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CHAPTER 5

Cannabinoids analysis: analytical methods for different biological specimens

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5.1 INTRODUCTION

Cannabinoids are a group of compounds unique to the cannabis plant (*Cannabis sativa* L.) of which Δ^9-tetrahydrocannabinol (THC) is the most active component that causes psychedelic activity. They are responsible for most of the pharmacological effects of the plant. These psychoactive constituents are present mainly in the flowering and fruiting tops and leaves of the plant.

Three cannabis preparations are illicitly trafficked: herbal cannabis (marijuana), cannabis resin with fine plant particles (hashish), and cannabis extract (cannabis oil or hash oil) [1].

Herbal cannabis (marijuana) is the most widely used illicit drug in the world [2–4]. It is prepared by collecting the flowering tops and leaves of the female plants and drying them in the air. The dried material may then be compressed into blocks or left as loose herbal material.

Cannabis resin (hashish) is prepared by threshing the herbal material, often against a wall, to separate the fibrous parts of the plant from the resin-producing parts, then compressing them into slabs. Alternatively, the flowering and fruiting tops are rubbed between the palms of the hands, which are then scraped periodically to remove the resin.

Cannabis oil (hashish oil) is an extremely potent preparation. It is prepared from the herbal or resin material by liquid extraction; the extract is often concentrated prior to trafficking and contains up to 60% of the active principle (THC).

The major active constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), was first characterized in 1964 by Gaoni and Mechoulam [5]. To date, 70 cannabinoids have been identified [6].

References pp. 235–241

The development of methods for the determination of cannabinoids is an area of increasing interest and a large number of publications appear every year describing a variety of analytical techniques, which vary in sensitivity, specificity, and instrumentation. Articles providing extensive reviews of the various analytical techniques have also been written [2,7–10]. This chapter will focus mainly on the methods published in the recent past with special emphasis on those methods that appear to be more practical and feasible for routine analysis of these compounds in various types of biological specimens. However, because of the large number of publications, this study is not meant to be exhaustive.

Various types of biological samples can be analyzed for cannabinoids to test for marijuana use.

5.2 ANALYSIS OF CANNABINOIDS IN URINE

Urine appears to be the biological fluid of choice to test for the presence of Δ^9-THC metabolites in the human body. Many THC metabolites are excreted in urine, but the major urinary metabolite is Δ^9-THC-11-oic-acid (THC-COOH), either free or conjugated as glucuronide [11]. Urinalysis has the advantage of being able to detect THC metabolites for a relatively long period of time. These metabolites, being highly lipophilic, are readily distributed to body tissues and are slowly eliminated in the urine [12]. THC metabolites, therefore, persist in urine for several days after smoking a single marijuana cigarette, and three to four weeks may be required for elimination of all metabolites in the case of heavy users [13].

The general approach for the analysis of THC metabolites in urine is to screen the samples by an immunoassay method such as radioimmunoassays (RIA), enzyme immunoassays (EIA) or fluorescence polarization immunoassays (FPIA). The presumed positive samples are then confirmed by another more specific method such as gas chromatography-mass spectrometry (GC-MS).

5.2.1 Immunoassays

Immunoassays are the most widely used screening methods for cannabinoids in urine. These methods are based on developing antibodies specific to the drug to be tested and/or one or more of its major urinary metabolites. EIA and RIA are among the most commonly used methods, although the RIA method has lost favor in the recent years.

5.2.1.1 Radioimmunoassay

RIA methods are very sensitive assays, which have been widely used for many years. However, the assays have the inherent disadvantages of limited stability of radiolabelled compounds and the need for special disposal of radioactive materials and

special handling to avoid health hazards [14]. Radiolabelling is usually carried out using either ^{3}H or ^{125}I. ^{125}I radiotracers are usually preferred since higher specific activity can be obtained, and separation and gamma counting are simpler than the liquid scintillation counting used for ^{3}H tracers.

A simple and sensitive RIA method using ^{125}I tracer was described by Law *et al.* [15], which required small sample volume and allowed the detection of cannabinoid metabolites many days after consumption. The sensitivity of the RIA method was then coupled with high performance liquid chromatography (HPLC) and the combined HPLC-RIA method was then used for the analysis of THC metabolites in urine and in blood [16]. Clatworthy *et al.* [17] compared the ^{125}I-RIA method of Law *et al.* [15] with another ^{3}H-RIA method and the results obtained were confirmed by GC-MS.

The specificity of the Abuscreen® RIA for cannabinoids, a method which was in commercial use for many years, was assessed by Jones *et al.* [18] who examined 41 cannabinoid and non-cannabinoid phenolic constituents for potential cross-reactivity and found that only cannabinoids of the dibenzopyran type structure cross-react with the antiserum. ElSohly *et al.* [19] tested the specificity of the assay with respect to indole carboxylic acids where none of the compounds tested showed any cross-reactivity. Altunkaya and Smith [20] reported false-positive and false-negative results of RIA for cannabinoids in urine samples. The interfering substance was not identified but the authors suggested that the cause was the presence of proteinaceous material in the urine.

Because of the limitations described above, RIA methods have been largely abandoned and their current use limited to specific research applications.

5.2.1.2 Enzyme immunoassays

EIA are the most commonly used screening methods for the detection of cannabinoids in urine today. EIA methods are rapid, simple, and do not require special precautions for handling and disposal.

Several publications describing the utilization of enzyme multiplied immunoassay techniques (EMIT) for the determination of cannabinoids in urine have been reported [21–23]. The initial screening by EMIT was followed by confirmation either by TLC [24,25], HPLC [26,27], or most commonly by GC-MS [28,29].

Rapid, cost-effective urine testing of a large number of urine samples with the EMIT urine cannabinoid assay was automated through the use of a centrifugal analyzer [26,30–32], Monarch analyzer [33], or a chemistry analyzer [34].

Specificity of the EMIT d.a.u. cannabinoid assay with respect to 162 drugs was studied by Allen and Stiles [35].The presence of nabilone, a synthetic cannabinoid used as an antinauseant, did not affect the results of the assay [36].

An enhanced chemiluminescent EIA for the detection of cannabinoids in urine samples was developed by Sharma *et al.* [14]. The assay is based on the horseradish peroxidase catalyzed oxidation of luminol by H_2O_2 in the presence of *p*-iodophenol under mildly basic conditions. The method is sensitive, simple, and suitable for the automation and routine screening of large numbers of samples.

The use of EMIT assays as a semi-quantitative method is controversial. Standefer and Backer [33] reported that quantitative results were obtained from the EMIT assays, using a multiple-point calibration curve which is updated regularly. However, other authors reported many factors that hinder this quantification including: cross-reactivity of the assay with several chemically related substances, sample carryover from highly concentrated samples, and absorbance changes that reached a plateau near the medium calibrator. Therefore, it was suggested that EMIT immunoassays be used only as a qualitative tool [32,37].

5.2.1.3 Fluorescence polarization immunoassays

Colbert *et al.* [38] developed two fluoroimmunoassays for the detection of cannabinoids in urine. The first was a single-reagent polarization immunoassay, which did not require a sample separation step but lacked sensitivity. The second assay had sensitivity comparable with RIA and could be automated. Stopped flow-FPIA (SF-FPIA) was also used for the determination of drugs of abuse in urine. They were suitable for routine screening programs, being faster and having lower detection limits, and better within- and between-assay precision than conventional FPIA [39].

ElSohly *et al.* [40] evaluated the cross-reactivity of the Abbott TDx® cannabinoid assay against a variety of cannabinoid and non-cannabinoid phenolic compounds. The antiserum was found to cross-react equally to 11-nor-Δ^9-THC-COOH, its glucuronide and to the corresponding Δ^8-isomer. The hydroxylated derivatives of Δ^9-THC and Δ^8-THC and other cannabinoids in general show limited binding potential toward the antibody.

The Abbott AxSYM assay for drugs of abuse was evaluated and compared with the Syva EMIT d.a.u./Roche Cobas Mira S Plus, Abbott TDx and ADx, Syva EMIT d.a.u./Syva ETS Plus, Syva EMIT II/Hitachi 717, and Roche Abuscreen OnLine/Roche Cobas Mira S Plus. The system's advantages, including stability of the calibration curves for 3–4 months, the possibility of providing semi-quantitative results, and the ability to process emergency samples, made it useful for routine analysis of drugs of abuse in urine samples [41].

5.2.1.4 Enzyme-linked immunosorbent assays

Microanalysis of cannabis components and their metabolites was also carried out by enzyme-linked immunosorbent assays (ELISA). The application of the method to the analysis of THC metabolites in plasma and urine was suggested [42].

Fraser *et al.* [43] used ELISA and EIA assays for the screening of urine samples for cannabinoids followed by GC-MS confirmation.

5.2.1.5 Kinetic interaction of microparticles in solution

Another type of immunoassay, which depends on the kinetic interaction of microparticles in solution (KIMS), is the Abuscreen OnLine assay. Hailer *et al.* [44] evaluated the Abuscreen OnLine cannabinoids assay using the COBAS FARA II

automatic analyzer where modifications were made in the cutoff definition, calibration curve and reagent volume to obtain maximum sensitivity and reagent economy. The results were compared with the EMIT d.a.u. assay, and the authors concluded that the OnLine cannabinoids assay was a good alternative to EMIT d.a.u. in terms of low detection limits, calibration curve stability, and cost effectiveness.

Armbruster *et al.* [45] compared the Roche OnLine assay, the Syva EMIT II assay, and the Abbott TDx FPIA with the Roche Abuscreen RIA assay. The OnLine assay and the EMIT II were reported to be better than the RIA procedure in terms of time and effort.

Microgenics' cloned enzyme donor immunoassay (CEDIA) and KIMS were evaluated for cannabinoids, amphetamines, barbiturates, benzodiazepines, benzoylecgonine, LSD, methadone, and opiates [46]. Cannabinoids showed 99.3% concordant results, where there was only one negative sample by KIMS (cutoff 50 μg/L) and positive by CEDIA at a cutoff level of 25 μg/L. The CEDIA and KIMS results for all eight drugs were in good agreement (93.3–100%).

Feldman *et al.* [47] developed four OnLine DAT II assays by modifying the original KIMS technology for the evaluation of cocaine, methadone, opiates, and THC for improved performance and enhanced ease of use. These assays are being applied to COBAS INTEGRA and Roche/Hitachi line of analyzers. Cutoffs for THC assay were 20, 50, and 100 ng/mL with 0–100, 0–300, and 0–300 ng/mL dynamic ranges, respectively.

5.2.1.6 On-site testing kits

Many on-site testing kits for the analysis of cannabinoids in urine are now commercially available. Compared with laboratory-based immunoassays, these kits have the advantages of being simple, easily performed, allow rapid access to the test results and do not need costly instrumentation or highly trained personnel. Several authors tested the performance of many of these kits and compared their results with other laboratory-based methods.

Armbruster and Krolak [48] evaluated the Abuscreen *ONTRAK* assay (Roche Diagnostic systems) and compared the results with those obtained using RIA, FPIA, and GC-MS confirmation. Results agreement was observed but the authors criticized the subjective nature of identifying the results and the absence of a positive control in the test kit.

