HANDBOOK OF FORENSIC DRUG ANALYSIS

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Elsevier Academic Press

30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
84 Theobald's Road, London WC1X 8RR, UK

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Library of Congress Cataloging-in-Publication Data
APPLICATION SUBMITTED

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

ISBN: 0-12-650641-8

For all information on all Elsevier/Academic Press publications
visit our Web site at www.academicpressbooks.com or www.books.Elsevier.com.

Printed in the United States of America
04 05 06 07 08 8 7 6 5 4 3 2 1

CHAPTER 3

CANNABIS: METHODS OF FORENSIC ANALYSIS

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Contents

The identification of marijuana or its chemical constituents has long been one of the most often performed analyses in the forensic drug laboratory. This includes analysis of the very common botanical samples, ranging from whole plants to finely chopped vegetation, as well as preparations and extracts, such as hashish and liquid hashish. Analytical issues do not end with merely identifying such exhibits. Occasionally the scientist is asked to compare exhibits to determine if they have a common provenance or what that provenance might be. This has resulted in a considerable body of literature devoted to profiling the constituents, both organic and inorganic, of *Cannabis* specimens. Evidence of the use of *Cannabis* as a drug is also of forensic interest both in drug-screening programs and in cases in which drug-induced impairment is an issue. The focus in the analytical toxicology of *Cannabis* has been on the major metabolite 11-nor-Δ^9-tetrahydrocannabinol carboxylic acid (THC-COOH). This chapter will address each of these topics separately and will include a historical perspective and cover specific widely accepted methodologies and recent advances.

Handbook of Forensic Drug Analysis
Frederick P. Smith, Editor

3.1 QUALITATIVE SEIZED-DRUG ANALYSIS OF *CANNABIS*, HASH, AND HASH OIL: CURRENT AND HISTORICAL PERSPECTIVES

3.1.1 INTRODUCTION

Seized-drug testing is a practice involving the examination and analysis of law enforcement submissions for the presence or absence of controlled substances. The amounts submitted are usually ample enough to be visible to the naked eye. They range in size from residues often found, for example, in smoking devices to tons of material seized from large transport or storage facilities. Such samples are seized by law enforcement from those possessing, selling, manufacturing, or attempting these acts. The practice is distinguished from toxicological analysis in that it is not concerned with metabolites, nor does it typically involve extraction from physiological matrices. It differs from toxicological and environmental analysis by the larger magnitude of analyte often present for testing (typically milligrams and larger) and because, unlike these two, it is not often concerned with elemental analysis. Seized-drug testing is concerned with both qualitative and quantitative determinations. Quantitative tests are often run for investigative purposes but may also be performed to meet statutory requirements, as in the setting of criminal charges (New York State has approximately 98 controlled-substance statutes of this type) or to aid the court in sentencing (see U.S. Federal Drug Statutes). Because it occurs in a forensic context, it frequently entails presentation and defense of test results by the analyst with case responsibility in criminal court. Common techniques employed for the analysis of seized drugs include color tests; microscopy; thin-layer, gas, and high-performance liquid chromatography (TLC, GC, HPLC); mass spectrometry (MS); and ultraviolet and infrared spectroscopy (UV, IR). In keeping with good laboratory practice, a positive identification should be based on at least two positive test results from two different test methodologies made on separate aliquots of the material. The test results should be reviewed by at least two individuals who are thoroughly familiar with the testing protocol. Two very good general discussions of seized-drug analysis that include a review of federal drug schedules, test methodologies for different drugs, and sound basic principles for creating analytical protocols are found in works by Siegel (1993) and Saferstein (2001).

Cannabis, according to a report from the Drug Enforcement Administration-sponsored National Forensic Laboratory Information System (*ASCLD News*, 2001), is the most frequently identified controlled substance in forensic laboratories in the United States, at 39.68% of all submissions. Cocaine was second at 30.65%, and all others tallied were at single-digit percentages or less. These numbers were based on a sampling of 165 individual laboratories at the state

and local levels. Testing for *Cannabis* typically involves identifying fresh plants or dried, crushed plant material as belonging to the genus *Cannabis* of the family Cannabinaceae. Where resins or oils of *Cannabis* are submitted, the aim is to establish that they contain constituents of *Cannabis*. Identifying any of several cannabinoids (See Figure 3.1) and remnants of the *Cannabis* plant present can accomplish this. In some jurisdictions the charge for oils and resins may be based simply on the presence of a cannabinoid like Δ^9-tetrahydrocannabinol (THC). In modern forensic laboratories, identification of the plant material may be made from an examination of macroscopic and microscopic

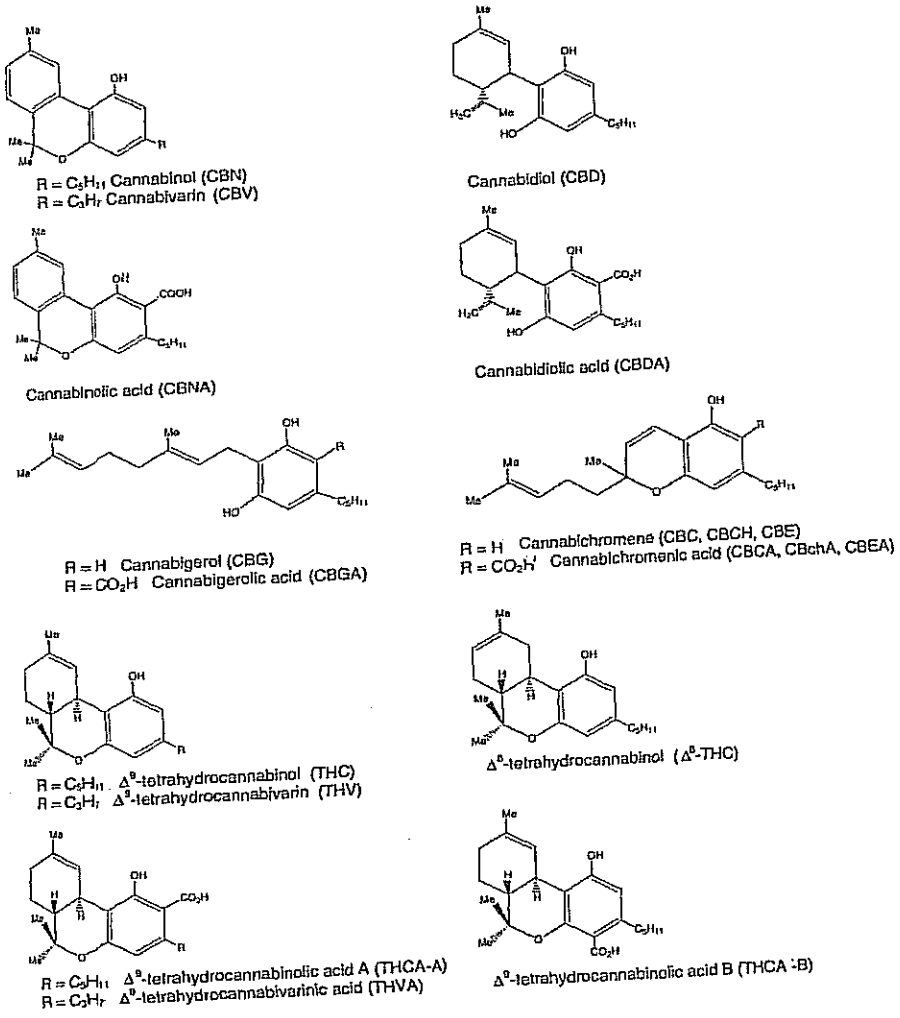


Figure 3.1
Structures of selected cannabinoids

botanical features along with chemical tests to establish the presence of various cannabinoids.

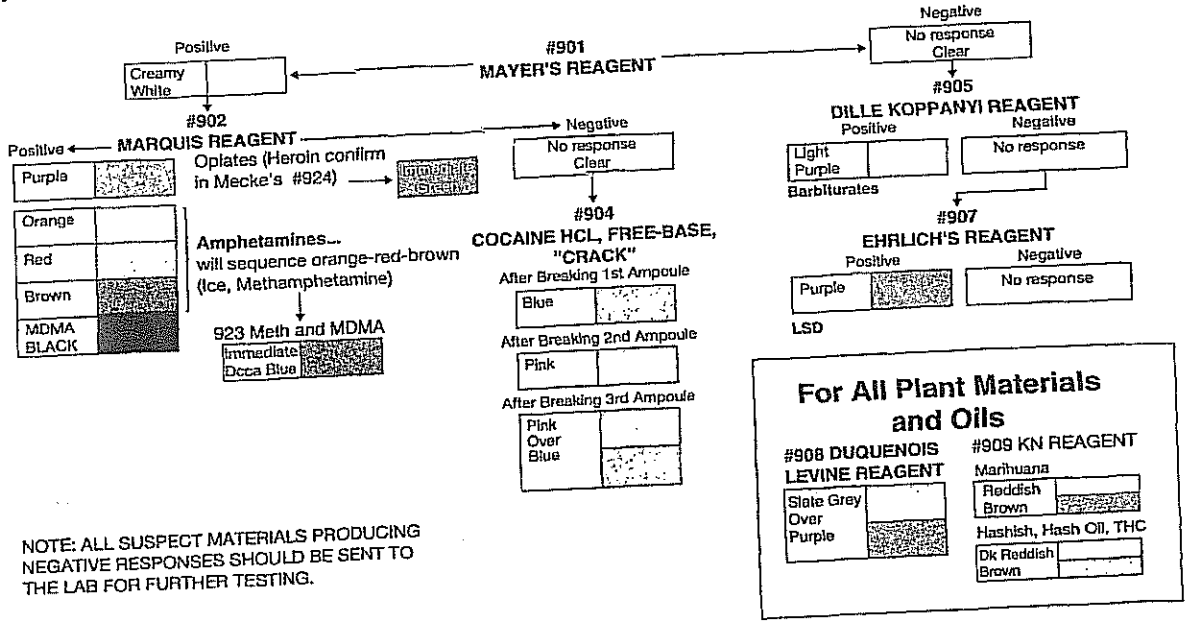
3.1.2 REVIEW OF FIELD AND LABORATORY TESTING

3.1.2.1 Current Field Testing

At least two companies in the United States (Sirchie of Raleigh, NC, and ODV of South Paris, ME) produce kits for testing materials suspected of containing controlled substances. These are often used by law enforcement in the field to establish probable cause for an arrest. For *Cannabis*, each company manufactures two different tests, one based on the Duquenois–Levine test and the other based on the fast blue B salt test. The test reagents are dispensed in two types of containers, a plastic pouch and a plastic tube.

The tests are performed as follows (Figure 3.2). A small amount of the suspected controlled substance (as plant material, resin, or liquid) is added to the pouch or tube. The container is closed. Squeezing the outside of the container crushes ampules located within that hold reagents. These actions are done in sequence to yield various colors that are then compared to a color chart for determination of whether the presence of a controlled substance is indicated. Neutralizing reagents are provided to render the contents safe for disposal. Users are instructed on how much suspected material to use and how to read the colors and about the presumptive nature of the tests (see Figure 3.2).

Figure 3.2
Simplified testing procedure for the major drugs of abuse utilizing field test reagents



3.1.2.2 Review of Laboratory Testing

What follows is a review of much of the analytical literature from approximately the 1970s until the present regarding the testing of *Cannabis* and its constituents. The references generally fit within the seized-drug context.

3.1.2.2.1 Identification of Cannabis by Multiple Tests

Traditionally, seized-drug identification of *Cannabis* relied on various combinations of wet chemical, microscopic, chromatographic, and spectroscopic testing methods. In recent years DNA profiling techniques for identification and individualization have been described, although we know of no instance where it is being performed on a routine basis. A series of publications document a combination of techniques (usually wet chemical, microscopic, and chromatographic) for the identification of *Cannabis*. Many studies regarding seized-drug identification of *Cannabis* were published in or around the 1970s.

Thornton and Nakamura (1972) provide an extensive review of the chemistry of the phenolic constituents of the resin from the leaves and flowering tops of the marijuana plant, the chemistry of the Duquenois–Levine color test, botanical features useful for identification, along with thin-layer chromatographic and infrared absorption methods for identifying the major cannabinoid constituents. They found, based on an earlier work by Nakamura (1969), that since at least 82 species of plants from families of the subclass dicotyledon possess cystolithic hairs and despite the usefulness of other morphological features such as trichomes, a Duquenois–Levine test was necessary for confirmation of identity. They further contend that chromatographic tests are indicated where morphological features are absent. In a subsequent article (Nakamura and Thornton, 1973), the authors review, in a question-and-answer format, issues pertinent to those testing for *Cannabis*. Topics include the issue of speciation of *Cannabis*, specificity of the Duquenois test, substances yielding false positives to the analytical scheme of the Duquenois test, TLC for cannabinoids, microscopic analysis, and the potency of *Cannabis*. They conclude that no set criteria exist for the identification of *Cannabis*; some analysts rely strongly on morphological characteristics, while others stress the importance of chemical tests for cannabinoids. They add that a Duquenois–Levine test, a microscopic examination, and a TLC test may be "more than sufficient to rule out plants other than marijuana (*Cannabis*)."

A pamphlet from the former U.S. Treasury Department Bureau of Narcotics (1948) provides textual descriptions with black-and-white photos of the mature plant along with stereophotomicrographs of microscopic features, all of which can be used as "identification characteristics" for the purposes of seized-drug analysis. Fairbairn (1972) uses scanning electron micrographs to view the

trichomes and glands of *Cannabis*, with emphasis on sessile glands found in abundance on the male, female, and monoecious plants studied. He notes that sessile glands provide an additional structure to be used in microscopic identification of *Cannabis*. DeForest and Morton (1972) describe a microscopic method for establishing the presence of marijuana in ash, such as from a pipe or ashtray. They show with photomicrographs how morphological structures of *Cannabis* ash differ from those of similar plant ash. This approach could be coupled with a TLC system, suggested by Kempe et al. (1972), that separates cannabinoids in charred *Cannabis*. They were able to distinguish cannabidiol, tetrahydrocannabinol, and cannabinol from residues, cinders, and paper. Hauber (1992), in an effort to avoid the hazardous waste generated by use of the Duquenois–Levine test, describes an unambiguous identification protocol that relies on the documentation of various botanical features and the running of two thin-layer systems. The systems indicate the presence of certain cannabinoids not discriminated by the Duquenois–Levine test.

3.1.2.2.2 Identifying Botanical Features

Seized-drug identification of *Cannabis* per se must include an examination of some of the plant's morphological characteristics as well as chemical tests to establish the presence of cannabinoids. *Cannabis* is classified, according to Nakamura (1969), as follows:

Division:	Spermatophyta (seed plants)
Class:	Angiospermae (flowering plants)
Subclass:	Dicotyledons (dicots); 31,874 species
Order:	Urticales (elms, mulberries, nettles, and hemps); 1753 species
Family:	Cannabinaceæ (hops and marihuana); 3 species
Genus:	*Cannabis*
Species:	*Sativa*

Morphological features may be micro- or macroscopically addressed. Since most submissions to forensic laboratories are in the form of crushed plant material, which no longer retains gross botanical features, and because most seized-drug analysts are not trained as botanists, an ability to recognize microscopic detail is critical for identification (Nakamura, 1969).

Microscopic Morphology

Some microscopic features are quite distinctive and the capacity to recognize them can be learned with moderate practice. Cystolithic trichomes and their hairs, other nonglandular trichomes, and glandular trichomes are especially useful for identification. Nakamura (1969) sampled 600 species of dicotyledons,

the botanical subclass of which *Cannabis* is a member, for those with cystolithic hairs. Many were found to possess cystolithic hairs, some with an appearance similar to *Cannabis*. He performed a Duquenois test on 80 species, with the result that many gave a positive Duqenois reaction, but none except *Cannabis* yielded a positive reaction to the Levine modification (i.e., purple color transferring to the $CHCl_3$ layer). Using a scanning electron microscope to look at *Cannabis* and other cystolith- and cystolithic hair-bearing plants, Mitosinka et al. (1972) found that the cystolithic hairs of *Cannabis* were much longer, given the broad size of their cystoliths, than they were for other plants possessing these structures. Figure 3.3 shows microscopic structures of *Cannabis*.

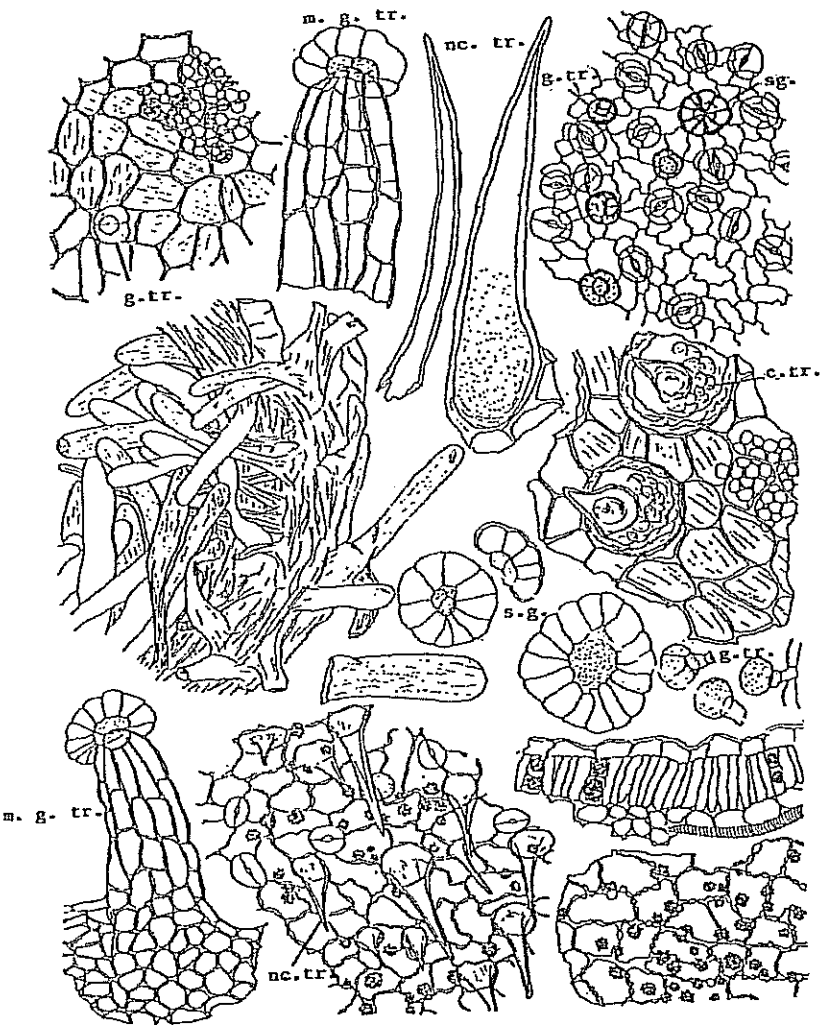


Figure 3.3
Microscopic characteristics of Cannabis g.tr. Small glanular trichomes m.g.tr. Multicellular multiseriate glanular trichomes s.g. sessile glands nc.tr. non-cystolithic trichomes c.tr. cystolithic trichomes

Microscopic detail can be viewed under a stereo- or compound light microscope. Cystoliths and their hairs are observable with a stereoscope at 10× to 25×. All of the structures shown in the Figure 3.3 sketches can be observed with a compound scope at 100× to 200×. Figures 3.3a and 3.3b are photomicrographs depicting such microscopic structures. Placing small fragments of leaf or seed husks on a microscope slide in an immersion medium facilitates observation of fine structure with the compound scope. A solution of chloral hydrate and water (5 g per 2 mL) works well as an immersion medium, particularly if the plant material is allowed to absorb the viscous liquid for a short period prior to viewing.

