vaporise from the needle while higher molecular weight analytes remain inside the needle. Therefore, lower molecular weight analytes show enhanced responses compared to higher weight analytes.

- Technique 1 – sample injected as rapidly as possible (high plunger and syringe speeds) to minimise the time the needle spends in the injection port therefore reducing the amount of heating experienced by the needle.
- Technique 2 – hot needle injection performed by artificially holding the needle in the injection port (injection port dwell time increased to 6 seconds)

allowing the needle to heat completely before ejecting the sample into the GC liner guaranteeing quantitative sample transfer from the syringe tip.

- Technique 3 – solvent flush injections introduced by drawing a small amount of solvent into the syringe, followed by a small amount of air, followed by the desired amount of sample. Again a hot needle injection heated the needle before expelling the contents of the syringe into the GC liner with the solvent effectively flushing the syringe barrel and needle ensuring complete transfer of the sample.

Huizer *et al.*, noted the benefits of hot needle injections through analysing standard solutions of diamorphine hydrochloride varying the time the needle remained in the injection port between 1, 2 and 3 seconds. An injection dwell time of 1 second resulted in a 5% lower response of diamorphine hydrochloride:internal standard compared to the longer injection dwell times.[118]

Ultimately split injections using liners without packing afforded erratic peak area responses. Intraday variations by diazepam peak area for six repeat injections of a diazepam sample (400µg/mL) with hexadecane (200µg/mL) in methanol were extremely high regardless of the injection technique (table 25).

Table 25 – Variation of intraday RSD values by compound peak areas using GC liners without packing and different injection techniques

Injection technique	**RSD (%) by hexadecane peak area**	**RSD (%) by diazepam peak area**
1	132.4	168.9
2	34.3	69.7
3	94.2	121.1

Packed GC liners were subsequently evaluated and used in future analyses.

4.1.4 – Packed GC liners

GC liners with packing (typically fused silica or glass wool) are essential for split methods to provide rapid and complete sample vaporisation and reproducible sample

transfer. The high split ratio quickly moves the vapour cloud through the liner, and the packing material provides added surface area on which to vaporise the sample into a homogeneous vapour cloud before it reaches the split point. Ensuring complete and uniform sample vaporisation also prevents molecular weight discrimination (discussed in chapter 4.1.9). The packing also avoids contact between the vaporised sample and the highly adsorptive hot metal inlet disc at the bottom of the injection port.

A wide range of GC inlet liners are reported to minimise sample adsorption during low level analyses providing maximum compound recoveries and responses.[134] The previously used Shimadzu straight focus liners packed with wool are intermediate polarity (IP) deactivated meaning that the glass surface is phenylmethyl deactivated ensuring good recovery of both polar and non polar compounds and compatibility with most common solvents.

Silanised wool packed liners were similarly tested where silanisation masks the polar silanol groups on the glass liner by chemically binding a non adsorptive silicone layer. Silanised liners are recommended for difficult matrices and reactive compounds providing inertness over a wide sample pH range.[135] Base deactivated wool packed liners were also tested where basic functional groups are bonded to the acidic silanol groups to minimise the thermal breakdown and adsorption of basic samples.[136] Despite their claims, the superior deactivated liners offered no improvement over the standard IP deactivated liner, the amount of wool also proved to be inconsequential. Diazepam calibration lines remained quadratic resulting in the inaccurate quantitation of the control samples with large percentage errors and considerable intraday/interday variation. IP deactivated Shimadzu straight focus liners packed with wool continued to be used in future analyses.

Manufacturers deactivate liners and wool in situ to prevent breaks in the wool as broken wool fibres can be adsorptive. To maintain consistency, no attempts were made to manually pack GC liners other than to reposition the wool ~28mm from the top of the injection port to coincide with the heated region of the Shimadzu GC

injection port. Similarly, deactivation of the GC liners by soaking in a 25% mineral acid solution to remove metal oxides from the glass surface followed by heating to remove water and silyl derivatisation was not attempted.

4.1.5 – Alternative GC liners

Uniliner inlet liners were not investigated as they are only suitable for splitless injections due to the samples being directly injected onto the column, minimising active sites in the sample pathway significantly improves active analyte recoveries.[135]. The column connects to the bottom of the liner via a press tight seal effectively eliminating sample contact and adsorption onto the stainless steel injection port.[133]

Improved sample vaporisation can also be achieved through the incorporation of mixing chambers and tortuous flow paths (CarboFrit packing, cyclosplitter or laminar cup liners).[6] These liners were not tested as they are specifically designed to retain high molecular weight components and prevent particulate matter reaching the head of the column, applications include analysing solvent residues within latex samples.

4.1.6 – GCMS inlet maintenance

Strömberg *et al*., found non-volatile matter contaminated the wall of the injection liner during GC analysis, negatively affecting repeatability, especially for longer sequences. To circumvent this they started each GC sequence with a new liner and limited the length of the series using control injections to monitor repeatability.[94]

To monitor the effect of GC liner usage, a diazepam sample (400µg/mL) with hexadecane (200µg/mL) in methanol was repeatedly analysed using an old liner before replacement with a new liner. Diazepam peak areas decreased to a greater extent than hexadecane peak areas suggesting increased adsorption of diazepam over hexadecane onto the active sites of the new liner, overall quantified diazepam concentrations appeared to decrease using a new liner.

An additional study was carried out replacing the liner, o-ring, gold seal and septum, cleaning the inlet using cotton wool sticks soaked in methanol, trimming both ends of the GC column with routine cleaning of the MS ion source. A diazepam sample (400µg/mL) with hexadecane (200µg/mL) in methanol was analysed before and after in situ deactivation of the GCMS system by multiple MSTFA injections (MS detector/filaments turned off to prevent the build up of silicone deposits on the mass filter). In theory silylating active sites in the sample pathway should reduce diazepam adsorption but the opposite effect was observed with quantified diazepam concentrations decreasing. This unexpected result questioned whether the trend of decreasing diazepam concentration is attributable to the new liner/MSTFA injections or some overriding factor possibly unrelated to inlet degradation/adsorption.

4.1.7 – Injection split ratio

The split ratio and hence the total gas flow were reduced in order to increase the residence time of the sample in the liner prior to injection aiming to promote more effective vaporisation. Injection split ratios of 100:1, 50:1 and 25:1 were employed diluting the samples with methanol by factors of zero, two and four to balance the increased sample amounts being applied to the column. Reducing the injection split ratio failed to improve the linearity of the diazepam calibration lines and a split ratio of 100:1 continued to be used in future analyses.

4.1.8 – Injection port temperature

Investigations were carried out to determine the effect of injection temperature on degradation/adsorption levels. Low injection temperatures may be insufficient for instantaneous sample vaporisation but high injection temperatures increase the risk of compound thermal degradation. The maximum injection temperature was determined to be 300°C as it cannot exceed the lower temperature from 470°C, the maximum oven temperature (300°C) and the maximum column temperature (350°C). Injection temperatures from 275°C to 300°C using 5°C increments including the default 290°C temperature were employed. Neither extreme of injection temperature provided linear diazepam calibration lines contradicting the concept of thermal (or

thermally catalysed) compound decomposition in the GC inlet. An injection port temperature of 290°C continued to be used in future analyses.

4.1.9 – Alternative injection techniques

Alternative injection techniques such as cold on-column and programmed temperature vaporising (PTV) injections were evaluated by Shimadzu method development specialists in Milton Keynes.[6]

Cold on-column injections introduce the sample directly into the unheated inlet of the capillary column without an intermediate evaporation step. During the course of the oven temperature programme the vapour pressure of the solutes increase and the chromatographic process begins. The absence of a heated injection system eliminates sample thermal degradation.

The cold on-column injection technique is suitable for trace analysis as the entire sample is introduced onto the column and also negates molecular weight discrimination. Samples containing compounds with a wide range of molecular weights/boiling points are prone to molecular weight discrimination. Inadequate sample volatilisation of the high molecular weight/high boiling point components produces aerosol droplets which are preferentially driven by the momentum of the carrier gas out of the injection port and through the split vent.

Figure 28 compares the GC responses obtained for an alkane mix acquired by normal GC injection at 340°C (plot 1) versus cold on-column injection at 40°C (plot 2). [6]

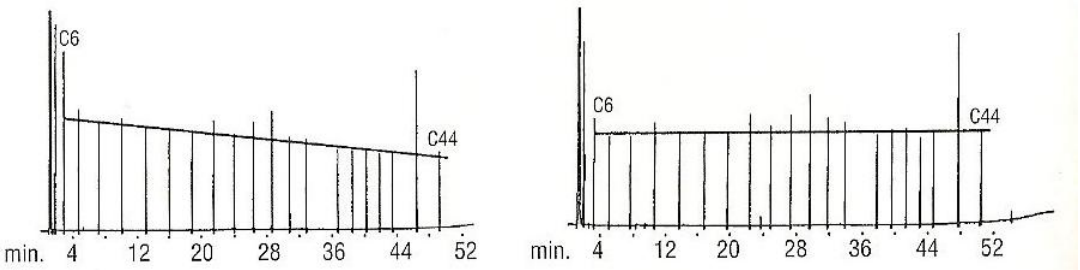


Figure 28 – Molecular weight discrimination varying injection technique [6]

Plot 1 responses do not correctly represent the real sample composition as early eluting compound peak heights are enhanced and later eluting compound peak heights are diminished. Quantitative transfer is only obtained for components with a boiling point below that of approximately *n*-C_{20} species (boiling point $C_{20}H_{42}$ = 343°C).[137]. In contrast, plot 2 peak heights are representative of the sample showing no molecular weight discrimination via the cold on-column injection.

PTV injectors include a cool injection step which prevents thermally unstable compounds from decomposing and isomerising. A controlled heated vaporisation then eliminates molecular weight discrimination effects and loss of high boiling point compounds as transfer of the sample into the chromatographic system is performed in the liquid state. This eliminates mass discrimination effects as well as greatly reducing changes due to thermal breakdown of heat labile substances thereby increasing accuracy and precision. The liner contains no wool and is porous so may eliminate compound adsorption.

Sperling utilised GC in combination with a PTV injector to determine diamorphine and common adulterants found in illicit heroin samples.[114] Morello *et al.,* similarly employed a PTV injector to analyse neutral and acidic heroin manufacturing impurities by GCMS.[107] The PTV inlet was shown to reduce potential analyte decomposition and provided better chromatography for later eluting components than conventional split/splitless injection techniques.

Diazepam calibration lines acquired by Shimadzu using cold on-column and PTV split injection techniques remained quadratic concluding that the lack of linearity is not attributable to compound degradation/adsorption by the GC inlet.

4.1.10 – GC column adsorption

A report by Gough and Baker in 1981 analysed mixtures of diamorphine, morphine, acetylcodeine, codeine and 6-MAM by GC and noted a uniform decrease in the level of components eluted as the flow rate decreased and their retention times increased. Column adsorption was thought to prevent the linear calibration lines passing

through the origin with morphine most influenced although all losses were found to be reproducible enabling satisfactory quantitation.[49]

The pattern of compound responses decreasing with increasing retention time is consistent with observations made in chapter 3.2.10. It is feasible that as the basic compounds spend longer periods of time on the column at higher temperatures the levels of adsorption/degradation increase resulting in quadratic behaviour. However, given the major advances in capillary GC column manufacture post 1981, column adsorption by extremely high specification inert GC columns is unlikely.

A new Phenomonex ZB-5 capillary column was initially tested to rule out column bleed during wear contaminating the MS and affecting the results. A higher specification more robust GC column manufactured by Thames Restek (Rxi-5ms, phase and dimensions identical to ZB-5 column) was also evaluated promising lower bleed levels specifically targeted for MS applications. Finally a Thames Restek base deactivated GC column was assessed where basic functional groups are bound to the analytical surface of the column in order to reduce the adsorption of basic heroin components.[136]

Further tests were conducted to determine whether reducing the compound times on column (compound retention times decreased by increasing the oven temperature ramp rates) incurs less compound adsorption/degradation improving linearity.

