ANALYSIS OF CANNABINOIDS IN POST-MORTEM BLOOD SAMPLES

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by

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To my wonderful parents
Abdul Rahman & Aini
who show me the meaning of life;

To my loving wife
Maimunah
who cares and support;

To my beautiful daughters
Maisarah & Athirah
who keep me going...

ACKNOWLEDGEMENTS.

CONTENTS

SUMMARY

This thesis is concerned with the analysis of cannabinoids, delta-9-tetrahydrocannabinol (THC) and its major metabolite, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THCCOOH), in post-mortem blood samples. It concentrates on sample preparation procedures and end-step detection techniques by High Pressure Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS).

A comparative evaluation was made of solid-phase (diatomaceous earth, Extrelut) and solvent extraction procedures. When extracted with hexane, mixtures of hexane and more polar solvents, or acetonitrile, the former gave low extraction yields, for example, with acetonitrile the recoveries were 70.4% for THC and 43.9% for THCCOOH. It was observed that clogging of the extrelut columns by precipitated blood proteins might have resulted in the poor results obtained.

On the other hand, solvent extraction procedures using acetonitrile gave better results. Recoveries obtained for THC and THCCOOH were 82.1% and 82.7% respectively. These values were found to be higher when deionised water was added to dilute the blood samples (83.2% for THC and 86.9% for THCCOOH). However, addition of buffers at pH 5.0 and 7.4 did not improve the recoveries obtained by addition of water. Calibration curves of the solvent extraction procedure produced linear plots within the 0-80ng/ml range for both THC and THCCOOH.

The solid-phase extraction material Bond Elut Certify II was developed recently for the extraction and purification of cannabinoids from biological matrices. It was incorporated into this study as a clean-up procedure for HPLC analysis of the solvent extracts.

Therefore, extraction procedure selected for further analysis involved deproteinization and extraction with acetonitrile, supernatant clean-up using the Bond Elut Certify II columns, evaporation to dryness and either reconstitution of the residue in mobile phase for HPLC analysis or derivatization of the residue prior to GC-MS analysis.

For HPLC analysis, acetonitrile-methanol-0.02N sulphuric acid (65:15:50, v:v:v) was chosen from four mobile phases examined. Detection of analytes was carried out using ultra-violet (UV) and electro-chemical (ECD) detectors at wavelengths 212nm, 220nm and at an applied potential of +1.10V respectively, after detector optimisation procedures. The clean-up procedure separated the cannabinoids into THC and THCCOOH fractions. Thus, two internal standards were needed for quantitation purposes. N-octyl p-hydroxy benzoate and phenylbutazone were found to be the most suitable choices for the two fractions, respectively. It was also observed that the THC fraction gave clean chromatograms while the THCCOOH fraction contained co-extracted interferences which eluted closed to the metabolite.

Average recoveries for THC, cannabidiol (CBD), cannabinol (CBN) and THCCOOH by this HPLC system were 91.1%, 93.6%, 90.1% and 91.4% detected by UV 212nm; 88.9%, 93.5%, 88.9% and 90.7% by UV 220nm and 93.4%, 94.7%, 98.7% and 89.1% by ECD +1.10V, respectively. Calibration curves were linear from 0-100ng/ml for both UV detection wavelengths, and within the range 0-200ng/ml for ECD detection. When eight test samples were analysed, which were positive when screened for cannabinoids by radioimmunoassay, none of the samples gave positive results for THC and CBN. Electrochemical detection but not UV detection gave five samples positive for CBD, while THCCOOH was found to be present in all of the test samples.

GC-MS analysis was performed under full scan, selected ion recording (SIR), multiple reaction monitoring (MRM) and negative ion chemical ionisation (NICI)

modes. Extracted samples were not cleaned up with Bond Elut Certify II in order to evaluate the sensitivity and selectivity of the different GC-MS modes. Full scan mass spectra of THC, THCCOOH and their derivatives were compared. They were found to produce parallel series of ion fragments through similar fragmentation mechanisms.

Analyses by SIR-MS were performed to confirm the identity of the analytes in blood extracts. Calibration curves for THC and THCCOOH were linear from 0-100ng/ml. When the same eight test samples were analysed, three of them were found to be positive for THCCOOH, but only two of the samples gave positive results for THC. In the MRM mode, the transitions to the most prominent daughter fragments $[M-15]^+$ formed by the parent analytes, THC and THCCOOH, were monitored. Linear calibration curves were obtained within the range 0-100ng/ml. Analysis of test samples showed that two samples were positive for both analytes, while another two samples were found to be positive for either THC or THCCOOH. Interference was observed for THC in the other four samples. This interference was also observed under NICI mode and all analyses for THC by NICI selected ion recording were unsuccessful. Case samples also suffered from the presence of interferences and could not be quantified.

In conclusion, the optimum method established in this study was acetonitrile extraction of blood samples followed by GC-MS in $EI^{(+)}$ selected ion recording mode used as the end-step analysis. Sample clean-up is not essential but would help in prolonging the column life.

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AIMS OF STUDY.

Modern techniques provide an oppurtunity to detect low levels of cannabinoids in body fluids. However, only a few published methods have dealt with routine screening and subsequent confirmatory analysis in whole or hemolysed blood samples, which are the most common form of exhibit material received in forensic toxicology laboratories. This analysis still poses problems because of interference caused by co-extracted blood components. Thus, the object of this study was;

(a) to compare and evaluate suitable extraction and clean-up techniques for delta-9-tetrahydrocannabinol and its metabolite, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol in whole blood,

(b) to evaluate two methods of analysis of the extracts which are suitable for routine laboratory use i.e. high-pressure liquid chromatography (HPLC) with non-specific but sensitive ultra-violet and electrochemical detectors, and also the more costly and technically demanding technique of gas chromatography-mass spectrometry (GC-MS), which offers high specificity and sensitivity in selected ion recording, metastable ion monitoring nad negative ion chemical ionisation detection modes; and finally,

(c) to evaluate the methods for use with authentic case samples.

CHAPTER 1. CANNABIS : AN OVERVIEW.

1.1 History.

The hemp plant and its products have been a valuable source for man's commercial, medical, religious and recreational use for thousands of years. For instance, its oil can be used in the composition of paint and varnish, its fiber can be made into rope, twine and bags, and the biologically active constituents can be used as relaxants and mild intoxicants.

Historians claim that cannabis was first used therapeutically in the Chinese and Indian cultures as an antiseptic and analgesic. Other medical uses were later developed and spread throughout the Middle East, Africa and Eastern Europe. It only became widely accepted by Western medical practitioners several years after the return of Napoleon's army from Egypt. However, cannabis began to lose the support of the medical profession in the latter half of the 19th century due to the growing superiority of other medications used in relation to their effects and more easily controlled as to dose.

In the early 20th century, perception of cannabis changed from that of being medically acceptable to being socially disreputable. Strong public reaction resulted in cannabis being "illegal" in many countries. Hence, cannabis drugs are generally regarded as obsolete and are rarely employed nowadays. In the United Kingdom under the Misuse of Drugs Act (1971), cannabis is considered to have no valid medical uses. Nevertheless, many U.S. drug companies are attempting to synthesize new analogs of tetrahydrocannabinol (THC) with the aim of developing some clinical useful preparations. As a result, delta-9-THC has recently re-appeared as an anti-emitic during chemotherapy in the United States.

1.2 Chemistry.

The plant material is quite complex, containing at least 421 individual compounds, where 61 of them have been identified as cannabinoids[1]. These include the principally psychoactive delta-9-tetrahydrocannabinol (delta-9-THC), its isomer delta-8-tetrahydrocannabinol (delta-8-THC), cannabidiol (CBD) and its by-product, cannabinol (CBN). Table 1.1 lists some of the chemical constituents found in cannabis.

When smoked, some of the chemicals contained are further transformed by pyrolysis into still other compounds. All but delta-8- and delta-9-THC, delta-9-THCV (delta-9-*trans*-tetrahydrocannabivarin) and CBN are psychomimetically inactive compounds[2], but several studies seem to indicate that some materials present in natural marihuana may act synergistically with THC and potentiate its psychologic effect[3].

The existence of two numbering systems confuses the chemical nomenclature of tetrahydrocannabinols. The monoterpenoid system considers the compounds as substituted terpenes (delta-1-THC) while the formal dibenzopyran system treats them as substituted dibenzopyrans (delta-9-THC). The formal system will be used hereafter (Figure 1.1).
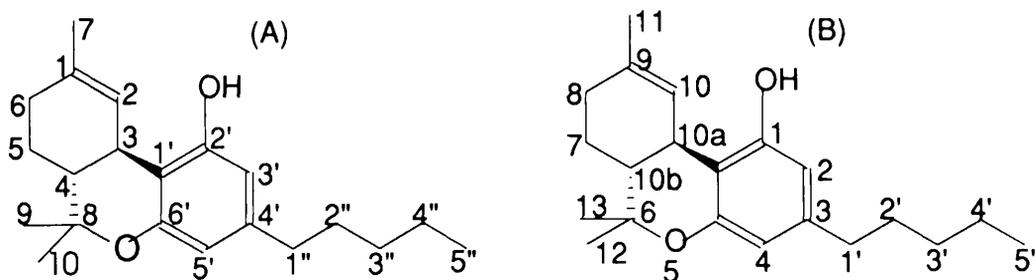
Figure 1.1 : Numbering Systems of Delta-9-Tetrahydrocannabinols.
(A) Monoterpene (B) Dibenzopyran

Table 1.1 : Chemical Constituents Found in Cannabis.[1]

Chemical Constituents	No. of Types Known
1. Cannabinoids	61
a) Cannabigerol (CBG)	6
b) Cannabichromene (CVC)	4
c) Cannabidiol (CBD)	7
d) Delta-9-Tetrahydrocannabinol (Delta-9-THC)	9
e) Delta-8-Tetrahydrocannabinol (Delta-8-THC)	2
f) Cannabicyclol (CBL)	3
g) Cannabielsoin (CBE)	3
h) Cannabinol (CBN)	6
i) Cannabinodiol (CBND)	2
j) Cannabitriol (CBT)	6
k) Miscellaneous	9
l) Others	4
2. Nitrogenous compounds	20
a) Quaternary bases	5
b) Amides	1
c) Amines	12
d) Spermidine alkaloids	2
3. Amino acids	18
4. Proteins, glycoproteins, and enzymes	9
5. Sugars and related compounds	34
a) Monosaccharides	13
b) Disaccharides	2
c) Polysaccharides	5
d) Cyclitols	12
e) Amino sugars	2
6. Hydrocarbons	50
7. Simple alcohols	7
8. Simple aldehydes	12
9. Simple ketones	13
10. Simple acids	20
11. Fatty acids	12
12. Simple esters and lactones	13
13. Steroids	11
14. Terpenes	103
a) Monoterpenes	58
b) Sesquiterpenes	38
c) Diterpenes	1
d) Triterpenes	2
e) Miscellaneous compounds of terpenoid origin	4
15. Noncannabinoid phenols	16
16. Flavanoid glycosides	19
17. Vitamins	1
18. Pigments	2

1.3 Botany.

Cannabis sativa L. or Indian hemp, ia a tall, annual, bushy plant that can be grown in any region with hot summers. It is widely distributed throughout the temperate and tropical zones of the world and is probably the oldest non-food plant ever cultivated.

Cannabis is a dioecious species with separate male and female plants, both producing flowers. The activity of the plants was about the same because the female plants yielded more but less potent resin than the male plants.[4] The drug content of the plant parts is variable. Generally, it decreases in the following order: bracts, flowering tops, leafy tops, small leaf, large leaf, stem. Practically no cannabinoids are found in the roots or seeds.

The plant can also be classified generally as either drug or fibre genotype. The drug type is high in THC but low in CBN and the fibre type is the converse. The intermediate cannabis has the percent by dry weight of CBD equal to or slightly greater than delta-9-THC. Cannabichromene (CBC) is always present in drug types.

The concentration of cannabinoids in the plant is a function of genetic and environmental factors (type of soil, water, temperature, growing space). Other factors include the time of collection (maturity of the plant) and the treatment of the sample taken (drying, storage, extraction and analysis).

Marihuana, bhang, ganja, kif and maconha are among the commonly used names for cannabis or its products in various countries. Differences in names refer to differences in the mixture of the leaves and flowering tops of the plant. For example, marihuana consists of the dried and crumbled stems, leaves and seed pods of the female plant. Bhang consists of specially dried leaves and flowering shoots of both male and

female plants, wild or cultivated, while ganja refers to a specially cultivated hemp plant that contains an unusually large quantity of active materials.

Meanwhile, hashish or charas are names often applied to the pure unadulterated resin from the top of the finest female plants of Indian hemp which also contains a very high concentration of active constituents. Less common and strongest of all, is cannabis oil or hash oil, a liquid prepared from the resin.

1.4 Routes of Administration.

The plant is usually cut, dried, chopped and rolled up into a cigarette and smoked, often combined with tobacco. It can also be smoked in a pipe, brewed into a drink or cooked in foods.

Conversion of THC precursor acids to active THC during marihuana smoking may activate and increase the potency of a cigarette, and a similar process may occur during the baking of brownies and other forms of cooking with marihuana plant material[5], although prolonged heating may destroy THC. Approximately 50% of the THC in a cigarette is absorbed by the lungs if the entire cigarette were smoked in ten minutes and each inhalation were retained for 30 seconds with no side-stream loss.[1]

Generally, the effects produced by ingested THC or ingested marihuana extract are comparable to those produced by nearly one third the amount of smoked and inhaled THC or marihuana[6]. Perez-Reyes et al.[7] indicated that it is the rate of THC absorption, and the duration of its action which is affected by the chosen route of administration.

Lemberger et al.[8] observed that by administering radiolabelled THC, the greatest and most prolonged radioactivity in the bloodstream was obtained following inhalation of smoke from spiked marihuana and the least after oral ingestion.

In addition, studies conducted by Cone et al.[9] and Law et al.[10] on the oral ingestion of THC showed behavioral and physiological effects similar to those produced from smoking marihuana cigarettes, but the onset and peak effect was delayed and the duration of effect was extended.

1.5 Metabolism.

Absorbed slowly and incompletely by the stomach, delta-9-THC is much more effective when smoked and absorbed by the lungs. It reaches a maximum blood level within a few minutes and then falls rapidly, due to metabolism and tissue storage, to approximately 10% of its peak concentration within the hour. The concentration of THC after this time normally being no more than a few nanograms per millilitre of blood. Figures 1.2 to 1.5 show the metabolic pathways of delta-9-THC, delta-8-THC, CBD, and CBN.

Agurell et al.[11] developed a method to identify and measure accurately nonlabelled delta-9-THC in the plasma of cannabis smokers. Results showed that within 10 minutes of smoking a cigarette containing 10mg of delta-9-THC, peak plasma levels of 19-26ng/ml were reached and later declined rapidly to < 5ng/ml within 2 hours.

CHO ⟶ COOH ⟶ Glucoronide

CH₂OH
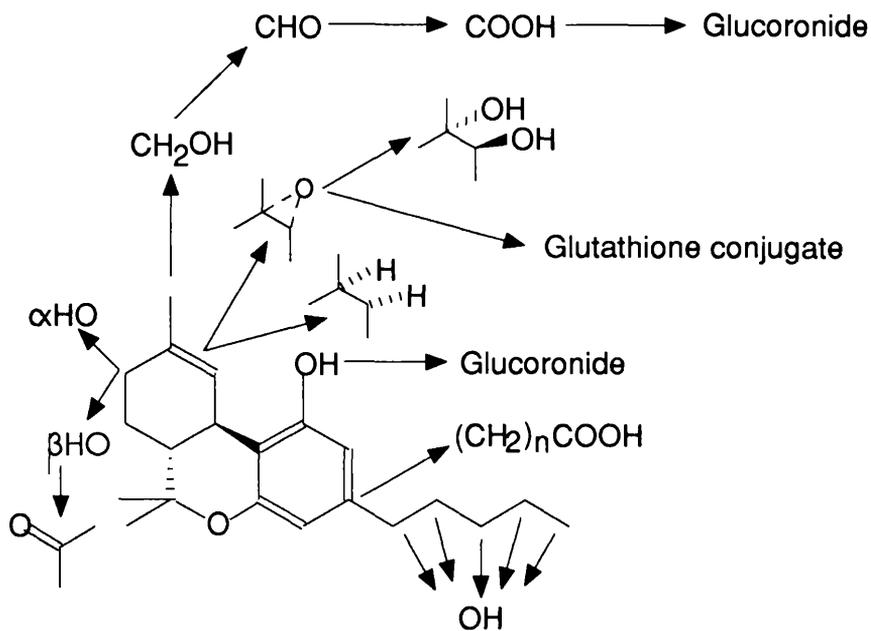
Figure 1.2 : Metabolic Pathways of Delta-9-THC.

Glucoronide

(CH₂)ₙCOOH

Glutathione conjugate
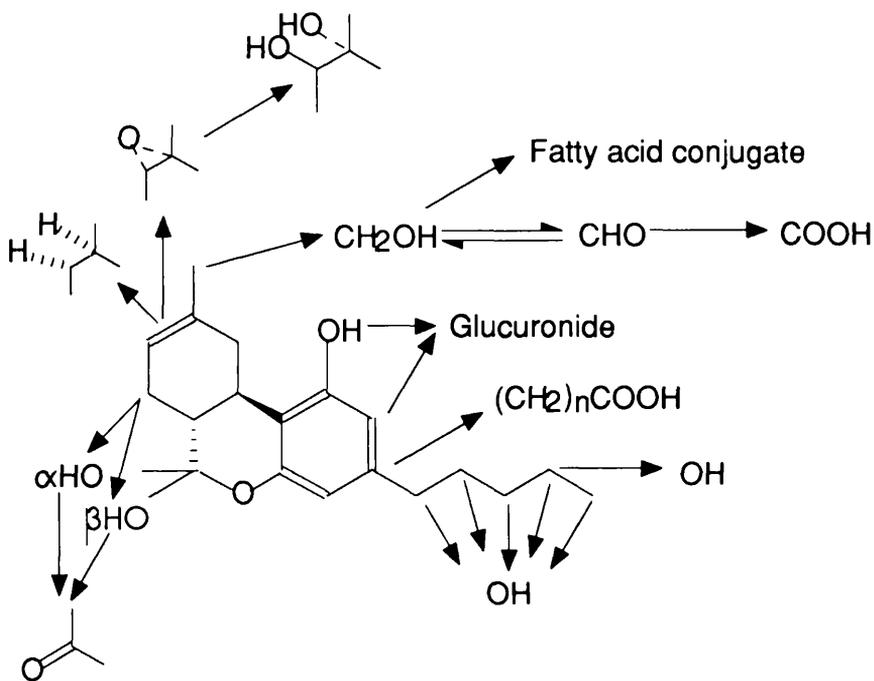
Figure 1.3 : Metabolic Pathways of Delta-8-THC

Fatty acid conjugate

CH₂OH ⟶ CHO ⟶ COOH

Glucuronide

(CH₂)ₙCOOH

OH

CH$_2$OH ⟶ COOH

OH ⟶ Glucuronide

αHO

βHO

O

(CH$_2$)$_n$COOH

OH

OH

OH

Figure 1.4 : Metabolic Pathways of Cannabidiol.

CH$_2$OCOR

CH$_2$OH ⟶ CHO ⟶ COOH

OH ⟶ Glucuronide

O

OH

OH ⟶ OCOR

(CH$_2$)$_n$COOH   OH

Figure 1.5 : Metabolic Pathways of Cannabinol.

Based on the chemistry of the phenolic group common to all cannabinoids, cannabinoid-like drugs and their metabolites, Rosenfeld et al.[12] developed and applied a mass fragmentographic assay for delta-9-THC in plasma of human volunteers. Results obtained were similar to those reported by Agurell et al.[11].

Lemberger[3] indicated that THC disappears from plasma in two phases. The initial rapid phase has two components and represents metabolism by the liver and redistribution from the blood to the tissues. The slower, second phase represents tissue retention and slow release and subsequent metabolism. THC is essentially insoluble in water and is protein bound.

Initial metabolism of cannabinoids from cannabis smoke or from orally consumed cannabis takes place in the lungs or in the liver, respectively. Since different enzymes are involved, different initial metabolites are produced. Major lung metabolites are usually hydroxylated derivatives of the cyclohexene ring system[13]. However, the major one is undoubtedly 11-nor-9-carboxy-delta-9-THC (THCCOOH) produced via the 11-hydroxy THC metabolite.

THC is rather unstable, being degraded by heat, light, acids and atmospheric oxygen, properties that account for the well known tendency of cannabis preparations to lose potency on storage.[14] Its terminal plasma half-live is 28-56 hours and has been seen to be significantly shorter in daily users than in nonusers. Urinary excretion and biliary excretion (reflected a day later in the faeces) was found to be greatest during the initial 24 hours, then gradually tapering off. Law et al.[10] pointed out that following a single, moderate, oral dose of cannabis resin in a meat sandwich (20mg delta-9-THC), significant concentrations of cannabinoid metabolites were detected in plasma samples for up to 5 days and in urine samples for at least 12 days.

1.6 Dose and Effects (Pharmacodynamics).

Pharmacologic actions of cannabis are highly subjective and are affected by a number of variables such as the quality of the drug, the dosage, the experience and expectations of the user and his environment.

The psychomimetic activity due to inhalation or ingestion of low doses (25ug/kg) of delta-9-THC may cause euphoria, relaxation, talkativeness, bouts of hilarity and sensory enhancement. Larger doses (250ug/kg) will give an intense hallucinogenic experience, perceptual distortion, temporal disintegration, confusion and can be distressfull.

Unlike the smoking route in which peak behavioral and physiologic effects occur within minutes after smoking, responses after ingestion of THC have a slower onset and greater variability with maximum effects occuring 1.5 to 3.5 hours later. This slow onset of effects is consistent with results reported by Perez-Reyes et al.[7], Ohlsson et al.[15], and Law et al.[10]

The most common physical responses to cannabis are an increase in pulse rate and reddening of the eyes. Also reported are dryness of the mouth and throat due to the irritancy of cannabis smoke. Its effects are more prominent on the central nervous system and the cardiovascular system. However, deaths due to an overdose of cannabis are rare and usually relate to large oral doses. The minimum fatal dose by oral injestion of charas, ganja and bhang was 2, 8 and 10ng/kg of body weight respectively.[4] The lethal dose of delta-9-THC, receiving intravenously, is 30mg/kg of body weight.[16]

Agurell et al.[17] pointed out the pharmacokinetics of delta-8-THC in man after smoking and the relationship of blood levels to physiological and psychological effects. It was shown that the increase in heart-rate was well correlated with the delta-8-

THC plasma levels but the alterations in mental performance were more delayed and prolonged than the peak plasma level.

Hollister et al.[18] used 11 volunteers to address the question of whether THC levels reflected the state of cannabis intoxication. The volunteers were given about 19mg THC by smoking, 20mg THC orally and 5mg THC i.v. The THC plasma levels were measured up to 4 hours after administration by GC-MS. They concluded that the relationship between pulse rate and plasma concentration of THC was similar to that reported by Agurell et al.[17], but they saw no clear-cut relationship between plasma concentrations of THC and the degree of intoxication, in contrast to the case for ethyl alcohol.

TABLE 1.2 : SUMMARY OF THE RELATIONSHIP BETWEEN DOSES AND EFFECTS OF CANNABIS ADMINISTRATION.

Doses	Effects	References
Delta-9-THC, ingestion 300-480ug/kg or smoking 200-250ug/kg. 10 subjects.	Marked distortion of visual & auditory perception and hallucination occurred in most subjects.	(19)
Marihuana extract, 66mg/kg of body weight. 12 subjects.	Produced a mild "high" in all subjects Reaction time & digit code memory tasks impaired.	(20)
THC, 4.5mg & 18mg, smoking. 9 naive subjects.	Impairment in simple intellectual and psychomotor performance tests	(21)
THC, 10mg, smoking	Significant decrements in human motor and mental performance.	(22)
THC, 15-25mg, ingestion or 5-10mg, smoking	Significant impairment of motor and mental performance	(23)

Cannabis is not physically addictive. Thus, abstinence or abrupt cessation of intake will not produce distinct withdrawal symptoms. The relationship between different doses and effects of cannabis is briefly summarized in Table 1.2. In relation to impairment of motor and mental performance, the dose and effect relationship is briefly summarized in Table 1.3.

TABLE 1.3 : SUMMARY OF DOSES AND EFFECTS OF MARIHUANA'S RELATIONSHIP IN RELATION TO PERFORMANCE IMPAIRMENT.

Doses	Effects	References
Marihuana, 1.7g (1.3% THC) smoking 36 subjects.	Significantly more speed errors on driving simulator	(24)
Delta-9-THC, 3.3 & 6.6mg smoking. 10 experience and 10 naive subjects.	Equal impairment in driving performance	(25)
Delta-9-THC, 350, 400 & 450ug/kg orally 54 subjects.	Impairment of attention and concentration capacities, prolongation of reaction time, increased frequency of wrong and inadequate responses, and smooth automation disturbances.	(26)
Delta-9-THC, 18mg, smoking. 58 subjects.	General impairment in Roadside Sobriety Tests.	(27)
Delta-9-THC, 6.3mg, smoking. 3 Professional and 3 private pilots.	Deterioration in flying performance.	(28)
THC, 0.09mg/kg, smoking. 10 certified pilots.	Significant deterioration in simulated instrument flying ability.	(29)
THC, 19mg, smoking. 10 private pilots.	Performance decrements on flight simulator.	(30)

1.7 Light vs Heavy Users.

Lindgren et al.[31] determined the plasma concentrations of THC given by smoking and i.v. administration to groups of light and heavy users of marihuana. The subjects were given marihuana cigarettes containing about 19mg THC or an i.v. dose of 5.0mg THC. Their smoking experiments were unable to determine any significant difference in plasma levels of THC between light and heavy users. But, they did note a tendency that heavy users showed higher THC levels suggesting more efficient smoking than light users.

Similarly, Perez-Reyes et al.[32] too found no differences in total plasma radioactivity or the amount of ^{14}C-THC needed i.v. to produce a maximal "high" in infrequent and frequent marihuana users. So far as maximal "high" is concerned, both studies found no difference in light vs heavy users and neither sensitivity nor tolerance to THC was observed.

Wall et al.[33] pointed out the extensive biotransformation of various cannabinoids in animals and man. They stressed the fact that due to some active metabolites of THC, no simple relationship between plasma levels and marihuana induced "high" would be expected.

Ohlsson et al.[34] studied the single dose kinetics of deuterium-labelled THC in light and heavy cannabis users by both smoking and i.v. administration. Plasma levels of ^{2}H$_3$-delta-9-THC were measured for 48 to 72 hours. The results showed little difference between the groups with regard to the amounts absorbed from the smoke, plasma levels or areas under the plasma concentration vs time curves. Thus, as in previous studies, no evidence was obtained for either sensitivity or tolerance to THC in heavy users.

1.8 Passive Inhalation.

Plasma concentrations of THC near the detection limits of most analytical methods may possibly be produced by either passive inhalation or from active cannabis smoking that occurred at some time earlier. The former may occur as demonstrated by the presence of THC in blood and cannabinoids in blood and urine of passive smokers.[35-40]

This presents a threat to the passive inhaler because secondary smoke too contains psychoactive delta-9-THC and other cannabinoids. However, the degree of threat depends on several factors which include the amount of cannabis smoked by the active user, the duration and frequency of exposure, the room size and ventilation conditions and the individual metabolic factors of the passive inhaler.

A study done by Morland et al.[35] showed that the highest concentration of plasma THC reached by passive inhalation was 13ng/ml which corresponds to the concentration measured in plasma approximately 30 minutes after active smoking of 15mg of THC.

Is there any way to distinguish between active and passive smokers? Mason et al.[36] suggested that it is possible to differentiate between them by determining the relative concentrations of 9-carboxy-THC and THC in plasma at different times. This is because their inhalation experimental findings showed that the concentration of 9-carboxy-THC was consistently less than the concentration of THC determined by GC-MS analysis, whereas the literature indicated that within 20 to 30 minutes after the initiation of active cannabis smoking, the concentration of 9-carboxy-THC becomes greater than the concentration of THC in plasma and remains higher for many hours.

Another method for avoiding unfair penalisation of passive smokers is to set up a cut-off level. For urine THC metabolites analysis, it is recommended to set up a cut-off level at about 20ng/ml, after considering the results of studies of passive smoking of THC under several conditions, as shown in Table 1.4. But, under extremely heavy passive smoking conditions, the distinction between active smoking and passive inhalation could be lost.

TABLE 1.4 : SUMMARIES ON STUDIES ON THE PASSIVE INHALATION OF THC.