The immunoassay TRIAGE^{TM} was applied to the detection of several classes of compounds including cannabinoids in postmortem urine samples [49]. Two difficulties were encountered. The first related to the nature of the postmortem urine samples, which contained significant amounts of sediment that reportedly blocked the nylon membrane, inhibiting complete absorption of the reaction mixture after spotting onto the detection area. This was overcome by removing the excess solution from the detection zone and increasing the amount of wash solution used. The second difficulty was the dependence of the color intensity produced on the drug concentration, making judgment of the results difficult, especially for inexperienced users. Nevertheless, the results obtained showed good agreement with the Abbott

ADx FPIA and when compared to GC-MS, a 95% confirmation rate for cannabinoids was reported.

Jenkins *et al.* [50,51] assessed the validity of the EZ-SCREEN® cannabinoid test and the accuPINCH™ THC test for the analysis of cannabinoids in urine. In both cases, 178 clinical urine samples, 72 urine samples containing known amounts of drug, and 50 drug-free urine samples were randomized and analyzed under blind conditions. The results were interpreted independently by three readers. The EZ-SCREEN® cannabinoid test showed high sensitivity for THC-COOH and low cross-reactivity to THC and 11-OH-THC. The LoD was reported to be much lower than the detection average specified by the manufacturer and that positive results should always be confirmed by GC-MS. The assay was easy to perform, provided rapid results, and could be used for on-site drug testing [50].

The accuPINCH™ THC test is a competitive EIA that is used for the detection of THC-COOH and shows relatively low cross-reactivity with THC and other cannabinoids. The assay was highly affected by sample turbidity, which interfered with color interpretation on the detection disk, but the assay was relatively insensitive to changes in sample temperature [51].

Triage® panel for drugs of abuse is a rapid immunoassay for the simultaneous detection of seven drugs in a single sample [52]. De La Torre *et al.* [53] evaluated the degree of concordance between the Triage® results and those obtained by FPIA and demonstrated that the performance of both assays was comparable and that the results of the assay were independent of the laboratory personnel's skills.

The Bionike One-Step tests for the detection of drugs of abuse in urine are used for on-site testing of amphetamines, methamphetamine, benzodiazepines, cannabinoids, methadone, and opiates. These tests were evaluated, and the results obtained were in good agreement with the EMIT d.a.u. assays [54].

Another simple and rapid test that screens for five different classes of drugs of abuse in urine samples is the Advisor™ drug screening system developed by Parsons *et al.* [55]. The system is composed of a multi-chambered vessel that automatically distributes the liquid reagent into distinct assay channels. Each of them tests for a specific class of drugs of abuse. The results of the tests compared well with other automated immunoassays for drugs of abuse.

Korte *et al.* [56] compared the results obtained with RapiTest THC for the detection of cannabinoids in urine with the results obtained with the EMIT d.a.u. and with gas chromatographic-mass spectrometric methods. The results correlate well together when operating above the cutoff concentrations of the methods. At low drug concentration, the color of the band is faint and inexperienced users may find it difficult to judge the results.

Two separate on-site testing kits for drugs of abuse, the ONTRAK TESTCUP and the Abuscreen ONTRAK, were compared, and the results obtained were further compared with another laboratory-based immunoassay, the Abuscreen OnLine [57]. The ONTRAK TESTCUP tests for three drug classes (benzoylecgonine, THC-COOH, and morphine) simultaneously, while the Abuscreen ONTRAK tests have a separate single kit for each drug class. Both systems agreed with the OnLine assays in identifying drug positive and drug negative samples.

The performance of the Abusign™ Drugs-of-Abuse Slide Tests was evaluated by Ros *et al.* [58]. Inter- and intra-individual agreement was tested by comparing the readings of four persons at different time intervals after incubation. Comparison with the FPIA-ADx method was also done and all the samples were confirmed by GC-MS.

For the Abusign cannabinoids (50 ng/mL) slide test, the method was found to be more sensitive than the FPIA-ADx test, but the specificity was lower. The drawback of this method was that the test results depended on the reader and on the time at which the test was read, especially when the concentration of the drug of abuse was near the cutoff. The authors, therefore, concluded that the test was not suitable for screening of drugs of abuse in situations in which a reliable test result was required. The test may be of value in emergency toxicology when a quick result is needed.

Wennig *et al.* [59] developed and evaluated the one-step dip-and-read immunochromatographic FRONTLINE® Rapid Tests for drugs of abuse testing in urine samples. Multicenter evaluation of the rapid tests was performed at six European sites, each following the same protocol, by comparing them with FPIA and EMIT assays. The evaluations showed reliable results for the rapid tests of cannabinoids, cocaine, and opiates as compared with the FPIA and EMIT.

Several publications comparing different types of immunoassays to each other and/or to chromatographic methods appearevery year.

Irving *et al.* [60] analyzed 200 urine specimens with two EIA (EMIT-st and EMIT-d.a.u.) and an RIA (Abuscreen RIA), and those samples found to be positive were further analyzed by gas–liquid chromatography with flame ionization detection (FID), gas–liquid chromatography/mass spectrometry, and an experimental RIA from the Research Triangle Institute. The aim of this study was to evaluate the two EIA by comparing the results with those obtained from other methods. The two assays were found to give 98–94% confirmation rates for positive results when compared with GC-MS. The authors noted that the high cutoff levels established eliminated false positives but allowed a high false-negative rate. Attempts to quantify the results of the RIA were unsuccessful.

Jones *et al.* [61] compared five methods, namely, Abuscreen RIA, EMIT d.a.u., HPLC, GC/electrochemical detection (ECD), and GC-MS, for the analysis of THC-COOH in urine. RIA and the EIA were used as screening procedures, and the other methods were used for confirmation of presumptive positives. Quantitative estimates obtained by the immunoassay procedure were always higher than those obtained by the chromatographic methods, probably because of the cross-reactivity of other THC metabolites with the antisera of both immunoassay procedures. The data obtained from the chromatographic methods were compared, and good correlation coefficients were obtained. The effect of storage of urine samples was studied and found to affect the concentration of THC-COOH.

Another comparative study between six cannabinoid metabolite assays was presented by Frederick *et al.* [62]. These assays were two EIA (EMIT-st and EMIT d.a.u.), two RIA (Abuscreen RIA and Immunalysis), one TLC assay (Toxi-Lab), and a new GC-MS method. The four immunoassays were used for screening purposes because of their simplicity and speed. When low levels of THC-COOH were

present, the Immunalysis RIA was recommended, while the EMIT-st and the Abuscreen were useful for screening higher levels of THC-COOH. The Toxi-Lab TLC and the GC-MS methods could both be used for confirmation.

Comparison between the TDx assay and the EMIT-Cobas assay for the detection of cannabinoids in urine from prison inmates was done by Karlsson and Stroem [63]. HPLC was used for confirmation. It was found that high background urine may affect the reliability of the results of the TDx assay, a problem that can be solved by diluting the samples and reanalyzing, or by setting the instrument background to a higher level. Apart from this, the TDx assay was reported to be reliable, with an excellent precision and curve stability. The EMIT-Cobas was reported to be faster, with the time to analyze one carousel being approximately 8 min, compared to 20 min for one TDx carousel. However, it was necessary to run the EMIT calibrators in each carousel because of the lack of curve stability.

Comparative results of five cannabinoid immunoassays were reported by Barnhill and Wells [64]. The five assays were the cannabinoid TLC assay (Toxi-Lab), the Syva EMIT urine cannabinoid assay, the DPC cannabinoids double antibody RIA, the Abuscreen RIA, TDx cannabinoids assay, and the urine THC direct RIA (Immunalysis). In general, the RIA gave a greater proportion of positive results than did the EIA or the FPIA.

Kogan *et al.* [65] compared the results of the Syva EMIT® d.a.u. and the Roche Abuscreen® RIA, which were the most widely used, commercially available immunoassays for detecting cannabinoids in urine. The results of both assays agreed qualitatively; however, there was no correlation between the semi-quantitative values obtained from both methods. The results of the immunoassays were confirmed by a modified bonded-phase adsorption/thin layer chromatography (BPA-TLC) and by GC-MS. The BPA-TLC was based on a visual color reaction between the developed spots and the spraying reagent, Fast Blue RR. It was a simpler non-instrumental technique, easier to interpret than quantitative GC-MS, and could be used successfully when only a qualitative confirmation is needed. However, the technique had limited utility for forensic purposes only.

Comparison of the Abbott FPIA and the Roche RIA for the analysis of 142 urine samples containing THC-COOH with subsequent confirmation by GC-MS was done by Budgett *et al.* [66]. The authors concluded that both immunoassays produced similar results and either of them could be used in a mass-drug-screening laboratory.

Weaver *et al.* [67] correlated the results of three commercial immunoassay kits, Abuscreen®, TDx®, and EMIT® with the concentration of THC-COOH determined by GC-MS. None of the methods studied showed perfect correlation with the results of GC-MS, but a significant correlation still exists. Attempts to select an appropriate cutoff value for each assay based on the derived regression equation were also done.

Another comparative study was conducted by Altunkaya *et al.* [68], who compared the results of four immunoassays, namely, EMIT d.a.u. Cannabinoid 20 (Syva Corp.), DPC cannabinoids RIA (Diagnostic Products Corp.), and the Roche Diagnostics System's Cannabinoids-1 RIA and Cannabinoids-2 RIA assays. The four

immunoassays correlated well with GC-MS, but the DPC-RIA was selected by the authors as the method of choice because it provided quantitative results that might be used to calculate the concentration of the extracts to be injected on the GC-MS.

Armbruster *et al.* [45] compared three non-radioisotopic immunoassays with the RIA (Roche Abuscreen) previously used in their laboratory and reported that the RIA tests had several drawbacks including short reagent shelf-life, a need for special handling and disposal of wastes, and the requirement of a fully automated system for analysis. The assays compared were the Syva EMIT II, the Abbott TDx FPIA, and the Roche OnLine. RIA and OnLine assays exhibited equivalent performance, detecting 99% of GC-MS marijuana confirmed samples. The TDx detected 95% of the samples, while the EMIT II assay detected 88%. The EMIT II and the OnLine assays were reported to be better than the RIA procedure in terms of time and effort.

A similar comparative study was conducted by Kintz *et al.* [69], where the results of the EMIT d.a.u., the Abbott ADx FPIA, and the Abuscreen OnLine assays were correlated with the GC-MS method. All methods compared favorably and could be successfully used for the screening of THC-COOH in urine samples. However, there was no correlation between the quantitative results obtained by the immunoassays and those by GC-MS, possibly due to the presence of different cross-reacting metabolites of THC.

Comparison between six immunoassays (EIA-EMIT and EZ-SCREEN, FPIA-ADx, RIA-Coat-A-Count, LI-Abuscreen ONTRAK, and CBI-Triage) and three chromatographic methods (TLC-Toxi-Lab, HPLC, and HPLC-REMEDI Drug Profiling System) with GC-MS confirmation of the results was done by Ferrara *et al.* [70]. The values of sensitivity, specificity, false-positive and false-negative rates were reported for each technique. Statistical analysis of the results allowed the determination of predictive positive and negative values for each single technique and for combinations of immunochemical and chromatographic techniques. A decision-making process for the determination of the best combination of these techniques was also presented.

Huestis *et al.* [71] studied the detection times of cannabinoids in urine following the administration of a single marijuana cigarette using different commercial cannabinoid immunoassays (EMIT® d.a.u.™ 100, EMIT d.a.u. 50, EMIT d.a.u. 20, EMIT II 100, EMIT II 50, Abuscreen® OnLine™ and Abuscreen RIA, DRI™, and ADx). The results were compared with GC-MS results at a 15 ng/mL cutoff concentration.