All of the GC columns/oven temperature programmes afforded quadratic diazepam calibration lines with inaccurate quantitation of the diazepam control samples indicating that the lack of linearity is not attributable to compound degradation/adsorption by the GC column.

4.1.11 – MS detector adsorption

The MS detector is tuned/GCMS system checked at the beginning of each week and the intended derivatised heroin calibration range (5µg/mL-500µg/mL) does not exceed the limits of the MS detector (reported to afford non linear responses over

concentration ranges greater than three orders of magnitude). However, having ruled out sample degradation/adsorption by the GC inlet and column, the quadratic behaviour and lack of reproducibility could only be attributed to the MS detector.

Calibration MS data was obtained in SIM mode as alternately acquiring both SIM and scan data in SIM/scan mode may split the duty cycle of the instrument but modifying the MS acquisition mode failed to improve linearity.

The stability and reproducibility of the MS detector was tested by repeatedly analysing a diazepam sample (400µg/mL) with hexadecane (200µg/mL) in methanol. Within a series of injections diazepam concentration either consistently decreased or increased. The trends were explained by early injections of the basic sample resulting in diazepam adsorbing onto the active sites of the detector effectively covering them (diazepam concentration decreased). Further repeat injections caused more and more active sites to be covered until equilibrium was reached. The diazepam response subsequently improved (diazepam concentration increased), but reproducibility was poor and the diazepam sample could not be accurately quantified. The 'priming effect' appeared temporary because the system reverted back to an adsorptive state after column conditioning periods or periods of inactivity.

4.1.12 – Calibration of diazepam using diazepam-d_5 internal standard

In order to compensate for the possible limited GCMS dynamic range and apparent priming effect, diazepam calibration studies were carried over a narrow concentration range (4-80µg/mL) using diazepam-d_5 as the internal standard. As diazepam and diazepam-d_5 are structurally related with almost identical chemical properties and retention times their responses should be equally affected by any MS detector fluctuation. The use of multiple deuterated internal standards also minimises the effects of molecular weight discrimination as they effectively mimic the range of standard molecular weights and boiling points present in the sample (discussed in chapter 4.1.9).

Diazepam-d_5 obtained as a 1 mg/mL in methanol from LGC Standards was found to elute immediately prior to diazepam, retention times 16.79 and 16.85 minutes respectively. Diazepam-d_5 was characterised by MS (supplied in appendices) and the quantitative and qualitative SIM target ions were selected (261.0 m/z (100%), 289.1m/z (72.74%), 287.1m/z (64.17%)). The deuterated internal standard was added to the drug profiling GCMS library and the heroin derivatised SIM/scan GCMS method.

Diazepam samples (4-80µg/mL) with diazepam-d_5 (40µg/mL) in methanol were subsequently analysed to generate a diazepam calibration line. A diazepam correlation coefficient of 0.999 signified an excellent linear relationship between diazepam:diazepam-d_5 peak area ratios and diazepam concentrations. Diazepam control samples (4µg/mL, 40µg/mL, 80µg/mL) also containing diazepam-d_5 (40µg/mL) were repeatedly analysed over six days to determine the diazepam quantitation accuracy and control percentage errors (table 26).

Table 26 – Measured diazepam control sample concentrations/percentage errors quantified with respect to the linear diazepam:diazepam-d_5 calibration line

Number of days between diazepam:diazepam-d_5 calibration and analysing control sample	**80µg/mL control**		**40µg/mL control**		**4µg/mL control**	
	µg/mL	**% error**	**µg/mL**	**% error**	**µg/mL**	**% error**
0	85.8	7.2	38.4	4.0	4.6	**16.1**
1	86.8	8.5	41.1	2.8	5.0	**24.6**
3	90.2	12.7	42.4	5.9	5.3	**31.3**
5	93.6	**17.0**	43.7	9.3	5.5	**37.4**

Over six days the measured diazepam control concentrations increased generating large percentage errors for the 4µg/mL and 80µg/mL concentration control samples. The 40µg/mL diazepam control sample consistently afforded percentage errors < 15% hinting that accurate diazepam quantitation is only possible if the diazepam concentration is close that of the diazepam-d_5 internal standard (40µg/mL).

As diazepam and diazepam-d_5 individually generate quadratic calibration lines, generation of a linear calibration line indicated that the diazepam and diazepam-d_5 responses initially cancelled one another out. The percentage increases in diazepam and diazepam-d_5 peak areas from the first control series (day of calibration) to the last control series (5 days after calibration) are compared in table 27.

Table 27 – Control sample diazepam/diazepam-d_5 peak area increases over 6 days

Control (µg/mL)	**Percentage increase in compound peak areas from control samples analysed 0 and 5 days following calibration (%)**	
	Diazepam	**Diazepam-d_5**
4	291	228
40	209	184
80	163	149

Evidently diazepam peak areas increased more than diazepam-d_5 peak areas over time particularly for lower concentration control samples. As the diazepam-d_5 concentration is constant within each control sample (40µg/mL), the significant control variations in diazepam-d_5 peak areas over time highlights the erratic results. Overall, as the diazepam and diazepam-d_5 responses change presumably due to MS detector fluctuations, the peak area ratios of diazepam:diazepam-d_5 randomly fluctuate with time preventing the accurate quantitation of diazepam control samples.

4.1.13 – Alternative GCMS instruments

Unable to determine why the Shimadzu GCMS instrument continuously afforded quadratic diazepam calibration lines, alternative in-house GCMS instruments were tested. The derivatised heroin method was transferred to two different instruments and Phenomonex ZB-5 columns were used to analyse diazepam calibration and control samples with hexadecane internal standard as discussed in chapter 4.1. The linearity of the diazepam calibration lines is expressed in terms of the correlation coefficient (r, measure of linear fit) and coefficient of determination (r^2, measure of quadratic fit).

Instrument 1 – Hewlett Packard (6890 GC and 5973 MSD), old model GCMS

The diazepam calibration line acquired in the first instance was linear (r = 0.996) but repeating analyses the following day afforded calibration data which favoured a quadratic over a linear fit (r = 0.989, r^2 = 0.999) and the diazepam control samples could not be accurately quantified.

Instrument 2 – Agilent (6890N GC and 5973 MSD), new model GCMS

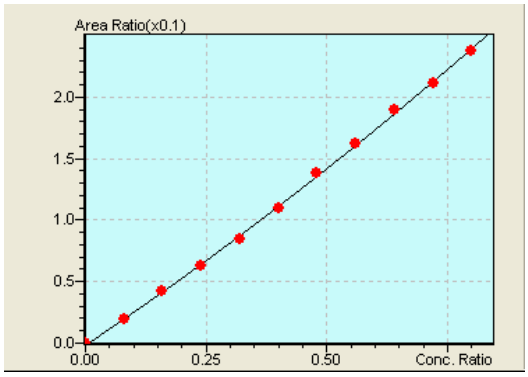
The linearity of the diazepam calibration line (r = 0.993) was improved over the analogous Shimadzu data (r = 0.984) although a quadratic fit was preferred (r^2 = 0.998) and again accurate quantitation of the diazepam control samples was not possible.

In an attempt to rationalise why the different instruments provided transient/improved diazepam linearity, differences between the set up of the Shimadzu and Hewlett Packard/Agilent MS detectors were scrutinised. It was noted that the ion sources of instruments 1 and 2 were both cleaned and dried in an oven (100°C) immediately before use (Hewlett Packard) and sometime prior to use (Agilent). In contrast the Shimadzu ion source is routinely cleaned and dried at room temperature as per the operating manual.

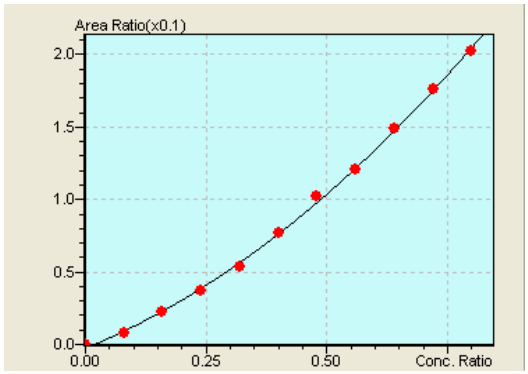
4.1.14 – Ion source cleaning procedure

Shimadzu report that drying the ion source at room temperature is sufficient for 99% of GCMS applications. On this occasion the additional optional step of heating the cleaned Shimadzu ion source in an annealing furnace at 400°C for 1 hour was incorporated. Cleaning the ion source exposes the active metal sites and the annealing process produces a deactivated inert surface and prevents bronzing.

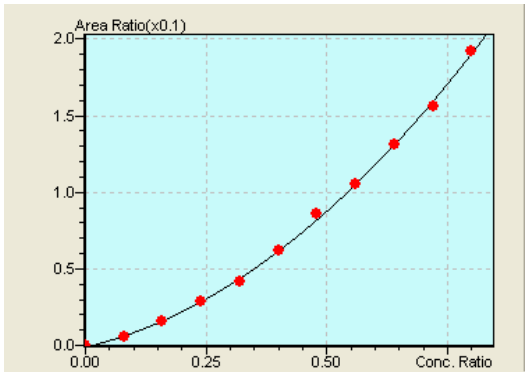
Diazepam calibration samples (40-400µg/mL) containing hexadecane (200µg/mL) were analysed followed by diazepam control samples, the analytical sequence was immediately repeated a further two times. The r (measure of linear fit) and r^2 (measure of quadratic fit) values obtained for the three diazepam calibration lines are recorded alongside their respective calibration plots in figure 29.



Diazepam calibration 1st analysis, r = 0.999, r^2 = 0.999



Diazepam calibration 2nd analysis, r = 0.991, r^2 = 0.999



Diazepam calibration 3rd analysis, r = 0.982, r^2 = 0.999

Figure 29 – Repeat diazepam calibration lines obtained after annealing the ion source

Evidently annealing the cleaned ion source immediately before use improved the initial diazepam linearity as was the case using the oven dried ion source for the Hewlett Packard instrument 1 experiments (chapter 4.1.13). However, as before

analysing repeat calibration lines saw the linearity wane in favour of quadratic behaviour with correlation coefficients decreasing from 0.999 to 0.982. With each successive calibration hexadecane peak areas gradually decreased and diazepam peak areas markedly decreased correlating to an overall decrease in the scale of the diazepam:hexadecane peak area ratios (figure 29).

The three series of diazepam control samples analysed after each calibration line were all quantified relative to the first calibration line. The non reproducibility of the falling diazepam:hexadecane peak area ratios translated to the determined diazepam concentrations decreasing over time (table 28).

Table 28 – Repeat diazepam control concentrations after annealing the ion source

Control (µg/mL)	**Measured diazepam control concentration (µg/mL)**		
	1st controls	**2nd controls**	**3rd controls**
40	27.2	20.5	18.4
200	156.5	125.1	107.7
360	374.4	318.9	291.6

The procedure of cleaning and annealing the ion source before analysing sequential diazepam calibration lines was repeated to confirm the above results. Ultimately annealing the ion source at 400°C for 1 hour afforded short-lived diazepam linearity but overall the method remained non reproducible. This could be explained by gradual accumulation of components in the initially inert ion source perpetuating additional active sites for further components to adhere to. The resulting decrease in compound responses affords non linear quadratic calibration lines negatively impacting on the ruggedness and long term method repeatability.

4.1.15 – Ion source temperature

In light of the above theory regarding contamination of the MS detector, additional experiments were carried out evaluating the effect of decreasing the ion source temperature from the default temperature of 300°C to 280°C and 150°C. Reducing the detector temperature minimises the thermal degradation of the compounds on the ion source but may equally encourage compounds to condense on the ion source.