Macroscopic Morphology

The sketch in figure 3.4 shows some of the gross botanical features of *Cannabis*. When present, these are useful as additional evidence of identity. Characteristic features include the serrated edges of the leaves, their compound palmate structure (i.e., several leaflets arise from the same point), and the ovoid mottled appearance of the seeds. Figure 3.4a is a photomicrograph depicting *Cannabis* seeds. Identification based on gross morphology requires large portions of the plant for examination because other plants possess these structures.

3.1.2.2.3 The Duquenois–Levine and Other Color Tests for Cannabis

Two fast versions (less than 3 minutes to perform both) of the Duquenois–Levine and fast blue B tests are described by De Faubert Maunder (1969). Only henna, of the dozens of botanicals tested, gave a false-positive reaction. Oddly, the author describes an actual submission to his laboratory of henna mixed with *Cannabis* resin. This speedy version contrasts with how Duquenois with Negm reported the test in 1938 (Mausolf, 2001). Duquenois contends that a petroleum ether extract, evaporated to dryness, will go through a series of colors for up to an hour after adding concentrated HCl. He adds that under these circumstances, the test is specific. Fulton (1970) describes a color test using furfural for the identification of *Cannabis* resin. He claims to have been using the furfural test for *Cannabis* resin for 30 years, a fact suggesting that he found it reliable. He fails, however, to provide mention of testing other substances for indications of the test's validity. Lau-Cam and McDonnell (1978) performed a validation of the furfural test by subjecting various botanicals to it and a modified version of the test. In the same study they also tested the Duquenois reaction with various brands of coffee. They found that the furfural test is simple to implement and very sensitive. It did not yield false positives with any of the coffees tested or with teas, some of which had been reported to generate false-positive reactions to the Duquenois test. Fochtman and Winek (1971) reported some of these false positives to the Duqenois–Levine test after testing

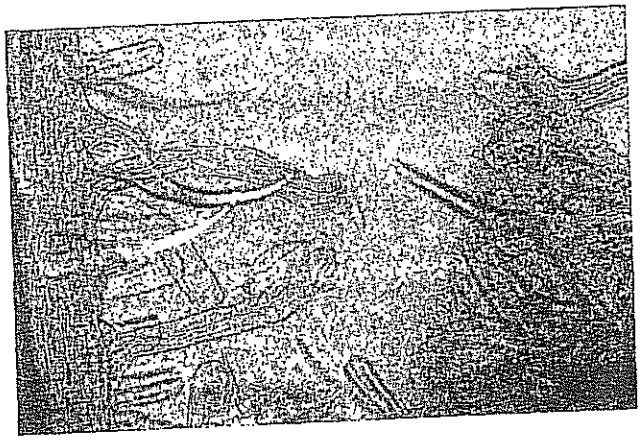


Figure 3.3a
Microscopic structures of Cannabis. *Note the cystolithic and glandular hairs. (micrograph, 100× with aqueous chloral hydrate used as mounting medium)*

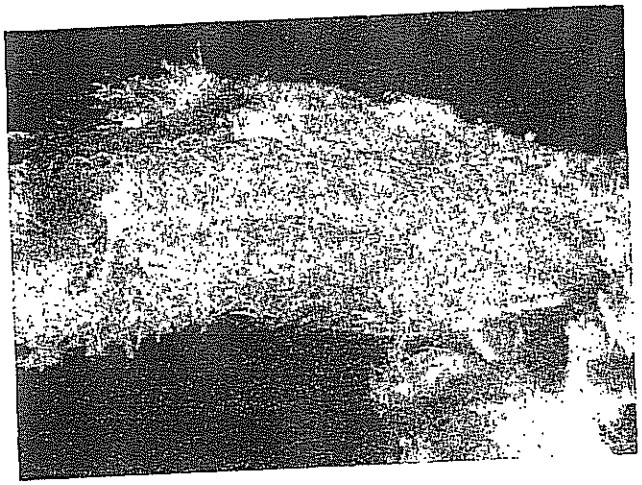


Figure 3.3b
Cystolithic hairs of Cannabis. *Note the "bear claw-like" morphology. (stereomicrograph, 25× taken with oblique light)*

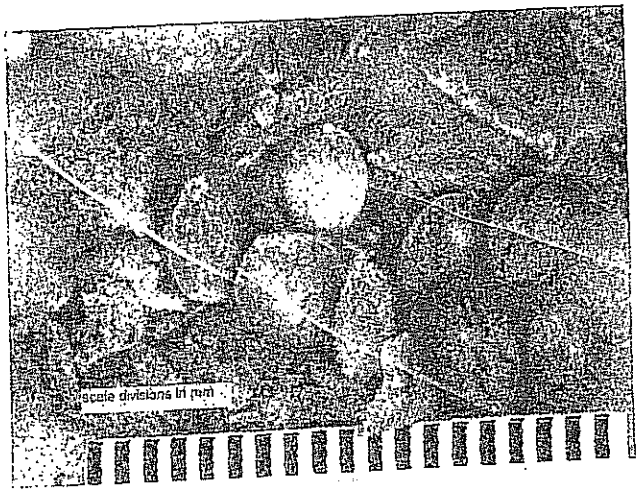


Figure 3.4a
Cannabis *seeds. Note the typical ovoid shape and mottled appearance. (stereomicrograph, 7.5×, taken with oblique light)*

Figure 3.4
Macroscopic plant morphology of Cannabis *sativa L.*
1. Flowering shoot
2. Male inflorescence
3. Male flower
4. Female inflorescence
5. Female flower
6. Fruit
7. Seed

several brands of coffee. They caution that microscopic examination and color tests constitute only screening tests for *Cannabis*; a positive identification should be based on these combined with thin-layer or gas chromatography. Lau-Cam (1978) was able to eliminate the false-positive reaction of the Duquenois–Levine test to various coffees by adding 5 to 10 drops of para-dioxane to the test mixture immediately following addition of hydrochloric acid. If only coffee is present, the colored products of the Duquenois reaction will be unstable and fade to brown within 1 minute under this procedure. The color reaction of *Cannabis*, however, will remain stable during this step, with the color being transferred to the chloroform layer during the Levine modification. El-Darawy et al. (1972) spotted thin-layer chromato-

graphic plates with six common cannabinoids (cannabinol, cannabidiol, tetahydrocannabinol, cannabichromene, cannabidiolic acid, and a cannabidiol isomer) and sprayed them with various visualizing reagents, including a Duquenois spray. When cinnamaldehyde was substituted for vanillin in the Duquenois spray, more of the cannabinoids responded with color reactions than reacted with vanillin. Substituting cinnamaldehyde for the aldehyde in a Duquenois and Blackie's test on hashish resin produced no appreciable improvement. Duke and Reimann (1973) compared various liquids for their efficiency in extracting Duquenois-positive cannabinoids. Alcohols, including methanol, ethanol, and propanol, were determined to be more efficient than other solvents tested. De Faubert Maunder (1974) describes an improved field test for *Cannabis* using dyes other than fast blue B, a dye suspected of posing health risks. Dyes that give similar colors to fast blue B and quick responses are fast blue BB, 1-diazo-4-benzoylamino-2,5-diethoxybenzene, and Corinth V. The testing procedure involves placing a small amount of liquefied material on filter paper, adding several reagents, and noting color changes at each step. The test can be rapidly performed. The author also comments on variables affecting the test, such as heat, different kinds of test papers, and the condition of the solvents. Hughes and Warner (1976) tested 67 compounds with a modified Duquenois–Levine test. They found that if 2 or 3 minutes is allowed to pass before adding chloroform, the selectivity of the test is greatly enhanced. They also found that only three types of coffee yield misleading test results following this procedure. In a follow-up study Jarzen (1977) observed that with a petroleum ether extract of the coffees (taken to dryness) the intense red-violet color generated with the Duquenois reagent for each brand of coffee was distinctly different than that produced with *Cannabis*. Additionally, he notes that the colors produced in the chloroform layer decomposed with time, whereas those generated from *Cannabis* became more intense over time. Contrary to the findings of Hughes and Warner, Jarzen found that the *immediate* addition of chloroform after the purple color begins to form in the aqueous layer increases the test's discrimination between coffee and *Cannabis*. He claims that when the Duquenois–Levine test is performed by a trained investigator on dried ether extracts with chloroform added immediately upon the appearance of a purple color in the aqueous layer, it is "a specific test for the presence of marijuana resin" and "will eliminate the possibility of a false-positive identification." Claims this strong for the discrimination power of the Duquenois–Levine test have not been noted elsewhere by us. Bailey (1979) reviewed three versions of the Duquenois test and their reactions to over 400 botanicals. He also looked at previous efforts to identify false positives to the Duquenois test. He observes, "There is no published report of an obviously botanical material apart from *Cannabis* that

gives a positive Duquenois–Levine test." Bailey concludes that an analytical scheme of Duquenois–Levine, botanical inspection (gross or microscopic features), and a TLC system are necessary for a complete identification. He also found that of the three versions of the Duquenois reaction tested (a rapid version, a traditional version, and one employing a solid reagent of 1% metaldehyde in vanillin), the traditional version was the most discriminating for the variety of plant materials tested. A comparison of the Duquenois–Levine and the fast blue B salt test using six forms of *Cannabis* and 10 other botanicals was made by Drover and Lacienta (1980). Their studies, employing a test tube version of the fast blue B salt test (similar to that used in the commercial field test kits described earlier), revealed no false positives with fast blue B for the samples tested. Several leaf and seed samples did yield false negatives to the Duquenois–Levine test. The authors cite previous work (Fochtman and Winek, 1971; DeFaubert Maunder, 1969) stating that coffee, nutmeg, and mace do yield false positives to the Duquenois–Levine test. O'Neal et al. (2000a), using THC as the only form of *Cannabis* tested, performed a validation of the modified Duquenois–Levine test. To standardize part of the test, they referenced the reaction colors formed to the Inter-Society Color Council and the National Bureau of Standards (ISCC-BBS) and to Munsell charts. They also described the colors using common color designations. Mace, nutmeg, and tea reacted with the modified Duquenois–Levine test, but only THC generated a deep purple color.

Pitt et al. (1972) using UV/visible (VIS) spectroscopy studied the chemical mechanism of the Duquenois color test in an effort to characterize its specificity. They found that a partial resorcinol structure was necessary, but not solely sufficient, to produce the characteristic purple color. They conclude that for an identification protocol, the Duquenois test is an acceptable screening step when combined with botanical evidence. They add, however, that because of the "ubiquitousness of phenols in nature" and when botanical evidence is absent, supplementing the color test with positive chromatographic data is mandatory. Forrester (1998), using UV/VIS, IR, MS, and nuclear magnetic resonance (NMR), studied the purple dye structure of the Duquenois reaction. An aromatic substitution of Δ^9-THC with *p*-dimethylaminocinnamaldehyde was indicated. He proposes a schematic of the Duquenois product with Δ^9-THC.

3.1.2.2.4 Chromatographic Methods for Identifying Constituents (Cannabinoids) of Cannabis

Often, to complete the analytical scheme of color testing and examination of plant morphology, a chromatographic method is employed for additional evidence of the presence of *Cannabis*. Identification testing of seized hash or

oils of *Cannabis* requires that the presence of various cannabinoids, many as monoterpenoids, be established. Evidence of their presence can be demonstrated with chromatographic methods such as TLC, GC, and HPLC. Most seized-drug testing for cannabinoids in *Cannabis*, its resins, and its oils typically involves only their identification.

Major cannabinoids are tetrahydrocannabinol (THC), cannabinol (CBN), and cannabidiol (CBD). Others include cannabinolic acid (CBNA), cannabidiolic acid (CBDA), cannabichromene (CBCh or CCEE), cannabichromenic acid (CBChA or CCEEA), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabivarin (CBV), tetrahydrocannabinolic acid (THCA), tetrahydrocannabivarin (THV), and tetrahydrocannabivarinic acid (THVA). The isolation of Cannabisativine from the root of the *Cannabis* plant was reported by C.E. Turner et al. (1976). C.E. Turner et al. (1974) state that CBCh, though thought to be a minor component of *Cannabis*, occurs more abundantly than CND in many *Cannabis* variants. Not all cannabinoids are psychoactive, as Siniscalco Gigliano (2001) notes with CBD and CBN and as Gaoni and Mechoulam (1966) report with CBCh. However, Fonseka et al. (1976) cite evidence that CBN may elicit a slight psychoactive effect in humans. Most of the nonmajor cannabinoids occur in quantities of less than 0.01% of total cannabinoids (Siniscalco Gigliano, 2001). Studies by R.N. Smith and Vaughan (1977) revealed that methanol is the most effective solvent of the four they tested (methanol, chloroform, light petroleum, and methanol-chloroform, 9:1) for extracting cannabinoids. Both neutral and acid cannabinoid solutions are relatively stable in darkness; acidic constituents tend to decompose in sunlight. J.M. Parker et al. (1974) report that cannabinoids, particularly THC, are unstable in various solvents like $CHCl_3$. Small and Beckstead (1973a) reported that some batches of *Cannabis* studied contained no THC. J.C. Turner and Mahlberg (1984) found that variation in extraction temperature (with $CHCl_3$ at 4°C and room temperature) had no significant effect on the amount of cannabinoids extracted. They observed that the amount of cannabinoids extracted from fresh plant material with $CHCl_3$ decreased over time (1.5h to 10h); no significant difference in amount extracted for these times was demonstrated with dried plant material.

Ohlsson et al. (1971), using chromatographic and mass spectrometric methods to study fresh *Cannabis* from different parts of the world, found cannabinoids present in all parts of the plant but is more abundant in the flowering tops and the small leaves around the flowers. They also found that both male and female plants have approximately the same amounts of cannabinoids and in similar ratios. The authors cite other research in support of the latter finding. ElSohly et al. (2000), in an analysis using gas chromatography of over 35,000 *Cannabis* preparations confiscated in the United States over an 18-year

period, determined that the percentages of Δ^9-THC has risen from less than 1.5% in 1980 to 4.47% in 1997. Also noted was that hashish and hash oil showed no significant potency trends and that other major cannabinoids (cannabidiol, cannabinol, and cannabichromene) showed no significant change in concentration during the period studied.

Thin-Layer Chromatography (TLC)

There are many thin-layer chromatographic systems listed in the professional literature that will separate cannabinoids. Generally they involve spotting an extract of the plant material, resin, or oil along with known cannabinoid standards on silica-coated glass plates and letting them develop in mobile phases composed of a single organic solvent or a combination of organic solvents. Early methods included partition chromatography, where plates were predeveloped, for example, in dimethylformamide, prior to development. Korte and Sieper (1964) suggest such a method using cyclohexane as the developer. Today it is probably the case that most seized-drug chemists practice adsorption chromatography. In either case, the plates are then dried and the separated cannabinoids observed in at least two ways. They can be viewed under ultraviolet light if the plates possess fluorescent agents that ascending compounds can quench or be visualized by spraying with reagents that color the separated cannabinoids. A visualizing spray used early on, fast blue B, was replaced by many practitioners with fast blue 2B because the former is thought to be carcinogenic. An alternate visualizing spray to fast blue B, a 1% methanolic solution of 2,6-dichloroquinone-4-chlorimide, was developed by Barbato (1978). He claims that it is less carcinogenic and more stable than fast blue B. TLC has the advantages for seized-drug analysis of being relatively rapid, inexpensive, and convenient to operate and interpret.

What follows is a listing of various TLC systems for separating cannabinoids.

Clarke (Moffat, 1986a) recommends two systems for separating CBN, CBD, and Δ^9-THC:

(TI) Plates of silica gel G are dipped or sprayed with a 10% solution of silver nitrate and dried. Mobile phase is toluene; plate is developed in an open tank under unsaturated conditions.

(TJ) Plates of silica gel G are sprayed with diethylamine immediately prior to use. Mobile phase is xylene/hexane/diethylamine, 25:10:1. Visualizing reagents for both TI and TJ are fast blue B or Duquenois reagent.

The *CRC Handbook of Chromatography Drugs* (Gupta, 1981) also recommends two systems for separating Δ^9-THC, Δ^8-THC, CBN, CBD, CBNA, CBDA, Δ^9-THCA, and CBChA:

(S-1) A two-dimensional system consisting of petroleum ether/diethyl ether/acetic acid, 40:10:1, in one dimension and *n*-heptane/diethyl ether, 80:10, for the second dimension on a silica gel G plate. Plate is sprayed with fast blue B salt in 0.1 *N* NaOH.

(S-2) Methanol/water, 95:5, on a silica gel plate. Cannabinoids can be visualized by spraying plates after development with a solution of sodium metal (8 g) in methanol (100 mL) and dimethyl sufoxide (8 mL) and viewing under ultraviolet light.

The United Nations (1991) Drug Control Program (UNDCP) recommends three systems for separating CBCh, CBV, CBN, THV, THC, CBD, THCN, and CBDA (the compound name that "THCN" abbreviates is not listed):

A. Petroleum ether/diethyl ether, 80:20
B. Cyclohexane/di-isopropyl ether/diethylamine, 52:40:8
C. (For cannbinoid acids) *N*-hexane/dioxane/methanol, 70:20:10

The UN treatise outlines minimum quantities of plant material, resin, and liquid suitable for extraction along with the advantages and liabilities of various organic solvents for extracting cannabinoids. Though not stated in the text, it is presumed that TLC plates are coated with silica gel. Plates can be visualized with a fast blue B or 2B solution and can be preserved for review purposes by a final spraying of diethylamine followed by sealing in plastic wrap. R_f values are cited for the eight cannabinoids and cannabinoid acids listed earlier.

Other TLC systems for separating cannabinoids include silica gel plates developed in petroleum ether and ether, 4:1 (Machata 1969), and a system using a mixture of pentane and ether, 88:12, described by Gaoni and Mechoulam (1971). Mechoulam (1973) notes that a 7:10 ratio of acetone/hexane on silica plates minimizes oxidative degradation of labile cannabinoids and also yields good separation. Parker and Fiske (1972), after a literature review of numerous TLC systems for separating six cannabinoids (Δ^9-THC, Δ^8-THC, CBN, CBD, CBDA, CBCh), suggest adsorption methods involving $CHCl_3$ or 1,4-dioxane as developing solvents and fast blue B as a visualizing spray.

Tewari and Sharma (1983) describe a two-dimensional TLC system that will resolve 47 *Cannabis* constituents. A 20 × 20-cm silica gel G plate was used with heptane/dichloromethane/butan-2-one, 83:5:12, as the solvent for the first dimension. After the solvent was allowed to rise 12 cm, the plate was dried, rotated 90°, run in hexane/acetone, 86:14, and sprayed with a 0.1% solution of fast blue 2B in 45% ethanol. They report that spots of the major cannabi-

noids, CBC, Δ^9-THC, *trans*-Δ^8-THC, and CBD, were clearly "distinct, prominent, and dense."

