A Comparison of Derivatisation Procedures for the Detection of Multiple Analytes in Systematic Forensic Toxicology

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by

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Dedication

For

My father who died in 1997 (1418H), whose wish it was to see me graduate, for my mother who has helped me and supported me by asking God to help me, for my wife and my daughter Rafeef who helped me a lot, also to my uncle Abdarahman who has supported me and helped me, and for all my family.

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Summary

Forensic Systematic Toxicological Analysis (STA) deals with the chemical analysis of biological samples in a logical sequence designed to detect a wide range of exogenous materials, including drugs and other chemical substances. The ideal approach would be to detect qualitatively and quantitatively all substances of interest in a single method involving a single extraction procedure and a single end-step analytical technique. In recent years solid phase extraction based on modified silica sorbents has become the most widely-used extraction procedure in forensic toxicology. More recently, new polymeric sorbents with different selectivities have become available which promise to provide the single extraction technique required for STA. Also, one of the principal end-step analytical techniques used for STA is gas chromatography-mass spectrometry, which is applicable to volatile and thermally-stable materials but which can be extended to cover a significantly wider range of toxicologically-relevant materials by use of chemical derivatisation procedures during sample preparation. The aim of this thesis was to investigate both the extraction and end-step components of STA.

The introductory section reviews the background to forensic toxicology and systematic toxicological analysis, whether dealing with the analysis of an individual drug or with a group of drugs within the same class, and introduces details about the blood matrix - the most commonly used sample type in forensic toxicology. Subsequently, some of the background theory of Gas Chromatography-Mass Spectrometry used in this project is examined including the historical development of chromatography. The detectors which are available, such as the mass spectrometer in combination with gas chromatography and the ionization methods used are also described. The background to drugs of abuse, which form an important group of drugs within STA, is discussed, including their chemistry and pharmacology and information on the main classes of drugs of abuse analysed in this thesis and their metabolism is given. Lastly, derivatisation methods are reviewed, including the different types of derivatisation used in this project.

Three different derivatisation procedures were evaluated for their general applicability to STA, involving (a) acylation with pentafluoropropionyl anhydride (PFPA) and pentafluoropropanol (PFP-OH), (b) acylation/esterification (methylation) with pentafluoropropionyl anhydride (PFPA) and a novel methylating agent trimethylsilyldiazomethane (TMS-diazomethane), used as a chromatographic

derivatisation reagent for the first time in this study, and (c) silylation with tertiary-butyldimethylsilyl-trifluoroacetamide (MTBSTFA). Model compounds were selected for the evaluation process including a primary amine (amphetamine), a secondary amine (methamphetamine), alicyclic and aromatic hydroxy compounds (morphine, tetrahydrocannabinol), and carboxylic acids (benzoylecgonine, 11-nor tetrahydrocannabinol-9-carboxylic acid). Microwave assisted derivatisation was evaluated as a replacement for conventional heating on a hotplate. Effective conditions for rapid derivatisation required only one minute in the microwave oven instead of 30 minutes in a heating block.

For method (a) derivatisation was successful for all of the test compounds and mass spectra were obtained for each of them. These derivatives gave good gas chromatographic behaviour, in terms of peak shape and thermal stability, and the mass spectra had prominent molecular ions and also prominent diagnostic fragment ions at high mass, suitable for use as qualifier ions in selected ion monitoring GC-MS. For method (b), the novel methylating agent trimethylsilyl-diazomethane was used to convert carboxylic acids into the corresponding methyl esters. This reaction was found to proceed rapidly and quantitatively at room temperature and holds potential for future use in toxicology to replace diazomethane, a hazardous and toxic material. The products of acylation of alcohol remained unchanged from method (a). However, the primary amine (amphetamine) and secondary amine (methamphetamine) gave less satisfactory results in terms of poor method linearity. The acylated/methylated products gave good gas chromatography and had mass spectra which are suitable for both identification and quantification by GC-MS-SIM. Method (c) gave derivatives with all test compounds except the secondary amine, methamphetamine, and the alcohol, morphine. The gas chromatographic behaviour of these derivatives was good and the mass spectra had prominent ions suitable for GC-MS-SIM, often characterised by prominent M-57 ions due to the loss of the butyl substituent in the silyl derivatives.

The extraction of multiple drugs from blood was evaluated using the novel polymeric SPE sorbent Strata-X. The same test compounds were used to assess the extraction step in terms of recovery and variation (within day and between days). The extracts were analysed by GC-MS-SIM using each of the three types of derivative. Recoveries of the test compounds were in the range of 50-100 percent depending on the analyte and its concentration in blood. All calibration curves were linear and had correlation coefficients

higher than 0.99. Within day variations and between day variations were in the range of 2-22% relative standard deviation. Limits of detection and quantitation were measured for the model compounds and were found to be in the ranges 0.4-7.3 ng/ml and 1.1-24.4 ng/ml respectively. The full method, combining extraction with each of the derivatisation reactions was finally evaluated for the presence of interferences with real case blood samples.

A particular application of extraction and derivatisation was investigated in which two isomers of hydroxybutyric acid (gamma-hydroxy butyric acid, GHB, and beta-hydroxybutyric acid, BHB) were analysed in small (0.2 ml) whole blood samples. GHB is of interest as an abused drug which has been implicated in drug assisted assault while BHB is of interest in alcohol related deaths as a potential marker of fatal alcoholic ketoacidosis. Both compounds were extracted with Clean Screen GHB solid phase extraction cartridges with recoveries in the range 44-89% depending on the analyte concentration. The extracts were analysed by GC-MS-SIM after derivatisation with bistrimethylsilyl-trifluoroacetamide. Calibration curves were linear, with correlation coefficients above 0.99. Limits of detection and quantification were acceptable for the intended application, being in the range of 0.1-4 mg/L depending on the analyte. The method was applied to authentic case samples from alcoholics and non-alcoholics. It was noted that when alcohol levels were high, the BHB levels were also high. When BHB levels were high, there was some indication that the GHB levels were affected, in that the higher the BHB levels, the lower the GHB levels. However this did not reach statistical significance.

Benzodiazepines form one class of drugs which is commonly encountered in STA and which often requires derivatisation prior to GC-MS analysis. The three derivatisation procedures were evaluated using four test compounds comprising diazepam plus its three metabolites nordiazepam, temazepam and oxazepam. The hydroxylated metabolites (temazepam and oxazepam) formed derivatives readily with all three reagent mixtures but nordiazepam (secondary aromatic amine) did not react except with MTBSTFA.

Based on the work of this study it is concluded that a method is possible for STA based on a polymeric sorbent, to give a general extract, followed by a generalised derivatisation procedure such as acylation, with PFPA/PFP-OH prior to GC-MS.

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List of Abbreviations

AM	Amphetamine
BZE	Benzoylecgonine
BHB	Beta-hydroxybutyrate
EI	Electron Impact
EDDP	2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine
GHB	Gamma-hydroxybutyrate
GC-MS	Gas Chromatography-Mass Spectrometry
LOD	limit of detection
LOQ	limit of quantitation
R^2	Linear Correlation Coefficient
m/z	mass-to-charge ratio
METH	Methamphetamine
µg/ml	micrograms per millilitre
mg/ml	milligrams per millilitre
MW	molecular weight
MOR	Morphine
ng/µl	nanograms per microlitre
MTBSTFA +1% TBDMCS	N-Methyl-N-(tert-butyl-dimethylsilyl)-trifluoroacetamide +1% Tert-butyl-dimethylchlorosilane
BSTFA	N, O-Bis-trimethylsilyl–trifluoroacetamide
THC-COOH	11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid
PFPA	Pentafluoropropionic anhydride
PFP-OH	2, 2, 3, 3, 3-pentafluoro-propanol

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pK_a	pH at which 50% of the analyte is ionised and 50% is non-ionised.
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
Strata ™X SPE	Solid phase extraction with Strata X™
STA	systematic toxicological analysis
THC-OH	delta-9-Tetrahydrocannabinol
D_3	tri-deuterated
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl-diazomethane

1 Introduction

1.1 Toxicology

Toxicology is the study of poisons which affect the function of the body and can cause death[1-3]. Forensic toxicology is part of general toxicology in which the results of toxicological analysis are used in legal proceedings. It is a branch of science that incorporates many areas of knowledge including pharmacology, analytical and organic chemistry, physiology and pathology. Traditionally, the forensic toxicology laboratory analyses biological specimens for the presence of alcohol, drugs and other toxic compounds as part of post-mortem investigations[4-9]. However, the field now also includes samples from living subjects, such as clinical cases, workplace drug testing and road traffic cases[10, 11]. The toxicology laboratory performs qualitative and quantitative chemical analysis of tissues and body fluids to determine the presence or absence of drugs and other toxic substances[12-19]. Drugs and poisons can be classified according to the method used for extraction. Seven major groups are usually considered: gases, volatile substances, drugs, metals, pesticides, anions and miscellaneous substances (Figure 1-1).[20, 21]

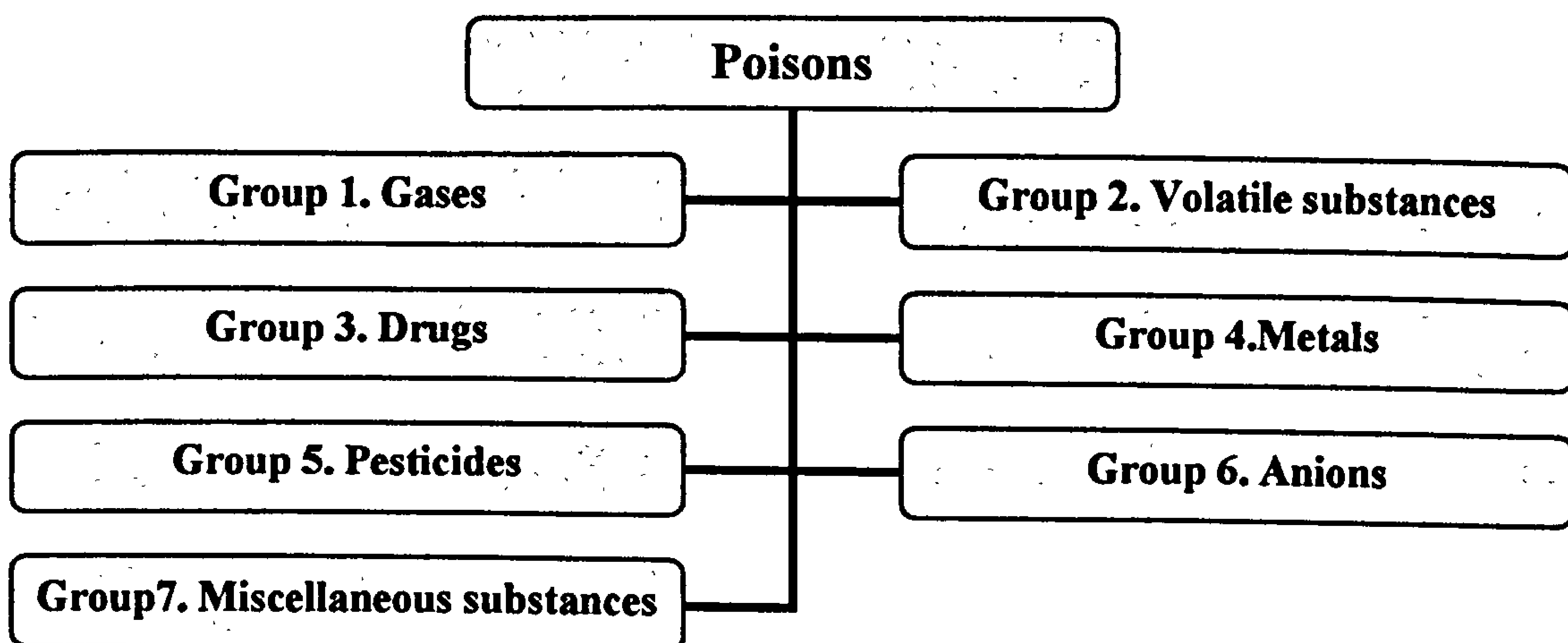


Figure 1-1:- The seven major groups of poisons.

Samples submitted to the toxicology laboratory for analytical tests can include blood, urine, stomach contents, tissues from all body organs, hair, nails, and, from

living subjects, oral fluid. Blood consists of plasma (55% by volume) and cells (45% by volume)[22]. There are three types of cells: the white blood cells, which are part of the immune response against bacteria and other invading organisms, red blood cells, which transport oxygen and carbon dioxide, and platelets, which are important for normal blood clotting (haemostasis)[22]. In general, the blood has many functions as it delivers nutrients from the digestive system, transports oxygen from the lungs to all of the cells in the body, transports waste from the body to the excretory organs, and carries hormones from the endocrine glands to target tissues to regulate body temperature and fluid balance, amongst others.[23] Blood is considered very important in forensic toxicology as it can indicate the amount of drug in the system at time of death and blood concentrations of drugs and poisons can in principle be interpreted in terms of their probable toxicity hazard by comparing them with published or in-house data for clinical subjects and post-mortem cases.[6] The amount of blood in the body is usually five litres (about 7% of body weight) and the normal pH of blood is slightly alkaline, ranging between 7.3-7.4. The toxicology laboratory routinely receives blood samples of more than 5 ml in volume. However, sometimes only small volumes of blood are available, as with live patients, especially infants and children, or conversely from much decomposed bodies: this makes it difficult to do a full toxicological screen.

1.2 Systematic Toxicological Analysis (STA)

Forensic systematic toxicological analysis is essentially a logical sequence of analytical methods which are applied to samples in order to detect and (usually) quantify a wide range of exogenous materials, including drugs and other chemical substances.11[24-29] This is in contrast to an analytical method which targets a specific substance, such as alcohol or carbon monoxide. STA is an important part of forensic toxicology since, in many cases; no information is available concerning which poisons may be present. The International Association of Forensic Toxicologists (TIAFT) has a specialist committee on STA.[25, 30-33]

Until recently, gas chromatography – mass spectrometry (GC-MS) was considered the most useful technique for identifying and quantifying poisons, including drugs of abuse. However, in the last few years, LC-MS-MS has become more widely available, more reliable and less expensive than previously and in future may replace

GC-MS as the analytical method of choice.[34-46] Nevertheless, many laboratories still depend on GC-MS as their gold standard technique, lacking equipment for LC-MS-MS. There remains a lot of interest and a great deal to be obtained by extending the capabilities of GC-MS in STA as far as possible as it provides higher chromatographic resolution than is currently available by liquid chromatography, is often more sensitive than LC-MS-MS and is more useful for screening for unknowns because of its library search capabilities.[47-57]

The limitations of GC-MS frequently result from thermal instability of analytes resulting in turn from their chemical structures. Some drugs contain polar functional groups which are thermally unstable and which will interact with the column or injection port, affecting peak shape and sensitivity. The most common approach to overcoming this problem is to modify the analytes chemically, usually referred to as chemical derivatisation.[58] Derivatisation improves separation and reduces tailing by increasing volatility, reducing polarity and increasing thermal stability. Depending on the functional group present, different derivatising mechanisms can be used, namely, silylation, acylation and alkylation, including methylation (esterification). For the analysis of drugs of abuse, individual extraction and derivatising procedures are required because different drugs need to be derivatised in different ways depending on the functional groups present. This is a major disadvantage because of the time required to identify and quantify a wide range of drugs. If a unified approach to the toxicological analysis could be developed which allows the detection of a wide range of substances of medicolegal interest in a minimum number of chromatographic analyses, this would be greatly advantageous in terms of time saved.[59]

1.3 Aims and Objectives

The aim of this project was to investigate the possibility of creating a unified analytical approach to toxicological screening. Different derivatising agents would be investigated using selected drugs of abuse with different functional group chemistry as model compounds. Based on the results of the evaluation of derivatisation chemistries, a method for the simultaneous detection and quantitation of multiple drugs and metabolites using only 1 ml of sample would be developed

and validated. The derivatives would also be evaluated for their use with benzodiazepines commonly encountered in forensic toxicology.

A particular application of derivatisation chemistry would also be investigated. Gamma–hydroxybutyrate (GHB) and beta-hydroxybutyrate (BHB) are endogenous to the human body and there is a relationship between GHB and BHB. When BHB concentrations are elevated, it can significantly inhibit the metabolism of GHB.[60] GHB has been abused recreationally for relaxation effects[61] and also has reputedly been used for drug facilitated sexual assault because of its sedative and amnesic effects at high doses.[62-65] BHB is one of the "ketone bodies" present in the body, the others being acetone and acetoacetate. When blood alcohol concentrations in the body are high, this will increase the production of BHB. This in turn increases the blood pH, causing death as a result of ketoacidosis. By measuring BHB in blood the forensic pathologist can obtain additional information to assist in assigning the cause of death in known alcohol abusers. The aim of this part of the project was to investigate and validate methods for the simultaneous determination of GHB and BHB in small blood samples, with particular emphasis on short processing times.

2 Gas Chromatography – Mass Spectrometry

2.1 Introduction

Gas chromatography-mass spectrometry (GC-MS, Figure 2-1) is an instrumental technique used in analytical chemistry which can separate, identify and quantify chemical substances in samples containing complex mixtures of substances, such as those extracted from biological specimens used in forensic toxicology.[66-70] GC-MS dynamically combines two techniques: gas chromatography (GC) which can separate the chemical mixtures into their separate chemical components and mass spectrometry (MS) which can identify and quantify the chemicals. It is a sensitive technique which has the ability to detect low concentrations of analytes. The use of chromatographic techniques to introduce samples dynamically to the mass spectrometer (as opposed to an off-line method of trapping materials eluting from the GC column and introducing them on a sample probe) first appeared in the 1960's, when all gas chromatography was performed using packed columns. The initial problem that needed to be solved was to remove the carrier gas, which for packed columns had a flow rate of 30 ml/min, which would destroy the vacuum needed for efficient mass spectrometry. The Ryhage jet separator achieved this but the problem subsequently disappeared when capillary columns became routinely available, as the pumping system used to maintain the vacuum in the mass spectrometer could easily cope with their flow rate of 1-2 ml/min.[71-81]

Gas chromatographic analysis is, however, restricted to a fairly narrow range of compounds which:

- are relatively volatile, having boiling points generally below 400°C. Substances with very high boiling points are usually not amenable to assay by GC-MS.
- Have low molecular weights, generally below 500 Daltons.
- Are relatively non-polar compounds. GC columns often have, or develop, undesirable active sites that interact with polar compounds,
- Are thermally stable: The bulk of GC analysis relies on the injection of samples into a vaporising inlet and thermally unstable compounds are liable to breakdown at

such temperatures, often resulting in inlets contaminated with non-volatile matrix products.

Despite its great capabilities, GC-MS has some disadvantages: most analyses are time consuming and involve labour-intensive steps.[71, 80, 82]

The separate techniques and principal applications of GC-MS are considered to be very important for drugs of abuse, and because of that, this section will provide details about gas chromatography and mass spectrometry.

Figure 2-1:- A modern instrument for Gas Chromatography – Mass Spectrometry

2.2 Chromatography: Overview

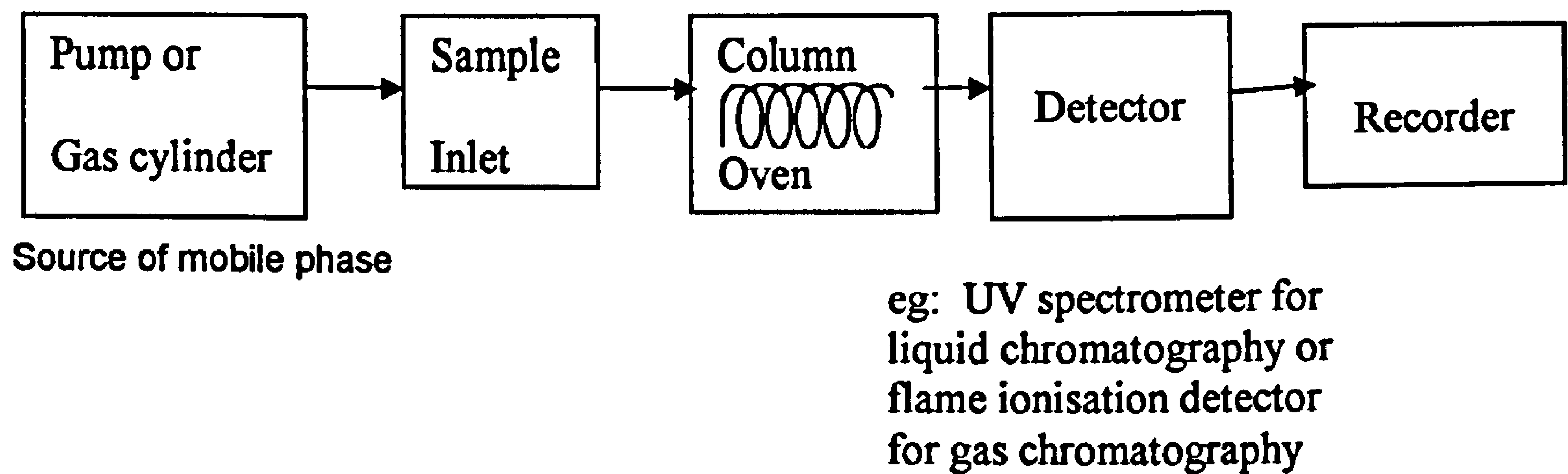


Figure 2-2:- Components of a chromatograph

Chromatography is a separation method that is used in many branches of science (Figure 2-2). It was invented by the Russian botanist Mikhail Tswett. [66, 80] He used the word chromatography to describe this technique, deriving it from two Greek words, *chroma* meaning colour and *graphy* meaning to write.[66, 83]

Chromatography basically involves the separation of a mixture of components due to differences in equilibrium distribution of sample components between two different phases.One of these phases is a mobile phase that does move and the other one is a stationary phase that does not move. All chromatographic separations are governed by distribution constant (K_D, see para 2.2.2)
Chromatography has advantages as it can separate very complex mixtures, cope with very small sample volumes, separated components can be collected individually and analyses can be highly accurate and precise.[80] It is used for determining concentrations where each chromatographic peak in a chromatogram corresponds to a separate component and therefore the area of each peak is proportional to its amount (Figure 2-3).[71, 80] There are general factors which will increase resolution, which describes the degree with which adjacent peaks are separated: longer column lengths, smaller column diameters, decreased flow rates, uniform stationary phase particle size (packing material in packed column chromatography), decreased sample size, selection of the correct stationary and mobile phases, using the correct pressure and using gradient elution. There are terms and symbols

which are very important for chromatography such as those described in the following paragraphs.[71, 80, 84]

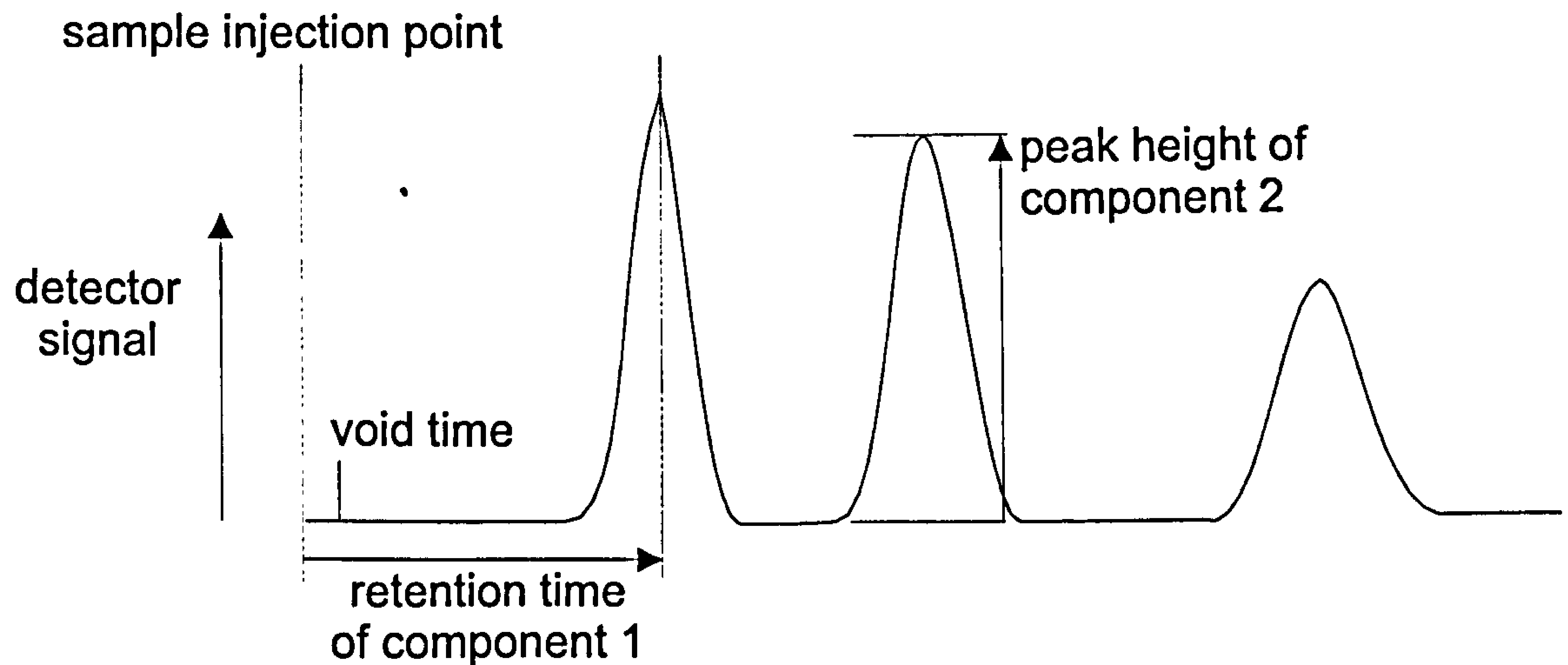


Figure 2-3:-Chromatogram nomenclature

2.2.1 Retention time

This is the time taken after sample injection for an analyte peak to reach the detector and is denoted by the symbol t_R.[66]

2.2.2 Distribution constant (K_D)

The distribution constant K_D is equal to the ratio of the concentrations of the solute C in stationary phase C_S divided by concentration in the mobile phases C_M (Figure 2-4).

$$K_D = C_S / C_M$$

Figure 2-4:- Distribution Constant

The value of K_D should remain constant across a wide range of solute concentrations where difference K_D to affect on the migration rates of solutes through a column[80, 83, 84]

2.2.3 Column

When selecting an analytical column, the basic choices to be made are in the stationary phase, column diameter, length and the thickness of stationary phase or stationary phase loading.[71, 80] A non polar stationary phase is often the most useful in analytical toxicology. The highest resolution is afforded by the narrowest columns with the thinnest stationary phase. Thin film, narrow bore columns are especially good for separating mixtures of high-boiling-point compounds that are retained too strongly on thick film columns. Short retention times provide high-speed analyses. However, thin film, narrow bore columns have very low sample capacity and require high sensitivity detectors. Thick film, narrow bore columns can be used with most detectors and with compounds of high volatility. Retention times are longer than on thin-film columns. Thick-film, wide-bore columns are required for use with thermal conductivity and infrared detectors. These detectors are relatively insensitive and require relatively large sample sizes to be loaded on the column, which usually requires the higher sample capacity of a thick film column. Columns used in gas chromatography are further discussed in section 2.3.3.[72, 80, 85]

2.2.4 Detectors

The detector can provide information on each compound as it elutes from the column. Its signals are proportionate to the quantity of each solute, making quantitative analysis possible (Figure 2-5).[80]

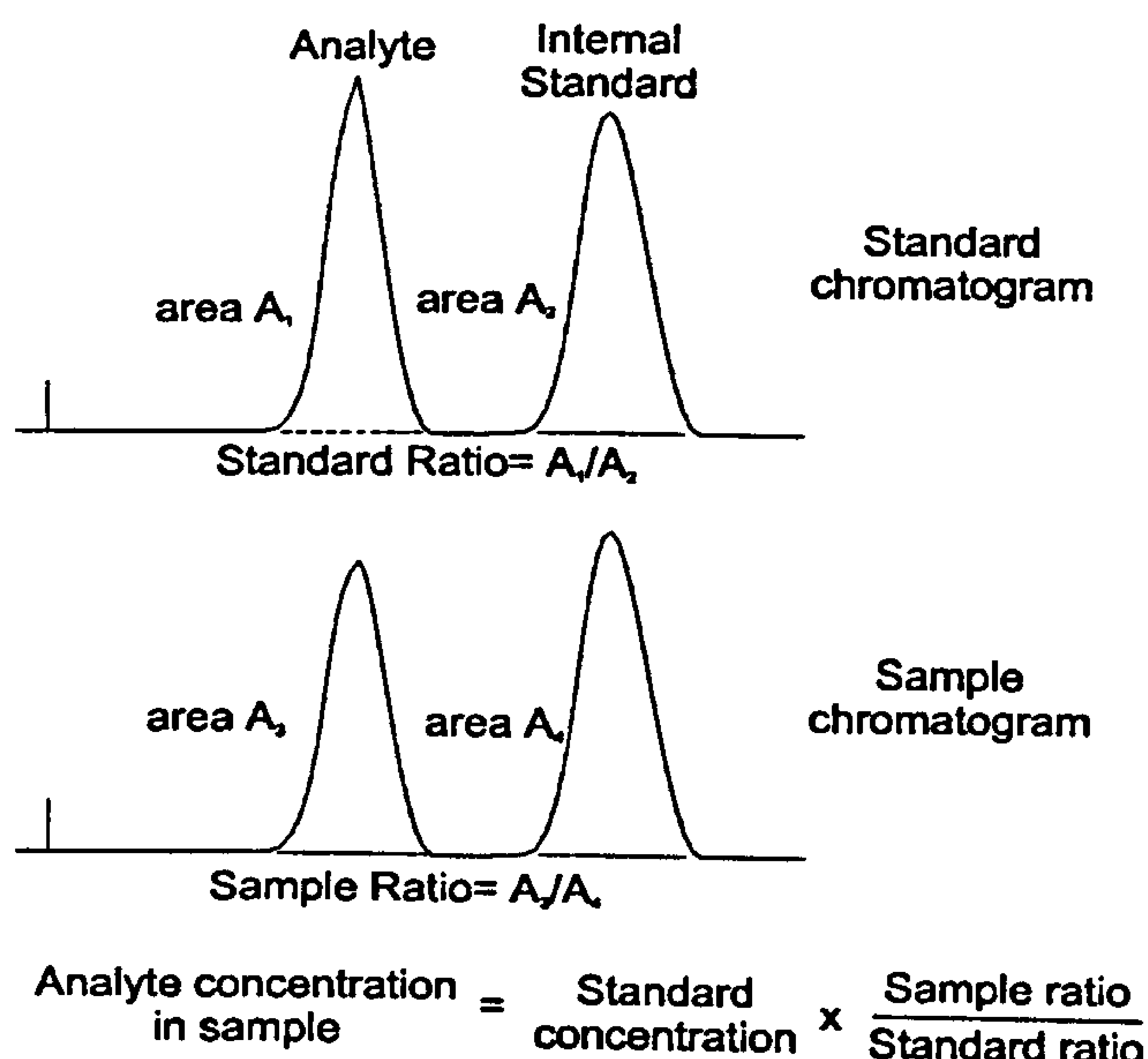


Figure 2-5:- Determination of analyte concentration

The most common detector is the flame ionisation detector (FID) but there are many other detectors such as the mass selective detector (MSD), the nitrogen-phosphorus detector (NPD), the electron capture detector (ECD), the flame photometric detector and the thermal conductivity detector (TCD). A common detector now is the mass spectrometer (MS), which will be explained in more detail in Section 2.4 .[72, 86]

2.3 Gas Chromatography

2.3.1 Introduction

Gas chromatography is one of the most widely used analytical techniques for quantitative analysis.[72, 87] Gases are used in gas chromatography as the mobile phase which transports the sample components through the column to the detector. Selection of the best carrier gas is important, because it affects both the column separation processes and detector performance. The carrier gas which is used should be inert chemically with respect to the column materials and sample components as well as being dry and free of oxygen. The carrier gas should be of high purity because impurities such as oxygen and water can chemically attack the liquid phase in the column and ultimately destroy the column. Gases with the smallest diffusion coefficients such as hydrogen (H_2) and helium (He) which are widely used will give better separation efficiencies than higher molecular weight gases such as nitrogen (N_2), carbon dioxide (CO_2) and argon (Ar).[80, 88] The ratio of viscosity to diffusion coefficient should be a minimum for rapid analysis. The choice of gas is often dictated by the type of detector. Generally, gas chromatography consists of three major components: an injection system, a chromatographic column and a detector.[89-91].

2.3.2 Injection system:

The sample injection port is connected to the top of the column and should be hot enough to vaporize the sample rapidly. The most common introduction techniques are split and splitless injection modes. Split injection can be considered to be the oldest and simplest technique (Figure 2-6). This procedure involves injection of approximately 1µl of the sample into a heated injection port and usually 1-2% of the vapour enters the column with the rest being lost to the waste outlet. There are advantages to split injections as the sample amount introduced to the column is very small, flow rate up to the split point is fast, there is no need to dilute the sample and the samples can be introduced by putting a

plug of deactivated glass wool in the inlet liner to trap non volatile compounds. One disadvantage is that trace analysis is limited.[72]

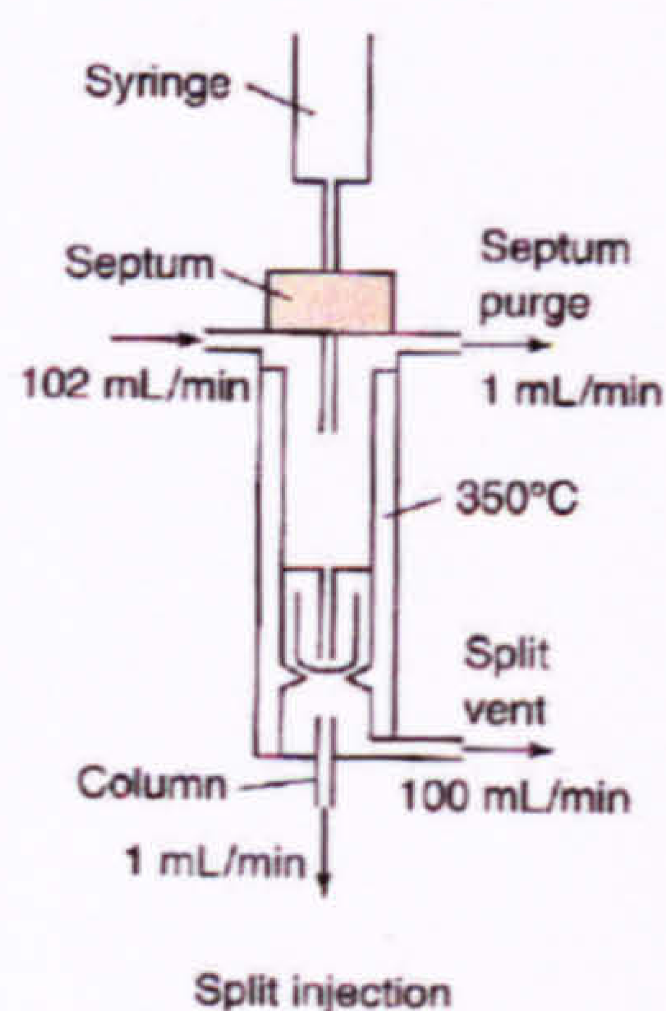


Figure 2-6:- Split Injection Mode.[72]

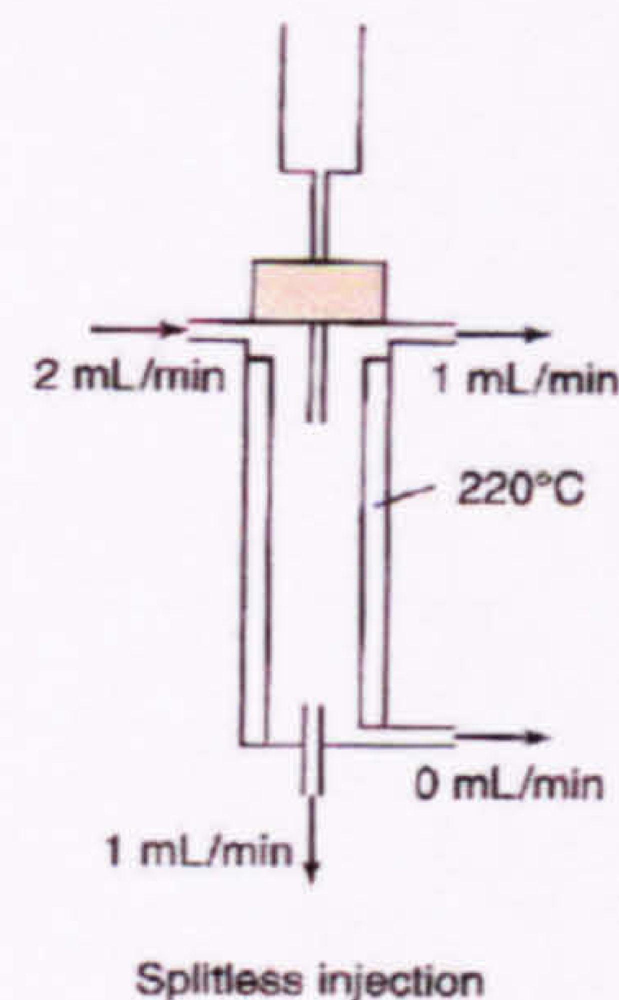


Figure 2-7:- Splitless Injection Mode.[72]

In splitless injection (Figure 2-7), the injector is kept hot enough to vaporize the specimen and solvent, but the column temperature remains below the boiling point of the analyte. It uses the same hardware as split injection, but the split valve is closed when the sample is injected. The sample is diluted with a volatile solvent and 1 to 5µl is injected in the heated injection port. The big advantage of splitless injection is the improved sensitivity over spilt injection and it is commonly used in trace analysis. There are some disadvantages, for Split , it is time-consuming as each chromatographic analysis starts with a cold column and uses a temperature programme, and also the sample must be diluted with a volatile solvent.[72, 80, 92].

2.3.3 GC Column:

Often the columns are coiled to allow housing within a fan-assisted thermostatically controlled oven. Two types of column are currently used in GC. Firstly, capillary columns (open tubular columns, Figure 2-8) which do not contain any packing material and are usually now constructed from fused silica with a polyimide protective coating. Fused silica capillary columns are extremely inert as the column wall is constructed of very high purity silica (<1ppm metals). Resistance to flow is very low therefore long lengths, coiled, up to 100m are possible. Internal diameters are in the range 0.1-0.7 mm, with a very thin layer (0.1-5µm) of a liquid or solid stationary phase coated or chemically bonded to the surface of the inner wall. They can be used with both spilt and splitless injection systems. The sample volume capacity of a narrow bore column is < 0.1µl, and the thinner the

coating the greater the efficiency, but the lower the sample capacity before overloading causes peak tailing and deterioration of resolution. By contrast, megabore columns having an internal diameter of 0.5mm or more can accommodate sample volumes up to 10μl. They are, however, less efficient, but have the highest sample capacity, so special injection techniques are not needed. The preferred carrier gas for capillary columns is helium or hydrogen. There are many advantages of capillary columns, such as they have very high efficiencies and resolving powers for complex mixtures of up to 100 or more components and solutes elute at lower temperatures than with corresponding packed column. However, capillary columns are expensive.[80, 93]

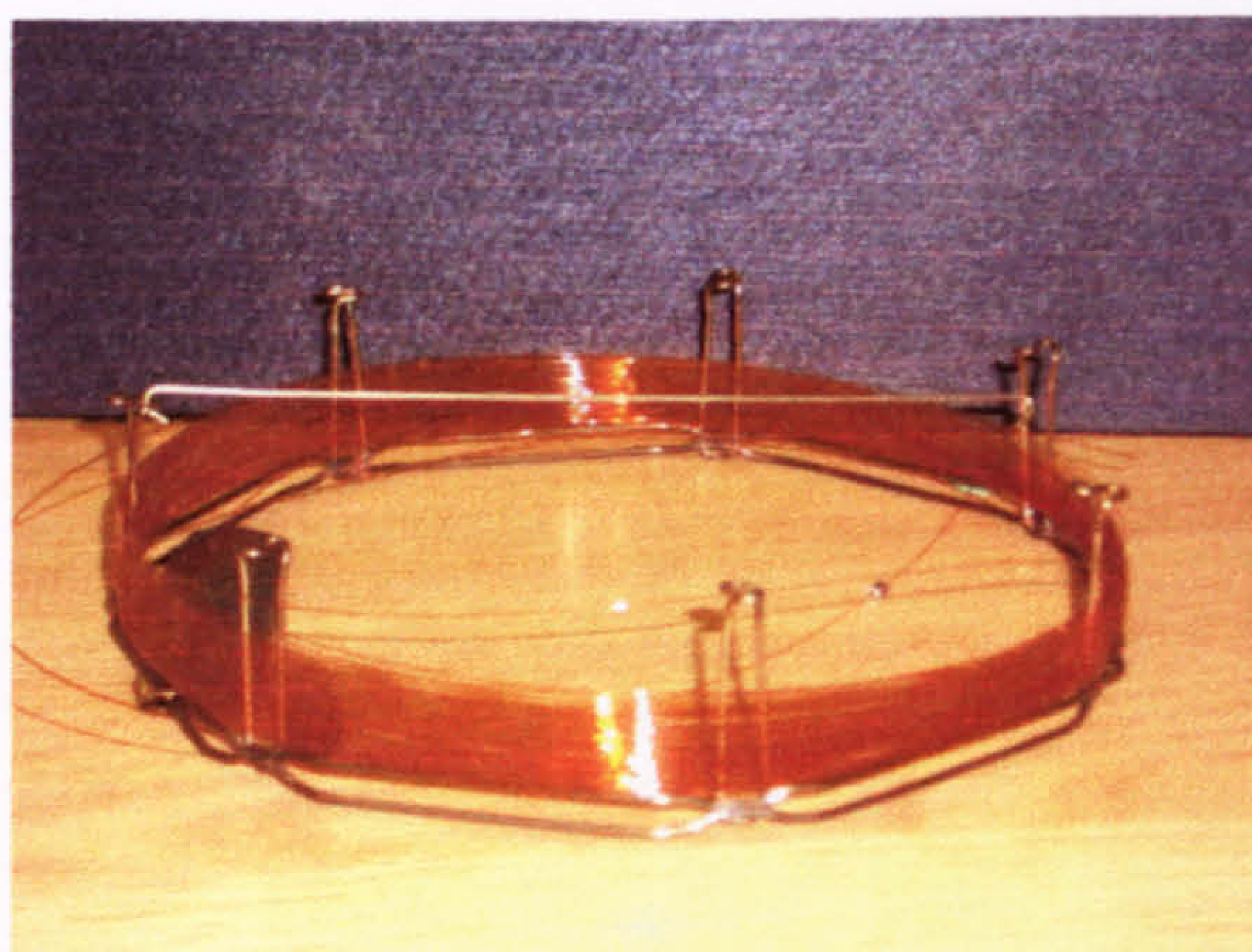


Figure 2-8:- Capillary Column (open tubular)

They can be further subdivided according to the method of supporting the stationary phase (Figure 2-9).[72]

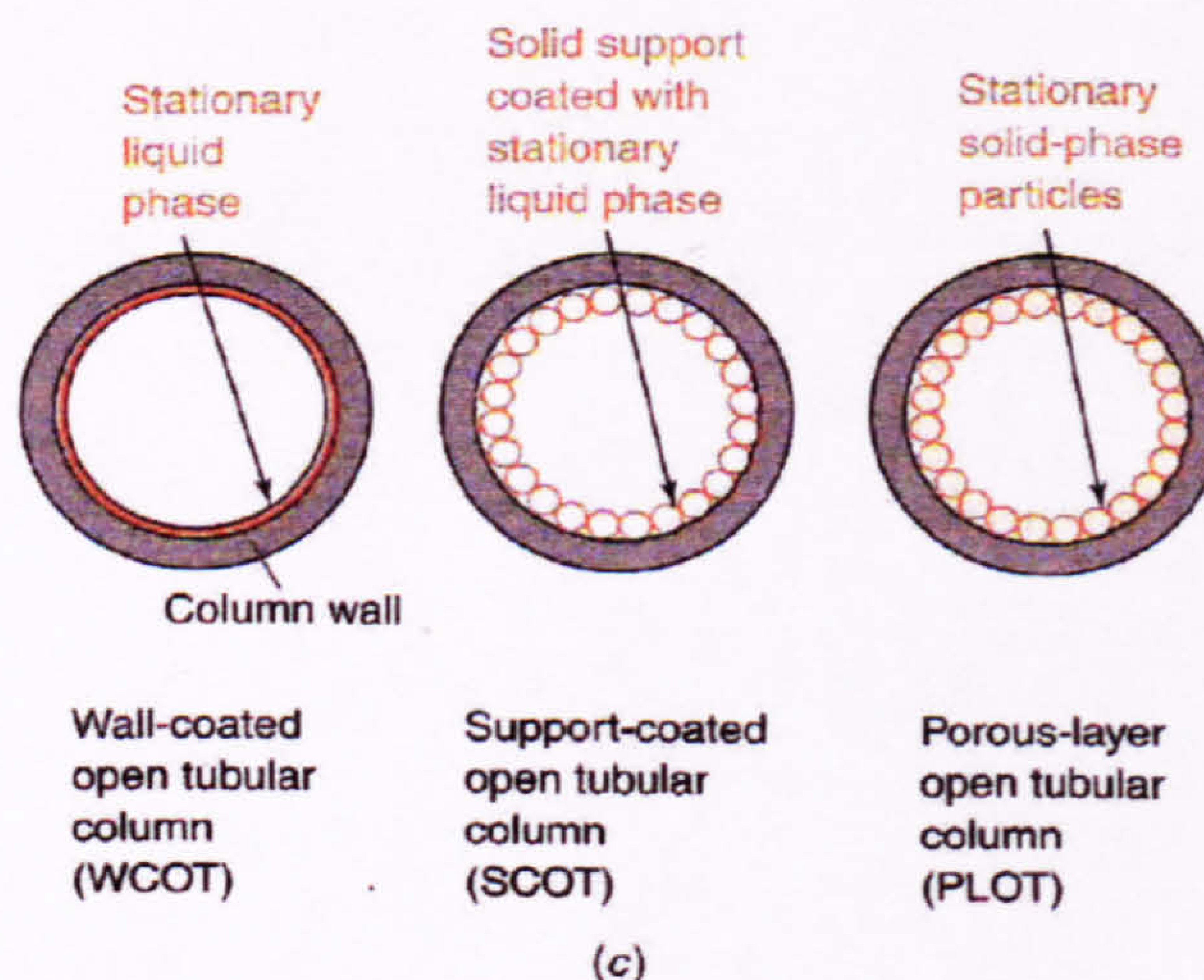


Figure 2-9:- Types of Stationary Phase.[72]

1- Wall coated open tubular (WCOT) columns:

These feature a 0.1-5 μm thick film of stationary liquid on the inner wall of the column and they provide the highest resolution of all gas chromatographic columns.[72] Tubing internal diameters commonly manufactured commercially are 0.1, 0.2, 0.3, 0.32 and 0.53mm. Lengths typically vary from 10 to 50 meters. Thin films provide high resolution and fast analysis, but they have limited sample capacity. Thicker films have higher sample capacity but show lower resolution and are typically used for only very volatile compounds.They also having the advantage that direct injection techniques without splitting may be used.[80]

2- Support coated open-tubular (SCOT) columns:

This type of column has solid particles coated with the stationary liquid phase and attached to the inner wall. SCOT columns can hold more liquid phase and have a higher sample capacity than the thin films common to the early WCOT columns but SCOT columns are less efficient. SCOT columns are not available in fused silica.[80]

3- Porous layer open-tubular (PLOT) columns:

These contain a porous layer of a solid adsorbent such as alumina or molecular sieve. PLOT columns are well suited for the analysis of light fixed gases and other volatile compounds. PLOT columns represent a small proportion (<5%) of capillary columns in use.[80]

2.4 Mass Spectrometry

2.4.1 Introduction

The general method of operation of a mass spectrometer is to create ions under vacuum then separate the ions based on their mass-to-charge (m/z) ratios and measure the quantity of ions of each mass-to-charge ratio.[94] In 1919 Francis Aston produced the first mass spectrometer as an instrument for determining the mass of an atom. He examined positive ions from neon and found that there were two types of ion present, with relative isotopic masses of 20 and 21. He had found a way of separating and identifying isotopes. Since

then, the method has been developed so that it is now a very valuable tool for analysing atoms and molecules of gases, liquids and solids (Figure 2-10).

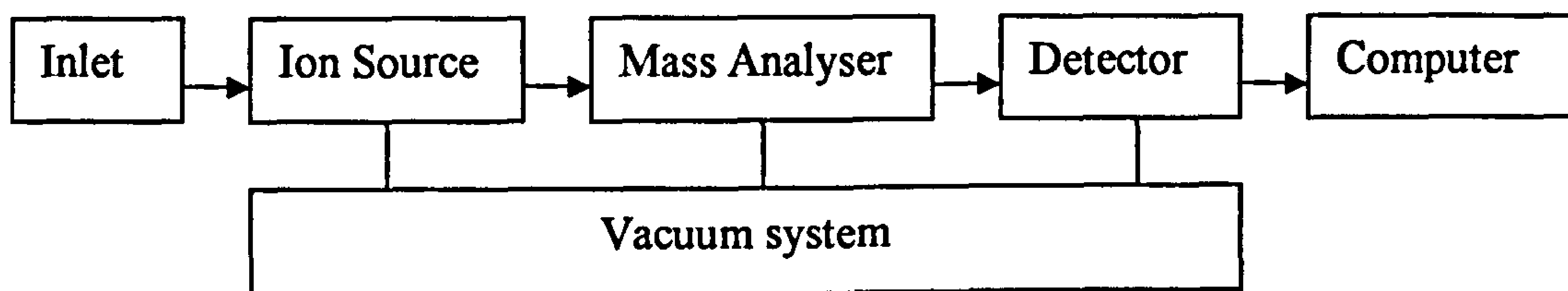


Figure 2-10:- Components of a mass spectrometer

Mass spectrometry (MS) is a technique in which materials are ionized and dissociated into fragments characteristic of the molecules or elements present in the sample. The numbers of ions at each m/z value provide information for qualitative and quantitative analysis. It is considered to be the most powerful detector for gas chromatography because the spectrometer is sensitive to low concentrations of analyte.[80]

2.4.2 Sample inlet:

When the sample is not a gas or a volatile liquid, it has to be heated to produce a vapour before it can be introduced into the mass spectrometer. The exceptional sensitivity of the instrument means that only picograms of sample are required.

2.4.3 Ion Source:

Once inside, the vaporized sample is ionized by bombardment with high energy electrons. The bombarding electrons scatter electrons from the atoms or molecules in the sample, creating positively charged ions (Figure 2-11, Figure 2-12, Figure 2-13):

These positive ions are expelled from the ion source into the mass analyser by the ion repeller plate, which is another metal plate carrying a slight positive charge.[95] There are two common ionization methods used in GC-MS.

2.4.3.1 Electron Impact (EI) ionization

The EI mass spectrum is obtained by the impact of a beam of electrons, usually generated from a tungsten filament, with the sample molecules in the gas phase, under vacuum. It is the oldest and best-characterized of all the ionization methods and it is usually considered to be a "hard" ionization method as sufficient energy is transmitted to the sample molecules to produce fragmentation in addition to simple ionisation. It can be used for GC-MS systems and direct inlet techniques. EI is best suited to relatively non-polar, volatile samples. An EI mass spectrum usually contains the molecular ion, $M^{+\cdot}$, and many fragment ions, making EI useful for structure characterization. A beam of electrons passes through the gas-phase sample. An electron that collides with a neutral analyte molecule gives a positively charged radical ion $M^{+\cdot}$. The ionization process can produce a molecular ion, which will have the same molecular weight and elemental composition as the starting molecule. The ionization potential is the energy required to produce a molecular ion. Most mass spectrometers use electrons with energy of 70 electron volts for EI, which is higher than the ionisation potential for most atoms or molecules and which results in the formation of energetic molecular ions containing enough excess energy to break internal bonds within the molecule and produce fragment ions. Decreasing the electron energy can reduce fragmentation but it also reduces the number of ions formed. The useful mass range for EI is generally up to 1,000 Daltons.[93, 96]

2.4.3.2 Chemical Ionization (CI)

This is known as a "soft" ionization method because the analyte is ionized without the transfer of excessive energy to the resultant ions and few fragments are produced. CI uses ion-molecule reactions to produce ions from the analyte and often a CI mass spectrum contains a protonated molecule, $[M+H]^+$, referred to as a pseudo-molecular ion. The chemical ionization uses a reagent gas such as methane, isobutane or ammonia at a relatively high pressure, which is ionized first by electron impact because of the large number of molecules of the gas which are present in the source.The high reagent gas pressure results in ion-molecule reactions between the reagent gas ions and uncharged reagent gas molecules. Some of the products of these ion-molecule reactions can react with the analyte molecules to produce analyte ions. The results depend on reagent gas type, reagent gas pressure or reaction time, and the nature of the sample. Because less fragmentation occurs in CI than with EI ionisation, a fragment pattern is obtained which is

not informative or reproducible enough for a library search. The mass range for CI is up to 1,000 Daltons. CI is better suited than EI to more polar compounds. The sample is heated to generate gas phase molecules which undergo proton transfer reactions with the CI gas.[93, 97]

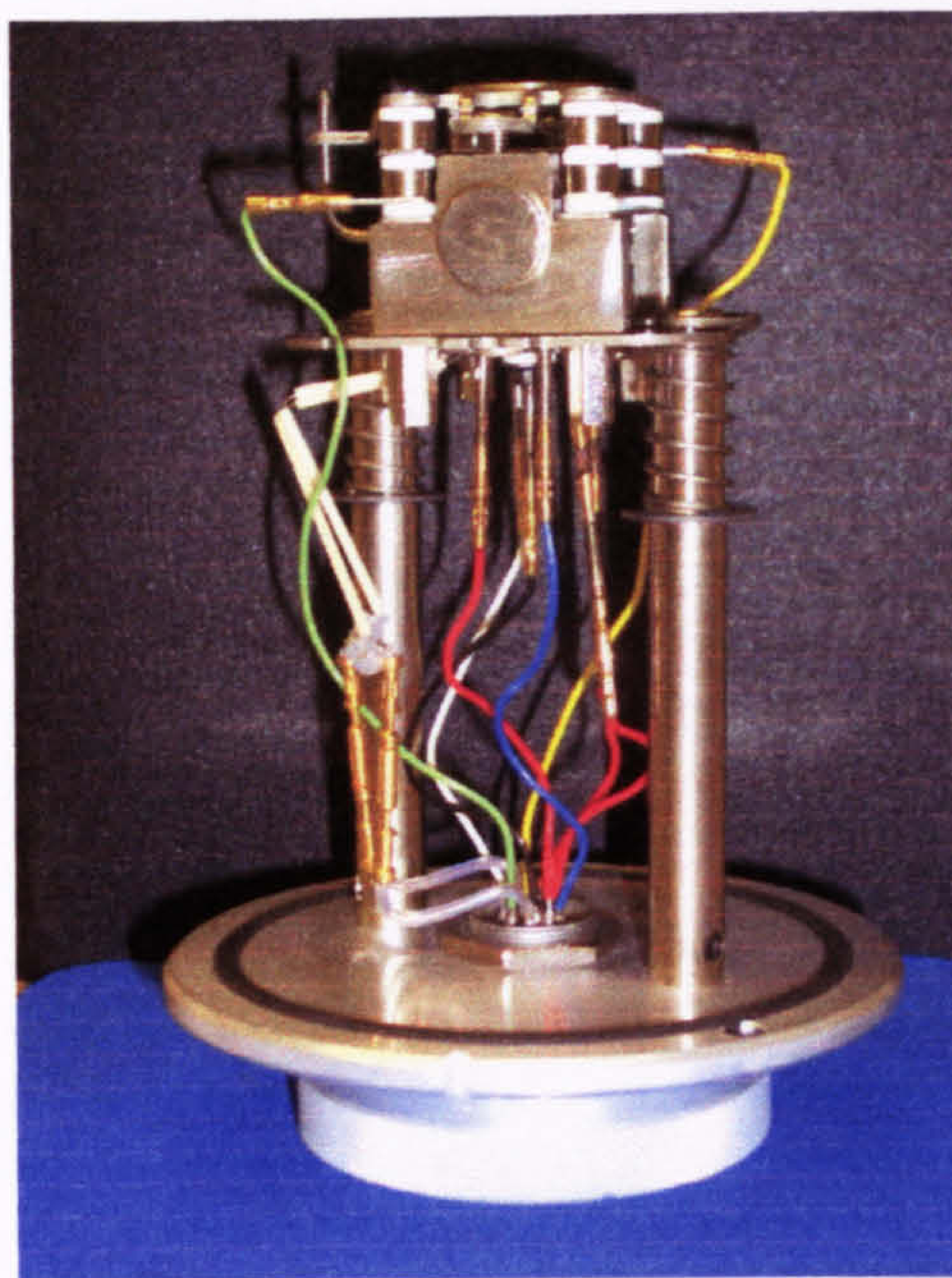


Figure 2-11:-Ion source (electron impact)

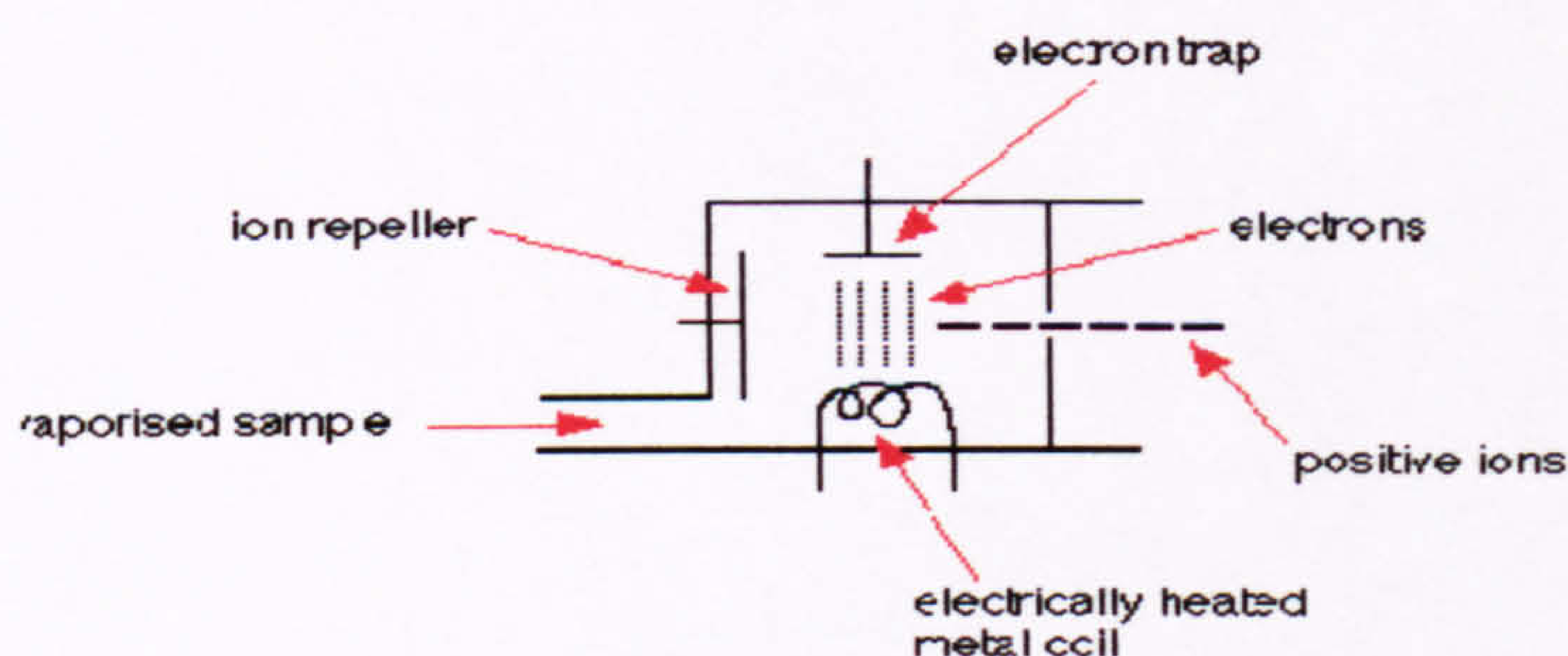


Figure 2-12:-A plan view of the ion source

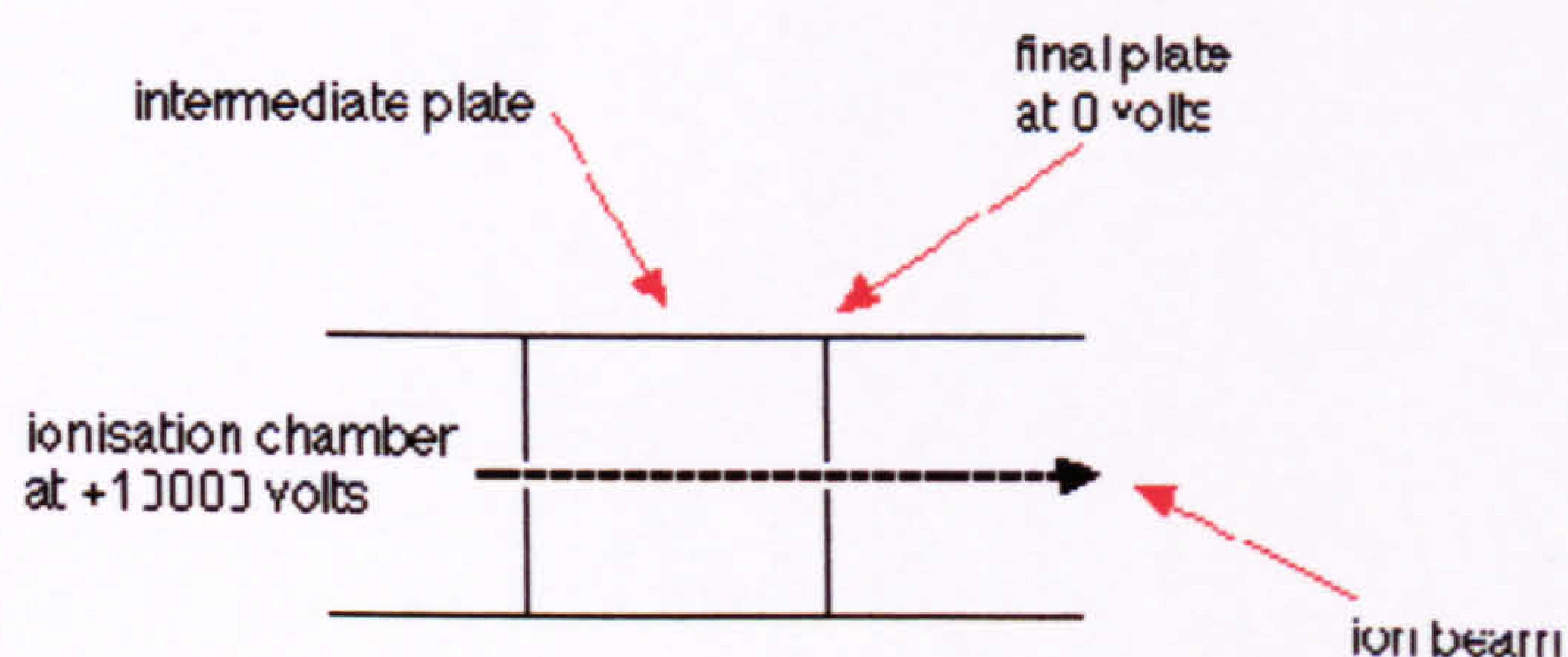


Figure 2-13:-Acceleration of ions from the ion source

2.4.4 Mass analyser:

The original method of separating ions according to their mass-to-charge ratios used a magnetic field or a combination of magnetic and electrostatic fields. More recently, alternative methods have become more common and these include the quadrupole, time of flight and ion trap mass analysers. The work carried out for this thesis used a quadrupole instrument.

In the original magnetic sector mass spectrometer, the positive ions formed are accelerated by an electric field of several thousand volts. The ions then enter a strong magnetic field, which deflects them by different amounts. The amount of deflection depends on firstly the mass of the ion - meaning lighter ions are deflected more than heavier ones, and secondly the charge on the ion - meaning ions with two or more positive charges are deflected more than ions with only one positive charge. These two factors are combined into the mass/charge ratio (m/z) (Figure 2-14).

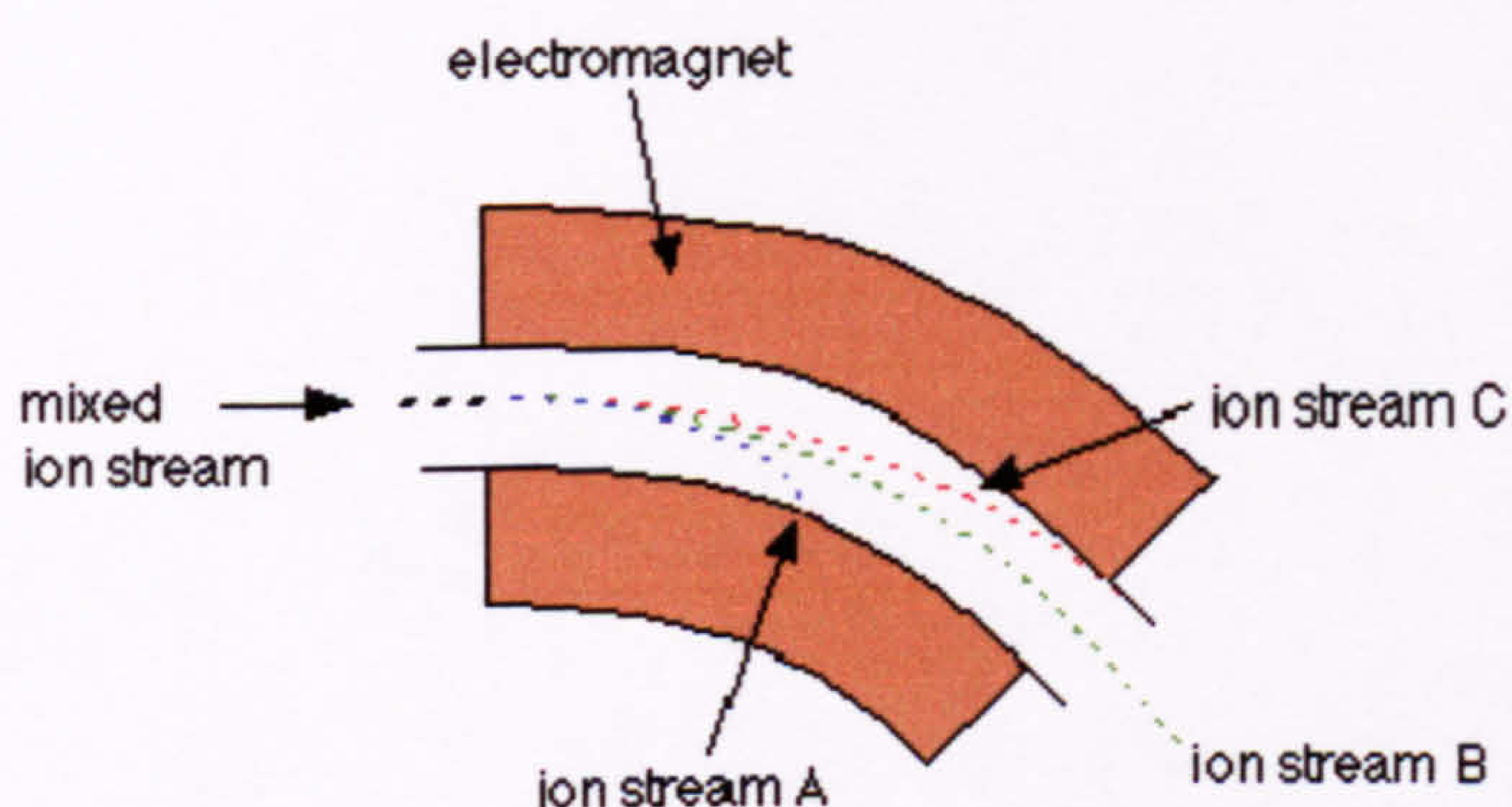


Figure 2-14:- Magnetic sector analyser

When the magnetic field is correct for a particular m/z value, ions with this m/z ratio are deflected to the collector and recorded. By continuously varying ("sweeping") the magnetic field, each separated positive ion beam can be focused on the collector in sequential order of mass/charge ratio, to build up the mass spectrum.

The quadrupole mass analyser is based on a quadrupole field created between four electrodes (the quadrupoles). Ions transit the quadrupole field at a velocity dependent on their mass, since each ion gains the same momentum during acceleration from the ion source. To enable the ions to transit the quadrupole region, the quadrupole field is switched in an alternating manner (at approximately radio frequency), otherwise the ions would simply collide with the quadrupole of opposite charge. The mass spectrum is recorded for

the desired range of m/z values by sweeping through a range of frequencies in the alternating quadrupole field. Major advantages of this type of analyser over the conventional magnetic sector include their smaller size, lower cost, lower operating voltages and ease of computer control.

The time of flight mass analyser has recently come to the fore in the field of proteomics, for the analysis of peptides. As mentioned earlier, ions leave the ion source at velocities dependent on their masses, i.e. they are travelling at different speeds. It follows that the time taken for them to reach the detector, if they travel in a straight line, can be used to measure the m/z ratio. Literally this is the time of flight between source and detector. The start time of the flight must be precisely known also and this is obtained by using a pulsed ionisation process, usually involving a laser.

2.4.5 Detector:

Ions impinging on a detector surface contain a significant amount of energy and this is used to generate a more easily recorded material, usually electrons or photons, which are detected using an electron- or photo-multiplier. Both of these multipliers can enormously amplify the signal created by the ions themselves. This amplified signal is converted into digital form with a high frequency analogue-to-digital converter and relayed to the computer data acquisition system.

3 Drugs of Abuse: Pharmacokinetics and Drug Metabolism

3.1 Introduction

Model compounds were chosen for this study from amongst the major groups of abused drugs, including the amphetamines, cocaine, opioids and cannabinoids. The following sections review some basic concepts in pharmacology and some background information on these drug groups.

3.2 Introduction to some pharmacological concepts

Generally, the toxicological study of chemicals or drugs requires a basic knowledge of how they affect the biological system (pharmacodynamics) and how the biological system absorbs and disposes of them (pharmacokinetics). The field of pharmacodynamics is one of the most important in pharmacology science which involves studying the behaviour of substances in the blood, whether the effects are desired effects or toxic side-effects.[98-102]

The people who take drugs may experience changes in mood and emotion, increase in body temperature, muscle tension, teeth clenching, nausea, anxiety, rapid eye movement, chills, sweating, faintness and blurred vision. However, the most serious health risks are depression, sleep problems, increase in heart rate and blood pressure, brain damage, muscle breakdown, liver damage, kidney failure, heart failure and psychological problems. People who need drug treatment have become addicted to drugs and have lost the ability to choose between using and not using the substance. Drug use becomes the only way that they feel normal. This is a very involved process, and we do not know exactly what each drug does. For example, alcohol is a drug and one of its effects is to dehydrate protoplasm. This prevents the cell wall from operating properly. This happens in every cell in the body, but it has its most noticeable effect on the central nervous system where it suppresses the higher cortical centres in the brain. This reduces a subject's normal ability to perceive the environment. There are many drugs of abuse but the more important ones will be discussed below as these were the model compounds used in this study.[103, 104] There are also terms which are very important for pharmacokinetics such as those described in the following paragraphs.

Pharmacokinetics includes *absorption* of the substances into the body, which describes how are the drugs absorbed through the skin, the intestine and the oral mucosa, *distribution*, which describes how the substances spread through the organism and *elimination*, which describes how the substances are metabolised and excreted (Figure 3-1).

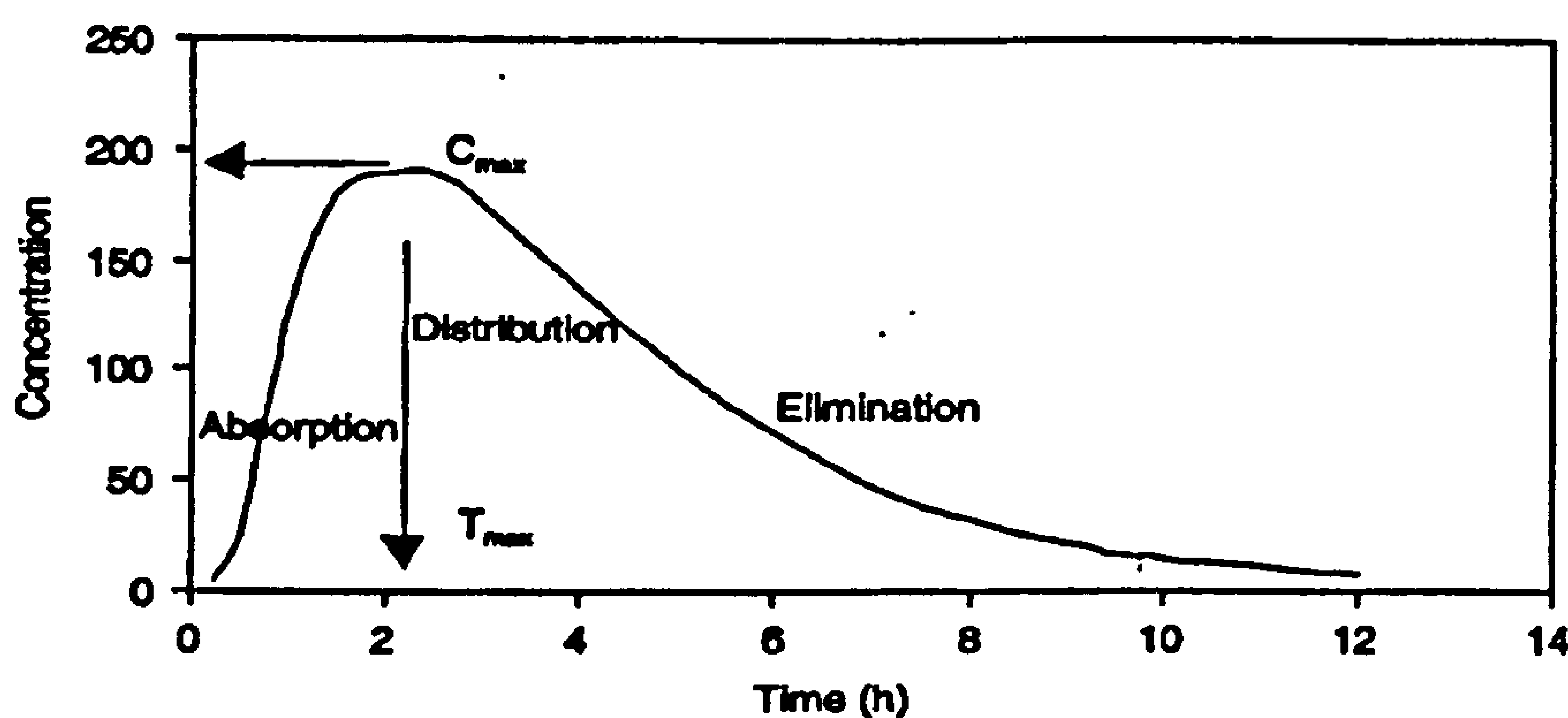


Figure 3-1:- Pharmacokinetic characteristics of an orally absorbed drug.[105]

Drugs can enter the body by several different routes including orally - by mouth, injection, or inhalation and by skin absorption. The transport of drugs across the plasma membrane of cells is one of the most important factors involved in delivery of drugs or other substances to the target organ. The cell is the basic building block of the body tissues. Each cell has a wall that protects the cell from harm, a nucleus which is the core of the cell and it also has a variety of other specialized parts with specialized functions called organelles. The nucleus contains deoxyribonucleic acid or DNA which determines how the cell is made and how it works. Drugs enter cells through the cell membrane, which is a phospholipids bilayer that is non-polar and so is hydrophobic. Most importantly, for the effective use of most drugs, non-polar molecules can cross cell membranes but less lipid soluble (lipophobic) molecules do not penetrate the lipid membrane and must enter cells by a different route, either by active transport or diffusion through specialised channels.

When a weakly acidic or basic drug is administered to the body, the drug will ionise to a greater or lesser extent depending on the pK_a and the pH of the body fluid in which it is dissolved.[106] Therefore the degree of ionization of a drug is most often determined by the relationship between its pK_a and the pH of its environment (as demonstrated in Equation 3-1 below). The pH of the body varies widely but the most important biological solution is the blood, which normally has a pH of 7.4.The relationship between pK_a and the ratio of

acid–base concentration to pH is expressed by the Henderson-Hasselbach equation (Equations 3-2 and 3-3 as shown below).[106-110]

$$pH = pKa - \log \frac{non-protonated\ species}{protonated\ species}$$ Equation 3-1

For acids: pH = pKa + log [ionized]/ [un-ionized] Equation 3-2

For bases : pH =pKa + log [un-ionized]/ [ionized] Equation 3-3

This equation is useful in determining how much drug will be found on either side of a membrane that separates two compartments that differ in pH. [101, 111] Examples of this include the absorption of drugs through the gastric mucosa (actually a series of membranes and layers) into blood. The pH of gastric juice is acidic (typically pH 3) and so basic drugs will be ionised (protonated) and therefore will be poorly absorbed from the stomach. By contrast, acidic drugs such as aspirin or ibuprofen will be largely non-ionised and will be more readily absorbed through the gastric mucosa. However, this situation is altered if an antacid is also administered, which increases the gastric pH. If the pH is increased by one unit, the percentage of a basic drug which is ionised will decrease by a factor of 10, which will affect its absorption from the stomach. Another common example is the effect of urine pH on the excretion of drugs. Basic drugs are more rapidly excreted through the kidney when the urine pH is acidic than when it is basic.

3.2.1 Drug metabolism

Drugs and other substances are usually metabolized by phase one reactions, which involve oxidation, reduction and hydrolysis processes which modify the molecule by changing the nature of a functional group or by adding a functional group.These can involve hydroxylation, oxidation and dealkylation routes of metabolism and may be considered degradation reactions. Phase two metabolism involves the production of compounds (conjugates) and may be considered conjugation reactions. Conjugation reactions often involve the addition of sulphuric acid or glucuronic acid to an existing functional group forming sulphates and glucuronides. However, other acids may be involved such as glycine

and glutamine conjugates.[105, 112] Phase two metabolism produces substances which are easily eliminated from the body through the kidney into the urine (Figure 3-2, and Figure 3-3).

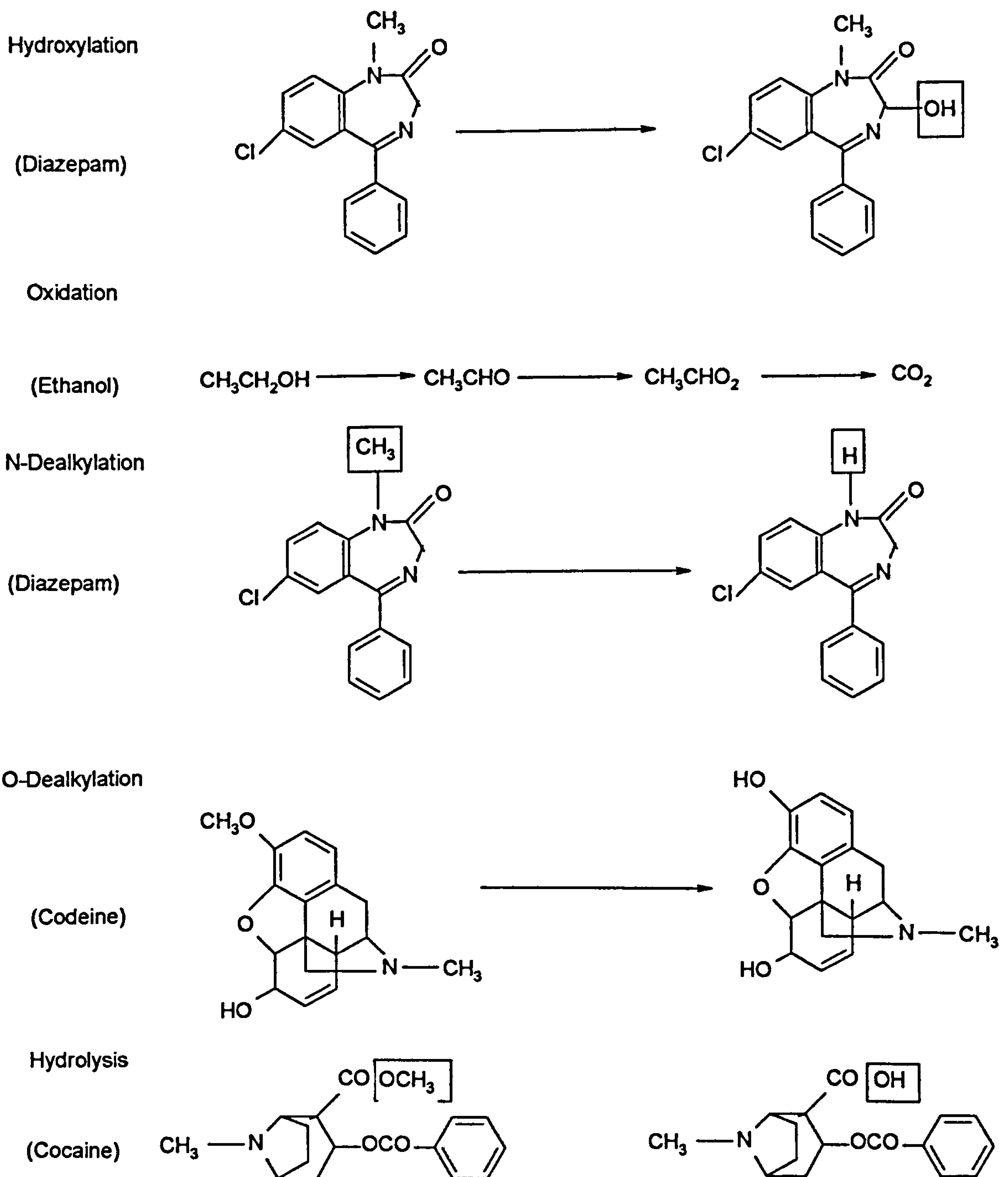
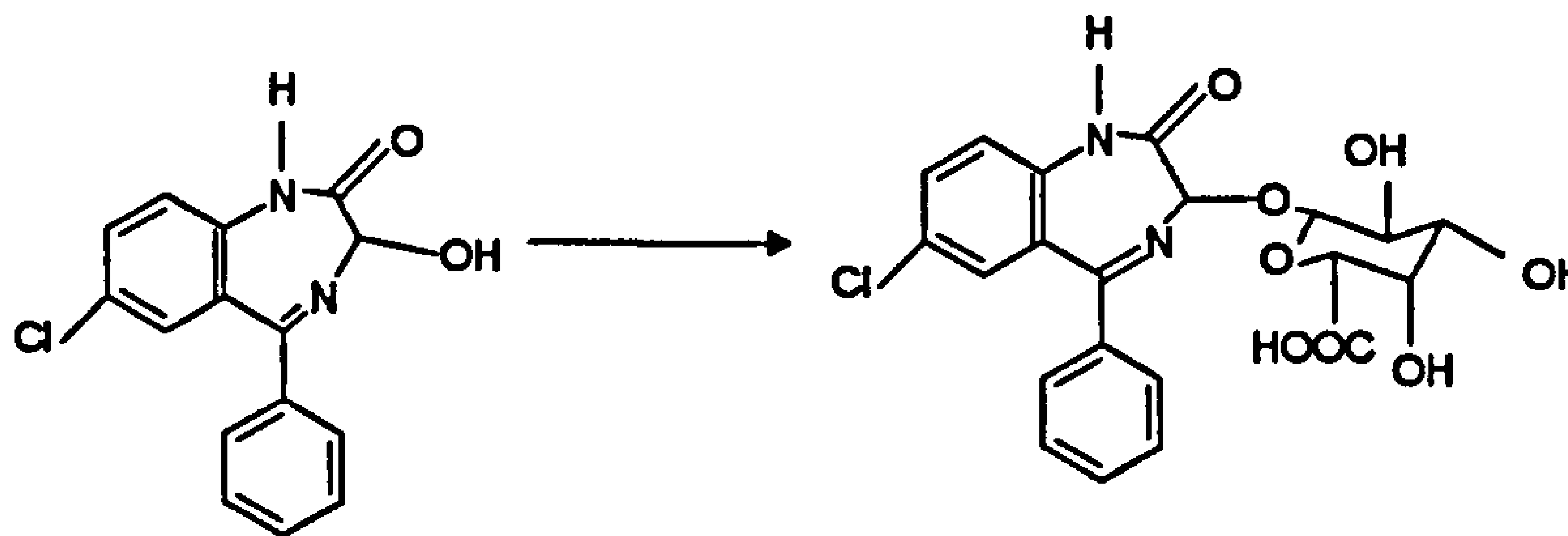


Figure 3-2:- Phase 1 metabolic pathways[105]

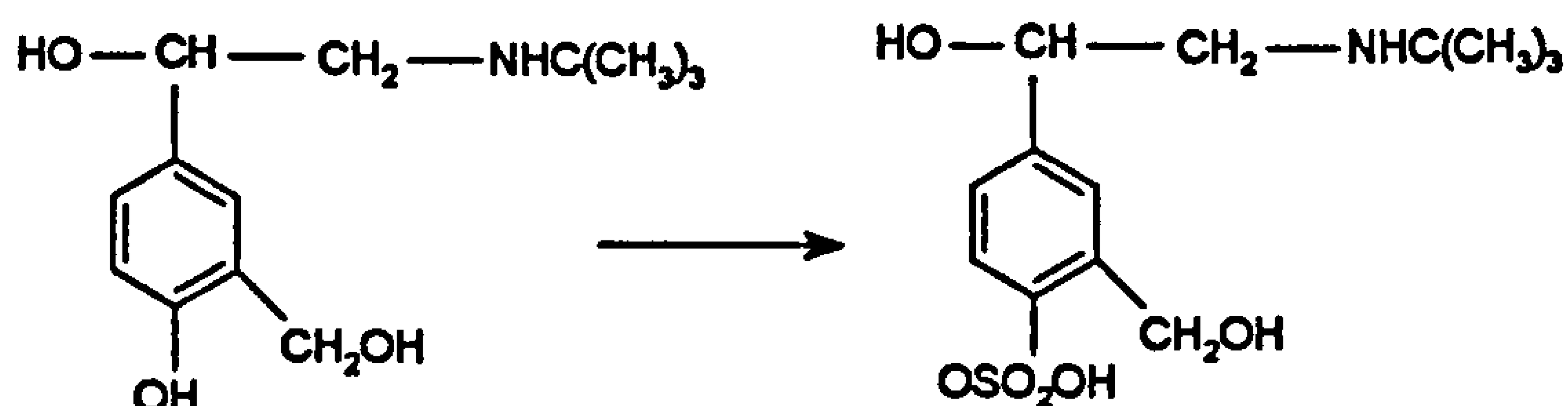
Glucuronidation

(Oxazepam)



Sulphation

(Salbutamol)



Acetylation

(Procainamide)

Methylation

(Captopril)

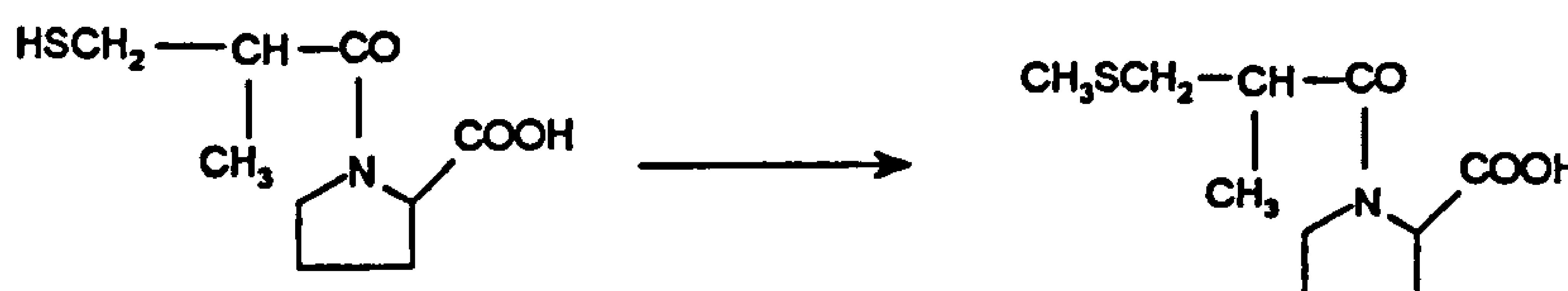


Figure 3-3:- Phase 2 conjugation reactions[105]

3.2.2 Clearance

Clearance is a term that defines the ability of the body to remove a drug from one part of the body e.g. kidneys, liver.[105] the most common definitions are the two expressions shown below in Equations 3-4 and 3-5:

$$CL = dose / AUC$$ **Equation 3-4[105]**

$$CL = K_0 / Css$$ **Equation 3-5[105]**

Where CL is the clearance in ml/min, dose is the actual dose absorbed by the body, AUC is the area under the plasma drug concentration versus time curve, K_0 is the infusion rate of the drug in a controlled clinical setting, delivering the drug intravenously, and Css is the steady state plasma concentration of the drug under these controlled conditions.