The effect of adulterants in urine samples on RIA and on FPIA was studied [72]. A number of readily accessible chemicals such as sodium chloride, bleach, potassium hydroxide, soap, 2-propanol, and ammonia, were added to test tubes containing urine samples, which were then analyzed by RIA and FPIA. For the THC-COOH RIA, false positives occurred with potassium hydroxide and bleach adulterants, while soap caused false-negative results. No adulterant caused FPIA false positives, but false negatives were observed with bleach.

A comparison was made for five non-instrumental urine drug testing devices (Syva RapidTest d.a.u. 8, Syva RapidCup d.a.u. 5, RocheTestcup 5, Biosite Triage, and

Casco-Nerl microLINE Drug Screen Card), using a challenging clinical specimen set with drug concentrations close to the immunoassay screening cutoffs [73]. Based on GC-MS confirmation cutoffs, the non-instrumental devices demonstrated an overall accuracy of 70% (66–74%) when compared with the Syva ETS analyzer (80%).

A comparison was made between on-site immunoassay drug-testing devices and GC-MS [74]. In this study, 800 people and two devices for oral testing and eight on-site devices for urine were used. Good results were obtained for the urine on-site devices, with accuracies of 83–99% for amphetamines, 97–99% for cannabinoids, 94–98% for opiates, and 90–98% for benzodiazepines. Detection of amphetamines and opiates was possible in oral fluids with the on-site devices, but these devices were not sensitive for the lower levels of benzodiazepines and cannabinoids.

5.2.2 Chromatographic methods

Chromatographic methods can be used for qualitative and quantitative screening and/or confirmation of cannabinoids in biological specimens [2]. For the analysis of urine specimens, these methods focus mainly on the major urinary metabolite, THC-COOH. A preliminary hydrolysis step is often required to analyze the free and the glucuronide forms, which increases the concentration of THC-COOH. Hydrolysis can be done enzymatically, using β-glucuronidase enzyme or with strongly alkaline solutions such as sodium or potassium hydroxides, since the majority of the THC-COOH exists as an ester glucuronide. Unlike immunoassays, chromatographic methods require extensive sample clean up using either liquid–liquid extraction methods or solid-phase extraction (SPE) methods.

5.2.2.1 Thin layer chromatography

Thin layer chromatography (TLC) has been used for the screening and identification of cannabinoids for many years. Immunoassays have almost replaced TLC as a screening method. However, TLC can still be used in developing countries where instrumentation and reagents required by other methods might be lacking. The availability of HPTLC plates, which improves the separation of compounds over that obtained by regular TLC plates and the development of densitometric techniques, which allow in situ determination of the separated compounds on the plate, may increase the use of TLC again. TLC methods have the advantage of being more specific to THC-COOH than immunoassays, which are known to cross-react to many THC metabolites. Several publications reported the use of TLC as either a screening or a confirmatory technique.

Nakamura *et al.* [75] used a TLC procedure previously described by Kaistha and Tadrus [76] as a screening and a clean-up procedure for the isolation of THC-COOH from urine samples. The spot corresponding to THC-COOH was visualized with Fast Blue B and then scraped off the plate and eluted with methanol for further analysis by GC-MS.

Kanter *et al.* [77] developed a sequential TLC method for the isolation and identification of THC-COOH from urine. In this method, the pH of a volume of urine containing 50 mg of creatinine was adjusted to 4.7–6.3; hydrolyzed with β-glucuronidase, extracted with ether, washed with 5% $NaHCO_3$, and then evaporated under nitrogen. The residue was dissolved in dichloromethane, spotted on a silica gel G plate, and chromatographed sequentially with two mobile phases, the first consisting of acetone–chloroform–triethlyamine (80:20:1) and the second consisting of petroleum ether–ether–glacial acetic acid (50:50:1.5). The plate was sprayed with a freshly prepared alkaline solution of Fast Blue B. A magenta red color of R_f approximately of 0.1 or corresponding to that of a reference standard indicated a positive response. The results obtained were compared with those produced by EMIT. Good correlation was obtained for samples having a THC-COOH concentration above the detection limit of the immunoassay technique or for completely negative samples; those samples in the borderline range gave mixed results, which could be explained by the fact that immunoassay measures total cannabinoids while TLC measures THC-COOH only.

Lillsunde and Korte [78] used TLC for preliminary screening of drugs of abuse in urine samples followed by confirmation by GC-MS. For screening of cannabinoids, samples were extracted with *n*-hexane–ethyl acetate (7:1) after alkaline hydrolysis with 10 N KOH. The extract was evaporated and the residue dissolved in 50 μL ethanol and applied onto a TLC plate. *n*-Hexane–1,4-dioxane–methanol (35:10:5) was used as mobile phase, while alkaline solution of Fast Blue B was used as the spraying reagent. THC-COOH was confirmed by GC-MS as its methylated derivative.

Commercially available TLC procedures for the detection of THC-COOH in urine are also available. These include the TOXI-LAB Cannabinoid Screen method, the TOXI-GRAMS MS (THC) and the Toxi.Prep THC metabolites. These methods have been evaluated by many authors [21,22,62,64,79,80].

In the TOXI-LAB procedure, urine samples were hydrolyzed at room temperature with KOH and then extracted with a mixture of ethyl acetate and hexane (1:9). The extracts were concentrated onto discs, and those discs were inserted into a toxigram together with a blank toxi disc and a standard disc containing 350 ng of Δ^8-THC-COOH. The plate was then developed using a mixture of heptane–acetone–glacial acetic acid (70:30:1) and visualized with Fast Blue BB salt. The TOXI-LAB method allowed simultaneous extraction of 10 samples with one control and one standard, using a disposable applicator cartridge. Frederick *et al.* [62] compared the Toxi-Lab cannabinoid screen method with four commercially available immunoassay procedures and a GC-MS method, while Wells *et al.* [64] compared it with five cannabinoid immunoassay systems. Foltz and Sunshine [22] compared it with the EMIT d.a.u. assay and with a reference GC-MS method. Sutheimer *et al.* [21] compared the TLC method with two EIA methods, EMIT-st and EMIT d.a.u. In general, the Toxi-Lab procedure was simple, easy to perform, and required minimal cost and instrumentation. The system did not provide the high throughput capacity of automated EMIT but was much better than conventional TLC [22,79]. The Toxi-Lab assay was reported to be successfully used as a screening method for urine samples or as a confirmatory technique to the immunoassays to minimize the need and cost of the GC-MS confirmation [21].

The TOXI-GRAMS MS (THC) procedure was described by King *et al.* [79]. It consisted of biphasic thin layer chromatograms made of glass-fiber paper impregnated with silica gel and chemically modified alkyl-silica layer along one edge. Urine samples were hydrolyzed at room temperature with KOH, then acidified with glacial acetic acid, and transferred to the cells of a cartridge applicator. The chromatogram was developed in *n*-heptane–acetone–glacial acetic acid (50:50:1) and visualized with Fast Blue BB salt. This method was reported to have the advantages of increased sensitivity and increased specificity. All the THC-COOH extracted from the sample was applied to the plate, minimizing sample loss associated with liquid–liquid extraction, drug adsorption into glass, and transfer of extracts to TLC plate. Therefore, the sensitivity was greatly enhanced and, when compared with the liquid–liquid extraction method of Sutheimer *et al.* [21], showed lesser interferences from co-extracted drugs and urinary artifacts; thus specificity was also increased [79].

The Toxi-Prep (TP) system is a semi-automated system that utilizes the SPE technique for the extraction of THC metabolites from urine. Steinberg *et al.* [80] compared the Toxi.Prep THC metabolites system with the Toxi-Lab cannabinoid screen method for evaluating THC metabolites in urine. In the TP method, urine samples were hydrolyzed, loaded onto a preconditioned column, and the columns were washed with 0.5 mL of 20% acetic acid followed by 0.5 mL hexane. Acid elution reagent (400 μL, hexane–ethyl acetate–glacial acetic acid (70:30:0.1)) were added to each SPE column and allowed to spot directly onto the chromatogram. The chromatograms were developed using heptane–acetone–glacial acetic acid (70:30:1) and visualized by Fast Blue BB salt followed by exposure to diethylamine vapors. The TP system was reported to have many advantages over the Toxi-Lab method including 40% labor reduction by automation of the different steps of extraction, washing, and spotting, leading to cost reduction, the need for less extraction solvent and less urine, and giving cleaner chromatograms, which result in increased sensitivity.

The BPA/TLC method for the determination of THC-COOH in human urine was developed by Kogan *et al.* [81]. In this method, 10 mL urine was hydrolyzed with NaOH, then the pH was adjusted to pH 1–3 and extracted with Bond-Elut THC columns. THC-COOH was eluted with acetone. Methylene chloride was added to the eluate, the mixture was vortexed, and the upper layer removed. The lower layer was then partitioned with hexane to get rid of any remaining water, the hexane was evaporated, and the residue reconstituted with 10 μL acetone and spotted on a TLC plate. The developing system was ethyl acetate–methanol–water-conc. ammonia (12:5:0.5:1) and the spraying reagent was Fast Blue RR. This method could be used as a confirmation method for the EMIT cannabinoid drug screen procedure.

The visualization step was modified by spraying the plate after developing with concentrated ammonium hydroxide then with Fast Blue RR spray [65]. The base intensified the color and made visualization of THC-COOH instantaneous. The authors used the modified method for confirmation of EMIT d.a.u. and Abuscreen RIA urine cannabinoids immunoassays, and the results were compared with GC-MS. The non-instrumental BPA-TLC assay was simpler to perform and interpret than the

GC-MS and could be used for qualitative confirmation of THC-COOH in urine after screening with immunoassays.

Vereby *et al.* [82] applied the method of Kogan *et al.* [65] to the confirmation of 100 urine samples which screened positive for cannabinoids by EMIT d.a.u. Another modification of the method was done by Vu Duc [25] who quantitated the method using scanning densitometry at 485 nm, and used petroleum ether (40–60°C): diethyl-ether–glacial acetic acid (5:5:0.1) as the developing system to obtain better separation of THC-COOH and 11-OH-Δ^9-THC. The author also reported that the thin layer plates could be stored in a freezer, wrapped in aluminum foil, for further analysis by GC-MS. This could be done by scraping the spots corresponding to THC-COOH and eluting with ethyl acetate followed by derivatization with TMS. This was advantageous since two confirmation methods could be applied to a single urine specimen.

High efficiency TLC (HETLC) together with an HPLC technique was used by Black *et al.* [26] for confirmation of EMIT urine cannabinoid assay. The method used for the isolation of THC-COOH from urine samples was that developed by ElSohly *et al.* [83] and consisted of the addition of an internal standard, followed by basic hydrolysis, then extraction on a Bond-Elut-THC column. Elution was done with acetonitrile. For HETLC, the eluant was evaporated and the residue reconstituted with methanol and applied on an HETLC plate. The plate was developed using hexane–acetone–glacial acetic acid as mobile phase, and the spots were visualized using an alkaline solution of Fast Blue B salt as the spraying reagent. The results of HPLC and HETLC were always in agreement, suggesting the use of HPTLC as a confirmatory technique for EMIT.