Gas Chromatography (GC)

Numerous qualitative methods published in the 1970s reference use of packed chromatographic columns with packings such as OV-1, OV-17, OV-225, OV-101, and SE-30 and a variety of detectors, including flame ionization (the most common at the time), MS, electron capture, and nitrogen/phosphorous detectors (Gupta, 1981). In a series of studies in the 1970s, Strömberg (1971, 1972a, 1974a, 1974b, 1976) characterized numerous (>30) minor components of hash and *Cannabis*, first with packed-column GC and later with packed-column GC/MS. Some major cannabinoids were also profiled. Packed-column GC, because of a resolution significantly lower than capillary-column GC, does not have the wide application today that it once enjoyed.

The UNDCP (1991) lists three gas liquid chromatographic systems along with operating parameters for qualitative and quantitative analysis of cannabinoids. Two involve packed columns; the third uses a 10-m OV-1 chemically bonded fused-silica capillary column. All use flame ionization detection. Mills and Roberson (1993), in a large compendium of analytical drug data, list UV, IR, Fourier-transform NMR, and MS data for Δ^8- and Δ^9-THC, CBCh, CND, CNB, CBG, cannabicyclol, and cannabispiran. The spectra were generated specifically for that publication. In a review of analytical methodology for identification and quantification of *Cannabis* products, Vollner et al. (1986) describe both a packed-column (involving derivatization) and capillary-column GC method for separating cannabinoids.

A modern seized-drug GC procedure for profiling cannabinoids utilizes a 15- to 50-m (I.D. 0.25 mm) capillary column of fused cross-linked methyl silicone (methyl siloxane, phenol siloxane, etc.). Plant material, hash, or hash oil can be prepared for injection by drying a filtered petroleum ether extract and solvating it in methanol. General GC screening parameters include starting with an injection port temperature (perhaps 250°C) and ramping up the column temperature from 110°C (at, for example, 20°C per minute) to approximately 290°C. A procedure based on these parameters should separate the three major cannabinoids: THC, CBN, and CBD. A mass selective detector can help to identify each.

The heat of GC will decarboxylize cannabinoid acids to their neutral form (Kanter et al., 1979). Cannabinoids and cannabinoid acids may be separated by using trimethylsilyl derivatives or HPLC. J.C. Turner and Mahlberg (1982) developed a method for the latter using variation in solvent pH to successfully separate neutral and acidic cannabinoids. This could be particu-

larly useful for fresh plant material, where the amounts of cannabinoid acids are high.

3.1.3 REVIEW OF TESTING METHODOLOGIES RECOMMENDED BY STANDARDIZING ORGANIZATIONS

Two international organizations have published standards specific to seized-drug analysis and *Cannabis* in particular. They are the United Nations (UN) and the Scientific Working Group for Seized Drug Analysis (SWGDRUG). The UN treatise describes specific testing procedures to analyze for *Cannabis* in its different dosage forms. SWGDRUG has made recommendations in the form of minimum standards for drug identification. These standards apply to both *Cannabis* as plant material and its chemical constituents. Specifically, SWGDRUG recommends a minimum number and combination of tests necessary for identification of controlled substances. As important as the specifics of testing procedures is that no testing protocol is complete if not performed in an analytically sound context. Accordingly, each organization has published guidelines on good laboratory practice and/or quality assurance. The following is a review of the analytical testing standards of each organization for *Cannabis* and its constituents.

3.1.3.1 The United Nations Drug Control Program (UNDCP)

The United Nations provides technical support for many forensic laboratories worldwide under the aegis of the UNDCP. The UNDCP, based in Vienna, Austria, publishes a series of monographs describing testing methodology for various controlled substances. Included in this series is *Recommended Methods for Testing Cannabis* (United Nations, 1991). It describes various tests for the identification of *Cannabis*, as plants, as resin (hashish), and as liquid. Except for its reliance on packed-column gas liquid chromatography, the methodologies described could easily find currency in modern forensic laboratories. After a discussion of how *Cannabis* is marketed in various locations, how the resin and liquids are prepared, and a description of the macro- and microscopic botanical composition of the plant, different testing methods, including a sampling plan, are described.

3.1.3.1.1 Sampling

The UN-recommended sampling plan can be applied to a single item, multiple items, or very large aggregates. In general, it recommends that, "sampling should be undertaken to conform to the principles of analytical chemistry, as laid down, for example, in national pharmacopoeias or by such organizations

as the Association of Official Analytical Chemists. For multiple items potentially containing *Cannabis*, emphasis is placed upon visual examination of all items needed for testing."

For quantitating a cannabinoid in single-package items it recommends homogenizing the plant material by passing it through progressively finer sieves. For qualitative analysis of multiple packages, it recommends the following:

a. For 10 packages or less: sample all 10.
b. For between 10 and 100: randomly select 10 packages for sampling.
c. For more than 100 packages: "select a number of packages equal to the square root of the total number of packages rounded to the next highest integer."
d. For sampling materials containing large aggregates that cannot be broken down: random samples should be taken from at least two different parts of an item.

3.1.3.1.2 Testing

A series of microscopic, wet chemical, and instrumental tests are described in cookbook fashion for identifying *Cannabis* and its cannabinoid constituents.

Macroscopic examination is based on identifying features of the male and female plant structure such as leaf and flower morphology. Drawings of these and more are provided. There is an abundance of microscopic structures that in conjunction with chemical tests can be used to reveal the presence of *Cannabis*. These include

a. Nonglandular hairs (trichomes), which include cystoliths
b. Glandular trichomes

Two color tests are described: the fast blue B salt test and the rapid Duquenois test (Duquenois–Levine test). Instructions are provided for performing the former either in a test tube or on filter paper. Essentially, an amount the size of a match head of plant material, resin, or liquid is put together with several reagents in stepwise fashion. The appearance of colors variously indicates the presence of the cannabinoids THC, CBN, and CBD. The rapid Duquenois test requires placing a small amount of suspected material in a test tube, adding several reagents, and noting color changes. According to this source, however, the appropriate colors indicate only "a *Cannabis* product." It observes that cannabinoids are not likely to be found in the stems or seeds of the plant but are abundant in its leaves and flowers. It is stressed that these

- In cases where hyphenated techniques are used (e.g., gas chromatography-mass spectrometry liquid chromatography-diode array ultraviolet spectroscopy), they will be considered as separate techniques provided that the results from each are used.
- *Cannabis* exhibits tend to have characteristics that are visually recognizable. Macroscopic and microscopic examinations of *Cannabis* will be considered, exceptionally, as uncorrelated techniques from Category B when observations include documented details of botanical features. Additional testing must follow the scheme outline in the first two items of this list.

  For exhibits of *Cannabis* that lack sufficient observable macroscopic and microscopic botanical detail (e.g., extracts or residues), Δ^9-tetrahydrocannabinol (THC) or other cannabinoids must be identified utilizing the principles set forth in the first two items of this list.
- Some examples of reviewable data include printed spectra, chromatograms, and photographs or photocopies of TLC plates. Contemporaneous documented peer review will suffice for microcrystalline tests and recording of detailed descriptions of morphological characteristics is sufficient for *Cannabis* (only).

Identification of *Cannabis* as plant material can be made, in the simplest way, using the two Category B macro- and microscopic examinations and a color test. Examples of the latter include a Duquenois–Levine test or a fast blue B salt test. As noted in the preceding list, resins or liquids require that Δ^9-THC be identified utilizing the principles set forth in the first two items in the list.

The recommendations stress the use of an analytical scheme consisting of multiple uncorrelated tests based on validated methods performed by competent analysts. Testing in excess of the minimums stated is not discouraged.

3.1.3.3 The International Association of Official Analytical Chemists (AOAC)

The AOAC (Cunniff, 1997) lists a Duquenois–Levine method for the identification of *cannabis* (marijuana) in drug powders. The reagent is prepared by dissolving 12 drops of acetaldehyde (fresh) and 1.0 g vanillin in 50 mL of alcohol. Approximately 100 mg of sample is extracted with 25 mL of petroleum ether, filtered into a white porcelain dish, and evaporated to dryness over a steam bath. Two milliliters of the Duquenois reagent is added to the dish and stirred to dissolve the residue. Two milliliters of HCL is then added, and the mixture is allowed to sit for 10 minutes. After noting the color, the solution is transferred to a test tube, 2 mL of $CHCL_3$ is added, and the tube is shaken. The liquids are allowed to separate. A purple color in the $CHCL_3$ layer reflects a positive test result.

3.1.4 REQUIREMENTS OF THE AMERICAN BOARD OF CRIMINALISTS' CERTIFICATION EXAMINATION REGARDING ANALYSIS OF CANNABIS

As a corollary to the topics covered relating to the seized-drug analysis of *Cannabis*, the American Board of Criminalists (ABC), a certifying body for those analyzing or examining forensic evidence, requires some mastery of these topics for applicants of their *Knowledge, Skills and Abilities Drug Analysis Specialty Examination.* In topics for review they list, under "Identification of *Cannabis*" (ABC, 2001),

A. Macroscopic and microscopic morphology
B. Duquenois–Levine test
C. Botanical characteristics of *Cannabis* (annual plant, two sexes, existence of several agronomic varieties of monospecific genera, etc.)
D. Hashish and hash oil
E. Major cannabinoid chemical components of the plant (two types of THC, other psychoactive and nonpsychoactive components)
F. TLC of cannabinoids

3.1.5 MINIMUM ANALYTICAL REQUIREMENTS OF THE NATIONAL LABORATORIES OF AUSTRALIA, ENGLAND, HOLLAND, CANADA, AND THE UNITED STATES FOR IDENTIFYING CANNABIS

An informal 2002 survey of senior scientists of the national laboratory systems in the United States, England, Australia, and Holland involved with seized-drug analysis revealed the following. Minimums of two tests are required for identification of *Cannabis*, with more being performed if there are difficulties in analysis. Canada requires four tests for plant material. For submissions that include plant material, all of the laboratories rely on microscopy as part of the analytical scheme, using either stereo or compound light scopes. All laboratories accept trace amounts of *Cannabis* for testing. With hash, hash oil, or trace amounts of material, all require at least two selections from the following approaches: a Duquenois test, microscopy, TLC, and GC/MS. England and Canada reported the occasional use of HPLC for individualizing samples of *Cannabis* to a common batch.

3.2 PROFILING—PROVENANCE

3.2.1 INTRODUCTION

In addition to identifying *Cannabis* plants and preparations or identifying the presence of THC, it has long been of interest to look at the other constituents

of the plant. Analysis of the concentrations and ratios of the major constituents THC, CBN, and CBD (Doorenbos et al., 1971; Fetterman et al., 1971a, 1971b; Small et al., 1975; Small and Beckstead, 1973b; Toffoli et al., 1966) was used to explore the taxonomy of *Cannabis* and to differentiate fiber-type (low THC) from drug-type (high THC) varieties. In addition, analytical profiles, or "fingerprints," of distributions of chemical constituents of *Cannabis* preparations have been used to provide answers to two recurring questions. The first, referred to by Perillo et al. (1994) as *strategic intelligence*, addresses the country or region of origin and is important in investigating distribution. The second addresses the question of whether two or more samples have a common origin. This is referred to as *tactical intelligence* (Perillo et al., 1994). Tactical intelligence involves a sample-to-sample comparison and is of particular utility in conspiracy investigations.

3.2.2 CHROMATOGRAPHIC METHODS

3.2.2.1 History

Early attempts to address one or both of these questions utilized TLC (Chiesa et al., 1973; Fowler et al., 1979; K.D. Parker et al., 1968; Tewari and Sharma, 1983; Tewari et al., 1974). Overpressured-layer chromatography has also been used (Oroszlan et al., 1987). Gas–liquid chromatography (GLC) using packed columns offered increased resolution and discrimination (Toffoli et al., 1966; Manno et al., 1974), and combination with mass spectrometry afforded positive identification of constituents (Vree et al., 1972). Differentiation of *Cannabis* of different origins by plotting of peak areas of CBD vs. CBN plus THC was first proposed by T.W.M. Davis et al. (1963). Small et al. (1975) and Small and Beckstead (1973b) used plots of %THC vs. %CBD to assign phenotypes. As awareness of coelution of CBD and cannabichromene (CBC) grew, comparison of THC to CBD + CBC was used by some investigators (Rowan and Fairbairn, 1977). However, it became apparent that the interpretation of analytical results and the appropriate choice of analytical tools are dependent on a wide range of factors. The most important factor arises from the chemical makeup of the *Cannabis* plant itself. The major components, THC, CBN, and CBD, are absent or in low concentration in the living plant (Fetterman et al., 1971a; Kimura and Okamoto, 1970; J.C. Turner and Mahlberg, 1982), where they exist as their carboxylic acid derivatives, which can be decarboxylated for use in profiling based on the major cannabinoids (Fetterman et al., 1971a). The degree to which the precursors are decarboxylated is dependent on the time between harvest and analysis and the environmental and storage conditions to which the samples are exposed (Vollner et al., 1986). Since the heat of the injection port causes decarboxylation (Fetterman et al., 1971a), the acid constituents cannot be

determined by GLC unless the samples are derivatized prior to analysis (C.E. Turner et al., 1974). In order to generate data that could be interpreted in terms of chemical strains (chemovars), samples were heated to decarboxylate the precursors and ratios, (THC + CBN)/CBD, were determined (Kimura and Okamato, 1970; Veress et al., 1990; Kanter et al., 1979). Even though cannabinoid ratios have been shown to vary with environmental factors (Coffman and Gentner, 1975; Fairbairn and Liebmann, 1974; Haney and Kutscheid, 1973; Latta and Eaton, 1975; Siniscalco Gigliano, 1984; Valle et al. 1978), sex of the plant (Siniscalco Gigliano, 1984; Ohlsson et al., 1971; Agurell, 1970), maturation (Krejčí, 1970; Hemphill et al., 1980; Latta and Eaton, 1975; J.C. Turner et al., 1977), amount of light (Mahlberg and Hemphill, 1983), part of the plant (Krejčí, 1970; Doorenbos et al., 1971; Fetterman et al., 1971a; Hemphill et al., 1980; Fairbairn and Liebmann, 1974; J.C. Turner et al., 1977), and season when collected (Latta and Eaton, 1975; Phillips et al., 1970), the bulk of the evidence indicates that the most important factor is heredity (Ohlsson et al., 1971; Siniscalco Gigliano, 2001). The interpretation of cannabinoid ratios was complicated by the inability to resolve CBN, CBC, and cannabivarin by packed-column GC (C.E. Turner and Hadley, 1973; C.E. Turner et al., 1975). The interpretation was further complicated by the observation that the ratios detected are affected by the storage and treatment of the material prior to analysis (C.E. Turner et al., 1973a; J.C. Turner and Mahlberg, 1984). R.N. Smith and Vaughan (1977) demonstrated that results are also affected by the solvents used to extract the samples and the method of storage of the extracts (J.M. Parker et al., 1974; R.N. Smith and Vaughan, 1977). Chloroform (C.E. Turner and Henry, 1975) and methanol or ethanol (R.N. Smith and Vaughan, 1977) are efficient in the extraction of cannabinoids. Storage of the extracts in the dark and in the cold is essential to minimize changes in the samples (R.N. Smith and Vaughan, 1977).

The presence in *Cannabis* of the alkanes *n*-heptacosane and *n*-nonacosane as well as other straight-chain alkanes, ranging from C_{16} to C_{32}, was reported by de Zeeuw et al. (1973). These compounds can interfere with cannabinoid analysis when using GC alone. The authors noted a variation in the hydrocarbon concentrations with area of origin. C.E. Turner et al. (1973b) noted that the propyl homologs of CBN (cannabivarin), CBD (cannabidivarin), and THC (tetrahydrocannabivarin), in which the pentyl side chain is replaced with propyl, showed variations with geographical origin. Turner and Hadley (1973) reported the absence of CBD in an African variant. This was followed by an extensive study of cannabinoids in *Cannabis* from South Africa (Field and Arndt, 1980). de Zeeuw et al. (1972) also noted geographical variation of the propyl side chains. The ratios of concentrations of CBC, CBD, and THC were also correlated with geographical origin (Holley et al., 1975).

The bulk of earlier work on cannabinoid ratios utilized packed-column GC. The packed-column GC of *Cannabis* constituents has been reviewed (Fish, 1974; J.M. Parker and Stembal, 1974). Methods for qualitative and quantitative analysis of *Cannabis* products have also been reviewed (Vollner et al., 1986).

Since the ratios of the concentrations of the major cannabinoids are more a reflection of the origin of the seed than of the region where the crop is grown, these ratios are more useful for separating chemical races and as tactical intelligence than they are for strategic intelligence. Other constituents have been studied in order to determine their value in determining provenance. In a series of papers, Strömberg (1971, 1972a, 1972b, 1974a, 1974b, 1976) used packed-column GC to explore the separation and identification of what he termed minor components of *Cannabis* resin. He proposed the use of chromatograms showing components with both shorter and longer retention times than CBD in comparing hashish samples (Strömberg, 1972b). Volatile components sampled from the sealed headspace of solid *Cannabis* samples were explored by Hood et al. (1973) and Hood and Barry (1978). Three different packed-column GC analyses were used to identify three fractions consisting of oxygenated compounds (MW < 100), monoterpenes (MW > 100), and sesquiterpenes (MW < 100) (Hood et al., 1973). Eighteen compounds were identified by retention times. Chromatograms of headspace volatiles from 14 samples having different geographical origins were compared (Hood and Barry, 1978). The authors concluded that headspace-volatile analysis may be useful in comparing two seizures but that since they vary with handling and history, their utility in determining geographic origin is doubtful. Both Strömberg and Hood were hampered by an inability to resolve many constituents using packed columns.

3.2.2.2 Extraction

A wide variety of extraction solvents and methods have been used to separate the cannabinoids and other compounds of interest from the plant material or the resins. They include soaking in petroleum ether (Barni Comparini and Centini, 1983; Stephanou et al., 1984), chloroform (Kanter et al., 1979; J.C. Turner and Mahlberg, 1984), or methanol (Björkman, 1982; Wheals and Smith, 1975; Nakahara and Sekine, 1985). Soxhlet extraction with cyclohexane has been used (Novotny et al., 1976). R.N. Smith and Vaughan (1976) used methanol–chloroform (9:1), as did Brenneisen (1984). Baker et al. (1980) also used methanol–chloroform (4:1). Brenneisen and ElSohly (1988) compared various solvents and methods and concluded that methanol–chloroform (9:1) gave the overall best extraction efficiency for the wide range of analytes in *Cannabis*. Sonication at room temperature of powdered sample with this solvent has become the method of choice for comparison studies with *Cannabis*.

Veress (1994) used *Cannabis* as an example for a method of optimizing extraction efficiency in supercritical fluid extraction (SCF) for quantification. An evaluation of the effect of particle size on the SCF extraction of *Cannabis* showed that selection of a given sieve fraction leads to erroneous conclusions about the sample as a whole (Eory et al., 2001a). SCF has been applied to the measurement of THC and THCA concentrations in plant material (Eory et al., 2001b).