Clearance will vary depending on the physiological state of the organs such that organ disease and organ damage may reduce clearance.[105] The concept of clearance is also extremely useful in clinical pharmacokinetics because clearance of a given drug is usually constant over the range of concentrations encountered clinically. This is true because systems for elimination of drugs are not usually saturated and the absolute rate of elimination of the drug is essentially a linear function of its concentration in plasma.[113, 114]

3.2.3 Plasma half - life ($T_{½}$):

This is the most commonly used pharmacokinetic term in court.[105] It defines the ability of the body to eliminate a drug from circulation in plasma and reflects how often a drug needs to be administered. The plasma half - life defines the time required for the body to remove 50% of the drug from plasma after absorption and distribution are complete.The half - life is usually in hours, although some drugs have a half - life of minutes or weeks.The half - life is calculated from the slope of the terminal region of the plasma drug concentration versus time curve from Equation 3-6:

$$\text{Half - life} = 0.693 / \beta \qquad \text{Equation 3-6}$$

Where β is the slope of the terminal part of the plasma concentration versus time curve. The half - life of a drug is dependent on its volume of distribution and its clearance.[105] The half – life is useful in estimating the concentration of drug at an earlier time by back calculation, for example, a drug with a half -life of 12 h and a blood concentration of 2.0 mg/L will be expected to have a concentration of 1.0 mg /L 12 h later. This will reduce to 0.5 mg/L at 24 h and 0.125 mg/L after two days.

3.3 Amphetamine type stimulants

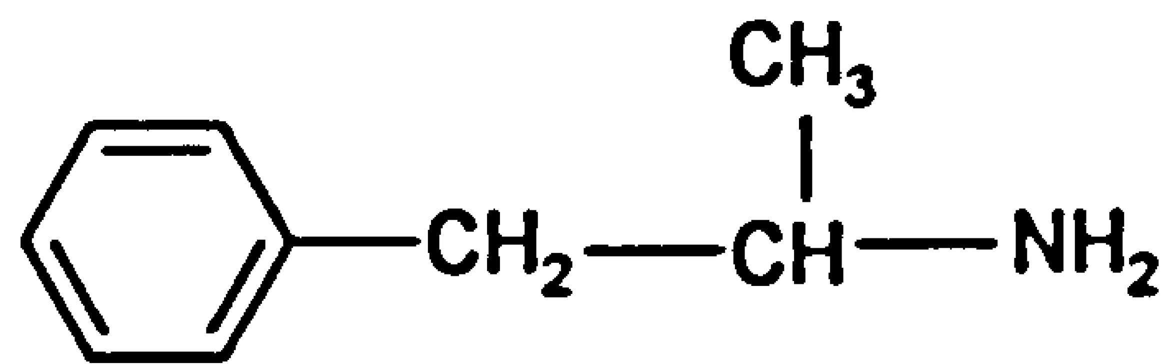


Figure 3-4:- Chemical structure of amphetamine ($C_9H_{13}N$, MW 135, pK_a 9.9).

Amphetamine (Figure 3-4) is used as a central nervous system stimulant in the treatment of certain conditions, such as attention deficit hyperactivity disorder, depression and narcolepsy and is abused illegally as a stimulant. It is a synthetic phenethylamine derivative available since 1935[115-117]. It appears as crystal, chunks and fine to coarse powders, off - white to yellow in colour, and is supplied loose or in capsules and tablets of various sizes and colours. The drug may be sniffed, smoked, injected or taken orally in tablet or capsule form. It is also a metabolite of a number of other drugs, including fenethylline, fenproporex and methamphetamine. The half-life of Amphetamine varies from a few hours to more than day depending on the value of the urine pH, where the half-life is normally 5-8 hours but decreases to 3-6 hours with low urine pH and increases to 9-16 hours with high urine pH.[105] Amphetamine is largely excreted unchanged but a percentage is also inactivated during metabolism, being deaminated to phenylacetone which is subsequently oxidized to benzoic acid and excreted as conjugates (Figure 3-5). However, a small amount is converted by oxidation to norephedrine and this compound and its parent are hydroxylated on the benzene ring in the *para* position. Following a single oral dose of 10 mg of *d*-amphetamine sulphate in a 66 kg adult, the blood concentration reached a peak of about 0.035 mg/L at 2 hours and declined with a half- life of 11-13 hours. Urine amphetamine concentrations are usually less than 4.5 mg/L in adults given a single 20 mg oral dose, while a patient taking 30 mg daily on a chronic basis had urine concentrations of 1.1-18 mg/L and amphetamine abusers often have urine concentrations in the range of 10-100 mg/L[117-120].

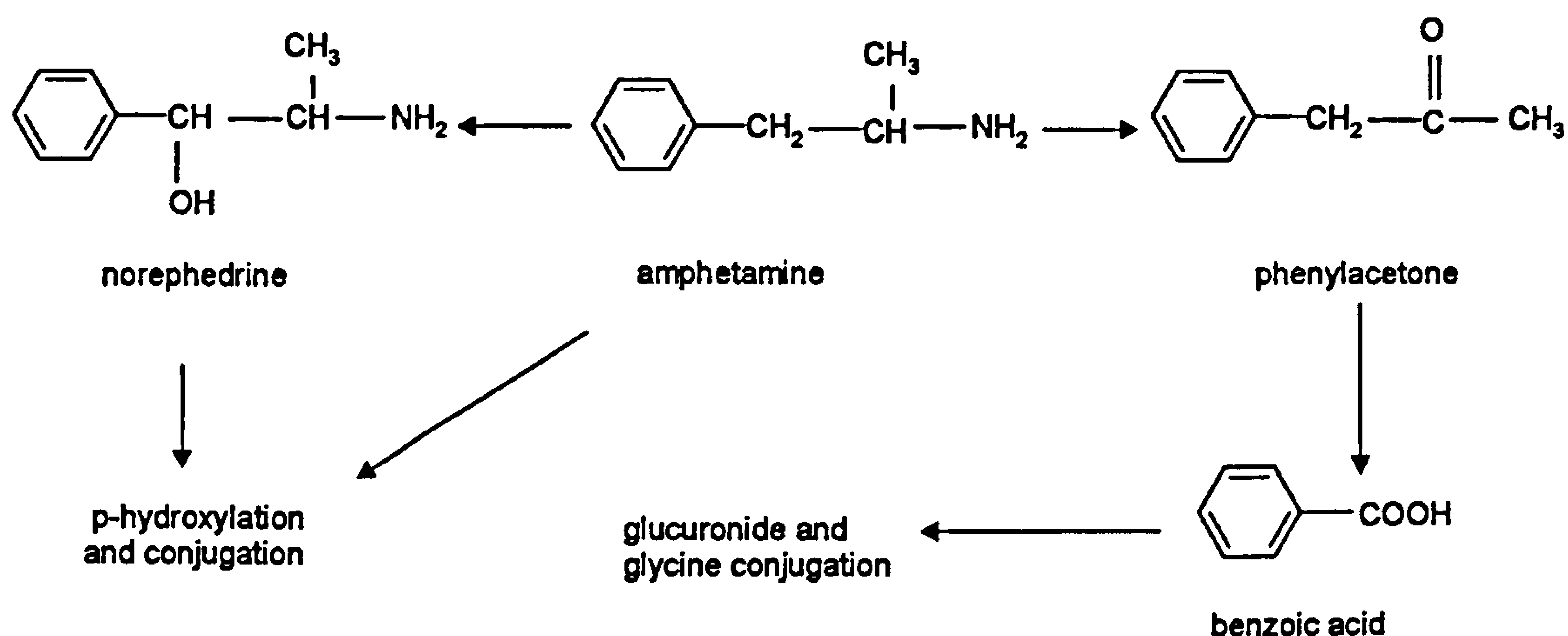


Figure 3-5:- Metabolic pathways for Amphetamine[118]

3.4 Methamphetamine

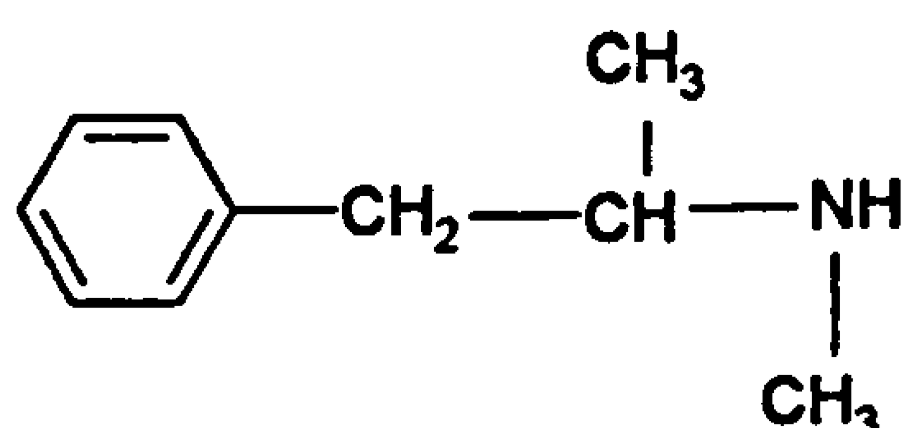


Figure 3-6:- Chemical structure of methamphetamine ($C_{10}H_{15}N$, MW 149, pK_a 10).

Methamphetamine (Figure 3-6) is an alkaloid similar in structure to amphetamine but with an N-methyl substituent. It was first synthesized in 1919 and is a powerful central nervous system stimulant. This stimulant can be swallowed, snorted, smoked or dissolved in water and injected. Generally it affects heart rate, body temperature, blood pressure, appetite, attention and mood. Methamphetamine users feel a short yet intense rush when the drug is initially administered. Methamphetamine hydrochloride is a salt commonly known as meth or speed. The smokeable form of the drug is called LA or, because of its clear, chunky crystals which resemble frozen water, ice, crystal, 64 glasses or quartz. A single oral methamphetamine dose of 0.125 mg/Kg (8.75 mg/70 Kg) given to 6 adults produced an average peak plasma concentration of 0.020 mg/L at 3.6 hours. The plasma elimination half-life averaged 10 hours (range 6-15 hours) for 24 subjects. Methamphetamine undergoes some N-demethylation to amphetamine, its major active metabolite (Figure 3-7). During normal physiological conditions, up to 45% of a dose is eliminated unchanged in the 24 hour urine, and about 4-7 % is excreted as amphetamine. In acidic urine, up to

76% is found in urine as unchanged drug and 7% as amphetamine in 24 hours, whereas in alkaline urine the corresponding values are 2% and less than 0.1% respectively.[62, 121, 122]

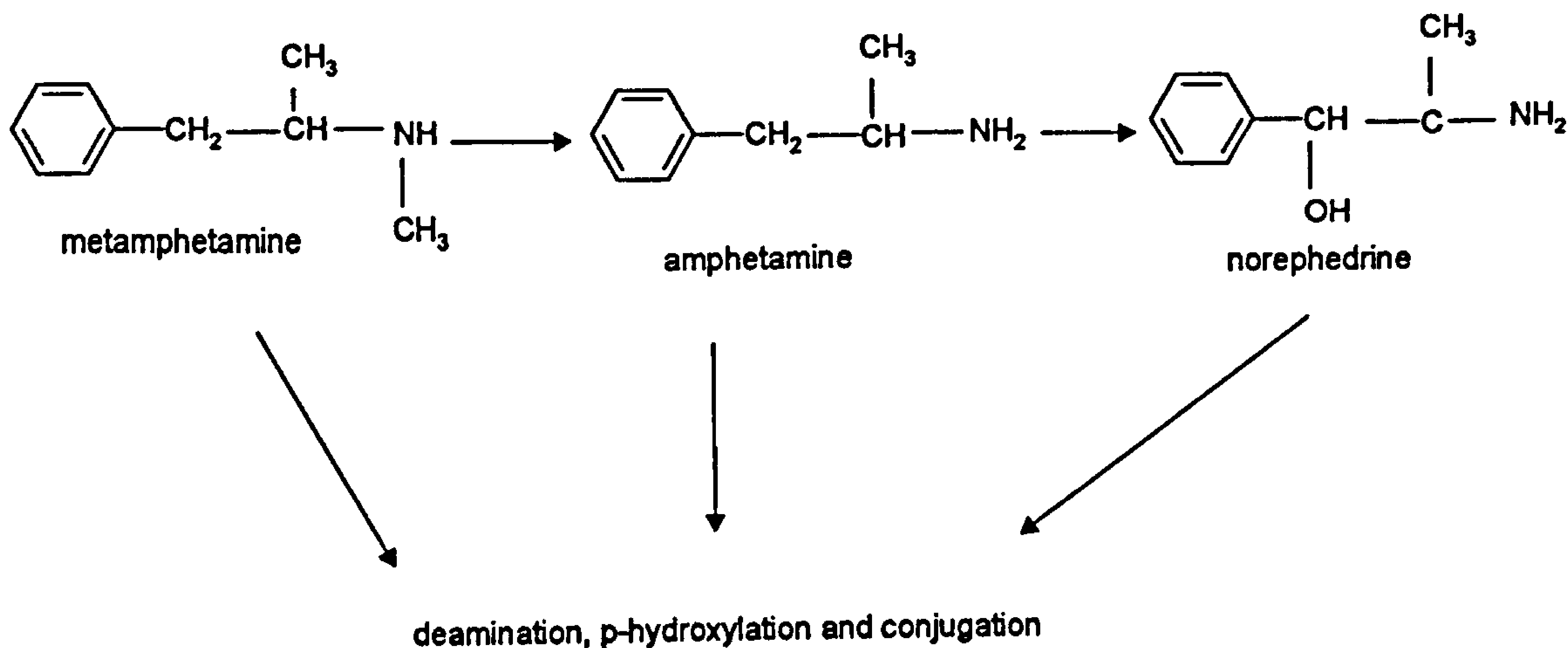


Figure 3-7:- Metabolic pathways for Methamphetamine[118]

3.5 Cocaine

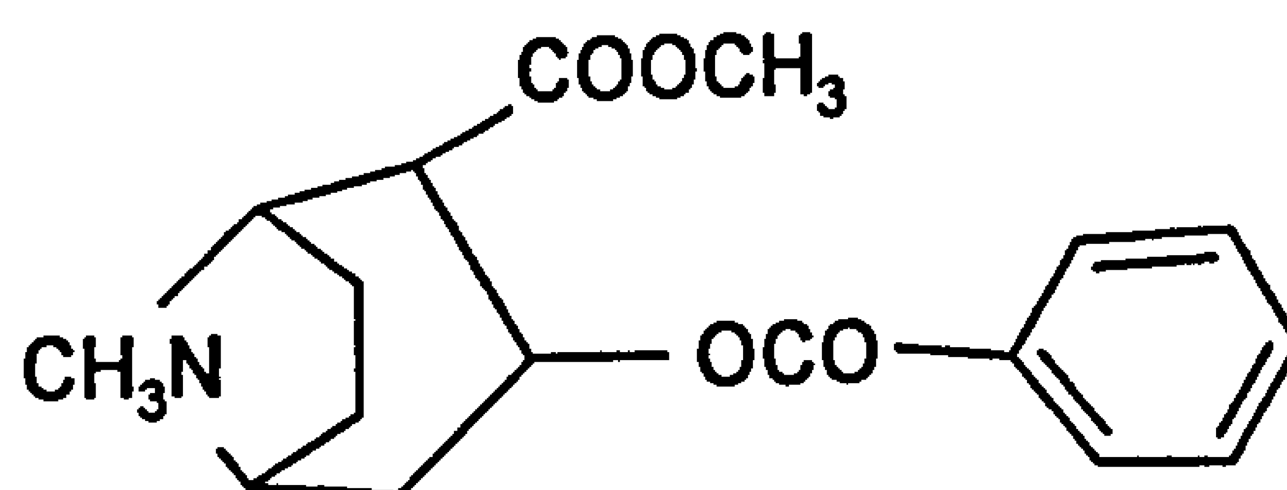


Figure 3-8:- Chemical structure of Cocaine ($C_{17}H_{21}NO_4$, MW 303, pKa 8.6)

Cocaine (Figure 3-8) is isolated from *Erythroxylon* coca which has been grown in numerous countries around the world but mostly in South America. There are more than 250 varieties of coca plants but only three are widely used in the illegal cocaine drug trade. These are *Huanuca* coca, grown in Bolivia and Peru, *Amazonian* coca, grown in the Amazon river basin and *Colombian* coca grown primarily in Colombia.[48, 123] The properties of cocaine include a molecular weight of 303 and a pK_a of 8.6 and it is considered to be a powerful stimulant. The routes of administration include chewing, snorting, injecting and smoking. It can be absorbed into the body through any mucous membrane. The chewing of powdered coca leaves containing 17-48 mg of cocaine by 6 volunteers produced peak plasma cocaine concentrations in the range 0.011-0.149 mg/L within 0.4-2 hours.[118] The half- life of cocaine ranges from 40 min to 4 h depending on how the elimination phase is estimated.[105] There are short term effects on the user, including increased energy, decreased appetite, mental alertness, increased heart rate and

blood pressure, constricted blood vessels and increased temperature.[124] There are also long term effects such as addiction, irritability and mood disturbances, restlessness, paranoia and auditory hallucination.[124, 125] Cocaine is considered a powerfully addictive drug. 80 to 90 % of cocaine is metabolised to ecgonine methyl ester by enzymatic hydrolysis and to benzoylecgonine by spontaneous hydrolysis at physiological and alkaline pH.[125] Rapid enzymatic hydrolysis by plasma and liver esterases yields ecgonine methyl ester. Other metabolites include norcocaine (Figure 3-8)[126-128].

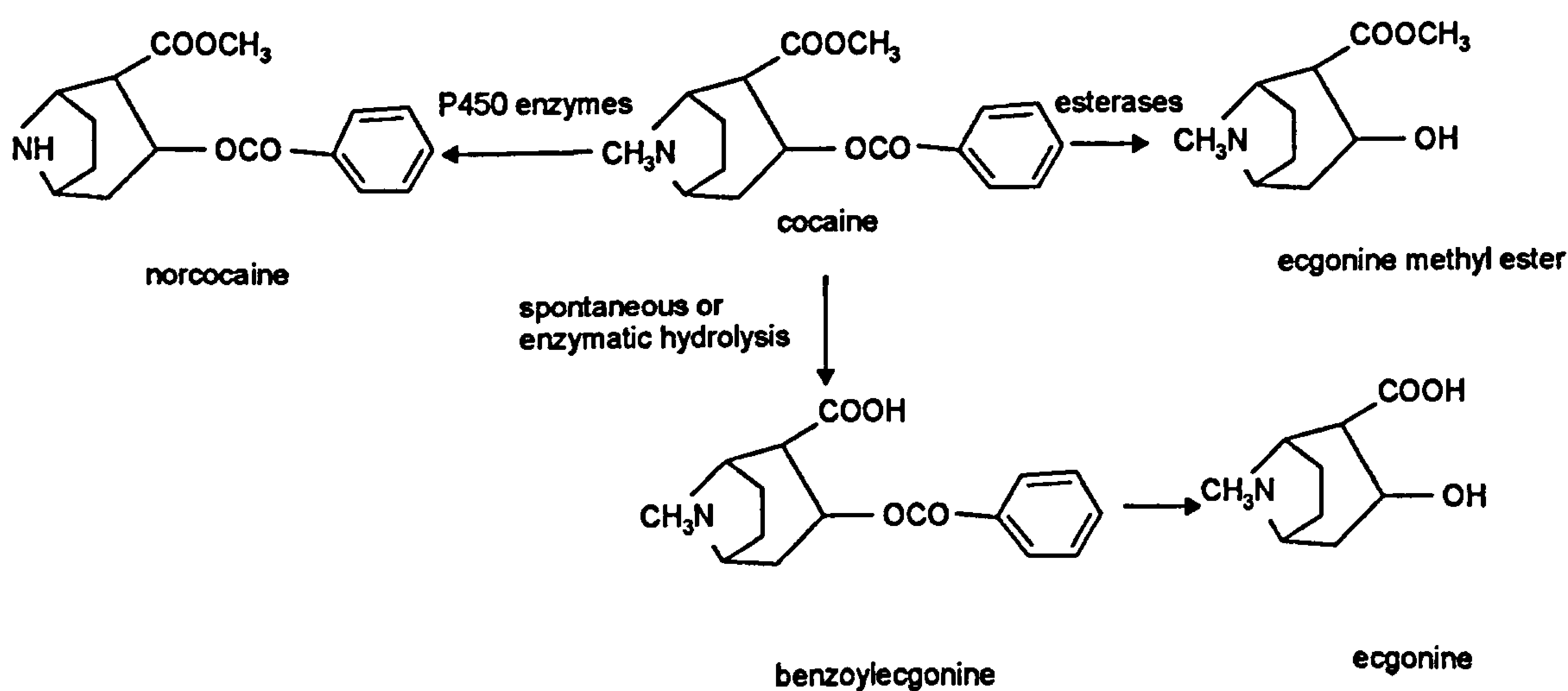


Figure 3-9:- Metabolic pathways for Cocaine[129].

3.6 Opioids

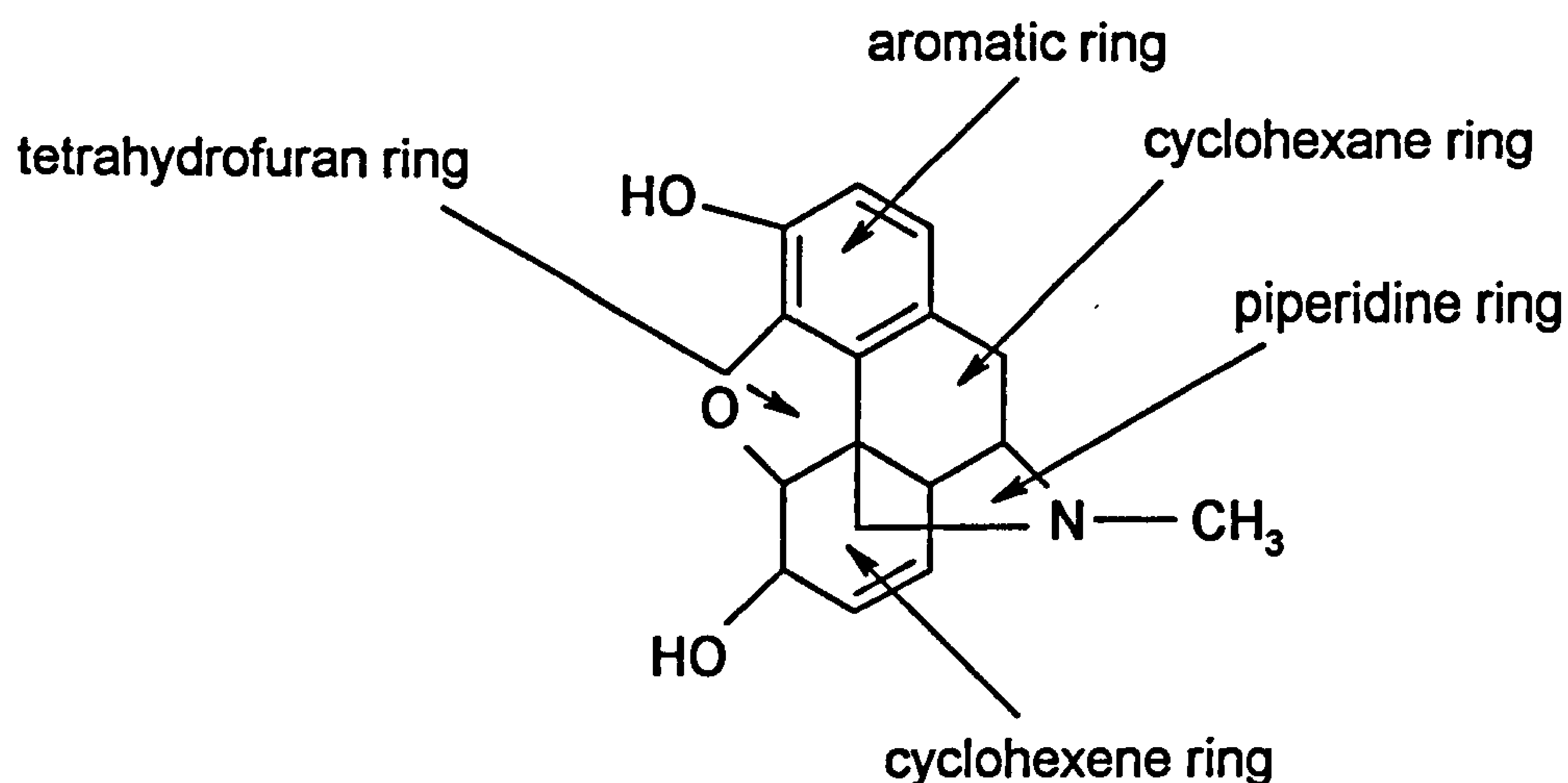


Figure 3-10:-Chemical structure of Morphine ($C_{17}H_{19}NO_3$, MW 285, pKa 9.85 and 7.87) showed with its heterocyclic rings distinguished.

The opioids are a large family of drugs and are the most important therapeutic components in the treatment of moderate to severe pain.[105] "Opiates" are drugs directly derived from the opium poppy and include opium, morphine and codeine. Opium is a very ancient drug which has been used as an analgesic for many thousands of years. The term *opioid* refers to all natural and synthetic drugs with morphine–like properties and so includes a wider range of drugs than the opiates. Morphine (Figure 3-10), hydromorphone, meperidine, heroin and fentanyl are agonists at opioid receptors and, as they are very lipid soluble, are rapidly and extensively distributed to tissues. Synthetic opioids like fentanyl, or semi-synthetic opioids such as hydromorphone, have higher lipid solubilities than morphine which allows them to cross the blood brain barrier quickly, leading to a more rapid onset of their effects. Due to the diversity of their chemical structures, the metabolism of opioids varies significantly between members (Figure 3-11).[105, 130]

Morphine is essentially completely metabolized by glucuronidation at the 3- and 6-positions, whereas methadone is dealkylated leading to a product which readily forms a cyclic structure. Dealkylation pathways are also important for drugs with larger substituents on the N-atom, e.g. buprenorphine, butorphanol and pentazocine.[105]

Morphine has the following properties: molecular weight 285, pKa values 9.85 and 7.87. It is an alkaloid narcotic drug extracted from opium and is a highly addictive analgesic which can be administered by several routes i.e. injected, smoked, sniffed or swallowed.[105, 118, 131] The trade names for morphine are Roxinal, MS contain, or

morphine sulphate.The common street name for morphine is Morph.[132] There are many symptoms of overdose which include cold clammy skin, flaccid muscles, fluid in the lungs, lowered blood pressure, pinpoint or dilated pupils, sleepiness, stupor, coma, slowed breathing and slow pulse rate.

The plasma half –life for a lot of the common opioids is relatively short; for instance, morphine which has a half-life only a few hours.[105] Morphine is rapidly excreted through the kidneys into the urine as glucuronides, with up to 85% of the dose recovered in urine within 24 hours. Only small amounts (2-10%) are excreted unchanged.[105]

The formation of glucuronide conjugates is an important feature of the pharmacokinetics and toxicology of morphine. Laboratories will often measure morphine as total morphine, which includes free morphine and the sum of the two glucuronide conjugates. This is due to the greater ease associated with measuring these higher concentrations of total morphine, since morphine levels are often undetectable after several hours[133-135]. However, since LC-MS became available, individual glucuronides are measured without hydrolysis. This is important since morphine-6-glucuronide is pharmacologically more active than morphine itself.[136]

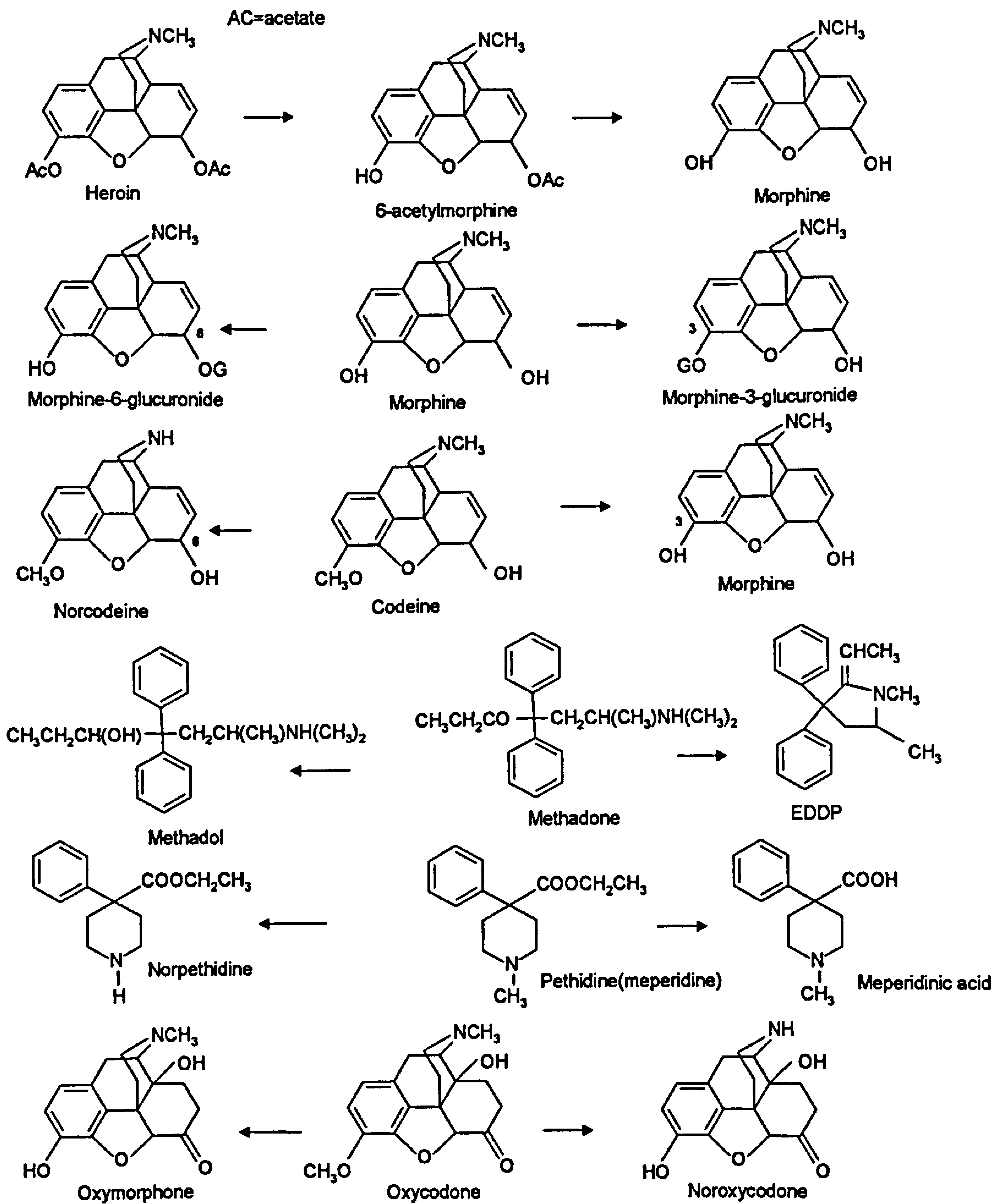


Figure 3-11:- Pathways for metabolism of selected opioids.[105]

3.7 Cannabis

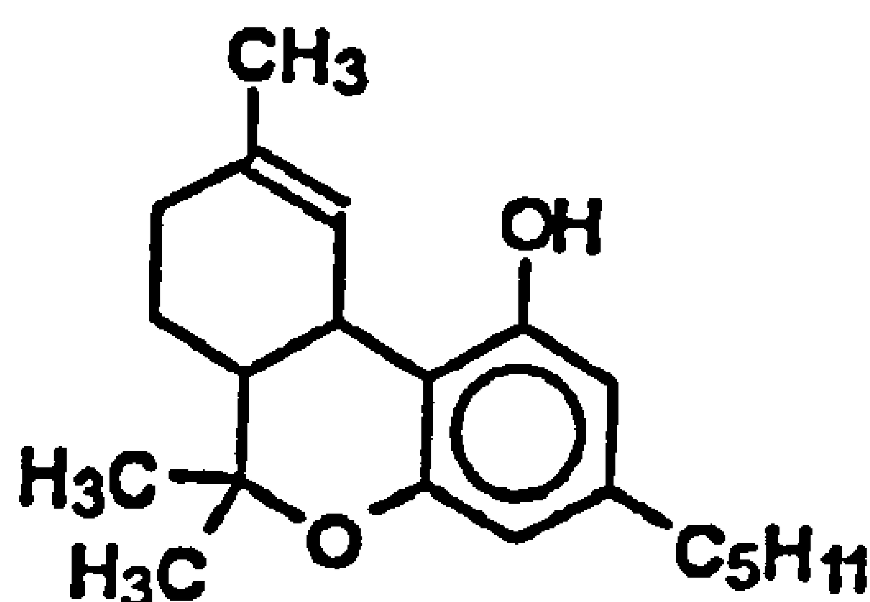


Figure 3-12:- Chemical Structure of delta-9-tetrahydrocannabinol, THC ($C_{21}H_{30}O_2$, MW 314, pK_a 10.6)

Cannabis is a green or grey mixture of dried leaves of the hemp plant *Cannabis sativa.* There are three drugs derived from the cannabis plant – herbal cannabis, hashish and hashish oil.[137, 138] There are over 200 slang terms for cannabis including boom, Mary Jane, gangster, cannabis and chronic. However, the names *marijuana* or *marihuana* are not used in UK legislation, which uses the name *cannabis* exclusively. The drug is usually smoked as cannabis cigarettes. The main active drug substance in cannabis is THC (delta - 9-tetrahydrocannabinol, Figure 3-12) Although most of the THC in cannabis plants is concentrated in the resin, all parts of the plant, apart from the seeds and roots, have been found to contain THC.[105, 118, 139]

After inhaling cannabis smoke for a few minutes, the bronchial passages relax and become enlarged and the blood vessels in the eyes expand, making the eyes look red. The heart rate, normally 70 to 80 beats per minute, may increase by 20 to 50 beats per minute or, in some cases, even double.[118] This effect can be greater if other drugs are taken with cannabis. The whole blood/plasma concentration ratio for THC is 0.55. Estimates of its elimination half-life have ranged from 20-57 hours in infrequent users and from 3-13 days in frequent users. The elimination plasma half-life of 11-carboxy-THC averaged 33 hours in infrequent users of cannabis and 40 hours in frequent users.[118] THC is metabolized to two monohydroxy compounds, 11-hydroxy-THC and 8-beta-hydroxy-THC (Figure 3-13) which, although pharmacologically active, do not achieve appreciable plasma concentrations and probably do not contribute significantly to the acute effects of the drug after smoking.[105] A third metabolite, and quantitatively the most important, 11-nor-9-carboxy-delta-9-THC, has been identified and is believed to be a product of further oxidation of 11-hydroxy-THC.[140] About 70% of a dose of THC is excreted within 72 hours in the faeces (40%) and urine (30%).[105] The urinary elimination half-life of 9-

carboxy-THC averaged 3 days (range 0.8-9.8) in heavy smokers of cannabis.[105] Passive inhalation of cannabis smoke has resulted in plasma THC levels of 1-7μg/L and urine 9-carboxy-THC levels as high as 39μg/L, as measured by a specific assay.[141, 142]

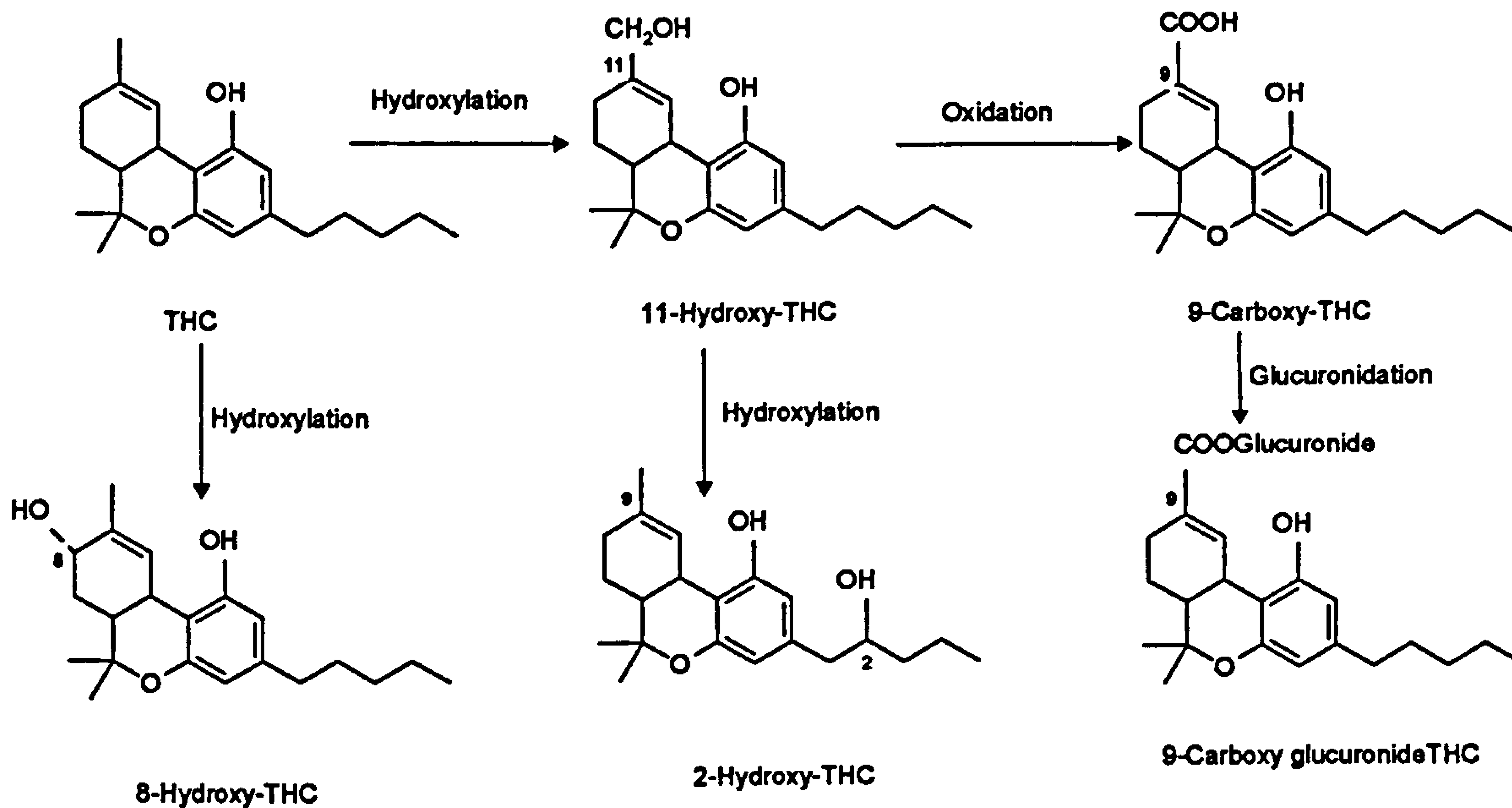


Figure 3-13:-Metabolic pathways for THC[105]

4 Comparison of Derivatisation Procedures

4.1 Introduction and Aims

Systematic forensic toxicology involves a logical sequence of analytical procedures designed to detect a wide range of drugs and poisons in cases for which there is no information about which substances might be present, i.e. a general unknown. Ideally, all possible substances will be detected but inevitably there are restrictions.Conventional methods used for the purpose have included UV spectroscopy, thin layer chromatography, gas chromatography and high pressure liquid chromatography, usually with a diode array detector.[11, 143-157] More recently, combined techniques involving a chromatographic technique and spectrometry have become more accessible to the majority of forensic toxicology laboratories and as a result are now the techniques of choice.[36, 39, 54, 158-173]

One of the main requirements in systematic toxicological analysis is often chemical derivatisation, which is the process of chemically modifying a molecule to produce a new compound which has properties that make it suitable for analysis.[152] Derivatives are made for most chromatographic techniques but are especially important for analyses involving GC-MS. Historically in the forensic sciences the use of derivatising agents with GC-MS has been largely within the scope of the forensic toxicology and chromatography of drugs and metabolites in biological fluids and tissues.[154, 174, 175]

There are several GC-MS methods for the determination of drugs of abuse which have been published, most of them dealing with determination of individual drugs of abuse or else with groups of related drugs. Derivatising procedures for systematic forensic toxicological analysis have included the silylation, acylation and esterification reactions described below in Section 4.2. [176-183] However, no systematic comparison has been made of the different derivatives for forensic toxicology.

The ultimate aim of this work was to create a unified procedure for systematic toxicological analysis which would enable a wide range of drugs and metabolites to be detected in a minimum number of analyses, thereby making the procedure suitable for the complete toxicological analysis of small samples. The intention was to develop a simple, fast method based on solid phase extraction of acid, neutral and basic analytes followed by a comprehensive derivatisation procedure which would enable the extracts to be analysed

by GC-MS. The study described in this chapter is concerned with the end-step derivatisation and analysis, which required to be set up before extraction procedures could be evaluated. Drugs were subsequently extracted from whole blood by solid phase extraction followed by derivatisation and analysis by GC-MS-SIM, and that study is described in Chapter 5.

The present study describes a comparison of three derivatisation methods involving silylation, acylation and esterification reactions for the analysis of drugs of abuse. Several existing reagents and a new reagent, trimethylsilyl–diazomethane were evaluated to establish which would be most suitable for a unified procedure. In addition, the literature records that derivatisation is more rapid when microwave irradiation is used rather than direct thermal heating, reducing the incubation time from more than thirty minutes to one minute,[184-190] and this was also evaluated in the present study.

Model compounds which feature each of the functional groups commonly found in target analytes of interest in forensic toxicology (amphetamine, methamphetamine, morphine, benzoylecgonine, THC-OH and THC-COOH) were used to evaluate the reagents. The justification for this approach is that functional groups usually react independently and are not significantly influenced by the molecule in which they are located.

4.2 Chemistry of Derivatisation Reactions

4.2.1 Introduction

Chemical derivatisation has long been a useful adjunct in the characterization of organic compounds using techniques that include chromatography and spectroscopy. Derivatisation is the process of chemically modifying a compound to produce a new substance which has properties that are suitable for analysis using a particular technique, such as GC.[80, 191-194] Volatility and thermal stability of the analytes are required in GC and GC-MS analysis. Other reasons for derivatisation are to enable analysis of functional groups, to provide selective detection, to produce mass shifts in mass spectra, to modify fragmentation and to improve chemical ionization.[80, 195]

Derivatisation prior to analysis involves an additional chemical step that is not only time consuming but also introduces potential impurities, uncertainties about the completeness of

the conversion and possible interpretation difficulties associated with the adopted chemical reaction. Many drugs are, however, derivatized prior to undergoing GC analysis to confer volatility to the analytes and improve their stability, separation and chromatographic properties. This is because most drugs, both licit and illicit, are organic in structure, and their analyses by chromatographic methodology often can be facilitated by chemical derivatisation.[80, 174, 191]

Generally, derivatisation is performed to convert polar N-H, O-H and S-H groups into thermally stable, nonpolar groups and derivatives are therefore used to increase sensitivity and chromatographic performance for specific compounds such as acids, alcohols and amines, which are difficult to analyse because of the presence of reactive hydrogen atoms. When attempting to analyse these types of compound, they can react with the surface of the injection port or the analytical column which can result in tailing peaks and low response. They may also be highly soluble in the sample matrix, causing very poor headspace content and low response.

GC derivatisation methods can be classified into 4 groups according to the reagents used and reaction achieved. These are silylation, acylation, alkylation and esterification reactions. Therefore the choice of a derivatising reagent is based on the functional group requiring derivatisation, the presence of other functional groups in the molecule, and the reason for performing the derivatisation. The chemical structure and properties of the molecule influence the reagent choice. In choosing a suitable derivatisation reagent certain criteria must be used as guidelines. A good reagent produces a derivatisation reaction that is 95-100% complete, will not cause any rearrangements or structural alterations during formation of the derivative, does not contribute to loss of the sample during reaction, produces a derivative that will not interact with the analytical (GC or HPLC) column, and one which is stable with respect to time.[183] The main requirements for a successful derivatisation reaction are that a single derivative should be formed for each compound, the derivatisation reaction should be simple and rapid and should occur under mild conditions.[196-207]

The following Sections review the derivatisation reactions used in this study.

4.2.2 Silylation

The term *silyl* refers to derivatives containing silicon and usually, but not exclusively, relates to the trimethylsilyl group, $(CH_3)_3Si$. Trimethylsilylation involves the introduction of the trimethylsilyl group into a molecule (Figure 4-1), usually in substitution for an active hydrogen atom.[194, 208]

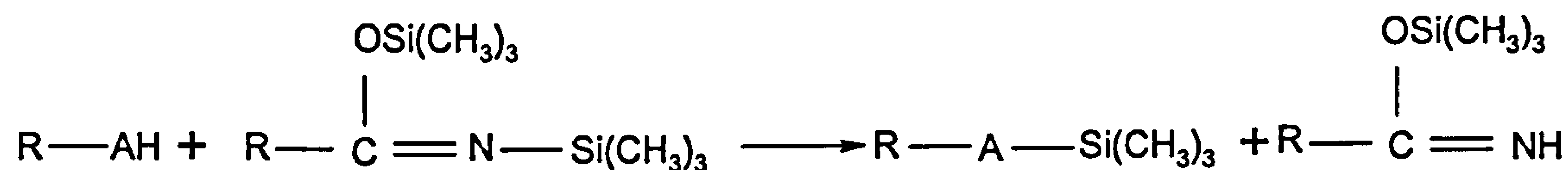


Figure 4-1:- Typical silylation reaction, in which A represents a heteroatom such as O or N.

The reagents that have been used for this purpose are derivatives of trimethylsilane, $(CH_3)_3SiH$. The replacement of active hydrogen by the trimethylsilyl group reduces the polarity of the compound and decreases the possibilities of hydrogen bonding (Figure 4-2).

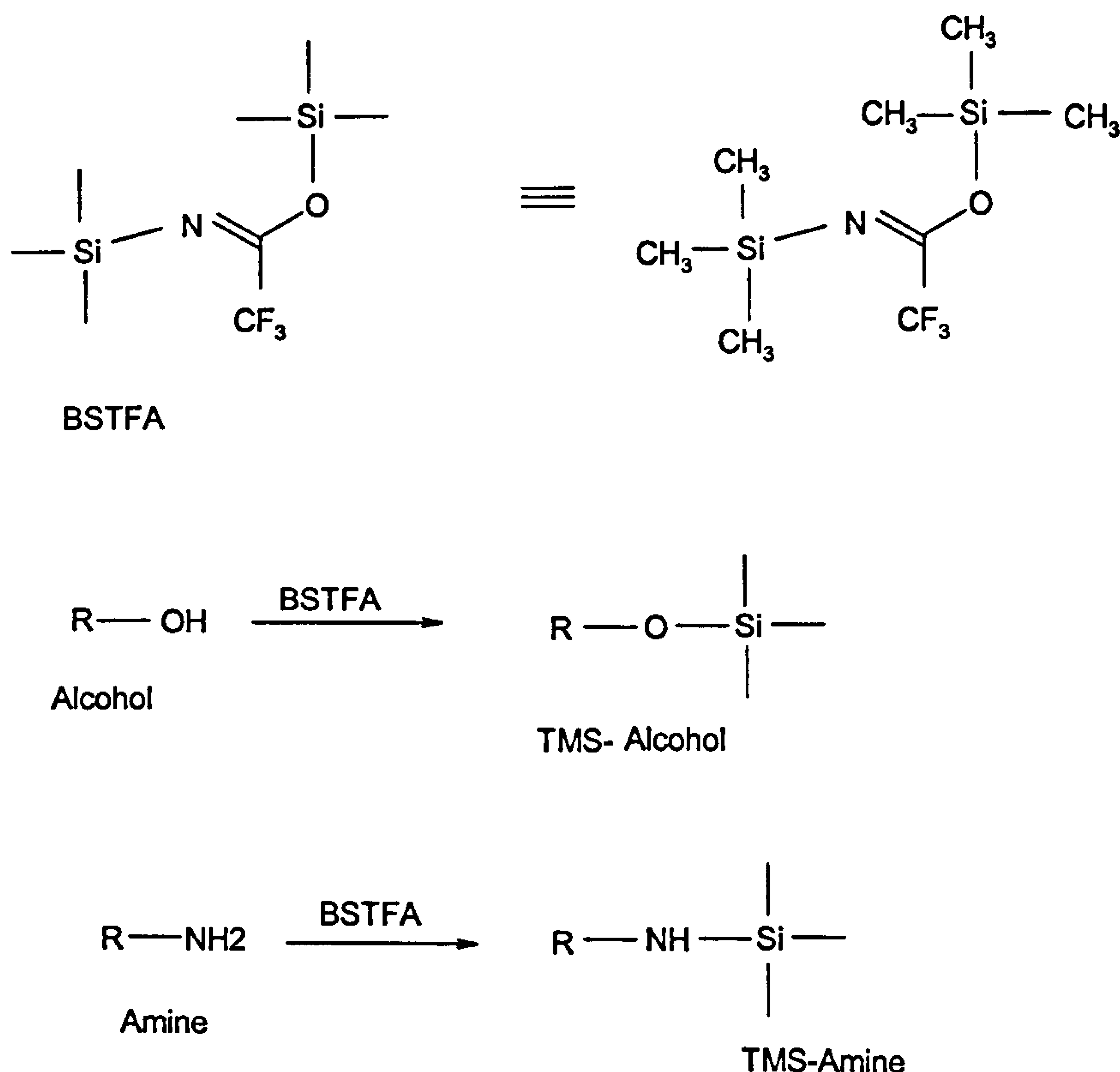


Figure 4-2:- Derivatisation by Silylation Reaction

The advantages of volatility and stability imparted by silylation make the process a natural tool for gas–phase purification and analysis[208-214]. The derivative is simply and conveniently prepared. In many case the reactants are mixed at room temperature and the reaction is complete in a few minutes. Nearly all functional groups which present a problem in gas chromatographic separation (hydroxyl, carboxylic acid, amine, thiol, phosphate) can be derivatized by silylation reagents. Even sugars and polar steroids have successfully been derivatised and analysed by GC-MS. Silylation reagents are influenced by both the solvent system and the addition of a catalyst. A catalyst such as trimethylchlorosilane or pyridine increases the reactivity of the reagent. [215]

Silylation has some disadvantages, as silylation reagents are moisture sensitive. The reaction must therefore be performed using anhydrous conditions. TMS derivatives are more sensitive to hydrolysis than other derivatives containing more sterically crowded alkyl substituents on the silicon atom and silylation must use aprotic organic solvents.[216-227]

A large number of silylating reagents is available commercially or else they are easily prepared, including N,O-bis(trimethylsily)acetamide (BSA), N,O-bis (trimethylsily)trifluoroacetamide (BSTFA), hexamethyldisilazane (HMDS), N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), N-*tert*-butyl-dimethylsilyl-N-methyl-trifluoracetamide (MTBSTFA), *tert*-butyl-dimethylchlorosilane (TBDMCS), N-*tert*-butyl-dimethylsilylimidazole (TBDMSIM), trimethylchlorosilane (TMCS), N-trimethylsilyl-diethylamine (TMSDEA) and trimethylsilylimidazole (TMSIM). MSTFA is the most volatile TMS amide available.

Typical reaction conditions involve heating samples with a silanising reagent on a hotplate at 60°C for 30-60 minutes, but molecules containing sterically-hindered hydroxyl or other groups often require prolonged exposure at high temperatures. Examples in forensic toxicology include buprenorphine, which has a sterically hindered alcohol group on the side chain and some anabolic steroids.

Silyl derivatives have been widely used in forensic toxicology and STA since their introduction in the 1950's.[144, 205, 211, 228-230] Formation of trimethylsilyl derivatives is commonly used before the GC-MS analysis of drugs, including the opiates, cocaine metabolites and cannabinoids.[231] However, trimethylsilylation is not often used for

amphetamines because incomplete conversion occurs unless suitable catalysts are used such as ethanethiol and ammonium iodide.[232]

In the present study, MTBSTFA reagent was selected for evaluation because previous work had shown that methyl-tertiary-butylsilyl derivative mass spectra are characterized by abundant ions at $[M-57]^+$ which are good for identification of drugs and for quantification.[228, 233, 234] MTBSTFA is a liquid at room temperature, and so can often be used for derivatisation without a solvent. It is sensitive to moisture[235]. The advantage of using MTBSTFA is enhanced reactivity including its ability to silylate carboxyls, hydroxyls, thiols and primary and secondary amines, short reaction times often at room temperature, and simplified work-up owing to derivatisation by-products being neutral and largely volatile, so providing the possibility for direct injection of the reaction mixture for GC analysis.[208] During the course of the work for this study, a literature publication appeared on the analysis of benzodiazepines in biological samples, based on the methyl-tertiary-butylsilyl derivative, in which the authors showed that MTBSTFA forms sensitive, reproducible and stable derivatives with benzodiazepines which have active hydrogen atoms in either a secondary amine (for example desmethyl diazepam) or in a hydroxyl group (for example, Temazepam).[236] Tertiary-butyl dimethylsilyl derivatives were found to be preferable to trimethylsilyl derivatives because stable trimethylsilyl derivatives are formed only with benzodiazepines which have a hydroxyl group.

4.2.3 Acylation

Acylation, an alternative to silylation, is the conversion of compounds with active hydrogen atoms such as OH, SH and NH into esters, thioesters and amides, respectively (Figure 4-3). A classic example of this method is the insertion of perfluoracyl groups into a substance to enable or enhance electron capture detection (ECD)[237] and negative ion chemical ionisation (NICI) mass spectrometry.[238, 239] Carbonyl groups adjacent to halogenated carbons enhance the response of ECD. A further benefit of acylation is the formation of fragmentation-directing derivatives for GC-MS analysis, which produce strong ions at high mass, suitable for GC-MS in the selected ion monitoring mode. Acylation reactions can be performed using three main types of reagents: acyl halides, acid anhydrides or reactive acyl derivatives such as acylated imidazoles. Acyl halides and acid anhydrides (including perfluoro acid derivatives) readily form derivatives of alcohols, phenols and amines which are both stable and highly volatile. Acyl imidazoles (including

perfluoracyl imidazoles) react readily with hydroxyl groups and secondary or tertiary amines to form acyl derivatives.

Figure 4-3:- Typical acylation reaction

As for silylation reagents, the acylation reagents can be used for highly polar, multifunctional compounds, such as carbohydrates and amino acids. Acylation is normally carried out in pyridine, tetrahydrofuran or another solvent capable of accepting the acid by-product. The tendency of acyl derivatives to direct the fragmentation patterns of compounds in MS applications provides helpful information on the structure of these materials. Other advantages of acylation are obtained because the derivatives are hydrolytically stable and give increased sensitivity and selectivity in GC-MS by increasing the molecular weight. Acylation can also be used as a first step to activate carboxylic acids prior to esterification, which was used in the present study.

However there are disadvantages with acyl derivatives as they can be difficult to prepare and reaction products (acid by-products) often need to be removed before analysis. Acylation reagents are moisture sensitive and are hazardous and odorous. There are many acylation reagents used, such as perfluorinated anhydrides, including trifluoroacetic anhydride (TFA), pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA), fluoroacylimidazoles, including trifluoroacetylimidazole (TFAI), pentafluoropropanoylimidazole (PFPA), and heptafluorobutyrylimidazole (HFBI), N-methyl-bis-trifluoroacetamide (MBTFA), and also acid chlorides such as pentafluorobenzoyl chloride (PFBCl)[240, 241]. PFPA is one of the most commonly used acylation reagents, as derivatives are suitable for both FID and ECD, the reagent reacts with alcohols, amines and phenols to produce stable and highly volatile derivatives. The acid by–product should be removed, via a stream of nitrogen, before injection on to the column. Bases, such as triethylamine, can be added as an acid receptor and promote reactivity.

Typical reaction conditions for acylation involve heating samples with an acylating reagent at 60°C for 30-60 minutes. The reagent can be used directly or can be diluted with a suitable solvent such as ethyl acetate. If necessary, a basic catalyst can also be added[242, 243]

Acyl derivatives have been used widely in forensic STA.[229, 230, 242, 243] In the present study, PFPA was selected for evaluation because of previous experience within Forensic Medicine and Science of its use for a range of drug types including amphetamines, antidepressants such as fluoxetine, opioids such as buprenorphine and acidic drugs.[228]

4.2.4 Alkylation

Alkylation involves adding an alkyl group to an active functional group(Figure 4-4) containing a replaceable hydrogen atom, such as those contained in carboxylic acids, alcohols, thiols, phenols, primary and secondary amines, amides and sulfonamides.[244-247] These are the main functional groups that can be subjected to alkylation reactions.

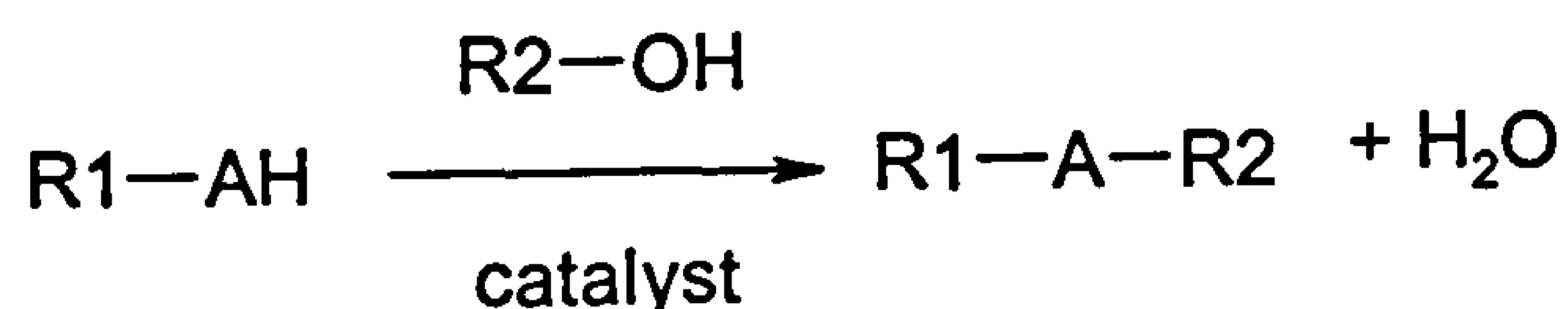


Figure 4-4:- Typical alkylation reaction, where R1 and R2 are organic groups and A represents a heteroatom

Replacement of hydrogen with an alkyl group is important because the derivative has lower polarity relative to the parent substance. Alkylation of weakly acidic groups such as alcohols requires strongly basic catalysts such as sodium or potassium methoxide.[244] More acidic OH groups such as those contained in phenols and carboxylic acids require less basic catalysts and can even be Lewis acids such as hydrogen chloride or boron trifluoride. Alkylation can be used alone to form esters, ethers and amides or it can be used in conjunction with silylation or acylation, as in the present study.[246]

Esterification is the most common type of alkylation and is used to derivatise carboxylic acids and other acidic functional groups. Esterification involves the condensation of the carboxyl group of an acid and the hydroxyl group of an alcohol, with the elimination of water.[244] Results are best in the presence of a catalyst, like BF_3 or HCl, which is then removed with the water. Alkyl esters have excellent stability and can be isolated and stored for long periods of time. There are many advantages for example, the wide range of alkylation reagents available, reaction conditions can vary from strongly acidic to strongly basic, some reactions can be done in aqueous solutions and alkyl derivatives are generally stable. Some disadvantages are that the reaction is limited to amines and acidic hydroxyls,

reaction conditions can be severe and the reagents are often toxic. Typical reaction conditions for esterification involve heating the acid with an alcohol and catalyst either under reflux (large scale) or in a sealed tube at 60°C for 30-60 minutes.[244, 247]

There are many alkylating reagents such as dimethylformamide (DMF - dialkylacetals), tetrabutylammonium hydroxide (TBH), BF_3 in methanol or butanol and pentafluorobenzyl bromide (PFBBr) (Figure 4-5). [245, 246, 248]

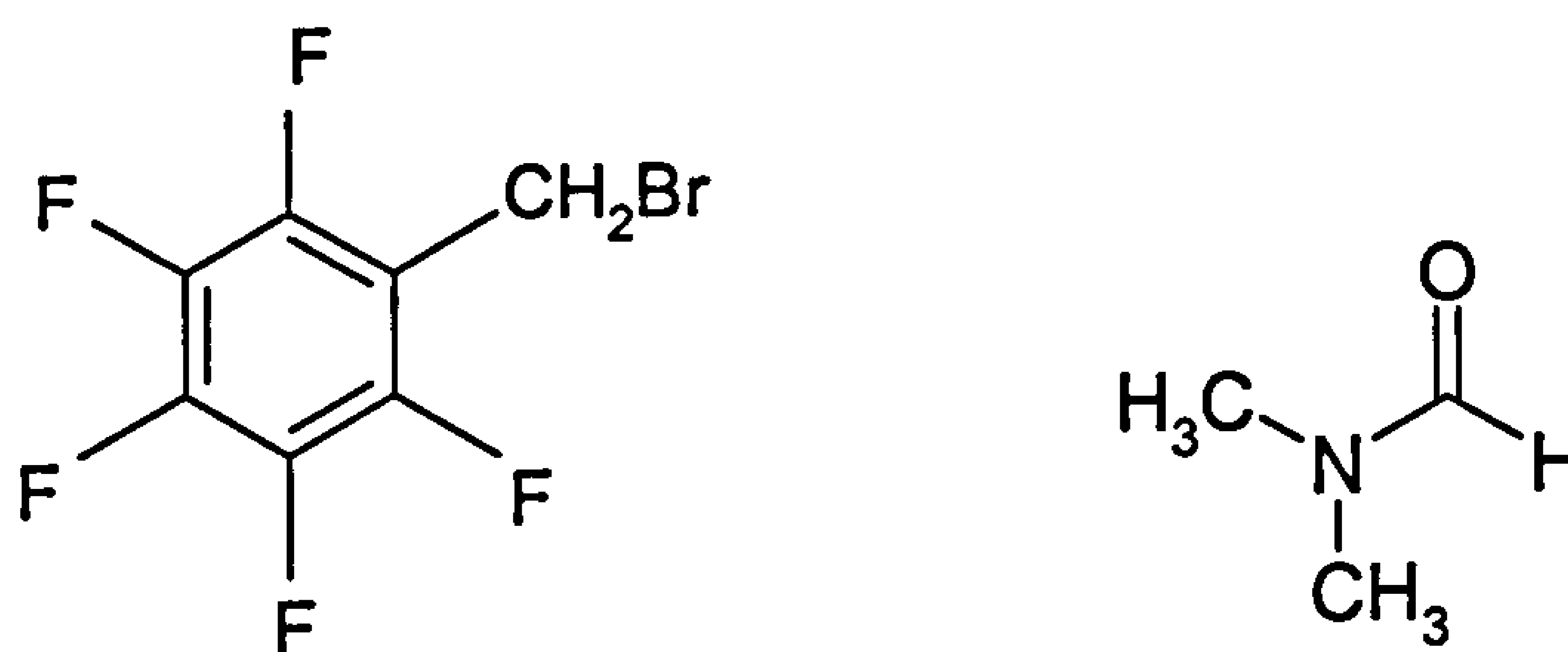


Figure 4-5:- Pentafluorobenzyl bromide (left) and N, N-Dimethylformamide (right)

In the present study, pentafluoropropanol was used to alkylate (esterify) carboxylic acid groups in the selected model compounds (THC-COOH and benzoylecgonine) with the corresponding acid anhydride (PFPA) as the catalyst. This combination has previously been used in forensic toxicology for the analysis of THC-COOH [249, 250].

4.2.5 Methylation (esterification)

Methylation is a particular form of the alkylation reaction in which a methyl group (CH_3-) is introduced into a molecule. A specific example is the replacement of a hydrogen atom by a methyl group. It is widely used for derivatisation of carboxylic acids. It is a stable reaction, the reagents are easy to evaporate, the retention time of the product is usually well-defined, a good mass spectrum is obtained and the reagents are readily available and inexpensive. On the other hand, methylation can not distinguish benzoylecgonine and cocaine.

Conventional reagents for methylation include boron trifluoride in methanol, tetramethylammonium hydroxide, methyl iodide and diazomethane.[251-257] Diazomethane is an ideal methylating agent in that it reacts quantitatively at room temperature and is

readily volatile. However, it is hazardous to prepare and use because of its toxicity and potential for detonation. More recently an alternative reagent, trimethylsilyl-diazomethane, became commercially available as a solution in hexane for applications in organic synthesis and this was evaluated in the present study.[251, 253, 254, 258-260]

4.3 Microwave assisted derivatisation

Microwaves are commonly used to heat things containing water but can also heat organic molecules as in microwave assisted derivatisation.[189, 190] Water molecules consist of two hydrogen atoms carrying a small positive charge and an oxygen atom carrying a negative charge. The oscillating electric field of the microwave interacts with these charged atoms to cause the molecule to twist. This rotation causes friction, producing heat.

Technically, in a microwave oven the mains voltage is stepped up by a transformer and capacitor to around 3000V, to power the magnetron and generate microwaves which are in effect high – frequency radio waves, at about 2.5GHz. The radiation is conducted through the waveguide into the heating chamber, and then the microwaves are reflected from the walls and door, and penetrate the material in the oven from the sides and above. A turntable rotates the target slowly to give even exposure. Some ovens achieve this by using a microwave stirrer instead.

The derivatisation reactions described in Section 4.2 are normally carried out using a dry-block (oven) for a period of typically 30 minutes up to several hours. This time delay is a major disadvantage of derivatisation.[187, 188, 261, 262] By using a microwave a much shorter time has been found to be needed for the same derivatisation, e.g. 1 minute at 900 Watts.[184-186]

4.4 Experimental Section

4.4.1 Reagents and materials

a) PFPA (pentafluoropropionic anhydride, $C_6F_{10}O_3$, MW 460, Figure 4-6) was supplied by Fluka Chemicals, The Old Brickyard New Road, Gillingham, Dorset, SP8 4JL, England.

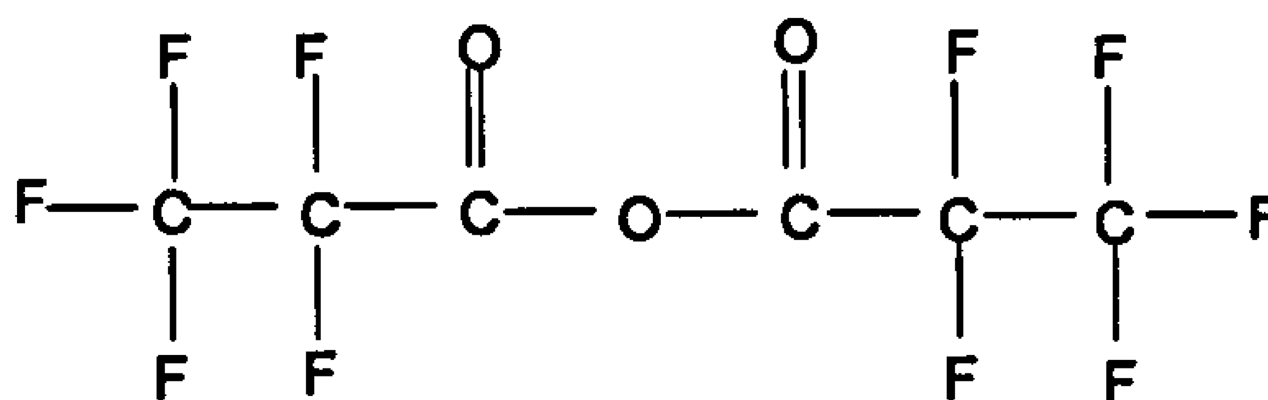


Figure 4-6:- Structure of PFPA anhydride

b) PFP-OH (2, 2, 3, 3, 3-pentafluoropropanol, 97%, $C_3F_5H_3O$, MW 150, Figure 4-7) and TMS (trimethylsilyl-diazomethane, 2.0 M solution in hexane, $C_4H_{10}N_2Si$. MW 114, Figure 4-8) were supplied by Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4OH, England.

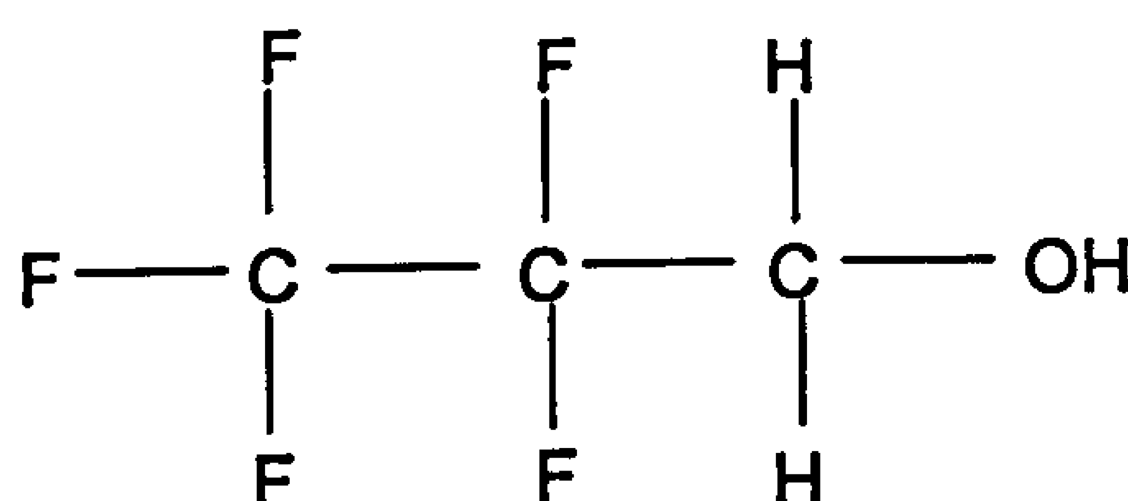


Figure 4-7:- Structure of PFP-OH alcohol

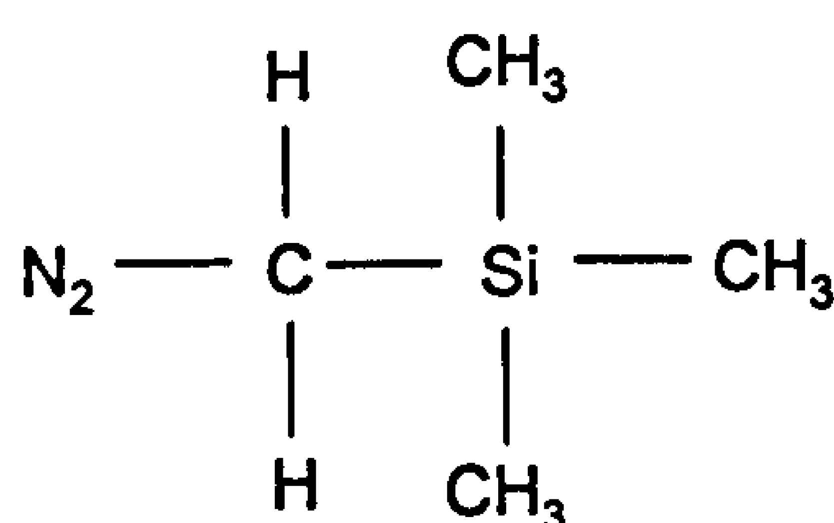


Figure 4-8:- Structure of trimethylsilyl-diazomethane

c) MTBSTFA, (N-Methyl-N-tert-butyldimethysilytrifluoroacetamide, $C_9H_{18}F_3NOSi$, MW 238, Figure 4-9) containing 1% by volume of TBDMCS was supplied by Perbio Science UK Limited, Century House, Tattenhall, Cheshire, CH3 9RJ.

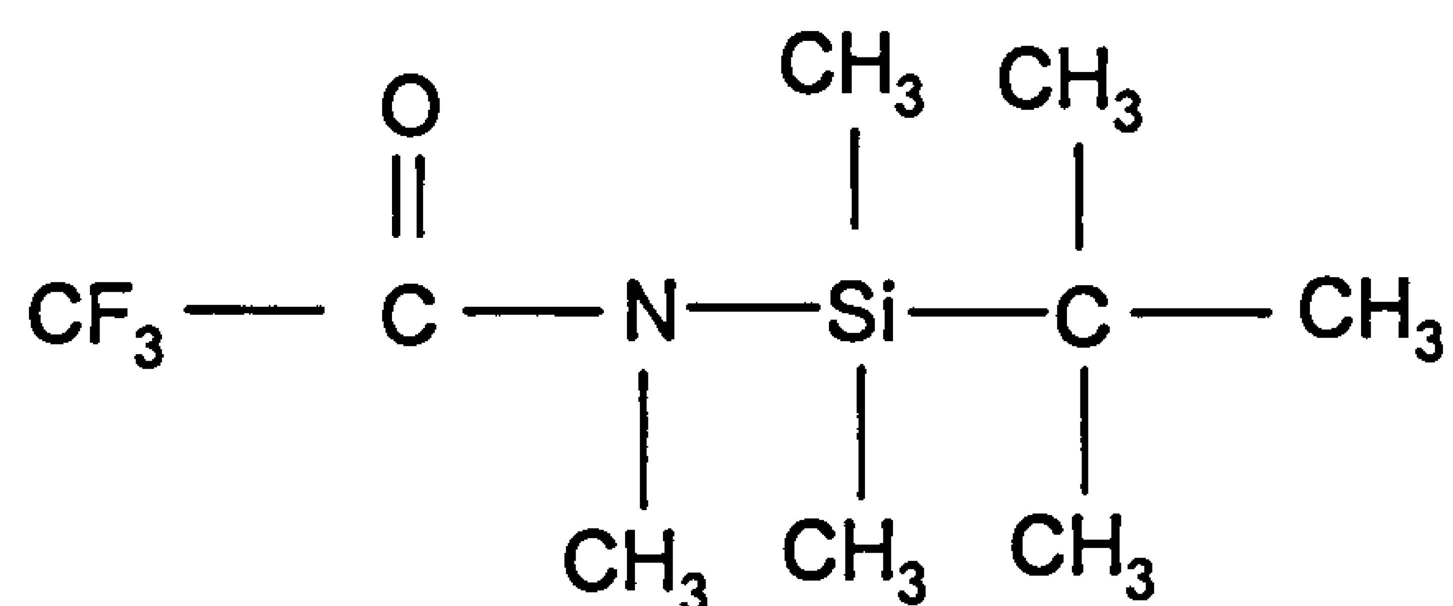


Figure 4-9:- Structure of MTBSTFA+1% TBDMCS

(d) ReactiVials® (1 ml and 3 ml sizes) for microwave-assisted derivatisation were obtained from Perbio Science UK Limited, Century House, Tattenhall, Cheshire, CH3 9RJ. These were sealed with the standard ReactiVial screw cap and septum.

4.4.2 Drug standards and internal standards

Drug standards and internal standards used were amphetamine, amphetamine–d_5 (Figure 4-10), methamphetamine, methamphetamine-d_5 Figure 4-11), morphine, morphine-d_3 (Figure 4-12), benzoylecgonine, benzoylecgonine-d_3 (Figure 4-13), Δ^9-tetrahydrocannabinol (THC-OH), tetrahydrocannabinol-d_3 (Figure 4-14) and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH, Figure 4-15). Stock standard solutions of all drugs were obtained at concentrations of 0.1 mg/mL in methanol from Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4OH, England. THC-OH and THC-COOH were supplied by LGC Promochem Group Company, Queens Road, Teddington, TW11 0LY, and Middlesex. Working standards were prepared at 10 µg/mL by dilution with methanol. Further details of standard solutions are given in Section 5.1.

Figure 4-10 :- Amphetamine and Amphetamine–d_5

Figure 4-11:- Methamphetamine and Methamphetamine–d_5

Figure 4-12:- Morphine and Morphine–d_3

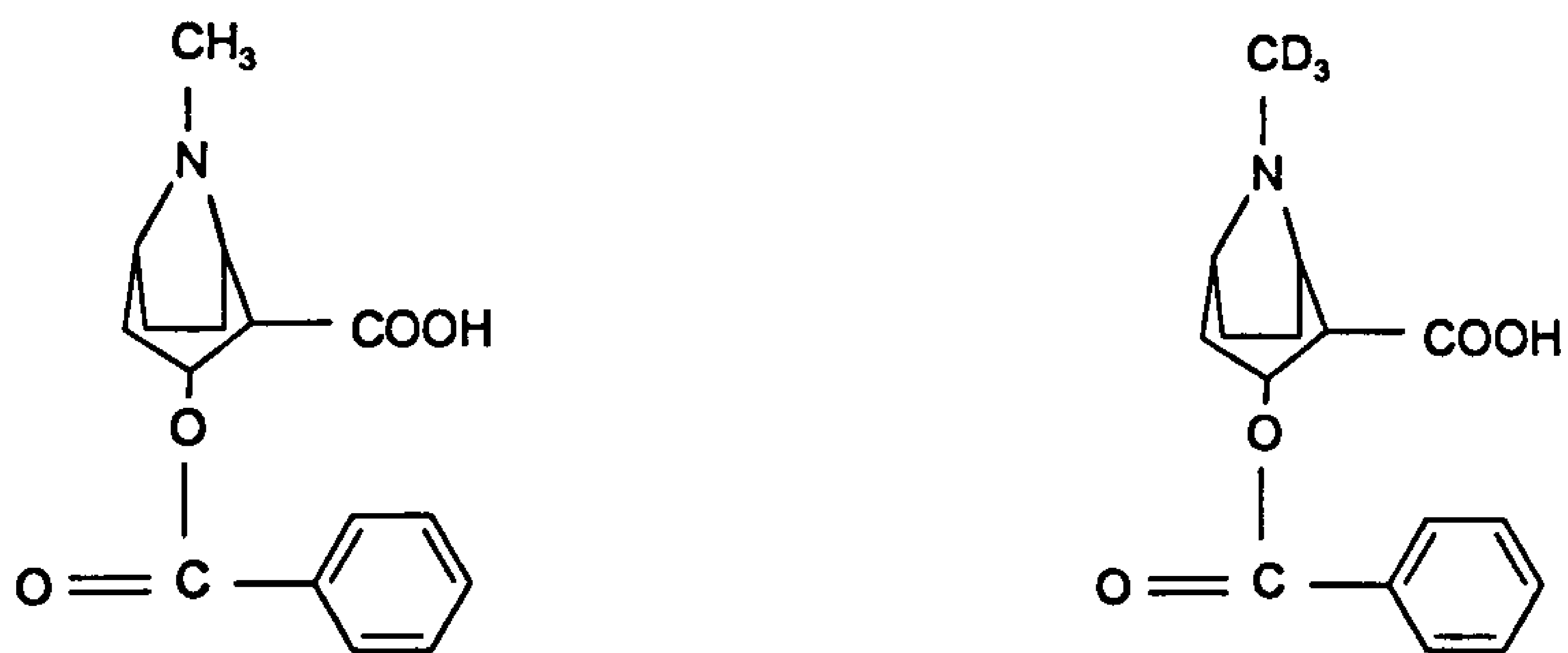


Figure 4-13:- Benzoylecgonine and Benzoylecgonine- d_3

Figure 4-14:- THC-OH and THC-OH-d_3

Figure 4-15:- THC-COOH and THC-COOH-d_3

4.4.3 Derivatisation procedures

During the initial studies of derivatisation reactions, standards were reacted individually rather than in mixtures. Aliquots of working standard solution (10 μg/ml, 100 μl) were pipetted into ReactiVials® and evaporated to dryness under a stream of nitrogen, without heating, to avoid evaporation of amphetamines. The residues were then dissolved in the three selected reagents and subjected to microwave heating as described in Section 4.4.4. Reagents were evaluated as follows

a) Acylation/alkylation was carried out with a mixture of PFPA and PFP-OH. Different ratios of PFPA and PFP-OH were prepared (0.5:1, 1:1 and 2:1 v/v) and different volumes of reagent mixture were reacted with the standards (50, 100 and 150 µl). In addition, the effect of adding the reagents sequentially instead of simultaneously was tested.

b) Acylation/methylation (esterification) was carried out with PFPA/TMS-diazomethane. Different volumes of PFPA (50, 100, 150 µl) and TMS-diazomethane (10, 20, 50 µl) were tried and the effect of adding the sequentially was tested.

c) Silylation was carried out with MTBSTFA containing 1% TBDMCS. Different volumes of reagent were tried (20, 30, 50 µl).

The yield of derivatisation reactions was indicated by the peak size of the products when measured by GC-MS. Comparisons were carried out within batches of samples and no internal standards were used except for morphine and benzoylecgonine TBDMS.

4.4.4 Microwave heating

The microwave oven used in this study was a Micro Chef Model ST23W manufactured by Proline, with a maximum power output of 750Watts. The irradiation intensity and length of the irradiation time is dictated by the power level chosen. Vials were arranged on the glass plate in the oven in a circle as shown in (Figure 4-16) to balance their exposure to the microwave radiation.

Figure 4-16:- Microwave Oven

Different power settings and reaction times were evaluated to establish practical conditions for derivatisation. In addition, different types of reaction vial were tested to identify a suitable type for use in the microwave oven, as initial tests found that some types of screw cap were damaged by the oven.

4.4.5 Instrumental methods

After heating in the microwave oven, the vials containing reaction mixtures were then cooled to room temperature and, except for those derivatised with MTBSTFA reagent, evaporated to dryness under a stream of N_2. The derivatised extracts were reconstituted in 50μl of ethyl acetate prior to analysis by GC-MS. MTBDMS derivatives were injected in the reagent.

GC-MS was carried out on a Thermo-Finnigan trace quadrupole instrument operated in EI+ repetitive full scan mode. Operational parameters used were based on those used in the Forensic Medicine and Science routine laboratory, many of which were the standard default settings recommended by the instrument manufacturer. Data was acquired and

processed using Excaliber™ software from Thermo-Finnigan. It was equipped with a fused silica capillary column (HP-5, 30m x 0.32 mm x 0.25 µm film thicknesses) and Supplied by J&W Scientific (Agilent Technologies). Helium was used as the carrier gases at flow rate of 1.2 ml/min. 1µl of samples were injected via a split-splitless injection mode with an injection temperature of 200 °C. The initial column temperature was 100 °C and was programmed to 300 °C at a rate of 10 °C/min. The spectrometer was used in the EI mode with electron energy of 70 eV and source temperature 200 °C. The emission current was 180µA with detector voltage 350V, as shown in Table 4-1.

Table 4-1:- Conditions for GC-MS

Parameter	Setting
Column	HP-5 column, 30 m x 0.32 mm x 0.25 µm film thickness. Supplied by J&W Scientific (Agilent Technologies)
Carrier Gas	Helium, 1.2 ml/min
Injector Temperature	280 °C
Injection volume	1µl,
Injection mode	split –splitless, purge time 0.7 min
Temperature Programme	100 to 300 °C at 10 °C / minute
Interface temperature	200°C
Electron energy	70 eV
Source temperature	200°C
Emission current	180µA
Detector voltage	350V

4.5 Results and Discussion

4.5.1 Microwave assisted derivatisation procedures

Microwave heating instead of a conventional heating block was used to assist derivatisation. It required less time to achieve complete derivatisation.

Initial tests of the microwave oven indicated that the conditions applied should be suitable for the vials and vial caps, particularly the power setting used. During trials of the procedure, it was found that conventional vial caps such as Bakelite caps (used on the standard laboratory ½-Drachm and 1-Drachm vials) or autosampler caps (used on screw-top autosampler vials) may melt at high power settings. Metallic crimp-top vials could not be used in a microwave oven. This problem was solved by using resistant ReactiVial®

type vials and caps, although these are relatively expensive and require to be re-used rather than being treated as disposable.

A disadvantage of using the microwave was that the distribution of power was uneven and the arrangement of the vials on the glass plate in the oven was found to be important. Regular laboratory test tube racks manufactured using plastic-coated wire could not be used because the plastic coating melted in the oven. However, ReactiVials are of heavy glass construction and were found to be stable in the oven without any additional support. It was found to be convenient to arrange the vials in a circle shape as shown in (Figure 4-16) so that the temperature distribution was balanced, which would help the derivatisation reaction.[263-265]

The use of a microwave oven instead of a conventional heating block gave a significant saving of time in the derivatisation step since tests showed that derivatisation reactions with all three reagents were complete in 1 minute instead of 30 minutes (usually) in a heating block. However, there were also disadvantages of using the microwave oven.

One disadvantage was that expensive vials were needed with caps that were able to resist the effects of the oven. Tests carried out with conventional vials such as those used in autosamplers showed that the caps melted or were damaged leading to loss of reagents by evaporation. The only suitable vials which were found were ReactiVials™ which are heavy-duty vials with thick bases and which cost several pounds each. This cost would prevent the vials being disposed of after use, which would lead to the risk of cross-contamination in forensic toxicology casework and which would be unacceptable unless a quality-controlled cleaning system was introduced by which vials were checked before use.

Another disadvantage was that volatile reagents were lost due to evaporation, even when ReactiVials were used. As a result, relatively large volumes of PFPA and PFPOH reagents was required so that the reaction mixture did not dry our during the derivatisation step. The use of 100 microlitres of this reagent mixture per sample would also be expensive for routine toxicology. This problem might be solved by adding a "keeper" solvent with a high boiling point, which has been done in the past when volatile analytes such as amphetamines are analysed, to prevent losses by evaporation. One example is the addition of dimethylformamide to SPE extracts of amphetamines before they are evaporated to a low volume.[231]

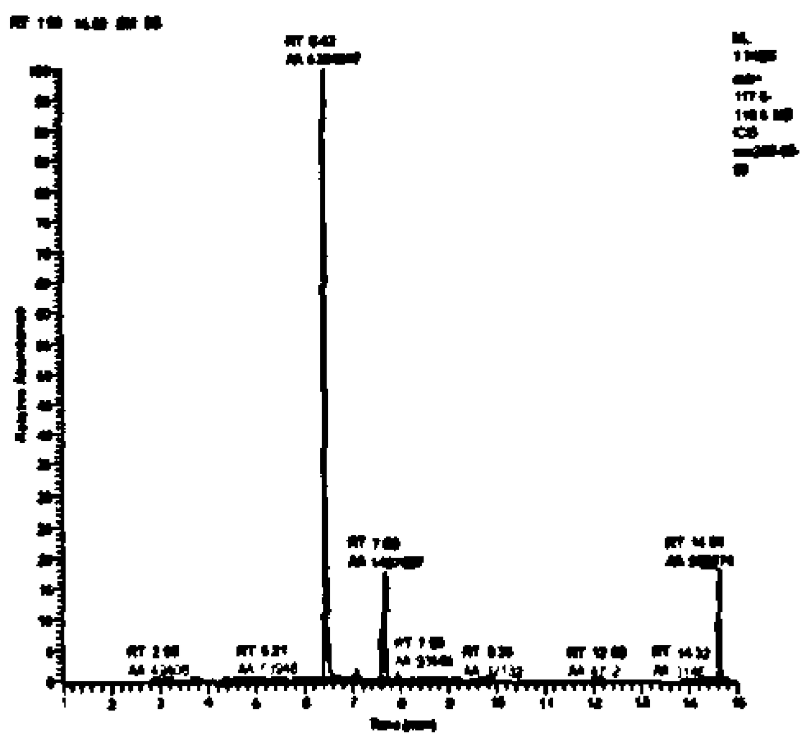
The aim of the investigation was to optimise the time for complete derivatisation especially for some drugs such as THC-COOH, which requires the derivatisation of two functional groups. The temperature, power and time of irradiation were investigated. The time to derivatise was varied from 1 to 5 minutes. It was found that using 3 or 5 minutes at the high power setting affected the integrity of the caps and the sample was lost. The optimum time was found to be 1 minute at the high power setting (750 Watts), which was found to be sufficient for all of the reactions which were under evaluation. Longer derivatisation time periods did not lead to higher yields of derivatives, based on the peak sizes of the products, but could lead to evaporation of the reagents. These settings were therefore retained for the rest of the study.