Another HPTLC procedure for the detection of THC-COOH in urine was described by Meatherall and Garriott [84]. This method involved alkaline hydrolysis of the urine sample followed by extraction of THC-COOH from acidified solution with hexane. The hexane was evaporated and the residue reconstituted with 50 μL of $CHCl_3/CH_3OH$ and spotted onto a Kieselgel 60 HPTLC. Development was done using heptane–butanol–acetic acid (90:9:1) as mobile phase, and visualization was done by sequential dipping of the plate in diethylamine, then in 0.1% Fast Blue BB solution. Cannabinol (CBN) was used as internal standard; although the R_f for THC-COOH and CBN were variable, the RR_f was consistent. Fast Blue B, Fast Blue RR, and Fast Blue BB were tried as visualizing reagents, and no differences in the color intensity were observed. Fast Blue B and RR dissolved more slowly in water and imparted a yellow background to the plate. Moreover, Fast Blue B is a potential carcinogen; therefore, 0.1% solution of Fast Blue BB was chosen for routine use.

A qualitative TLC method for the identification of cannabis metabolites in human urine was described by Haensel and Stroemmer [85]. Quantitation of THC-COOH can be done using densitometry [86].

5.2.2.2 High performance liquid chromatography

Combining the separating power of HPLC with different detectors has led to the development of several methods that can be used for the detection of cannabinoids in urine samples.

HPLC with immunoassay detection is a powerful tool that couples the specificity and the separation power of HPLC and the sensitivity of the RIA. It was first introduced by Twitchett *et al.* [87] and was used for the analysis of LSD in body fluids. The combined technique was then used for the analysis of THC and its metabolites in urine and plasma [88–90]. The coupling of the two techniques overcame the problems of cross-reactivity of the RIA and allowed the use of a sensitive and relatively non-specific antigen in the RIA [90]. For the analysis of urine, hydrolyzed samples were injected onto the HPLC column and a stepped solvent elution program was used. The concentrations of THC, CBN, mono-hydroxylated metabolites, di-hydroxylated metabolites, Δ^9-THC-11-oic acid, Δ^9-THC-11-oic acid ester glucuronide can be quantified in the eluting fraction by RIA. The method cannot be used, however, for routine use because of the low sample throughput. A modified method using single acidic elution instead of the stepped gradient elution [91] and a ^{125}I-RIA method [15] was then used by Law *et al.* [16] for the confirmation of cannabis use by the analysis of blood and urine samples. Peat *et al.* [92] studied the HPLC-IA profiles for the analysis of cannabinoid metabolites in urine samples. The samples were chromatographed on a reverse-phase system using a gradient of acetonitrile in water (pH 3.3). Four different antisera, three different RIA procedures, and one EMIT were used for the detection of the eluting fractions.

An HPLC method with UV detection for the determination of THC-COOH was developed by ElSohly *et al.* [83]. Hydrolyzed urine samples were cleaned up using Bond-Elut®-THC columns, and then injected on a reverse-phase column with acetonitrile–phosphoric acid (50 mM) (65:35) as the mobile phase. The clean-up procedure using Bond-Elut® columns had the advantages of saving time and reagents, and the final eluate was clean and could be injected directly onto the HPLC column without evaporation or derivatization. The HPLC method described was rapid and reproducible and could be used as an alternative to GC. This method was compared with four other previously published methods, namely, RIA, EIA, GC/ECD, and GC-MS [61] and was adopted by Black *et al.* [26] for the confirmation of positive results obtained using the EMIT Urine Cannabinoid assay.

Preliminary sample preparation using SPE methods followed by HPLC analysis with UV detection was also used by many authors [93–96].

Bourquin and Brenneisen [93] used Bond-Elut®-THC-SPE columns for the isolation of THC-COOH, which was analyzed by HPLC on C_8 column using acetonitrile–aqueous phosphoric acid (50 mM) (68.5:31.5) as eluting solvent followed by photodiode-array detection. The method was used to confirm 100 urine samples screened positive by immunoassays.

Parry *et al.* [94] used Supelclean DrugPak-T SPE tubes for the isolation of THC-COOH from urine samples prior to analysis by HPLC or GC and reported absolute and relative recoveries higher than 85% and 92%, respectively. HPLC analysis was then performed using a C_{18} column and 55:45 mixture of acetonitrile and 2% acetic acid in water as the mobile phase followed by UV detection at 280 nm.

Ferrara *et al.* [95] used various types of SPE columns for the isolation of metabolites of drugs of abuse from urine samples. Adsorbex RP8 100-mg columns

(Merck) were used for the isolation of THC-COOH and chromatographic separation was done on C_8 column using 0.05 M phosphoric acid–acetonitrile (35:65, v/v) as the mobile phase.

Bianchi and Donzelli [96] used disposable C_{18} SPE cartridges (100 mg) from Bio-Rad Labs and a reversed-phase column with acetonitrile–phosphate buffer (0.125 M) (55:45) as the mobile phase. The proposed method was reported as being precise, sensitive, and linear over a wide range of concentrations, did not require more than 30 min, and could, therefore, be used for routine analysis of large numbers of samples.

THC-COOH can be determined in urine samples by a combination of liquid chromatography with UV detection and gas chromatography (GC) with electron-capture detection [97]. Delta-8-THC-11-oic acid was used as the internal standard, and the pentafluoropropyl-pentafluoropropionyl derivatives were used for GC. HPLC served as a clean-up step for the GC analysis, leading to an increase in the selectivity and sensitivity of the method. Moreover, the LC step could be used alone for the determination of THC-COOH in high concentrations. However, HPLC remained a sophisticated tool for use in sample clean up; therefore, another procedure was presented by L. Karlsson [98]. The author described a fully automated HPLC system in which hydrolyzed urine samples were directly injected onto a CN pre-column, followed by chromatographic separations on two different columns (CN and C_8) in series by means of a column-switching technique. Two detectors were used: an UV detector after the first column, and an electrochemical detector after the second column. This method was reported to have the advantages of selectivity, low detection limit (2 ng/mL), and minimum sample pre-treatment; however, a long time was needed for each run and the sample throughput was therefore low (two urine samples per hour).

Another HPLC method with EC detection for the determination of THC metabolites in urine was presented by Nakahara *et al.* [99]. The method involved automatic sample extraction with ODS-minicolumns followed by separation of THC, THC-COOH, and 11-OH-THC on a reversed-phase silica C8 column with acetonitrile–methanol–H_2SO_4 (0.02 N) (35:15:50) as the mobile phase. The method was linear in the concentration range of 10–500 ng/mL, and the limit of detection was 0.5 ng/mL.

5.2.2.3 Liquid chromatography/mass spectrometry

Weinmann *et al.* [100] developed a method using automated SPE and LC coupled to tandem mass spectrometry (LC/MS-MS) with negative atmospheric chemical ionization (APCI) for the detection of THC-COOH in urine samples. Prior to SPE, conjugates of THC-COOH were hydrolyzed. No derivatization step was needed and the run time was 6.5 min. Thus, this method reduces the sample preparation step and also provides a shorter analysis time. The LoD and LLoQ were 2.0 and 5.1 ng/mL, respectively. Another method was developed for the detection of THC-COOH and THC-COOH-glucuronide [101]. THC-COOH and THC-COOH-glucuronide were extracted in one step using ethyl acetate–diethylether (1:1, v/v). The generation of

molecular ions of THC-COOH (MH^+, m/z 345) and THC-COOH-glucuronide (MH^+, m/z 521) was achieved using a PE/SCIEX turboionspray source in positive ionization mode. THC-COOH-d_3 was used as the internal standard.

5.2.2.4 Gas chromatography

5.2.2.4.1 Gas chromatography/flame ionization detection

Irving *et al.* [60] used gas–liquid chromatography with FID and GC-MS for the confirmation of the positive results of immunoassays. The authors concluded that the GLC/FID method was not sufficiently sensitive, and a more sensitive assay was needed if higher confirmation rates were to be attained.

Parry *et al.* [94] used GC/FID for the analysis of urine samples after extraction using Supelclean DrugPak-T SPE tubes and derivatization with BSTFA.

5.2.2.4.2 Gas chromatography/electrochemical detection

ElSohly *et al.* [102] developed a gas chromatographic/electron-capture detection GC/ECD procedure for the determination of THC-COOH in urine samples. Samples were hydrolyzed with 10 N KOH, shaken with 2 mL hexane–ethyl acetate (7:1), and the organic phase was discarded. The pH of the aqueous phase was adjusted to 2–2.5 and the THC-COOH and CBN-COOH (used as internal standard) were extracted with hexane–ethyl acetate (7:1). Derivatization was done with pentafluorobenzyl bromide (PFBBr) in a biphasic system using benzyl tributylammonium hydroxide as a phase transfer catalyst. Jones *et al.* [61] compared the previously described procedure with four other published methods, namely RIA, EIA, HPLC, and GC-MS. The described procedure was sensitive, accurate, and reproducible and needed only a small volume of urine. Another GC/ECD for the determination of THC-COOH in human urine was presented by Rosenfeld *et al.* [103]. They increased the specificity of the assay by selective derivatization of the phenolic group using PFBBr in pentanol in alkaline medium (0.1 N NaOH), and by purification by chromatography on XAD-2 resin to produce an extract almost free from interference.

Micellar electrokinetic capillary chromatography (MECC) with on-column multiwavelength detection was used for the analysis of THC-COOH in urine samples. This technique required concentrated samples; therefore, the extraction and concentration steps were very important for the analysis. Four different SPE columns, namely, Bond-Elut THC cartridges, Bond-Elut Certify II columns, Clean Screen THC columns, and Bond-Elut Certify columns were investigated. The first two SPE columns provided a simple and clean electropherogram but the recovery of THC-COOH was low. Clean Screen THC and Bond-Elut Certify columns provided a more complex electropherogram but the peak corresponding to THC-COOH was well separated and the extraction efficiency was good ($80 \pm 10\%$). Therefore, these columns were used for the confirmation of urine samples screened positive by FPIA [12].

5.2.2.5 Gas chromatography-mass spectrometry

GC-MS is the method of choice for the confirmation of cannabinoids in urine [104]. It has the highest sensitivity and specificity of all the techniques. GC-MS methods are usually used as reference for evaluating other cannabinoid assays [2].

A modified GC-MS procedure for the detection of past and recurrent marijuana use was described by Joern *et al.* [105]. The method, a modification of the methods of Karlsson *et al.* [106] and Foltz *et al.* [107], included preparing the standards in alkaline solution to minimize adsorption onto glass and plastic surfaces and using potassium hydroxide–methanol (1:4) for hydrolysis to obtain a cleaner extract. The internal standard was d_3-THC-COOH, and the derivatizing agents used were pentafluoropropionic anhydride (PFPA) and pentafluoropropanol (PFPOH). The new GC-MS method was reported to be more indicative of recent marijuana use than the EMIT semi-quantitative concentration values. Stout *et al.* [108] used PFPA and PFPOH for derivatization of THC and THC-COOH in the evaluation of the performance of d_3-THC-COOH and d_9-THC-COOH as internal standards. The method utilized a positive pressure manifold anion-exchange polymer-based SPE, which was followed by elution directly into the automated liquid sampling (ALS) vials. The LoD for THC-COOH was 0.875 ng/mL by GC-MS. To answer the question of whether a positive drug test for marijuana was the result of the sole use of Marinol®, ElSohly *et al.* [109] used Tetrahydrocannabivarin-9-carboxylic acid (THCV-COOH) as a marker for marijuana ingestion. THCV is the C3 homolog of THC, commonly found as a companion cannabinoid to THC in the cannabis plant, which is metabolized by human hepatocytes to THCV-COOH.