3.2.2.3 Derivatization

One approach to achieve increased resolution in packed-column GC and to allow simultaneous analysis of acid components is to derivatize the phenolic and carboxylic acid functions of the cannabinoids. Claussen et al. (1966) first used TMS derivatives to separate the cannabinoids from their acidic derivatives. TMS and trifluoroacetyl derivatives were described by Caddy and Fish (1967). Fetterman et al. (1971b) and K.H. Davis et al. (1970) described TMS derivatives in separations of THC, CBN, and CBD. The use of TMS derivatives to separate and identify cannabidiolic acid (CBDA) (Paris and Paris, 1973) and to separate CBD and CBC (C.E. Turner et al., 1974) was also reported. The use of silyl derivatives in routine analysis was advocated by C.E. Turner et al. (1974). Rasmussen (1975a, 1975b) explored the use of GC with solid injection and with on-column silation following solid injection with cold trapping.

Harvey and Paton (1975) combined packed-column GC with MS to explore silyl derivatives with longer alkyl groups to separate dihydroxy from monohydroxy cannabinoids. The tri-*n*-butyl silyl derivatives afforded complete separation. *t*-Butyldimethylsilyl, trimethylsilylacetate, and diethylphosphate derivatives were used in a study of the cannabinoids by GC, GC/MS, and HPLC (Knaus et al., 1976). The *t*-butyldimethylsilyl and trimethylsilylacetate derivatives were stable enough for use in HPLC. On-column methylation using dimethylformamide dimethylacetal in pyridine enabled Björkman (1982) to separate and identify at least 16 components of *Cannabis* extracts, by packed-column GC/EIMS, compared with 6 without derivatization.

3.2.2.4 High-Performance Liquid Chromatography and Capillary Column Gas Chromatography

An alternative to derivatization of polar and acidic functions is to use a method that does not cause decomposition or rearrangement in thermally labile compounds. High-performance liquid chromatography (HPLC) fits this need. Although HPLC does not offer the resolving power of capillary-column GC, it is on a par with packed-column GC for the resolution of the main cannabinoids. The identification of the separated components requires either comparison to standards or isolation and analysis by mass spectrometry (Wheals and

Smith, 1975; R.N. Smith, 1975). Reversed-phase HPLC with UV detection has been utilized for provenance determination and specimen comparison (R.N. Smith and Vaughan, 1976; P.B. Baker et al., 1980; J.C. Turner and Mahlberg, 1982; Brenneisen, 1984, 1986). Nakahara and Sekine (1985) developed a method using electrochemical (EC) detection. Kanter et al. (1979) used HPLC in a method to quantitate THC and THCA based upon comparison of raw samples vs. decarboxylated samples, since the normal-phase separation being used did not enable direct measurement of the THCA. Veress et al. (1990) also used normal-phase HPLC and optimized the decarboxylation process to determine cannabinoid acids.

Novotny et al. (1976) used an 11-m by 0.26-mm glass capillary coated with SE-52 (methylsilicone) to separate cannabinoids. Soxhlet extraction with cyclohexane, followed by washing with nitromethane, was used to obtain samples for analysis. The cyclohexane was evaporated to dryness, and dichloromethane was used as the solvent for chromatography. The sample (4 μL) was injected into a precolumn that was flushed with helium to remove solvent. The precolumn was placed in the modified injection port of a GC, where it was thermally striped (250°C) onto the analytical column (room temperature). Temperature programming from 70°C to 240°C at 2°C was used for both FID and MS detection. The analysis lasted 110 minutes. The method separated 70 components, 38 of which were identified by EIMS. The authors demonstrated the discrimination of two samples that appeared identical by comparison of CBN, THC, and CBD using the same column under different conditions. Stephanou et al. (1984) used capillary-column GCEIMS to separate and identify the TMS derivatives of *Cannabis* extracts. Mass spectra of 18 components, including the TMS derivative of tetrahydrocannabinolic acid A (THCA-A), were reported. Barni Comparini and Centini (1983) compared packed-column GC, capillary-column GC, and HPLC for the analysis of *Cannabis* constituents and advocated the combination of capillary GC and HPLC.

Brenneisen and ElSohly (1988) used a comprehensive approach to developing chromatographic and spectral profiles of *Cannabis* of different origins. The samples (100 mg herb, 50 mg resin) were extracted by sonication in 1.0 mL of methanol–chloroform (9:1) containing 0.2 mg/mL of phenanthrene as internal standard. This solvent was chosen for its ability to rapidly extract, over a wide polarity range, the highest amount of cannabinoids and noncannabinoids compared with methanol, chloroform, dichlormethane, and cyclohexane. It was specifically recommended *not* to use a Soxhlet extraction, which can cause decomposition of thermolabile compounds. Gas chromatography was performed on 30-m × 0.25-mm fused silica columns coated with DB-1. Column temperature was programmed from 70°C to 250°C at 5°/min. The same column was used with an FID for generating profiles and with a GCEI (ion trap)

MS for identification. More than 100 different compounds were separated in a 70-minute analysis (Figures 3.5 and 3.6). This same GCMS procedure was used to identify TMS derivatives of acidic cannabinoids and polar noncannabinoids isolated by HPLC. HPLC profiles were generated using an isocratic mobile phase ($CH_3OH:H_2O$ + 1% HOAc\77:23) in a 750- × 4.6-mm column packed with 3µm ODS-1. Detection was by UV at 230nm. Up to 45 different com-

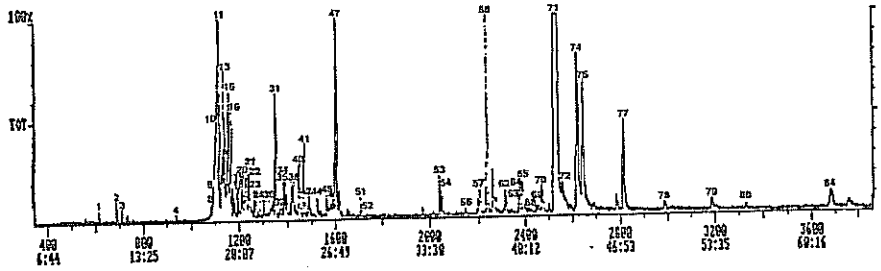


Figure 3.5
GCMS profile (reconstructed ion chromatogram) of Cannabis "Mexico" Copyright ASTM. Reprinted with permission from Brenneisen and ElSohly (1988).

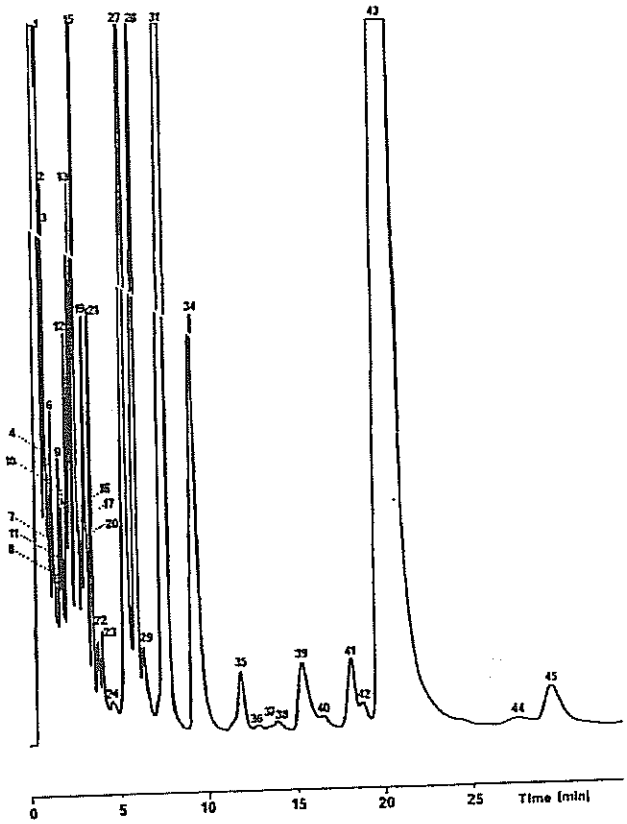


Figure 3.6
HPLC profile of Cannabis "Mexico" Copyright ASTM. Reprinted with permission from Brenneisen and ElSohly (1988).

pounds were separated in a 35-minute analysis (Figure 3.6). Isolation followed by GCMS was used to identify 20 of these. The authors concluded that most of the diagnostically important peaks in the GC profiles are in the terpene region and that these profiles might be useful for determining geographic origin. They also concluded that HPLC is the preferred method of acquiring profiles of thermolabile and polar compounds such as THCA, CBDA, and CBCA and that these profiles are more useful for assessing the history of the samples. The combination of these two methods provided a very powerful tool for comparing samples (tactical intelligence) and showed great improvements over earlier methods.

Lehmann and Brenneisen (1995) developed an HPLC method with photodiode array detection (DAD) that allows qualitative and quantitative analysis of the neutral and acidic cannabinoids. Peaks were identified by comparison to standards and by DAD-UV spectra. A 200- × 2.0-mm column and a 20- × 2.0-mm precolumn packed with ODS-1 (C_{18}), 3μm, was used in combination with a complex-gradient elution to separate compounds with a wide variety of polarities (Figure 3.7). A limit of detection (LOD) of about 25 ng of cannabinoid per 1 mL of extract was reported. The method easily classifies the chemotypes and can be used to measure the psychotropic potency and to compare samples.

Much of the recent research in analytical methods for cannabinoids has focused on reducing the time of analysis and increasing the resolution and specificity. For developing profiles of *Cannabis*, including the terpenes and sesquiterpenes as well as the main cannabinoids, capillary-column GC and GCMS are excellent. However, in order to obtain a complete profile, a method that allows identification and quantification of the acid components without derivatization or decarboxylation is desirable. The method of Lehmann and Brenneisen (1995) is excellent for establishing chemotypes and provides a very good profile; however, it does not resolve all of the acidic components, and it requires a 60-minute run. Weinberger and Lurie (1991) explored the use of micellar electrokinetic capillary chromatography for the analysis of clandestinely manufactured drugs. They found the method provided great improvement over HPLC methods for heroin and cocaine seizures but lacked sufficient resolving power for *Cannabis* samples. Lurie et al. (1998) used capillary electrochromatography (CEC) to achieve excellent resolution of acidic, highly polar, and neutral cannabinoids in a 40-minute analysis (Figures 3.8 and 3.9).

Rustichelli et al. (1996) proposed HPLC-MS as a rapid method for separation and identification of hashish constituents. Reversed-phase C-18 columns were used with isocratic MeOH–H_2O (80:20) as the mobile phase. Excellent resolution of THC, CDB, and CBN was achieved, but the acid components were poorly resolved. Mass spectrometry (EI) utilized a Finnigan MAT SSQ 710A

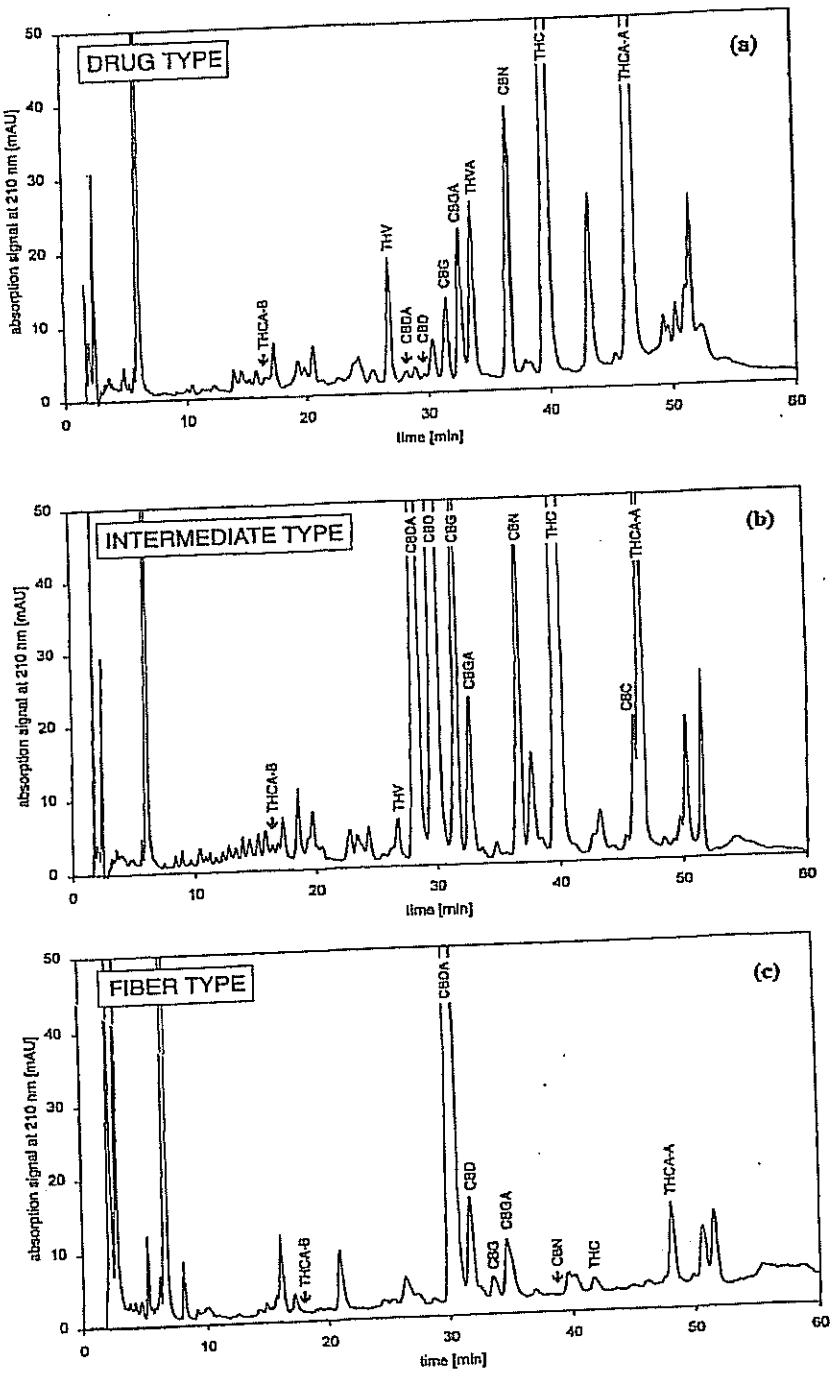


Figure 3.7
HPLC profiles of chemotypes of Cannabis sativa L.: (a) Chemotype I (drug type); (b) chemotype II (intermediate type); (c) chemotype III (fiber/industrial type) Reprinted from Lehmann and Brenneisen (1995) by courtesy of Marcel Dekker, Inc.

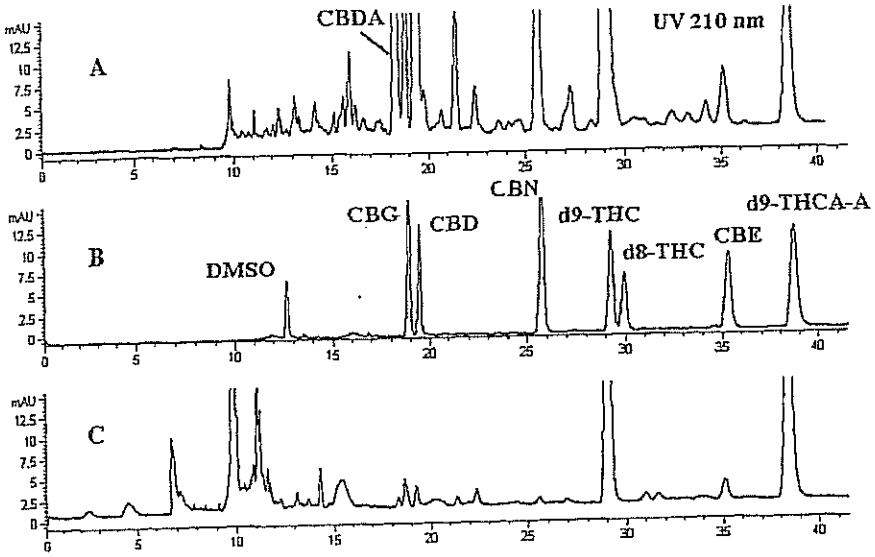


Figure 3.8
Capillary electrochromatography of (A) concentrated hashish extract, (B) standard mixture of cannabinoids, and (C) concentrated marijuana extract Reprinted with permission from Lurie et al. (1998). Copyright 1998 American Chemical Society.

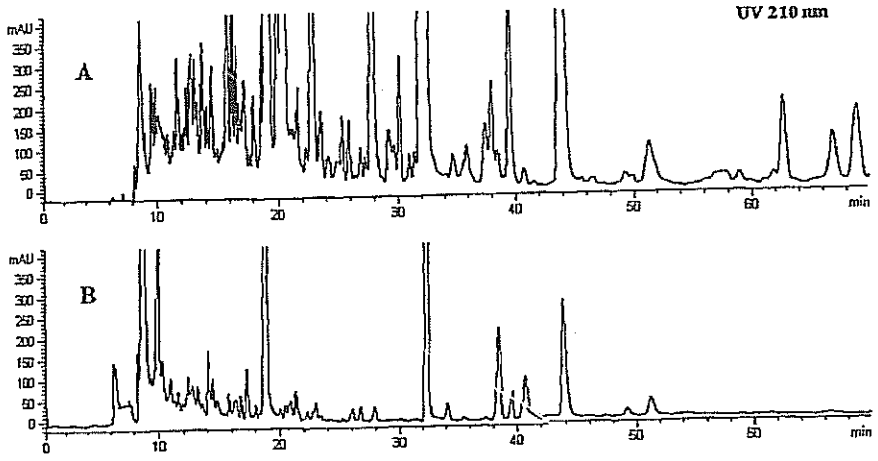


Figure 3.9
Capillary electrochromatography of (A) concentrated hashish extract and (B) concentrated marijuana extract Reprinted with permission from Lurie et al. (1998). Copyright 1998 American Chemical Society.

equipped with an interface particle beam (Finnigan) and a Jasco PU980 pump. Under the conditions used, the acid cannabinoids were decarboxylated. CBDA and CBNA coeluted. The same chromatographic system with UV detection was used (Rustichelli et al., 1998) in the analysis of cannabinoids in fiber hemp plant varieties. These analyses may be sufficient in differentiating chemotypes but would not suffice for forensic comparisons, due to the lack of resolution of acidic cannabinoids.

Ndjoko et al. (1998) used HPLC thermospray mass spectrometry (HPLC-TSMS) and HPLC-TSMSMS to separate and identify the major cannabinoids

at the 100-pg level. A gradient elution (acetonitrile–H_2O, 50:50 to 100:0) was used to resolve the cannabinoids that gave intense molecular ions ($[M + H]^+$) in the TSMS with no fragment ions. Compounds recorded included THC (m/z 315), CBD (m/z 315), CBC (m/z 333), THCA (no attempt to define A or B; m/z 359), CBDA (m/z 359), CBN (m/z 311), and cannabichromevarinic acid (CBCVA, m/z 331). MSMS was used to obtain the characteristic fragments shown in Table 3.2.