THC burn (mg)	Room size (L)	Exposure time (h)	Sample/ Compound	Detection/ Sensitivity	References
52	15,500	1	urine cannabinoids	EMIT 0ng/ml	(36)
52	large car	1	urine cannabinoids	EMIT 20ng/ml	(36)
104.8	15,500	1	plasma 9-THCCOOH	RIA <3ng/ml	(35)
103	27,950	3	urine metabolites	RIA <7ng/ml	(37)
27	(10x10) inches	0.25 0.50	saliva THC	RIA/18ng/ml RIA/0ng/ml	(38)
90	small car	0.50	blood THC	GC/MS <7ng/ml	(34)
98	12,226	1	urine cannabinoids THCCOOH THCCOOH	EMIT/<7ng/ml GC/MS<26ng/ml RIA/ <42ng/ml	(39)
398	12,226	1	urine cannabinoids THCCOOH THCCOOH	EMIT/<71ng/ml GC/MS<90ng/ml RIA/<145ng/ml	(39)

1.9 Stability of Cannabinoids in Body Fluids.

A number of studies on the stability of THC in body fluids during storage have been published. Wong et al.[41] evaluated the effects of long-term storage on the concentrations of THC in blood and serum stored between -20 and $5^{o}C$. They found that THC concentrations in blood and serum samples had decreased markedly after 17 and 19 weeks respectively.

The stabilities of THC and two of its metabolites, 11-hydroxy-THC and THCCOOH, were studied by Johnson et al.[42] in blood and plasma stored at -10, $4^{o}C$ and room temperature. Their results are similar to those reported by Wong et al.[41] for storage periods up to 17 weeks. However, after 26 weeks of storage at room temperature, the concentrations of THC and 11-OH-THC in blood and plasma were significantly lower, but the THCCOOH concentration did not change.

While THC in dry form, chloroform, hexane and carbon tetrachloride deteriorated about 10% each month, it was reasonably stable for more than 75 days at room temperature and for more than one year at $0-5^{o}C$ when stored in ethanol solution.[43]

In addition, since THC is a lipophilic molecule and tends to bind readily to hydrophobic surfaces, its concentration will be greatly reduced if stored in certain types of containers. This is supported by results obtained from Garrett and Hunt[44], who reported absorption of THC to rubber stoppers, but in unsilanized glass, its absorption was apparently prevented by proteins in blood that bind over 97% of THC. However, in the study cited above, Johnson et al.[42] found no absorption to rubber stoppers in their blood collection tubes.

Christophersen[45] investigated the stability of THC in whole blood during storage in unsilanized glass vials with rubber septa and in plastic polystyrene tubes over 4 days and 4 weeks at room temperature and -20°C respectively. He noted that blood concentrations of THC were stable in the glass vials, but 60-100% of THC stored in the plastic tubes was lost. Even the recovery of internal standard from plastic tube samples was low, varying from 30-50% of the value in the corresponding glass vial samples. These losses were due principally to absorption to the plastic that could not be prevented by binding proteins in the blood. Thus, it is advisable and important to carry out stability tests for biological samples in the actual containers used in each laboratory and with the same sample handling and storage procedures used for real samples.

1.10 Forensic Toxicology.

The toxicity of marihuana is low and fatalities due to its poisoning are very rare. It is impossible to prove anything safe, but smoking marihuana appears to be safer than overindulgence with alcohol, tobacco, barbiturates, heroin, aspirin, and many other drugs.[46] This is because deaths and withdrawal symptoms attributed to heroin, alcohol and barbiturates do not occur with marihuana. Moreover, respiratory diseases and cancer associated with chronic marihuana smoking have not been reported and cardiac effects of caffeine of coffee, tea and soft drinks have not been attributed to marihuana.

In addition, there is no conclusive evidence that short-term marihuana use alone directly results in any physical damage to humans.[1] Many fatal overdoses are caused by drug interactions due to consumption of cannabis with other drugs. Cannabis has been shown to alter the effects of alcohol, barbiturates, benzodiazepines, nicotine, amphetamines, cocaine, phencyclidine (PCP), opiates and other drugs.[47] This alteration

will enhance or prolong behavioral, physical and psychological effects, thus may result in fatalities such as road accidents, drowning, fires and injuries due to falls or fights.

1.11 Conclusion.

It is frequently of considerable concern to know whether someone was under the influence of cannabis at the time of his death. Since circulation ceases at death, the metabolism of delta-9-THC in the liver would be expected to be halted and the levels determined by analysis should reflect those present at death. Thus, this definitive identification in blood from an autopsy sample presents direct and unquestionable evidence of the deceased's use of this drug.

Development of improved methods gives an opportunity to detect and estimate low levels of delta-9-THC or its metabolites. However, only a few have dealt directly with the development of reliable methods for the routine screening and subsequent confirmatory and quantitative analysis in whole or hemolysed blood samples. Because of the forensic nature of this study, its objective was therefore to develop screening, confirmatory and quantitative methodologies for the analysis of cannabinoids in autopsy blood samples.

CHAPTER 2.   EXPERIMENTAL REVIEW.

2.1 Introduction.

For forensic purposes, analysis of cannabis drugs of abuse can be divided into three operations: screening, identification and quantitation of the cannabis materials or products in question. Many studies have been extensively pursued, but most of them have been developed for plasma and urine from living patients or volunteers.

The major problem in the development of an accurate method is that active physiological levels of cannabis, particularly delta-9-THC, are usually quite low, rarely exceeding 100ng/ml of whole blood.[48,49] Autopsy samples are most likely to be lower than this and clear plasma is never available. Thus, sensitive methods are needed which should also be specific and characteristic for delta-9-THC, to be valid in forensic science applications.

Analysis of cannabinoids in autopsy samples may also be considered as two parts: (1) extraction and clean-up procedures, and (2) the end-step analysis which may also involve derivatisation, depending on the instrument used.

2.2 Sample Preparation.

2.2.1 Urine Hydrolysis.

The major metabolite of THC in urine is THCCOOH, which exists in both a free acid or a conjugated glucuronide form. Thus, prior to extraction, the urine sample should be decomposed by hydrolysis to convert the conjugated metabolite to the free acid

form for extraction. Most hydrolysis steps involve the use of potassium hydroxide (KOH) or *beta*-glucuronidase, processes known as alkaline or enzyme hydrolysis respectively. Among those who used alkaline hydrolysis were Nakahara et al.[50], Karlsson[51], Parry et al.[52] and Meatherall and Garriott[53], while Kanter et al.[54], McBurney et al.[55] and Craft at al.[56] used the enzyme to hydrolyse their urine samples.

2.2.2 Blood (Plasma/Serum) Pretreatment.

Because THC and its metabolites bind strongly to blood proteins, it is difficult to extract them completely. A deproteinization step is necessary to improve extraction efficiency. Nakahara et al.[57] have evaluated the recoveries of THC metabolites obtained in plasma after deproteinization with various solvents, and results showed that acetonitrile-pyridine (10:1) gave the highest recoveries while 10% trichloroacetic acid gave the lowest recoveries.

2.2.3 Solvent Extraction.

Extractions by solvents (liquid-liquid) are most commonly used since many solvents can extract THC and its metabolites from plant materials and body fluids. But it has been shown that hexane alone extracts cannabinoids very efficiently and in some cases gives clean extracts suitable for direct analysis by gas-phase techniques.[45,53,58-62] However, some extraction techniques used single solvents such as ether[54] acetonitrile[63-65], and acetone[66], while others used mixed solvents such as hexane-ethyl acetate[55] and heptane-isoamyl alcohol.[67-69]

2.2.4 Solid-Phase Extraction.

Solid-phase extraction (SPE) has emerged as a powerful tool for chemical isolation and purification, where the analyte is selectively absorbed from the biological matrix on to one of a variety of commercially available solid phases (eg. silica or bonded silica) contained within a disposable cartridge. The use of SPE columns has gained popularity over recent years due to reported excellent recoveries and ease of use. Furthermore, its advantages include the simultaneous extraction of a number of samples, the prevention of emulsion formation as often occurs with solvent extraction, rapid sample preparation, removal of evaporation steps and potential automation.

There are many types of SPE depending on the specific properties of the bonded cartridge. Schermann et al.[70] and Hattori[71] have used a diatomaceous earth (Extrelut) column to extract urinary and blood cannabinoids respectively, while Craft et al.[56], Foltz et al.[72] and Zweipfenning et al.[73] have extracted cannabinoids using bonded-silica C_{18} columns. However, Parry et al.[52] have recently reported a procedure using Supelclean DrugPak-T SPE (anion exchange) tubes for the isolation of THCCOOH, where they obtained an absolute recovery of greater than 85% for the metabolite from a urine sample matrix.

2.2.5 Clean-up Procedure.

Some extracted samples do contained coextracted interferences such as endogenous urine compounds or blood lipids. These will result in lower extraction recoveries and detection limits. Thus, extensive sample clean-up procedures are essential in most chromatographic assays, especially HPLC, in order to prevent ruining the octadecasilica (ODS) column used during the analysis. Clean-up procedures are usually based on some form of wet chromatography such as TLC, as used by Bachmann et al.[74],

or HPLC as used by Garrett and Hunt[75] and Peat et al.[76] Others have also used column clean-up like C_{18} columns[63] and Sephadex LH-20 columns.[11,66]

2.3 End-Step Analytical Methods.

It is important that cannabinoid assays should be specific and sensitive. If these are achieved, the assays can avoid lengthy clean-up and derivatisational procedures.

2.3.1 Thin Layer Chromatography (TLC).

Separation of cannabinoids by TLC is difficult because of the close similarity in structure between the different compounds, and it is much more difficult to detect and measure them in biological fluids. Silica-gel is the most widely used adsorbent together with single or mixed solvents of low polarity. Although many spray reagents have been used, Fast blue B/BB gave the most sensitive detection. Generally, TLC is only used as a screening tool and it suffers from a lack of both sensitivity and specificity.

However, many improvements have been made to TLC systems with respect to detection and measurement of THC and its metabolites. A hexane extract of cannabis material was examined on silica gel G plates previously soaked in diethylamine. The toluene-developed plates were dried and sprayed with Fast blue 2B salt. Five spots corresponding to delta-9-THC, CBD, CBN, CBC and CBG, each with a different R_f value and a distinctive colour were observed.[77]

In the procedure for detection of THCCOOH in urine described by Kanter et al.[54], a silica-gel plate was used and developed first with acetone-chloroform-

triethylamine (80:20:1) and then with petroleum ether-ether-acetic acid (50:50:1.5). The spray used was Fast blue B giving a detection limit in the 20-50ng/ml range.

Vinson et al.[67] have developed a method based on fluorescence detection in which THC extracted from serum was derivatised with 2-p-chlorosulphonylphenyl-3-phenylindone prior to TLC. The developed plate was sprayed with an alkoxide to produce the fluorescent derivative and a detection limit of 0.2ng/ml was claimed.

Furthermore, it should be noted that screening procedures based on TLC are more specific for THCCOOH.[78-81] Stated limits of THCCOOH detection from urine are 25ng/ml using 10ml[78], 20ng/ml using 10ml[79], 50ng/ml using 20ml[80] and 2ng/ml using 2ml of urine.[81]

2.3.3 High Pressure Liquid Chromatography (HPLC).

HPLC has been proposed for the determination of cannabinoids in biological fluids. The methods employed either normal- or reversed-phase separations with either Ultra-Violet (UV), Electro-Chemical (EC) or Fluorescence detection. Unfortunately, while UV detection lacks sensitivity and selectivity due to co-extracted interference, fluorescence detection requires the derivatization of THC. It seems that EC detection might be better since it is highly sensitive and it can eliminate the need for derivatization.

Kanter et al.[82] reported that greater sensitivity for UV detection could be obtained by monitoring at shorter wavelengths, for example, the sensitivity at 215nm is thirty times that at 280nm. However, Valentine et al.[83] reported that by reducing the wavelength from 274 to 209nm, the detection limit decreased from 100 to 2ng/ml of delta-9-THC in human plasma.

Analysis of cannabinoids in tissues which involved post-column derivatisation with Fast blue B salt and detection by UV at 490nm was reported by Borys and Karler[84]. A detection limit of better than 50ng/ml was claimed. On the other hand, Abbott et al.[85] reported the detection at the low picogram range of delta-8-THC and its 7-hydroxy metabolite using fluorescence detection of their dansyl derivatives.

An electrochemical detector at +1.1V was used by Nakahara et al.[50] in combination with a reversed-phase silica C_8 column and a mobile phase of acetonitrile-methanol-0.02N sulphuric acid (35:15:50) at 1.8ml/min to detect delta-9-THCCOOH in urine and plasma. The detection limit obtained was under 0.5ng/ml (S/N >3).

HPLC has also been widely used in cleaning-up extracts or separating metabolites from unchanged cannabinoids in more sensitive techniques such as MS or RIA. Garrett and Hunt[86] have investigated reversed and normal phase HPLC systems for separating delta-9-THC from its 11-hydroxy metabolite and have used electron capture GLC as the detection system. A detection limit of 0.2ng/ml of plasma was claimed. Meanwhile, Williams et al.[87] have developed a specific and sensitive RIA method for delta-9-THC in plasma based on HPLC separation of cross-reacting metabolites. They have reported a detection limit of 0.1ng/ml.

2.3.4 Immunoassay.

Immunoassay techniques, either Radioimmunoassay (RIA) or Homogeneous Enzyme Multiplied Immunoassay Technique (EMIT), have the advantages of being more rapid, simpler to operate, requiring minimal sample size and not requiring complex equipment.

RIA antibodies are raised by covalently linking the drug (hapten component) to a large molecule such as a protein to form the immunogen, which is then injected into animals (rabbit, cow, goat) to stimulate formation of antibodies. The binding site of the antibody accommodates the hapten with some ambiguity around the area which corresponds to the point of linkage in the immunogen.

The EMIT method is related to simple immunoassay techniques, and relies on the observation that certain enzymes can be inhibited by antihapten antibodies when the hapten is covalently bonded to the enzyme.

The major drawback of immunoassay methods is cross-reactivity, especially with non-specific antisera, which occurs between substances within the cannabinoid group, their metabolites and other drugs. This can reduce both the specificity and interassay precision and will definitely cause concern about the sole use of this technique. But, if employed with a subsequent confirmatory technique, such as HPLC or GC-MS, immunoassay is a valuable screening tool especially for batch samples. However, with specific antisera, it is possible to measure delta-9-THC directly.

Recently, Roche Diagnostics developed a latex agglutination immunoassay technique (ONTRAK). This technique provides the sensitivity of an immunoassay without the need for expensive equipment. It is based on the principles of latex agglutination-inhibition, where the test relies on the competition for binding to antibody between latex-drug conjugate and drug which may be present in the urine being tested.

Gross et al.[88] produced an antiserum in goats following immunization with THC coupled to protein through an azo link to the aromatic ring and reported an assay limit of 25 to 50ng/ml. They also found that a cross-reactivity of 47% occurred with 11-hydroxy-delta-9-THC. Teale et al.[89] used a delta-9-THC-protein complex with THC

attached by the phenol group to immunize sheep and have achieved a detection limit of 7.5ng/ml in plasma and 1.0ng/ml in urine for delta-9-THC.

Owens et al.[90] presented a radioimmunoassay for delta-9-THC in plasma and whole or hemolyzed blood. An aliquot of the supernatant sample, extracted by methanol, was incubated with RIA buffer, radiolabelled delta-8-THC and rabbit anti-THC serum. Solid-phase goat anti-rabbit immunoglobulins were added to separate bound from free THC and the supernatant fluid was later aspirated, after centrifugation. Radioactivity of the precipitate was counted in a gamma counter. The level of delta-9-THC in 0.1ml of sample was determined with a sensitivity of 1.5ng/ml in plasma and 3.0ng/ml in hemolyzed blood.

An HPLC/RIA method for the measurement of cannabinoids in plasma and urine was described by Moffat et al.[91] The method consisted of chromatographing a plasma extract or hydrolysed urine sample by HPLC and quantifying the eluted cross-reacting cannabinoids by RIA with non-selective antisera. Results obtained showed that the plasma levels of cannabinoids for volunteers who smoked 10mg THC were between 70 and 6ng/ml over the 2-hour period examined.

2.3.5 Gas-Liquid Chromatography (GLC).

GLC methods offer more selectivity and in many cases, sensitivity, provided that a highly sensitive detection system such as electron capture or flame photometry is employed. But, it has been argued that GLC offers little advantage over TLC and it is less sensitive with flame-ionisation detection.[92]

Many stationary phases have been used for packed-column GLC separations which can separate all of the major cannabinoids and many of the minor ones, although the

cannabinoid acids require derivatisation to prevent on-column decarboxylation. Moreover, glass packed-columns have been recommended in preference to stainless-steel ones which can cause up to 30% losses.[93]

Novotny et al.[94] demonstrated that because of the large number of cannabinoids present in many samples of cannabis material, capillary GLC can be used to advantage, where samples were concentrated on a pre-column and the resulting chromatograms could be used to "fingerprint" samples of marihuana.

Although good separations of the major neutral cannabinoids can be achieved on most phases, superior results can usually be obtained after derivatisation, particularly as TMS ethers.[93] The introduction of a derivatisation step results in improved peak shapes, reduced tailing and much higher sensitivities because sample losses through absorption are reduced. Derivatisation can also lead to large increases in sensitivity since it can be designed to suit specific sensitive detection systems. Moreover, TMS derivatives have the advantage of keeping the column and other parts of the system well silanised and thus nonpolar.

Fenimore et al.[68] have compared the relative sensitivity of eight electron-capturing derivatives and obtained the best results for the heptafluorobutyryl ester (HFB), which was analysed on a dual-column chromatograph to minimise interference from co-extracted material. The effluent was monitored by a flame ionization detector at the exit of the first column and only the effluent containing the THC peak was diverted into the second high-resolution capillary column for detection by electron capture. A detection limit of 100pg/ml of blood was claimed.

Garrett and Hunt[95] used a packed column (OV-225) and pentafluorobenzoates in their GLC assay of delta-9- and delta-8-THC. They claimed a 40

to 125pg/ml detection limit with electron capture detection. Bachmann et al.[74] formed the HFB derivative prior to separation by TLC, and GLC detection using electron capture gave a detection limit of 8pg for the pure THC and 1ng/ml from plasma.

As an alternative to electron capture detection, McCallum[96] has used flame photometric detection of the diethylphosphate derivative to give a detection limit of 2ng/ml on a 3% OV-1 column. Later, McCallum and Shaw[97] extended the method to measure THC in brain, where 10ng/g could be measured. In addition, Ritchie et al.[62] have determined delta-9-THC in whole blood that employs hexane extraction followed by identification using GC with nitrogen-phosphorous detection. Their method was sensitive to 2ng/ml and linear for concentrations up to 120ng/ml.

2.3.6 Gas Chromatography-Mass Spectrometry (GC-MS).

GC-MS represents the most sensitive and selective of all analytical techniques for cannabinoid analysis. It offers a very high degree of reliability, accuracy and precision, but the complexity and high cost of the instrument has restricted its use. The mass spectra of cannabinoids and in particular, their TMS derivatives are very characteristic due to the fragmentation pattern which is directed mainly by charge localisation on the pyran oxygen. However, silyl ethers having substituents capable of forming stable radicals following electron impact may produce additional ions representing loss of these radicals.[98]

Detection limits of pure THC injected into a GC-MS instrument are in the upper picogram range[99], but these can be improved by derivatisation of the phenol group, thus reducing polarity. A detection limit of 500fg has been obtained by derivatisation as the TMS ether.[58]

Rosenfeld[100] developed a GC-MS method for the simultaneous determination of delta-9-THC and 11-hydroxy-delta-9-THC (11-OH-delta-9-THC) in plasma using BSTFA:TMCS (9:1) as the derivatization reagent. It was noted that standard curves produced were linear from 0-200ng/ml of cannabinoid in plasma and recoveries obtained for delta-9-THC and 11-OH-delta-9-THC were 42% and 83% respectively. When the method was tested in an animal study, Rosenfeld observed that the maximum concentration of the metabolite obtained was less than 10ng/ml and the metabolite seemed to be disappearing quite rapidly from the plasma.

The first GC-MS method based on selected ion monitoring for the measurement of delta-9-THC in plasma was published by Agurell et al.[11] in 1973. The sample was cleaned-up using Sephadex LH-20 and measured to 1ng/ml of plasma in the underivatised state using $1',1'-^2H_2$-delta-9-THC as the internal standard. Other deuterated standards have been investigated but it was emphasized that deuterated standards prepared by a nonexchange method were superior to other standards.

Rosenthal et al.[59] used hexane as the extracting solvent, rather than polar solvents, to reduce pre-GLC clean-up by reducing the amount of co-extracted lipid material. Reasonable extraction efficiencies and detection limits of 0.5ng/ml were achieved. Hexane was also used by Harvey et al.[58] to extract delta-9-THC from plasma and tissues with a reported detection limit of 5pg/ml. This was achieved by measuring THC as its TMS derivative with a double-focussing MS tuned to a metastable ion rather than to the molecular or fragment ion, which greatly increased the selectivity and sensitivity of the instrument. Additional clean-up was not necessary.

The most sensitive GC-MS technique reported was the detection of pentafluorobenzoyl ester with electron-capture negative-ion chemical-ionization (NICI)

MS, where as little as 10fg could be detected.[101] GC-NICI-MS techniques were also used by other workers.[65,71,72]

2.4 Conclusion.

Sample preparation techniques applied in the methods reviewed above employed either solvent or solid-phase extraction procedures. The former may lead to emulsion formation and a requirement for centrifugation leading to prolongation of analysis time, while the latter may provide rapid and clean sample preparation.

Many end-step analytical techniques have been utilised. TLC and RIA/EMIT lack specificity and are mainly used for screening purposes. HPLC and GLC have been widely used although they are less specific than GC-MS. However, the use of proper derivatives made them a powerful tool for confirmatory and quantitation purposes. On the other hand, GC-MS provides the most sensitive and specific instrumental technique, but its complexity and high cost have restricted its use.

In view of the capabilities of all the instruments reviewed above, HPLC and GC-MS were chosen for this study. Results with each technique will be compared and evaluated in order to establish the most reliable detection method for the analysis of cannabinoids in blood samples.

CHAPTER 3.   SAMPLE PREPARATION.

3.1 Introduction.

Cannabinoids can be extracted from plant, resin and biological materials. But, it is very difficult to extract them completely from biological fluids, and it is particularly challenging for the Forensic Scientist to extract them fully from blood samples.

Many solvents have been used, which include hexane and acetonitrile. Christophersen[45] used hexane to extract THC from whole blood in his stability study and Bergman et al.[61] have successfully extracted THC from whole blood samples with two 15ml portions of hexane.

However, Foltz et al.[64] reported that greater reproducibility and efficiency of cannabinoid extraction procedure can be obtained if the cannabinoids in blood are first separated from proteinaceous material using a polar water-miscible solvent such as acetonitrile. Their extraction procedure involved an addition of deuterium-labelled internal standard to hemolysed blood followed by 3N sulphuric acid and high purity acetonitrile, which permitted quantitation of THC and its metabolites at concentrations as low as 0.2ng/ml of sample.

Whole or hemolysed blood samples, usually several days old, are the most common form of exhibit material received in most forensic Laboratories. Thus, the object of this study was;

(a)   to establish suitable methods for the analysis of cannabinoids in whole or hemolysed blood samples from post-mortem cases, and

(b)   to compare and evaluate suitable solvents and extraction techniques for the sample preparation procedure.

3.2 Experimental.

3.2.1 Materials.

Delta-9-THC and its trideuterated analog were obtained from Sigma Chemical Company while 11-nor-9-carboxy-delta-9-THC and its trideuterated analog were obtained from Alltech-Applied Science Laboratories. Delta-8-THC was obtained from Macor, Jerusalem. Stock and working standards were prepared by appropriate dilutions with methanol at a concentration of 1mg/ml and 1ug/ml respectively. The internal standards were the trideuterated analogs similarly prepared as above. All standards were stored in the freezer below $0^{O}C$.

All chemicals were A.C.S. grade and all solvents were HPLC grade obtained from BDH Laboratory, England. The derivatization reagent used was N,O-bis(Trimethylsilyl)-trifluoroacetamide (BSTFA), which was obtained from Pierce & Warriner (UK) Ltd. Extrelut (Merck) was washed with dichloromethane and dried before use.

Glassware used was silanised by rinsing with a 5% (v/v) solution of dimethyldichlorosilane in toluene followed by methanol and then acetone and dried. This treatment cut down the loss of cannabinoids due to adsorption on glass surfaces.

3.2.2 Apparatus.

GC-MS was carried out with an integrated VG 70-250S/SE system equipped with Hewlett-Packard HP 5890 gas chromatograph and HP 7673 automatic sampler unit.

The VG 70-250S/SE system has been developed from two separate units, the VG 70S/SE mass spectrometer and the VG 11-250 data system. The system utilizes all the facilities of the two separate units in a more compact form and is a complete mass spectrometer, control, data acquisition, data reduction and processing system.

The GC column was a Chrompack WCOT Fused Silica Column (25m x 0.32mm ID) with chemically bonded CP-SIL5CB coating material. It was connected to the mass spectrometer with the transfer line temperature held at 270°C. The GC temperature program was 200-300°C at 10°C/min. The injector temperature was 270°C and injections were done under splitless mode. Purified helium was used as the carrier gas at an average linear flow velocity of 60 cm/sec.

The MS was used in the electron impact (EI^{+}) selected ion recording mode (SIR), programmed to detect ion peaks at m/z 371.24, 386.26, 473.25, 488.28, and for the trideuterated analogs, at m/z 389.28 and 491.29. Electron impact spectra were recorded at 70eV ionization energy and a source temperature of 220°C. Photomultiplier was set at 500V. Perfluorokerosene (PFK) was used as a mass marking calibrator for daily tuning of the instrument.

3.3 Evaluation of Extraction with Diatomaceous Earth.

3.3.1 Procedure.

A blood sample (0.5ml) was diluted with 0.5ml of deionized water and was spiked with 50ul (50ng) of the delta-8-THC working standard. The sample was then poured into a glass column containing 1.5g of diatomaceous earth (Extrelut) and was allowed to equilibrate for at least 30 min. Extraction solvent (6.0ml) was added and the

eluent was collected into a separate tube. The eluent was evaporated to dryness under a nitrogen stream at 60°C on a hot plate. The dried residue was reconstituted in 50ul of hexane and 50ul of d3-delta-9-THC (IS) solution (50ng) was added, before storage in the freezer, until analysed by GC-MS.

Before analysis, the sample was again evaporated to dryness and 50ul of BSTFA was added to the residue. This derivatization process was carried out on a hot plate at 60°C for at least 30 min. A 1ul volume of the derivatized sample was injected into the GC-MS instrument.

The effects of the eluting solvent on the recoveries of THC were examined by using hexane, hexane-acetone (1:1), hexane-cyclohexane (1:1), hexane-dichloromethane (1:1), hexane-ethyl acetate (1:1), and hexane-isopropanol (1:1). Recoveries were measured by comparison of the ratio of delta-8-THC to the IS in the extract with the corresponding ratio in an unextracted standard.

3.3.2 Results and Discussion.

Because cannabinoids are highly lipophilic and have a strong affinity to blood proteins, it was difficult to extract them completely from blood samples. Table 3.1 gives the recoveries of delta-8-THC obtained with various solvent combinations by extrelut extraction.

**Table 3.1 : Recoveries of delta-8-THC from blood samples
by Extrelut extraction.**

Solvents	Total Recovery (%)		
	Batch 1	Batch 2	Average
hexane	27.6	30.4	29.0
hexane-acetone (1:1 v/v)	20.0	22.4	21.2
hexane-cyclohexane (1:1 v/v)	10.5	14.7	12.6
hexane-dichloromethane (1:1 v/v)	9.5	7.4	8.5
hexane-ethyl acetate (1:1 v/v)	7.7	11.6	9.7
hexane-isopropanol (1:1 v/v)	17.7	26.3	22.0
acetonitrile (n=6)			68.3 ± 8.2

From the above results, it was shown that recoveries were very poor. This was probably due to poor equilibration and mass transfer between the absorbed blood held in the matrix of the diatomaceous earth and the eluting solvent. Also, although all glassware had been previously silanised, losses due to adsorption on the walls of the vessel in which the solutions were prepared and used cannot be ignored.

Nakahara and Cook[57] explained that due to strong binding between cannabinoids and blood proteins, it is essential to precipitate the proteins in order to obtain good recoveries. Thus as recommended by Foltz et al.[64], a polar water-miscible solvent, acetonitrile (CH_3CN), was used for further extraction studies.

When CH_3CN was used to elute the spiked delta-8-THC and delta-9-THCCOOH, extracted as above using extrelut, recoveries obtained (n=6) were in the range of 58-82% and 23-55% respectively. Results were better than for the hexane-based

systems, but the reproducibility was poor. This was due to clogging of some of the extrelut columns by the precipitated proteins, thus giving lower extraction yields.

3.4 Solvent Extraction.

3.4.1 Procedure.

A blood sample (0.5ml) was diluted with 0.5ml of deionized water and was spiked with 50ul (50ng) of delta-8-THC working standard solution. CH_3CN (5.0ml) was added, followed by vortexing for 2 min. The solution was centrifuged for 10 min at 4000 rpm. The supernatant solution was collected and the process was repeated with 2ml of CH_3CN. The combined supernatant was evaporated to dryness under a nitrogen stream at 60^oC. The residue was reconstituted in 50ul of n-hexane and 50ul (50ng) of the deuterated analog internal standard solution was added. The sample was stored in the freezer until analysed. Derivatization and injection conditions were similar to those described earlier (Para. 3.3.1).

