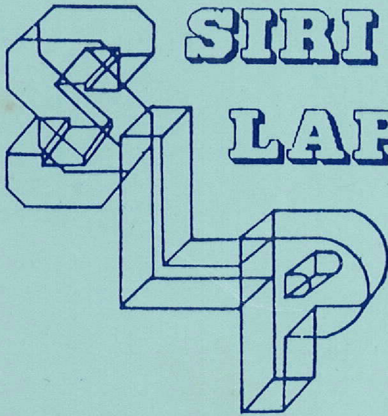


SIRI LAPORAN PENYELIDIKAN

23

# Chromatographic Methods For The Detection Of The Principal Cannabinoid Metabolite In Urine

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# CHROMATOGRAPHIC METHODS FOR THE DETECTION OF THE PRINCIPAL CANNABINOID METABOLITE IN URINE

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| Acet. | : | Acetone |
| AR | : | Analytical reagent |
| AUFS | : | Arbitary unit full scale |
| BPA | : | Bonded phase adsorption |
| BSA | : | N, O-Bis-(trimethylsilyl)-acetamide |
| BSTFA | : | N, O-Bis-(trimethylsilyl)-trifluoroacetamide |
| BuA | : | Butylamine |
| CBD | : | Cannabidiol |
| CBN | : | Cannabinol |
| CI | : | Chemical ionization |
| CI/MS | : | Mass spectrometer with chemical ionization |
| DAD | : | Diode array detector |
| DD | : | Double development |
| DEA | : | Diethylamine |
| DMF | : | Dimethylformamide |
| DMSO | : | Dimethylsulfoxide |
| DNS | : | Dibenzopyran numbering system |
| ECD | : | Electron capture detector |
| EChD | : | Electrochemical detector |
| EI | : | Electron impact |
| EI/MS | : | Mass spectrometer with electron impact ionization |
| EMIT | : | Enzyme Multiplied Immunoassay Technique |



| | | |
|---|---|---|
| $Et_2O$ | : | Diethyl ether |
| EtoAc | : | Ethyl acetate |
| FBB | : | Fast Blue B |
| FBBB | : | Fast Blue BB |
| FBRR | : | Fast Blue RR |
| FID | : | Flame ionization detector |
| GC | : | Gas chromatography |
| GC-CI/MS | : | GC with CI/MS |
| GC-ECD | : | GC with ECD |
| GC-EI/MS | : | GC with EI/MS |
| GC-FID | : | GC with FID |
| GC-MS | : | GC with mass spectrometer |
| GC-NICI/MS | : | GC with negative ion CI/MS |
| GPR | : | General purpose reagent |
| Hept. | : | Heptane |
| HETLC | : | High efficiency TLC |
| Hex. | : | Hexane |
| HFIP | : | Hexofluoroisopropanol |
| HPLC | : | High performance liquid chromatography |
| HPLC-EChD | : | HPLC with EChD |
| HPLC-RIA | : | HPLC with radioimmunoassay |
| HPLC-UV | : | HPLC with uv |
| HPTLC | : | High performance TLC |
| I.D. | : | Inner diameter |
| LLE | : | Liquid-liquid extraction |
| MeCN | : | Acetonitrile |

MID : Multiple ion detector

min : Minute

MNS : Monoterpene numbering system

MS : Mass spectrometer

MSD : Mass selective detector

MSTFA : N-methyl-N-trimethylsilyltrifluoroacetamide

MTBSTFA : N-methyl-N-(tertiary-butyldimethylsilyl)-trifluoroacetamide

NI : Negative ion

NICI : Negative ion chemical ionization

NICI/MS : MS with NICI

O.D. : Outer diameter

PBM : Probability based matching

Pet. ether : Petroleum ether

PFb-Br : Pentafluorobenzyl bromide

PFPA : Pentafluoropropionic anhydride

PFP-OH : Pentafluoropropanol

PICI : Positive ion chemical ionization

PNS : Pentyl side chain numbering system

RIA : Radioimmunoassay

rpm : Rotations per minute

s : Second

s.c.c.t. : Screw capped culture tube

SIM : Selected ion monitoring

S/N : Signal-to-noise

SPE : Solid phase extraction



TBDMS : Tertiary-butyldimethylsilyl

TBDMS-Cl : Tertiary-butyldimethylsilyl chloride

TEA : Triethylamine

TFAA : Trifluoroacetic anhydride

THC : $\Delta^9$-Tetrahydrocannabinol

THC-COOH : 11-Nor-THC-9-carboxylic acid

THC-OH : 11-Hydroxy-THC

TLC : Thin-layer chromatography

TMAM : Tetramethylammonium hydroxide

TMCS : Trimethylchlorosilane

TMS : Trimethylsilyl

UV : Ultraviolet

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## ABSTRAK


Kanabinoid utama yang bertanggung-jawab terhadap kesan-kesan psikoaktif bagi persediaan-persediaan dadah kanabis adalah $\Delta^9$-tetrahidrokanabinol (THC) yang mana asid 11-nor-$\Delta^9$-tetrahidrokanabinol-9-karboksilik (THC-COOH) merupakan metabolit utamanya didalam air kencing. Dua jenis kaedah yang digunakan untuk mengesan THC-COOH adalah kaedah-kaedah imunoasai dan kromatografi. Walaupun kaedah-kaedah kromatografi memerlukan perlakuan sampel yang terdahulu, ia digunakan dengan lebih meluas kerana ia lebih spesifik dan sensitif.

Kajian ini adalah bertujuan untuk menilai dan membentukkan kaedah-kaedah pengekstrakan dan kromatografi yang lebih sesuai untuk pengesanan THC-COOH dalam air kencing. Dalam kajian ini nilai-nilai $pK_1$ dan $pK_2$ untuk THC-COOH yang telah ditentukan dengan menggunakan kaedah HPLC dan kaedah spektrofotometri masing-masingnya adalah 6.1 dan 10.9 dan nilai-nilai ini telah digunakan untuk pembentukan suatu kaedah pengekstrakan secara sistematik. Etil asetat:isooktan (90:10, v/v) pada nilai pH 9 untuk air kencing telah menghasilkan pengekstrakan THC-COOH yang paling kuantitatif dan selektif. Kaedah pengekstrakan ini seterusnya telah ditingkatkan lagi dengan mencuci ekstrak yang diperolehi dengan larutan tampan fosfat pada pH 3.00. Nilai min pengembalian sebanyak 81.02% dan kejituan 5.67% telah diperolehi untuk THC-COOH dalam air kencing dan kaedah ini juga boleh digunakan untuk pengekstrakan morfina.

Sistem TLC dengan fasa bergerak yang terdiri daripada kloroform:metanol:larutan ammonia pekat (70:30:2, v/v/v) telah memberi resolusi yang optimum untuk THC-COOH daripada bahan-bahan endogenus air kencing dan juga $\Delta^9$-tetrahidrokanabinol (THC), kanabinol (CBN) dan kanabidiol (CBD). Teknik penglihatan yang terbaik adalah dengan menyembur dietilamina diikuti olih larutan bermetanol garam Fast Blue B yang telah menghasilkan suatu warna berciri amat merah untuk THC-COOH. Nilai $R_f$ yang telah diperolehi adalah 0.4 dan banyaknya yang dapat dikesankan sekurang-kurangnya adalah 37.5 ng THC-COOH.

Kaedah HPLC dengan turus fasa terbalik oktilsilana (RP-8) dan suatu fasa bergerak yang terdiri dari 47.5% asetonitril dan 52.5% air (pH 3.00) dengan kadar pengaliran sebanyak 1.00 ml $min^{-1}$ telah dapat mengesan 0.8 ng THC-COOH pada panjang gelombang 214 nm dan isyarat output pengesan sebanyak 0.005 AUFS. Resolusi yang baik telah diperolehi untuk THC-COOH daripada kebanyakan bahan-bahan endogenus air kencing. Isyarat pengesan didapati berkadaran linear untuk larutan bermetanol THC-COOH dari kepekatan 40 ng $ml^{-1}$ hingga 100 µg $ml^{-1}$ dengan pekali regrasi 0.999.

Sistem GC-MSD dengan turus rerambut silika rangkaian silang SE 54 telah memberi spektrum berciirian untuk trimetilsilil THC-COOH. Sistem tersebut juga dapat mengesan 2.5

$\mu g$ $ml^{-1}$ dan 25 ng $ml^{-1}$ trimetilsilil THC-COOH masing-masingnya didalam mod imbasan penuh dan mod SIM. Had pengesanan didalam mod imbasan penuh adalah 5 $\mu g$ $ml^{-1}$ yang telah memberi mutu bandingan spektrum sebanyak 80%.




## ABSTRACT

The principal cannabinoid responsible for psychoactive effects of cannabis drug preparations is $\Delta^9$-tetrahydrocannabinol (THC) of which the major metabolite in urine is 11-nor-$\Delta^9$-tetrahydrocannabinol-9-carboxylic acid (THC-COOH). Both immunoassay and chromatographic methods have been used for the detection of THC-COOH. Although chromatographic methods require sample pretreatment, they are more widely used because of their specificity and sensitivity.

The purpose of this investigation was to evaluate and develop suitable extraction and chromatographic methods for the detection of THC-COOH in urine. The $pK_1$ and $pK_2$ of THC-COOH which were determined by a HPLC and a spectrophotometric method were 6.1 and 10.9 respectively and these values were used in the systematic development of an extraction procedure. Ethyl acetate : isooctane (90 : 10, v/v) at urine pH 9 gave the most quantitative and selective extraction of THC-COOH. The selectivity was further enhanced by washing the extract with pH 3.00 phosphate buffer. A mean recovery of 81.02% and a precision of 5.67% was obtained and the method could also be used for the extraction of morphine.

The TLC system with chloroform:methanol:concentrated ammonia (70:30:2, v/v/v) as the mobile phase gave optimum resolution of THC-COOH from urinary endogeneous materials as well as from $\Delta^9$-tetrahydrocannabinol (THC), cannabinol (CBN) and cannabidiol (CBD). The best visualisation technique was spraying with diethylamine followed by methanolic solution of Fast Blue B salt which produced a characteristic intense red colour for THC-COOH. The $R_f$ value was 0.4 and the minimum detectable amount was 37.5 ng of THC-COOH.

The HPLC method with a reversed phase octylsilane (RP-8) column and a mobile phase consisting of 47.5% acetonitrile and 52.5% water (pH 3.00) at a flow rate of 1.00 ml $min^{-1}$ was capable of detecting 0.8 ng of THC-COOH on-column at a wavelength of 214 nm and detector output signal of 0.005 AUFS. Good resolution of THC-COOH from the bulk of the urinary endogeneous materials was obtained. The detector response was linear for methanolic THC-COOH solutions from 40 ng $ml^{-1}$ to 100 $\mu g$ $ml^{-1}$ concentrations with a coefficient of regression of 0.999.

The GC-MSD system with a SE-54 cross-linked fused silica capillary column gave characteristic spectrum for trimethylsilylated THC-COOH.The system was capable of detecting 2.5 $\mu g$ $ml^{-1}$ and 25 ng $ml^{-1}$ of trimethylsilylated THC-COOH in the scan and SIM modes respectively. The limit of detection in the scan mode was 5 $\mu g$ $ml^{-1}$ which gave a 80% match quality.

# Chapter 1

## INTRODUCTION

### 1.1 Chemistry, pharmacokinetics, pharmacology and metabolism

Many drug preparations from the hemp plant Cannabis sativa are in common use. A list of some of these preparations and the parts of the plant from which they are derived from are given in table 1.1. Cannabis is a plant intoxicant consisting of a complex mixture of about 421 constituents [2, 3]. The classes and sub-classes of those constituents that have been identified are given in table 1.2. However the constituents associated with the pharmacological effects of cannabis are the cannabinoids of which 61 have been identified [2, 3]. The cannabinoids are compounds containing 21 carbon atoms that are unique to the cannabis plant and are present as carboxylic acid analogs and transformation products derived from them. Cannabinoids show similar chemical characteristics to terpenophenolic compounds [4] and are regarded as substituted monoterpenoids. Consequently the monoterpene numbering system (MNS) is used for cannabinoid nomenclature besides the dibenzopyran numbering system (DNS). Figure 1.1 shows both the numbering systems as well as the pentyl side chain numbering system (PNS). The principal cannabinoids that have been the subject of extensive research are $\Delta^9$-tetrahydrocannabinol (THC), cannabinol (CBN) and cannabidiol (CBD) (figure 1.2). However THC and its biotransformation products will be named using the dibenzopyran system in this thesis. The above cannabinoids as well as others are also present as carboxylic acid derivatives in plant material [5, 6, 7].

The relative as well as absolute amounts of the above principal cannabinoids and other minor cannabinoids that have been quantitated by researchers were found to vary widely in different species of the cannabis plant. Three species of the cannabis plant which have been reported are Cannabis sativa, Cannabis indica and Cannabis ruderalis [4]. The cannabis plant has also been divided into 3 types of chemovariants which possess different amounts of THC and CBD [2]. They are the fibre type which has a high content of CBD, the intermediate type which contains percent CBD equal to or greater than THC and the drug type which has a high content of THC and very little or none of CBD. Another classification based on relative amounts of THC and CBD as well as phenotypic differences have also been reported [8].

Table 1.1: Cannabis drug preparations and their percent THC content (1)

| Preparation | Part(s) of the plant | Percent THC (%) |
|---|---|---|
| Marijuana (also known as bhang) | All the plant material dried | 0.5 - 3.0 |
| Ghanja | Dried flowers and top leaves only | up to 5.0 |
| Hashish (also known as charas) | Resin | 10.0 |
| Hash oil | Liquid or tarry substance obtained by percolating a solvent such as ether through marijuana to extract the THC | up to 63.0 |
| Kif | Mixture of ghanja and tobacco | |
| Marijuana tea | Beverege brewed from marijuana in water | |
| "Alice B. Toklas" fudge or brownies | Fudge or brownies containing marijuana | |



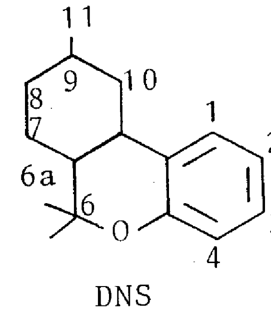
Table 1.2: Chemical constituents of cannabis preparations (2)

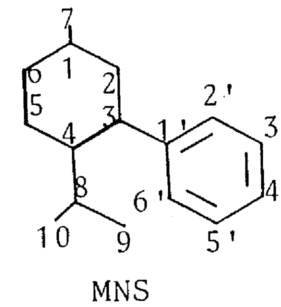
| Constituents | Number of constituents identified |
|---|---|
| 1. Cannabinoids: | 61 known |
| a. Cannabigerol (CBG) type | 6 known |
| b. Cannabichromene (CBC) type | 4 known |
| c. Cannabidiol (CBD) type | 7 known |
| d. $^{9}$-Tetrahydrocannabinol ( $^{9}$-THC) type | 9 known |
| e. $^{8}$-Tetrahydrocannabinol ( $^{8}$-THC) type | 2 known |
| f. Cannabicyclol (CBL) type | 3 known |
| g. Cannabielsoin (CBE) type | 3 known |
| h. Cannabinol (CBN) type | 6 known |
| i. Cannabinodiol (CBND) type | 2 known |
| j. Cannabitriol (CBT) type | 6 known |
| k. Miscellaneous types | 9 known |
| l. Other cannabinoids | 4 known |
| 2. Nitrogenous compounds | 20 known |
| a. Quarternary bases | 5 known |
| b. Amides | 1 known |
| c. Amines | 12 known |
| d. Spermidine alkaloids | 2 known |
| 3. Amino acids | 18 known |
| 4. Proteins, glycoproteins, and enzymes | 9 known |
| 5. Sugars and related compounds | 34 known |
| a. Monosaccharides | 13 known |
| b. Disaccharides | 2 known |
| c. Polysaccharides | 5 known |
| d. Cyclitols | 12 known |
| e. Aminosugars | 2 known |
| 6. Hydrocarbons | 50 known |
| 7. Simple alcohols | 7 known |
| 8. Simple aldehydes | 12 known |
| 9. Simple ketones | 13 known |
| 10. Simple acids | 20 known |
| 11. Fatty acids | 12 known |

Contd. Table 1.2

| Constituents | Number of constituents identified |
|---|---|
| 12. Simple esters and lactones | 13 known |
| 13. Steroids | 11 known |
| 14. Terpenes | 103 known |
| a. Monoterpenes | 58 known |
| b. Sesquiterpenes | 38 known |
| c. Diterpenes | 1 known |
| d. Triterpenes | 2 known |
| e. Miscellaneous compounds of terpenoid origin | 4 known |
| 15. Noncannabinoid phenols | 16 known |
| 16. Flavanoid glycosides | 19 known |
| 17. Vitamins | 1 known |
| 18. Pigments | 2 known |





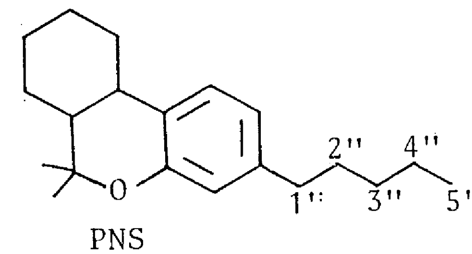
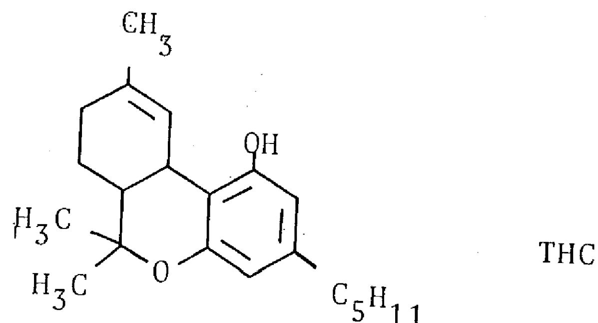
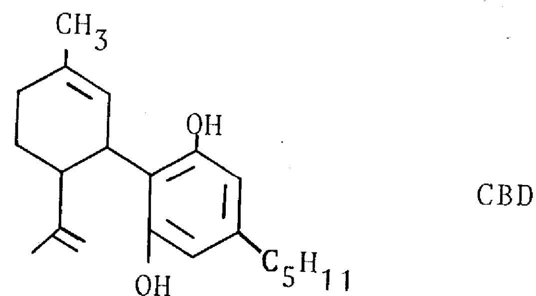




Figure 1.1: Nomenclature systems for Cannabinoids



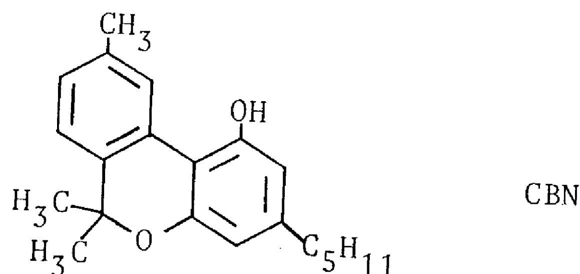




Figure 1.2: The Principal Cannabinoids



There are many routes of administration of cannabis drug preparations. The usual method used by occasional users or abusers is either by smoking or by oral consumption. Cannabis is usually smoked directly or as a mixture with tobacco in cigarettes or with various smoking appliances. Oral ingestion is usually in the form of a beverage, fudges or brownies and capsules containing THC. Other routes of administration, which are normally used for research purposes only, are parenteral administration by intravenous or intramuscular injections, opthalmic preparations and rectal suppository formulations [9].

Smoking is the most widely used mode among abusers. The process of smoking results in pyrolysis of the cannabinoids and in the case of THC, which is regarded as the principal psychoactive component, it has been reported that about 30% of the original THC dose was degraded. Furthermore after taking into account the volume and duration of an inhalation of a typical smoker, the actual amount being delivered was estimated to be about 16% to 19% of the original dose in the cigarette [9]. The carboxylic acid derivatives of the cannabinoids usually present in fresh plant material are also decarboxylated due to heat on smoking [10, 11]. This process also takes place on storage and exposure to light [6]. This would imply that the absolute amount of cannabinoids available on smoking is higher than when ingested since there is no transformation of inactive carboxylated cannabinoid to active uncarboxylated cannabinoid on oral consumption.

THC is the principal psychoactive component of cannabis and as a consequence research on the psychoactivity of cannabis has been mainly directed towards THC [12, 13, 14]. Smoking cannabis results in rapid absorption of THC, as well as other cannabinoids, through the lungs and respiratory tract into the systemic circulation. Peak THC concentration in plasma is reached very rapidly [9]. However the bioavailability of THC varies and is 16% to 40% and 13% to 14% for heavy and occasional users respectively after smoking a cigarette containing 1% to 2% THC. In the case of oral administration, peak THC levels in plasma are reached in about 2 h due to the slow absorption of the lipophilic THC through the gastrointestinal tract. The bioavailability of THC is 10% to 20% and this low value is primarily due to "first-pass effects" during absorption [9]. The THC is metabolised during absorption in the liver before it reaches the systemic circulation [9, 14]. In the case of intravenous administration the time taken to reach peak plasma levels are dependent on the vehicle used and is usually reached in about 15 to 30 min when injected in the right vehicle [9].

The initial metabolism of the cannabinoids on smoking takes place in the lungs whereas it occurs in the liver when consumed orally [2]. Different enzymes are involved in the metabolism of cannabinoids in the lungs and the liver and consequently different initial metabolites are formed [2]. Major metabolites of the lung are side chain hydroxylated metabolites whereas that of the liver are hydroxylated derivatives of the cyclohexene ring system. More than 35 metabolites have been reported for THC, 22 metabolites for CBN and 22 metabolites for CBD [2]. Some of the known metabolic pathways for THC which are schematically represented in figure 1.3 are allylic and aliphatic hydroxylations (A), oxidation of methyl groups to acids, aldehydes and ketones (B), epoxydation of double bonds (C), reduction of the terpene double bond (D) and conjugation with fatty acids or β-glucuronic acid (E). The major metabolite of THC (I) is 11-nor-$\Delta^9$-tetrahydrocannabinol-9-carboxylic acid (IV,THC-COOH), which undergoes conjugation with β-glucuronic acid (V) to give its O-ester glucuronide (VI) whereas 11-hydroxy-$\Delta^9$-tetrahydrocannabinol (II,THC-OH), is an intermediate metabolite, which is oxidised to 11-oxo-THC (III) and finally to THC-COOH (figure 1.4). THC-COOH is the predominant metabolite after smoking and THC-OH is present only in very low concentrations, less than 10% of the THC concentration. However in intravenous and oral dosing, THC-COOH concentrations are 2 to 3 times and 6 to 10 times more than THC concentrations respectively whereas THC-OH constitute 10% to 15% and 50% of the THC-COOH concentrations respectively [9]. The majority of plasma metabolites are polar acidic compounds and neutral (mainly hydroxy) metabolites represent only about 10%. Some of the metabolites that have been reported for THC (figure 1.5) are allylic hydroxylated metabolites such as 8α - and 8β -hydroxy- $\Delta^9$-tetrahydrocannabinol (8α- and 8β -OH-THC), aliphatic hydroxylated metabolites of the pentyl side chain such as 1"-, 2"-, 3"-, 4"- and 5"-hydroxy- $\Delta^9$- tetrahydrocannabinol (1"-, 2"-, 3"-, 4"- and 5"-OH-THC), mixture of allylic and aliphatic hydroxylated metabolites such as 8 ,11-dihydroxy-THC [9, 15, 16, 17]. These metabolites were detected in animal studies but a few of them were also present in human subjects. Furthermore, studies on human subjects showed that 80% to 90% of the initial THC dose was excreted as its metabolites and 65% of the excreted amount was found in faeces and 18% to 23% in urine [9, 18]. The faeces contained neutral and acidic metabolites whereas urine contained mainly acidic metabolites. Twenty acidic metabolites have been identified in urine of which 18 were unconjugated acids and 2 were conjugated with β-glucuronic acid. With the exception of THC-COOH all the unconjugated acids had their side chains oxidised or degraded to form various hydroxylated and carboxylated metabolites. However THC-COOH, the major metabolite in faeces and urine, is excreted mainly as the glucuronic acid conjugate [19, 20, 20].



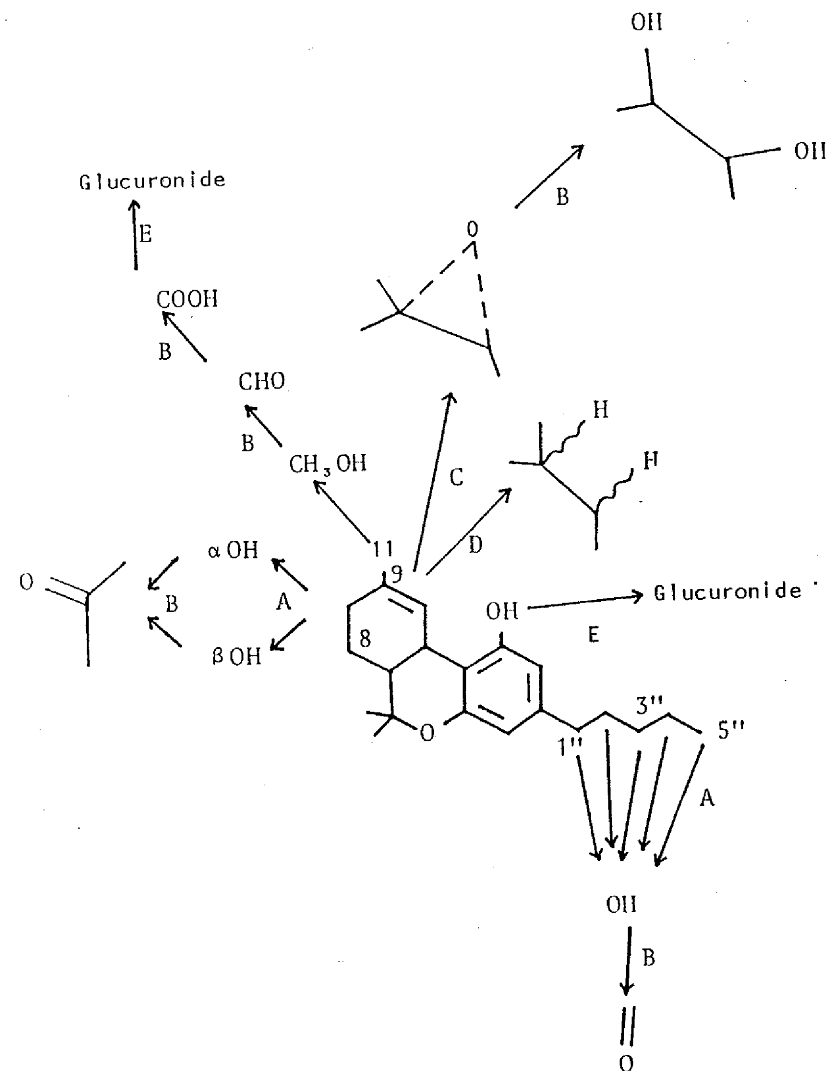



Figure 1.3: Metabolic pathways of THC

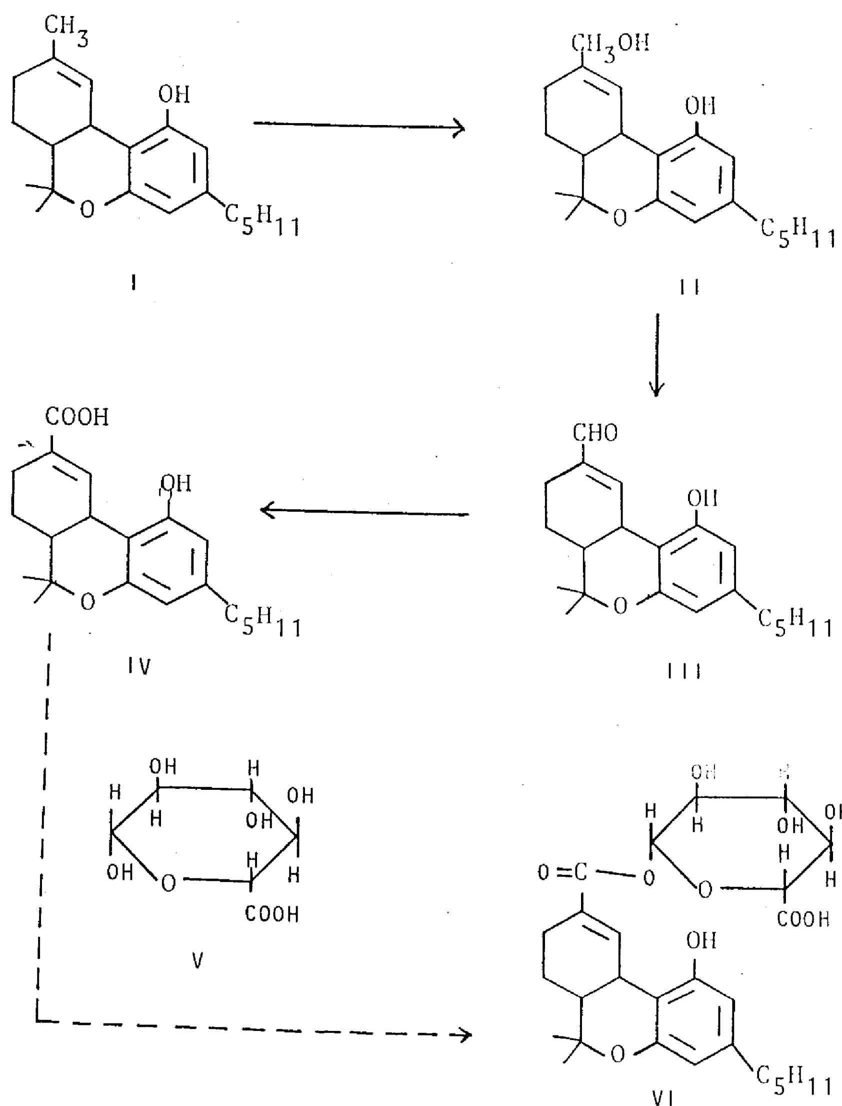



Figure 1.4: Metabolic pathway of THC to THC-COOH and its glucuronide ester

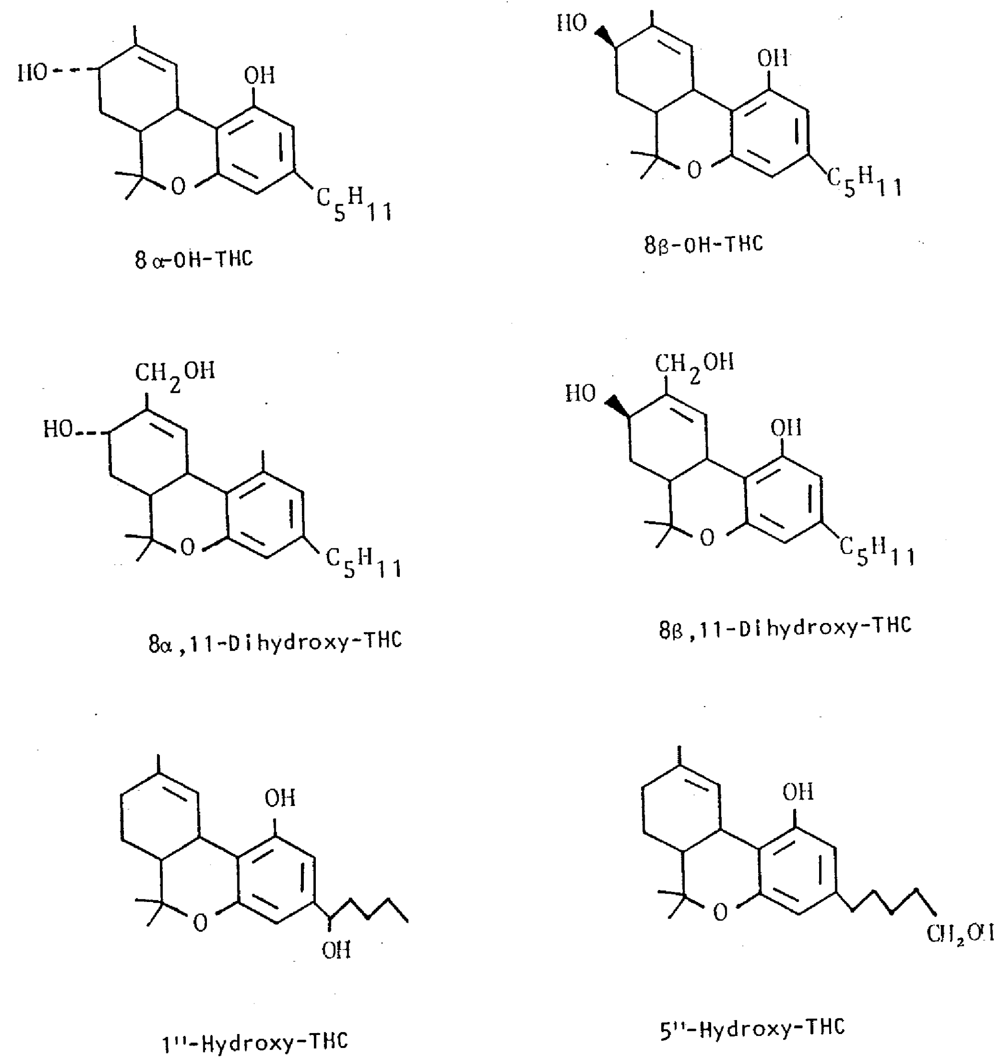



Figure 1.5: Metabolites of THC

The majority of the metabolites of THC are highly plasma bound and the plasma terminal half-life is approximately 50 hours [9]. The rate of elimination of the metabolites is therefore much longer than that of THC and is due to their slower rates of formation resulting from the slow return of THC from sequestered tissues to form metabolites [9]. The metabolites have been reported to persist in plasma up to 72 h after consumption [19, 21, 22]. The other reasons reported for the slow elimination of the metabolites include enterohepatic recirculation and renal reabsorption [14, 18]. Figure 1.6 shows the plasma concentration of THC and THC-COOH as a function of time after smoking and oral administration. The rapid decline of the THC concentration has been attributed to its distribution into body tissues. Both 3 compartment [12] and 4 compartment [9, 13] pharmacokinetic models have been proposed to explain the distribution of THC into different types of tissues with different binding strengths. THC is returned to blood from the sequestered tissues only after a pseudo-equilibrium is reached between plasma and the tissues. The terminal half-life of THC is approximately 1 day. On returning to blood THC is then metabolised to its metabolites and this distribution and slow return of THC has been cited as one of the reasons for the longer half-life of its metabolites. THC metabolites have been detected in urine 12 days after consumption [19]. Thus the detection of THC-COOH, which is the major metabolite of THC [9, 18], would be a good indication of consumption of THC or cannabis based drug preparations.

## 1.2 Analytical methods

Analytical techniques which have been reported in literature for analysis of cannabinoids in body fluids can be broadly classified as either immunoassay or chromatographic methods.

Immunoassay methods include radioimmunoassay (RIA) and enzyme multiplied immunoassay technique (EMIT). Chromatographic methods include thin-layer chromatography (TLC), high performance TLC (HPTLC) or high efficiency TLC (HETLC), high performance liquid chromatography (HPLC), gas chromatography (GC) and GC - mass spectrometer (GC-MS). The practical range of sensitivities for some of these techniques are given in figure 1.7.