Another approach to analysis of the thermolabile components of *Cannabis* without the need for derivatization was proposed by Bäckström et al. (1997). Supercritical fluid chromatography was used to separate CBD, CBN, Δ^8-THC, and Δ^9-THC in 8 minutes. THC-d_3 was used as an internal standard. Separation was achieved with a gradient elution consisting of 2% methanol in CO_2 going to 7% methanol in CO_2 over 15 minutes. A 250- × 4.6-mm cyanopropyl silica column was used. Analytes were detected by atmospheric pressure chemical ionization mass spectrometry (APCI-MS). At low cone voltages, intense molecular ions ($[M + H]^+$) ideal for quantitation were obtained. At higher voltages, characteristic fragmentation was observed. The authors noted in their introduction the limitation placed on GC analysis by decarboxylation of the cannabinoid acids but did not include any acids in their study. Application of this method to separation of mixtures such as those explored by Lurie et al. (1998) (Figure 3.8) and Lehmann and Brenneisen (1995) (Figure 3.7) should be of great interest.

The use of pyrolysis–gas chromatography to classify and compare hashish is another novel approach to determination of provenance (Hida et al., 1995). Dendrograms were developed from cluster analysis of the peaks in pyrograms of hashish from different sources and were effective in discriminating the samples. The method of pattern recognition of pyrograms has been widely used in forensic science to perform comparisons of polymeric materials such as paint, rubber, and plastic. In the case of hashish, however, it lacks the ability to focus on the individual components of the complex mixture.

Table 3.2
Thermospray MSMS of cannabinoids

	Parent Ion m/z	Daughter Ion m/z
THC	315	259, 247, 193
CBD	315	259, 247, 193
THCA	359	341, 295, 316
CBDA	359 (low intensity)	
CBN	311	296, 242, 232, 195, 181, 164
CBCVA	331	205
CBC	333	298, 287, 275, 263, 207, 166, 153

Source: Ndjoko et al. (1998).

The application of GC coupled with infrared spectrophotometric detection (GC-IR) has been investigated by Idilbi et al. (1985).

3.2.3 NONCHROMATOGRAPHIC APPROACHES

3.2.3.1 Carbon-13

J.H. Liu et al. (1979) explored variations in the ratio of ^{13}C to ^{12}C in *Cannabis* samples. Variations were found among the parts of the plant as well as among samples from different sources. In spite of the authors' suggestion that the addition of this approach to other (chromatographic) methods of comparing and sourcing *Cannabis* would "increase the chance of success," this approach has not attracted further research.

3.2.3.2 Inorganic Constituents in Determination of Provenance

The presence and relative concentrations of trace elements were used by Fagioli et al. (1986) to compare five hashish submissions. Atomic adsorption (AA) with sampling in a carbonaceous slurry was used to determine Na, K, Ca, Mg, Fe, Cu, Mn, and Zn. The submissions consisted of five different batches made up of multiple cakes of hashish. The within-cake variations and the within-batch variations were determined, and they demonstrated relative homogeneity within each. The elemental data clearly differentiated all of the batches. Lahl and Henke (1997) and Tenhagen et al. (1998) have used metals analysis to differentiate hashish samples. The authors quantified the elements in hashish samples by both inductively coupled plasma atomic absorption spectrometry (ICP-AES) and neutron activation analysis and found no significant difference in their power of discrimination when neural networks were used to interpret the data. Watling (1998) used laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) to source the provenance of *Cannabis* crops in western Australia. LA-ICP-MS is not suitable for determining precise, accurate elemental concentrations, but it is excellent for developing "fingerprints" of elemental association patterns. The sample preparation is simple and does not require ashing or dissolution. The water-washed specimens are freeze-dried and ground to fine powder under liquid N_2. The powder is then compressed into a tablet in a cardboard mount. The cardboard mount is placed inside the laser cell, where a small portion is removed by multiple ablation (20 shots) and transferred to the plasma. The data can be presented as plots of raw data (Figure 3.10), as histograms (Figure 3.11), or as ternary ratio percentage plots that represent the direct comparison of the relationship between three analytes (Figure 3.12). This method should prove exceptionally valuable in determining provenance. Ferioli et al. (2000) used AA as one component of a multiple-method comparison of hashish samples.

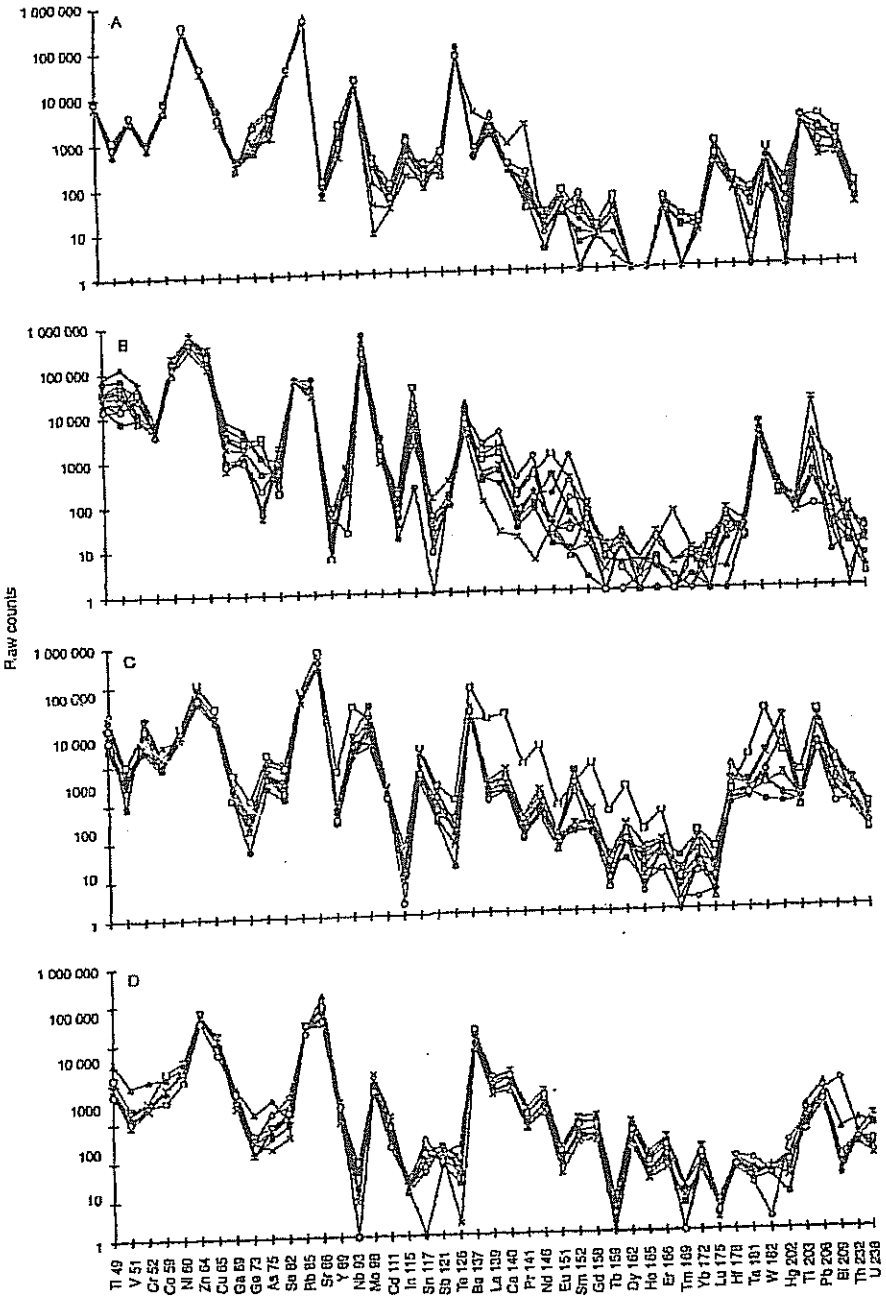


Figure 3.10
Raw count data from laser ablation ICP-MS of four samples showing significant similarity and probable single source Reproduced by permission of the Royal Society of Chemistry from Watling (1998).

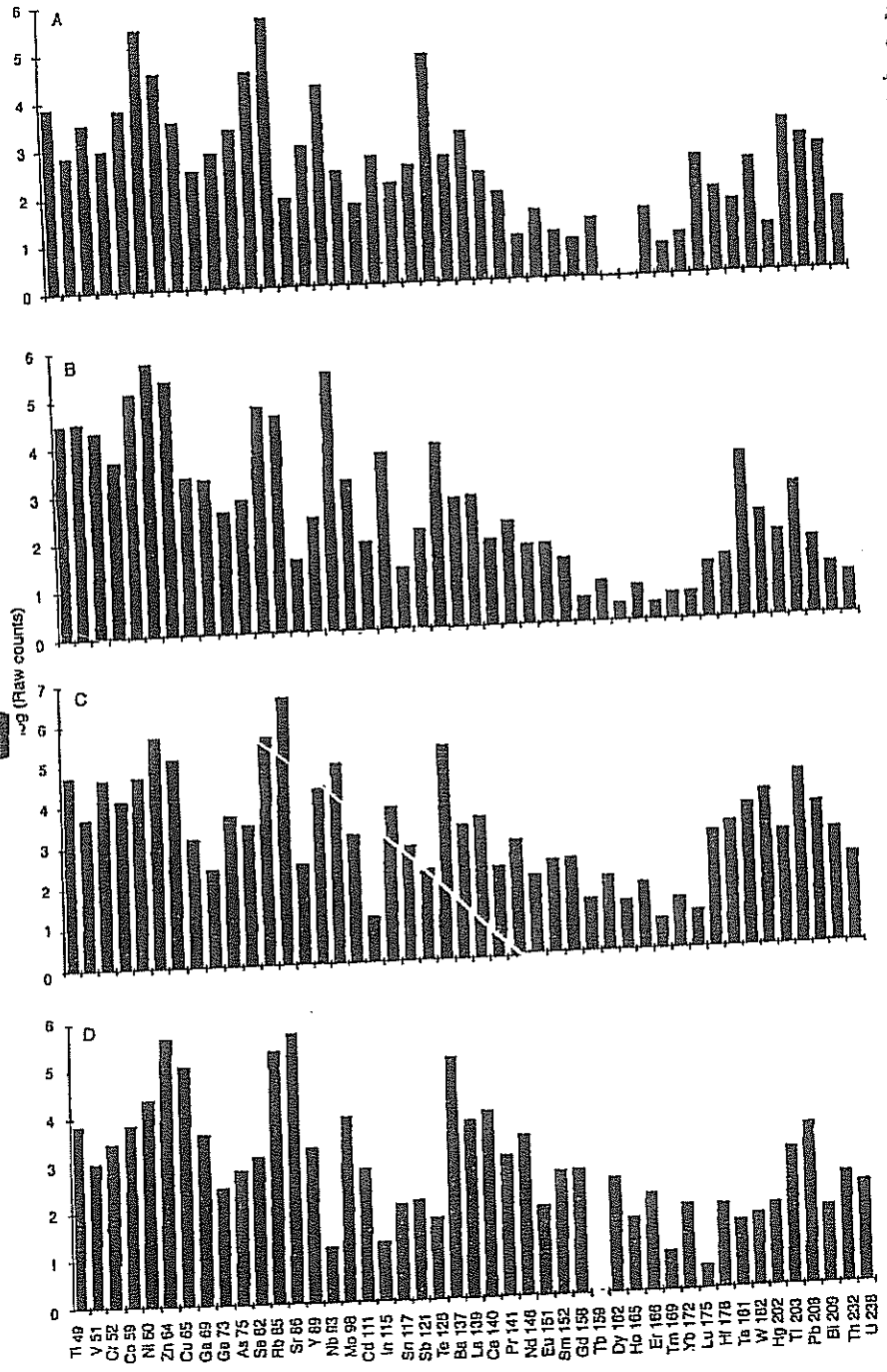
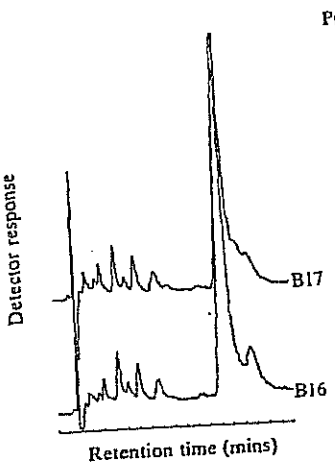


Figure 3.11
Simplified histogram plots of median data from four samples
Reproduced by permission of the Royal Society of Chemistry from Watling (1998).

1998; Silistato Gignano and Dimizio, 1997;



Primer	Plant B16	Plant B17
P31	290	290
	270	270
P32	270	270
	250	250
	<7 bands in each at 210–120 bp length	
P33	250	250
	230	230
	190	190
	170	170
P34	250	250
	180	180
P37	350	240
	290	180
	280	170
	250	
	240	
	180	
	170	

Figure 3.13
Example of differentiation by RAPD of two Cannabis samples that could not be differentiated by HPLC alone Reprinted from Gillan et al. (1995) by permission of the Forensic Science Society.

discrimination. Using these three primers (Genosys, 33: 5′-CTTGAGTGGA-3′, 34: 5′-GGATCTGAAC-3′, 37: 5′-CCACTTT-3′), all but 2 of the 17 samples could be distinguished. This included samples that were indistinguishable by HPLC (Figure 3.13) up to 10.

Kojoma et al. (2002) reported differentiation of *Cannabis* samples that were compared by HPLC using inter-simple sequence repeat (ISSR) amplification. The authors claim the method is easier than either RAPD or restriction fragment length polymorphism (RFLP) analyses.

Coyle et al. (2003) have reported a simplified method of DNA extraction using a commercially available plant DNA extraction kit manufactured by QIAGEN to replace a more difficult and time-consuming hexadecyltrimethylammonium bromide extraction (Doyle and Doyle, 1990). Amplified fragment length polymorphism was used to assess the quality of the extracts and the reproducibility of profiles from clonal *Cannabis*.

Single-strand conformation polymorphism (SSCP) using primers designed for the intergenic spacer region between *trnL* and *trnF* genes of *Cannabis sativa* chloroplast DNA was used to differentiate strains was reported by Kohjyouma et al. (2000).

3.2.4 SUMMATION

The determination of provenance is a complex undertaking for which there is no single ideal procedure that meets all the needs of the forensic science

community. For the determination of chemotypes (differentiation of fiber- and drug-type material), HPLC procedures such as that published by Lehmann and Brenneisen (1995) are adequate (see Figure 3.7). For determining compliance with laws governing allowable concentrations of cannabinoids in crops, seeds, or seed-derived products, methods that concentrate on determination of THC alone or THC and THCA can be sufficient. HPLC procedures already described or recently published (Zoller et al., 2000) are ideal for determining the concentrations of THC and THCA without derivatization. GC or GC/MS methods in which THCA is converted to THC by decarboxylation (i.e., heating) work well when total THC is needed. Such a method (Ross et al., 2000) has been applied to the study of THC in seeds. When the task at hand is to determine whether samples could have a common origin or may be from a particular geographical location, high-resolution GC or GC/MS revealing not only the main cannabinoids but also the terpenes, sequiterpenes, and minor cannabinoids is needed. An additional profile by a method suitable for analysis of thermolabile compounds must also be used. These would include high-resolution HPLC, SCFC, or CEC. Elemental analysis may be called for, especially if a geographical location is the target of the investigation. An example of the combined approach is presented by Ferioli et al. (2000), who used HPLC, GC, GC/MS, and AA in an analytical characterization of hashish samples.

3.3 IMMUNOASSAYS FOR THE DETECTION OF CANNABINOIDS IN BIOLOGICAL MATRICES

3.3.1 OVERVIEW

Immunoassays for detecting *Cannabis* abuse are generally called cannabinoids assays or THC assays. Cannabinoids immunoassays are used to detect Δ^9-THC and its metabolites in biological and forensic matrices; therefore, the design and utility of immunoassays for different types of matrices have to take into consideration Δ^9-THC metabolism and pharmacokinetics. The administration, absorption, metabolism, and excretion profiles of cannabinoids have been extensively studied and reported (Hawks, 1982; Wall et al., 1983; Chiang and Barnett, 1984; Harvey, 1984; Law et al., 1984b; Alburges and Peat, 1986; Johansson et al., 1990; Moody et al., 1992b; Huestis et al., 1992a, 1992b, 1995, 1996; Cone and Huestis, 1993; Huestis and Cone, 1998a, 1998b; Smith-Kielland et al., 1999). It is generally concluded that THC is rapidly absorbed following marijuana smoking. The peak THC concentration in plasma is reached within 30 minutes and quickly declines due to redistribution into tissues, lipid stores, and metabolism. THC is extensively metabolized to a large number of compounds in humans; however, most of them are inactive. Notably, THC is trans-

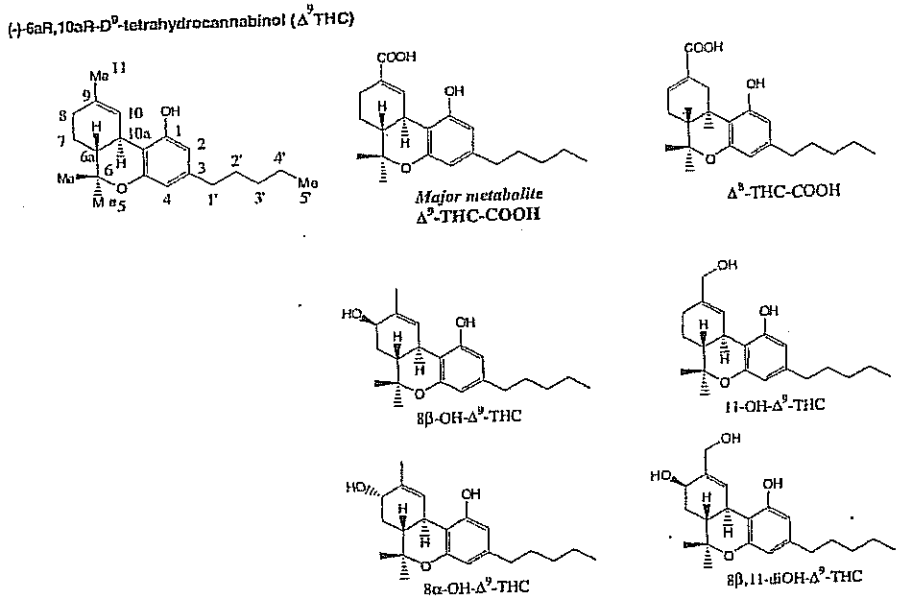


Figure 3.14
Structures of THC and selected THC metabolites

formed to 11-hydroxy-Δ^9-THC (11-OH-THC), which is subsequently oxidized to 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH). The major THC metabolite in urine is THC-COOH, which is present as both the free acid and its glucuronide conjugate (Williams and Moffat, 1980; Kanter et al., 1982b; Wall et al., 1983). Although the unconjugated THC-COOH is the "target analyte" for cannabinoids screening and confirmation, its glucuronide conjugate is present in considerably higher concentrations than the parent drug in urine and blood (Skopp and Potsch, 2002a, 2002b). Other metabolites found in urine (Figure 3.14) are 8-α-hydroxy-Δ^9-THC, 8-β-hydroxy-Δ^9-THC, 8-β,11-dihydroxy-Δ^9-THC, 11-hydroxy-Δ^9-THC, and a group of acid metabolites.