3.4.2 Results and Discussion.

The recovery for delta-8-THC was found to be 80.7%. This higher recovery obtained is due to the liquid-phase extraction which has been effectively employed for the determination of THC and its metabolites in minute volumes of blood samples ranging from clear serum to hemolyzed blood.[45,55,59-64,65-69] Acetonitrile deproteinization breaks the lipophilic bonds between the blood proteins and the cannabinoids, leaving the cannabinoids freely dissolved within the supernatant layer. Moreover, vortexing facilitates the extraction/equilibration process while centrifugation separates the extracted

cannabinoids from the precipitated blood sample. These factors account for a higher reproducibility in the extraction yields that were obtained.

3.5 Comparison of Diatomaceous Earth and Solvent Extraction.

3.5.1 Procedure.

A comparison between solid-phase and solvent extraction methods was done simultaneously using blood spiked with 50ng of delta-9-THC and delta-9-THCCOOH. Extraction procedure was as described earlier (Para. 3.3.1 & 3.4.1), with acetonitrile as the extraction reagent.

3.5.2 Results and Discussion.

The extrelut extraction procedure gave lower recoveries of 70.35% and 43.88% for delta-9-THC and delta-9-THCCOOH, respectively. Better results were obtained with the solvent extraction procedure despite the large variation of values obtained for delta-9-THCCOOH. Full recovery results are listed in Table 3.2.

**Table 3.2 : Recoveries of THC and THCCOOH by two
extraction procedures (n=4).**

Analyte	Extrelut (%)	Solvent (%)
THC		
sample : 1	75.19	79.80
2	70.81	86.17
3	69.62	80.80
4	65.80	81.49
average	70.36	82.07
THCCOOH		
sample : 1	55.09	111.21
2	24.91	92.82
3	41.01	55.70
4	54.51	71.22
average	43.88	82.74

Large variations in extraction efficiency did occur occasionally in blood analysis since cannabinoids and their deuterated analogs were tightly bound to the proteins. The extraction ratio between the two cannabinoids (THC/THCCOOH) was variable and ranged from 0.73 to 1.28. Thus, homogeneity of spiked samples is important for extraction efficiency. Moreover, since the samples were run in two batches on two different days, the variation of results obtained from those two days has contributed to the high values. The within-day and between-day variances were not studied in more detail.

3.6 Effect of pH on Recovery.

3.6.1 Procedure.

Further analyses were conducted to find out whether pH has any effect on the recoveries by solvent extraction. Acetate buffer of pH 5.0 and phosphate buffer of pH 7.4 were prepared and added to the blood samples separately, before the additions of

standards. Blood samples with deionized water added were also prepared for comparison. Extractions were carried out similarly to those described in Para. 3.4.1.

3.6.2 Results and Discussion.

Nakahara and Sekine[102], in their extraction analysis using disposable ODS-Minicolumns, reported that recoveries of drugs from biological samples were affected by the pH of the solution. They found that cannabinoids were absorbed maximally at pH 5.0 and minimally at pH 8.0. However, results obtained in the present study (Table 3.3) showed the opposite, with pH 5.0 having the lowest recovery with a large CV while deionized water gave better results.

Table 3.3 : Comparison of THC and THCCOOH recoveries by pH of solutions (n=5).

| | | Assays | |
Analyte	pH 5.0 (%)	water (%)	pH 7.4 (%)
THC			
mean recovery	80.13	83.23	82.45
std. deviation	14.01	3.57	4.15
CV	17.48	4.29	5.03
THCCOOH			
mean recovery	74.18	86.90	115.98
std. deviation	19.06	17.31	39.67
CV	25.69	19.92	34.20

Erratic results were obtained for THCCOOH at pH 7.4 with recoveries above 100%. However, it seemed that the binding between cannabinoids and blood proteins was not affected markedly by the pH of the solution. This can also be confirmed when comparing recovery results between Tables 3.2 and 3.3.

3.7 Preparation of Calibration Curves.

3.7.1 Procedure.

Calibration curves for THC and THCCOOH were determined as peak area ratios of drug/IS plotted against their corresponding spiked concentrations in blood. Since THC persists in blood of users at the low nanogramme level, standards were spiked in the range 0-80ng/ml and assayed. A solvent extraction procedure was followed as described before (Para. 3.4.1).

3.7.2 Results and Discussion.

The plots for THC and THCCOOH (Figure 3.1) were linear within the 0-80ng/ml range with linear regression correlation coefficient values of 0.9997 and 0.9939 respectively. This linear relationship of the solvent extraction procedure provides confidence that the method can be utilised as a preliminary step for further analysis.

3.8 Clean-Up Procedure.

The Bond Elut Certify II (Analytichem Int'l) extraction columns was specifically designed for the extraction of THC and its metabolite from biological samples. It provides two extracts containing THC and THCCOOH, respectively. The clean-up procedure as recommended by the manufacturer, with slight modifications, is as follows;

Figure 3.1 : Calibration Curves of Solvent Extracted Cannabinoids.
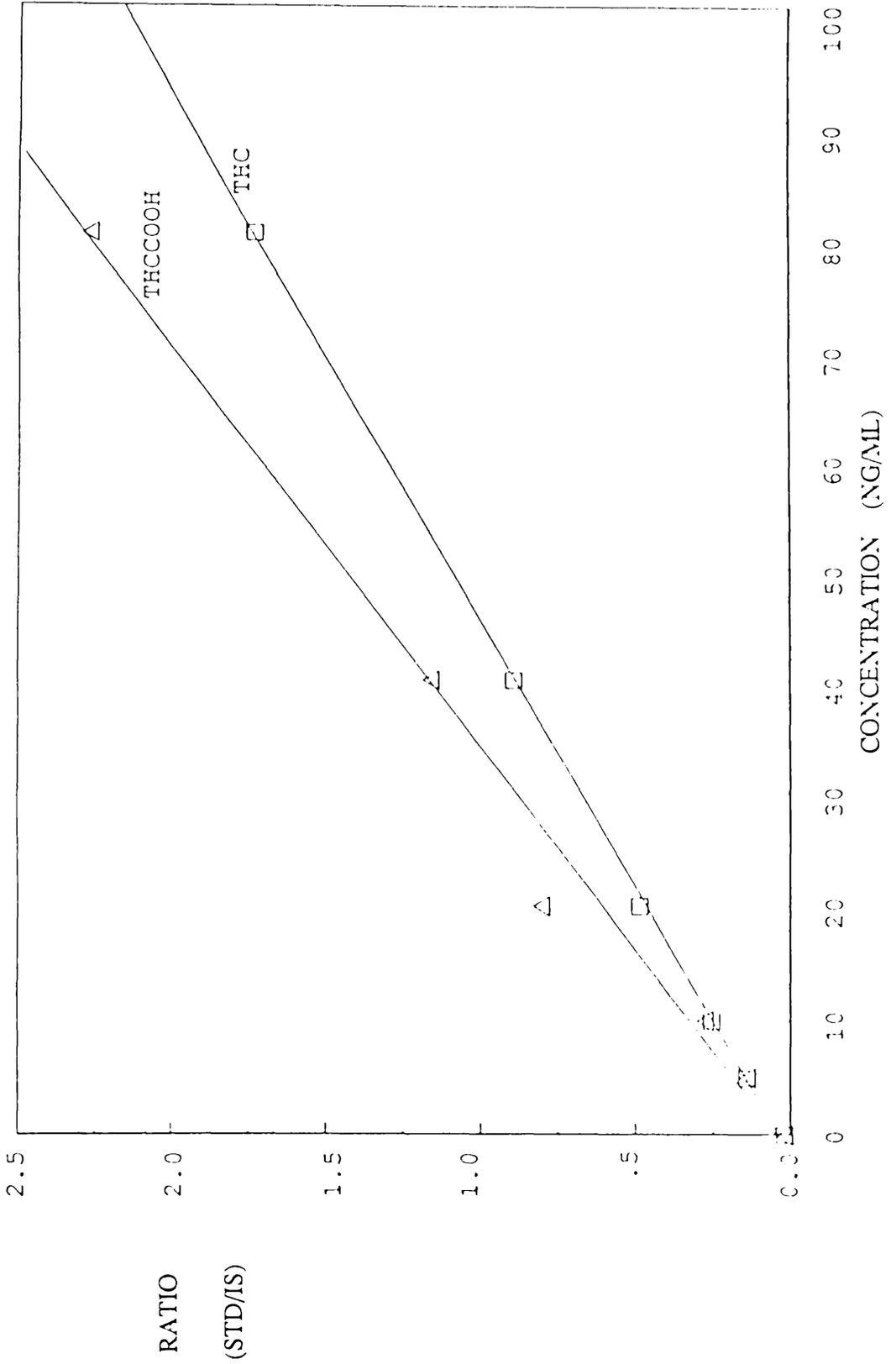
a) Solutions required.

    (1)   1.0M HCl (500 ml).

    (2)   0.1M Sodium acetate buffer, pH 7.0 with 5% methanol.

           In 450 ml deionized water was dissolved 6.8g of sodium acetate (MW 136.08). The pH was adjusted to 7.0 (+/-0.1) with 1.0M HCl while stirring. The volume was brought up to 500ml with deionized water and was mixed well. 25ml of the buffer was removed and discarded, while approximately 25ml of MeOH was added to bring back the total volume to 500ml. The buffer was mixed well and stored at room temperature.

    (3)   MeOH-H_2O (1:1) (200 ml).

    (4)   Hexane-ethyl acetate (95:5) (100 ml).

    (5)   Hexane-ethyl acetate (75:25) with 1% acetic acid solution (100 ml).

b) Specimen Preparation.

           Solvent extraction and deproteinization of spiked blood samples were done as before. However, instead of evaporating the supernatant to dryness, the samples were evaporated down to approximately 1ml under a slow stream of nitrogen at 30°C. To the samples were added 4.0ml of the prepared buffer.

c) Bond Elut Certify II Preparation.

           The column was placed into a Vac Elut Box, plugging any unused ports. To the column was added 2.0ml MeOH and 2.0ml buffer sequentially, making sure that the vacuum was turned off as soon as the solvent/buffer reached the top of the sorbent bed to prevent column drying.

d) Specimen Application.

           The specimen was poured into the column reservoir. The brass Vac Elut flow valve was opened in order to reduce the vacuum so that the specimen would be drawn

down slowly through the column. It should take at least two minutes to pass the specimen through the column.

e) Column Rinse.

Buffer (1.0ml) was added and passed through. The column was then dried under full vacuum (15 in. Hg) for five minutes.

f) Elution of Free Cannabinoids.

A labelled vial was placed into the Vac Elut which, contained a rack, and the tip of the Vac Elut delivery needles was wiped clean. 2.0ml of hexane-ethyl acetate (95:5) were passed through the column slowly, taking at least two minutes. The vial containing the eluant was removed.

g) Column Rinse.

The column was rinsed with 5.0ml MeOH-H_2O (1:1) and was then dried under full vacuum for five minutes.

h) Elution of Acidic Cannabinoid.

Another labelled vial was placed into a rack in the Vac Elut. The delivery needle was wiped clean and 2.0ml of hexane-ethyl acetate (75:25) with 1% acetic acid was added. The eluant was passed through the column slowly and was collected in the vial.

i) Sample Concentration and Injection.

Eluants collected in both vials were evaporated to dryness under a slow stream of nitrogen at $30^{o}C$ and were then reconstituted into either mobile phase or derivatization reagent for HPLC or GC-MS analysis respectively.

3.9 Conclusion.

Extraction using hexane and combinations of hexane with other organic solvents gave low recoveries. Acetonitrile proved to be a better extraction solvent since it can also deproteinize the blood sample, freeing the cannabinoids from the precipitated blood proteins.

The precipitates formed caused clogging of the extrelut columns. This resulted in variable extraction yields. However, the problem could be eliminated by employing a solvent extraction procedure, where the centrifugation step divides the cannabinoids in the supernatant acetonitrile solution from the precipitated blood sample. GC-MS analyses showed that more acceptable recoveries were obtained and the procedure was linear from 0 to 80ng/ml of THC and THCCOOH in blood samples. In addition, the overall recoveries of THC, CBD, CBN and THCCOOH after extraction and clean-up procedures were found to be above 85% (Table 4.6). Figure 3.2 shows a flow diagram for the recommended procedure for the solvent extraction of cannabinoids from blood samples.

Figure 3.2 : Recomended Solvent Extraction Procedure.
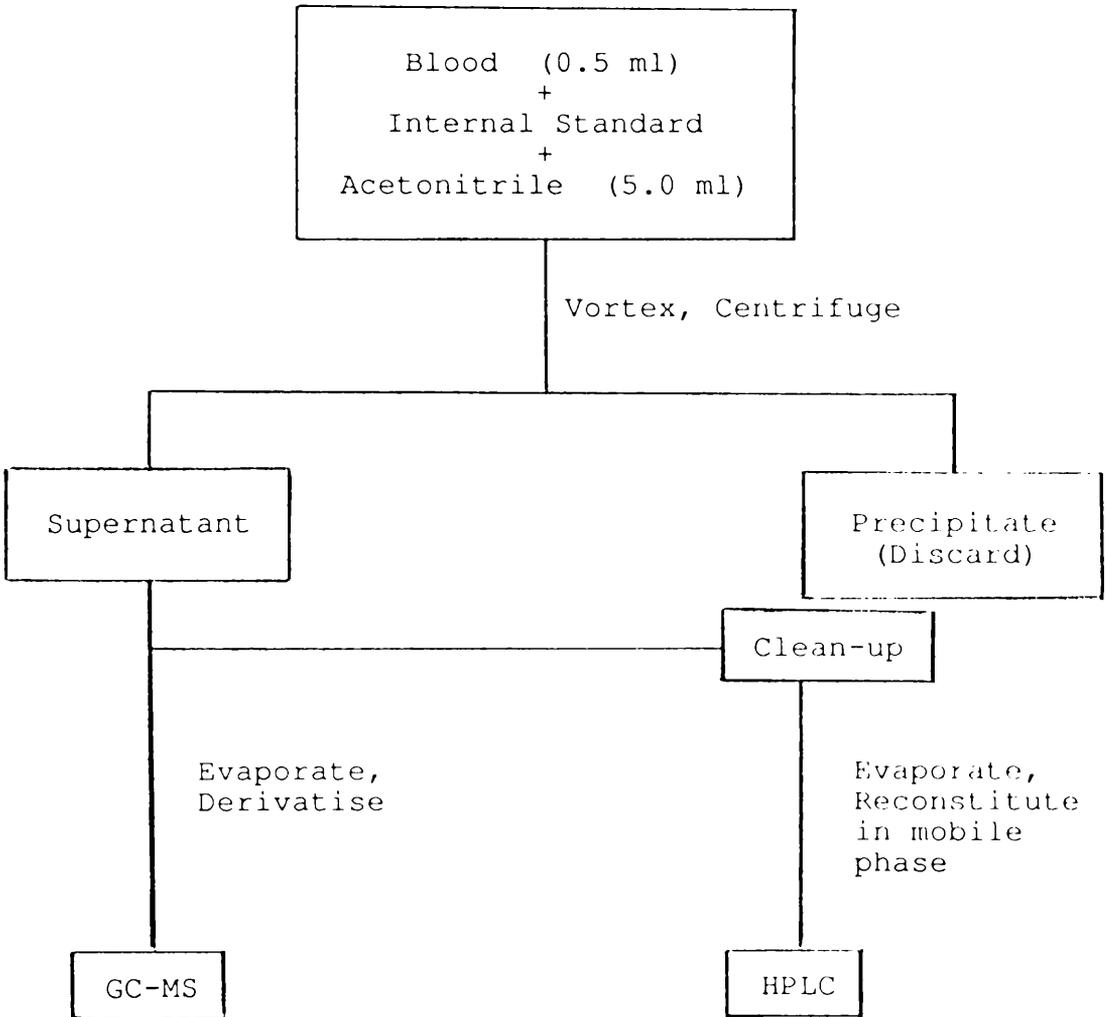
CHAPTER 4. HPLC ANALYSIS.

4.1 Introduction.

High pressure or high performance liquid chromatography (HPLC) has been developed and used since the late 1960's. It is a complementary technique to gas chromatography (GC) in that HPLC can be used to analyse compounds which are thermally labile. HPLC technique relies on interactions (adsorption, partition, ion exchange and molecular exclusion) between the mobile and stationary phases to cause differences in the migration rates of the components of substances analysed, thus achieving a separation.

The range of appropriate solvents for the mobile phase is extensive and the selection depends on many factors including the type of sample analysed and the detection system available. The strength and polarity of the solvent used will determine the type of chromatography involved. In adsorption and normal-phase liquid chromatography, the solvent strength increases with polarity while in a reversed-phase system, the solvent strength decreases with increasing polarity. The latter is particularly suitable for the separation of a homologous series of compounds with the same functional group.[103]

A variety of detectors is available for HPLC. These include the UV-visible spectrometer, the fluorescence and the electro-chemical detectors which can detect compounds that absorp in the UV-visible wavelengths, that can fluoresce and that can be readily oxidised or reduced within the voltage range of the electrodes, respectively. Other principles like the refractive index, radiochemical, atomic absorption spectrometry and the mass spectrometry have also been utilised as HPLC detectors.

Electrochemical detection is dependent upon some electroactivity of the solute in the mobile phase through oxidation or reduction, or the ability to act as a

conductor of an electrical charge. The electrochemical (coulometric) detector used is based on current measurements at fixed electrodes. Its principle is the application of a potential difference across two electrodes resulting in an electrochemical reaction at the surface of the working electrode. The electrons produced are attracted to the working electrode and the resultant current flow is measured, which is directly proportional to the number of molecules oxidised.

Generally, the sensitivity of electrochemical detection is good and is capable of detecting picograms of many electroactive species.[104] Moreover, it is also considered to be selective since only electroactive species will provide a response, and the selectivity can be varied by altering voltages for oxidation-reduction reactions. For example, easily oxidizable solutes could be selectively detected in the presence of solutes that are more difficult to oxidize by maintaining the potential below that necessary for oxidation of the latter but high enough to cause a reaction with the former.

At present, most HPLC analyses are carried out by direct means without the need to derivatise compounds. However, in some instances, direct analyses are not possible at the desired level of sensitivity. Thus, chemical derivatisation can be used to increase the sensitivity and selectivity of a detector, particularly in absorbance and fluorescence detection.

4.2 Materials.

4.2.1 Chemicals and Analytical Standards.

All solvents were of HPLC grade obtained from BDH Laboratory, England while all chemicals were Analar grade materials.

Four analytical standards were used in this study. Three of them, delta-9-THC, cannabinol and cannabidiol were obtained from Sigma Chemical Co., while the other , delta-9-THCCOOH was obtained from Alltech-Applied Science. The internal standards used, n-octyl p-hydroxybenzoate and phenylbutazone, were obtained from Lancaster Synthesis and Ciba-Geigy Pharmaceuticals, respectively. Stock and working standards were prepared in methanol by appropriate dilutions to give a concentration of 100 and 1ug/ml. Internal standards were also prepared the same way to give concentrations of 10ug/ml and 50ug/ml respectively.

4.2.2 Apparatus.

The system consisted of an LKB Bromma 2150 HPLC pump, a Rheodyne injection valve with a 20ul loop and two stainless steel columns from HPLC Technology, connected to each other with a 2cm stainless steel tubing. Sizes of the columns were 5cm by 4.6mm (pre-column) and 25cm by 4.6mm (analytical column) internal diameter, both packed with Lichrosorb RP8 (5 micron).

The detectors, both connected in series with the column, were a programmable Ultra-Violet multiwavelength detector (Waters model 490-MS) and an amperometric detector model LC-4B from Bioanalytical Systems (BAS, West Lafayette, USA). The electrochemical detector consists of a glassy carbon working and a silver/silver chloride reference electrodes. Glassy carbon electrodes were used because they showed much promise for detecting electrochemically oxidizable compounds with high current yield.[104] Both detector responses were recorded on a quadruple pen INSEIS chart recorder.

4.3 Selection of Mobile Phase.

4.3.1 Introduction.

Four mobile phases were evaluated, all containing the same components but at different ratios. In three of the mobile phases, the percent of CH_3CN was changed against a constant ratio of methanol-0.02N sulphuric acid (15:50), in order to find the optimal conditions for the separation of the cannabinoids. This determined the capacity factors (k') which were measured as a function of the organic modifier content. The fourth mobile phase had a higher methanol content.

All mobile phases were degassed by purging with helium gas for 10 min before use. The four mobile phases, evaluated at a flow rate of 1.5ml/min, were;

(1) CH_3CN-MeOH-0.02N H_2SO_4 (15:15:50, v:v:v)

(2) CH_3CN-MeOH-0.02N H_2SO_4 (35:15:50, v:v:v)

(3) CH_3CN-MeOH-0.02N H_2SO_4 (65:15:50, v:v:v)

(4) CH_3CN-MeOH-0.02N H_2SO_4 (16:7:6, v:v:v)

4.3.2 Procedure.

Blood (0.5ml) was spiked with 100ul of delta-9-THC, CBD, CBN and delta-9-THCCOOH. Extraction and clean-up procedures were followed as recommended earlier (Para. 3.6). Both extracts of the sample from the clean-up procedure were evaporated to dryness under a nitrogen stream at $60^{\circ}C$ and were later reconstituted into 30ul of mobile phase. A 25ul sample was injected into the HPLC system.

4.3.3 Results and Discussion.

All four mobile phases were examined with the standards at a flow rate of 1.5ml/min (Figure 4.1). They all eluted the cannabinoids with capacity factors (k') of THC ranging from 1.57 (with mobile phase 4) to 13.36 (with mobile phase 3). Table 4.1 gives the complete list.

Table 4.1 : Comparison of k' values for THC, CBD, CBN, THCCOOH and IS with different mobile phases.

| Mobile | Capacity Factors (k') of Analyte | | | |
Phase	THC	CBD	CBN	THCCOOH
4	1.57	1.00	1.36	0.64
1	2.80	2.20	2.60	0.80
2	8.86	6.86	7.86	3.14
3	13.36	8.64	11.71	4.43

From the above list, mobile phases 2 and 3 gave the desired retention volume of eluted analytes with good separations. However, it was observed that sharper peaks were obtained with mobile phase 3 due to its higher CH_3CN content. Thus, mobile phase 3 was used for the HPLC system.

(a) Mobile Phase 1
      UV 212nm, AUFS 0.02

(b) Mobile Phase 2
      UV 212nm, AUFS 0.02

(c) Mobile Phase 3
      UV 212nm, AUFS 0.05

(d) Mobile Phase 4
      UV 212nm, AUFS 0.1

Retention Time (min)
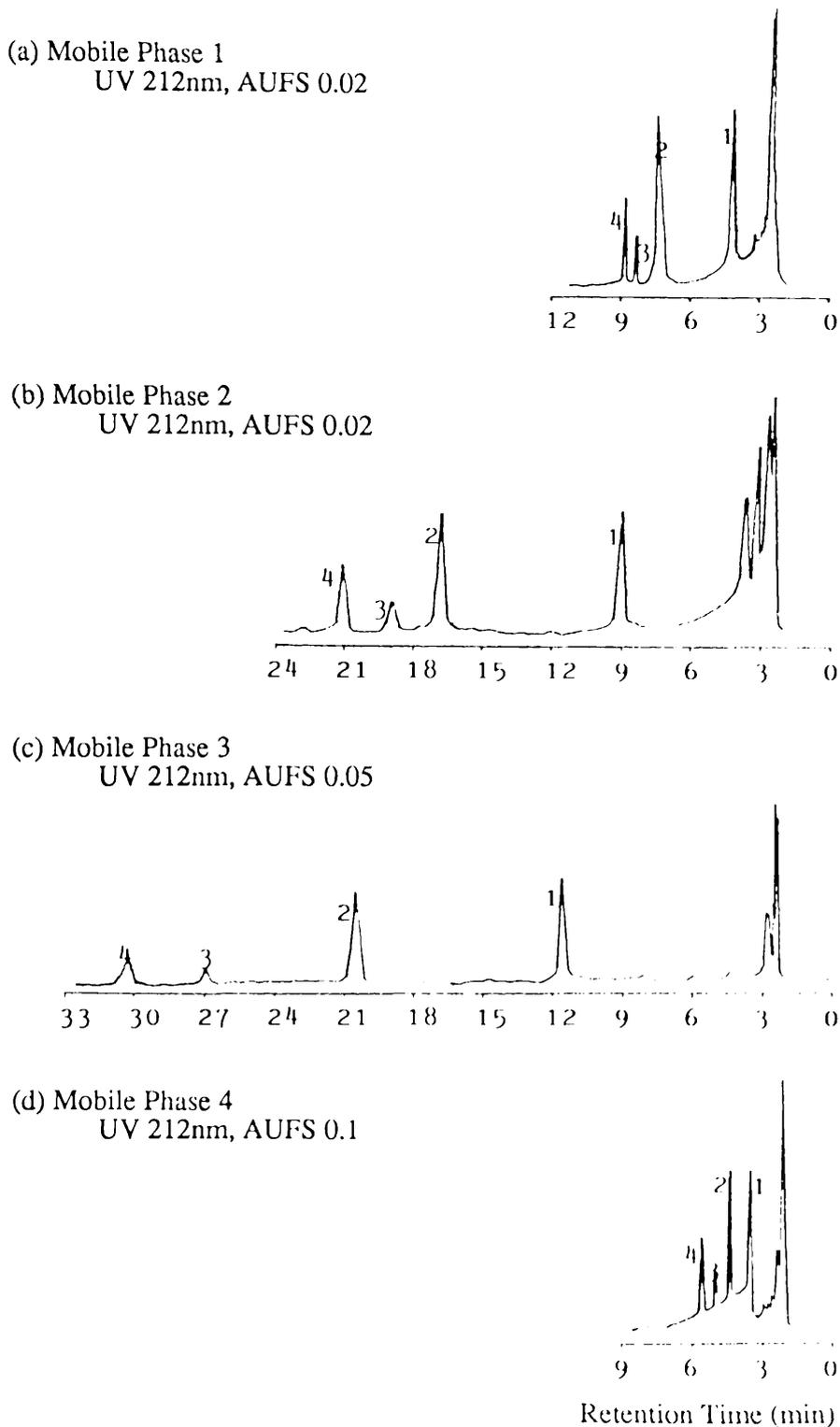
Figure 4.1 : Cannabinoid Chromatographs by Different Mobile Phases.
(1) THCCOOH   (2) CBD   (3) CBN   (4) THC.

4.4 Detector Optimisation.

4.4.1 Selection of UV Wavelengths.

4.4.1.1 Procedure.

The absorbance maxima in the UV spectra of the four standards used were obtained by running the standards on a Hewlett-Packard UV diode array spectrophotometer model HP 1845A. A solution containing 100ul of the working standard in 5.0ml of mobile phase was used (Figures 4.2a - d).

4.4.1.2 Results and Discussion.

All cannabinoids analysed showed maximum absorbances in the range of 200-280nm (Table 4.2). Since Kanter et al.[82] and Valentine et al.[83] have reported that greater sensitivity for UV detection could be obtained by monitoring at shorter wavelengths, the THC maximum absorbance at 212nm was chosen for the UV detector. Besides that, 220nm was also used for comparison since the wavelength has been extensively used by researchers.[61,66-7]

Table 4.2 : UV absorbance wavelengths for THC, CBD, CBN, and THCCOOH.