## 1.3 Immunoassay methods

Immunoassay techniques are relatively popular screening methods due to their ease of operation. They require minimal or no sample pre-treatment, small volume of sample (25 ul in the case of EMIT), short analysis time and are able to generate semi-quantitative data. Several papers have been published correlating EMIT semi-quantitative data with those of other methods such as



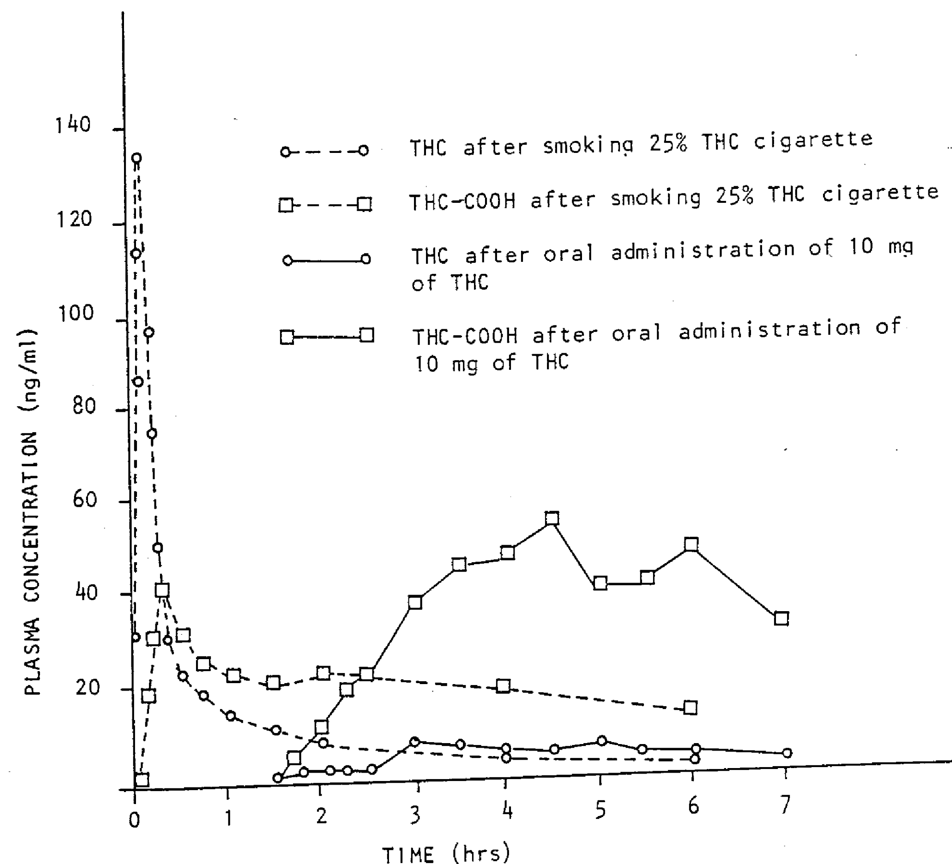



Figure 1.6: Plasma concentrations of THC and THC-COOH after smoking and oral consumption (18)

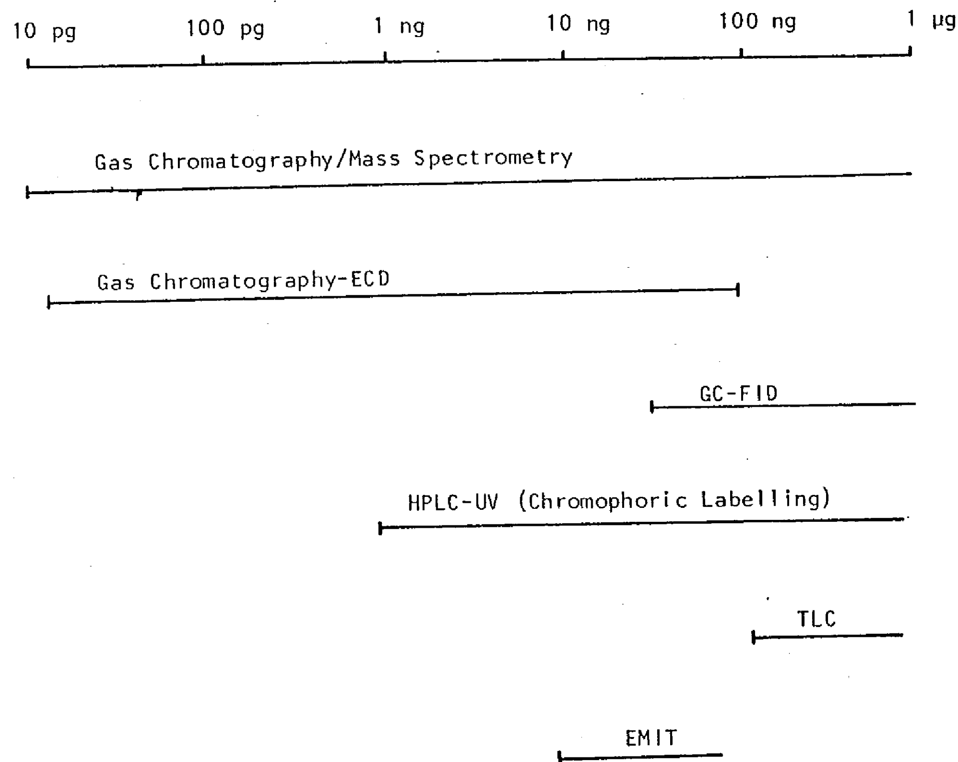



Figure 1.7: Sensitivity range of cannabinoid detection methods



GC with flame ionization detector (GC-FID) and GC-MS [23, 24]. Studies of urinary excretion patterns on smoking cannabis cigarettes [25] and passive or accidental inhalation of cannabis smoke [26, 27] by the EMIT have also been reported but such assays could be unreliable due to cross-reaction with endogeneous urinary materials and other drugs [28, 29], as well as being dependent on the ionic strength of the sample [28]. In view of this, it has been suggested that all samples giving positive responses with EMIT should be verified by an independent second method equally sensitive but utilizing a different principle for detection [28, 30 - 32]. As a consequence a wide variety of methods capable of giving qualitative and quantitative results to confirm EMIT positive responses were developed. These methods include TLC, HETLC and HPLC [32], RIA and GC-MS [33, 36], HPLC-RIA [20, 34, 35] and bonded phase adsorption (BPA)-TLC [31, 36]. RIA has also been reported to be nonspecific because its specificity depends on the radiotracers [37]. However the method is much more sensitive with reported sensitivities of 3.3 ng $ml^{-1}$ of cannabinoids and metabolites [38] and 0.1 ng $ml^{-1}$ of THC and metabolites [39] in urine. The problem of nonspecificity has been partially overcome by the use of HPLC prior to RIA analysis. The resolving power of HPLC coupled with the sensitivity of RIA has made it a popular method for metabolic and pharmacokinetic studies [19 - 21, 39, 40] as well as for detection of drug use [35, 38].

## 1.4 Chromatographic methods

Chromatographic methods are widely used for analysis of cannabinoids but due to their limited sample loading capacity and the complex nature of body fluids, sample pre-treatment is necessary.

### 1.4.1 Sample pre-treatment

In general, sample pre-treatment is used mainly for the following three reasons:

i. isolate drugs or compounds of interest from a larger concentration of endogeneous materials that might interfere with the final analysis by clean-up steps;

ii. concentrate samples particularly for drugs present in trace amounts as well as to increase sensitivity of those that do not have high response factors with respect to the mode of analysis; and

iii. reduce analysis time for complex mixtures which might require longer analysis time for effective separation of the drug from other materials.

Sample pre-treatment steps that have been reported for THC-COOH include liquid-liquid extraction (LLE), solid-phase extraction (SPE) and preparative HPLC methods. Some of the methods employed by previous workers are summarised in table 1.3. LLE methods are the most widely used [23, 41 - 55] followed by SPE [31 - 33, 56 - 61] and HPLC [20, 21, 35, 38, 40, 62]. SPE methods have only gained popularity recently due to the availability of commercially packed SPE cartridges and extraction systems and possibility of automation for routine analysis of large number of samples. HPLC as a preparative method has also been used previously to provide a greater degree of selectivity to immunoassay methods which are sensitive but lack selectivity [20, 21, 35, 38, 40]. A HPLC method using preparative cartridges has also been reported recently which is capable of analysing urine containing THC-COOH with minimum sample pretreatment [62]. Almost all of the sample pretreatment procedures also include a hydrolysis step because a major portion of the THC-COOH excreted is in the O-ester glucuronide form [19 - 21]. The hydrolysis step usually involves using an aqueous solution of a base [31, 32, 36, 39, 45, 50, 53, 56 - 59, 61] or an alcohol solution of a base [20, 21, 40, 42, 44, 46, 49, 51, 55, 60, 62]. Enzyme hydrolysis with β - glucuronidase [21, 23, 46, 47] or β-glucuronidase-aryl sulphatase [33, 43] have also been utilised but base hydrolysis is much more efficient and reproducible [57]. However, transesterification of the THC-COOH glucuronide conjugate to the corresponding methyl or ethyl ester has been reported with methanolic or ethanolic hydroxides [20, 44, 55].

Liquid-liquid extraction or solvent extraction is a widely used procedure mainly because it does not require elaborate equipment. In contrast SPE methods require a vacuum manifold with a vacuum pump and HPLC procedures utilize more sophiscated instrumentation. Open-column chromatography, the predecessor to HPLC and SPE, can also be used for sample pre-treatment and does not require special instrumentation. However it requires a longer time for elution of samples especially if longer columns are used to obtain clean samples. Longer elution time implies more solvent when compared to the smaller volumes used in LLE or SPE.

The efficiency of a LLE procedure depends on a number of factors which include the nature of the extraction solvent, the pH of the matrix being extracted, the miscibility of the extracting solvent and matrix, the solubility of the solute or extractant and the adsorption of solute on glass [63].



Table 1.3: Summary of sample pre-treatment methods from literature

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 41 | 20 | LLE | 1. 2 ml methanolic KOH (10% w/v)<br>2. 10-15 mins at 100$^{o}$C | 3 ml glacial acetic acid to pH 3-4 | 15 ml cyclohexane: ethyl acetate (96:4) | TLC | |
| 42 | 20 | LLE | 1. 2 ml methanolic KOH (10% w/v)<br>2. 10-15 mins at 100$^{o}$C | 3 ml glacial acetic acid to pH 3-4 | 15 ml cyclohexane: ethyl acetate (96:4) | TLC and GC-MS | 1. Extract was passed through a bed of anhydrous sodium sulphate prior to drying.<br>2. TLC also served as a preparative step prior to GC-MS |

contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 43 | Volume equivalent to 50 mg creatinine | LLE | 1. pH 4.7 to 6.3 with 6N HCl<br>2. 0.1 ml Boehringer-Mannheim β-glucuronidase-aryl sulfatase<br>3. 30 mins at 55-60$^{o}$C | pH 4.7 - 6.3 | 2 x 15 ml anhydrous diethyl ether | TLC | 1. Ether extract was washed twice with 5% sodium bicarbonate solution and dried with 1g anhydrous sodium sulphate after discarding the bicarbonate<br>2. Urine containing more than 50mg of creatinine reportedly found to yield emulsions frequently. |
| 44 | 10 | LLE | 1. 8 ml methanol & 2 ml 10 M KOH.<br>2. 15 mins at 50$^{o}$C. | 2 ml 1 M $KH_2PO_4$ buffer and conc. HCl to pH 2.0 - 2.5. | 20 ml hexane : ethyl acetate (7:1) | GC-FID | 1. Extract purified by back-extraction with 0.5 M KOH.<br>2. Recovery of method reported as 76 $\pm$ 5.8 % for 50 ng $ml^{-1}$ and 66 $\pm$ 4.6 % for 200 ng $ml^{-1}$ urine THC-COOH concentrations. |



contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 45 | 1 | LLE | 1. 0.2 ml 10 M KOH.<br>2. 15 mins at 50$^{o}$C. | 0.2 ml $KH_2PO_4$ buffer at pH 2.5 and 2.0 N $H_2SO_4$ to pH 2.0 to 2.5. | 2 ml hexane : ethyl acetate (7:1) | GC-ECD | 1. Extract purified by extracting urine once with 2 ml extraction solvent and discarding the extract before acidification for actual extraction.<br>2. Method capable of detecting 1 to 2 ng $ml^{-1}$ in urine. |
| 46<br>47 | 1 | LLE | 1. 0.8 ml 0.5 M acetate buffer at pH 4.5.<br>2. 2300 U β-glucuronidase.<br>3. 30 mins at 56$^{o}$C. | pH 4 to 5 | 4 ml diethyl ether : hexane (1:1). | HPLC-UV, GC-ECD and GC-MS | 1. HPLC used for sample clean-up step prior to GC-ECD analysis.<br>2. Recovery reported as 77% and 79% for 2 separate determinations on 68 ng of spiked THC-COOH.<br>3. Detection limit was 20 ng $ml^{-1}$ |

contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 48 | 1 | LLE | NIL | 1. 0.2N NaOH<br>2. 0.1M HCl | 2 ml hexane :ethyl acetate (9:1). | GC-MS | 1. Method involves addition of MeCN and separation of supernatant followed by evaporation to less than 1 ml.<br>2. Concetrated supernatant then extracted for basic and neutral cannabinoids prior to acidification and extraction of THC-COOH. |
| 49 | 1 | LLE | 1. Equal volume of 1 M NaOH in methanol.<br>2. 12 mins at 100°C. | 1 ml 1 N $H_2SO_4$ and 1 ml pH 2 buffer. | 4 ml hexane : ethyl acetate (7:1). | GC-MS | 1.Extracts washed with 2 ml 0.2 N $H_2SO_4$ before drying. |



contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 50 | 5 | LLE | 1. 1 ml 1 M KOH.<br>2. 15 mins at 37°C. | 3 ml phosphate buffer at pH 1.8. | 5 ml hexane : ethyl acetate (7:1). | GC-MS | |
| 23 | 3 | LLE | 1. 50% acetic acid and 3 ml 0.1 M sodium acetate buffer (pH 5.0) to give pH 5.0.<br>2. 6 ml - glucuronidase (9800 Fishman units).<br>3. 24 hours at 37°C. | pH 5.0. | 30 ml acetone (added slowly while sonicating derivatized mixture). | GC-FID and GC-MS. | 1. Preparative HPLC was used to purify samples after extraction and evaporation. |

contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 51 | 1 | LLE | 1. 1 ml 45% (w/w) KOH : methanol (1:4). 2. Rock for 30 mins. | 0.5 ml 6.0 M HCl. | 0.5 ml hexane : ethyl acetate (5:1). | GC-MSD | 1. Extraction method with clean-up step involving extraction with hexane : isoamyl alcohol under basic conditions and then discarding. 2. Absolute recovery reported as 70%. |
| 52 | 10 | LLE | 1. 11.8 M KOH. | Maleic acid. | Ethyl acetate : hexane (1:9). | TOXI - LAB TLC system. | 1. Sample clean - up involved back - extraction using 5 ml 5% $NaHCO_3$: MeCN (5:1) and re-extraction with hexane : $CH_2Cl_2$ : EtOAc (1:1:1). 2. TOXI - LAB THC procedure used. |



contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 53 | 5 | LLE | 1. 1 ml 10 M KOH. 2. 15 to 30 mins at 56°C. | 3 ml phosphate buffer at pH 5.2 and 1 ml conc. HCl. | 8 ml isooctane : ethyi acetate (80 : 20). | HPLC - UV | 1. Recovery reported as 80% to 84%. 2. Limit of detection was 20 ng $ml^{-1}$. 3. Reproducibility was 6.5% for 130 ± 16 ng/ml and 5.9% for 150 ± 17 ng $ml^{-1}$ of pooled urine samples. |
| 54 | 5 | LLE | NIL | 5 ml 1 M citrate - HCl buffer at pH 4.1. | 7.5 ml diethyl ether. | GC-MS | |

contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 55 | 10 | LLE | 1. 8 ml ethanol & 2 ml 10 M KOH.<br>2. 15 mins at $60^{o}C$ | 1 M $NaH_2PO_4$ buffer (pH 2.5) and conc. HCl to pH 2 to 3. | 3 ml hexane : ethyl acetate (7:1). | GC-MS | 1. Following clean-up step was used:-<br>a) Hydrolysed and acidified urine passed through celite 500 diatomaceous tube.<br>b) Tube was then eluted with 4 % 15 ml hexane : ethyl acetate (7:1).<br>c) Eluent concentrated to 10 ml and 5 ml 0.5 M KOH added.<br>d) Organic phase aspirated and aqueous phase extracted after acidification. |



contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 31<br>36<br>56 | 10 | SPE | 1. 0.9 ml 10 M NaOH.<br>2. 15 mins at $100^{o}C$ | Approx. 0.7 ml conc. HCl to pH 1 to 2. | 1 ml acetone | TLC | 1. Water in eluent removed by vortexing eluent with $CH_2Cl_2$ and collecting the resulting organic phase, leaving behind water.<br>2. Bond - Elut THC column (500 ng) was used. |
| 32<br>57<br>58 | 10 | SPE | 1. 2 ml 10 M KOH<br>2. 15 mins at $50^{o}C$. | Conc. HCL added to pH 5 to 6. | 1.5 ml MeCN | 1. HPLC-UV<br>2. HPLC-UV & HETLC.<br>3. HPLC-EChD & GC-MS. | 1. Bond - Elut THC column used.<br>2. Recoveries were 90% [32], 25.6% [57], 90 ± 5% [58].<br>3. Reproducibility was 3.6% and 12.9% for 100.4 and 31.0 ng $ml^{-1}$ urine samples respectively [32]. |

contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 33 | NA | SPE | Glucuronidase - sulphatase overnight at pH 5. | NIL | $CH_2Cl_2$ : acetone mixture. | GC-MS | 1. XAD - 2 resin (PREP 1 system by DuPont) was used. 2. Detection limit was 2 ng $ml^{-1}$. |
| 59 | 10 | SPE | 1. 1 ml 10 M KOH. 2. 15 mins at $50^oC$. | Conc. HCl or 12 M NaOH to pH 8.2 to 8.7. | Ethyl acetate : methanol : acetic acid (90:10:1). | GC-MS | 1. Type A cartridge containing strongly basic anion exchange resin in dry form (Du Pont) was used. 2. Extraction efficiency was 50 to 60% |
| 60 | 5 | SPE | 1. 1 ml 10 M KOH & 1 ml. methanol. 2. 15 mins at $50^oC$. | 2 ml phosphate buffer (pH 7) & conc. HCl. | 10 ml acetone : water (95:5). | GC-MS | 1. Sepharose 4B coupled with THC antibody was used. 2. Reported to be specific in binding. 3. Recovery was 92.3± 3.9%. 4. Capable of detecting 0.5 ng $ml^{-1}$. |



contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 61 | 2 | SPE | 1. 0.2 ml 6 M NaOH. 2. 20 mins at $60^oC$. | 0.3 ml acetic acid. | 1.5 ml ethyl acetate : isopropanol (85:15). | HPTLC with densitometer | 1. Pre-packed XAD - 2 resin used. 2. Recovery was 95.9% with average precision of 6.3%. Sensitivity was 15 ng $ml^{-1}$. |
| 20 40 | 0.1 to 3.0 | HPLC | 1. Methanolic NaOH (equal volume to urine). 2. Evacuate $O_2$ and stand | Acetic acid to pH 5.5. | Sample diluted to 6 ml with methanol : 0.1 M acetic acid at pH 5.5 (50:50) and injected into HPLC. | HPLC-RIA & GC-MS | 1. HPLC eluent was fraction collected and freeze dried before analysis by RIA or GC-MS. |

contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 21 | NA | HPLC | 1. a) Methanol & 1 M NaOH (1:1). b) 20 mins at RT. c) Terminated with glacial acetic acid. 2. a) 0.075 phosphate buffer (pH 5). b) 2000 units ml glucuronidase. c) 2 hours at 37°C. | Phosphoric acid added to pH 3. | 1. Half volume of diethyl ether : n-propanol (8:2). 2. Extract dried and dissolved in methanol (100 ul methanol per 200 ml urine extracted). and injected into HPLC. | HPLC-RIA & GC-MS | 1. Eluate fraction from HPLC was analysed by RIA and GC-MS. |
| 35 | 2 | HPLC | NIL | NA | 1. Sample mixed with 2 ml $CH_3OH$ & 2 ml $CH_3OH$:water at pH 5.5 (1:1) and centrifuged. 2. 5 ml supernatant injected into HPLC. | HPLC with immunoassay & GC-MS | 1. Immunoassay methods used were EMIT and RIA. |



contd. Table 1.3

| Reference | Urine Volume Used (ml) | Extraction Method | Hydrolysis Conditions | Extraction Buffer and pH | Extraction/Elution Solvent(s) | Detection Method | Comments |
|---|---|---|---|---|---|---|---|
| 38 | < 3 | HPLC | NIL | NA | 1. Sample diluted to 3 ml with water : $CH_3OH$ (1:1) and acidified to pH 2 ± 0.5 with 1 M HCl. 2. 6 ml diluted sample was injected into HPLC. | HPLC-RIA | 1. Recovery after RIA was 85%± 16%. 2. Sensitivity was 3.3 ng $ml^{-1}$. |
| 62 | 1 | HPLC | 1. 0.1 ml saturated KOH in methanol. 2. 15 mins at 110°C. | 0.3 ml conc. acetic acid to pH 4 to 5. | Injected into HPLC without further treatment. | HPLC-uv, HPLC EChD and GC-MS | 1. Pre-column and valve switching technique used. |

Key To Table 1.3

| | | |
|---|---|---|
| EMIT | : | Enzyme multiplied immunoassay technique |
| EtOAc | : | Ethyl acetate |
| GC-ECD | : | Gas chromatography with electron capture detector |
| GC-FID | : | GC with flame ionization detector |
| GC-MS | : | GC with mass spectrometer |
| HETLC | : | High efficiency thin-layer chromatography |
| HPLC | : | High performance liquid chromatography |
| HPLC-EChD | : | HPLC with electrochemical detector |
| HPLC-RIA | : | HPLC with radioimmunoassay |
| HPLC-UV | : | HPLC with ultraviolet detector |
| HPTLC | : | High performance thin-layer chromatography |
| LLE | : | Liquid-liquid extraction |
| MeCN | : | Acetonitrile |
| RIA | : | Radioimmunoassay |
| SPE | : | Solid phase extraction |
| THC | : | $\Delta$9-Tetrahydrocannabinol |
| THC-COOH | : | 11-nor-$\Delta$9-Tetrahydrocannabinol-9-carboxylic acid |
| TLC | : | Thin-layer chromatography |



The pH of the matrix to be used is determined by the $pK_a$ of the solute and is usually chosen so that the solute is predominantly in the unionized molecular form in order to exclude strong interactions with the aqueous matrix. Various methods have been reported for the determination of ionization constants which include potentiometric titration, ultraviolet spectrometry, conductimetry, kinetic methods and solubility or distribution methods [64, 65]. The most precise method is by potentiometric titration but for substances which are too insoluble for potentiometric determination, spectrometric method is used. Ang [66] and others [67] have described spectrometric methods for the determination of ionization constants of compounds with overlapping ionization constants. Ang obtained the following relationship for a dibasic acid:

$$a^2(\varepsilon - \varepsilon_1) + ak_1(\varepsilon - \varepsilon_2) + k_1k_2(\varepsilon - \varepsilon_3) = 0 \qquad (1.1)$$

where $\varepsilon_1$, $\varepsilon_2$ and $\varepsilon_3$ refer to the molar extinction coefficients of the diprotonated, monoprotonated and nonprotonated species respectively; $k_1$ and $k_2$ are the first and second ionization constants; $\varepsilon$ and 'a' refer to the molar extinction coefficient and hydrogen ion activity for a particular buffer respectively. However if the ionization constants of the two functional groups are well separated, i.e. $k_1/k_2 > 1000$, then the second group is assumed to ionize only after the first has ionized completely and equation 1.1 can be simplified to:

$$k_1 = \frac{\varepsilon - \varepsilon_1}{\varepsilon_2 - \varepsilon}\, a \qquad (1.2)$$

$$k_2 = \frac{\varepsilon - \varepsilon_2}{\varepsilon_3 - \varepsilon}\, a \qquad (1.3)$$

The two ionization constants can now be calculated separately provided $\varepsilon_1$, $\varepsilon_2$, $\varepsilon_3$ and are known. $\varepsilon_1$ is the absorbance obtained in acidic condition where only the unionized molecules are present. $\varepsilon_3$ is obtained under basic conditions and $\varepsilon_2$ at an intermediate pH where the monoprotonated species is predominant. $\varepsilon$ is determined at a pH close to the $pK_a$ value expected for the particular ionization constant and 'a' is

the hydrogen ion activity at that pH. If the $pK_a$ values are known then a suitable pH can be selected for the matrix that will give the best quantitative and selective extraction of the drug or solute.

A second important factor is the choice of a suitable extraction solvent system either consisting of a single solvent or a mixture of solvents. Two properties of a good solvent system are inertness and immiscibility with the aqueous matrix. In addition, the solvents must possess suitable specific gravity and volatility. The ability of a solvent to extract the solute will depend on its polarity which takes into account four types of interactions namely, dispersion, dipole, hydrogen bonding and dielectric interactions [68]. Snyder [69] used the polarity index, $P'$, as a measure of the polarity of a solvent and obtained $P'$ values of common solvents for extraction and chromatography. The $P'$ values which were based on the ability of the solvents to interact with ethanol, dioxane and nitromethane were calculated using the following equation:

$$P' = \log(K''_g)_{ethanol} + \log(K''_g)_{dioxane} + \log(K''_g)_{nitromethane} \quad (1.4)$$

Snyder also defined selectivity parameters by the following equations:

$$x_e = \log(K''_g)_{ethanol}/P' \quad (1.5)$$

$$x_d = \log(K''_g)_{dioxane}/P' \quad (1.6)$$

$$x_n = \log(K''_g)_{nitromethane}/P' \quad (1.7)$$

where $K''_g$ is the solute solubility constant corrected for the molecular weights of both solvent and solute; $x_e$, $x_d$ and $x_n$ defined by equation 1.5, 1.6 and 1.7 represent the fraction of $P'$ contribution from interactions with ethanol, dioxane and nitromethane respectively. The values of the selectivity parameters $x_e$, $x_d$ and $x_n$ can also be considered to approximately reflect the ability of a solvent to function as a proton acceptor, proton donor or a strong dipole interactor respectively. The total selective interaction strength of a solvent with ethanol, dioxane and nitromethane is given by $P'x_e$, $P'x_d$ and $P'x_n$ respectively. The common solvents have been classified into 8 groups within a selectivity triangle according to the magnitudes of $x_e$, $x_d$ and $x_n$. Solvents in groups I, VIII and V constitute compounds which can be classified as good



proton acceptors, good proton donors and as having large dipole moments respectively. The solvents in the remaining groups reflect the relative importance of the different intermolecular interactions with no single interaction outweighing the other two. The polarity index, $P'$, of a solvent mixture of A and B can also be determined by using the following simple additivity rule [68, 69]:

$$P' = \phi_A P_A + \phi_B P_B \quad (1.8)$$

where $\phi_A$ and $\phi_B$ are volume fractions of A and B in the mixture and $P_A$ and $P_B$ are the polarity indices of pure A and B respectively.

### 1.4.2 Thin-layer chromatography (TLC)

Table 1.4 lists some of the more recent TLC systems published in the literature for analysis of THC-COOH in urine. TLC has been used for screening purposes [41, 43], as confirmatory methods for immunoassay results [31, 32, 36, 52, 56, 61] and as a clean-up procedure for GC-MS analysis [42]. All the TLC systems in table 1.4 were visualised by spraying a solution of a diazonium compound such as 4-benzamido-2,5-dimethoxybenzenediazonium or Fast Blue RR or FBRR [31, 36, 41, 56], 4-benzoylamino-2, 5-diethoxyanaline or Fast Blue BB or FBBB [38, 46] and O-dianisidine tetrazolium chloride or Fast Blue B Salt or FBB salt [32, 42, 43]. The TLC systems used either conventional precoated or high performance TLC plates [32, 61].

The solvent systems listed in table 1.4 gave different $R_f$ values for THC-COOH because the relative migration of a solute with respect to the solvent front depends on the solvent adsorption strength, $\varepsilon^o$, which is the adsorption energy per unit area of the solvent [68]. Snyder et. al. [68, 70] showed that $\varepsilon^o$ of a binary solvent system is given by the equation,

$$\varepsilon_{AB} = \varepsilon_A + \frac{\log(N_b 10^{\alpha' n_b(\varepsilon_B - \varepsilon_A)} + 1 - N_B)}{\alpha' n_B} \quad (1.9)$$

where $\varepsilon_A$ and $\varepsilon_B$ are the $\varepsilon^o$ values for weak solvent A and strong solvent B; $N_B$ is the mole fraction of B; $n_b$ is the cross-sectional area of a molecule of B adsorbed on the adsorbent surface and $\alpha'$ is the adsorbent activity coefficient and is constant for a particular type of adsorbent with a specific amount of adsorbed water. Snyder et. al. [71] also extended the treatment for calculating the $\varepsilon^o$ value of a multicomponent system.

Table 1.4: Summary of thin-layer chromatography (TLC) systems from literature

| Reference | Plate | Solvent System | Visualisation | Comments |
|---|---|---|---|---|
| 41 | Gelman precoated silica gel glass microfibre sheet, 20x20 cm, 250 µm | Chloroform:methanol:concentrated (28-30%) ammonium hydroxide (85:15:2, v/v/v) | Sprayed with 0.5% (w/v) solution of Fast Blue RR in methanol:water (50:50) | 1. THC-COOH appeared as a pink spot at $R_f$ of 0.25-0.38 |
| 42 | Analtech Uniplate silica gel G plate, 250 µm thick | Chloroform:methanol:ammonia (70:30:2) | Sprayed with Fast Blue B | 1. THC-COOH observed at $R_f$ of approx. 0.25. |
| 43 | Analtech precoated silica gel G plate, 250 µm thick | Double development: 1. alkaline system acetone:chloroform:triethylamine (80:20:1) 2. acidic sysytem petroleum ether (b.p. 35 - 60°C) : diethyl ether:glacial acetic acid (50:50:1.5) | Sprayed with 0.1% solution of Fast Blue salt B in 2 M sodium Hydroxide | 1. THC-COOH appeared as a magenta zone at $R_f$ of approx. 0.1. 2. Sensitivity was 0.5 µg of THC-COOH in urine. 3. Plate was placed in fume hood for 5 mins between developments using the two systems. |



contd. Table 1.4

| Reference | Plate | Solvent System | Visualisation | Comments |
|---|---|---|---|---|
| 31 36 56 | E. Merck precoated silica gel 60 TLC plate, 25x75 mm | Ethyl acetate:methanol:water:conc. ammonium hydroxide (12:5:0.5:1) | Sprayed with 0.5% Fast Blue RR solution in methanol: water (1:1) mixture | 1. THC-COOH appeared as a red spot at $R_f$ 0.43 to 0.50. |
| 32 | Analtech HETLC-HL plate, 10x10 cm | Hexane:acetone:glacial acetic acid (70:30:1) | Sprayed with 0.1 g Fast Blue B salt in 100 ml deionised water with 3 drops of 0.45 M sodium hydroxide | 1. THC-COOH appeared as a red spot at $R_f$ 0.38. |
| 61 | Whatman LHP-K silica gel HPTLC plates with pre-adsorbent area, 20x10 cm or 10x10 cm | Heptane:diethyl ether:acetic acid (80:10:4) | Dipped in 0.3% solution of Fast Blue BB in water | 1. THC-COOH appeared as rose-red band at $R_f$ 0.32 to 0.34. 2. Sensitivity of the method was 15 ng $ml^{-1}$ for a 5 ml urine sample. |
| 52 | TOXI-GRAM with TOXI-DISC (TOXI-LAB THC procedure) | Heptane:acetone:glacial acetic acid (70:30:1) | Dipped in 0.1% (w/v) solution of Fast Blue BB in dichloromethane and dried then followed by exposure to diethylamine vapour (stage 1) and hydrochloric acid vapour (stage 2) | 1. At stage 1 of visualization rose or red-orange colour was observed which during stage 2 turned purple. 2. Sensitivity of the method was 25 ng $ml^{-1}$ urine sample. |

Key to Table 1.4

HETLC : High efficiency thin-layer chromatography

HPTLC : High performance thin-layer chromatography

THC-COOH : 11-nor- $\Delta^9$-tetrahydrocannabinol-9-carboxylic acid



### 1.4.3 High performance liquid chromatography (HPLC)

HPLC systems were initially used for sample pre-treatment for sensitive RIA methods [20, 21, 34, 35, 38, 40], gas chromatography (GC) methods with FID [23] and electron-capture detectors [47] and for mass-spectrometers [21, 23]. HPLC systems have also been reported for detection of cannabinoids and their metabolites with ultraviolet (UV) [32, 35, 47, 53, 57, 62] and more recently with electrochemical detectors [58, 62]. Table 1.5 summarizes a selection of HPLC systems published in literature. The major advantage of HPLC is that in general no additional sample pre-treatment such as derivatization is required after the extraction step. Early methods for the analysis of the cannabinoids and metabolites used normal phase sytems [72] because of the similarities in structures of the cannabinoids. However the methods for analysis of THC-COOH in biological fluids have generally employed reversed phase systems probably because THC-COOH possesses a substantial hydrocarbonaceous moiety which can enter into hydrophobic interactions with the nonpolar stationary phase. Such interactions would bring about separation from the polar endogenous urinary materials namely, amino acids, phenolic and indolic acids [89].

