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 to the liquid–liquid extraction method of Sutheimer et al. [21], showed fewer interferences from co-extracted drugs and urinary artifacts; thus specificity was also increased [74].

The Toxi-Prep (TP) system is a semi-automated system that utilizes a solid-phase extraction technique for the extraction of THC metabolites from urine. Steinberg et al. [75] compared the Toxi-Prep THC metabolites system to the Toxi-Lab cannabinoid screen method for evaluating THC metabolites in urine. In the Toxi-Prep method, urine samples were hydrolyzed, loaded onto a preconditioned column, and the columns were washed with 0.5 ml 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 solid-phase extraction 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 Toxi-Prep 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 requirement of less extraction solvent and less urine, and gives cleaner chromatograms which lead to increased sensitivity.

Bonded-phase adsorption/thin-layer chromatographic (BPA–TLC) method for the determination of THC-COOH in human urine was developed by Kogan et al. [76]. In this method, 10 ml urine were 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 [62]. 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 the qualitative confirmation of THC-COOH in urine after screening with immunoassays.

Vereby et al. [77] applied the method of Kogan et al. [62] to the confirmation of 100 urine samples 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° to 60°C)–diethylether–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 ethylacetate followed by derivatization with TMS. This was advantageous since two confirmation methods could be applied to a single urine specimen.

High-efficiency thin-layer chromatography (HETLC), together with a high-performance liquid chromatography (HPLC) technique were used by Black et al. [26] for confirmation of EMIT urine cannabinoid assay. The method used for isolation of THC-COOH from urine samples was that developed by ElSohly et al. [78] 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 a HETLC plate. The plate was developed using hexane–acetone–glacial acetic acid as mobile phase, and the spots were visualized using alkaline solution of Fast Blue B salt as spraying reagent. The results of HPLC and HETLC were always in agreement suggesting the use of HETLC as a confirmatory technique for EMIT.

Another HPTLC procedure for the detection of THC-COOH in urine was described by Meatherall and Garriott [79]. The 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 the 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. CBN was used as internal standard. Although the R_f values 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 Strommer [80]. Quantitation of THC-COOH can be done using densitometry [81].

5.2.2.2 High-performance liquid chromatography (HPLC)

Combining the separating power of HPLC with different detectors 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. [82] 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 [83–85]. 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 [85]. 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, cannabinol, 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 radioimmunoassay. 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 [86] and an ^{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. [87] studied the HPLC-IA profiles for the analysis of cannabinoid metabolites in urine samples. The samples were chromatographed on a reversed-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. [78]. Hydrolyzed urine samples were cleaned up using Bond-Elut® -THC columns then injected on a reversed-phase column with acetonitrile–50 mM phosphoric acid (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 described HPLC method 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 [58] 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 solid-phase extraction methods followed by HPLC analysis with UV detection was also used by many authors [88–91].

Bourquin and Brenneisen [88] used Bond-Elut® -THC-SPE columns for the isolation of THC-COOH which was analyzed by HPLC on a C_8 column using acetonitrile–aqueous 50 mM phosphoric acid (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 [89] 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. [90] 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 a C_8 column using 0.05 M phosphoric acid–acetonitrile (35 : 65, v/v) as the mobile phase.

Bianchi and Donzelli [91] used disposable C_{18} SPE cartridges (100 mg) from Bio-Rad Labs and a reversed-phase column with acetonitrile–0.125 M phosphate buffer (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 with electron-capture detection [92]. Δ^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 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 Karlsson [93]. 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, a 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. Therefore, the sample throughput was low (two urine samples/h).

Another HPLC method with EC detection for the determination of THC metabolites in urine was presented by Nakahara et al. [94]. 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 C_8 column with acetonitrile–methanol–0.02 N H_2SO_4 (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 Gas chromatography (GC)

GC–FID. Irving et al. [57] used gas–liquid chromatography with flame ionization detection 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. [89] used GC–FID for the analysis of urine samples after extraction using Supelclean DrugPak-T SPE tubes and derivatization with BSTFA.

GC–ECD. ElSohly et al. [95] 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 in a biphasic system using benzyl tributylammonium hydroxide as a phase transfer catalyst. Jones et al. [58] 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. [96]. 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 multi-wavelength 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 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 ± 10%). Therefore, these columns were used for the confirmation of urine samples screened positive by FPIA [12].