Based on the trails of different reagent mixture compositions, the following were found to be the optimum amounts.

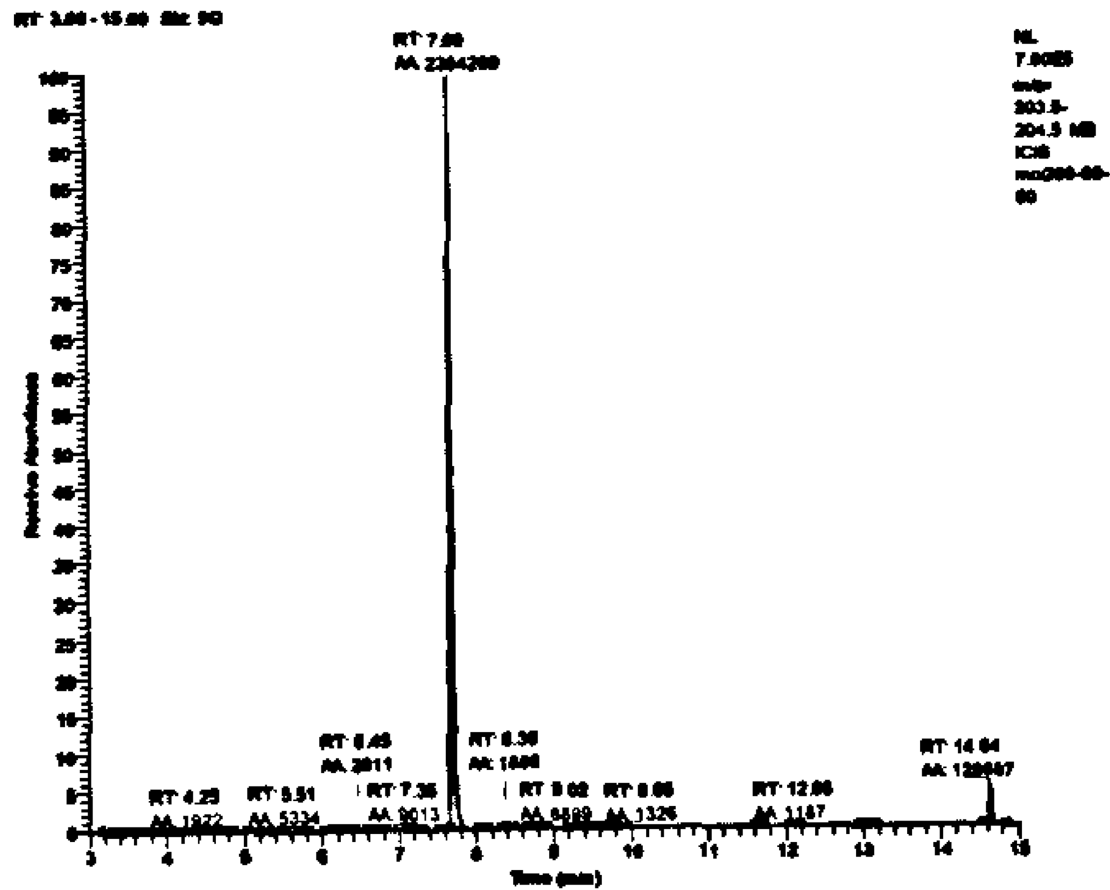
a) Acylation/alkylation:

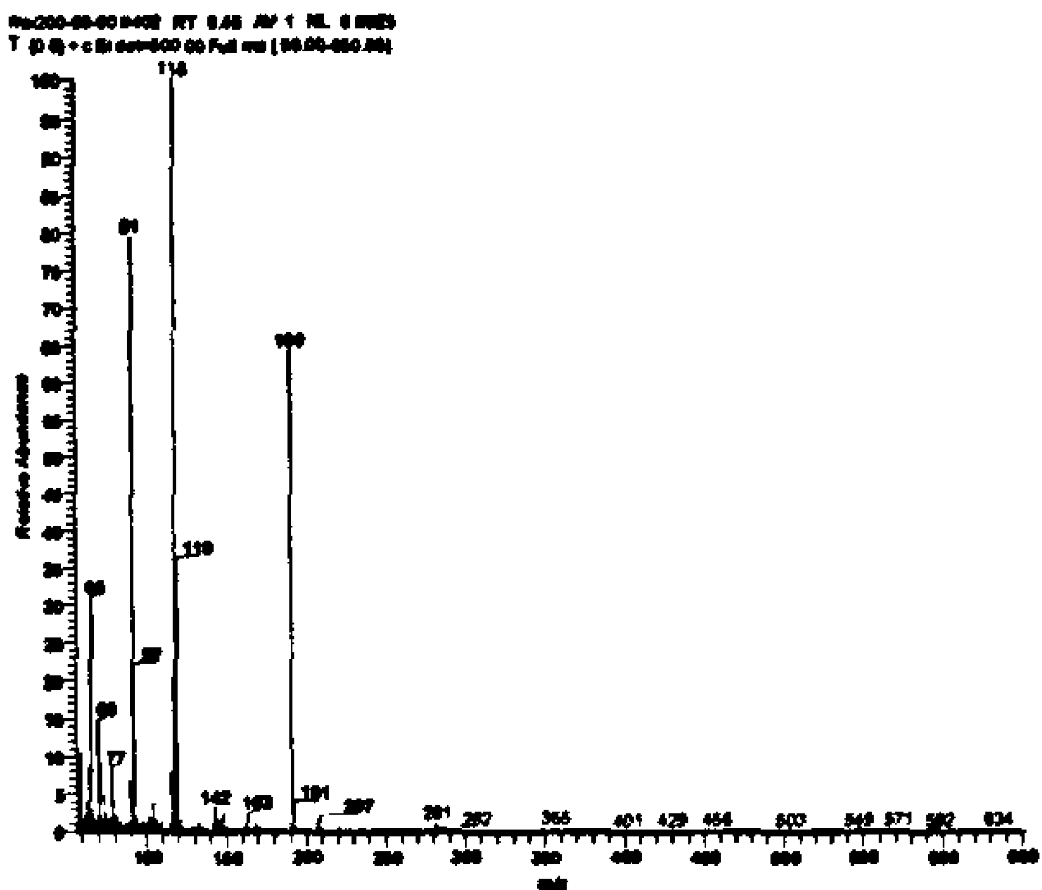
Acyl derivatives were formed using 100 μl PFPA and 50 μl PFP-OH. Better results were obtained when these reagents were added individually and consecutively, beginning with PFPA, which reacts with alcohols and amines. PFP-OH added subsequently reacts with carboxylic acids. Trials were carried out of different volumes of the reagents as it was important to know the effect of the reagent amount on the reaction. Chromatograms were recorded of. the derivatives formed by the test drugs after reaction with 200μL PFPA, 50 μL PFP-OH and 60 μL ethyl acetate, which showed that all the drugs reacted well except THC-OH and THC-COOH (Figure 4-17).

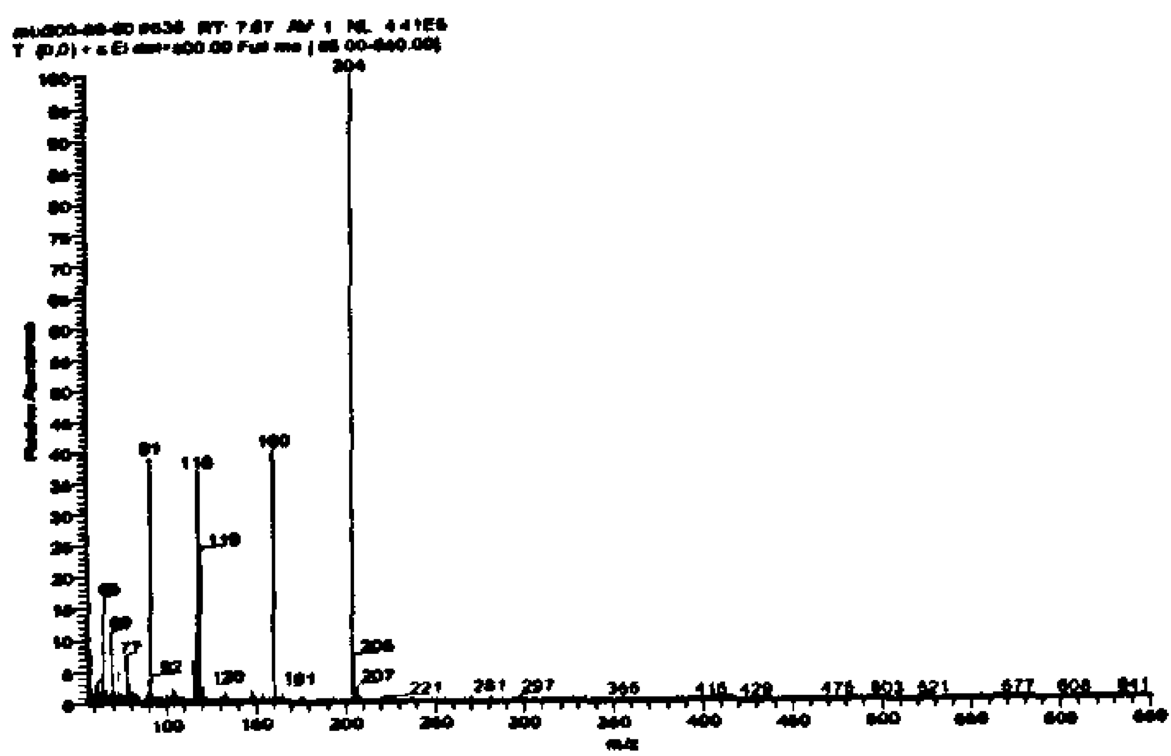
Amp



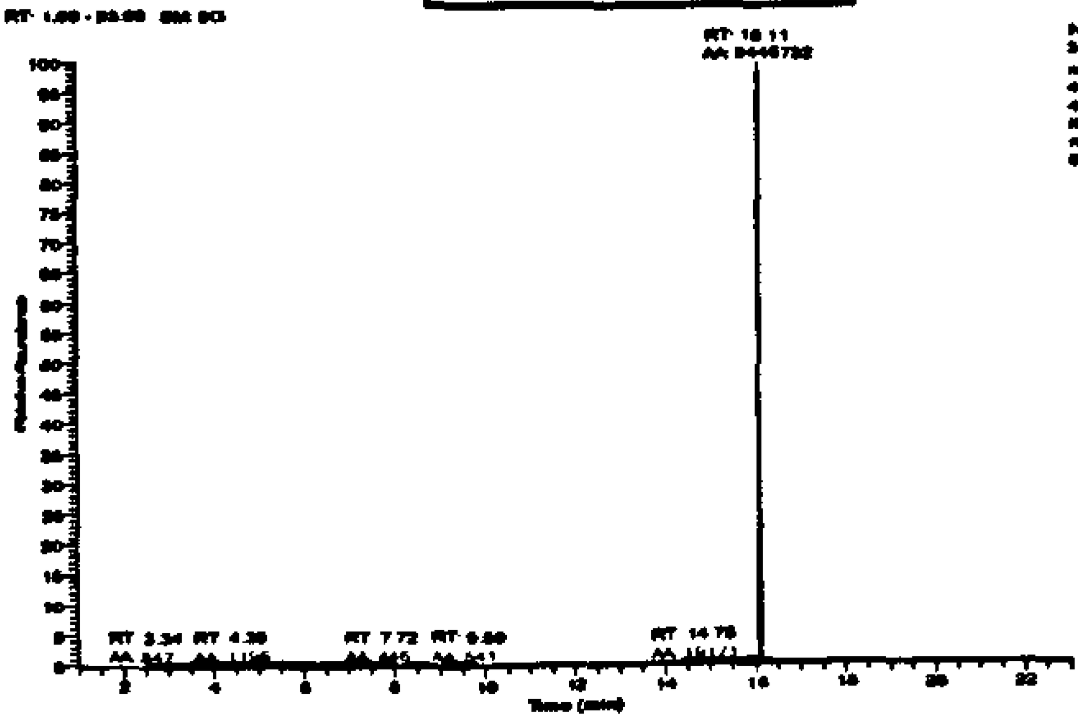
Meth



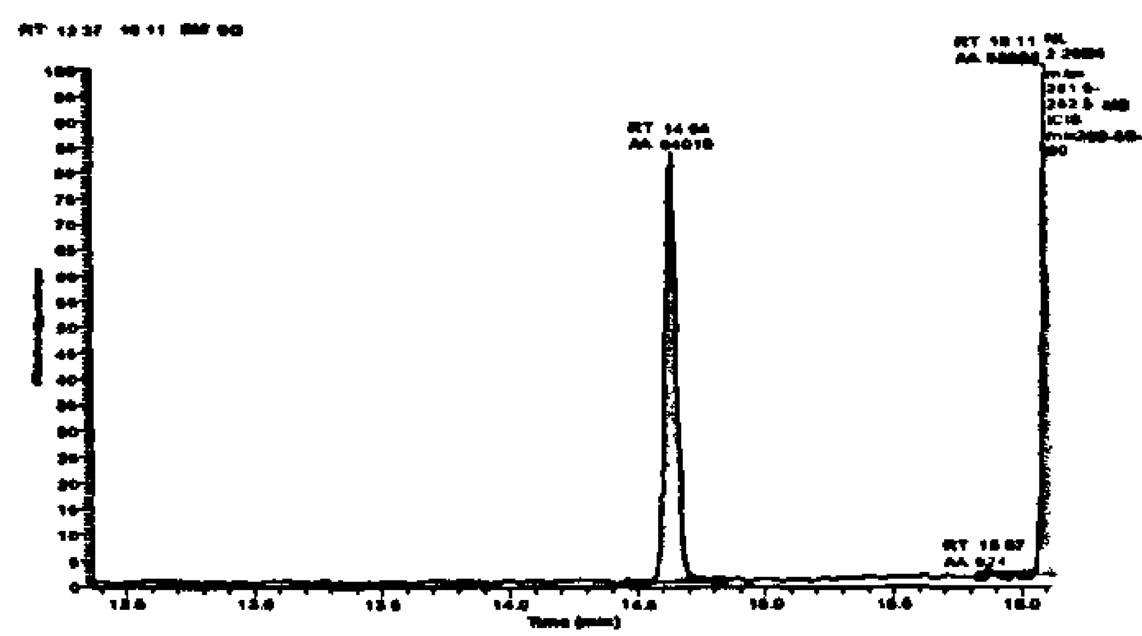


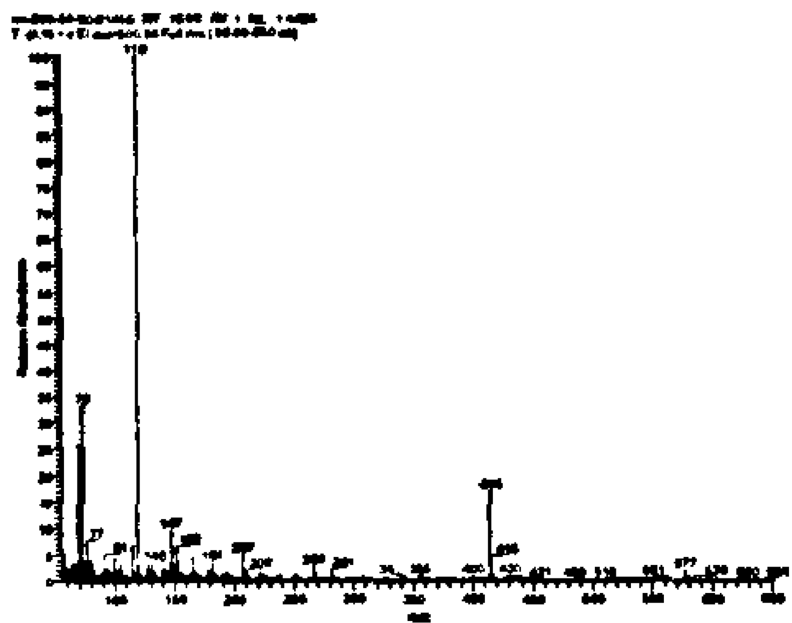


Mor



Bze





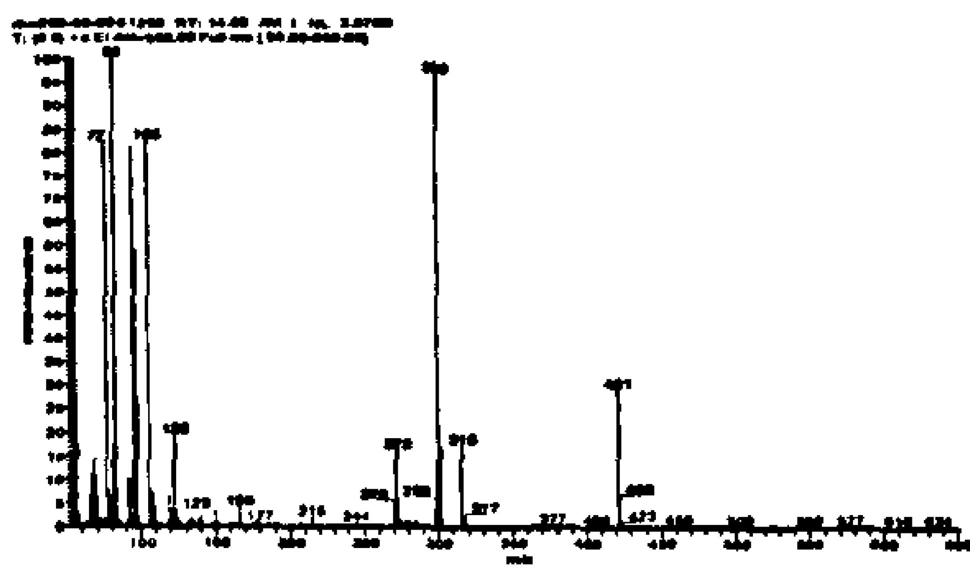


Figure 4-17:- TIC Chromatograms and Mass Spectra for test drugs following reaction with PFPA, PFP-OH and ethyl acetate.

b) Acylation/methylation (esterification):

Acyl derivatives were formed using 100 µl PFPA and methylation was subsequently carried out using 20 µl TMS-diazomethane in hexane (2M solution).

c) TBDMS derivatives:

These were prepared using 30 µl of MTBSTFA reagent containing 1% TBDMS, which was enough to dissolve the sample for derivatisation in the microwave without evaporation to dryness during the heating process.

4.5.2 GC-MS analysis of derivatives of model compounds

A summary of the products obtained using the three derivatisation reagents with each of the model compounds is given in Table 4-2 .The derivatives formed by the deuterated internal standards are similar to those formed by the unlabelled compounds and have similar retention times, which were only 1-2 seconds less than those of the unlabelled compounds. The chromatography and fragmentation patterns observed in their mass spectra are discussed in detail below.

Table 4-2:- Results of derivatisation reactions

Model Compound	Reagent	Product	Retention Time
Amphetamine (AM)	PFPA/PFP-OH	AM-PFP	4.54
	PFPA/TMS-diazomethane	AM-PFP	4.54
	MTBSTFA	AM-TBDMS	6.92
Methamphetamine (METH)	PFPA/PFP-OH	METH-PFP	5.73
	PFPA/TMS-diazomethane	METH -PFP	5.73
	MTBSTFA	None formed	-
Morphine (MOR)	PFPA/PFP-OH	MOR-bis-PFP	13.98
	PFPA/TMS-diazomethane	MOR-bis-PFP	13.98
	MTBSTFA	BZE-bis-TBDMS	14.76
Benzoylecgonine (BZE)	PFPA/PFP-OH	BZE-PFP ester	12.36
	PFPA/TMS-diazomethane	Cocaine	13.98
	MTBSTFA	BZE-TBDMS ester	15.86

THC	PFPA/PFP-OH	THC-PFP	13.01
	PFPA/TMS-diazomethane	THC-PFP	13.01
	MTBSTFA	THC-TBDMS	15.86
THC-COOH	PFPA/PFP-OH	THC-COOH-PFP PFP ester	14.35
	PFPA/TMS-diazomethane	THC-COOH-PFP methyl ester	15.29
	MTBSTFA	THC-COOH-bis-TBDMS	19.33

4.5.3 PFPA/PFP-OH Derivatives:

Derivatisation was successful for all of the test compounds and mass spectra were obtained for each of them. These derivatives gave good gas chromatographic behaviour, in terms of peak shape and thermal stability, and the mass spectra had prominent molecular ions and also prominent diagnostic fragment ions at high mass, suitable for use as qualifier ions in selected ion monitoring GC-MS. The ions used for the identification and quantification are shown in Table 4-3.

The optimum derivatisation reagent composition was regarded as the one which produced a good chromatographic peak with the correct fragmentation pattern. These reagents react with alcohols, amines and phenols (Figure 4-18, Figure 4-19, Figure 4-22 and Figure 4-26). PFPA will replace the proton (H^+) of a functional group with $-C_3F_5O$. In the case of benzoylecognine and THC-COOH, which contain carboxylic acid groups (Figure 4-24 and Figure 4-28) the reaction will be with PFP-OH with addition of $-C_3H_2F_5$.

Table 4-3:- Selected Ions used for GC/MS-SIM with PFPA-PFP-OH

Target analyte	Retention time (min)	Qualifier Ions (m/z)	Internal Standard	Selected Ions (m/z)
Amphetamine	4.51	118	Amp-D_5	122
Methamphetamine	5.71	204	Meth-D_5	208
Morphine	13.86	414	Mor-D_3	417
Benzoylecgonine	12.43	82	Bze-D_3	85
THC-OH	12.94	417	THC-OH-D_3	420

THC-COOH	14.35	459	THC-COOH-D_3	462

(a) Amphetamine and Methamphetamine:

The mass spectra of amphetamine and methamphetamine PFP derivatives are shown in Figures 4-17 and 4-18.

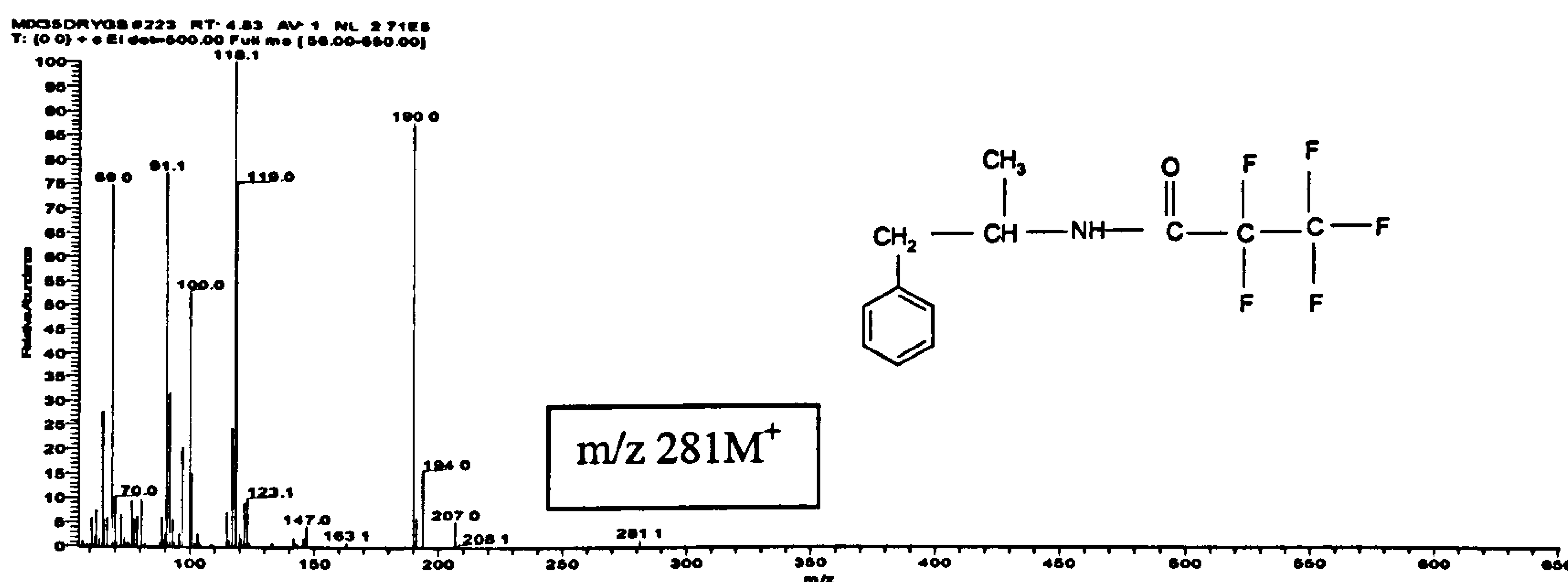


Figure 4-18:- Mass Spectrum of Amphetamine –PFP

The molecular ions for amphetamine and methamphetamine PFP derivatives are at m/z 281 and m/z 295 respectively. These molecular ions fragment as shown in Figures 4-19 and 4-20. Amphetamine and methamphetamine PFP derivatives produce base peaks at m/z 118 and m/z 204 repectively and correspond to the fragments $[C_6H_5\text{-}CH_2\text{-}C_2H_3]^+$ for amphetamine and $[CH\ CH_3\ NCH_3\ C_2OF_5]^+$ for methamphetamine. PFP derivatives of both amphetamine and methamphetamine share a fragment ion at m/z 119 which corresponds to the loss of C_3F_5ONH and $C_4H_3F_5NO$ for amphetamine and methamphetamine respectively.

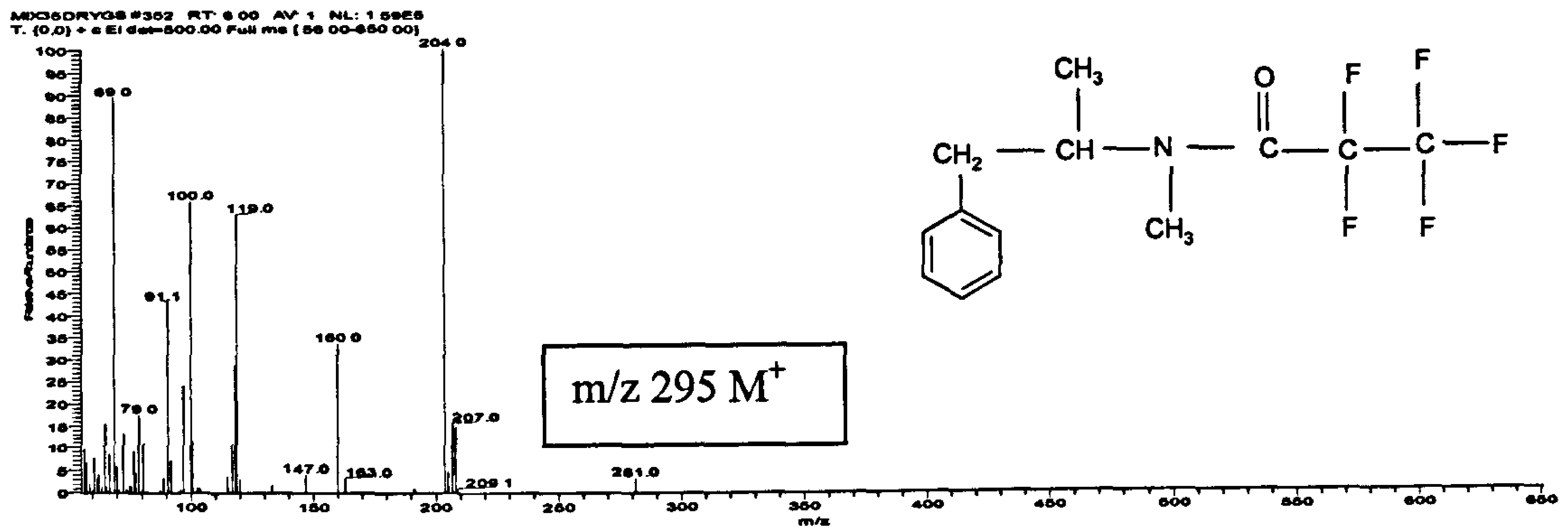


Figure 4-19:- Mass Spectrum of Methamphetamine –PFP

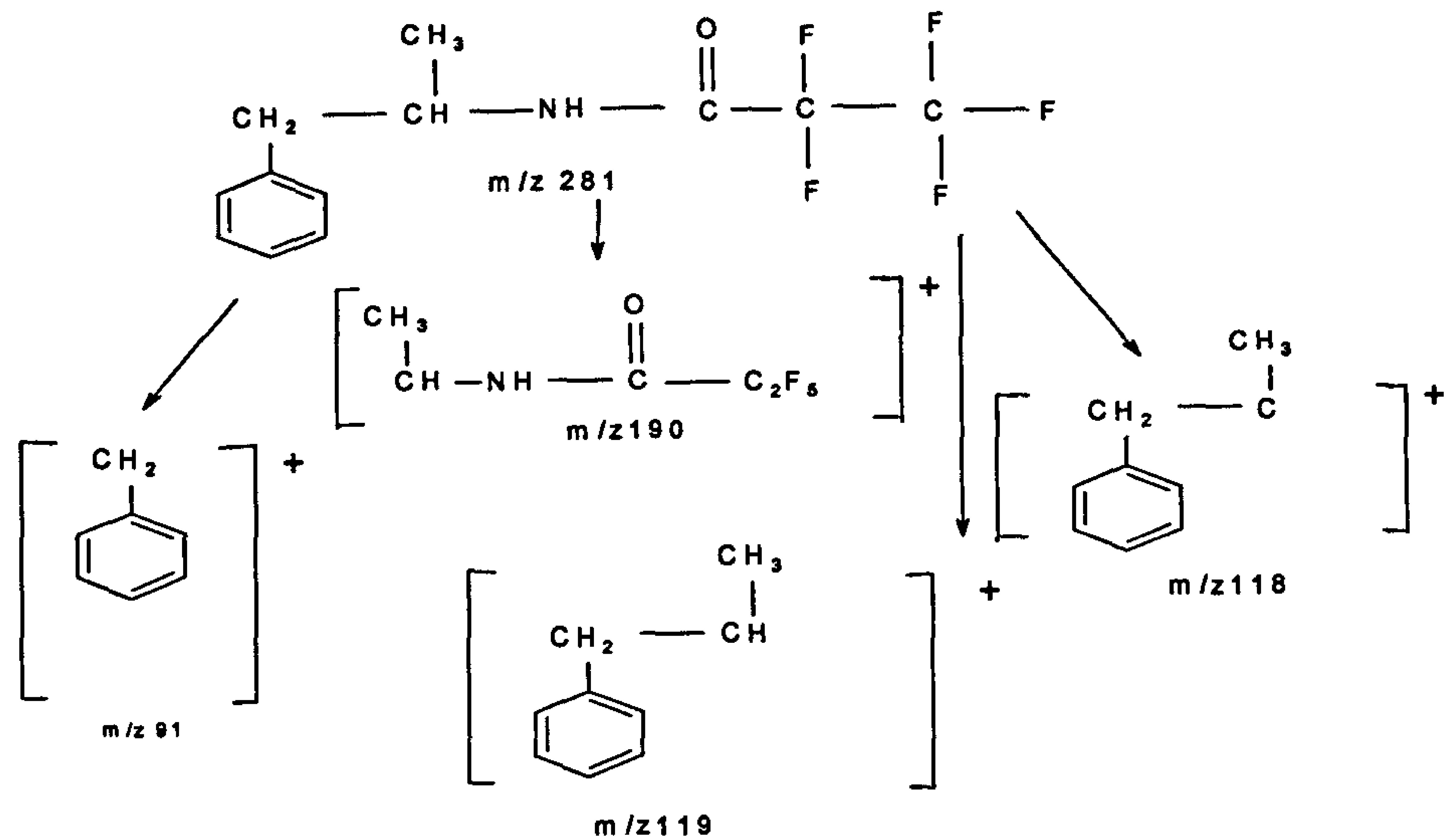


Figure 4-20:- Fragmentation of Amphetamine PFP derivative

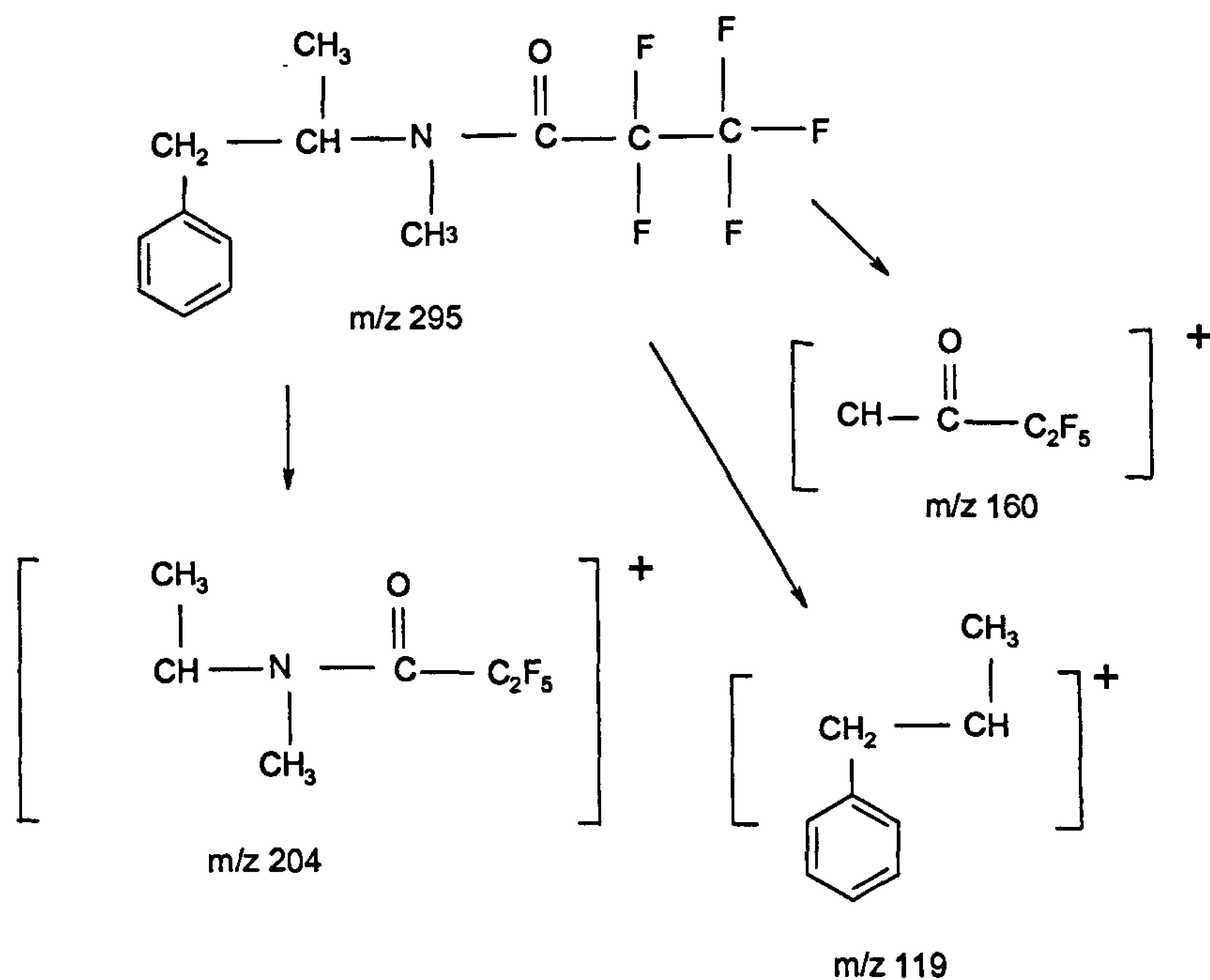


Figure 4-21:- Fragmentation of Methamphetamine PFP derivative

The fragmentation reactions can therefore be classified according to where the cleavage occurs in the molecule: amphetamine derivatives show a typical alpha cleavage reaction adjacent to the carbon holding the nitrogen atom (charge retention on nitrogen) as well as a charge migration reaction resulting in cleavage between the carbon atom and the nitrogen atom plus loss of 1 proton Table 4-3:

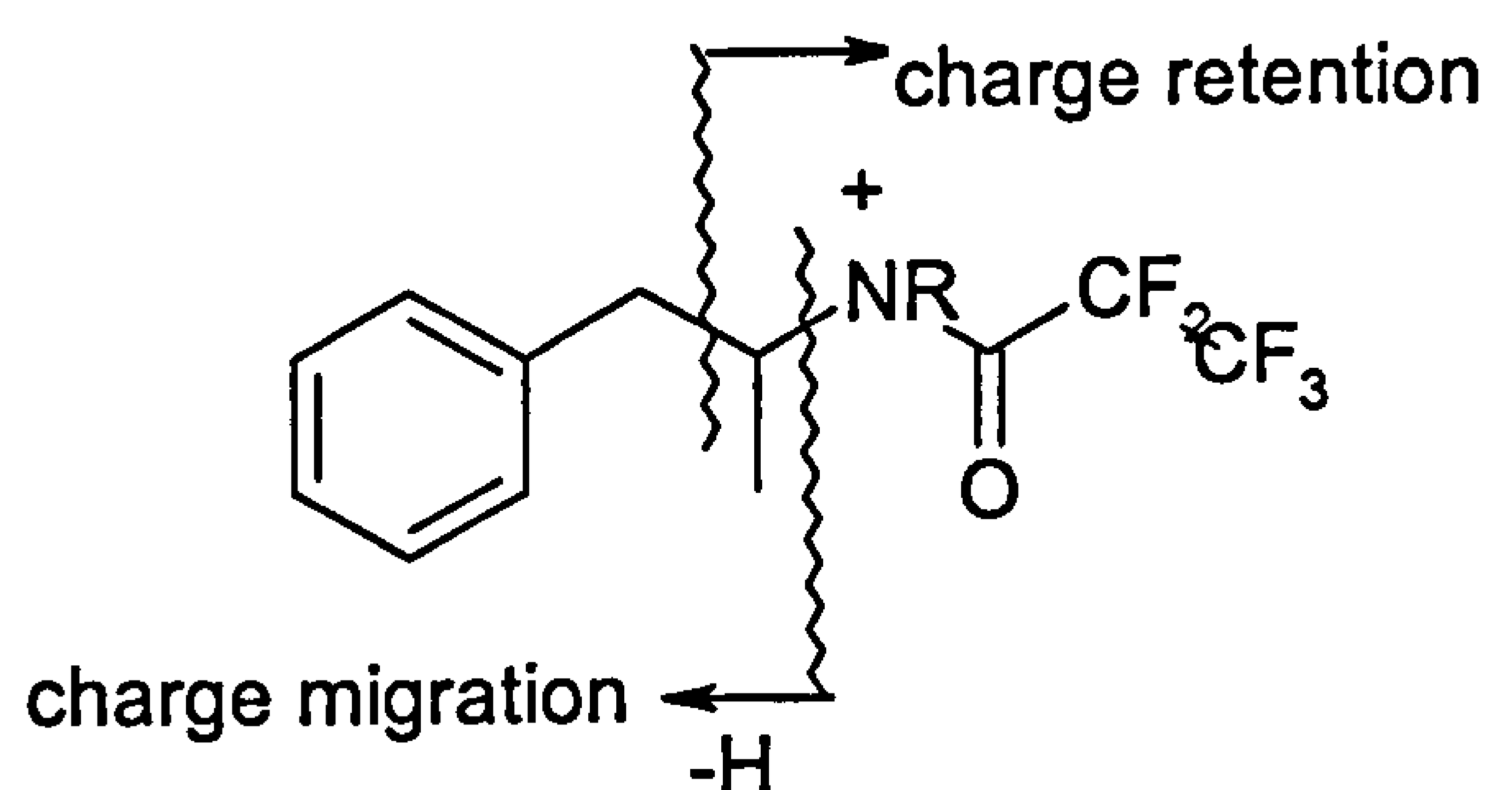


Table 4-3:- retention and migration of charge

Compound	Reaction	
	Charge retention	Charge migration
AM	190	118
AM-d5	194	122
METH	204	118
METH-d5	208	122

Fragment ions at m/z 118, 122, 204 and 208 were selected for use as quantitation ions in subsequent work.

(b) Morphine:

The mass spectrum of morphine is shown in Figure 4-21. Morphine has 2 hydroxyl groups and so will react with PFPA producing a molecular ion at m/z 577. This ion fragments as shown in Figure 4-22. Significant reactions involve loss of the pentafluoropropanoyl ion from the phenolate hydroxyl group and of the pentafluoropropionyl ion from the alicyclic hydroxyl group to give the base peak at m/z 414. This fragment ion and the corresponding one at m/z 417 for morphine-d_3 were used as quantitation ions.

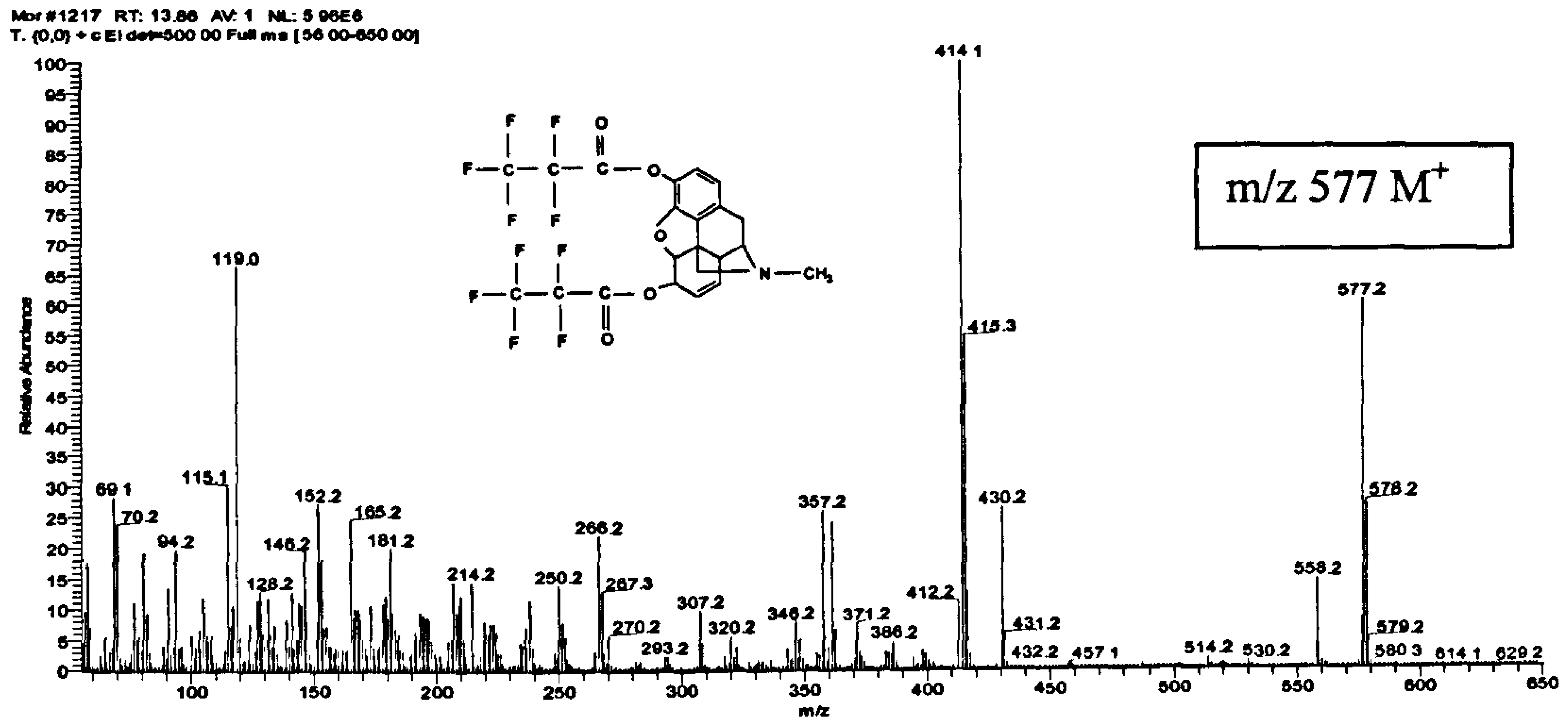


Figure 4-22:- Mass Spectrum of Morphine – PFP

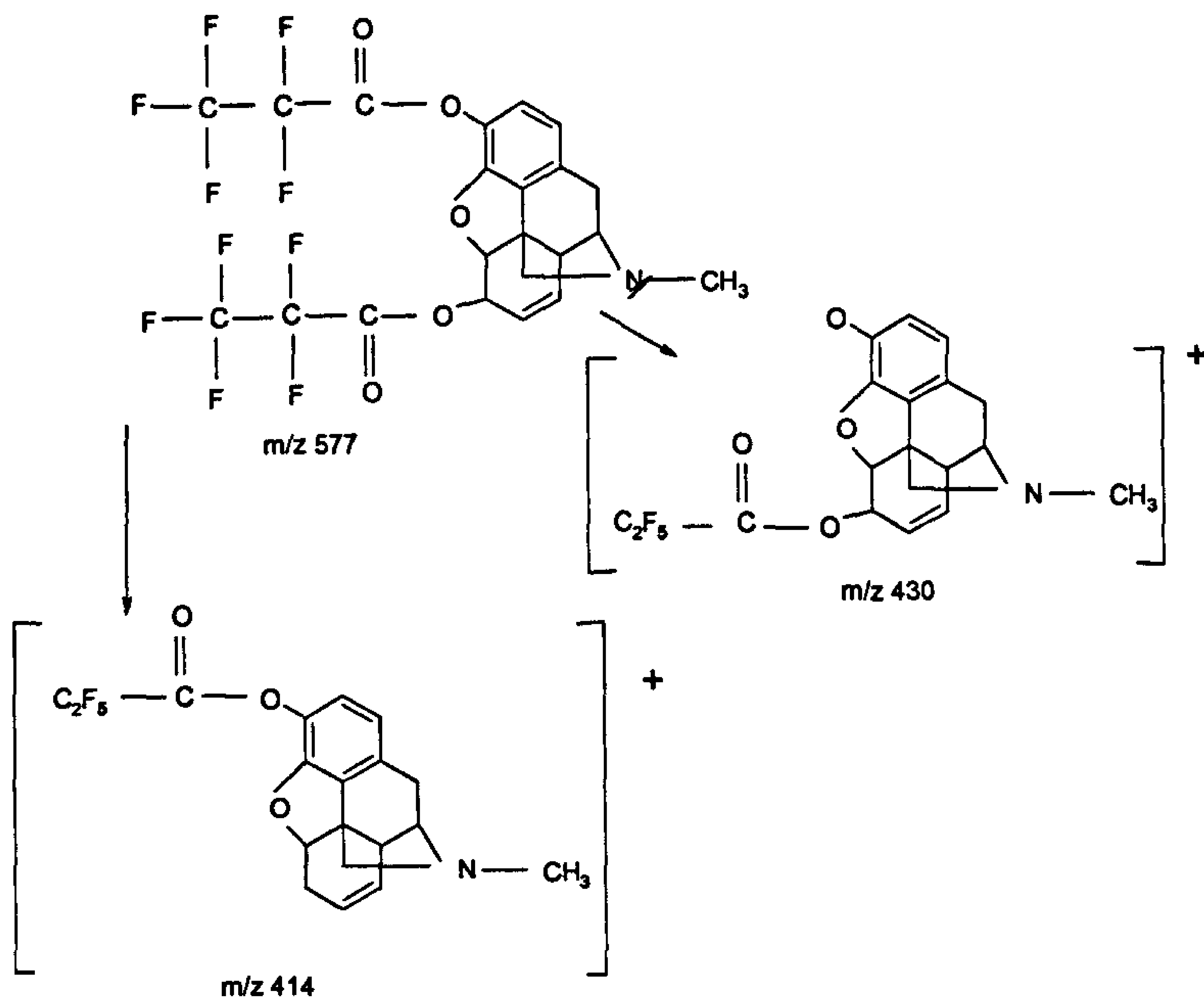


Figure 4-23:- Fragmentation of Morphine PFP derivative

(c) Benzoylecgonine:

Benzoylecgonine has a carboxylic acid group which reacts with PFP-OH to form the corresponding ester with a molecular ion at m/z 421 as shown in Figure 4-24. The molecular ion for benzoylecgonine fragments as shown in Figure 4-25. The base peak is at m/z 82, which consists of part of the ecgonine ring system, and the intense ion at m/z 300 is formed by loss of benzoic acid. Notably, the loss of the pentafluoropropanol fragment is not favoured and gives a relatively weak ion at m/z 272. The base peak ion and the corresponding ion in benzoylecgonine-d_3 at m/z 85 were used as quantitation ions.

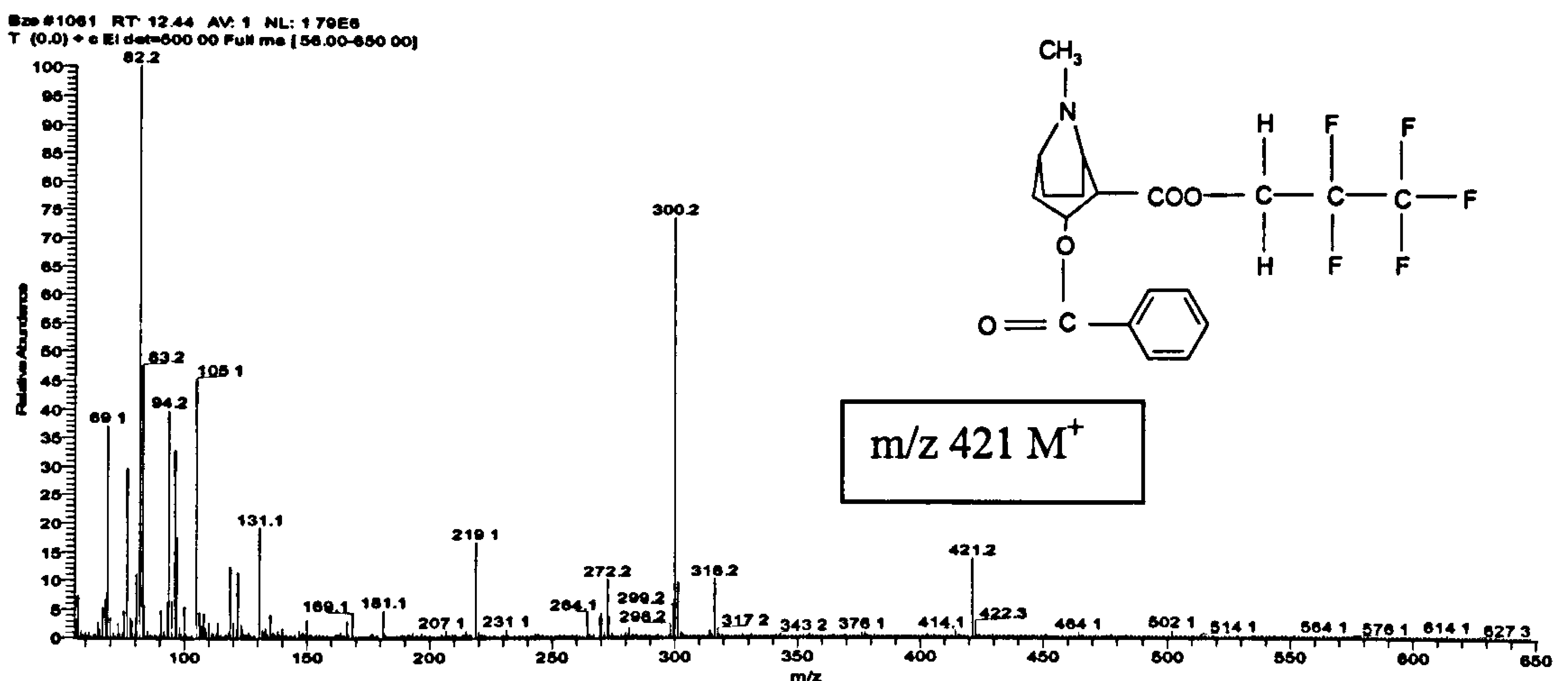


Figure 4-24:- Mass spectrum of Benzoylecgonine –PFP

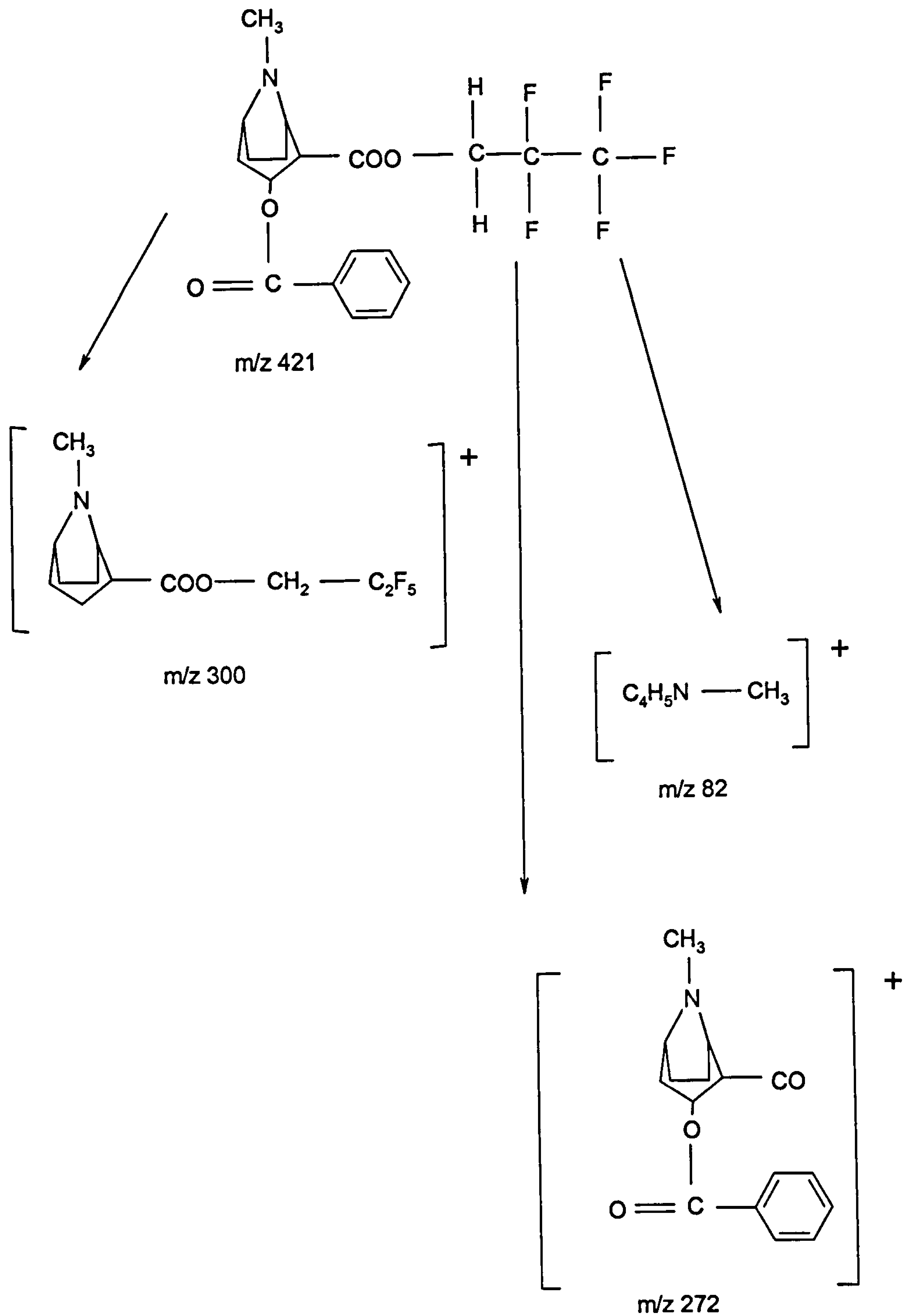


Figure 4-25:- Fragmentation of Benzoylecgonine PFP derivative

(d) THC-OH and THC-COOH

THC-OH has a phenolic hydroxyl group so will react with PFPA producing a PFP derivative with a molecular ion at m/z 460 as shown in Figure 4-26, which is also the base peak. This ion fragments as shown in Figure4-27. A number of strong ions are present at high mass, which are useful as qualifier ions

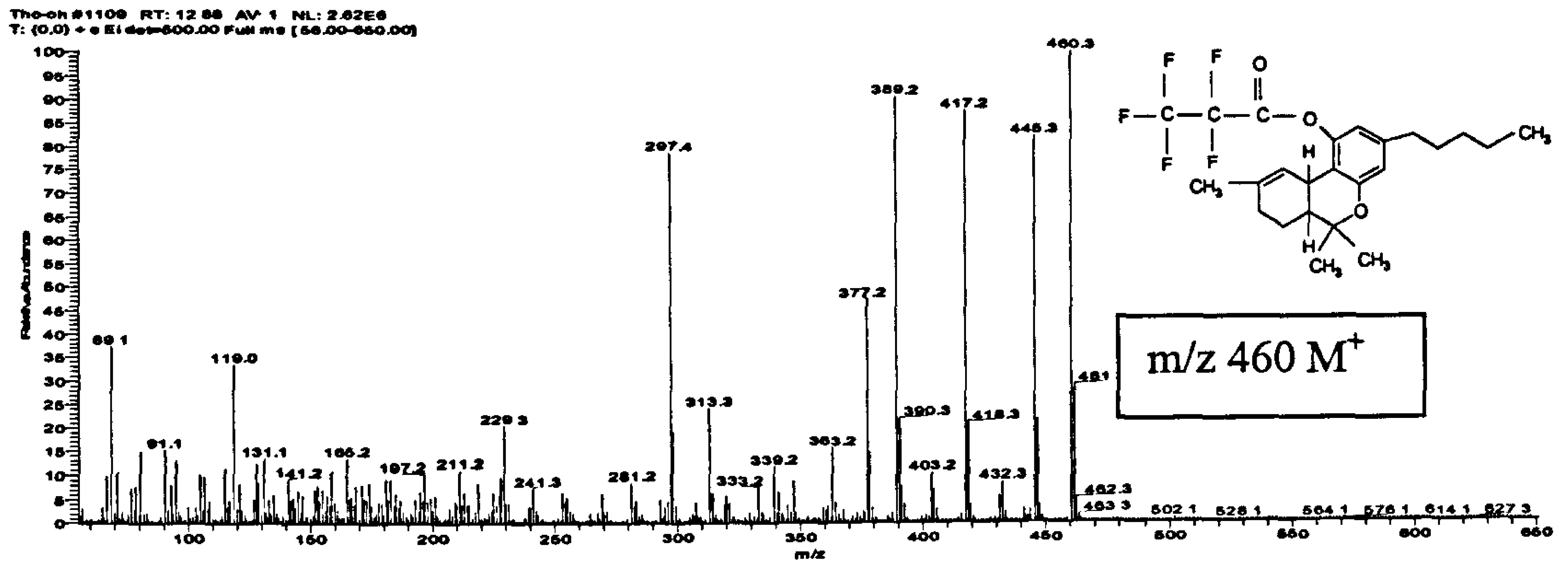


Figure 4-26:- Mass Spectrum of THC-OH-PFP

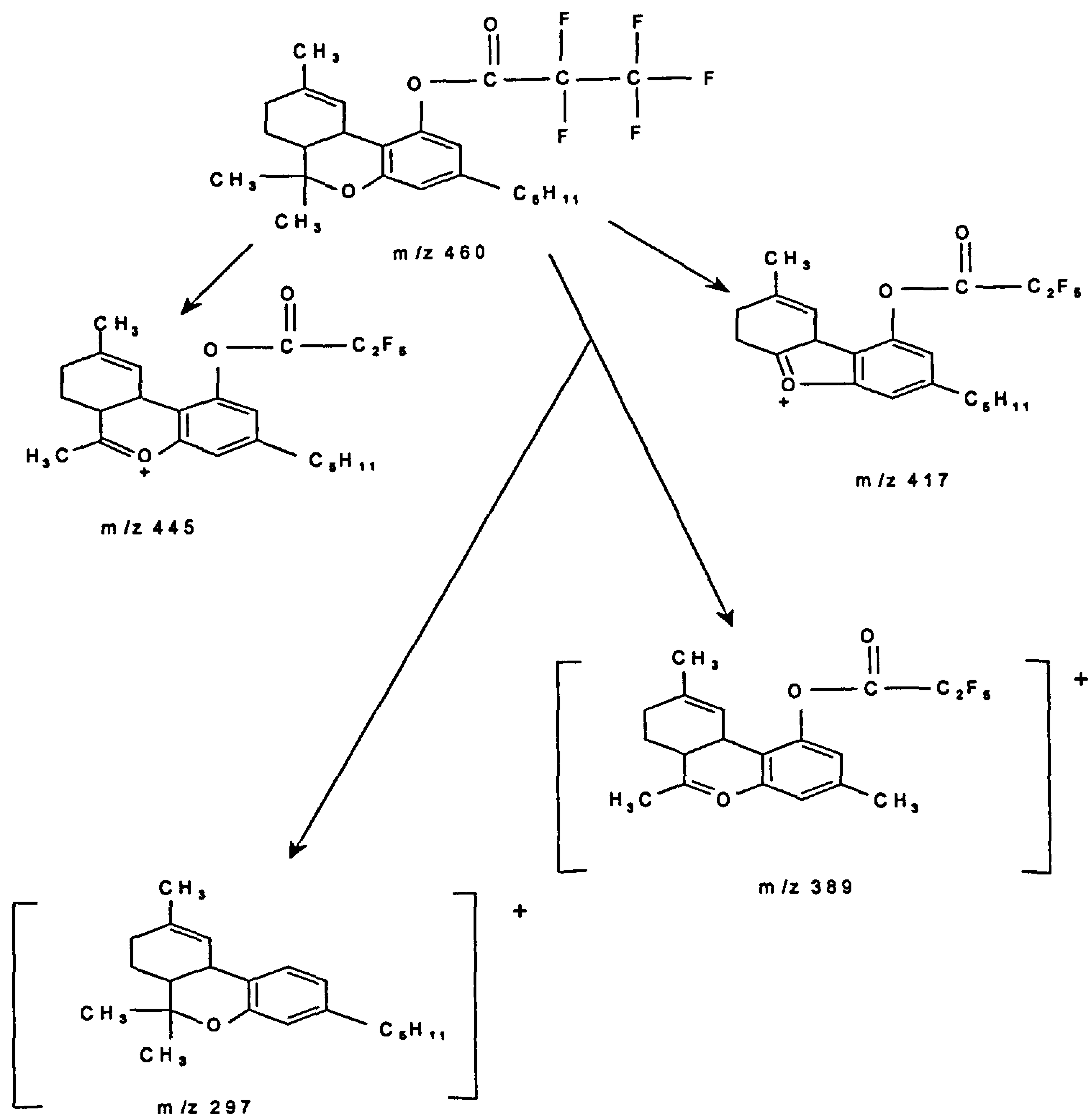


Figure4-27:-Fragmentation of THC-OH PFP derivative

THC-COOH also has a carboxylic acid group which reacts with PFP-OH and PFPA to produce a bis-PFP derivative with a molecular ion at m/z 622 as shown in Figure 4-28. The molecular ion for THC-COOH will fragment as shown in Figure 4-29. The base peak ion at m/z 459 is formed by loss of pentafluoropropanoate, and this ion together with the corresponding ion for THC-COOH-d_3 at m/z 462 was used as quantitation ions.

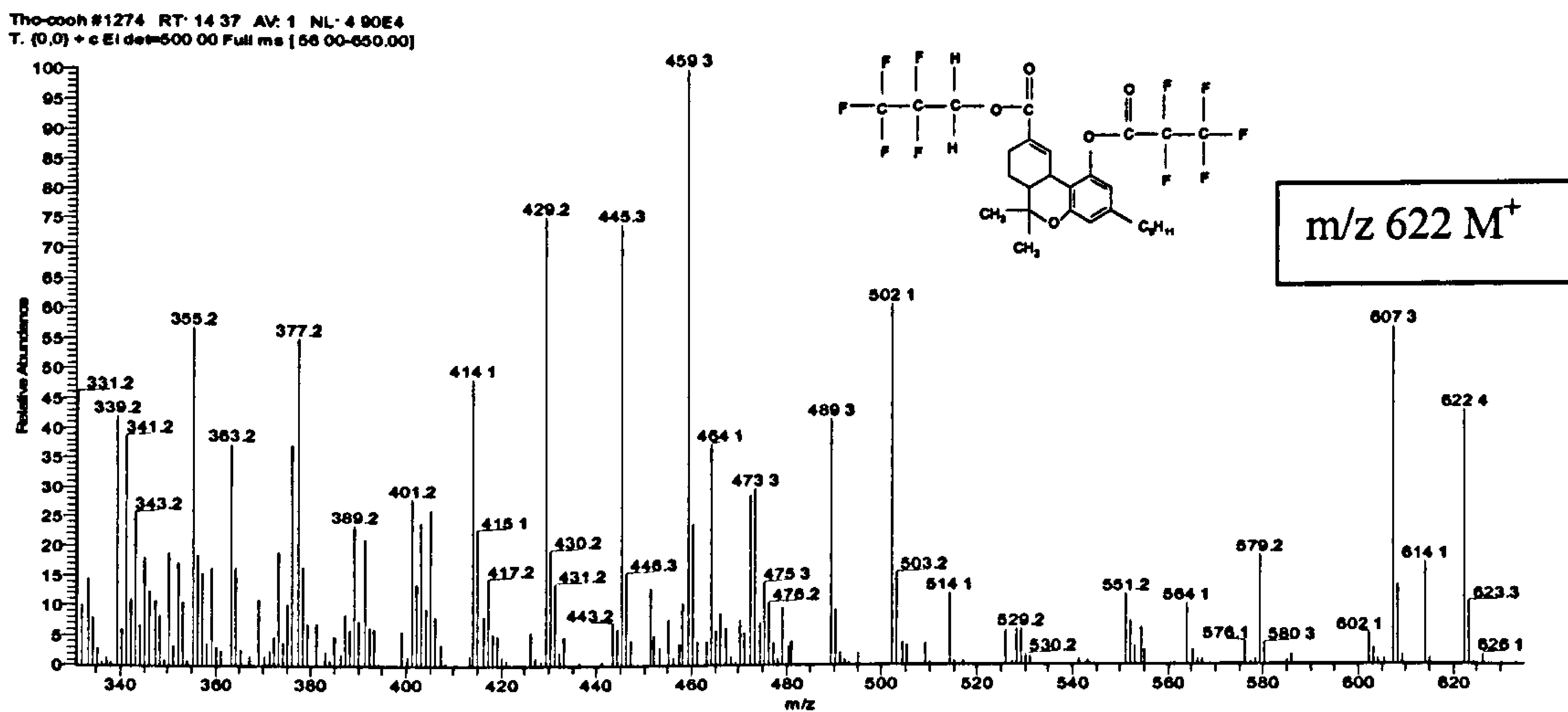


Figure 4-28:- Mass Spectrum of THC-COOH –PFP derivative

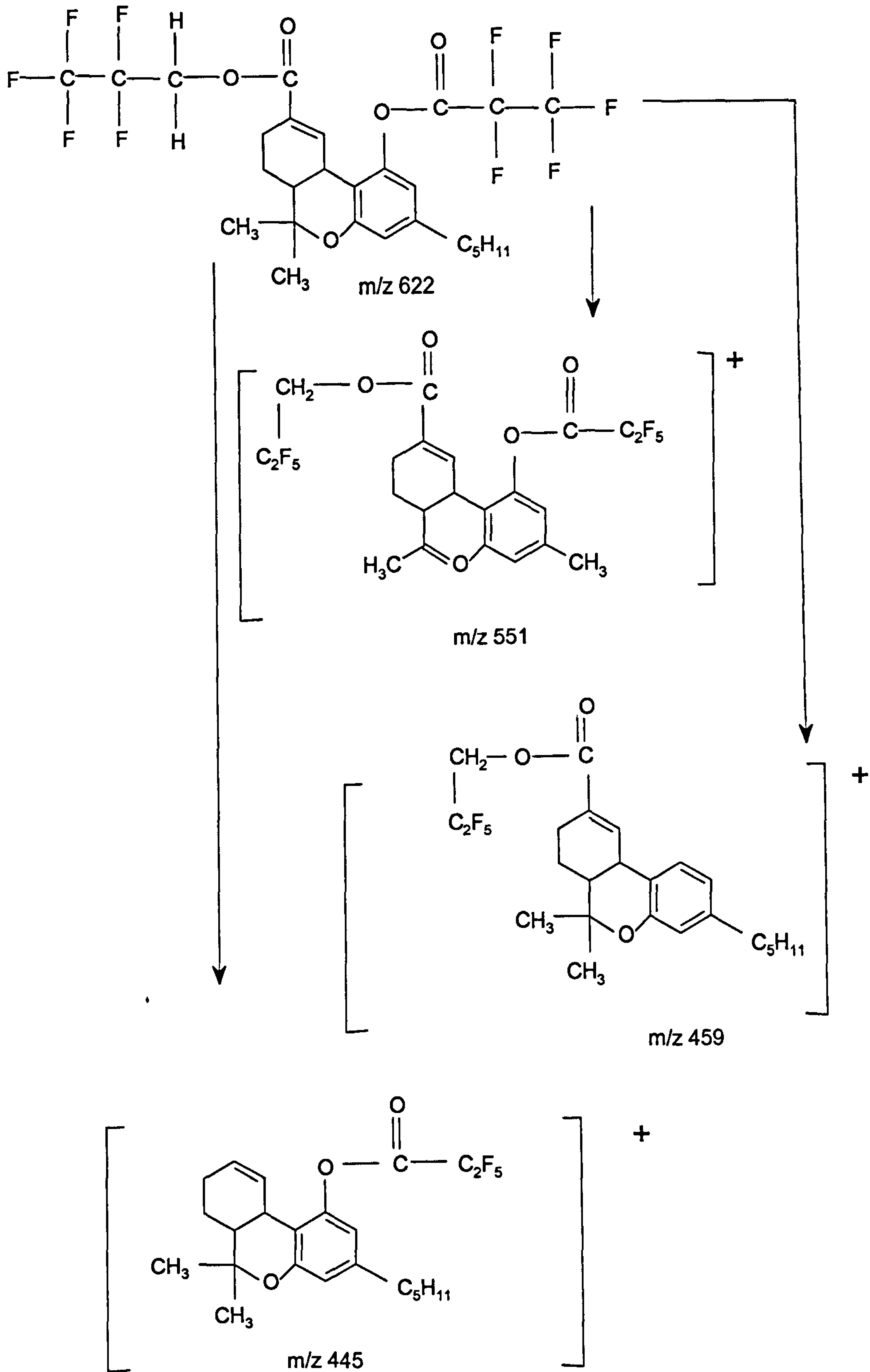


Figure 4-29:- Fragmentation of THC–COOH PFP derivative

4.5.4 PFPA/TMS-diazomethane Derivatives:

The second derivatisation procedure investigated used PFPA and TMS-diazomethane. PFPA does not react with carboxylic acid groups but the novel methylating agent TMS-diazomethane was added in order to convert these to methyl esters. This reaction was found to proceed rapidly and quantitatively at room temperature and holds potential for future use in toxicology to replace diazomethane, a hazardous and toxic material[252, 255].

Trimethylsilyl-diazomethane potentially provides a safer alternative to diazomethane, which is prepared in small batches just before use because of its high toxicity and explosion risk. Diazomethane is an almost ideal reagent for methylation because it is a gaseous reagent used in solution which reacts with carboxylic acids immediately and can even be used with aqueous samples. Trimethylsilyl-diazomethane is available commercially, dissolved in hexane or ether as a 2M solution, and so avoids the hazards and risks of preparing diazomethane in the laboratory. The present study found that a small volume of the reagent (20 microlitres) was sufficient to methylate the test compounds used.

However, the commercial product is not of "derivatisation grade" as are other reagents used for trace analysis and it contains impurities derived from its manufacture. The originally-published method for this involves a Grignard rection in ether or hexane and distillation to obtain a solution of trimethylsilyl-diazomethane.[266] Some of the reaction bi-products and reagents are present in the trimethylsilyl-diazomethane solution which is obtained and these may potentially cause interference. Interferences due to the reagent in toxicological analyses were assessed in the second phase of this study (Chapter 5). However, the reagent has also been advocated for the analysis of acidic pesticides in wastewater.[255] Also, the reagent was used for the analysis of acidic metabolites of the riot control agent 2-chlorobenzylidene malononitrile (CS gas) in post mortem liver samples.[267] In the present study, no interference was found in the GC-MS analysis of the test compounds.

The products of acylation of amines and alcohol remained unchanged from those obtained with PFPA/PFP-OH but the acylated/methylated products also gave good gas chromatography and had mass spectra which are suitable for both identification and quantification by GC-MS-SIM. The ions for use in selected ion monitoring are given in Table 4-4.

Table 4-4:- Selected Ions used for GC/MS-SIM with PFPA-TMS-Diazomethane

Target analyte	Retention time (min)	Selected Ions (m/z)	Internal Standard	Selected Ions (m/z)
Amphetamine	4.51	118	Amp-d_5	122
Methamphetamine	5.71	204	Meth-d_5	208
Morphine	13.86	414	Mor-d_3	417
Benzoylecgonine	12.43	82	Bze-d_3	85
THC-OH	12.94	417	THC-OH-d_3	420
THC-COOH	14.35	489	THC-COOH-d_3	492

Therefore, in this reaction PFPA reacts with all drugs as before (Figure 4-18, Figure 4-19, 4-21 and Figure 4-26) except benzoylecgonine and THC-COOH which contain carboxylic acid groups. These acid groups are derivatised with TMS-diazomethane, substituting a proton from the functional group with a methyl group (Figure 4-36, Figure 4-38).The mass spectra of PFP acyl derivatives of AM, METH, MOR and THC are not repeated here but specimen chromatograms are given in Figure 4-30 to Figure 4-35, which illustrate the symmetrical peak shapes obtained with this reagent.

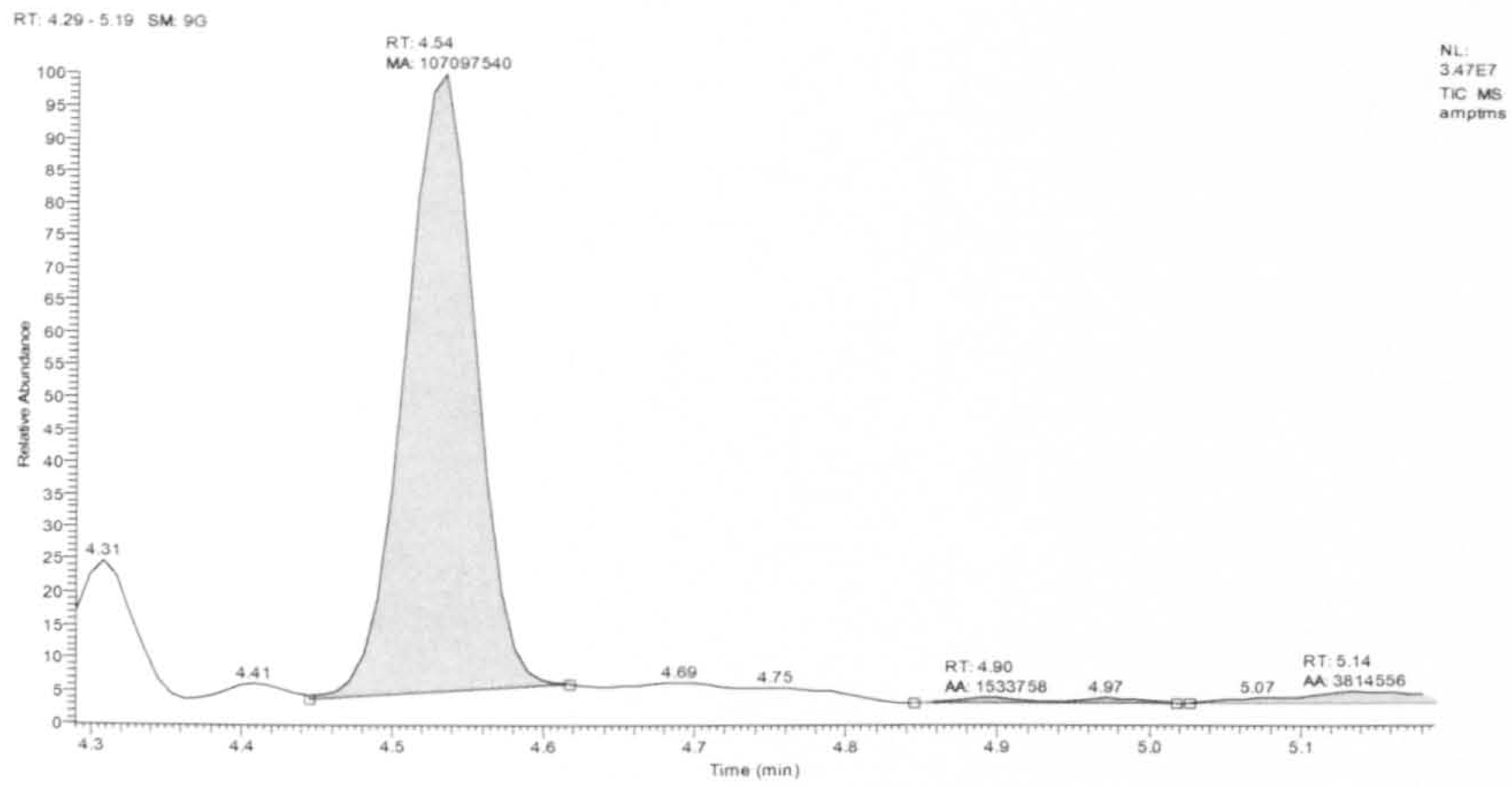


Figure 4-30:- TIC chromatogram of amphetamine PFP derivative

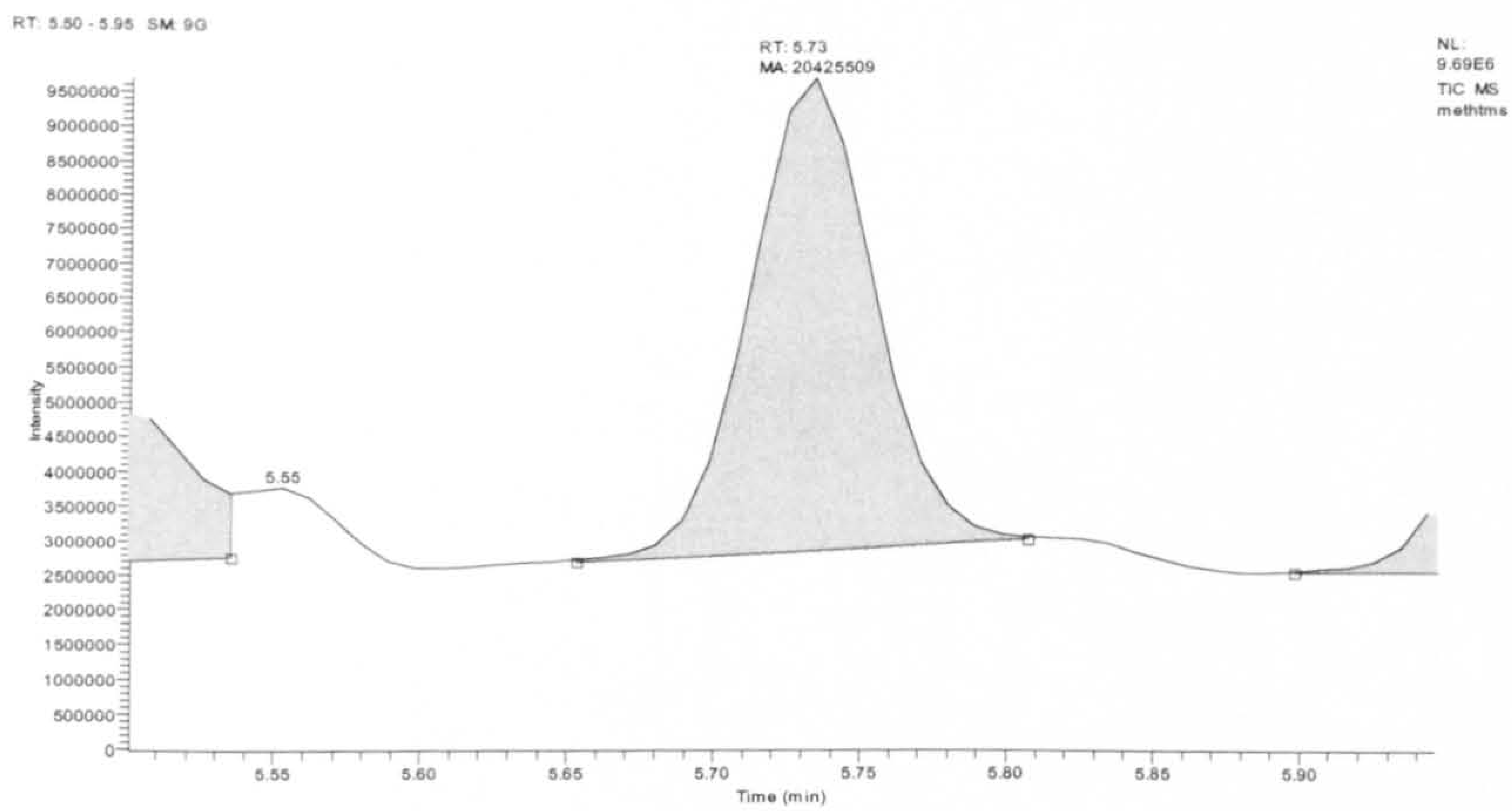


Figure 4-31:- TIC chromatogram of methamphetamine PFP derivative

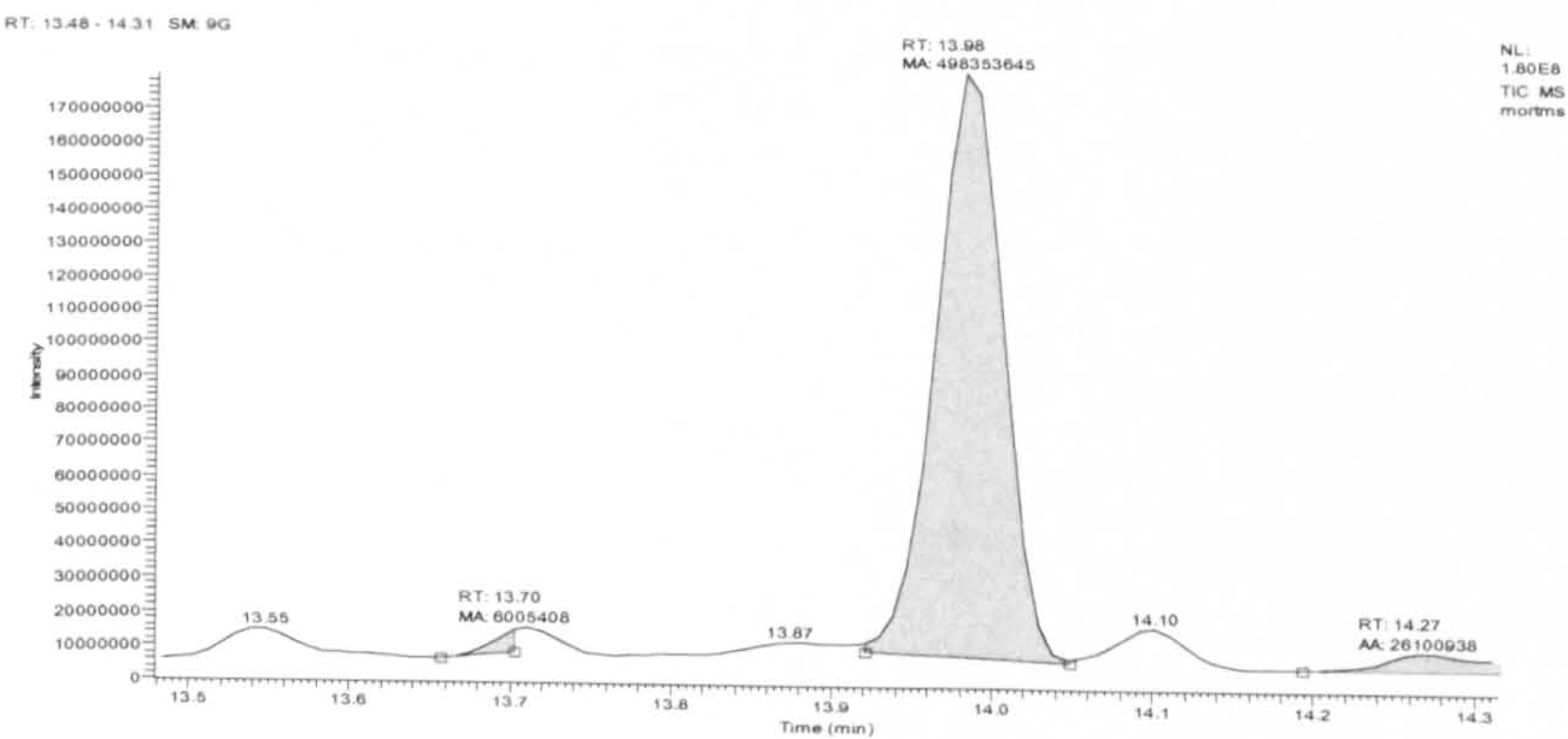


Figure 4-32:- TIC Chromatogram and Mass Spectrum for Morphine-PFP

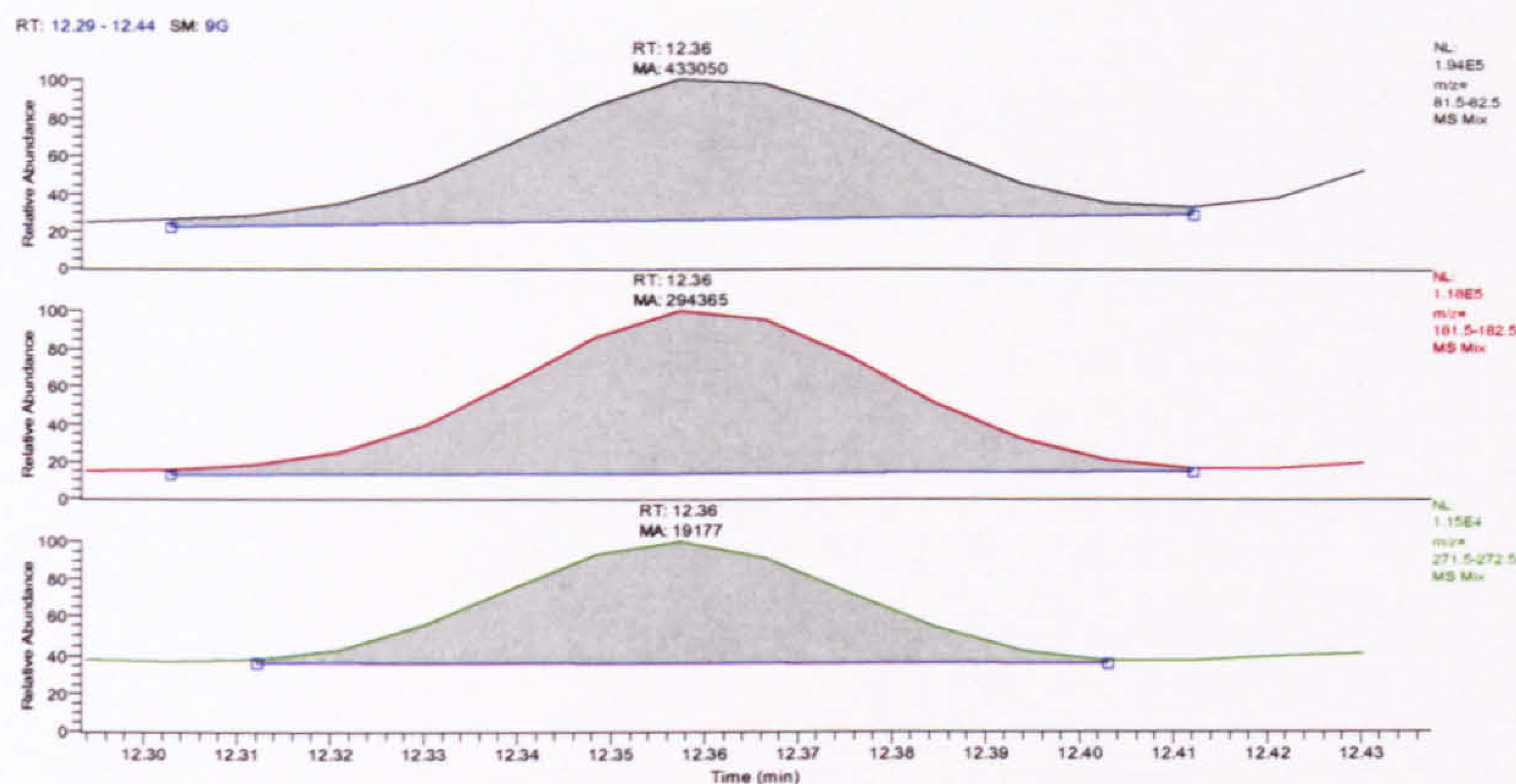


Figure 4-33:- Selected ion chromatograms for benzoylecgonine methyl ester (cocaine)

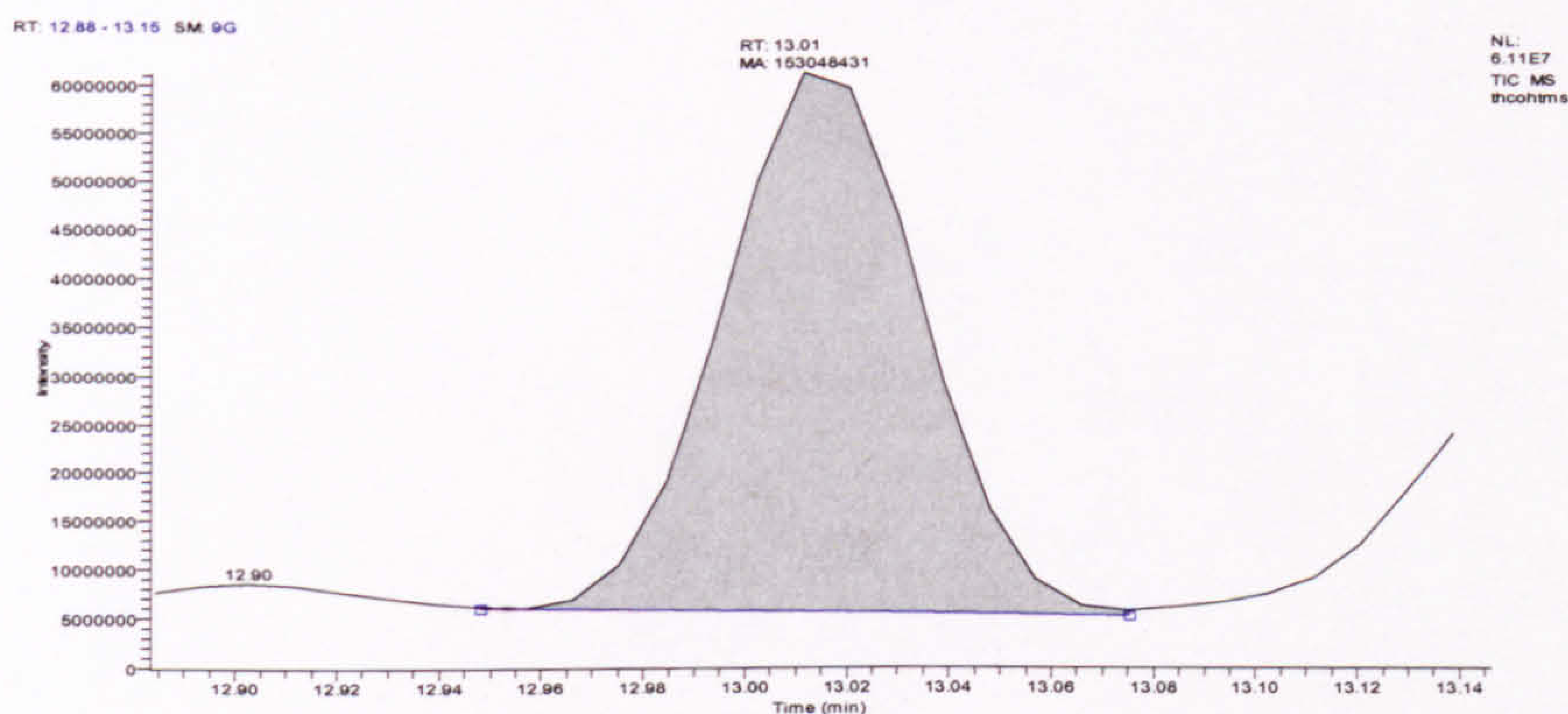


Figure 4-34:-TIC chromatogram for THC-OH PFP derivative

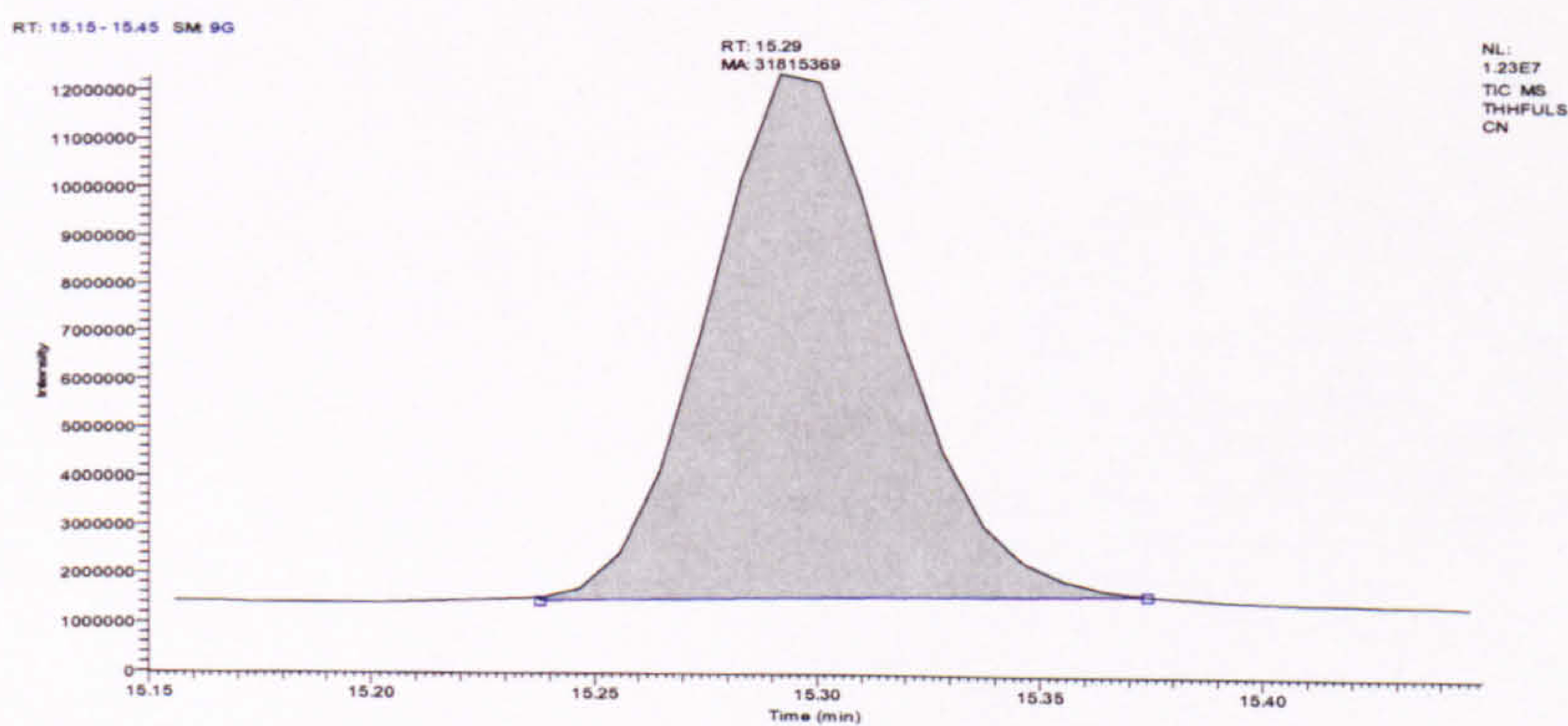


Figure 4-35:- TIC chromatogram for THC-COOH PFP/methyl ester derivative

The molecular ion for benzoylecognine with PFPA/TMS- diazomethane was m/z 303, i.e. methylation of benzoylecgonine produces cocaine as shown in Figure 4-36. This is a disadvantage of methylation compared to other derivatising agents as the product cannot be distinguished from cocaine which was in the sample. This problem could be overcome by using an analogue of TMS-diazomethane such as TMS-diazopropane. TMS-diazoethane would also cause a problem because cocaethylene may also be present in blood following simultaneous administration of cocaine and ethanol, which is a common practice.