### 1.4.4 Gas chromatography (GC)

GC-MS is the most widely used method for confirmation of THC-COOH in body fluids because of the good resolution from interfering substances during separation by the GC, coupled with the unequivocal identification by the MS. However, due to the low volatility and strong adsorption on GC columns, THC-COOH is first modified chemically to form a suitable derivative. Table 1.6 summarizes commonly used derivatization procedures which have been reported in literature. The derivatization procedures include alkylation, silylation and acylation. The most widely used alkylation procedure is methylation which converts THC-COOH at room temperature to its 1-O-methyl ether, 11-carboxylic acid methyl ester [20, 23, 42, 44, 48, 50, 54, 59, 60, 73, 74]. Other methods of alkylation which can be carried out at room temperature are ethylation [55] and pentafluorobenzylation [45]. Silylation with trimethylsilyl ether as the derivatizing agent has also been extensively used to form the trimethylsilyl ether of THC-COOH [20, 21, 23, 49, 50, 54, 73]. However, some workers [58] have prefered tertiary-butyldimethylsilyl (TBDMS)

Table 1.5: Summary of high performance liquid chromatography (HPLC) systems from literature

| Reference | Column | Mobile Phase | Detector | Comments |
|---|---|---|---|---|
| 47 | Merck Hibar 125x4 mm Licrosorb RP-8, 5 µm | Acetonitrile:0.05M ammonium dihydrogen phosphate (50:50, v/v) at 1.0 ml min$^{-1}$ | UV at 220/225 nm | 1. Method was used for detection as well as clean-up for further analysis by GC-ECD.<br>2. THC-COOH eluted out at about 9 mins. |
| 57 | 25 cm x 4.6 mm I.D. column packed with C8 RP, 5 µm | Acetonitrile:50 mM phosphoric acid (65:35) at 1.6 ml min$^{-1}$ | UV at 214 nm | 1. $R_t$ of THC-COOH was 4.30 mins.<br>2. Minimum detectable limit was 25 ng ml$^{-1}$ at a S/N ratio of 3:1. |
| 23 | Partisil 10 C18 | Methanol:water with 0.05% ammonium acetate (w/v), (63:37) | NIL | 1. Method used for purification of extracts for analysis by GC-FID or GC-MS. |
| 32 | Adsorbosphere C8 column | Acetonitrile:50 mM phosphoric acid (65:35) at 1.5 ml min$^{-1}$ with 40°C column and 28°C solvent temperature. | UV at 214 nm | 1. $R_t$ of THC-COOH was 4.55 min.<br>2. Able to detect 20 ng ml$^{-1}$ THC-COOH in urine samples. |
| 20<br>40 | Spherisorb-5 ODS 10 cm x 4.6 mm I.D. | Stepped mobile phase conditions:-<br>1. 10 ml methanol:water (50:50)<br>2. 10 ml methanol:water (62.5:37.5)<br>3. 20 ml methanol:water (72.5:27.5)<br>Flow rate was 1 ml min$^{-1}$. | RIA | 1. Eluent fractions were collected every 30 secs and analysed by RIA.<br>2. Retention volume of THC-COOH was 16.5 ml. |



contd. Table 1.5

| Reference | Column | Mobile Phase | Detector | Comments |
|---|---|---|---|---|
| 21 | Hypersil-ODS 10 cm x 4.6 mm I.D. | 1. THC-COOH glucuronide: acetonitrile:water at pH 3 (45:55) at 2 ml min$^{-1}$.<br>2. THC-COOH from urine: acetonitrile:water at pH 3 (35:65) at 4 ml min$^{-1}$.<br>3. Hydrolysed metabolite:<br>a. base hydrolysed: acetonitrile:water at pH 3 (50:50) at 4 ml min$^{-1}$<br>b. enzyme hydrolysed: acetonitrile:water at pH 2 (45:55) at 2 ml min$^{-1}$. | RIA and GC-MS | 1. HPLC was used as a sample preparative method.<br>2. Elution volume for THC-COOH glucuronide was 4 ml, 29 ml and 4.5 ml using mobile phase systems (1), (2) and (3) respectively.<br>3. Elution volume of free THC-COOH was 16 ml using system (3). |
| 35 | Spherisorb-ODS,5 µm, 10 cm x 4.6 mm I.D. | Acetonitrile:water with phosphoric acid at pH 3 and 4 ml min$^{-1}$ using following acetonitrile (B) gradient compositions:<br>0 to 10 min at 36% B,<br>10 to 20 min from 36% to 70% B,<br>20 to 25 min at 70% B. | EMIT, RIA and UV at 215 nm | 1. Method was used for sample preparation prior to analysis by EMIT and RIA. |

contd. Table 1.5

| Reference | Column | Mobile Phase | Detector | Comments |
|---|---|---|---|---|
| 53 | St[illegible]less steel 30 cm x 3.[illegible]m I.D. column packed wi[illegible] u-Bondpack-C18 (W[illegible]rs) or ASI-C18 (A[illegible]ytical Sciences). | Acetonitrile:phosphate buffer at pH 6.0 (45:55) at 2 ml min$^{-1}$ and column temperature of 45$^{o}$C. | UV at 205 nm | 1. $R_t$ of THC-COOH was 7.1 min. |
| 38 | St[illegible]less steel 16 cm x 5 [illegible] I.D. column packed wi[illegible] Hypersil 5-ODS (or Hy[illegible]sil SAS-Shandon, or [illegible]herisorb 5-ODS). | 85% methanol in pH 1.95 buffer (3.5 g glycine and 5 ml conc. HCl in 1650 ml methanol). | RIA | |
| 58 | 15[illegible]m x 4.6 mm I.D. column sl[illegible]y packed with Spl[illegible]isorb ODS-2, 3 µm (1[illegible] carbon, fully capped). | Methanol:5% aqueous acetic acid (76:24) at 1.5 ml min$^{-1}$. | EChD | 1. Detection limit was 5 ng ml$^{-1}$ at S/N ratio of 5:1. |

contd. Table 1.5

| Reference | Column | Mobile Phase | Detector | Comments |
|---|---|---|---|---|
| 62 | Col[illegible]ns were:<br>1. [illegible]ard-Pak$^{TM}$ cartridge [illegible] column (Waters)<br>2. [illegible]cleosil CN, 5 µm, [illegible] x 4.0 mm I.D.<br>3. [illegible]cleosil C8, 5 µm, [illegible]0 x 4.0 mm I.D. | Eluents used were:<br>1. column (1): 40 mM phosphoric acid,<br>2. column (2): acetonitrile:40 mM phosphoric acid (1:2),<br>3. column (3): acetonitrile:40 mM phosphoric acid (1:1),<br>at a flow rate of 1 ml min$^{-1}$ for all the columns. | UV at 220 nm, and EChD | 1. Detection limit was 2 ng ml$^{-1}$ at SN ratio of 3:1 with EChD detector. |

## Key to Table 1.5

| | | |
|---|---|---|
| EChD | : | Electrochemical detector |
| EMIT | : | Enzyme multiplied immunoassay technique |
| GC-ECD | : | Gas chromatography with electron capture detector |
| GC-FID | : | GC with flame ionization detector |
| GC-MS | : | GC with mass spectrometer |
| HPLC | : | High performance liquid chromatography |
| RIA | : | Radioimmunoassay |
| $R_t$ | : | Retention time |
| S/N | : | Signal-to-noise |
| THC-COOH | : | 11-nor- $\Delta^9$-tetrahydrocannabinol-9-carboxylic acid |
| UV | : | Ultraviolet detector |



Table 1.6: Summary of derivatization procedures for GC from literature

| Reference | Technique | Reagents | Reaction Conditions | Detector | Comments |
|---|---|---|---|---|---|
| 42 | Alkylation | 1. 25% tetramethylammonium hydroxide (TMAH).<br>2. Dimethylsulfoxide (DMSO).<br>3. 1-Iodomethane ($CH_3I$). | 1. Room temperature (RT).<br>2. Reaction terminated by adding 0.2 ml 0.1 M HCl.<br>3. Derivatives extracted from reaction mixture with hexane. | GC-MS | |
| 44 | Alkylation | 1. TMAH.<br>2. DMSO.<br>3. $CH_3I$. | 1. RT.<br>2. Reaction terminated with 0.2 ml 0.1 M HCl.<br>3. Derivatives extracted with isooctane. | GC-FID, GC-MS | 1. TMAH acts as catalyst to methylation of THC-COOH by $CH_3I$ in DMSO as solvent.<br>2. Product was 1-O-methyl ether, 11-carboxylic acid methyl ester of THC-COOH. |
| 45 | Alkylation | 1. Pentafluorobenzyl bromide (PFb-Br).<br>2. Benzyl tributylammonium chloride.<br>3. 0.1 M NaOH.<br>4. 5% $CH_2Cl_2$ in hexane. | 1. RT. | GC-ECD | 1. Derivatization procedure used PFb-Br and NaOH in a biphasic system with benzyl tributylammonium chloride as a catalyst.<br>2. 5% $CH_2Cl_2$ in hexane gave high yield as well as clean chromatograms on analysis. |

contd. Table 1.6

| Reference | Technique | Reagents | Reaction Conditions | Detector | Comments |
|---|---|---|---|---|---|
| 46<br>47<br>62 | Acylation | 1. Pentafluoropropionic anhydride (PFPA).<br>2. Pentafluoropropanol (PFP-OH). | 1. 15 min at 70°C. | GC-ECD, GC-MS | 1. Pentafluoropropyl-pentafluoropropionyl derivative of THC-COOH formed |
| 49 | Silylation | 1. N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethyl-chlorosilane (TMCS). | 1. 1 hour at 90°C. | GC-NICI/MS | 1. Trimethylsilyl derivative easily hydrolysed and exposure to air should be minimized.<br>2. Derivatized mixture may be frozen until analysed. |
| 73 | Alkylation - silylation | 1. Diazomethane.<br>2. N,O-bis-(trimethylsilyl)-acetamide (BSA) containing 1% TMCS. | 1. Diazomethane in ether added until yellow colour persists in reaction mixture which is then heated at 50°C for 30 min, cooled and dried.<br>2. Residue from above silylated using BSA with 1% TMCS at 90°C for 1 hour. | GC-CI/MS | 1. Diazomethane used to form methyl ester of carboxyl functional group.<br>2. BSA used to form trimethyl-silyl (TMS) ether of phenolic (OH) group. |



contd. Table 1.6

| Reference | Technique | Reagents | Reaction Conditions | Detector | Comments |
|---|---|---|---|---|---|
| 48 | Alkylation - acylation | 1. Boron trifluoride ($BF_3$)-methanol (14%, w/v).<br>2. Trifluoroacetic anhydride (TFAA). | 1. $BF_3$-methanol reaction at 70°C for 10 min followed by addition of 1 ml water and 2 ml hexane, hexane layer was then transferred and dried.<br>2. Residue from above acylated with TFAA at 70°C for 10 min. | GC-NICI/MS | 1. Methylation with $BF_3$-methanol was necessary to esterify carboxylic group of THC-COOH before acylation of phenolic OH group by TFAA. |
| 50 | Alkylation and/or silylation. | 1. 25% TMAH.<br>2. DMSO.<br>3. $CH_3I$.<br>4. N-methyl-N-trimethylsilyltri-fluoroacetamide (MSTFA).<br>5. BSTFA.<br>6. TMCS.<br>7. Diazomethane. | 1. Dimethyl derivative at RT.<br>2. Methyl-TMS derivative:-<br>a) methylation at RT,<br>b) trimethylsilylation at 90°C for 1 hour.<br>3. Di-TMS derivative at 90°C for 1 hour. | GC-EI/MS | |
| 23 | Silylation-methylation. | 1. Regisil with 1% TMCS. | 1. Silylation at 110°C for 8 hours.<br>2. Methylation similar to reference 43 (above). | GC-FID, GC-MS | |

contd. Table 1.6

| Reference | Technique | Reagents | Reaction Conditions | Detector | Comments |
|---|---|---|---|---|---|
| 33 | Acylation | 1. PFPA.<br>2. Hexafluoroisopropanol (HFIP). | | GC-MS | |
| 74 | Alkylation | 1. Methyl-8 (dimethylformamide [DMF] dimethylacetal). | | GC-MS | 1. Method involves on-column derivatization. |
| 60 | Alkylation | 1. TMAH.<br>2. DMSO.<br>3. $CH_3I$. | 1. RT. | GC-MS | |
| 51 | Acylation | 1. PFPA.<br>2. PFP-OH. | 1. 20 min at 45°C. | GC-MSD | |
| 59 | Alkylation | 1. TMAH.<br>2. DMSO.<br>3. $CH_3I$. | 1. RT. | GC-MSD | 1. Methylated derivative reported to be stable for a minimum of 72 hours. |



contd. Table 1.6

| Reference | Technique | Reagents | Reaction Conditions | Detector | Comments |
|---|---|---|---|---|---|
| 20 | Alkylation-silylation | 1. BSTFA with 1% TMCS.<br>2. Ethereal diazomethane. | 1. Silylation at 80°C for 10 mins.<br>2. Methylation at RT. | GC-MS | |
| 21 | Silylation | 1. BSTFA with 1% TMCS. | 1. 10 min at 90°C. | GC-MS | |
| 54 | Alkylation-silylation | 1. Diazomethane.<br>2. BSA. | 1. Methylation at RT.<br>2. Silylation at 50 to 60°C for 10 min. | GC-MS | |
| 58 | Silylation | 1. N-methyl-N-(tertiary-butyl-dimethylsilyl)-trifluoro-acetamide (MTBSTFA).<br>2. Tertiary-butyldimethylsilyl chloride (TBDMS-Cl). | 1. 1 hour at 60°C. | GC-MS | 1. TBDMS-Cl used as a catalyst.<br>2. TBDMS derivatives reported to be more stable and provide better yield than TMS derivatives. |
| 55 | Alkylation | 1. Trimethylanilinium hydroxide<br>2. DMSO.<br>3. Ethyl iodide ($CH_3CH_2I$). | 1. 1 min at 60°C followed by 4 min at RT. | NA | |

Keys To Table 1.6

| | | |
|---|---|---|
| BSA | : | N, O-bis-(trimethylsilyl)-acetamide |
| BSTFA | : | N, O-bis-(trimethylsilyl)-trifluoroacetamide |
| DMF | : | Dimethylformamide |
| DMSO | : | Dimethylsulfoxide |
| GC-CI/MS | : | Gas chromatography with chemical ionization mass spectrometer |
| GC-ECD | : | GC with electron capture detector |
| GC-EI/MS | : | GC with electron impact ionization mass spectrometer |
| GC-FID | : | GC with flame ionization detector |
| GC-MS | : | GC with mass spectrometer |
| GC-NICI/MS | : | GC with negative ion chemical ionization mass spectrometer |
| HFIP | : | Hexafluoroisopropanol |
| MSTFA | : | N-Methyl-N-trimethylsilyltrifluoroacetamide |
| MTBSTFA | : | N-methyl-N-(tertiary-butyldimethylsilyl)-trifluoroacetamide |
| PFb-Br | : | Pentafluorobenzyl bromide |
| PFPA | : | Pentafluoropropionic anhydride |
| PFP-OH | : | Pentafluoropropanol |
| RT | : | Room temperature |
| TBDMS | : | Tertiary-butyldimethylsilyl |
| TBDMS-Cl | : | Tertiary-butyldimethylsilyl chloride |
| TFAA | : | Trifluoroacetic anhydride |
| THC-COOH | : | 11-nor- $\Delta^9$-tetrahydrocannabinol-9-carboxylic acid |
| TMAH | : | Tetramethylammonium hydroxide |
| TMCS | : | Trimethylchlorosilane |
| TMS | : | Trimethylsilyl |



derivative of THC-COOH for analysis due to its greater stability and better yield when compared to trimethylsilyl derivatives. Acylation procedures which have been used include the formation of pentafluoropropyl-pentafluoropropionyl derivatives [46, 47, 51, 62], hexafluoroisopropyl-pentafluoropropionyl derivative [33] and trifluoroacetyl-methyl ester derivative [48]. Almost all the methylation procedures reported have been carried out at room temperature, while silylation and acylation procedures have been carried out at higher temperatures suggesting that methylation is easier to perform. However the choice of a derivatizing agent also depends on other factors besides minimizing column adsorption and increasing volatility. Other factors which need to be considered in the selection of the derivatizing agent are enhancement of sensitivity, introduction of certain groups to give characteristic responses for better identification (usually with GC-MS), separation from the endogeneous materials by specific derivatization and the limitations of the detector itself. Alkylation to give pentafluorobenzyl [45] and acylation to give pentafluoropropyl-pentafluoropropionyl [46, 47, 62] derivatives have been used to add electrophoric functional groups to THC-COOH in order to increase its sensitivity on GC-ECD. Electrophores have been added to THC-COOH for the same purpose for GC-MS procedures utilizing negative ion chemical ionization (NICI) mode [48]. The mass scan range of the MS detector can also be a limiting factor for choosing certain derivatives [51]. Most of the GC-MS procedures reported in literature either involve methylation or silylation steps because the main objectives were to prevent adsorption on the GC column and for the resulting derivative to possess a mass which was sufficiently high to distinguish it from endogenous materials in urine. In addition the mass fragmentation patterns of the derivatives with electron impact (EI) ionization MS were characteristic and easy to interpret.

Several GC procedures have been reported for analysis of THC-COOH derivatives and some of these methods are listed in table 1.7. Various detectors have been used with these procedures such as FID [44], ECD [45 - 47, 62, 75] and MS utilizing EI ionization mode [20, 21, 23, 42, 44, 46, 50, 51, 54, 55, 58 - 60, 62, 74] and chemical ionization (CI) mode [48, 49, 73, 75].

Table 1.7: Summary of gas chromatography systems from literature

| Ref | Column | GC Condition | | | | | | Detector | Comments |
|---|---|---|---|---|---|---|---|---|---|
| | | Injector (°C) | Det./TL (°C) | Oven Temp. 1 | Rate (°C/min) | Oven Temp. 2 | Carrier Gas | | |
| 42 | 6'x1/4", OV-17 on Chromosorb WHP 80/110 mesh. | NA | NA | 220 | 10 | 270 | NA | MS | |
| 44 | 1. 6'x 2 mm I.D., 3% OV-17 on 100/120 mesh Gas-chrom Q.<br>2. 3' x 2 mm I.D., 3% OV-101 on 100/120 mesh Gas-chrom Q. | 1. 260<br>2. NA | 1. 275<br>2. NA | 1. 255<br>2. 230 | 1. NIL<br>2. NIL | 1. NIL<br>2. NIL | 1. Helium at 30 ml $min^{-1}$.<br>2. Helium at 30 ml $min^{-1}$. | FID<br>MS | 1. Molecular jet separator used at GC-MS interface. |
| 45 | 1.8 m x 2 mm I.D., 2% OV-25 on 100/120 mesh Chromosorb W. | 300 | 300 | 270 | - | - | 5% methane in argon at 30 ml $min^{-1}$. | ECD | |



contd. Table 1.7

| Ref | Column | GC Condition | | | | | | Detector | Comments |
|---|---|---|---|---|---|---|---|---|---|
| | | Injector (°C) | Det./TL (°C) | Oven Temp. 1 | Rate (°C/min) | Oven Temp. 2 | Carrier Gas | | |
| 47 | 25 m x 0.3 mm I.D., fused silica SE 54. | a. 250<br>b. 250 | a. 300<br>b. 300 | a. 220<br>b. 200 | a. 5<br>b. 10 | a. 280<br>b. 280 | a. Helium at 45 cm $s^{-1}$.<br>b. Helium at 40 cm $s^{-1}$. | ECD | 1. Split injection technique was used at a split ratio of 1:30.<br>2. Make-up gas was nitrogen at 46 ml $min^{-1}$.<br>3. $R_t$ of THC-COOH was 3.0 - 4.0 min and 4.5 - 5.0 min with conditions (a) and (b) respectively. |
| 49 | 1.8 m x 2 mm I.D., 3% OV-17 on 100/120 mesh Gas-chrom Q. | 280 | 280 | 190 | 20 | 310 | Methane at 15 - 20 ml $min^{-1}$. | NICI/MS (SIM mode) | 1. $NH_3$ was used as reagent gas for NICI.<br>2. $R_t$ of THC-COOH was 4 - 5 min. |

contd. Table 1.7

| Ref | Column | GC Condition: Injector ($^oC$) | Det./TL ($^oC$) | Oven Temp. 1 | Rate ($^oC$/min) | Oven Temp. 2 | Carrier Gas | Detector | Comments |
|---|---|---|---|---|---|---|---|---|---|
| 50 | 6'x 2 mm I.D., 3% SP 2250 on 100/120 Supelcoport. | 270 | 270 | 260 | | | Helium at 30 ml $min^{-1}$. | EI/MS (SIM mode) | |
| 73 | 1. 1.8 m x 2 mm I.D., 3% OV-17 on 100/120 mesh Gas-chrom Q.<br>2. 15 m x 0.25 mm I.D., glass capillary column coated with SE 52. | 1. 280<br>2. NA | 1. 320<br>2. NA | 1. 250<br>2. 160 | 1. NIL<br>2. 'rapid' | 1. NIL<br>2. 250 | 1. $H_2$ at 15 ml $min^{-1}$.<br>2. NA | CI/MS | 1. $NH_3$ was used as reagent gas.<br>2. Splitless injection was used utilizing the 'solvent effect' with n-tetradecane as the solvent. |
| 48 | 15 m x 0.32 mm I.D., fused silica with 1.0 μm film thickness dimethylsilicone. | 250 | NA | 200 (1 min) | 25 | 280 (1 min) | $H_2$ at 60 $cm/s^{-1}$ (at oven temp. of 200$^oC$). | NICI/MS | 1. Splitless injection used with cold trapping.<br>2. $R_t$ of derivatized THC-COOH was 3.8 min. |



contd. Table 1.7

| Ref | Column | GC Condition: Injector ($^oC$) | Det./TL ($^oC$) | Oven Temp. 1 | Rate ($^oC$/min) | Oven Temp. 2 | Carrier Gas | Detector | Comments |
|---|---|---|---|---|---|---|---|---|---|
| 23 | 1. Refer to ref. 34.<br>2. 25 m CP Sil-5, WCOT (wide bore) Chrompack. | 2. 265 | 2. NA | 2. 270 | 2. NIL | 2. NIL | 2. Helium at 1.5 ml $min^{-1}$. | MS | |
| 74 | 196 cm x 2 mm I.D., 3% OV-1 on 80/100 mesh Chromosorb W HP. | 300 | 250 | 270 | NIL | NIL | Helium at 25 ml $min^{-1}$. | EI/MS, CI/MS | 1. $R_t$ of methylated THC-COOH was 1.2 min. |
| 60 | 25 m x 0.23 mm I.D., chemical-bonded fused silica SE 54. | 280 | NA | 75 | 15 | 300 | NA | EI/MS (MID mode) | 1. $R_T$ for methylated THC-COOH was 15.9 min. |
| 51 | 30 m x 0.25 mm I.D., J&W DB-5 bonded phase, 0.25 um. | 260 | 280 | 180 | 15 | 270 | Helium at 0.9 $ml/min^{-1}$ (32 $cm\ s^{-1}$. | MSD (SIM) | 1. Open-split interface between GC and MSD. |

contd. Table 1.7

| Ref | Column | GC Condition: Injector ($^oC$) | Det./TL ($^oC$) | Oven Temp. 1 | Rate ($^oC$/min) | Oven Temp. 2 | Carrier Gas | Detector | Comments |
|---|---|---|---|---|---|---|---|---|---|
| 59 | 15 m x 0.25 mm I.D., DB-5 fused silica. | 265 | 255 | 245 | NIL | NIL | Helium at 1.6 ml $min^{-1}$. | MSD (Scan mode) | 1. Split injections made with split ratio of 1:10. 2. Direct interface between GC and MSD. |
| 20 | 0.3 m x 2 mm I.D., 3% OV-17 on 80/100 mesh Gas-chrom Q. | NA | NA | 220 | NIL | NIL | Helium at 20 ml $min^{-1}$. | MS | 1. Jet separator interface between GC and MS. |
| 21 | 0.3 m x 2 mm I.D., 3% OV-17 on 80/100 mesh Gas-chrom Q. | NA | NA | 270 | NIL | NIL | Helium at 30 ml $min^{-1}$. | MS | 1. Jet separator interface used. |
| 54 | 3% SE 30 on 100/120 mesh Gas-chrom Q. | NA | NA | 220 | NIL | NIL | NIL | MS (MID mode) | |



contd. Table 1.7

| Ref | Column | GC Condition: Injector ($^oC$) | Det./TL ($^oC$) | Oven Temp. 1 | Rate ($^oC$/min) | Oven Temp. 2 | Carrier Gas | Detector | Comments |
|---|---|---|---|---|---|---|---|---|---|
| 58 | 25 m x 0.2 mm I.D., fused silica cross-linked HP 5 (5% phenylmethylsilicone). | NA | NA | 250 | NIL | NIL | Helium at 0.6 ml $min^{-1}$. | MS | 1. Splitless (SIM) injection. |
| 62 | 15 m x 0.25 mm I.D., fused silica DB-5. | 250 | NA | 150 (2 min) | 45 | 280 | NA | MSD (SIM) | 1. Splitless injection. |
| 46 | 1. 25 m x 0.3 mm I.D., HP fused silica SE 54. 2. 25 m x 0.2 mm I.D., HP fused silica SE 54. | 1. 260 2. 260 | 1. 250 2. 250 | 1. 220 2. 250 | 1. NIL 2. NIL | 1. NIL 2. NIL | 1) Helium at 55 cm $s^{-1}$ 2) Helium at 50 cm $s^{-1}$ | MS (SIM) | 1. EI, PICI and NICI modes used. 2. Methane used as reagent gas for CI modes. |
| 55 | 30 m x 0.25 mm I.D., DB-5, 0.25 µm. | NA | NA | 40 | 30 | 270 | NA | MS (SIM) | |

contd. Table 1.7

| Ref | Column | GC Condition | | | | | | Detector | Comments |
|---|---|---|---|---|---|---|---|---|---|
| | | Injector ($^{o}C$) | Det./TL ($^{o}C$) | Oven Temp. 1 | Rate ($^{o}C/min$) | Oven Temp. 2 | Carrier Gas | | |
| 75 | 1. 6'x 1/8", 1.5% OV-17 on 80/100 mesh Chromosorb W. | 1. NA | 1. 350 | 1. 270 | 1. 8 | 1. 300 | 1. 15% argon in $CH_4$ at 15 ml $min^{-1}$ | 1. ECD | 1. Capillary ECD with on-column injection. 2. Splitless injection used for MS analysis. |
| | 2. 15 m J&W DB-17N, 0.15 µm. | 2. NA | 2. NA | 2. 260 | 2. 6 | 2. 300 (5 mins) | 2. $H_2$ at 40 cm $s^{-1}$ (at 210$^{o}$C). | 2. ECD | |
| | 3. 10 m x 0.32 mm I.D., DB 1701, 0.25 µm. | 3. 290 | 3. 290 | 3. 200 | 3. 20 | 3. 295 | 3. $H_2$ at 78 cm $s^{-1}$ (at 200$^{o}$C). | 3. NICI/MS | |



Key to Table 1.7

CI : Chemical ionization

CI/MS : Mass spectrometer with chemical ionization

ECD : Electron capture detector

EI : Electron impact ionization

EI/MS : Mass spcetrometer with electron impact ionization

FID : Flame ionization detector

GC : Gas chromatography

GC-MS : GC with mass spectrometer

ID : Inner diameter

MID : Multiple ion detector

MS : Mass spectrometer

MSD : Mass selective detector

NA : Not available

NICI : Negative ion CI

NICI/MS : Mass spectrometer with NICI

PICI : Positive ion CI

SIM : Selected ion monitoring

THC-COOH : 11-nor- $\Delta^9$-tetrahydrocannabinol-9-carboxylic acid

GC-MS with EI mode generates spectra that have many fragment ion mass peaks and are suitable for structure verification of an unknown sample. The base peak in such spectra are usually a fragment ion. The spectra obtained can be compared with standard spectra to give probability of fit which can be used to confirm the presence or absence of THC-COOH derivative [76, 77]. On the other hand, GC-MS systems utilizing CI mode usually generate predominantly molecular ions and very little fragments. The degree of fragmentation, which is usually less than for EI, is dependent on the reagent gas [78]. Confirmation is usually by comparison of ion ratios of the derivatized deuterated analogs of THC-COOH to that of the derivatized THC-COOH in samples. The ratios chosen are those of the intense mass peaks. This technique is also utilized with the EI mode. GC-MS using negative ion CI mode has also been reported to give low background and sometimes higher sensitivity than EI or positive ion CI [79].

The GC methods which have been reported in literature have utilized both packed [20, 21, 23, 42, 44, 45, 49, 50, 54, 73, 74] and capillary columns [23, 46 - 48, 51, 55, 58 - 60, 62, 73]. In the case of capillary GC for analysis, split injection [47, 59], on-column injection [75] and splitless injection techniques [48, 58, 62, 73] have been used. The preference for the splitless technique, which is usually employed for trace compound analysis, is probably due to the presence of low levels of THC-COOH in biological samples. Furthermore most MS are coupled to the GC by means of open-split interfaces where additional splitting of GC separated components takes place. Although such an interface prevents contamination of the MS ion source, there is a substantial loss in sensitivity when used with the split injection technique. Both the methods utilizing split injection technique in table 1.7 were developed for either an ECD, or a MS coupled to the GC by a direct interface.

In conclussion, the literature survey suggests that the detection of THC-COOH which is the main metabolite of the principal psychoactive constituent of cannabis is a good choice for indicating use of cannabis drug preparations. Furthermore, THC-COOH is present in sufficiently high concentrations and is eliminated from the body over a long period of time. The literature survey also indicates that chromatographic methods are more widely used for analysis of THC-COOH. This study firstly proposes to develop systematically a sample



pre-treatment step for extraction of THC-COOH from urine by determining the ionization constants and by varying the polarity and selectivity of the extracting solvents. A TLC method with a diazo dye spray reagent will then be developed for screening purposes since both the colour reaction and retention factor can be used for identification. As an alternative, a HPLC method will also be considered for routine analysis since both methods do not involve derivatization in the sample pre-treatment step. Finally a GC-MS method will be developed for confirmation of THC-COOH in positive urine samples after establishing a suitable derivatization procedure.

Chapter 2

EXPERIMENTAL

## 2.1 Extraction method for THC-COOH from urine

### 2.1.1 Materials and instrumentation

All solvents and chemicals used were of analytical reagent (AR) grade. 11-nor-$\Delta^9$-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) standard (100 µg $ml^{-1}$ ampules) was obtained from Research Triangle Institute (RTI), USA. Pooled urine from an addict tested positive and containing about 870 ng $ml^{-1}$ of THC-COOH was used as stock urine (A). All extractions were carried out using 10 ml screw-capped culture tubes (s.c.c.t.).

The following solutions and buffers were prepared:

i. borate buffer B prepared by mixing approximately 21 ml of 0.10 M NaOH solution with approximately 50 ml of a solution of 0.10 M boric acid and 0.10 M KCl to give pH 9.00;

ii. solution C prepared by mixing approximately 50 ml of 0.20 M KCl with approximately 97 ml of 0.20 M HCl to give pH 1.00;

iii. solution D prepared by mixing approximately 50 ml of 0.20 M KCl with approximately 11 ml of 0.20 M HCl to give pH 2.00;

iv. buffers E, F, G, H and I prepared by mixing approximately 81, 61, 49, 36 and 13 ml of 0.10 M citric acid with approximately 20, 39, 51, 64 and 87 ml of 0.20 M $Na_2HPO_4$ to give solutions with pH values of 3.00, 4.00, 5.00, 6.00 and 7.00 respectively;

v. buffer J prepared by mixing approximately 5 ml of 0.20 M $NaH_2PO_4$ with approximately 95 ml of 0.20 M $Na_2HPO_4$ to give a solution of pH 8.00;

vi. buffer K prepared by mixing approximately equal volumes of 0.25 M $Na_2CO_3$ and 0.25 M $NaHCO_3$ to give pH 10.00.

### 2.1.2 Method

A liquid-liquid extraction method which employed the following procedure was used:



(a) 0.3 ml of 10.00 M NaOH solution was added to 3.0 ml of urine sample in a 10 ml s.c.c.t. After vortexing for about 15 s, the tube was left in an oven at 50°C for 30 min and finally cooled to room temperature.

(b) The hydrolysed urine was adjusted to pH 9 with 50% HCl (v/v) and 0.20 M NaOH solutions using universal pH indicator strips. 0.5 ml borate buffer B (pH 9) followed by 1.0 g of solid NaCl was added and the sample was then vortexed for 15 s.

(c) 4.0 ml of the extraction solvent, ethyl acetate:isooctane (90:10, v/v) was added to the sample and vortexed for 2 min.

(d) The sample was then centrifuged at 3000 rpm for 20 min and the organic phase transferred to another s.c.c.t. 4.0 ml of 1.00 M phosphate buffer at pH 3.00 was added to the organic extract and vortexed for 2 min.

(e) The sample was centrifuged at 3000 rpm for 10 min and the organic phase transfered to another tube and was dried in a stream of nitrogen at about 60°C.

Hexane, cyclohexane, isooctane, diethylether, dichloromethane, ethyl acetate, benzene, toluene and chloroform were evaluated for extraction. THC-COOH was extracted from samples of the stock urine (A) using the above procedure with minor modifications. The different extraction solvents were used in step (c) and acidification in step (b) was carried out with citric acid for pH 3 to 8 while 50% HCl (v/v) was used for pH 1, 2, 9 and 10. The respective buffers C to K (section 2.1.1) were then added without addition of NaCl. Step (d) was omitted. Three replicate extractions were carried out for each of the solvents using stock urine (A). All the extracts were dried and their residues dissolved in 100 µl methanol. After centrifuging at 3000 rpm for 20 min, 20 µl aliquots were injected into the HPLC for analysis.

To determine the optimum extraction solvent, three replicate extractions by the same procedure were carried out for each solvent at that particular pH which gave the maximum recovery.

Ethyl acetate which was found to be the best solvent was then diluted with isooctane to investigate the effect of varying polarity on extraction. The ratios of ethyl acetate:isooctane used were 10:90, 25:75, 50:50, 70:30, 90:10, 95:5 and 100:0. Three replicate extractions using stock urine A were performed for each solvent

mixture by the same procedure except step (d) and addition of NaCl in step (b) were omitted.

Salting-out effect was studied by adding approximately 1.0 g NaCl to the acidified urine in step (b), omitting step (d) and using the optimum mixture of ethyl acetate and isooctane for extraction in step (c).

Various clean-up methods were evaluated. The methods were acid-wash with 4.0 ml of 0.20 N $H_2SO_4$, 5% $NaHCO_3$ wash, phosphate buffer-wash (pH 7) and back-extraction with 0.20 M NaOH. The procedure given above was used with the modification that the various clean-up methods were substituted for 1.00 M phosphate buffer (pH 3) in step (d). The buffer-wash method, which was chosen, was first optimized for pH. Salting-in effects were then studied by varying the concentrations of the phosphate buffer solution at the optimum pH. The same extraction procedure was used with stock urine A except that 0.20 M phosphate buffers at pH 3.00, 4.00, 5.00, 6.00 and 2.00, 1.00, 0.50, 0.20, 0.10 and 0.05 M phosphate buffer at optimum pH 3.00 were used in step (d).

The recovery of the optimized extraction method was quantitated by HPLC. Spiked urine samples were prepared by serial dilution of a stock urine sample spiked with 1000 ng $ml^{-1}$ THC-COOH with blank urine to give 500, 250, 125 and 50 ng $ml^{-1}$. Methanolic standard solutions were likewise prepared by serial dilution of 100 µg $ml^{-1}$ stock standard solution of THC-COOH to give 30, 15, 7.5, 3.7(5) and 1.5 µg $ml^{-1}$. The concentration of the standard solution corresponded to those of the aliquots of extracted spiked urine solutions (assuming total recovery of THC-COOH) which were injected into the HPLC. 20 µl of the solution of THC-COOH extracted from spiked urine and standard solutions were used for the HPLC analysis. The data was used for plotting extracted and direct standard curves of THC-COOH.

A study was also conducted to determine whether the extraction method could also be used for morphine. 3.0 ml of urine spiked with morphine and THC-COOH to give 1000 and 300 ng $ml^{-1}$ respectively was extracted using the optimized extraction procedure, with the exception of step (d). The extract was dried and the residue was silylated with 50 µl of BSTFA at 90°C for 90 min. 3 µl of the derivatized mixture was injected into the GC-MSD.

## 2.2 Determination of $pK_a$ values of THC-COOH

### 2.2.1 Materials and instrumentation

All chemicals were analytical reagent grade and both $KH_2PO_4$ and $NaHCO_3$ were dried in a desiccator containing silica gel. All other salts were dried in an



oven at 100°C for 2 h prior to use. THC-COOH standard (100 µg $ml^{-1}$) was obtained from Research Triangle Institute, USA.

A Waters Millipore HPLC filtration kit with 0.45 um regenerated cellulose membrane filter was used to filter all stock solutions. A Corning pH Meter 113 and a Orion Research Digital Ionalyzer 501 were used to measure the pH of the buffer solutions.

A Hewlett-Packard (HP) 1090 Series M HPLC with a HP 1040 A diode array detector (DAD), an auto-injector and a Merck Hibar 125 x 4 mm stainless steel column packed with 5 µm, RP-18 (octadecylsilane) were used for all chromatographic analyses. The HPLC was coupled to a HP 79994A chemstation. All spectrophotometric measurements were carried out using a HP 8450A Diode Array Spectrophotometer.

### 2.2.2 Preparation of stock solutions

About 50 g of NaOH pellets was dissolved in approximately 50 ml of deionised water. The resulting solution was centrifuged at 3000 rpm for about an hour. Two ml of the clear solution was diluted to 1 liter with deionised water and standardized by potentiometric titration with a 0.50 M standard solution of potassium hydrogen phthalate. The concentration of the stock NaOH solution (L) was 0.38 M.

The following stock solutions were also prepared: 0.41 M NaCl (M), 0.44 M $KH_2PO_4$ (N), 0.36 M $K_2HPO_4$ (O), 0.51 M $NaHCO_3$ (P), 0.45 M $Na_2CO_3$ (Q), 0.47 M $CH_3COONa$ (R), 0.50 M HCOOH (S), 0.50 M $CH_3COOH$ (T), 0.50 M HCl (U) and 0.50 M $H_3PO_4$ (V).

### 2.2.3 Method

$pK_1$ of THC-COOH was determined using the HPLC. The mobile phase consisted of 60% (v/v) HPLC grade methanol in the appropriate buffer solutions. The detector was monitored at 214 nm and simultaneously programmed to obtain spectra for the range 200 - 400 nm. For each analysis 20 µl of a 50 µg $ml^{-1}$ solution of THC-COOH diluted from a stock solution of 100 µg $ml^{-1}$ in the appropriate methanol-buffer solution was injected.

Table 2.1

Buffer solution composition and their pH values

| Volume of stock solution 1 (ml) | Volume of stock solution 2 (ml) | Volume of water (ml) | pH |
|---|---|---|---|
| 135 V | 104 N | 161 | 2.26 |
| 9.0 V | 91 N | 200 | 3.07 |
| 86 S | 78 L | 136 | 4.04 |
| 0.4 V | 136 N | 464 | 4.43 |
| 35 T | 84 R | 281 | 5.03 |
| 81 V | 110 L | 209 | 5.43 |
| 79 V | 120 L | 201 | 5.83 |
| 44 N | 19.0 O | 337 | 6.39 |

Table 2.1 shows the buffer solutions which were used and the method of preparation from the stock solutions (section 2.2.2).

Spectrophotometric measurements with 1 cm quartz cells were used for determining $pK_2$ of THC-COOH. For each analysis, an ethanolic stock standard solution of 100 µg $ml^{-1}$ THC-COOH was diluted to give a final concentration of 1 µg $ml^{-1}$ in the buffer solutions. The reference cell contained ethanol and buffer solution with the volume of ethanol being equal to the volume of the ethanolic standard solution of THC-COOH used. Table 2.2 shows the buffer solutions and their method of preparation from the stock solutions (section 2.2.2).