3.3.1.1 Cutoff Considerations

Commercial cannabinoids immunoassays can detect the presence of several THC metabolites via antibody cross-reactivities, although most assays for urine drug screening are calibrated only with THC-COOH. The overall sensitivity and specificity of an immunoassay is, to a certain extent, related to the characteristics of the antibody used in the assay (Teale et al., 1974a; Jones et al., 1984a; Peat, 1984; ElSohly et al., 1990; Salamone et al., 1998). The quantified value (assay result), as expressed in "apparent THC-COOH concentration" (i.e., calibrator-equivalent unit), is the "sum" of antibody immunoreactivities toward

THC-COOH and other structurally related THC metabolites in the testing specimen. Therefore, the "administrative cutoff" levels for THC initial tests are set at higher quantities than the associated confirmatory cutoff concentrations in order to accommodate the total contribution of antibody cross-reactivities. However, the correlation of total immunoreactivities to a single GC/MS value of the THC-COOH compound can vary to some extent. Such variations may influence the balance of clinical sensitivity and specificity for cannabinoids immunoassays, especially for testing specimens that contain near-cutoff concentrations of THC-COOH.

Regardless of the technology used, the analytical performance for a cannabinoids immunoassay is calibrated relative to the specified cutoff concentration and optimized for its comparative performance to the confirmation technologies. The screening cutoff of 100 ng/mL (or 100 μg/L) cannabinoids in urine was chosen at the inception of the SAMHSA drug-testing guidelines (53 FR 11970, 1988). This immunoassay cutoff was set partially due to the risk of passive exposure to marijuana smoke (Perez-Reyes et al., 1983; Law et al., 1984c; Morland et al., 1985; Cone and Johnson, 1986; Moffat, 1986b; Mulé et al., 1988). A comprehensive study of passive inhalation conducted by NIDA illustrated that it takes extensive exposure to extremely high concentrations under unrealistic conditions to cause a positive result (Cone et al., 1987). Additional factors, such as the cost and goals of the specific drug-testing programs, also influence the choice of cutoff levels (Sunshine, 1988). Several studies have since been conducted to demonstrate that lowering the initial testing cutoff in urine would increase the positive rates for marijuana detection (Wells and Barnhill, 1989; Rowland et al., 1994; Huestis et al., 1994; Wingert, 1997). Smith et al. (1989) showed that marijuana test sensitivity increased from 47% to 88% and specificity increased from 91% to 94% when the screening and confirming were lowered from 100 ng/mL and 15 ng/mL to 20 ng/mL and 5 ng/mL, respectively.

The current U.S. *Mandatory Guidelines* (59 FR 29916, 1994) specify a 50 ng/mL screening cutoff and a 15 ng/mL confirmation cutoff for cannabinoids testing. In case a retest is required for a specimen or for the testing of "Bottle B" of a split specimen, the retest quantification is not subject to a cutoff requirement; however, the retest "must provide data sufficient to confirm the presence of the drug or metabolite" (59 FR 29916, 1994). In general, many drug-testing programs in the nonregulated sectors also follow the cutoff defined by the *Federal Guidelines*. Depending on the drug-testing program goals and preference, four major cutoff concentrations have been used for urinary cannabinoids immunoassays: 20 ng/mL, 25 ng/mL, 50 ng/mL, and 100 ng/mL. Moreover, the screening immunoassay cutoff could be further decreased for detecting maternal and neonatal drug exposure (Hattab et al., 2000).

For any given cutoff, there can be substantial variability between subjects and between doses in the excretion profiles of THC-COOH. Huestis et al. (1996) demonstrated that mean detection times in urine following smoking varied considerably between individuals, even in highly controlled smoking studies. In addition, consecutive urine specimens may fluctuate below and above the cutoff during the terminal elimination phase, when THC-COOH concentrations approach the cutoff (Ellis et al., 1985; Huestis et al., 1996; Smith-Kielland et al., 1999). The "normalization" of drug excretion to urine creatinine concentration has been employed to predict new drug use and to reduce the variability of drug measurement attributable to urine dilution (Lafolie et al., 1991, 1994; Simpson et al., 1993; Huestis and Cone, 1998b; Fraser and Worth, 1999).

3.3.2 STABILITY OF CANNABINOIDS IN BIOLOGICAL FLUIDS

Results obtained from any given immunoassay for drugs of abuse can be determined either by qualitatively comparing the resulting signal output of the specimen to that of the "cutoff-level standard" solution or by semiquantitatively comparing to the calibration curve. Consequently, the stability of the analyte in calibrator solutions and in testing specimens is critical to the accuracy of the analytical system. The hydrophobic nature of the cannabinoids molecules can lead to the loss of the drugs in the specimen because of surface adsorption to the specimen-handling devices and storage containers. Thus the stability of cannabinoids in biological fluids has been evaluated in various container materials stored at different temperatures.

Dextraze et al. (1989) observed a 27% reduction in THC-COOH concentration due to adsorption to glass and reported that foaming of spiked urine caused by vigorous mixing resulted in a reversible 89% apparent reduction in THC-COOH concentration. Blanc et al. (1993) investigated cannabinoids loss from calibrators during the immunoassay testing process and found significant losses attributable to both the kind of pipette used and the surface contact in the analyzer cup. The loss of THC-COOH can be reduced by using appropriate pipette and maintaining a minimal surface-to-volume ratio in the analyzer cup. Roth et al. (1996) investigated the effects of solution composition and an assortment of container material types on the loss of THC-COOH using immunoassay and x-ray photoelectron spectroscopy. The authors also evaluated the effects of sample volume and sample handling and found that THC-COOH loss due to pipetting ranged from 1.1 ng to 7.9 ng per aliquot. Stout et al. (2000) observed rapid loss of THC-COOH at 4°C for polypropylene (maximal 14% loss) and polyethylene (maximal 17% loss), as well as a small loss (<5%) in polyethylene bottles at 25°C. All losses stabilized within 1 hour, and no further losses were seen over 1 week.

Paul et al. (1993) examined the effect of freezing on the concentration of abused drugs in urine and observed no significant loss of compounds except for THC-acid, which showed an average loss of 11% (ranging from 0 to 34%). Golding et al. (1998) observed appreciable losses (>22.4%) in some urine samples stored at room temperature for 10 days and approximately 8% loss when the samples were refrigerated for 4 weeks. The authors observed cannabinoids loss in frozen samples and postulated that the loss may be due to the decrease of the solubility of THC-COOH or the absorption process of cannabinoids molecules to the storage containers. Dugan et al. (1994) reanalyzed urine that had been stored at −20°C for 12 months and reported no extensive change in the average drug concentrations for THC-COOH. In contrast, Romberg and Past (1994) retested previously analyzed and frozen samples and found that 85 THC-COOH positive samples stored frozen for 1 to 10 months declined an average of 25% (ranging from −80% to +30%). The authors found that drugs partition into strata when frozen in urine because of the thermodynamics of the freezing process.

Skopp and Potsch (2002b) assessed the stability of THC-COOH glucuronides in urine and plasma by LC-tandem mass spectrometry. The glucuronide was stable in both matrices when stored frozen, whereas the glucuronide concentrations decreased at all other storage conditions. The authors reaffirmed that stability data derived from a particular biological matrix are important for reliable interpretation of the analytical results. The antibodies used in different cannabinoids immunoassays cross-react with THC-COOH and its glucuronide to different extents. Thus the specimen transportation and storage conditions following the collection procedure may affect the immunoassay results; however, the effect in general should not interfere with the screening result interpretation.

Johnson et al. (1984) reported that THC in blood was stable for up to 4 months at 4°C and −20°C. At room temperature, THC in blood decreased significantly at 2 months and dropped 90% after 6 months. By contrast, the concentration of THC-COOH was not significantly different from that of the control. McCurdy et al. (1989) assessed the stability of THC-COOH in whole blood while stored in four different kinds of blood collection tubes for up to 30 days at refrigeration and room temperature. Utilizing both radioimmunoassay and GC/MS, the authors reported that THC-COOH was stable in blood under all conditions studied. Skopp et al. (2000) demonstrated that cannabinoids usually measured in hair analysis are more affected by solar radiation than other drugs of abuse detected in hair. In addition to the deleterious effect of sunlight on the stability of cannabinoid constituents in hair, the weathering of hair, which damages the hair fiber at the ultrastructural level, may cause additional changes in drug concentrations in hair.

3.3.3 SPECIMEN VALIDITY AND INTEGRITY

Purposeful invalidation of the specimen by the donor can compromise specimen validity and integrity and, subsequently, negatively impact the accuracy of drug-testing results. Cook et al. (2000) reviewed the characterization of human urine for specimen validity determination. Cone et al. (1998) reported that the average detection times for marijuana metabolites appeared to be slightly shorter following ingestion of 1 gallon of fluids compared with ingestion of 12 oz of water. A popular means of sample adulteration is the addition of exogenous chemicals, such as glutaraldehyde, detergent, bleach, and various oxidizing agents (Baiker et al., 1994; Wu et al., 1995, 1999; George and Braithwaite, 1996; ElSohly et al., 1997; Urry et al., 1998; Tsai et al., 2000, 2001). THC-COOH is sensitive to oxidizing agents such as nitrite, peroxide, and chlorochromate. Because of the effect of oxidants on the cannabinoids molecules, by and large all immunoassay technologies can be affected. Specimens adulterated with oxidizing agents may give false-negative screening and escape further confirmation. For those that remain positive at the time of initial testing, the presence of oxidizing agents can interfere with subsequent GC/MS confirmation unless samples are treated with sodium bisulfite prior to the extraction procedure (ElSohly et al., 1997). The effectiveness of oxidizing agents can be affected by sample pH, original drug concentration, and the time between sample collection and sample testing. For example, Tsai et al. (2000) reported that significant decreases in the immunoassay results could be observed shortly after nitrite treatment in samples with acidic urinary pH values. In contrast, samples with neutral or higher pH values may remain immunoassay positive 3 days post-nitrite spiking, even though some of these adulterated urine samples exhibited significant decrease in GC/MS recoveries following bisulfite treatment. Moreover, the decrease or loss of immunoassay-detectable cannabinoid cross-reactives in acidic "THC positive samples" can be attenuated by chemically increasing the pH value of the samples to the basic pH range (Lewis et al., 1999; Tsai et al., 2000).

3.3.4 ALTERNATIVE MATRICES

The most commonly used biological matrices for cannabinoids analysis are urine, blood, serum, and plasma samples. Urine can readily be applied to various immunoassay analyses without sample pretreatment. Also, urine specimens can be obtained in relatively large quantities in comparison to other biological fluids. Therefore urine remains the most widely used specimen for initial screening tests, despite various issues such as variability of excretion profile, relationship to impairment, cutoff considerations, detection window, and col-

lection and adulteration concerns. The use of alternative matrices may offer advantages in addressing some of the issues concerning urine drug testing (Schramm et al., 1992; Cone, 1993, 1997, 2001; Moeller, 1996; Kidwell et al., 1998; Skopp and Potsch, 1999; Jehanli et al., 2001; Caplan and Goldberg, 2001; Niedbala et al., 2001).

Analyses of THC and major metabolites in blood, including those involving immunoassay techniques, have been applied in a variety of pharmacokinetic and pharmacodynamic investigations, driving-impairment studies, and forensic cases (Hanson et al., 1983; Gjerde, 1991; Moody et al., 1992a, 1992b; Goodall and Basteyns, 1995). Interpretation of the significance of blood cannabinoids testing results and level of impairment or cannabis exposure has not been clearly established. However, various controlled studies have been conducted to correlate and predict these relationships (Huestis et al., 1992b; Cone, 1993).

Analysis of drugs in hair provides a longer detection window than urinalysis even though hair analysis for cannabinoids is one of the most difficult analyses (Nakahara, 1999). On the other hand, the detection of THC in saliva/oral fluids may indicate very recent marijuana use (Cone, 1993; Jehanli et al., 2001; Niedbala et al., 2001). It is generally postulated that cannabinoids in oral fluids were from residuals left in the oral cavity during the use of cannabis (Cone, 1993; Jehanli et al., 2001). Therefore, an ideal immunoassay for oral fluid THC should employ antibody produced against the parent THC compound instead of the traditional THC-COOH target analyte. Maseda et al. (1986) reported that saliva THC concentrations in subjects who drank beer after smoking marijuana were lower than those of nondrinking subjects. An earlier study in humans using radiolabeled THC administered by intravenous injection did not detect any radioactivity in saliva samples (Hawks, 1982). Although an advantage of oral fluid is the relative ease of collection, currently there is no standardized method for specimen collection. O'Neal et al. (2000b) demonstrated the impact of various collection devices on the measured drug concentrations in oral fluids. The chemical nature of the cannabinoids warrants caution in the specimen-collection mechanism. Alternative matrices are considered less vulnerable to adulteration due to the observed procedures of sample collection. However, there exist issues of environmental contamination, passive exposure, and bias concerns for some of the matrices (Nakahara, 1999; Kidwell et al., 2000; Skopp et al., 2000; Kidwell and Smith, 2001). A very small percentage of oral fluid sample substitution with canine or feline saliva was noticed when oral fluid sample integrity was checked for the presence of human IgG, indicating that the collection was not witnessed (Peat, 2000).

Compared to the urine matrix, oral fluid testing and sweat drug testing may have the limitations of the small amount of matrix collected and the lower levels of drugs in the specimen (Kintz et al., 2000). Oral fluid concentrations of THC

vary over a wide range immediately after marijuana exposure and then rapidly decline over the first few hours. Thus the selection of a low cutoff for THC in oral fluid can increase the diagnostic sensitivity and allow a longer window of detection time. Niedbala et al. (2001) compared oral fluid THC testing to urine testing utilizing a screening cutoff of 1 ng/mL and a confirmation (GC/MS/MS) cutoff of 0.5 ng/mL. Although currently there is no standardized cutoff decision for alternative matrices, SAMHSA has been drafting a revision of the *Mandatory Guidelines* that will set cutoff levels and define requirements for alternative specimen drug screening and confirmation tests. The low drug concentration and hydrophobic feature of THC can present challenges for some of the screening technologies when applied to alternative matrices testing. Nevertheless, alternative matrix testing is an actively pursued area and more studies and reports are to be expected in the foreseeable future.

3.3.5 IMMUNOASSAY TECHNOLOGIES

Immunoassays utilize the high affinity and specificity of antibody–antigen binding interactions to detect minute amounts of molecules in complex biological materials. Immunoassays for drugs-of-abuse testing are generally used to eliminate negative samples, and to maximize the likelihood of finding the presence of a drug or a group of structurally related drugs in the specimen at, or above, a predetermined cutoff concentration. The following provides a brief overview of the commonly used cannabinoids immunoassay. The principles of these technologies were reviewed in greater detail in Chapter 2. Because most of the commercial immunoassays have been extensively evaluated in various comparative studies, the overall comparison of cannabinoids immunoassay technologies will be reviewed collectively in Section 3.3.6.

3.3.5.1 Radioimmunoassay (RIA)

An array of RIA tests has been developed or evaluated in the past three decades for the quantification of cannabinoids in urine, blood, serum, and plasma (Teale et al., 1974a, 1974b, 1975; Marks et al., 1975; Gross et al., 1974; Wall et al., 1976; Owens et al., 1981; Bergman et al., 1981; Zimmerman et al., 1983; Mason et al., 1983; Childs and McCurdy, 1984; Jones et al., 1984a, 1984b; D.E. Smith et al., 1989; Clatworthy et al., 1990; Altunkaya et al., 1991; Moody et al., 1992a). Hanson et al. (1983) compared ^{3}H-RIA and ^{125}I-RIA for cannabinoids with GC/MS and found that the three methods gave parallel but significantly different quantitative results. However, each technique was capable of measuring THC concentrations in blood and serum up to 3 hours after usage. Law et al. (1984a) described the confirmation of cannabis use through the analysis of blood and urine for THC-COOH and its O-ester glucuronide using combined

HPLC/RIA. An assortment of cannabinoids RIA kits was developed by Roche (Abuscreen RIA; now discontinued), Diagnostics Products Corporation (DPC, Los Angeles), the Immunalysis Corporation (Pomona, CA), and by Research Triangle Institute. The cross-reactivity profile, GC/MS comparison and regression analysis of RIA kits from different times and manufacturers have been analyzed in a number of studies (Bergman et al., 1981; Jones et al., 1984a; Peat, 1984; Weaver et al., 1991; R.H. Liu et al., 1994; R.H. Liu and Goldberger, 1995; Brendler and Liu, 1997).

In addition to blood and urine, RIA has been applied to the analysis of cannabis in oral fluids (Gross et al., 1985), fingernails (Lemos et al., 1999), hair (Mieczkowski, 1995), and meconium (Ostrea et al., 1989). Although RIA has the advantages of high analytical sensitivity for quantifying cannabinoids in a variety of biological and forensic matrices, there have been increasing concerns regarding the handling of radioactive materials and the disposal of radioactive waste. Currently the double-antibody cannabinoids RIA can be purchased from DPC. The THC Direct RIA available from Immunalysis Corporation has a 100 ng/mL cutoff for urine cannabinoids and claims a sensitivity of 2.5 ng/mL and a sharp and linear plot through 50 ng/mL from the low point through the high concentration point.

3.3.5.2 *Enzyme-Linked Immunosorbent Assay (ELISA)*

ELISA-based cannabinoids assays have the advantages of low detection limits and high versatility for various forensic and toxicological analyses. Diverse commercial ELISA kits can be used or tailored to test matrices such as urine, blood, serum, oral fluid, sweat, meconium, bile, vitreous humor, and tissue extracts (Perrigo and Joynt, 1995; K.A. Moore et al., 1999; Kerrigan and Phillips, 2001; Niedbala et al., 2001). ELISA has also been used to detect cannabinoids in plant tissue culture systems (Kanaka, et al., 1996).

Kerrigan and Phillips (2001) compared the performance of ELISA kits to detect drugs of abuse in blood with a selected cutoff for cannabinoids of 30 ng/mL. In comparative analysis of ELISAs from STC (now OraSure Technologies, Inc., Bethlehem, PA) and Immunalysis Corporation for six drugs-of-abuse classes in whole blood and urine, the authors concluded that Immunalysis assays offered superior binding characteristics and detection limits, whereas STC assays offered improved overall precision and lot-to-lot reproducibility. The STC serum cannabinoids assay is directed toward the carboxylic acid metabolite but is also reactive with parent THC. The percent cross-reactivity to Δ^9-THC for the ELISA kits used in the study (Kerrigan and Phillips, 2001) was 24.2% for STC and <5% for Immunalysis. By comparison, the respective percent cross-reactivity to Δ^9-THC and Δ^8-THC is reported as 10.4% and 125% for the Diagnostix cannabinoids assay and 35% and 200% for the Neogen cannabinoids

Table 3.3
Example of cross-reactivity profile of ELISA cannabinoids immunoassays

Compound	Approximate Percent (%) Cross-Reactivity Relative to the Specified THCA Equivalent Concentration of Each Assay			
	Immunalysis Direct ELISA (equivalent to 30 ng/mL THCA)[a]	Immunalysis Direct ELISA (equivalent to 10 ng/mL THCA)[b]	Immunalysis Ultrasensitive ELISA (equivalent to 25 pg/mL THCA)[b]	Immunalysis Sweat/Oral fluid ELISA (equivalent to 1 ng/mL THCA)[b]
	April 1998[c]	May 2001[c]	July 2001[c]	May 2001[c]
11-nor-9-Carboxy-Δ^9-THC	100	100	100	100
11-nor-9-Carboxy-Δ^8-THC	110	110	110	125
8 11-Dihydroxy-Δ^9-THC	<5	<5	<5	<1
11-Hydroxy-Δ^9-THC	<5	<5	16.6	<1
Δ^9-THC	<5	21	4.1	60
Δ^8-THC	NA[d]	45	NA[d]	66
Cannabinol	<5	<5	<1	<1
Cannabidiol	<5	<5	<1	<1

[a] Information was published by Kerrigan and Phillips (2001).
[b] Information was obtained from the specified package inserts, Immunalysis Corporation.
[c] Date of package insert or package insert revisions.
[d] NA—Not available.

assay (package inserts: 2001). In general, the cross-reactivity profile may vary in kits from the same company at different times and can be optimized for the specific requirements of different kit configurations. For example, the cross-reactivity profile of various ELISA kits from Immunalysis is shown in Table 3.3.