Needleman *et al.* [110] developed a liquid–liquid extraction method followed by GC-MS for the determination of THC-COOH in urine. The extraction procedure used isobutanol–hexane (1:9) for initial extraction from urine samples followed by back extraction into 0.1 N NaOH. The aqueous layer was again extracted with methylene chloride, which was evaporated to dryness. The sample was derivatized with tetramethylammonium hydroxide–dimethyl sulfoxide (1:1) followed by the addition of iodomethane.

Clouette *et al.* [111] developed a GC-MS with electron ionization mode for the determination of THC-COOH utilizing its *t*-butyldimethylsilyl derivative. Trideuterated THC-COOH was added to the samples followed by alkaline hydrolysis and extraction with hexane–ethyl acetate (7:1.5) from acidic solution. Derivatization was done with MTBSTFA at 110°C for 15 min. The derivative obtained was more stable than the trimethylsilyl derivative and could be used for routine analysis of THC-COOH in urine samples.

Most of the GC-MS procedures developed focused on the determination of THC-COOH as a marker for marijuana use, with little or no attention given to other metabolites. Kemp *et al.* [112,113] developed a GC-MS method for the simultaneous determination of THC and six of its metabolites, namely, 8α-OH-THC, 8β-OH-THC, 11-OH-THC, 8α,11-diOH-THC, 8β,11-diOH-THC, and THC-COOH, in addition to CBN and cannabidiol (CBD). The different steps described in the procedure were optimized to achieve cleaner extracts, maximum recovery of the analytes and

adequate chromatographic resolution of the extracted compounds. Therefore, the influence of hydrolysis conditions (base hydrolysis or enzyme hydrolysis, enzyme concentration and incubation time), solvent combinations used for extraction, and type of derivatizing agent were studied. Optimum results were obtained using enzyme hydrolysis with 5000 units of bacterial β-glucuronidase from *Escherichia coli* incubated at pH 6.8 for 16 h [113]. Extraction was done with hexane–ethyl acetate (7:1) and derivatization was done with BSTFA in 1% TMCS.

Szirmai *et al.* [114] described a GC-MS method for the determination of three major acidic metabolites of Δ^1-THC, namely, THC-7-oic acid, 1,4″, 5″-bisnor-Δ^1-THC-7,3″-dioic acid, and 4″-hydroxy-Δ^1-THC-7-oic acid. Five derivatization systems (CH_2N_2-BSTFA, CH_2N_2-MBTFA, BSTFA, TFE-PFPA, and TMAH-methyl iodide) were examined.

All the procedures previously mentioned used liquid–liquid extraction method for the isolation of THC metabolites from urine samples. SPE methods were developed in an attempt to produce cleaner and more concentrated extracts. Comparison between four extraction procedures for the isolation of THC-COOH from urine samples was presented by Congost *et al.* [115]. The procedures presented were two solid–liquid methods and two liquid–liquid methods. The first solid–liquid procedure used octadecylsilane-bonded silica resin while the second procedure used an ion exchange (NH_4^+ Cl^- resin). In one liquid–liquid procedure, the acidified urine samples were extracted with hexane–ethyl acetate (7:1), the organic layer was extracted with alkali, and the solution was acidified and re-extracted with hexane–ethyl acetate (7:1). The other liquid–liquid extraction method involved a one-step extraction with hexane–ethyl acetate (9:1) from alkaline solution. The best results were obtained with the last procedure. The authors also suggested a derivatizing agent consisting of a mixture of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), trimethyliodosilane (TMIS), and dithioeritrithol (100:0.2:1, v/v/w) and compared it with MSTFA.

SPE methods are gaining increasing use in sample preparation techniques, and many publications appear each year utilizing and/or evaluating SPE cartridges. Nakamura *et al.* [116] used Sep-PAK cartridges for clean-up of urine samples prior to GC-MS analysis. McCurdy *et al.* [117] used C_{18} bonded-phase adsorption (BPA) columns for the extraction of THC-COOH in evaluating the suitability of the ion-trap detector for the detection of THC-COOH, while Paul *et al.* [118] used cartridges containing strongly basic anion-exchange resin (E.I. Du Pont de Nemours & Co) for the detection of THC-COOH using GC-MS. Supelclean DrugPak-T SPE tubes were evaluated by Parry *et al.* [94], CLEAN SCREEN® reduced solvent volume (RSV) SPE columns were evaluated by O'Dell *et al.* [119], and Empore extraction disk cartridges (C_{18}) were evaluated by Singh and Johnson [120]. The Toxi-lab SPEC extraction discs were used by Wu *et al.* [121] for the extraction and simultaneous elution and derivatization of THC-COOH to produce the trimethylsilyl derivatives.

Quantitative interpretation of the results of chromatographic methods necessitates the use of internal standards like 11-nor-9-carboxy-CBN [61,83,102], CBN [93], oxyphenbutazone [122], and ketoprofen [115]. The most commonly used internal standard is the trideuterated derivative of Δ^9-THC-COOH [105,111,112,119,120]. The trideuterated isomer has the disadvantage of having a fragment in common with

the natural metabolite at m/z 316 when using the methyl derivative [123]. This results in distortion of the ion ratio of the internal standard and limits the dynamic range of the analysis. Therefore, ElSohly *et al.* [123,124] developed a new internal standard, hexadeutero-Δ^8-THC-9-COOH, having the advantages of a wider linear dynamic range and having no common ion with THC-COOH using different derivatives. The d_6-THC-COOH was used by Wu *et al.* [121] for the analysis of THC-COOH in urine samples by GC-MS.

A new internal standard, $^2H_{10}$-Δ^1-THC-7-oic acid was evaluated by Szirmai *et al.* [114] and can be used as an alternative to the previous internal standards. Stout *et al.* [108] evaluated the performance of d_3-THC-COOH and d_9-THC-COOH as internal standards. The authors determined that d_9-THC-COOH was the preferred internal standard for their method.

5.3 ANALYSIS OF CANNABINOIDS IN BLOOD

The analysis of cannabinoids in blood is an alternative to urine analysis, where THC and its metabolites can be detected for a relatively short time after intake. Therefore, the detection of THC along with its metabolites indicates the recent use of cannabis and their levels may correlate with an actual state of intoxication.

5.3.1 Immunoassays

Immunoassay methods for screening blood samples for cannabinoids are now widely used. The methods employed are often based on the use of those tests primarily developed for use with urine samples.

5.3.1.1 Enzyme multiplied immunoassay techniques

In 1978, E. L. Slightom [125] first reported the application of homogenous EIA to the analysis of drugs in biological fluids other than urine. This was followed by many attempts to refine the EMIT assays for use with blood samples.

Asselin *et al.* [126] described a simple method for the detection of THC in methanolic extract of blood using EMIT d.a.u. cannabinoids urine assay. This method had the advantage of requiring only 1 mL of whole blood, and it also avoided the lengthy extraction procedure previously used. The results obtained encouraged many authors to use methanolic blood extracts for the detection of cannabinoids [127–129].

Perrigo and Joynt [127] made two modifications in the procedure suggested by Syva in the 3M619 Kit product literature to improve the sensitivity of the assays. These modifications included increasing the amount of the sample in the measurement kit and increasing the flow cell temperature. Coupling the advantages of using the methanolic blood extraction procedure with those of using an automatic analyzer allowed the processing of a large number of samples in a short period of time

and at low cost. Moreover, the small volume requirements of the automatic analyzer resulted in a five to ten-fold drug enrichment [129,130].

The addition of *N*,*N*-dimethylformamide (DMF) to serum, plasma, or blood resulted in a clear, colorless supernatant, which does not cause light scattering or irrelevant absorbance in the spectrophotometric measurements of the EMIT analysis [131].

Another procedure for the extraction of THC metabolites from whole blood was suggested by Lewellen and McCurdy [132]. This procedure involved precipitation of the blood proteins with acetone, followed by evaporation and reconstitution of the residue in a 1:1 ratio of EMIT buffer and methanol.

5.3.1.2 Fluorescence polarization immunoassays

Bogusz *et al.* [133] determined drugs of abuse in whole blood by FPIA (FPIA–Abbott TDx and ADx) after protein precipitation with acetone. The results obtained were compared with the acetone precipitation EMIT d.a.u. method. The authors concluded that FPIA was less influenced by matrix effects and was not affected by the decomposition of blood, which means that it could be utilized to analyze autopsy blood samples.

FPIA was also used for the analysis of blood samples for the presence of cannabinoids, and the confirmation and quantitation of THC, 11-hydroxy-THC, and 11-nor-9-carboxy-THC was done by GC-MS [134].

5.3.1.3 Radioimmunoassays

RIA were also used for the determination of THC and THC-COOH in blood and serum samples [15,135]. Hanson *et al.* [135] compared ^{3}H- and ^{125}I-RIA and GC-MS for the determination of cannabinoids in blood and serum. They concluded that both RIA methods could be used to detect THC and THC-COOH, and that serum was a better specimen than blood in terms of accuracy, sensitivity, reproducibility, and specificity.

Moody *et al.* [136] compared the results obtained for the analysis of cannabinoids by RIA using methanol-extracted blood with those obtained using non-extracted blood. The results of both methods were compared with GC-MS analysis. Both procedures were qualitatively similar, but the methanol extract procedure proved to be superior in providing semi-quantitative results that could be correlated with those obtained by GC-MS.

5.3.1.4 KIMS assays

Moody and Medina [137] used the Roche OnLine® KIMS assay to detect cannabinoids in serum. They modified the KIMS method used by Armbruster *et al.* [45] for the detection of abused drugs in urine. Modifications were made to increase the sensitivity of the assay because drug concentrations in serum are usually lower than in urine. Direct measurement of unextracted sera was not possible. Therefore, extraction of the samples was done by the addition of 7 mL of chloroform–isopropanol

(9:1), the organic phase was then separated, dried, and the residue was reconstituted with ethanol and potassium phosphate (pH 7.4).

5.3.1.5 Enzyme-linked immunosorbent assays

THC metabolites can be detected by ELISA [42,138]. When ELISA procedures were applied to the detection of drugs of abuse in whole blood, they were found to be more sensitive and less time-consuming than the EMIT procedures [138].

5.3.1.6 Cloned enzyme donor immunoassay

Another type of immunoassays used for the analysis of cannabinoids in whole blood is the Microgenics CEDIA DAU. Cagle *et al.* [139] compared the CEDIA DAU assay (EIA) and the Abbott AxSym system (FPIA) for the analysis of whole blood. Protein precipitation with acetone was used for the CEDIA assay, while for the FPIA addition of acetonitrile at a ratio of 1:2 (blood–acetonitrile) was found to give the best results. The results obtained were confirmed by GC-MS, which was found to correlate better with FPIA ($r = 0.75$) than with EIA($r = 0.22$).

5.3.2 Chromatographic methods

5.3.2.1 Thin layer chromatography

Quantitative separation and analysis of THC, CBN, and CBD can be done by separation on silica gel HPTLC plates followed by densitometric scanning of the separated compounds [140]. This procedure, however, uses two extraction steps, initial SPE using C_{18}-Sep-Pak cartridge. The eluate obtained was evaporated, reconstituted with acetone and derivatized with dansyl chloride. The dansyl derivatives were then extracted with diethyl ether. The final extract, almost free of interfering compounds, was then spotted on HPTLC plates and developed using isooctane–ethyl acetate–acetic acid (30:10:1).