5.2.2.4 Gas chromatography/mass spectrometry (GC–MS)

GC–MS is the method of choice for the confirmation of cannabinoids in urine [97]. It has the highest sensitivity and specificity of all other 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 [98]. The method, a modification of the methods of Karlsson et al. [99] and Foltz et al. [100], included preparing the standards in alkaline solution to minimize adsorption onto glass and plastic surfaces and using potassium hydroxide–methanol (1 : 4) for hydrolysis in order to obtain a cleaner extract. The internal standard was d_3-THC-COOH, and the derivatizing agents used were pentafluoropropionic acid (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.

Needleman et al. [101] 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. [102] 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 MTB-STFA 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. [103,104] 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 cannabinol and cannabidiol. 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 [104]. Extraction was done with hexane–ethyl acetate (7 : 1) and derivatization was done with BSTFA in 1% TMCS.

Szirmai et al. [105] 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. Solid-phase extraction 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. [106]. 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. 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 dithioerytrithol (100 : 0.2 : 1, v/v/w) and compared it with MSTFA.

Solid-phase extraction methods are gaining increasing use in sample preparation techniques, and many publications appear each year utilizing and/or evaluating SPE cartridges. Nakamura et al. [107] used Sep-PAK cartridges for clean up of urine samples prior to GC–MS analysis. McCurdy et al. [108] used C_{18} bonded-phase adsorption 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. [109] used cartridges containing strongly basic anion-exchange resin (E.I. Du Pont de Nemours and Co.) for the detection of THC-COOH using GC–MS. Supelclean DrugPak-T SPE tubes were evaluated by Parry et al. [89], CLEAN SCREEN® reduced solvent volume (RSV) SPE columns were evaluated by O'Dell et al. [110], and Empore extraction disk cartridges (C_{18}) were evaluated by Singh and Johnson [111]. The Toxi-lab SPEC extraction discs were used by Wu et al. [112] 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-cannabinol [58,78,95], cannabinol [88], oxyphenbutazone [113], and ketoprofen [106]. The most commonly used internal standard is the trideuterated derivative of Δ^9-THC-COOH [98,102,103,110,111]. 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 [114]. This results in distortion of the ion ratio of the internal standard and limits the dynamic range of the analysis. Therefore, ElSohly et al. [114,115] developed new internal standards, hexadeutero-Δ^8-THC-9-COOH [114] and hexadeutero-Δ^9-THC-9-COOH [115] having the advantages of wider linear dynamic range and having no common ion with THC-COOH using different derivatives. The Δ^6-THC-COOH was used by Wu et al. [112] 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. [105] and can be used as an alternative to the previous internal standards.

5.3 ANALYSIS OF CANNABINOIDS IN BLOOD

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 recent use of cannabis and their levels may correlate with an actual state of intoxication.

5.3.1 Immunoassays

Immunoassay methods for screening of 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 (EMIT)

In 1978, Slightom [116] first reported the application of homogenous enzyme immunoassay 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. [117] 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 [118–120].

Perrigo and Joynt [118] 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 five- to ten-fold drug enrichment [120,121].

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 [122].

Another procedure for the extraction of THC metabolites from whole blood was suggested by Lewellen and McCurdy [123]. 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 (FPIA)

Bogusz et al. [124] determined drugs of abuse in whole blood by fluorescence polarization immunoassays (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 [125].

5.3.1.3 Radioimmunoassays (RIA)

Radioimmunoassays were also used for the determination of THC and THC-COOH in blood and serum samples [15,126]. Hanson et al. [126] compared ^{3}H- and ^{125}I-radioimmunoassays 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. [127] 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 Kinetic interaction of microparticles in solution (KIMS) assays

Moody and Medina [128] used OnLine® kinetic microparticle immunoassay (KIMS) to detect cannabinoids in serum. They modified the KIMS method used by Armbruster et al. [44] 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 (ELISA)

THC metabolites can be detected by ELISA [42,129]. 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 [129].