Table 2.2

Buffer solution composition and their pH values

| Volume of stock solution 1 (ml) | Volume of stock solution 2 (ml) | Volume of water (ml) | pH |
|---|---|---|---|
| 2.0 U | 21.9 M | 76 | 1.88 |
| 5.6 N | 2.4 O | 92 | 6.36 |
| 2.2 N | 3.8 O | 94 | 7.03 |
| 0.5 N | 4.5 O | 95 | 7.79 |
| 0.4 L | 24.0 M | 76 | 11.25 |
| 2.6 L | 22.0 M | 75 | 12.11 |

## 2.3 High performance liquid chromatographic method for THC-COOH in urine

### 2.3.1 Materials and instrumentation

All solvents used were HPLC grade and all chemicals were analytical grade. A Gilson modular HPLC system consisting of 2 Gilson model 303 pumping units with a Waters Millipore Lambda-Max model 481 UV detector was used. Chromatographic analysis were carried out with a Merck LiChrosorb Hibar 125 x 4 mm standard cartridge column packed with 7 µm, RP-8 (octylsilane) particles and Merck Hibar LichroCart PVDF RP-8 guard column cartridges. The mobile phase was filtered with a Waters Millipore solvent filtration kit with 0.45 µm RC 55 regenerated cellulose membrane filter (Schleicher and Schuell) before ultrasonication for 15 min.

### 2.3.2 Method

The HPLC method was developed using stock urine A. The THC-COOH was extracted by the procedure given in section 2.1.2 and the residue obtained after drying the solvent was dissolved in 100 µl of methanol and transferred to microvials. The microvials containing the methanolic solution of the residues were then centrifuged at 3000 rpm for 20 min before injecting 20 µl aliquots into the HPLC.

The HPLC conditions were arrived at by systematically varying the percentage of acetonitrile in the aqueous mobile phase from 40.0 to 52.5, the pH from 2.50 to 4.00 and the flow rate from 1.00 to 1.75 ml $min^{-1}$. The optimum conditions obtained for the mobile phase was 47.5% acetonitrile and 52.5% water (pH 3.00) and a flow rate of 1.00 ml $min^{-1}$. The eluent was monitored at 214 nm with the detector output signal of 0.005 AUFS. The eluent was adjusted to pH 3.00 with phosphoric acid and a Merck RP 8 cartridge guard column was used when the method was applied to developing the extraction procedure (section 2.1.2). For all other application the pH of the mobile phase was adjusted with $KH_2PO_4$.

## 2.4 Thin-layer chromatographic method for THC-COOH in urine

### 2.4.1 Materials and instrumentation

Acetone, chloroform and methanol were all analytical reagent (AR) grade; ammonia solution was general purpose reagent (GPR) grade; anisidine, butylamine, diethylamine and triethylamine were synthesis grade. Di-O-anisidine tetrazolium chloride or Fast Blue B salt (FBB) obtained from BDH was microscopy material grade. THC-COOH (100 µg $ml^{-1}$ ampules) were obtained from RTI, USA.) Development of TLC plates was carried out in twin trough glass tanks whose dimensions were 27 x 27 x 7 cm. Samples were spotted with 5 µl micropipettes (Drummond) on either 10 x 20 cm or 20 x 20 cm glass backed pre-coated silica gel $60F_{254}$ TLC plates (Merck) with a layer thickness of 0.25 mm. The mobile phase consisted of chloroform, methanol and ammonia in the proportion 70:30:2 respectively (solvent system 1).

The spray reagent was prepared prior to use by dissolving 20 mg of FBB in 20 ml of a solution prepared by mixing 25 ml of water with 75 ml of methanol. All densitometric measurements were carried out on a Shimadzu CS-920 High Speed TLC Scanner equipped with a tungsten lamp for scanning the wavelength range between 350 and 630 nm.

### 2.4.2 Method

Samples from the stock urine (A) containing about 870 ng $ml^{-1}$ THC-COOH were used. 3.0 ml of urine samples were first hydrolysed (step (a) in section 2.1.2) before extraction. The hydrolysed urine was adjusted to pH 2 to 3 using 50% HCl (v/v) and 0.20 M NaOH solutions followed by addition of 0.3 ml of 1.00 M $KH_2PO_4$ at pH 2.5. The acidified urine was extracted with 3.0 ml of a mixture of cyclohexane:ethyl acetate (92:8) and the extract was dried in a stream of nitrogen at approximately 60°C. The residue was dissolved in 50 µl of acetone and spotted on the plate using a 5 µl micropipette. The tube was rinsed



with 50 µl acetone and the solution was transferred to the plate on to the same spot. The acetone was evaporated with a hair dryer during spotting. Spots of different relative concentration of the extract were obtained by combining 3.0 ml urine extracts. The extracting solvent mixture was removed by evaporation with a vacuum rotary evaporator. The residue obtained was dissolved in a suitable volume of methanol and different volumes of this solution were then used for preparing solutions of different relative concentrations of the extract. A known volume of the methanol solution of the extract was first transferred into a conical test tube and dried in a stream of nitrogen at about 60°C. The residue was dissolved in 50 µl of acetone and transferred to the plate using the same spotting procedure.

The TLC tanks, which were lined on one side with chromatographic paper, were allowed to equilibrate with the solvent system for about 20 min before inserting the plates. The plates were removed for spraying after the solvent front had migrated three-quarters of the way up the plate.

The effect of spraying diethylamine which is used as mordanting/bleaching agent prior to spraying the chromogen, FBB, was investigated. Two plates which were spotted with solutions containing 1.5 and 3.0 ml extracts of the stock urine (A) were developed with solvent system A (section 2.4.1). One of the plates was sprayed with 10 ml of FBB only, after drying the plate for 15 min, and the other was sprayed with 10 ml of diethylamine (DEA) after 2 minutes followed immediately by 10 ml of FBB. Both plates were scanned with the densitometer after visual observation.

The DEA-FBB spraying sequence was further investigated to determine the optimum time for spraying. Six plates were each spotted with solution containing 1.0 and 3.0 ml extracts of the stock urine. The plates were developed using the solvent system in section 2.4.1 and sprayed with 10 ml of DEA followed by 10 ml of FBB after 30, 60, 90, 120, 150 and 180 s. The optimum time for spraying was determined by visual observation. The effect of using different proportions of DEA and FBB was also investigated. Four plates spotted with solution containing 1.0 and 3.0 ml extracts of the stock urine were developed and sprayed separately at the optimum time with 10 ml DEA followed by 10 ml FBB, 10 ml DEA followed by 20 ml FBB, 20 ml DEA followed by 10 ml FBB, and 20 ml DEA followed by 20 ml FBB. The optimum proportion was determined by visual observation. The effect of repeated spraying at the optimum time and with the optimum proportion of DEA and FBB was investigated. Four plates were developed as above and sprayed individually once, twice, three and four times respectively. Visual

observation of the intensity of colour of the spots was used for evaluation.

A study was also carried out to compare the effectiveness of DEA as a mordanting/bleaching agent with ammonia solution, butylamine (BuA), triethylamine (TEA), DEA with 1.0, 2.5 and 5.0% anisidine and DEA with 5.0, 10.0 and 25.0% BuA.

The detection limit of spraying with the optimized technique and with FBB was compared by spotting two plates with 5 µl of serially diluted standard solutions of 15, 7.5 and 3.7(5) µg $ml^{-1}$ of THC-COOH to give spotted amounts of 75.0, 37.5 and 18.7(5) ng respectively. The plates were developed with solvent system A and evaluation of the two techniques was by comparing visually the intensity of the colour of the spots. The linear range of quantitation with the densitometer was also studied by spotting 5 µl of serially diluted standard solutions of 600, 300, 150, 75, 30, 15, 7.5 and 3.7(5) µg $ml^{-1}$ of THC-COOH to give spotted amounts of 3000, 1500, 750, 375, 150, 75, 37.5 and 18.7(5) ng respectively. The plates were developed and sprayed using the optimized technique.

The seven mobile phase systems listed in table 2.3 were selected from the TLC systems reviewed in section 1.4.2. The systems were evaluated for separating and identifying simultaneously THC-COOH in urine as well as the plant cannabinoids THC, CBN and CBD. 5 µl aliquots of 1 mg $ml^{-1}$ solution of THC, CBN and CBD and a solution containing THC-COOH from a 3.0 ml extract of stock urine were spotted on 5 x 20 cm TLC plates and developed with each of the mobile phases. The plates were sprayed with 10 ml of DEA followed by 10 ml of FBB solution.

## 2.5 Evaluation of derivatization procedures for GC

### 2.5.1 Materials and instrumentation

Acetonitrile, cyclohexane, hexane, isooctane, methyl iodide ($CH_3I$) and 25% tetramethylammonium hydroxide (TMAH) were all analytical reagent (AR) grade. Dimethylsulphoxide (DMSO) and N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) were of silylation grade. All reactions were carried out in Pierce 5 ml and 1 ml reacti-vials with teflon lined screw caps. THC-COOH (100 µg $ml^{-1}$ ampules) were obtained from RTI, USA.



Table 2.3

Seven mobile phase systems for TLC of THC-COOH in urine

| | Solvent system | Reference |
|---|---|---|
| A | $CHCl_3:CH_3OH:NH_3$ (70:30:2) | 42 |
| B | $CHCl_3:CH_3OH:NH_3$ (85:15:2) | 41 |
| C | $EtOAc:CH_3OH:H_2O:NH_3$ (12:5:0.5:1) | 31, 36, 56 |
| D | $Hex:Acet:CH_3COOH$ (70:30:1) | 32 |
| E | $Hept:Et_2O:CH_3COOH$ (80:10:4) | 61 |
| F | $Hept:Acet:CH_3COOH$ (70:30:1) | 52 |
| G | DD: i. $Acet:CHCl_3:TEA$ (80:20:1)<br>ii. Pet. ether:$Et_2O:CH_3COOH$ (5:5:0.15) | 43 |

### 2.5.2 Method

The derivatization methods evaluated were methylation and silylation.

i. Methylation

The methylation procedure involved the following steps:

(a) The dried residue of a standard solution or urine extract containing THC-COOH was dissolved in 70 ul of a solution of TMAH:DMSO (1:20) and vortexed.

(b) After 2 min, 5 ul of $CH_3I$ was added and the solution was vortex-mixed.

(c) After 5 min at room temperature (25°C) the reaction was stopped by adding 0.2 ml of 0.10 M HCl.

(d) The reaction mixture was extracted twice with cyclohexane. For each extraction, 1.0 ml of cyclohexane was added and the mixture was vortexed - mixed for 1 min.

(e) The combined extract was evaporated to dryness at 60°C in a stream of nitrogen and the resulting residue dissolved in 50 µl of cyclohexane. 1 and 3 µl aliquots of the solution were then analysed with GC-FID and GC-MSD respectively.

The above procedure was optimized by varying the reaction temperature, reaction time, concentration of $CH_3I$ and by using different extraction solvents. All experiments were carried out with samples from standard solutions of THC-COOH which were evaporated to dryness at 60°C in a stream of nitrogen in 5 ml reacti-vials to yield 1000 ng of THC-COOH.

The effect of varying the reaction temperature and time was investigated by carrying out the reactions for 5, 15, 30 and 60 min at 25°C (RT) and at 60°C. However in this series of experiments isooctane was substituted for cyclohexane in the extraction step (step d). The effect of varying the concentration of $CH_3I$ was studied by using 5, 10 and 20 µl of $CH_3I$ in step (b) of the procedure. For evaluation of extraction solvents, hexane and isooctane were used in step (d) after following the procedure from steps (a) to (c). Each of the experiments described above was repeated with duplicate samples.

ii. Silylation

The silylating agent was BSTFA. All reactions were carried out by adding 50 µl of BSTFA to the evaporated residue of a standard solution or extract. The reaction mixture was heated at 90°C for 90 min. The reaction time was selected after carrying out the reaction at 90°C for 60, 90, 120 and 150 min. The experiments relating to the optimization of reaction time were carried out with duplicate samples from standard solutions of THC-COOH which were evaporated to dryness to yield 1000 ng THC-COOH. The samples were analysed with the GC-FID and GC-MSD systems.

## 2.6 Gas chromatographic method for detection of THC-COOH in urine

### 2.6.1 Materials and instrumentation

All analyses with a model HP 5880A GC (Hewlett Packard) and the flame ionization detector (FID) were carried out with a HP 5880/90 series 6 ft. x 1/4 in. O.D.



x 2 mm I.D. glass column packed with 100/120 mesh chromosorb WHP coated with 3% OV 101, methyl silicone fluid, liquid phase (Hewlett Packard). The nitrogen used as carrier gas was further purified by passing through moisture and oxygen traps. A 30 m x 0.25 mm I.D. cross-linked fused silica column (Supelco) coated with 5% SE 54, phenylmethyl silicone gum, liquid phase and having a film thickness of 25 µm was used for analysis performed on a model HP 5890 GC equipped with a HP 5970A Mass Selective Detector (MSD) and a HP5970C Chemstation (Hewlett Packard). THC-COOH (100 µg $ml^{-1}$ ampules) were obtained from RTI, USA, and Mirex (1, 1a, 2, 2, 3, 3a, 4, 5, 5, 5a, 5b, 6 - dodecachlorooctahydro-1,3,4-metheno-1H-cyclobuta[c,d]pentalene) was obtained from Supelco, USA.

### 2.6.2 Method

A. The GC-FID conditions used were:

| | | |
|---|---|---|
| i. | injection port temperature | : 300°C, |
| ii. | detector temperature | : 300°C, |
| iii. | oven temperature | : 250°C for 7.5 min, ramp to 280°C at 5°C $min^{-1}$ 280°C for 1 min and |
| iv. | carrier gas flow rate | : 20 ml $min^{-1}$ (nitrogen) |

Isothermal analyses at column (oven) temperatures of 230, 240 and 250°C were used before selecting the above conditions. A 10 µl Hamilton GC syringe was used for injection of samples.

B. The GC-MSD conditions used were

| | | |
|---|---|---|
| i. | injector port temperature | : 290°C, |
| ii. | transfer-line temperature | : 290°C, |
| iii. | oven temperature | : 160°C for 1 min, ramp to 280°C at 35°C $min^{-1}$, 280°C for 10 min, |

iv. carrier gas flow rate : 0.9 and 0.8 ml $min^{-1}$ (helium) at oven temperatures of 160 and 280°C respectively,

v. splitless injection time : 0.80 min,

vi. open-slit interface flow rate : 0.60 and 0.20 ml $min^{-1}$ at oven temperatures of 160 and 280°C respectively and

vii. injector port purge gas flow rate : 60 ml $min^{-1}$.

The effect of initial oven temperatures, oven ramp rates and splitless times were investigated by carrying out the analysis at 50, 65, 70, 80, 90, 100, 130, 140, 150, 160, 170, 180 and 200°C, 15, 35 and 70°C $min^{-1}$, and 0.30, 0.40, 0.50, 0.60 and 0.80 min respectively. A study was also conducted to optimize sample vapourization by injecting different volumes of the silylated THC-COOH mixture by itself as well as with acetonitrile, hexane, heptane and toluene as solvents. The solvents were used to study the effectiveness of concentrating the vapour plug of sample components by 'solvent effect'. Mirex was added to the samples, prior to derivatization, as an internal standard during optimization of splitless time.

Samples were introduced into the injector port manually with a 10 µl GC syringe. The needle of the syringe was left in the injector port for the duration of the splitless time and until the injector purge was restored.

Quantitation with the MSD was carried out using the scan mode using the following parameters.



i. Solvent delay time : 4.00 min

ii. Electron multiplier voltage : 1600 V.

iii. Mass scan range : 70 to 500 u.

iv. Scan threshold : 500 counts

iv. A/D samples : 4 (number of times the abundance of a particular mass is recorded)

vi. Scan rate : 1.00 scan $s^{-1}$

The sensitivity of the GC-MSD method using the scan mode was investigated by injecting silylated THC-COOH mixtures of 50, 25, 12.5, 5, 2.5 and 1 µg $ml^{-1}$ THC-COOH in 50 µl BSTFA prepared from 100 µl aliquots of serially diluted methanolic standard solutions of 25, 12.5, 6.2(5), 2.5, 1.2(5) and 0.5 µg $ml^{-1}$ of THC-COOH which were evaporated to dryness at 60°C in a stream of nitrogen. The sensitivity of the method using selected ion monitoring (SIM) was investigated by injecting mixtures of 12.5, 5.0, 2.5 and 1 µg $ml^{-1}$ and 50, 25 and 12.5 ng $ml^{-1}$ THC-COOH in 50 µl BSTFA prepared as for the scan mode from serially diluted methanolic standard solutions of 6.2(5), 2.5, 1.2(5) and 0.5 µg/$ml^{-1}$ and 25, 12.5 and 6.2(5) ng $ml^{-1}$ of THC-COOH respectively. The SIM parameters were as follows:

i. Solvent delay time : 9.00 min

ii. Electron multiplier voltage : 1600 V.

iii. Ions monitored : m/z 488, 473 and 371

iv. Dwell time : 150 ms.

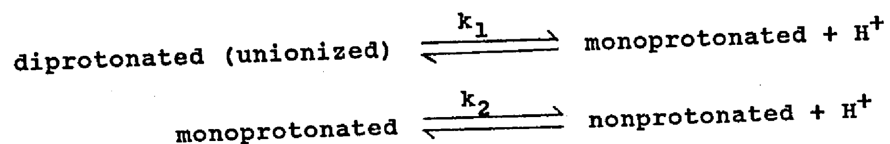
v. Scan rate : 1.8 cycles $s^{-1}$

# Chapter 3

## RESULTS AND DISCUSSION

### 3.1 Determination of $pK_a$ values of THC-COOH

Ionization constants or $pK_a$ values are useful for the systematic development of extraction methods of ionizable drugs and other substances from a complex sample matrix. Various methods [64, 65] have been reported for determination of ionization constants of monobasic compounds as well as dibasic compounds [65 - 67] with overlapping $pK_a$ values. The ionization process for a dibasic compound can be represented as follows:




where $k_1$ and $k_2$ are the first and second ionization constants. However if the first and second ionization constants differ greatly in magnitude, i.e. $k_1/k_2 > 1000$, they can be determined separately since only one step of the ionization process would be occuring at a given pH. THC-COOH (figure 1.3) has a carboxyl and a phenol functional group at the 11 and 1 position respectively. The phenolic group of THC-COOH is identical to that of THC (figure 1.1) and therefore should have a $pK_a$ value similar to that reported for THC [49]. However, since the $pK_1$ value of the carboxylic group was unavailable in literature, the ionization constants of both groups of THC-COOH were determined using a HPLC and a spectrophotometric method respectively.

#### 3.1.1 Determination of the $pK_1$ value for the carboxyl functional group of THC-COOH

Various models [80 - 84] have been proposed for explaining the retention behaviour of solutes on reversed phase materials in HPLC. Horvath [81, 82] suggested that the interaction between solute molecules and the hydrocarbonaceous ligands of the stationary phase was a reversible association resulting in formation of solute - ligand complexes. This association was due to solvophobic or hydrophobic interactions which originate essentially from the net repulsion between water and the nonpolar ligand as well as the nonpolar moiety of the solute. Therefore, for unionized solute, the degree of interaction would depend solely on the water content of the mobile phase since water had a tendency to reduce the nonpolar surface area of the solute in contact with the solvent component of the mobile phase. However for ionogenic solutes the pH of the aqueous mobile phase also played a



role in the retention behaviour since solute molecules could undergo ionization under suitable pH conditions. As a consequence electrostatic interactions of the solute with the aqueous mobile phase would increase considerably. Thus the plot of capacity factor, $k'$, which is a measure of retention and hence partition equilibrium versus pH of the mobile phase would result in a sigmoidal curve and the pH value at the point of inflexion of the curve would be equal to the $pK_a$ value of the solute. The measurement of $pK_a$ values of carboxylic acids from inflexion points of plots of $k'$ values versus pH of eluent have also been described by other investigators [85, 86, 87]. However this method can only be used if the pH of the eluent is between 2 and 8 because reversed phase materials are unstable beyond this range.

The capacity factor, $k'$, can be calculated from

$$k' = \frac{t_R - t_o}{t_o} \qquad (3.1)$$

where $t_R$ and $t_o$ are the retention times for THC-COOH and that of an unretained eluite. The value of $t_o$ was conveniently evaluated from the position of the baseline distortion at the begining of the chromatogram since the "peak" was caused by disturbances in the mobile phase during injection of the sample. Figure 3.1 shows a typical HPLC chromatogram of THC-COOH in a methanolic buffer and table 3.1 gives the values of $k'$ as a function of the measured pH values, $pH^{app}$. The observed decrease in $k'$ with incresing $pH^{app}$ of the mobile phase can be explained by the increase in ionization of the dibasic acid, THC-COOH, hence reducing its retention. Figure 3.2 shows a plot of $k'$ values versus the thermodynamic pH values, $pH^*$. The $pH^*$ values were calculated from the corresponding $pH^{app}$ values which were the pH values of the aqueous methanol buffer solutions referred to aqueous standard solutions by utilizing a correction term $\delta$ given by

$$\delta = pH^{app} - pH^* \qquad (3.2)$$

Bates et. al. [88], evaluated experimentally values which were essentially differences between liquid - junction and glass electrode potentials in neat aqueous and methanolic buffer solutions. Figure 3.3 gives a plot of $\delta$ against weight percentage of methanol in methanol - water mixtures. The $\delta$ value for methanol content of the mobile phase (60.0%, v/v or 54.2%, w/w) was 0.13 and this value was used for calculating $pH^*$ values given in table 3.1. The $pK_1$ value of the carboxyl group which is equal to the $pH^*$ value at the point of inflexion of the sigmoid curve in figure 3.2 is 6.1.

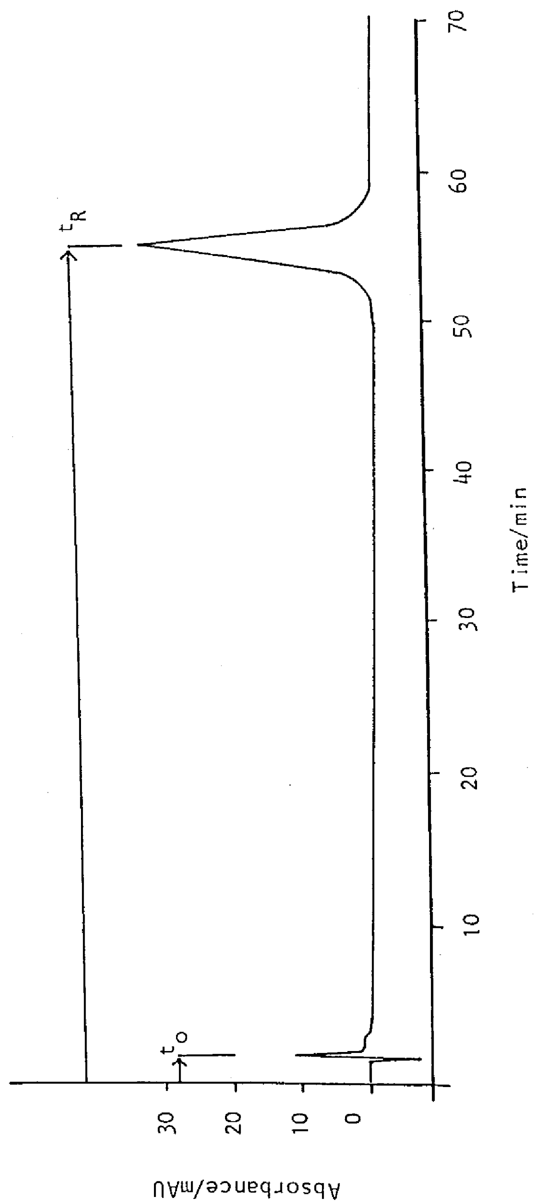



Figure 3.1: HPLC chromatogram of THC-COOH with pH* 3.54 methanolic buffer



Table 3.1: Capacity factor (k') values of THC-COOH at different buffer pH values

| Aqueous buffer pH | 60% (v/v) methanolic buffer pH | | Retention time (min) | | Capacity factor (k') |
|---|---|---|---|---|---|
| | measured $pH^{app}$ | calculated $pH^{*}$ | $t_o$ | $t_R$ (THC-COOH) | |
| 2.26 | 3.67 | 3.54 | 1.346 | 54.812 | 39.7 |
| 3.07 | 4.46 | 4.33 | 1.332 | 53.100 | 38.9 |
| 4.04 | 4.97 | 4.84 | 1.298 | 49.574 | 37.2 |
| 4.43 | 5.81 | 5.68 | 1.355 | 42.234 | 30.2 |
| 5.03 | 6.15 | 6.02 | 1.319 | 34.289 | 25.0 |
| 5.43 | 6.87 | 6.74 | 1.338 | 21.749 | 15.3 |
| 5.83 | 7.27 | 7.14 | 1.336 | 15.696 | 10.7 |
| 6.39 | 7.87 | 7.74 | 1.334 | 12.073 | 8.1 |

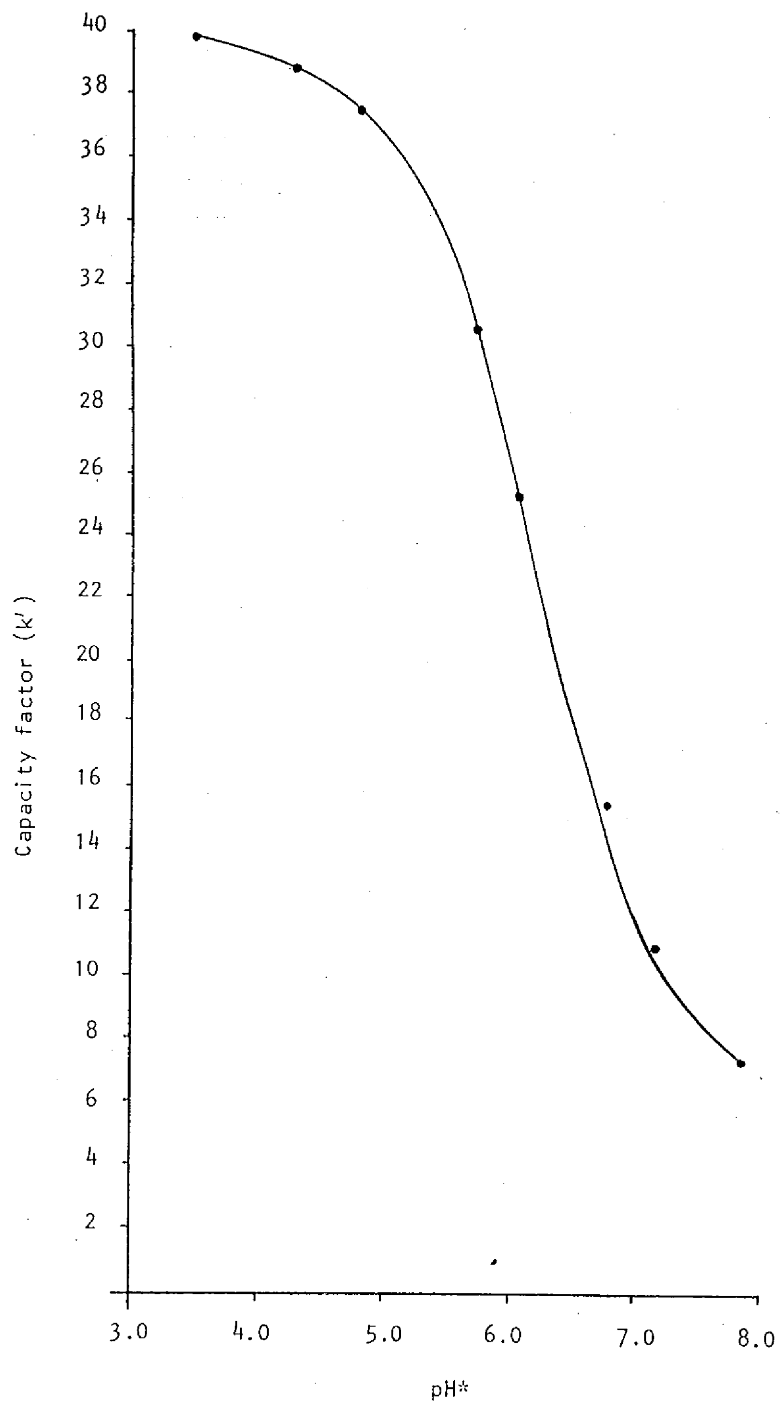



Figure 3.2 Plot of capacity factor (k') versus thermodynamic pH values (pH*) of methanolic buffers



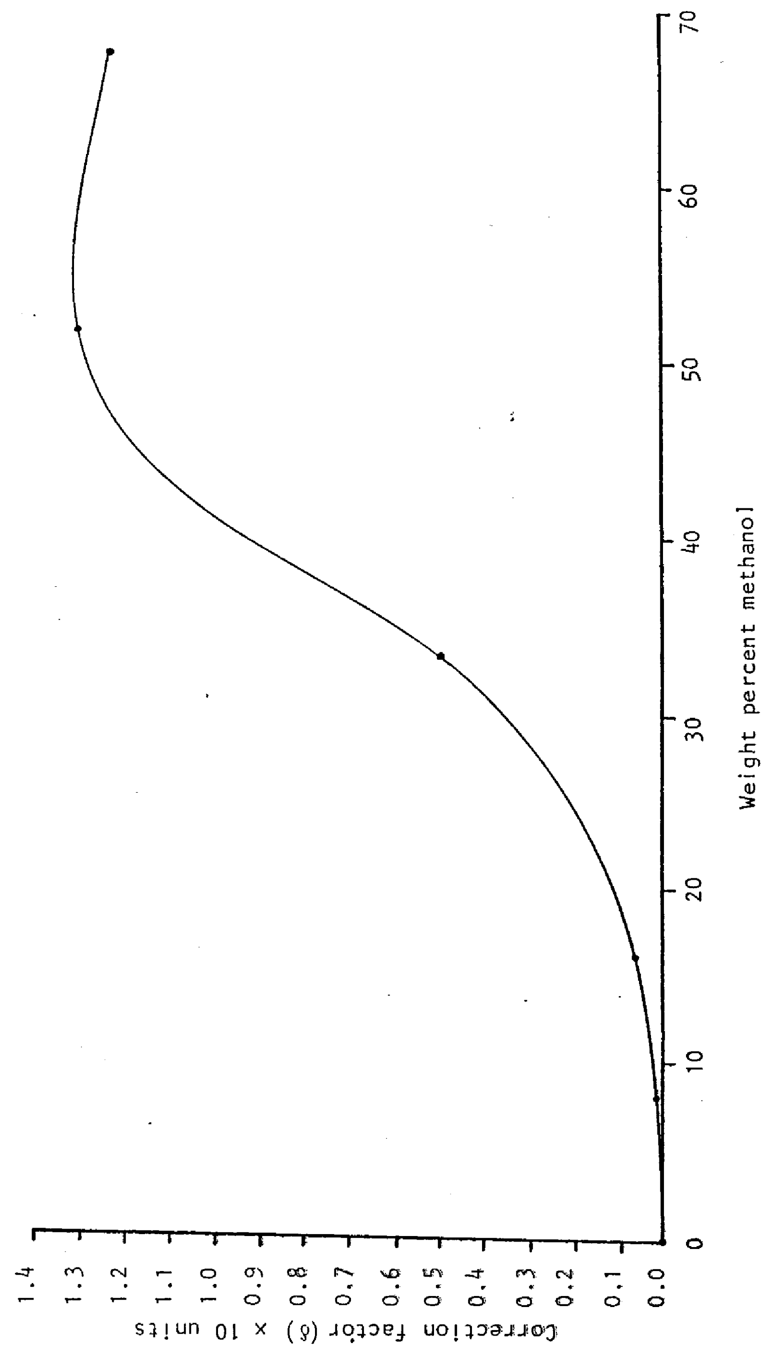



Figure 3.3: Plot of correction factor (δ) versus weight percent of methanol

Horvath [85] and van de Venne [86] have shown that $pK_a$ values of carboxylic acids obtained chromatographically with HPLC systems are in good agreement with titrimetric values using similar solvents. The $pK_a$ values obtained with neat aqueous chromatographic systems were found to be similar to those reported by other methods. However $pK_a$ values obtained chromatographically and by titration with mixed aqueous/organic solvents were at least 0.5 $pK_a$ units higher than values obtained with neat aqueous solvents [86].

### 3.1.2 Determination of the $pK_2$ value for the phenol functional group of THC-COOH

The $pK_2$ value of the phenol functional group of THC-COOH was determined using a spectrophotometric method. If $pK_2$ = 10.6 of THC is taken as the approximate value for the phenol functional group of THC-COOH together with $pK_1$ = 6.1 for the carboxyl group (section 3.1.1) then the ratio of the ionization constants $k_1/k_2$ > 30000. Therefore the ionization constants are well separated and they can be determined separately [65 - 67] since then only one stage of ionization is operative at a given pH.

Figure 3.4 shows the UV spectra of THC-COOH at constant concentration (1 µg $ml^{-1}$) in 6 aqueous buffer solutions. It is clear from the figure that the absorbance of THC-COOH increases with increasing pH values until pH 7.03 indicating ionization of the carboxyl group in this pH range. Since $pK_1$ = 6.1 for the carboxyl group, about 90% of the carboxyl group would be ionized at pH 7. Thus the spectra obtained with pH 7.03 and 7.79 buffer solutions would be predominantly of the monoprotonated species and should be similar as observed in figure 3.4. The absorbance values increases again with increase in pH indicating ionization of the phenol functional group and at pH 12.11 it is reasonable to assume that ionization is complete and the nonprotonated THC-COOH species predominates. The analytical wavelength was selected at 212 nm since maximum accuracy for calculating the ionization constant, $k_2$, could be obtained at this wavelength. Table 3.2 gives the absorbance values of the various solution at the analytical wavelength.

The ionization constant of the phenol functional group is given by equation 1.3:

$$k_2 = \frac{\varepsilon - \varepsilon_2}{\varepsilon_3 - \varepsilon} a$$

where $\varepsilon$, $\varepsilon_2$ and $\varepsilon_3$ are the absorbance values at the analytical wavelength for pH 11.25, either pH 7.03 or pH 7.79, and pH 12.11 solutions respectively and 'a' is the



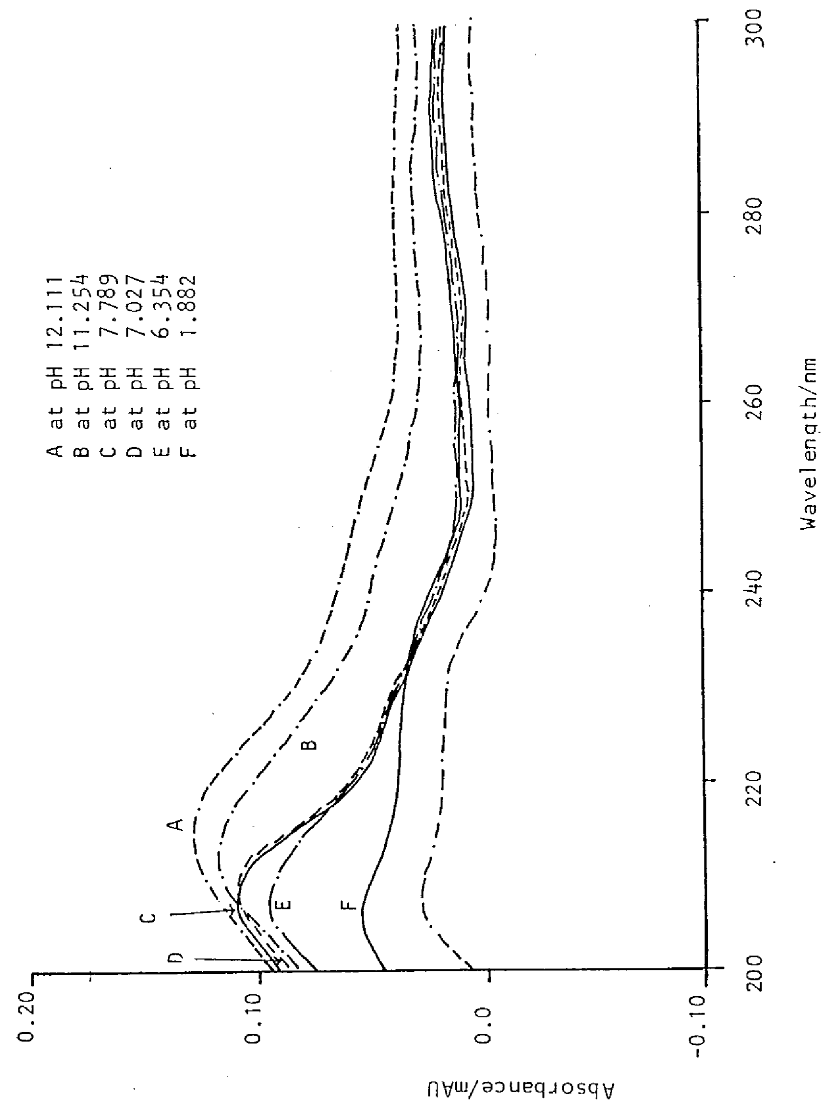



Figure 3.4: UV spectra of THC-COOH at different buffer pH values

Table 3.2: UV absorbance measurements of THC-COOH at 212 nm for different buffer pH values

| Aqueous buffer pH | Absorbance at 212 nm |
|---|---|
| 1.88 | 0.0440 |
| 6.36 | 0.0798 |
| 7.03 | 0.0896 |
| 7.79 | 0.0878 |
| 11.25 | 0.1153 |
| 12.11 | 0.1279 |



hydrogen ion concentration of the pH 11.25 solution. An average $pK_2$ value of 10.9 was obtained for the phenol functional group from the two calculated values.