Diazepam calibration samples (40-400µg/mL) containing hexadecane (200µg/mL) were analysed twice using an ion source temperature of 150°C, the analytical sequence was immediately repeated a further two times using an ion source temperature of 280°C. The r (measure of linear fit) and r^2 (measure of quadratic fit) values obtained for the four diazepam calibration lines alongside some diazepam:hexadecane peak area ratios are given in table 29.

Table 29 – Repeat diazepam calibration lines varying the ion source temperature

Ion source Temperature (°C)	**r**	**r^2**	**Diazepam:hexadecane peak area ratio 40µg/mL standard sample**	**Diazepam:hexadecane peak area ratio 400µg/mL standard sample**
150	0.998	0.998	0.017	0.206
150	0.993	0.999	0.007	0.155
280	0.997	0.998	0.015	0.225
280	0.995	0.999	0.012	0.208

Clearly each diazepam calibration line favoured quadratic behaviour ($r^2 \geq r$) although linearity improved on increasing the ion source temperature from 150°C to 280°C. Similarly, the large initial fall in diazepam:hexadecane peak area ratios at 150°C recovered on increasing the ion source temperature to 280°C and subsequent peak area ratio losses were smaller at 280°C (table 29). These results imply that the levels of compound condensation at 150°C exceed those of compound thermal degradation at 280°C, as such an ion source temperature of 300°C continued to be used in future analyses to improve linearity/reproducibility.

4.1.16 – Additional variables affecting GCMS linearity/reproducibility

Further GCMS parameters were modified in an attempt to improve method linearity and reproducibility:

- On column compound concentrations increased by either reducing the split ratio (100:1 to 50:1 and 25:1, total flow also reduced) or doubling the sample concentrations (total flow unchanged). Degradation/adsorption of equivalent

compound amounts corresponds to smaller relative proportions of higher concentration samples.

- GCMS emission current reduced (150µA to 60µA) to lower instrument sensitivity as it may be too high for this application.
- Compound velocity into the ion source amplified by increasing the flow rate (1.0mL/min to 2.0mL/min) to minimise diffusion losses in the electron beam.
- GC column inserted further into the ion source (0.5cm and 1.0cm) focussing the compound direction into the ion source limiting possible diffusion.

The above modifications failed to afford linear diazepam calibration lines and accurate diazepam quantitation remained impossible. Ultimately as transient linearity could only be achieved using an annealed clean ion source, other potential ion source modifications were considered to improve reproducibility:

- Silanising the ion source would add an organic layer to the surface effectively rendering it non conducting.
- Base deactivated ion sources are not commercially available.
- Continually base deactivating the ion source by introducing ammonia as a chemical ionisation (CI) carrier gas at low concentrations while still running in electron impact mode was not feasible as the Shimadzu GCMS instrument could not be adapted.

Having exhausted the GCMS method development options it was concluded that use of GCMS for the quantitation of major derivatised heroin components is not viable.

4.2 – SUITABILITY OF GC FID SYSTEM FOR QUANTIFYING HEROIN SAMPLES

The vast majority of heroin profilers use GC FID to quantify major heroin components, this technique is then combined with GCMS using the same method conditions if samples are unusual or complex to obtain additional qualitative data. The UNODC brought together relevant heroin profiling experts and compiled their

methods used to profile major heroin components, none of the renowned laboratories used GCMS favouring HPLC, GC or CE techniques.[24]

Quantitative analyses favours GC FID as the dynamic linear range is very large and the detector is exceptionally stable and robust giving almost identical responses with excellent reproducibility. In contrast, GCMS offers a limited linear range due to the nature of the ionisation process and compound responses depend on the instrument tuning and condition. GCMS reproducibility demands accurate instrument tuning and calibration and custom autotuning may be required to ensure the instrument is in perfect condition before meaningful data can be obtained.

4.2.1 – GC FID results

Mixed derivatised heroin samples were prepared using the optimised derivatised major opiates sample preparation method given in appendices. The calibration and control samples were analysed using a Shimadzu GC FID instrument based at the companies method development site in Milton Keynes. The GC FID instrument was set up using the optimised derivatised major opiates GC method parameters given in appendices. GC FID method parameters included a hydrogen flow rate of 40mL/min and an air flow rate of 400mL/min, no makeup gas was used. The split ratio was reduced from 100:1 to 30:1 to compensate for the lower sensitivity of the GC FID (emission current 60µA) compared to the GCMS (emission current 150µA).

The derivatised compound elution order remained unchanged (figure 30) although diazepam and codeine TMS coeluted as a result of the flow rate modifications and their peak areas were summed for the purposes of the calibration. The derivatised paracetamol calibration data was similarly obtained by plotting the peak area ratio of the summed paracetamol derivatives to hexadecane against paracetamol concentrations using excel.

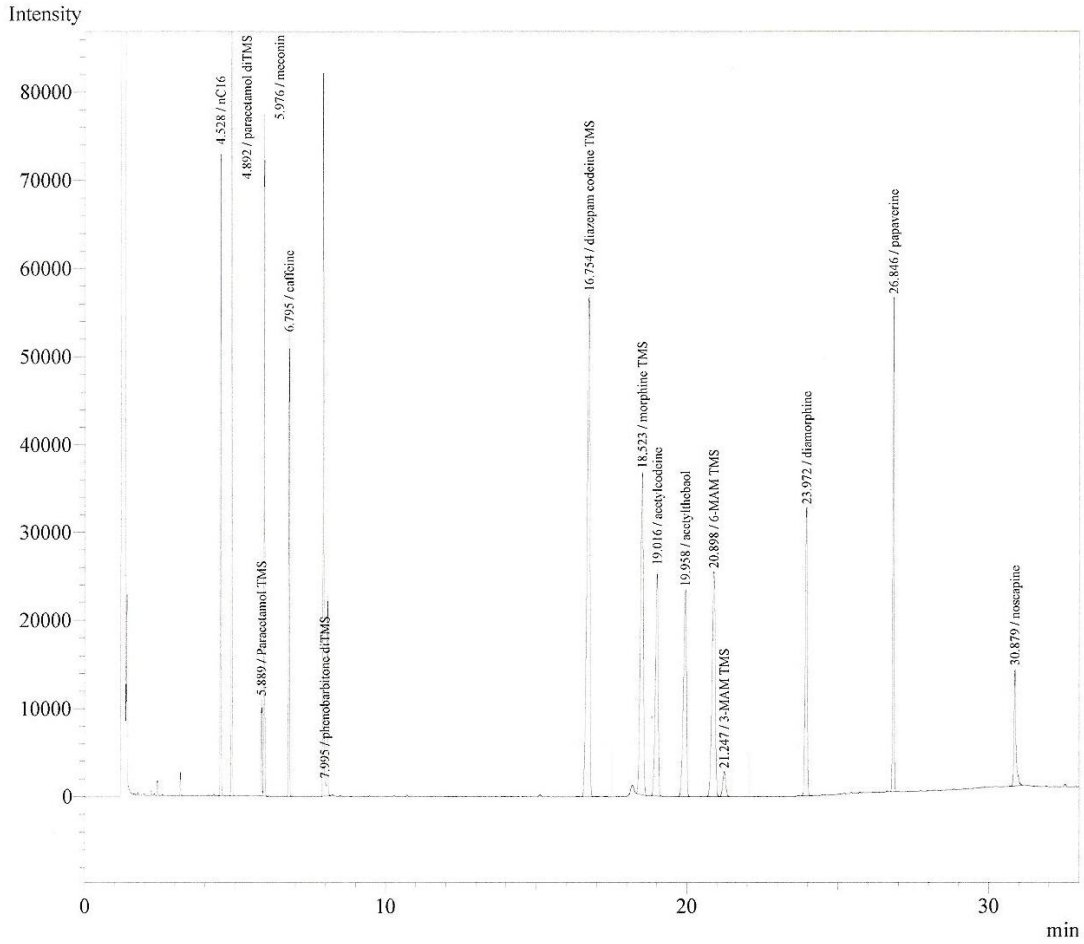


Figure 30 – Derivatised major heroin component GC FID chromatogram

All compounds afforded linear calibration lines for the calibration range 4.9-500µg/mL. The correlation coefficients, measured control sample concentrations, percentage errors and limits of quantitation are given in table 30, r values ≥ 0.995 and percentage errors $\leq 15\%$ throughout signify excellent linearity and accuracy respectively.

Table 30 – GC FID linear calibration results 4.9-500µg/mL

Compound	**r**	**449.2 µg/mL control**		**249.2 µg/mL control**		**24.9 µg/mL control**		**LOQ µg/mL**
		µg/mL	**% error**	**µg/mL**	**% error**	**µg/mL**	**% error**	
Sum paracetamol derivatives	0.9997	450.1	0.1	248.5	0.6	25.5	2.2	2.5
Meconin	0.9998	447.5	0.6	249.2	0.3	24.9	0.2	10.0
Caffeine	0.9998	447.6	0.5	250.4	0.2	25.2	0.1	10.0
Phenobarbitone di-TMS	0.9997	451.3	0.3	251.5	0.6	24.4	2.0	24.9
Diazepam and codeine TMS	0.9998	447.5	0.6	249.8	0.1	24.8	0.4	10.0
Morphine di-TMS	0.9998	448.4	0.4	250.4	0.2	25.1	0.8	10.0
Acetylcodeine	0.9998	447.4	0.6	249.6	0.2	24.4	2.1	10.0
Acetylthebaol	0.9998	447.0	0.7	249.1	0.3	24.6	1.2	10.0
6-MAM TMS	0.9998	447.3	0.6	250.0	0.1	24.4	2.0	10.0
3-MAM TMS	0.9997	445.9	0.9	252.3	0.9	25.1	0.6	10.0
Diamorphine	0.9998	447.2	0.6	250.0	0.1	24.7	1.1	10.0
Papaverine	0.9998	448.6	0.3	248.2	0.7	25.0	0.3	10.0
Noscapine	0.9997	452.5	0.6	251.1	0.5	26.3	5.7	24.9

A typical calibration line obtained for diamorphine is shown in figure 31, the remaining calibration plots were identical in their high levels of linearity.

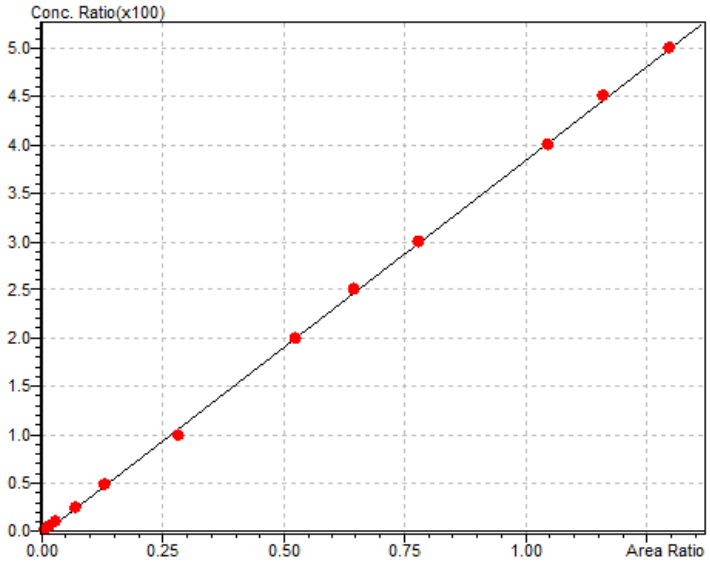


Figure 31 – Diamorphine GC FID linear calibration line 4.9-500µg/mL

As the GC FID and GCMS inlet and column parameters are identical, the individual MS detectors are entirely accountable for the different calibration behaviours. The MS detector afforded quadratic calibration lines resulting in inaccurate and non reproducible quantitation, whereas the FID detector achieved linear calibration lines enabling accurate and reproducible quantitation.

Only two days were available for GC FID instrument testing, further work would include optimising the resolution/sensitivity, intraday and interday variation studies, incorporation of retention indices and full method validation. Ideally the Shimadzu GCMS instrument would be modified using a splitter to integrate a FID detector allowing single injection heroin samples to simultaneously be quantified by GC FID and qualified by GCMS.