5.3.1.6 CEDIA

Another type of immunoassays used for the analysis of cannabinoids in whole blood is the Microgenics CEDIA DAU. Cagle et al. [130] 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 to 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 (TLC)

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 [131]. This procedure, however, uses two extraction steps, initial solid-phase extraction 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–ethylacetate–acetic acid (30 : 10 : 1).

5.3.2.2 High-performance liquid chromatography (HPLC)

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 [83,84,86], 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.

High-performance liquid chromatography with electrochemical detection (HPLC–ECD) was also used for the analysis of plasma samples [94,132]. Both methods utilized a preliminary solid-phase extraction. Zweipfenning et al. [132] used Bond-Elut C_{18} solid-phase extraction columns for the isolation of THC, followed by HPLC analysis on a C_{18} column using tetrahydrofuran–methanol–0.005 M sodium citrate buffer, pH 7.0 (7.5 : 68 : 24.5, v/v) as the mobile phase. Nakahara et al. [94] 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 a Zorbax C_8 column using a mobile phase composed of acetonitrile–methanol–0.2 N H_2SO_4 (35 : 15 : 50).

5.3.2.3 Gas chromatography (GC)

Gas chromatography with electron-capture detector was used for the determination of cannabidiol, the most abundant cannabinoid in hashish and in fiber-type *Cannabis*, in plasma [133]. Tetrahydrocannabidiol was used as 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 pentafluorobenzyl 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 a nitrogen-selective detector was proposed by Ritchie et al. [134]. 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 pentafluorobenzyl bromide (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 [135].

5.3.2.4 Gas chromatography/mass spectrometry (GC–MS)

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 [17,103,126,127,136–138], or by solid-phase extraction [139,140].

Derivatization of the samples is also necessary. Hanson et al. [126] utilized trimethylphenyl ammonium hydroxide to form the methyl derivative of THC which was then analyzed by electron-impact selected-ion monitoring GC–MS. Gariott et al. [136] used trimethylanilium 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 [137]. This detector improved the limit of detection of THC in plasma by 6.25-fold, over that obtained with the same GC–MS system without the new detector. Moody et al. [127] 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. [103] for the analysis of THC and six metabolites, namely, 8α-hydroxy-Δ^9-tetrahydrocannabinol, 8β-hydroxy-Δ^9-tetrahydrocannabinol, 11-hydroxy-Δ^9-tetrahydro-cannabinol, 8α-11-dihydroxy-Δ^9-tetrahydrocannabinol, 8β-11-dihydroxy-Δ^9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol. The method of Kemp et al. [103] had also the advantage of being able to detect cannabidiol and cannabinol in plasma. Simultaneous quantitation of THC and THC-COOH in serum by GC–MS using tetrabutylammonium hydroxide in DMSO was also reported [139]. Trimethylsilyl derivatization was also used for the determination of cannabidiol (CBD) in plasma utilizing gas

chromatography/ion-trap mass spectrometry in positive-ion chemical ionization mode [141].

GC–MS–MS method was used to confirm the unusually high levels of THC in two postmortem samples [142]. 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.

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 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 [143,144]. Balabanova et al. [145] was the first author that published 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 [146,147]. Since this time, many papers have been published describing the use of GC–MS methods for 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 [148] or with pentafluoropropionic anhydride (PFPA) and pentafluoropropionyl alcohol (PFP-OH), with levallorphan as internal standard [149]. Alternatively, liquid–liquid extraction and deuterated internal standards were used for the determination of THC-COOH in hair [150] and for the determination of THC and THC-COOH in human hair and pubic hair [151]. 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. Cirimele et al. [152] proposed a simpler method for the simultaneous identification of tetrahydrocannabinol, cannabinol and cannabidiol in hair samples, using THC-d_3 as internal standard. This method is a rapid screening method that does not require derivatization prior to analysis. Jurado et al. [153] 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 one is an acid hydrolysis followed by organic solvent extraction of opiates and cocaine, followed by alkaline hydrolysis and extraction of the cannabinoids with organic solvent after addition of maleic acid. Wilkins et al. [154] utilized a liquid–liquid extraction procedure prior to quantitative analysis of THC, 11-OH-THC, and THCCOOH in human hair by GC–MS. The extraction procedure included digestion of the sample with NaOH, followed by extraction with hexane–ethyl acetate (9 : 1, v/v), the organic phase was then further extracted for THC and 11-OH-THC and the aqueous phase was used for THC-COOH.