Analyte	Maximum UV Absorbance Wavelengths (decending order)
THC	212, 280.
CBD	202, 206, 212, 226.
CBN	220, 224, 228, 286, 204.
THCCOOH	258, 230, 234, 212, 218.
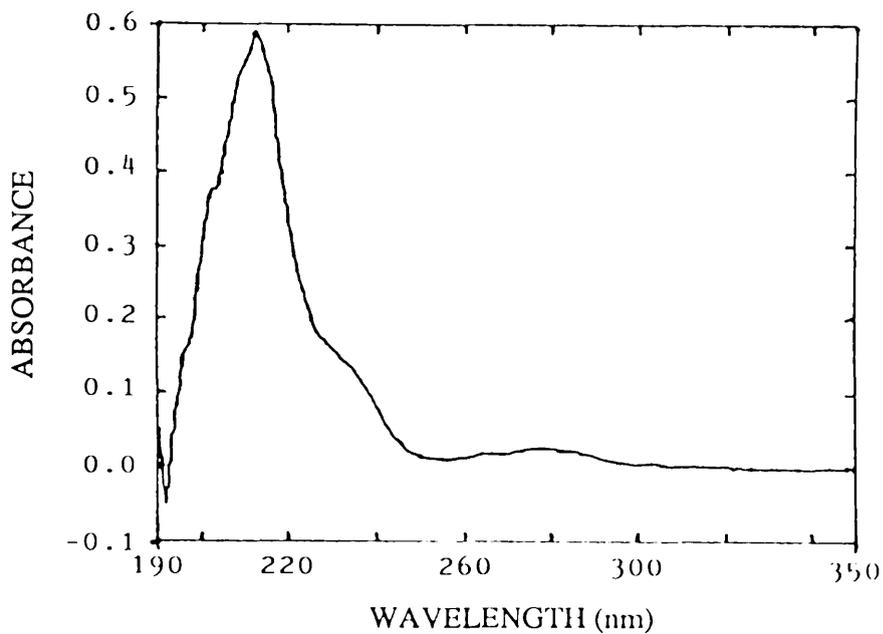
Figure 4.2a : UV Spectra of THC in Mobile Phase 3.
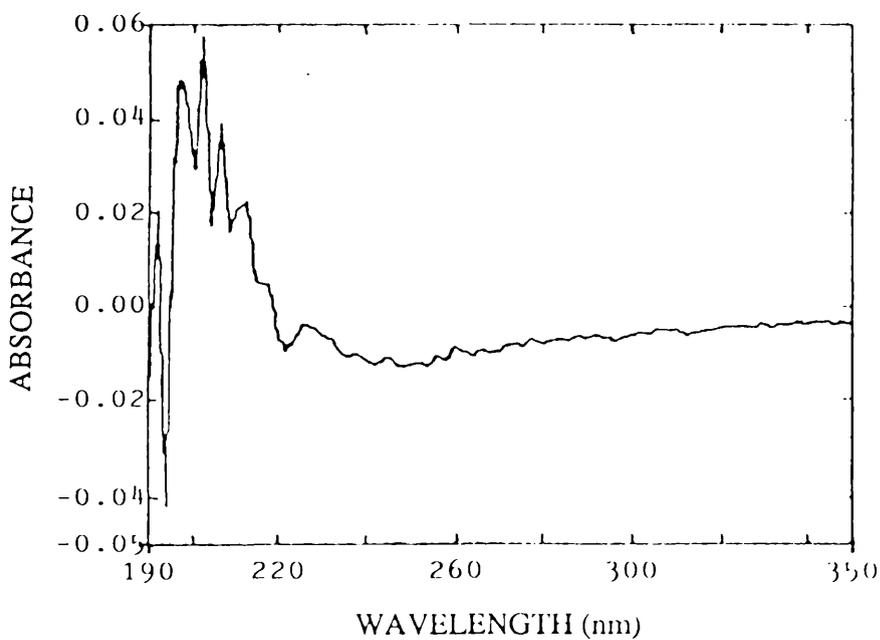
Figure 4.2b : UV Spectra of CBD in Mobile Phase 3.
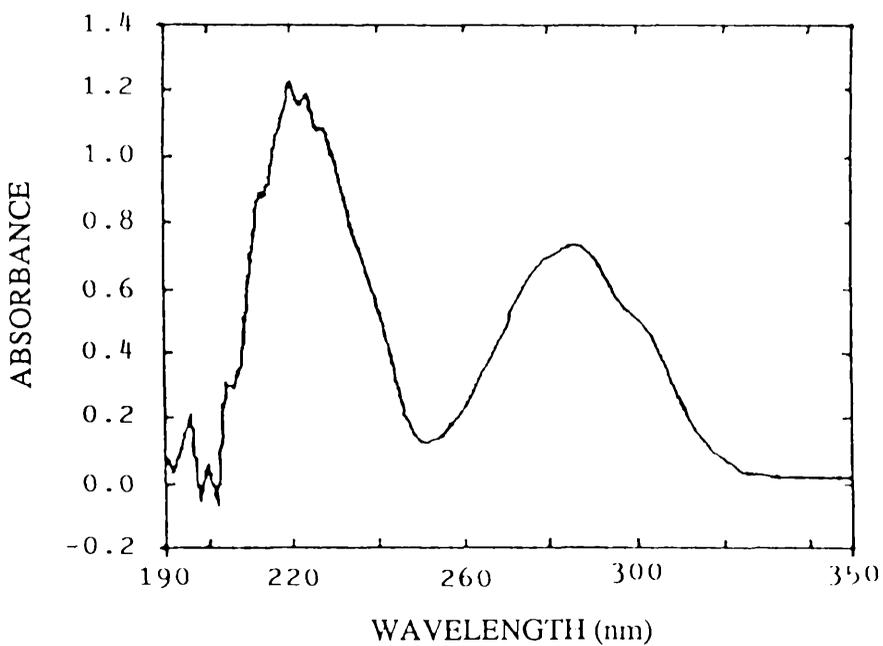
Figure 4.2c : UV Spectra of CBN in Mobile Phase 3.
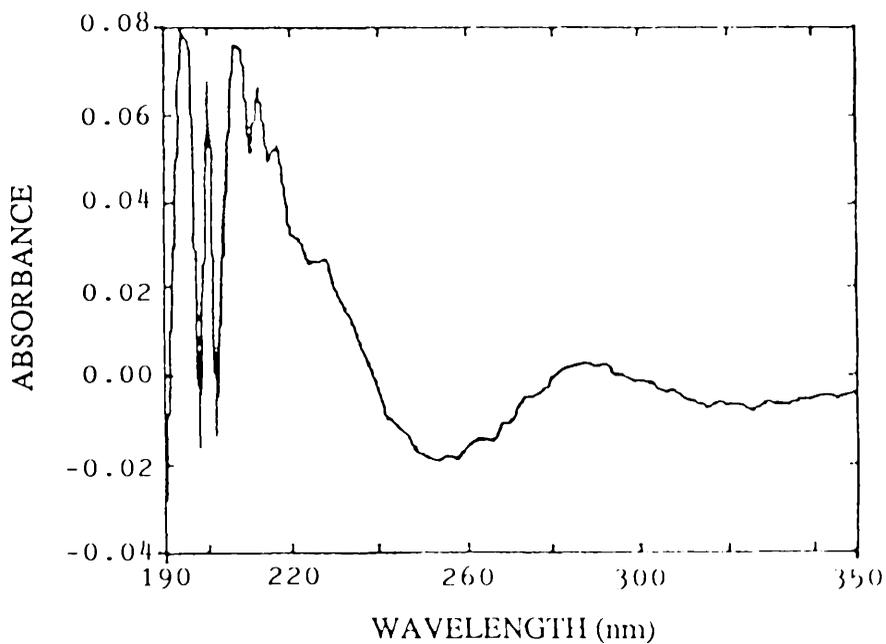
Figure 4.2d : UV Spectra of THCCOOH in Mobile Phase 3.

4.4.2 Variation of ECD Potential.

4.4.2.1 Procedure.

Electrochemical detection of standards was evaluated at an applied potential of +1.10 Volts vs the reference electrode. However, the potential applied to the working electrode was varied in order to observe its effects on sensitivity since electrode responses vary with the applied potential. A full cyclic voltamogram was not carried out because the required equipment was not available.

4.4.2.2 Results and Discussion.

At an applied potential of +1.10V, all cannabinoid peaks were at a maximum height. When the potential was lowered to +1.00V, no peaks were observed for THC and THCCOOH while those for CBD and CBN were reduced significantly. Increasing the potential from +1.10V to +1.20V only results in decreasing the sensitivity. Hence, an applied potential of +1.10V was chosen for the electro-chemical detector because it gave high response with low background noise. Full results are listed in Table 4.3.

Table 4.3 : Effect of Potential Variation on Sensitivity of Standards.

Applied Potential (V)	Peak Height (cm)			
	THC	CBD	CBN	THCCOOH
1.00	-	0.20	0.30	-
1.10	1.50	2.60	2.70	7.90
1.15	0.65	1.20	1.10	7.70
1.20	0.25	0.50	0.45	3.25

4.4.3 Detection Limits (Sensitivity).

4.4.3.1 Procedure.

With UV and EC detectors set at 212nm (AUFS 0.01), 220nm (AUFS 0.01) and +1.10V (range 5nA) respectively, the detection limits (signal-to-noise ratio > 3) of the standards were determined. Standard mixtures of concentration 100ng/ml were extracted and analysed on the HPLC system. The concentration was subsequently decreased by a factor of two for further injections.

4.4.3.2 Results and Discussion.

Results showed that THCCOOH gave the lowest possible detection limits for both detectors while CBN had the poorest sensitivity (Table 4.4). As expected, electro-chemical detection was more sensitive, with detection limits ranging from 1.0-2.5ng/ml of cannabinoids while the detection limits with the UV detector ranged from 10-100ng/ml and 10-60ng/ml of cannabinoids at wavelengths 212nm and 220nm respectively.

Table 4.4 : Detection Limits of Cannabinoids by UV and EC Detectors.

Analyte	Limits of Detection (ng/ml)		
	UV 212nm	UV 220nm	EC +1.1 V
THC	20	20	2.5
CBD	20	20	1.0
CBN	100	60	2.5
THCCOOH	10	10	1.0

4.5 Selection of Internal Standards.

4.5.1 Procedure.

The internal standard (IS), n-octyl p-hydroxybenzoate, as recommended by Nakahara[105] was used. It was found that the IS was separated by the Bond Elut Certify II column into the THC portion. This left the acidic metabolite without any IS.

Analgesic drugs and chemicals with either hydroxyl or carboxyl functional groups and also those with long carbon chains were tested as internal standards for THCCOOH analysis. Ten test compounds were prepared with appropriate dilutions to give concentrations of 10ug/ml in methanol. These were injected into the HPLC system in order to select the best possible IS for the THCCOOH extract.

4.5.2 Results and Discussion.

It can be seen that UV detection gave a good response for all of the possible IS examined. However, for EC detection, only six out of the ten tested were oxidized at the electrode surface, thus giving good responses (Table 4.5). When the k' values of these six IS were compared with chromatograms of the acidic extracts of blank blood, phenylbutazone was observed to be the best choice since it was eluted within 6.45 min, clear of the solvent front and in a range where not many interfering peaks occurred. Hence, phenylbutazone was chosen as the IS for the acidic cannabinoid assays.

Table 4.5 : Retention Times and Responses of some Analgesic Drugs and Chemicals in Mobile Phase 3.

Drug/Chemical (obtained from)	Retention Time (min)	Method of Detection		
		UV 212nm	UV 220nm	ECD +1.10V
3,3-Naphthalene dicarboxylic acid (Aldrich Chem. Co.)	3.00	**	**	*
Aspirin (Hopkin & Williams Ltd.)	3.30	**	**	**
Salicylic acid (unknown)	3.45	**	**	**
Naproxen (Syntex Pharm. Ltd.)	4.35	**	**	**
Ketoprofen (May & Baker Ltd.)	4.35	**	**	ND
Anthracenecarboxylic acid (Aldrich)	4.65	**	**	**
Fenbufen (Lederle Lab.)	4.80	**	**	ND
Oxyphenbutazone (Ciba-Geigy Pharm.)	5.55	**	**	**
Ibuprofen (Boots Co.Ltd.)	5.85	**	**	ND
Phenylbutazone (Ciba-Geigy)	6.45	**	**	**

　　** = good response
　　 * = poor response
　ND = not detected

4.6 Recovery and Calibration Curves.

4.6.1 Procedure.

Standards of concentration 100ng/ml were analyzed according to the extraction and clean-up procedures described earlier (Para 3.6). Before extraction, all

samples were also spiked with 50ul of internal standards (n-octyl p-hydroxybenzoate, 10ug/ml and phenylbutazone, 50ug/ml). Analytical recoveries were calculated by comparing peak height ratios of the extracted standards to the non-extracted standards.

To determine the calibration curves for cannabinoids, spiked blood samples of concentration 0, 5, 25, 50, 75, 100, 150, and 200ng/ml were prepared. The detectors were set at UV AUFS of 0.08 and ECD range of 100nA. These values were lowered to 0.03 and 20nA, respectively, as the cannabinoids concentrations decreases. Peak height ratios of cannabinoids/IS were plotted against the concentration.

4.6.2 Results and Discussion.

The high recoveries obtained indicate that most of the cannabinoids were recovered during the extraction and clean-up procedures (Table 4.6). All recoveries were obtained without many co-extracted interferences, especially the THC extract. Recoveries of delta-9-THC and delta-9-THCCOOH did not vary greatly from those results obtained for solvent extraction studies (Table 9). In fact, UV and ECD detection for this HPLC system gave higher calculated recoveries than GC-MS under electron impact mode.

The calibration plots (Figure 4.3a to 4.3c) showed that THC, CBD and CBN were linear from 0-200ng/ml. At UV wavelengths 212nm, 220nm and ECD working potential of +1.10V, linear regression analysis indicated that the correlation coefficients for THC were 0.9995, 0.9962 and 0.9989; CBD were 0.9983, 0.9979 and 0.9990; and CBN were 0.9868, 0.9909 and 0.9965 respectively.

On the other hand, only electrochemical detection gave a linear calibration curve for THCCOOH from 0-200ng/ml. UV detection at both wavelengths was linear from

**Table 4.6 : Recoveries of Cannabinoids (n=3) Determined After Extraction
and Clean-Up Procedures.**

Analyte	Recoveries (%)		
	UV/212nm	UV/220nm	EC/+1.1V
THC			
sample : 1	92.30	92.30	94.60
2	85.71	80.00	87.88
3	95.35	94.44	97.62
average	91.12	88.91	93.36
CBD			
sample : 1	102.30	104.80	102.00
2	93.75	87.50	100.00
3	84.62	88.24	81.97
average	93.56	93.51	94.66
CBN			
sample : 1	92.30	93.30	105.00
2	83.33	83.33	94.83
3	94.74	90.00	96.30
average	90.12	88.88	98.71
THCCOOH			
sample : 1	85.48	83.33	78.20
2	98.44	98.00	94.44
3	90.14	90.74	94.74
average	91.35	90.69	89.13

0-100ng/ml. But, from 100-200ng/ml, it gave a concave isotherm which is rarely observed in liquid chromatography. These non-ideal isotherms with increasing quantity of sample injected, usually result in a slight change of the retention times of the peaks.[104] However, these slight changes were not observed.

Poor extraction yields for the internal standard which gave rise to higher ratios might be one of the answers for the results obtained at concentrations 100-200ng/ml. Other reasons might be due to interference from co-extracted materials or even possibly due to wrongly prepared spiked samples. Therefore, UV detection for THCCOOH at wavelengths 212 and 220nm is considered to be linear only from 0-100ng/ml with correlation coefficients and slopes of 0.9972, 0.0156 and 0.9746, 0.0150 respectively.

Figure 4.3a : Calibration Curves of Cannabinoids by UV 212nm.
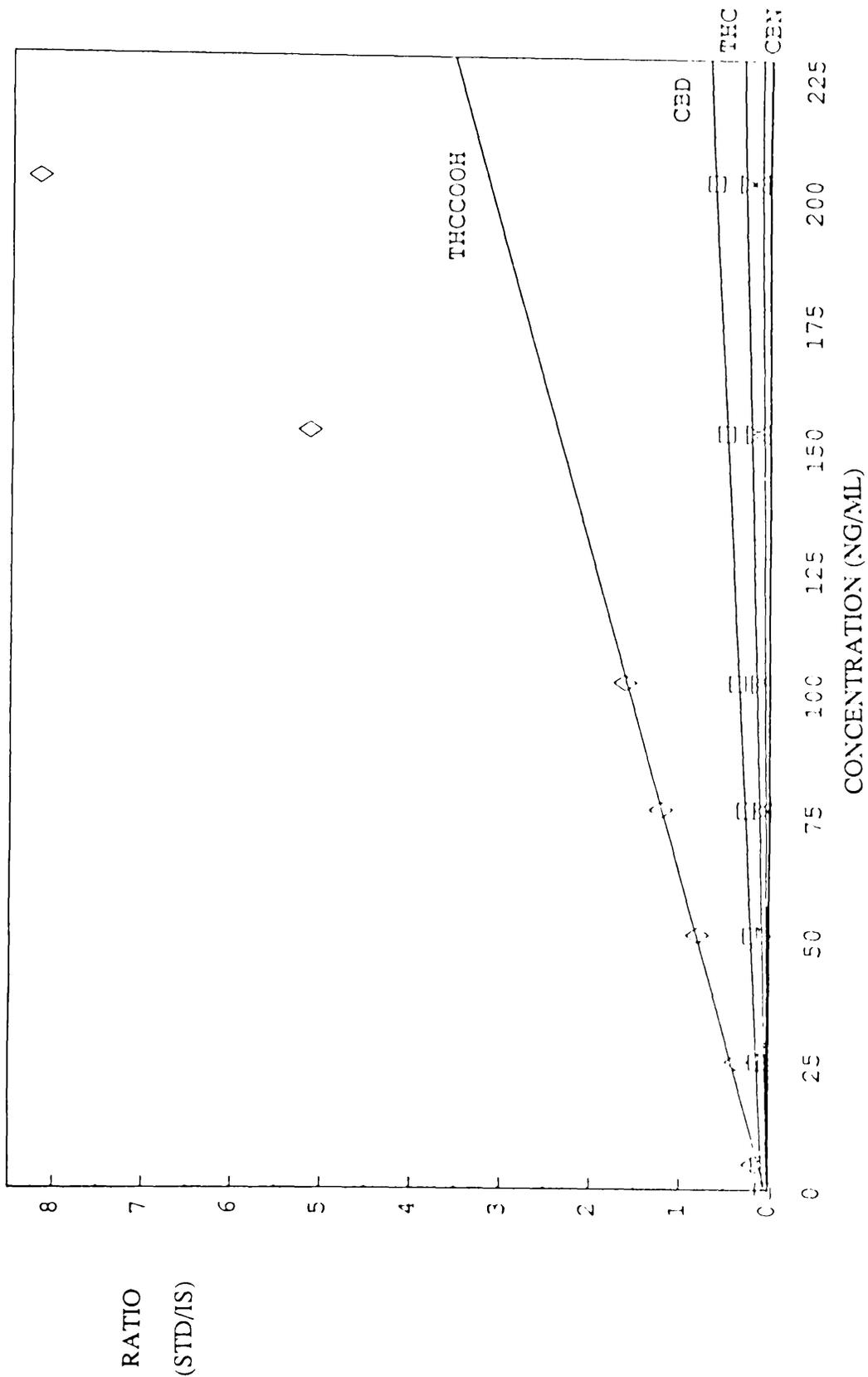
Figure 4.3b : Calibration Curves of Cannabinoids by UV 220nm.

RATIO
(STD/IS)

CONCENTRATION (NG/ML)

THCCOOH

CBD
THC
CBN
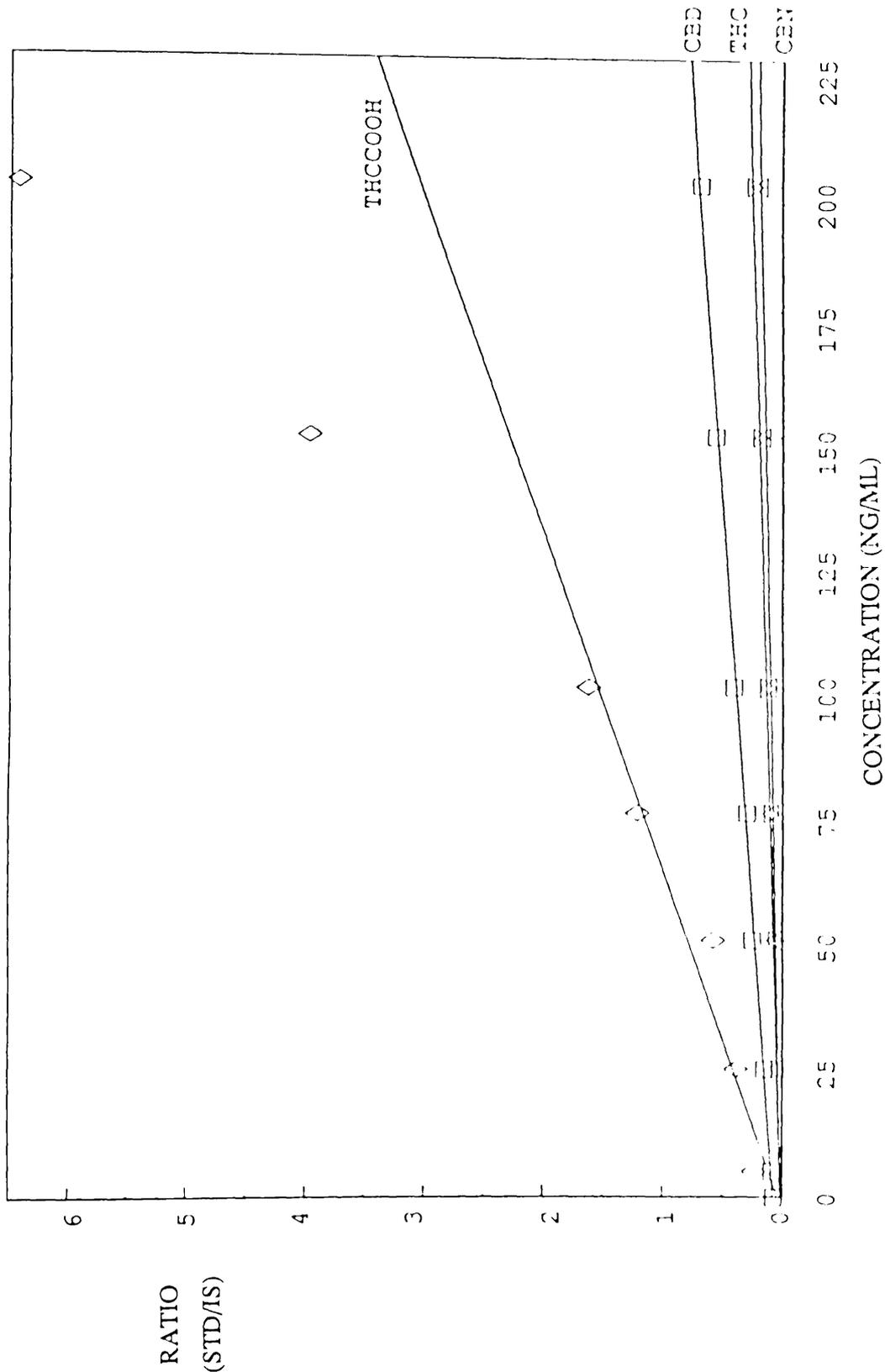
Figure 4.3c : Calibration Curves of Cannabinoids by ECD +1.10V.
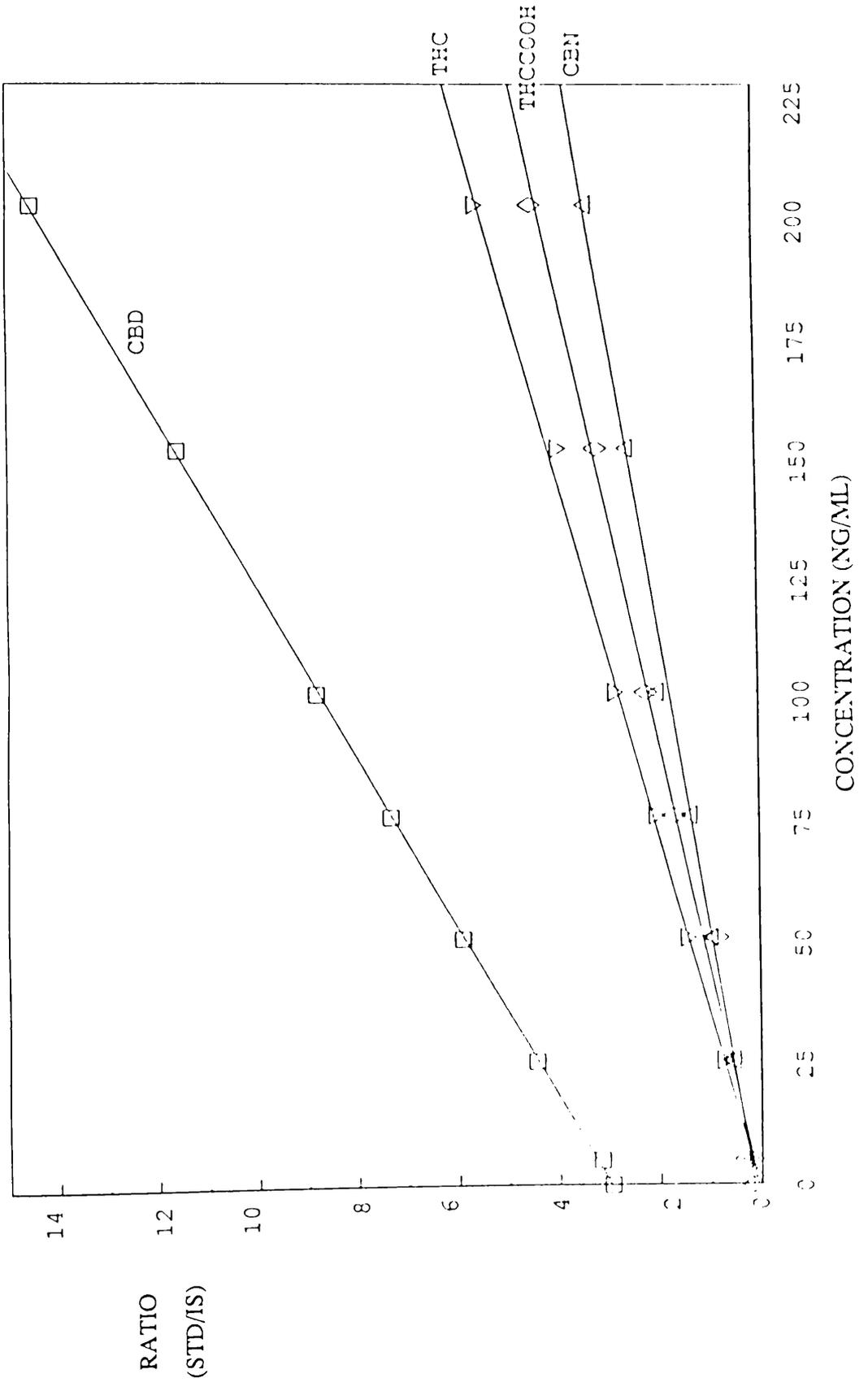
4.7 Case Sample Analysis.

4.7.1 Sample Collection.

The Toxicology section of the Department of Forensic Medicine & Science at Glasgow University undertakes analyses of drugs in samples of blood and urine received from pathologists employed by the Crown Office, the police, clinicians and solicitors. These samples can be divided into cases such as homicides, suicides, and road traffic accidents.

A total of eight post-mortem blood samples were received for this study. The age distribution ranged from 16 to 71, and five of the samples were from male victims. Cases involved were deaths either due to road traffic accidents, drug overdoses or inhalation of vomitus. All samples had given positive results with the routine RIA screen.

4.7.2 Procedure.

Internal standards (50ul of working standard solution; 50ng) were added to 0.5ml of the case blood samples. Deionised water was added to make the volume up to 1.5ml. All extraction and clean-up procedures were conducted exactly as recommended (Para 3.6), together with a blood sample spiked with standards of concentration 100ng/ml and a blank sample spiked with internal standards. The extracts were analysed by HPLC using the instrument described earlier, with solvent system 3 (Para. 4.3.1).

4.7.3 Results and Discussion.

It is to be expected that most samples will show negative results for the parent compound, THC and positive results for the metabolite. When smoked, THC

reaches a maximum blood level within a few minutes and then falls rapidly to a few ng/ml of blood. Only 3% of THC present in the blood is in the free state, the remaining 97% is bound mainly to proteins, with about 9% bound to blood cells, but THCCOOH is even more strongly bound with values up to 99% have been reported.[106] Most workers in this field report that a percentage of samples showing positive in the initial immunoassay screen subsequently show negative in the confirmatory analysis for THC and THCCOOH, presumably because of the presence of other cross-reacting substances which are not detected in the confirmatory test.

Results obtained for the eight samples (Table 4.7) showed that THC and CBN were absent for all of the test samples. Electro-chemical detection of CBD gave five positives whereas negative results were obtained with UV detection. These were unexpected and very surprising because CBD is usually the major cannabinoid found in fiber type cannabis plant. However, elevated values due to interference cannot be ignored since the blank too shows a peak at the same retention time as CBD, which was not present during earlier studies. Figures 4.4a to 4.4c shows the HPLC chromatographs of cannabinoid sample detected by UV and ECD..

Detection of THCCOOH by both detectors showed positive results for all test samples. However, some of them were below the safe detection limits of the method and had to be considered as negative. It was also observed that results obtained by UV detection at 220nm gave higher values than at wavelength 212nm, thus showing that the former wavelength is more sensitive than the latter. But, when comparing results obtained by UV detection at 220nm with those from ECD, it was observed that neither UV or ECD proved to be reliable in giving a true picture of the metabolite level in blood. Thus, half of the samples gave a higher result for ECD compared to UV at 220nm, whereas the other half were lower than UV at 220nm.

Table 4.7 : Concentration of Cannabinoids (ng/ml) detected in test samples by HPLC.

		Case Samples						
Analyte	1 M/16	2 F/18	3 F/71	4 F/64	5 M/49	6 M/59	7 M/54	8 M/30
THC								
212nm	–	–	–	–	–	–	–	–
220nm	–	–	–	–	–	–	–	–
ECD	–	–	–	–	–	–	–	–
CBD								
212nm	–	–	–	–	–	–	–	–
220nm	–	–	–	–	–	–	–	–
ECD	13.0	6.0	25.2	–	60.2	–	–	1.2
CBN								
212nm	–	–	–	–	–	–	–	–
220nm	–	–	–	–	–	–	–	–
ECD	–	–	–	–	–	–	–	–
THCCOOH								
212nm	1.7*	4.8*	1.7*	3.5*	21.9	6.6*	0.5*	5.7*
220nm	5.4*	12.7	2.4*	12.6	23.0	12.1	3.5*	8.4*
ECD	1.2	13.0	3.8	12.4	23.8	1.4	1.6	16.0

–   Not detected
*   Values were below the safe detection limits measured earlier (Para 4.4.3).

