M.J. Bogusz (Ed.), *Forensic Science*
Handbook of Analytical Separations, Vol. 2

CHAPTER 5

Cannabinoids analysis: analytical methods for different biological specimens

Mahmoud A. ElSohly [a,b] and Maissa Salem [c]

[a] *ElSohly Laboratories, Incorporated (ELI), 5 Industrial Park Drive, Oxford, MS 38655, USA*
[b] *National Center for Development of Natural Products, School of Pharmacy, University of Mississippi, University, MS 38677, USA*
[c] *Department of Analytical Chemistry, Faculty of Pharmacy, University of Cairo, Kasr El-Aini, Cairo, Egypt*

5.1 INTRODUCTION

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

Three cannabis preparations are found in the illicit traffic; these are herbal cannabis (marijuana), cannabis resin (hashish) and liquid cannabis (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 cannabis plant and allowing to dry 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 into slabs. Alternatively, the flowering and fruiting tops are rubbed between the palms of the hands, which are then scrapped 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, over 60 cannabinoids have been identified [6].

Development of methods for the determination of cannabinoids is an area of increas-

TABLE 5.1

SUMMARY TABLE OF REFERENCES PERTAINING TO THE USE OF DIFFERENT METHODS USED FOR THE ANALYSIS OF THE DIFFERENT BIOLOGICAL SPECIMENS

Variable	Urine	Blood	Hair	Meconium	Others
Immunoassays					
RIA	[15–20,83–86]	[15,16,86,126,127]	[145]	[159,160]	[167]
EIA	[14,21–37]	[117–123]		[161,164]	
FPIA	[36,38–41]	[124,125,130]		[162,163]	
ELISA	[42]	[42,129]			
KIMS	[43,44]	[128,144]			
CEDIA		[130]			
On-site testing kits	[45–57]				
Chromatographic methods					
TLC	[21,22,24–26,59,61,62,70–76,79–81]	[131]			
HPLC	[15,16,26,27,83–94]	[16,86,94,132]		[162]	[175]
GC–FID	[57,89]				
GC–ECD	[92,95,96]				
GC–NPD		[133,135]			
GC–MS	[17,28,29,57,71,73,89,98–112,125,126,130]	[134,17,103,126,127,135–140]	[145,148,150–154]	[163–165]	[171,176]
GC–MS–MS		[142]	[155]		
Extraction methods					
LLE	[21,72,73,79,95,101,103,104,106]	[17,103,126,127,131,133,134,136–138]	[150–153]	[162,163,165]	[176]
SPE	[12,75,76,78,88–91,94,106]	[94,131,132,135,139,140]			[175]
Derivatization reagents					
BSTFA	[89,103–105]	[17,103,141,142]			
PFBBr	[95,96]				
PFPA–PFPOH	[99]		[148,150,151]		
TMAH-CH_2I_2	[101,105]	[126]			
CH_2N_2-BSTFA	[105]				
CH_2N_2-MBTFA	[105]				
TFE-PEPA	[105]				
MTBSTFA	[102]			[163]	
OTHERS	[106,133,134]	[136,137,139]	[148]		

ing interest. A large number of publications appear each year describing a variety of analytical techniques which vary in sensitivity, specificity, and instrumentation. Articles representing extensive reviews of the various analytical techniques have also been presented [2,7–10]. This work will focus mainly on the methods published in the last 15 years with special emphasis given to those methods which appear to be more practical and feasible for routine analysis of these compounds in various types of biological specimens (Table 5.1). In addition, because of the large number of publications, this review is not meant to be all inclusive.

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-tetrahydrocannabinol-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 3 to 4 weeks may be required for elimination of all metabolites in 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), and presumed-positive samples are then confirmed by another more specific method such as 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. Enzyme immunoassays and radioimmunoassays are among the most commonly used methods.

5.2.1.1 Radioimmunoassays (RIA)

Radioimmunoassays 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 requirement of 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 detection of cannabinoid metabolites many days after consumption. The sensitivity of the RIA method was then coupled to 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® Radioimmunoassay 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 radioimmunoassays for cannabinoids in urine sample. The interfering substance was not identified but the authors suggested the presence of proteinaceous material in urine to be the cause.

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)

Enzyme immunoassays are the most commonly used screening methods for detection of cannabinoids in urine today. Enzyme immunoassays 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]. Also, 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, suitable for automation and routine screening of a large number 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 that 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 which 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 (FPIA)

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 sample separation step but lacked sensitivity. The second assay had a sensitivity comparable to RIA and could be automated. Stopped flow-fluorescence polarization immunoassay (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 to 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 advantages including stability of the calibration curves for 3–4 months, possibility of providing semi-quantitative results, and ability of processing emergency samples, made it useful for routine analysis of drugs of abuse in urine samples [41].