3.3.5.3 Enzyme-Multiplied Immunoassay Technique (EMIT)

The original formats of EMIT cannabinoids assay included the Emit-st assays, which utilized a single 100 ng/mL calibrator containing Δ^8-THC-COOH, and the Emit d.a.u. assays, which utilized a negative control, a low calibrator, and a medium calibrator (Irving et al., 1984; Bastiani, 1984; Ellis et al., 1985). O'Conner and Rejent (1981) confirmed EMIT cannabinoids assay with RIA and GC/MS and postulated that for routine screening applications, the heterogeneity of the EMIT cannabinoid antibody may be more sensitive than the other methods in detecting cannabinoid metabolites. Black et al. (1984) adapted EMIT for high-volume urine cannabinoids testing. Foltz and Sunshine

(1990) compared the analysis of urine specimens with TLC, EMIT, and GC/MS and reported that 63% of the urine specimens shown by GC/MS to contain greater than 20 ng/mL of THC-COOH were identified as positive by the Emit d.a.u. assay at the 100 ng/mL cannabinoids cutoff. Standefer and Backer (1991) investigated the precision, linearity, accuracy, and stability of quantitative results for five drugs of abuse by using Emit d.a.u. reagents and reported that the within-day and between-day coefficients of variation were between 10% and 20% for THC-COOH. The formulation of Emit II differs from earlier ones in the use of new drug–G6P-DH conjugates and new antibodies to improve performance at the cutoff level (Armbruster et al., 1993a). A comparative study indicated that both Emit 700 and Emit II assays detected approximately 90% of the urine samples screened positive by RIA and confirmed positive for marijuana (Armbruster et al., 1994). Smith-Kielland et al. (1999) compared results of the 1992 and 1993 testing of up to 20,000 urine specimens and concluded that there was no major difference in performance with the new formulation.

Currently the THC assays for both Emit d.a.u. (lyophilized) and Emit II Plus (liquid) are available in three cutoff levels: 20 ng/mL, 50 ng/mL, and 100 ng/mL.

In addition to Dade Behring Syva Emit products, cannabinoids assays that utilize similar enzyme immunoassay principles for urinary drug screening are also available from the companies Beckman Coulter, as Synchron THC-cannabinoids assay (Dietzen et al., 2001), and Microgenics, as DRI (formerly Diagnostic Reagents Inc.) cannabinoid (THC) assay (Broussard and Hanson, 1997). Besides urinary drug testing, EMIT assays have been applied to matrices such as blood and plasma (Peel and Perrigo, 1981; Mason and McBay, 1984; Asselin et al., 1988; Lewellen and McCurdy, 1988; Perrigo and Joynt, 1989; Blum et al., 1989; Bogusz et al., 1990; Gjerde et al., 1990), saliva (Peel et al., 1984), and meconium (Wingert et al., 1994; ElSohly et al., 1999). Gjerde (1991) reported the use of EMIT d.a.u. cannabinoid assay to test methanolic extracts of blood as a screening method in cases of suspected impairment by cannabis, provided that THC was analyzed in the subsequent assay. When a cutoff limit corresponding to 50 nM THC-COOH (17 ng/ml) was used, 86% of the EMIT positive blood samples contained THC concentrations above the cutoff limit of 1 nM (0.3 ng/mL).

3.3.5.4 Fluorescence Polarization Immunoassay (FPIA)

The Abbott (Abbott Park, IL) FPIA Cannabinoids assay is calibrated using either a master calibration (2-point) or a six-point calibration curve (0, 25, 40, 60, 80, and 135 ng/mL). Master calibrator concentrations were chosen that most accurately adjust the 0–135 ng/mL calibration curve with only two points. The FPIA cannabinoids assay is designed to perform at a variety of commonly

used threshold levels. Analyzers for Abbott FPIA such as TDx, TDxFLx, and AXSYM have been "factory set" at a specified cutoff concentration. Instrument procedures are available for the users to configure the cutoff level if necessary. The reagent pack consists of bottles that contain antiserum, pretreatment solution, and cannabinoids fluorescein tracer, respectively. Previously the cross-reactivities of the Abbott TDx assay to various cannabinoid metabolites and a group of cannabinoids and noncannabinoid phenolic constituents of cannabis were also analyzed by ElSohly et al. (1990). In addition to urine samples, FPIA-based drug assays have been applied for the analysis of blood (Bogusz et al., 1990; Goodall and Basteyns, 1995; Cagle et al., 1997; Keller et al., 2000), meconium (ElSohly et al., 1999), synovial fluid of the knee joint and in vitreous humor (Felscher et al., 1998), and hair (Kintz et al., 1992).

3.3.5.5 Kinetic Interaction of Microparticles in Solution (KIMS)

The Roche (Indianapolis, IN) Abuscreen ONLINE and ONLINE DAT II products are KIMS-based immunoassays (Armbruster et al., 1993a, 1993b; Hailer et al., 1995; Crouch et al., 1998b; Boettcher et al., 2000). The cannabinoids qualitative applications utilize calibrators for cutoff at 20, 50, or 100 ng/mL, whereas the semiquantitative applications employ either four or five calibrators as appropriate for the respective cutoff levels. For example, calibrators 0, 20, 50, 100, and 300 ng/mL are used for the 50 ng/mL cutoff assay. For semiquantitative COBAS INTEGRA applications, the change in absorbance for each calibrator is plotted against its concentration and a lineal interpolation model is used to construct a calibration curve. For semiquantitative Roche/Hitachi applications, the analyzer computer constructs a calibration curve from absorbance measurements of the standards using a four-parameter logit-log fitting function, which fits a smooth line through the data points. The absorbance measurements of samples are then used to calculate drug or drug metabolite concentration by interpolation of the logit-log fitting function. The resulting curves are retained in analyzer memory and recalled for later use. In addition to urine drug testing, the ONLINE assays have also been applied to drugs-of-abuse analysis in serum (Moody and Medina, 1995) and other applications can be developed by the users for their specific drug-testing needs.

The design and selection of antibody have a significant impact on the specificity of an immunoassay. The immunogen structures can be designed to generate antibodies with different selectivity towards the cyclohexyl ring of the cannabinoid structure. Immunogens from benzpyran derivatives have been developed to elicit antibodies with broad cross-reactivity to cannabinoid metabolites (Salamone et al., 1998). The total cross-reactive cannabinoid values obtained with the benzpyran-elicited antibodies were 49% higher than the values obtained using the traditional immunogen structure. The broad-

spectrum antibody has been used to develop the ONLINE DAT II cannabinoids assay. This antibody has also been utilized in an immunoaffinity extraction procedure for the simultaneous analysis of THC and its major metabolites in urine, plasma, and meconium by GC/MS (Feng et al., 2000).

3.3.5.6 Cloned Enzyme Donor Immunoassay (CEDIA)

The Microgenics (Fremont, CA) CEDIA DAU Multi-Level THC assays can be used for qualitative or semiquantitative determinations of cannabinoids at 25, 50, or 100 ng/mL cutoff levels. The data analysis using the Roche/Hitachi analyzers has been described in the previous section. The calibrators used for a 50 ng/mL cannabinoids assay are 0, 25, 75, and 100 ng/mL. The performance of CEDIA for urine drug testing was compared to that of RIA, TDx, ONLINE, and EMIT II (Armbruster et al., 1995).

Wu et al. (1995) compared CEDIA to EMIT II for its use in drug screening in urine and investigated the effect of various adulterants on the immunoassay performance. Cagle et al. (1997) evaluated CEDIA and FPIA for their combined effectiveness in the analysis of cannabinoids in acetone-pretreated whole blood. The authors reported that all blood samples that screened positive could be confirmed for the presence of THC-COOH by GC/MS at concentrations greater than the 10 ng/mL cutoff. However, the GC/MS results were found to correlate significantly better with those of the FPIA cannabinoids assay. Iwersen-Bergmann and Schmoldt (1999) demonstrated that the use of the CEDIA urine-screening technique without any adaptation can provide a sensitive serum/whole blood screening for several drugs of abuse, including cannabinoids.

3.3.5.7 Onsite (Point-of-Collection) Immunoassays

Various immunoassay technologies have been applied to develop onsite drug-testing products since the late 1980s. In a study that involved volunteers with a history of marijuana use, Jenkins et al. (1993) compared results of the enzyme immunoassay based EZ-SCREEN (Environmental Diagnostics, Burlington, NC) with GC/MS and reported that the test produced positive results at a standard THC-COOH concentration of 5 ng/mL. Overall agreement between the three analysts was approximately 80%. Delayed readings and photocopy readings tended to be less accurate than readings obtained at 3 minutes. Another example of multistep, point-of-collection drug screening is the Triage drugs-of-abuse testing panel (Biosite Diagnostics Inc., San Diego, CA) (Buechler et al., 1992; Wu et al., 1993; Rohrich et al., 1994; de la Torre et al., 1996).

The Roche Abuscreen OnTrak THC assay was based on the visual interpretation of qualitative results following a KIMS-like reaction (Schwartz et al., 1990; Cone et al., 1991; Armbruster et al., 1993a; Crouch et al., 1998a, 1998b). The

manual, qualitative immunoassay system was shown to be sufficiently sensitive, and the results were found to agree well with those obtained from instrument-based screening and GC/MS confirmation (Armbruster and Krolak, 1992). (The product line was recently discontinued.) With the advantages of simple and rapid one-step testing as well as room temperature storage, a variety of lateral-flow assays have been gaining popularity in various drug-testing programs in the past few years. Most of these devices have been evaluated for onsite screening of abused drugs, including cannabinoids, in urine (Jenkins et al., 1995; Towt et al., 1995; Ros et al., 1998; Crouch et al., 1998b; Buchan et al., 1998; Wennig et al., 1998; Peace et al., 2000; Leino et al., 2001; Gronholm and Lillsunde, 2001). The lateral flow immunochromatographic assays were also adapted to drug screening for alternative matrices such as oral fluids (Kintz et al., 2000; Samyn and van Haeren, 2000). For example, the Cozart RapiScan (Abingdon, Oxfordshire, UK) is a hand-held reader that couples a lateral-flow test strip with digital photography for the detection of drugs of abuse in saliva. Jehanli et al. (2001) compared the use of Cozart RapiScan with that of enzyme immunoassays and GC/MS methods in blind clinical trials. The authors reported that the cutoff of the marijuana test at 10 ng/mL THC-COOH was too high to detect marijuana use for more than a few hours after smoking.

3.3.6 COMPARATIVE EVALUATIONS

With plenty of technological choices, a wide variety of studies have been carried out over the past decades to evaluate and compare the performance of various immunoassays. When comparing results from these studies, it is always important to recognize the variables that can influence the outcome and interpretation of the immunoassays and comparative studies (Baselt, 1984; Kricka, 2000). The contributing variations include, but are not limited to, physiological and biological factors (Huestis and Cone, 1998a, 1998b; Vandevenne et al., 2000) and potential interference with the assays by food or medication (Berkabile and Meyers, 1989; Rollins et al., 1990; Colbert, 1994; Wagener et al., 1994; Linder and Valdes, 1994; Joseph et al., 1995; ElSohly and Jones, 1995; Costantino et al., 1997; Lehmann et al., 1997; Struempler et al., 1997). Additional factors to be taken into consideration include market-segment goals on sensitivity and specificity, percentage of "near-cutoff" specimens evaluated, sample size, prevalence, the type of population selected for evaluation, and the study protocols.

As discussed in previous sections, the utility of combining a 50 ng/mL immunoassay cutoff and a 15 ng/mL GC/MS cutoff has been supported by several studies and chosen as the rule by the U.S. *Mandatory Guidelines*. However, the composition of total THC metabolites relative to the amount of THC-

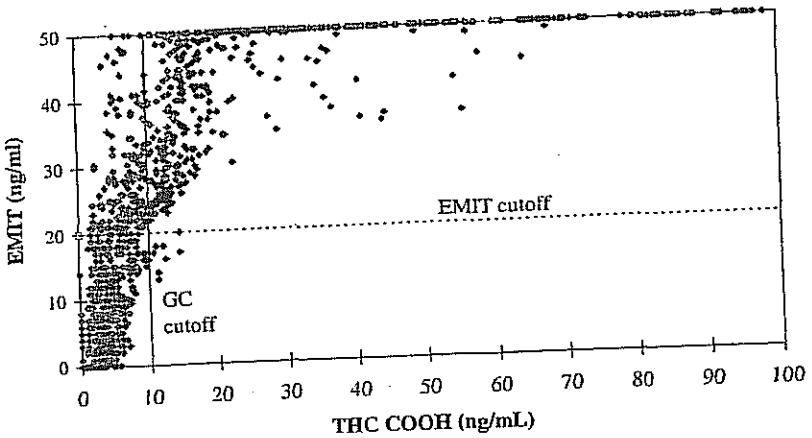


Figure 3.15
Correlation of GC and immunoassay results for THC-COOH Reproduced with permission from Smith-Kielland et al. (1999).

COOH can vary for any given specimen donor that provides a sample at any given time. Therefore, the balance of "analyte detection rate" and "confirmation rate" for samples that exhibit GC/MS values between the GC/MS cutoff and immunoassay cutoff can differ from study to study. Smith-Kielland et al. (1999) demonstrated the relationship between the GC analysis of urinary THC-COOH concentration (using a 10 ng/mL cutoff) and Syva Emit immunoassay values (using a 20 ng/mL cutoff). The results were obtained by screening 1432 samples, of which 1248 samples were further analyzed by GC. Although the cutoff levels chosen for EMIT and GC/MS were lower than those mandated by the current SAMHSA guidelines, the scatter plot (Figure 3.15) exemplifies a field scenario where there is correlation but not a directly linear relationship between the two types of results. Comprehensive studies that utilize regression analysis to explore the relationship of immunoassay screening and GC/MS confirmation have been presented in a series of scatter graph plots published by Weaver et al. (1991), R.H. Liu et al. (1994), R.H. Liu and Goldberger (1995), and Brendler and Liu (1997).

Baselt (1989) and Haver et al. (1991) raised questions regarding the utility of immunoassays as a quantitative tool. When used as a qualitative assay, the amount of drugs and metabolites detected in any given sample cannot be estimated from immunoassay results. Semiquantitative determinations of cannabinoids concentrations are possible by plotting the ΔA or mP values of the calibrators and comparing the ΔA or mP value of the positive sample to the standard curve. Armbruster et al. (1993a) reported that the slopes for the immunoassay calibration curves of KIMS and FPIA assays were significantly greater than those of the Emit II assays. Wu et al. (1995) reported that the rate separations by CEDIA assays between the negative and cutoff calibrators were greater than corresponding Emit II assays.

Frederick et al. (1985) compared GC/MS and five commercial cannabinoids immunoassays (Emit-st, Emit d.a.u., Abuscreen RIA, Immunalysis RIA, and Toxi-Lab TLC). The GC/MS method provided confirmation for all procedures except 2% or 3% of the positive EMIT-d.a.u. results. Abercrombie and Jewell (1986) evaluated EMIT and RIA "high volume test procedures" for THC metabolites in urine utilizing GC/MS confirmation and reported that EMIT and RIA results agreed for 91% of samples. The authors found that there is no relationship between quantitations determined by the two tests.

Budgett et al. (1992) compared the qualitative detection of cannabinoids in urine using Abbott FPIA and Roche RIA and concluded that the two technologies give comparable results. Karlsson and Strom (1988) applied the FPIA using TDx and the Emit assay using Cobas analyzer for detection of cannabinoids in urine from prison inmates. Their results indicated that the Emit assay detects a few more positive samples but also yields a higher rate of unconfirmed positive results compared to the TDx. Those additional "positives" by Emit had THC-COOH concentrations below 10 ng/mL.

Kintz et al. (1995c) compared GC/MS and immunological methods, including Syva Emit, Abbott FPIA, and Roche ONLINE immunoassay, for the determination of THC-COOH. The immunological methods compared favorably and are acceptable for detecting the presence of cannabis metabolites in urine. The authors stated that these results support the concept that all immunoassays for cannabinoids should be considered screening procedures. No concentration correlation between GC/MS and the immunoassays could be established because of the different cross-reactivities of the metabolites.

K.A. Moore et al. (1999) compared double antibody RIA kits (DPC and Immunalysis) and ELISA (STC Microplate EIA) for the screening of postmortem blood and tissues for nine cases of drugs of abuse. The cutoff used for THC immunoassay and confirmation was 25 ng/mL and 10 ng/mL, respectively. The performances of EIA and RIA were comparable when 239 samples were tested for cannabinoids. Niedbala et al. (2001) compared ELISA-based cannabinoids assays for oral fluid testing (by using a 1 ng/mL cutoff) to ELISA-based cannabinoids assays for urine testing (by using a 50 ng/mL cutoff) in subjects who were administered single doses of marijuana by smoked and oral routes. The results supported the utility of oral fluid testing with the chosen cutoff concentration and hinted at the dependence of the detection time window for various matrices or assays on their cutoff level selection.

A few large-scale evaluations were carried out to examine and compare diverse drugs-of-abuse immunoassays that were conducted in the same study setting or comparable conditions. Ferrara et al. (1994) compared six immunochemical techniques and three chromatographic techniques and demonstrated the statistical approach and experimental comparison of these

nine techniques. Among the seven immunoassays evaluated (the assays have different cutoffs), sensitivity for cannabinoids detection ranged from 56.8% to 97.6%, whereas specificity for corresponding tests ranged from 94.0% to 98.9%. The sensitivity results were inversely related to the cutoff concentrations for the assays evaluated.

Armbruster et al. (1993a) compared several immunoassays and showed that RIA, TDx, ONLINE, and EMIT II detected 99%, 95%, 99%, and 88% of the GC/MS-confirmed marijuana samples, respectively. In a separate study, Armbruster et al. (1995) showed that RIA, TDx, ONLINE, EMIT II, and CEDIA detected 100%, 87.2%, 88.8%, 85.5%, and 88.8% of the GC/MS-confirmed marijuana samples, respectively.