Figure 4.4a : Chromatographs of Cannabinoid Samples Detected by UV 212nm.
(1) phenylbutazone   (2) THCCOOH   (3) n-octyl p-hydroxy benzoate
(4) CBD   (5) CBN   (6) THC.

(a) Unextracted Standards

AUFS 0.05

(b) Extracted THC Fractions:

(i) Blank with IS

AUFS 0.04

(ii) Spiked Sample 100ng/ml

AUFS 0.07

(iii) Case Sample

AUFS 0.15

(c) Extracted THCCOOH Fractions:

(i) Blank with IS

AUFS 0.03

(ii) Spiked Sample 100ng/ml

AUFS 0.06

(iii) Case Sample

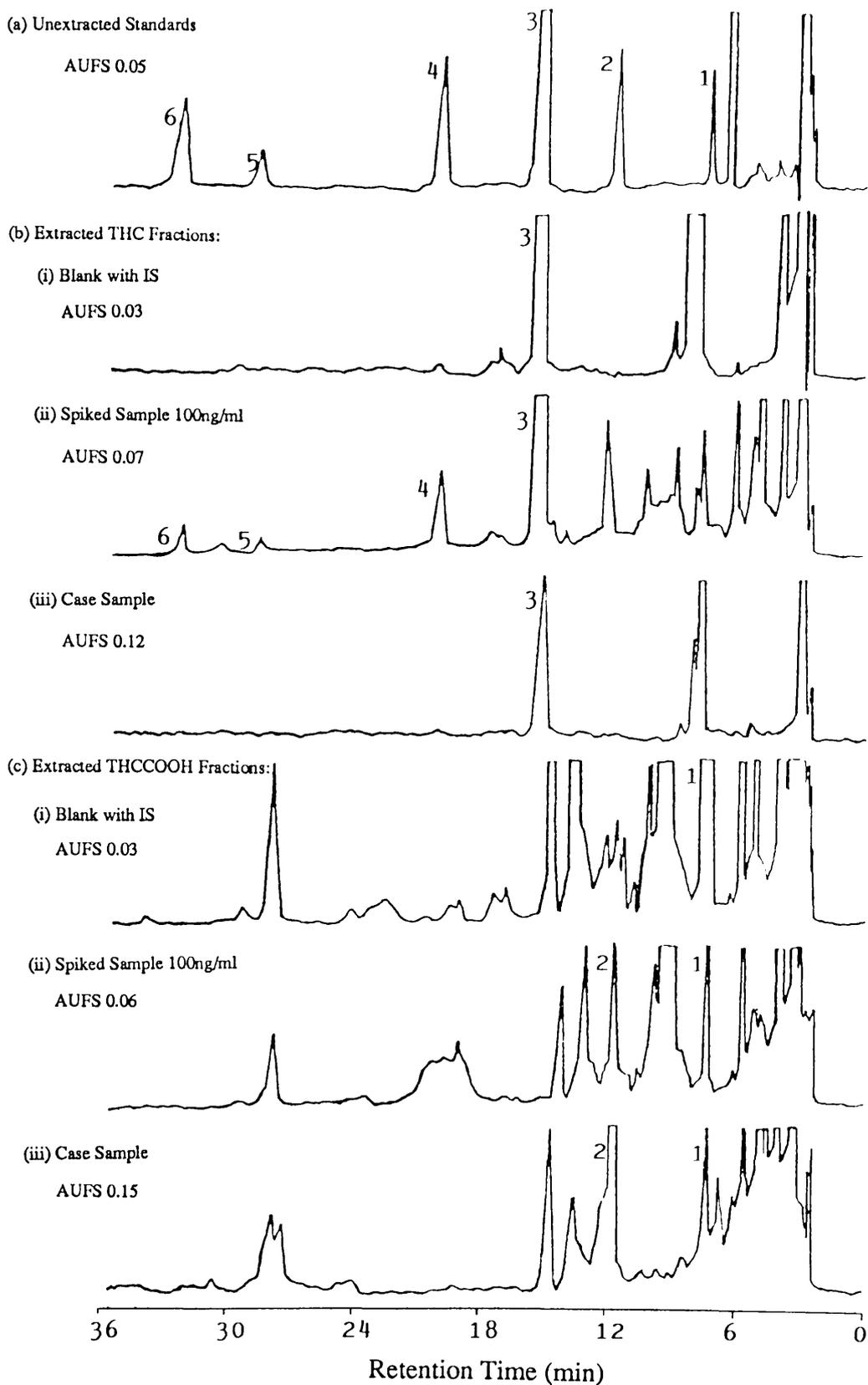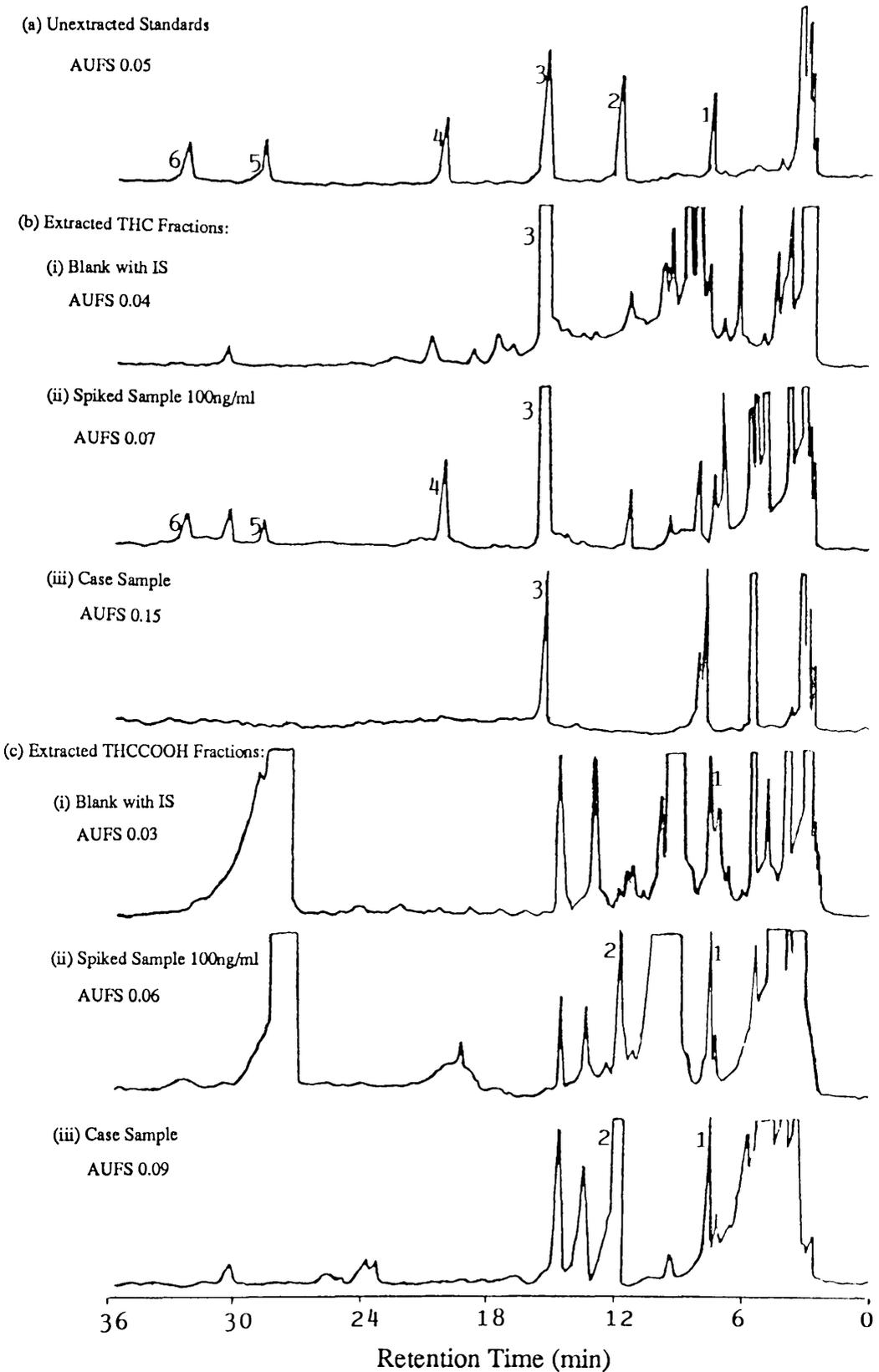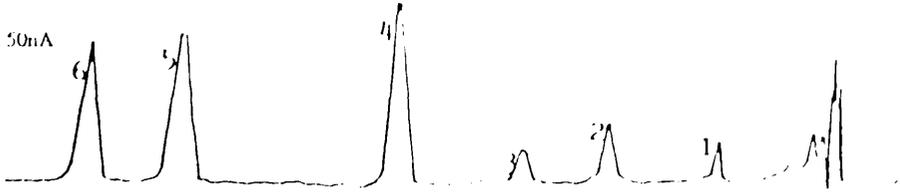AUFS 0.09

Retention Time (min)

Figure 4.4b : Chromatographs of Cannabinoid Samples Detected by UV 220nm.
(1) phenylbutazone (2) THCCOOH (3) n-octyl p-hydroxy benzoate
(4) CBD (5) CBN (6) THC.
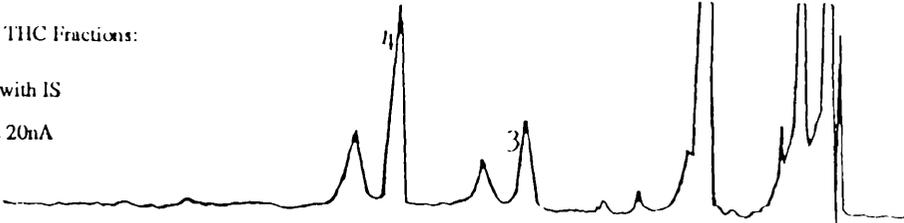
(a) Unextracted Standards

Range 50nA

(b) Extracted THC Fractions:

(i) Blank with IS

Range 20nA

(ii) Spiked Sample 100ng/ml

Range 100nA
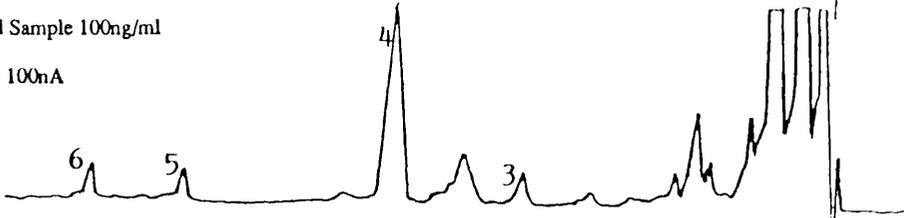
(iii) Case Sample
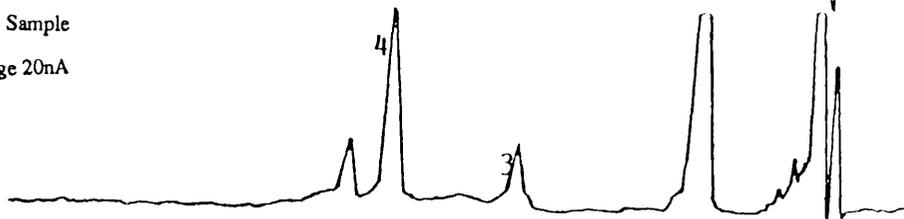
Range 20nA

(c) Extracted THCCOOH Fractions:

(i) Blank with IS

Range 20nA

(ii) Spiked Sample 100ng/ml

Range 50nA

(iii) Case Sample

Range 20nA

36   30   24   18   12   6   0

Retention Time (min)

Figure 4.4c : Chromatographs of Cannabinoid Samples Detected by ECD +1.10V.
(1) phenylbutazone   (2) THCCOOH   (3) n-octyl p-hydroxy benzoate
(4) CBD   (5) CBN   (6) THC.

If HPLC is the only method available for the analysis of cannabinoids in blood and urine, the use of more than one type of detector is clearly advisable to improve the degree of reliability of identification and quantitation of the target analytes, especially when dealing with an inhomogeneous complex sample matrix such as autopsy blood.

Since the sensitivity of ECD detection for the analysis of cannabinoids is over 400 times greater than that of UV at 220nm,[107] it is assumed that those results by UV at 220nm which are higher than ECD are due to interferences from co-extracted materials. These interferences differ from sample to sample reflecting different dietary intake of each individual. In addition, the possibility of co-extracted materials that might resemble cannnabinoids by having a phenolic group, which may also undergo oxidation at the electrode surface, thus producing interferences, cannot be ignored.

4.8 Conclusion.

Use of UV and ECD detectors in the described HPLC system produced linear calibration curves from 0-100ng/ml. Total delta-9-THCCOOH levels in test blood samples have been quite low (1-24ng/ml), whereas delta-9-THC and CBN were not detected and only ECD gave positive results for CBD. UV at 220nm proved to be more sensitive than at 212nm, but when compared to ECD, neither of them showed to be better than the other.

The THC extracts gave chromatograms clear of interference except for ECD where an interference peak was observed at the same retention time as THC. The metabolite portion contained many nearby interferences. Thus, further work is necessary to improve the clean-up procedure before this HPLC method can be used for routine analysis.

CHAPTER 5. GC-MS ANALYSIS.

5.1 General Introduction.

Mass spectrometry is the basis of the most specific and sensitive detector for gas chromatography. It has been particularly useful for bio-analysis. Recently, HPLC coupled with an MS detector has also been developed for analysis of biological samples.

The technique relies upon three basic functions. These are (1) to vapourise compounds of widely varying volatility, (2) to ionise and produce fragment ions from molecules in the gas phase, and (3) to separate fragmented ions according to their mass-to-charge ratios (m/z), detect and record them in a form of a mass spectrum, a graph of mass-to-charge ratios of the fragments plotted against relative abundance. The basic components of a double-focussing mass spectrometer are shown in Figure 5.1.
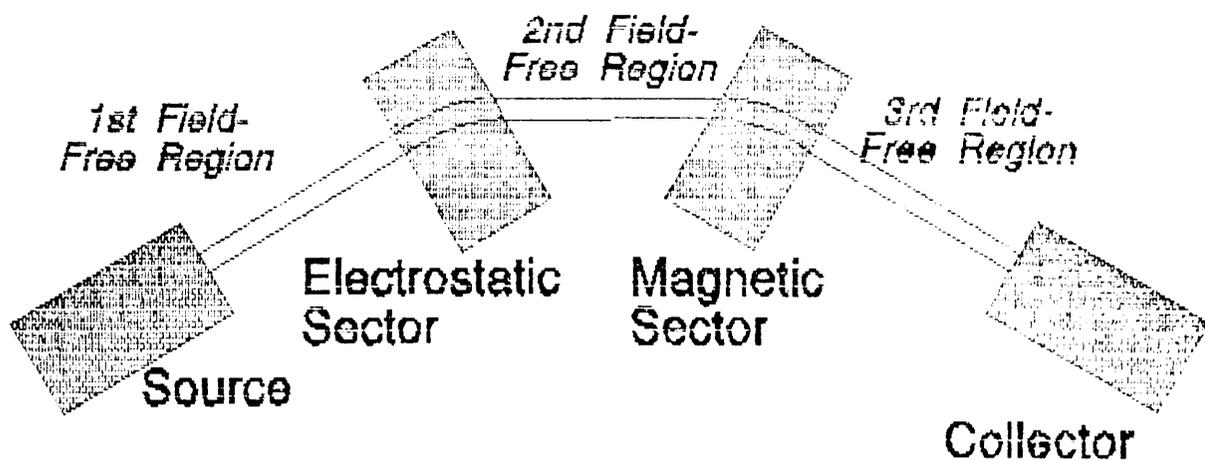

Figure 5.1 : Components of a Double-Focussing Mass Spectrometer.

Sample inlet systems allow the introduction of samples - solids, volatile liquids or gases, into the ionisation source. The instrument used in the present study was equipped with three inlets including a direct capillary (GC) inlet and a septum inlet. The latter was used regularly for the introduction of volatile liquid samples such as perfluorokerosene (PFK), used to calibrate the mass range of the spectrometer. A molecular leak, within the system, regulates the constant volume flow rate of vapourised sample into the ion chamber.

In the ion source, electrons are emitted from a hot filament and are accelerated across the ion chamber to the collector anode. The vapourised sample molecules are bombarded by this stream of electrons at energies in the range 0-100eV, thus producing ions.

$$M + e^- \ \text{-----> } \ M^+ + 2e^-$$
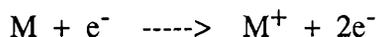
The electron energy supplied is usually higher than the ionization energy required to remove an electron from the highest occupied molecular orbital of the sample. This excess energy remains in the molecular ion and may be used to break one or possibly more bonds.

The ions formed are repelled out of the source using a small repeller voltage (< 50V), transmitted through an accelerating high potential difference (about 8KV) between the source block and the accelerating slit, and then injected into a mass analyser which separates the ions into groups of different mass-to-charge ratios. Once this has been accomplished, the ions arrive at the collector, where they are detected, measured and recorded, either electrically or photographically. This type of measurement distinguishes the instrument used, either mass spectrometer or mass spectrograph, respectively.

Most modern GC-MS instruments are smaller and cheaper than in previous years and need little experience to operate and maintain. They consist of a quadrupole analyser which has two particular advantages compared with magnetic sector instruments, that is, ease of data system control and small size. However, the Federal Drug Agency in the USA requires high-resolution MS for drug toxicology/pharmacology studies, which is only available with a double focusing magnetic sector instrument. Also, the sensitivity of a more powerful instrument may be higher and research instruments may have a number of facilities which provide more specificity than a smaller, routine instrument. This could be used to advantage for difficult analyses such as THC in whole blood.

The normal operating modes of the MS are the full scan mode and selected ion recording. A full scan is obtained by variation of the magnetic field between the source and collector slits. The scan law is;

$$m/z = r^2H^2/2V$$

where r is the radius of curvature through the magnetic field of strength H, and V is the accelerating voltage. By varying V also, ions of different m/z ratio separated by the magnetic field can be made to reach the collector. This is used in SIR mode to bring the selected m/z values into focus, thus achieving a high sensitivity since SIR focusses on a few ions. For instance, SIR has been used to effect the separate recording of THC and THCCOOH with their corresponding [2]H-IS, which eluted from the GC with closely similar retention times. There is a limit to the mass range accessible by voltage switching of approximately 50% of the mass of the lowest ion monitored.

However, there is a need for specificity in SIR mass spectrometry since it may not be possibble for the analyser to detect a high abundance of molecular ions. By focussing on high masses especially with the use of derivatives, specificity could be

enhanced. Moreover, if the resolution of the MS is increased to moderate or high resolution, the cross contribution between one mass and another is reduced and the interference from background or other sample components at the mass being monitored is reduced accordingly. This would achieve the specificity needed but sensitivity is lost since fewer ions pass through the mass analyser.

A computer control and data system is generally interfaced to the MS for quick data acquisition and processing. Moreover, the computer-aided instrument can monitor about 10-12 ions in groups at pre-set times during a GC-run.

5.2 Full Scan MS.

5.2.1 Introduction.

Selection of suitable derivatives and fragment ions for use in GC-SIR-MS requires an initial examination of the full spectra of the parent compound and the derivatives. Criteria for the selection, as mentioned earlier, are (a) intense ions at high mass, (b) ions free from background interference, and (c) at least two suitable ions within the mass range accessible by voltage switching. Also, this initial evaluation process allows the GC retention times of the analytes to be recorded for subsequent use in creating SIR-MS method files (Para. 5.3).

5.2.2 Experimental.

5.2.2.1 Materials.

Delta-9-THC, delta-9-THCCOOH and their deuterated IS, obtained earlier, were prepared as before to give a concentration of 1ug/ml in methanol.

In addition to those chemicals and solvents previously used, Analar grade benzene was obtained from Rathburn Chemicals Ltd. while triethylamine was obtained from BDH Laboratories Ltd. The benzene was dried over calcium hydride and redistilled in order to remove any moisture present that would react with anhydrides. The derivatization reagents evaluated were trifluoroacetic acid anhydride (TFA) obtained from Koch-Light Laboratories Ltd., heptafluorobutyric acid anhydride (HFBA) and pentafluoropropionic acid anhydride (PFPA) both obtained from Pierce & Warriner (UK) Ltd., and pentafluorobenzyl bromide (PFB) which was obtained from Sigma Chemical Co. Ltd.

5.2.2.2 Apparatus.

All full scan was carried out with the integrated VG 70-250S/SE system equipped with an HP 5890 GC and HP 7673 autosampler, described earlier. Helium was used as the carrier gas at a head pressure of 35kPa (5psi). The conditions for GC were as described earlier (Para. 3.2.2), while the scanning parameters were as listed in Table 5.1.

The MS was programmed to detect ion peaks ranging from m/z 20 to 750. This gave the full spectra of the compound analysed. PFK was used for calibration and tuning of the MS instrument.

```
 SYSTEM:RAA2           Parameters for Scanning acquisition         (Sector)
 IAV  Maximum volts              10000  IMR  Maximum mass at IAV           1791
 DAT  Data filename             D:D9THC  SVD  Solvent delay(mm:ss)        02:00
 CAL  Calibration filename      18JULY  COT  Centroid or Top                 C
 INS  Instrument               1:70-250S  HOA  Heights or Areas               H
 ACN  Customer account                  HDR  High dynamic range             N
 ACV  Accelerating volts          8000  PBT  Probe Threshold            0 BPK
 SCS  MAG KVE ESA/P/2 MS2         MAG   CON  Continuum or Mca               N
 HIM  High mass                    758  HDT  Hardware tic                   N
 LOM  Low mass                      20  DSC  Digital scanner                Y
 MHI  Monitor high                   0  AOD  Analogue or Digital            A
 MLO  Monitor low                    0  MAT  Store mass and time            N
 TAM  Tic above mass                41  SCL  Scan(EL,DU,BIN,SG)          EDIS
 STH  Signal threshold              10  RES  Instrument resolution        1000
 MPW  Minimum peak width             6  RND  Duration(Time/#Scans)    1:30:00
 MTH  Multiplet threshold          250  ATR  Range(ABC,ABC) AA      AA      AA
 TIM  Scan time(s/d,s)            1.00  MOD  Ion Modes EI+   1 ---   0 ---   0
 IST  Interscan time(s)          0.50  GAS  Gas types
 TXT  DELTA-9-TETRAHYDROCANNABINOL
        'H=hardcopy 'T=tune RETURN=next ESC=prev NAME=select CTRL/A=abort
        'G=go 'A=Agmm 'R=Rpt 'Q=quit 'C=create 'DEL=delete 'O=overwrite 'Z=zero
```

Table 5.1 : GC Scanning Parameters.

5.2.3 Preparation of Derivatives.

Trimethylsilyl derivatives were prepared by reacting 40ng of THC and THCCOOH with a solution (50ul) of BSTFA + 1% TMCS for 30 min at 60°C.

Fluorinated acid anhydride derivatives were prepared by combining 50ng of standards dissolved in 500ul dry benzene with 100ul 0.05M triethylamine in dry benzene and 10ul of acid anhydride.[111] The mixture was heated as above and then analysed by GC-MS.

Pentafluorobenzyl derivatives were prepared by mixing 50ng of standards with 2.0ml of dichloromethane, 10ul of PFB-Br, 0.5ml of 0.4M tetrabutylammonium sulphate and 0.5ml of 0.8M sodium hydroxide.[111] The mixture was mixed using a rotator for at least 30 min and the dichloromethane layer was then washed twice with 2.0ml of 0.1M sulphuric acid. Before injection, the washed dichloromethane layer was evaporated to dryness and reconstituted in 50ul dry benzene.
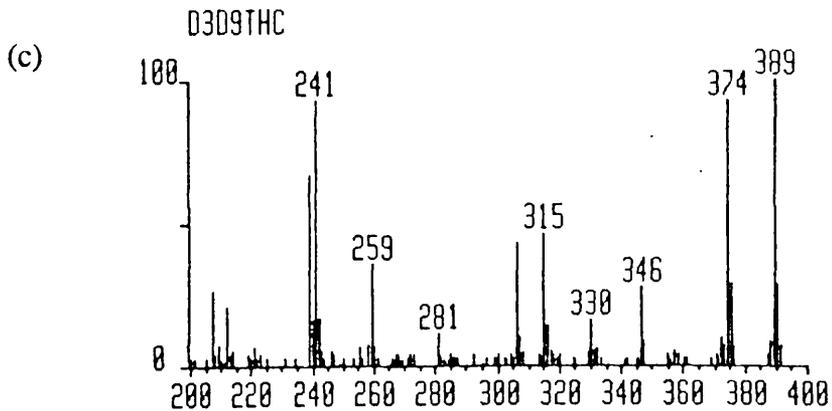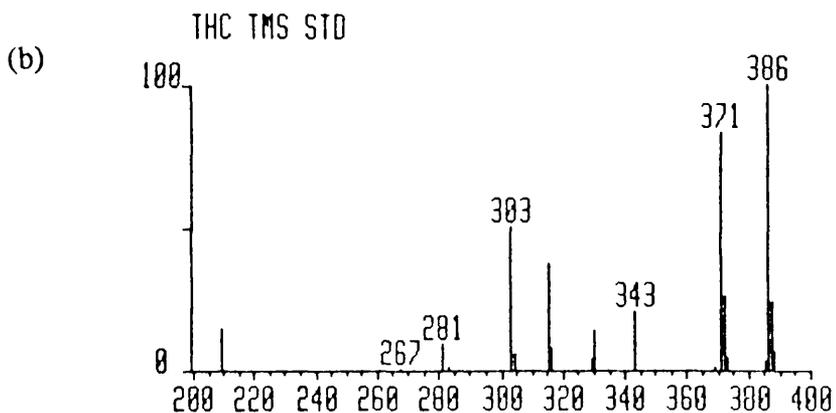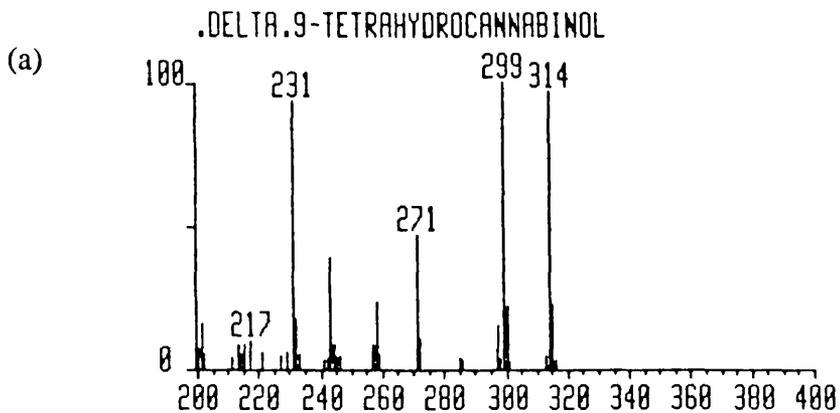
5.2.4 Results and Discussion.

The full scan mass spectra of delta-9-THC and its derivatives, including the deuterated analog, are given in Figures 5.2a to 5.2g, and a summary of the fragment ions is given in Table 5.2.

The THC spectrum shows a prominent molecular ion at m/z 314, which is good for identification and SIR. The pyran ring is the centre of charge localization, and elimination of an electron from this nucleus gives the molecular ion m/z 314.[112] The principal fragments are at m/z 299 [M-15]$^+$ and 271 [M-43]$^+$. The former is produced by the loss of a methyl group, probably from one of the geminal methyl groups attached to the pyran ring[113], indicating charge localization on the heterocyclic oxygen, or from the carbon seven (C-7) position (Figure 5.3a). The latter involves the elimination of a carbon at the eight position (C-8), both the geminal methyl groups and a hydrogen atom, either from the C-3 or C-5 position[112] (Figure 5.3b).

Other fragments are the ion at m/z 258 [M-56]$^+$ which corresponds to the elimination of a 1-butene molecule from the pentyl side chain via a McLafferty rearrangement (Figure 5.3c), and the ion at m/z 243 [M-15-56]$^+$ which has been assumed to derive from the loss of a geminal methyl group from the [M-56]$^+$ ion or vice versa.[112] Formation of ion at m/z 231 [M-83]$^+$ appears to be important in the mass spectra of cannabinoids because its relative intensity varies between 30 and 100%, depending upon the structure of the compound.[112] Its formation mechanism is not clear due to the complicated structures of the many naturally occurring cannabinoids.[112] It is likely that the fragment contains the phenolic ring moiety of the molecule, as a corresponding fragment is formed for the TMS ether derivative at m/z 294 (ie at m/z 231 plus 72 amu for the TMS group).