### 3.1.3 Factors affecting determination of $pK_a$ values and precautions taken

The dependence of the thermodynamic ionization constants of a monobasic acid, $K_a^T$, on activity coefficient of the anion, $f_{A^-}$, is given by

$$K_a^T = \frac{\{H^+\}\,[A^-]}{\{HA\}} \cdot f_{A^-} \qquad (3.3)$$

where $\{H^+\}$ and $\{HA\}$ are the molar activities of the hydrogen ion and unionized molecule HA respectively, and $[A^-]$ is the stoichiometric molar concentration of the anion. The activity coefficient of the anion, $f_{A^-}$, is related to the ionic strength, I, by the Debye - Huckel equation:

$$-\log f_{A^-} = \frac{Az^2\sqrt{I}}{1 + Ba_{A^-}\sqrt{I}} \qquad (3.4)$$

where A and B are constants which vary with the dielectric constant and temperature of the solvent, $a_{A^-}$ is the ionic size parameter and z is the valency of the anion. Equations 3.3 and 3.4 show that the ionization constant is dependent on the ionic strength and in order to use the spectrometric absorbance values obtained with different buffers the ionic strength of the solutions must be kept constant. Horvath [85] has shown theoretically and experimentally the effect of ionic strength on elution of solutes as a result of its effect on surface tension and electrostatic interactions of the eluent. This effect has also been described as salting effects by Snyder and Kirkland [68]. The effect of ionic strength on the $pK_a$ of a solute is also important especially when the eluent pH is in the region of the $pK_a$ [86, 87]. Thus the buffer solutions used for determination of $pK_1$ and $pK_2$ of THC-COOH were all prepared at a constant ionic strength of 0.10 M. The buffer solutions were all freshly prepared in the same room where pH, HPLC and spectrometric measurements were made and the temperature was 26 $\pm$ 1$^{o}$C.

## 3.2 Liquid - liquid extraction method for THC-COOH in urine

Snyder and others [63, 68, 89] have described the steps involved in the systematic development of a liquid - liquid or solvent extraction method. Some of the factors which need to be considered include the properties of the solvent system, the pH of the extraction matrix, the selectivity of a solvent system with respect to extraction pH, miscibility and inertness of the solvent and the partition coefficient of the solute between the matrix and solvent system. Snyder used the polarity index, $P'$, and the selectivity factors $x_e$, $x_d$ and $x_n$ to describe the properties of a solvent to extract a solute quantitatively and selectively from a matrix (section 1.4.1). However, in addition to solvent selectivity, the selectivity of the overall extraction method can be improved further by the use of specific clean-up steps. Some of the steps include back-extraction of THC-COOH [44, 52], extract washing steps where some of the unwanted materials from the organic extract are removed with either 5% $NaHCO_3$ [43] or 0.20 M $H_2SO_4$ [49] and extraction before acidification of the hydrolysed urine so that some of the unwanted endogeneous materials are removed from the THC-COOH in the urine [45, 51].

### 3.2.1 Determination of a solvent system for extraction of THC-COOH

Eight solvents were selected with increasing $P'$ values and from different selectivity groups and their ability to extract THC-COOH in urine was evaluated over a range of urine pH values. Some of the relevant properties of the solvents are given in table 3.3. The $P'$ and selectivity values given in table 3.3 are recently published values [90] corrected for errors in the old values given by Snyder [68, 69]. The peak height counts obtained for the solvents are given in tables A1 to A8 in the appendix. The peak height counts of the extracted THC-COOH were plotted against the urine pH values (figures 3.5 to 3.7). The plots (figure 3.5) obtained for the neat hydrocarbon solvents namely isooctane, hexane and cyclohexane show similar extraction profiles. The inflexion points of the sigmoidal curves approximately correspond to pH 6 which is close to the $pK_a$ value for the carboxyl functional group of THC-COOH ($pK_1 = 6.1$, section 3.1.1). Therefore the ability of these neat hydrocarbon solvents to extract decreased significantly after the first ionization constant of THC-COOH, indicating the interactions occuring between these solvents and THC-COOH are weak van der Waals interactions.

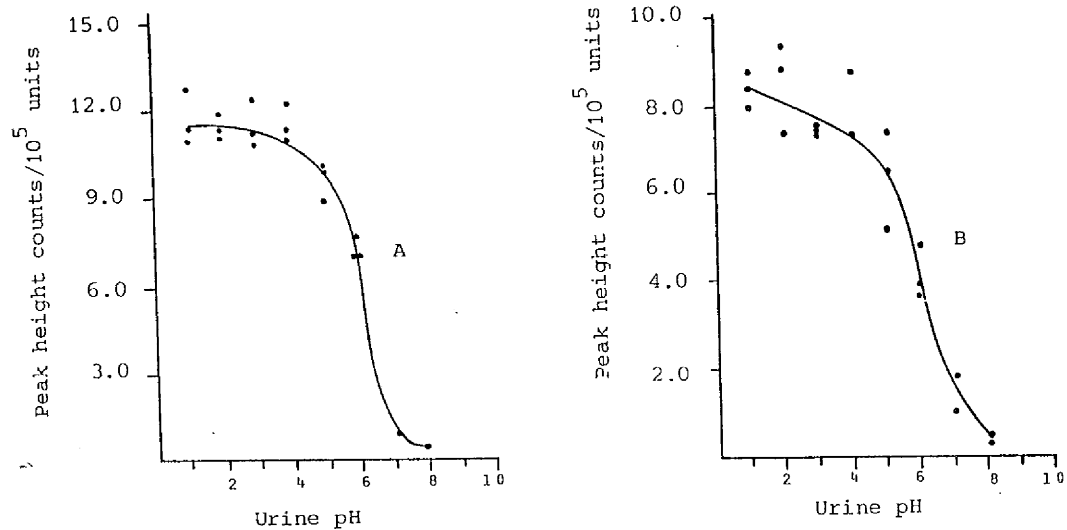
The other solvents with larger $P'$ values yielded plots with more complex extraction profiles (figure 3.6 and 3.7). Ethyl acetate and diethyl ether (curves D and E, figure 3.6) which are relatively strong proton acceptors showed a significant increase in the amount extracted

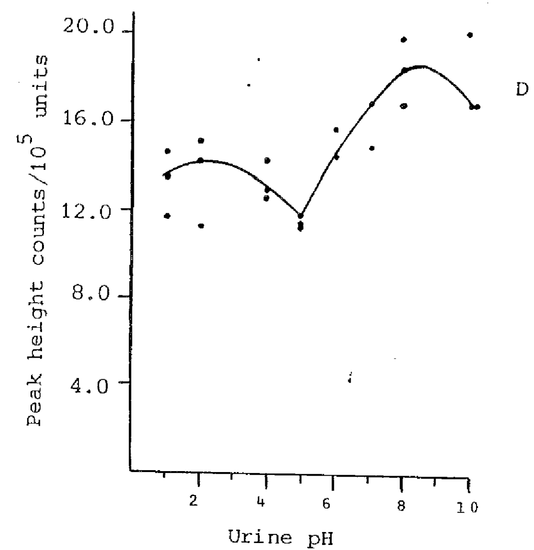


Table 3.3: Table of solvents evaluated for extraction of THC-COOH and their solvent properties (90)

| Solvent, | Dielectric constant, | Polarity Index, P' | Selectivity group | Selectivity parameters* | | |
|---|---|---|---|---|---|---|
| | | | | $x_e$ (acceptor) | $x_d$ (donor) | $x_n$ (dipole) |
| Hexane | 1.88 | -0.14 | | | | |
| Isooctane | 1.94 | -0.03 | | | | |
| Cyclohexane | 2.02 | 0.17 | | | | |
| Diethyl ether | 4.3 | 3.15 | I | 0.53 (1.67) | 0.13 (0.41) | 0.34 (1.07) |
| Benzene | 2.3 | 3.19 | VII | 0.27 (0.86) | 0.28 (0.89) | 0.45 (1.44) |
| Ethyl acetate | 6.0 | 4.24 | VI | 0.36 (1.53) | 0.22 (0.93) | 0.42 (1.78) |
| Dichloromethane | 8.9 | 4.29 | VII | 0.27 (1.16) | 0.33 (1.42) | 0.40 (1.72) |
| Chloroform | 4.8 | 4.31 | VIII | 0.31 (1.34) | 0.35 (1.51) | 0.34 (1.47) |

* The values for the total selective interaction strength ($P x_e$, $P x_d$ and $P x_n$ respectively) are given in brackets.



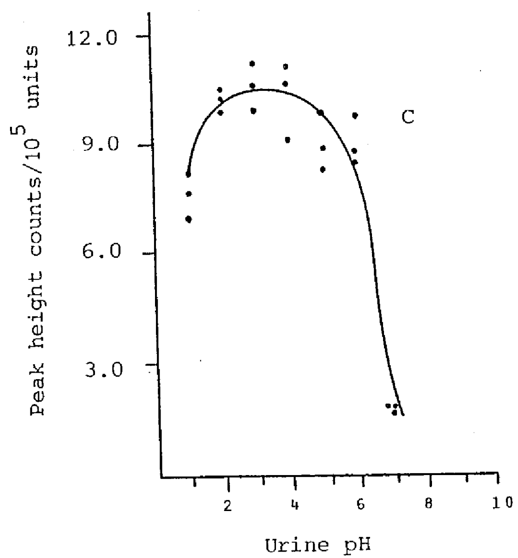




Figure 3.5: Plot of peak height counts of THC-COOH versus urine pH for isooctane (A), cyclohexane (B) and hexane (C)

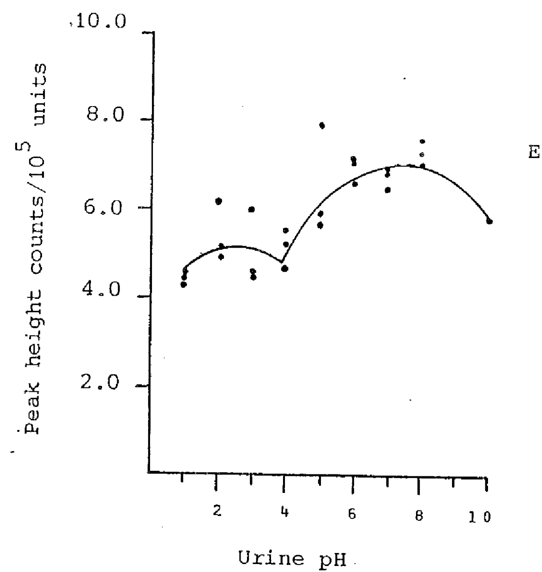



Figure 3.6: Plot of peak height counts of THC-COOH versus urine pH for ethyl acetate (D) and diethyl ether (E)

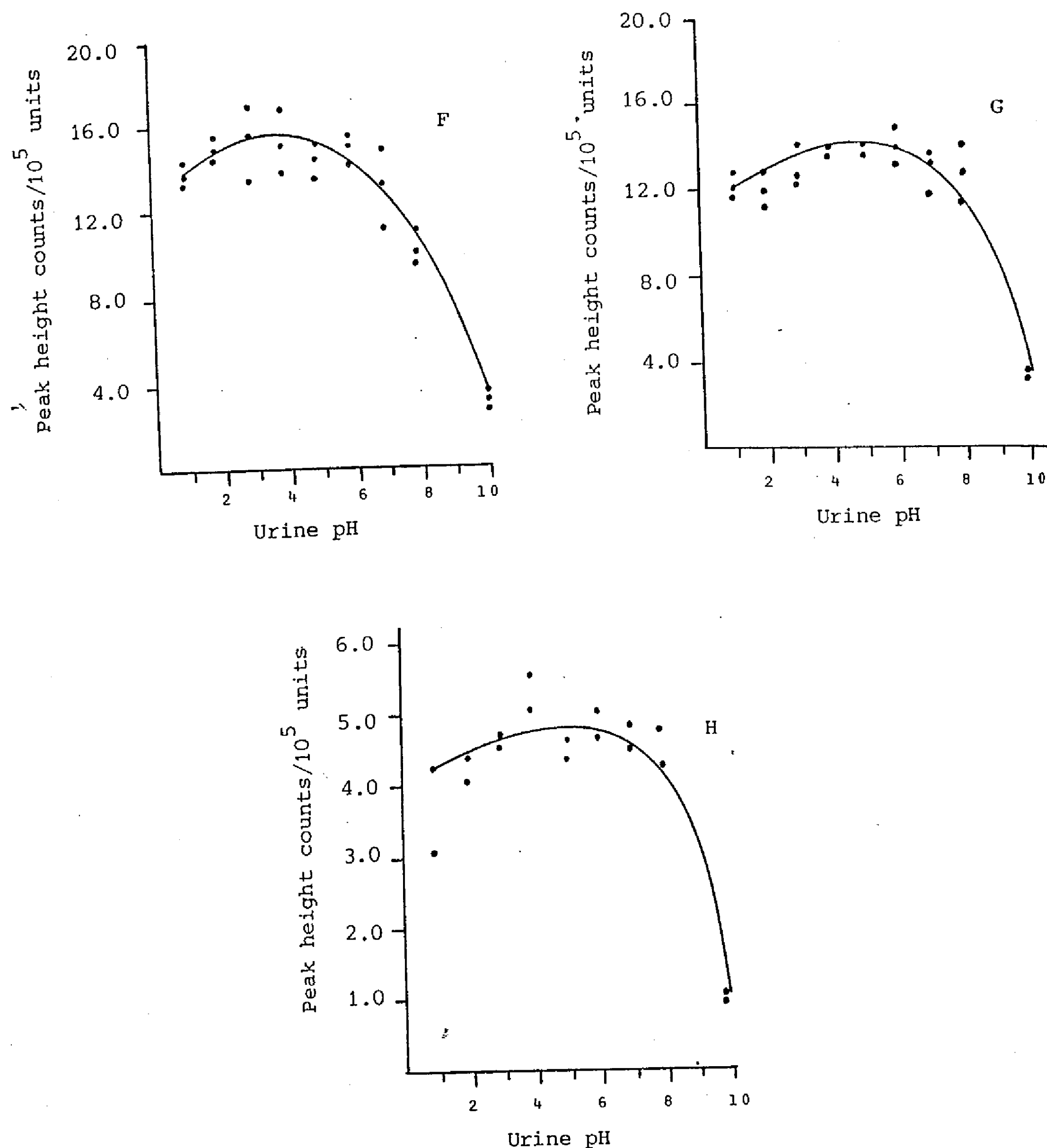



Figure 3.7: Plot of peak height counts of THC-COOH versus urine pH for benzene (F), chloroform (G) and dichloromethane (H)



between pH 5 and pH 7. This would suggest that these two solvents were able to interact more strongly with the monoprotonated THC-COOH species than with the undissociated molecule. This is in contrast to the behaviour of dichloromethane and chloroform which are a slightly acidic and a moderately strong proton donor respectively (curves G and H, figure 3.7). These two solvents did not show any significant change in the amount extracted between pH 2 and pH 7 although dichloromethane unlike chloroform is also a strong dipole interactor. Benzene (table 3.3) which has roughly the same ability to function as a proton acceptor and donor with a P' value similar to that of diethyl ether shows an extraction profile (curve F, figure 3.7) which has certain features similar to ethyl acetate and diethyl ether (curves D and E) as well as chloroform and dichloromethane curves (curves G and H). In all cases there was a decrease in the amount extracted at pH = 10 which suggests that formation of increasing amounts of nonprotonated THC-COOH species inhibited extraction ($pK_2$ = 10.9). Further with chloroform, dichloromethane and benzene as solvents, the amount extracted at pH = 10 where both monoprotonated and some nonprotonated species occur was only about 19 to 28% of the amount extracted at pH = 2 where the diprotonated species predominates. In contrast, if the same comparison is made for ethyl acetate, there is an increase of about 23% and for diethyl ether the amount extracted is about the same.

The low precision obtained for some of the solvents, namely cyclohexane, diethyl ether and benzene (table A2, A5 and A6 respectively), was due to emulsions which were formed when the organic and aqueous urine phases were vortexed. The emulsions could not be broken in spite of prolonged centrifugation. The addition of a salt prior to centrifugation might have resulted in better separation of the phases as a result of increased surface tension of the aqueous phase. However this was not attempted since it would also cause salting-out effects and the amount of drug extracted would then not be a true refelction of the solvents' extraction ability. The random precision values obtained for the other solvents were most probably due to salting effects that would have resulted from variations in ionic strength. These variations in ionic strength resulted from small differences in the amounts of acid and base that were added during pH adjustment.

The extraction capacity and selectivity of the 8 solvents at their optimum urine pH values were compared by extraction of a pooled urine sample (A, section 2.1.1). Table 3.4 gives the urine pH for extraction together with the mean peak height counts obtained. Figures 3.8 to 3.10 show representative HPLC chromatograms obtained for the solvents. Diethyl ether and benzene not only gave the

Table 3.4: Mean peak height counts of THC-COOH at optimum urine pH conditions for 8 solvents

| Solvent | Urine pH | Peak height counts | | |
|---|---|---|---|---|
| | | Mean | S.D. | R.S.D. |
| Hexane | 3 | 1466254 | 126141 | 8.60% |
| Cyclohexane | 2 | 1492661 | 123106 | 8.25% |
| Isooctane | 3 | 1529924 | 119569 | 7.82% |
| Diethyl ether | 8 | 1703714 | 54224 | 3.18% |
| Benzene | 3 | 1832811 | 50982 | 2.78% |
| Dichloromethane | 4 | 1408343 | 111988 | 7.95% |
| Ethyl acetate | 9 | 1446561 | 42338 | 2.93% |
| Chloroform | 4 | 1368341 | 46188 | 3.38% |



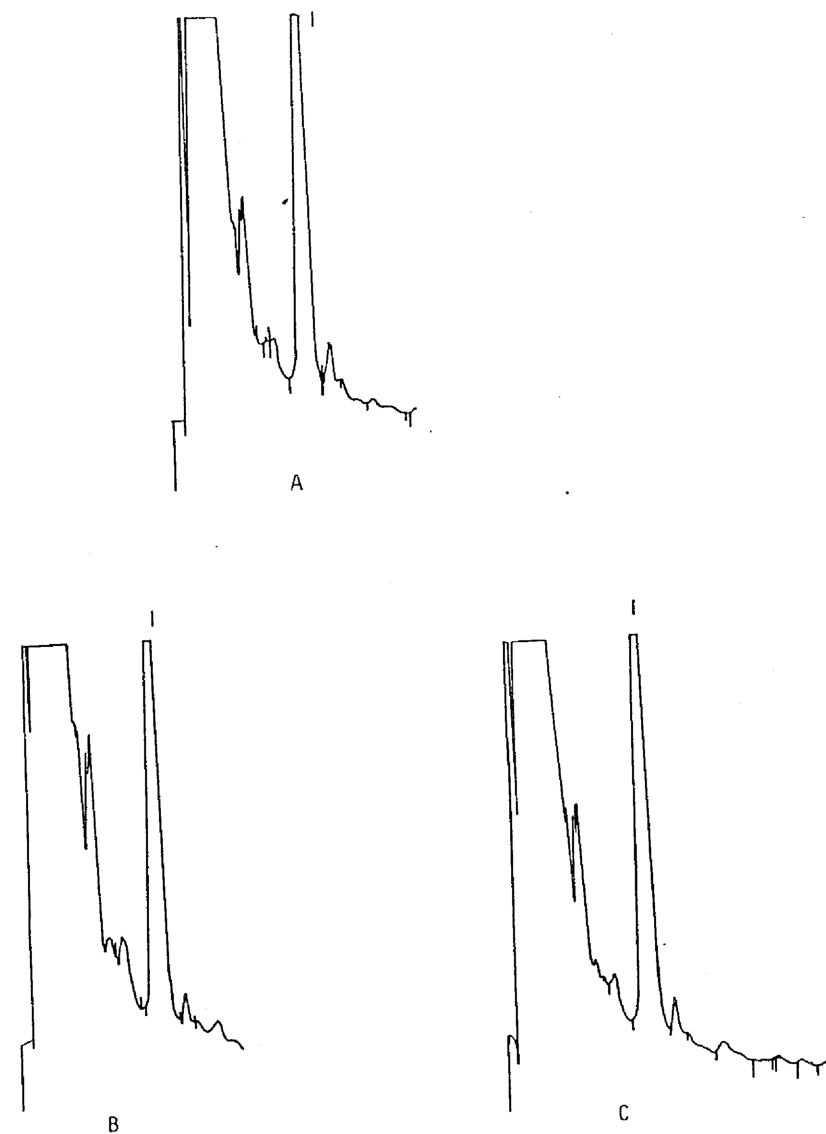
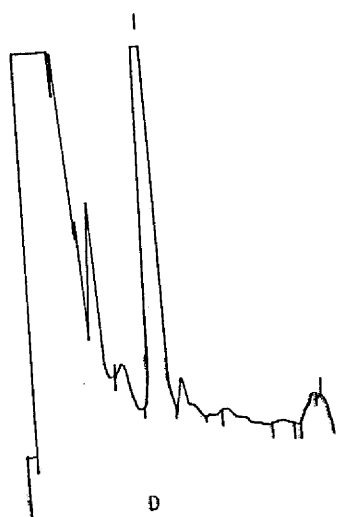
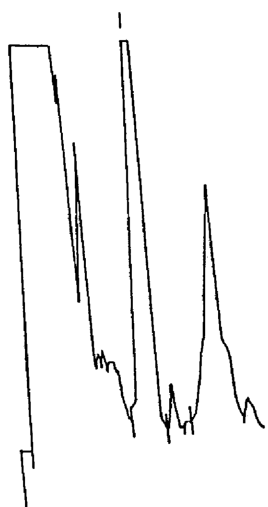



Figure 3.8: HPLC chromatograms of urine extracts containing THC-COOH (I) extracted with isooctane (A), cyclohexane (B) and hexane (C)



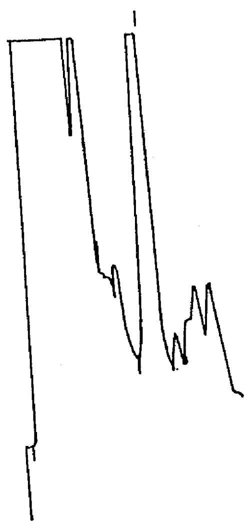
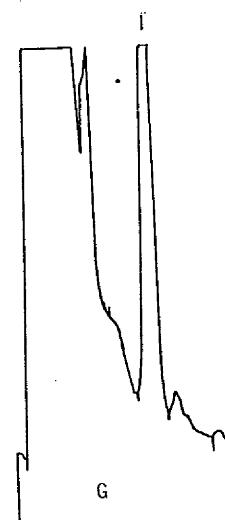




Figure 3.9: HPLC chromatograms of urine extracts containing THC-COOH (I) extracted with ethyl acetate (D), diethyl ether (E) and benzene (F)





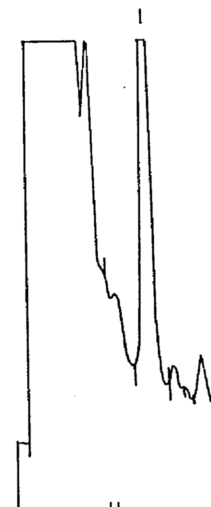




Figure 3.10. HPLC chromatograms of urine extracts containing THC-COOH (I) extracted with chloroform (G) and dichloromethane (H)

highest recovery (mean peak height counts) but also co-extracted larger amounts of endogeneous materials (figure 3.9). The other solvents gave comparable recoveries but chloroform and dichloromethane extracts also had more endogeneous peaks (figure 3.10). Ethyl acetate was therefore chosen as the best solvent from the remaining four because it gave a relatively cleaner extract (figures 3.8 and 3.9).

Binary mixtures of ethyl acetate and isooctane were also evaluated as extraction solvents because would result in smaller P' dilution of ethyl acetate values without altering selectivity. Solutions with smaller P' values should co-extract smaller amounts of endogeneous solutes which are weakly polar. The recovery (mean peak height counts) of THC-COOH with different mixture compositions are given in table 3.5. Figure 3.11 gives the plot of recovery versus percentage of isooctane (% isooctane) in the binary mixture. Figure 3.11 shows that the amount of THC-COOH extracted did not vary significantly when ethyl acetate was diluted with up to 10% isooctane. Furthermore the chromatogram obtained for the extract with 10% isooctane was much cleaner than with neat ethyl acetate (figure 3.12). Therefore a binary mixture of ethyl acetate containing 10% isooctane (v/v) was chosen as the extraction solvent system because of its relatively cleaner extracts and high recovery.

### 3.2.2 Determination of clean-up steps for extraction of THC-COOH

Several clean-up steps were evaluated in order to impart greater selectivity to the extraction method for THC-COOH. Table 3.6 lists these steps together with salting-out effect by the addition of 1.0 g of NaCl and the mean peak height values for THC-COOH extracted. Table 3.6 also gives mean peak height value for extracts not treated with any of the clean-up steps so as to make a better comparison of the recovery. Salting-out with NaCl gave higher recovery as well as better reproducibility. Back-extraction with 0.20 M NaOH resulted in the lowest recovery (6a, table 3.6). In view of this, the original extract which is normally discarded was analysed in order to evaluate the efficiency of the step. The result in table 3.6, 6(b) show that most of the THC-COOH was left in the original extract. These observations can be explained if hydrolysis of ethyl acetate occured during back-extraction with 0.20 M NaOH. The formation of acetic acid would then increase the acidity of the aqueous basic solution thereby reducing its ability to extract THC-COOH out of the original organic extract. The effectiveness of the other 3 clean-up steps were comparable. Figures 3.13 and 3.14 show the chromatograms obtained for the clean-up steps.



Table 3.5: Mean peak height counts of THC-COOH for different mixtures of isooctane and ethyl acetate

| % Isooctane (v/v) | Peak Height Counts | | |
|---|---|---|---|
| | Mean | S.D. | R.S.D. |
| 90 | 248396 | 30623 | 12.33% |
| 75 | 636354 | 52259 | 8.21% |
| 50 | 1011244 | 37142 | 3.67% |
| 30 | 1041005 | 38638 | 3.71% |
| 10 | 1099300 | 101562 | 9.24% |
| 5 | 1001838 | 44770 | 4.47% |
| 0 | 1033030 | 42556 | 4.12% |

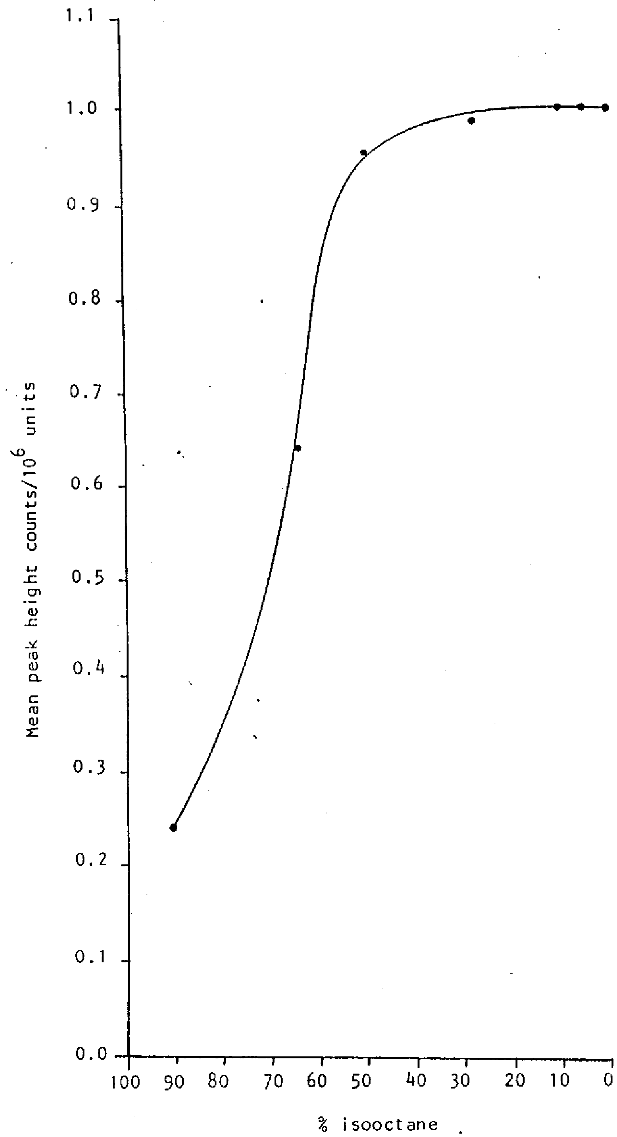



Figure 3.11: Plot of mean peak height counts of THC-COOH versus percentage of isooctane (% isooctane) in a solution of ethyl acetate



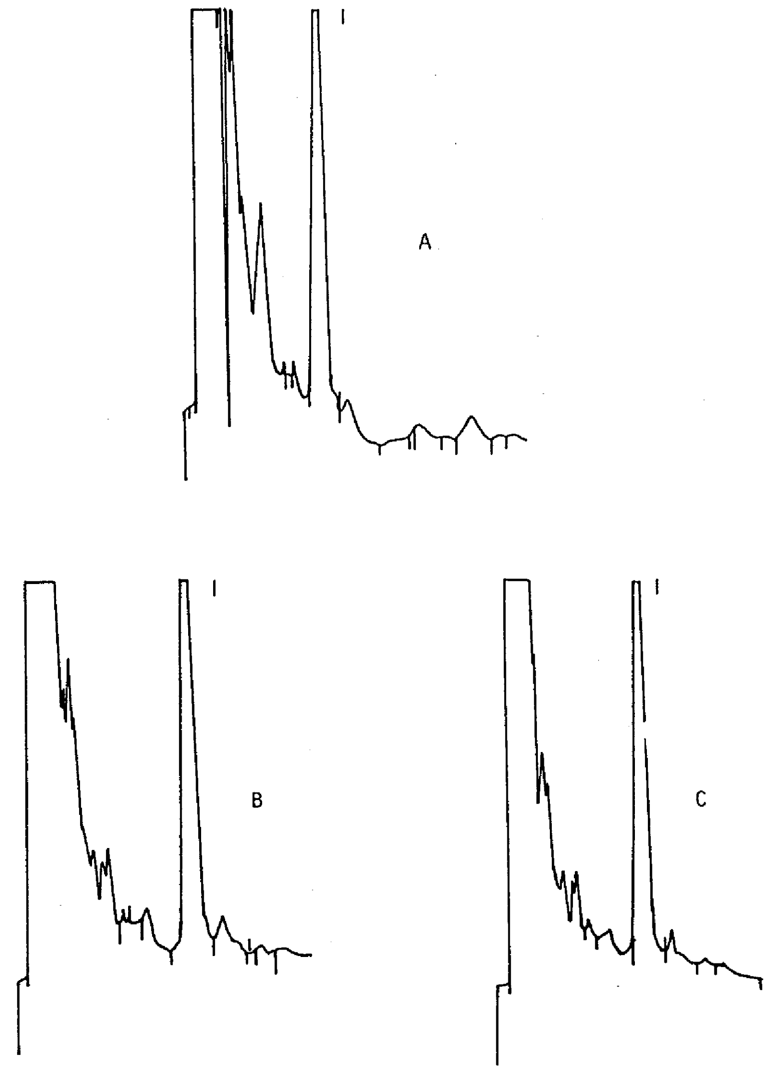



Figure 3.12: HPLC chromatograms of urine extracts of THC-COOH (I) extracted at pH 9 with ethyl acetate containing percentage isooctane of 0% (A), 10% (B) and 30% (C)

Table 3.6: Mean peak height counts of THC-COOH after clean-up and salting-out effects

| Procedure | Peak height counts | | |
|---|---|---|---|
| | Mean | S.D. | R.S.D. |
| 1. No clean-up or salting-out | 1590531 | 63610 | 4.00% |
| 2. Salting-out | 1765207 | 3180 | 0.18% |
| 3. 0.20 M HCl acid washing | 1554121 | 92035 | 5.92% |
| 4. 5% $NaHCO_3$ washing | 1586874 | 65300 | 4.12% |
| 5. pH 7 phosphate buffer washing | 1474643 | 73453 | 4.98% |
| 6. Back-extraction | 273304 | 17149 | 6.29% |
| a. final cleaned-up extract | 273304 | 17149 | 6.29% |
| b. original extract (normally discarded) | 1180172 | 32794 | 2.78% |



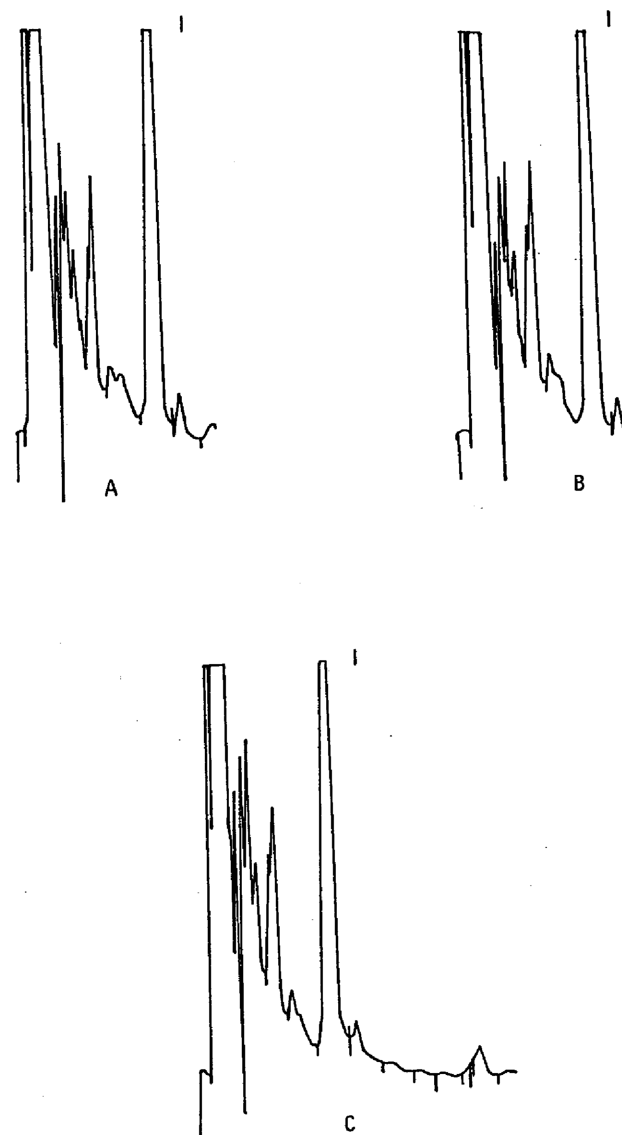



Figure 3.13: HPLC chromatograms of urine extracts of THC-COOH (I) after clean-up with 0.20 M HCl (A), 5% $NaHCO_3$ (B) and pH 7 phosphate buffer (C)

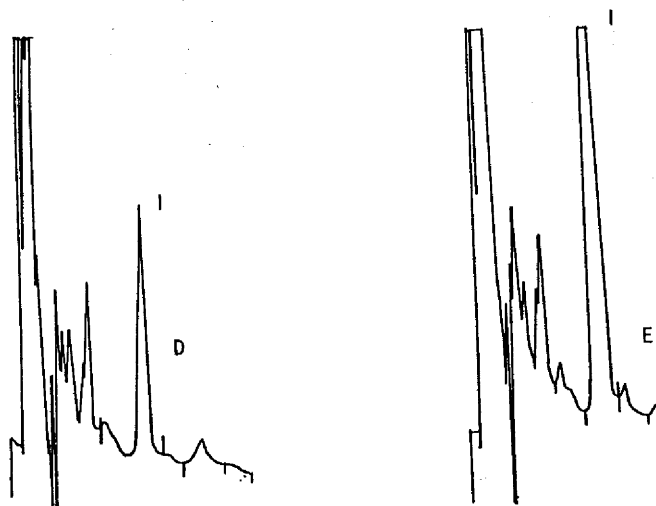



Figure 3.14: HPLC chromatograms of urine extracts of THC-COOH (I) after back-extraction (D) and the normally discarded original extract (E)



The effect of varying the pH and the ionic strength of the aqueous phase used for washing was then evaluated with phosphate buffer. Tables 3.7 and 3.8 summarizes the results and figures 3.15 to 3.18 show the HPLC chromatograms of the resulting extracts. It is clear from tables 3.7 and 3.8 that the optimum buffer for clean-up was 1.00 M phosphate buffer at pH 3.00. Figures 3.15 to 3.16 show that at lower pH more of the endogeneous materials were selectively partitioned into the buffer. This is because most of the endogeneous solutes extracted from the urine at pH 9 would be basic in nature and when mixed with the acidic buffer would ionize and selectively dissolve into the aqueous phase. The effect of varying the molarity of the buffer would result in different salting effects. When the ionic concentration of an aqueous phase is increased, the solubility of a polar solute will also correspondingly increase and this is termed salting-in effect [68]. However beyond a certain ionic concentration the solubility of the solute will decrease with further increase in ionic concentration and this is called salting-out effect. Figure 3.17 and 3.18 show that on increasing the molar concentration of the phosphate buffer at pH 3.00, a salting-in effect for the partitioning of endogeneous solutes into the aqueous buffer phase had occured. However between buffer concentrations of 1.00 and 2.00 M this effect was negligible or appeared to be reversing (salting-out effect).