A GC–MS–MS method was used by Mieczkowski [155] 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, yet the results should be confirmed by GC–MS–MS methods.

5.5 ANALYSIS OF CANNABINOIDS IN MECONIUM

Analysis of meconium for the presence of drugs of abuse gained interest in the last few 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 [156].

Ostrea et al. [157–159] 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 to stand at room temperature for 10 min, centrifuging and testing the supernatant for cannabinoid metabolites by RIA [159]. 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 [159].

Nair et al. [160] used the procedure of Ostrea et al. [159] for the analysis of 141 meconium samples and also concluded that a meconium sample is superior to 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 [161]. 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 [161].

FPIA followed by HPLC with diode-array detection was also used for the analysis of THC-COOH in meconium samples [162]. The extraction of THC-COOH from meconium samples was done with 5 ml water and 1 drop 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. [163]. 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 for the determination of THC-COOH in meconium was the low confirmation rate. Wingert et al. [161] failed to confirm any of the positive specimens screened by EMIT, Moore et al. [163] reported a 20% confirmation rate for samples analyzed by FPIA and confirmed by GC–MS, while ElSohly et al. [164] 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 [165]. 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 are therefore necessary to increase the confirmation rate of samples screening positive for cannabinoids by immunoassays.

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 driving while intoxicated and in surveying populations for illicit drug use [166].

RIA and mass spectrometry were used for the analysis of methadone, cocaine, tetrahydrocannabinol, benzodiazepine, barbiturates, morphine and cotinine in apocrine sweat and the data obtained indicated depositions of those drugs in axillary hair [167]. The effect of pilocarpine stimulation on the concentration of THC in perspiration samples obtained from THC smokers was also determined [168]. 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 for the degree of exposure to drugs for a whole week [169,170].

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 [171]. Drug residues on the hands of human subjects were also detected using a sampling method based on aspirating and trapping of the drug microparticles on a filter plug followed by ion-mobility spectrometry [172].

The detection of cannabinoids in breath and saliva may be particularly useful in traffic control where a non-invasive and simple 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 [173]. A breath analyzer consisting of a tube containing Fast Blue Salt B, NaOH and silica gel and a mouth piece was developed by Volkmann et al. [174]. Consumption of hashish or marijuana can be detected by a change in the color of the indicator into red when the person blows into the mouthpiece.

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 [175]. Kircher and Parlar

[175] 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/ml using a UV detector at 220 nm.

5.7 AUTOPSY MATERIALS

Blood and urine are the most widely used autopsy samples. The determination of THC in forensic blood samples [121,123,124,127,134,136,139,142] and postmortem urine samples [46,136] was discussed before 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. [176] 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. 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 with electrochemical detection 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–0.01 M H_2SO_4 (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 involved gas chromatography with flame ionization detection (GC–FID), gas chromatography/mass spectrometry (GC–MS), and high-performance liquid chromatography (HPLC). The

following summarizes some of the procedures described over the last few years for the analysis of these preparations.

Morita and Ando [177] described a GC–MS procedure for the analysis of the different cannabinoids in hash oil in which eleven components 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 [178] described a high-resolution capillary GC–FID and 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 the 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. [179] reported on the classification of hashish by pyrolysis–gas chromatography 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. [180]. 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 direct analysis of the extract on a DB-1 column.

Analysis of neutral cannabinoids by HPLC was reported by Veress et al. [181], 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 reversed-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 United Nations, Division of Narcotic Drugs [182]. The manual is a compilation of methods for sampling and analysis of cannabis products, recommended for use by the National Narcotics Laboratories.

Quantitation of the individual cannabinoids was accomplished through 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 (dibenzylphthalate or di-*n*-octylphthalate).

HPLC was used for the analysis of THC, CBD, and CBN along with their acid precursors (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 [183]. The authors carried out standardized storage conditions with hashish samples along with the pure cannabinoids and concluded that the total values of CBD–CBDA, CBN–CBNA, and THC–THCA were important in the judgment of hashish samples.

Elias and Lawrence [184] 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 chromatography, 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 putting this chapter together was to provide an overview of the technologies available with references 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 that the readers can find it a useful and easy reference for the information sought.

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