The molecular ion fragments as shown in Figure 4-37. The mass spectrum has a base peak at m/z 182 and with elemental composition $[C_{10}H_{12}NO_2]^+$.The PFPA/TMS-diazomethane and PFPA/PFP-OH derivatives of benzoylecgnine share the m/z 82 and m/z 272 ions, which have elemental composition $[C_5H_8N]^+$ and $[C_{18}H_8O_3]^+$, because the ester group is lost when each of these ions is formed.

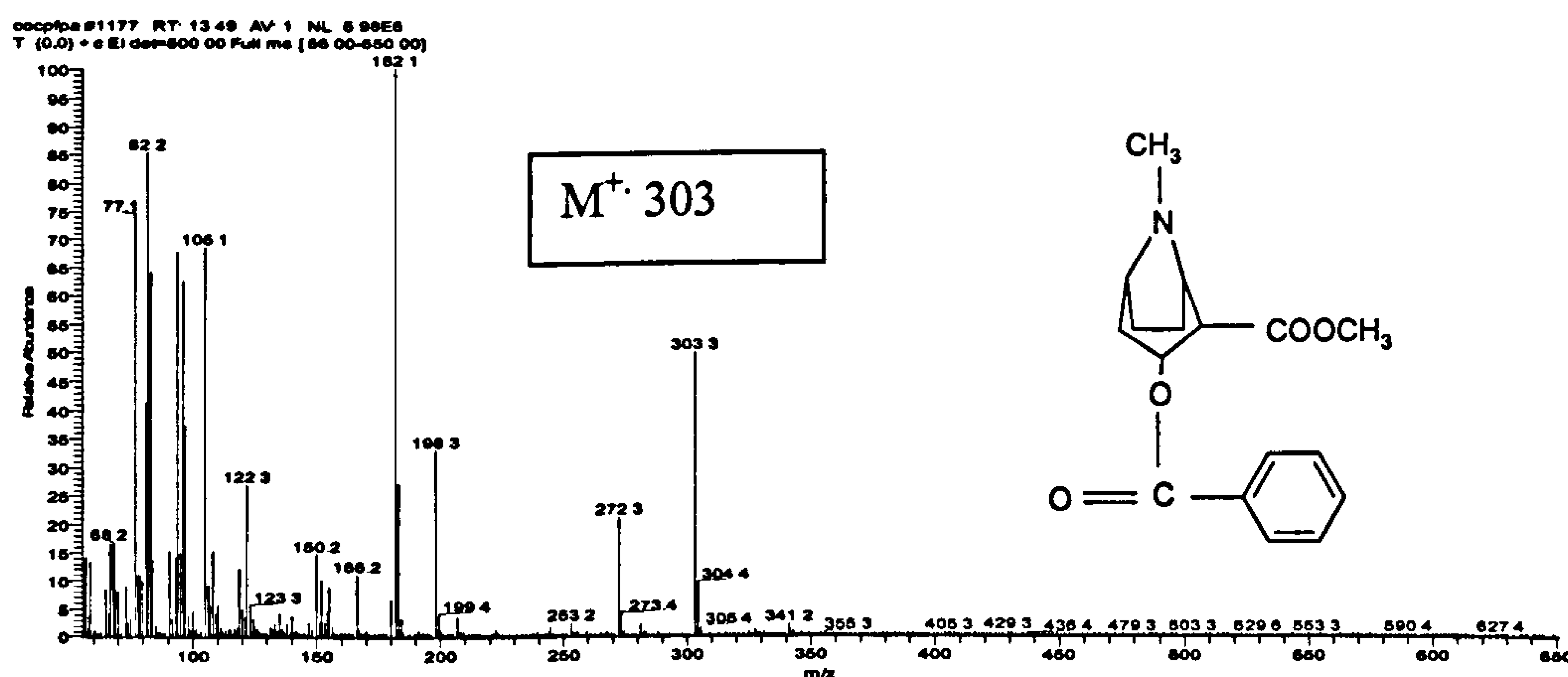


Figure 4-36:- Mass Spectrum Benzoylecgonine PFP-TMS-diazomethane derivative (Cocaine)

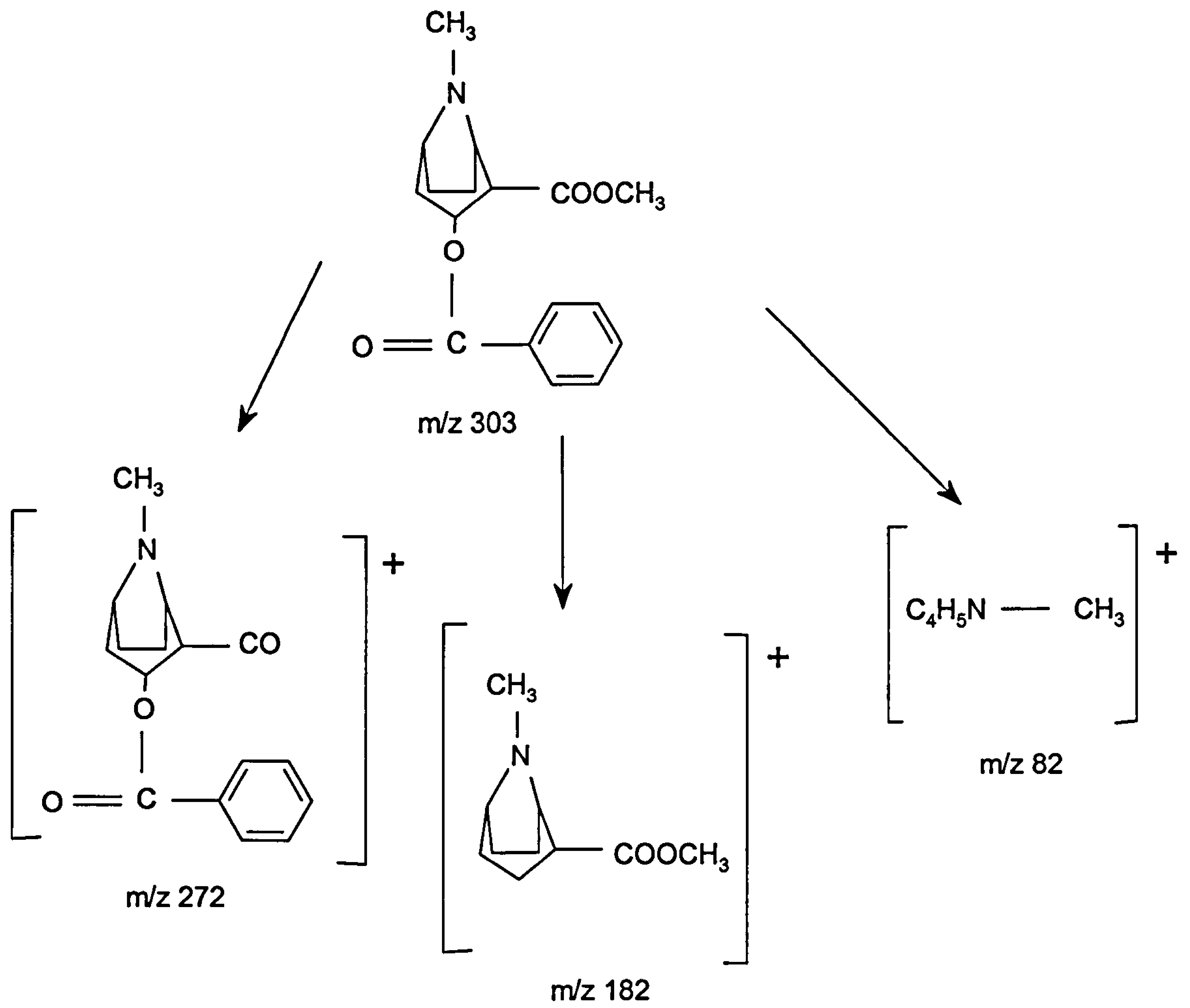


Figure 4-37:- Fragmentation for benzoylecgonine PFP/TMS- diazomethane derivative (cocaine)

The molecular ion for THC-COOH with PFPA/TMS-diazomethane was at m/z 504 as shown in Figure 4-38.This ion fragments as shown in Figure 4-39. It has a base peak at m/z 489 and has the composition [$C_{24}H_{26}O_5F_5$

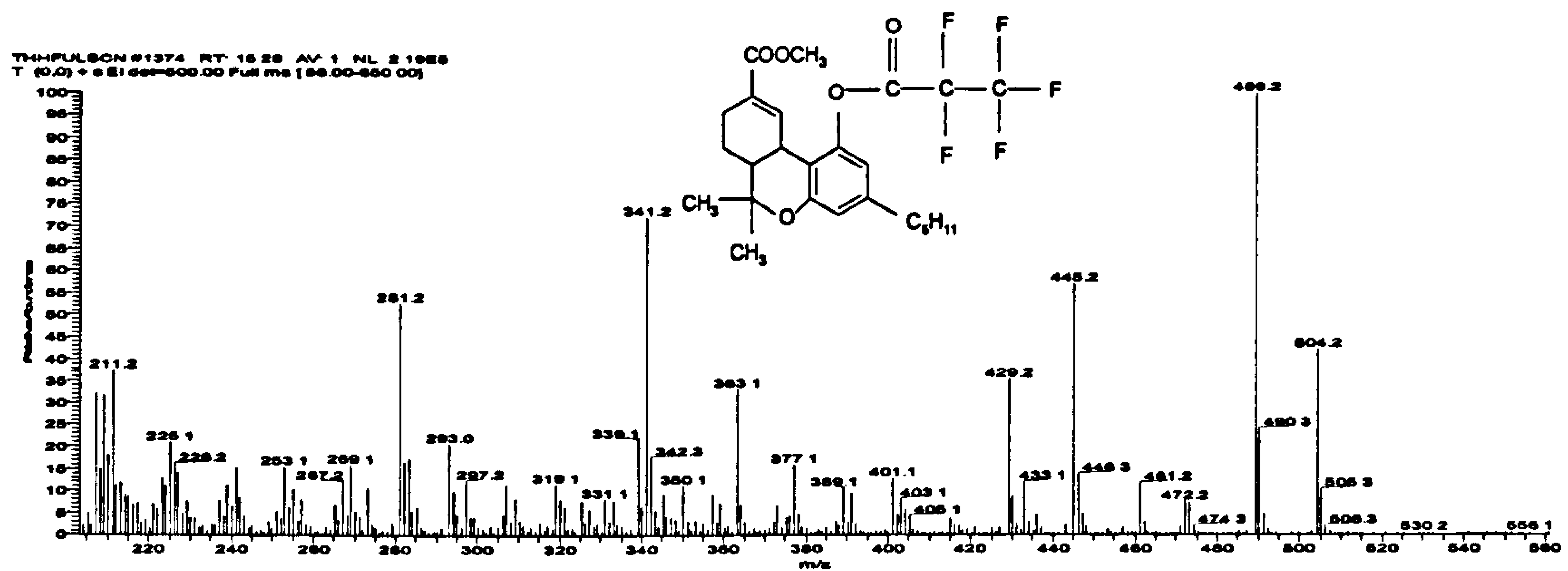


Figure 4-38:- Mass Spectrum for THC–COOH-PFP-TMS-diazomethane

The methyl ester/PFP derivative and PFP-OH ester/PFP derivative share a common ion at m/z 489, which indicates that the ester substituent is lost, as this removes the structural difference between the two derivatives.

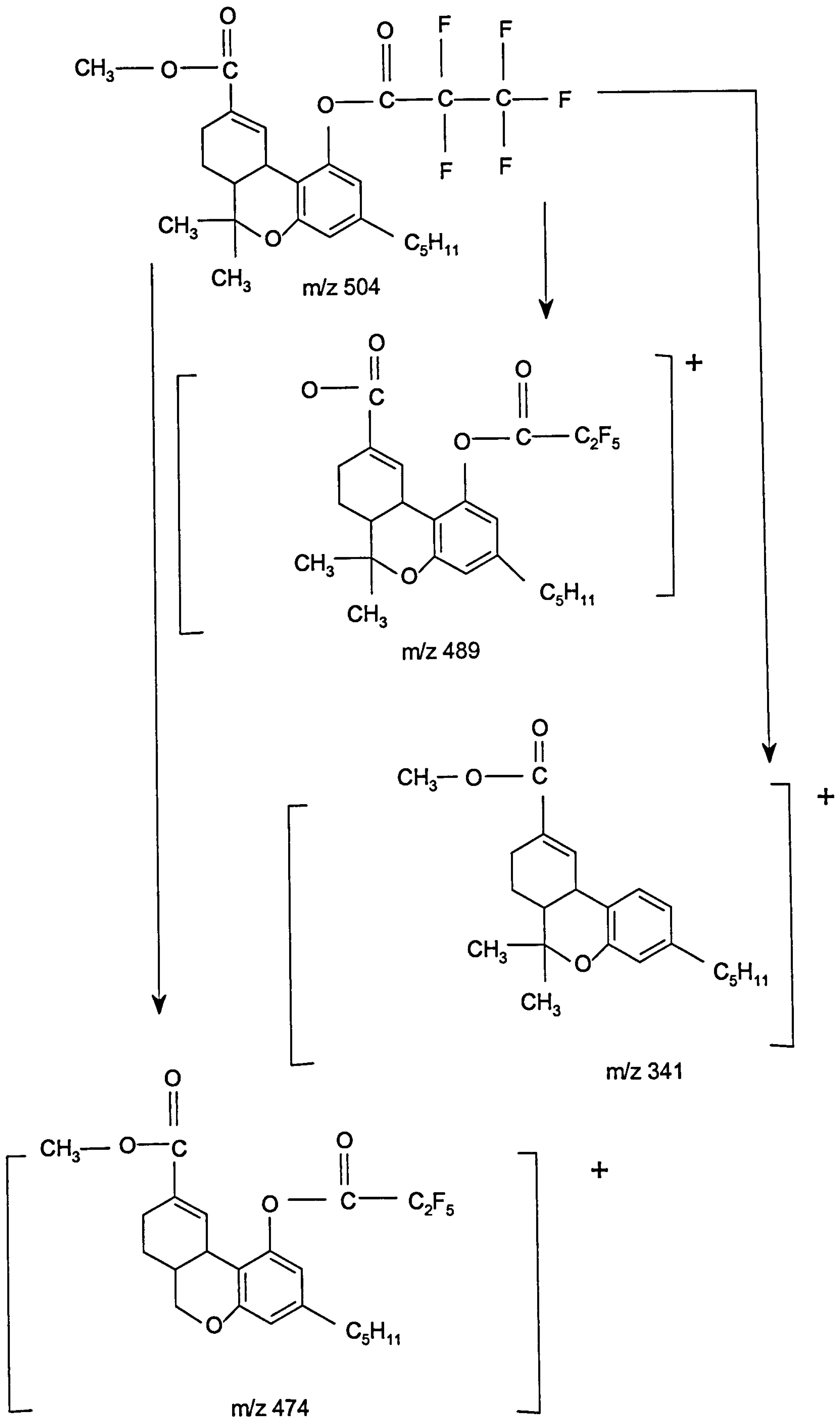


Figure 4-39:- Fragmentation for THC-COOH-PFP/TMS- diazomethane

4.5.5 TBDMS Derivatives:

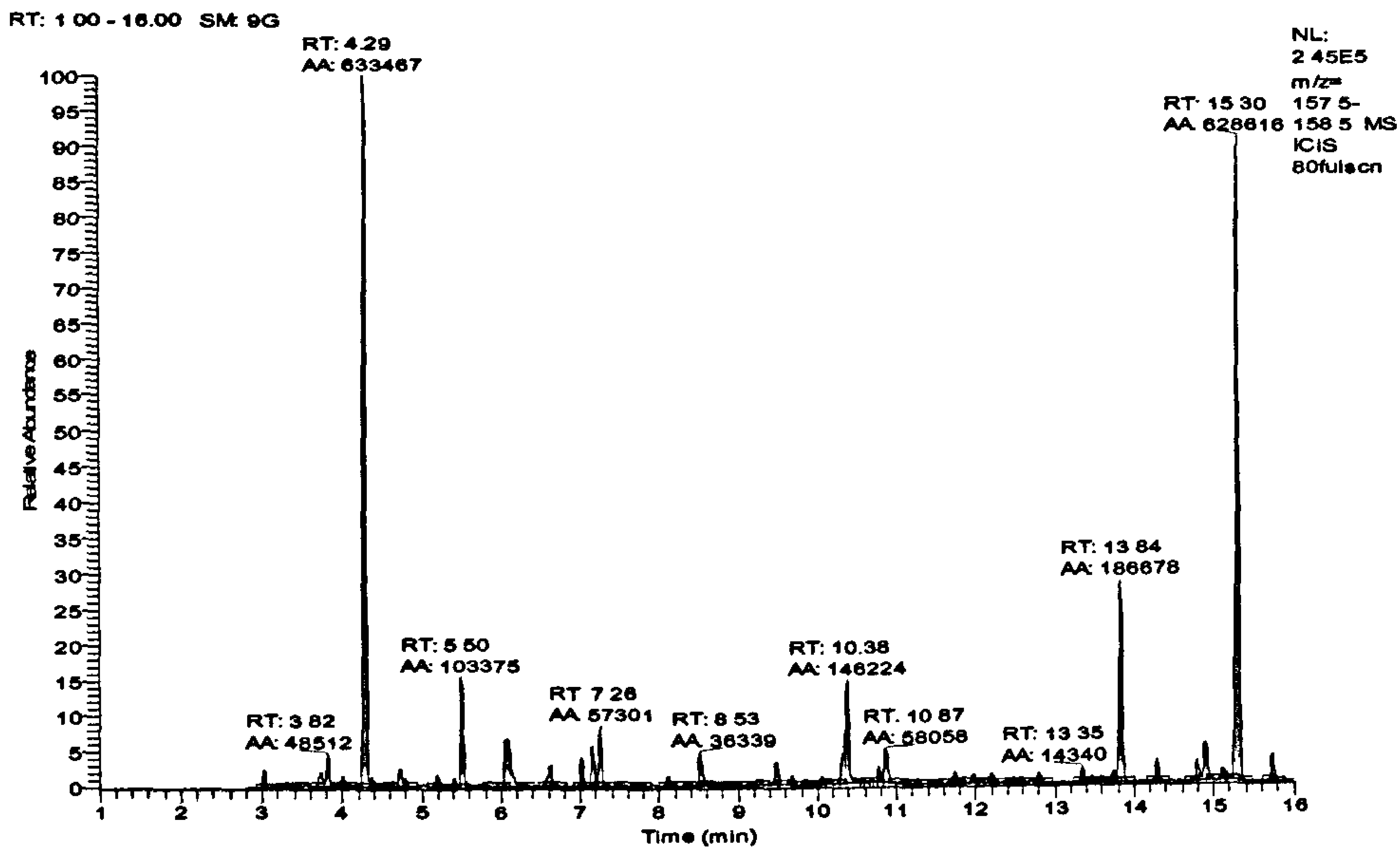
The third derivatisation method investigated was using MTBSTFA. In this reaction analytes react with $(CH_3)_2SiC(CH_3)_3$ from MTBSTFA reagent to produce the structures shown in (Figure 4-41, Figure 4-43, Figure 4-45, Figure 4-47 and Figure 4-49).The advantage of this reagent is its ability to react with alcohols, primary amines, phenols and carboxylic acid groups and this method gave derivatives with all test compounds except the secondary amine, methamphetamine, despite the addition of a catalyst, MTBDMCS,Figure 4-40. Gas chromatographic behaviour of these derivatives was good, but although using MTBSTFA to derivatise morphine produced good peak shapes and looked to be sensitive, these results were for a standard at a high concentration. At a low concentration, it was difficult to detect morphine. The mass spectra had prominent ions suitable for GC-MS-SIM, often showing ions due to the loss of the butyl substituent in the silyl derivatives. Ions for use in selected ion monitoring are given in Table 4-5.

Table 4-5:- Selected Ions used for GC/MS with MTBSTFA derivatisation

Target analyte	Retention time(min)	Selected Ions(m/z)	Internal Standard	Selected Ions (m/z)
Amphetamine	6.92	158	Amp-D_5	162
Morphine	15.72	342	Mor-D_3	345
Benzoylecgonine	14.76	282	Bze-D_3	285
THC-OH	15.86	342	THC-OH-D_3	345
THC-COOH	19.33	515	THC-COOH-D_3	518

The molecular ions for amphetamine, morphine, benzoylecgonine, THC-OH and THC-COOH TBDMS derivatives were at m/z 249, m/z 513, m/z 403, m/z 428 and m/z 572 respectively. These ions fragment as shown in (Figure 4-42, Figure 4-44, Figure 4-46, Figure 4-48 and Figure 4-50).

(A)



(B)

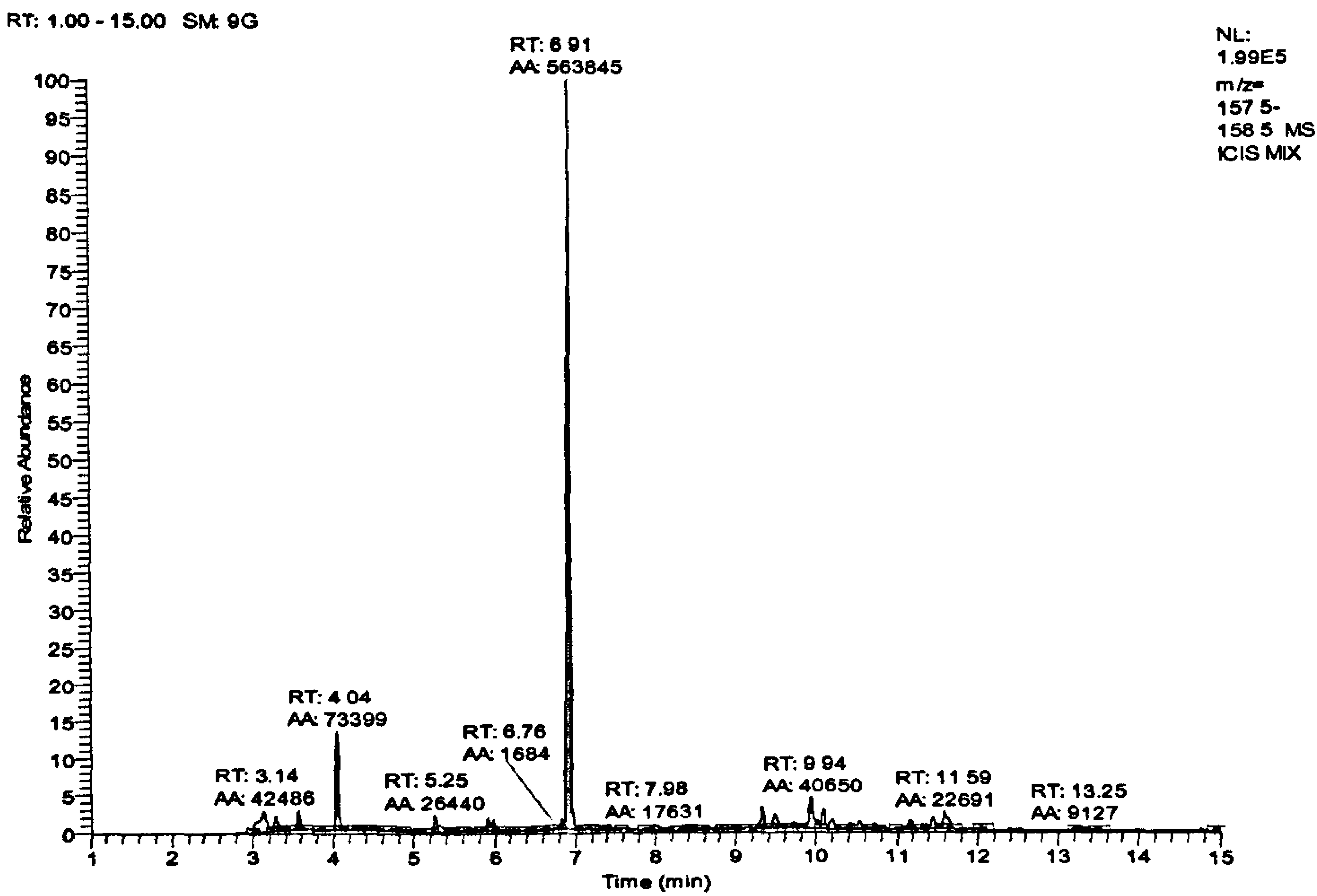
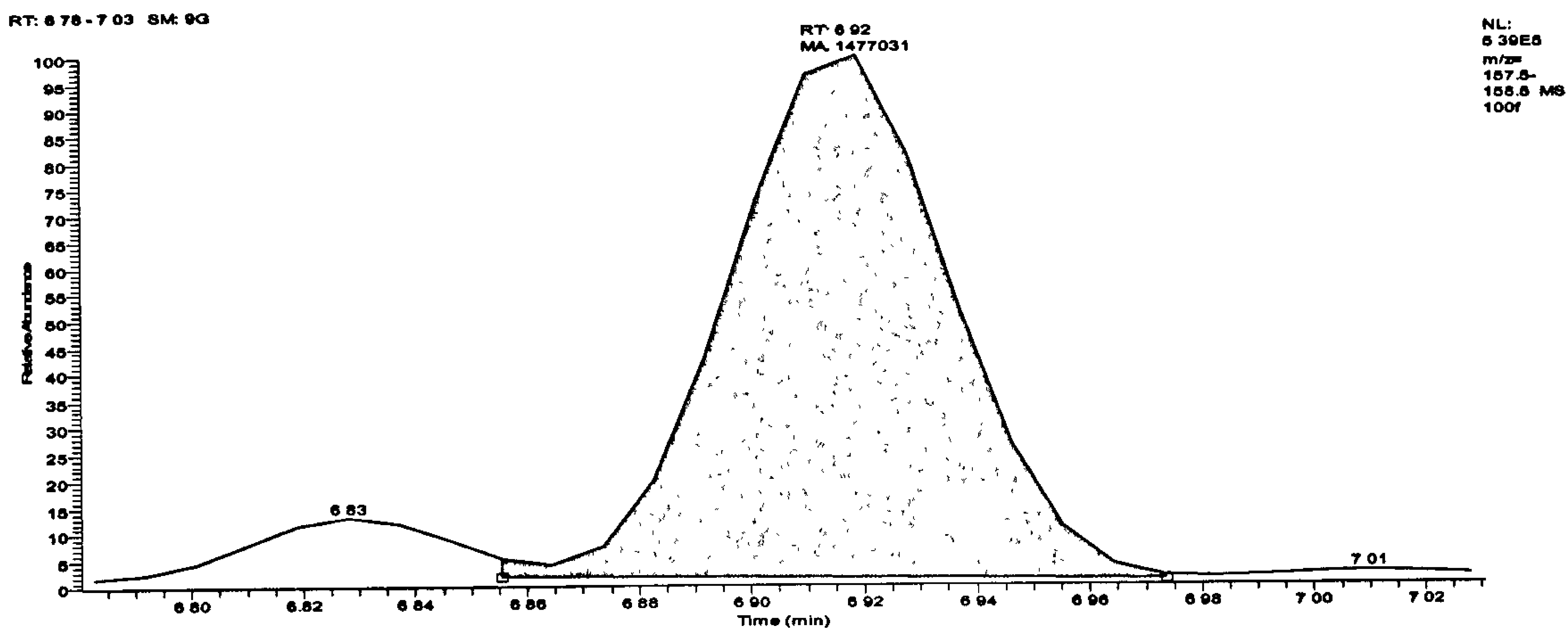


Figure 4-40:- TIC Chromatogram for Amphetamine –TBDMS, (A) without Catalyst (B) with Catalyst



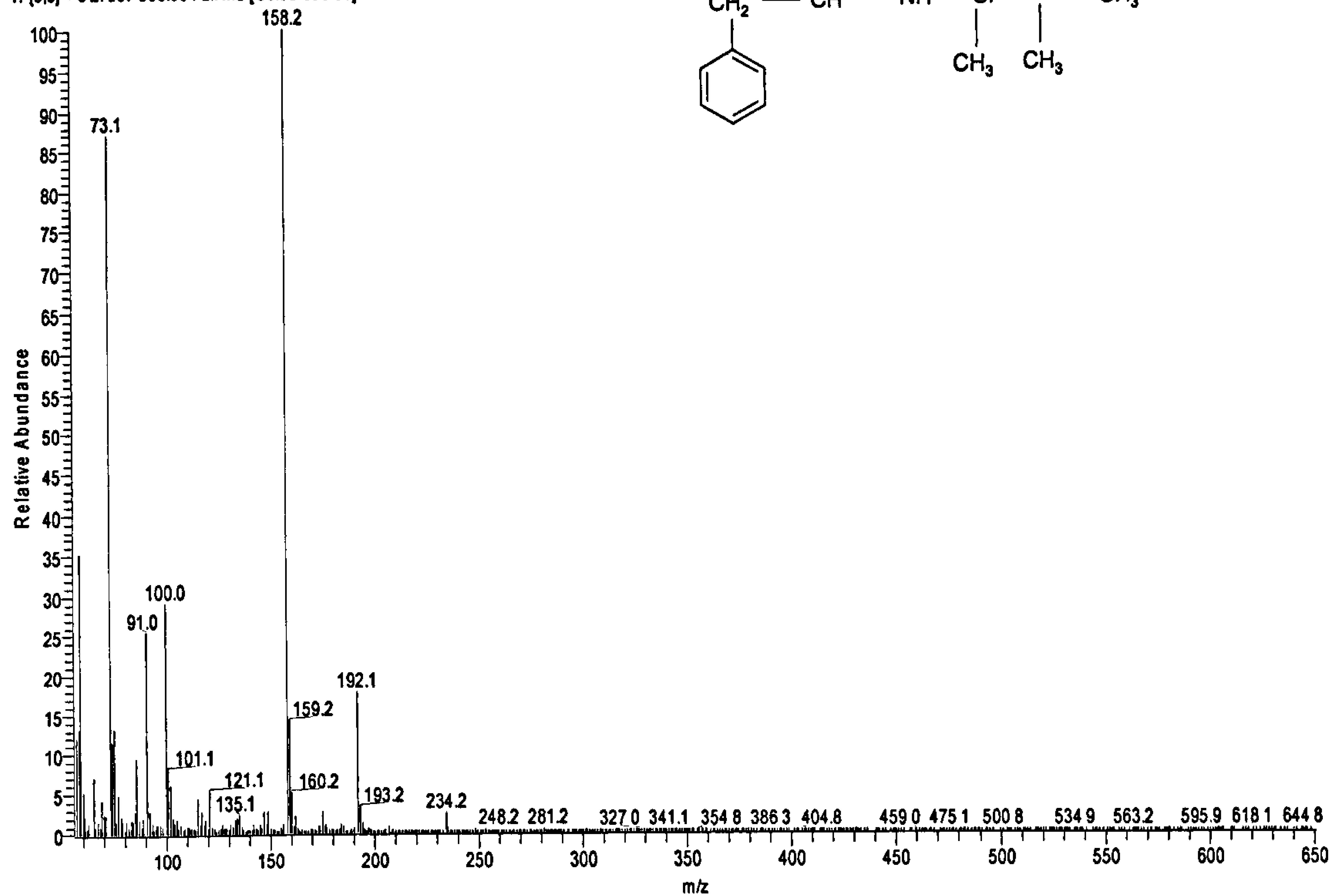


Figure 4-41:- Ion Chromatogram for m/z 158 and Mass Spectrum for Amphetamine -TBDMS

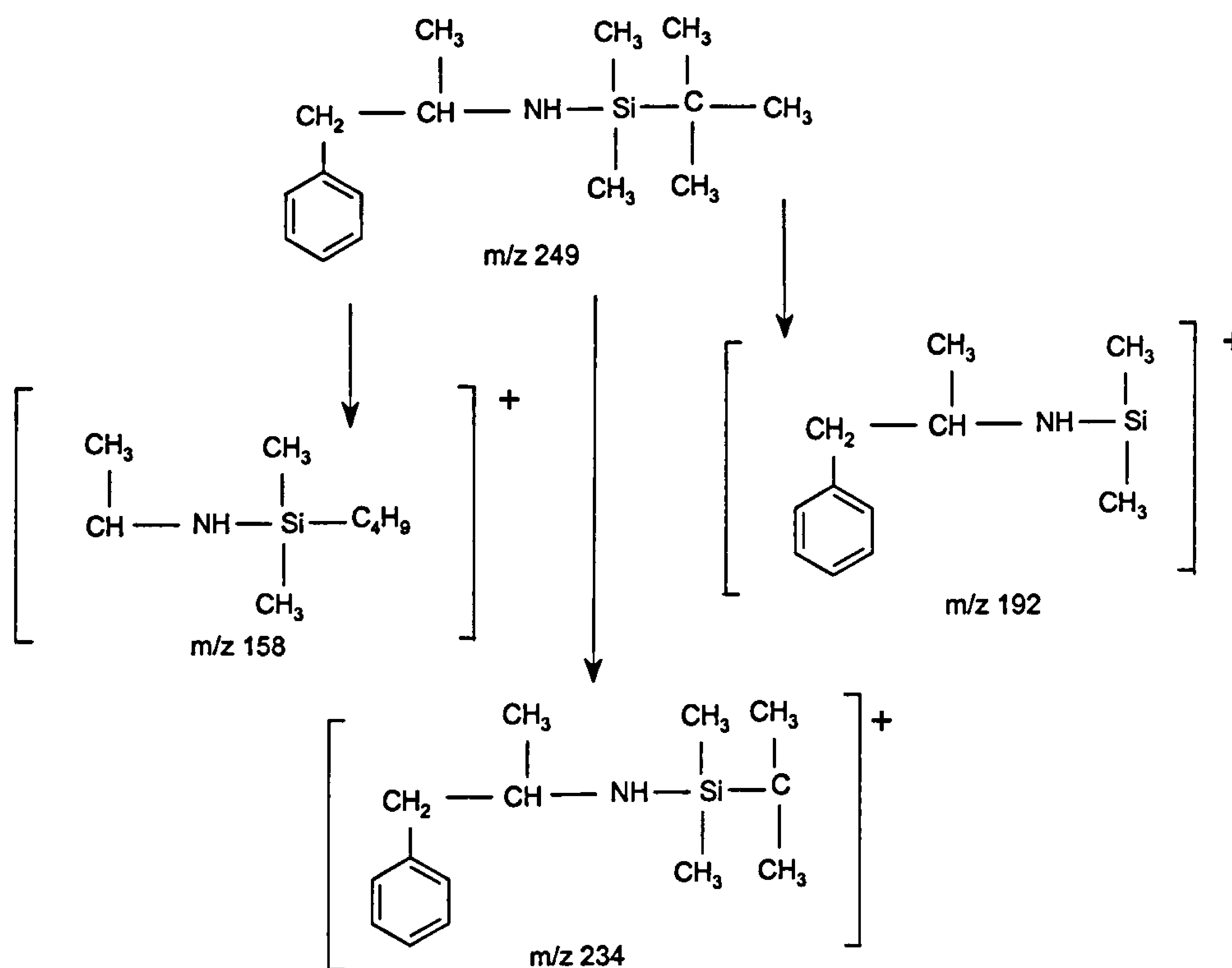
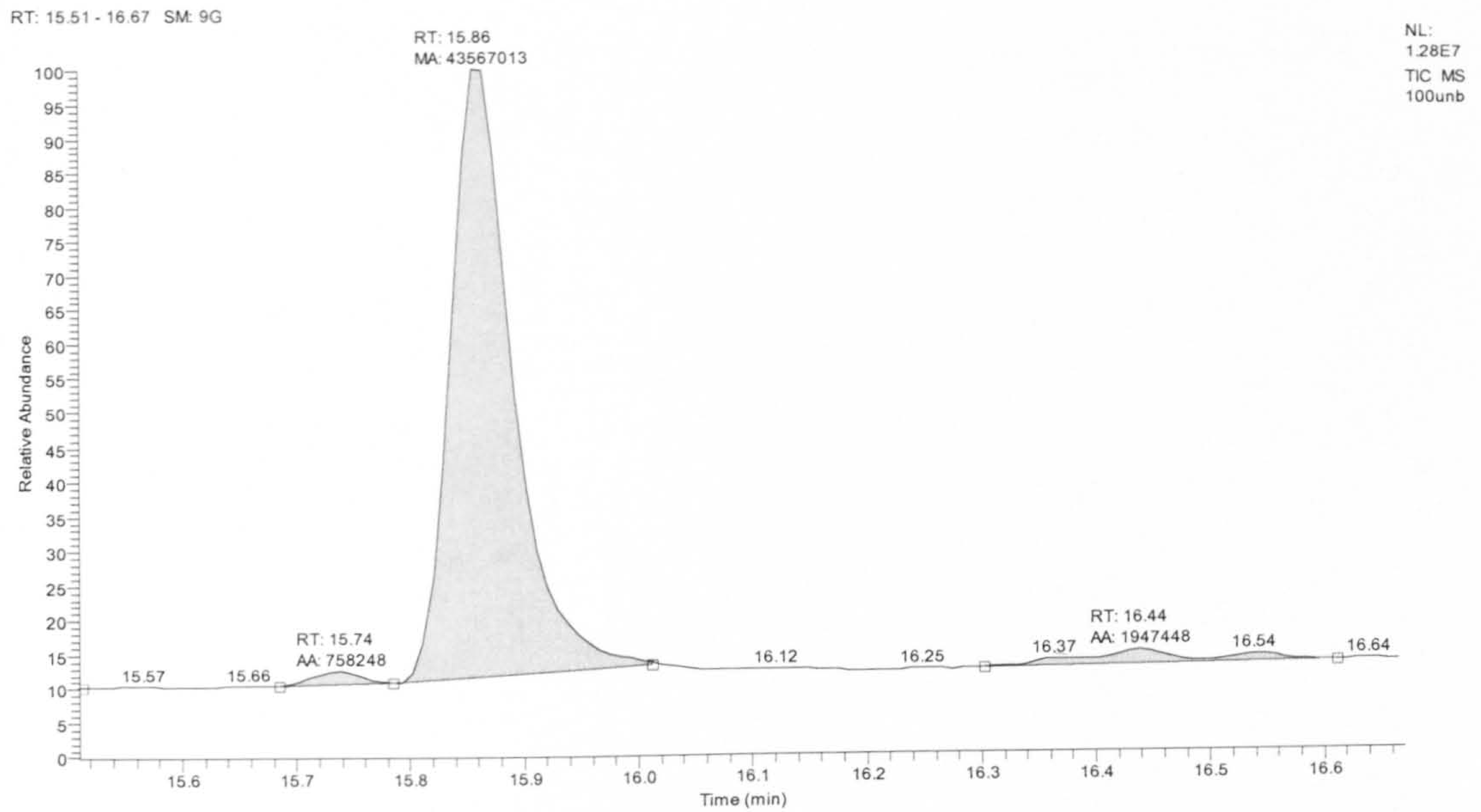
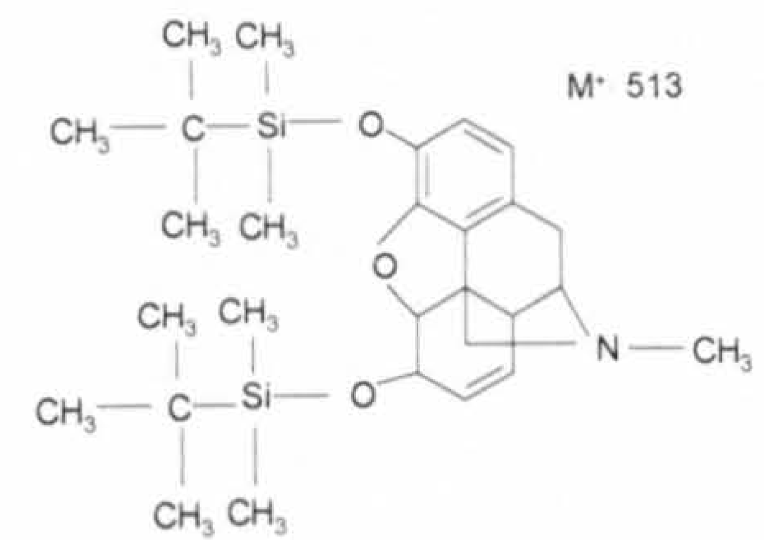


Figure 4-42:- Fragmentation of Amphetamine -TBDMS

Amphetamine has a primary amine and produces a base peak at m/z 158 assigned the fragment composition $[C_8H_{20}SiN]^+$. This ion is formed by the characteristic reaction of amphetamines, alpha cleavage, beside the carbon holding the nitrogen atom. The other high-mass fragment ion, at m/z 192, is formed by loss of the tertiary butyl group from the derivative. The molecular ion at m/z 249 is not visible but there is a weak ion at m/z 234 due to loss of a methyl group.





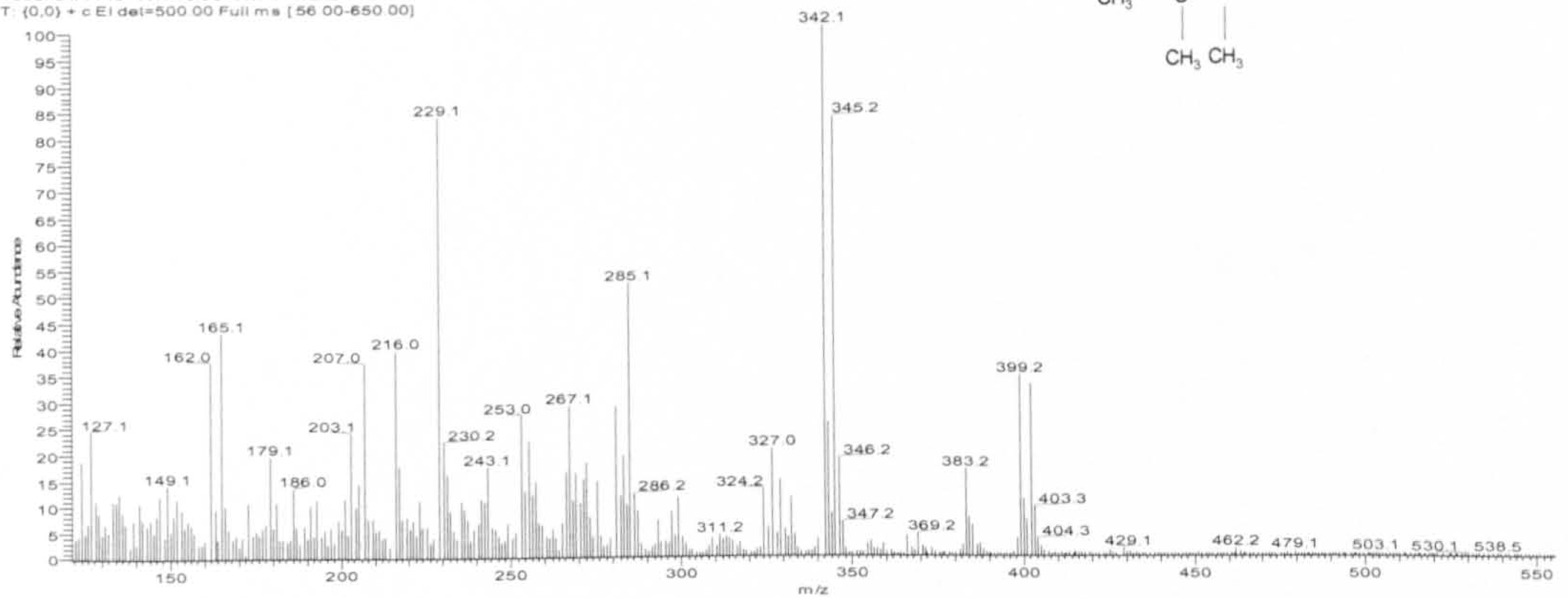


Figure 4-43:- TIC Chromatogram and Mass Spectrum for Morphine-TBDMS (also containing the internal standard Morphine-d_3).

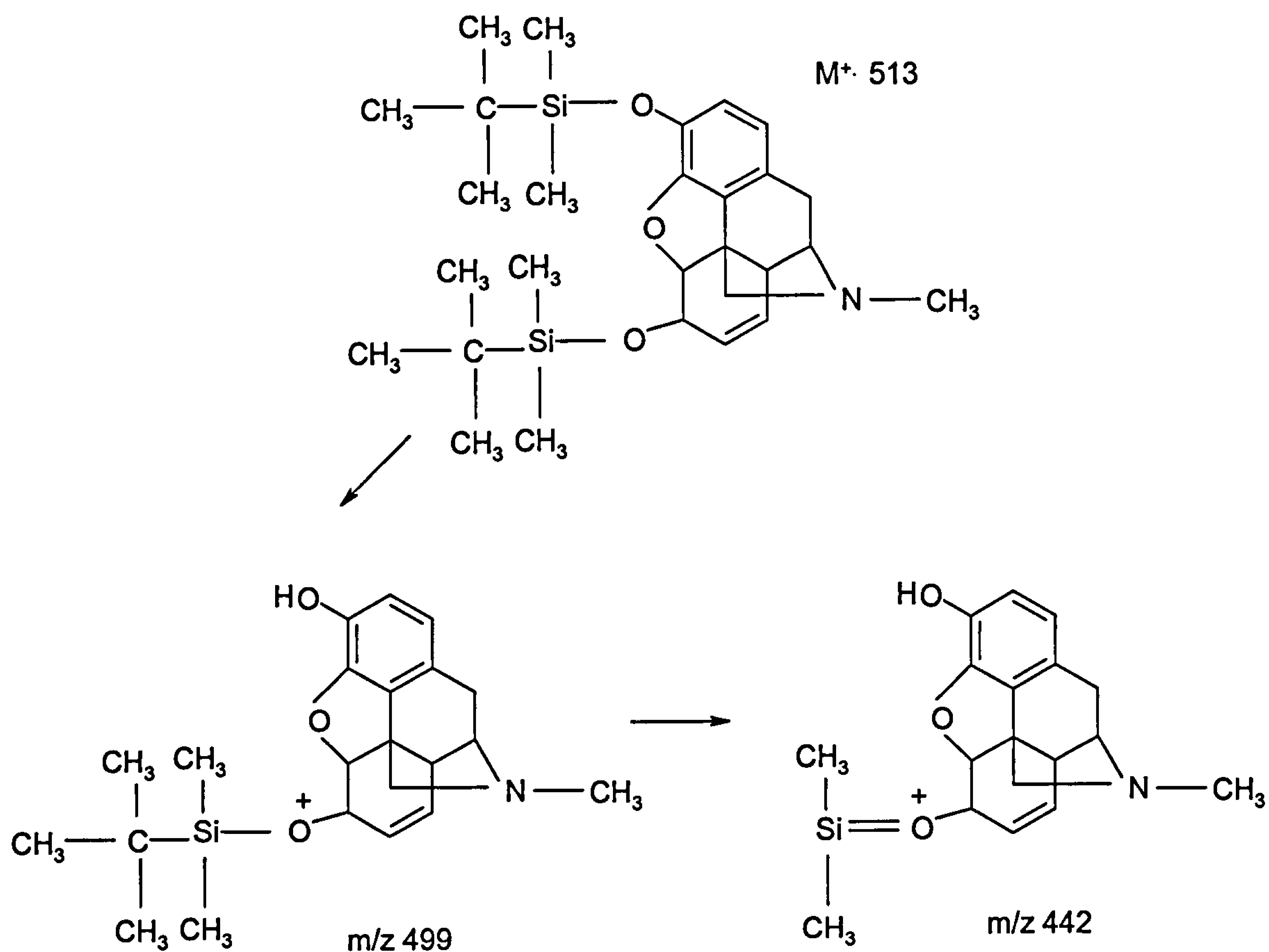
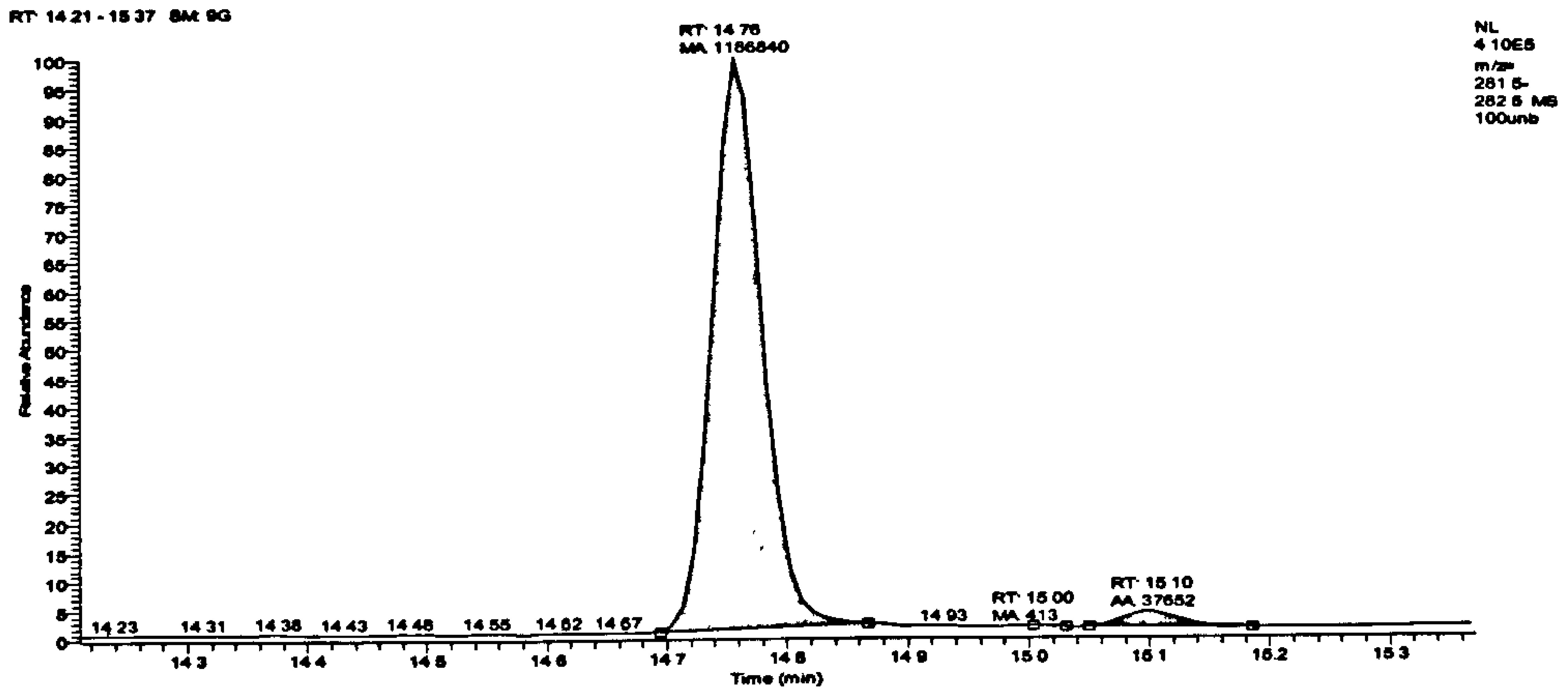


Figure 4-44:- Fragmentation of Morphine-bis-TBDMS derivative

The mass spectrum of morphine-bis-TBDMS derivative contains strong ions at m/z 399 and m/z 342. Note that the internal standard, present in the GC peak, gives analogous ions 3 mass units higher at m/z 502 and m/z 345, but other ion doublets are not clearly visible, suggesting that other fragments have lost the N-methyl group which contains the deuterium atoms. The ion at m/z 499 is formed by loss of the silyl group on the phenolic hydroxyl group together with a proton transfer to the ion (a similar reaction occurs with diamorphine). The ion at m/z 442 is then formed by loss of the tertiary butyl group from the initial product. This is suggested by the absence of an ion corresponding to loss of the tertiary butyl group from the molecular ion, which would have appeared at m/z 456.



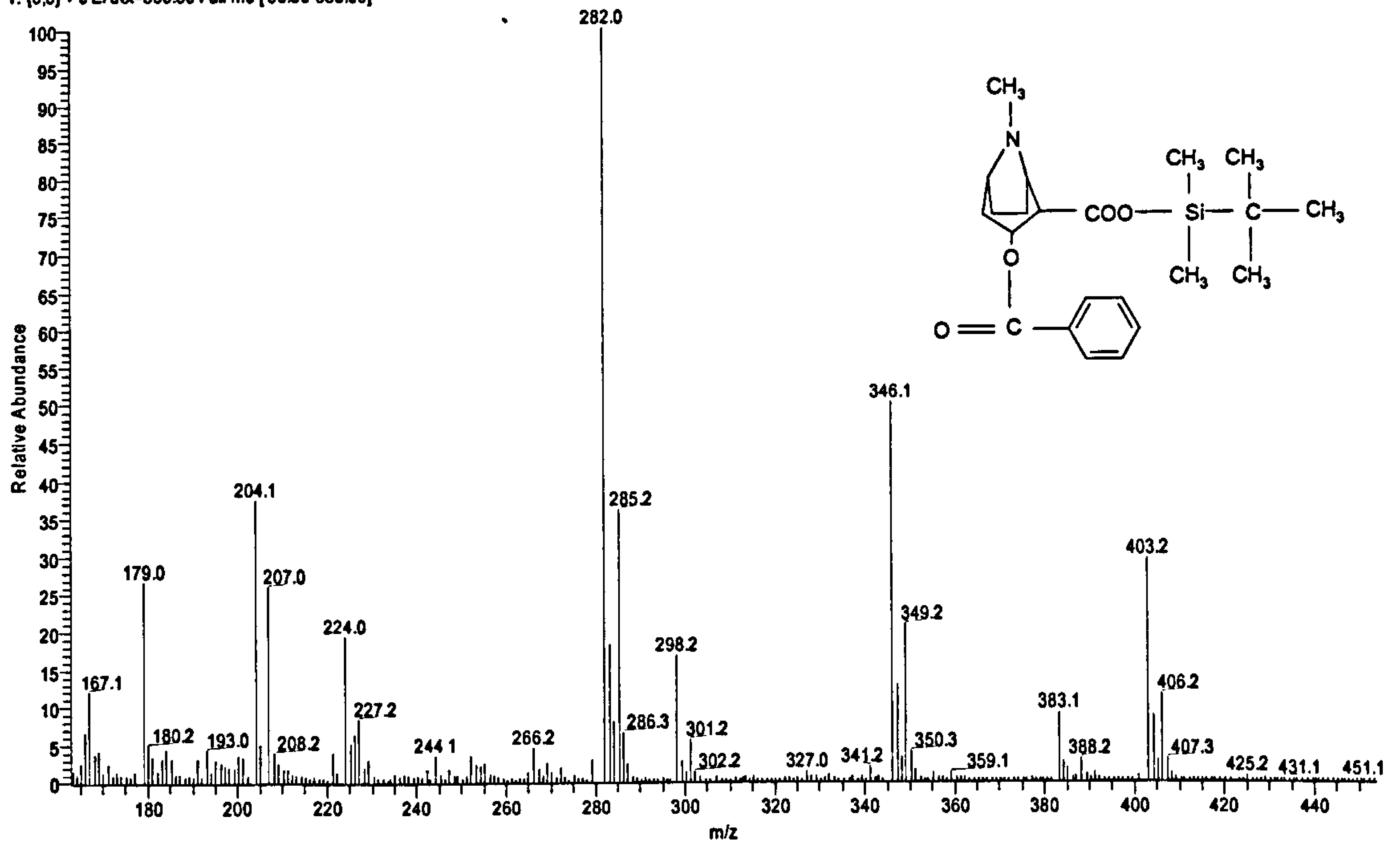


Figure 4-45:-Ion Chromatogram m/z 282 and Mass Spectrum for Benzoylecgonine TBDMS (also containing the internal standard Benzoylecgonine-d_3)

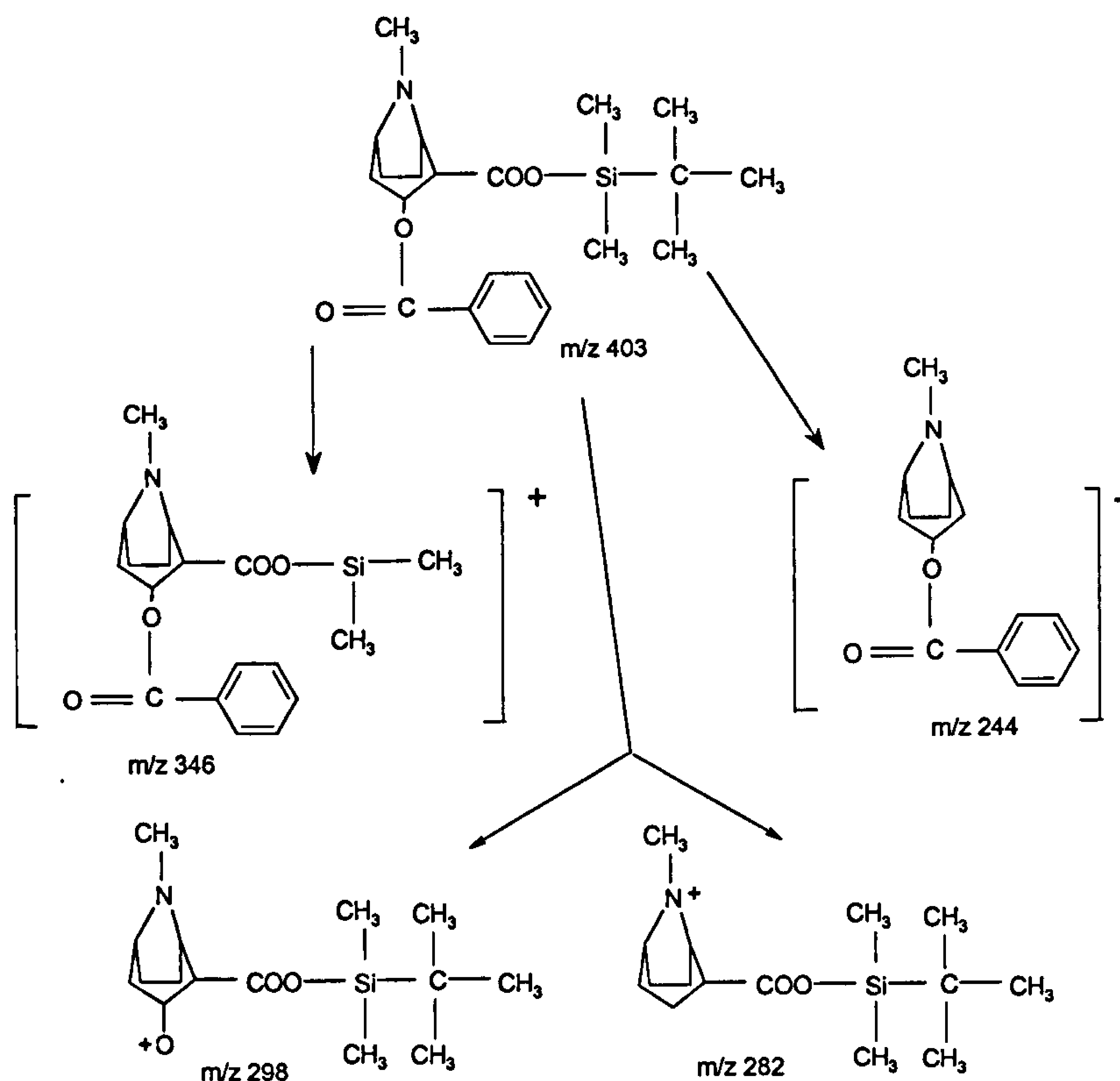
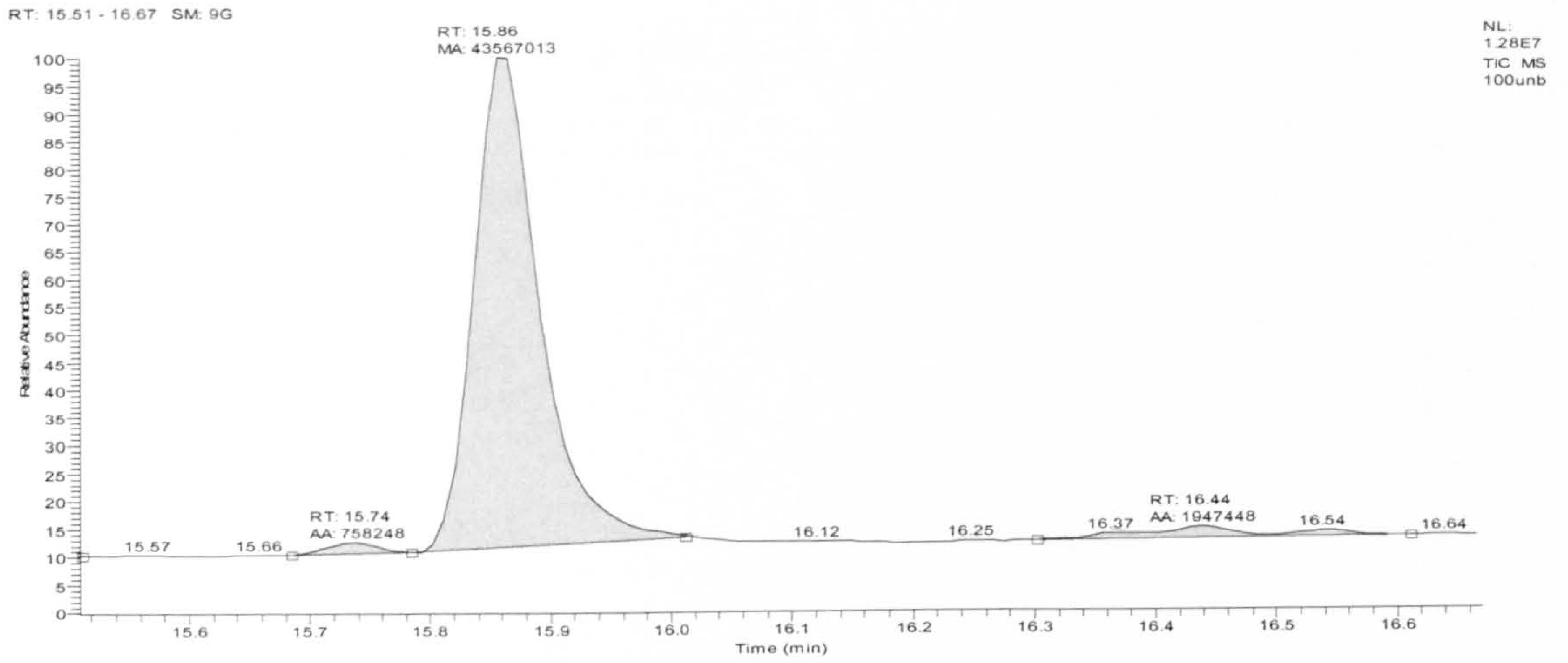
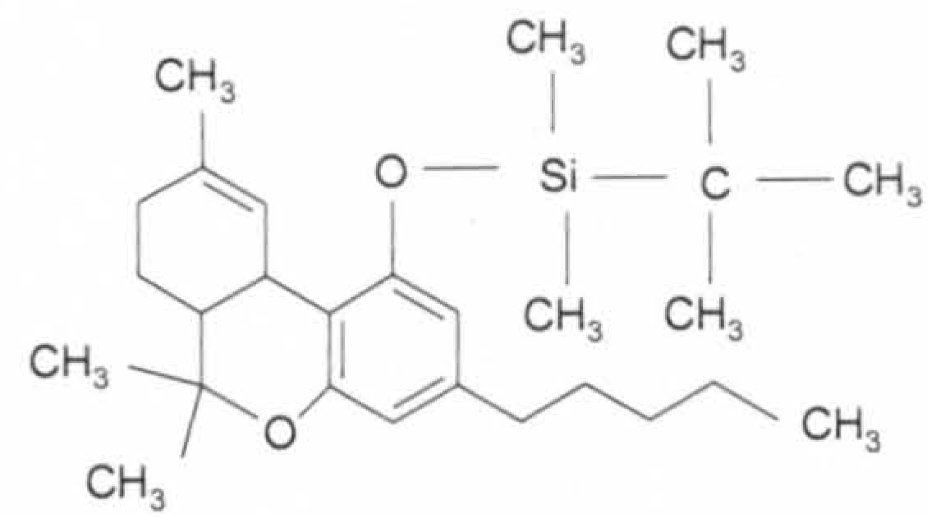


Figure 4-46:- Fragmentation of Benzoylecgonine -TBDMS

Benzoylecgonine has a carboxylic acid group and forms an MTBDMS ester. The base peak in the mass spectrum is at m/z 282 and is assigned to the fragment $[C_{14}H_{22}O_4Si]^+$ .It is formed by loss of the benzoyl group, as in cocaine, which produces the ion at m/z 182 in the mass spectrum of cocaine. The loss of the tertiary butyl group occurs directly from the molecular ion to give the fragment at m/z 346. The presence of the internal standard helps in identifying the fragmentation reactions by providing a parallel series of ions for those fragments which retain the deuterium, which is held by the N-methyl group.





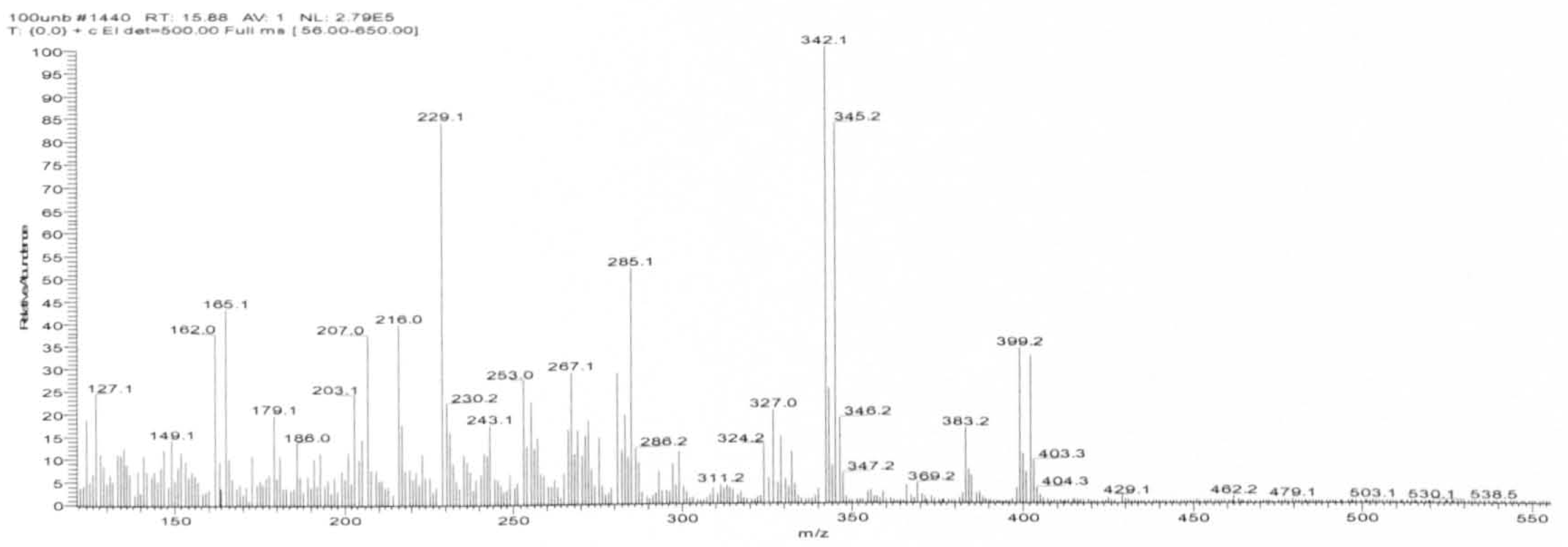


Figure 4-47:- TIC Chromatogram and Mass Spectrum for THC-OH TBDMS

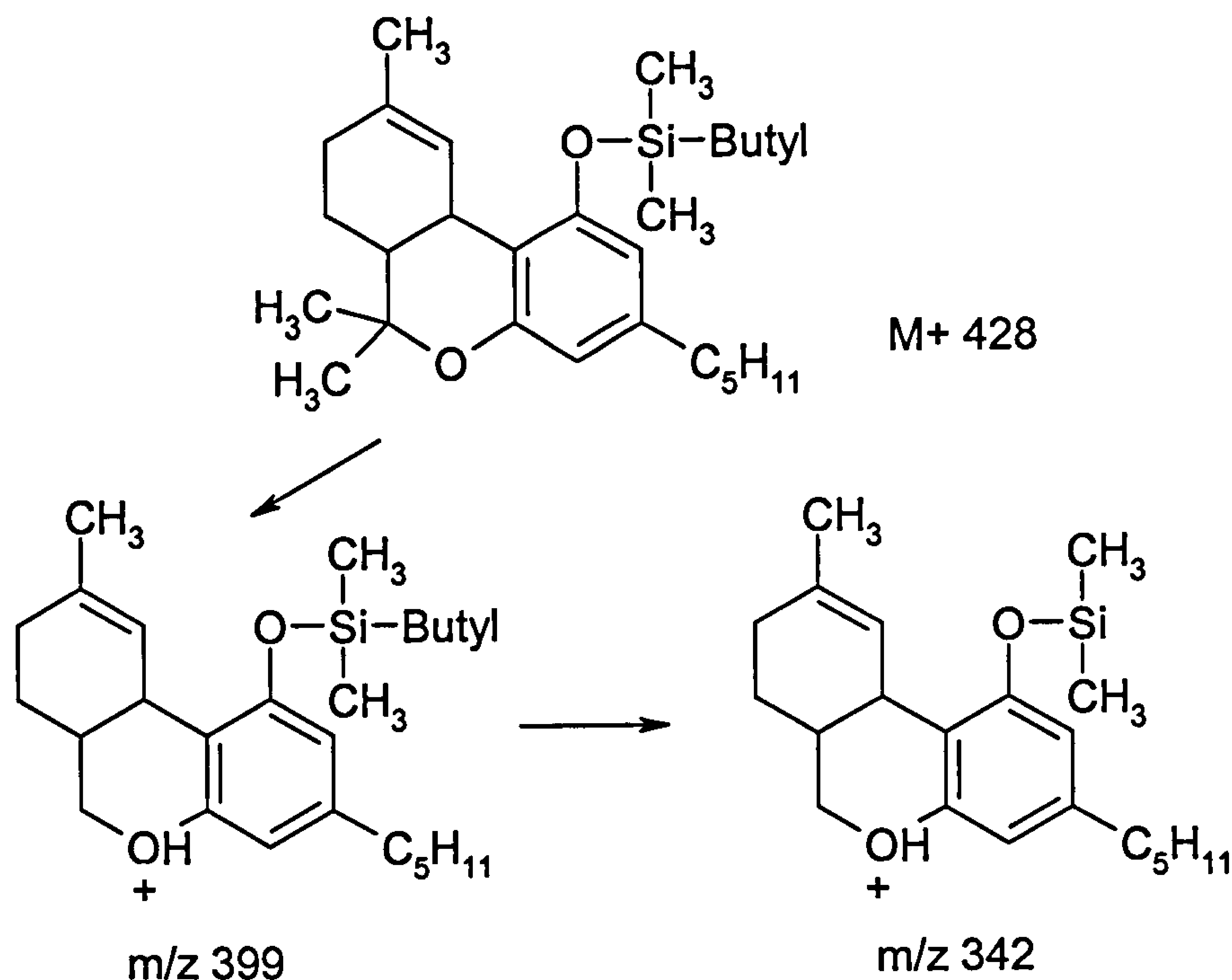


Figure 4-48:- Fragmentation of THC-OH-TBDMS

The molecular ion of THC-OH TBDMS derivative is too small to be visible in the mass spectrum. High mass fragment ions are present at m/z 399 and m/z 342, corresponding to the loss of C_2H_5 and then C_4H_9 fragments. This sequence is indicated by the absence of a strong M-57 ion, which would have appeared at m/z 371. The source of the C_2H_5 fragment is suggested in Figure 4-48 but is not known with certainty. The source of the C_4H_9 fragment could either be the tertiary butyl group or else the side-chain, as there is a loss of 57 in the mass spectrum of THC which comes from the side chain.[192] However, if the fragment came from the side chain this would result in the loss of deuterium from the internal standard, as the deuterium is on the end carbon of the side chain. The mass spectrum clearly shows that this does not happen as there is an ion at m/z 345 for the internal standard.

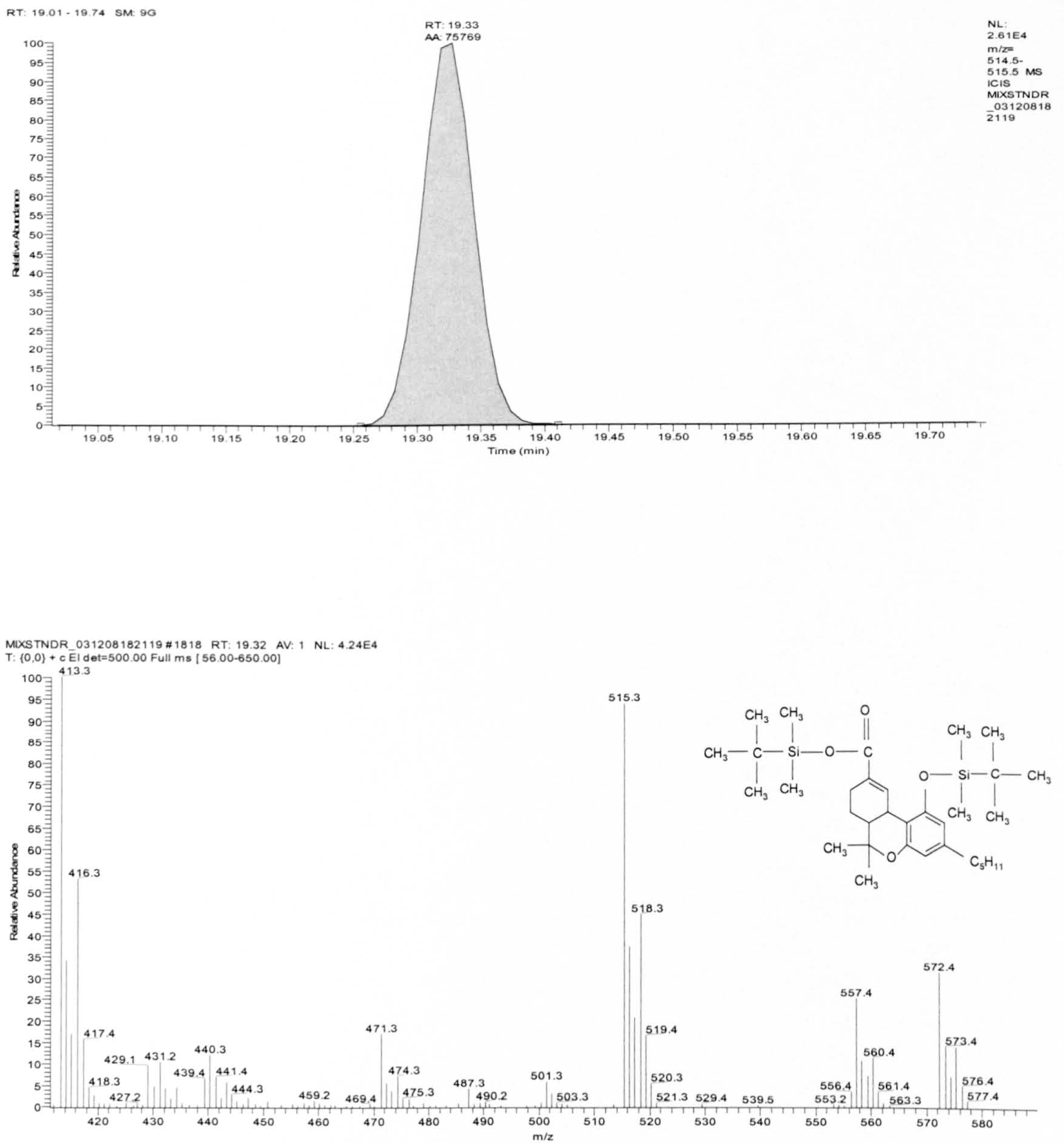


Figure 4-49:- Ion Chromatogram m/z 515 and Mass Spectrum for THC-COOH TBDMS

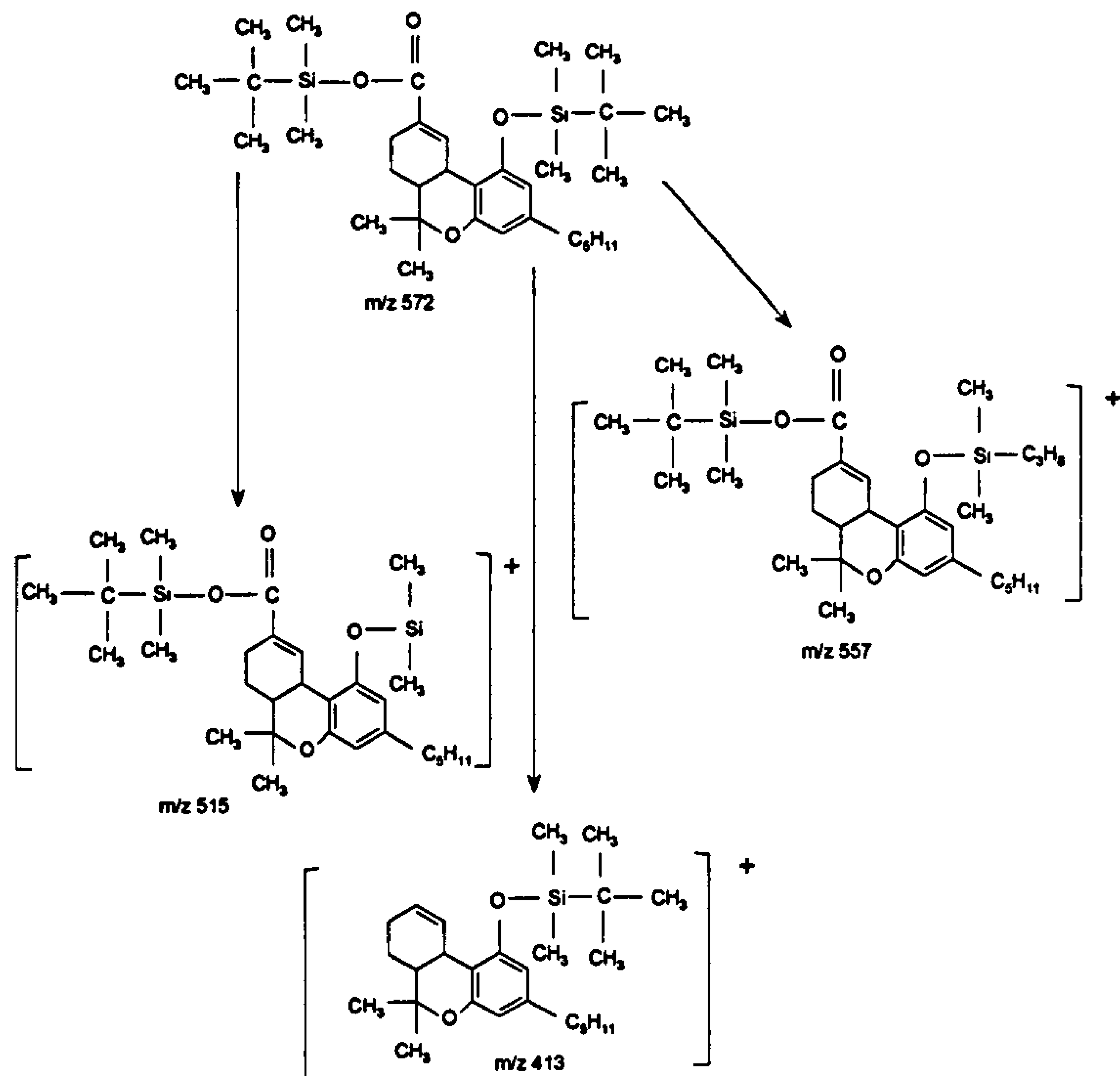


Figure 4-50:- Fragmentation of THC-COOH-TBDMS

Benzoylecogonine and THC-COOH have carboxylic acid groups and base peaks at m/z 282 and m/z 515 and are assigned to the fragments $[C_{14}H_{22}O_4Si]^+$ and $[C_{29}H_{47}O_4Si_2]^+$. THC-OH has a hydroxyl group and a base peak at m/z 342 assigned to the fragment $[C_{21}H_{30}SiO_2]^+$.