5.3.2.2 High performance liquid chromatography

Law *et al.* [16] described a method for the confirmation of cannabis use by the analysis of blood and urine samples by combined HPLC and RIA. This method, which resulted from the modification and improvements of already published methods [88,89,91], coupled the separation power of HPLC and the sensitivity of RIA. It allowed the complete analysis of at least six samples per day and could, therefore, be used for routine toxicological analysis of Δ^9-THC-11-oic acid and its glucuronide derivative in methanol extracts of blood samples.

HPLC with ECD (HPLC/ECD) was also used for the analysis of plasma samples [99,141]. Both methods utilized a preliminary SPE. Zweipfenning *et al.* [141] used Bond-Elut C_{18} SPE columns for the isolation of THC, followed by HPLC analysis on C_{18} column using tetrahydrofuran–methanol–sodium citrate

buffer (0.005 M), pH 7.0 (7.5:68:24.5, v/v) as the mobile phase. Nakahara *et al.* [99] used an automatic extractor equipped with ODS-minicolumn for the extraction of THC and its major metabolites (THC-COOH and 11-OH-THC), followed by analysis on Zorbax C_8 column using a mobile phase composed of acetonitrile–methanol–H_2SO_4 (0.2 N) (35:15:50).

5.3.2.3 Liquid chromatography-mass spectrometry and LC-MS/MS

Guinea pig plasma was analyzed by LC-MS using negative mode electrospray ionization detection for Δ^8-THC and Δ^8-THC-COOH [142]. Yang and Xie [143] used solid-phase microextraction membrane (SPMEM) and detected THC and CBD in blood and brain of injected male mice, and in spiked human urine by using LC-MS. Maralikova and Weinmann [144] used LC-MS/MS for the detection of THC, 11-OH-THC, and THC-COOH in human plasma. Automated silica-based SPE was used for sample clean up. LC-MS/MS was equipped with a turbo ion spray interface and triple quardrupole mass analyzer using positive electrospray ionization and multiple-reaction monitoring. The LoD was 0.2 ng/mL for THC and 11-OH-THC and 1.6 ng/mL for THC-COOH, while LoQ was 0.8 ng/mL for THC and 11-OH-THC and 4.3 ng/mL for THC-COOH.

5.3.2.4 Gas chromatography

GC with electron-capture detector was used for the determination of CBD, the most abundant cannabinoid in hashish and in fiber-type *Cannabis*, in plasma [145]. Tetrahydrocannabidiol was used as the internal standard. Liquid–liquid extraction with hexane-1.5% isoamyl alcohol was used. The extracts were concentrated, washed with NaOH, then with HCl, and evaporated to dryness. The pentaflurobenzyl derivatives were then analyzed by GC using an electron-capture detector.

Another liquid–liquid extraction method for the determination of THC in blood by GC with nitrogen selective detector was proposed by Ritchie *et al.* [146]. The procedure comprised hexane extraction of whole blood, followed by re-extraction into alkaline methanol, and derivatization of THC and the internal standard (Δ^8-THC) using 3-pyridinediazonium chloride solution. The mixture was then acidified and back extracted into hexane. The hexane was evaporated, and the residue was reconstituted with methanol. The phenolic groups of THC and the internal standard were methylated by on-column flash alkylation with TMAH and then injected onto the GC.

A solid support reagent, consisting of PFBBr deposited upon XAD-2 resin, was used to extract and derivatize Δ^9-THC, 11-hydroxy-Δ^9-THC, and 11-nor-9-carboxy-Δ^9–THC from plasma samples. The pentafluorobenzyl derivatives could then be analyzed by GC/ECD or GC-MS/ NICI [147].

5.3.2.5 Gas chromatography-mass spectrometry

GC-MS methods are the most widely used confirmatory techniques for the detection of cannabinoids in whole blood, serum, or plasma. Sample clean up before analysis is necessary and is usually done by liquid–liquid extraction [17,112,135,136, 148–150], or by SPE [151,152].

Derivatization of the samples is also necessary. Hanson *et al.* [135] utilized trimethylphenyl ammonium hydroxide to form the methyl derivative of THC, which was then analyzed by electron-impact selected ion monitoring GC-MS. Garriott *et al.* [148] used trimethylanilinium hydroxide as derivatizing agent for the determination of Δ^9-THC, 11-hydroxy-Δ^9-THC, and 11-nor-Δ^9-THC-9-carboxylic acid in blood. Trifluoroacetic anhydride derivatization procedure was used for the determination of THC in plasma using a GC-MS operated in the negative chemical-ionization mode and retrofitted with a High Energy Dynode detector system [149]. This detector improved the limit of detection of THC in plasma by 6.25fold, over that obtained with the same GC-MS system without the new detector. Moody *et al.* [136] compared RIA and GC-MS for the analysis of forensic blood specimens for cannabinoids. Blood specimens were analyzed by negative ion chemical ionization GC-MS with deuterated internal standards for the trifluoroacetyl derivative of THC and the methyl ester trifluoroacetyl derivative of THC-COOH. Bis(trimethylsilyl) trifluoroacetamide (BSTFA) was used for derivatization of THC-COOH by Clatworthy *et al.* [17] for the development of a GC-MS method for the detection of THC-COOH in blood, and by Kemp *et al.* [112] for the analysis of THC and six metabolites, namely, 8α-hydroxy-Δ^9-THC, 8β-hydroxy-Δ^9-THC, 11-hydroxy-Δ^9-THC, 8α-11-dihydroxy-Δ^9-THC, 8β-11-dihydroxy-Δ^9-THC, and 11-nor-9-carboxy-Δ^9-THC. The method of Kemp *et al.* [112] had also the advantage of being able to detect CBD and CBN in plasma. Simultaneous quantitation of THC and THC-COOH in serum by GC-MS using tetrabutyl-ammonium hydroxide in DMSO was also reported [151]. Trimethylsilyl derivatization was also used for the determination of CBD in plasma utilizing GC/ion-trap mass spectrometry in positive ion chemical ionization mode [153].

The GC-MS-MS method was used to confirm the unusually high levels of THC in two postmortem samples [154]. In this method, electron-impact mass fragmentation of the trimethylsilyl derivatives yielded a full scan mass fragmentation pattern. The most abundant ions are again fragmented to produce another spectrum characteristic of THC.

Chi *et al.* [155] used PFPA in PFPOH derivatization for the analysis of THC in whole blood using GC-MS in electron-impact mode.

An automated SPE method (using Zymark RapidTrace SPE Workstation with a RSV SPE copolymer cartridge) was developed for the simultaneous extraction, confirmation, and quantitation of THC and THC-COOH from whole blood [156]. Quantitation was done by GC-MS using electron ionization mode with selected ion monitoring of 3 ions for each analyte. The LoD for THC and THC-COOH were 1.6 and 0.8 ng/mL while the LoQs were 2 and 1 ng/mL, respectively.

Steinmeyer *et al.* [157] validated a method for the quantification of THC, 11-OH-THC, and THC-COOH in serum. SPE was used to isolate the analytes, which were derivatized by methylation and analyzed in the selected ion mode using GC-MS. The LoD for THC, 11-OH-THC, and THC-COOH were 0.52, 0.49, and 0.65 ng/mL, respectively.

THC concentrations in human plasma from three individuals who smoked marijuana were 151, 266, and 99 ng/mL drawn immediately after the end of smoking while THC-COOH concentrations were 41, 52, and 171 ng/mL [158]. SPE was used for plasma samples, while trifluoroacetic anhydride and hexafluoroisopropanol were used for derivatization. THC and THC-COOH were detected using GC-MS in the negative ion chemical ionization mode with LoQs of 0.5 and 2.5 ng/mL for THC and THC-COOH, respectively.

Schutz *et al.* [159] developed a GC-MS method for the detection of THC, THC-CCOH, 11-OH-THC morphine, codeine, cocaine, benzoylecgonine, methylecgonine, cocaethylene, amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymetamphetamine, and *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine in small blood samples and blood stains using solid-phase SPE columns and a pipetting robot (Gilson Aspec XL). The LoDs are in the order of 0.15–0.82 ng/50 μL spot (cannabinoids), 1.62–4.10 ng/50 μL spot (amphetamines), 1.67–4.70 ng/50 μL spot (cocaine and derivatives), and 4.53–4.91 ng/50 μL (opiates). A GC-MS method was used for the analysis of THC, 11-OH-THC, THC-COOH, CBD, and CBN in plasma after oral application of small doses of THC and cannabis extract [160]. The LoDs were between 0.15 and 0.29 ng/mL for THC, 11-OH-THC, THC-COOH, CBD, and 1.1 ng/mL for CBN.

A GC-MS method was used for the detection of THC and THC-COOH in whole blood samples [161]. In this method, conventional solvent extraction was followed by a clean up using solid-phase cartridges. The LoD was better than 1 ng/mL with extraction efficiencies greater than 80% for THC and 70% for THC-COOH.

A simple extraction procedure for THC, and its three metabolites (11-OH-THC, THC-COOH, and 8β-11OH-diOH-THC) from urine, plasma, and meconium was developed based on immunoaffinity chromatography [162]. Using the affinity resin prepared by immobilization of THC antibody onto cyanogen bromide-activated Sepharose 4B, THC and its three metabolites were extracted from urine and plasma. The same procedure was used for analysis of meconium with some modifications. After derivatization of the samples, GC-MS was used for analysis in the electron impact ionization (EI) mode with SIM monitoring. The LoDs ranged from 0.5 to 2.5 ng/mL in plasma and urine and from 1.0 to 2.5 ng/g in meconium. The extraction recovery from meconium, however, was lower than that of plasma and urine, ranging from 52 to 72% at 10 ng/g level.

SPE (C_{18}) cartridges were used to extract THC, 11-OH-THC and THC-COOH from serum and their trimethylsilyl derivatives were analyzed by GC-MS-MS system based on an ion trap with external ionization [163]. The quantitation of three analytes was achieved in relation to trideuterated internal standards in dual MS-MS

mode. Confirmation of these analytes was done by registering the daughter spectra in full scan mode. The LoDs for THC, 11-OH-THC, and THC-COOH were 0.25, 0.5, and <2.5 μg/L, respectively.

For the identification and quantification of THC in rabbit plasma, two ionization techniques were utilized for GC-MS [164]. EI (TMS derivatized) was used after intravenous administration, while negative chemical ionization (NCI) (TFA derivatized) was used after sublingual administration with deuterated internal standard in both cases. The method was successful in analyzing THC from rabbit plasma.

The method used by Richard *et al.* incorporates *E. coli* β-glucuronidase hydrolysis of plasma samples to cleave glucuronic acid moieties and simultaneous SPE of THC, 11-OH-THC, and THC-COOH [165]. After addition of deuterated analogs for each analyte as internal standards, quantification was done on a bench top positive chemical ionization (PCI) GC-MS. LoDs for THC, 11-OH-THC, and THC-COOH were 0.5, 0.5, and 1.0 ng/mL. Plasma samples were collected from individuals participating in a controlled oral THC administration study and analyzed by this method.