Horvath [ 85 ] has described salting effects of solutes in terms of the free energy of transfer from gas phase into solution $\Delta G_{solv}$ which is the sum of the energy required to make a suitable cavity in the solvent, $\Delta G_{cav}$, and the free energy change resulting from interactions between the solute and surrounding solvent molecules, $\Delta G_{int}$. $\Delta G_{int}$ is the result of free energies due to van der Waals, $\Delta G_{vdw}$, and electrostatic, $\Delta G_{es}$, interactions. Horvath has shown that the solubility of ionized amines increase with increase in ionic strength, up to about 1.5 m, as a result of increasing electrostatic interactions and hence $\Delta G_{es}$. However at higher ionic strength, or at any ionic strength for unionized solutes, increasing ionic strength resulted in decreasing solubility due to an increase in $\Delta G_{cav}$ with increase in surface tension of the solvent.

The results of the above experiment shows a similar behaviour for the partitioning of the endogenous solutes into the phosphate buffers of increasing molarity. Increased partitioning of the endogenous solutes were observed up to a buffer concentration of 1.00 M but appeared to be reducing at 2.00 M.

Table 3.7: Mean peak height counts of THC-COOH after clean-up with 0.20 M phosphate buffer of varying pH values

| Buffer pH | Peak height counts | | |
| --- | --- | --- | --- |
| | Mean | S.D. | R.S.D. |
| 3.00 | 1998927 | 80494 | 4.03% |
| 4.00 | 2085693 | 75840 | 3.64% |
| 5.00 | 1954637 | 71840 | 3.68% |
| 6.00 | 2004844 | 244115 | 12.18% |

Table 3.8: Mean peak height counts of THC-COOH after clean-up with pH 3.00 phosphate buffer of varying concentrations (M)

| Concentration of buffer (M) | Peak height counts | | |
| --- | --- | --- | --- |
| | Mean | S.D. | R.S.D. |
| 0.05 | 944249 | 82349 | 8.72% |
| 0.10 | 980783 | 18195 | 1.86% |
| 0.20 | 978498 | 120558 | 12.32% |
| 0.50 | 1123542 | 202324 | 18.01% |
| 1.00 | 1089594 | 61957 | 5.69% |
| 2.00 | 1095824 | 29727 | 2.71% |



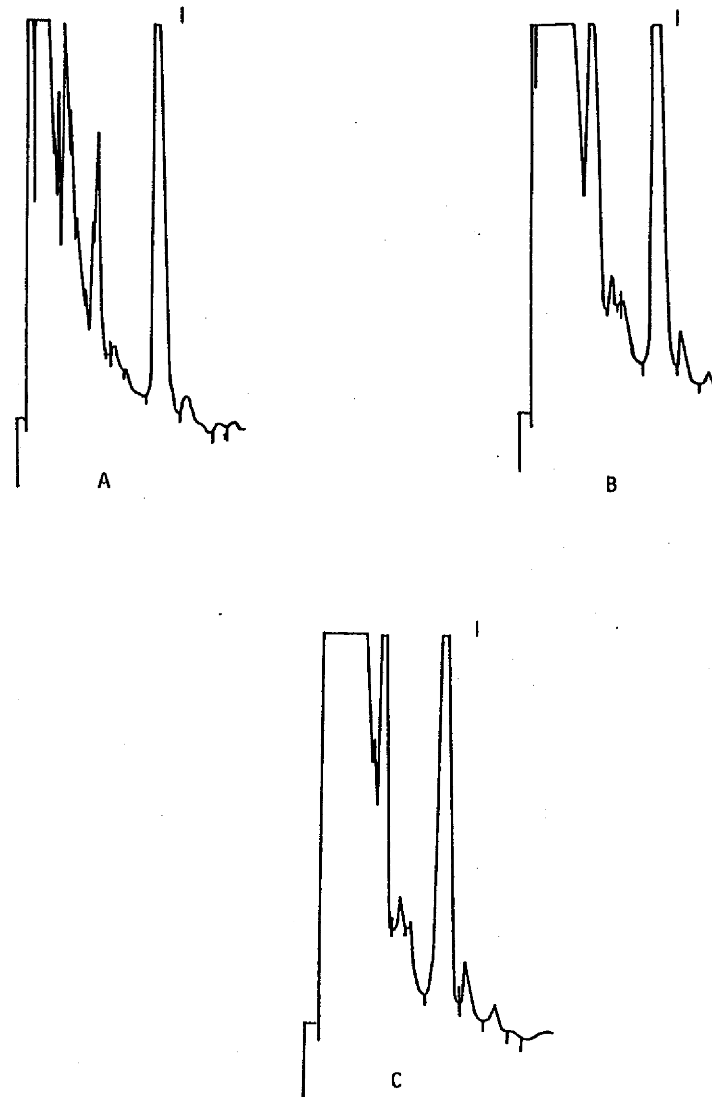



Figure 3.15: HPLC chromatograms of urine extracts of THC-COOH (I) after clean-up with 0.20 M phosphate buffer at pH 3.00 (A), 4.00 (B) and 5.00 (C)

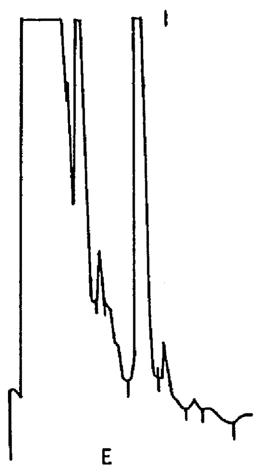
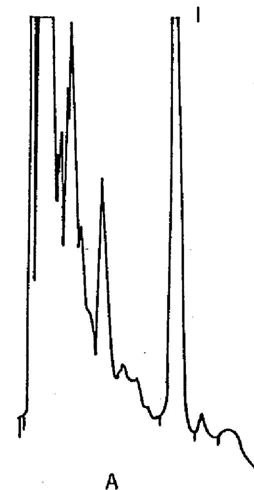
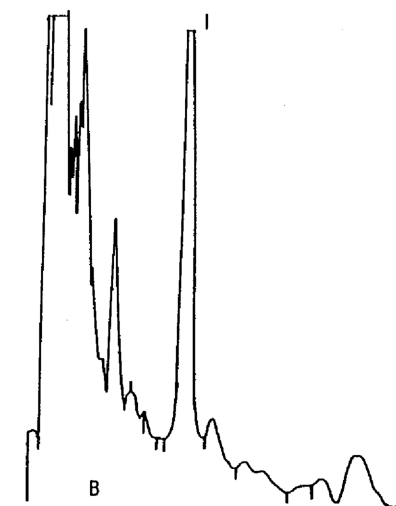



Figure 3.16: HPLC chromatograms of urine extracts of THC-COOH (I) after clean-up with 0.20M phosphate buffer at pH 6.00 (D) and 7.00 (E)





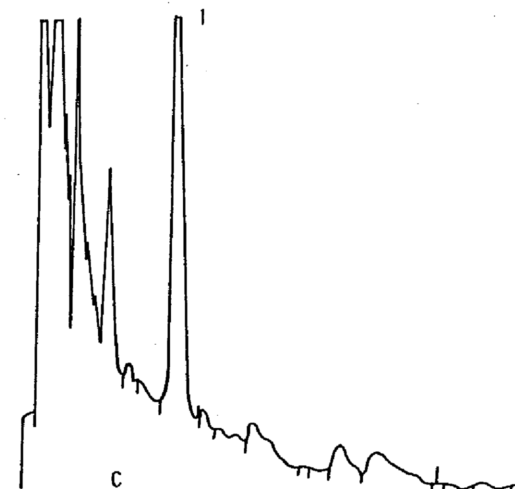




Figure 3.17: HPLC chromatograms of urine extracts of THC-COOH (I) after clean-up with pH 3.00 phosphate buffer at a concentration of 0.05 M (A), 0.10 M (B) and 0.20 M (C)

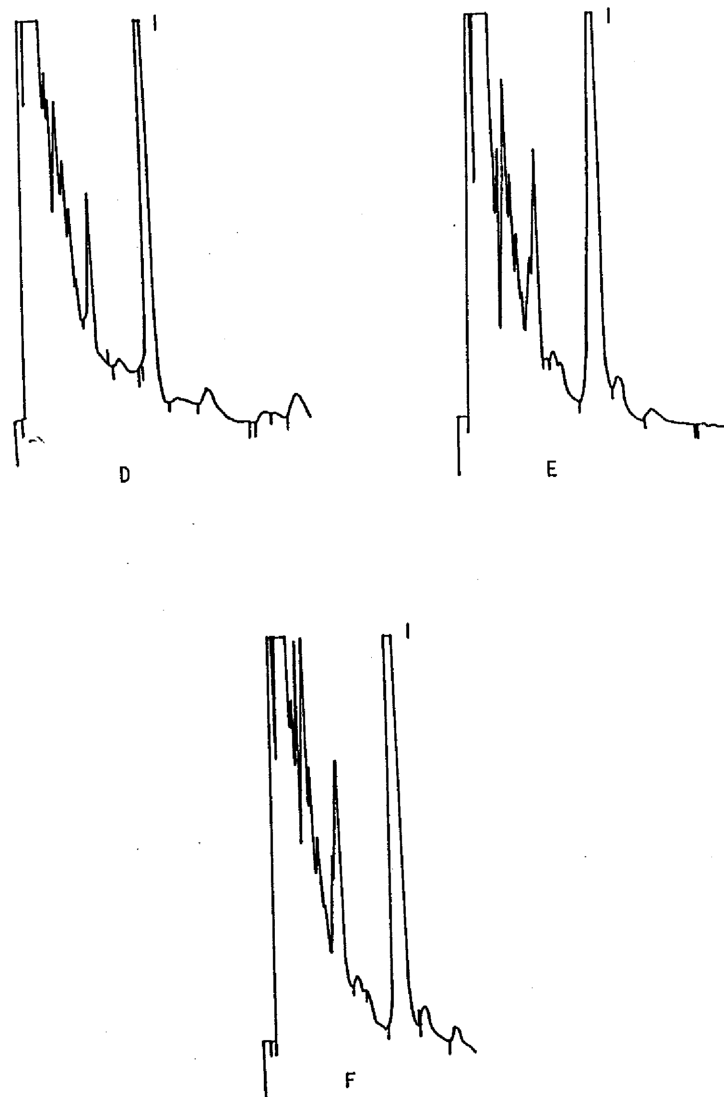



Figure 3.18: HPLC chromatograms of urine extracts of THC-COOH (I) after clean-up with pH 3.00 phosphate buffer at a concentration of 0.50 M (D), 1.00 M (E) and 2.00 M (F)



### 3.2.3 Efficacy of the extraction method for THC-COOH

The optimized extraction method is given in section 2.1.2. 10.00 M NaOH was selected as the hydrolysis agent because previous workers [57] have reported that hydrolysis by a base was faster than enzymic hydrolysis.

Blank urine spiked with increasing concentrations of THC-COOH was extracted and analysed together with methanolic THC-COOH standard solutions of corresponding concentrations by the HPLC system (section 2.1.2). The average peak height counts obtained for the standard solutions and spiked urine extracts are given in tables 3.9 and 3.10. The concentration of THC-COOH versus the corresponding mean peak height counts were plotted for the standard solutions of THC-COOH (figure 3.19) and spiked urine extracts of THC-COOH (figure 3.20) using a HP 85 desk top computer with Standard Pac programme. The coefficients of regression for the standard curve (figure 3.19) and extracted standard curve (figure 3.20) were 1.000 and 0.994 respectively. The percentage recoveries of THC-COOH for the spiked urine concentrations were determined and are given in table 3.11. The precision of the assay was less than 10% R.S.D. for all the spiked urine concentrations. The average percentage of recovery for the whole range of spiked urine concentrations was 81.02% with 5.67% R.S.D.

### 3.2.4 Simultaneous extraction of morphine and THC-COOH

The extraction method in the preceeding section was evaluated for the simultaneous extraction of THC-COOH and morphine which is the main metabolite excreted after consumption of heroin, morphine and opium. Urine spiked with 1000 ng $ml^{-1}$ of morphine and 300 ng $ml^{-1}$ of THC-COOH was extracted and after evaporation of the solvent, the resulting residue was silylated with BSTFA (section 2.5.2). The samples were analysed using the GC-MSD system (section 2.6.2, B) and the resulting total ion chromatogram (figure 3.21) showed presence of silyl derivatives of both morphine and THC-COOH. However the efficacy of the method for extraction of morphine was not investigated because other parameters would also have to be optimized.

## 3.3 High performance liquid chromatographic system for THC-COOH in urine

A mechanism for retention in reversed phase (RP) HPLC was discussed in section 3.1.1. The main factors responsible for retention by a particular RP column are the solvent composition, pH of the aqueous component, eluent flow rate through the column and column temperature. The separation of THC-COOH from urine endogeneous materials on a RP 8 HPLC column was investigated by systematically varying the percentage of acetonitrile (solvent),

Table 3.9: Mean peak height counts for standard solutions of THC-COOH

| THC-COOH standard solution (μg $ml^{-1}$) | Peak height counts | | |
|---|---|---|---|
| | Mean | S.D. | R.S.D. |
| 1.5 | 40856 | 1431 | 3.50% |
| 3.7(5) | 95520 | 4029 | 4.22% |
| 7.5 | 194062 | 7912 | 4.08% |
| 15 | 388037 | 9173 | 2.36% |
| 30 | 763745 | 18114 | 2.37% |

Table 3.10: Mean peak height counts for spiked urine extracts of THC-COOH

| Urine THC-COOH Concentration (ng $ml^{-1}$) | Peak height counts | | |
|---|---|---|---|
| | Mean | S.D. | R.S.D. |
| 50 | 35423 | 71 | 0.20% |
| 125 | 82005 | 3571 | 4.35% |
| 250 | 143032 | 11292 | 7.89% |
| 500 | 317017 | 8181 | 2.58% |
| 1000 | 615795 | 20930 | 3.40% |



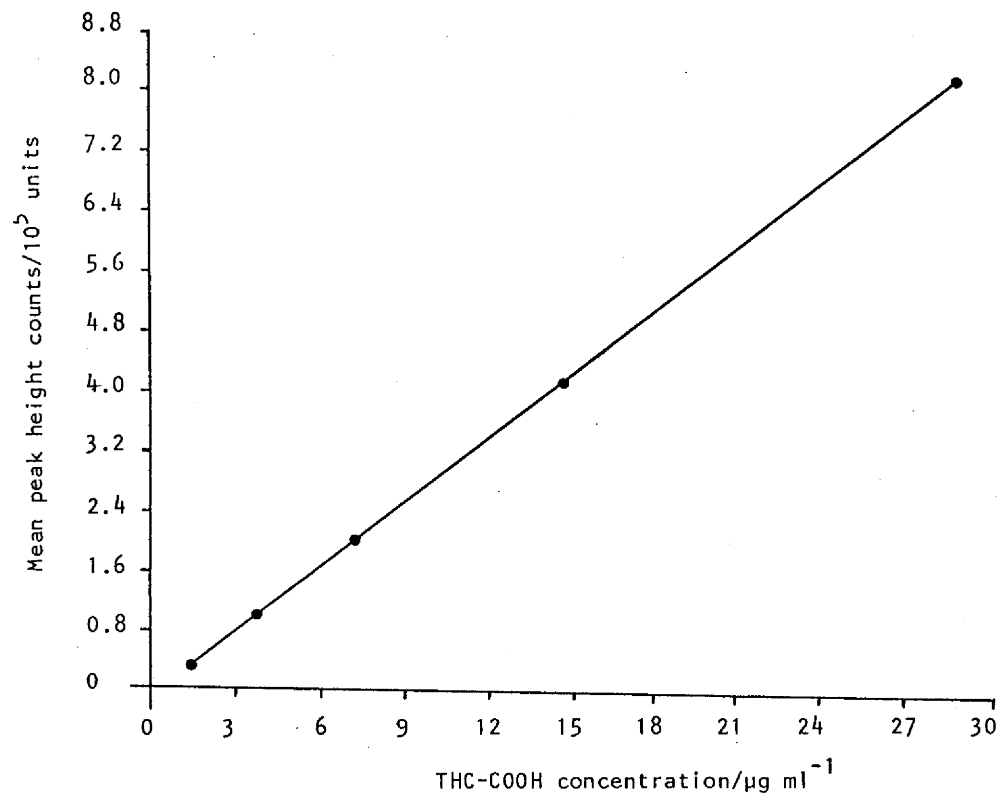



Figure 3.19: Standard curve of methanolic solutions of THC-COOH for the concentration range between 1.5 and 30 μg $ml^{-1}$ with the HPLC system

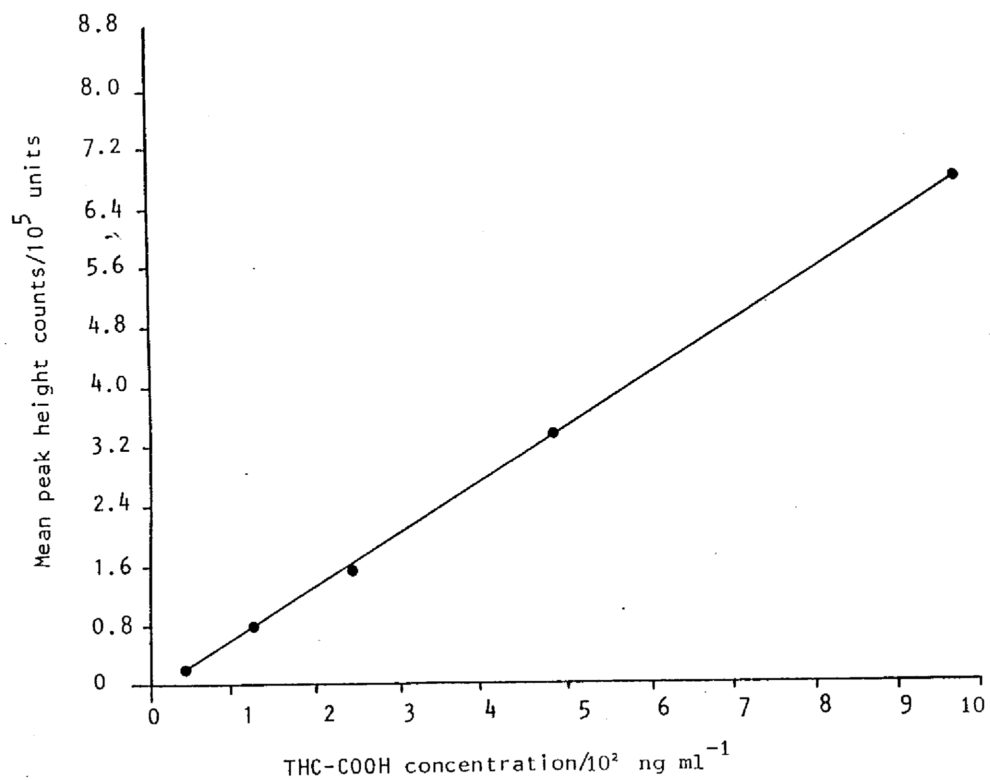



Figure 3.20: Standard curve of extracted THC-COOH from spiked urine with the HPLC system



Table 3.11: Recoveries of THC-COOH for spiked urine samples of varying concentrations

| Spiked urine concentration (ng $ml^{-1}$) | Recovered urine concentration (ng/ml) | | | Percentage recovery |
|---|---|---|---|---|
| | Mean | S.D. | R.S.D. | |
| 50 | 43 | 0.2 | 0.55% | 85.64% |
| 125 | 104 | 4.8 | 4.63% | 83.14% |
| 250 | 184 | 14.8 | 8.08% | 73.53% |
| 500 | 412 | 10.8 | 2.63% | 82.40% |
| 1000 | 804 | 27.4 | 3.42% | 80.37% |

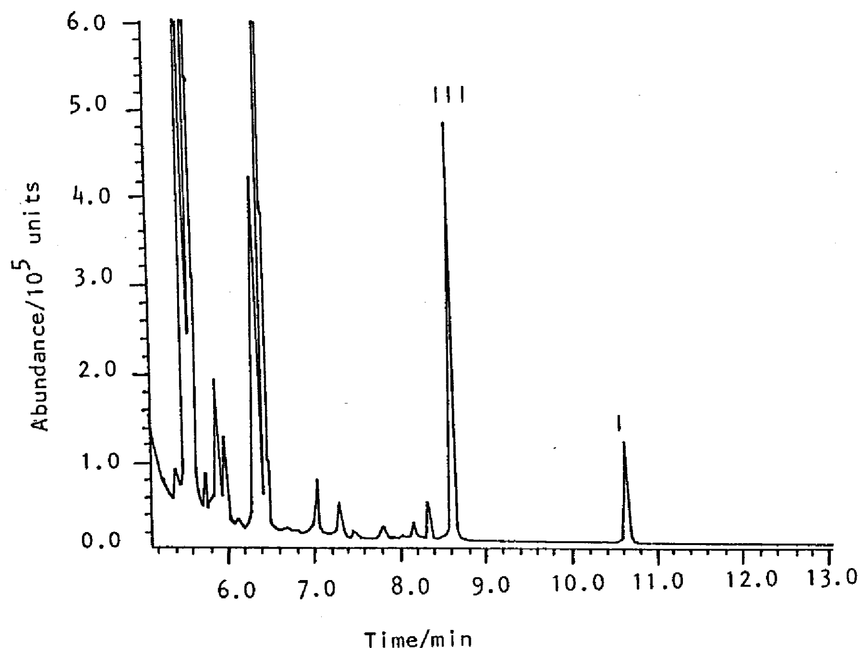



Figure 3.21: Total ion chromatogram of TMS derivatives of THC-COOH (I) and morphine (III) from urine extract with the GC-MSD system



of the aqueous component and flow rate of the mobile phase ile maintaining the column at ambient temperature. Urine racts of pooled urine samples containing THC-COOH (section 1.2) were used in order that the method could be utilized for alysing forensic samples.

### 3.3.1 Optimization of mobile phase

The HPLC system was optimized by first varying the pH of the aqueous component from 2.50 to 4.01 at two different acetonitrile (B) compositions namely 40.0% and 42.5% B. Table 3.12 gives the capacity factor values ($k'$) which were calculated using equation 3.2 for THC-COOH (I) and a closely eluting endogeneous peak (II). Figure 3.22(B) shows a chromatogram of an urine extract containing THC-COOH and peak II which was obtained with 47.5% of acetonitrile and 52.5% water (pH 3.00) at a flow rate of 1.00 ml min$^{-1}$. Selectivity factor values ($\alpha$) were also calculated for the two peaks (table 3.12) using the following equation :

$$\alpha = k'_2/k'_1 \qquad (3.5)$$

where $k'_1$ and $k'_2$ are the capacity factors of THC-COOH and peak II respectively. The $k'$ and $\alpha$ values did not vary significantly with pH at both 40.0% and 42.5% B. The percentage of acetonitrile was then varied from 40.0% to 52.5% with buffer pH 3.00 and the mobile phase flow rate at 1.00 ml min$^{-1}$. Table 3.12 shows that when the percentage of acetonitrile in the mobile phase increased, $\alpha$ slightly increased while $k'$ significantly decreased. This decrease in $k'$ is expected since the increase in acetonitrile composition would result in greater interaction between the mobile phase and the nonpolar moiety of THC-COOH as well as with the nonpolar ligands resulting in greater solubility of THC-COOH in the mobile phase. However, at higher % B, THC-COOH was not well resolved from the early eluting endogeneous peaks (figure 3.22). The optimum acetonitrile composition was 47.5%. The effect of increasing flow rate was also investigated (figure 3.23). High flow rate resulted in poor resolution of THC-COOH from early eluting endogeneous peaks. A flow rate of 1.00 ml min$^{-1}$ gave adequate resolution. The final conditions for the HPLC system were given in section 2.3.2.

### 3.3.2 Method efficacy

The minimum limit of detection for standard methanolic solutions of THC-COOH was 40 ng ml$^{-1}$ which is equivalent to a calculated value of 0.8 ng on-column, with a signal-to-noise (S/N) ratio of 2:1 at detector output signal of 0.005 AUFS. The detector linearity for serially

Table 3.12: Capacity factor ($k'$) and selectivity factor ($\alpha$) values of THC-COOH (I) and an endogeneous peak (II) for varying mobile phase conditions on HPLC

| Buffer pH | Acetonitrile composition (% B) | Capacity factor, $k'$ | | Selectivity factor ($\alpha$) |
|---|---|---|---|---|
| | | THC-COOH (I) | Endogeneous peak (II) | |
| 3.00 | 52.5 | 5.44 | 6.71 | 1.23 |
| | 50.0 | 7.61 | 9.18 | 1.21 |
| | 47.5 | 9.80 | 11.69 | 1.19 |
| | 45.0 | 12.32 | 14.63 | 1.19 |
| | 42.5 | 17.33 | 20.13 | 1.16 |
| | 40.0 | 23.95 | 28.39 | 1.16 |
| 2.50 | 40.0 | 24.42 | 28.51 | 1.18 |
| | 42.5 | 17.13 | 20.22 | 1.18 |
| 3.50 | 40.0 | 24.23 | 28.52 | 1.19 |
| | 42.5 | 16.93 | 20.12 | 1.19 |
| 4.01 | 40.0 | 24.49 | 28.91 | 1.18 |
| | 42.5 | 17.30 | 20.66 | 1.20 |



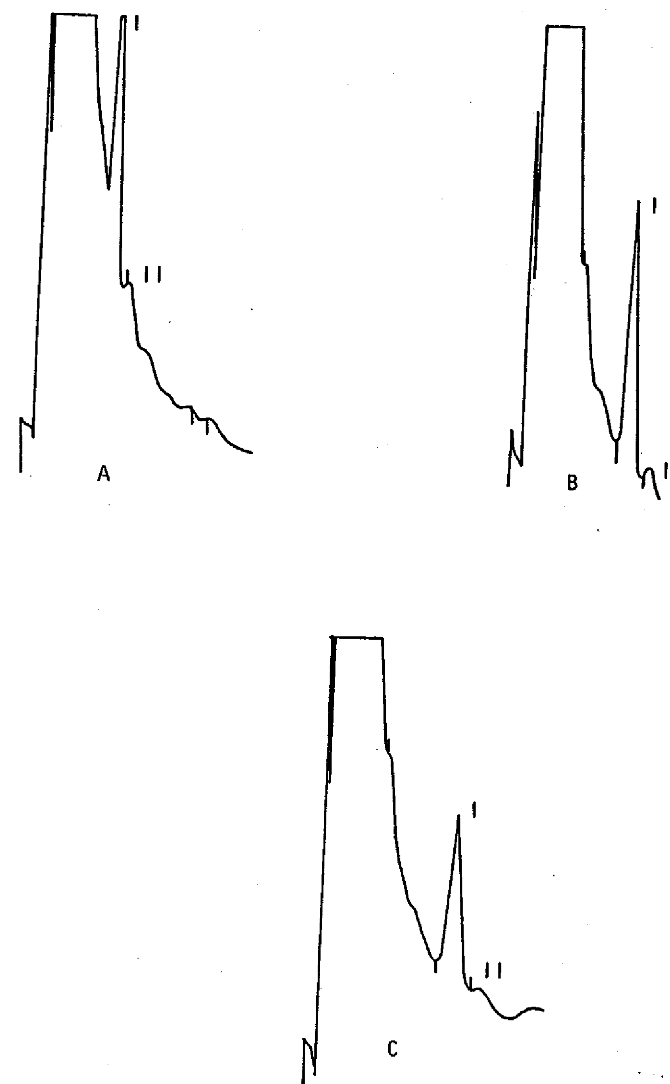



Figure 3.22: HPLC chromatograms of urine extracts of THC-COOH (I) for different percentages of acetonitrile (%B); (A) 50.0, (B) 47.5 and (C) 45.0 %B

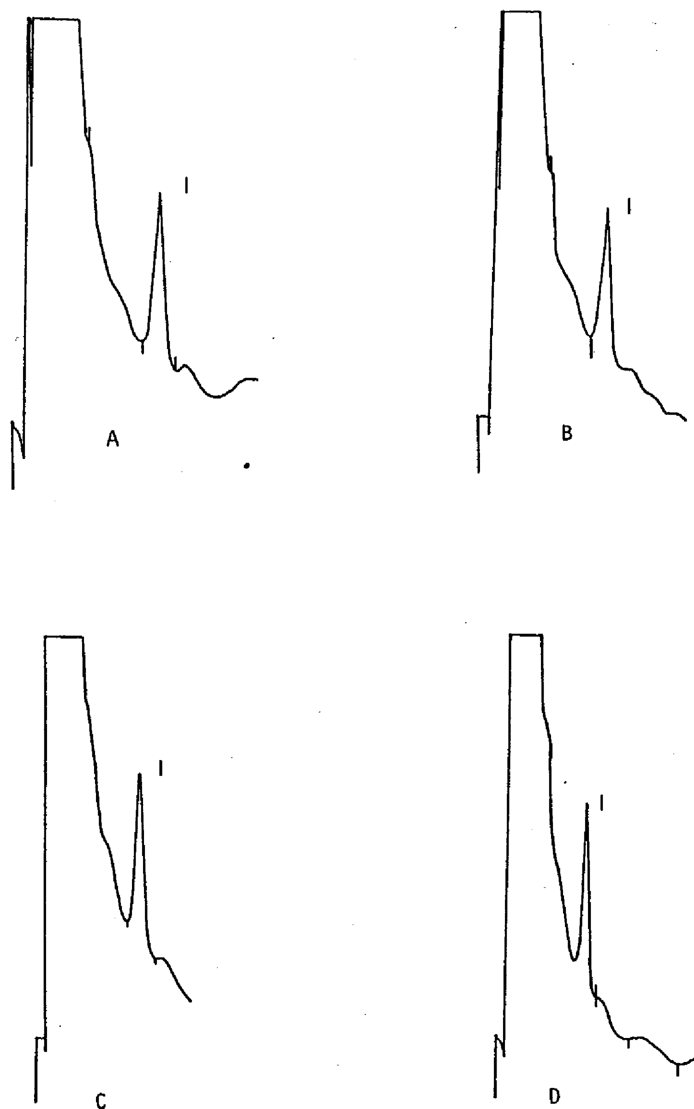



Figure 3.23: HPLC chromatograms of urine extracts of THC-COOH (I) at different mobile phase flow rates; (A) 1.00, (B) 1.25 (C) 1.50 and (D) 1.75 ml $min^{-1}$



diluted THC-COOH concentrations between 40 ng $ml^{-1}$ and 100 µg $ml^{-1}$ (figure 3.24), which also included the concentration range for the standard curve (figure 3.19), gave a coefficient of regression of 0.999. The reproducibility of the system was determined for triplicate injections of a 0.9 µg $ml^{-1}$ methanolic solution of THC-COOH. The within-day precision was 3.58% and the between-day precision for 3 consecutive days was 4.55%.

## 3.4 Thin-layer chromatographic system for THC-COOH in urine

### 3.4.1 Visualisation technique

The diazonium dye reagents which are the most commonly used visualising spray reagents for cannabinoids, are Fast Blue RR [31, 36, 41, 56], Fast Blue BB [38, 46] and Fast Blue B salt [32, 42, 43]. In view of this, an aqueous methanolic solution of Fast Blue B salt (O-dianisidine tetrazolium chloride) was used for the visualisation of the TLC chromatograms.

The colours observed after spraying are the result of coupling reactions between the diazonium reagent and the cannabinoids. The coupling reaction is an electrophilic aromatic substituition reaction at the para position to an activating group which is either a phenol or an amine [91, 92]. The presence of the activating group is necessary to increase the reactivity of the ring for attack by the weak diazonium electrophile.

Maunder [93] reported the preservation of developed colours of TLC chromatograms by spraying with a base. The base improved dye mordanting to acidic sites on the silica gel to yield more intense colours. Maunder also reported improved response speed on spraying with the base. This is probably due to conversion of the phenol group to the more reactive phenoxide ion. In addition, over-spraying of the base was found to produce a bleaching effect of the plate background. Maunder concluded that chromogen basicity, residual solvent on the plate and chromogen dyestuff were the major factors determining colour intensity and stability.

Two methods were considered for the introduction of the base and these were spraying of the base separately and addition of the base to a solution of the dye. The former method was selected because addition of the base to a methanolic solution of the dye not only induces self-coupling of the dye rendering it inoperative but also aqueous solutions of the diazonium dye are prone to cause excessive wetting of the plates leading to spot diffusion [93].