Figure 5.2 : Full Scan Mass Spectra of (a) THC   (b) THCTMS   (c)D$_3$-THCTMS
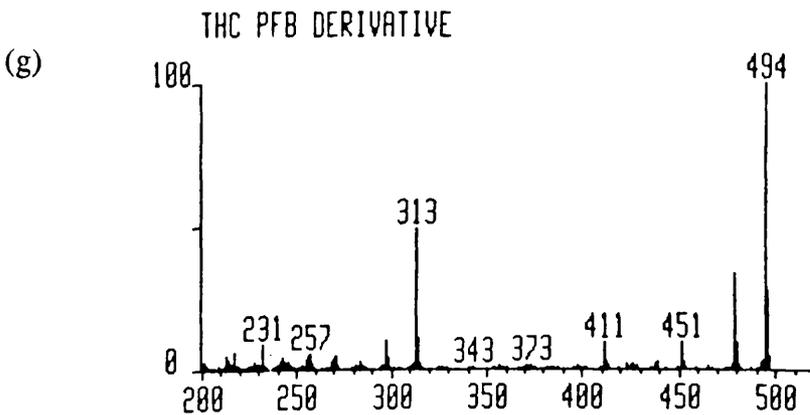(d) THCTFA   (e) THCPFP   (f) THCHFB   and   (G) THCPFB.

(a) .DELTA.9-TETRAHYDROCANNABINOL

(b) THC TMS STD

(c) D3D9THC

(d)

DELTA-9-THC TFA DERIVATIVE

(e)

DELTA-9-THC PFP DERIVATIVE

(f)

DELTA-9-THC HFB DERIVATIVE

(g)

THC PFB DERIVATIVE
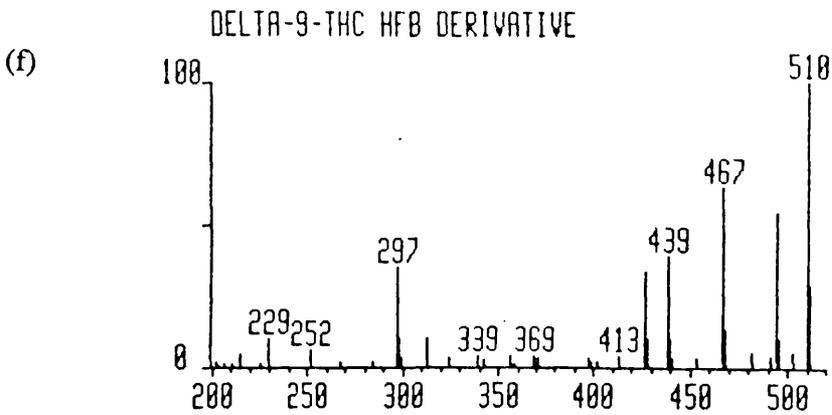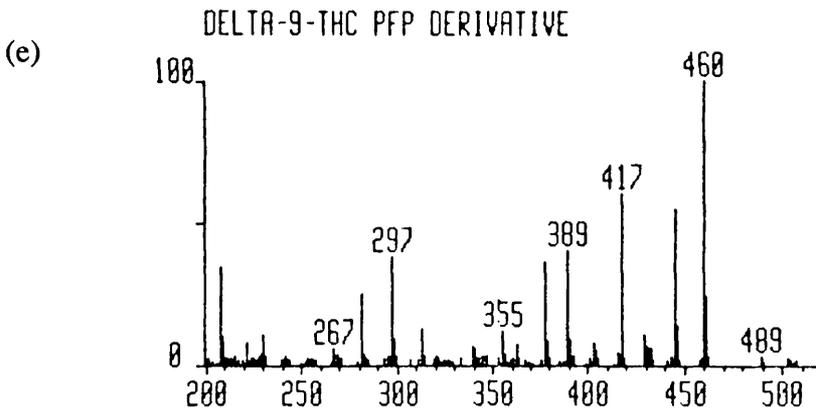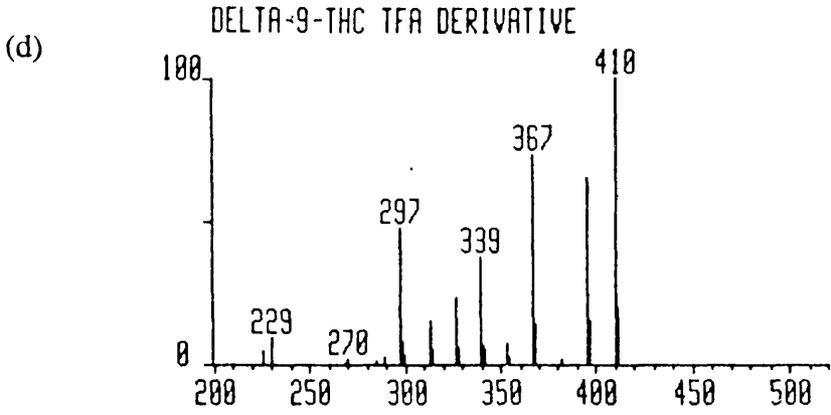
Figure 5.2 : Summary of Fragment Ions Detected by Full Scan GC-MS.

Analytes	M^+	$[M-15]^+$	$[M-43]^+$	$[M-56]^+$	$[M-(56+15)]^+$	$[M-83]^+$	$[M-(O-Derivative)]$
Delta-9-THC	314	299	271	258	243	231	-
THCTMS	386	371	343	330	315	303	297
D3-THCTMS	389	374	346	330	315	306	300
THCTFA	410	395	367	354	339	327	297
THCPFP	460	445	417	404	389	377	297
THCHFB	510	495	467	454	439	427	297
THCPFB	494	479	451	438	423	411	297

m/z 314 $-CH_3 \cdot$ m/z 299
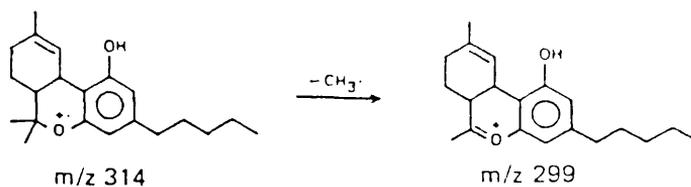
Figure 5.3a : Mechanism for the Formation of Fragment Ion $[M-15]^+$.
(Loss of a geminal methyl group attached to the
pyran ring)

Figure 5.3b : Mechanism for the Formation of Fragment Ion $[M-43]^+$.
(Elimination of a C-8 carbon, both geminal methyl
groups and a hydrogen atom at C-10a position)

m/z 314 $-C_4H_8$ m/z 258

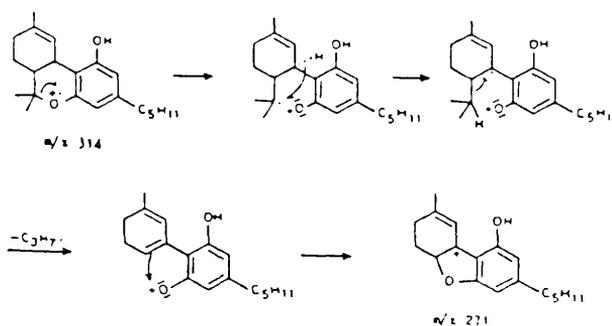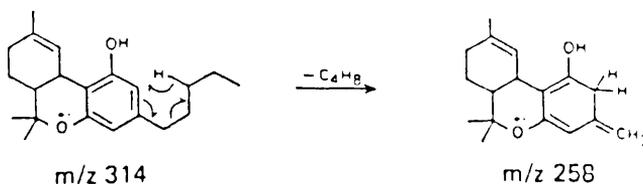Figure 5.3c : Mechanism for the Formation of Fragment Ion $[M-56]^+$.
(Elimination of a 1-butene molecule from the
pentyl side chain via a McLafferty rearrangement)

The mass spectrum of THC-TMS derivative showed a high relative intensity of the molecular ion m/z 386. Principle fragments are found at m/z 371 and 343, corresponding to the elimination of -CH$_3$ and -C$_3$H$_7$, similar to those of underivatized THC.

Comparison between TMS derivatives of THC and d$_3$-THC showed similar formation of ion fragments. The fragment ions in the deuterated compound were found at m/z values three a.m.u. higher than in the unlabeled compound, except the [M-56]$^+$ and [M-15-56]$^+$ fragments, where their m/z values are the same, that is at m/z 330 and 315 respectively. This is due to the removal of the 1-butene molecule from the pentyl side chain that contains the deuterated methyl group, thus confirming the elimination mechanism. Other fragments do not involve loss of the deuterated methyl group.

In addition, the mass spectra of fluorinated acyl and benzylate derivatives showed similar mechanisms of ion formation to those of the TMS derivative and underivatized THC (Fig. 5.2a-g, Table 5.2). This confirmed that fragmentation, initiated by charge localization, occurred favourably at the pyran or aryl rings rather than at the acyl or benzyl group. Hence, the derivatization process is considered to be necessary only when higher sensitivity and specificity is to be achieved. Moreover, if derivatisation is needed, TMS derivatives seem to be a good choice because the reagents are cheaper and easily available.

On the other hand, full scan mass spectra of THCCOOTMS and d$_3$-THCCOOTMS (Figures 5.4a & b) showed fragmentations similar to the THC results above. However, only three high intensity peaks were prominent. These corresponded to the molecular ions [M]+ at m/z 488 and 491; [M-15]+ at m/z 473 and 476; and [M-117(COOTMS)]$^+$ at m/z 371 and 374, respectively. The former fragment is probably due to the loss of a methyl group as stated earlier, while the latter might be due to the
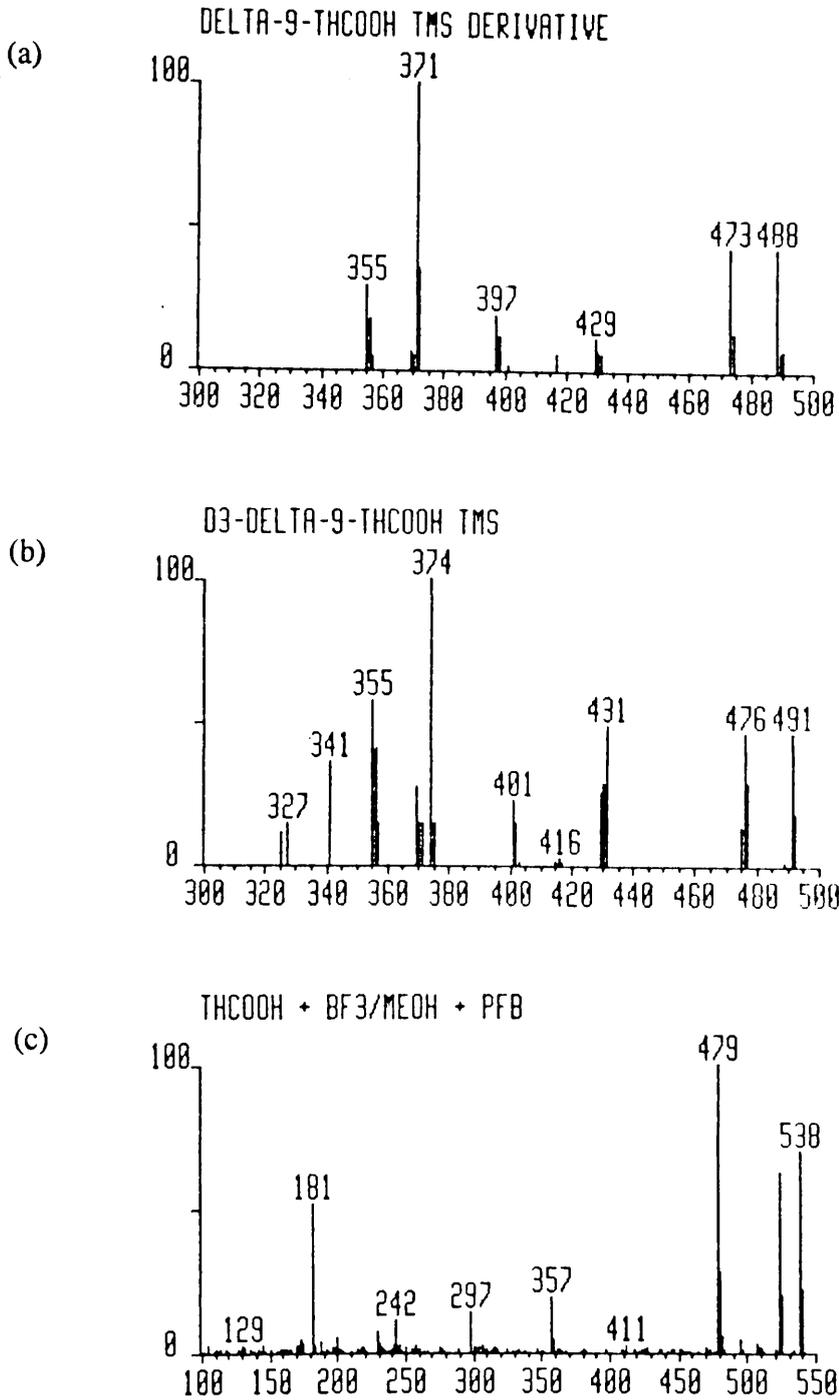
Figure 5.4 : Full Scan Mass Spectra of (a) THCCOOTMS, (b) D3-THCCOOTMS and (c) THCCOOPFB.

elimination of the COOTMS group. The removal of the other derivatized group, 3'-OTMS, might have resulted in the formation of the weak fragment ion at m/z 397, thus showing that elimination of the former derivatized group is favoured. A mixed derivative, the methyl ester-pentafluorobenzylate was also prepared. This gave the expected ions (Figure 5.4c) corresponding to the molecular ion (m/z 538), M-15 (m/z 523), M-carboxymethyl (m/z 479) and M-pentafluorobenzyl (m/z 357)

5.3 Selected Ion Recording Mass Spectrometry.

5.3.1 Introduction.

Following evaluation of the full-scan mass spectra of THC, THCCOOH and their derivatives, the TMS derivatives were selected for further study by SIR-MS. The ions selected for monitoring were the molecular ions of the cannabinoids and deuterated internal standards plus the corresponding M-15 and M-117 fragment ions to provide confirmation of the identity of the analytes in blood extracts.

5.3.2 Calibration Curves of SIR.

5.3.2.1 Procedure.

The operating conditions for the GC-MS instrument are summarised in Tables 5.3(a) and (b). The most intense ion peaks corresponding to the analytes, THC and THCCOOH, were chosen for SIR. Spiked blood samples of concentration 0, 5, 10, 20, 40, 80, 160, and 240ng/ml were prepared with the addition of 40ul of deuterated IS. These were extracted according to the recommended procedure as described earlier (Para. 3.6).

(a)

```
* SYSTEM:HASH        Parameters for Selective Ion Recording [G# 1 KVE] (Sector)
DAT Data filename        D:THC    IAV Maximum volts                        10000
REF Reference filename      PFK    IMR Maximum mass at IAV                   1773
INS Instrument        1:70-250S    GTM Time 0:04:00 0:05:00 Mode EI+IS Gas
ACN Customer account                CHN  M(amu) S(ms) D(ms)     M(amu) S(ms) D(ms)
ACV Accelerating volts       8000   A  371.2406    50    20
RES Instrument resolution    3000   B  380.9760    50    20
SMP #Samples                    1   C  386.2641    50    20
INJ #Injections                 2   D  389.2829    50    20
GRP #Groups                     2
CLS #Calibration scans          2
CST Cal. scan time (s)         10
CTL Cal. tolerance (ppm)       50
CEX Cal. examination            Y
PEX Peak examination            Y
LMS Lock span (peak widths)   2.0
LST Lock step (peak widths)  0.02
FLO Fast lock on                N
TXT Sample# 1:  DELTA-9-TETRAHYDROCANNABINOL TMS DERIVATIVE
        'I=sort masses 'H=hardcopy RETURN=next ESC=prev CTRL/A=abort
        'G=go 'Q=quit 'C=create 'DEL=delete 'O=overwrite 'Z=zero <group,sample>
```

(b)

```
* SYSTEM:HASH        Parameters for Selective Ion Recording [G# 2 KVE] (Sector)
DAT Data filename     D:THCCOOH    IAV Maximum volts                        10000
REF Reference filename      PFK    IMR Maximum mass at IAV                   1773
INS Instrument        1:70-250S    GTM Time 0:06:30 0:08:00 Mode EI+IS Gas
ACN Customer account                CHN  M(amu) S(ms) D(ms)     M(amu) S(ms) D(ms)
ACV Accelerating volts       8000   A  371.2406    50    20
RES Instrument resolution    3000   B  380.9760    50    20
SMP #Samples                    1   C  473.2543    50    20
INJ #Injections                 2   D  489.2778    50    20
GRP #Groups                     2   E  491.2966    50    20
CLS #Calibration scans          2
CST Cal. scan time (s)         10
CTL Cal. tolerance (ppm)       50
CEX Cal. examination            Y
PEX Peak examination            Y
LMS Lock span (peak widths)   2.0
LST Lock step (peak widths)  0.02
FLO Fast lock on                N
TXT Sample# 1:  11-NOR-9-CARBOXY-THC TMS DERIVATIVE
        'I=sort masses 'H=hardcopy RETURN=next ESC=prev CTRL/A=abort
        'G=go 'Q=quit 'C=create 'DEL=delete 'O=overwrite 'Z=zero <group,sample>
```

Table 5.3 : SIR-MS Operating Conditions for (a) THC and (b) THCCOOH.

Calibration curves were determined as peak area ratios of THC and THCCOOH with their deuterated IS, plotted against their corresponding spiked concentrations in blood samples.

5.3.2.2 Results and Discussion.

The SIR plots for THC and THCCOOH produced linear curves from 0-100ng/ml, but lost significant linearity from 100-240ng/ml (Figure 5.5). Linear regression analysis indicated that the correlation coefficients were 0.9896 for THC and 0.9996 for THCCOOH. In the latter range, values obtained were far lower than expected.

This might be due to either the lack of sample homogeneity, wrongly prepared sample or interference. Those errors associated with the actual MS determination such as stability of ion source conditions, stability of the mass scale, thresholding in the data acquisition, and ion statistics may also contribute to some extent.[109] However, sample handling and treatment accounts for the larger proportion of the sources of errors resulting in the sample losts. But, from the curves, it was observed that a flat plateau was obtained at higher concentrations which indicated a more probable cause as being a problem of detector saturation where the high amount of sample injected has overloaded the detector.

5.3.3 Case Sample Analysis.

5.3.3.1 Procedure.

The same test samples used for HPLC analyses were used again. The volume of each blood sample used was 0.5ml and each was spiked with 40ul of deuterated IS. Extraction procedure was followed as recommended (Para. 3.6).

Figure 5.5 : Calibration Curves of Cannabinoids by SIR-MS.
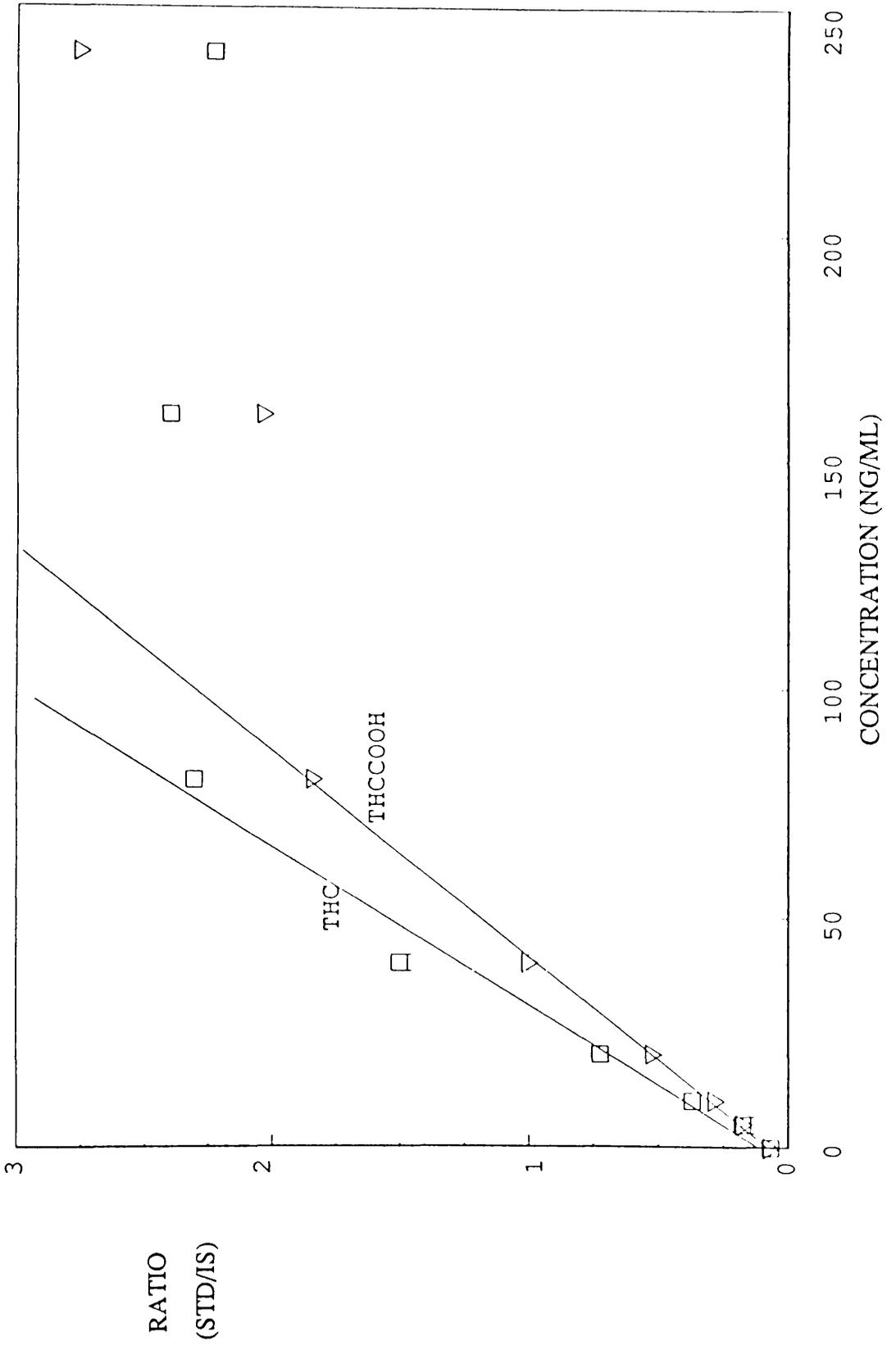
5.3.3.2 Results and Discussion.

Two samples were found to be positive for THC and three for THCCOOH (Table 5.5). When comparing the results with those from HPLC analyses, it shows that GC-MS by SIR is more sensitive and selective than HPLC with UV and EC detectors since the values obtained were higher by SIR with more samples being confirmed positive. In addition, SIR chromatograms did not suffer from interference by co-extracted materials, thus producing clean chromatograms, even though the extracts were not cleaned up prior to analyses.

Table 5.4 : Concentration of Cannabinoids (ng/ml) in Case Samples Detected by SIR-MS.

Case Sample No.	THC	THCCOOH
1	7.4	10.3
2	1.2	9.7
3	-	-
4	-	-
5	-	-
6	-	-
7	-	-
8	-	5.4
- Not detected		

5.4 Metastable Ion/Multiple Reaction Monitoring.

5.3.1 Introduction.

Generally, there are three types of fragment ions that may be detected by MS equipped with magnetic analyzers. These are the molecular ions, daughter ions and metastable ions. Molecular ions are transmitted intact through the MS. Meanwhile, daughter ions arise by fragmentation of the molecular ions or by decomposition of other daughter ions in the ion source region. They are then transmitted through the instrument.

$$M^+ \longrightarrow d_1^+ + d_2$$
$$d_1^+ \longrightarrow d_3^+ + d_4$$
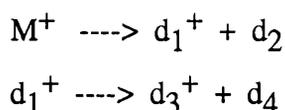
However, metastable ions only arise when ions dissociate in one of the three field-free regions after leaving the source but before reaching the collector of the instrument (Figure 5.1). The three field-free regions are situated before the first analyzer, between the two analyzers, and after the second analyzer. These regions are so called because ions are not acted on by either magnetic or electric fields, but are moving linearly through the regions. In the present study, only metastable ions formed in the first field free region were examined.

Metastable ion monitoring involves the adjustment of the electrostatic analyser voltage and magnetic field to the point where daughter ions formed in a metastable transition in the first field free region are brought into focus at the collector. Other fragment ions, including those arising from endogenous contaminants by different fragmentation mechanisms, are trapped by the electrostatic analyser leaving an almost clean background.

Three types of metastable ion reactions can be selectively measured by the appropriate selection of the instrumental parameters. The reactions are (1) daughter ions originating from a specified parent ion, (2) all parent ions producing a specified daughter ion, and (3) all transitions of specified neutral fragment loss characteristic of a particular compound class. The scan laws are as follows;

Daughter H/E = Constant

Parent H^2/E = Constant

Constant Neutral Mass Loss $H/E \, [1-E/E_o]^{1/2}$ = Constant

where H and E are the magnetic field strength and electroststic sector voltage respectively, while E_o is the corresponding value of a normal ion when the instrument was tuned before analysis.

The measurement of metastable ions in an MS provides information related to the structure of the ions and the structure of the compounds from which they originate. This information will provide a fingerprint spectrum for a specific compound which may be recorded, even in the presence of a matrix giving many other ions, in the normal spectrum. Thus, its application can provide a more specific and sensitive structural probe especially for the differentiation of isomers. Moreover, a sample component may be detected with high sensitivity by monitoring selected metastable ions.

5.4.2 Experimental.

5.4.2.1 Materials.

All solvents and chemicals were obtained as before and all standards were prepared as previously described (Para. 3.2.1). Trimethylsilyl derivatives of standards were prepared as described earlier (Para. 5.2.3).

5.4.2.2 Apparatus.

All conditions on the VG 70-250S/SE system were the same (Tables 5.3 and 5.4) except that the MS was used in the multiple reaction monitoring (MRM), programmed to detect the transitions m/z 386.26 to 371.24 for delta-9-THC and m/z 488.28 to 473.25 for delta-9-THCCOOH.

5.4.3 Metastable Scan.

Metastable spectra were recorded in full scan mode for daughter ions formed from the molecular ions of THCTMS and THCCOOTMS. The spectra obtained (Figures 5.6a & b) showed many fragment ions but only the daughter ion $[M-15]^+$ peak at m/z 371 and 473 respectively, was prominent. Hence, the transition $M^+ \rightarrow [M-CH_3]^+$ of the TMS derivative was chosen for further analysis. Moreover, previous work has indicated that this derivative gives the best GC-MS characteristics.(114)

5.4.4 Calibration Curves for Metastable Ion Monitoring.

5.4.4.1 Procedure.

Deuterated IS (40ng) was added to spiked blood samples of concentrations 0, 5, 10, 20, 40, 80, 160, and 140ng/ml. These were extracted as recommended (Para. 3.6) and derivatized with TMS (Para. 5.2.3). Calibration curves were determined as peak area ratios of THCTMS and THCCOOTMS with their deuterated IS, plotted against corresponding spiked blood concentrations.