5.4 ANALYSIS OF CANNABINOIDS IN HAIR

Hair is another sample that can be analyzed for the presence of drugs of abuse. Drugs persist in hair for months after consumption; therefore, hair analysis can be used as a tool for detection of drug use in forensic sciences, in traffic, and occupational medicine and in clinical toxicology [166,167]. Balabanova *et al.* [168] was the first author to publish a method for the RIA detection of cannabinoids in hair followed by GC-MS confirmation of Δ^9-THC. However, this paper was subject to criticism because the SIM chromatograms shown in the publication were very poor [169,170]. Since this time, many papers have been published describing the use of GC-MS methods for the detection of cannabinoids in hair samples. THC-COOH was determined in hair by GC-MS after alkaline hydrolysis and extraction from acid solution on Baker C_{18} columns, followed by derivatization with methyl iodide [171] or with PFPA and pentafluoropropionyl alcohol (PFP-OH), with levallorphan as the internal standard [172]. Alternatively, liquid–liquid extraction and deuterated internal standards were used for the determination of THC-COOH in hair [173] and for the determination of THC and THC-COOH in human hair and pubic hair [174]. In both methods, hair samples were first decontaminated with methylene chloride, then pulverized and incubated in NaOH to destroy the protein matrix of the hair. Samples were then extracted with *n*-hexane–ethyl acetate (9:1) after acidification with acetic acid. The organic phase was washed with 1 mL 0.1 N NaOH followed by 1 mL 0.1 N HCl, then evaporated to dryness and derivatized with PFPA and PFP-OH. Young *et al.* developed an analytical method for the evaluation of CBD, CBN and THC level in human hair using GC-MS [175].

Hair samples were washed with isopropanol and, after the addition of deuterated internal standard, the hair samples were incubated in 1.0 M NaOH for 10 min at 95°C. These hydrolyzed (digested) samples were then extracted with *n*-hexane–ethyl

acetate (7.5:2.5), evaporated, derivatized, and analyzed by GC-MS. Baptista *et al.* [176] used β-glucuronidase/aryl sulfatase for hydrolysis and found that, for the quantification of THC-COOH, GC-MS-NCI (negative ion chemical ionization mode) using methane gas as reagent gas is more sensitive than the GC-MS-EI method, which may give rise to false negatives.

Cirimele *et al.* [177] proposed a simpler method for the simultaneous identification of THC, CBN, and CBD in hair samples, using THC-d_3 as the internal standard. This method is a rapid screening method that does not require derivatization prior to analysis. Jurado *et al.* [178] described a method for the simultaneous quantification of opiates, cocaine, and cannabinoids in hair. In this method, the sample was decontaminated with dichloromethane, then two consecutive hydrolyses were done: the first is an acid hydrolysis followed by organic solvent extraction of opiates and cocaine; this is followed by alkaline hydrolysis and extraction of the cannabinoids with organic solvent after addition of maleic acid. Wilkins *et al.* [179] utilized a liquid–liquid extraction procedure prior to quantitative analysis of THC, 11-OH-THC, and THC-COOH in human hair by GC-MS. The extraction procedure included digestion of the sample with NaOH, followed by extraction with hexane–ethyl acetate (9:1v/v), the organic phase was then further extracted for THC and 11-OH-THC and the aqueous phase was used for THC-COOH. Sabina and Maecello described a method for application of solid-phase microextraction (SPME) to cannabis in hair [180]. Hair samples were washed with petroleum ether, hydrolyzed with NaOH, neutralized, deuterated internal standard was added and directly submitted to SPME. The SPME elute was analyzed by GC-MS. The LoD for both CBN and THC was 0.1 ng/mg while CBD had 0.2 ng/mg LoD.

A GC-MS-MS method was used by Mieczkowski [181] for the confirmation of the presence of THC and THC-COOH in hair samples screened by RIA for cannabinoids. He concluded that although RIA screening of hair samples for cannabinoids is efficient, the results should be confirmed by GC-MS-MS methods.

Sachs and Dressler developed a method for the detection of THC-COOH in hair by GC-MS after HPLC clean up [182]. After the sample was digested with 2 M NaOH at 95°C and the neutralized liquid was extracted with a mixture of *n*-hexane and ethyl acetate, the dried residue was reconstituted in acetonitrile–methanol–sulfuric acid (0.01 M) (49:21:30, v/v/v) and the cannabinoids were separated by HPLC, derivatized and analyzed by GC-MS. The LoD and LoQ for THC-COOH were 0.3 and 1.1 pg/mg, respectively.

Musshoff *et al.* [183] developed a fully automated procedure using alkaline hydrolysis and headspace SPME (HS-SPME) followed by on-fiber derivatization with *N*-methyl-*N*-trimethylsilyl-trifluoroactamide (MSTFA) and detection of cannabinoids by GC-MS. The authors concluded that this automated HS-SPME-GC-MS procedure is substantially faster than the conventional methods of hair analysis. Headspace solid-phase dynamic extraction (HS-SPDE) was also used for detection of cannabinoids in human hair samples [184]. SPDE is a further development of SPME, based on an inside needle capillary absorption trap.

5.5 ANALYSIS OF CANNABINOIDS IN MECONIUM

The analysis of meconium for the presence of drugs of abuse has gained interest in recent years. It is now a widely accepted alternative to infant's blood and maternal urine to detect prenatal exposure to these drugs. Although meconium appears to be a more difficult sample to analyze because of the additional steps required to disrupt the tissues and to extract and clean up the samples, it has the advantages of being easier to collect than blood and urine, and it increases the window of detection to the last months of gestation [185].

Ostrea *et al.* [186–188] were the first authors to publish methods for the screening of drugs of abuse in meconium. The analysis of cannabinoid metabolites in meconium was done by mixing the sample with methanol, allowing it to stand at room temperature for 10 min, then centrifuging and testing the supernatant for cannabinoid metabolites by RIA [188]. The authors analyzed the meconium and urine of 20 infants of drug-dependent mothers for the metabolites of heroin, cocaine, and cannabinoids and concluded that meconium contains more drug metabolites than urine and is therefore more useful in detecting fetal exposure to drugs-of-abuse [188].

Nair *et al.* [189] used the procedure of Ostrea *et al.* [188] for the analysis of 141 meconium samples and also concluded that meconium is a superior sample than urine for the detection of fetal exposure to drugs.

EMIT was also used for the screening of meconium samples for the presence of cocaine, cannabinoids, opiates, and methadone [190]. The method consisted of extracting 0.5–1 g meconium with methanol and evaporating the extract to dryness. The residue was reconstituted with 1 mL methanol and divided into two portions, one used for the EMIT and the other saved for confirmation of the results by GC-MS. Comparison between meconium, maternal urine, and neonatal urine was also done and the authors found that maternal urine is more useful than meconium for the detection of THC metabolites [190].

FPIA followed by HPLC with diode-array detection was also used for the analysis of THC-COOH in meconium samples [191]. The extraction of THC-COOH from meconium samples was done with 5 mL water and one drop of NaOH and the supernatant was assayed by FPIA. For the HPLC method, the aqueous extract was partitioned with hexane–ethyl acetate (80:20), then the organic phase was evaporated and the residue reconstituted with the mobile phase, which is composed of acetonitrile–phosphoric acid (50 mM) (65:35), then injected onto a C_{18} column.

Another method for the determination of THC-COOH in meconium was presented by Moore *et al.* [192]. Extraction of meconium samples was done using acetic acid. Diphenylamine in acetone was then added and the mixture was centrifuged. The supernatant was filtered, evaporated to dryness, and the residue was reconstituted with the appropriate buffer and analyzed by FPIA. Confirmation of the results was done by GC-MS using deuterated internal standards and *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide as derivatizing agent.

One problem encountered in the determination of THC-COOH in meconium was the low confirmation rate. Wingert *et al.* [190] failed to confirm any of the positive specimens screened by EMIT, Moore *et al.* [192] reported a 20% confirmation rate

for samples analyzed by FPIA and confirmed by GC-MS, while ElSohly *et al.* [193] reported a 26% confirmation rate for samples screened by EMIT and confirmed by GC-MS.

A study of the elimination profile of Δ^9-THC in meconium was therefore conducted by ElSohly and Feng [194]. The authors found that in addition to THC-COOH, two other major metabolites of THC, namely 11-OH- 9-THC and 8β–11-diOH-Δ^9-THC, are found in meconium, mainly as their glucuronides. Enzymatic hydrolysis of meconium samples followed by determination of the three metabolites is therefore necessary to increase the confirmation rate of samples screening positive for cannabinoids by immunoassays.

Coles *et al.* [195] analyzed meconium samples for THC, 9-carboxy-THC, and 11-OH-THC using GC-MS with LoD of 5 ng/g for 9-carboxy-THC and 11-OH-THC with more than 66% recovery at 100 ng/g for both metabolites. A GC-MS method was developed for the analysis of 24 meconium specimens, which showed that 11-OH-THC is an important metabolite in meconium [162].

5.6 OTHER BIOLOGICAL SPECIMENS

Sweat, skin, saliva, and breath are other biological matrices that can be analyzed for the presence of cannabinoids.

Sweat and saliva are easier to collect than urine and blood but drug concentrations are lower and the window of detection is often shorter than urine. Their use may be of value for detecting if someone is driving while intoxicated and for surveying populations for illicit drug use [196].

RIA and mass spectrometry were used for the analysis of methadone, cocaine, THC, benzodiazepine, barbiturates, morphine, and cotinine in porcine sweat and the data obtained indicated depositions of those drugs in axillary hair [197]. The effect of pilocarpine stimulation on the concentration of THC in perspiration samples obtained from THC smokers was also determined [198]. The use of sweat patches for detection of drugs of abuse may be advantageous over urine analysis because the patch can be worn for a week without discomfort and can therefore provide a cumulative estimate of the degree of exposure to drugs for a whole week [199,200].

Skin swabs were also used for the detection of cannabinoids, opiates, and cocaine on the skin of drug abusers using an on-the-spot immunological test and GC-MS [201]. Drug residues on the hands of human subjects were also detected using a sampling method based on aspirating and trapping the drug microparticles on a filter plug followed by ion-mobility spectrometry [202].

Lemos *et al.* [203] evaluated fingernail clippings as analytical specimens for the detection and quantitation of cannabinoids. Detergent, water and methanol washes followed by alkaline hydrolysis and liquid–liquid extraction were used. The mean cannabinoid concentration in fingernail clippings of six known cannabis users was 1.03 ng/mg detected by RIA. When GC-MS was used, the mean THC concentration was 1.44 ng/mg in fingernail clippings of 14 known cannabis users. The average

THC-COOH concentrations in fingernail clippings of three known cannabis users was 19.85 ng/mg by GC-MS when extracted in acidic pH.

The detection of cannabinoids in breath and saliva may be particularly useful in traffic control where a non-invasive and simple method of sample collection is required.

The concentration of THC in breath ranges from 10 to 56 ng/sample taken 15 min after smoking and can be detected for about 1 h later [204]. A breath analyzer consisting of a tube containing Fast Blue Salt B, NaOH, and silica gel and a mouthpiece was developed by Volkmann *et al.* [205]. Consumption of hashish or marijuana can be detected by the color of the indicator changing to red when the person blows into the mouthpiece.

5.6.1 Oral fluid

In saliva, the concentration of THC may reach 1000 ng/mL after the administration of 5–20 mg THC and then fades to 50 ng/mL after 3–4 h [206]. Kircher and Parlar [206] developed an HPLC method for the determination of THC in human saliva. They prepared an immunoaffinity column by covalent immobilization of cannabinoid specific IgG on epoxy-activated silica and utilized it for sample clean up and enrichment. This was followed by the transfer of the cannabinoid fraction to an analytical RP column using a column-switching procedure. The authors were able to separate THC from CBN and CBD and achieved a limit of quantification of 20 ng THC/milliliter, using an UV detector at 220 nm.