4.6 Conclusions

Several conclusions were drawn from this study, which was the initial step in the development of a method for use in systematic forensic toxicology.

1. PFPA/PFP-OH derivatisation:

This reagent produced derivatives with all of the test compounds, including the secondary amine (methamphetamine) and the two carboxylic acids (benzoylecgonine and THC-COOH). The derivatives gave good gas chromatographic properties in terms of peak shape and had mass spectra with strong ions which were suitable for selected ion monitoring either as the quantification ion or else as qualifier ions. The reagent is volatile which allows it to be evaporated before GC-MS analysis, which would help to preserve the GC column, injector liner and mass spectrometer source from contamination.

It was concluded that this reagent had a potentially wide range of applications in STA and it was investigated further in phase 2 of this study.

2. PFPA/trimethylsilyl-diazomethane derivatisation:

Comments on trimethylsilyl- diazomethane as a reagent are given above. When used as a derivatising agent in combination with PFPA, all of the test compounds gave derivatives with good chromatographic and mass spectrometric properties. The mass spectra of amines and alcohols were identical to those obtained with PFPA and PFP-OH described above. Methyl esters were easily formed from benzoylecgonine and THC-COOH and these derivatives had good mass spectra with suitable ions for selected ion monitoring. The only potential disadvantages with this reagent was that benzoylecgonine was converted to cocaine and so could not be distinguished from it and there is a risk of interference in GC-MS analysis due to the impurities in the TMS-diazomethane reagent mentioned above.It was concluded that trimethylsilyl-diazomethane is a useful reagent for forensic toxicology but care must be taken to check for potential interference from the reagent during method validation.

3. MTBSTFA derivatisation:

This reagent gave good derivatives with all of the test compounds except methamphetamine where secondary amines not react very well with MTBSTFA. Chromatographic and mass spectrometric properties were good. However, although morphine with MTBSTFA derivatision had a good peak shape and the presence of strong ions gave the potential for sensitive detection by GC-MS, it was found that low concentration standards were not well detected in practice. This reagent had previously been used for analysis of carboxylic acids in urine of racing greyhounds and was effective in identifying unknown acids because the mass spectra had strong ions at m/z values corresponding to loss of the tertiary butyl group from the molecular ion, thereby allowing the molecular weight to be obtained.[228] The reagent was also found to be effective in a subsequent part of this research project for the analysis of benzodiazepines (Chapter 7).

The conclusion from this study is that conventional heating would be preferable to microwave heating as the time saved is usually less important than the overall cost of a routine analysis, especially if large numbers of samples are being analysed. This might be different under different circumstances, for example in emergency toxicology in hospital, where rapid turn-around is potentially life saving. Each of the reagents used in this study showed some advantages and all three of them were considered to have potential use in forensic STA. In phase 2 of the study, the reagents were applied to the derivatisation of extracts of the test compounds from whole blood.

5 Study of a Unified Method

5.1 Introduction

Methods of analysis generally have the steps shown in Figure 5-1 consisting of sample preparation followed by extraction of the substances of interest, derivatisation if required and then the end step analysis.

Sample pre-treatment	pH, protein precipitation, homogenisation, dialysis, digestion, hydrolysis of conjugates
↓	
Extraction and clean-up	Liquid-liquid extraction, solid phase extraction, headspace analysis
↓	
Chemical derivatisation if required	Formation of esters, ethers, oximes etc
↓	
End-step Analysis	Spectrometric and chromatographic methods

Figure 5-1 :- Components of an analytical method

As mentioned previously, the ultimate aim of this work was to create a unified procedure for systematic toxicological analysis which would enable a wide range of drugs and metabolites to be detected in a minimum number of analyses, thereby making the procedure suitable for the complete toxicological analysis of small samples. The usual way of developing a new method is to start at the end step, so that knowledge is gained about the possibility of detecting extracted analytes with instrumental techniques. This part of the method is carried out with standards of the substances of interest. Similarly, derivatisation methods can be tested using reference standards without the need to extract them from a biological material such as blood. When the end detection method is available, it can be used to test extraction procedures.

The previous Section described the investigation of the derivatisation and GC-MS analysis steps. This section deals with the extraction step for selected drugs in whole blood, with the aim being to develop a general extraction procedure which would work with most types of drug.

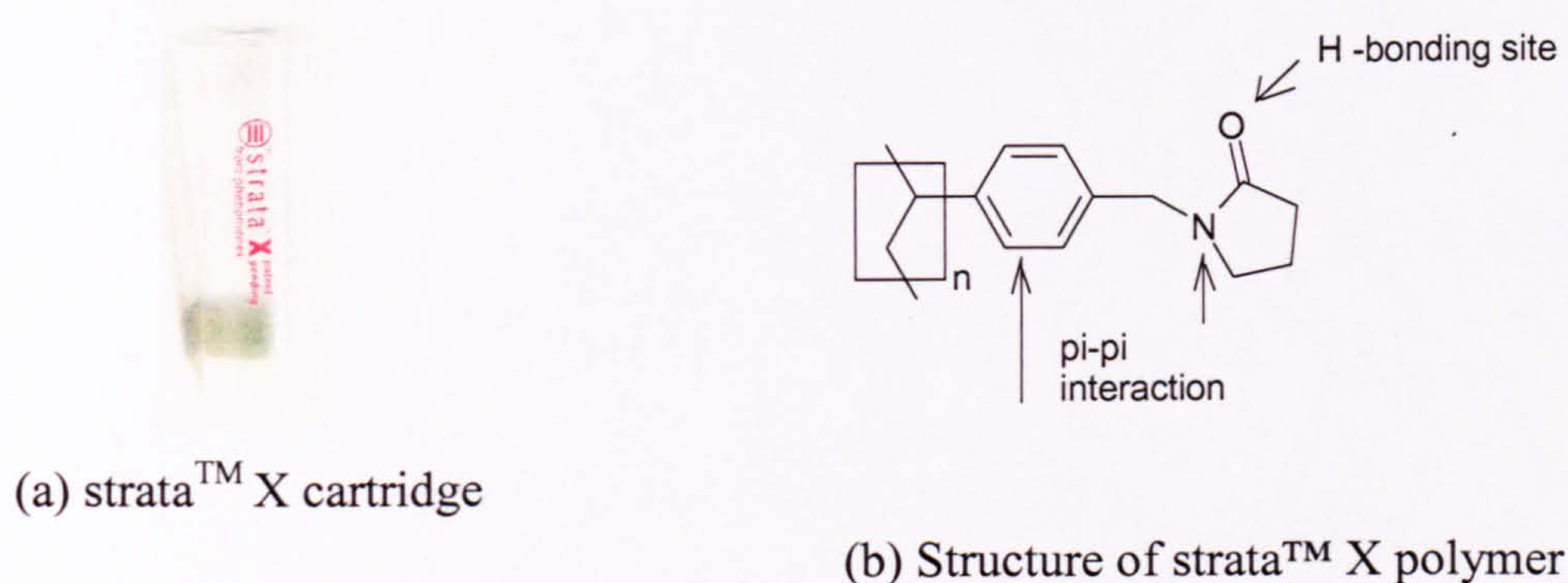
Solid Phase Extraction (SPE) is an extraction method that uses a solid phase and a liquid phase to isolate one or many type of analyte from a solution is usually used to clean up a sample before using a chromatographic technique. The separation properties of the components in a mixture are constant under constant conditions.[249, 268-271] SPE is a well-known method for rapid sample preparation in which a solid stationary phase is typically packed into a syringe barrel and used to selectively extract, concentrate, and purify target analytes prior to analysis by HPLC or GC[272]. SPE methods usually have four steps in which (i) the cartridge is conditioned, (ii) the sample for analysis is loaded on the cartridge, (iii) the cartridge is washed to remove as many impurities as possible and then (iv) the analytes of interest are eluted and collected for analysis.

It is an increasingly useful sample preparation technique. Many of the problems associated with liquid /liquid extraction (LLE), such as incomplete phase separations, can be avoided using SPE. Other disadvantages of using LLE include incomplete recoveries, use of expensive and specialised glassware, and disposal of large quantities of organic solvents. SPE is more efficient than liquid / liquid extraction and yields quantitative extractions that are easy to perform. It is rapid and can be automated so solvent use and lab times are reduced. SPE is used most often to prepare liquid samples and extract semivolatile or nonvolatile analytes, but it can also be used with solids that are pre-extracted into solvents. SPE products are excellent for sample extraction, concentration, and cleanup. They are available in a wide variety of chemistries, adsorbents, and size. Selecting the most suitable product for each application and sample is important.[273-278]

There are many types of SPE including cartridges which are more efficient, have fewer steps, and work with acidic and basic drugs and which are especially suitable for forensic laboratories.[279] .

The method is not ideal and an improved extraction procedure would be desirable which gave a single fraction containing all drugs which was clean enough to be analysed by GC-MS. Recent developments in SPE have included the introduction of new techniques such as microextraction techniques[280] and also new types of, polymeric sorbent, including molecularly imprinted polymers[281, 282]

In this study, a new sorbent, strata™ X polymeric reversed phase sorbent for polar and non-polar compounds was investigated as a replacement for the Certify sorbent.[283] The SPE strata™ X procedure was developed for the isolation of acidic, neutral and basic drugs from whole blood. The sorbent in strata™ X is composed of a styrene divinylbenzene polymer that has hydrophilic, hydrophobic and pi-pi retention mechanisms. Full details of the composition of strata™ X have not been released by the manufacturer, Phenomenex, but it has substituents on a carbon chain as shown inFigure 5-2 (b).



(a) strata™ X cartridge

(b) Structure of strata™ X polymer

Figure 5-2:- strata™ X

Strata™ X (Figure5- 2a) uses a novel surface chemistry in the sorbent and offers significant advantages over the traditional silica-based SPE products. The selectivity of the strata™X resin provides high and reproducible recoveries for a wide range of acidic, neutral and basic compounds, simplifying the sorbent selection process for method development. Not only is development simple but the unique surface properties of the resin remain "conditioned" even if it accidentally runs dry during the extraction, a condition that would significantly impair silica-based sorbents.

Strata™ X has been used for extraction of drugs from different types of biological samples including tissues, plasma and urine and seemed like a good alternative to the silica-based sorbent Certify.[284-287]

This chapter describes work carried out to develop the extraction step of the proposed unified method and the validation of the whole method, including the extraction, derivatisation and GC-MS analysis steps. Three versions of a possible unified method were investigated based on different types of derivatising agent as described in Chapter 4.

These investigations were carried out using the same test standards as described in Chapter 4. The final test of the method was to apply it to case samples.

5.2 Method Validation - Introduction

Method validation: this term consists of two parts. Firstly, "method": this is a procedure for the analysis of a specific analyte. Secondly, "validate": to support or corroborate on a sound or authoritative basis.[288] Method validation is a way of testing a particular analytical method to see if it is acceptable for its intended purpose.[289] Methods which have been validated by one laboratory need to be validated before they are implemented in another laboratory. Commercial methods also need to be checked even if the manufacturer's recommended procedures are followed. Validation is also needed if any method is modified or used for a new application, for example, a method for the analysis of urine specimens should be validated if used for blood. Minor changes do not need re-validation, for example, changing the column in a gas chromatograph for another of the same type.

The validation of a method follows a standardised set of experimental tests which produce data relating to accuracy, precision, linearity, limit of detection, limit of quantification and recovery.[290] Guidelines have been published by several organisations.[291-293]

Definitions of these terms are as follows.[294] Accuracy is the ability of a method to get the true result. For quantitative tests the accuracy expresses the closeness of agreement between the true value and the value obtained by applying the test procedure a number of times.

Precision is the closeness of agreement between independent test results obtained under specified conditions. In this study precision was measured Intra-day and Inter-day. Precision is usually affected by the analyte concentration and so should be measured at different concentrations. In this study three different concentrations were used. Precision is usually expressed in terms of the percentage relative standard deviation of the test results.

Linearity is defined as the range of concentrations of analyte for which the procedure provides test results that are in direct correlation to amount of analyte in the sample. This

is determined from the calibration curve and values of R^2 where R^2 is the correlation coefficient between X and Y in the graph.

LOD is defined as the lowest amount of analyte in a sample that can be detected, but not necessarily quantified.[295] usually the limit of detection is evaluated as the amount of analyte that gives a signal-to-noise ratio of 3. Alternatively, it is equivalent to the blank reading plus 3 times the standard deviation of the noise or standard error of the regression line. The following equation was used:

$$LOD = Y_B + 3Sy/x \qquad \textbf{Equation 5-1}$$

where Y_B is the intercept on the Y-axis (equivalent to the blank reading) and Sy/x is the standard error of the calibration linear regression line.

Similarly, The LOQ is the smallest amount of analyte in a sample that can be quantified with suitable precision and accuracy.[295] Usually the quantitation limit is evaluated as the quantity of analyte that gives a signal-to-noise ratio that is equivalent to 10. Alternatively, it is the blank reading plus 10 times the standard deviation of the noise or standard error of the regression line. .

Recovery is the percentage of the drug, metabolite, or internal standard originally in the specimen that reaches the end of the procedure.

Additional parameters have tended to be added to the list of those required for full method validation[289] including selectivity. Selectivity was examined in this project by analysing negative control blood samples from real cases, to determine if interferences were present.

In the present study, these validation parameters were measured for three possible versions of a unified method based on solid phase extraction with strata™ X followed by derivatisation with the three reagents described in Chapter 4 and GC-MS analysis.

5.3 General Experimental Section

5.3.1 Introduction

Drug-free blood samples were spiked with different amounts of drug mixture including amphetamine, methamphetamine, morphine, benzoylecgonine, THC-OH and THC-COOH as model compounds. The blood was then extracted by SPE using the novel strata™ X

cartridges. Three different derivatisation methods were then applied to the extracts including PFPA with PFP-OH, PFPA with TMS-diazomethane and MTBSTFA.

5.3.2 Preparation of drug mixtures

a) All stock solutions were purchased or prepared at a concentration of approximately 0.1mg/mL for spiking blood samples. Less concentrated solutions were prepared at 1µg/mL by dilution with methanol.

A stock solution of amphetamine sulphate (salt) was prepared at 0.11mg/ml; correcting for amphetamine free base the concentration was 0.08mg/ml = 80ng/µl. However a concentration of 1µg/ml was needed, therefore 100µl was taken from 80ng/µl stock and 900µl of methanol was added. This gave a 8µg/ml solution which was diluted again by taking 500µl into a 5ml mixture flask solution and making up to volume with methanol, giving a final concentration of 0.8µg/ml = 0.8 ng/µl.

A stock solution of methamphetamine hydrochloride was prepared at 0.105 mg/ml; correcting for methamphetamine free base the concentration was 0.08 mg/ml. The same dilution procedure was carried out as before, giving a final concentration 0.8 µg/ml = 0.8 ng/µl.

A stock solution of benzoylecgonine was prepared at 0.105 mg/ml. 50µl of this solution was taken and 950µl of methanol was added to give a solution of 5.25µg/ml. 250µl of 5.25 µg/ml solution was taken and 4.75ml of methanol added to give a final concentration of 0.263µg/ml = 0.263 ng/µl.

The concentrations of stock solutions of THC-OH and THC-COOH were 100µg/ml. 50µl of 100µg/ml solution was added to 4.95ml of methanol resulting in a final concentration of 1 µg/ml = 1 ng/µl. The final concentrations of working drug standards are summarized in Table 5-1.

Table 5-1:- Final concentrations of drug standards

Drug	Final concentration of drug standards(ng/μl)
Amphetamine	0.8
Methamphetamine	0.8
Benzoylecgonine	0.261
Morphine	0.91
THC-OH	1
THC-COOH	1

b) Preparation of Internal Standard Solutions.

Stock internal standard solutions of amphetamine–d_5, methamphetamine-d_5, benzoylecgonine-d_3, cocaine-d_3, THC-OH-d_3, THC-COOH-d_3 and morphine-d_3 were purchased at concentrations of 100μg/ml in methanol. Each of the stock solutions was diluted with 10ml methanol to give standard solutions at 1μg/ml = 1 ng/μl.

5.3.3 Preparation of drug mixtures in blood

Time expired transfusion blood supplied from a local hospital, which was kept in the fridge at 4°C, was used to prepare drug standards. A drug mixture was prepared containing amphetamine, methamphetamine, benzoylecgonine, cocaine, THC-OH, THC-COOH and morphine with a final concentration of approximately 1μg/ml=1ng//μl of each drug are listed in Table 5-1.

Samples used for method validation were prepared by adding 1ml blood to 3.5 ml buffer solution and then adding standard solutions and 100μl of deuterated internal standard solution. The blood standard was mixed thoroughly and centrifuged for ten minutes before being applied to the extraction cartridge.Blank samples were prepared with and without internal standard.

5.3.4 Preparation of buffer solution

A buffer is a solution characterized by the ability to resist changes in pH when limited amounts of acid or base are added to it. A buffer solution for the study was prepared by weighing 6.81g of potassium dihydrogen orthophosphate (KH_2PO_4, MW 136.09) into a 500ml beaker with 450ml of distilled water. The pH was adjusted to pH 6 with 0.1 M potassium hydroxide solution and made up to 500ml with distilled water in a volumetric flask.

5.3.5 Extraction method: SPE on Strata™ X

The method used followed the manufacturer's instructions and was as follows:

A- Conditioning: prior to loading the sample, the SPE column was washed and wetted with 1 ml methanol.

B- Equilibration: in this study, the SPE column was equilibrated with 1ml distilled water.

C- Sample loading: blood standards containing different concentrations of the test drug compounds were applied to the SPE columns.

D- Washing out of impurities. 5% methanol in distilled water was prepared by mixing 95ml of distilled water with 5ml methanol. 1.5 ml of this solution was applied before drying for 30 seconds at full vacuum.

E- Elution was carried out in one step with 1.5ml of methanol.

The extracts were then evaporated to dryness under a stream of nitrogen, without heating.

5.3.6 Derivatisation

Extracts were derivatised with each of the three reagents described in Chapter 4 before being analysed by GC-MS

5.3.7 GC-MS analysis

Derivatised extracts were analysed by GC-MS using the conditions described in Chapter 4, Table 4-1.Data was collected in the SIM mode using the ions listed in Tables 4-2, 4-4 and 4-5.

5.3.8 Validation parameters

The parameters measured for each of the three derivatisision procedures were:- Linearity, Intra-day Precision, Inter-day Precision, Limit of Detection, Limit of Quantification and Recovery. Each parameter was measured using 5 replicate standards.

5.3.9 Application to case samples

Each version of the poroposed unified method was applied to real case samples taken at autopsy and received by the Section of Forensic medicine and Science, University of Glasgow. Cases were selected for analyses which were known to be positive for the drug groups of interest which were used as test substances in this study, to serve as positive controls. In addition, a second group of cases was analysed which had not been found to contain the drugs of interest to serve as negative controls. These were kept frozen until analysed.

5.4 Method Validation for Drug Derivatisation with PFPA-PFPOH

5.4.1 Introduction

These reagents produce pentafluoropropionyl (acyl) derivatives of amines and alcohols and simultaneously the anhydride activates carboxylic acids so that PFPOH will react with them to produce the pentafluoropropyl ester derivatives. The following paragraphs describe the validation procedures carried out.

5.4.2 Linearity

Five replicate analyses were carried out at each concentration and linearity was determined from the calibration curve and values of R^2 where R^2 is the correlation coefficient between X and Y in the graph. Calibration curves for the target analytes were linear over a concentration range of 16-80 ng/ml of blood for amphetamine and methamphetamine, 4.55-22.75 ng/ml of blood for morphine, 5.25-26.25 ng/ml of blood benzoylecgonine and 20-100 ng/ml blood for THC-OH, THC-COOH. The correlation coefficient (R^2) values

are listed in Table 5-2, and curves (averaged values of 5 replicates) are shown below in Figure 5-3 to Figure 5-8.

Table 5-2:-Summary of Values of correlation coefficient (R^2) for all compounds analysed as the PFP-PFP-OH derivatives

Compound	R^2 Values
Amphetamine	0.9966
Methamphetamine	0.9947
Morphine	0.9992
Benzoylecgonine	0.9945
THC-OH	0.9989
THC-COOH	0.9934

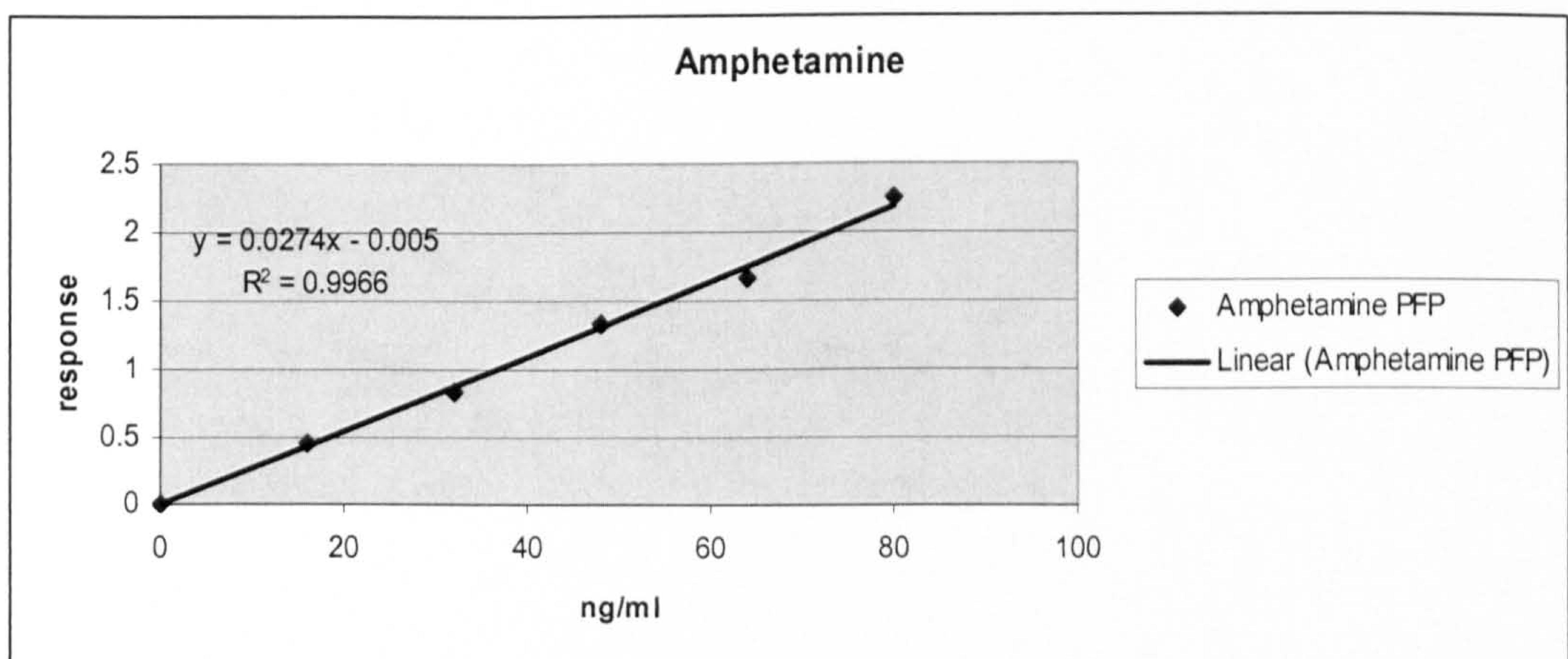


Figure 5-3:- Calibration curve for Amphetamine analysed as the PFP derivative

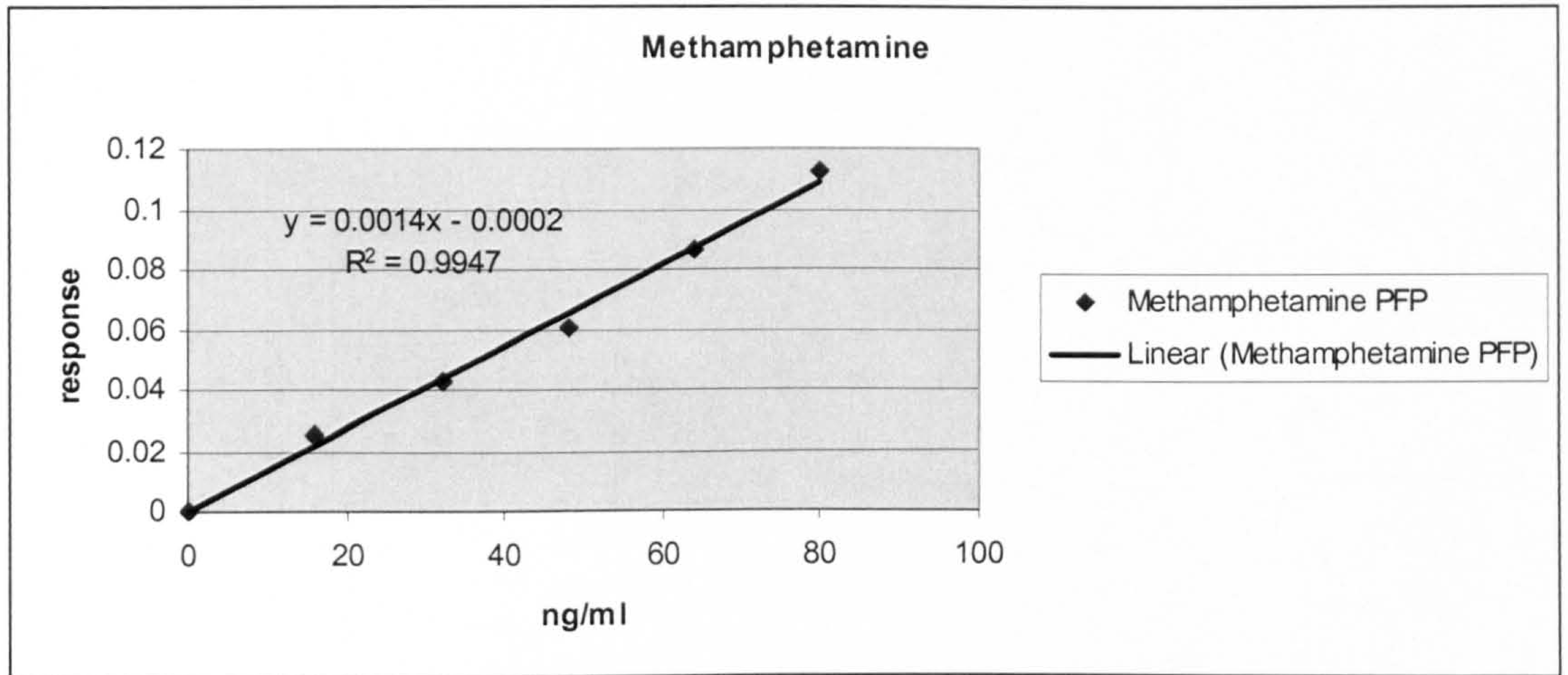


Figure 5-4:- Calibration curve for Methamphetamine analysed as the PFP derivative.

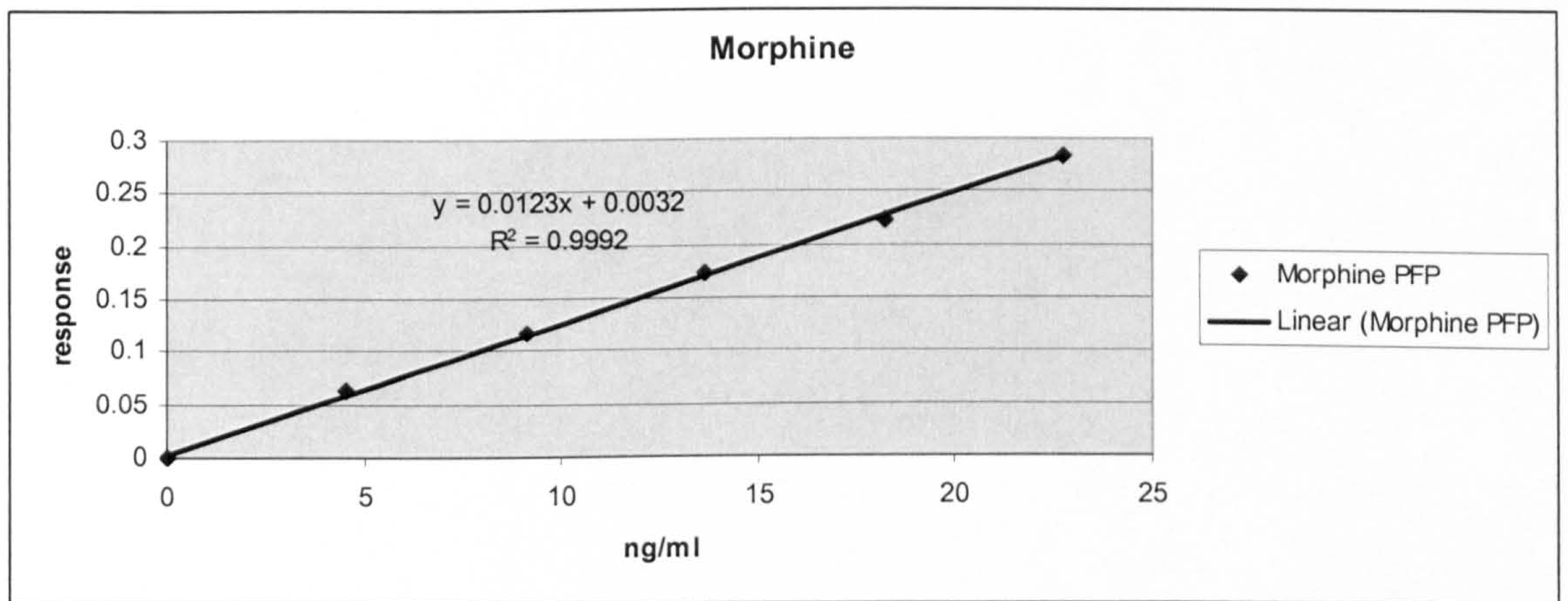


Figure 5-5:- Calibration curve for Morphine analysed as the bis-PFP derivative

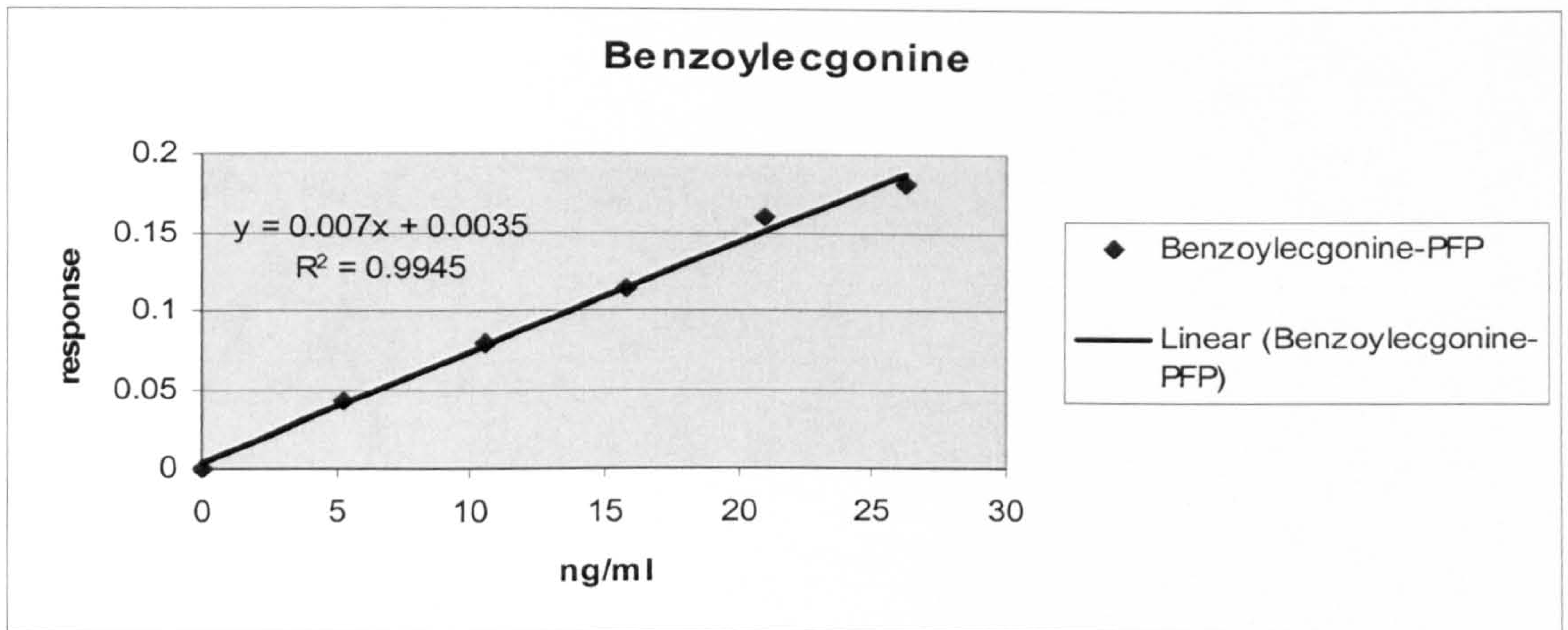


Figure 5-6:- Calibration curve for Benzoylecgonine analysed as the PFP ester derivative

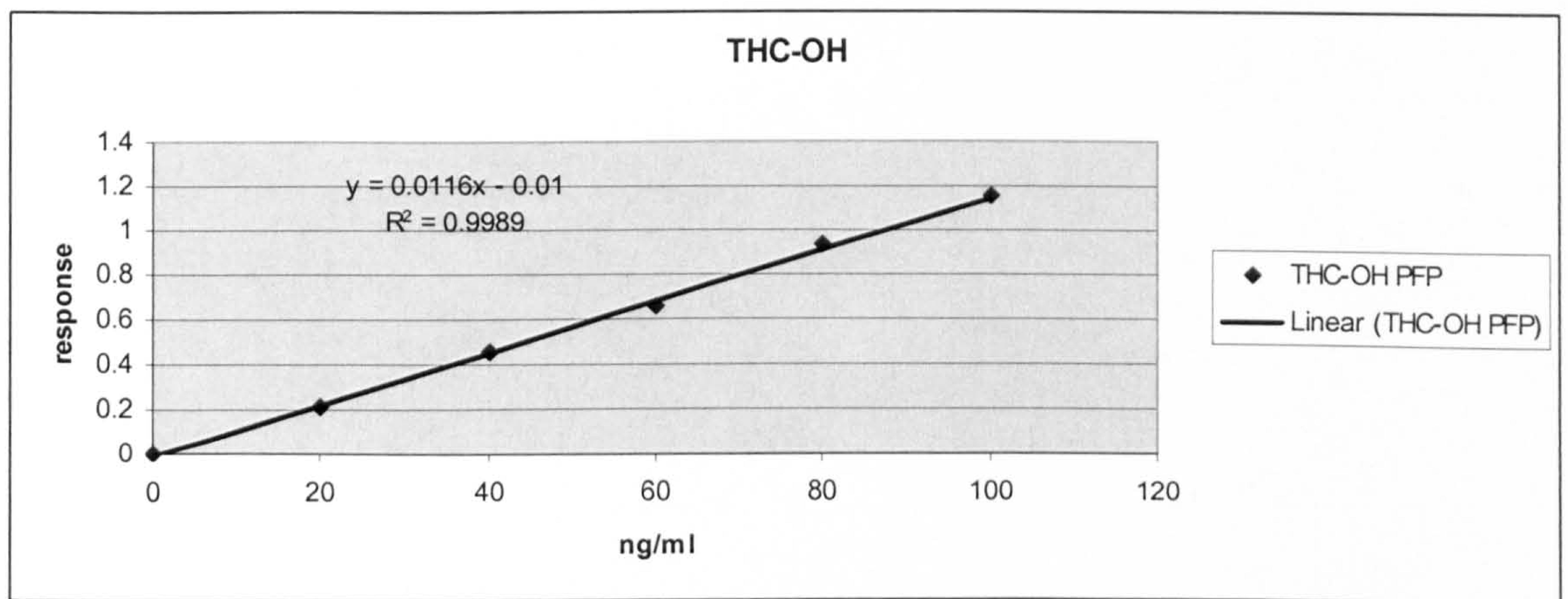


Figure 5-7:- Calibration curve for THC-OH analysed as the PFP derivative

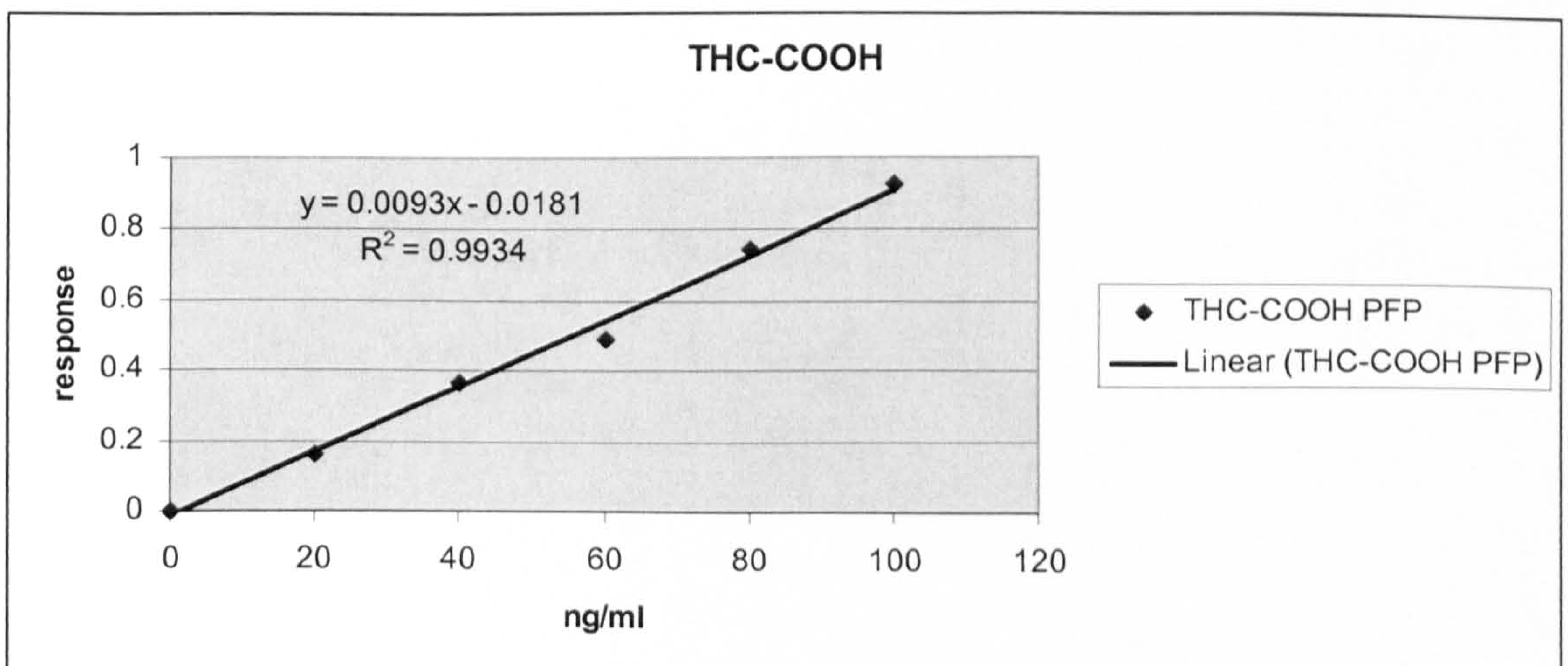


Figure 5-8:- Calibration curve for THC-COOH analysed as the PFP/PFP ester derivative

5.4.3 Intra-Day variation

Different concentrations for the test drugs in the range 16-80ng/ml of blood for amphetamine and methamphetamine, 4.55-22.75ng/ml of blood for morphine, 5.25-26.25ng/ml of blood benzoylecgonine and 20-100ng/ml blood for THC-OH, THC-COOH were extracted five times to calculate the intra–day variation of the assay. Average drug/internal standard ratios and relative standard deviations (%RSD) were calculated for each concentration as shown from Table 5-3 to Table 5-6.

Table 5-3:- Intra-Day variation for Amphetamine and Methamphetamine analysed as the PFP-PFP-OH derivatives

Added Concentration (ng/ml of blood)	Average area Ratio (%RSD)	
	Amp/Amp-D_3	Meth/Meth-D_5
16	0.2 (22.3)	0.01 (5.8)
48	0.9 (10.4)	0.03 (10.9)
80	1.6 (10.3)	0.05 (6.8)

Table 5-4:- Intra-Day variation for Morphine analysed as the bis-PFP derivative

Added Concentration (ng/ml of blood)	Average area Ratio Mor/Mor-D3 (%RSD)
4.6	0.05 (3.0)
13.7	0.1 (8.1)
22.8	0.2 (13.5)

Table 5-5:- Intra-Day variation for Benzoylecgonine analysed as the PFP ester derivative

Added Concentration (ng/ml of blood)	Average area Ratio Bze/Bze-D3 (%RSD)
5.3	0.07 (10.8)
15.8	0.13 (4.8)
26.3	0.21 (7.2)

Table 5-6:- Intra-Day variation for THC-OH and THC-COOH analysed as the PFP/PFP ester derivatives

Added Concentration (ng/ml of blood)	Average area Ratio (%RSD)	
	THC-OH /THC-D_3	THC-COOH/THC-COOH-D_3
20	0.4 (6.5)	0.14 (15.6)
60	0.7 (4.8)	0.61 (10.3)
100	1.3 (6.2)	1.3 (16.5)

5.4.4 Inter-Day variation

A set of calibration standards in blood containing six drugs was extracted five times during different days with the same concentration as before (16-80ng/ml of blood for amphetamine and methamphetamine, 4.55-22.75ng/ml of blood for morphine, 5.25-26.25ng/ml of blood benzoylecgonine and 20-100ng/ml blood for THC-OH, THC-COOH). Calibration curves were constructed and the concentration of each standard was measured using the calibration curve, based on the ratio of analyte to internal standard. The inter-day variation for each test substance was measured and these values are displayed in Table 5-7 to Table 5-10.

Table 5-7:- Inter-Day variation for Amphetamine and Methamphetamine analysed as the PFP derivatives

Added Concentration (ng/ml of blood)	Mean Measured Concentration (%RSD)	
	Amp	Meth
16	15.9 (6.9)	16.8 (27.0)
48	47.7 (5.4)	40.6 (12.9)
80	80.4 (2.5)	81.0 (5.1)

Table 5-8:- Inter-Day variation for Morphine analysed as the bis-PFP derivative

Added Concentration –Morphine (ng/ml of blood)	Mean Measured Concentration (%RSD)
4.6	4.5 (12.3)
13.6	14.1 (10.3)
22.7	23.0 (2.4)

Table 5-9:- Inter-Day variation for Benzoylecgonine analysed as the PFP ester derivative

Added Concentration –Benzoylecgonine (ng/ml of blood)	Mean Measured Concentration (%RSD)
5.3	5.5 (3.9)
15.8	15.3 (0.8)
26.3	26.6 (1.6)

Table 5-10:- Inter-Day variation for THC-OH and THC-COOH analysed as the PFP/PFP ester derivatives

Added Concentration (ng/ml of blood)	Mean Measured Concentration (%RSD)	
	THC-OH	THC-COOH
20	21.2 (8.5)	19.8 (21.4)
60	58.4 (7.5)	60.9 (9.0)
100	103.5 (1.9)	98.4 (3.1)

5.4.5 Recovery

The recovery of the method, using strata™ X, was calculated using spiked blood samples at three different concentrations. Each concentration was extracted five times without internal standard present. Two unextracted standards were also prepared at each concentration without internal standard and were kept in the fridge throughout the extraction. Before blowing down the samples under a stream of nitrogen, 100μl of internal standard was added to each vial including the unextracted standards. The recovery calculated was obtained from the peak area ratio of the extracted standard divided by the peak area ratio of the unextracted standard, multiplied by 100. Recovery for all the drugs is summarized in Table 5-11 and Figure5-9.

Table 5-11:- Recovery of six drugs analysed as PFPA-PFP-OH derivatives

Analyte	Concentration(ng/ml)	Recovery %
Amphetamine	16	74
	48	89
	80	89
Methamphetamine	16	23
	48	15
	80	18
Morphine	4.6	96
	13.7	60
	22.8	71
Benzoylecgonine	5.3	86
	15.8	91
	26.3	95
THC-OH	20	62
	60	49
	100	44
THC-COOH	20	61
	60	58
	100	59

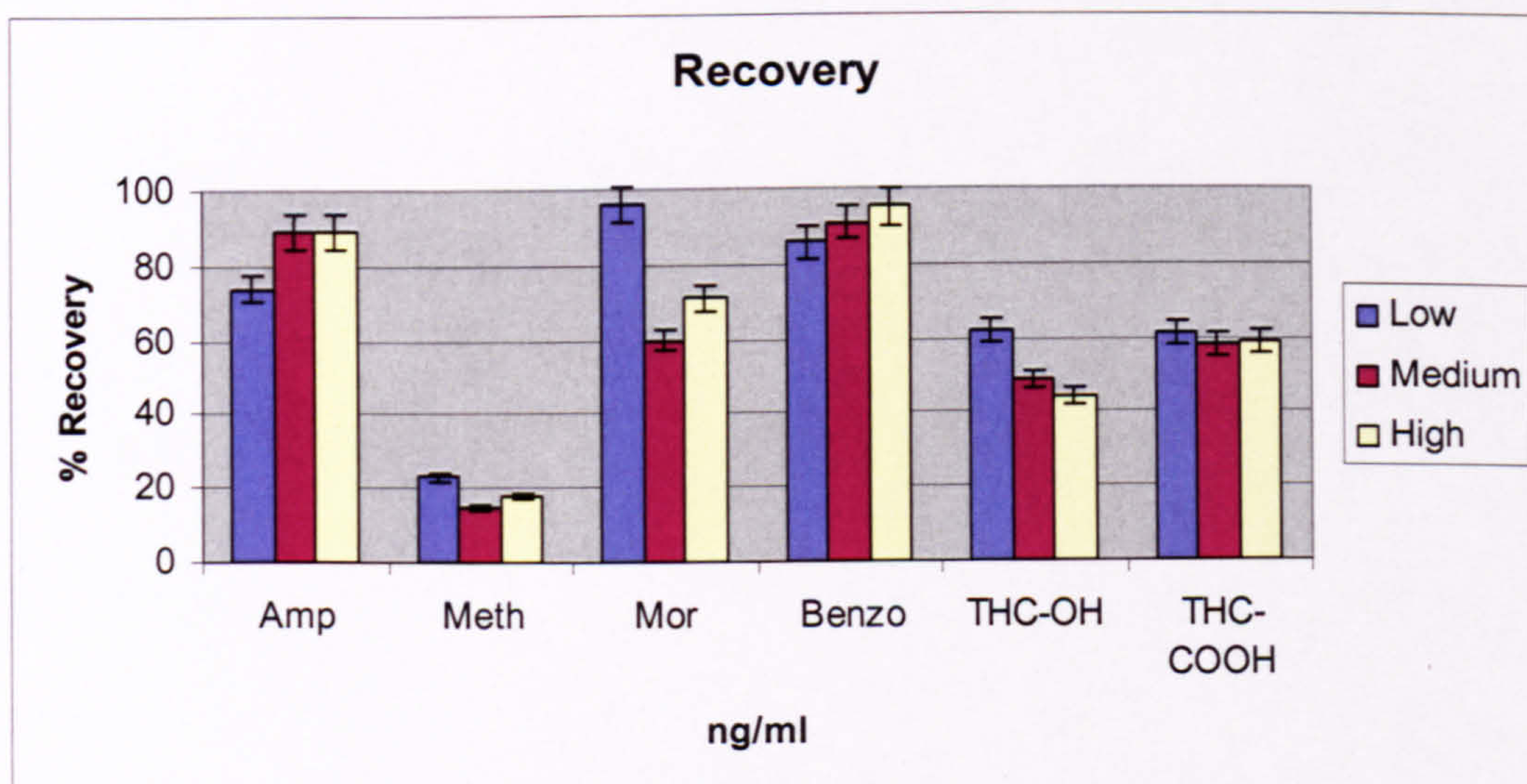


Figure 5-9:- Recoveries of drugs from blood using Strata™X and analysed as the PFP-PFP-OH derivatives

5.4.6 Limit of Detection (LOD)

Blood was spiked to give concentrations of 20, 15, 10, 5, 2.5, 1 and 0.5 ng/ml bloods for amphetamine, methamphetamine, morphine, benzoylecgonine, THC-OH and THC-COOH. Five replicates were analysed at each concentration. The equation of the line was

calculated for each drug using Microsoft Excel. The regression analysis program within Microsoft Excel was also used to calculate Sy/x for each drug.[295] Equation 5-2 was then applied to find the limit of detection based on Equation 5-1

LOD = intercept + (3 x standard error) **Equation 5-2**

Rearranging the equation of the line for x gives the following:

y= mx + b **Equation 5-3**

Answer equation (2) applied in Equation (3) for getting limit of detection as

x = y-b/m **Equation 5-4**

5.4.7 Limit of Quantitation (LOQ)

As presented before, Equation 5-5 was then applied to find the limit of quantitation and rearranging the equation of the line from x Equation 5-3 for finding limit of quantitation.

LOQ = Y_B + 10Sy/x **Equation 5-5**

The limits of detection and quantitation were calculated and are shown below in Table 5-12.

Table 5-12:- Limits of Detection and Limits of Quantitation of the six drugs analysed as the PFP-PFP-OH derivatives

Drugs	LOD (ng/ml)	LOQ (ng/ml)
Amphetamine	1.2	4.0
Methamphetamine	0.8	2.7
Morphine	0.8	2.6
Benzoylecgonine	0.4	1.5
THC-OH	1.7	5.8
THC-COOH	3.7	12.4

5.4.8 Application to real case samples

The developed method was applied to 51 cases samples of which 36 samples had previously been found to be positive for drugs of abuse. Case results are summarized below in Table 5-13 and Table 5-14.

Table 5-13:- Results of GC/MS for drug-positive cases analysed as PFPA-PFP-OH derivatives

Case No	Drug Concentration (mg/L)						
	Amp	Meth	Mor	Cocaine	Bze	THC	THC-COOH
Blank	-	-	-	-	-	-	-
1	0.28	-	-	-	-	-	-
2	-	-	0.5	-	-	-	-
3	-	-	0.34	-	-	-	-
4	-	-	1.74	-	-	-	-
5	-	-	-	-	0.3	-	-
6	0.02	-	-	-	-	-	-
7	-	-	0.7	-	-	-	
8	-	-	0.01	-	-	-	-
9	-	-	0.46	-	-	-	-
10	-	-	0.11	-	-	-	-
11	-	-	0.32	-	-	-	-
12	-	-	0.15	-	-	-	-
13	-	-	-	-	0.0005	-	-

14	-	-	-	-	0.07	-	-
15	-	-	-	-	0.1	-	-
16	-	-	-	-	0.03	-	-
17	-	-	-	-	-	0.09	0.23
18	-	-	0.02	-	-	-	-
19	0.001	-	-	-	-	-	-
20	-	-	0.39	-	-	-	-
21	-	-	0.39	-	-	-	-
22	-	-	0.001	-	-	-	-
23	-	-	0.31	-	-	-	-
24	-	-	0.94	-	-	-	-
25	-	-	0.56	-	-	-	-
26	-	-	0.19	-	-	-	-
27	-	-	0.22	-	-	-	-
28	-	-	0.18	-	-	-	-
29	-	-	0.34	-	-	-	-
30	-	-	0.34	-	-	-	-
31	-	-	0.423	-	-	-	-
32	-	-	-	-	-	-	
33	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-

35	-	-	-	-	-	-	-
36	-	-	-	-	-	-	-

Table 5-14:- Results of GC/MS analysis of extracts containing other drugs using PFP/PFP-OH reagent

Case No	Drug Concentration (mg/L)								
Drugs	Amitrip	Metha	Code	Propox	Dothi	Indo	Fluox	Bupre	Parcet
Blank	-	-	-	-	-	-	-	-	-
1	1.4	-	-	-	-	-	-	-	-
2	-	0.84	-	-	-	-	-	-	-
3	-	-	0.1	-	-	-	-	-	-
4	-	-	0.3	-	-	-	-	-	-
5	-	-	-	0.2	-	-	-	-	-
6	-	-	-	2.2	-	-	-	-	-
7	-	-	-	0.3	-	-	-	-	-
8	-	-	-	0.01	-	-	-	-	-
9	-	-	-	-	2.5	-	-	-	-
10	-	-	0.1	-	-	-	-	-	-
11	-	-	0.02	-	-	-	-	-	-
12	-	-	0.014	-	-	-	-	-	-
13	-	-	-	-	-	0.02	-	-	-

14	2.3	-	-	-	-	-	-	-	-
15	2.8	-	-	-	-	-	-	-	-

(Amitrip = Amitriptyline, Metha = Methadone, Code = Codeine, Propox = Propoxyphene, Doth = Dothiepin, Indo = Indomethacin, Fluox = Fluoxetine, Bupre = Buprenorphine, Parcet = Paracetamol)

5.4.9 Discussion

This version of the proposed unified method was validated using PFP-OH-PFPA as the derivatising agent. The validation parameters, including linearity, precision, recovery, limit of detection and limit of quantification were measured and are summarised in Table 5-15 below.

Table 5-15:- Recovery, intra and inter-day assay precision with PFPA-PFP-OH at different concentration

Compound- Product	Concentration (ng/ml)	Recovery % (n=5)	Intra-day RSD%(n=5)	Inter-day RSD%(n=5)
Amphetamine-PFP	16	74	22.3	6.9
	48	89	10.4	5.4
	80	89	10.3	2.5
Methamphetamine-PFP	16	23	5.8	27.0
	48	15	10.9	12.9
	80	18	6.8	5.1
Morphine-PFP	4.55	96	3.0	12.3
	13.65	60	8.1	10.3
	22.75	71	13.5	2.4
Benzoylecgonine-PFP ester	5.25	86	10.8	3.9
	15.75	91	4.8	0.8
	26.25	95	7.2	1.6
THC-OH-PFP	20	62	6.5	8.5
	60	49	4.82	7.5
	100	44	6.2	1.9
THC-COOH PFP ester	20	61	15.6	21.4
	60	58	10.3	9.0
	100	59	16.5	3.1

The intra- and inter-day precision were evaluated at low, medium and high concentrations for five replicates and the % RSD was subsequently calculated. The %RSD values were mostly within the suggested acceptable limit of 15% (20% at the LOQ).[289] Intra-day assay %RSDs ranged from 0.3 to 22.3 %. The highest intra-day %RSD was 22.3% for amphetamine in blood at a concentration of 16 ng /ml while the lowest %RSD was 3% for morphine at 4.55 ng/ml blood. The inter-day assay %RSDs ranged from 0.8 to 27 %. The highest inter-day %RSD was with methamphetamine while lowest %RSD was with Benzoylecgonine at 15.8 ng/ml blood as shown in Table 5-15.

Calibration curves prepared with with six standards including blank experiments showed that the response was linear (R^2 > 0.99) for all the analyzed compounds which is acceptable for the analysis of biological specimens. Also, although there was matrix interference observed using the Strata™X SPE cartridge, good calibration graphs were obtained.

Benzoylecgonine produced the lowest detection limit whereas THC-COOH produced a higher detection limit. The limits of detection and quantitation were listed in Table 5-12. Two conclusions can be made from these results. Firstly, the derivatisation procedure worked well with both amines and carboxylic acids in the same extract so it fulfils the requirements of a unified procedure. Secondly, the detection limits for THC-COOH and benzoylecgonine reflect the fragmentation patterns seen in their mass spectra. The selected ions for both compounds are the base peaks in their mass spectra. However, the percentage of the ion current carried by the base peak ion of benzoylecgonine is larger than that for THC-COOH, i.e. the mass spectrum of the latter shows more ions, and so the sensitivity is expected to be better for benzoylecgonine. The detection limits for THC-OH and THC-COOH would need to be improved if the method was used routinely since the recommended cut-off level for THC in oral fluid is 4 ng /ml.

The recovery ranged between 15-96 %. The lowest recovery was with methamphetamine and the highest with morphine. The reason for the poor recovery of methamphetamine is not clear, as more polar compounds such as amphetamine, and less polar compounds such as THC-OH had higher recoveries. Possible reasons may relate to failure of the chemical derivatisation step for the secondary amine or else loss of methamphetamine during the evaporation step. Comparison with the recovery of methamphetamine measured using other reagents, for example the PFPA-methyl ester (Figure 5-10) shows that the recovery is good and that the loss of methamphetamine must have occurred outside the extraction step itself. However, the limit of detection and limit of quantification of methamphetamine were acceptable.

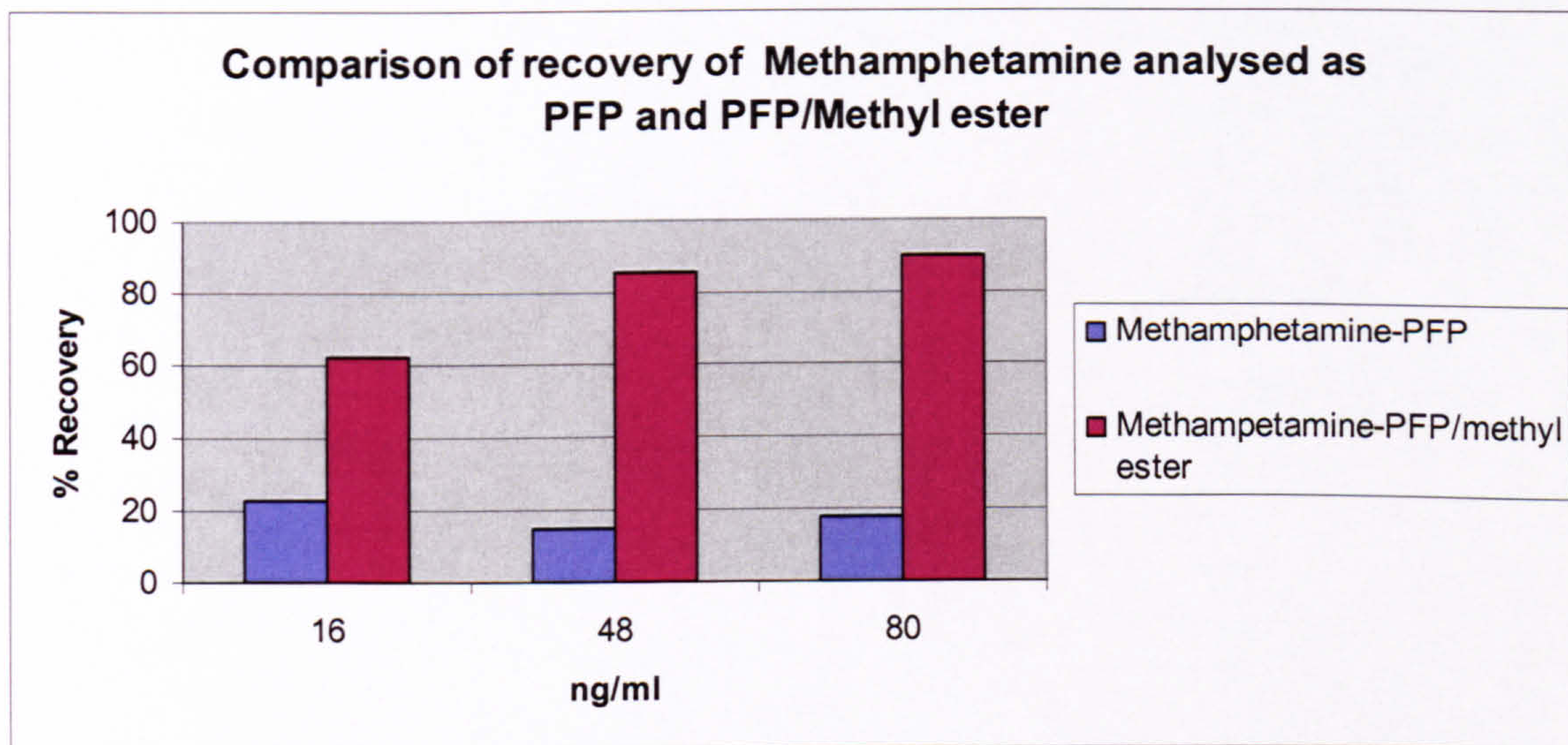
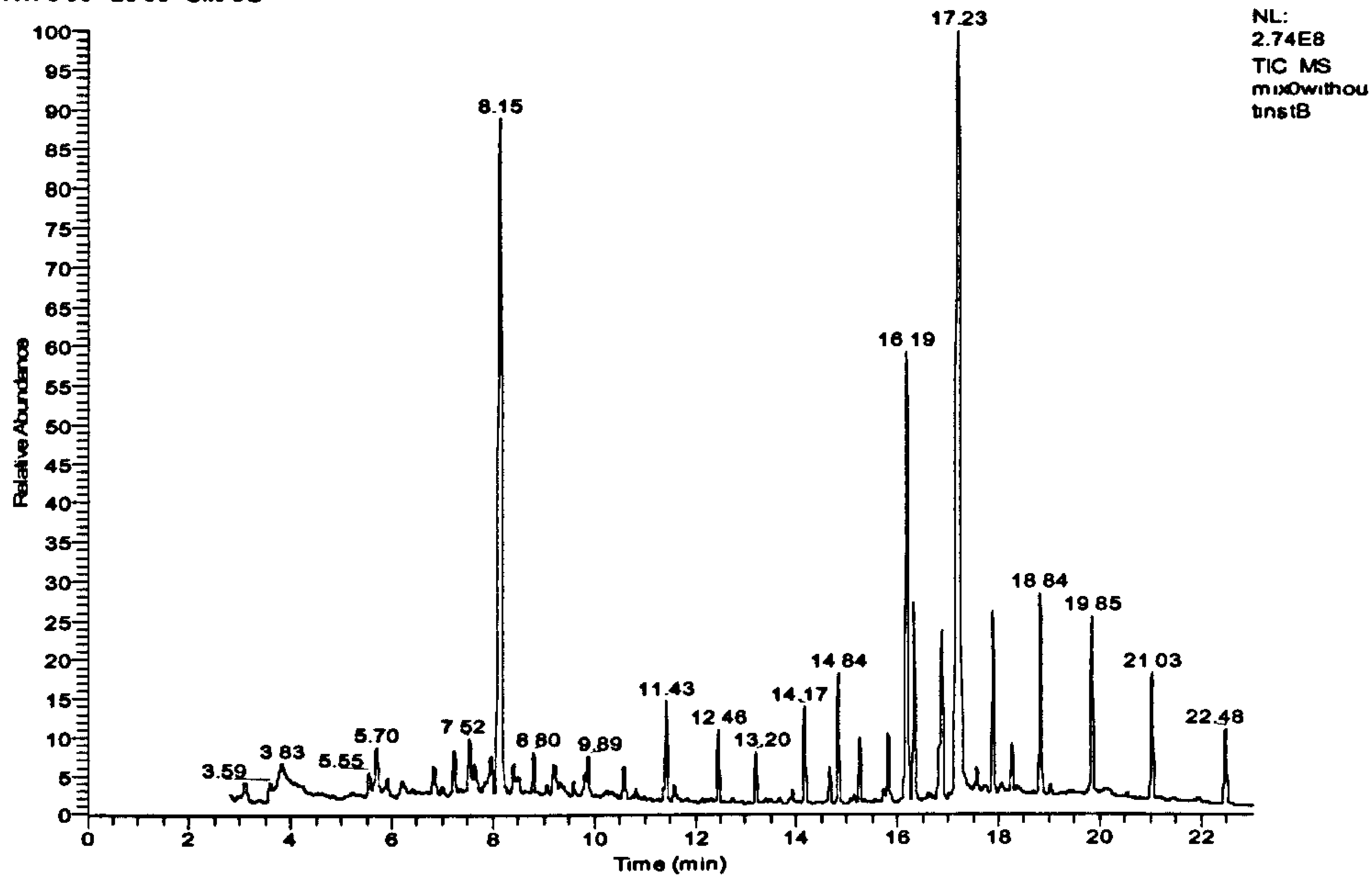


Figure 5-10:- Recovery of methamphetamine from blood using Strata ™X and analysed after derivatisation with PFPA-PFP-OH and PFPA/Methyl ester

The validated method was applied to blood samples collected from Forensic Medicine and Science at the University of Glasgow. In general the method worked very well with strata™ X. However there was a great deal of interference in the GC-MS analysis that could potentially obscure the analyte peaks. Because of the interferences observed using the strata ™ X SPE cartridge, washing of the SPE cartridge with more than 1.5 ml of 5% methanol in distilled water could perhaps resolve this problem Figure 5-11. The method

was found to be able to detect and quantify both basic and acidic drugs including drugs of abuse and prescribed drugs.

(A)



(B)

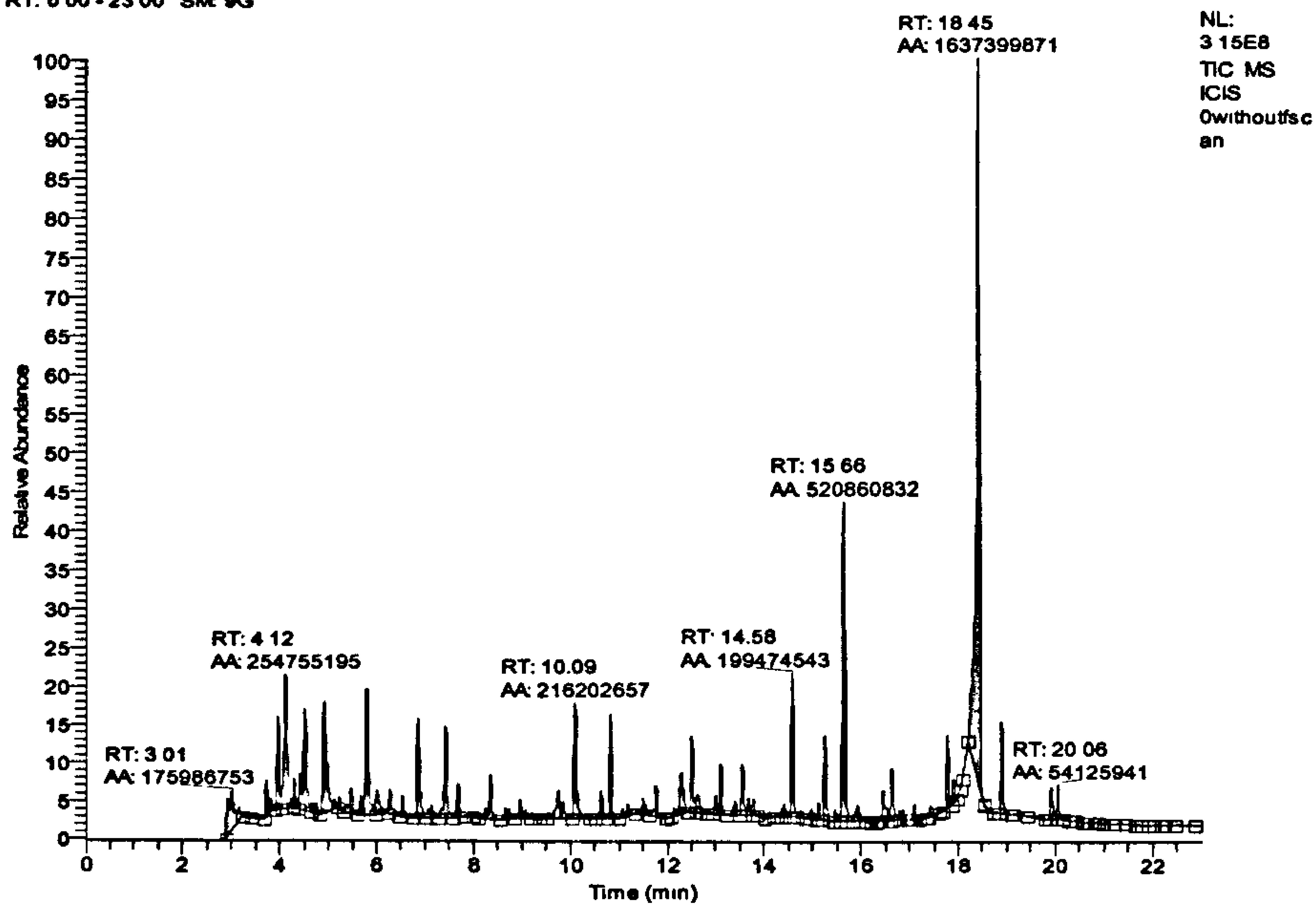


Figure 5-11:- TIC Chromatogram blank for Strata ™ X SPE (A) with 1ml methanol (B) with 1.5 methanol

5.4.10 Conclusion

This version of the method showed promise as a unified procedure for general use because of its ability to detect and meaure both acidic and basic drugs. Strata™ X also was able to extract both of these types of analyte from whole blood, although with different efficiencies and with co-extracted interferences, but nevertheless with acceptable limitis of quantification.

5.5 Method validation for drug derivatisation with PFPA and TMS-diazomethane

5.5.1 Introduction

These derivatising agents produce pentafluoropropionyl (acyl) derivatives of alcohols and amines, as for the PFPA-PFPOH reagent but the pentafluoropropyl esters are replaced with the methyl ester function. In principle, it would not be necessary to repeat the validation parameters for drugs which lack a carboxylic acid group. However, the possibility arises that changes in the reaction conditions might affect other drugs in unexpected ways and changes in the products obtained might also affect both the subsequent gas chromatography and mass spectrometry. For this reason, the same set of drug standards was used to model the analytical method, allowing direct comparison with the other versions of the procedure. The following paragraphs describe the validation procedures carried out.

5.5.2 Linearity

Standards in blood were prepared and each concentration was extracted five times and calibration graphs of each compound were produced. The concentration ranges were the same as listed in Paragraph 5.4.2. As seen before, the calibration curves of peak area ratio against the concentration of the target analyte were linear and these are shown in Figure 5-12 to Figure 5-17.The correlation coefficient (R^2) values are listed in Table 5-16.

Table 5-16:- Correlation coefficient (R^2) for calibration curves for all compounds analysed as the PFP/methyl ester derivatives

Compound	R^2 Values
Amphetamine	0.9888
Methamphetamine	0.9089
Morphine	0.9995
Benzoylecgonine	0.9862
THC-OH	0.9966
THC-COOH	0.9994

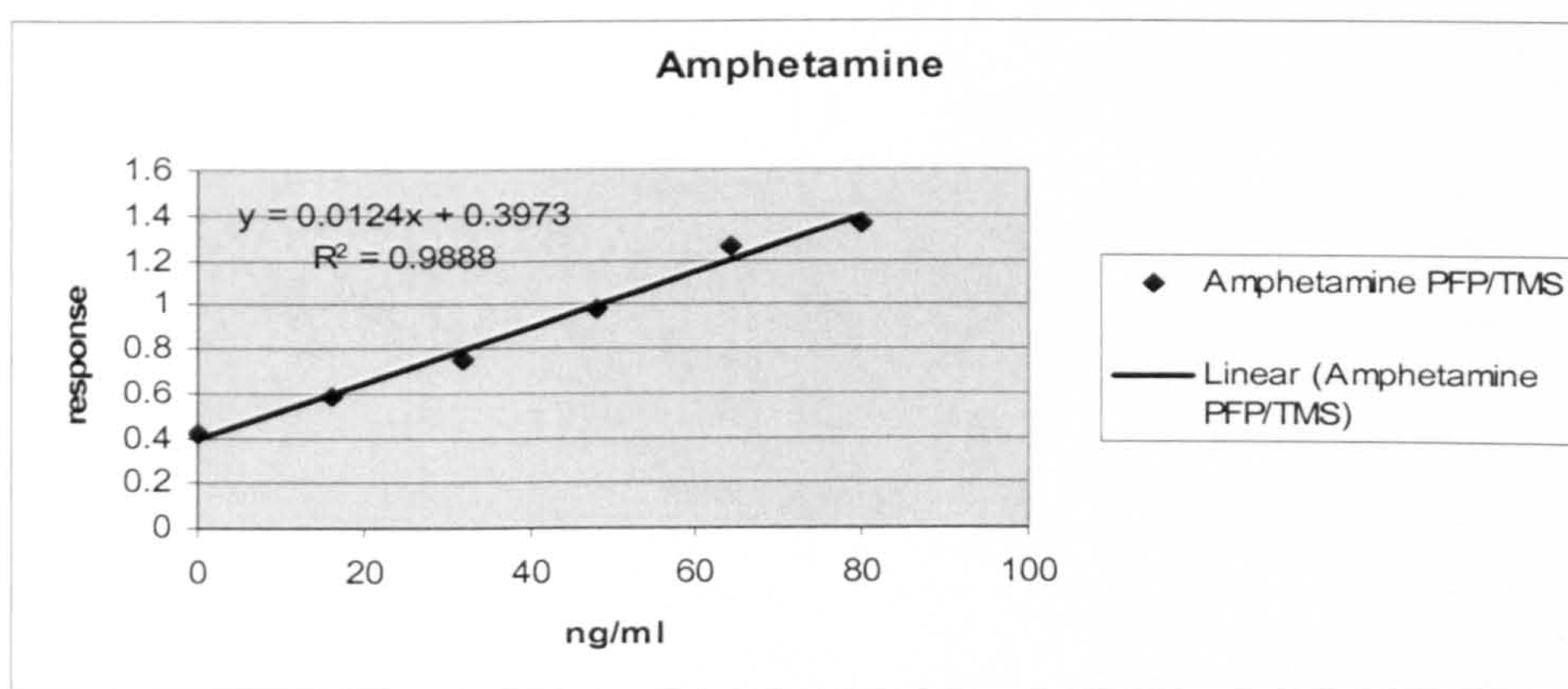


Figure 5-12:- Calibration curve for Amphetamine analysed as the PFP derivative

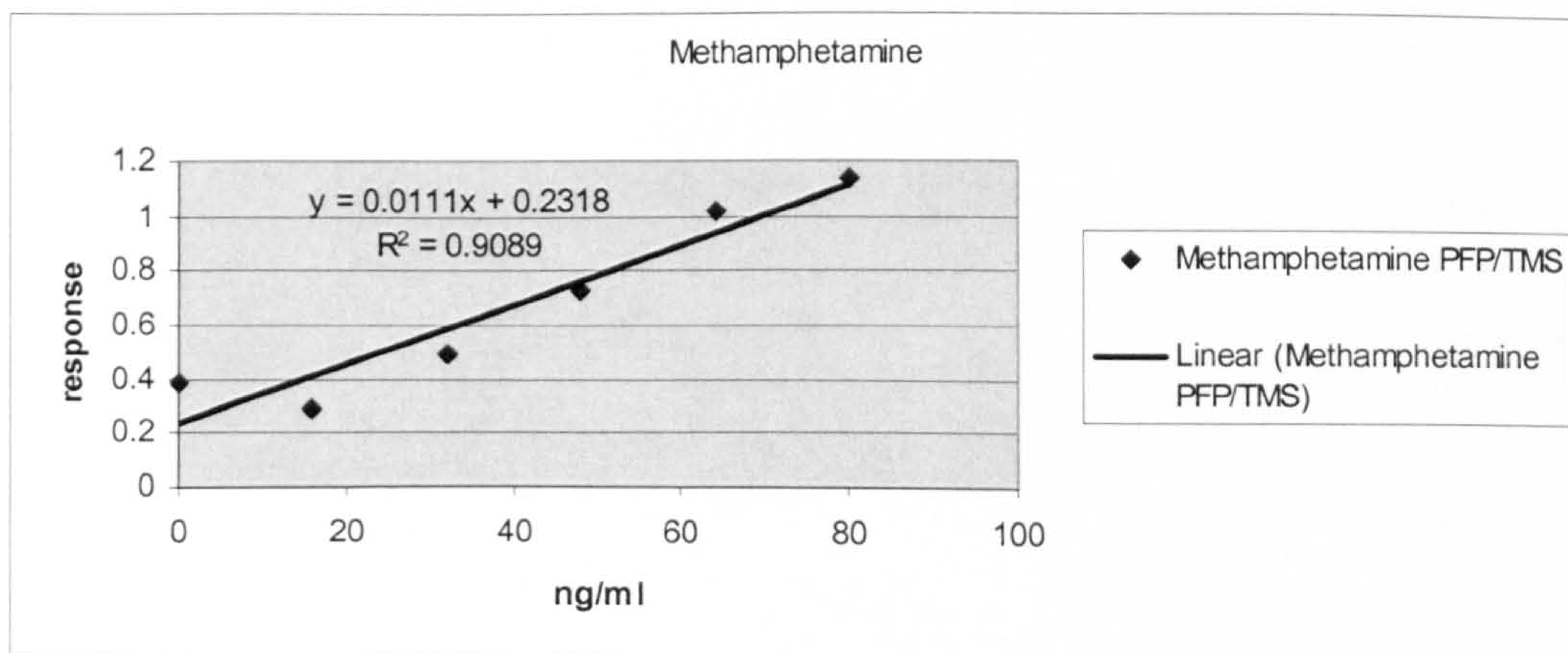


Figure 5-13:- Calibration curve for Methamphetamine analysed as the PFP derivative

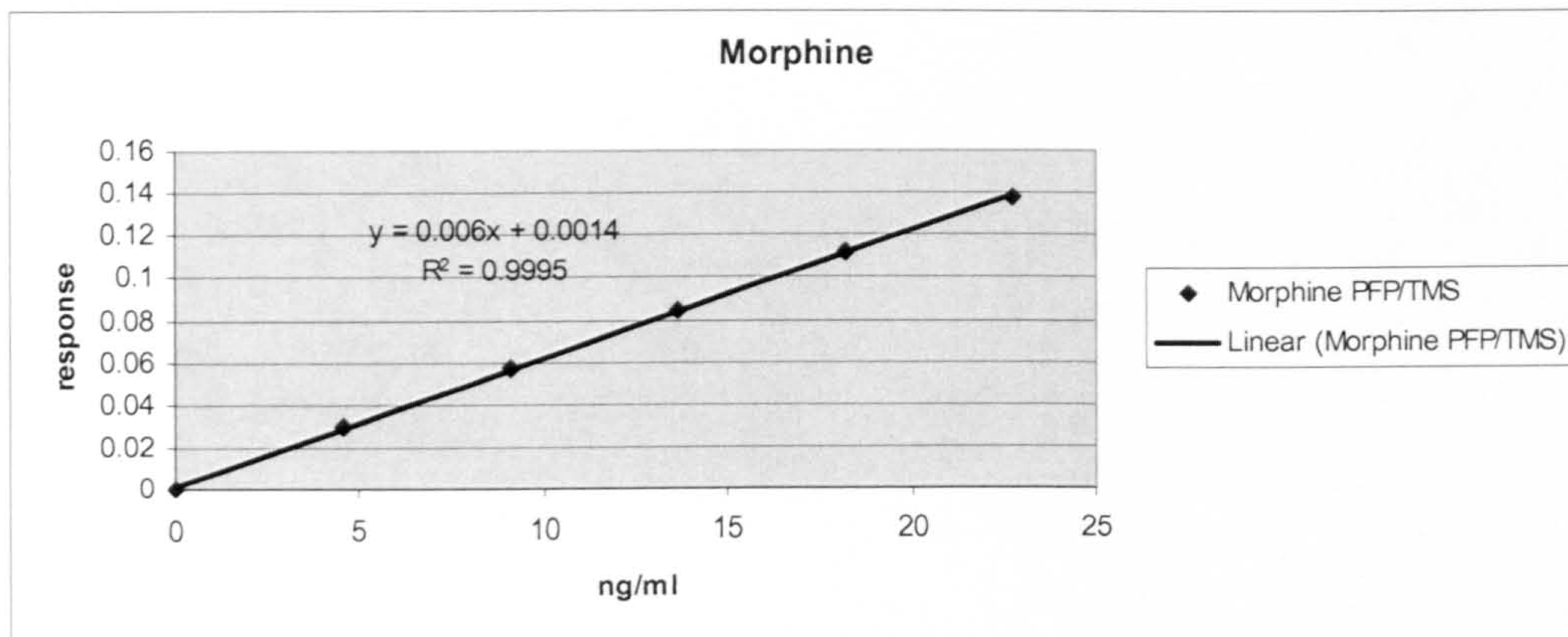


Figure 5-14:- Calibration curve for Morphine analysed as the bis-PFP derivative

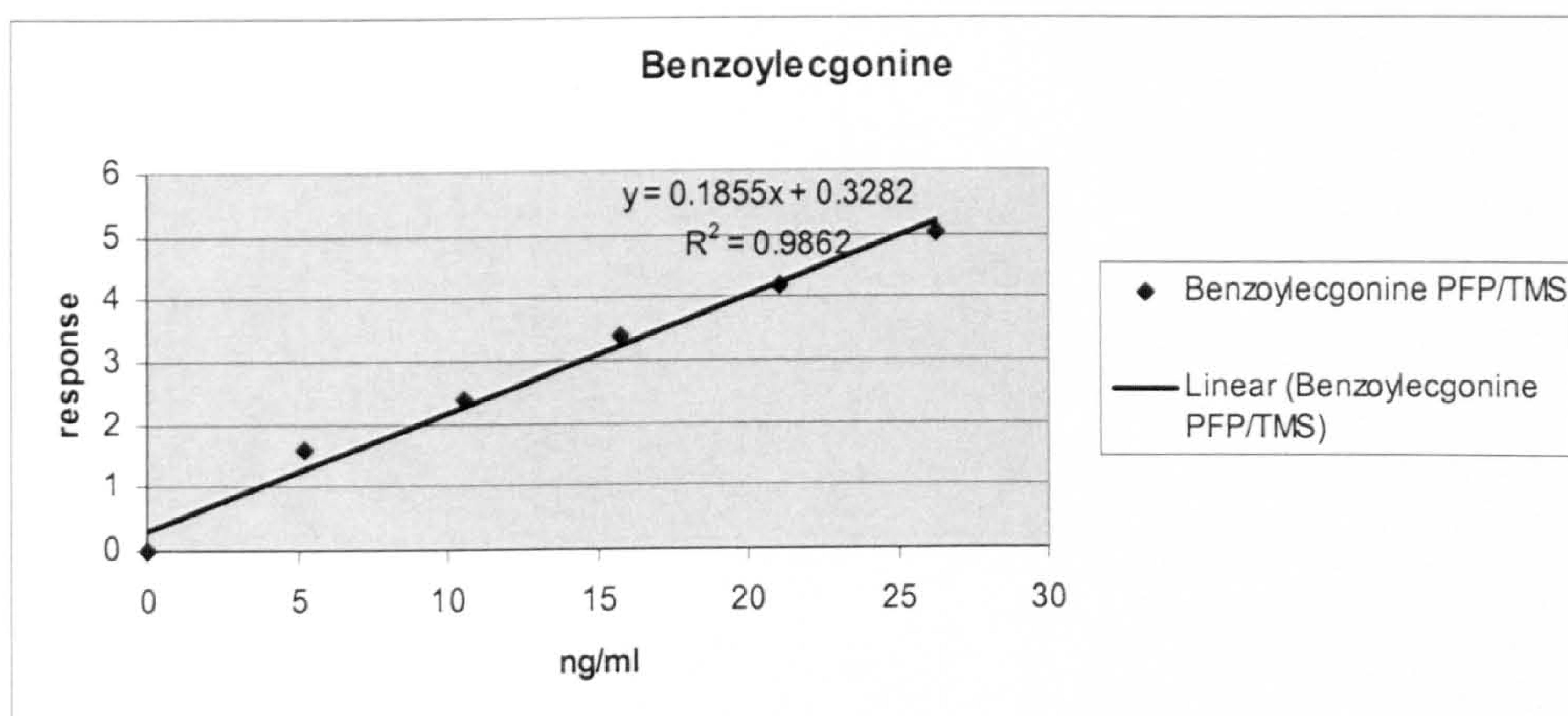


Figure 5-15:- Calibration curve for Benzoylecgonine analysed as the methyl ester derivative

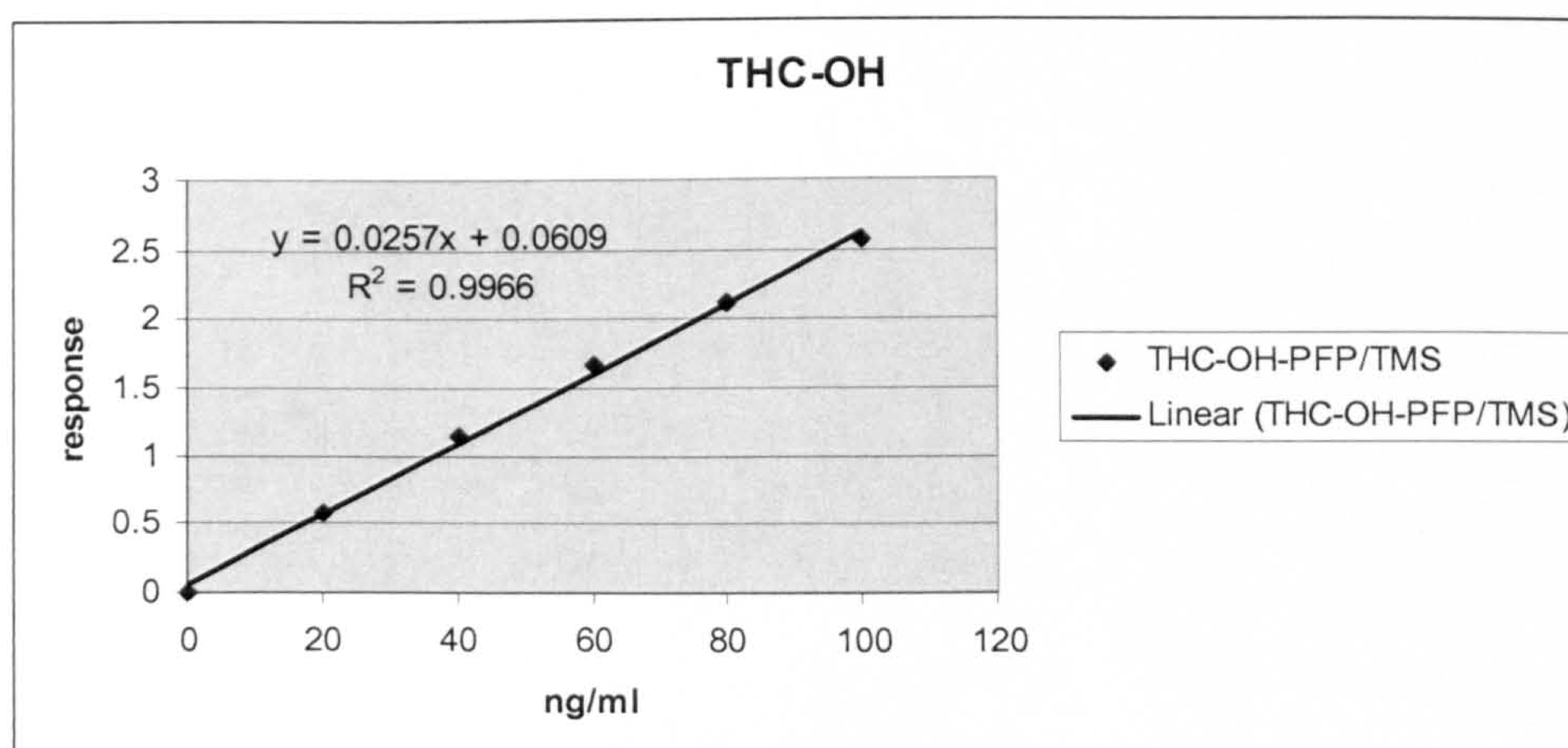


Figure 5-16:- Calibration curve for THC-OH analysed as the PFP derivative

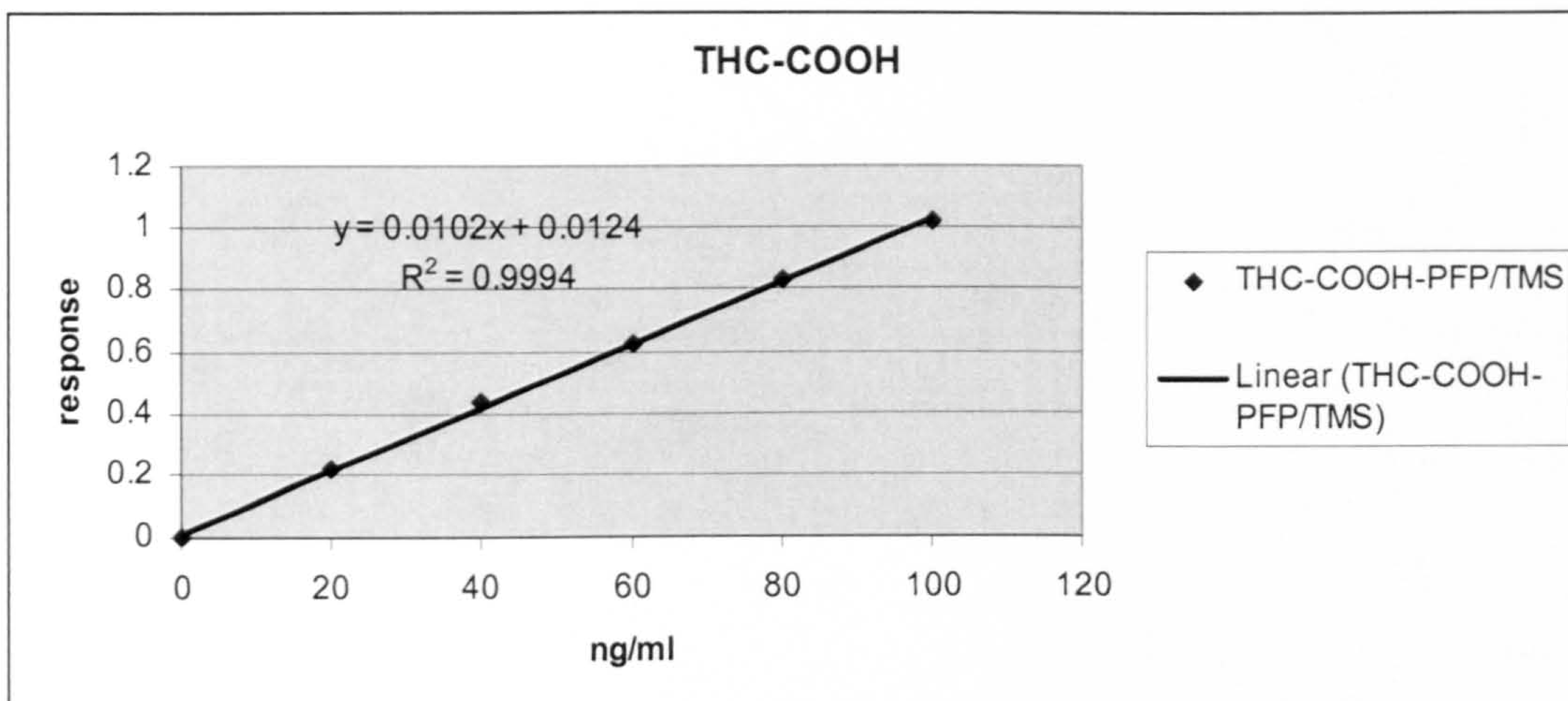


Figure 5-17:- Calibration curve for THC-COOH analysed as the PFP/methyl ester derivative

The calibration curves obtained in this validation work were similar to those obtained with the PFP-PFPOH reagent except for amphetamine and methamphetamine. For both of these, the blank showed significantly positive. The amphetamine curve was however, a reasonable straight line whereas for methamphetamine the blank reading reduced the correlation coefficient to 0.9089.Excluding the blank results in a better linear correlation with an R^2 value of 0.9675.