5.4.4.2 Results and Discussion.

The plots produced linear curves from 0-100ng/ml, but lost significant linearity from 100-240ng/ml (Figure 5.7). Linear regression analysis indicated that the correlation coefficients were 0.9988 for THC and 0.9977 for THCCOOH. Results obtained were similar to those of GC-SIR-MS. Values at the higher range were lower than anticipated, showing again that apparent sample losses were occurring at spiked concentrations of 100-240ng/ml. This loss of linearity is probably due to detector saturation where the high concentration of the sample injected has overloaded the dynamic range of the analogue-to-digital converter used in the detector/computer interface.
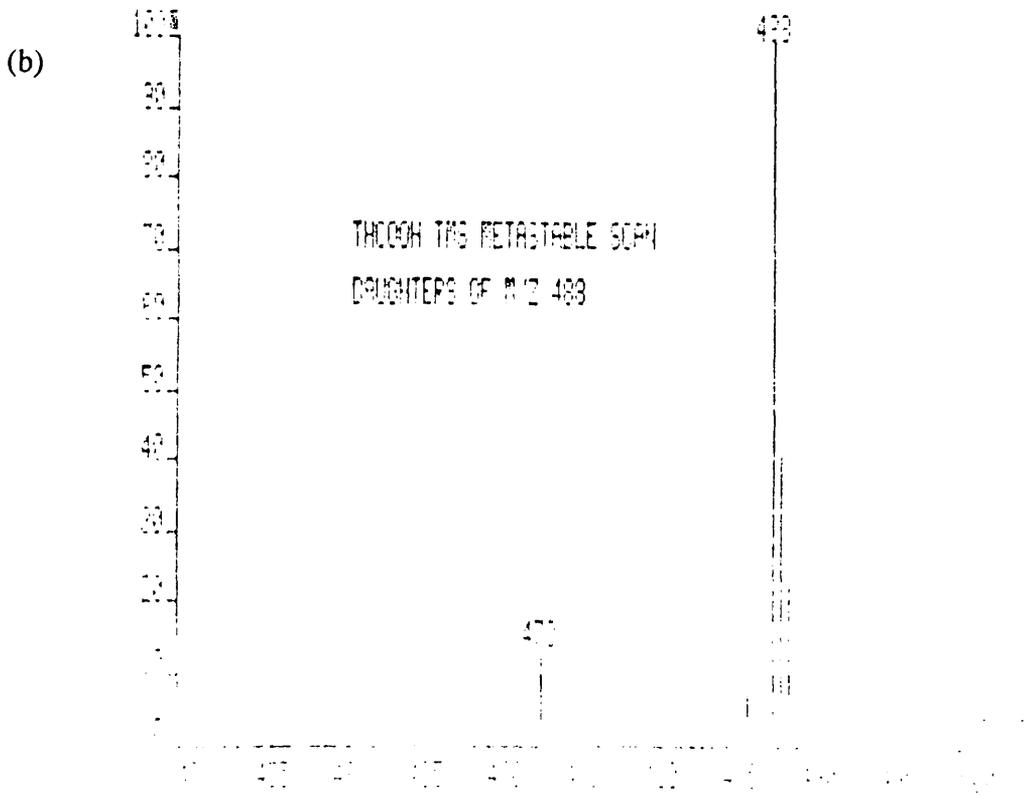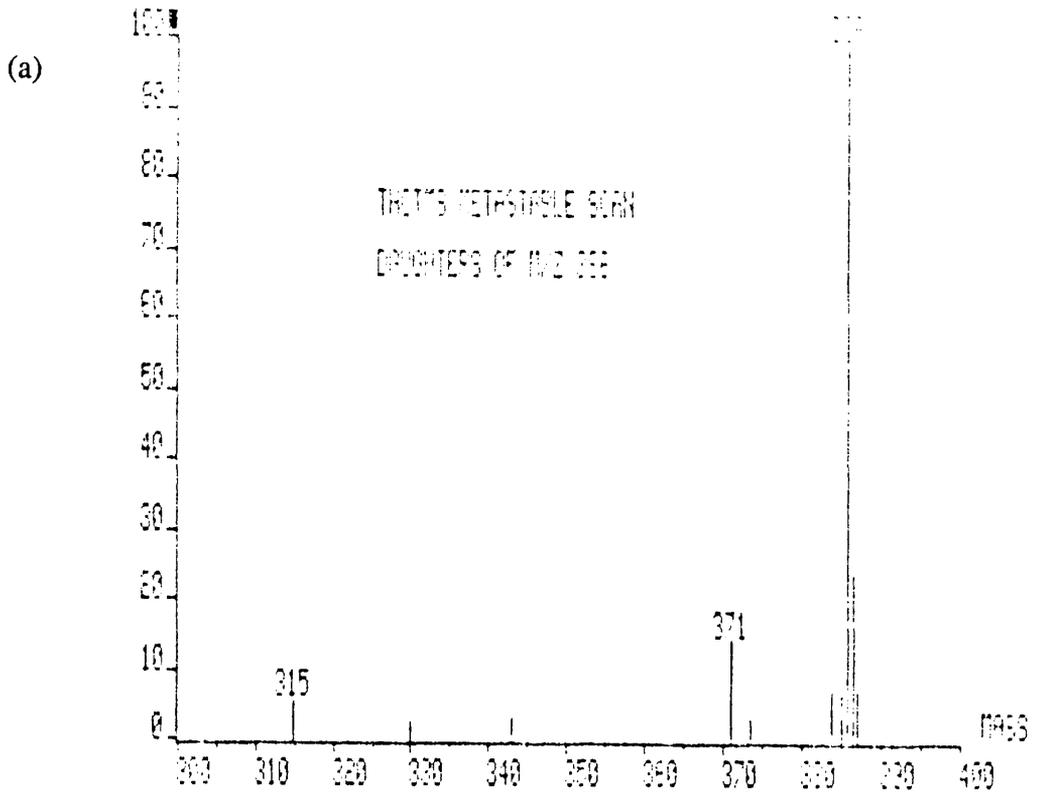
(a)

(b)

Figure 5.6 : Metastable Scan Mass Spectra of (a) THCTMS and (b) THCCOOTMS.

5.4.5 Case Sample Analysis.

5.4.5.1 Procedure.

The same case samples used for HPLC and GC-SIR-MS analyses were used again. Each blood sample (0.5ml) was spiked with 40ng of deuterated IS. The extraction procedure was followed as recommended (Para. 3.6).

5.4.5.2 Results and Discussion.

It was observed that most of the THC-metastable peaks had interference, resulting in inaccurate quantitation of the cannabinoid. Three samples were found to be positive for THC and THCCOOH (Table 5.6). For THC, MRM gave higher calculated levels than SIR, but the opposite was found to be true for the metabolite. This is because the test samples, which might contain certain endogenous compounds having similar extractive and analytical properties to THC in giving $[M-CH_3]^+$ fragments, were not submitted to the clean-up procedure prior to GC-MRM-MS analyses.

More samples could have been confirmed positive if not for the presence of co-extracted interference. However, the interference did not occur under SIR or even during the calibration curve determinations. Hence, the clean-up procedure was found to be necessary for detection of cannabinoids in blood samples by MRM, although Harvey et al[58] had reported earlier that sample clean-up was not required for their cannabinoid-metastable ion detection in plasma samples.

Figure 5.7 : Calibration Curves of Cannabinoids by MRM.
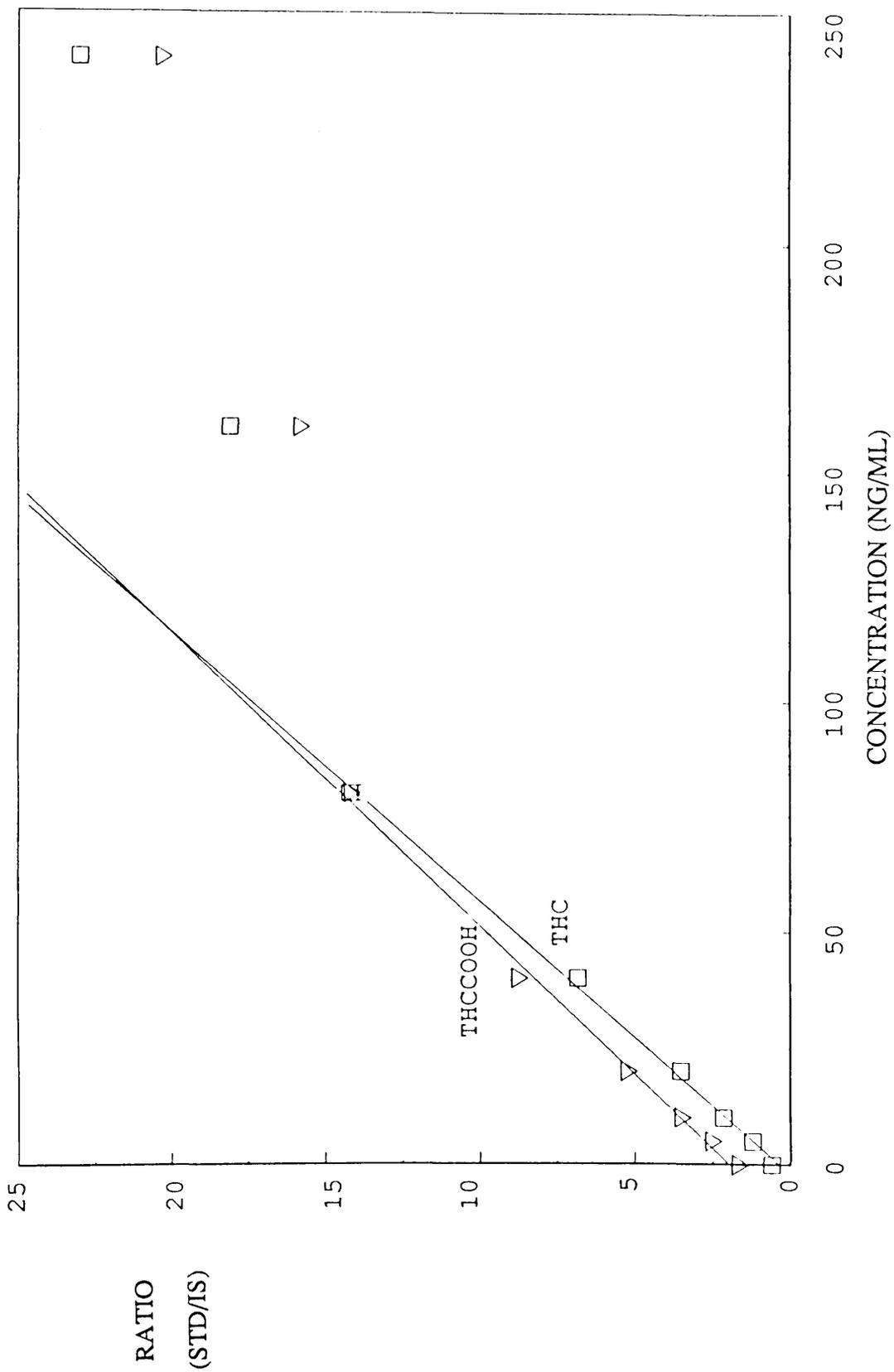
Table 5.5 : Concentration of Cannabinoids (ng/ml) in Case Samples Detected by Multiple Reaction Monitoring.

Test Sample No.	THC	THCCOOH
1	9.1	8.4
2	5.2	8.8
3	2.8	-
4	*	-
5	-	-
6	*	-
7	*	-
8	*	2.4

-	Not detected
*	Interference

Another way to exclude any interference is to monitor other transitions, although they are not prominent. For instance, the fragment [M-56]+ corresponding to the elimination of a 1-butene molecule from the pentyl side chain could produce a cleaner chromatogram since not many endogeneous compounds will have the same side chain giving rise to a similar fragmentation pattern.

5.5 Negative Ion Chemical Ionisation.

5.5.1 Introduction.

Chemical ionisation (CI) occurs when sample molecules are ionized by a chemical reaction (charge transfer) rather than by electron bombardment. The technique requires a high pressure of a reagent gas (e.g. methane, isobutane, ammonia) in the ion source and the gas molecules are usually present in high concentration compared to the sample molecules, in order to minimize the occurrence of any electron impact ionisation.

The principal applications of CI derive from its ability to produce information about the molecular weight of compounds analysed and their structural and stereochemical information, where EI^+ often fails to do so.[109] Moreover, the structural information obtained can be controlled by varying the reagent gas used since the characteristics of the CI mass spectrum produced are highly dependent on the nature of the reagent gas used to ionize the sample.

There are two types of CI, positive ion (CI or PICI) and negative ion (NICI). Both ions are formed during an ionization process, but the negative ions usually remain undetected because ion source and focussing potentials allow only the extraction of positive ions from the source. The total ionization in PICI is at least an order of magnitude less than that recorded by EI^+, and the sensitivity of NICI is several orders of magnitude lower than PICI.[109]

Negative ion (NICI) normally occurs by electron capture (associative resonance $AB + e^- \longrightarrow AB^-$ and dissociative resonance $AB + e^- \longrightarrow A^- + B$) and ion pairing ($AB + e^- \longrightarrow A^+ + B^- + e^-$) mechanisms. Reagent ions are formed in the source by electron bombardment of suitable reagent gases which will then react chemically with sample molecules to form negative ions. In order to achieve detection limits in the low

femtogram (10^{-15}g) region, the sample must naturally possess or acquire through the preparation of a suitable derivative, a positive electron affinity.[110] However, trace quantities of impurities with high electron affinities (for example, molecules containing halogens) can seriously deplete the population of electrons in the ion source available for sample ionization, thus a sharp drop in sample sensitivity will result.[110]

In the present study, ammonia was chosen as the reagent gas because proton transfer from NH_4^+ is restricted to compounds with a proton affinity greater than that of ammonia, although methane and isobutane too contribute very little to the background.(109) For comparison purposes, the isobutane NICI mass spectra were also examined, as were the effects of the electron energy.

5.5.2 Experimental.

5.5.2.1 Materials.

Standards were prepared as before to give a concentration of 1ug/ml in methanol. Similarly, chemicals, solvents and derivatization reagents were obtained and used as before.

5.5.2.2 Apparatus.

GC-NICI-MS was carried out with the same integrated VG 70-250S/SE system equipped with an HP 5890 GC and HP 7673 autosampler. The conditions for GC were as described earlier (Para. 3.2.2).

The MS was used in the negative ion chemical ionisation (NICI) mode using ammonia and isobutane as reagent gases. Full scan mass spectra in NICI mode were

recorded at the mass range m/z 700-20 for the TFA, PFP, HFB and PFB derivatives of THC. The mass spectrum of the TFA derivative was recorded at 70eV and 32eV to assess the effect of electron energy on the fragmentation pattern. In the selected ion recording mode, the instrument was programmed to detect ion peaks at m/z 410 for THC-TFA, 460 for THC-PFP, 494 for THC-PFB, and 510 for THC-HFB. Ammonia was used as the NICI reagent gas in the SIR mode at a source pressure and temperature of 1×10^{-5} torr and 180°C, respectively. The photomultiplier was set at 500 Volts and NICI spectra were recorded at an ionization energy of 70eV. PFK was used again as a mass marking calibrator for daily calibration. Tuning of the MS instrument in the negative ion mode was conducted using PFK at m/z 331.

5.5.3 Full Scan NICI Mass Spectra.

Full scan NICI spectra were recorded for the TFA, PFP, HFB and PFB derivatives of delta-9-THC to permit selection of suitable prominent negative ions for quantitation of THC by NICI selected ion recording. The results (Figures 5.8a - d) showed that PFB derivative gave stronger high mass negative ions than the other derivatives in which the most prominent ions were characteristic of the acyl fragment and not the cannabinoid molecule. This was true for both ammonia and isobutane reagent gases. For one derivative, THC trifluoroacetate, the electron energy was reduced to 32eV to try and reduce the degree of fragmentation but this had little or no effect on the intensity of the high mass ions. Thus, in view of its sensitivity, PFB derivatization was used for further NICI analysis.

In the present initial assessment, only delta-9-THC was examined. The *bis*-derivatives of THCCOOH could not be detected, presumable because of their high molecular weights. The mixed derivative (methyl ester-pentafluorobenzyl derivative)
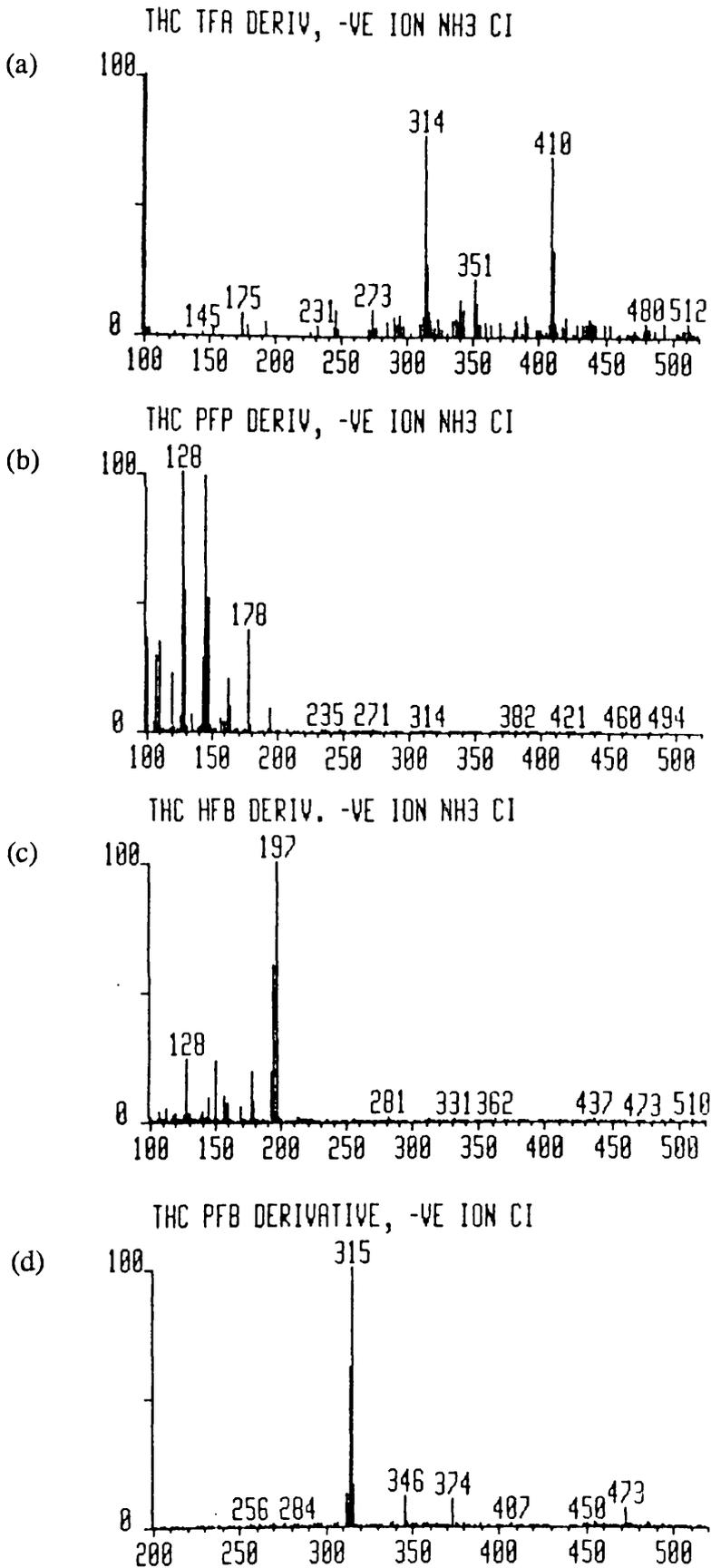
Figure 5.8 : Full Scan NICI Mass Spectra of (a) THCTFA
(b) THCPFP   (c) THCHFB   and   (d) THCPFB.

would be suitable but requires the use of diazomethane for the preparation of the ester. Alternate esterification reactions, for example, using BF_3/methanol were evaluated briefly but were found to be unsuccessful substitutes for diazomethane. In view of the hazards of this material, the mixed derivative was not examined further in this pilot study.

5.5.4 Calibration Curve for NICI Selected Ion Recording.

5.5.4.1 Procedure.

Spiked blood samples of concentration 0, 1, 5, 10, 20, 40, 80, and 160ng/ml were prepared with the addition of 40ng deuterated IS. These were extracted as recommended (Para. 3.6) and derivatized with PFB-Br (Para. 5.2.3).

5.5.4.2 Results and Discussion.

The SIR traces obtained showed severe interference due to co-extracted interferences (Figure 5.9a to 5.9d). None of the spiked blood samples could be used for calibration curve determination. This observation showed that a clean-up procedure for blood extracts is necessary for NICI analysis.

5.5.5 Case Sample Analysis.

5.5.5.1 Procedure.

Two of the test blood samples that gave positive results under MRM were analysed used under NICI mode. The test samples (0.5ml) were mixed with 40ng of
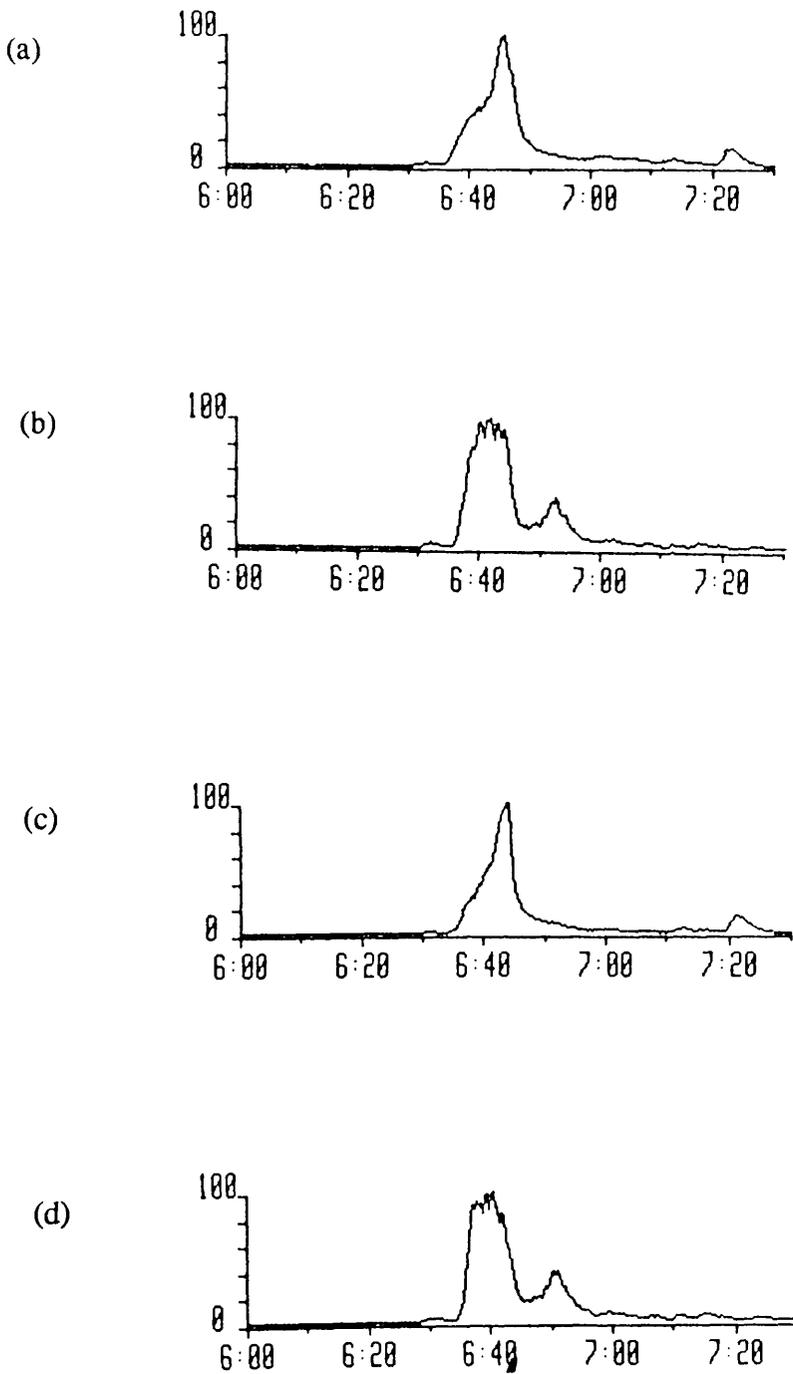
Figure 5.9 : NICI-SIR Chromatograms of (a) THCPFB 20ng/ml Std.
(b) D_3-THCPFB 20ng/ml IS   (c) Blank   (d) Blank & IS.

deuterated THC and were then extracted and derivatized with PFB-Br, as recommended. A 1ul aliquot was injected into the GC-MS system.

5.5.5.2 Results and Discussion.

Both test samples, no. 1 and 2, showed THC GC peaks. Unfortunately, the latter had the THC peak at a shoulder of an interference peak while in the former, the THC peak could not be quantified since the preparation of a calibration curve was unsuccessful (Figure 5.10). Thus, the NICI method, although it is sensitive and selective, was not found to be particularly useful due to presence of co-extracted materials, unless the samples were cleaned up prior to analysis.

5.6 Conclusion.

The purpose of this GC-MS study was to select the best detection method for the quantitation of THC and THCCOOH in autopsy blood without extensive sample clean-up. Results obtained showed that only selected ion recording at moderate resolution under the electron impact ionisation mode gave good SIR chromatograms, free from interference. Other detection methods including metastable ion monitoring and negative ion chemical ionisation SIR, produced chromatograms which were unsatisfactory due to the presence of co-extracted interferences. Further studies must be conducted, especially with sample clean-up prior to analysis, before these methods can be utilised.

Consequently, a GC-MS method for the analysis of cannabinoids in blood samples has been established using the selected ion recording mode. This method is recommended for daily analysis because it was proven to be sensitive and selective, to need only a small sample volume and to be suitable for the automated analysis of batch samples.
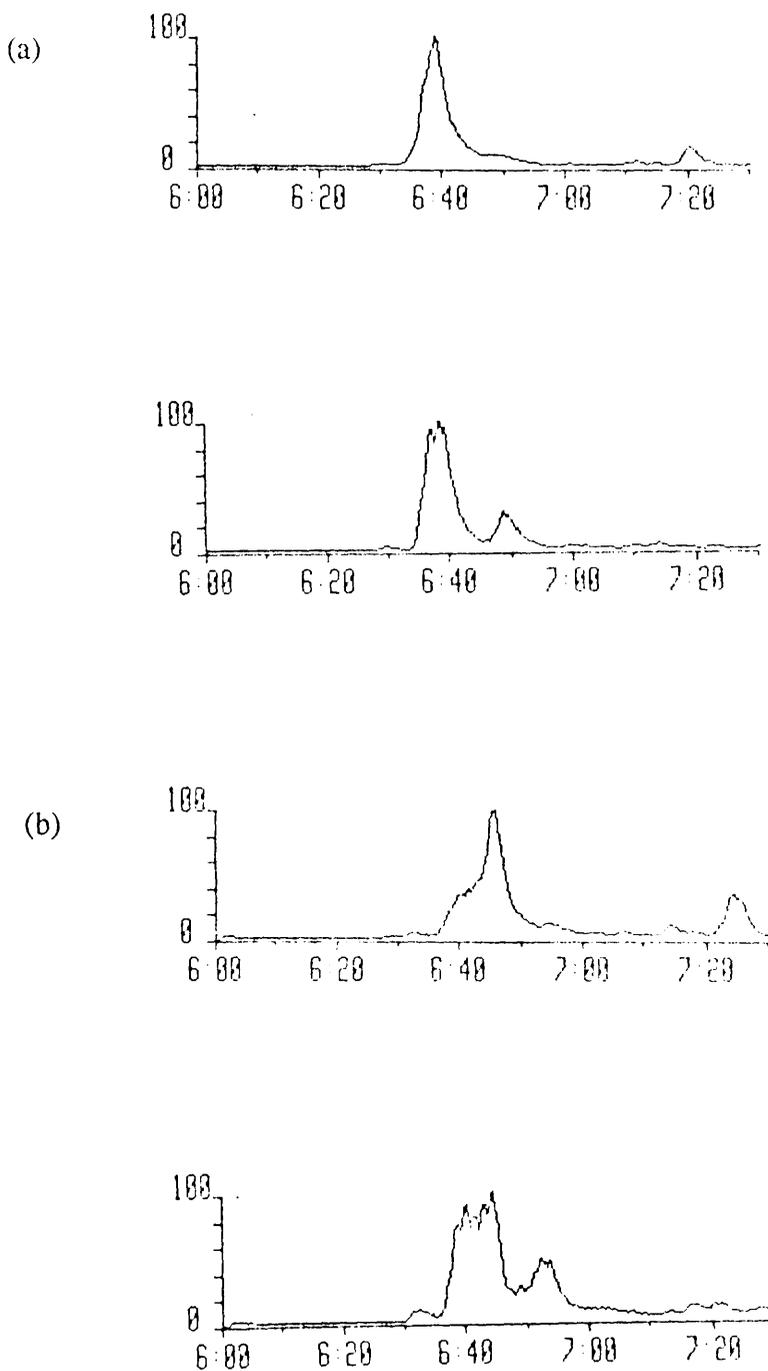
(a)

(b)

Figure 5.10 : NICI-SIR Chromatograms of Case Samples.
(a) Case No. 1 and D_3-THCPFB 20ng/ml IS.
(b) Case No. 2 and D_3-THCPFB 20ng/ml IS.

CHAPTER 6: CONCLUSIONS.

6.1 AIM OF STUDY.

Cannabis has been widely known for both its narcotic qualities and its fiber for thousand of years. It is undoubtly the most widely misused drug in UK and most of the people who have ever used any illegal drug have used cannabis. From the British Crime Survey[115] conducted in 1981, it was found that 5% of 16,000 people interviewed admitted having used cannabis. In addition, according to a 1982 Household Survey done by the US Department of Justice, there are about 20 million annual users and about 4 million daily users, most of whom are adolescents and young adults.[47]

Detection of cannabis in body fluids provides unquestionable evidence of its misused. Many methods have been developed and are widely used routinely, especially for the detection of the psychoactive component delta-9-tetrahydrocannabinol (delta-9-THC) and its major metabolite, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (delta-9-THCCOOH) in urine samples. However, only few have dealt with routine screening and subsequent confirmatory analysis in whole and hemolysed blood samples. This is because blood samples often contain co-extracted lipid materials that give rise to interferences. Moreover, it is difficult to extract the cannabinoids from blood proteins and inhomogeneous samples will lead to unreliable results. Thus, it is a challenging problem for the forensic toxicologist to use blood samples for the detection of cannabinoids.

Modern analytical and instrumental techniques provide an opportunity to detect low levels of cannabinoids in body fluids. However, funds are usually restricted and often unavailable, especially for developing countries, to set up a modern well-equipped laboratory, making their forensic work restrictd in its range of capabilities. An alternative to costly techniques is needed.

In the present study, high-pressure liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) were chosen for evaluation. The former was used with ultra-violet (UV) and electrochemical (ECD) detectors. Although both detectors are non-specific, ECD will provide the sensitivity needed. The latter was used under full scan, selected ion recording (SIR), metastable ion monitoring (MRM) and negative ion chemical ionisation (NICI) modes which are specific and sensitive. Gas-liquid chromatography with electron capture detector was not available for evaluation.