CHAPTER 5 – CONCLUSIONS AND FUTURE WORK

A non derivatised heroin GCMS method was developed analysing the components in methanol. Method parameters were modified to optimise resolution obtaining good intraday variation but interday variations were poor (morphine, papaverine and noscapine RSD values > 5%). Sample instability in methanol was attributed to the transesterification of heroin components in the GC injection port affording on column artefacts.

To eliminate transesterification and improve method reproducibility, a derivatised heroin GCMS method was developed. Heroin components were derivatised by silylation with MSTFA heating in a 5:1 chloroform/pyridine mixture at 80°C for 45 minutes. Method parameters were modified to optimise resolution obtaining good intraday and interday variations. The derivatisation conditions were modified to enable reproducible paracetamol silylation (summing paracetamol TMS and paracetamol di-TMS) and the processing parameters were changed to allow consistent phenobarbitone di-TMS integration.

The derivatised heroin GCMS method was converted from a qualitative TIC method to a quantitative SIM/scan method. Heroin component calibration lines using hexadecane internal standard (concentration ranges 200-1000µg/mL or 100-500µg/mL) only proved linear if the origin was omitted as a calibration point. Further calibration studies revealed that the derivatised heroin components favour quadratic over linear calibration behaviour (paracetamol derivatives found to prefer power regression and linear calibration lines). Using two quadratic lines (concentration ranges 5-100µg/mL and 100-500µg/mL) enabled accurate control sample quantitation and good intraday/interday variations, sample preparation was reproducible and LOQ and LOD were measured.

Additional evaluation of the quadratic calibration model highlighted the inability to accurately and reproducibly quantity low concentration samples days after calibration. Compound degradation/adsorption was proposed as the source of quadratic behaviour and resulting non reproducibility. A systematic and thorough

review was conducted modelling the calibration of diazepam with respect to hexadecane to try and determine where degradation/adsorption may be occurring:

- Sample preparation
  - Glassware silanised
  - Methanol added to samples
  - Diazepam-d_5 trialled as an internal standard

- Inlet parameters
  - Liner and solvent vapour volumes checked
  - Non packed, packed and alternative liners tested
  - Inlet rigorously cleaned and silanised with MSTFA
  - Injection split ratio and port temperature varied
  - On-column and programmed temperature vaporising injections evaluated

- Column parameters
  - New column, low bleed high specification MS column and base deactivated column assessed
  - Compound retention times/time spent on column reduced
  - On column concentrations increased

- MS parameters
  - Priming effect monitored
  - Alternative GCMS instruments compared
  - Ion source annealed post cleaning
  - Ion source temperature and emission current varied
  - Flow rate and column insertion depth into source increased

It was established that compound degradation/adsorption was not attributed to the method of sample preparation and was not occurring in the GC inlet or GC column. Ultimately linear calibration lines could only be achieved using an annealed clean ion source but linearity was only transient. Repeat analyses afforded quadratic calibration lines, samples could no longer be accurately quantified and it was concluded that the use of GCMS for the quantitation of major derivatised heroin components was not reproducible and therefore not viable.

Having identified the MS detector as the source of the compound degradation/adsorption, a GC instrument fitted with a FID detector was investigated. Each of the derivatised heroin components generated linear calibration lines within the calibration range 4.9-500µg/mL enabling the accurate quantitation of control samples. Continuation of the project would include optimising the GC FID parameters and full method validation. The Shimadzu GCMS instrument would be modified using a splitter to integrate a FID detector allowing single injection heroin samples to simultaneously be quantified by GC FID and qualified by GCMS.

5.1 – IMMEDIATE PLAN

As previously discussed modification of the Shimadzu instrument would enable the parallel acquisition of quantitative data by GC FID with qualitative data by GCMS. The existing derivatised GCMS method parameters would be used and adapted for the GC FID with careful balancing of the respective vacuums and flow rates. Finally the GCMS and GC FID methods would require optimisation prior to full validation:

- Retention times, relative retention times, resolution
- Incorporation of retention indices software
- Intraday/interday variation using low/medium/high sample concentrations
- Linear calibration range determinations
- Control sample quantitations and percentage error measurements
- Determination of LOQ and LOD
- Reproducibility of sample preparation

The latter point incorporates the reproducibility of the sample preparation in terms of the derivatisation but also includes the heroin sampling method. Representative homogenous heroin samples must be taken to ensure that the analytical results are representative of the total heroin sample.

Samples are typically homogenised to break aggregates into a powder by grinding using a pestle and mortar. The coning and squaring technique is often adopted to generate a representative sample. The ground powder poured onto a flat surface to form a cone shape and the cone is divided at right angles using a ruler forming four quarters. Each quarter is then assigned a number from 1 to 4 and a random number between 1 and 4 is generated using a calculator. The identified quarter is selected and if further sample size reduction is required the coning and squaring process is repeated on this quarter.

Anglada *et al.*, developed a statistically correct and practical sampling procedure for large heroin blocks considering the intra-variability within heroin blocks and the inter-variability between heroin blocks in terms of drug purity and chemical profile.[98]

Finally additional research could be carried out to enable the indirect quantitation of expensive and difficult to obtain drug standards (acetylthebaol shipped from Australia costing £170.00/10mg) relative to more commercially available reliable standards. For example, 3-MAM TMS could be approximately quantified relative to morphine di-TMS using the Shimadzu software as the derivatised compounds possess similar response factors.

5.2 – LONG TERM OPTIONS

The heroin profiling project could be extended to include impurity profiling of one or more additional analyses designed to target the minor trace components. Positive correlation linkage determinations for evidential purposes cannot be based solely on the results of a single method.

The importance of incorporating trace component analysis was highlighted by Collins *et al.,* during the analysis of heroin samples seized from a vessel in Australian waters. The major alkaloid and occluded solvent profiles were typical of Southeast Asian heroin but the acid/neutral components found were not consistent with heroin from this origin.[52] Further stable isotope analysis confirmed that the seizures originated from a new region or new illicit process.[86]

Similarly, Besacier *et al.,* analysed heroin samples using a three step procedure including the identification and quantitation of major components by GC, the GC analysis of trace level impurities and GC-IRMS isotopic analysis to determine common batch samples with a high degree of certainty.[1] Chiarotti *et al.,* also employed multiple techniques including HSGC, GCMS, TLC, HPLC and AA to help attribute or exclude common sources of separate heroin samples.[65] Finally, the Australian heroin chemical profiling programme determine major alkaloid ratios using CE with minor components by GCMS and occluded solvents by HSGC to establish the geographic origin of seizures at the Australian border.[138]

Another project extension could involve simulating the dealers chain to monitor the effect (if any) of diluents on the analytical heroin results as carried out by Klemenc.[122] Strömberg *et al.,* also measured the influence on heroin profile by the addition of caffeine, paracetamol, phenobarbitone, diazepam and phenolphthalein adulterants where only phenobarbitone caused considerable interference.[94]

Ultimately there is the possibility of automating the sample preparation to aid the harmonisation of analytical methods for the quantitation and comparison of heroin samples. The Federal Government in Germany introduced robotics (heroin samples weighed, evaporated, solvents dispensed, samples shaken and mixed, centrifuged, pipetted, evaporated and capped) improving both the repeatability and productivity of the heroin profiling procedure compared to manual operations.[37]

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APPENDICES

Optimised derivatised major opiates sample preparation:

A 1mg/mL mixed standard solution in methanol (stock A) was prepared by transferring the 10mg free base equivalents of each compound to a 10mL volumetric flask. The vials were rinsed with methanol (2 x 250µL), added to the flask and the flask sonicated for 5 minutes before making the total volume up to 10mL with methanol (stock A).

1mg/mL mixed standard solution in methanol (stock A) was diluted with methanol (900µL) affording a 0.1mg/mL mixed standard solution (stock B).

A 0.39mg/mL internal standard solution in chloroform was prepared by weighing hexadecane (97.4mg) into a 250mL volumetric flask, chloroform was added and the flask sonicated for 5 minutes before making the total volume up to 250mL with chloroform.

Calibration and control samples were prepared by transferring aliquots of stock A and stock B mixed standard methanol solutions to individual vials as detailed in the table below. The methanol was immediately removed by evaporation at 30°C under nitrogen.

Hexadecane 0.39mg/mL in chloroform (417µL), pyridine (83µL) and MSTFA (150µL) were added to each evaporated vial. A blank was also prepared by adding chloroform (417µL), pyridine (83µL) and MSTFA (150µL) to a separate vial. Final hexadecane sample concentrations of 250µg/mL were achieved. Each of the vials were capped, heated at 80°C for 45 minutes, cooled to room temperature and analysed.

Table – Preparation of calibration and control heroin standard samples

Sample	**Volume mixed standard solution (μL)**	**% w/w**	**Concentration (μg/mL)**
Standard 0	0 [a]	0	0
Standard 1	32 B	0.985	4.92
Standard 2	65 B	2	10
Standard 3	162 B	4.985	24.92
Standard 4	32 A	9.846	49.23
Standard 5	65 A	20	100
Standard 6	130 A	40	200
Standard 7	195 A	60	300
Standard 8	260 A	80	400
Standard 9	325 A	100	500
Control 1	162 B	4.985	24.92
Control 2	162 A	49.85	249.23
Control 3	292 A	89.85	449.23

[a] 325μL methanol added

GCMS instrument parameters:

Shimadzu QP2010 Plus GCMS using helium carrier gas fitted with an automated split/splitless injector and Shimadzu straight focus liners with wool (092062SH).

Generic GC parameters:

Solvent rinses	0 pre run, 6 post run
Sample rinses	0
Plunger speed	high
Syringe speed	high
Injection mode	normal
Injection volume	1μL
Injection mode	split
Split ratio	100:1
Flow control mode	linear velocity

Generic MS parameters:

Tuning	detector voltage set relative to tuning result
Ion source temperature	300°C
Emission current	150μA
Solvent cut time	1.2 min
Solvent start time	3.35 min

Optimised non derivatised major opiates GC method parameters:

Column	Phenomonex ZB-5 capillary column (30m × 0.25mm internal diameter × 0.25μm film thickness)
Flow rate	1.0mL/min
Injection temperature	280°C
Oven programme	200°C for 1 min 200°C to 248°C at 2°C/min 248°C to 290°C at 10°C/min 290°C for 6 min
Method run time	35 min
Detector temperature	310°C

Optimised derivatised major opiates GC method parameters:

Column	Thames Restek Rxi-5ms capillary column (30m × 0.25mm internal diameter × 0.25μm film thickness)
Flow rate	1.4mL/min
Injection temperature	290°C
Oven programme	150°C for 1 min
	150°C to 224°C at 12°C/min
	224°C to 228°C at 0.25°C/min
	228°C to 275°C at 25°C/min
	275°C to 300°C at 5°C/min
	300°C for 2.95 min
Method run time	33 min
Detector temperature	325°C

Optimised derivatised major opiates GC method integration parameters:

Base	area
Slope	2/min
Width	3 sec
Drift	0/min
Min area/height	0
Number smoothing times	2
Smoothing width	2 sec
Reference ion mode	absolute
Default allowance	30%

Optimised derivatised major opiates GC method calibration parameters:

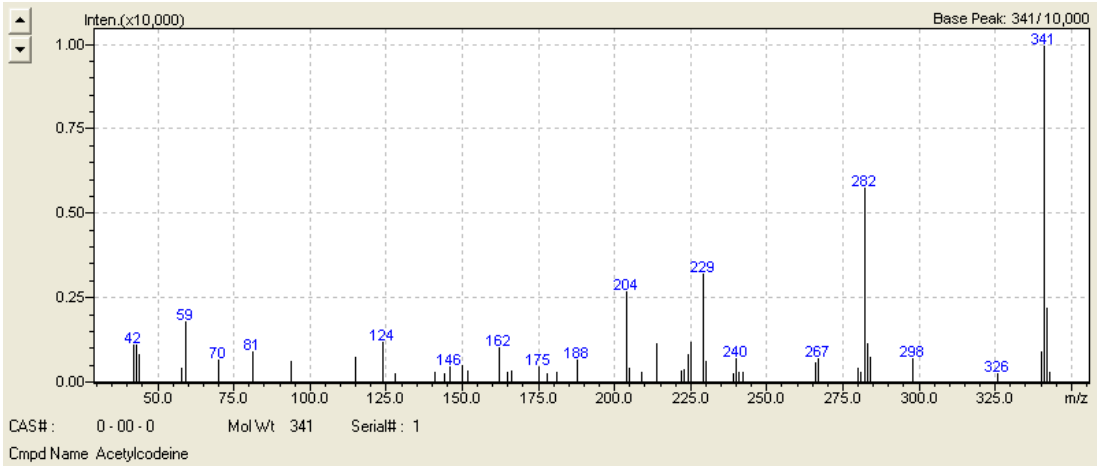
Base	area
Curve fit type	linear/quadratic/power regression
Origin	not forced
Weighted regression	none
Concentration unit	% w/w
Grouping method	sum concentration

Optimised derivatised major opiates GC FID method parameters:

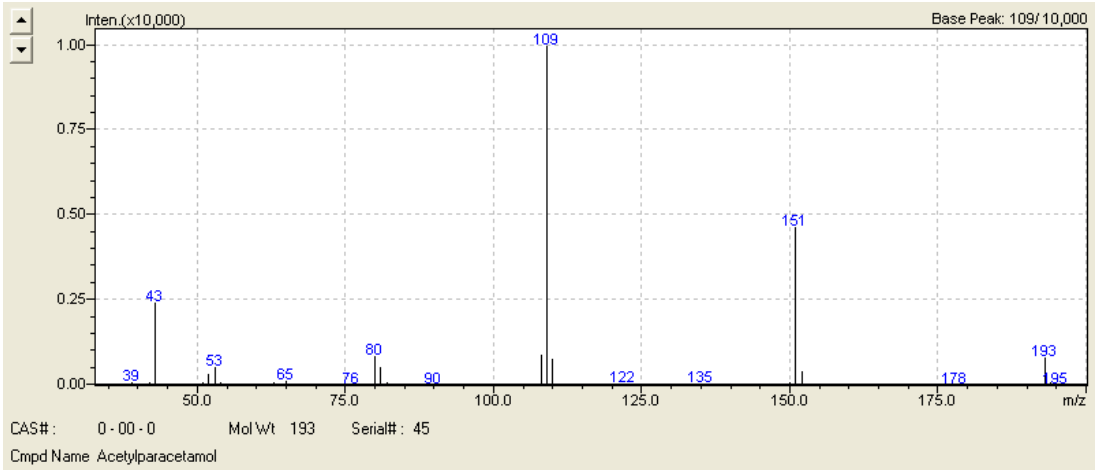
Hydrogen flow rate	40mL/min
Air flow rate	400mL/min
Makeup gas	none
Split ratio	30:1
Emission current	60µA

Experimental mass spectra of analysed components:

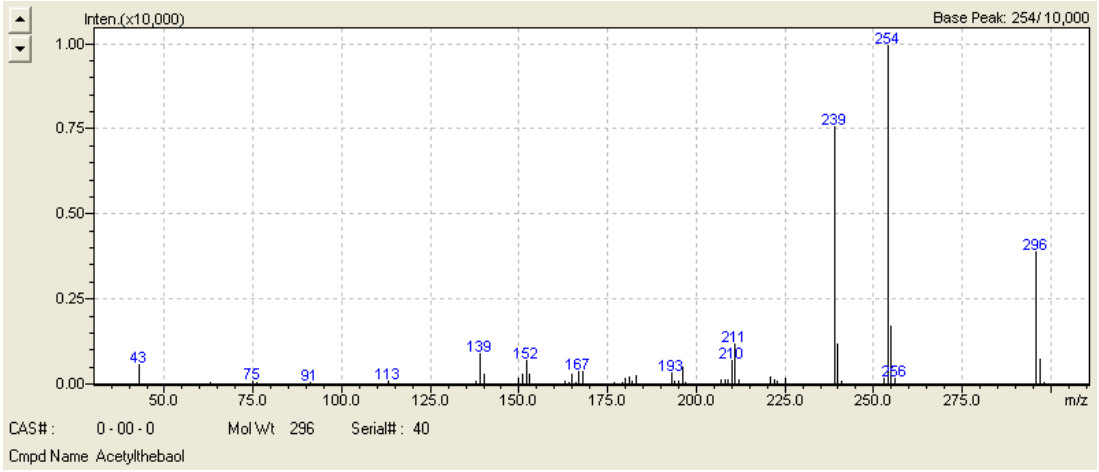
Acetylcodeine



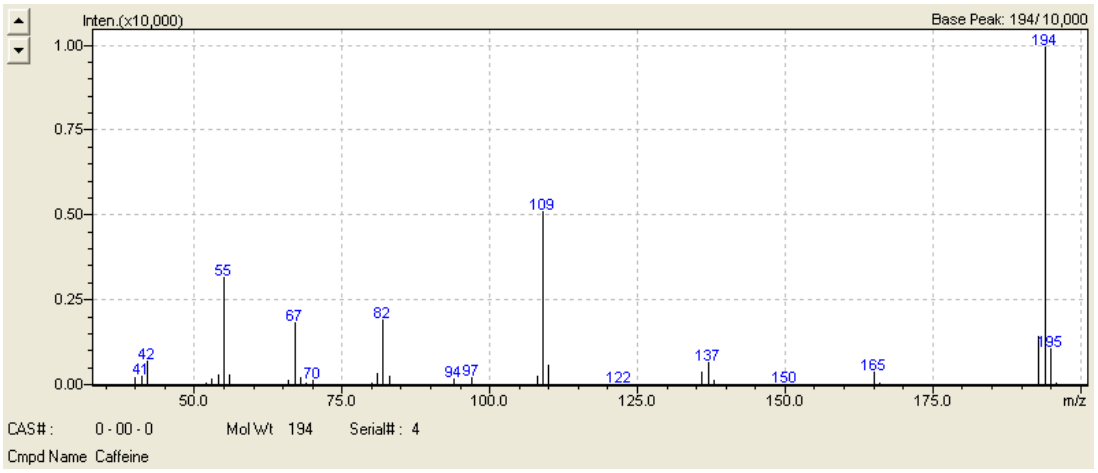
Acetylparacetamol



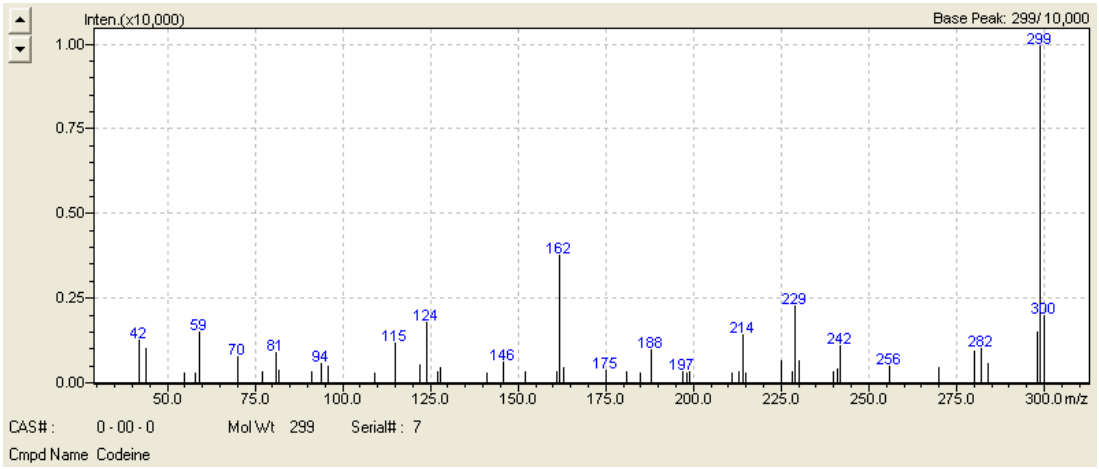
Acetylthebaol



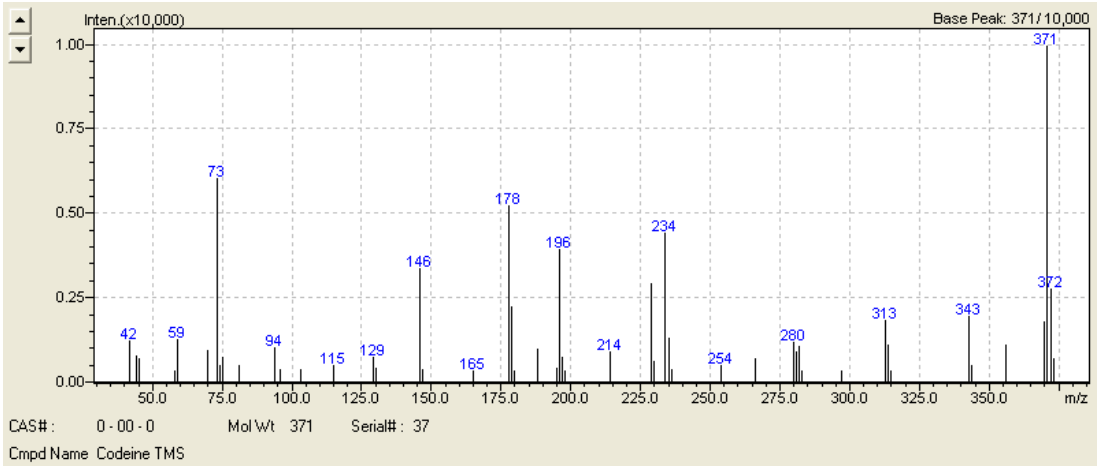
Caffeine



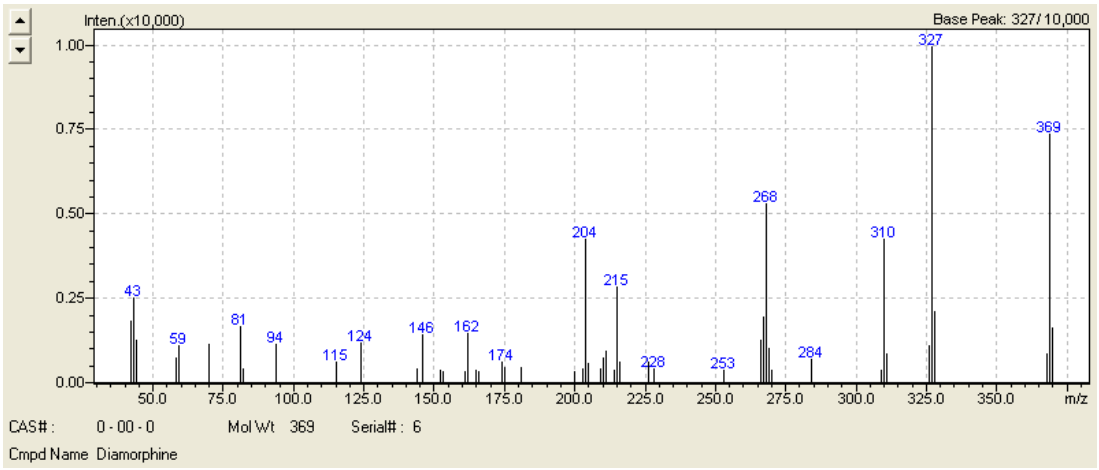
Codeine



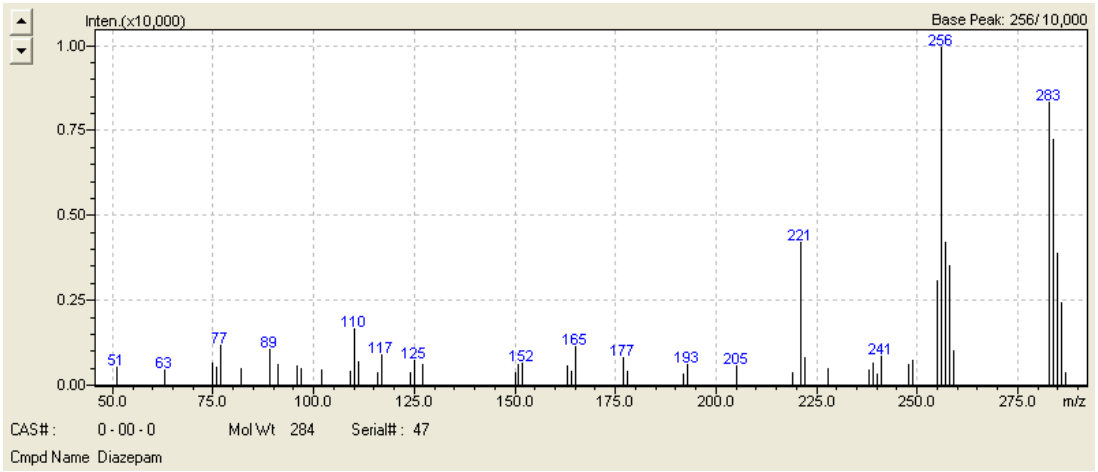
Codeine TMS



Diamorphine



Diazepam



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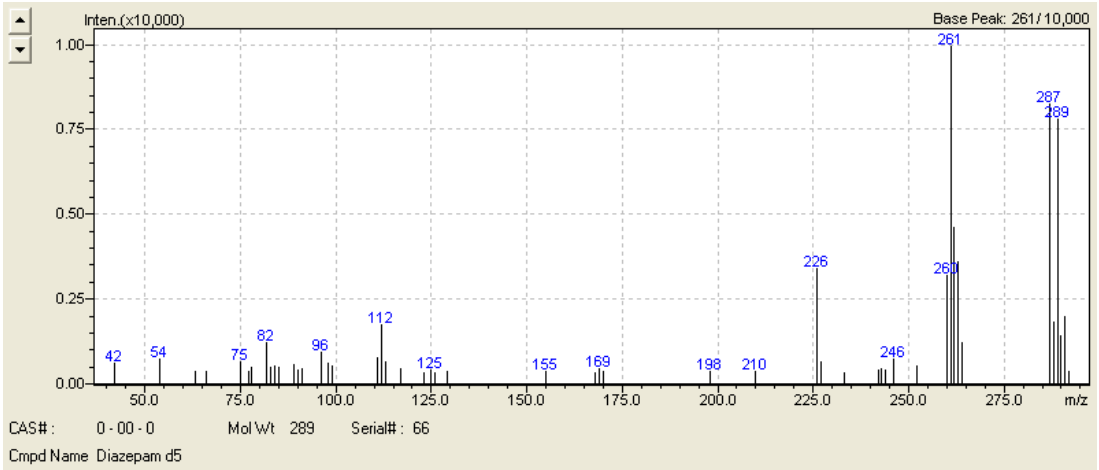

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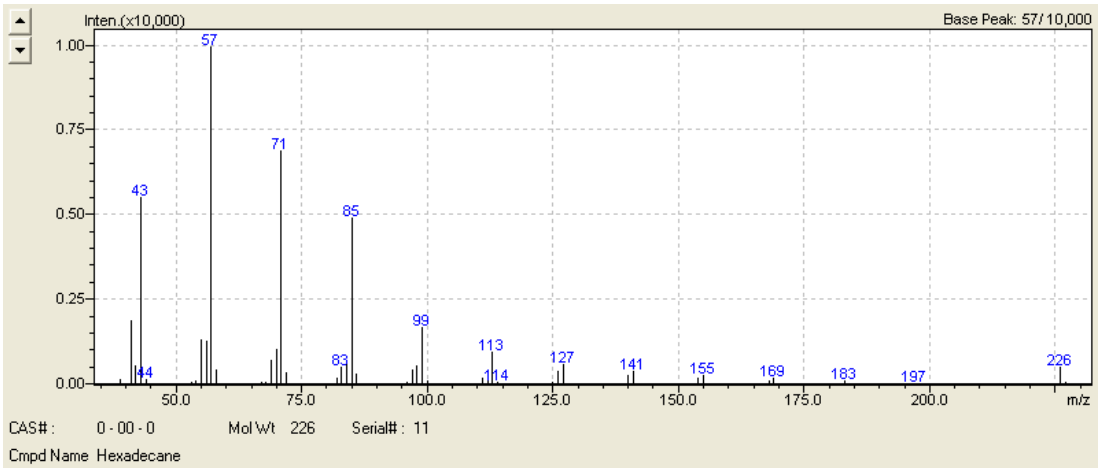

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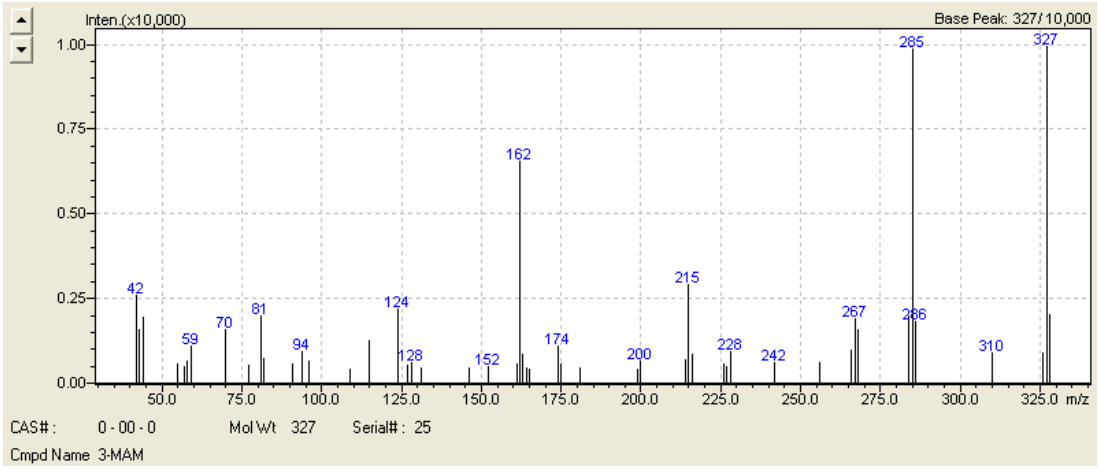

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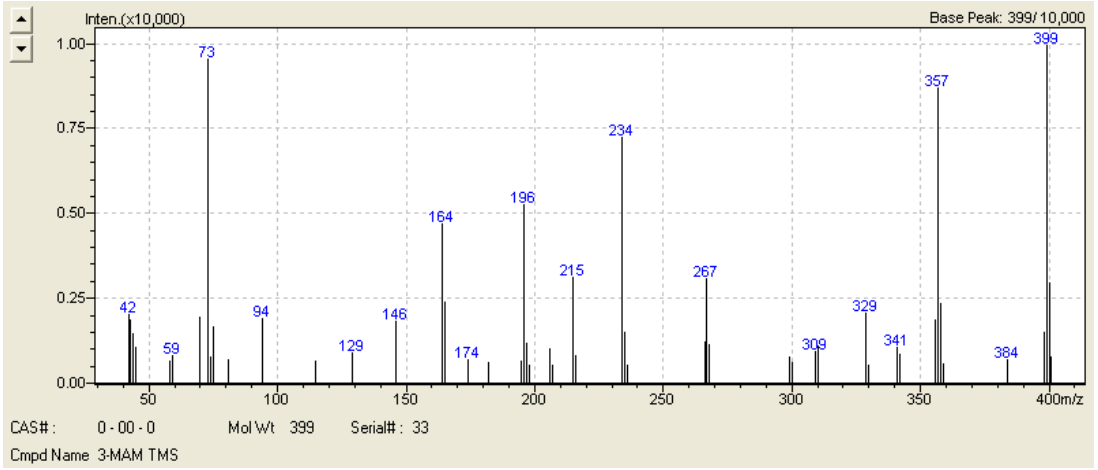

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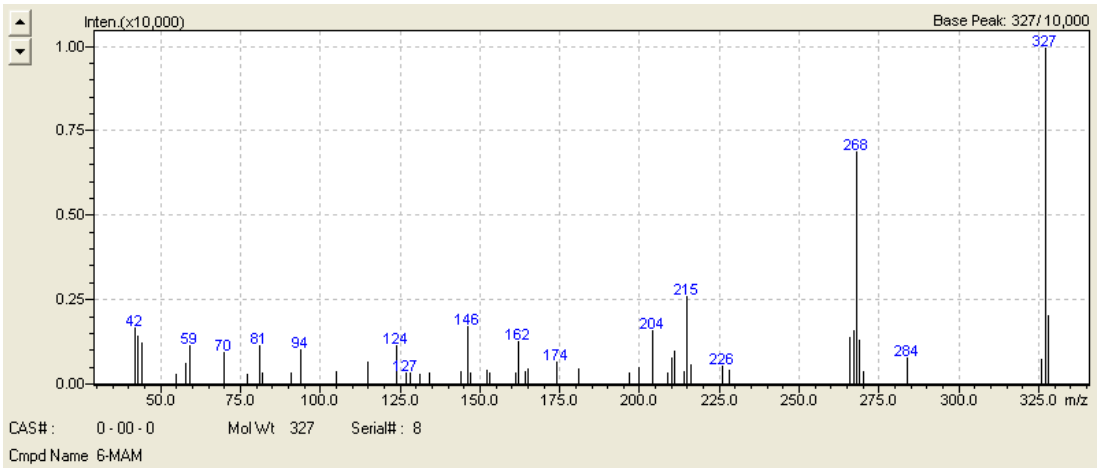