5.5.3 Intra –day variation

Using the same procedure as for PFPA-PFP-OH derivatives, five sets of blood standards were extracted and analysed as PFPA-methyl derivatives. The main potential disadvantage of this reagent is that benzoylecgonine is converted to cocaine so information on extent of metabolism in casework samples would be lost. The average area ratios were calculated and are displayed from Table 5-17 to Table 5-20.

Table 5-17:- Intra-day variation for Amphetamine and Methamphetamine analysed as the PFP derivatives

Concentration (ng/ml of blood)	Average area Ratio (%RSD, n=5)	
	Amp/Amp-d3	Meth/Meth-d5
16	0.6 (1.5)	0.3 (6.0)
48	1.0 (1.4)	0.8 (3.2)
80	1.3 (4.4)	1.14 (1.4)

Table 5-18:- Intra-day variation for Morphine analysed as the PFP derivative

Concentration (ng/ml of blood)	Average area Ratio Mor/Mor-d3 (%RSD, n=5)
4.6	0.03 (0.4)
13.7	0.1 (1.1)
22.8	0.12 (0.74)

Table 5-19:- Intra-day variation for Benzoylecgonine analysed as its methyl ester

Concentration (ng/ml of blood)	Average area Ratio Bze/Bze-d3 (%RSD, n=5)
5.3	2.25 (10.2)
15.8	5.91 (10.6)
26.8	11.7 (10.4)

Table 5-20:- Intra-day variation for THC-OH and THC-COOH analysed as their PFP/methyl ester derivatives

Concentration (ng/ml of blood)	Average area Ratio (%RSD, n=5)	
	THC-OH/THC-OH-d3	THC-COOH/THC-COOH-d3
20	0.59 (5.6)	0.21 (8.6)
60	1.6 (5.1)	0.64 (3.2)
100	2.4 (3.7)	0.9 (7.2)

The within-day variations as shown by the %RSD are mostly less than 10%, with benzoylecgonine lying between 10 and 11 %RSD. These are all within the suggested limits for precision.[289]

5.5.4 Inter – day variation

The blood standards used to obtain the linearity curve were each extracted five times during one month for all the drugs and the average concentrations were calculated using the calibration curves and these are displayed from Table 5-21 to Table 5-24. The concentrations were the same as those used previously.

Table 5-21:-Inter-day variation for Amphetamine and Methamphetamine analysed as their PFP derivatives

Concentration (ng/ml of blood)	Mean Measured Concentration (%RSD, n=5)	
	Amp	Meth
16	15.7 (2.4)	15.7 (4.8)
48	48.7 (1.8)	48.4 (1.0)
80	79.8 (1.6)	79.4 (1.6)

Table 5-22:-Inter-day variation for Morphine analysed as the bis-PFP derivative

Concentration –Morphine (ng/ml of blood)	Mean Measured Concentration (%RSD, n=5)
4.6	4.7 (2.4)
13.7	13.6 (1.9)
22.8	22.7 (0.6)

Table 5-23:- Inter-day variation for Benzoylecgonine analysed as the methyl ester derivative

Concentration –Benzoylecgonine (ng/ml of blood)	Mean Measured Concentration (%RSD, n=5)
5.3	5.4 (10.5)
15.8	15.6 (2.9)
26.26	26.43 (1.6)

Table 5-24:-Inter-day variation for THC-OH and THC-COOH analysed as the PFP/methyl ester derivatives

Concentration (ng/ml of blood)	Mean Measured Concentration (%RSD, n=5)	
	THC-OH	THC-COOH
20	19.8 (5.9)	20 (5.1)
60	60.3 (4.2)	59.4 (1.4)
100	99.5 (0.84)	99.5 (1.3)

5.5.5 Recovery

Recoveries of drugs by the method were calculated using spiked blood samples with low, medium and high concentrations (Table 5-25, Figure 5-18).

Table 5-25:- Recovery for six drugs analysed as the PFP/methyl ester derivatives

Analyte	Concentration(ng/ml)	Recovery %
Amphetamine	16	99
	48	92
	80	95
Methamphetamine	16	62
	48	86
	80	90
Morphine	4.55	88
	13.65	91
	22.75	87
Benzoylecgonine	5.25	85
	15.75	85
	26.25	83
THC-OH	20	66
	60	50
	100	56
THC-COOH	20	56
	60	60
	100	68

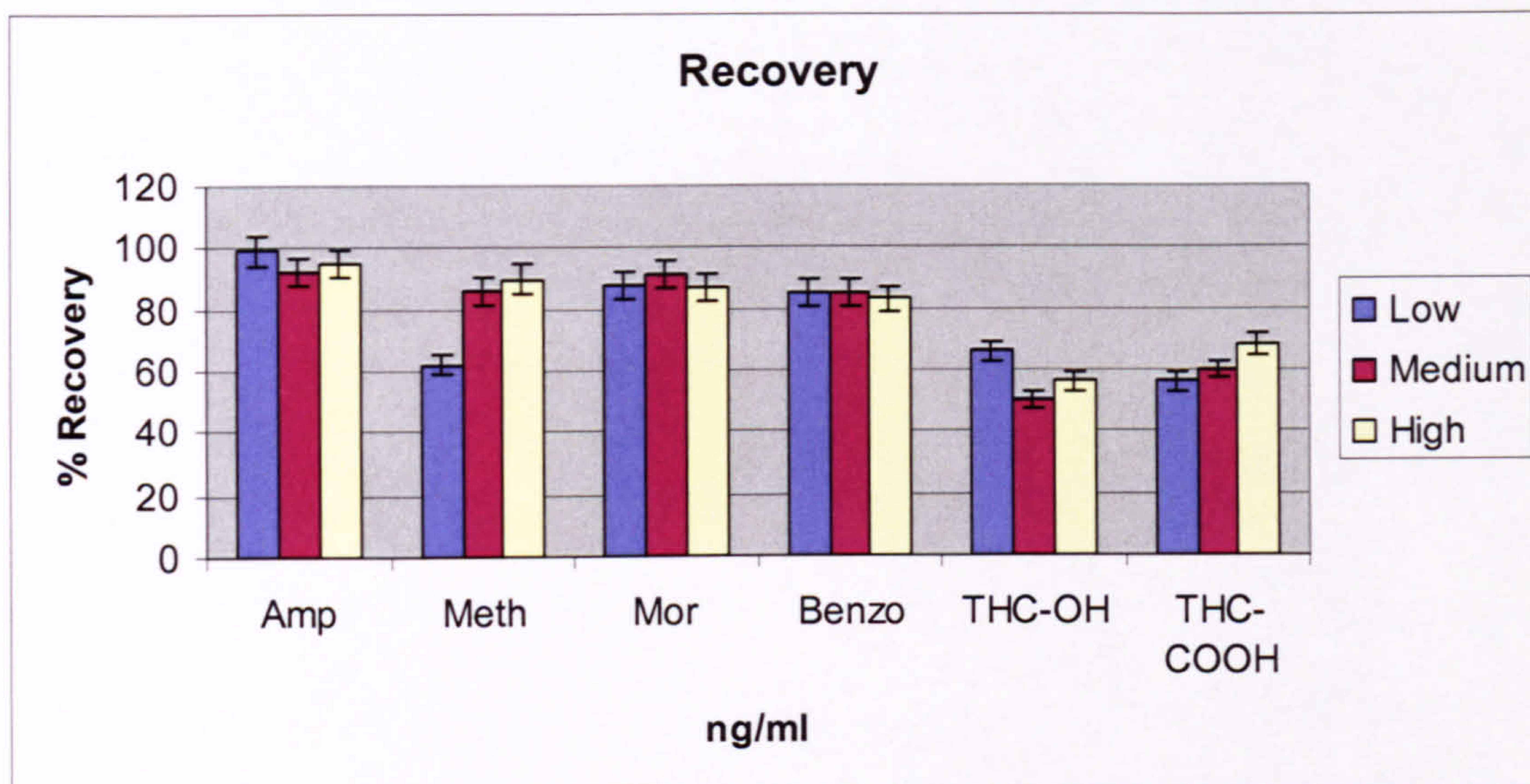


Figure 5-18:- Recoveries of drugs from blood using Strata ™X

It is noted that the recovery of methamphetamine in this set of tests was much better than in the previous set.

5.5.6 Limit of Detection and Limit of Quantification

The methods used for obtaining the LOD and LOQ were the same as mentioned in Sections 5.4.6 and 5.4.7 and the results are shown below in Table 5-26.

Table 5-26:- LOD and LOQ for Test Drugs analysed as the PFP/methyl ester derivatives

Drugs	LOD (ng/ml)	LOQ (ng/ml)
Amphetamine	0.6	2.1
Methamphetamine	0.6	2.1
Morphine	0.3	1.1
Benzoylecgonine	0.3	1.2
THC-OH	1.3	4.3
THC-COOH	1.4	4.7

The LOD and LOQ values show some small improvements compared to the first set of tests. The LOQ of THC-COOH, however, is better when it is analysed as the PFP acyl /methyl ester derivative.

5.5.7 Application to real case samples

46 case samples were analysed with the PFPA/TMS-diazomethane reagent of which 36 samples were previously found to be positive for some drugs of abuse (Table 5-27 and Table 5-28). These cases were not related to the cases mentioned in Table 5-13 and 5-14.

Table 5-27:- Results of GC/MS for cases analysed using the PFP/TMS diazomethane reagent

Case No	Drug Concentration (mg/L)					
	Amp	Meth	Mor	Cocaine/Bze	THC	THCCOOH
Blank	-	-	-	-	-	-
1	0.32	-	-	-	-	-
2	-	-	0.5	-	-	-
3	-	-	0.24	-	-	-
4	-	-	0.11	-	-	-
5		-	0.54	-	-	-
6	-	-	0.18	-	-	-
7		-	0.36	-	-	
8	0.01	-		-	-	-
9	0.13	-		-	-	-
10	0.01	-	-	-	-	-
11	0.02	-	-	-	-	-

12	0.014	-	-	-	-	-
13	-	-	-	0.007	-	-
14	-	-	-	0.11	-	-
15	-	-	-	0.11	-	-
16	-	-	-	0.022	-	-
17	-	-	-	0.01		
18	-	-	0.32	-	-	-
19		-	0.26	-	-	-
20	-	-	0.1	-	-	-
21	-	-	0.5	-	-	-
22	-	-	0.04	-	-	-
23	-	-	0.1	-	-	-
24	-	-	0.2	-	-	-
25	-	-	0.1	-	-	-
26	-	-	0.5	-	-	-
27	-	-	0.41	-	-	-
28	-	-	0.1	-	-	-
29	-	-	0.2	-	-	-
30	-	-	0.2	-	-	-
31	-	-	0.4	-	-	-
32	-	-	0.4	-	-	

33	-	-	0.4	-	-	-
34	-	-	0.54	-	-	-
35	-	-	0.2	-	-	-
36	-	-	0.01	-	-	-

Table 5-28:- Results of GC/MS analysis of extracts containing other drugs using PFP/TMS diazomethane reagent

Drugs	Amitrip	Metha	Code	Propox	Dothi	Indo	Fluox	Bupre	Paracet
Blank	-	-	-	-	-	-	-	-	
1	0.3	-	-	-	-	-	-	-	
2	2.4	-	-	-	-	-	-	-	
3	-	0.2	-	-	-	-	-	-	
4	-	-	0.03	-	-	-	-	-	
5	-	-	0.6	-	-	-	-	-	
6	-	-	-	0.8	-	-	-	-	
7	-	-	-	2.5	-	-	-	-	
8	-	-	-	-	-	-	-	-	
9	-	-	-	-	-	-	-	-	
10	-	-	-	-	-	-	-	-	

(Amitrip = Amitriptyline, Metha = Methadone, Code = Codeine, Propox = Propoxyphene, Doth = Dothiepin, Indo = Indomethacin, Fluox = Fluoxetine, Bupre = Buprenophine, Parcet = Paracetamol)

5.5.8 Discussion

The validation parameters including linearity, precision, recovery, limit of detection, and limit of quantification were evaluated as described previously and recoveries and precision are summarised in Table 5-29.

The R^2 values of calibration curves were ≥0.99 for morphine and the cannabinoids but for amphetamine, methamphetamine and benzoylecogonine $R^2 \leq 0.98$). The interference effects on amphetamine and methamphetamine appeared to come from SPE using Strata - ™X and from the TMS-diazomethane reagent so the intercepts for amphetamine and methamphetamine were high.

The intra-day and inter-day precision were evaluated at low, medium and high concentrations for five replicates. Intra-day assay %RSDs ranged from 0.4 to 10.6 % while the inter-day assay RSDs ranged from 0.64 to 10.5 %. The highest intra-day %RSD was 10.8% for Benzoylecgonine at a concentration of 15.8 ng/ml blood while the lowest %RSD was 0.4% for morphine at a concentration of 4.6 ng/ml blood. A similar pattern was seen for inter-day precision:, the highest inter-day %RSD was 10.5% for benzoylecgonine at a concentration of 5.3 ng/ml blood while lowest %RSD was 0.64% for morphine at a concentration of 22.8 ng/ml as shown in Table 5-29. It is noted that some intra-day precisions are worse than for the corresponding inter-day values, although they mostly lie in the same range. These variations may represent basic problems in reproducibility of the SPE procedure, of the derivatisation procedure (due to impurities in the TMS-diazomethane reagent) or else of the GC-MS instrument. Comparing this derivatisation procedure with the previous one, it can be seen that there are some differences in precision between the methods for the test substances but neither is clearly better than the other one.

Recoveries ranged between 50 and 99%.The lowest recovery was for THC-OH and the highest was for amphetamine. As noted earlier, the recovery of methamphetamine was higher in this series of tests than in the first set.

Morphine and benzoylecgonine produced the lowest detection limits and THC-COOH produced the highest detection limit. The limits of detection and quantitation are listed in Table 5-26.

Table 5-29:- Accuracy, intra- and inter-day assay precision for the test substances analysed at different concentrations with PFPA-TMS-diazomethane derivatisation

Compound-Product	Concentration (ng/ml)	Recovery % (n=5)	Intra-day RSD% (n=5)	Inter-day RSD% (n=5)
Amphetamine-PFP	16	99	1.5	2.4
	48	92	1.4	1.8
	80	95	4.4	1.6
Methamphetamine-PFP	16	62	6.02	4.8
	48	86	3.2	1.03
	80	90	1.4	1.6
Morphine-bis-PFP	4.6	88	0.4	2.4
	13.7	91	1.1	1.9
	22.8	87	0.74	0.6
Benzoylecgonine-Methyl ester	5.25	85	10.2	10.5
	15.8	85	10.6	2.9
	26.8	83	10.4	1.6
THC-OH-PFP	20	66	5.63	5.9
	60	50	5.13	4.2
	100	56	3.7	0.84
THC-COOH PFP/Methyl ester	20	56	8.6	5.12
	60	60	3.2	1.41
	100	68	7.16	1.33

The validated method was applied to blood samples collected from Forensic Medicine and Science at the University of Glasgow. In general the method worked well with this derivatising reagent. However as found in the first set of tests, there were interferences in the GC-MS analyses due to the Strata™ X cartridges and also due to the TMS-

diazomethane reagent. However, the method was found to be able to detect and quantify both basic and acidic drugs including drugs of abuse and prescribed drugs.

5.5.9 Conclusion

This version of the method also showed promise as a unified procedure for general use because of its ability to detect and meaure both acidic and basic drugs. This derivatisation procedure would be very useful if a pure form of the TMS-diazomethane solution could be prepared. The limits of quantification for cannabinoids would need to be improved if the method was to be used routinely.

5.6 Method validation for drug derivatisation with MTBSTFA

5.6.1 Introduction

The third version of the proposed unified method was validated as before, using the same approach to the measurement of the validation parameters. Neither methamphetamine nor morphine could be detected as their TBDMS derivatives, as noted in Chapter 4.

5.6.2 Linearity

Using the procedure as before, each drug concentration in blood was extracted five times and the calibration curves for the target analytes were prepared. These were found to be linear. The correlation coefficient (R^2) values are listed in Table 5-30, and the curves are shown below in Figure 5-19 to Figure 5-22. The ions for these derivatives used for GC-MS-SIM were at high mass values and were selective, so there was no interference in the GC-MS analysis.

Table 5-30:- Correlation coefficients (R^2) for all compounds analysed as TBDMS derivatives.

Compound	R^2 Values
Amphetamine	0.9995
Benzoylecgonine	0.9971
THC-OH	0.999
THC-COOH	0.9976

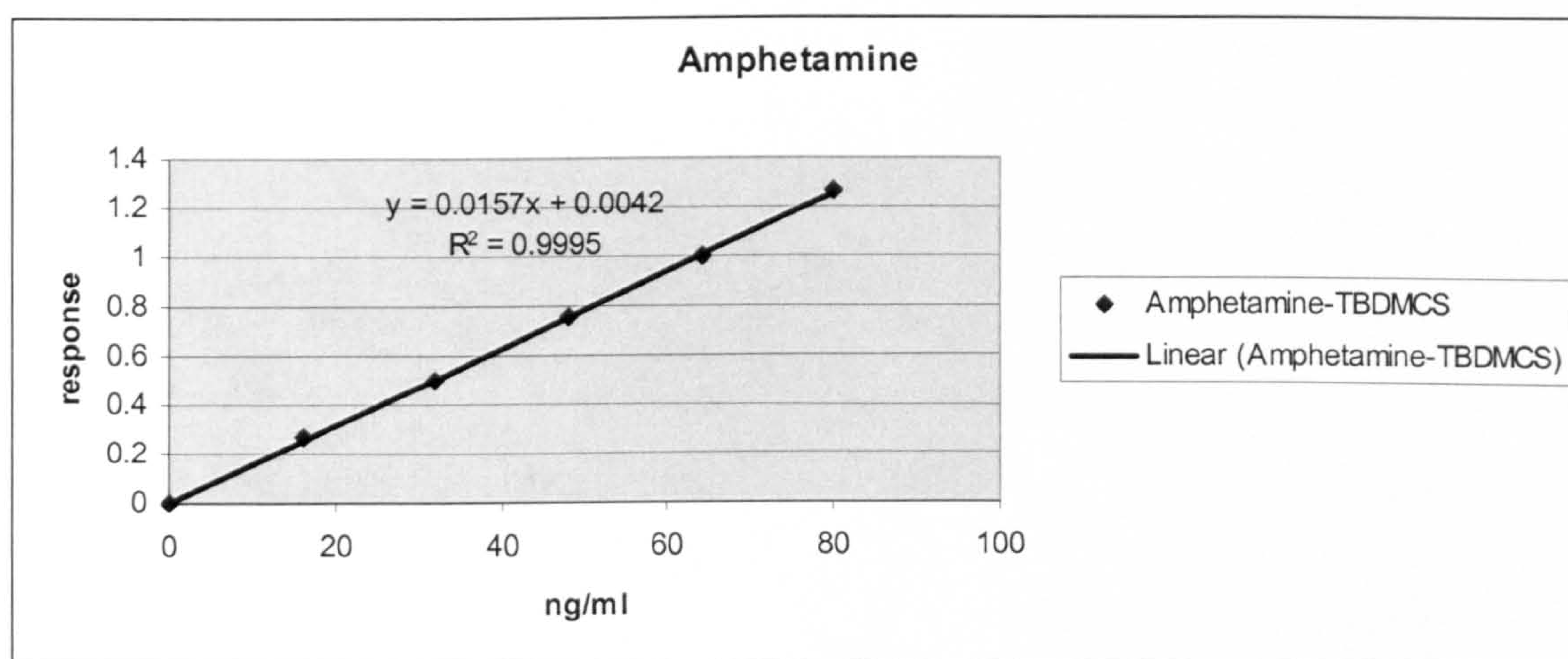


Figure 5-19:- Calibration curve for Amphetamine-TBDMCS derivative

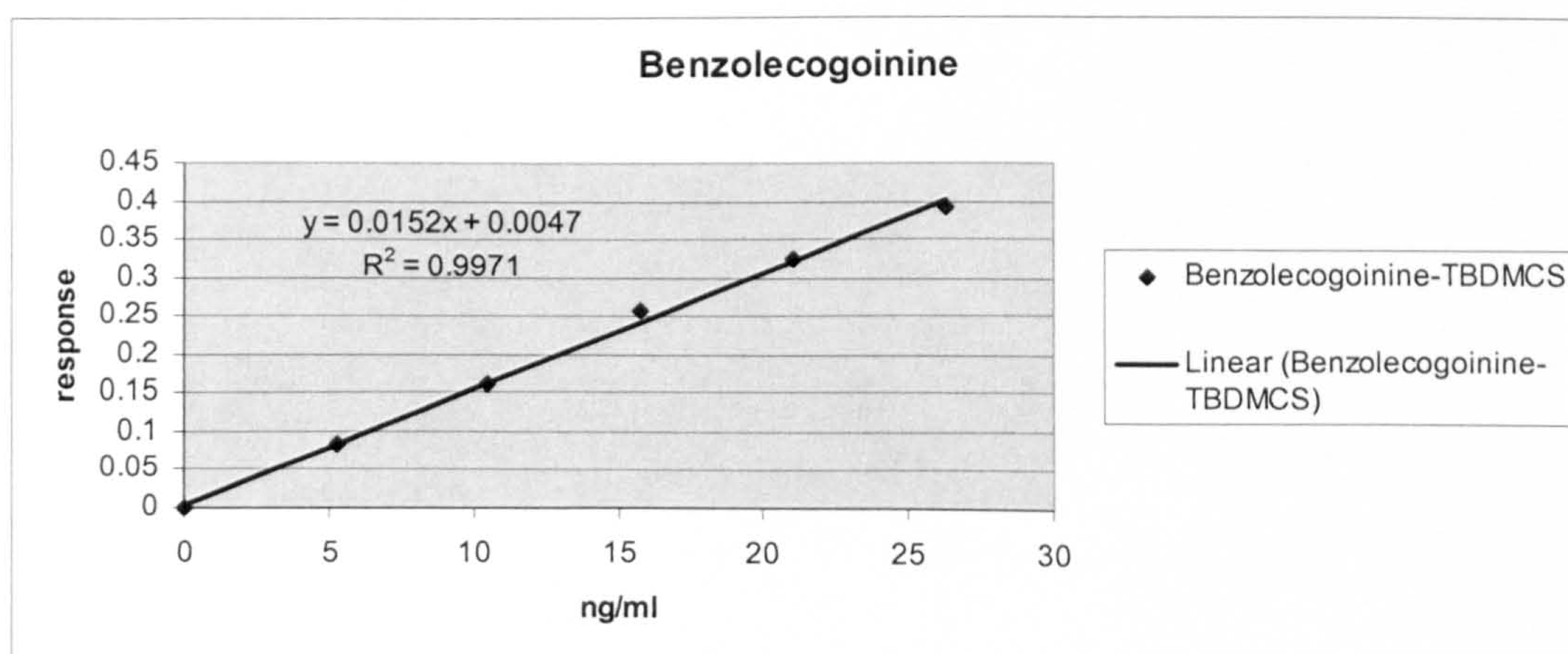


Figure 5-20:- Calibration curve for Benzoylecgonine-TBDMCS derivative

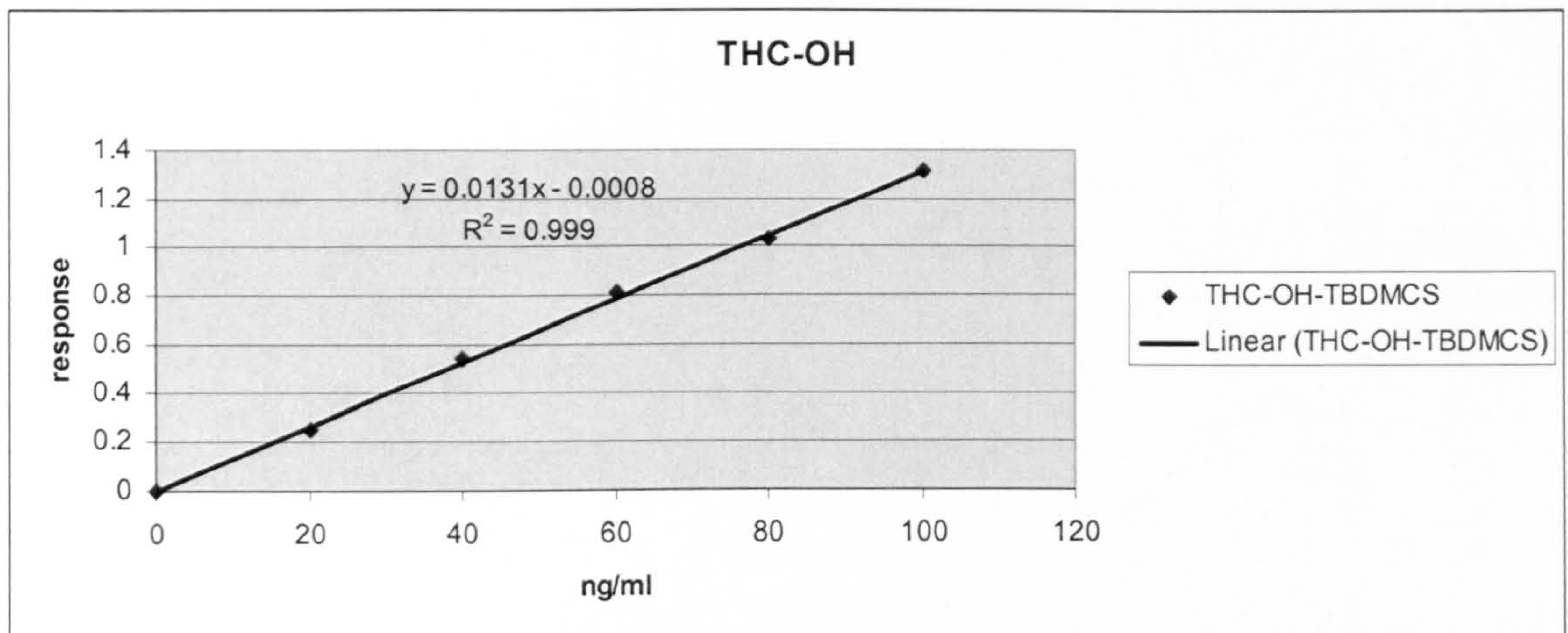


Figure 5-21:- Calibration curve for THC-OH-TBDMCS derivative

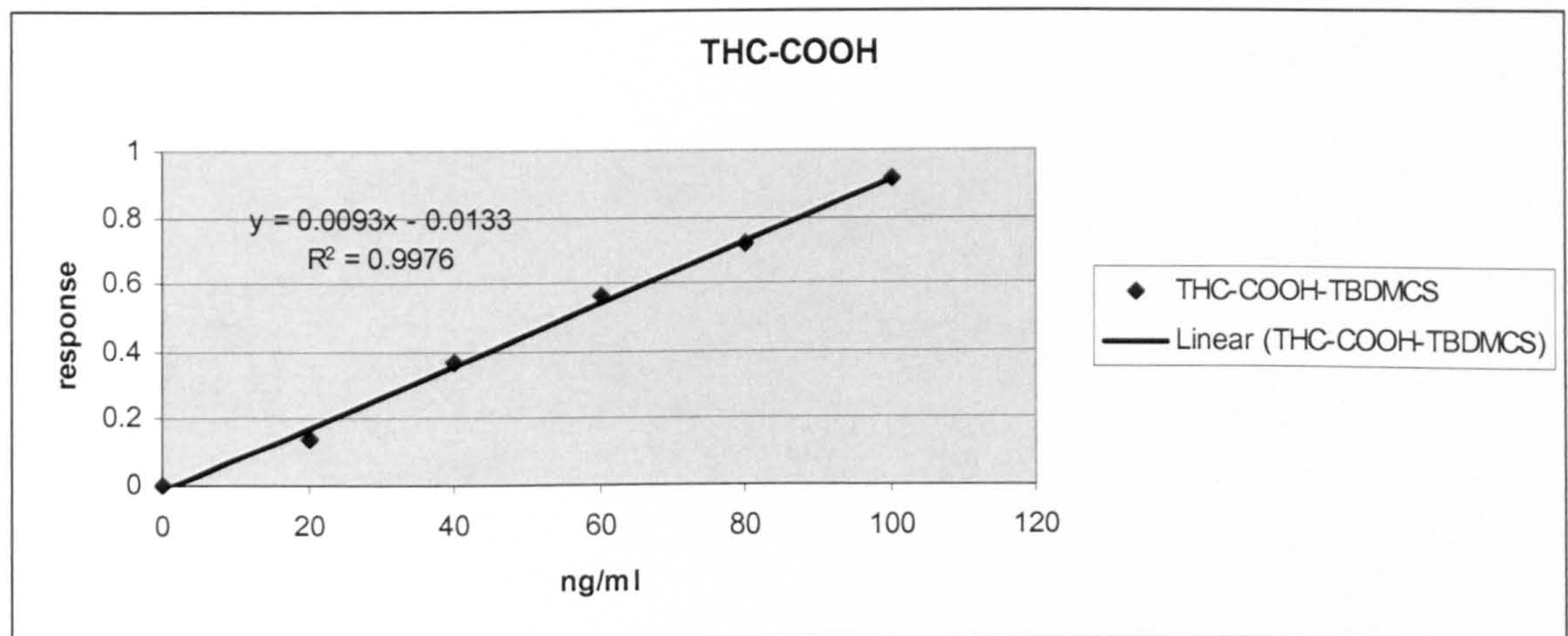


Figure 5-22:- Cailbration curve for THC-COOH-TBDMCS

5.6.3 Intra –Day Variation

The set of calibration curve standards containing all six drugs was extracted five times during one day and the results of GC-MS analysis are displayed in Table 5-31 to Table 5-33.

Table 5-31:- Intra-Day variation for Amphetamine analysed as the TBDMS derivative

Concentration (ng/ml of blood)	Average Area Ratio of Amp/Amp-d5 (%RSD, n=5)
16	0.07 (8.9)
48	0.5 (6.4)
80	0.97 (10.3)

Table 5-32:- Intra-Day variation for Benzoylecgonine analysed as the TBDMS derivative

Concentration (ng/ml of blood)	Average area Ratio Bze/Bze-d3 (%RSD, n=5)
5.3	0.32 (10.7)
15.75	0.9 (6.7)
26.25	0.42 (10.3)

Table 5-33:- Intra-Day variation for THC-OH/THC-COOH analysed as the TBDMS derivatives

Concentration (ng/ml of blood)	Average area Ratio (%RSD, n=5)	
	THC-OH	THC-COOH
20	0.21 (5.2)	0.15 (1.0)
60	0.7 (1.9)	0.52 (1.9)
100	1.2 (10.3)	0.86 (10.0)

5.6.4 Inter-Day Variation

The average area ratios were calculated for five sets of drug standards which were extracted five times over a period of 1 week and are displayed in Table 5-34 to Table 5-36.

Table 5-34:- Inter- Day variation for Amphetamine analysed as the TBDMS derivative

Concentration –Amp (ng/ml of blood)	Mean Concentration (%RSD, n=5)
16	16.3 (4.1)
48	50.3 (12.6)
80	79.9 (2.2)

Table 5-35:- Inter- Day variation for Benzoylecgonine analysed as the TBDMS derivative

Concentration –Bze (ng/ml of blood)	Mean Concentration (%RSD, n=5)
5.3	5.5 (12.0)
15.75	15.3 (8.4)
26.25	25.9 (2.7)

Table 5-36:- Inter- Day variation for THC/THC-COOH

Concentration (ng/ml of blood)	Mean Concentration (%RSD, n=5)	
	THC-OH	THC-COOH
20	19.2 (9.36)	18.1 (5.3)
60	58.4 (6.48)	57.8 (7.5)
100	101.2 (2.45)	99.5 (3.3)

5.6.5 Recovery

The recoveries of the method for drugs extracted from spiked blood samples at low, medium and high concentrations were measured as before. The results are shown below in Table 5-37 and Figure 5-23.

Table 5-37:- Recovery for test substances analysed as MTBSTFA derivatives

Analyte	Concentration (ng/ml)	Recovery %
Amphetamine	16	90
	48	85
	80	64
Benzoylecgonine	5.25	85
	15.75	56
	26.25	80
THC-OH	20	65
	60	56
	100	62
THC-COOH	20	64
	60	72
	100	73

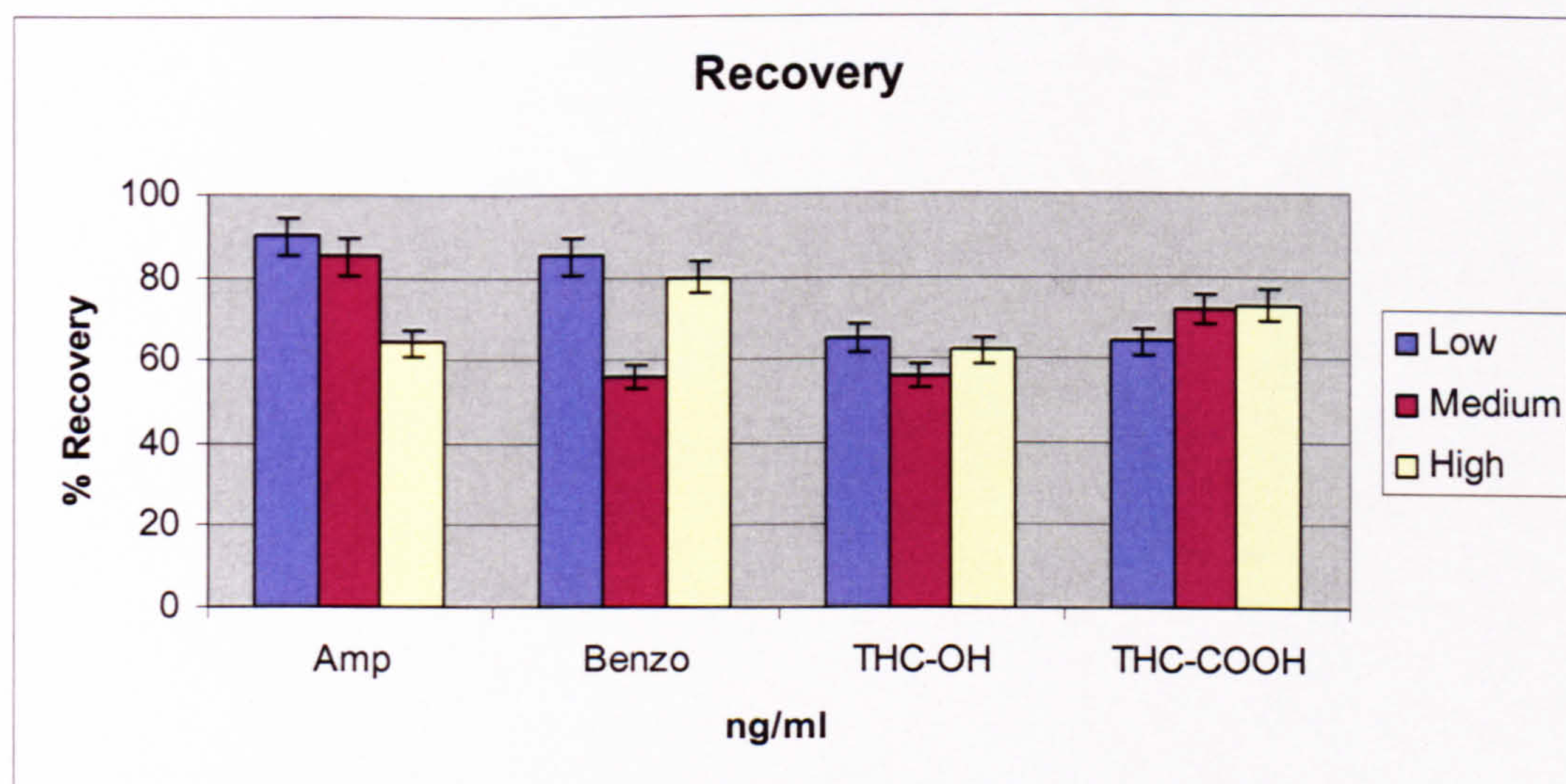


Figure 5-23:- Recoveries of drugs from blood using strata ™ X and analysed as the TBDMS derivative

5.6.6 Limits of Detection and Quantification

The method for obtaining the LOD and LOQ was described in Sections 5.4.5 and 5.4.6. The results for the test substances analysed as the TBDMS derivative are shown below in Table 5-38.

Table 5-38:- Limits of detection and quantitation for TBDMS derivatives

Drug	LOD (ng/ml)	LOQ (ng/ml)
Amphetamine	3.3	10.8
Benzoylecgonine	1.9	4.9
THC-OH	7.3	24.4
THC-COOH	5.9	19.8

5.6.7 Application to real cases

The developed method was applied to 26 case samples of which 16 cases were previously found to be positive for drugs of a buse. These were collected from Forensic Medicine and Science, University of Glasgow. They are summarised below in Table 5-39. No prescription drugs were detected in any of the blood samples using this derivatising reagent and all these cases different from other cases.

Table 5-39:- Results of GC/MS for cases analysed with MTBSTFA derivatisation

Case No	Drug Concentration (mg/L)						
	Amp	Meth	Mor	Cocaine	Bze	THC	THC-COOH
Blank	-	-	-	-	-	-	-
1	0.54	-	-	-	-	-	-
2	0.43	-		-	-	-	-

3	0.7	-	-	-	-	-	-
4	0.9	-	-	-	-	-	-
5	-	-	-	-	-	-	0.02
6	-	-	-	-	-	-	0.01
7	-	-	-	-	-	-	0.04
8	-	-		-	-	-	0.03
9	-	-	-	-	0.35	-	-
10	-	-	-	-	0.11	-	-
11	-	-	-	-	-	-	-
12	-	-		-	-	-	-
13	-	-	-	-	0.02	-	-
14	-	-	-	-	0.023	-	-
15	-	-	-	-	0.03	-	-
16	-	-	-	-	-	-	

5.6.8 Discussion

The validation parameters including linearity, precision, recovery, limit of detection (LOD) and limit of quantification (LOQ) were evaluated as for the other two derivatising reagents and the recoveries and precision measurements are summarised in Table 5-40.

The MTBSTFA reagent produced $R^2 \geq 0.99$ for all analytes and the GC-MS analyses were less affected by interferences than the previous reagent. The intra- and inter-day precisions were evaluated at low, medium and high concentrations for five replicates. Intra-day assay %RSDs ranged from 1 to 10.7%. The highest intra-day %RSD was for benzoylecgonine at

a concentration of 5.3 ng/ml blood while the lowest %RSD was for THC-OH at a concentration of 4.55 ng/ml blood. However, the inter-day assay %RSDs ranged from 2.45 to 12.6 %. The highest inter-day %RSD was for amphetamine at a concentration of 48 ng/ml while the lowest %RSD was for amphetamine at 80 ng/ml blood as shown in Table 5-40.

The recovery ranged between, 56 and 90 %. The lowest recovery was for benzoylecgonine at a concentration of 15.75 ng/ml blood and the highest was for amphetamine at a concentration of 16 ng/ml blood.

The limits of detection and quantitation are listed Table 5-38 which show that benzoylecgonine produced the lowest detection limit and THC-OH gave the highest detection limit.

Table 5-40:- Accuracy, intra and inter-day assay precision with MTBSTFA at different concentration

Compound-Product	Concentration(ng/ml)	Recovery % (n=5)	Intra-day RSD%(n=5)	Inter-day RSD%(n=5)
	16	90	8.9	4.1
Amphetamine-TBDMS	48	85	6.4	12.6
	80	64	10.3	2.2
	5.25	85	10.7	12
Benzoylecgonine-bis-TBDMS	15.75	56	6.7	8.4
	26.25	80	10.3	2.7
	20	65	5.2	9.36
THC-OH- TBDMS	60	56	1.9	6.5
	100	62	10.3	2.5
	20	64	1.0	5.3
THC-COOH-TBDMS	60	72	1.9	7.5
	100	73	10	3.3

The method was applied to blood samples collected from Forensic Medicine and Science at the University of Glasgow. Drugs of abuse were detected but no prescription drugs were found in any of the samples.

5.6.9 Conclusion

The TBDMS derivatives gave good linearity for the 4 test compounds. However, this derivative was found to be unsuitable for methamphetamine and morphine, which could not be detected. These compounds were used as model compounds which represented the behaviour of other drugs with similar functional groups and so it can be concluded that many other drugs and metabolites would not be detected using this reagent. Also, the limits of detection and quantification were too high with this reagent for the method to be usable in routine toxicology.

5.7 General Discussion and Conclusions

Analytical chemistry is a very important field particularly when applied to forensic toxicology in which gas chromatography-mass spectrometry, extraction and derivatisation techniques are used. This study looked into the effects of different chemical derivatisation procedures on separation and identification of illicit drugs such as amphetamine, methamphetamine, morphine, benzoylecgonine, THC-OH and THC-COOH. A single method was used for the extraction of these drugs from whole blood and three different methods were used for derivatisation.

Extraction procedures are used to clean up the sample by removing interfering substances and concentrating the drug for injection into the GC-MS instrument. Solid phase extraction was used in this case. There are different types of solid phases available depending on what kind of drug needs to be extracted, acidic, basic or neutral. If drugs of different types are to be extracted simultaneously then two fractions can be eluted from the solid phase cartridge, one for acidic drugs and one for basic drugs.This can be time consuming.

The aim of this study was to find a single extraction method for the simultaneous extraction of acidic and basic drugs. Solid phase extraction cartridges produced by Phenomenex, called Strata™X were investigated. The disadvantage of using Strata™X was that the eluate was relatively dirty so investigation into the use of more solvents in the washing steps to remove more impurities was carried out: the original method used 1.5 ml methanol but because of the problem with interferences, the volume was increased to 2 ml methanol.

Using a microwave instead of a conventional heating block was used to assist derivatisation. It required less time to achieve complete derivatisation. Special heat

resistant vials and caps were required as the normal caps used melted in the microwave. The microwave temperature is mentioned in section 5.2.8. The temperature, power and time of irradiation were investigated. The time for derivatisation was varied from 1 to 5 minutes. It was found that using 3 or 5 minutes at the high power setting affected the integrity of the caps and sample was lost. One and two minutes were similar but high temperature and to keep the elute by prevention evaporate under high temperature. The optimum time was found to be 1 minute at the high power setting. A disadvantage of using the microwave was that the distribution of power was uneven and therefore it was important to distribute the vials in a circle as shown in Figure 4-16.

A validated, sensitive and specific method for the extraction and quantification of drugs abuse in blood is presented. For this project three different reagents were compared. Acylation with PFP-OH/PFPA was applied with 6 drugs which have different functional groups. Amphetamine had problems due to evaporation, as it is volatile, so it is recommended that the evaporation to dryness stage should be carried out slowly without heating to prevent loss of analyte. Methylation and acylation with PFPA-TMS-diazomethane was considered better than the acylation reaction because the reagent gave good reactions and improved the shape of chromatographic peaks. However the best sequence for addition of the reagents is first to put PFPA then TMS–diazomethane for safety reasons. Silylation with MTBSTFA was only successful with amphetamine, benzoylecgonine, THC-OH and THC-COOH but did not work with morphine and methamphetamine, which formed derivatives but these had poor fragmentation patterns and bad chromatographic peak shapes. However the sample can be injected directly to GC/MS without the need to use ethyl acetate like the other reagents.

It is important to know the chemical structure and functional groups of the analyte to be derivatised when choosing a derivatising agent. No single reagent was optimum for every analyte. Amphetamine gave optimum results with PFPA/PFP-OH whereas methamphetamine, morphine, benzoylecgonine, THC-OH and THC-COOH gave optimum results with TMS–diazomethane/PFPA. However, using acylation-methylation gave the best sensitivity and improved chromatography.

Finally, the derivatistion and validation of methods for different drugs containing different functional groups was interesting because the reactions are dependent on both function groups and reagents. Amphetamine, methamphetamine, benzylecgonine, THC-OH and

THC-COOH derivatised well and gave good validation results with PFPA-PFP-OH and PFPA-trimethylsilyl-diazomethane derivatisation. However, derivatisation of amphetamine, benzoylecgonine and THC-OH was optimum with MTBSTFA. Morphine and methamphetamine did not derivatise with MTBSTFA. PFPA-trimethylsilyl-diazomethane as the derivatising reagent was best for morphine and THC-COOH. Table 5-41 summarises the validation parameters for each drug for each of the three derivatisation reagents.

Table 5-41: Validation parameters for each drug according to the three derivatising reagents

		PFPA/PFPOH	PFPA-ME	TBDMS
Linearity (R^2)				
AMP		0.9966	0.9888	0.9995
MA		0.9947	0.9089	
MOR		0.9992	0.9995	-
BZE		0.9945	0.9862	0.9971
THC		0.9989	0.9966	0.999
THCOOH		0.9934	0.9994	0.9976
Precision, Intra day, %RSD*				
AMP		22.3-10.3	1.5-4.4	8.9-10.3
MA		5.8-6.8	6.0-1.4	-
MOR		3.0-13.5	0.4-0.74	-
BZE		10.8-7.2	10.2-10.4	10.7-10.3
THC		6.5-6.2	5.6-3.7	5.2-10.3
THCOOH		15.6-16.5	8.6-7.2	1.0-10.0
Precision, Inter day, %RSD*				
AMP		6.9-2.5	2.4-1.6	4.1-2.2
MA		27-5.1	4.8-1.6	-
MOR		12.3-2.4	2.4-0.64	-
BZE		3.9-1.6	10.5-1.6	12-2.7
THC		8.5-1.9	5.9-0.84	9.4-2.5
THCOOH		21.4-3.1	5.1-1.3	5.3-3.3
Recovery %*				
AMP		74-89	99-95	90-64
MA		23-18	62-90	-
MOR		96-71	88-87	-
BZE		86-95	85-83	85-80
THC		62-44	66-56	65-62
THCOOH		61-59	56-68	64-73
LOD (ng/ml)				
AMP		1.2	0.6	3.3

MA		0.8	0.6	-
MOR		0.8	0.3	-
BZE		0.4	0.3	1.9
THC		1.7	1.3	7.3
THCOOH		3.7	1.4	5.9
LOQ (ng/ml)				
AMP		4	2.1	10.8
MA		2.7	2.1	-
MOR		2.6	1.1	-
BZE		1.5	1.2	4.9
THC		5.8	4.3	24.4
THCOOH		12.4	4.7	19.8

* Parameters measured at different concentrations are given as ranges, from the lowest to the highest concentration values.

The summary table allows the effect of the derivatisation method to be compared. Each of the derivatising methods gave acceptable linearities, although the MTBSTFA reagent did not work with methamphetamine or morphine. However, for all of the other measured parameters, the PFPA/TMS-diazomethane combination gave generally better results for each of the test compounds and this combination warrants further investigation, especially with respect to developing an analytical grade of the TMS-diazomethane reagent.

6 Gamma-Hydroxybutyric Acid and Beta-Hydroxybutyric Acid

6.1 Introduction

This study concerned a specific application of derivatisation techniques to compounds of interest in forensic toxicology, although for different reasons, as described below. These had previously been measured individually but not simultaneously.

The full chemical name of GHB is gamma-hydroxybutyrate (4-hydroxybutyrate) and for BHB the extended chemical name is (beta-hydroxybutyrate, 3-hydroxybutyrate). These isomers are normal components of mammalian metabolism; they occur naturally in every cell in the human body and are most properly considered nutrients[296, 297]. BHB is one of the ketone bodies which will affect GHB metabolism when they reach elevated concentrations. High concentrations could significantly inhibit the metabolism of GHB[298]. GHB is a metabolite of the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) and is an endogenous compound found in central nervous system (CNS) and peripheral tissues. At moderate doses, GHB induces a state of relaxation, euphoria and disinhibition similar to alcohol [298-300].

GHB was first synthesized in 1960[301] by the French researcher Dr. Henry. He was interested in exploring the effects of GABA on the brain, because little or no GABA crosses the blood-brain barrier. His laboratory synthesized GHB, which substitutes a hydroxyl group for an amino group. This difference allows GHB to cross the blood-brain barrier where some of it is metabolized into GABA. GHB was initially used as an anaesthetic to aid in surgery because of its ability to induce sleep and reversible coma[302, 303]. In the late 1980's, GHB was marketed and sold in the health food industry as a growth hormone stimulator to help body builders promote muscle mass and maintain weight [304]. People take GHB because it helps to fight stress and depression, induces deep sleep, relieves anxiety, enhances sexual feelings and enhances athletic performance[301]. As an anti-depressant GHB is typically dissolved in water, so it appears as an odourless, colourless and nearly tasteless liquid and it is therefore easily dissolved in alcoholic beverages. Sometimes the substance is available as a powder, or in capsule form. GHB has more than 30 common street or slang names such as soap, liquid x, Gamma OH, salt

water, Georgia home body, Grievous bodily harm, Great Hormones at Bedtime, G-caps, GGHBA, Easy lay, Oxy-sleep, Natural sleep 500. In recent years, GHB has gained a reputation as an illicit drug and has become a popular drug of abuse[305-311] when used alone or in combination with other substances, as when mixed with alcohol and other drugs to increase its euphoric effects. GHB is also one of the drugs reputedly used in drug-facilitated assault and drug-facilitated sexual assault, more popularly referred to as "date rape" [312].

GHB is a naturally-occurring substance that is present in small amounts in every cell in the body but is found in greater concentrations in kidney, heart, skeletal muscles and brown fat tissues. However liver, lung, blood, brain and white fat have low concentrations and it is an endogenous compound found in central nervous (CNS) and peripheral tissues that has been hypothesized to have a role as a neurotransmitter. GHB has been used as an anaesthetic and as a treatment for sleep disorders, alcohol withdrawal (alcoholism), and narcolepsy. GHB and BHB are both very important, especially BHB, which is thought to be responsible for many cases of sudden death, and therefore more information will be given below. BHB is considered to be one of the ketone bodies[313] (Figure 6-1) which are normally present in urine and blood, but in very low concentrations. Ketone bodies consist of the three substances acetoacetate, beta-hydroxybutyrate and acetone[297, 314, 315].

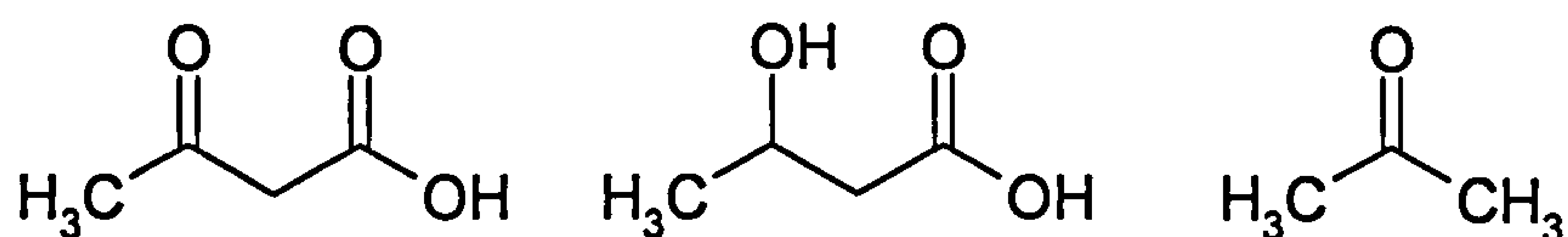


Figure 6-1 Ketone Bodies

The body typically uses glucose for energy and it supplies the tissues. Glucose, under the influence of insulin, is converted to glycogen and body fat. However, when there are excess ketone bodies available for energy, more than is required by the tissues, they cannot be converted into fat and so accumulate in the blood, and are excreted in the urine. This process is known as ketosis. Ketone production results from the breakdown of body fats Figure 6-1. When fats are absorbed through the walls of the small intestine, the glycerol is separated from the fatty acids, and the fatty acids are broken into pieces in the liver. The pieces are known as ketone bodies.The excretion of the excess ketones in the urine is important because the presence of large amounts of ketone bodies in the blood threatens to

upset the acid-alkaline balance of the blood, and thereby, the balance in the tissues[316]. Actually, BHB and acetoacetate chemically are not ketones: they are considered to be physiologically equivalent to ketones because they are readily interconverted in the body. Therefore acetone is a true ketone, and the more general term is "ketone bodies". Ketones are by-products of fatty acid metabolism which are increased in diabetes, alcoholic ketoacidosis and starvation[317] .Metabolism occurs in the liver mitochondria. Ketone bodies are oxidized by most tissues except those of the liver[318] to provide an energy source from stored lipids at times of low carbohydrate availability. Therefore normal glucose concentrations inhibit ketone body synthesis, and the ketone bodies only begin to be synthesized in high concentrations as serum glucose concentration falls (Figure 6-2) [319].

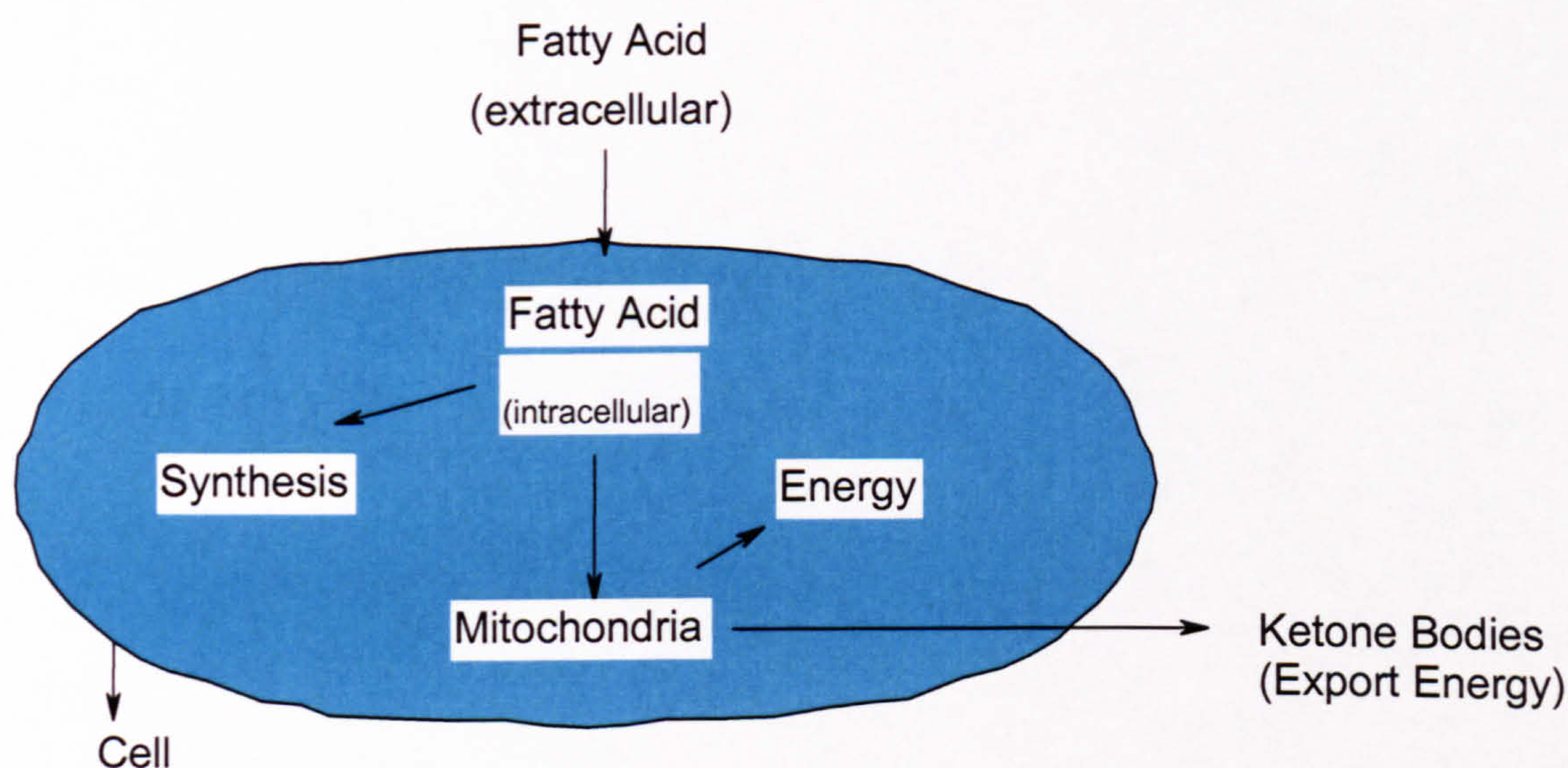


Figure 6-2:- The cells produce ketone bodies for energy.

6.2 GHB and BHB in the body

6.2.1 Chemistry, synthesis and pharmacokinetics of GHB

GHB's chemical name is gamma–hydroxybutyrate (4-hydroxybutrate, the sodium salt being sodium oxybutyrate) Figure 6-3. It consists of four carbons and is a small polar molecule, soluble in water and a short chain fatty acid[301]. It is formed primarily from the precursor aminobutyric acid by the enzyme monoamine oxidase.

Figure 6-3:- Chemical structures of GABA and GHB

The properties of GHB include its having a neuromodulatory role in the brain and the ability to induce several pharmacological and behavioural effects. Following oral ingestion, GHB is rapidly absorbed from the gastrointestinal tract and transported by the portal circulation to the liver where most of it gets metabolized to carbon dioxide and water by first-pass metabolism pathways. A certain amount crosses the blood-brain barrier. GHB overdose has been reported to result in coma, cardiorespiratory depression, seizures and death[301]. GHB has a half–life of 20-53 min, which appears to increase with higher doses. Because the drug is rapidly eliminated from the body, GHB exposure is difficult to document.

The simplest way to produce GHB is by the hydrolysis of the corresponding lactone (a cyclic intramolecular ester) to the desired hydroxy acid. Ester hydrolysis can be done in two ways: an acid catalyzed reaction or a base catalyzed reaction. The base catalyzed reaction is preferred, because the reaction is not reversible, unlike the acid catalyzed one, and therefore higher yields are obtained. Alkaline hydrolysis gives the sodium salt of GHB. The free acid is not stable and will immediately cyclize into gamma –butyrolactone again. The reaction proceeds equimolarly (the number of moles of each substance that reacts), and there are no by products produced in this reaction, such as hydrogen gas or water Figure 6-4·

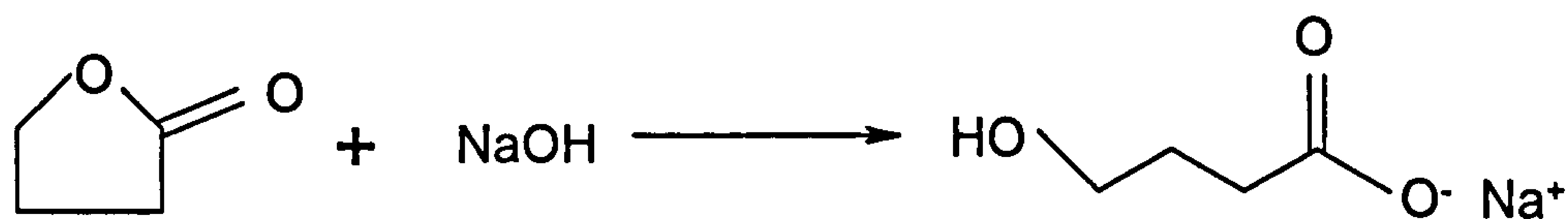


Figure 6-4:- Chemistry and Synthesis Sodium Gamma–Hydroxy Butyrate (NaGHB).

GHB is formed in the brain from GABA, derived in turn from succinic semialdehyde (SSA) via a specific succinic semialdehyde reductase (SSR, Figure 6-5). GHB can be reconverted back to SSA via GHB dehydrogenase, and GHB can be converted back to GABA. Succinic semialdehyde can also be metabolized by succinic semialdeyde dehydrogenase (SSADH) to succinic acid. Mutant mice in which the SSADH enzyme is deleted display high levels of GHB and GABA[301].

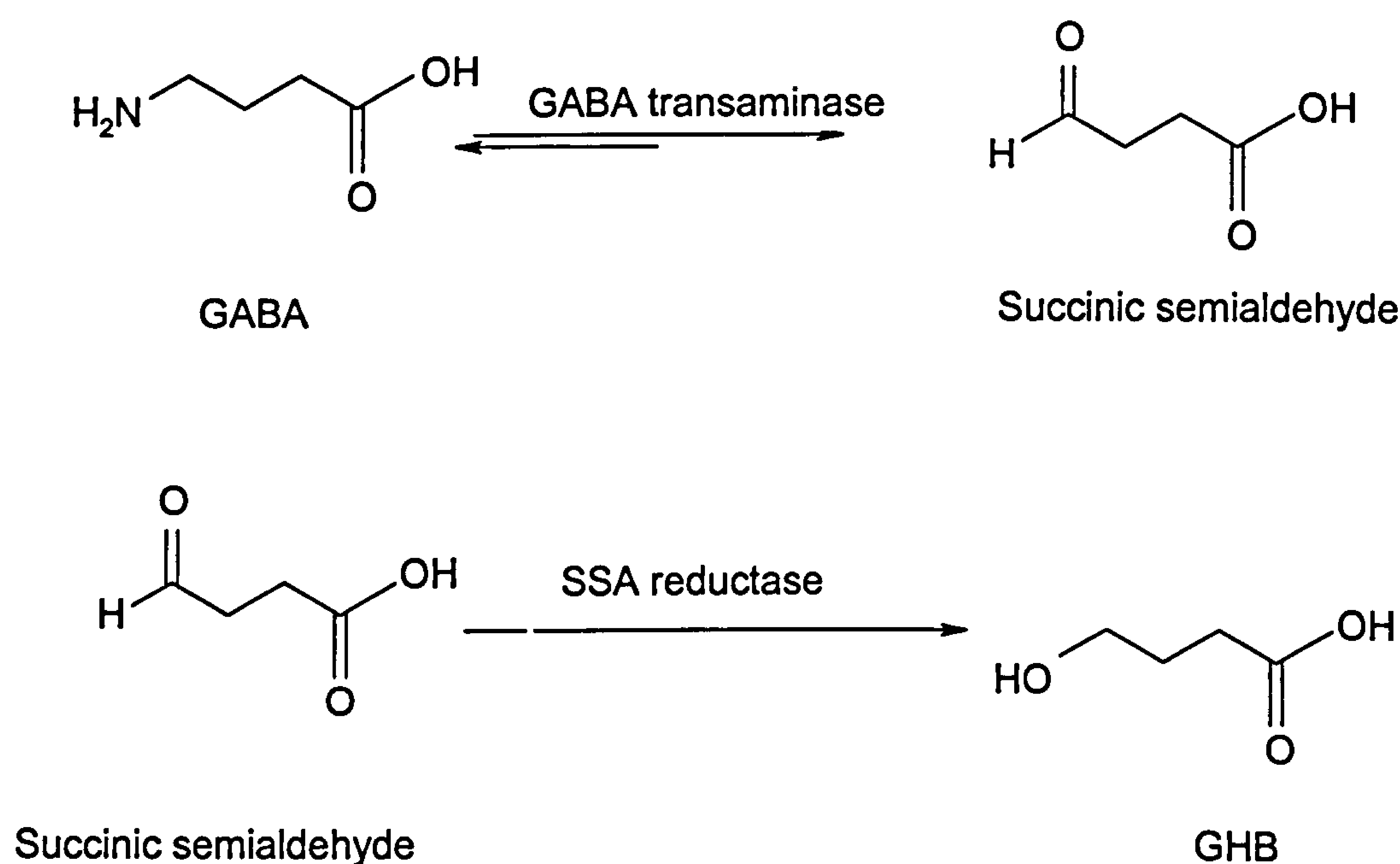


Figure 6-5:- GABA metabolism to GHB in body

When GHB became illegal in the United States of America, drug users started looking for similar chemicals. Gamma-butyrolactone (GBL) and 1.4-butanediol (1,4-BD) are two such analogues of GHB. The body will convert them to GHB where GBL is metabolized to GHB via lactonase while 1.4-BD is first metabolized to gamma-hydroxybutyraldehyde via alcohol dehydrogenase, then to GHB via aldehyde dehydrogenase (Figure 6-6).

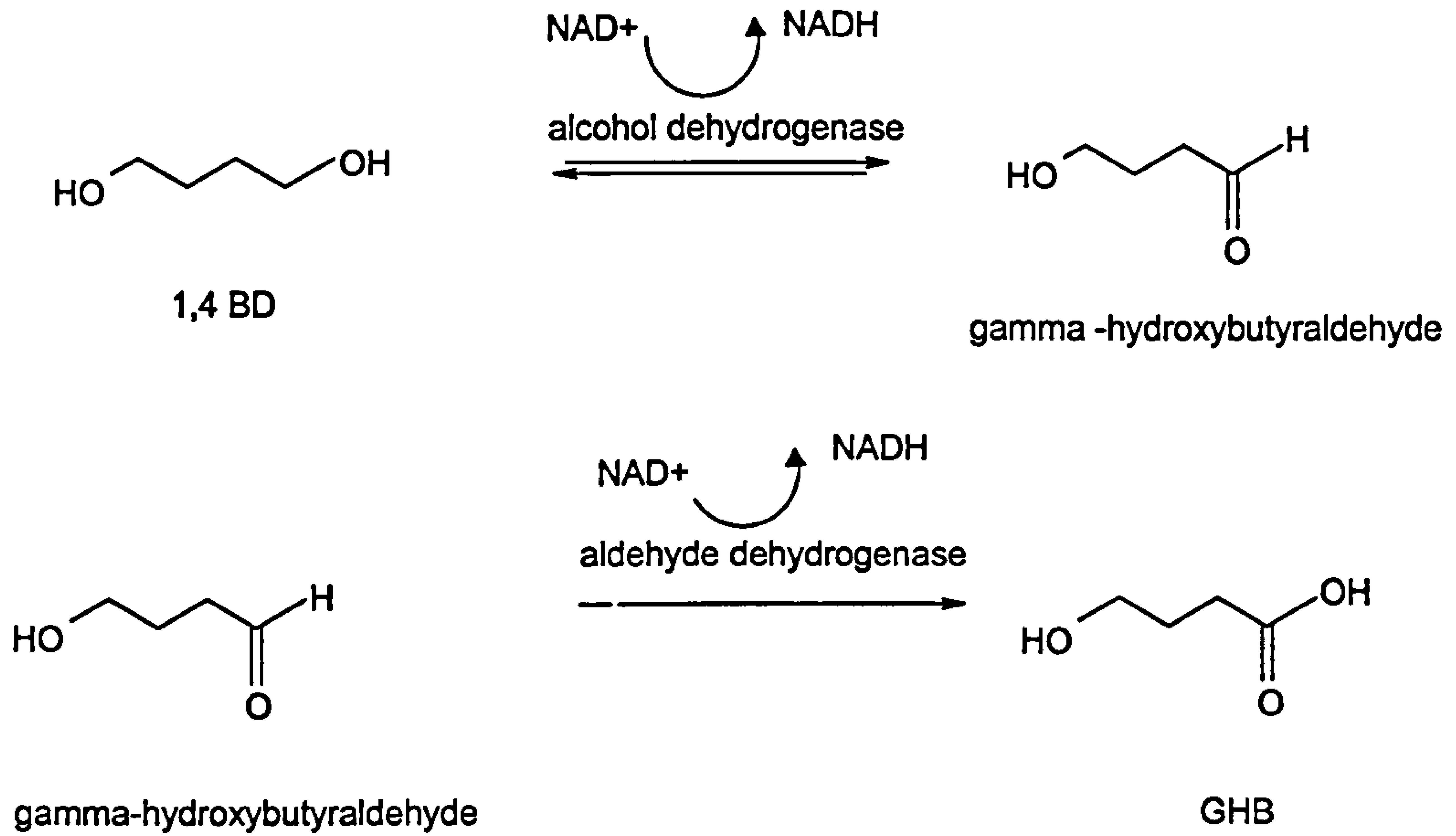


Figure 6-6:- Metabolism of 1, 4 BD to GHB in the body.

6.2.2 Chemistry and pharmacokinetics of BHB

Beta-hydroxybutyrate and other ketone bodies are synthesized from acetyl CoA (Figure 6-7) and this occurs in hepatic mitochondria. The thioester bond which links the acyl group to coenzyme A has a large negative standard free energy of hydrolysis, therefore an acyl group attached to COA in this manner is considered to be activated.

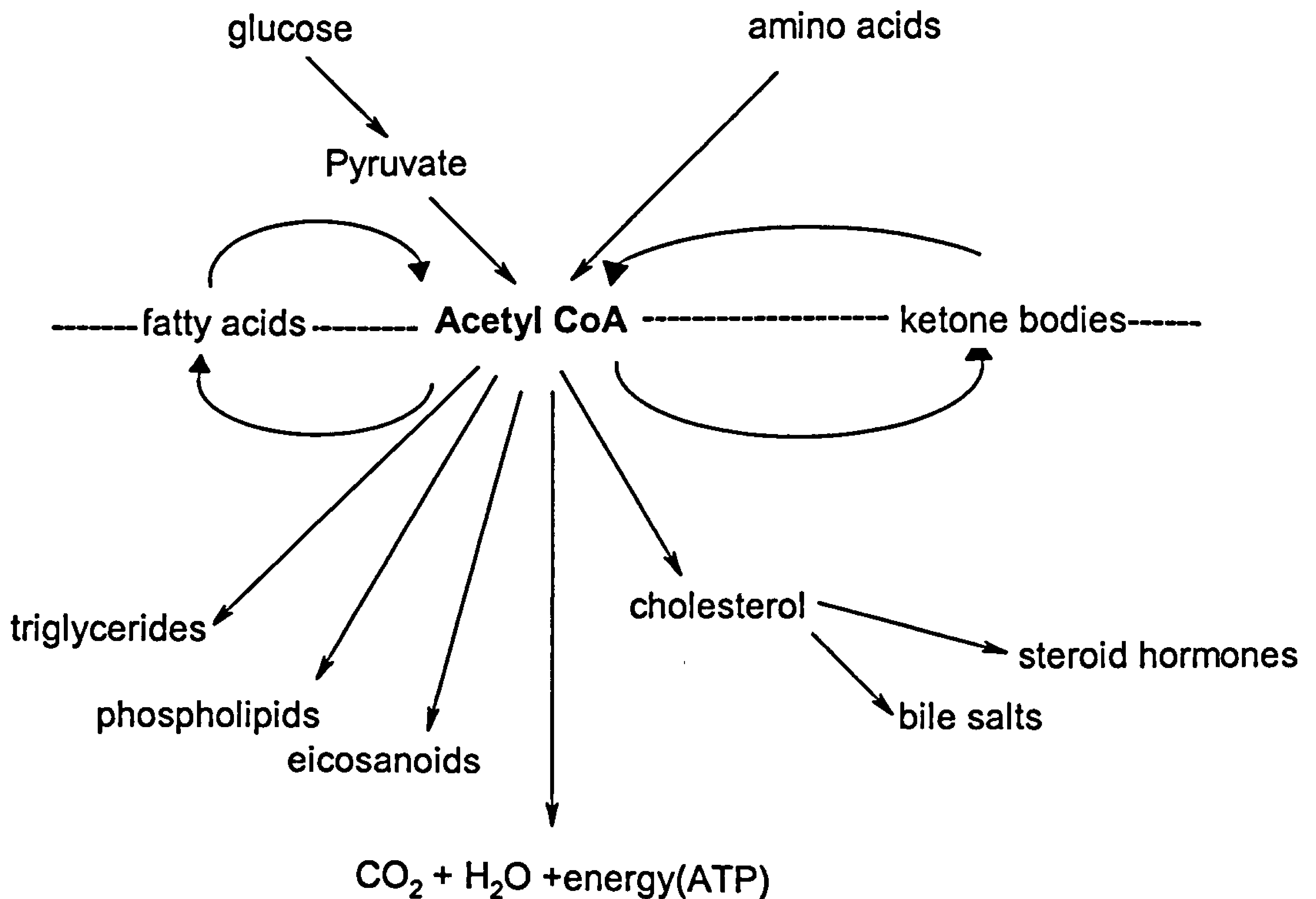


Figure 6-7:- Major metabolic sources of acetyl CoA.

Acetoacetate is produced in a three step process. However, it can be reduced to beta-hydroxybutyrate, and acetone also arises in small amounts as a biologically inert side product. When mobilization of fatty acids from adipose tissue is high, hepatic beta-oxidation will occur at a high rate and so will synthesis of ketone bodies from the resulting acetyl CoA. The first step is the formation of acetoacetyl CoA in a reversal of the thiolase step of beta oxidation (Figure 6-8).

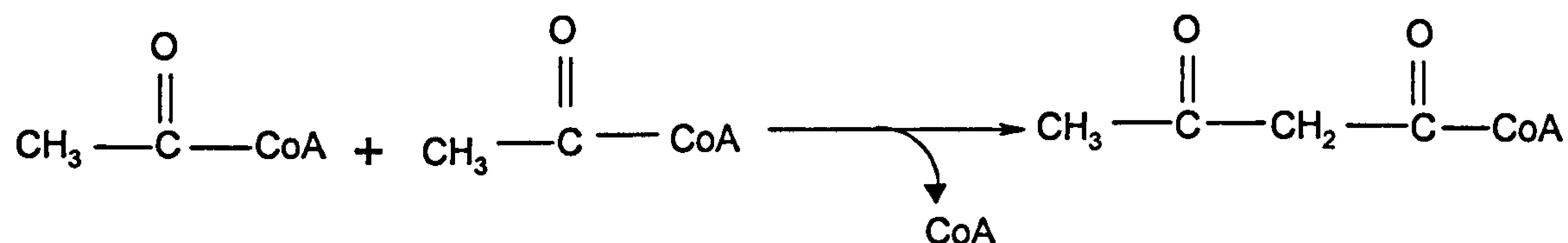


Figure 6-8:- First step is formation of acetoacetate-CoA

In the second step, a third molecule of acetyl CoA condenses with the acetoacetyl CoA, forming 3-hydroxy-3-methlglutaryl CoA (HMG CoA) in a reaction catalyzed by HMG CoA synthase Figure 6-9.

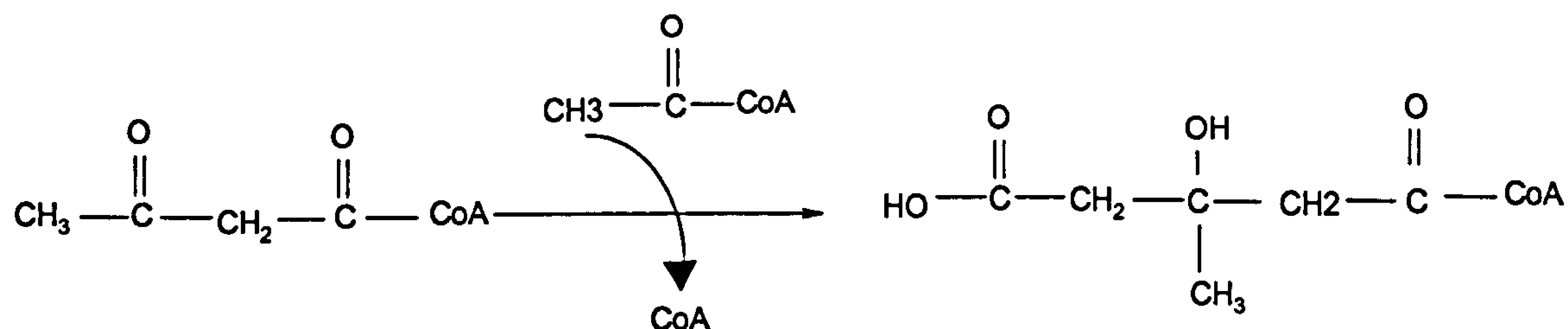


Figure 6-9:- Second step is formation of 3-hydrox-3-methlglutaryl-CoA (HMG CoA)

In the third step HMG CoA is cleaved to yield acetoacetate (ketone body) in a reaction catalyzed by HMG CoA lyase. One molecule of acetyl CoA is also produced (Figure 6-10). Subsequently acetoacetate can be reduced to beta-hydroxybutyrate by beta-hydroxybutyrate dehydrogenase in a reaction requiring NADH. The extent of this reaction depends on the state of the NAD pool of the cell: when it is highly reduced, most or all of the ketones can be in the form of beta-hydroxybutyrate (Figure 6-11). Some acetoacetate spontaneously decarboxylates to yield acetone. The odour of acetone can be smelled on the breath of individuals with severe ketosis (Figure 6-10 to Figure 6-12) [320].

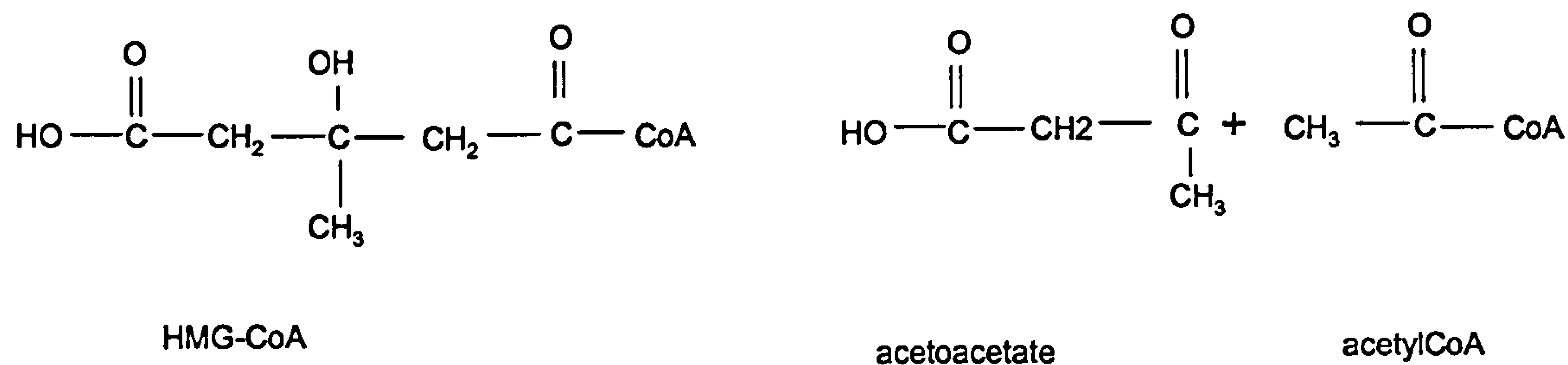


Figure 6-10:- Third step is formation of acetoacetate and acetyl CoA

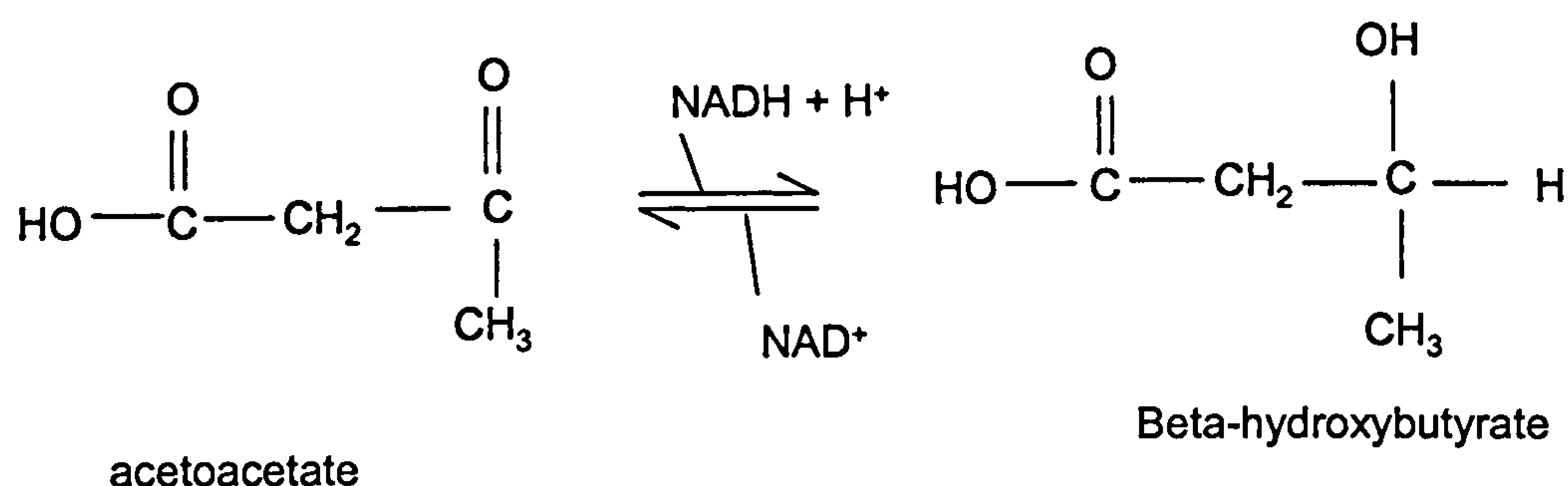


Figure 6-11:- Fourth step is formation of B-hydroxybutyrate

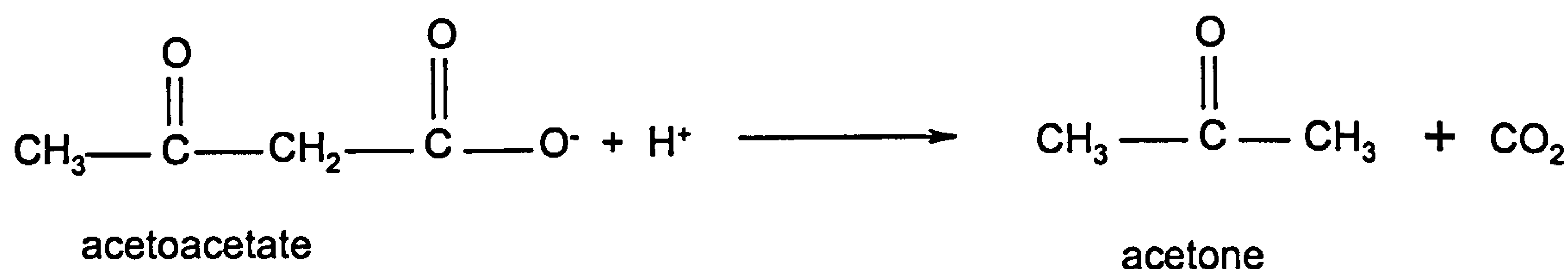


Figure 6-12:- Formation of acetone from acetoacetate by decarboxylation

6.2.3 Toxicity

Gamma-hydroxybutyric acid is readily absorbed after oral administration and rapidly metabolised in the liver by oxidative enzymes. Conversion to gamma-butyrolactone can occur and elimination is rapid, via the kidneys, with urine recovery virtually complete within 8 hours of administration. Only negligible amounts of the parent drug are recovered unchanged in urine (<5%) and after 12 hours, the drug is not detectable. Effects of GHB

including drowsiness, euphoria, dizziness, nausea, visual disturbances and unconsciousness, are usually manifested by 15 minutes after administration and persist for about 3 hours on average. Serious adverse reactions have included hypotension, bradycardia, respiratory depression, seizures, coma and possibly death. In many forensic cases alcohol abusers were found dead and the cause of death could not be ascertained. In order to examine the possible role of ketoacidosis for the cause of death the concentration of ketone bodies (acetone, acetoacetate and D-β-hydroxybutyrate) have to be determined in postmortem blood specimens[321]. However, beta-hydroxbutyrate is not detected by the screening test for ketones which detects only acetoacetic acid. Because beta-hydroxybutyrate is stable and also is usually present at concentrations higher than the other ketones, it provides a more sensitive test to use when monitoring the treatment of diabetic ketoacidosis. The mechanism of these deaths is obscure but could be related to a critical fall in blood pH to around pH 7[322, 323].