5.2.1.4 Enzyme-linked immunosorbent assays (ELISA)

Microanalysis of cannabis components and their metabolites was also done by ELISA. Application of the method to the analysis of THC metabolites in plasma and urine was suggested [42].

5.2.1.5 Kinetic interaction of microparticles in solution (KIMS)

Another type of immunoassay, which depends on the kinetic interaction of microparticles in solution (KIMS) is the Abuscreen OnLine assay. Hailer et al. [43] 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 in order 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. [44] 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.

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 to laboratory-based immunoassays, these kits have the advantages

of being simple, easily performed, allow rapid access to the test results and they 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 [45] 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™ was applied to the detection of several classes of compounds including cannabinoids in postmortem urine samples [46]. Two difficulties were encountered. The first one 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 judgement 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, 95% confirmation rate for cannabinoids was reported.

Jenkins et al. [47,48] 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 [47].

The accuPINCH™ THC test is a competitive enzyme immunoassay 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 [48].

Triage® panel for drugs of abuse is a rapid immunoassay for the simultaneous detection of seven drugs in a single sample [49]. De La Torre et al. [50] 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 [51].

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. [52]. 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. [53] 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 difficulty to judge the results.

Two separate on-site test kits for drugs of abuse, the ONTRAK TESTCUP and the Abuscreen ONTRAK, were compared, and the results obtained were further compared to another laboratory-based immunoassay, the Abuscreen Online [54]. 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. [55]. Inter- and intra-individual agreement were 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. [56] developed and evaluated the one-step dip-and-read immuno-chromatographic 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 are presented each year.

Irving et al. [57] analyzed 200 urine specimens with 2 enzyme immunoassays (EMIT-st and EMIT-d.a.u.) and a radioimmunoassay (Abuscreen RIA), and those samples found to be positive were further analyzed by gas–liquid chromatography with flame ionization detection, gas–liquid chromatography/mass spectrometry, and an experimental RIA from Research Triangle Institute. The aim of this study was to evaluate the two enzyme immunoassays 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 radioimmunoassay were unsuccessful.

Jones et al. [58] compared five methods, namely, Abuscreen RIA, EMIT d.a.u., HPLC, GC–ECD, and GC–MS, for the analysis of THC-COOH in urine. The 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. [59]. These assays were two enzyme immunoassays (EMIT-st and EMIT d.a.u.), two radioimmunoassays (Abuscreen RIA and Immunanalysis), 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 Immunanalysis 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 Ström [60]. 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 reliable, with an excellent precision and curve stability. The EMIT-Cobas was reported to be faster, with the time to analyze one carousel 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 Wells and Barnhill [61]. 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 (Immunanalysis). In general, the radioimmunoassays gave a greater proportion of positive results than did the enzyme immunoassay or the fluorescence polarization immunoassay.

Kogan et al. [62] 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.

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. [63]. The authors concluded that both immunoassays produced similar results and either of them could be used alone in a mass-drug-screening laboratory.

Weaver et al. [64] 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. [65] 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 which might be used to calculate the concentration of the extracts to be injected onto the GC–MS.

Armbruster et al. [44] 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, 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 better than the RIA procedure in terms of time and effort.

A similar comparative study was conducted by Kintz et al. [66] 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. [67]. 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 those techniques was also presented.

Huestis et al. [68] studied the detection times of cannabinoids in urine following 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 the radioimmunoassay and on the fluorescence polarization immunoassay was studied [69]. A number of readily accessible chemicals, e.g. 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 radioimmunoassays, 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.

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 methods.

5.2.2.1 Thin-layer chromatography (TLC)

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 improved 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. Thin-layer chromatographic methods have the advantage of being more specific to THC-COOH than immunoassays which are known to cross-react to many THC metabolites. In the last 15 years, several publications appeared which used TLC as either a screening or confirmatory technique.

Nakamura et al. [70] used a TLC procedure previously described by Kaistha and Tadrus [71] 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. [72] 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 silica gel G plate, and chromatographed sequentially with two mobile phases, the first one consisting of acetone–chloroform–triethylamine (80 : 20 : 1) and the second one 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 0.1 or corresponding to that of a reference standard indicated a positive response. The results obtained were compared with those obtained 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 [73] 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 [21,22,59,61,74,75].

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. [59] compared the Toxi-Lab cannabinoid screen method with four commercially available immunoassay procedures and a GC–MS method, while Wells et al. [61] compared it to five cannabinoid immunoassay systems. Foltz and Sunshine [22] compared it to the EMIT d.a.u. assay and to a reference GC–MS method. Sutheimer et al. [21] compared the TLC method to two enzyme immunoassay 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 [22,74]. 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. [74]. 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