The point-of-collection oral fluid drug testing devices Oratect (Branan) and Uplink (OraSure) were evaluated for their ability to detect cannabinoids, amphetamines, cocaine, and opiates [207]. For cannabinoids and cocaine, Drugwipe (Securtec) was also evaluated. The performance of all three devices in THC detection was poor, but Branan and OraSure detected well THC-COOH, amphetamine, methamphetamine, and opiates. Nine saliva specimens were positive for cannabis using the On-site OraLine® IV s.a.t. device, with THC concentrations ranging from 3 to 265 ng/mL and confirmed by GC-MS [208]. One OraLine® device positive was not confirmed by GC-MS, which gave a LoQ of 1 ng/mL.

RIA was used for the analysis of oral fluid specimens, while plasma specimens were analyzed by GC-MS [209]. The similarity in oral fluid and plasma concentrations indicated that there is a physiological link between these specimens. This evidence suggested that during cannabis smoking, THC is deposited in the oral cavity.

Moore *et al.* [210] screened oral fluid specimens by ELISA and confirmed by GC-MS for THC and THC-COOH. Quantisal™ oral fluid collection device was used for the first time by Moore *et al.* [211].

Saliva samples were collected by the EPITOPE system and after an SPME step were analyzed on GC-MS [212]. THC and CBD showed positive results up to 13 h after use. SPME and direct immersion-SPME (DI-SPME) followed by GC-MS were also used for the detection of THC, CBD, CBN, cocaine, EDDP,

cocaethylene, amphetamine, methamphetamine, MDMA, MDEA, and MBDB in saliva samples [213].

The effects of adulterants and foodstuffs were investigated using the Oral Fluid drug screen, Oratect, on oral fluid drug tests [214]. This study revealed that common foods, beverages, food ingredients, cosmetics, and hygienic products do not cause false positive results when tested 30 min after their consumption.

5.6.1.1 Chromatographic methods

5.6.1.1.1 Gas chromatography-mass spectrometry-MS

Cone *et al.* [215] did comparative studies of the oral fluid testing using intercept immunoassay and GC-MS-MS confirmation versus urine testing and determined that oral fluid testing produces equivalent results to urine testing.

Oral fluid specimens collected from cannabis-free volunteers but exposed to cannabis smoke were screened by EIA for cannabinoids (cutoff concentration is 3 ng/mL) and tested by GC-MS-MS (LoD and LoQ is 0.75 ng/mL) [216]. This study concluded that the risk of positive oral fluid tests from passive cannabis inhalation is limited to a period of approximately 30 min following exposure.

Cannabinoid Intercept MICRO-PLATE EIA was used for the analysis of oral fluid samples from passive cannabis exposure, while the LoD and LoQ for THC in GC-MS-MS assay was 0.3 and 0.75 ng/mL, respectively [217].

5.6.1.1.2 Liquid chromatography-mass spectrometry and LC/MS-MS

In contrast to existing GC-MS methods, no extensive sample clean up and time-consuming derivatization steps are needed to analyze the samples by LC-MS. LC-MS was used to detect THC in oral fluid samples with a LoD and LoQ of 1.0 and 2.0 ng/mL, respectively [218]. The oral fluid was extracted using Bond-Elut LRC-Certify SPE columns and THC was analyzed by LC-MS [219]. Concheiro *et al.* [220] analyzed THC in oral fluid by using 200 μL of sample and achieved a LoD of 2 ng/mL.

Laloup *et al.* [221] developed a simple and rapid method for the analysis of THC in oral fluid using LC-MS-MS. The use of liquid–liquid extraction by hexane was highly effective and decreased the interferences present in the matrix. XTerra MS C18 column was used for chromatographic separation using 1 mM ammonium formate–methanol (10:90, v/v) as the solvent system isocratically. By using 100 and 500 μL of oral fluid, the LoQs were 0.5 and 0.1 ng/mL, respectively.

5.7 AUTOPSY MATERIALS

Blood and urine are the most widely used autopsy samples. The determination of THC in forensic blood samples [130,132,133,136,146,148,151,154] and postmortem urine samples [49,148] has been discussed above under the analysis of cannabinoids in blood and urine.

Other autopsy materials include human solid tissues such as liver, kidney, brain, spleen, stomach, and intestine. Kudo *et al.* [222] developed a simple and sensitive

method that can be used for routine forensic analysis of THC in human solid tissues. Tissue samples were homogenized in acetonitrile, the sample was then centrifuged and the supernatant made alkaline by the addition of NaOH. The alkaline solution was shaken with hexane–ethyl acetate (9:1), the organic phase was then separated and shaken again with 0.1 M HCl. Finally, the organic layer was evaporated, derivatized by methylation and analyzed by GC-MS. The application of the method to samples taken from an autopsied individual allowed the study of the distribution of THC in human tissues. THC was found in all tissues except urine. The highest concentration was found in adipose tissues, then in the lungs and the lowest concentration was in the whole blood and liver.

An HPLC/ECD was developed for the determination of THC in rat brain tissue. Methanol was used for protein precipitation and initial extraction of THC from brain tissues. After evaporation of the methanolic extract, the residue was dissolved in hexane–ethyl acetate (7:3) and the solution washed with 0.05 M H_2SO_4. The organic phase was then evaporated and the residue reconstituted with mobile phase–methanol (25:10) then injected onto a C_{18} column. The internal standard used was 4-dodecylresorcinol and the mobile phase was methanol–acetonitrile–H_2SO_4 (0.01 M) (21:24:55).

5.8 ANALYSIS OF CANNABINOIDS IN CRUDE CANNABIS PREPARATIONS

Crude cannabis preparations include marijuana (the dried leaves and flowering tops of the female plants), hashish (the dried resin with fine plant particles), and hash oil (the concentrated extract of the plant material).

The most commonly used methods of analysis over the last two decades have involved GC with FID (GC-FID), GC-MS, and HPLC. The following summarizes some of the procedures described over the last few years for the analysis of these preparations.

Morita and Ando [223] described a GC-MS procedure for the analysis of the different cannabinoids in hash oil in which 11 compounds were separated and identified. These included Δ^9-THC, CBD, CBC, and CBN, along with some C_3 homologs. The composition of major mass spectral fragments of Δ^9-THC were proposed.

In 1988, Brenneisen and ElSohly [224] described a high-resolution capillary GC-FID and a GC-MS procedure for the identification of the different components of a cannabis extract to establish the chemical profiles (chemical signature) of samples of different geographical origin. The components analyzed included terpenes, alkanes, cannabinoids, and non-cannabinoid phenols. Over 100 different components were identified, and the procedure proved to be of forensic value in tracing the geographical origin of a cannabis sample through its chemical profile. In addition, the separation of free cannabinoids and their carboxylic acid precursors was accomplished by HPLC analysis of the samples using a Beckman Ultrasphere

3 μm ODS column (75 mm × 4.6 mm). More than 40 components were detected using a UV detector in the HPLC tracing.

In 1995, Hida *et al.* [225] reported on the classification of hashish by pyrolysis–GC in the presence of powdered chromium, followed by cluster analysis of the normalized pyrograms (the peaks in each pyrogram were normalized against the highest peak in that pyrogram). The results of the cluster analysis were presented in easily interpreted visual representations known as dendograms. The dendograms were used to compare unknown hashish samples with those of samples from different sources for classification purposes.

A GC-FID procedure for the routine analysis of confiscated marijuana samples and quantitation of several cannabinoids including Δ^9-THC, CBD, CBC, CBN, CBG, and THCV was described by Ross *et al.* [226]. The procedure involved the extraction of a small amount of sample (100 mg) with a methanol–chloroform mixture (99:1) containing the internal standard (4-androstene-3, 17-dione) followed by the direct analysis of the extract on a DB-1 column.

Analysis of neutral cannabinoids by HPLC was reported by Veress *et al.* [227], using two types of bonded-phase columns. An amino-bonded-phase column was used, which allows the extraction of plant material with non-polar solvents followed by direct injection of the extract without pre-separation. The results obtained by the amino-bonded column were compared with those obtained by a reverse-phase method, which required sample clean up using a C_{18}-Sep-Pak cartridge prior to HPLC analysis. The authors concluded that the amino-bonded-phase HPLC procedure was superior to that using the reversed phase for the quantitation of neutral cannabinoids.

Several analytical procedures (TLC, GC-FID with both packed and capillary columns, and HPLC) have been described in detail for the analysis of cannabinoids (neutral and acidic) in different cannabis products (marijuana, hashish, and hashish oil) in a manual prepared by the Division of Narcotic Drugs of the United Nations [228]. The manual is a compilation of methods for sampling and analysis of cannabis products, recommended for use by National Narcotics Laboratories. Bosy and Cole [229,230] used GC-MS for the determination of THC amounts in hemp seed oil. HPLC was used for the determination of THC and THC-COOH in hemp-containing foods [231]. Ross *et al.* [232] analyzed the total THC content of both drug- and fiber-type cannabis seeds by GC-MS.

The quantitation of the individual cannabinoids was accomplished by the use of internal standards, which varied depending on the method and included the use of long-chain hydrocarbons (e.g. *n*-tetradecane or *n*-docosane), steroids (androst-4-ene-3,17-dione and cholestane), and phthalates (dibenzyl phthalate or di-*n*-octyl phthalate).

HPLC was used for the analysis of THC, CBD, and CBN along with their acid precursor (THCA, CBDA, and CBNA), using a reversed-phase column (7 μm particle size) and a mixture of methanol and 0.01 M sulfuric acid (80:20) as the mobile phase [233]. The authors carried out standardized storage conditions with hashish samples along with pure cannabinoids and concluded that the total values of CBD-CBDA, CBN-CBNA, and THC-THCA were important in the judgment of hashish samples.

Hazekamp *et al.* [234] developed a ^{1}H-NMR method for the quantitative analysis of cannabinoids present in *C. sativa* plant material. The distinguishable signals of cannabinoids were in the range of δ 4.0–7.0 in the ^{1}H-NMR spectrum. Anthracene was used as the internal standard. The quantitation of the target compound was performed by calculating the relative ratio of the peak area of selected proton signals of the target compound to the known amount of the internal standard. This method allows the simple and rapid quantitation of cannabinoids without any chromatographic purification with 5 min analysis time.

Elias and Lawrence [235] summarized different instrumental methods used in drug interdiction. These methods used for detecting concealed drugs were categorized into two main techniques based on bulk detection and air sampling. The bulk detection techniques included X-ray imaging, gamma backscattering, thermal neutron activation, and other systems, while the air sampling techniques included acetone vapor detection, mass spectrometry, gas spectrometry, and ion-mobility spectrometry. The authors concluded that these methods have their limitations and pointed to the continued need for other more effective and selective methods.

5.9 CONCLUDING REMARKS

The scientific literature today is rich in methods to analyze (both qualitatively and quantitatively) for the presence of cannabinoids in biological specimens with a variety of techniques. The diversity of the techniques available to the analyst is such that one could carry out the task without the need for adding new instrumentation to a modestly equipped laboratory. Therefore, the objective of this chapter is to provide an overview of the technologies available with reference to such technologies so that the analyst reviewing this information can find it easy to follow and be directed to information pertinent to the problem at hand. It is hoped that this chapter has met this goal and thatreaders will find it a useful and easy reference to the information sought.

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