6.2.4 Methods of Analysis of GHB and BHB

There are several methods for the detection of GHB and BHB in blood samples by gas chromatography mass spectrometry (GC/MS).[324, 325] These have been used to investigate the relationship between GHB and depression of the central nervous system where effects were obsered similar to alcohol as well as induced relaxtion and tranquillity, libido enhancement and euphoria .[326, 327]

The blood samples were analysed by GC/MS using liquid-liquid extraction or solid phase extraction and disilyl-derivatisation with or without conversion to the gamma-butyrolactone (GBL).[324, 328, 329] Gamma hydroxybutyrate (GHB) is naturally present in both blood and urine but the role of GHB remains unclear.[330, 331] Some authors extract GHB from urine because GHB remains in urine longer than blood. Of these papers the post-mortem blood GHB concentration range was 13.8-86.3 mg/l.[332-336]

BHB has also been analysed by headspace gas chromatography following conversion to acetone,[337, 338] One paper reports a method to analyse both of them in urine but not blood, using a single method, based on solvent extraction and silylation before GC-MS.[339] LC-MS methods have also been published for GHB either as the free acid[340] or as its n-butyl ester.[341]

6.2.5 *Aims*

Gamma-hydroxybutric acid is a central nervous system depressant that has been abused recreationally for its purported euphoric and relaxant effects and for the purposes of drug-facilitated sexual assault due to its sedative and amnesic affects at high doses. The dramatic increase in the abuse of GHB and association in criminal investigation over the past decade has created the need for forensic laboratories to develop analytical methods to detect GHB. The 4-hydroxy isomer of GHB is also of forensic interest. In some cases it may be difficult for the forensic pathologist to assign a cause of death for a known alcohol abuser who has been found dead, and alcoholic ketoacidosis has been implicated. One diagnostic test for ketoacidosis is to measure the concentration of beta-hydroxybutyric acid in blood. Therefore it is beneficial to have a GC/MS method which can be used in the forensic lab for the determination of physiological concentrations of beta-hydroxbutyrate in postmortem blood, so creating a method for measuring both GHB and BHB at the same time with the same instrumental conditions. The aims of this study were to evaluate the reagents for derivatisation, evaluate microwave assisted derivatisation and evaluate suitable chromatographic analytic procedures, and so to develop a method for the simultaneous quantification of GHB and BHB in blood.

6.3 Experimental Section

6.3.1 *Chemicals and reagents*

N, O-Bis (trimethylsilyl) trifluoroacetamide with 1% Trimethylchlorosilane (BSTFA + 1% TMCS, Figure 6-13), is a silylation reagent. It has some desirable properties such as reacting faster and more completely than other reagents because of the presence of the trifluoroacetyl group; it has high volatility, as do its by-products; it results in separation of early eluting peaks, has excellent solubility in organic solvents and the addition of TMCS catalyzes reactions of hindered functional groups in secondary alcohols and amines[342]. The reagent was purchased from Fluka. Methanol was purchased from VWR International Ltd. Ammonia solution, NH_4OH, and potassium dihydrogen orthophosphate were purchased from BDH Laboratory supplies.

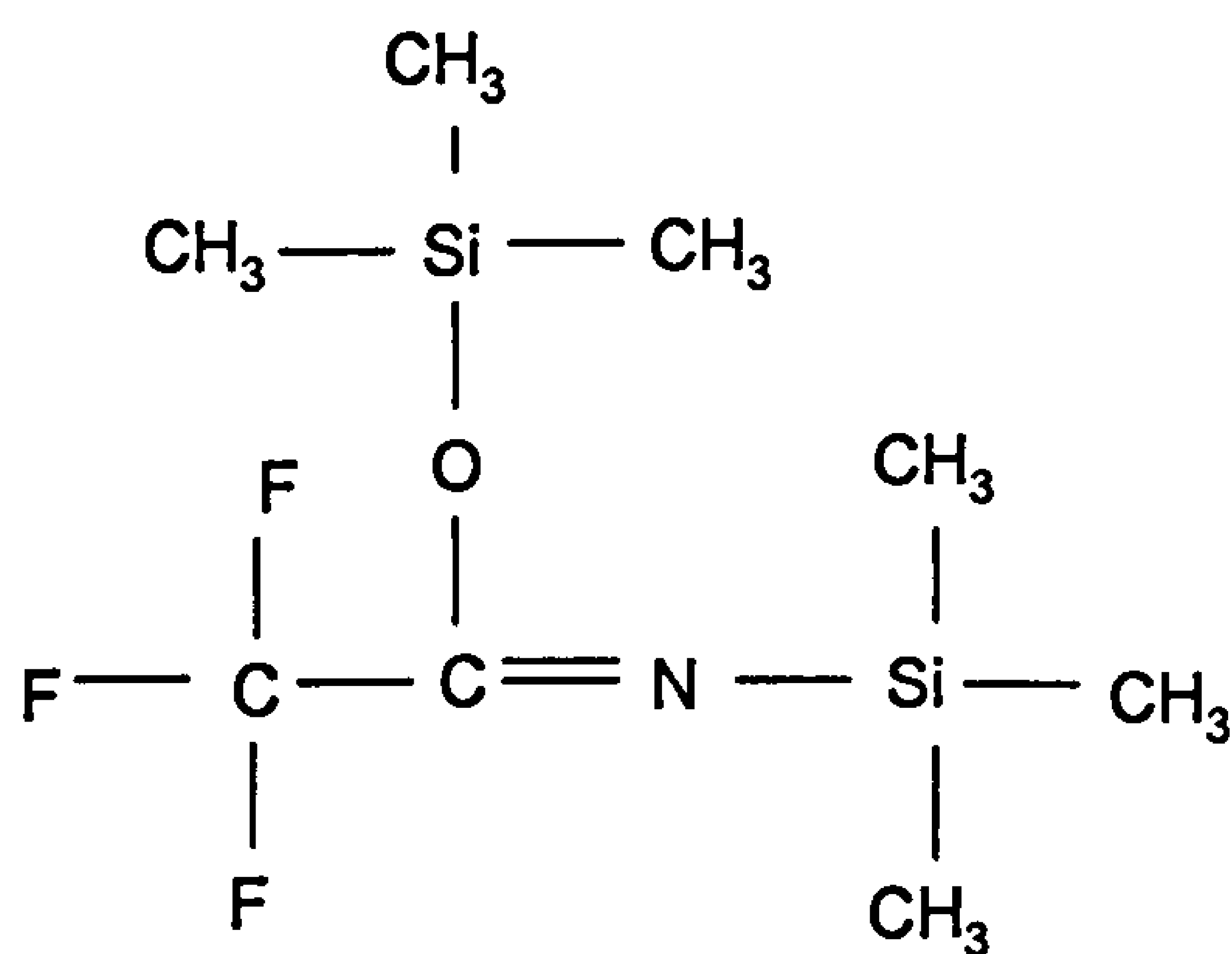


Figure 6-13:- Chemical structure of BSTFA

6.3.2 Standards

The drug standards used were GHB, 1 mg/ml in methanol and GHB-d6, 1mg/ml in methanol, as the internal standard for GHB and BHB. Both were produced by Cerilliant and supplied in the UK by LGC Promochem. Beta-hydroxybutyric acid sodium salt was obtained from Fluka.

6.3.3 Preparation of standard solutions of GHB

GHB working standard, 100µg/ml (0.1mg/ml) in methanol, was prepared from the stock 1mg/ml solution by diluting 100µl of the stock solution with 900µl of methanol. The internal standard was GHB-d6, 100µg/ml, prepared from the stock solution. 25µl of internal standard solution was added to each sample. Blood standards were prepared by adding different amounts of GHB and GHB-d6 to blood as indicated in Table 6-1.

Table 6-1:- Preparation of different concentrations of GHB

Working Standard Volume (µl)	Whole Blood Volume (µl)	Concentration (µg/ml)
10	200	5
20	200	10
40	200	20
60	200	30
80	200	40
100	200	50

6.3.4 Preparation of standard solutions of BHB

1.0052g of BHB was weighed into 1L deionised water. This gave a free BHB concentration of 8.3mg/ml. The concentration range used for analysis was from 0-2 mg/L, therefore 12.05 ml of an 8.3 mg/ml solution was added to 50 ml flask and made up to volume with blank blood, resulting in a BHB working solution with a concentration of 2 mg/ml Table 6-2. 25µl of internal standard solution was added to each sample.

Table 6-2:- Preparation of different concentrations of BHB

Number	Standard in Blood (ml)	Blank Blood (ml)	Concentration (mg/ml)
1	10	0	2
2	7.5	2.5	1.5
3	5	5	1
4	2.5	7.5	0.5
5	1	9	0.2
6	0.5	9.5	0.1

6.3.5 Extraction method based on SPE clean screen

Solid phase extraction (SPE) was carried out using Clean Screen GHB® cartridges (200mg/3ml, Figure 6-14), supplied by United Chemical Technologies Inc (UCT CSGHB203) which contained phenyl and propylsulphonic acid substituents. The extraction mechanism of Clean Screen was efficient, robust and clean. When a sample is loaded onto the column at pH6, many functional groups present in the sample are ionized. This creates repulsion between the column and many sample borne interferences, thereby reducing the likelihood of their adsorbing onto the column. The SPE column can then be washed with water or weak aqueous buffers at or below pH6 without risking loss of the analytes. Many compounds of intermediate polarity and potential interferences will also remain on the column. The majority of these potential interferences can be removed by using a methanol wash. Anionic analytes bound to the column can be eluted after another drying step. The drying steps are necessary to remove water which would have prevented water immiscible elution solvents from optimally interacting with the analytes. To elute the anionic analytes, an organic solution with a high pH between 11 and 12 should be used.

A methylene chloride-isopropanol-ammonium hydroxide mixture will simultaneously disrupt these ionic interactions and successfully elute the desired compound.

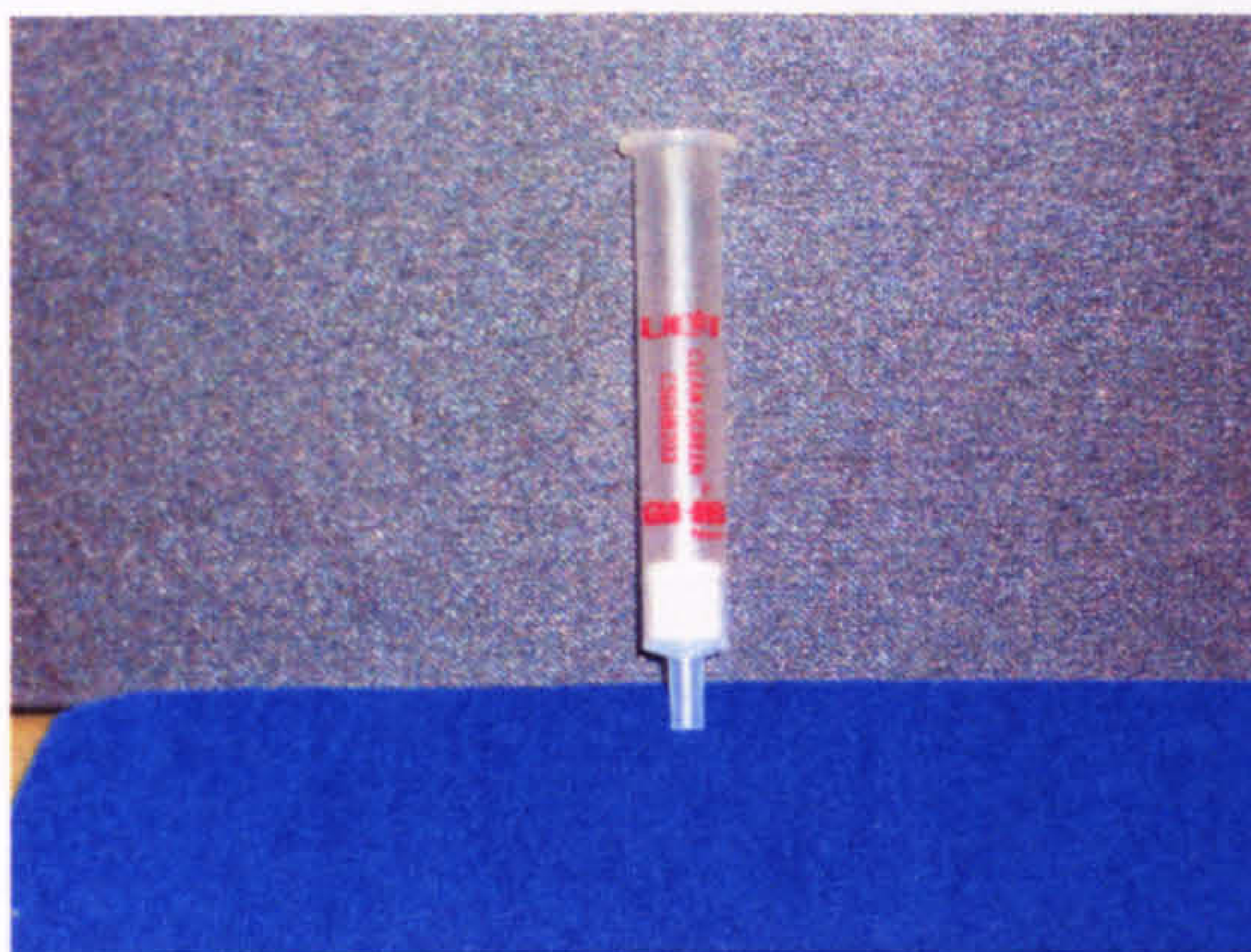


Figure 6-14:- UCT-GHB-SPE Solid Phase Extraction

6.3.6 Instrumental method

The GC was equipped with a fused silica capillary column (HP-5, 30 m × 0.32 mm i.d., 0.25μm film thickness) from J&W Scientific. The injection port was operated in the splitless mode at 280°C, with the purge being initiated at 0.5 minutes. The initial oven temperature was 65°C for 0.5 minute at which time a 15°C/min ramp was instituted until reaching a final temperature of 300°C. The mass spectrometer was operated using the same conditions listed in Table 4-1.

The ions used for selected ion monitoring are given in Table 6-3. Representative SIM and full scan chromatograms and the mass spectrum for GHB are given in

Figure 6-15 and Figure 6-16. The fragmentation pattern of GHB as its TMS derivative is explained in Figure 6-17.Corresponding diagrams for BHB are given in Figures 6-18 to 6-19.

Table 6-3:- selected Ions used for GC-MS SIM

Target analyte	Retention time	Selected Ions (m/z)	Internal standard	Selected Ions(m/z)
GHB	4.78	117, 233	GHB-d6	239
BHB	4.13	191, 233	GHB-d6	239

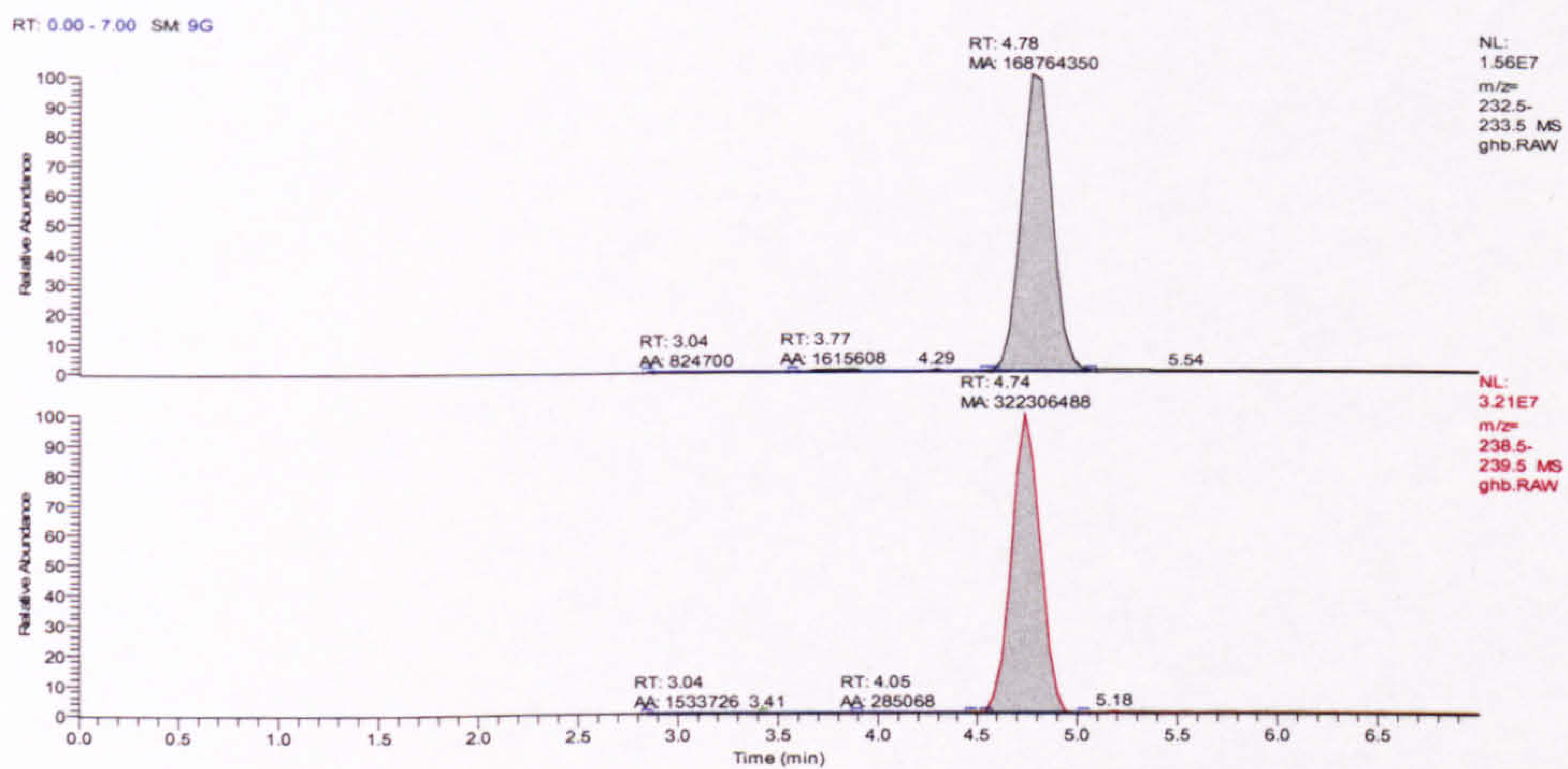
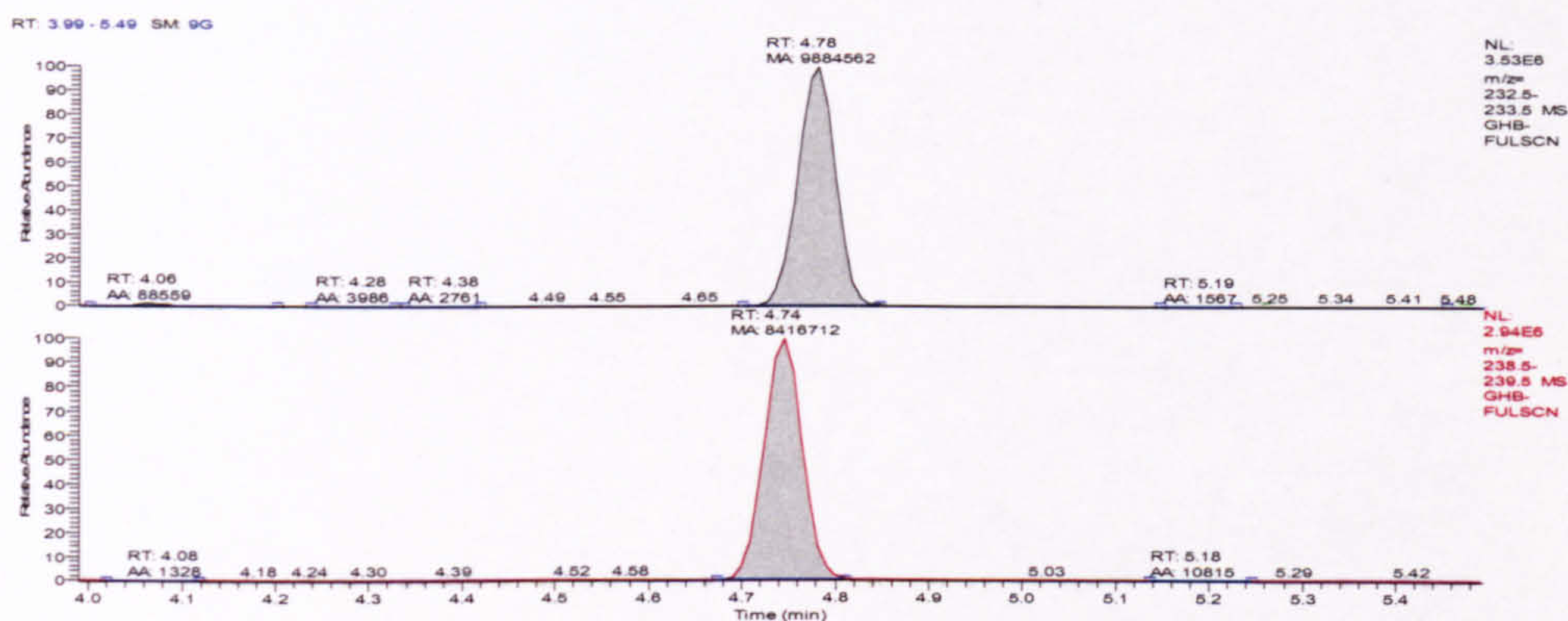


Figure 6-15:- SIM Chromatograms for GHB

(a)



(b)

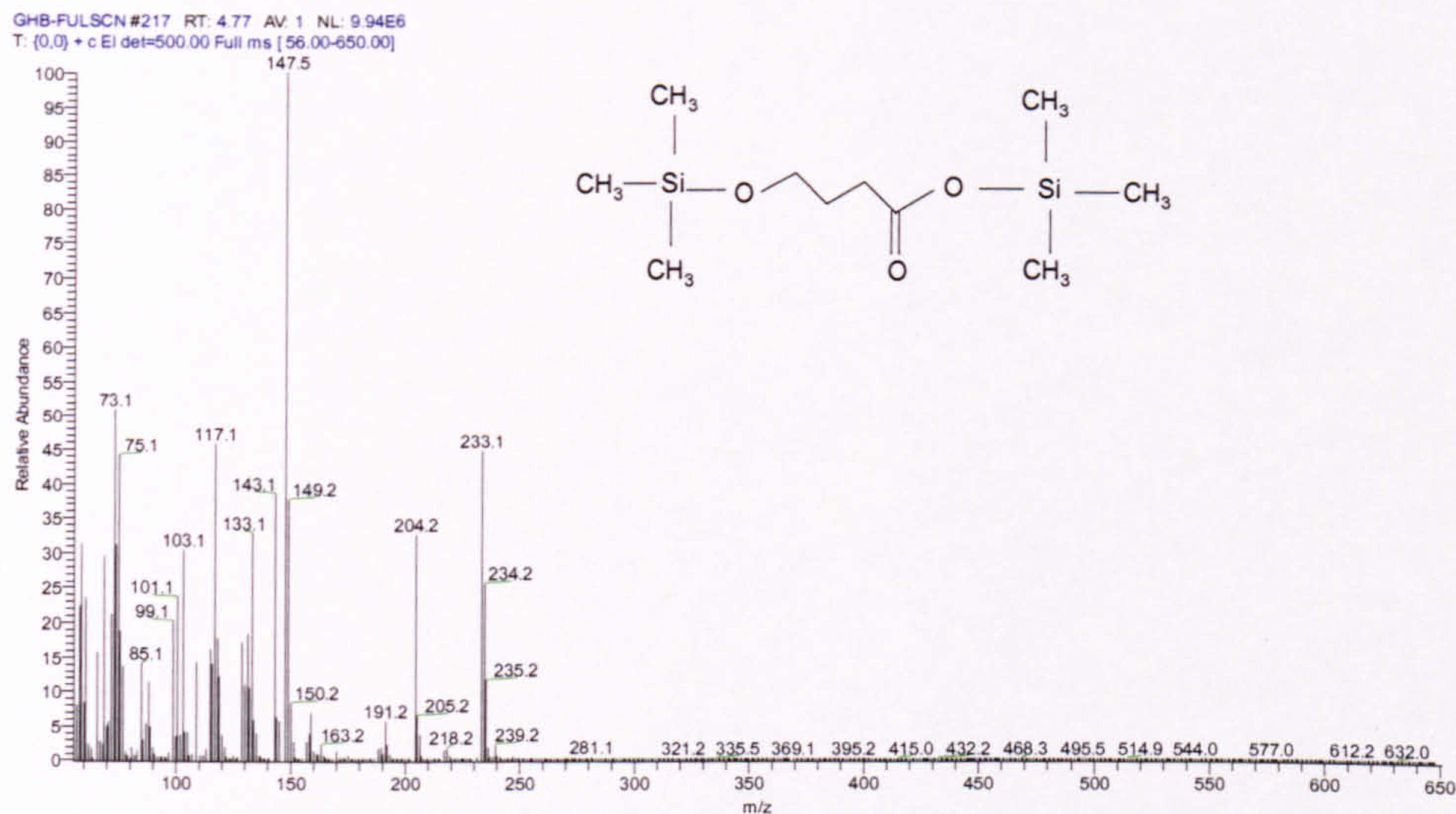


Figure 6-16:- (a) Selected ion chromatograms from repetitive full scan GC-MS and (b) mass spectrum for GHB (M^+248) - TMS derivative

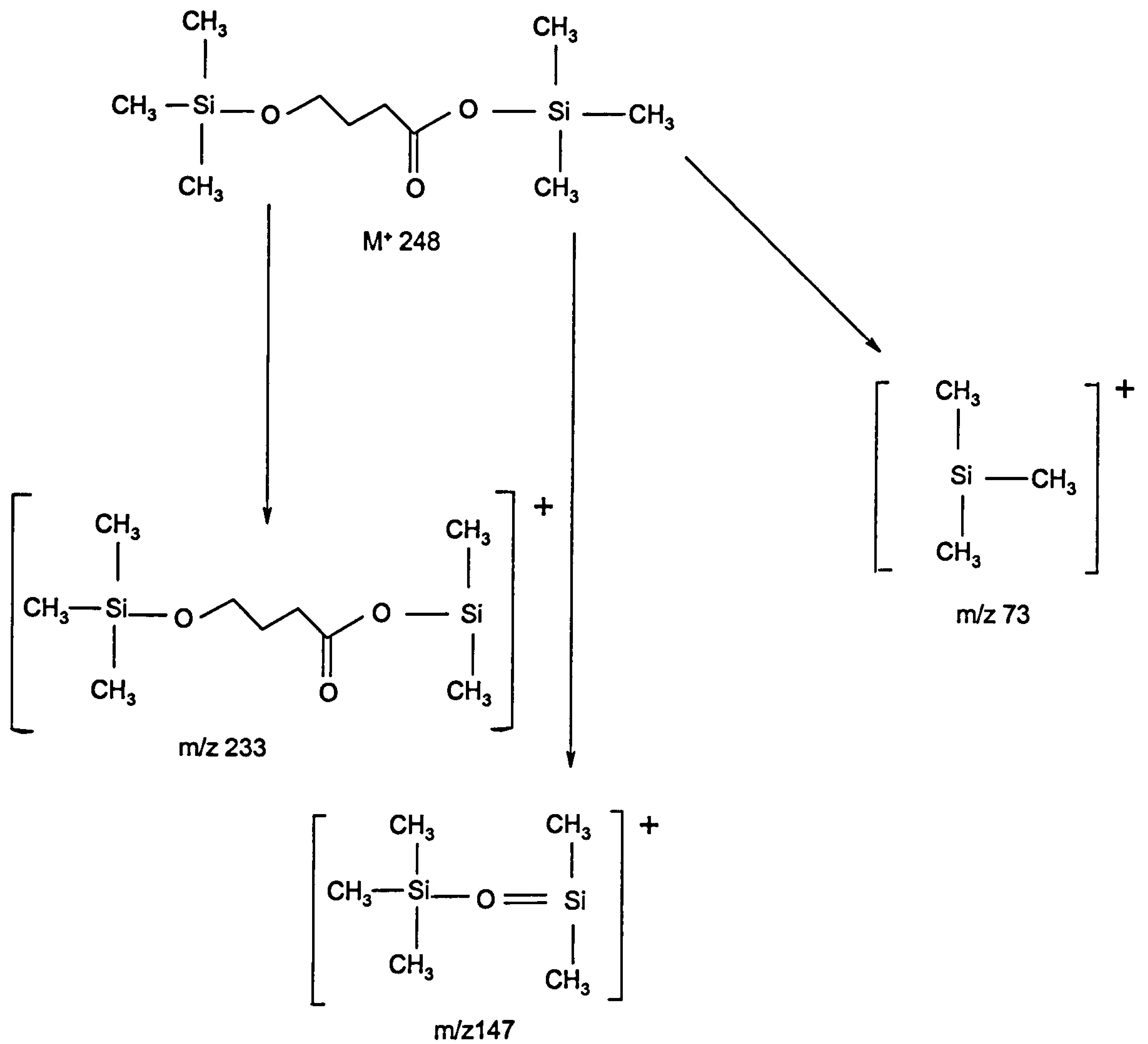
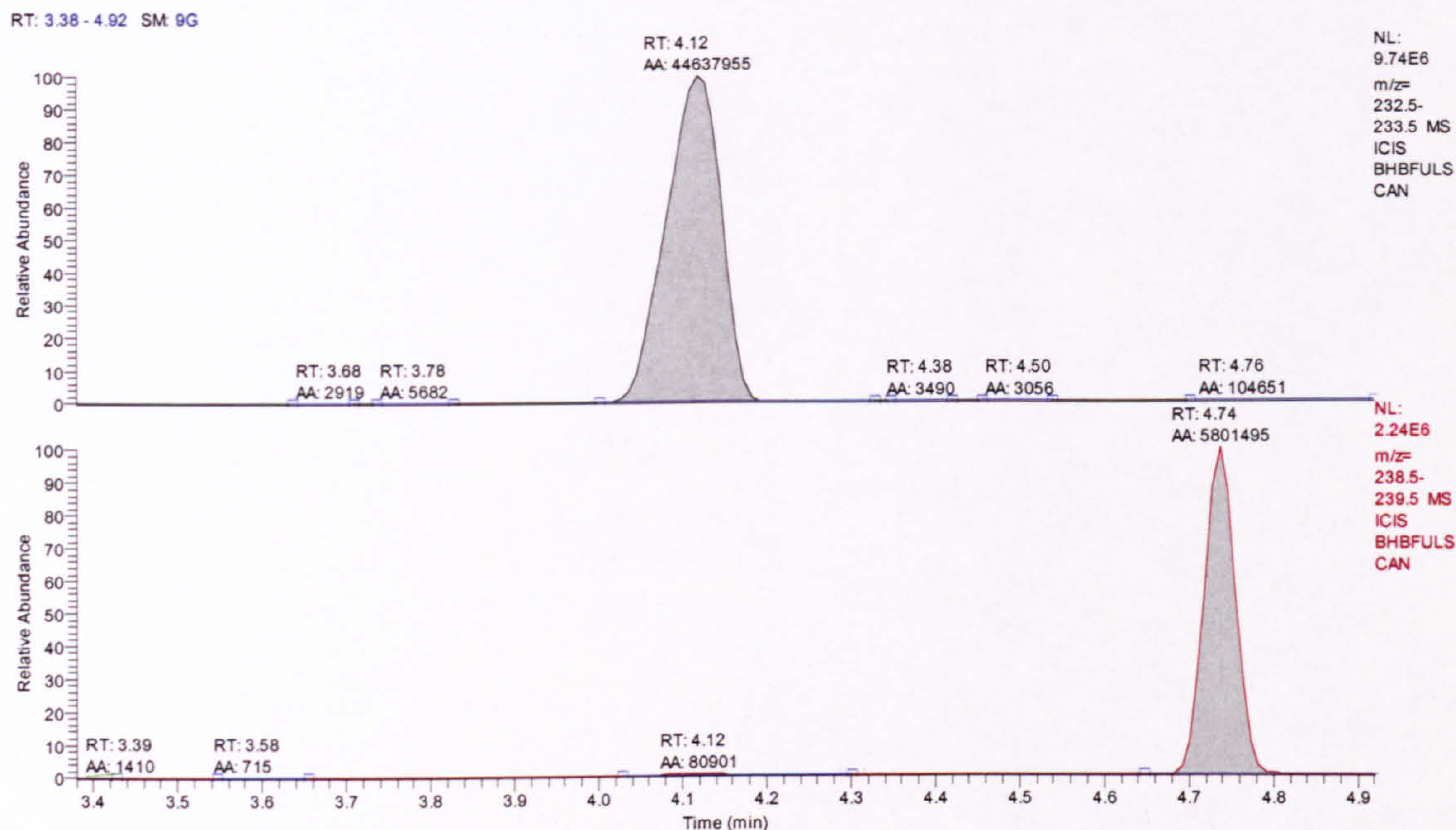


Figure 6-17:- Fragmentation for GHB TMS derivative



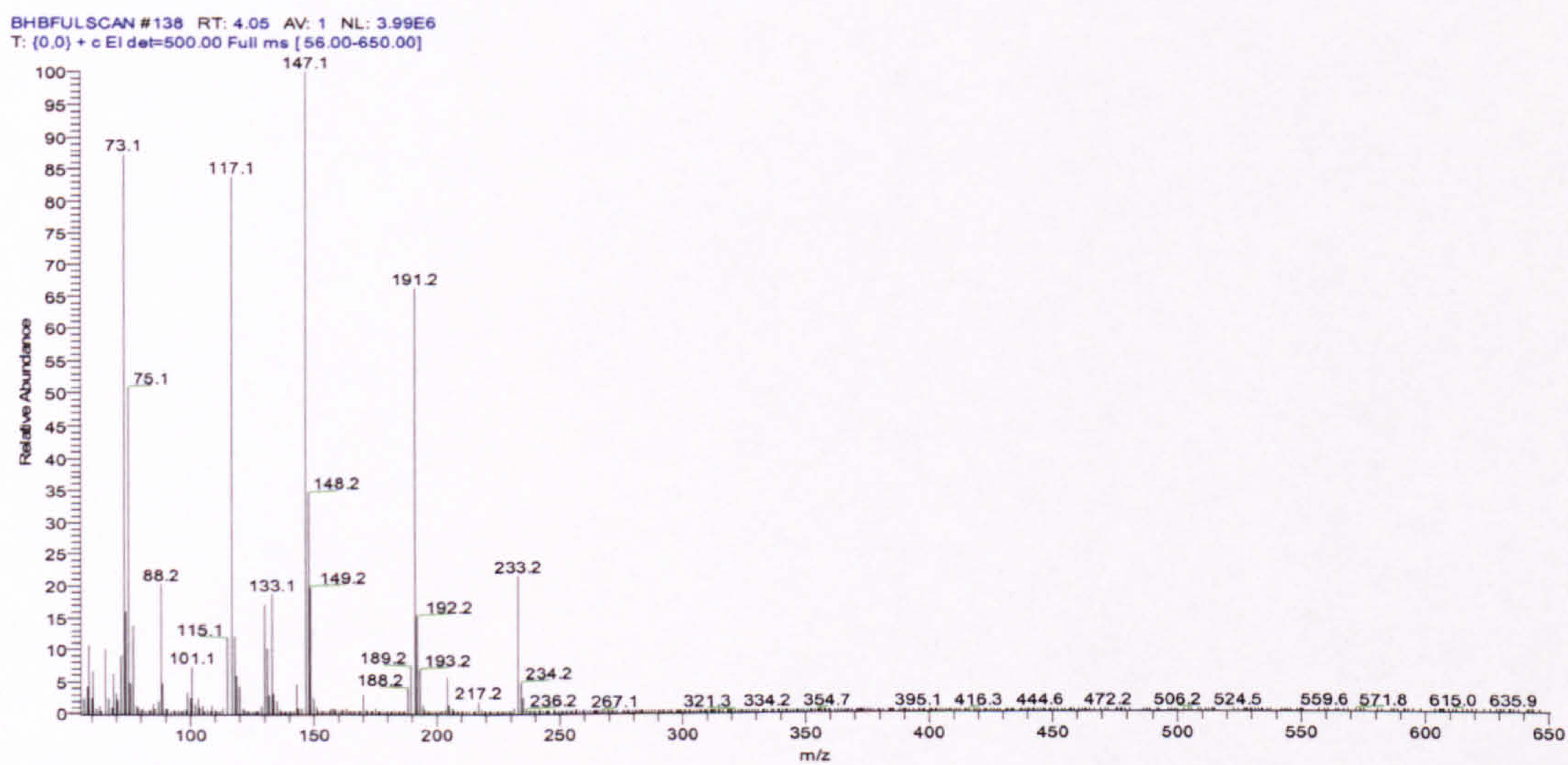


Figure 6-18:- Full scan chromatograms and Mass spectrum for BHB-BSTFA/TMCS

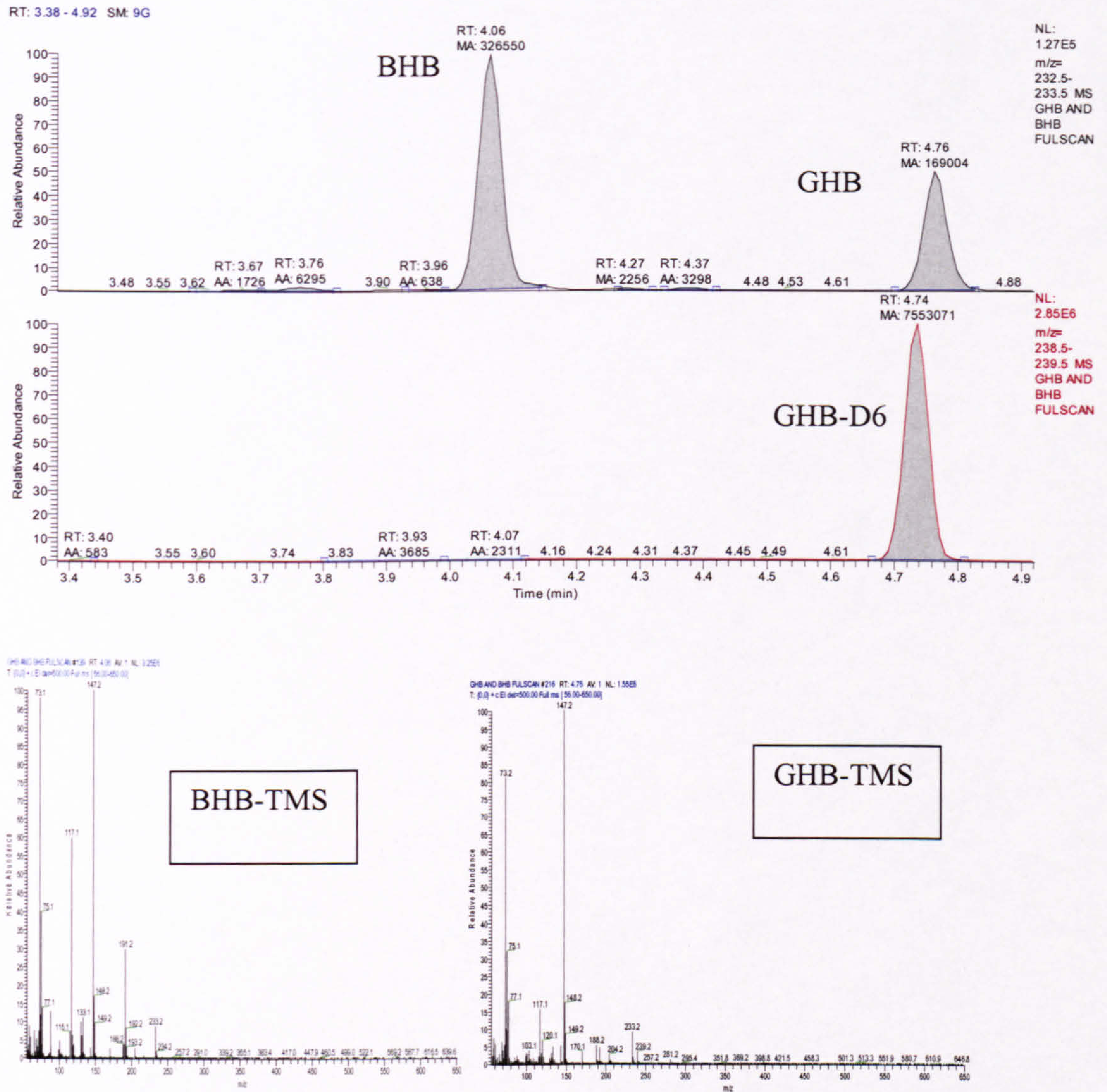


Figure 6-19:- Ion chromatograms and mass spectra for GHB and BHB as their TMS derivatives from full scan GC-MS analysis.

6.3.7 Method validation for GHB

6.3.7.1 **Linearity**

Linearity was determined for the concentration range of 0 to 50µg/200µl blood. The calibration curve for the target analyte was linear and had a linear correlation coefficient (R^2) equal to 0.9993 as shown in Figure 6-20

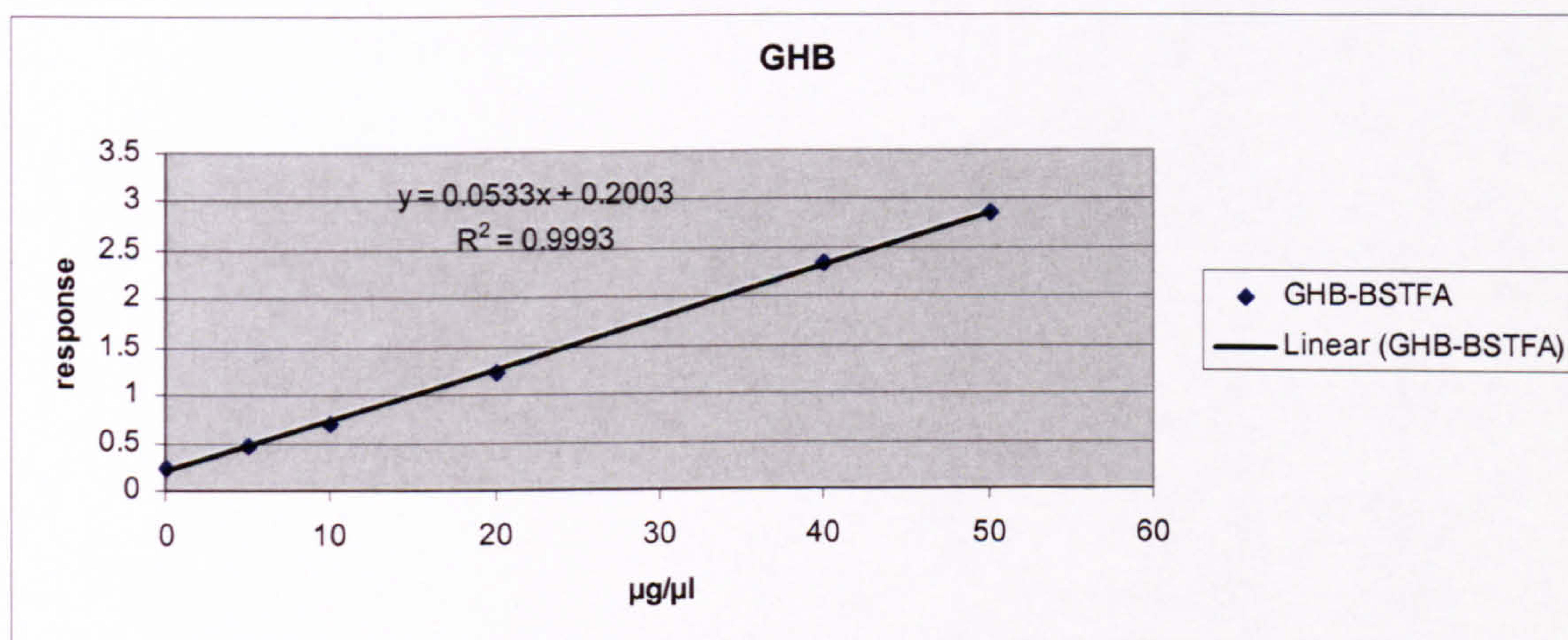


Figure 6-20:- Calibration curve for GHB

6.3.7.2 **Recovery**

The recovery of GHB from spiked blood samples was determined as described earlier in Chapter 5. Recoveries for the analyte were in the range 44-76% (Table 6-4, Figure 6-21).

Table 6-4:- Recovery of GHB from blood using Clean Screen GHB cartridges

Concentration (µg/ml)	GHB % Recovery
10	44
30	49
50	76

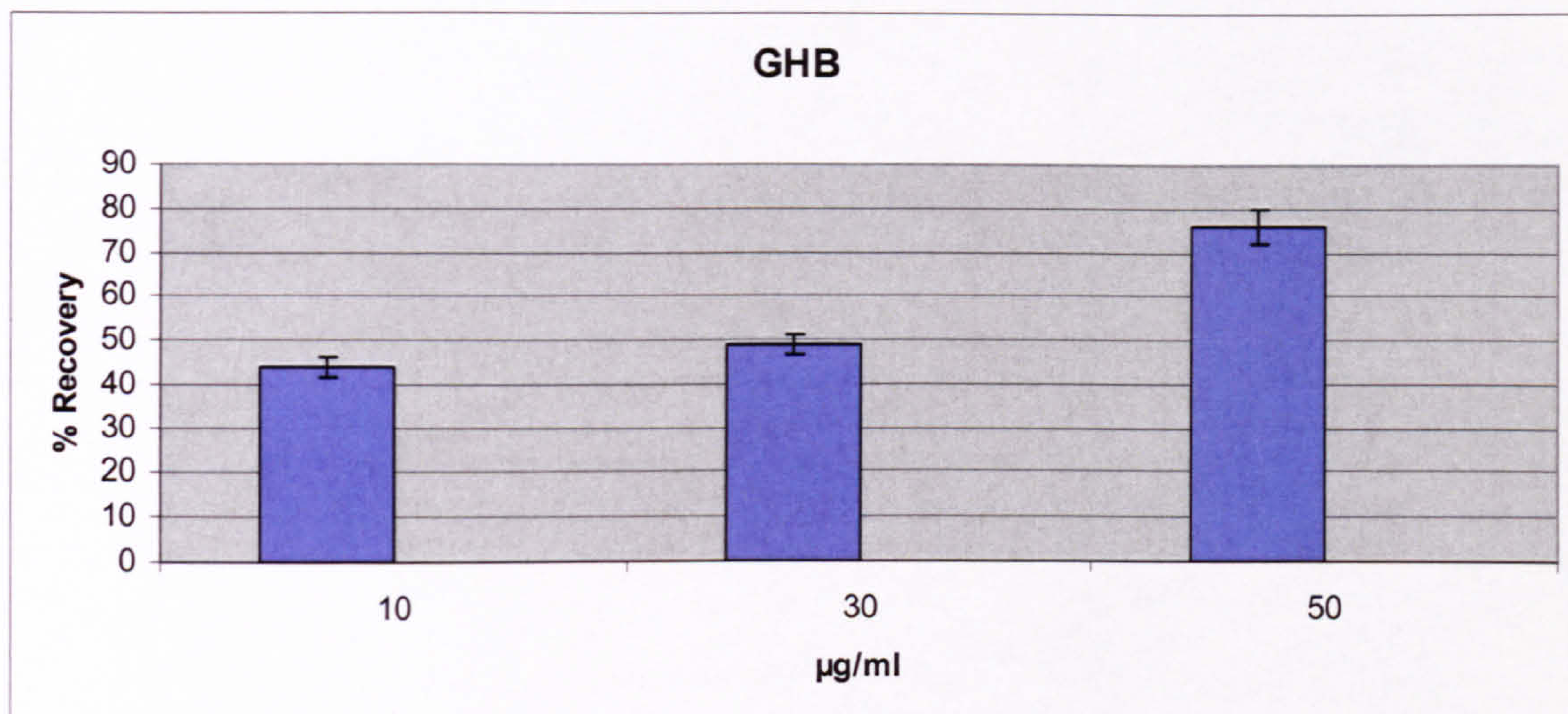


Figure 6-21:- Recovery of GHB from blood using Clean Screen GHB cartridges

6.3.7.3 **Limits of Detection and Quantitation**

The methods for determining limits of detection and quantitation of GHB from spiked blood samples have already been described in Chapter 5 (Paragraph 5.4.6 and 5.4.7). Blank blood was spiked with GHB to give concentrations of 1, 2.5, 5, 10 and 20µg/ml. These were extracted as described previously. The detection limit was evaluated as the amount of analyte that gives a signal-to-noise ratio of 3 times the background noise. The limit of quantitation was evaluated as the signal-to-noise ratio that is equivalent to 10 times the noise (Table 6-5).

Table 6-5:- Limit of Detection and Quantitation for GHB

	GHB (µg/ml)
LOD	**0.4**
LOQ	**1.30**

6.3.7.4 **Inter-Day Precision**

The calibration curve standards were extracted five times during different days and the inter–day variation at each concentration was assessed and is displayed below in Table 6-6.

Table 6-6:- Inter-Day precision

Concentration GHB (µg/ml)	Mean concentration (%RSD)
5	5.1 (10.6)
10	10.0 (18.3)
20	19.7 (11.8)
30	30.1 (2.9)
40	39.9 (0.9)
50	50.0 (0.4)

6.3.7.5 Intra- Day Precision

The average area ratios (drug/internal standard ratio) and relative standard deviations were calculated after the linearity curve standards were extracted five times to determine the intra-day variation of the assay over the calibration range and this is displayed in Table 6-7.

Table 6-7:- Intra-Day Precision

Concentration in blood (µg/ml)	m/z 233/239 GHB/GHB-d6 (RSD %)
5	0.8 (9.7)
10	1.3 (6.3)
20	4.7 (9.6)
30	6.3 (10.5)
40	6.5 (9.8)
50	7.6 (8.8)

6.3.8 Method validation for BHB

6.3.8.1 Linearity

Linearity was determined for concentration range of 0 to 2mg/ml. The calibration curve for the target analyte was linear and the linear correlation coefficient (R^2) was 0.9988 (Figure 6-22).

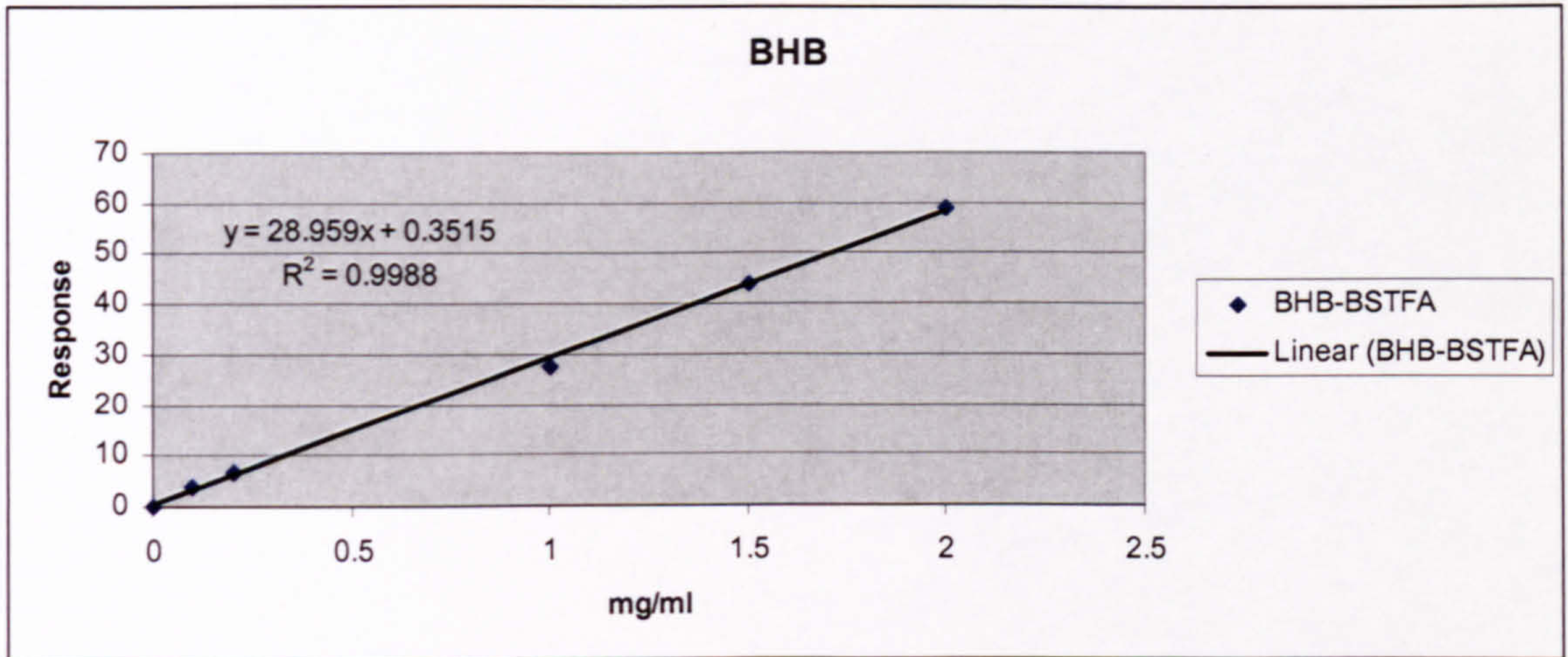


Figure 6-22:- Calibration Curve for BHB

6.3.8.2 **Recovery**

The method for determining the recovery of the analyte from spiked blood samples has already been described in Chapter 5. Recoveries for the analyte were in the range 47-89% (Table 6-8, Figure 6-23).

Table 6-8:- Recovery of BHB from blood samples

Concentration (mg/ml)	Recovery %
0.1	47
1	84
2	89

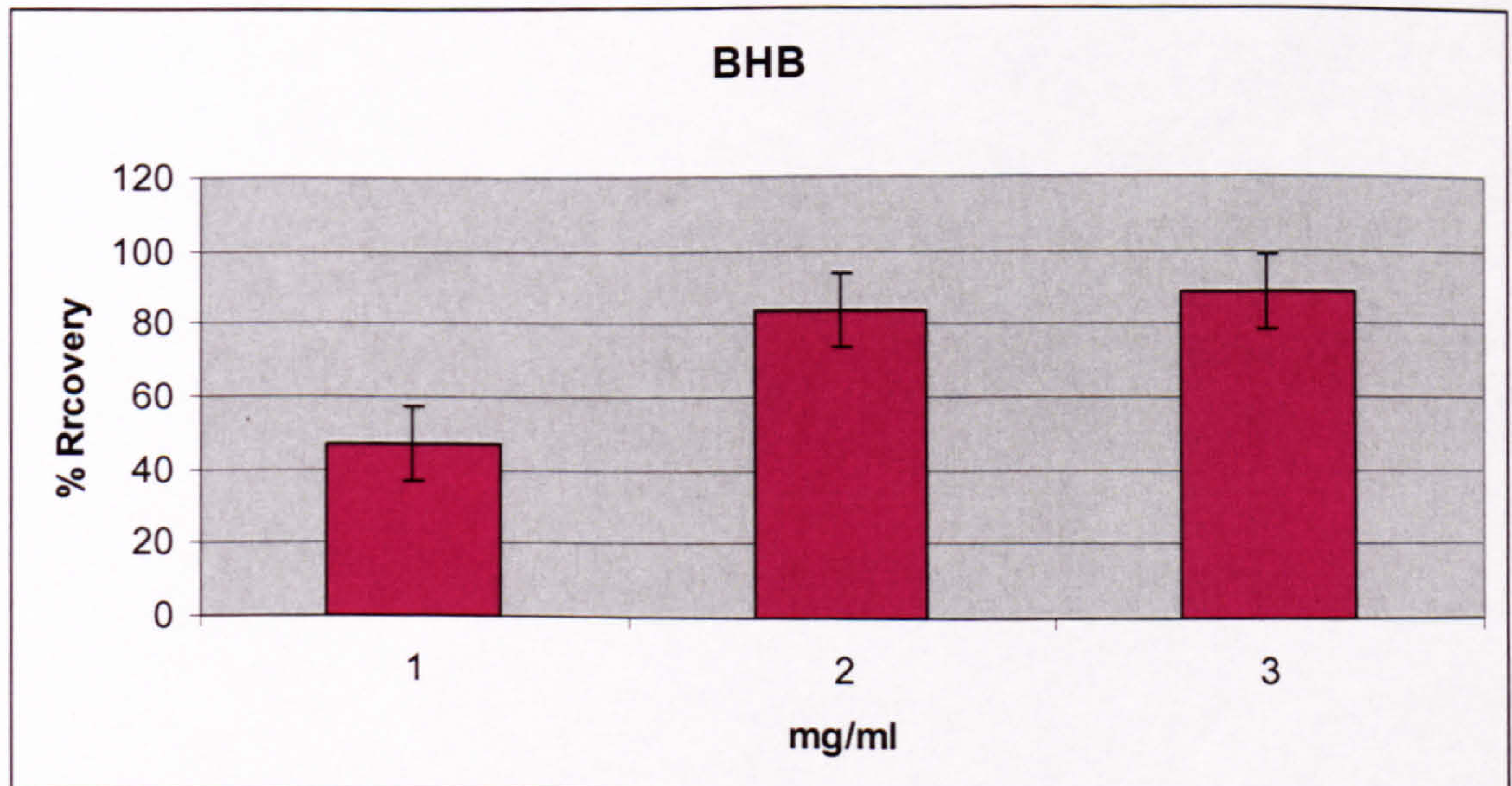


Figure 6-23:- Recovery of BHB from blood using Clean Screen GHB cartridges

6.3.8.3 Limits of Detection and Quantitation

Limit of detection and quantitation of the method for spiked blood samples were measured as described previously in Chapter 5. Blank blood was spiked with BHB to give concentrations of 0.0025, 0.005, 0.01, 0.02, 0.06 mg/ml. these were extracted as described previously. The limit of detection was evaluated as the concentration of analyte that gave a signal-to-noise ratio of 3 times the noise. The limit of quantitation was evaluated as the concentration of analyte that gave a signal-to-noise ratio that was equivalent to 10 or else it is blank reading plus 10 times the standard deviation of the noise or standard error of the calibration curve (Table 6-9).

Table 6-9:- Limits of Detection and Quantitation

	BHB (mg/ml)
LOD	0.001
LOQ	0.004

6.3.8.4 Intra-Day precision

The average area ratio and relative standard deviations were calculated for each concentration and are displayed in Table 6-10

Table 6-10:- Intra-Day precision

Concentration in blood (mg/ml)	m/z 233/239 BHB/GHB-d6 (RSD %)
0.1	4.3 (8.5)
0.2	6.2 (9.7)
0.5	16.2 (7.8)
1	26.2 (7.1)
1.5	27.1 (0.4)

6.3.8.5 Inter-Day Precision

The set of standards used for the calibration curve was extracted five times during different days and the inter-day variation was assessed and is displayed in Table 6-11.

Table 6-11:- Inter-Day precision

Concentration in blood (mg/ml)	m/z 233/239 BHB/GHB-d6 (RSD %)
0.1	0.13 (8.1)
0.2	0.21 (10.9)
0.5	0.46 (6.0)
1	0.1 (4.0)
1.5	1.5 (0.94)

6.4 Application to Case Samples

The developed method was applied to 89 forensic autopsy case samples which were collected from Forensic Medicine and Science, University of Glasgow. The samples were kept frozen until analysed by GC/MS.

These real case samples were subdivided into four groups: the first group contained cases which did not have a history of alcohol abuse (i.e. not known to be alcoholics) and which had a negative alcohol result. The second group were not known to be alcoholics but had a positive alcohol result. The third group had a history of alcohol abuse but had a low blood alcohol concentration (less than 200/100ml) at the time of death. The fourth group had a history of alcohol abuse and had a high blood alcohol concentration at the time of death. The results of the analyses of case samples are given in Table 6-12.

Table 6-12:- Results of GC/MS analysis of cases for GHB/BHB

Case Number	BHB Concentration (mg/ml)	GHB Concentration (mg/L)	Group
Blank	-	-	-
1	0.4	76	Non-alcoholic, Neg alc
2	0.4	101	Non-alcoholic, Neg alc
3	0.3	72	Non-alcoholic, Neg alc
4	1.1	236	Non-alcoholic, Neg alc
5	0.3	91	Non-alcoholic, Neg alc
6	0.33	207	Non-alcoholic, Neg alc
7	0.3	123	Non-alcoholic, Neg alc
8	0.4	123	Non-alcoholic, Neg alc
9	0.4	189	Non-alcoholic, Neg alc
10	0.34	65	Non-alcoholic, Neg alc

11	0.42	211	Non-alcoholic, Neg alc
12	0.4	312	Non-alcoholic, Neg alc
13	0.34	89	Non-alcoholic, Neg alc
14	0.4	150	Non-alcoholic, Neg alc
15	0.32	81	Non-alcoholic, Neg alc
16	0.31	237	Non-alcoholic, Neg alc
17	0.31	88	Non-alcoholic, Neg alc
18	0.31	56	Non-alcoholic, Neg alc
19	0.3	102	Non-alcoholic, Neg alc
20	0.34	112	Non-alcoholic, Neg alc
21	0.34	24	Non-alcoholic, Neg alc
22	0.42	100	Non-alcoholic, Neg alc
23	0.42	185	Non-alcoholic, Neg alc
24	0.4	143	Non-alcoholic, Neg alc
25	0.4	88	Non-alcoholic, Neg alc
26	0.34	9	Non-alcoholic, Neg alc
27	0.35	103	Non-alcoholic, Neg alc
28	0.38	93	Non-alcoholic, Neg alc
29	0.35	285	Non-alcoholic, Neg alc
30	0.4	72	Non-alcoholic, Neg alc
31	0.4	236	Non-alcoholic, Neg alc
32	0.34	84	Non-alcoholic, Neg alc
33	0.33	126	Non-alcoholic, Neg alc
34	0.37	187	Non-alcoholic, Neg alc
35	0.32	24	Non-alcoholic, Neg alc
36	0.31	186	Non-alcoholic, Neg alc
37	1.3	251	Non-alcoholic, Pos alc
38	1.1	256	Non-alcoholic, Pos alc
39	0.6	138	Non-alcoholic, Pos alc
40	0.3	242	Non-alcoholic, Pos alc
41	0.4	509	Non-alcoholic, Pos alc
42	1.5	123	Non-alcoholic, Pos alc
43	0.49	509	Non-alcoholic, Pos alc
44	0.4	177	Non-alcoholic, Pos alc
45	0.4	310	Non-alcoholic, Pos alc

46	0.5	182	Non-alcoholic, Pos alc
47	0.4	145	Non-alcoholic, Pos alc
48	0.3	210	Non-alcoholic, Pos alc
49	0.63	161	Non-alcoholic, Pos alc
50	0.36	44	Non-alcoholic, Pos alc
51	0.38	75	Non-alcoholic, Pos alc
52	0.34	55	Non-alcoholic, Pos alc
53	0.38	98	Non-alcoholic, Pos alc
54	0.4	132	Non-alcoholic, Pos alc
55	0.31	216	Non-alcoholic, Pos alc
56	0.39	131	Non-alcoholic, Pos alc
57	0.37	186	Non-alcoholic, Pos alc
58	0.4	216	Non-alcoholic, Pos alc
59	0.36	147	Non-alcoholic, Pos alc
60	0.35	144	Non-alcoholic, Pos alc
61	0.39	226	Non-alcoholic, Pos alc
62	0.31	34	Non-alcoholic, Pos alc
63	0.4	216	Non-alcoholic, Pos alc
64	0.36	40	Non-alcoholic, Pos alc
65	0.35	86	Non-alcoholic, Pos alc
66	0.38	83	Non-alcoholic, Pos alc
67	0.37	172	Alcoholic, Low alc
68	0.37	218	Alcoholic, Low alc
69	0.39	411	Alcoholic, Low alc
70	0.35	53	Alcoholic, Low alc
71	0.76	243	Alcoholic, Low alc
72	0.4	103	Alcoholic, Low alc
73	0.3	91	Alcoholic, Low alc
74	0.4	54	Alcoholic, Low alc
75	0.4	253	Alcoholic, Low alc
76	0.38	348	Alcoholic, Low alc
77	1.5	123	Alcoholic, Low alc
78	0.35	123	Alcoholic, Low alc
79	0.35	118	Alcoholic, Low alc
80	2.2	118	Alcoholic, High alc

81	4.2	169	Alcoholic, High alc
82	5.7	97	Alcoholic, High alc
83	3.5	172	Alcoholic, High alc
84	0.87	255	Alcoholic, High alc
85	0.49	190	Alcoholic, High alc
86	7.15	137	Alcoholic, High alc
87	0.36	186	Alcoholic, High alc
88	0.37	38.2	Alcoholic, High alc
89	6.4	76.9	Alcoholic, High alc

Analyses of the data for these cases was carried out by regression analysis, looking for any correlation between blood alcohol and either BHB or GHB or between BHB and GHB, and also using the Student's t-test to test for the occurrence of any significant differences between the four groups (two-tailed test, unequal variance). No significant correlations were found between alcohol concentration and either BHB or GHB concentration in blood or between BHB and GHB concentrations in blood (Figure 6-18, Figure 6-19). The significance level did not increase by selecting either alcoholics or non-alcoholics for this analysis.

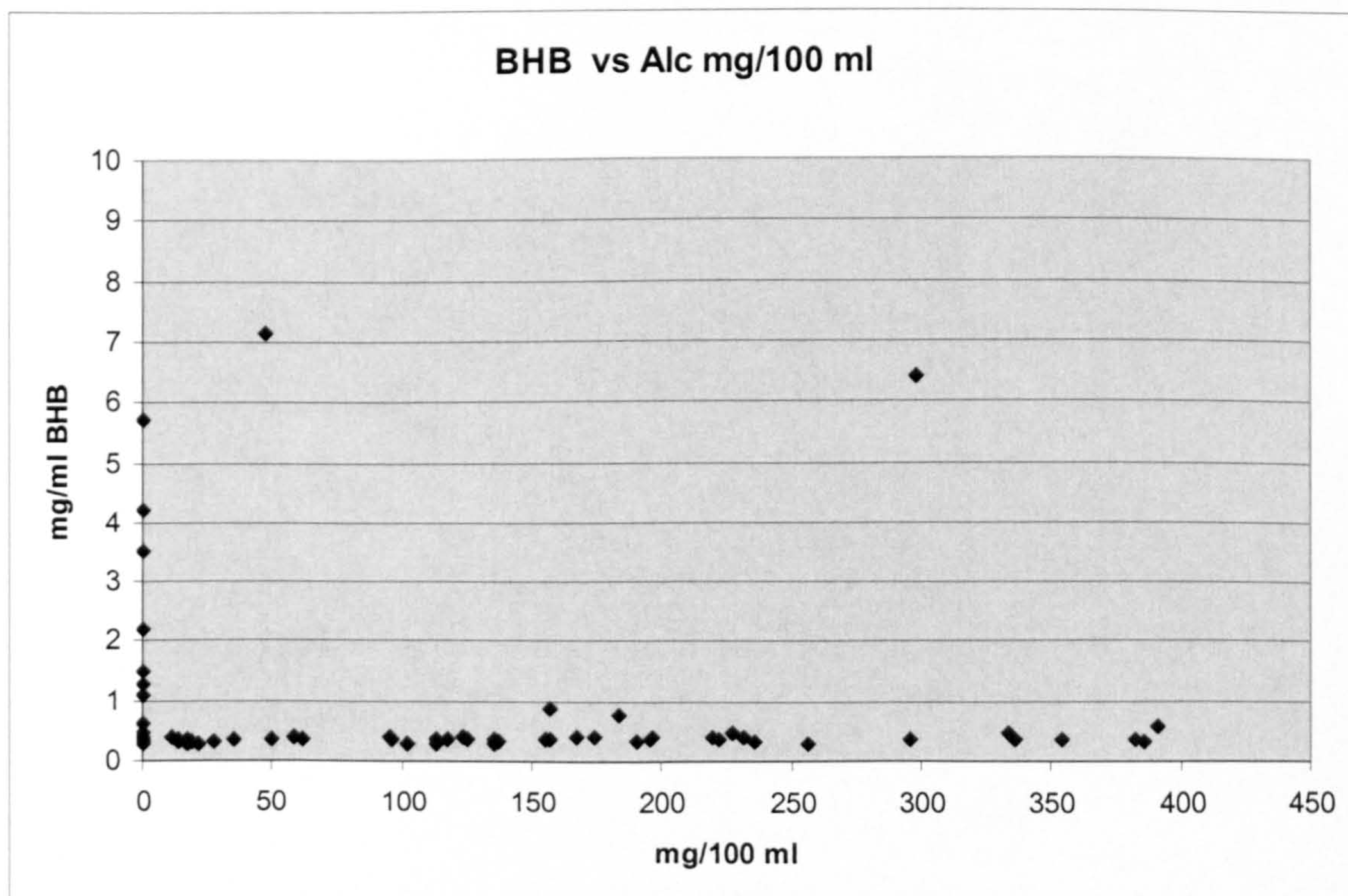


Figure 6-18:- Scatter plot of blood alcohol concentration *versus* blood BHB concentration.

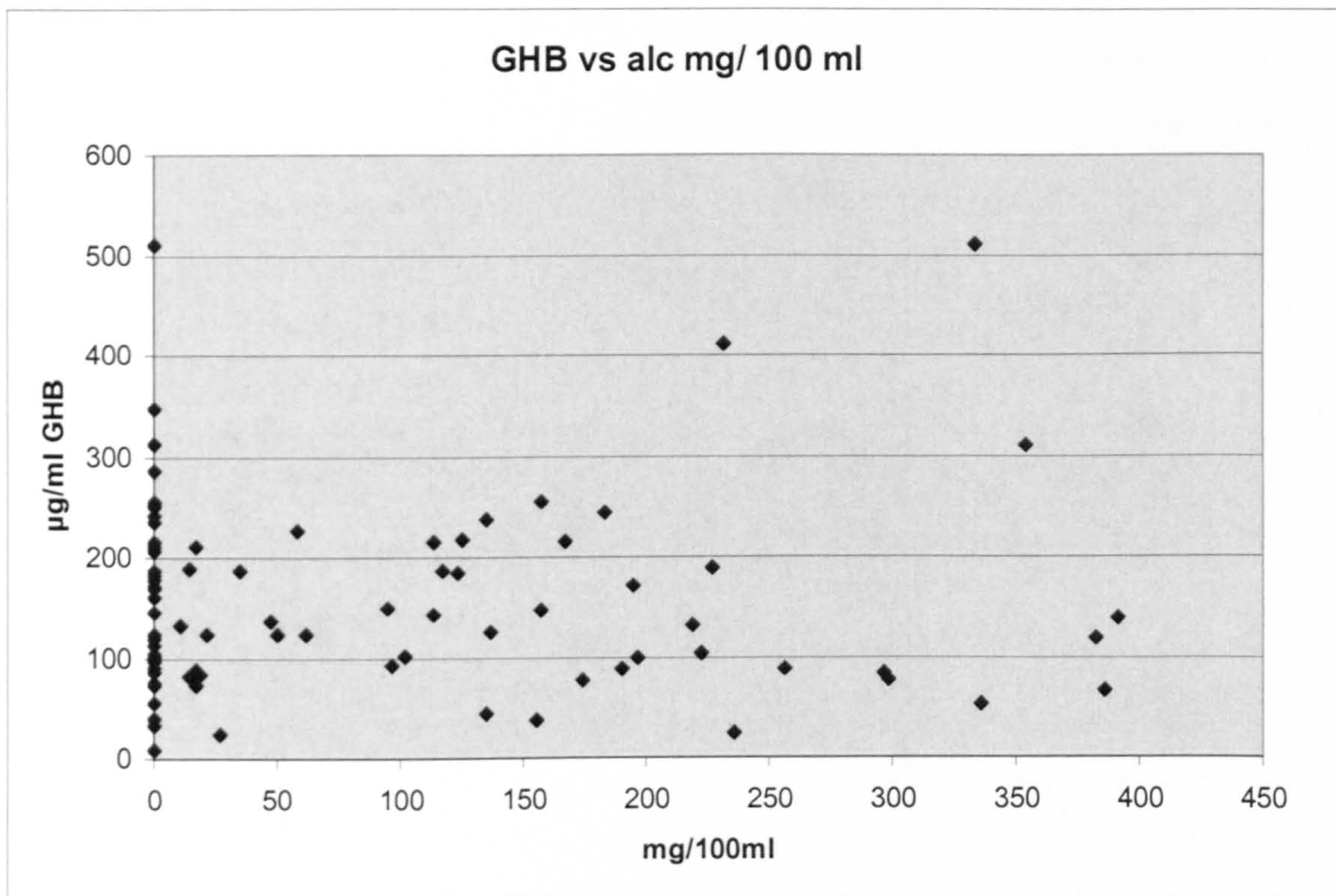


Figure 6-19:- Scatter plot of blood alcohol concentration *versus* blood GHB concentration.

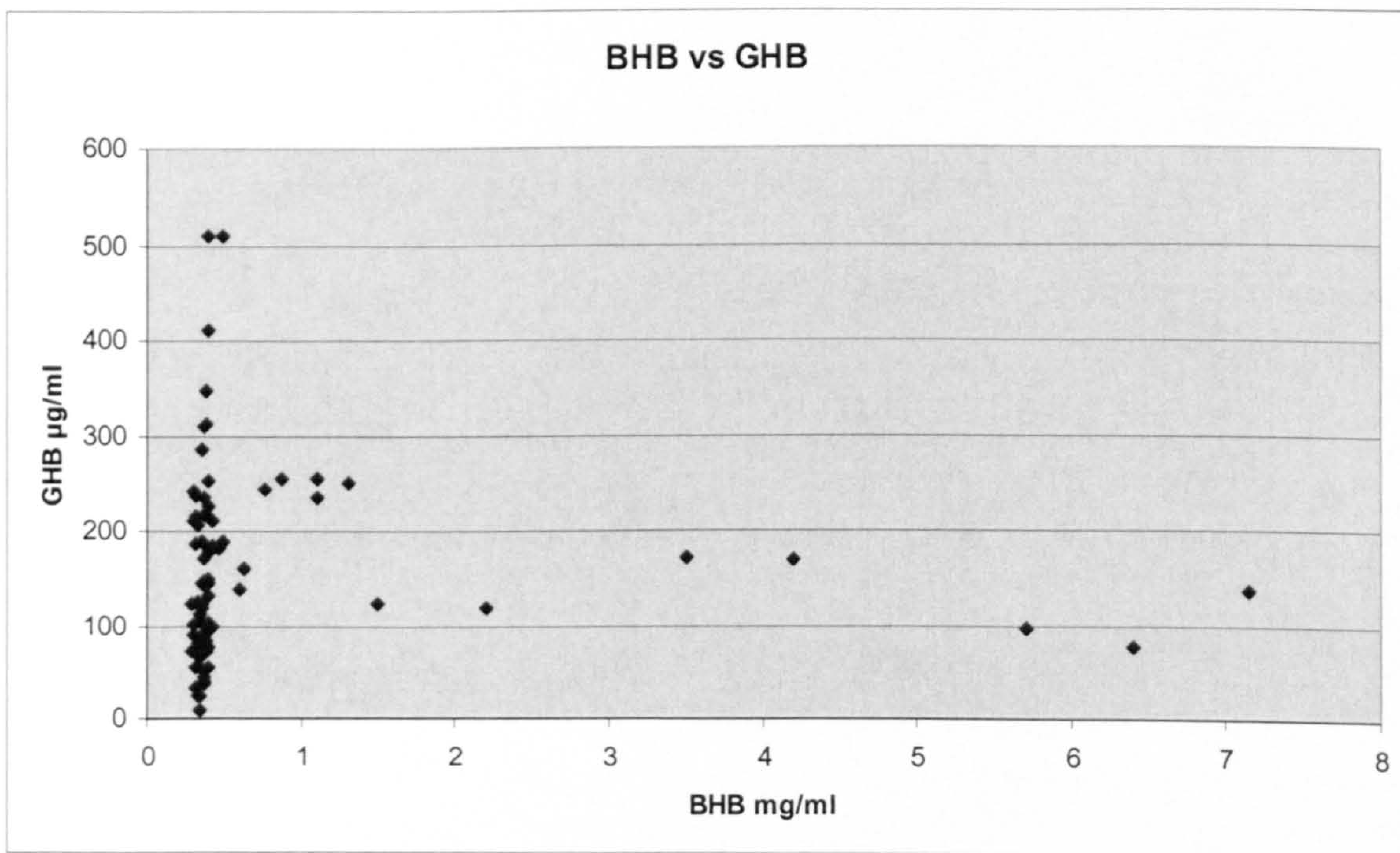


Figure 6-20:- Scatter plot of blood BHB concentration *versus* blood GHB concentration.

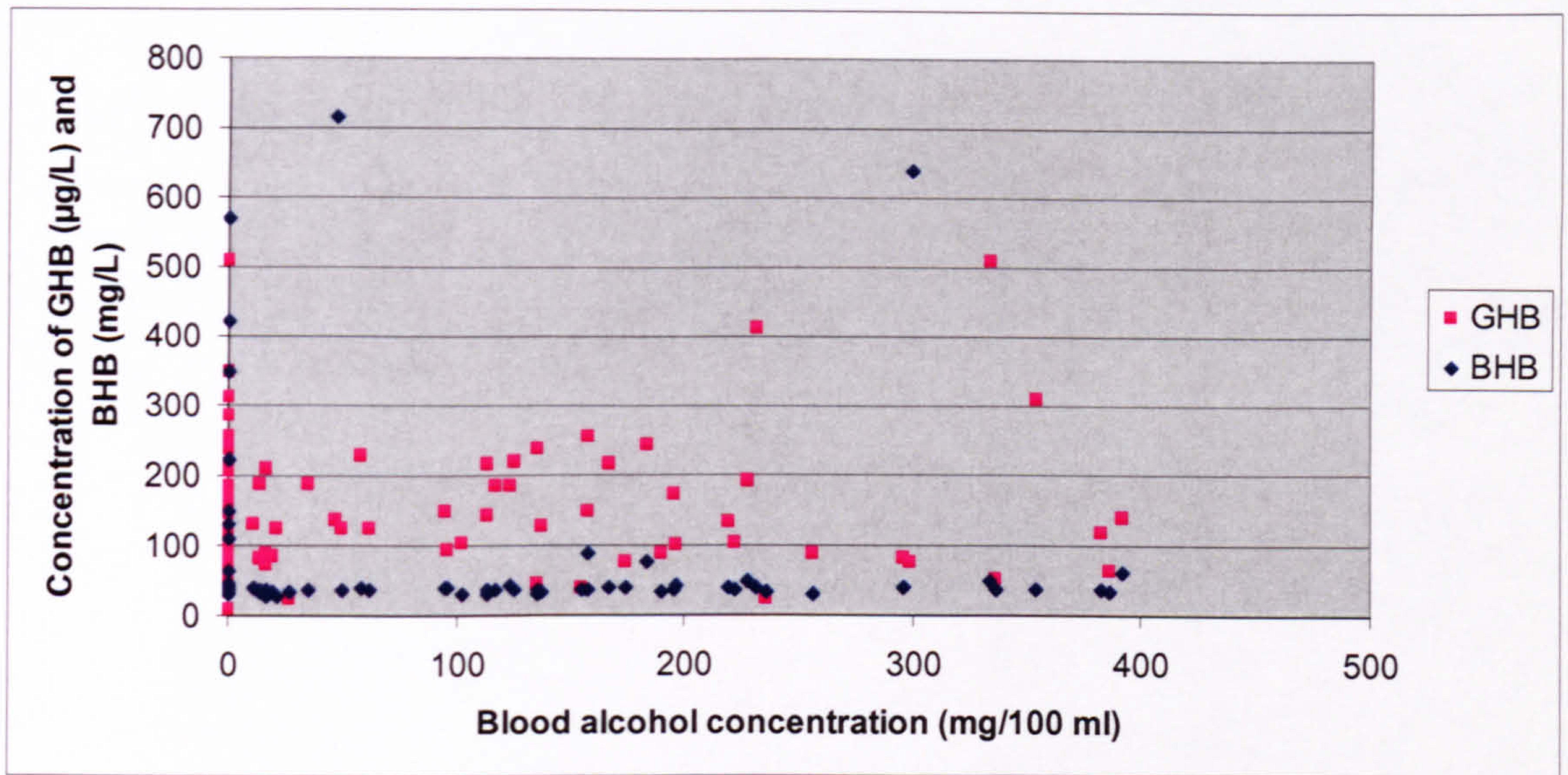


Figure 6-21:- GHB and BHB combined with alcohol

By contrast, t-test results indicated significant differences between some of the groups (Table 6-13 and 6-14). At least in the cases used for this study, the alcoholics group with high blood alcohol concentrations has significantly higher BHB concentrations than the other groups.

Table 6-13:- Statistical summary of the four groups:

Group	N	BHB		GHB	
		Average (µg/ml)	Std Dev	Average (µg/ml)	Std Dev
1	36	0.37	0.131	130	73.6
2	30	0.48	0.284	174	113
3	13	0.49	0.324	178	111
4	10	3.81	2.51	152	57

Table 6-14:- P-values from T-test comparison of the four groups:

BHB	
Group 1 vs Group 2	0.060
Group 1 vs Group 3	0.229
Group 1 vs Group 4	0.006*
Group 2 vs Group 3	0.912
Group 2 vs Group 4	0.007*
Group 3 vs Group 4	0.007*
GHB	
Group 1 vs Group 2	0.065
Group 1 vs Group 3	0.153
Group 1 vs Group 4	0.363
Group 2 vs Group 3	0.920
Group 2 vs Group 4	0.446
Group 3 vs Group 4	0.492

* Statistically significant ($p<0.05$)

6.5 Discussion

GHB and BHB were derivatised by silylation using BSTFA/TMCS. In the silylation process a trimethylsilyl (TMS) group replaces each active hydrogen of GHB and BHB creating one method for determination of GHB and BHB by GC/MS in a single run[61, 343]. However, the same internal standard and ion at m/z 239 was used for both GHB and BHB which might reduce accuracy and precision for BHB and also the same ion (m/z 233) was used for both BHB and GHB, which creates the potential for confusion or mistaken identity if the retention time window is not carefully monitored.

The fragmentation and derivatisation of GHB and BHB are very similar (Figure 6-17), because BHB has a similar structure to GHB. The retention times of GHB and BHB were 4.78, 4.13, respectively. The quantification ions were m/z 233 and 239 for the analytes GHB and BHB and Internal standard, respectively. The base peak at m/z 147 cannot be used because the ion appears as a fragment in the mass spectrum of both the drug and the internal standard. In addition, this ion is considered non-selective, because it contains only parts of the derivative molecule coming from the derivatising reagent.

It has been discussed previously (section 6.1 and 6.2) that GHB and BHB are naturally produced in the body in small quantities. The human body produces BHB in times of starvation, or if the individual is a diabetic in order to produce energy in place of glucose. Another theory proposes that there is a relationship between alcohol and BHB concentrations. Table 6-12 shows four classification groups for alcohol levels. These involve non-alcoholic subjects with negative and positive alcohol concentrations and alcoholic subjects with low or high alcohol concentrations. It was noted that when alcohol levels were high, the BHB levels were also high. When BHB levels are high, there is some indication that GHB levels are affected, in that the higher the BHB levels, the lower the GHB levels. However this did not reach statistical significance.[343, 344]

6.6 Conclusion

In the method developed, SPE was used to extract both GHB and BHB from blood samples, followed by BSTFA /TMCS derivatisation and then 1µl in GC/MS. This method was effective and was found to be a simple, fast and sensitive procedure for determination of GHB and BHB. The silylation derivatisation method with microwave was carried out and improved the chromatography. There is a relationship between alcohol level and BHB concentration in the body. Where the alcohol level becomes high the BHB will also be high. In non-alcoholics with either positive or negative alcohol levels the concentration of BHB was less than 1.5mg/ml. However, with high alcohol levels, the BHB concentration was more than 0.49 mg/ml. There is some evidence for a relationship between GHB and BHB where each concentration affects the other, but in a normal situation the body keeps the balance by controlling the production mechanism.

7 Benzodiazepines

7.1 Introduction

Benzodiazepines, sometimes called "benzos", are based on the fusion of a benzene ring and a 7-membered diazepine ring. They are believed to act on the gamma-aminobutyric acid (GABA) receptor $GABA_A$. Receptors for benzodiazepines are located throughout the central nervous system, including the cerebral cortex, cerebellum, hippocampus, striatum and spinal cord, so benzodiazepines offer a clinical therapy of primary importance in treating neurological disorders such as anxiety, sleep disturbance, muscle spasms and epilepsy[163-165, 176, 177, 345-348] The benzodiazepines are a particularly large family of drugs[349].

They can be taken orally or, when abused, oral formulations can be ground up for injection. They are used as anticonvulsants, anxiolytics, hypnotics or muscle relaxants with different durations of action[145]. Since Sternbach synthesised the first benzodiazepine in 1955 by an unexpected ring extension of a quinazoline-3-N-oxide derivative, a number of structurally similar compounds have been marketed by drug companies. Chlordiazepoxide was the first medical benzodiazepine, introduced in 1961 and followed in 1963 by diazepam and in 1965 by oxazepam. More than fifty of these drugs are presently marketed for clinical use throughout the world. Thirty-five are subject to international control under the 1970 United Nations Convention on Psychotropic Substances. The most significant benzodiazepines in the last decade have been diazepam, lorazepam, alprazolam, temazepam, chlordiazepoxide and lormetazepam . Benzodiazepines are strongly associated with opioid users and with drug-related deaths, and are one of the main drug classes found in poisoning–related hospital admissions. These drugs are frequently encountered in clinical and forensic toxicology and they have featured in an increasing number of misuse situations over the past years [146, 182]

Large doses are rarely fatal unless other drugs are taken concomitantly, because of their relative safety. Benzodiazepines and benzodiazepine–like substances such as zopiclone are frequently abused. Analysis of benzodiazepines, their active metabolites and benzodiazepine–like substances in blood samples may be indicated in many forensic cases such as driving under the influence of drugs, cases of date rape or violent crime and cases in which the cause of death is unknown. These include the 1, 4-benzodiazepines such as

diazepam, temazepam and oxazepam and the often more potent, diazolo- and triazolo – groups represented by alprazolam, midazolam, triazolam. In high doses benzodiazepines can cause persons to exhibit classical features of central nervous system (CNS) depressant drugs such as nystagmus, ataxia, slurred speech and impaired divided attention skills[178, 179, 350].

Benzodiazepine abuse is a large problem in poly drug users. They are often used to reduce anxiety, help sleep or counter the negative effects of other drugs. They are addictive and can cause significant withdrawal symptoms. Short term prescribing of benzodiazepines may have some benefit in supporting drug users to control their intake of other drugs when first coming into treatment and to stabilise their lives[161, 179]. Consequently, toxicologists need to be able to detect these drugs in biological specimens and to understand their toxicology. The most commonly encountered benzodiazepines are diazepam and its three metabolites N-desmethyldiazepam, temazepam and oxazepam. The derivatisation procedure for each of these drugs with three different reagents was investigated.