A sample preparation procedure has been established. However, the extra clean-up step was only used for the non-specific end-step analysis while for GC-MS analysis, the step was omitted. The use of diatomaceous earth (Extrelut), although quicker than solvent extraction, was found to be useless. It has been found to give good reproducibility and recovery with other drugs such as morphine and amphetamines, but it did not work with cannabinoids. Perhaps the *log* K (partition coefficient of THC between octanol and water, i.e. the lipid solubility) is too high for effective extractions to be made.

Many clean-up procedures have been reported. However, the use of solid-phase extraction (SPE) tubes are more favourable than the lengthy liquid-liquid extraction procedures. SPE has the advantages of rapid sample preparation, especially batch samples, prevention of emulsion formation and potential automation. Recently, Bond Elut Certify II SPE tubes have been developed specially for the extraction and purification of cannabinoids from biological samples. Availability of these tubes, which were incorporated in this study, provide a clean and quick clean-up procedure. New developments in this field must be pursued in order to offer analysts a unique SPE which can give high drug recoveries, complete removal of interfering materials and rapid sample preparation times.

The quality of results obtained by HPLC and GC-MS analyses were compared. The former under both detection methods showed cleaner chromatograms for the THC fraction but the metabolite fraction contained many nearby co-extracted materials. GC-MS, on the other hand, under selected ion recording electron impact mode gave good SIR chromatograms, free from interferences. Other detection modes showed interferences and gave unsatisfactory results. None of these detection systems gave any false negative result but HPLC was found to give false positive results for the cannabinoids. Moreover, GC-MS provided the accuracy and precision needed, whereas HPLC was found to give large variation of calculated results with low precision.

From the above observations, it was concluded that GC-MS under SIR-EI$^+$ mode can provide the reliability of end-step detection needed for legal purposes. But, whether it can be used routinely by an inexperienced analyst to give reproducible, reliable, accurate and precise results is yet to be questioned.

6.2 FUTURE WORK.

Further studies need to be performed to provide the most sensitive and selective method for routine screening and subsequent confirmatory of cannabinoids in blood samples. Rapid single step sample preparations are needed, especially with the development of unique solid-phase extraction tubes, which can separate the cannabinoids efficiently from a blood matrix.

Evaluation of HPLC detection systems must be extended with the addition of a more specific and sensitive detector such as a fluorescence detector which is very selective when a fluorescence derivative is being used. Gas chromatography can also be

utilised as an end-step analysis. It will provide a very sensitive method when electron capture or nitrogen phosphorous detector is employed with the use of suitable derivatives.

Other sophisticated and rapidly growing mass spectrometry techniques such as LC-MS and MS-MS can also be evaluated. The former does not require any derivatization while the latter does not need any clean-up or chromatography. However, LC-MS is less sensitive than GC-MS with current instrumentation. Moreover, problems associated with interfacing both systems have been recognised. Introduction of samples using a direct probe technique makes MS-MS a very sensitive instrument, but its use is restricted mainly for research studies due to the high costs and the expertise needed to operate it.

Whatever future methods are developed and used for the detection of cannabinoids in blood samples, must be sensitive and specific. The techniques can either utilise a very sensitive instrument such as GC-MS or two independent non-specific techniques like radioimmunoassay and HPLC. But, it must give reliable results which the expert can confidently present in the court of law.

REFERENCES

1. Blum, K. Handbook of Abusable Drugs. Gardner Press, Inc., New York, pp. 447-533 (1984).

2. Hollister, L.E. Structure-Activity Relationships in Man of Cannabis Constituents and Homologs and Metabolites of Delta-9-THC. Pharmacology 11: 3 (1974).

3. Lemberger, L. The Metabolism of the THC. Adv. Pharmacol. Chermother. 10: 221 (1972).

4. Martindale, The Extra Pharmacopoeia. 26th Ed. The Pharmaceutical Press, London. pp. 375-77 (1972).

5. Cone, E.J., Huestis, M.A. Urinary Excretion of Commonly Abused Drugs Following Unconventional Means of Administration. Forensic Sci. Rev. 1: 121 (1989).

6. Hollister, L.E. Status Report on Clinical Pharmacology of Marihuana. Annual New York Academy Sci. 191: 132 (1971).

7. Perez-Reyes, M., Lipton, M.A., Timmons, M.C., Wall, M.E., Brine, D.R, and Davis, K.H. Pharmacology of Orally Administered Delta-9-THC. Clin. Pharmacol. Ther. 14: 48-55 (1973).

8. Lemberger, L., Weiss, J.L., Watanabe, A.M., Galanter, I.M., Wyatt, R.J., and Cardon, P.V. Delta-9-THC. Temporal Correlation of the Psychologic Effects and Blood Levels After Various Routes of Administration. N. Engl. J. Med. 286: 685-688 (1972).

9. Cone, E.J., Johnson, R.E., Paul, B.D., Mell, L.D., and Mitchell, J. Marijuana-Laced Brownies: Behavioral Effects, Physiological Effects and Urinalysis in Humans Following Ingestion. J. Anal. Toxicol. 12: 169 (1988).

10. Law, B., Mason, P.A., Moffat, A.C., Gleadle, R.I., and King, L.J. Forensic Aspects of the Metabolism and Excretion of Cannabinoids Following Oral Ingestion of Cannabis Resin. J. Pharm. Pharmacol. 36: 289-94 (1984).

11. Agurell, S., Gustafsson, B., Holmstedt, B., Leander, K., Lindgren, J.E., Nilsson, I., Sandberg, F., and Asberg, M. Quantitation of Delta-9-THC in Plasma from Cannabis Smokers. J. Pharm. Pharmacol. 25: 554-58 (1973).

12. Rosenfeld, J.J., Bowins, B., Roberts, J., Perkins, J., and MacPherson, A.S. Mass Fragmentographic Assay for Delta-9-THC in Plasma. Anal. Chem. 46: 2232-34 (1974).

13. Turner, C.E. Chemistry and Metabolism, in "Marihuana Research Finding : 1980," (R.C. Peterson, ed.), National Institute on Drug Abuse Research Monograph #31, Rockville, Maryland, p. 81 (1980).

14. Fairbairn, J.W., Liebmann, J.A., and Rowan, M.G. The Stability of Cannabis and its Preparations on Storage. J. Pharm. Pharmacol. 28: 1-7 (1976).

15. Ohlsson, A., Lindgren, J.E., Wahlen, A., Agurell, S., Hollister, L.E., and Gillespie, H.K. Plasma Delta-9-THC Concentrations and Clinical Effects After Oral and

Intravenous Administration and Smoking. Clin. Pharmacol Ther. 28: 409-16 (1980).

16. Nahas, G.G. Lethal Cannabis Intoxication. N. Engl. J. Med. 284: 792 (1971)

17. Agurell, S., Levander, S., Binder, M., Bader-Bartfai, A., Gustafsson, B., Leander, K., Lindgren, J.E., Ohlsson, A., and Tobisson, B. Pharmacokinetics of Delta-9-THC in Man After Smoking -- Relations to Physiological and Psychological Effects, in "Pharmacology of Marihuana," Vol. 1 (M.C. Braude and S. Szara, Eds.), Raven Press, New York, pp. 93-113 (1976).

18. Hollister, L.E., Gillespie, H.K., Ohlsson, A., Lindgren, J.E., Wahlen, A., and Agurell, S. Do Plasma Concentration of Delta-9-THC Reflect the Degree of Intoxication? J. Clin. Pharmacol. 21: 171S-177S (1981).

19. Isbell, H., Gorodetzsky, C.W., Jasinki, D., Claussen, Spulak, F.V., and Korte, F. Effects of (-)Delta-9-THC in Man. Psychopharmacologia 11: 184-188 (1967).

20. Clark, L.D. and Nakashima, E.N. Experimental Studies of Marihuana. Amer. J. Psychiat. 125: 379-384 (1968).

21. Weil, A.T., Zinberg, N.E., and Nelsen, J.M. Clinical and Psychological Effects of Marihuana in Man. Science 162: 1234-42 (1968).

22. Manno, J.E., Kiplinger, G.F., Haine, S.E., Bennett, I.F., and Forney, R.B. Comparative Effects of Smoking Marihuana or Placebo on Human Motor and Mental Performance. Clin. Pharmacol. Ther. 11: 808-15 (1970).

23. Nahas, G.G. Clinical Pharmacology of Cannabis Sativa with Special Reference to Delta-9-THC. Bull. Narcot. 25: 9-39 (1973).

24. Crancer, A.Jr., Dille, J.M., Delay, J.C., Wallace, J.E., and Haykin, M.D. Comparison of the Effects of Marihuana and Alcohol on Simulated Driving Performance. Science 164: 851-54 (1969).

25. Casswell, S. and Marks, D. Cannabis Induced Impairment of Performance of a Divided Attention Task. Nature 241: 60-61 (1973).

26. Kielholz, P., Hobi, V., Ladewig, D., Mierst, P., and Richter, R. An Experimental Investigation About the Effect of Cannabis on Car Driving Behaviour Pharmakopsychiat. 6: 91-103, (1973).

27. Reeve, V.C., Robertson, W.B., Grant, J., Soares, J.R., Zimmermann, E.G., Gillespie, H.K., and Hollister, L.E. Hemolyzed Blood and Serum Levels of Delta-9-THC : Effects on the Performance of Roadside Sobriety Tests. J. Forens. Sci. 28: 963-71 (1983).

28. Meacham, M.P., Janowsky, D.S., Blaine, J.D., Bozzetti, L.P., and Schorr, M. Effects of Marihuana on Flying Ability. J. Am. Med. Asso. 230: 1258 (1974).

29. Janowsky, D.S., Meacham, M.P., Blaine, J.D., Schorr, M., and Bozzetti, L.P. Marihuana Effects on Simulated Flying Ability. Am. J. Psychiatry 133: 384-88 (1976).

30. Yesavage, J.A., Leirer, V.D., Denari, M., and Hollister, L.E. "Carry-Over Effects of Marihuana Intoxication on Aircraft Pilot Performance : A Preliminary Report." Am. J. Psychiat. 142: 1325-29 (1985).

31. Lindgren, J.E., Ohlsson, A., Agurell, S., Hollister, L., and Gillespie, H.K. Clinical Effects and Plasma Levels of Delta-9-THC in Heavy and Light Users of Cannabis. Psychopharmacol. 74: 208-212 (1981).

32. Perez-Reyes, M., Timmons, M.C., and Wall, M.E. Long Term Use of Marihuana and the Development of Tolerance or Sensitivity to Delta-9-THC. Arch. Gen. Psychiat. 31: 89-91 (1974).

33. Wall, M.E., Brine, D.R., and Perez-Reyes, M. Metabolism of Cannabinoids in Man, in "Pharmacology of Marihuana," Vol. 1 (M.C. Braude and S. Szara, Eds.), Raven Press, New York, pp. 93-113 (1976).

34. Ohlsson, A., Lindgren, J.E., Wahlen, A., Agurell, S., Hollister, L.E., and Gillespie, H.K. Single Dose Kinetics of Deuterium Labelled Delta-9-THC in Heavy and Light Cannabis Users. Biomed. Mass Spec. 9: 6-10 (1982).

35. Morland, J., Bugge, A., Sketerud, B., Steen, A., Wethe, G.H., and Kjeldsen, T. Cannabinoids in Blood and Urine After Passive Inhalation of Cannabis Smoke. J. Forens. Sci. 30: 997-1002 (1985).

36. Mason, A.P., Perez-Reyes, M., and McBay, A.J. Cannabinoid Concentrations in Plasma After Passive Inhalation of Marijuana Smoke. J. Anal. Toxicol. 7: 172-174 (1983).

37. Perez-Reyes, M., DiGuiseppi, S., Mason, A.P., and Davis, K.H. Passive Inhalation of Marihuana Smoke and Urinary Excretion of Cannabinoids. Clin. Pharmacol. Ther. 34: 36-41 (1983).

38. Law, B., Mason, P.A., Moffat, A.C., King, L.J., and Marks, V. Passive Inhalation of Cannabis Smoke. J. Pharm. Pharmacol. 36: 578 (1984).

39. Gross, S.J., Worthy, T.E., Nerder, L., Zimmermann, E.G., Soares, J.R., and Lomax, P. Detection of Recent Cannabis Use by Saliva Delta-9-THC Radioimmunoassay. J. Anal. Toxicol. 9: 1-5 (1985).

40. Cone, E.J., Johnson, R.E., Darwin, W.D., Yousefnejad, D., Mell, L.D., Paul, B.D., and Mitchell, J. Passive Inhalation of Marihuana Smoke : Urinalysis and Room Air Levels of Delta-9- THC. J. Anal. Toxicol. 11: 89 (1987).

41   Wong, A.S., Orbanosky, M.W., Reeve, V.C., and Beede, J.D. Stability of Delta-9-THC in Stored Blood and Serum, in "Cannabinoid Analysis in Biological Fluids," (R.L. Hawks, ed.), National Institute on Drug Abuse Research Monograph #42, Rockville, Maryland, pp. 119-124 (1982).

42. Johnson, J.R., Jennison, T.A., Peat, M.A., and Foltz, R.L. Stability of Delta-9-THC, 11-Hydroxy-THC, and 11-Nor-9-Carboxy-THC in Blood and Plasma. J. Anal. Toxicol. 8: 202-204 (1984).

43. Levine, B. and Smith, M.L. Stability of Drugs of Abuse in Biological Specimens. Forens. Sci. Rev. 2: 147 (1990).

44. Garrett, E.R. and Hunt, C.A. Physicochemical Properties, Solubility and Protein Binding of Delta-9-THC. J. Pharm. Sci. 63: 1056 (1974).

45. Christophersen, A.S. THC Stability in Whole Blood: Plastic Versus Glass Containers. J. Anal Toxicol. 10: 129-31 (1986).

46. McBay, A.J. Marihuana : Current Assessment. J. Forens. Sci.

47. Morgan, R. The Emotional Pharmacy, How Mood-Altering and Psychoactive Drugs Work. The Body Press, Los Angeles. pp.148-55 (1988).

48. Lemberger, L., Silberstein, S.D., Axelrod, J., and Kopin, I.J. Marihuana Studies on the Disposition and Metabolism of Delta-9-THC in Man. Science 170: 1320-22 (1970).

49. Lemberger, L., Tamarkin, L.R., Axelrod, J., and Kopin, I.J. Delta-9-THC : Metabolism and Disposition in Long-Term Marihuana Smokers. Science 173: 72-4 (1971)

50. Nakahara, Y., Sekine, H., and Cook, C.E. Confirmation of Cannabis Use II. Determination of THC Metabolites in Urine and Plasma by HPLC with ECD. J. Anal. Toxicol. 13: 22-4 (1989).

51. Karlsson, L. Direct Injection of Urine on a HPLC-Switching System for Determination of Delta-9-THC-11-oic Acid with Both UV and EC Detection.

52. Parry, R.C., Nolan, L., Shirey, R.E., Wachob, G.D., and Gisch, D.J. Pretreatment of Urine Samples for the Analysis of 11-Nor-Delta-9-THCCOOH Using Solid-Phase Extraction. J. Anal. Toxicol. 14: 39-44 (1990).

53. Meatherall, R.C. and Garriott, J.C. A Sensitive TLC Procedure for the Detection of Urinary 11-Nor-Delta- 9-THCCOOH. J. Anal. Toxicol. 12: 136-40 (1988).

54. Kanter, S.L., Hollister, L.E., and Musumeci, M. Marihuana Metabolites in Urine of Man. 10 : Identification of Marihuana Used by Detection of Delta-9-THC-11-oic Acid Using TLC. J. Chromatogr. 234: 201-8 (1982).

55. McBurney, L.J., Bobbie, B.A., and Sepp, L.A. GC-MS and EMIT Analyses for Delta-9-THC Metabolites in Plasma and Urine of Human Subjects. J. Anal. Toxicol. 10: 56-64 (1986).

56. Craft, N.E., Byrd, G.D., and Hilpert, L.R. Preparation and Certification of Standard Reference Material 1507 : 11-Nor-Delta-9-THCCOOH in Freeze-Dried Urine. Anal. Chem. 61: 540-44 (1989).

57. Nakahara, Y. and Cook, C.E. Confirmation of Cannabis Use III. Simultaneous Quantitation of Six Metabolites of Delta-9-THC in Plasma by HPLC with Electrochemical Detection. J. Chromatogr. 434: 247-52 (1988).

58. Harvey, D.J., Leuschner, J.T.A., and Paton, W.D.M. Measurement of Delta-9-THC in Plasma to the Low Picogram Range by GC-MS Using Metastable Ion Detection. J. Chromatogr. 202: 83-92 (1980).

59. Rosenthal, D., Harvey, T.M., Bursey, J.T., Brine, D.R., and Wall, M.E. Comparison of GC-MS Methods for the Determination of Delta-9-THC in Plasma Biomed. Mass Spectrom. 5: 312-16 (1978).

60. Harvey, D.J. Stereospecific Elimination of Deuterium as a Method for Determining the Stereochemistry of a Number of Metabolites of the THC. Biomed. Mass. Spectrom. 7: 28-34 (1980).

61. Bergman, R.A., Lukaszewski, T., and Wang, S.Y.S. The Detection of THC in Blood : A Comparative Study. J. Anal. Toxicol. 5: 85-9 (1981).

62. Ritchie, L.K., Caplan, Y.H., and Park, J. Delta-9-THC Analysis in Forensic Blood Specimens Using Capillary Column GC with Nitrogen Sensitive Detector. J. Anal. Toxicol. 11: 205-9 (1987).

63. Samara, E. and Bialer, M. Rapid HPLC assay with Pharmacokinetic Application for Monitoring Cannabidiol in Plasma. J. Chromatogr. 416: 370-4 (1978).

64. Foltz, R.L., McGinnis, K.M., and Chinn, D.M. Quantitative Measurement of Delta-9-THC and Two Major Metabolites in Physiological Specimens Using Capillary Column Gas Chromatography Negative Ion Chemical Ionisation Mass Spectrometry. Biomed. Mass Spectrom. 10: 316-23 (1983).

65. Fysh, R.R. and Curry, C.J. The Measurement of Delta-9-THC and its Major Metabolite, Delta-9-THCCOOH in Small Samples.

66. Rosenthal, D. and Brine, D. Quantitative Determination of Delta-9-THC in Cadaver Blood. J. Forens. Sci. 24: 282-90 (1979).

67. Vinson, J.A., Patel, D.D., and Patel, A.H. Detection of THC in Blood and Serum Using a Fluorescent Derivative and TLC. Anal. Chem. 46: 163-5 (1977).

68. Fenimore, D.C., Freeman, R.R., and Loy, P.R. Determination of Delta-9-THC in Blood by Electron Capture Gas Chromatography. Anal. Chem. 45: 2331-35 (1973).

69. Joern, W.A. Detection of Past and Recurrent Marijuana Use by a Modified GC-MS Procedure. J. Anal. Toxicol. 11: 49-52 (1987).

70. Scherrmann, J.M., Hoellinger, H., Sonnier, M., Hoffelt, J., and Nguyen-Hoang-Nam. Simple, Rapid Method for Extraction of Urinary Cannabinoids by Liquid-Solid Chromatography. J. Chromatogr. 196: 342-6 (1980).

71. Hattori, H. Detection of Cannabinoids by GC-MS. Part III. NI-CI GC-MS of Cannabinoids in Human Materials. Jpn. J. Legal Med. 38: 294-301 (1984).

72. Foltz, R.L., Clarke, P.A., Hidy, B.J., Lin, D.C.K., Graffeo, A.P., and Petersen, B.A. Quantitation of Delta-9-THC and 11-Nor-Delta-9-THCCOOH in Body Fluids by GC/CI-MS. In Vinson, J.A., (ed.), Cannabinoid Analysis in Physiological Fluids A.C.S. Symp. Ser. No. 98: 59-71 (1979).

73. Zweipfenning, P.G.M., Lisman, J.A., Van Haren, A.Y.N., Dijkstra, G.R., and Holthuis, J.J.M. Determination of Delta-9-THC in Plasma Using Solid-Phase Extraction and HPLC with Electrochemical Detection. J. Chromatogr. 456: 83-91 (1988).

74. Bachmann, E.W., Hofmann, A.A., and Waser, P.G. Identification of Delta-9-THC in Human Plasma by Gas Chromatography. J. Chromatogr. 178: 320-3 (1979).

75. Garrett, E.R. and Hunt, C.A. Pharmacokinetics of Delta-9-THC in Dogs. J. Pharm. Sci. 66: 395-407 (1977).

76. Peat, M.A., Deyman, M.E., and Johnson, J.R. HPLC-RIA of Delta-9-THC and its Metabolites in Urine. J. Forens. Sci. 29: 110-9 (1984).

77. Mechoulum, R., Ed., in Marihuana : Chemistry, Pharmacology, Metabolism and Clinical Effects. Academic Press, New York. p.144 (1973).

78. Sutherland, C.A., Yarborough, R., Hepler, B.R., and Sunshine, I. Detection and Confirmation of Urinary Cannabinoids. J. Anal. Toxicol. 9: 156-60 (1985).

79. Kogan, M.J., Newman, E., and Willson, N.J. Detection of Marihuana Metabolite 11-Nor-Delta-9-THCCOOH in Human Urine by Bonded-Phase Adsorption and TLC. J. Chromatogr. 306: 441-3 (1984).

80. Kaistha, K.K. and Tardrus, R. Semi-Quantitative Thin-Layer Mass Screening Detection of 11-Nor-Delta-9-THCCOOH in Human Urine. J. Chromatogr. 273: 528-33 (1982).

81. Vinson, J.A. and Lopatofsky, D.J. A Semi-Automated Extraction and Spotting System for Drug Analysis by TLC. I. Procedure for Analysis of the Major Metabolite of Delta-9-THC in Urine. J. Anal. Toxicol. 9: 6-9 (1985).

82. Kanter, S.L., Hollister, L.E., and Loeffler, K.O. Marihuana Metabolites in the Urine of Man. VIII. Identification and Quantitation of Delta-9-THC by TLC and HPLC. J. Chromatogr. 150: 233-7 (1978).

83. Valentine, J.L., Gan, O.H.M., Niu, H.C., and Thompson, E.D. HPLC Analysis of Delta-9-THC and Delta-9-THCCOOH in Human Plasma. In : Cannabinoid Analysis in Physiological Fluids, J.A. Vinson, ed., A.C.S. Symp. Ser. 98: 175-205 (1979).

84. Borys, H.K. and Karler, R. Post Column Derivatization Procedure for the Calorimetric Analysis of Tissue Cannabinoids Separated by HPLC. J. Chromatogr. 205: 303-23 (1981).

85. Abbott, S.R., Abu-Shumays, A., Loeffler, K.O., and Forrest, I.S. HPLC of Cannabinoids as Their Fluorescent Dansyl Derivatives. Res. Commun. Chem. Pathol. Pharmacol. 10: 9-19 (1975).

86. Garrett, E.R. and Hunt, C.A. Separation and Analysis of Delta-9-THC in Biological Fluids by HPLC and GLC. J. Pharm. Pharmacol. 66: 20-6 (1977).

87. Williams, P.L., Moffat, A.C., and King, L.J. Combined High-Pressure Liquid Chromatography and Radioimmunoassay Method for the Quantitation of Delta-9-THC and some of its Metabolites in Human Plasma. J. Chromatogr. 155: 273-83 (1978).

88. Gross, S.J., Soares, J.P., Wong, S.L.R., and Schuster, R.E. Marijuana Metabolites Measured by a Radioimmune Technique. Nature 252: 581-2 (1974).

89. Teale, J.D., Forman, E.J., King, L.J., Piall, E.M., and Marks, V. The Development of a Radioimmunoassay for Cannabinoids in Blood and Urine. J. Pharm. Pharmacol. 27: 465-72 (1975).

90. Owens, S.M., McBay, A.J., and Reisner, H.M. Radioimmunoanalysis of Delta-9-THC in Blood by Means of an [125]I Tracer, in "Cannabinoid Analysis in Biological

Fluids," (R.L. Hawks, ed.), Nat'l. Inst. on Drug Abuse Research Monogr. #42, Rockville, Maryland, pp.33-43 (1982).

91. Moffat, A.C., Williams, P.L., and King, L.J. Combined HPLC and RIA Method for the Analysis of Delta-9-THC and its Metabolites in Plasma and Urine, in "Cannabinoid Analysis in Biological Fluids," (R.L. Hawks, ed.), Nat'l. Inst. on Drug Abuse Research Monogr. #42, Rockville, Maryland, pp.56-68 (1982).

92. Gough, T.A. and Baker, P.B. Identification of Major Drugs of Abuse Using Chromatography. J. Chromatogr. Sci. 20: 289-314 (1982).

93. Turner, C.E., Hadley, K.W., Henry, J., and Mole, M.L. Constituents of Cannabis Sativa L. 7 : Use of Silyl Derivatives in Routine Analysis. J. Pharm. Sci. 63: 1872-6 (1974).

94. Novotny, M., Lee, M.L., Low, C.E., and Raymond, A. Analysis of Marihuana Samples from Different Origins by High-Resolution GLC for Forensic Applications Analyst. Chem. 48: 24-9 (1976).

95. Garrett, E.R. and Hunt, C.A. Picogram Analysis of THC and Application to Biological Fluids. J. Pharm. Sci. 62: 1211-4 (1973).

96. Mc Callum, N.K. The Measurement of Cannabinols in the Blood by GC. J. Chromatogr. Sci. 11: 509-11 (1973).

97. McCallum, N.K. and Shaw, S.M. Chromatographic Analysis for Delta-1-THC in Blood and Brain. J. Anal. Toxicol. 5: 148-9 (1981).

98. Harvey, D.J. Comparison of Fourteen Substituted Silyl Derivatives for the Characterization of Alcohols, Steroids and Cannabinoids by Combined GLC and MS. J. Chromatogr. 147: 291-8 (1978).

99. Harvey, D.J., Martin, B.R., and Paton, W.D.M. Identification and Measurement of Cannabinoids and Their in vivo Metabolites in Liver by GC-MS, in, "Marihuana : Biological Effects", (G.G. Nahas and W.D.M. Paton, eds.), Pergamon, Oxford. pp. 45-62 (1979).

100. Rosenfeld, J. The Simultaneous Determination of Delta-9-THC and 11-Hydroxy-Delta-9-THC in Plasma. Analyt. Letters 10: 917-30 (1977).

101. Hunt, D.F. and Crow, F.W. Electron-Capture Negative Ion Chemical Ionisation Mass Spectrometry. Anal. Chem. 50: 1781-4 (1978).

102. Nakahara, Y. and Sekine, H. Automatic Extraction Using the Mini-Column Liquid Chromatography Method for Analysis of Drugs of Abuse in Biological Fluids. Chromatographia 19: 131-6 (1984).

103. Lawrence, J.F. Organic Trace Analysis by Liquid Chromatography. Academic Press, New York. pp. 235-8 (1981).

104. Lawrence, J.F., ibid. pp. 86-116 (1981).

105. Nakahara, Y. Summary of the Information and Comments Related with the Analysis of Cannabinoids and Opiates in Body Fluids. National Inst. of Hygenic Sciences, Tokyo. pp. 2-19 (1989).

106. Widman, M., Agurell, S., Ehrnebo, M., and Jones, G. Binding of (+)- and (-)-Delta-9-THCs and (-)-7-Hydroxy-Delta-9-THC to Blood Cells and Plasma Proteins. J. Pharm. Pharmacol. 26: 914-6 (1974).

107. Nakahara, Y. and Sekine, H. Studies on Confirmation of Cannabis Use I. Determination of the Cannabinoid Contents in Marijuana Cigarette, Tar, and Ash Using HPLC with Electrochemical Detection. J. Anal. Toxicol. 9: 121-4 (1985).

108. Howe, I., Williams, D.H., and Bowen, R.D. Mass Spectrometry Principles and Applications. 2nd Ed., Mc Graw-Hill, London. pp. 4-9 (1981).

109. Chapman, J.R. Practical Organic Mass Spectrometry. John Wiley & Sons Ltd. London. pp.79-100 (1985).

110. Howe, I., Williams, D.H., and Bowen, R.D. Mass Spectrometry. Principles and Applications. 2nd Ed., Mc Graw-Hill, London. pp. 186-9 (1981).

111. Pierce & Warriner (UK) Ltd., Handbook & General Catalog, pp. 165-71 (1989).

112. Vree, T.B. Mass Spectrometry of Cannabinoids. J. Pharm. Sci. 66: 1444-50 (1977).

113. Lee, T.K. Mass Spectra of Delta-1- and Delta-6-THC and Their Metabolites. J. Singapore Natl. Acad. Sci. 16: 99-103 (1987).

114. Harvey, D.J., Martin, B.R. and Paton, W.D.M., in G.G. Nahas and W.D.M. Paton (Eds.), Marihuana : Biological Effects, Pergamon, Oxford. p. 45 (1979).

115. The Institute for the Study of Drug Dependence (ISDD), National Audit of Drug Misuse Statistics : Drug Misuse in Britain, 1990.