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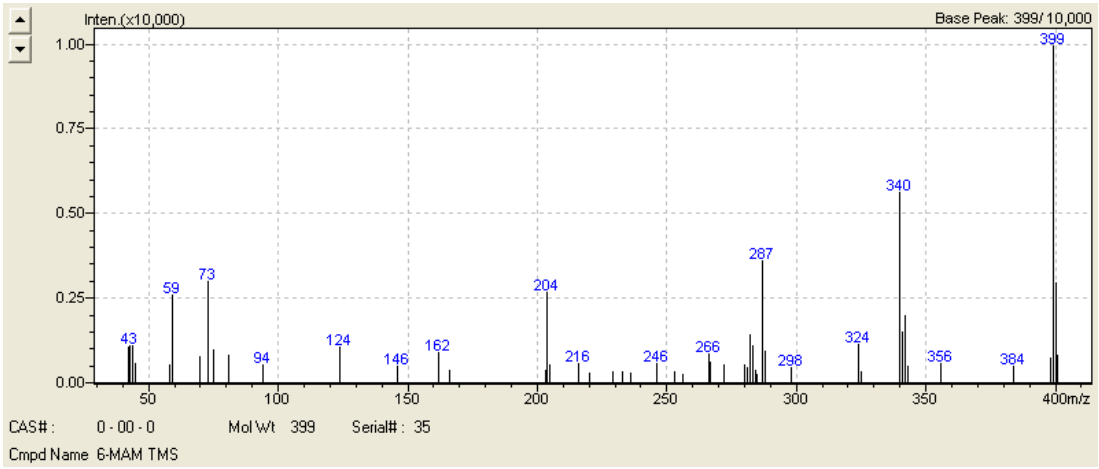
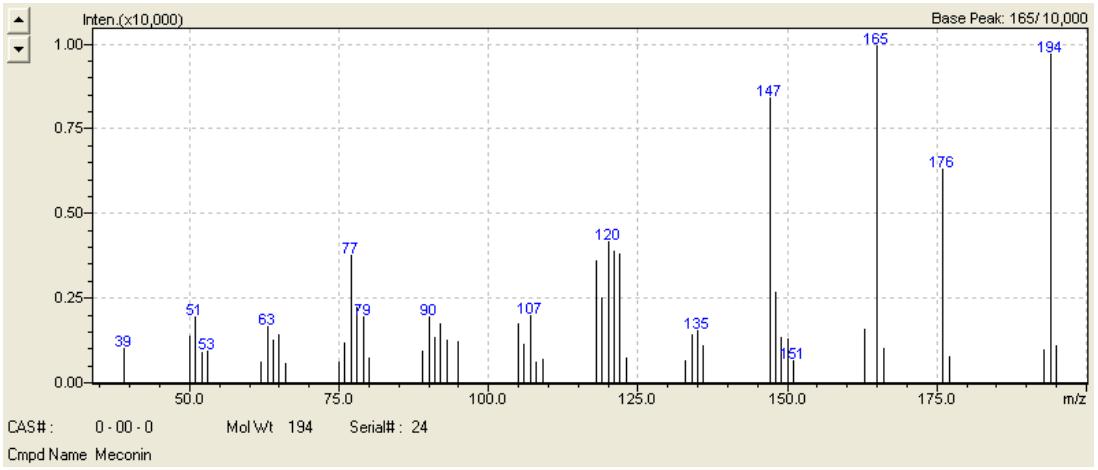
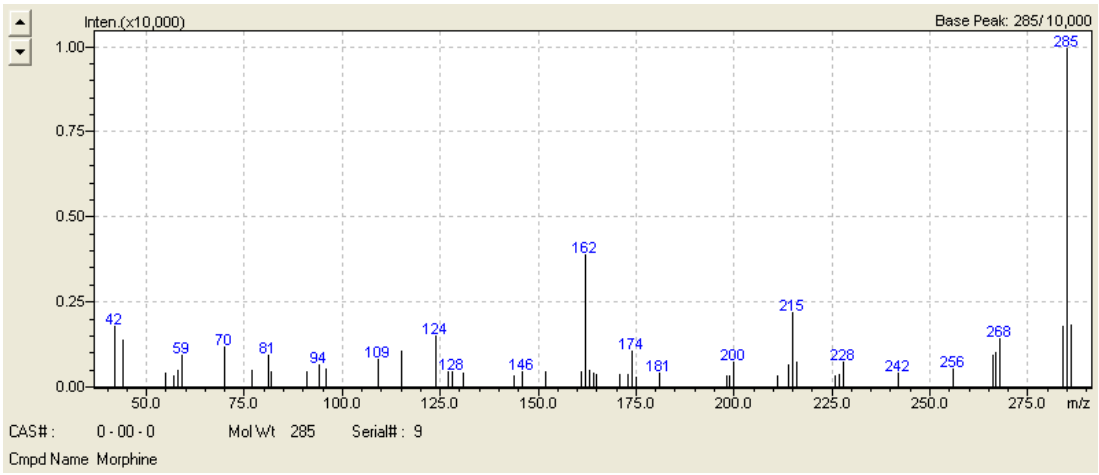