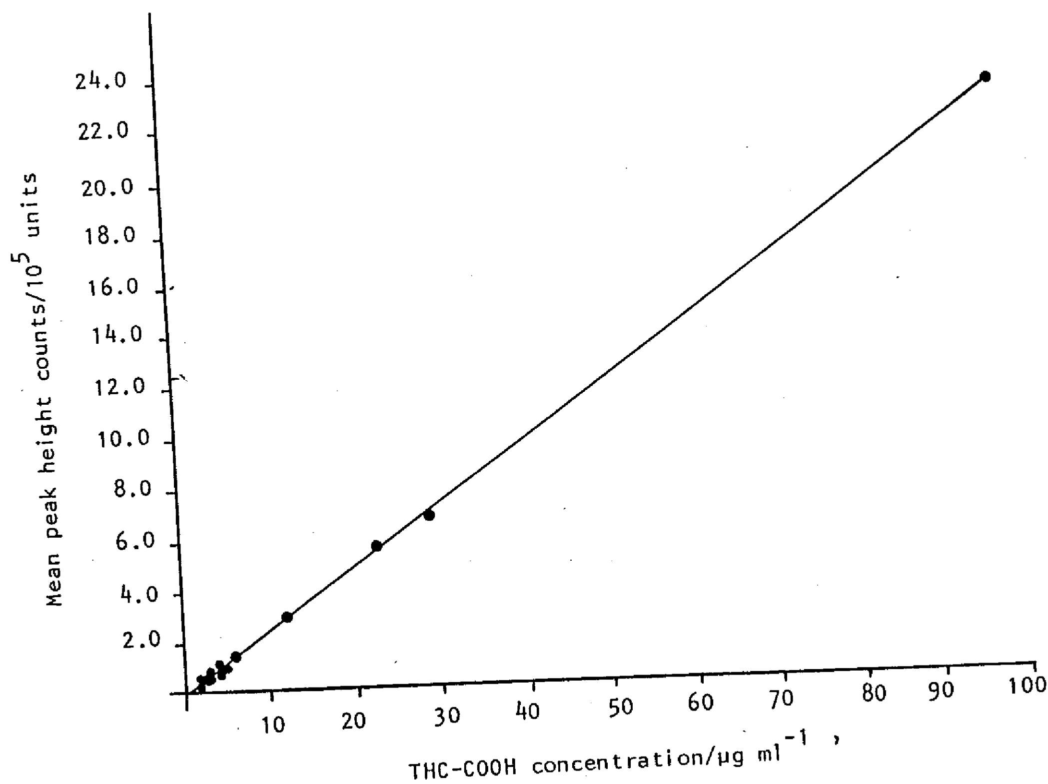



Figure 3.24: Linearity plot of mean peak height counts versus THC-COOH concentrations between 40 ng ml$^{-1}$ and 100 µg ml$^{-1}$



The mordanting and bleaching effects of the base were evaluated by spraying two developed TLC plates, one with 10 ml of diethylamine (DEA) followed by 10 ml of Fast Blue B salt (FBB) solution (section 2.4.1 and 2.4.2) and the other with only 10 ml of FBB solution. The chromatogram sprayed with DEA prior to FBB yielded more intense colours and the plate background was not coloured. The chromatogram sprayed with FBB not only gave less intense colours but also a brownish plate background which became darker with time (photodarkening). The plates were also scanned between 400 and 600 nm with a densitometric scanner and plots of the signal (absorbance units) versus wavelength are given in figure 3.25. The signals for the plate sprayed with DEA prior to FBB (curves A and B) were greater than those sprayed with FBB only (curves C and D) probably because the clearer plate background in the former case yielded lower background measurements.

The effect of different amounts of the residual chromatographic solvent on the plate was evaluated by spraying developed plates with DEA followed by FBB 30, 60, 90, 120, 150 and 180 s after removal from the developing tanks. The optimum spraying time was between 90 and 120 s. The occurence of the optimum spraying time has been explained by assuming that the residual solvent bound to the active sites of the support allowed more effective competition between dye - THC-COOH complex and water molecules on the hydrophilic silica gel [93]. Too much water on the silica surface however could result in precipitation rather than adsorption of the dye complex. The optimum volumes of DEA and FBB solutions for spraying a 10 x 10 cm plate were 10 ml of each. A larger volume of DEA did not result in significant changes to the chromatogram when observed visually. Larger volume of FBB solution yielded more intense spots but the excess water from the FBB solution caused wetting of the plates with consequent spot diffusion. Repeated spraying of DEA and FBB also resulted in spot diffusion with slight darkening of the plate background.

Spraying with other organic bases, namely triethylamine, butylamine and anisidine, resulted in darkening of the plate background. Dilution of anisidine with DEA also resulted in darkening of the plate background from a brownish black to a dark black colour. Dilution of butylamine with DEA also caused darkening of the plate background although to a much lesser extent. However the colours of the spots were not significantly more intense than those obtained by spraying with DEA. Thus the method adopted consisted of spraying 10 ml of DEA followed by 10 ml of FBB, 90 s after removal of the plate from the developing tank.

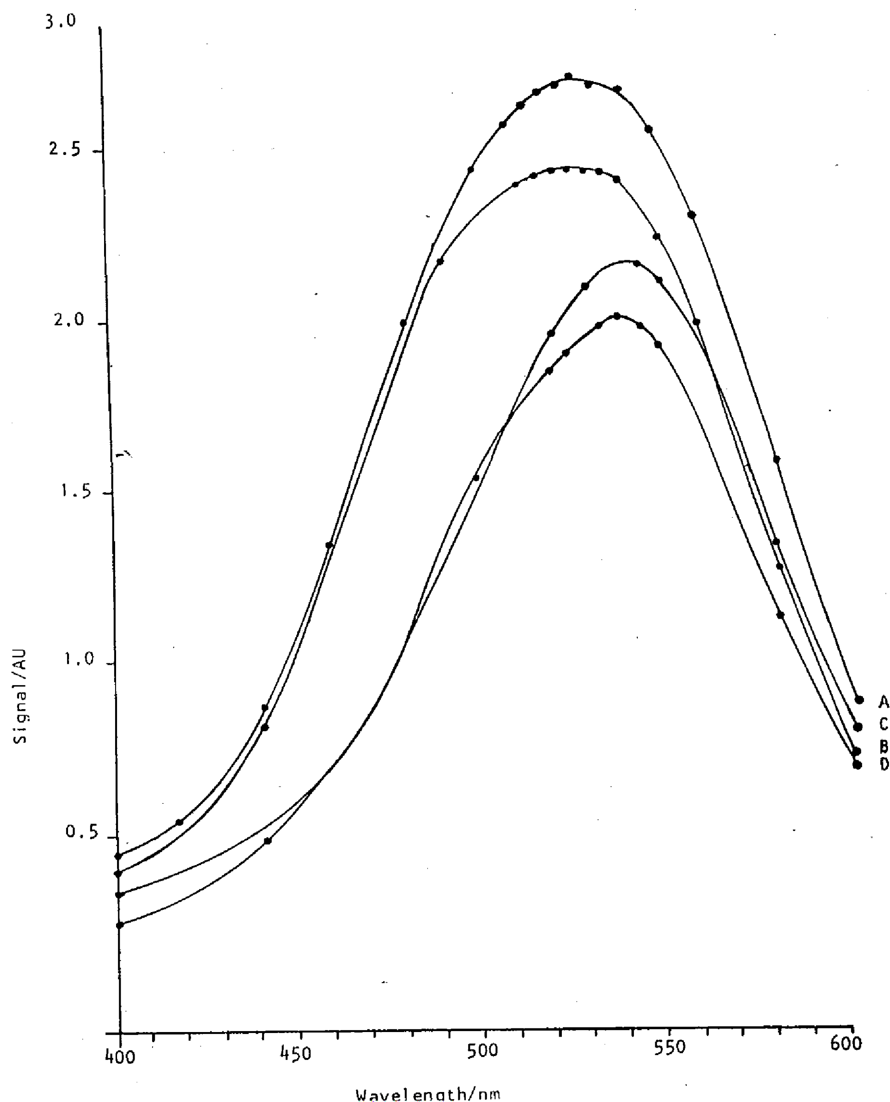



Figure 3.25: Plot of signal (absorbance units, AU) versus wavelength (nm) for 1.5 ml (B and D) and 3.0 ml (A and C) urine extracts; A and B sprayed with DEA and FBB; C and D sprayed with FBB



#### 3.4.2 Mobile phase solvent systems

Various TLC solvent systems have been reported for THC-COOH (table 1.4). The seven TLC systems in table 3.13 were selected for evaluation to resolve THC-COOH from urinary endogeneous materials as well as from THC, CBN and CBD which are cannabinoids with closely related structures. Table 3.13 gives the $R_f$ values for THC-COOH and the other cannabinoids for the seven systems and figure 3.26a to 3.26d shows the photographs of the developed chromatograms. Solvent system 1 not only gave the best resolution of THC-COOH from endogeneous materials and from THC, CBN and CBD but in addition no streaking of the spot was observed. The colours with the optimized spraying technique was red, reddish orange, purple and orange for THC-COOH, THC, CBN and CBD respectively.

#### 3.4.3 Method efficacy

The minimum detectable limits when observed visually were 75 and 37.5 ng of THC-COOH when sprayed with FBB solution and with DEA followed by FBB solution respectively. The standard curve for serially diluted THC-COOH concentrations between 18.7(5) and 3000 ng versus densitometric spot area counts in figure 3.27 gave a coefficient of regression of 0.998. Therefore the optimized system can be used for quantitative screening of a solution of THC-COOH with a limit of detection of 18.7(5) ng.

### 3.5 Derivatization procedures for gas chromatographic analysis of THC-COOH

The two most extensively used derivatization procedures for THC-COOH are methylation [20, 23, 42, 44, 48, 50, 54, 59, 60, 73, 74] and trimethylsilylation [20, 21, 23, 49, 50, 54, 73]. These two procedures were optimized and their relative responses compared on GC-FID and GC-MSD systems.

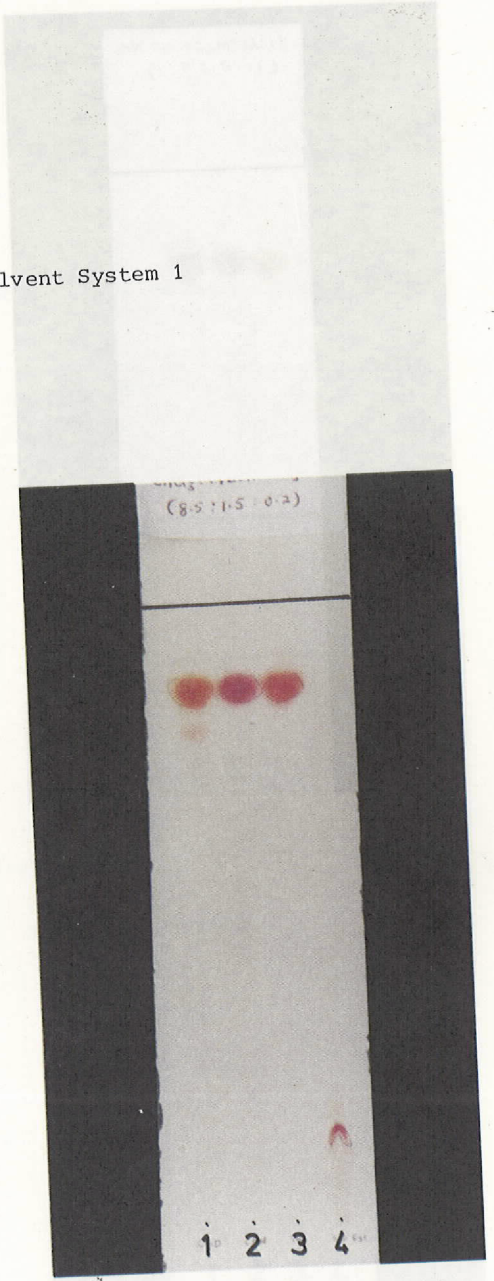
#### 3.5.1 Optimization of methylation conditions

Methylating reagents which have been reported in literature are methyl iodide [23, 42, 44, 50, 59, 60], ethereal diazomethane [20, 54, 73], boron trifluoride - methanol [48] and dimethylformamide dimethylacetal [74]. Diazomethane is considered to be both explosive and carcinogenic [94]. Methyl iodide is however the most widely used reagent and methylation is usually carried out in the presence of tetramethylammonium hydroxide (TMAH) as catalyst and dimethylsulfoxide (DMSO) as the reaction medium [44]. Therefore this procedure was selected and optimized for maximum yield of methylated THC-COOH.

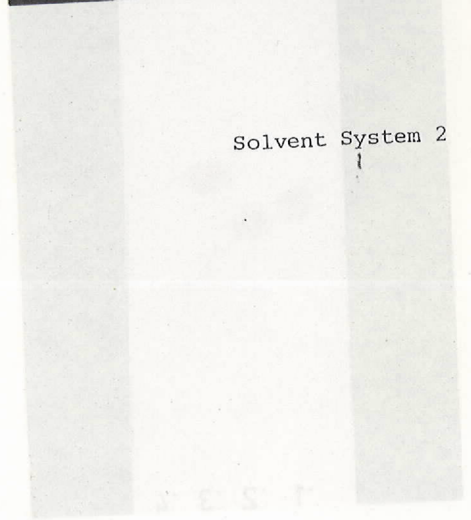
Optimum reaction time and temperature were determined with 1000 ng amounts of THC-COOH residue. The

Table 3.13: Experimental and reported $R_f$ values for 7 selected TLC systems

| Solvent system | Reference | Reported $R_f$ value for THC-COOH | Experimental $R_f$ values THC-COOH | THC | CBN | CBD |
|---|---|---|---|---|---|---|
| 1. $CHCl_3:CH_3OH:NH_3$ (70:30:2) | 42 | approx. 0.25 | 0.44 | 0.96 | 0.96 | 0.95 |
| 2. $CHCl_3:CH_3OH:NH_3$ (85:15:2) | 41 | 0.25 - 0.38 | 0.13 | 0.86 | 0.85 | 0.85 |
| 3. $EtOAc:CH_3OH:H_2O:NH_3$ (12:5:0.5:1) | 31, 36, 56 | 0.43 - 0.50 | 0.25 | 0.83 | 0.83 | 0.83 |
| 4. $Hex:Acet:CH_3COOH$ (70:30:1) | 32 | 0.38 | 0.31 | 0.48 | 0.41 | 0.44 |
| 5. $Hept:Et_2O:CH_3COOH$ (80:10:4) | 61 | 0.32 - 0.34 | 0.08 | 0.32 | 0.29 | 0.33 |
| 6. $Hept:Acet:CH_3COOH$ (70:30:1) | 52 | NA | 0.12 | 0.32 | 0.31 | 0.36 |
| 7. DD i. $Acet:CHCl_3:TEA$ (80:20:1) | | | | | | |
| ii. Pet. ether:$Et_2O$:$CH_3COOH$ (5:5:0.15) | 43 | approx. 0.1 | 0.12 | 0.82 | 0.79 | 0.83 |

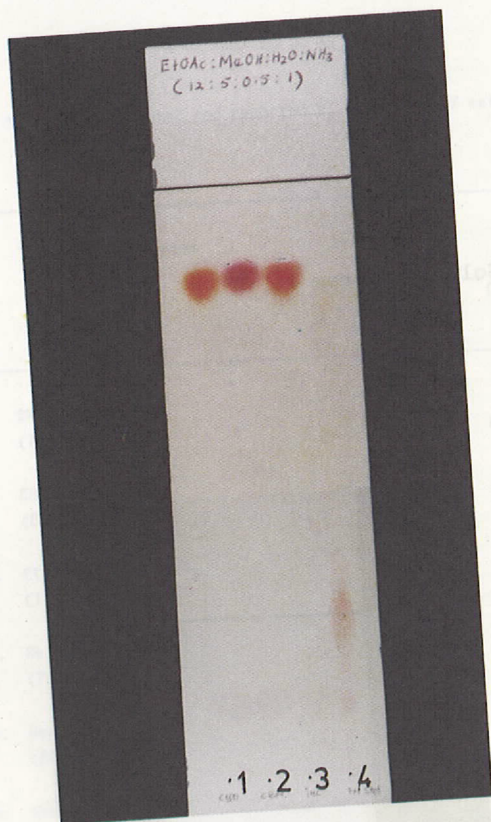



Solvent System 1

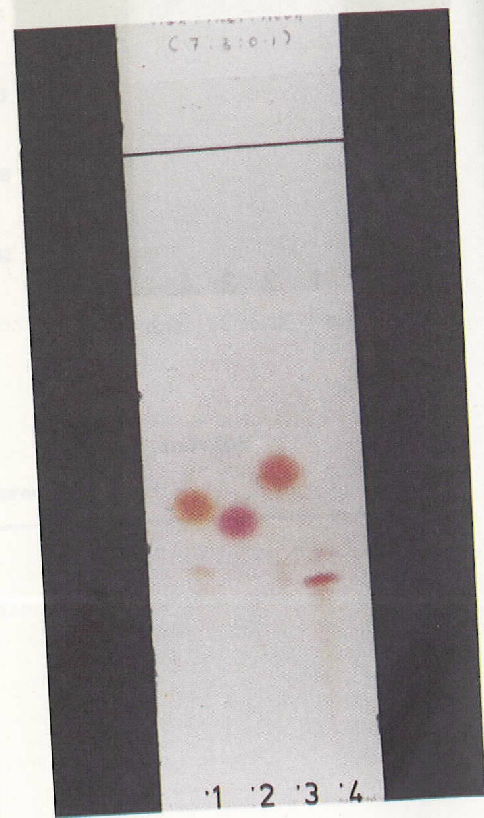


Solvent System 2

Figure 3.26a: Photographs of TLC chromatograms developed with solvent systems 1 and 2 (1 = CBD, 2 = CBN, 3 = THC, 4 = THC-COOH extract)



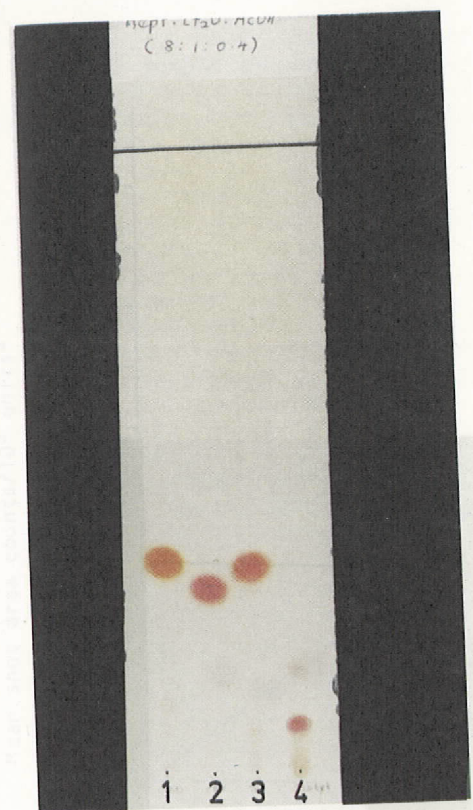

Solvent System 3



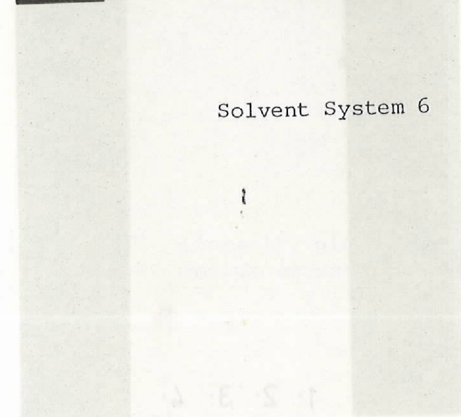

Solvent System 4

Figure 3.26b: Photographs of TLC chromatograms developed with solvent systems 3 and 4.
(1 = CBD, 2 = CBN, 3 = THC, 4 = THC-COOH extract)






Solvent System 5



Solvent System 6

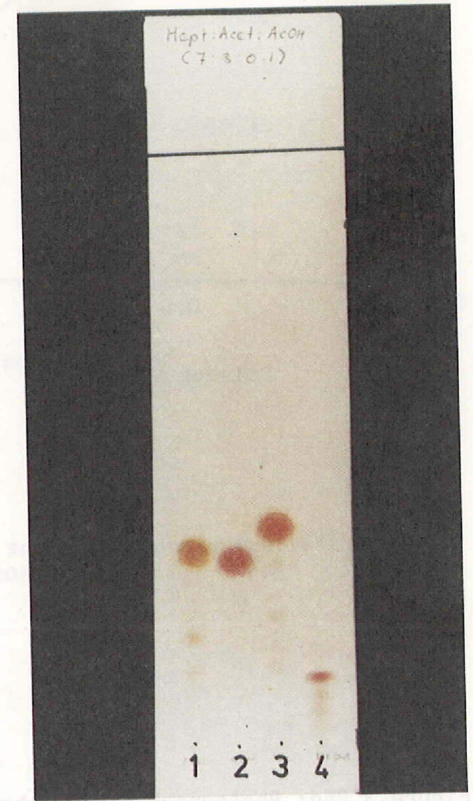



Figure 3.26c: Photographs of TLC chromatograms developed with solvent systems 5 and 6.
(1 = CBD, 2 = CBN, 3 = THC, 4 = THC-COOH extract)

Solvent System 7

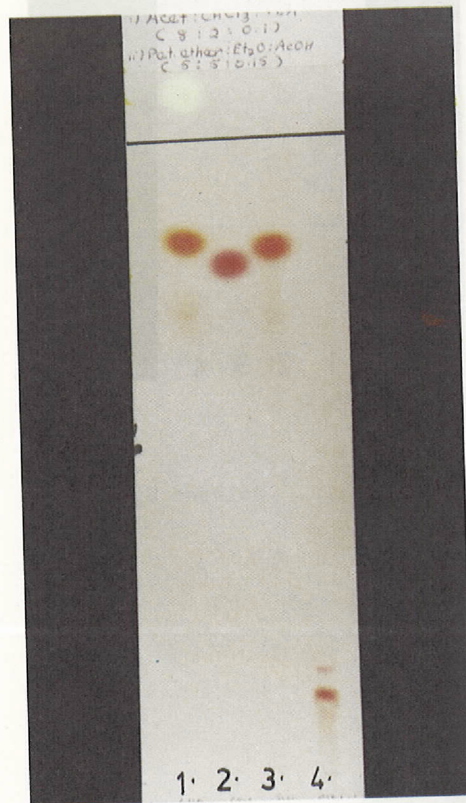



Figure 3.26d: Photograph of TLC chromatogram developed with solvent system 7.
(1 = CBD, 2 = CBN, 3 = THC, 4 = THC-COOH extract)



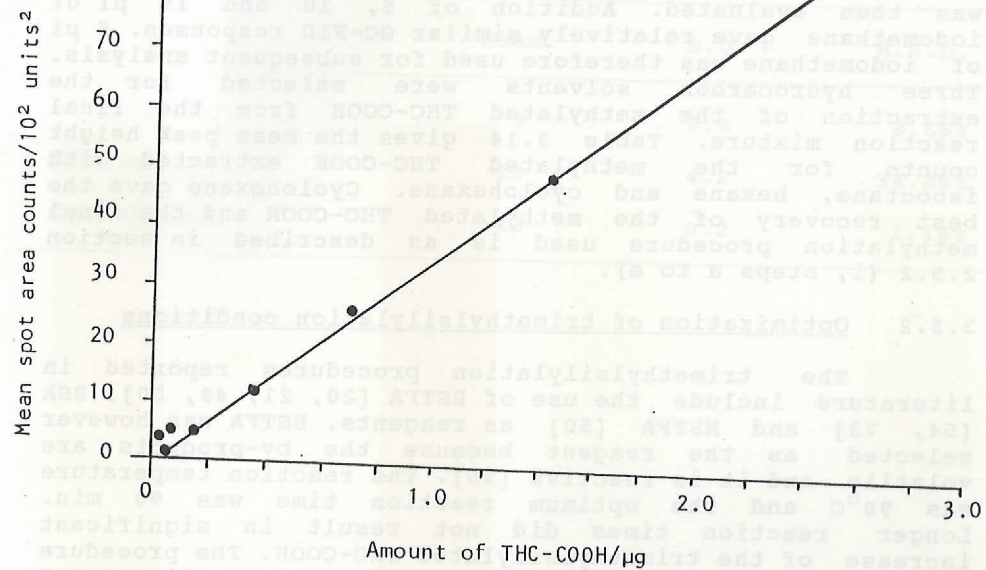



Figure 3.27: Linearity plot of mean spot area counts (units²) versus amount of THC-COOH

derivatization yield, represented by GC-FID peak height response, was the same for all the reaction times at room temperature. However at 60°C, a reaction time of 5 min yielded the methylated product while no significant response was obtained for reaction times of 15, 30 and 60 min. This indicated possible breakdown of one of the reagents used. A reaction time of 5 min at room temperature (25°C) were therefore chosen as the optimum reaction conditions which were the same as those reported in literature [23, 42, 44, 50, 59, 60]. The optimum concentration of iodomethane required for optimum yield was then evaluated. Addition of 5, 10 and 15 µl of iodomethane gave relatively similar GC-FID responses. 5 µl of iodomethane was therefore used for subsequent analysis. Three hydrocarbon solvents were selected for the extraction of the methylated THC-COOH from the final reaction mixture. Table 3.14 gives the mean peak height counts for the methylated THC-COOH extracted with isooctane, hexane and cyclohexane. Cyclohexane gave the best recovery of the methylated THC-COOH and the final methylation procedure used is as described in section 2.5.2 (i, steps a to e).

### 3.5.2 Optimization of trimethylsilylation conditions

The trimethylsilylation procedures reported in literature include the use of BSTFA [20, 21, 49, 50], BSA [54, 73] and MSTFA [50] as reagents. BSTFA was however selected as the reagent because the by-products are volatile and it is reactive [95]. The reaction temperature was 90°C and the optimum reaction time was 90 min. Longer reaction times did not result in significant increase of the trimethylsilylated THC-COOH. The procedure used for trimethylsilylation is given in section 2.5.2 (ii).

### 3.5.3 Optimum derivatization procedure for GC-MSD analysis of THC-COOH

The relative response factors of the methylation and trimethylsilylation procedures were compared with the GC-MSD and the GC-FID systems. Table 3.15 gives the mean peak area counts after derivatization of 20 µg $ml^{-1}$ THC-COOH solutions. Methylated THC-COOH gave better response than the trimethylsilylated THC-COOH on the GC-FID. However, trimethylsilylated THC-COOH gave larger responses on the GC-MSD as well as cleaner chromatograms for both the GC systems. Figures 3.28 and 3.29 show the chromatograms obtained with the GC-FID and GC-MSD systems respectively. Therefore the trimethylsilylation procedure was used for subsequent analyses with the GC-MSD system.



Table 3.14: Mean peak height counts for methylated THC-COOH extracted from the reaction mixture

| Extraction solvent | Peak height counts | | |
|---|---|---|---|
| | Mean | S.D. | R.S.D. |
| Isooctane | 9 | 0.4 | 4.66% |
| Hexane | 12 | 4.2 | 34.86% |
| Cyclohexane | 13 | 0.2 | 1.16% |

Table 3.15: Peak area counts for 20 µg $ml^{-1}$ solution of methylated and trimethylsilylated THC-COOH

| Derivatization procedure | Response (peak area counts) | |
|---|---|---|
| | GC-FID | GC-MSD |
| Methylation | 224 | $2.2 \times 10^6$ |
| Trimethylsilylation | 47 | $9.6 \times 10^6$ |

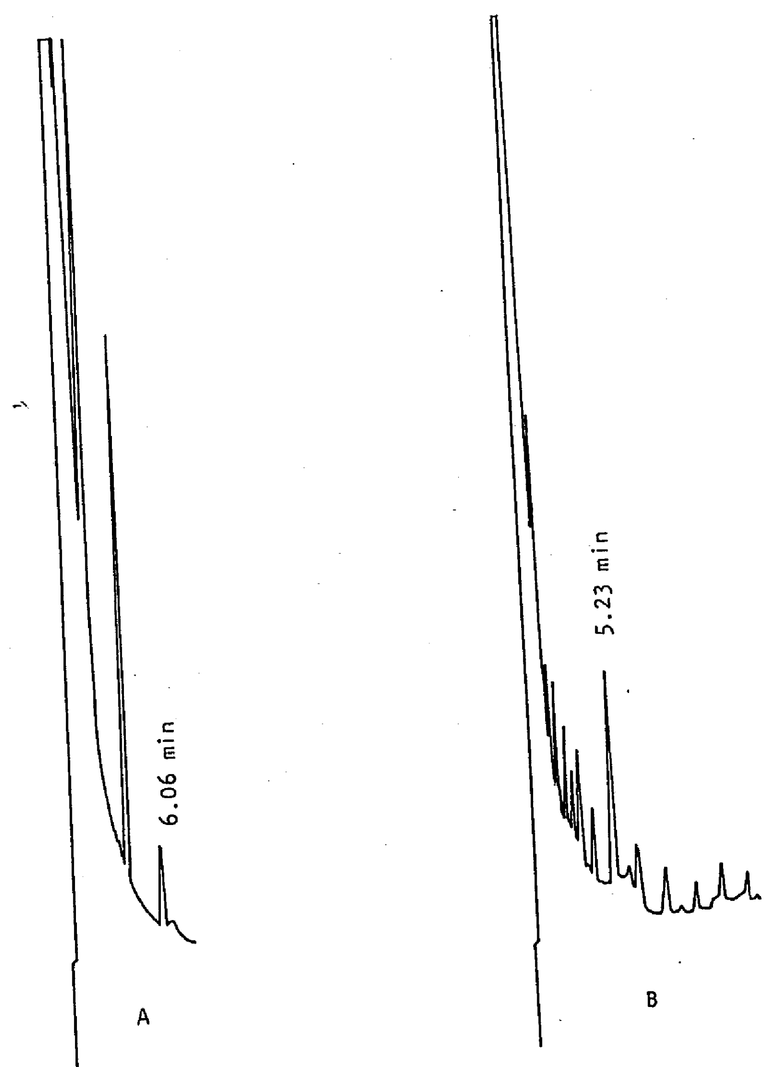



Figure 3.28: GC-FID chromatograms of (A) trimethylsilylated and (B) methylated THC-COOH



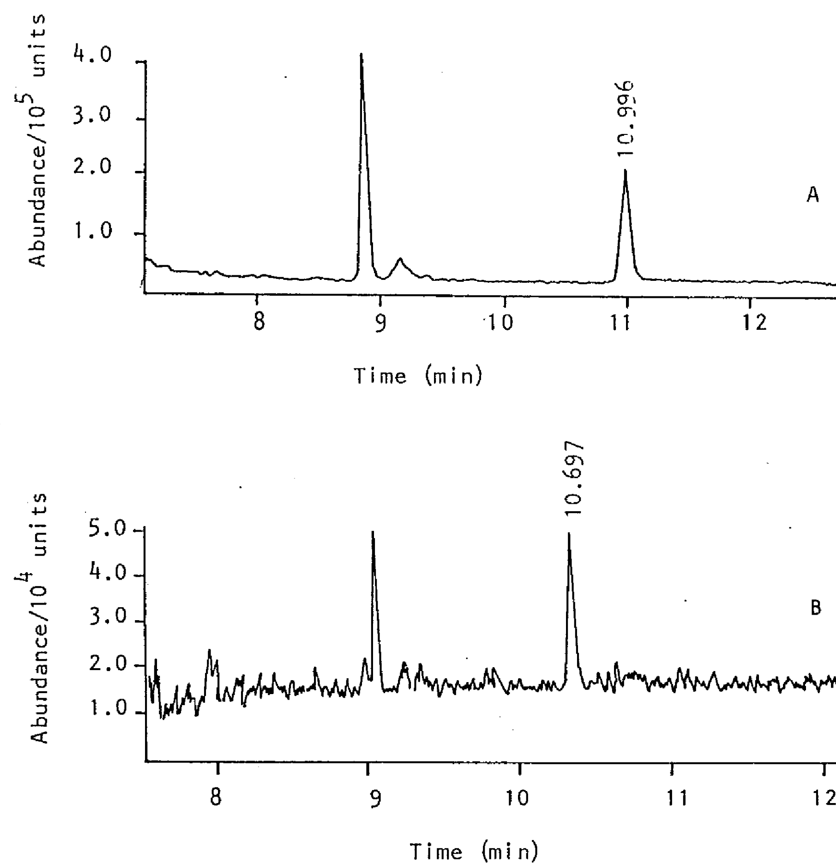



Figure 3.29: GC-MSD total ion chromatograms of (A) trimethylsilylated and (B) methylated THC-COOH

## 3.6 Gas chromatographic systems for THC-COOH in urine

Both GC-FID and GC-MSD systems were used for the analysis of THC-COOH. The GC-FID system was used for quantitation during optimization of the derivatization procedures (section 3.5) whereas the GC-MSD system was developed for confirmation of the presence of THC-COOH.

### 3.6.1 GC-FID system for THC-COOH

The final optimized GC-FID conditions are given in section 2.6.2(A). The temperature was ramped up after elution of the methylated THC-COOH in order to elute strongly retained extraneous peaks in the derivatization mixture (figure 3.28, B). The retention times for the methylated and trimethylsilylated derivatives of THC-COOH were 5.23 and 6.06 min respectively (figure 3.28).

### 3.6.2 GC-MSD system for confirmation of THC-COOH in urine

The GC-MSD system, which requires a capillary column for the GC, was chosen as the method for confirmation of THC-COOH due to its sensitivity and specificity. Capillary column stationary phases which have been used for the analysis of THC-COOH in urine include 100% methyl silicone gum [23, 48], 5% phenylmethyl silicone gum [46, 47, 55, 58, 59, 60, 62, 73] and 50% phenylmethyl silicone [75]. Since the most widely used stationary phase was 5% phenylmethyl silicone, a SE 54 column was selected. The splitless technique with a quartz splitless liner of 2 mm I.D. was used for introduction of the sample onto the column. During injection of the sample, the injection port purge gas was diverted in order to maintain a gas flow rate equivalent to the column flow rate through the liner so as to prevent splitting of the sample. The purge was however activated after a fixed time interval, the splitless time, in order to flush out excess solvent in the liner which otherwise would result in large solvent tailing [96]. To avoid sample mass discrimination during injection, the sample was injected at a rate 1.0 ul $s^{-1}$ and the syringe needle was left in the injector port until the purge gas was activated. However due to the slow rate of sample introduction onto the GC column, a sample reconcentration step was necessary to avoid sample band broadening. Two methods, both the "solvent effect" [73] and cold trapping [48] have been used for this purpose in the case of THC-COOH.

Different volumes of the derivatized THC-COOH mixture were injected to minimize dilution of the sample by the carrier gas. The analyses were carried out with an initial injector and transfer line at 290°C and an initial column temperature of 65°C for 1 min followed by ramping to 280°C at 70°C $min^{-1}$ and held at the final



temperature for 10 min. From table 3.16, injection volumes of 3 and 4 µl were both found to give satisfactorily reproducible results. However an injection volume of 3 µl was chosen for all subsequent analyses as too much solvent can cause striping of the liquid phase off the column.