7.2 Chemistry

Benzodiazepines (Figure 7-1) are based on the fusion of a benzene ring with the diazepine ring. The subsequent molecule as a benzodiazepine is characterized by two nitrogen atoms on the diazepine ring. These nitrogen atoms are usually in the 1 and 4 positions of the ring, according to the numbering convention of atoms around the molecule. Differences in the benzodiazepines are based on the presence of additional substituents at the 1, 2, 3, 5 and 7 positions of the molecule (refer to Table7-1). Variations in these substituents lead to a change in their pharmacological potencies and efficacies, variations in the physiochemical properties and of course variations in the ring.[105] A number of modifications in the benzodiazepine ring structure have resulted in a number of new compounds with similar pharmacological activities, but differences in lipophilicity, basicity and chemical reactivity. These differences are also responsible for the different response of benzodiazepines to analytical methods (Figure 7-2)[351].

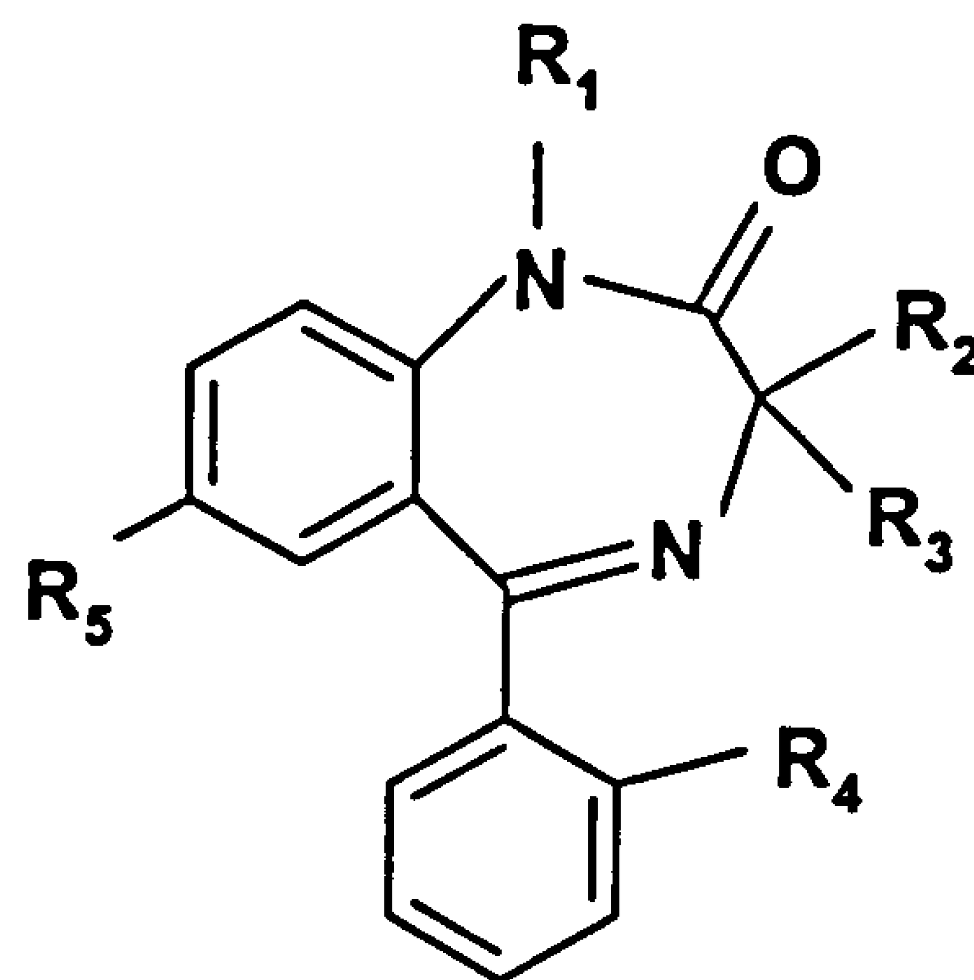


Figure 7-1:- General benzodiazepine structure

Table 7-1:- Chemical structures of selected 1, 4-benzodiazepines

Benzodiazepine	Trade name	R_1	R_2	R_3	R_4	R_5
Diazepam	Valium	$-CH_3$	-H	-H	-H	-CI
Nordiazepam	Calmday	-H	-H	-H	-H	-CI
Temazepam	Restoril, Levanxol	$-CH_3$	-H	-OH	-H	-CI
Oxazepam	Adumbran, Praxiten	-H	-H	-OH	-H	-CL
Clonazepam	Ritrovil	-H	-H	-H	-CI	$-NO_2$
Flunitrazepam	Rohypnol	$-CH_3$	-H	-H	-F	$-NO_2$
Nitrazepam	Mogadon	-H	-H	-H	-H	$-NO_2$

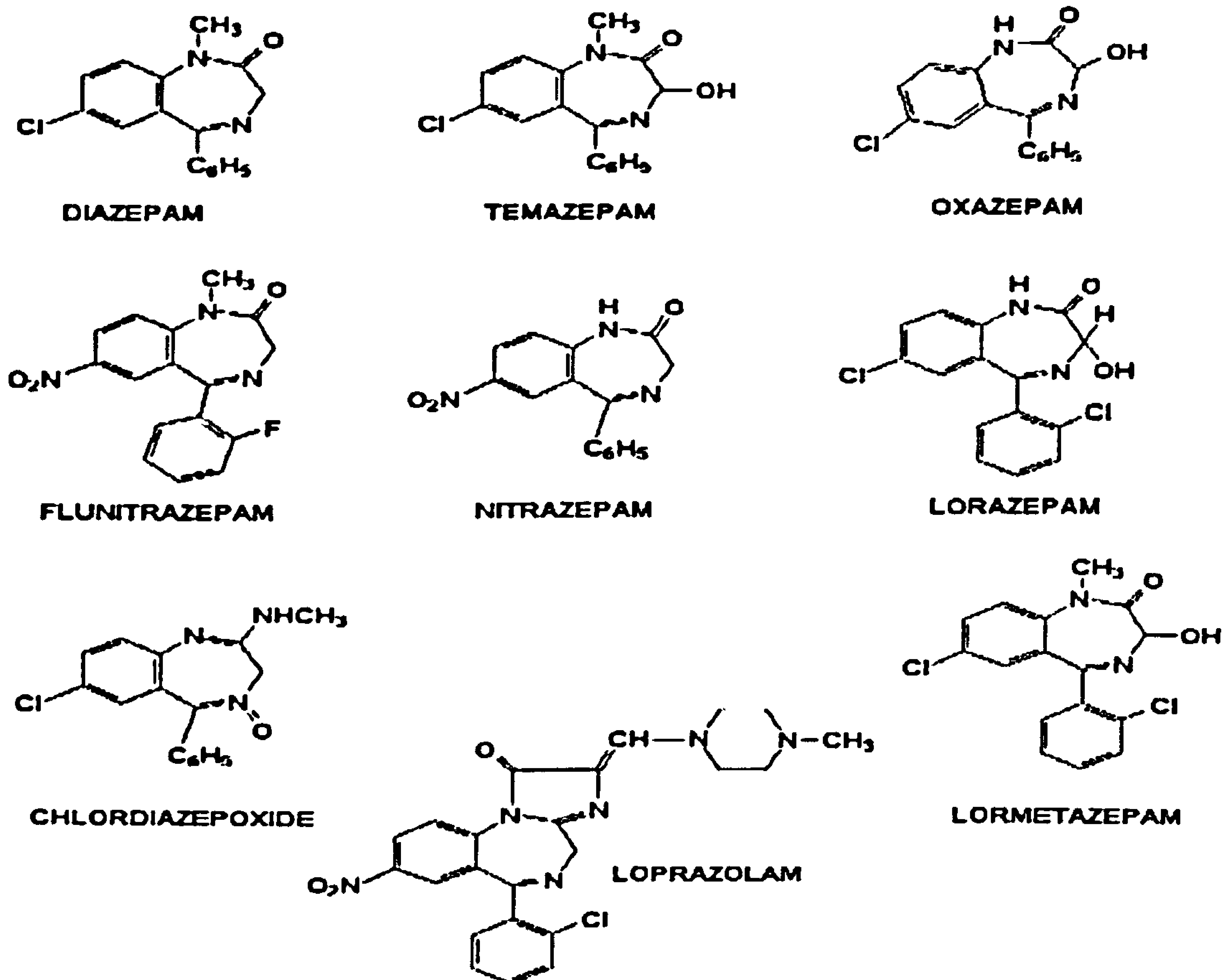


Figure 7-2:- Structures of some benzodiazepines

7.3 Pharmacokinetics of benzodiazepines

Benzodiazepines have important pharmacokinetic differences with respect to speed of onset and duration of action and all benzodiazepines are metabolized in the liver through oxidation, glucuronidation, or nitrogen reduction and are eliminated by the kidneys. The rate of onset of action depends on the mode of administration, the dissolution of the formulation, the rapidity of absorption, and the rate of entry into the brain. Diazepam is rapidly absorbed after oral use and enters the brain quickly thus promptly relieving anxiety. Given intravenously, it can rapidly stop repeated epileptic seizures. N-Desmethyldiazepam is absorbed more slowly than diazepam. Oxazepam is quite slowly absorbed and takes some time to penetrate the brain. Lorazepam is also slow to enter the brain but it can be used intravenously to sedate during investigative or minor surgical procedure.

The hepatic metabolism of drugs can be phase I, mainly oxidation and de-alkylation, and phase II, conjugation to form glucuronides, and sulfates compounds. Benzodiazepines can undergo both phase I and phase II metabolism (diazepam, chordiazepoxide and

flurazepam) or phase II alone (lorazepam, oxazepam, and temazepam). Drugs metabolized via phase II processes alone are better tolerated than phase I - phase II drugs by patients such as alcoholics with liver impairment. In addition, phase I metabolism slows with age so that the elderly are more likely to suffer adverse effects when taking phase I - phase II drugs than phase II only drugs. The elimination half lives of benzodiazepines vary greatly, and usually lengthen in the aged patient for two essential reasons: firstly, the volume of distribution is increased and secondly, the clearance rate is decreased. N-desmethyldiazepam (nordiazepam) is the major active metabolite of diazepam (Figure 7-3) It has a prolonged half-life, over 100 hours, consequently it accumulates over the first month of treatment, reaching higher plasma concentrations than the parent compound, diazepam. N-desmethyldiazepam is also the major metabolite of clorazepate, prazepam, ketazolam, halazepam, medazepam and to some extent chlordiazepoxide. In contrast to these long-lasting drugs, lorazepam, oxazepam, temazepam and lormetazepam have half-lives of about 6-24 hours. As the body eliminates much of each dose between each administration, these compounds are suitable for acute, short-lived anxieties, fluctuating levels of anxiety, and for insomnia (Table 7-2)

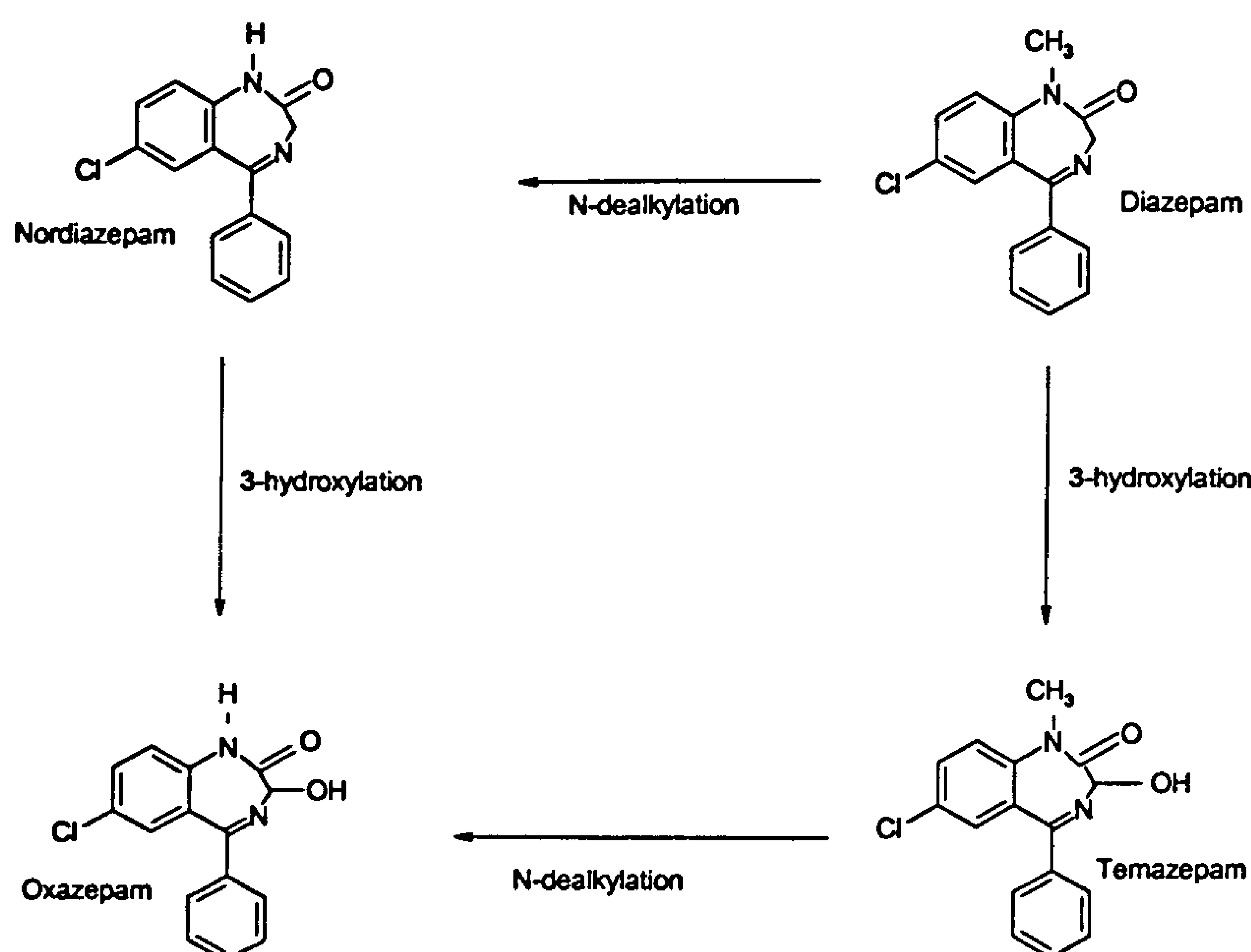


Figure 7-3:- Metabolic pathway of diazepam

Table 7-2:- Pharmacokinetic data of some of benzodiazepines of interest

Benzodiazepine (MW)	Half-life(h)	Key active metabolite	pKa	Vd (L/kg)	Protein binding (%)
Diazepam (284)	20-50	Nordiazepam	3.4	0.7-2.6	98
Nordiazepam (270)	50-99	Oxazepam	3.5, 12.0	-	97
Oxazepam (286)	4-15	None	1.7, 11.6	0.7-1.6	97
Temazepam (300)	5-15	Oxazepam	1.3	0.8-1.0	96

7.4 Methods of analysis of benzodiazepines: previous work

A large number of papers have been published on methods of analysis of benzodiazepines. HPLC methods and more recently LC-MS based methods have generally been preferred because of chromatography problems in GC based methods. Benzodiazepines have nevertheless been analysed by GC/MS.[236, 352, 353] Several papers describe methods for diazepam and its metabolites. Some of these papers discuss analysis of whole blood samples and others the analysis of hair using liquid – liquid or solid phase extraction.[354] One paper describes derivatisation using a silylating reagent such MTBSTFA, MSTFA or BSTFA containing 1% TMCS. The target range concentrations were 25-6000 ng/ml of diazepam and oxazepam when analysed as TBDMS derivatives.[236]

7.5 Aims

As explained in previous chapters, chemical derivatisation is very useful for modifying compounds to produce a new compound which is suitable for analysis using GC/MS. Benzodiazepines are a very important class of drug to analyse, therefore when analysing by GC-MS the best derivatising reagent needs to be assessed and an evaluation made of

microwave assisted derivatisation. However, as mentioned above, GC-MS has not been successfully applied to benzodiazepine analysis because many of them are thermally unstable and do not chromatograph well by gas chromatographic-based techniques. For this reason, derivatisation procedures mentioned in Chapter 5 were applied to the most frequently-encountered benzodiazepines in the UK, diazepam and its three metabolites and this pilot study was aimed at an initial investigation of derivatisation methods which might extend the range of benzodiazepines which could be analysed by GC-MS. As before, these compounds were used as model substances as an indicator of the applicability of the derivatisation methods to benzodiazepine screening by GC-MS within a general STA procedure. Diazepam itself has no available derivatisation sites but was included to assess whether the derivatisation conditions would result in molecular changes to the parent substances.

7.6 Derivatisation procedures

7.6.1 Reagents

Refer to Chapter 4 and 5

7.6.2 Drug standards and internal standards

The drug standards and internal standards used were diazepam, diazepam-d_5, N-desmethyldiazepam, nordiazepam-d_5, temazepam, temazepam-d_5 oxazepam, and oxazepam-d_5. Stock standard solutions were obtained at a concentration of 100μg/ml in methanol, supplied by Cerilliant.

7.6.3 Preparation of drug mixtures and internal standard solutions

A mixed drug solution containing a concentration of 1 μg/mL of each drug was prepared by taking 100μl of 100μg/mL solution and making up to 10mL with methanol. The internal standard solution was prepared similarly using the deuterated standards.

7.6.4 Method and Results

Preparation of buffers and spiked standards, and extraction, instrumentation and derivatisation procedures were the same as in Chapter 4 and 5. The chromatograms and mass spectra of the test compounds are given in Figures 7- 4 to 7-9 and the fragmentation reactions of some derivatives are given in Figures 7-10 to 7-14.

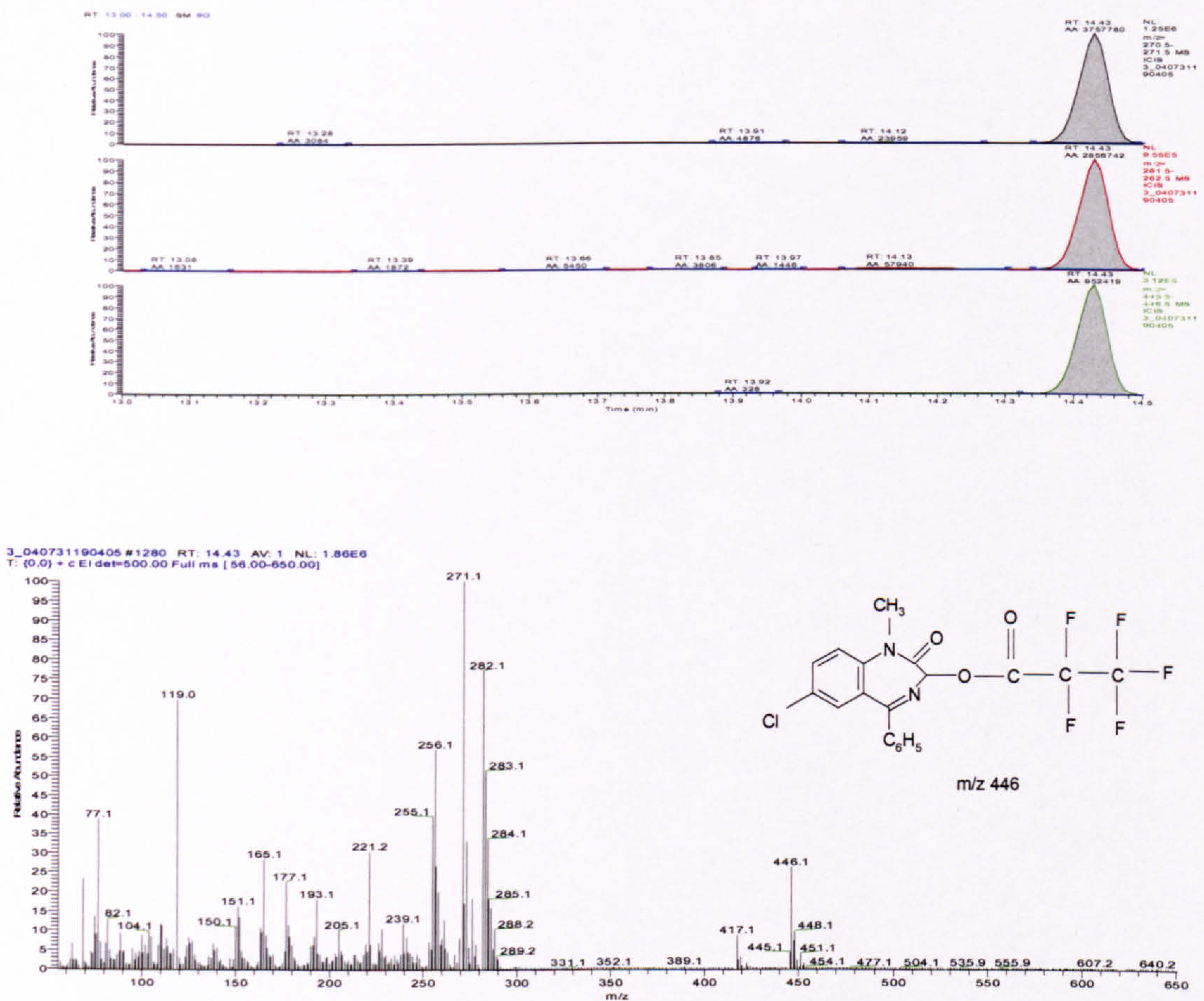
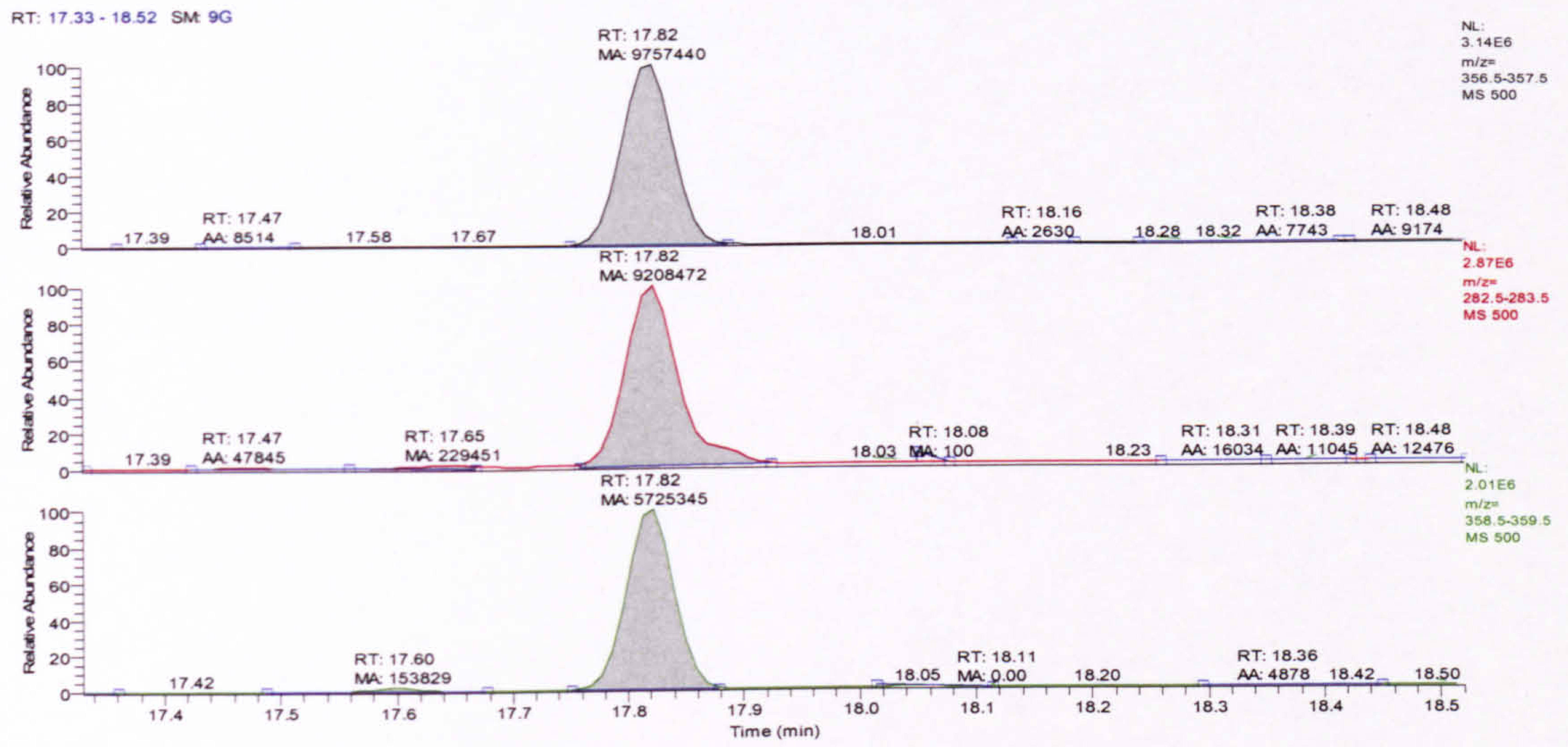


Figure 7-4:- Ion Chromatogram and Mass Spectrum for Temazepam with PFPA-PFP-OH



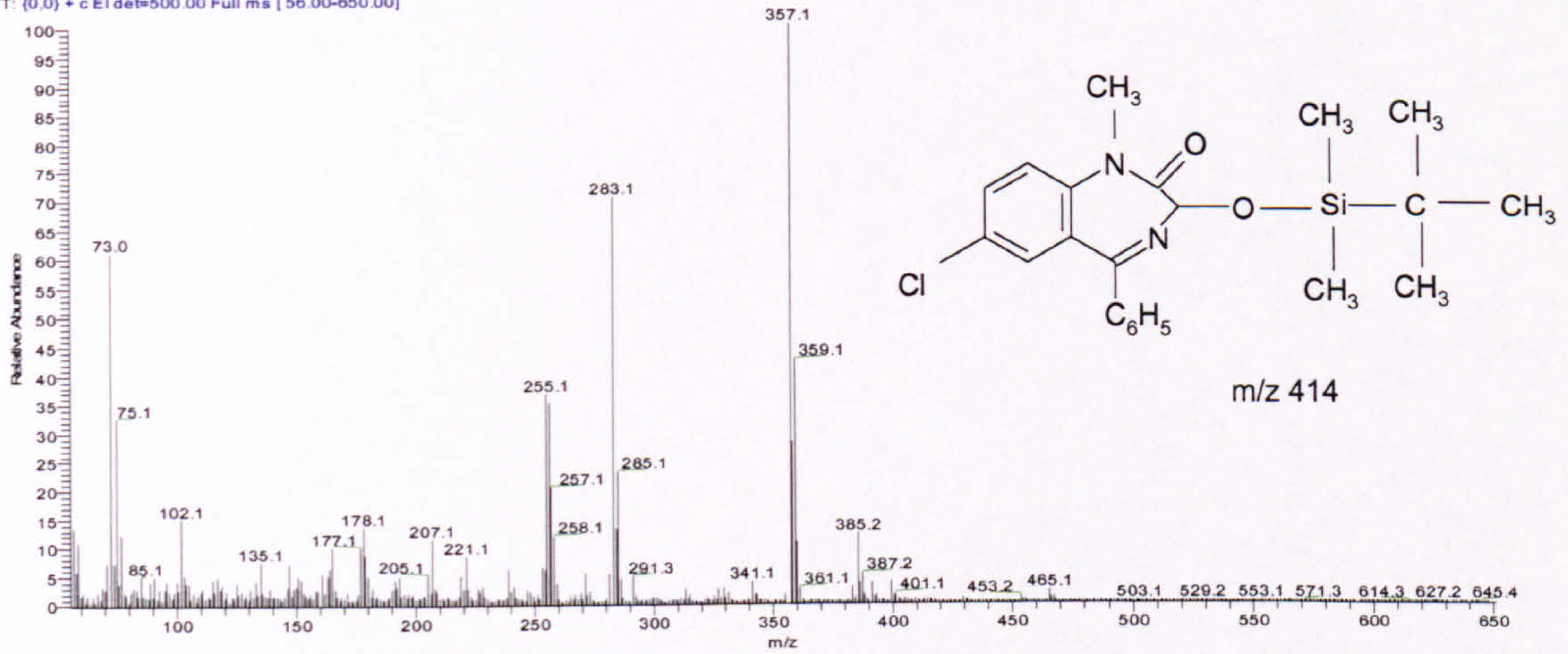
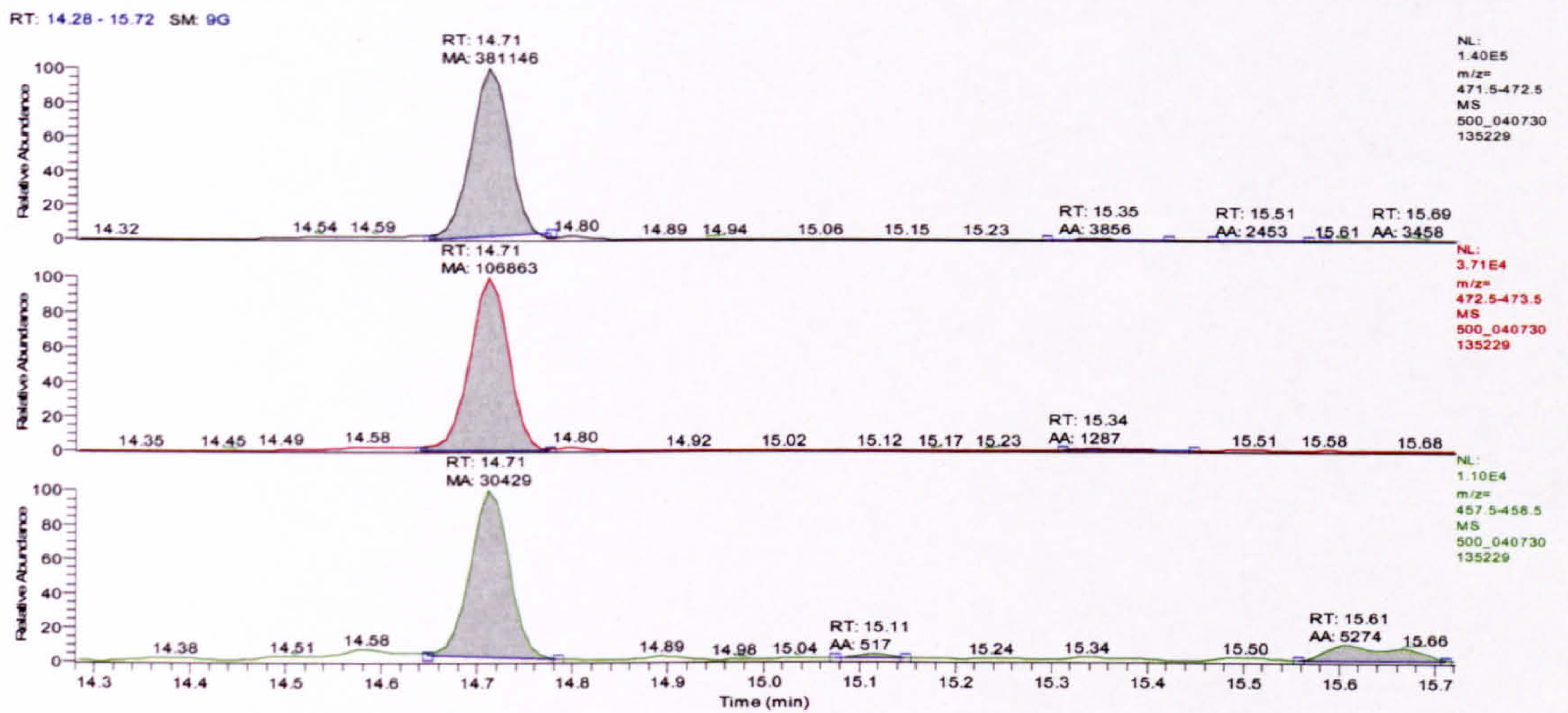


Figure 7-5:- Chromatogram and Mass Spectrum for Temazepam with TBDMS



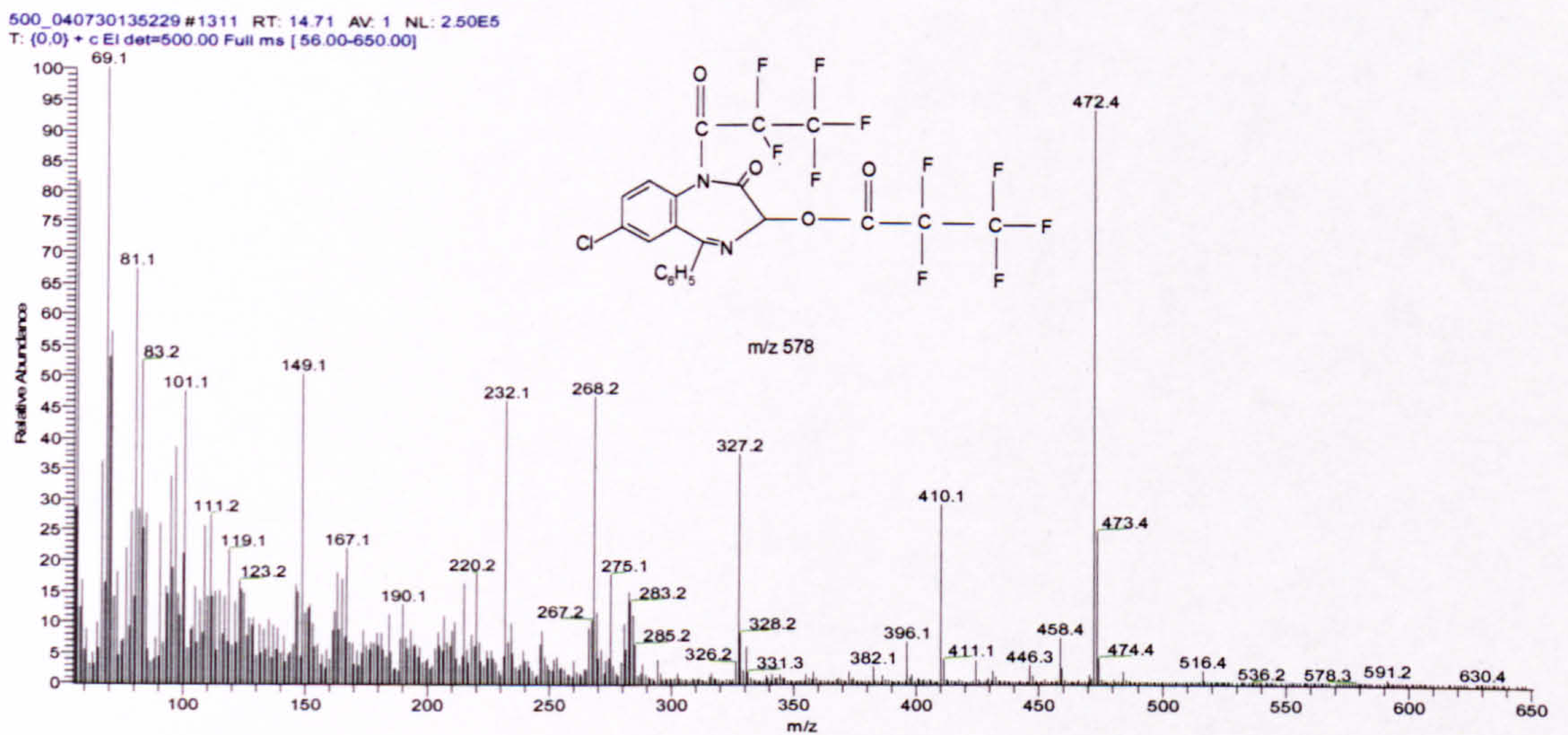
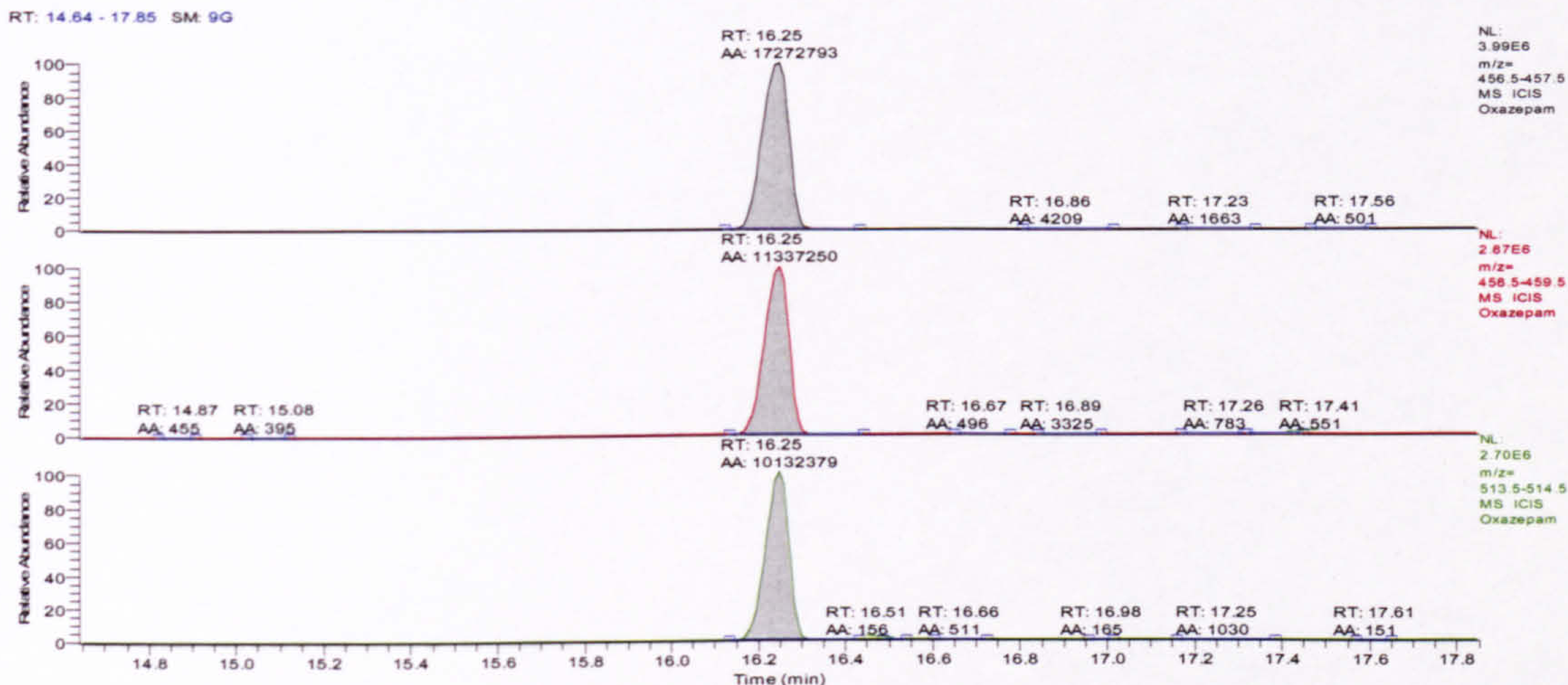


Figure 7-6:- Chromatogram and Mass Spectrum for Oxazepam with PFPA- PFP-OH



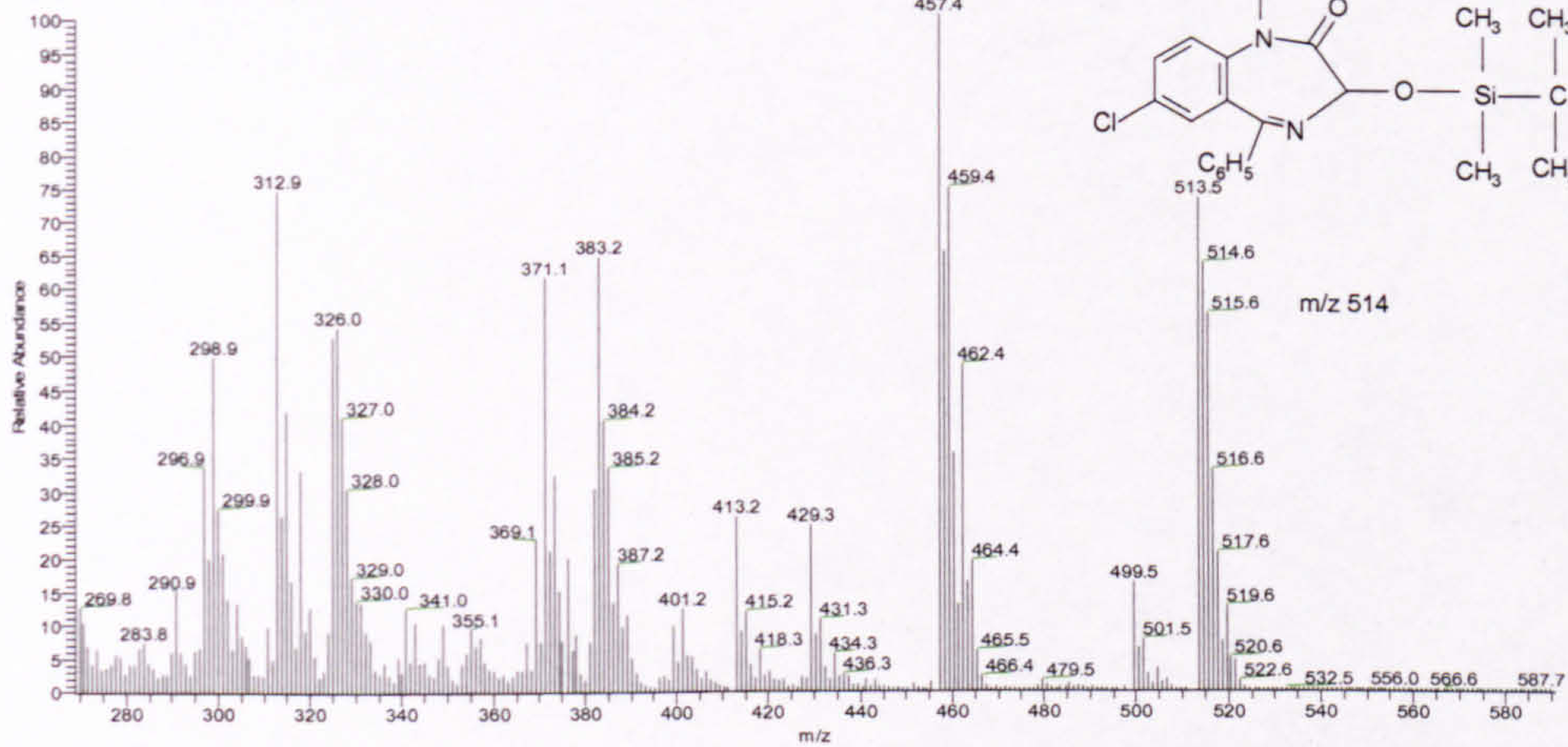
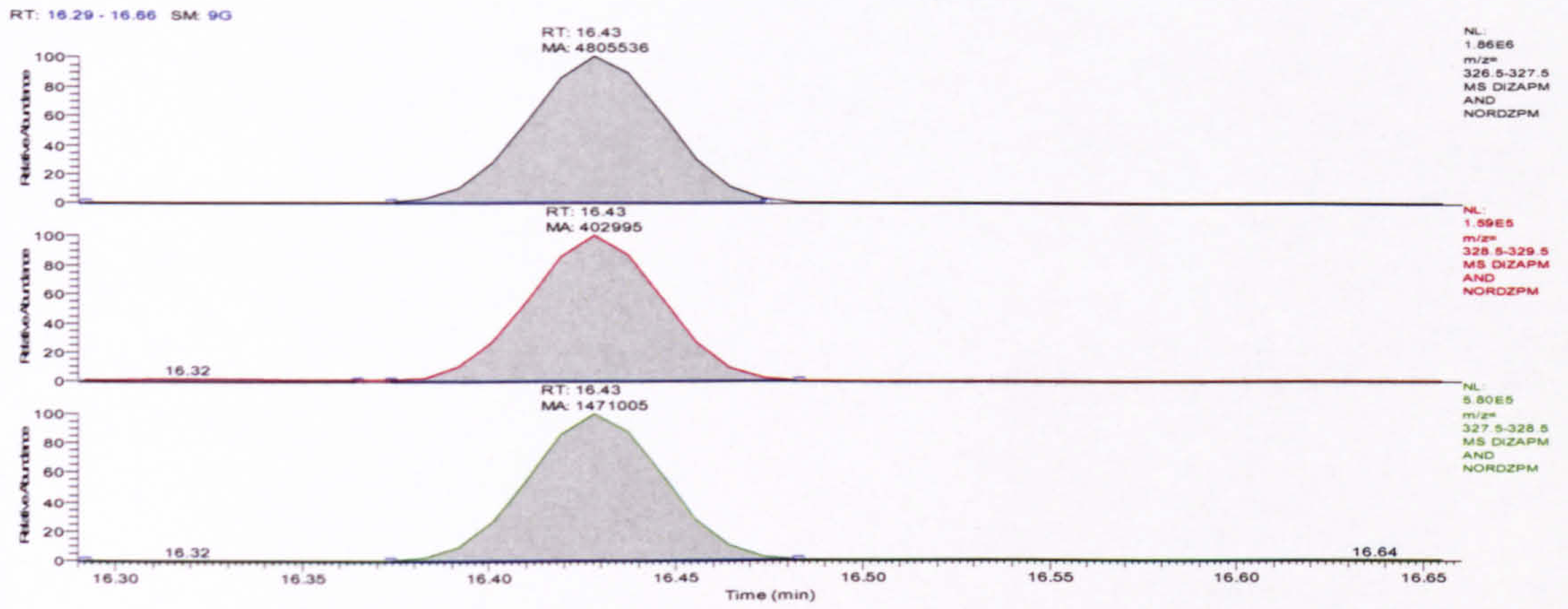


Figure 7-7:- Chromatogram and Mass Spectrum for Oxazepam with TBDMS (also contains oxazepam-d_5)



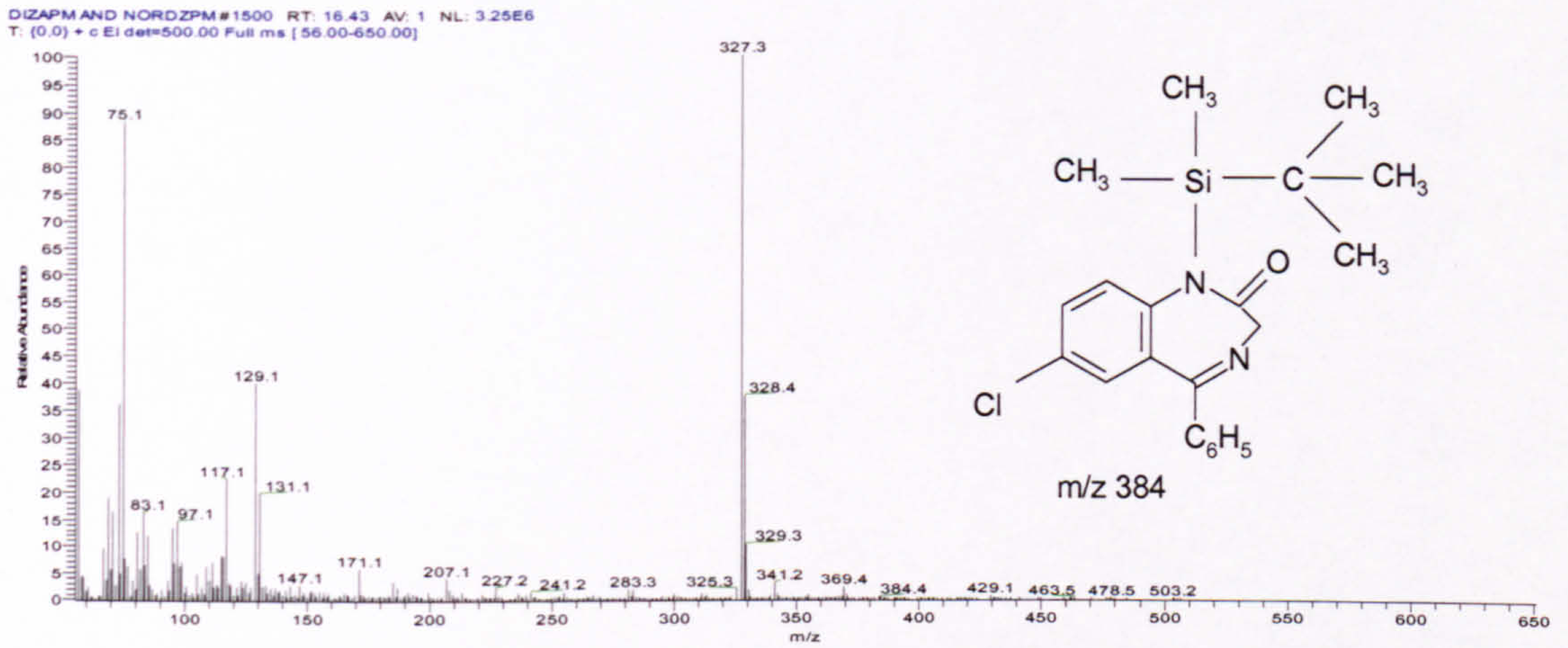
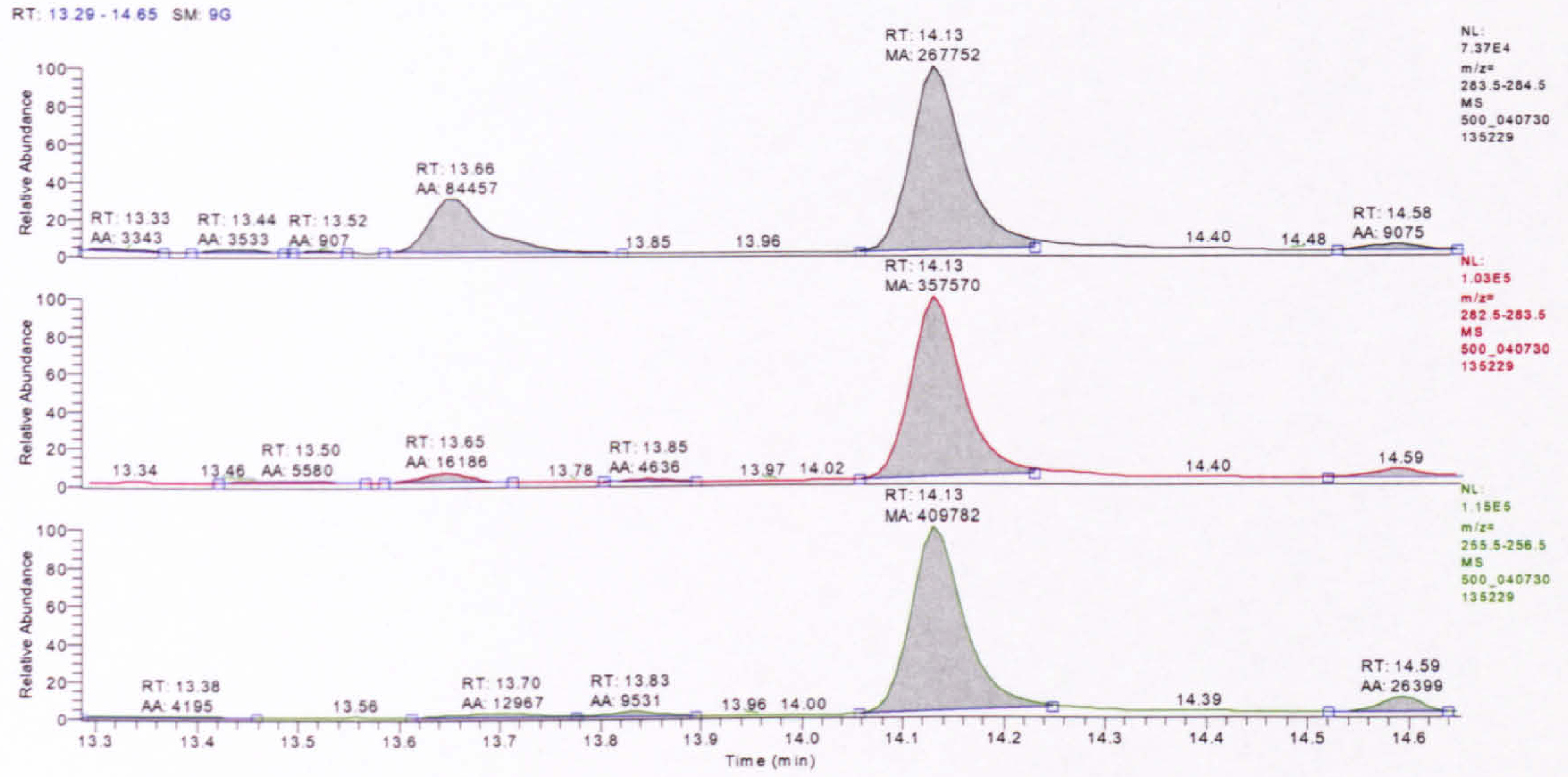


Figure 7-8:- Chromatogram and Mass Spectrum for Desmethyldiazepam with TBDMS



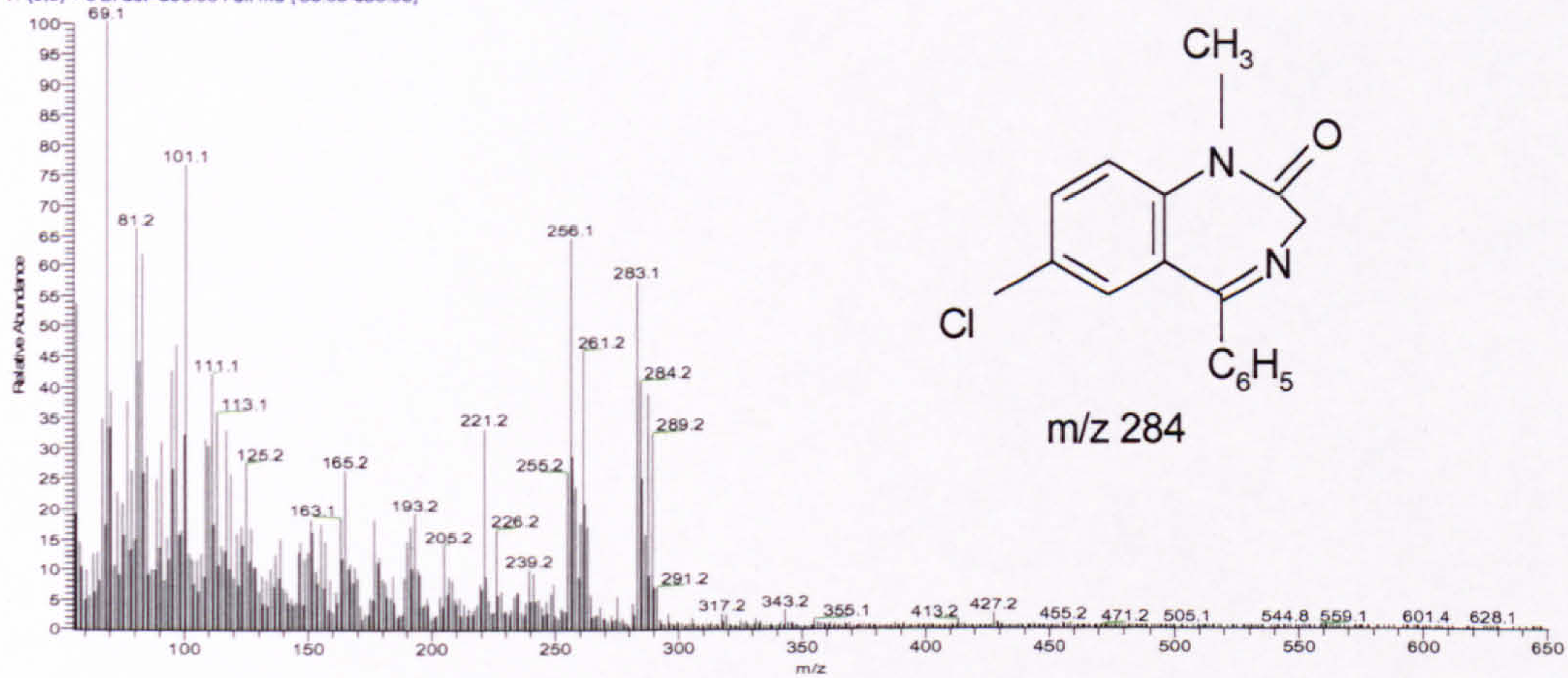


Figure 7-9:- Chromatogram and Mass Spectrum for Diazepam with PFPA - PFP-OH (also contains diazepam-d_5)

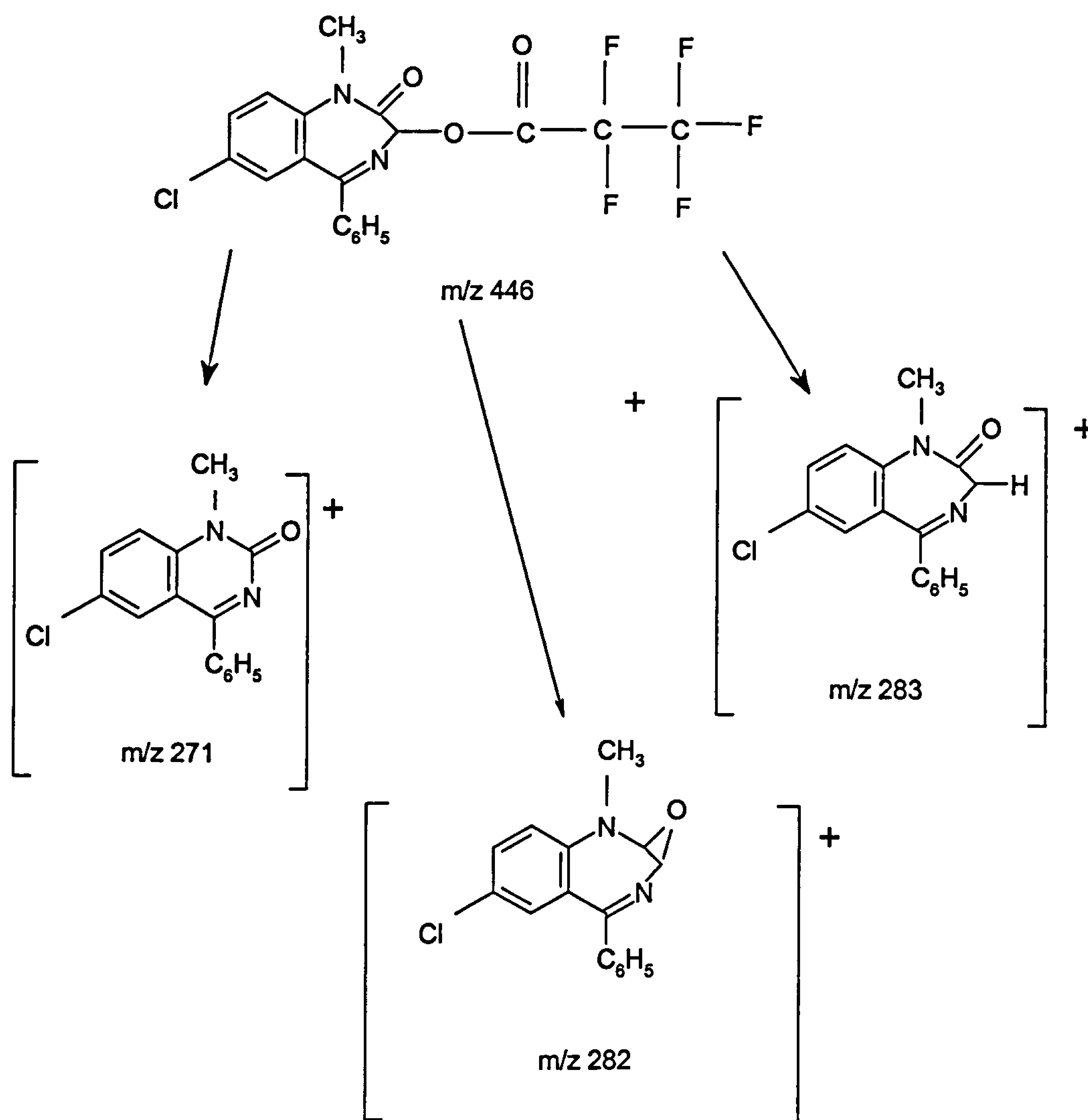


Figure 7-10:-Fragmentation of Temazepam with PFPA-PFP-OH

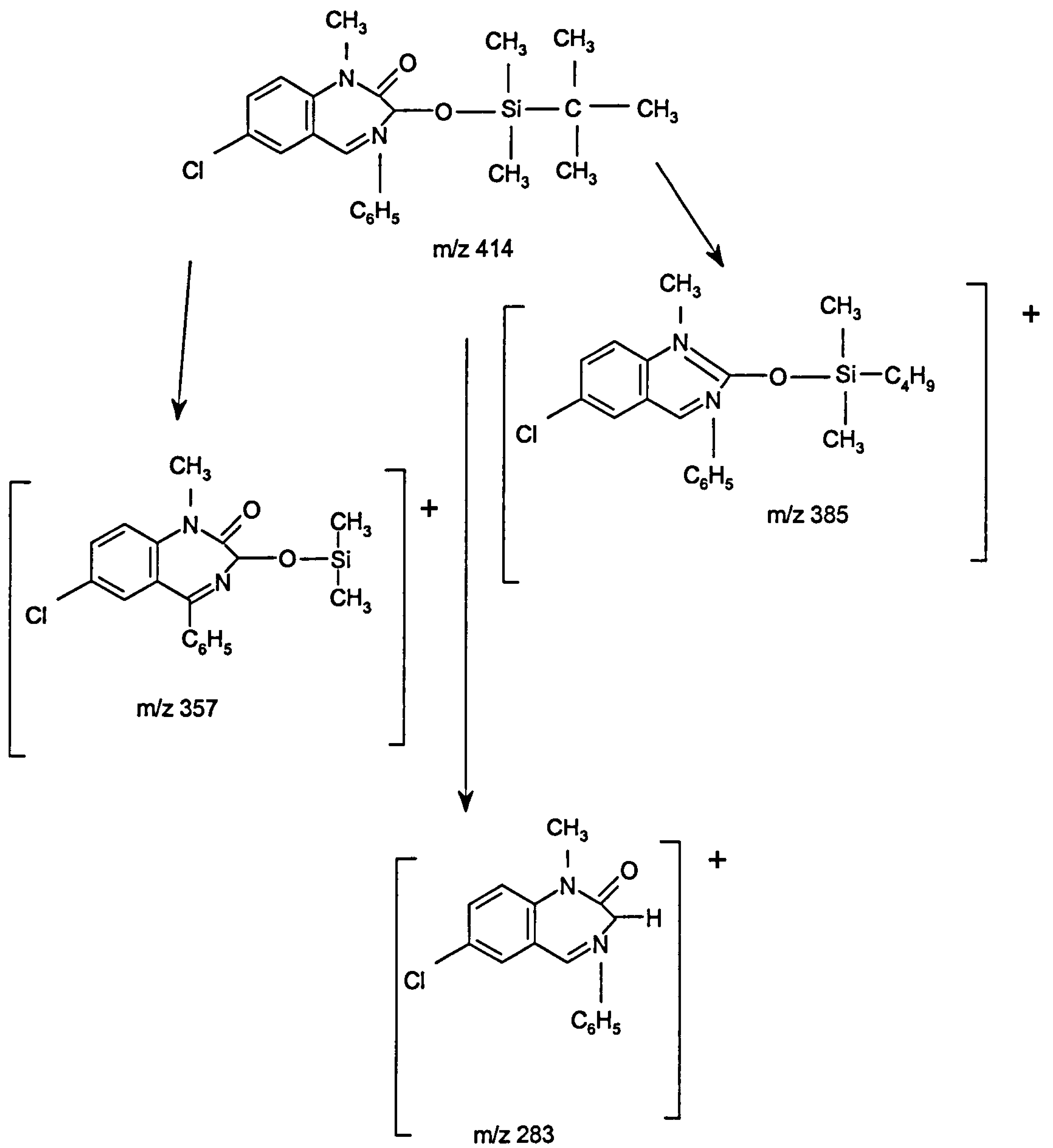


Figure 7-11:-Fragmentation of Temazepam -TBDMS

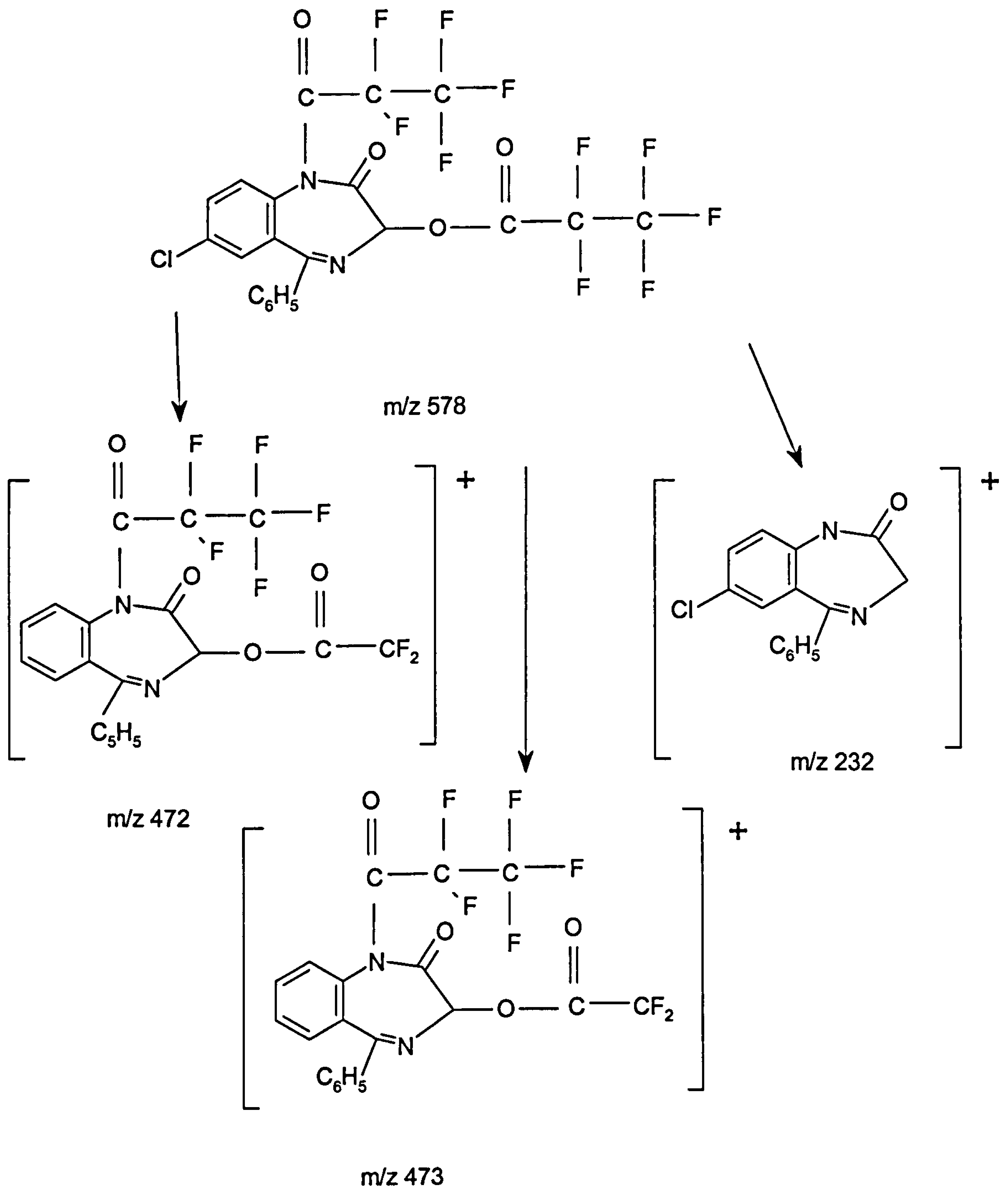


Figure 7-12:-Fragmentation of Oxazepam with PFPA-PFP-OH

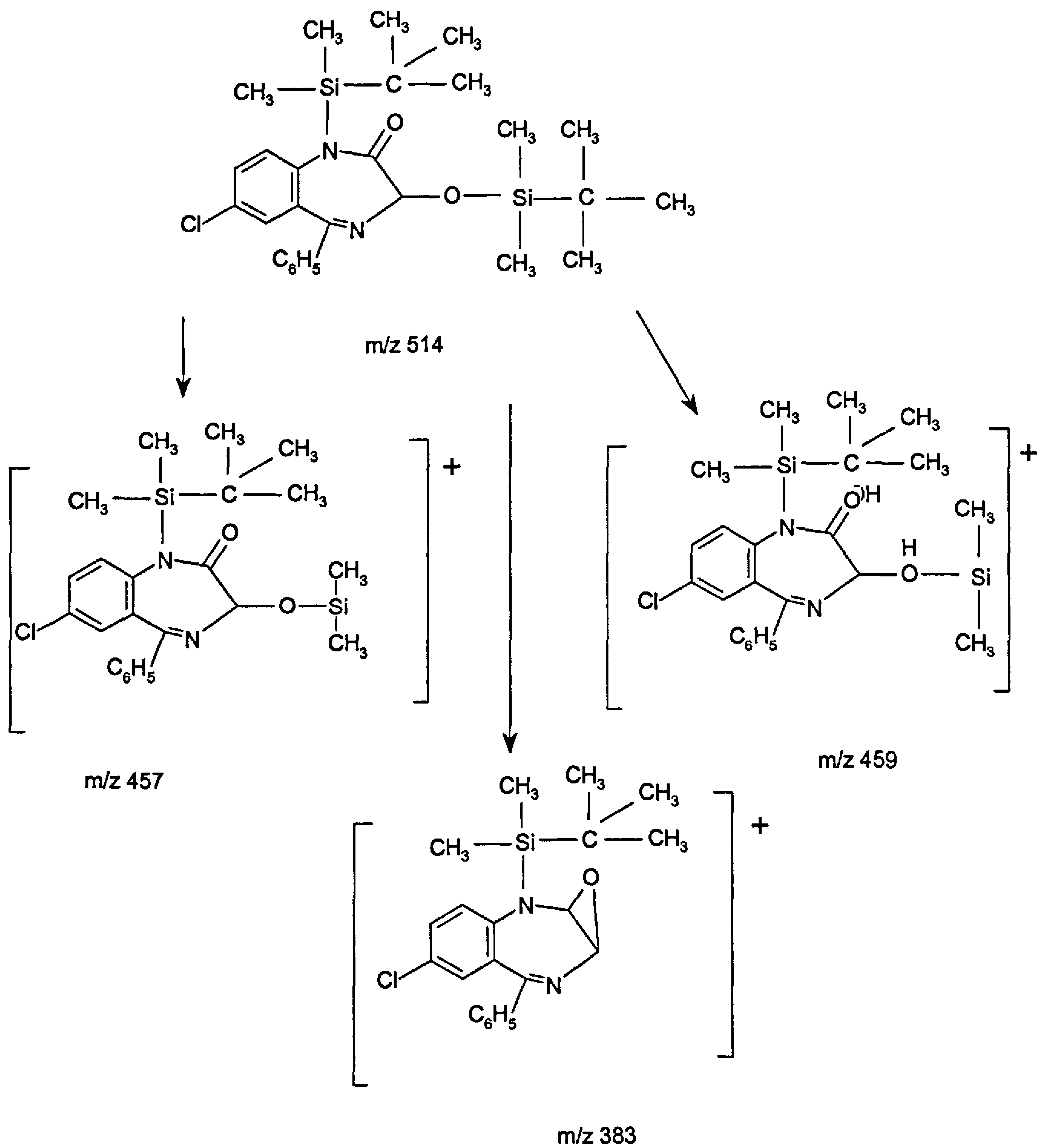


Figure 7-13:-Fragmentation of Oxazepam -TBDMS

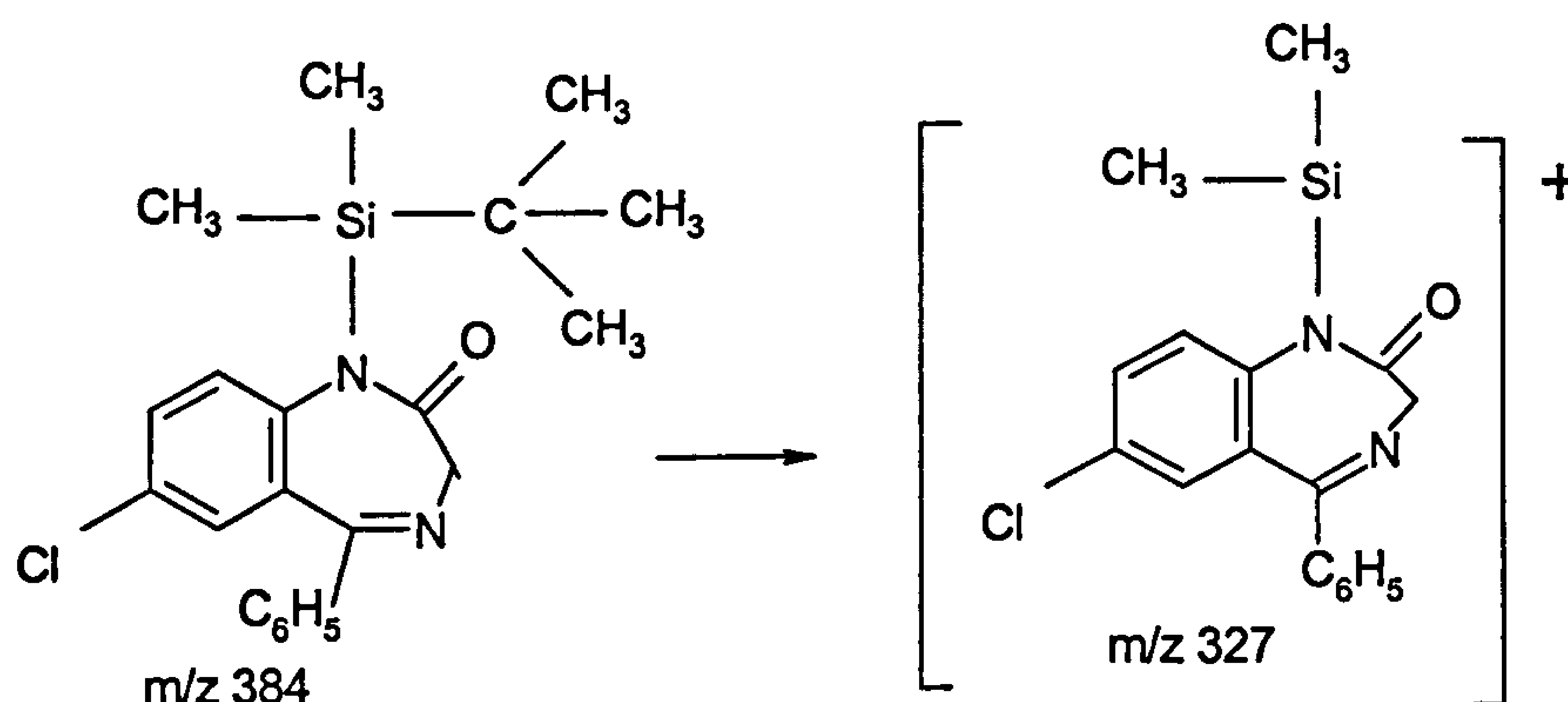


Figure 7-14:-Fragmentation of Nordiazepam –TBDMS

7.6.5 Discussion

This study was aimed at giving some information about GC-MS behaviour of diazepam and its metabolites with three derivatising reagents. These reactions were conducted under the same GC-MS conditions and derivatisation methods and extraction already mentioned in Chapters 4 and 5. Table 7-3 shows the retention times and ions selected to identify the derivatised benzodiazepines.

The solid phase extraction (SPE) required fewer steps and worked with acidic and basic drugs, which are especially suitable for forensic laboratories. The SPE cartridge called Strata™X employs a novel surface chemistry in the sorbent, offering significant advantages over the traditional silica-based SPE products.

Generally, temazepam and oxazepam reacted with PFPA and MTBSTFA. The molecular ions for temazepam PFP and TBDMCS were m/z 446 and m/z 414 respectively. The ions and fragmentation patterns with PFP-OH and TMS-diazomethane were the same because the same reaction had occurred with both reagents. These ions gave fragmentation patterns as shown in Figure 7-10 and

Figure 7-11. Temazepam derivatives had base peaks at m/z 271 and m/z 357 which were assigned to the fragments $[C_{15}H_{12}N_2ClO]^+$ and $[C_{20}H_{22}N_2ClO2]^+$, using PFP and TBDMCS, respectively. Oxazepam has two different functional groups (-OH) and (-NH)

so during derivatisation the protons in these groups were replaced by (C_3F_5O) and ($SiC(CH_3)_5$) respectively. The molecular ions for oxazepam PFP and TBDMCS were at m/z 578 and m/z 514 and the fragmentation patterns are shown in Figure 7-12 and Figure 7-13. The base peaks at m/z 472 and m/z 457 were as signed to the fragment $[C_{20}H_7F_7N_2O_4]^+$ and $[C_{25}H_{34}SiClO_2N_2]^+$, respectively. The molecular ion for nordiazepam (desmethyldiazepam) with MTBSTFA was at m/z 384 and the fragmentation pattern is shown in (Figure 7-14). It has a base peak at m/z 327, assigned to the fragment $[C_{16}H_{12}Si\ CLO_2N_2]^+$. The PFP and MTBSTFA (TBDMCS) derivatives of temazepam by loss of ($C_3F_5O_2$) and (SiC $(CH_3)_5O$) consecutively share the ion at m/z 283.

Table 7-3:- Retention times and selected ion data for diazepam and its metabolites with different derivatising reagents

Compound	Active group	Reagent	Retention Time (min)	Ions monitored
Diazepam	-	PFPA-PFP-OH	14.13	256, 283, 284
Nordiazepam	-NH-	PFPA-PFP-OH	-	
Oxazepam	-NH-, -OH-	PFPA-PFP-OH	14.71	472, 473,232
Temazepam	-OH-	PFPA-PFP-OH	14.43	271, 282, 283
Diazepam	-	PFPA-TMS	14.13	256, 283, 284
Nordiazepam	-NH-	PFPA-TMS	-	-
Oxazepam	-NH-, -OH-	PFPA-TMS	14.78	472, 473, 232
Temazepam	-OH-	PFPA-TMS	14.78	271, 282, 283
Diazepam	-	MTBSTFA	14.13	256, 283, 284
Nordiazepam	-NH-	MTBSTFA	16.43	327, 328, 329
Oxazepam	-NH-, -OH-	MTBSTFA	16.25	457, 459, 383
Temazepam	-OH-	MTBSTFA	17.82	357, 385,283

- = underivatived

As expected, diazepam did not react with any of the derivatising reagents nor was it affected structurally by any of them. The mass spectrum in Figure 7-9 is given as one

example of the reaction product, in this case, with PFP-PFP-OH reagent, which is the mass spectrum of unchanged diazepam.

The acylation derivatisation reagent used for diazepam and its three metabolites is one of the most commonly used acylation reagents. This reagent reacts with alcohols, amines and phenols to produce derivatives with good chromatographic peak shapes. Nordiazepam was the exception and did not work as well with this reagent, most likely because of the secondary amine group. However, temazepam and oxazepam both gave derivatives. The spectrum of temazepam-PFP derivative has a base peak corresponding to loss of the derivatised hydroxyl group. There is no clear interpretation of the mass spectrum of oxazepam bis-PFP derivative and the proposed fragmentation pathway in Figure 7-12 is tentative.

The methylation (esterification) reagent only reacts with carboxylic acid (-COOH) groups and was not expected to react with any of the benzodiazepines, which was confirmed experimentally. However, it was important to evaluate this reagent with these common benzodiazepine structures because the diazepine ring could in theory open and react with the derivatising agents. This mixture produced good peak shapes for all drugs except for oxazepam which can be explained by the presence of secondary amine groups. As expected, desmethyldiazepam did not react with the acylating reagent in this mixture either, also as a result of the secondary amine group.

MTBSTFA reagent had enhanced reactivity as a result of its ability to silylate carboxyls, hydroxyls, thiols and primary and secondary amines; hence it reacted with diazepam and its three metabolites and improved the chromatography. The mass spectra showed the expected M-57 ions due to loss of the tertiary butyl group, and this reaction produced the base peak in the mass spectrum of desmethyldiazepam.[236]

7.7 Conclusion

Derivatisation procedures with three different reagents for some common benzodiazepines, including temazepam, oxazepam, diazepam and nordiazepam, were investigated. The extraction of diazepam and its three metabolites in blood is presented. These drugs have different functional groups such as NH and OH, so generally the best derivatisation method suitable for these functional groups used acylation or silylation reagents. PFPA-PFP-OH

and PFPA- TMS did not work with nordiazepam, but MTBSTFA did however work well with nordiazepam. Overall, the silylation derivatisation method gave the best sensitivity and most improved chromatography for all analytes. During the course of the work for this study, a literature publication appeared for the analysis of benzodiazepines in biological samples based on the methyl-tertiary-butylsilyl derivative. The results of this study were similar to those obtained in the present work except that different retention times were obtained for the derivatives.[236] As mentioned earlier in Section 4.2.2, the authors of that study found the derivatives to be reliable, sensitive and reproducible for the analysis of benzodiazepines in biological samples.

8 Conclusions and Further Work

8.1 Conclusions

The use of blood in forensic toxicology was investigated by developing a reliable method of derivatisation for multiple drugs based on the model compounds amphetamine, methamphetamine, morphine, benzoylecgonine, THC-OH and THC-COOH, GHB, BHB, and some commonly-encountered benzodiazepines.

The aim of this project was to develop a suitable analytical method for the qualitative and quantitative analysis of drugs in blood using derivatisation prior to gas chromatography-mass spectrometry. Derivatisation is very important for modifying a compound to produce a new substance which has different properties from the original compound and which is suitable for GC-MS. Different derivatisation reagents and procedures were investigated with the various analytes mentioned earlier. Prior to derivatisation a simple and rapid solid phase extraction was developed using a novel mixed-mode sorbent. This extraction procedure was used for both acidic and basic drugs.

The first part of the study was the investigation of different derivatising reagents including PFPA plus PFP-OH, PFPA-TMS and MTBSTFA with different drugs of abuse. These were analysed by gas chromatography-mass spectrometry. The method gave good recoveries for the target drugs and the method validation parameters were acceptable.

The second part of the study concerned a particular application of derivatisation reactions and was the investigation of the analysis in autopsy blood of GHB and BHB, which are both endogenous to the human body. A small amount of blood (0.2mL) was used with the solid phase extraction procedure developed earlier. The extraction was found to be simple, efficient, robust, clean and fast. The derivatising reagent which gave the best results was silylation by BSTFA with TMCS as catalyst. This reagent showed excellent solubility and derivatised faster and more completely than any other reagent. The SPE procedure worked very well with BHB, with recoveries shown in Table 6-8. The method was simple and efficient; recoveries were good resulting in a very sensitive procedure for qualitative and quantitative analysis.

Finally a derivatisation procedure for benzodiazepines was investigated. Benzodiazepines as a class encompass a wide variety of drugs and therefore it is a very important class of drug for analysis. Different derivatisation procedures were applied to diazepam and its three metabolites, nordiazepam, temazepam and oxazepam. These compounds have different functional groups and by using different reagents with them the best fragmentation patterns produced from each analyte and those which gave the most sensitivity were found. Some of these reagents did not work with analytes, for example, nordiazepam did not react with PFPA-PFP-OH or PFPA-TMS but worked well with MTBSTFA. Silylation with BSTFA containing TMCS gave the best sensitivity and chromatography.

Generally, when more information is obtained about an unknown sample this will help to select the best and most suitable derivatisation reagent for the target drugs because different classes and function groups have different requirements. GC-MS parameters are very important to obtain stable, reproducible sensitivity during analyses. Selection of an SPE cartridge suitable for volume of samples is also important to avoid loss of any sample during the extraction step.

8.2 Further work

This study has worked well with blood analysis including many of drugs of abuse, but there are still many aspects of analysis of blood which have not yet been done. There are many drugs included in the benzodiazepine group and more investigation into the relationship with derivatisation reagents is still needed. Also, the derivatisation chemistry used is very important in obtaining clean, quantitative, reactions and the selection of suitable reagents for use with different types of drug needs further investigation, especially with respect to drugs containing a wider range of functional group types.

The derivatisation and extraction procedures developed here could be applied to other sample matrices, including urine, hair or nail. The investigation into the use of alternative catalysts may help to analyse a wider range of drugs.

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Appendix 1: Publication in support of this thesis

COMPARISON OF CHEMICAL DERIVATIVES FOR SYSTEMATIC TOXICOLOGICAL ANALYSIS OF AUTOPSY BLOOD USING GAS CHROMATOGRAPHY – MASS SPECTROMETRY

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Abstract

Aims: Different derivatisation techniques have been developed for drug analysis in whole blood using GC-MS. The method was validated for six drugs of abuse and their metabolites.

Method: A mixed standard stock solution was prepared to give a final concentration of 1µg/ml for each analyte and this was used to spike whole blood. Solid phase extraction (SPE) was carried out using the polymeric phase Phenomenex Strat-X (60 mg/3ml cartridges containing 33µm particles). Blank blood (1ml) was mixed with internal standard solution and different concentrations of mixed standard stock solution, and phosphate buffer (pH 6, 3.5ml). This was centrifuged for 10 minutes and transferred to SPE columns. The extracts, in methanol, were evaporated to dryness and derivatised using three different methods: (a) acylation-esterification with PFPA/ PFP-OH (2:1 v/v, 150 µl); (b) acylation–methylation with PFPA/ trimethylsilyldiazomethane (TMS) (5:1 v/v, 120 µl); (c) silylation with MTBSTFA containing 1% TBDMSCl (30 µl). Reaction vials were heated with a microwave oven for one minute. After derivatisation, vials for methods (a) and (b) were cooled to room temperature and evaporated to dryness under a stream of nitrogen. The derivatised extracts were reconstituted in 50µl of ethyl acetate prior to analysis by GC-MS. Extracts derivatised by method (c) were analysed directly. A Thermo-Finnigan Trace GC-MS instrument was used in selected ion monitoring (SIM) mode except method (c) which used full scan mode. The GC was equipped with an HP5 column (30m x 0.32 mm x 0.25µm) from J&W scientific and split/splitless injection port at 280°C. The oven temperature was at 100°C for 2 min, programmed at 12°C/min to 300°C. Ions monitored for quantitative analysis were as follows :

Derivative	Amp	MA	MOR	BZE	THC	THC-COOH
PFPA/PFP-OH	118	204	414	82	417	459
PFPA/TMS	118	204	414	82	417	489
MTBSTFA	158	172	341	282	371	515

The method was subsequently applied to 35 forensic autopsy case samples.

Results: Recoveries for all drugs of interest were found to be over 70%. Limits of detection (LOD) were calculated as 3 times the standard error of the regression line plus the intercept. LOD's in blood ranged from 0.4 ng/ml to 3.7 ng/ml with PFPA/PFP-OH, 0.3ng/ml to 1.4ng/ml with PFPA/TMS and with MTBSTFA were from 1.9 ng/ml to 7.3 ng/ml. Limits of quantification (LOQ) were calculated as 10 times the standard error. They were ≤12.4 ng /ml with PFPA/PFP-OH, ≤4.7 ng/ml with PFPA/TMS and ≤19.8 ng/ml with MTBSTFA of blood. The case samples analysed were found to contain various drugs of abuse and prescription drugs. The different derivatisation techniques gave varying results; however acylation-methylation gave the best sensitivity and chromatography.

Conclusion: A validated, sensitive and specific method for the extraction and quantification of drugs abuse in blood is presented. An alternative derivatisation method (acylation–methylation) is proposed which gives better sensitivity and improved chromatography for detection and quantification of drugs of abuse compared to silylation or acylation alone.

Keywords: Drugs of abuse, Derivatisation, GC-MS

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