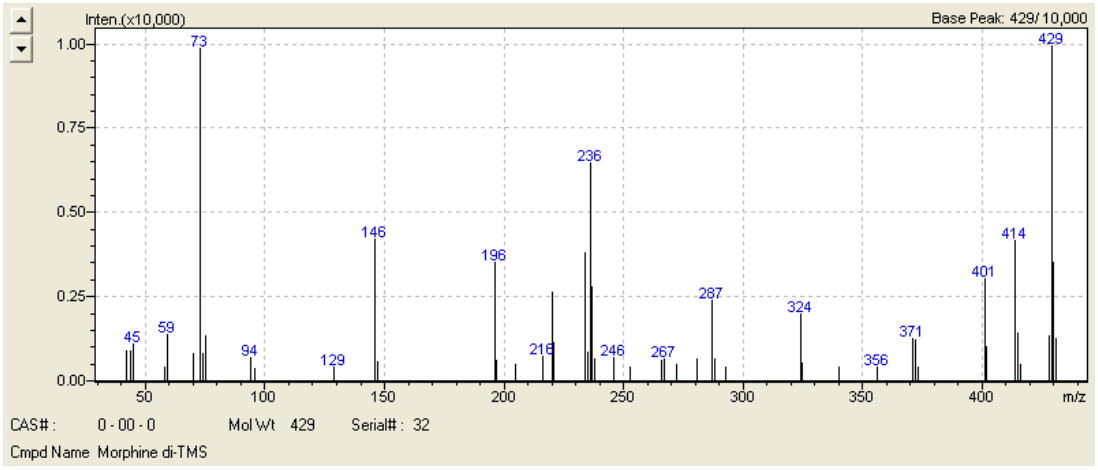
Meconin



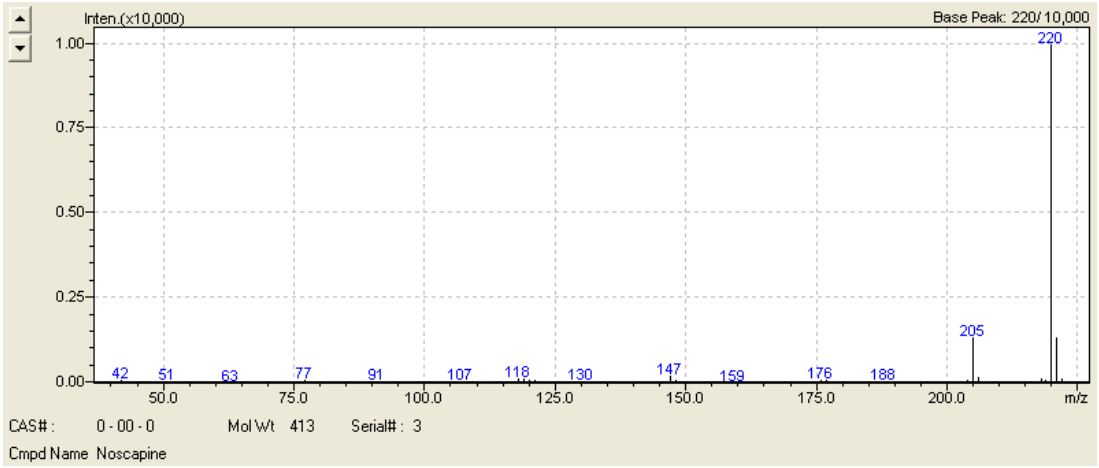
Morphine



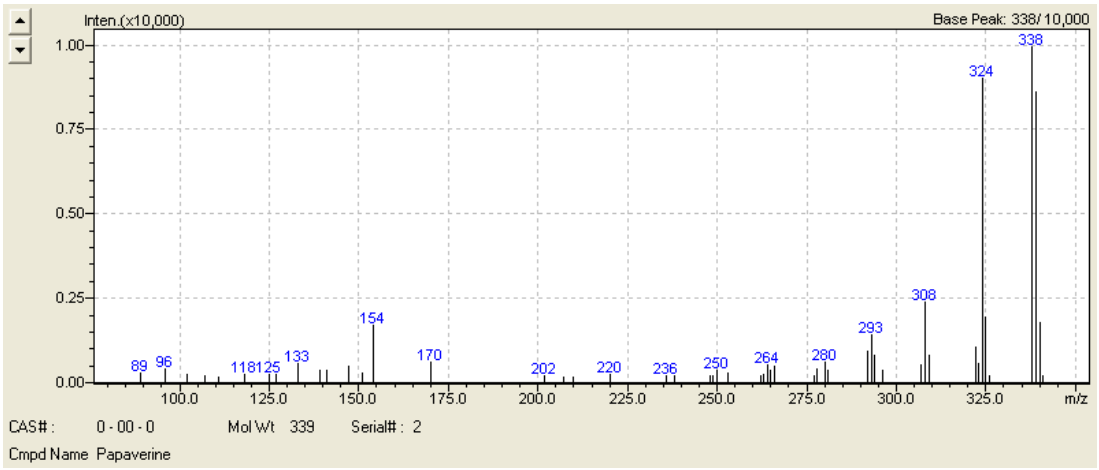
Morphine di-TMS



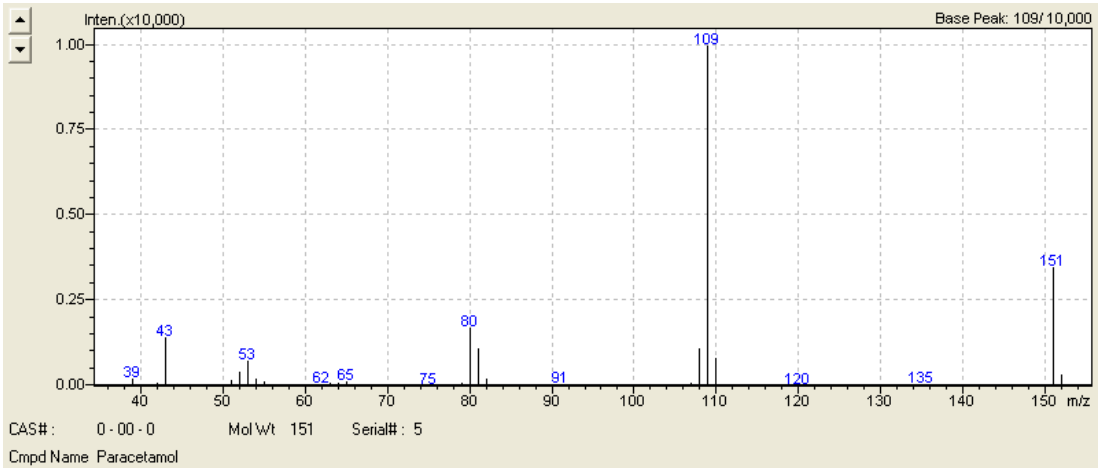
Noscapine



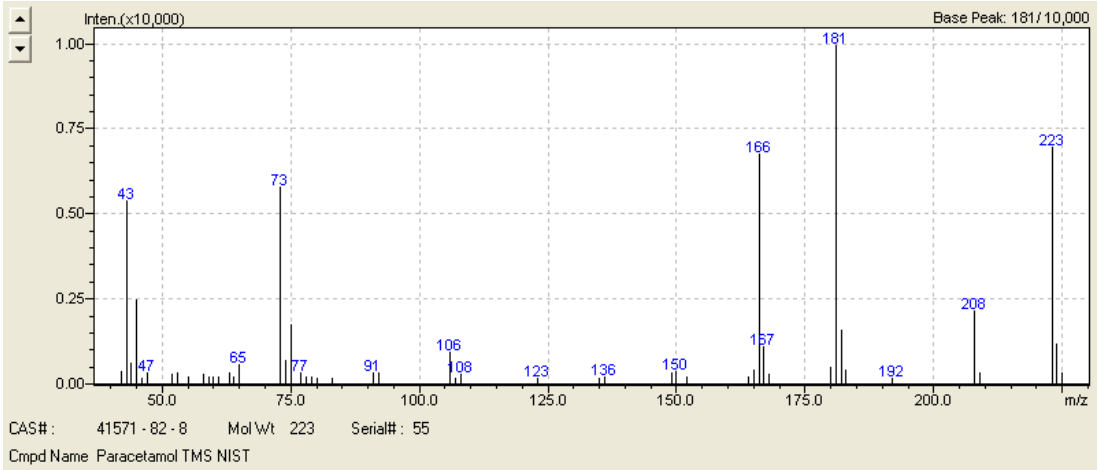
Papaverine



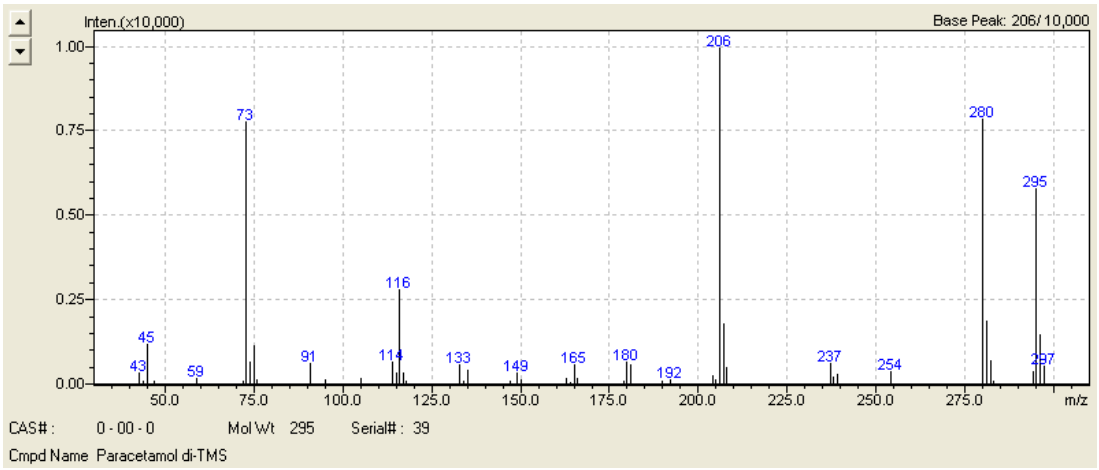
Paracetamol



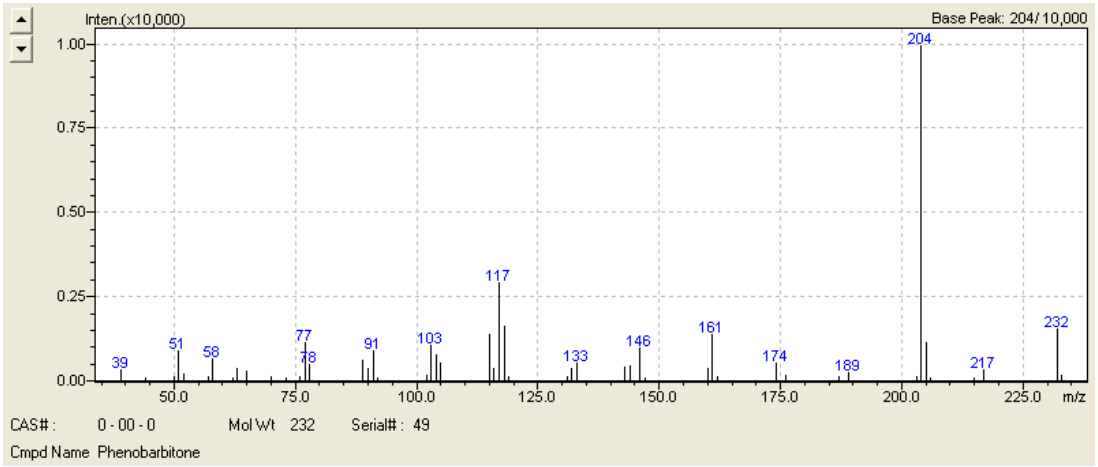
Paracetamol TMS



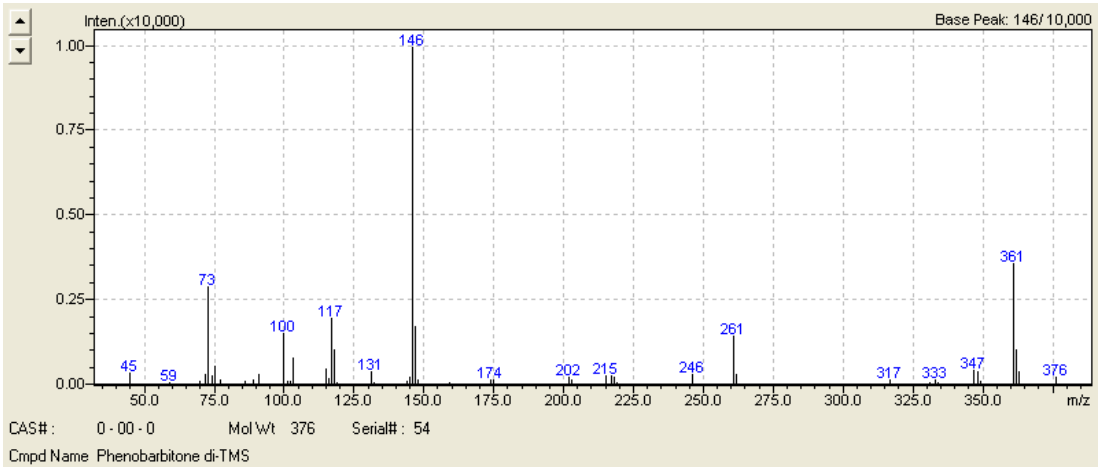
Paracetamol di-TMS



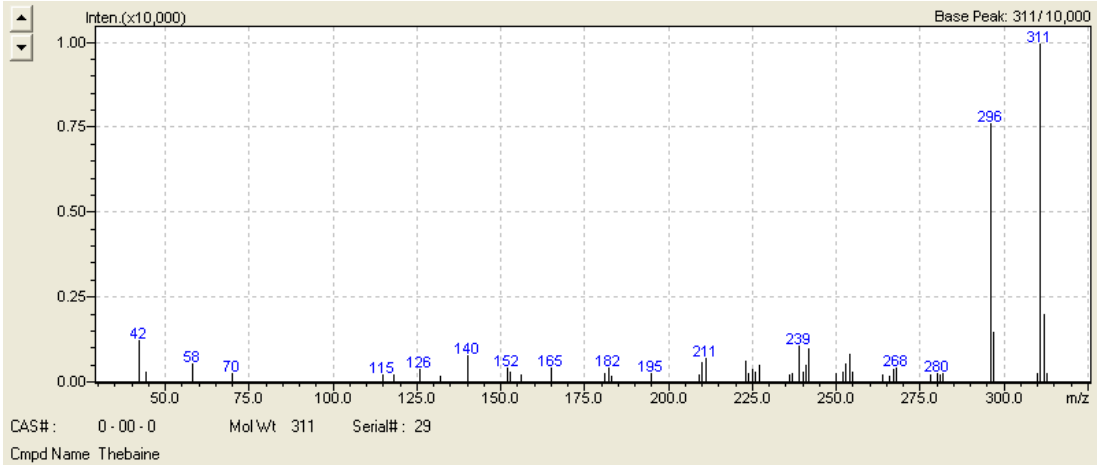
Phenobarbitone



Phenobarbitone di-TMS

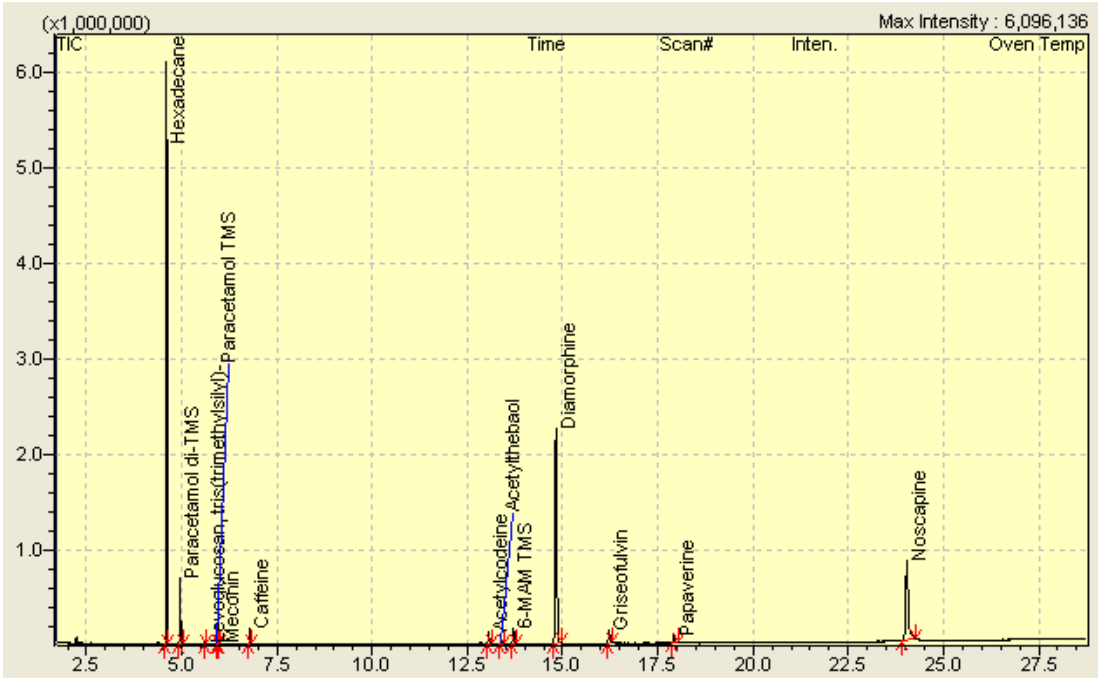


Thebaine

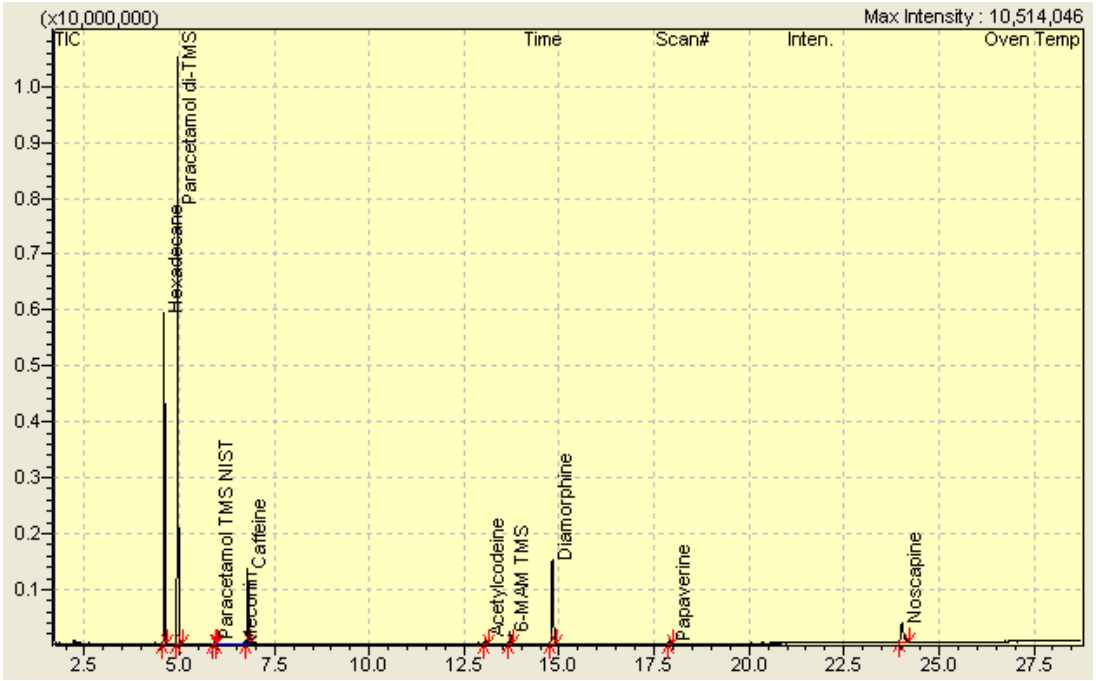

Inten.(x10,000)
Base Peak: 311/10,000
1.00
0.75
0.50
0.25
0.00
42
58
70
115
126
140
152
165
182
195
211
239
268
280
296
311
50.0
75.0
100.0
125.0
150.0
175.0
200.0
225.0
250.0
275.0
300.0
m/z
CAS# : 0 - 00 - 0
Mol Wt 311
Serial# : 29
Cmpd Name Thebaine

Chromatograms of analysed heroin samples (chapter 3.2.3):

Case sample 1



Case sample 2



Case sample 3