Grob [97] and Freeman [96] have described the experimental conditions for an effective "solvent effect". Partition of solutes between the gaseous mobile phase and liquid stationary phase of the GC column can be represented by the equation

$$K_D = k\beta \qquad (3.6)$$

where $K_D$, $k$ and $\beta$ are the partition constant, partition ratio and phase ratio respectively. The partition ratio and phase ratio are given by

$$k = W_L/W_G \qquad (3.7)$$

$$\beta = V_G/V_L \qquad (3.8)$$

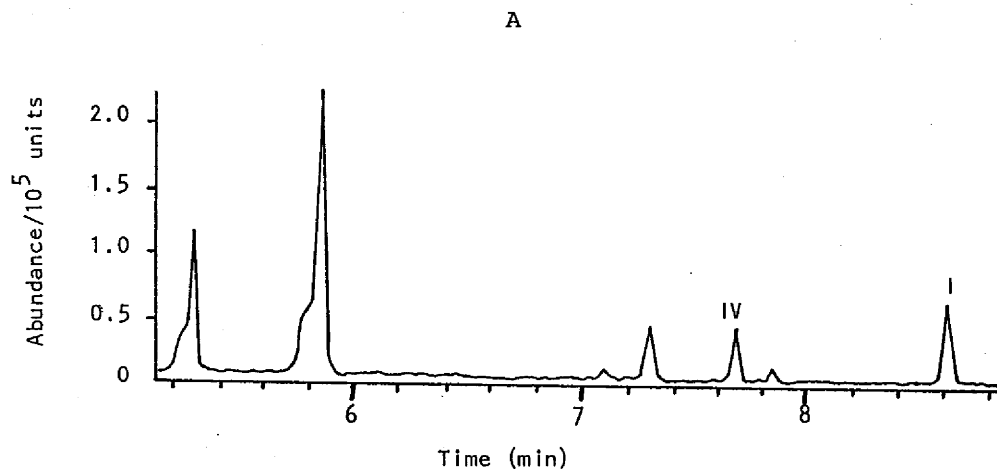
where $W_L$ and $W_G$ refer to the weights of the sample in the liquid and gas phases and $V_G$ and $V_L$ refer to the volumes of the gaseous and liquid phases. During "solvent effect" the low initial column temperature causes condensation of the solvent thereby decreasing the value of $\beta$. This results in an increase in the value of k which implies greater retention. Since the volume of condensed solvent increases in the direction of migration of the sample, the leading edge of the sample band will undergo greater retention than the preceeding end resulting in compression of the sample band. Various solvents were used at initial oven temperatures which were selected to bring about condensation of the solvents on column. These were methanol and n-hexane at 50°C, acetonitrile at 65°C, n-heptane at 80°C and toluene at 80° and 90°C. The best effect was obtained with toluene at 90°C (figure 3.30, B) whereas toluene with initial oven temperature of 80°C resulted in distortion of early eluting extraneous peaks (figure 3.30, A). The trimethylsilylated THC-COOH peak eluted at 8.6 min with an initial oven temperature of 90°C. The splitless time was varied from 0.60 to 1.30 min in order to determine the optimum time for maximum transfer of the vapourised sample onto the column. Mirex was added as an internal standard to THC-COOH prior to derivatization. The peak area ratios for THC-COOH to mirex obtained for different splitless times over two days are given in table 3.17. The results indicate variable precisions for the two days at a particular splitless

**Table 3.16: Mean peak area counts for different volumes of the trimethylsilylated THC-COOH reaction mixture with the GC-MSD system**

| Injection volume (µl) | Peak area counts (X $10^6$ units$^2$) | | |
|---|---|---|---|
| | Mean | S.D. | R.S.D. |
| 2 | 17.5 | 4.10 | 23.38% |
| 3 | 29.4 | 3.42 | 11.63% |
| 4 | 51.6 | 4.85 | 9.34% |



A




B

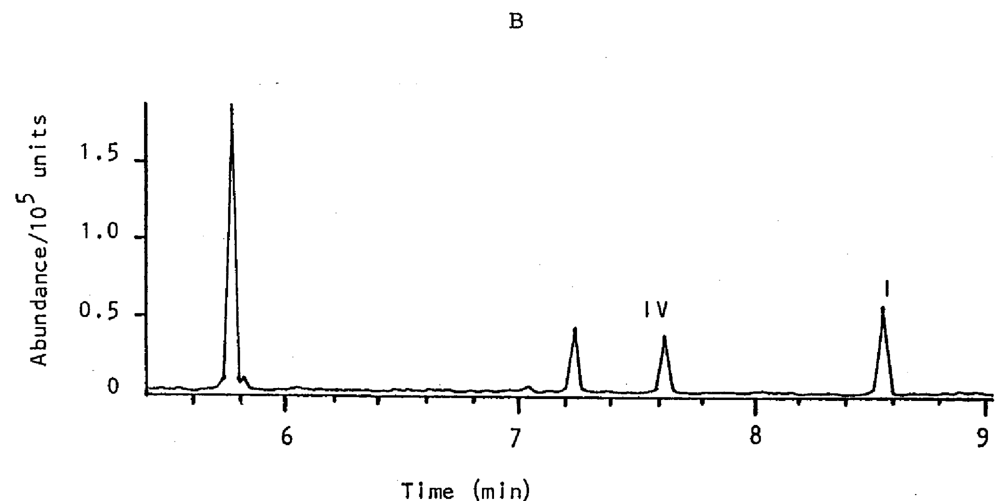



Figure 3.30: GC-MSD total ion chromatograms of trimethylsilylated THC-COOH (I) and mirex (IV) to illustrate "solvent effect" with toluene at initial oven temperatures of 80°C (A) and 90°C (B)

Table 3.17: Peak area ratios of THC-COOH to mirex for different splitless times

| Splitless time (min) | Peak area ratio | | | | | |
|---|---|---|---|---|---|---|
| | Day 1 | | | Day 2 | | |
| | Mean | S.D. | R.S.D. | Mean | S.D. | R.S.D. |
| 0.30 | 1.8 | 0.24 | 13.79% | 2.4 | 0.19 | 8.18% |
| 0.40 | 1.8 | 0.24 | 13.60% | 2.5 | 0.44 | 17.27% |
| 0.50 | 1.9 | 0.15 | 7.69% | 2.5 | 0.14 | 5.85% |
| 0.60 | 2.2 | 0.14 | 6.33% | 2.2 | 0.16 | 7.62% |
| 0.80 | 2.3 | 0.26 | 11.26% | 2.4 | 0.02 | 0.93% |



time. Table 3.18 shows that the peak area counts obtained for THC-COOH and mirex were also not reproducible. The splitless time used for subsequent analysis was 0.8 min since it would allow more THC-COOH to be transferred to the column before activation of injector port purge.

The effect of cold trapping was evaluated by varying the initial column temperature from 120$^{o}$ to 200$^{o}$C by 10$^{o}$C increments. The precision of the peak area counts was between 7.24% and 27.09%.

The lack of reproducibility of the peak area responses obtained with "solvent effect" and cold trapping was eventually found to be due to fluctuations in the carrier gas flow rate at the open-split interface. Flow rate measurements with a soap film flowmeter showed that the interface purge flow rate varied between 0.21 and 0.31 ml min$^{-1}$ with a mean flow rate of 0.25 ml min$^{-1}$ and precision of 13.73% at a column temperature of 280$^{o}$C. A possible reason for this fluctuation is the reduction in column flow rate with increase in column temperature. The flow fluctuations at the interface would bring about variable splitting of separated sample bands prior to their introduction into the MS source chamber through the restrictor. The final GC conditions and the MSD parameters for both the scan and SIM modes are given in section 2.6.2.

### 3.6.3 Limits of detection for THC-COOH with the MSD system

The minimum limit of detection of trimethylsilylated THC-COOH derivative in the scan mode was 2.5 µg ml$^{-1}$ with a S/N ratio of 3:1 (figure 3.31). However the minimum limit for confirmatory identification based on the probability based matching (PBM) of the spectra with that of the standard trimethysilylated THC-COOH spectrum was 5 µg ml$^{-1}$ which gave a match quality of 81%. At 2.5 µg ml$^{-1}$ no value for match quality was obtained. The PBM matches were done with the aid of the PBM programme supplied with the GC-MSD system software.

The minimum detection limit for trimethylsilylated THC-COOH by the SIM mode was 25 ng ml$^{-1}$ (figure 3.32). The spectra obtained in the SIM mode could not be used for PBM matches since a minimum of 10 mass peaks are required for a match to be made. However the SIM mode can be used for confirmatory identification by using labelled isotopes of THC-COOH [48, 50, 59].

### 3.6.4 Mass spectra of the THC-COOH derivatives

The mass spectra obtained from the total ion chromatogram in figure 3.29 for the 1-O-trimethylsilyl

Table 3.18: Peak area counts for THC-COOH and mirex for different splitless times

| Compound | Splittless time (min) | Peak area counts (X $10^6$ units$^2$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Day 1 | | | Day 2 | | |
| | | Mean | S.D. | R.S.D. | Mean | S.D. | R.S.D. |
| THC-COOH | 0.30 | 3.3 | 0.55 | 13.79% | 4.4 | 0.97 | 20.80% |
| | 0.40 | 3.0 | 0.29 | 9.51% | 4.4 | 0.25 | 5.52% |
| | 0.50 | 4.1 | 0.78 | 19.02% | 5.3 | 0.15 | 2.80% |
| | 0.60 | 4.0 | 0.35 | 8.91% | 4.3 | 0.53 | 12.29% |
| | 0.80 | 4.1 | 0.70 | 17.15% | 7.1 | 0.67 | 9.41% |
| Mirex | 0.30 | 1.9 | 0.32 | 16.85% | 1.8 | 0.25 | 13.61% |
| | 0.40 | 1.8 | 0.31 | 17.58% | 1.8 | 0.37 | 20.56% |
| | 0.50 | 2.1 | 0.28 | 13.25% | 2.1 | 0.13 | 5.92% |
| | 0.60 | 1.8 | 0.09 | 5.22% | 2.0 | 0.15 | 7.35% |
| | 0.80 | 1.8 | 0.24 | 13.35% | 2.4 | 0.02 | 0.93% |



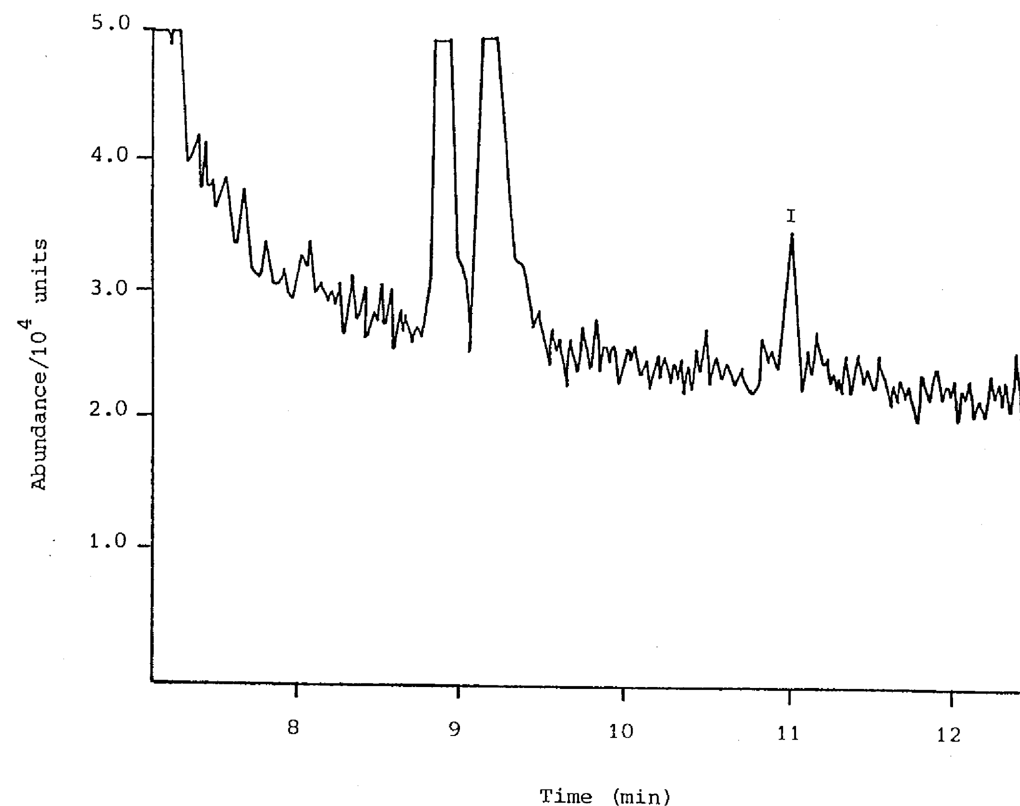



Figure 3.31: GC-MSD total ion chromatogram of 2.5 μg $ml^{-1}$ of THC-COOH (I) after trimethylsilylation in the scan mode

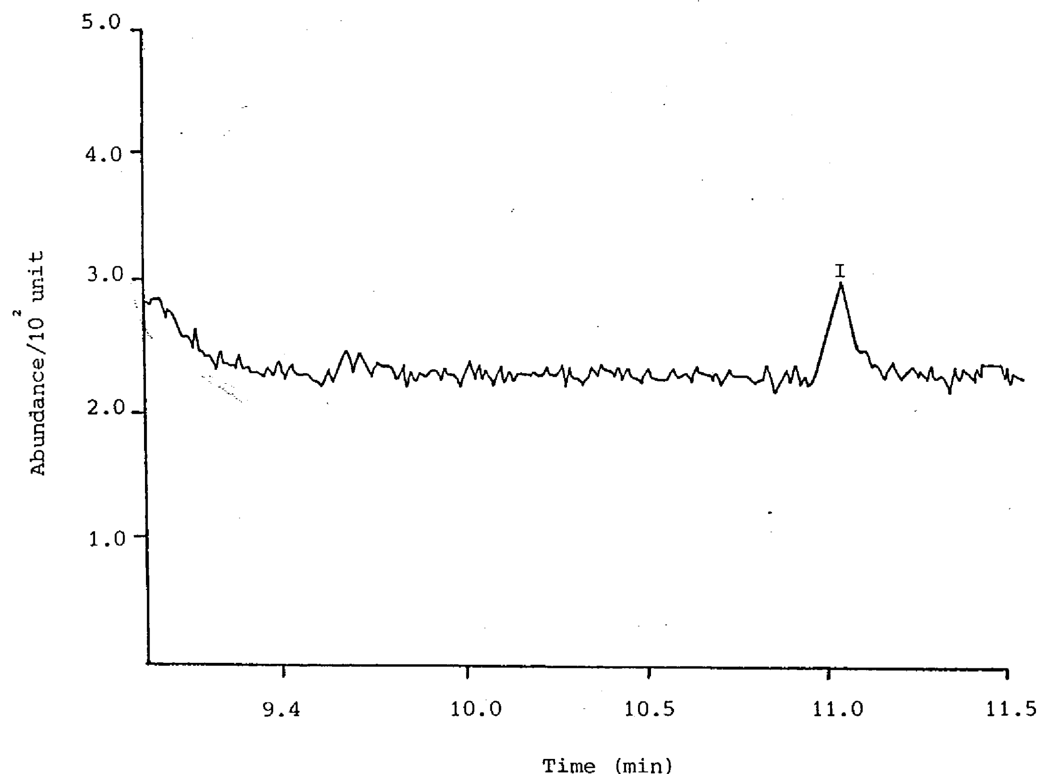



Figure 3.32: GC-MSD total ion chromatogram of 25 ng ml$^{-1}$ of THC-COOH (I) after trimethylsilylation in the SIM mode



(TMS) ether, 11-carboxyl TMS ester and 1-O-methyl ether, 11-carboxyl methyl ester of THC-COOH are given in figures 3.33 and 3.34 respectively. The 1-O-TMS ether, 11-carboxyl TMS ester of THC-COOH standard spectrum used for PBM matches is given in figure 3.35.

The origin of the mass peaks of the trimethysilylated THC-COOH used for PBM matches (figure 3.35) can be explained as follows. The mass peak m/z 488, structure I in figure 3.36, was the molecular ion $M^+$ and loss of a methyl group, 15 u, would result in m/z 473 whereas loss of the trimethylsiloxycarbonyl, $[(CH_3)_3SiOCO]^+$ = 117 u, would result in the mass peak at m/z 371. Poole [95] and others [98 - 101] have discussed the characteristic fragment groups of trimethylsilylated compounds. These characteristic fragments include $[(CH_3)_3Si]^+$ = 73 u, $[HO{=}Si(CH_3)_2]^+$ = 75 u and $[(CH_3)_3SiO{=}Si(CH_3)_2]^+$ = 147 u, the last fragment only occurs when more than one TMS group is present adjacent to one another. The loss of fragment $[HO{=}Si(CH_3)_2]^+$ from m/z 473 would have resulted in m/z 398 and loss of fragment $[(CH_3)_3SiH]$ = 74 u from the base peak, m/z 371, would have resulted in m/z 297. Vree [102] has shown that fragmentation of the pentyl side chain by McLafferty rearrangement yields the butylene fragment , 56 u. The loss of the butylene fragment from m/z 473 would result in m/z 417. The probable fragmentation scheme for formation of the mass peak at m/z 209 is given in figure 3.36. Vree [102, 103] has discussed the transformation of the alicyclic double bond in trans(3,4)-para-9,10-THC, $\Delta^9$-THC, to trans(3,4)-para-8,9-THC, $\Delta^8$-THC, as being due to either a phenolic proton transfer or a double bond shift proposed by Budzikiewicz. The phenolic proton transfer however has been shown not to occur when the phenol group is derivatized to an ether function by trimethylsilylation, methylation or other derivatization procedures. Therefore it is reasonable to assume that trimethylsilylated $\Delta^9$--THC-COOH (I) underwent a alicyclic double bond shift to give trimethylsilylated $\Delta^8$-THC-COOH (II) which then fragmented to m/z 318 (III) by means of a retro-Diels-Alder reaction. Loss of a methyl group from structure (III) gave m/z 303 (IV) which then lost $C_3H_2$ by ring cleavage and rearrangement to give m/z 265 (V). Loss of a butylene fragment from the side chain of structure V would give m/z 209 (VI). Vree and others [102 - 107] proposed a similar scheme for THC.

Bjorkmann [74] has elucidated the fragmentation pathway of the major fragments of the mass spectrum of methylated THC-COOH in figure 3.34. Loss of a methyl group or methoxycarbonyl group from the molecular ion m/z 372 resulted in the formation of m/z 357 and 313 respectively. In conclussion, comparison of figures 3.34 and 3.35 show

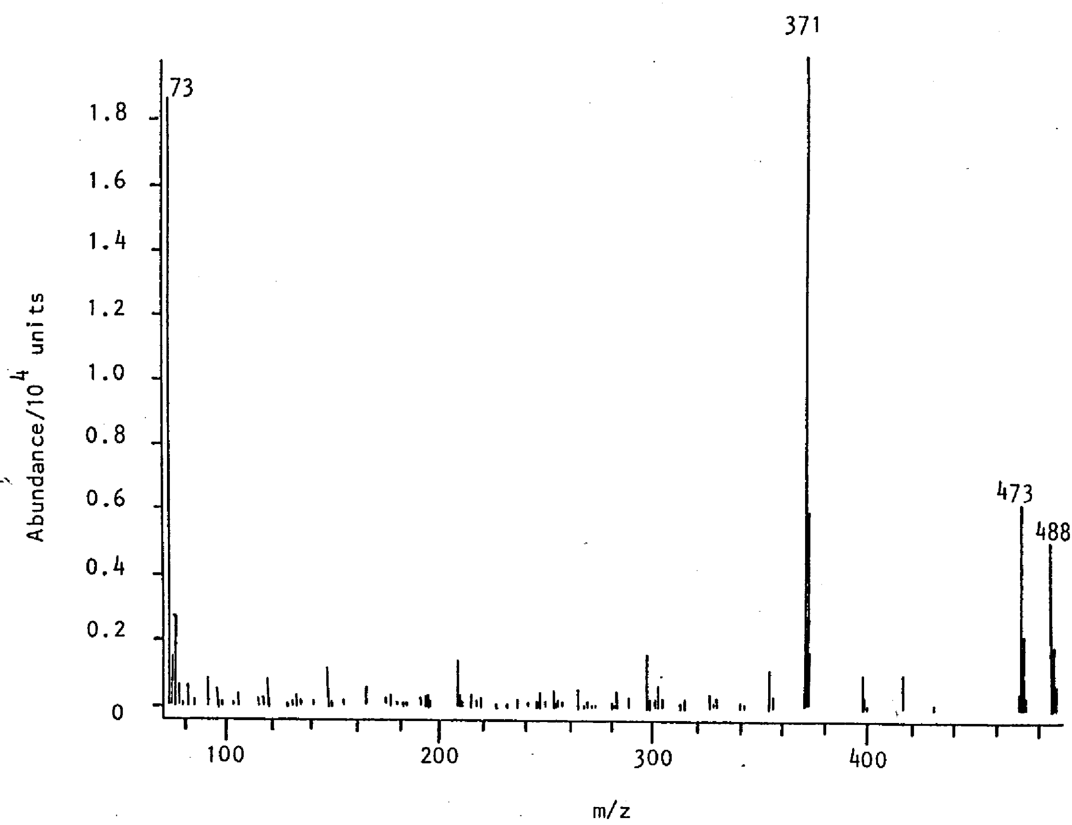



Figure 3.33: Mass spectrum of 1-O-TMS ether, 11-carboxyl TMS ester of THC-COOH

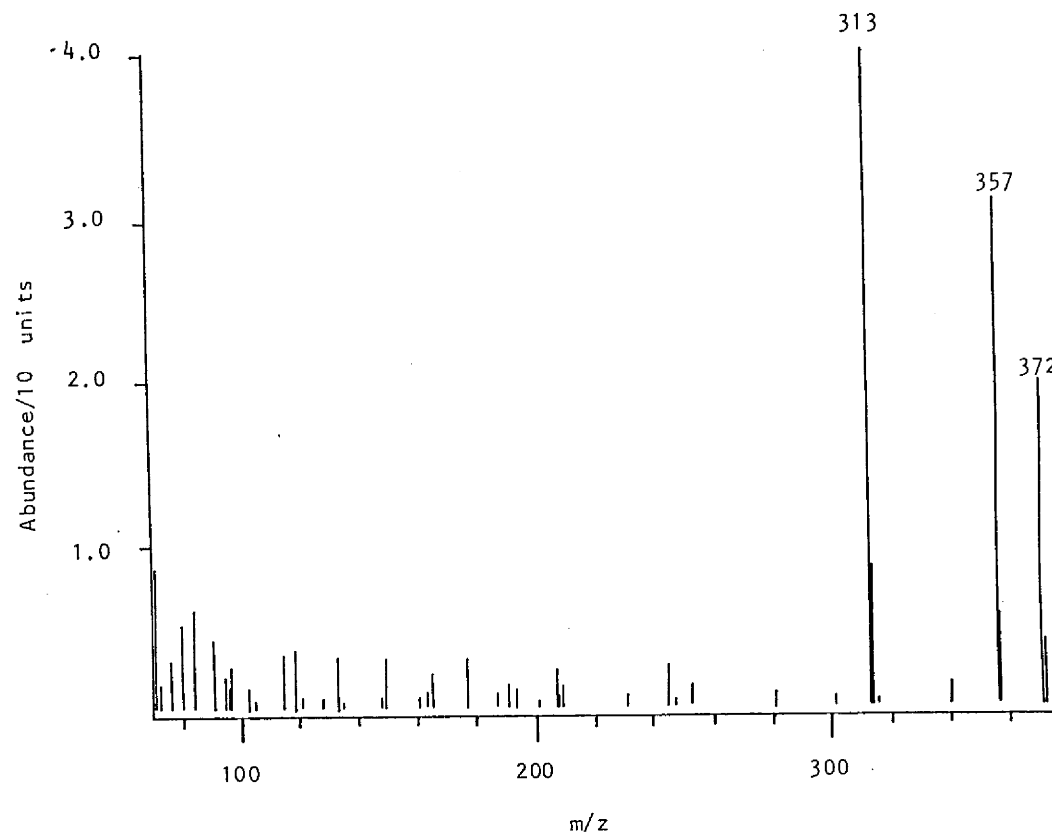



Figure 3.34: Mass spectrum of 1-O-methyl ether, 11-carboxyl methyl ester of THC-COOH

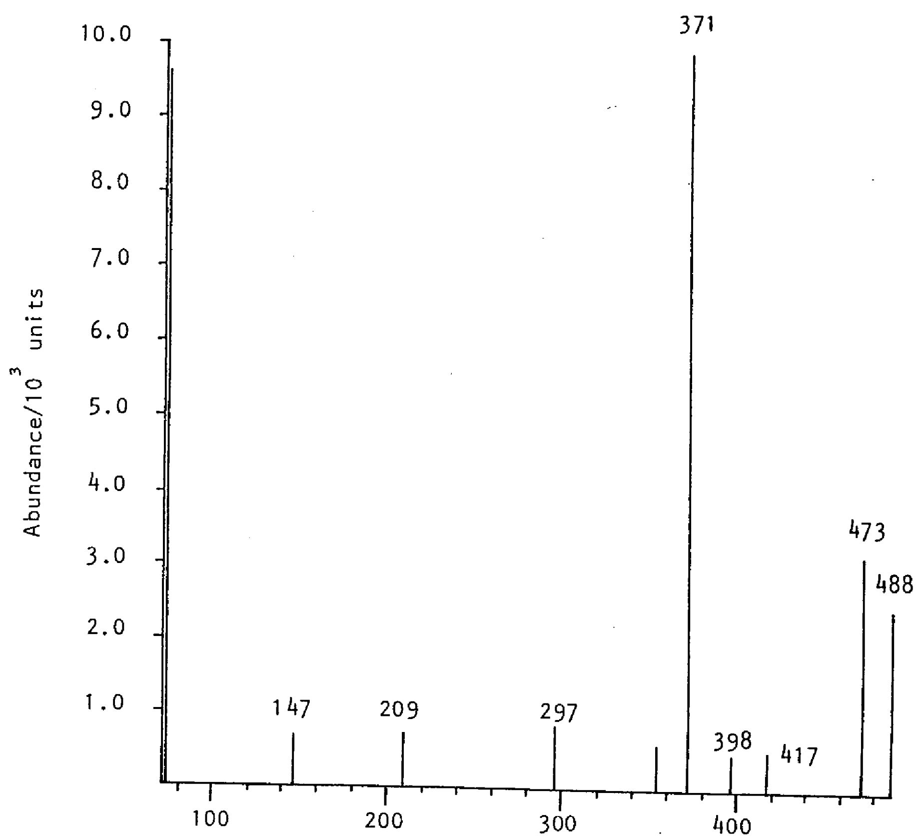



Figure 3.35: Mass spectrum of 1-O-TMS ether, 11-carboxyl TMS ester of THC-COOH used for PBM matching

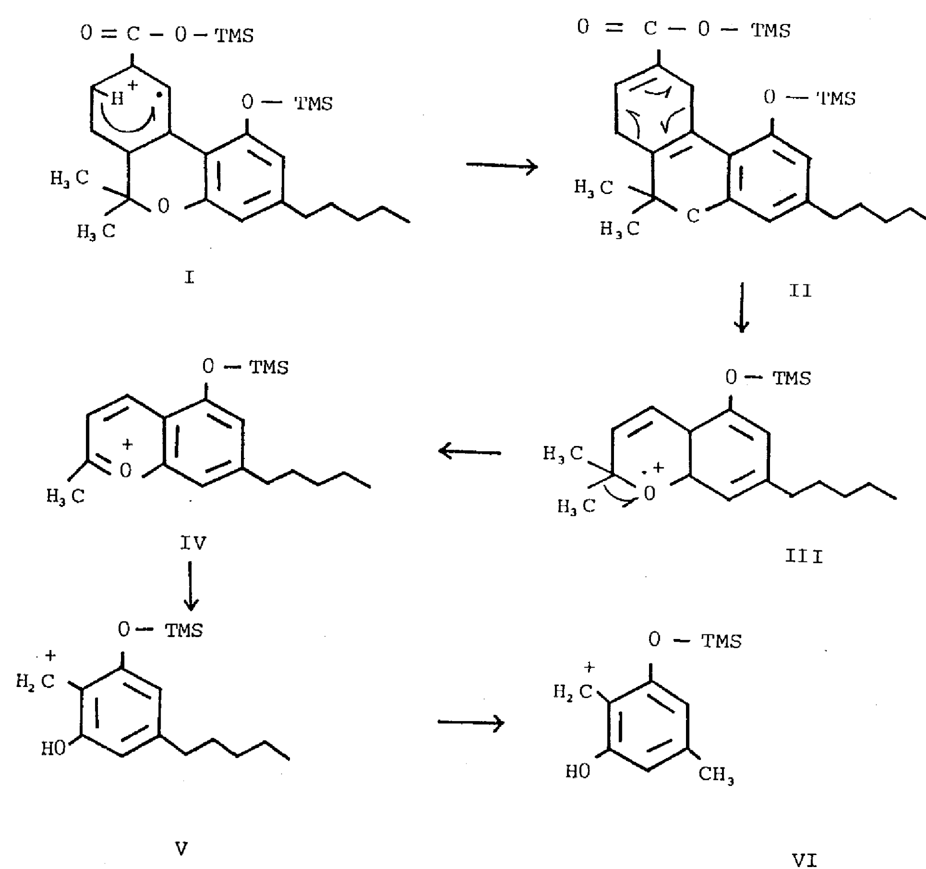



Figure 3.36: Fragmentation scheme for formation of m/z 209 from the molecular ion of trimethylsilylated THC-COOH

that the higher sensitivity obtained for trimethylsilylated THC-COOH is reflected in greater abundance values of the major mass peaks when compared to those for the methylated derivative.

## 3.7 Conclusion

In conclusion, the extraction procedure and the chromatographic systems were both selective and sensitive for forensic drug screening. The HPLC system was suitable for quantitative screening of THC-COOH in forensic urine samples. However for general forensic laboratory mass screening pruposes, the TLC system was found to be the best method. In addition to its selectivity, sensitivity and larger number of samples assayed per unit time, the TLC chromatogram can also be used for semi-quantitative determination of the metabolite with the aid of the densitometer. Finally, the GC-MSD system is capable of providing unequivocal identification down to 5 µg $ml^{-1}$ of THC-COOH with a 80% match quality.

Further work will however be required in order to validate the above methods for forensic laboratory applications. Firstly, the methods will have to be subjected to analysis of a large number of different forensic urine samples. This is to ensure that variations in the urine samples do not interfere with the procedures which were developed for screening. The data obtained, qualitative or quantitative, can be further correlated to that obtained for the same samples with established methods. Such a correlation would serve as a measure of the methods' efficacy compared to the established methods currently in use.

One other area that would be beneficial will be a clinical study to determine the urine excretion profiles for THC-COOH. This study should include volunteers who are casual users as well as regular users and of different racial mix. The results of the study will be useful in determining the time frame for detection of the metabolite utilizing the developed screening procedures.



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Table A1: Peak height counts of THC-COOH with isooctane for different pH values

| Urine pH | Peak height counts | | | |
|---|---|---|---|---|
| | Replicates | Mean | S.D. | R.S.D |
| 1 | 1135721<br>1099204<br>1274650 | 1169858 | 92571 | 7.91% |
| 2 | 1116580<br>1143605<br>1188846 | 1149677 | 36514 | 3.18% |
| 3 | 1133366<br>1083272<br>1227889 | 1148176 | 73437 | 6.40% |
| 4 | 1224244<br>1099923<br>1129511 | 1151226 | 64943 | 5.64% |
| 5 | 996791<br>970253<br>893674 | 953573 | 53544 | 5.62% |
| 6 | 769088<br>706250<br>706175 | 727171 | 36301 | 4.99% |
| 7 | 83820<br>93606 | 88713 | 6918 | 7.80% |
| 8 | 54636<br>54229 | 54433 | 288 | 0.53% |
| 10 | N.E. | - | - | |

N.E. = THC-COOH not extracted at this pH using the above solvent



Table A2: Peak height counts of THC-COOH with cyclohexane for different pH values

| Urine pH | Peak height counts | | | |
|---|---|---|---|---|
| | Replicates | Mean | S.D. | R.S.D |
| 1 | 788783<br>834089<br>868536 | 830469 | 39999 | 4.82% |
| 2 | 928035<br>875720<br>735506 | 846420 | 99553 | 11.76% |
| 3 | 727311<br>754188<br>739330 | 740276 | 13463 | 1.82% |
| 4 | 869307<br>729133<br>718506 | 772315 | 84165 | 10.90% |
| 5 | 518818<br>743452<br>637585 | 633285 | 112379 | 17.75% |
| 6 | 386964<br>476617<br>367544 | 410375 | 58183 | 14.18% |
| 7 | 176459<br>90624 | 156379 | 28397 | 18.16% |
| 8 | 44413<br>50549 | 47481 | 4339 | 9.14% |
| 10 | N.E. | - | - | |

N.E. = THC-COOH not extracted at this pH using the above solvent

Table A3: Peak height counts of THC-COOH with hexane for different pH values

| Urine pH | Peak height counts | | | |
|---|---|---|---|---|
| | Replicates | Mean | S.D. | R.S.D |
| 1 | 821439<br>758772<br>678975 | 753062 | 71403 | 9.48% |
| 2 | 1041594<br>975336<br>1019796 | 1012242 | 33769 | 3.34% |
| 3 | 1061526<br>988014<br>1118306 | 1055949 | 65325 | 6.19% |
| 4 | 1063642<br>898102<br>1114006 | 1025250 | 112956 | 11.02% |
| 5 | 823317<br>880357<br>992674 | 898783 | 86169 | 9.59% |
| 6 | 970749<br>860631<br>833450 | 888277 | 72705 | 8.18% |
| 7 | 180899<br>179594<br>155975 | 172156 | 14028 | 8.15% |
| 8 | N.E. | - | - | |
| 10 | N.E. | - | - | |

N.E. = THC-COOH not extracted at this pH using the above solvent.



Table A4: Peak height counts of THC-COOH with ethyl acetate for different pH values

| Urine pH | Peak height counts | | | |
|---|---|---|---|---|
| | Replicates | Mean | S.D. | R.S.D |
| 1 | 1162216<br>1445764<br>1340932 | 1316304 | 143369 | 10.98 |
| 2 | 1111717<br>1508007<br>1416472 | 1462239 | 64725 | 4.43 |
| 4 | 1298260<br>1423131<br>1257973 | 1326455 | 86113 | 6.49 |
| 5 | 1130813<br>1183654<br>1135342 | 1149936 | 29288 | 2.55 |
| 6 | 1445859<br>1561657<br>1428642 | 1478719 | 72340 | 4.89 |
| 7 | 1681187<br>1483327<br>1690676 | 1618397 | 117070 | 7.23 |
| 8 | 1838200<br>1967152<br>1669810 | 1825054 | 149106 | 8.17 |
| 10 | 1678361<br>2059358<br>1684857 | 1807525 | 218118 | 12.07 |

Table A5: Peak height counts of THC-COOH with diethyl ether for different pH values

| Urine pH | Peak height counts | | | |
|---|---|---|---|---|
| | Replicates | Mean | S.D. | R.S.D |
| 1 | 461678<br>437729<br>455585 | 451664 | 12447 | 2.75% |
| 2 | 516582<br>621136<br>478569 | 538762 | 73826 | 13.70% |
| 3 | 602259<br>451970<br>464605 | 506278 | 83362 | 16.47% |
| 4 | 510619<br>477602<br>552349 | 513523 | 37458 | 7.29% |
| 5 | 592861<br>573283<br>790934 | 652359 | 120408 | 18.46% |
| 6 | 671248<br>707876<br>704137 | 694420 | 20155 | 2.90% |
| 7 | 704029<br>649768<br>695586 | 683128 | 29197 | 4.27% |
| 8 | 726926<br>709495<br>763334 | 733252 | 27471 | 3.75% |
| 10 | 343002<br>587913<br>418249 | 449721 | 125452 | 27.90% |



Table A6: Peak height counts of THC-COOH with benzene for different pH values

| Urine pH | Peak height counts | | | |
|---|---|---|---|---|
| | Replicates | Mean | S.D. | R.S.D |
| 1 | 1297275<br>1413912<br>1334865 | 1348684 | 59534 | 4.41% |
| 2 | 1457863<br>1292360<br>1508798 | 1419674 | 113160 | 7.97% |
| 3 | 1660508<br>1300513<br>1518266 | 1493096 | 181313 | 12.14% |
| 4 | 1640687<br>1326842<br>1467554 | 1478361 | 157201 | 10.63% |
| 5 | 1401599<br>1475885<br>1298383 | 1391956 | 89143 | 6.40% |
| 6 | 1503352<br>1459052<br>1364729 | 1442378 | 70800 | 4.91% |
| 7 | 1426075<br>1062203<br>1268562 | 1252280 | 182482 | 14.57% |
| 8 | 894516<br>1060940<br>952920 | 969459 | 84436 | 8.71% |
| 10 | 273324<br>216202<br>317940 | 269155 | 50997 | 18.95% |

Table A7: Peak height counts of THC-COOH with chloroform for different pH values

| Urine pH | Peak height counts | | | |
|---|---|---|---|---|
| | Replicates | Mean | S.D. | R.S.D |
| 1 | 1156128<br>1269980<br>1198355 | 1208154 | 57555 | 4.76 |
| 2 | 1097721<br>1172004<br>1283368 | 1184364 | 93439 | 7.89 |
| 3 | 1257586<br>1413975<br>1233682 | 1301748 | 97924 | 7.52 |
| 4 | 1399619<br>1339698<br>1699775 | 1479731 | 192897 | 13.04 |
| 5 | 1342512<br>1340904<br>1405300 | 1362905 | 36724 | 2.69 |
| 6 | 1290109<br>1366911<br>1477910 | 1378310 | 94418 | 6.85 |
| 7 | 1306704<br>1339575<br>1150450 | 1265576 | 101048 | 7.98 |
| 8 | 1248186<br>1380785<br>1122995 | 1250655 | 128913 | 10.31 |
| 10 | 320253<br>331043<br>358406 | 336567 | 19667 | 5.84 |



Table A8: Peak height counts of THC-COOH with dichloromethane for different pH values

| Urine pH | Peak height counts | | | |
|---|---|---|---|---|
| | Replicates | Mean | S.D. | R.S.D |
| 1 | 311928<br>423454 | 367691 | 78861 | 21.45 |
| 2 | 444315<br>405014 | 424665 | 27790 | 6.54 |
| 3 | 455199<br>469917 | 462558 | 10407 | 2.25 |
| 4 | 548007<br>509230 | 528619 | 27420 | 5.19 |
| 5 | 426100<br>461918 | 444009 | 25327 | 5.70 |
| 6 | 462203<br>509315 | 485759 | 33313 | 6.86 |
| 7 | 484276<br>447080 | 465678 | 26302 | 5.65 |
| 8 | 478843<br>423169 | 451006 | 39368 | 8.73 |
| 10 | 100721<br>85826 | 93274 | 10532 | 11.29 |