Huestis et al. (1995) evaluated the use of RIA, EMIT, FPIA, and KIMS immunoassays (at two cutoff concentrations) to monitor urine samples from six healthy subjects with a history of marijuana use when they had resided in the clinical ward of the Addiction Research Center for 4 to 6 weeks. Using 50 ng/mL and 15 ng/mL as the respective cutoff for immunoassays and GC/MS, the respective efficiency of these immunoassays ranged from 91.4% to 94.7%. In another study, Huestis et al. (1994) determined detection times of cannabinoids in urine using five cannabinoid immunoassays (EMIT, ONLINE, RIA, DRI, and ADx) with different cutoff concentrations and GC/MS and reported that urinary cannabinoid detection times varied substantially across assays, subjects, doses, and cutoff concentrations.

von Meyer et al. (1997) evaluated the performance of the following systems in accordance with the guidelines of the European Committee for Clinical Laboratory Standards (ECCLS): Abbott TDx and ADx (using Abbott AxSYM analyzer), Syva Emit d.a.u. (using Roche MIRA S Plus analyzer), Syva Emit d.a.u. (using Syva ETS Plus analyzer), Syva Emit II (using Hitachi 717 analyzer), and Roche Abuscreen (using MIRA S Plus analyzer). The test analytes, including cannabinoids, were each investigated in three laboratories on different systems. The authors reported that the imprecision of all systems in the series and from day to day was good, with CV values of less than 5% and 10%, respectively.

Studies have also been carried out to evaluate diverse onsite devices. A SAMHSA-sponsored study was conducted by the Center for Substance Abuse Prevention Division of Workplace Programs to evaluate 15 onsite devices and the instrument-based Emit assays (http://workplace.samhsa.gov/ResourceCenter/r362.htm). The evaluation was designed to challenge the devices on their accuracy around the cutoff. The report did emphasize that actual specimens from the field have much fewer specimens with drug concentrations near the cutoff. This means that a much higher percentage of confirmed positive

results and fewer false-negative results should occur during actual testing in the field.

Another large study, a field test of onsite drug detection devices, was sponsored by the Department of Transportation National Highway Safety Administration (http://www.nhtsa.dot.gov/people/injury/research/pub/onsitedetection/Drug_index.htm). The study identified 30 onsite devices and rated 16 devices based on 14 criteria. From the rating results, 5 devices were selected to evaluate 800 samples in two high prevalence counties in New York and Texas, respectively. For THC assay, there were no false negatives for the samples that tested negative on all devices. However, false-negative results were obtained on samples that tested negative on some, but not all, of the devices for a given drug. These false-negative rates ranged from 0.12% to 0.37% for drug present in concentrations greater than the screening cutoff and ranged from 0.25% to 0.87% for drug present in concentrations greater than the confirmatory cutoff. The report indicated that when cutoff concentration and additional drugs are taken into consideration, the devices were accurate in identifying positive samples and rarely failed to identify a driver who had the target drugs in his/her urine. The report also stated that police officers who participated in the study generally favored the use of onsite devices in the enforcement of impaired driving laws, although the use of these devices should not supplant the officer's judgment regarding impairment.

Buchan et al. (1998) reported a field evaluation of onsite, multianalyte drug-testing devices to determine their accuracy, efficiency, and cost-effectiveness as a tool for identifying impaired drivers and determining prevalence of illicit drugs in reckless drivers in a county in Florida. For THC, results from testing 303 voluntary urine specimens indicated that the accuracy ranged from 97.4% to 98.0%. The authors observed that the four kits were in very close agreement on prevalence (15.5–15.8% for THC). Gronholm and Lillsunde (2001) evaluated the accuracy of 10 onsite testing devices for drug screening using urine or oral fluid specimens. The onsite test results were compared with GC/MS. A total of 800 people and eight onsite devices for urine and two for oral fluid testing were included in the study. The accuracy of the devices was in the range of 97% to 99% for cannabinoids, although there were differences in the ease of performance and interpretation of test results. For oral fluid onsite devices, the cannabinoids assay did not fulfill the needs of sensitivity. Leino et al. (2001) evaluated eight commercially available onsite drugs-of-abuse testing devices and reported sensitivities ranging from 88% to 98% and specificities ranging from 95% to 100% for THC-COOH. However, the devices differed markedly with respect to the interpretation of test results and to the ease of test performance, leading to the suggestion that different criteria should be used for selecting

onsite devices for either emergency laboratories in hospitals or police stations and prisons. The authors also emphasized the importance of confirming any positive screening test result.

In 1989, Frings et al. reported results of a blind study designed to determine the accuracy of drugs-of-abuse testing in urine in 31 laboratories across the United States. The authors concluded that urine drug testing could be accurate when performed by qualified staff, using up-to-date screening and confirmation methods, appropriate quality assurance measures, and a chain of custody. The fundamental conclusions remain valid more than 10 years later. However, because the immunoassay reagent formulations may change over time and the evaluation protocols, goals, and sample populations may vary significantly, the relative performance of one immunoassay over the other may vary from study to study. Even though most studies showed that there is no absolute relationship between quantifications of various cannabinoids immunoassays, the majority of cannabinoids immunoassay evaluations demonstrated comparable performance. Most important, these immunoassays are cost-effective initial tests for the screening of abused drugs, provided that confirmatory tests of presumptive positives are performed to ensure reliability of forensic drug analysis results.

3.4 CONFIRMATION (CONFIRMATORY TEST) OF CANNABINOIDS IN URINE SPECIMENS

3.4.1 INTRODUCTION

Confirmations of drugs or metabolites detected by immunoassay require methods capable of quantifying a single chemical species and of excluding all other relevant species. By far the most commonly performed analysis in the toxicology of cannabinoids is the confirmation of THC-COOH in urine. This is the preponderant cannabinoid in urine, and it is the target of commercially available immunoassays used in screening.

In order for an analytical method to be of value in forensic cases, preemployment testing, postemployment testing, or probation testing it must be highly specific and it must be sensitive. In the United States the required cutoff for confirmation of THC-COOH in federally regulated drug testing is 15 ng/mL (Federal Register 59, 29908–29931, 1994). The method used must be capable of detecting THC-COOH at 6 ng/mL (40% of the cutoff) in reanalysis of challenged cases. In Europe, cutoff concentrations from 1 to 400 ng/mL are used, depending on the type of laboratory and the purpose of the analysis (Badia et al., 1998a, 1998b). A cutoff of 15 ng has been recommended in the European Union (de la Torre et al., 1997). Methods of analysis have been reviewed by ElSohly and Salem (2000).

3.4.1.2 Sample Preparation

3.4.1.2.1 Glassware/Plasticware/Pipettes

Adsorption of cannabinoids by the surface of containers and equipment has been reported (Garrett and Hunt, 1974; Fenimore et al., 1976a; Jones et al., 1984a; Christophersen, 1986; Joern, 1987; Dextraze et al., 1989; Blanc et al., 1993; Bond et al., 1990; Dugan et al., 1994). Such adsorption of THC-COOH can lead to a lack of linearity in analysis (Joern, 1987) and is one explanation for loss of THC-COOH in storage (Jones et al., 1984a). Joern (1992c) reported that loss occurs from standard solutions of THC-COOH in borosilicate glass tubes whether or not they are silanized and the adsorption is highly variable. The adsorption of THC-COOH is greatly reduced or nonexistent from basic solutions and organic solutions (Joern, 1987). Joern (1992c) proposed that standard THC-COOH solutions be prepared in drug-free urine made basic by addition of 10 M sodium hydroxide to yield a final concentration of sodium hydroxide of 0.10 M. The solution must be centrifuged or filtered to remove precipitate. Additionally, it was suggested that the basic solutions used in hydrolysis be added to tubes before the addition of patient or control urines.

Roth et al. (1996) reported a comprehensive study on the effects of solution composition and container material type on the loss of THC-COOH. The authors measured losses in relation to surface area (ng/cm^2) for glass, acrylic, silanized glass, Kynar, Teflon-S, polystyrene, polypropylene, and high-density polyethylene and for three solvents: water, urine, and Abbott cannabinoids diluent. The authors' conclusions provide insight into methods to minimize loss of standard or analyte. The losses were greatest for high-density polyethylene and least for untreated glass. Water solutions were subject to greater loss than urine. The smaller the volume of solution (with a greater surface-to-volume ratio), the greater was the observed loss. Of particular interest is the observation that no loss was observed beyond the first hour.

Losses in pipetting were least for unsilanized glass and were determined by time of exposure and temperature, with less loss at lower temperatures. The authors concluded that exposure of THC-COOH solutions to new surfaces should be avoided during sample handling. The stability of THC in urine in high-density polyethylene is addressed by Giardino (1996).

3.4.1.2.2 Hydrolysis

Since THC-COOH is found in urine as both the free acid and the glucuronic acid conjugate (Kanter et al., 1982b; Law et al., 1984a, 1984b), the analysis generally starts with the hydrolysis of the sample. Either basic solutions or enzymes can be used to free the acid from its conjugate. A wide variety of conditions have been reported for the basic hydrolysis. Baker et al. (1984) compared recoveries of THC-COOH using a variety of hydrolysis conditions and

concluded 1 mL of 1 N KOH for 5 mL of urine heated at 37°C for 15 minutes gave optimum recovery. They also concluded that β-glucuronidase (bovine liver) could completely hydrolyze the sample in 30 minutes at 37°C. It should be noted that many reported β-glucuronidase hyrolyses are carried out overnight (16 h) and that results are dependent on the source and the particular batch. Kemp et al. (1995b) reported that 2 N NaOH (0.5 mL) when added to a solution of unhydrolyzed urine (1 mL) and phosphate buffer (1 mL, for volume adjustment) followed by hexane : ethyl acetate extraction gave complete hydrolysis of THC-COOH without heat or incubation time. Kemp et al. (1995b) also demonstrated that base hydrolysis is ineffective in hydrolyzing ether glucuronides (as opposed to esters) and that hydrolysis with bacterial β-glucuronidase revealed significant concentrations of THC and 11-OH-THC in urine. The β-glucuronidase from bacteria (*Escherichia coli*) was shown to be much more effective than β-glucuronidase from mollusks (*Helix pomatia*) in hydrolyzing ether conjugates, such as found in THC-glucuronide. The choice of method will depend on the purpose of the analysis, with β-glucuronidase being essential if analytes other than THC-COOH are of interest. For routine analysis of THC-COOH, basic hydrolysis is the most widely used method.

3.4.1.2.3 Extraction

The extraction of cannabinoids from biological matrices can be achieved by liquid–liquid extraction or solid-phase extraction. The most commonly used solvent for extraction from urine is hexane–ethyl acetate (7:1, 9:1). For extraction from blood, plasma, serum, and other tissues, acetone and/or acetonitrile are often used. The supernatant after centrifugation is generally evaporated, and the extract is dissolved in base and the neutral and basic components extracted into an organic solvent, typically hexane–ethyl acetate (9:1). The aqueous layer is then acidified, and the acid components are extracted into organic solvent. Liquid–liquid extraction is still widely used in research studies. Solid-phase extraction is now the most frequently used method in urine drug testing (Gere and Platoff, 1995) and in many research studies. A widely used method for THC-COOH in urine (Paul et al., 1987) employs anion exchange resin. Solid-phase extraction (SPE) systems are commercially available from numerous manufacturers. Gere and Platoff (1995) have reviewed all facets of SPE, including the cartridges of specific manufacturers and automation of the process.

The trend in workplace and regulatory drug testing is toward systems that reduce the volume of organic solvents (O'Dell et al., 1997) and that can be automated. In this regard extraction discs in which the solvent is enmeshed in inert microfibrils have proved useful (*Singh and Johnson, 1997*). *Wu et al.*

(1993a) utilized such discs in a procedure where the THC-COOH that had been extracted onto the disc was eluted and derivatized by MSTFA in one step.

Solid-Phase Extraction (SPE)

A typical analysis for THC-COOH using SPE will include the following steps:

1. Measurement of aliquot of urine (1–3 mL)
2. Addition of base and internal standard (hydrolysis)
3. Adjustment of pH (acid)
4. Transfer of solution to appropriately prepared extraction cartridges
5. Passage of solution through the cartridge
6. One or more washes with appropriate solvents (dependent upon solid phase used)
7. Drying of solid phase
8. Elution with appropriate solvent
9. Evaporation
10. Derivatization (may be multiple steps)
11. Chromatography

Preparation of the SPE cartridges requires one or more washings with appropriate solutions, which are dependent on the specific packing. The cartridge preparation and steps 5 to 8 are often preformed by placing the cartridges in the top of a vacuum manifold that draws the liquids through the cartridges. These commercially available vacuum systems allow processing of 12 or more samples simultaneously.

An actual application of SPE is shown in the following example from Langen et al. (2000) using Bakerbond SPD NARC-1 3-mL extraction cartridges.

Step 1. 1 mL urine.

Step 2. 300 µL 10 M KOH, 2 mL H_2O, 25 µL IS (4 µg/mL THC-COOH-d_9) 15 min/60°C.

Step 3. 350 µL acetic acid (96%), adjust to pH 2.5 with 2 mL 50 mM phosphate buffer.

Step 4. Preparation of cartridges:
   a. 3 mL methanol
   b. 3 mL 50 mM phosphate buffer

Step 5. Draw sample through cartridge—do not dry.

Step 6–7. Wash—2 mL acetonitrile/0.1 M HCl (2:3 v/v)—dry (1 min)—0.5 mL hexane—dry 5 min.

Step 8. Elute—3 mL hexane/ethyl acetate (1:1 v/v).

Automation

Automation of the entire process or significant components of the process offers the possibility of improving throughput, reducing errors, reducing labor costs, and improving precision. One automation system, the Dupont Prep-I, has been widely used (Paul et al., 1987; Abercrombie and Jewell, 1986) in this regard but is no longer manufactured. Creative Technology has developed a successor (Xtrx Automated Processor) to the Prep-I. An early version of the Zymate, a complete robotic system manufactured by Zymark, was found by one author (CT) to take up too much space and to perform too slowly to justify its cost. A less ambitious instrument, Rapid Trace, by Zymark has, on the other hand, been successful in efficiently performing all the steps usually performed manually on the vacuum manifold (Polyniak, 2001). Stonebraker et al. (1998) reported the use of Rapid Trace to automate the SPE and GCMS analysis of THC in blood. Zymark also manufactures Confir Mate for robotic preparation of samples for GCMS.

Instruments designed to automate all or part of the sample preparations are manufactured by Tecan, Waters, Savant, Gilson, Hamilton, and Agilent. Whitter et al. (1999) have reported successful improvement of laboratory efficiency and reduction of costs using a Six-Head Probe Hamilton Microlab 2200 system to automate steps 1 through 8. Langen et al. (2000) have reported on the use of ASPECXL (Gilson) to automate the extraction procedure. The authors encountered difficulty in controlling absorption of the analyte in the tubing and glassware during the procedure. Throughput was slow, but the system could operate 24 h a day. The instrument can be used to perform the hydrolysis, evaporation, derivatization, and possibly the injection steps; however, no evaluation of these functions has been reported. Extraction procedures for analytes from matrices other than urine will be covered under specific matrices. Steinberg et al. (1997) evaluated Toxi-Prep to semiautomate SPE extractions of drugs in urine.

3.4.1.3 Standards

Certified urine-based standard reference material (SRM) for 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (SRM 1507b) is available from the U.S. National Institute of Standards and Technology (NIST). The SRM 1507b consists of three concentration levels (approximately 12, 25, and 50 ng/mL). These certified reference standards can also be obtained from the College of American Pathologists (CAP). Standards of Δ^9-THC-COOH as well as Δ^8-THC-COOH, 11-hydroxy-Δ^9-tetrahydrocannabinol, cannabinol, and cannabidiol are available from Sigma (St. Louis, MO). Standards are also available in the United States from Research Triangle Institute (Research Triangle Park, NC), Alltech-Applied Science (State College, PA), and Cerilliant (Austin, TX).

3.4.1.4 Analytical Methods

Confirmation of THC-COOH by GC/MS is by far the most widely used method, especially for forensic examination and for regulated analyses (Bronner and Xu, 1992; Goldberger and Cone, 1994; Badia et al., 1998a). However, a wide variety of methods are used in unregulated testing or in countries where regulated testing does not mandate mass spectrometry (Badia et al., 1998a).

3.4.1.4.1 Thin-Layer Chromatography

The use of TLC to identify THC-COOH is widespread in clinical laboratories and has substantial use in forensic laboratories (Badia et al., 1998a). Kaistha and Tadrus (1982) advocated the use of silica gel plates with chloroform–methanol–concentrated ammonium hydroxide (85:15:2) as the mobile phase. Fast blue RR (0.5% w/v, in equal volumes of methanol and water) was used for detection. The limit of detection is approximately 50 ng/mL. Kanter et al. (1982b) developed a method for simultaneously detecting THC-COOH and THC-COOH glucuronide by extracting the free acid prior to hydrolysis of the glucuronide. The extracts were sequentially developed in two different solvent systems and detected with fast blue salt B. A method (Kanter et al., 1982a) for identifying total THC-COOH utilized silica gel G plates and sequentially developed them in acetone–chloroform–triethylamine (80:20:1) followed by petroleum ether–ether–glacial acetic acid (50:50:1.5). The procedure could detect a spot containing 0.5 µg of THC-COOH. High-efficiency thin-layer chromatography (HETLC) was utilized by Black et al. (1984) to aid in the confirmation of EMIT results. An internal standard (IS) (Δ^8-THC) was added to 10 mL of urine before basic hydrolysis. Solid-phase extraction (Bond-Elut-THC) was used to isolate the analyte and IS for HPLC and HETLC. Hexane–acetone–glacial acetic acid (70:30:1) was used to effect the separation on 10 × 10-cm HETLC-HL plates (Analtech). Visualization utilized alkaline fast blue B salt. The LOD was 20 ng/mL. Kogan et al. (1984) used SPE and 25 × 75-mm E. Merck silica gel 60 plates. A mobile phase of ethyl acetate–methanol–water–ammonium hydroxide (12:5:0.5:1) was used to chromatograph the extracts, and fast blue RR was used to visualize the cannabinoids. The LOD was 20 ng/mL in 10 mL of urine. Meatherall and Garriott (1988) used HPTLC plates from three different manufactures (Analtech, Whatman, and Merck Science) to detect THC-COOH with a LOD of 5 ng/mL in 2 mL of urine. Fast blue BB was used to visualize the analyte, and heptane–butanol–acetic acid (90:9:1) was used as the mobile phase. The hydrolyzed samples were made acidic and extracted with hexane. Foltz and Sunshine (1990) evaluated the Toxi-MS cannabinoid test (Toxi-Lab, Inc.). In this system the hydrolyzed samples are aspirated through a SPE layer and then through a silica gel phase

to achieve separation. The first layer concentrates the sample at the beginning of the TLC plate, and a rapid development separates the THC-COOH from other components. Fast blue BB is used to visualize the analyte. The method was compared directly via EMIT and GC/MS. A LOD of 10 ng/mL was reported.

Brandt and Kovar (1997) developed a TLC method that is sensitive, quantitative, and specific enough for forensic identification of THC-COOH. SPE was performed with Isolute C_8-(EC), 500 mg, 10-mL columns. After passing the hydrolyzed urine through the column, the column was washed first with acetonitrile–water (4:6) and then with dichloromethane–*n*-hexane (2:8). Elution with diethyl ether–*n*-hexane (2:8) gave very clean extracts. Separation was achieved on 0.1-mm layers of silica gel 60 WRF_{254^s} (Merck). Online detection and quantitation was carried out by UV (LOD 4 ng/mL) and IR (LOD 14 ng/mL). An IR spectrum of the THC-COOH is obtained.

3.4.1.4.2 High-Performance Liquid Chromatography

Between 10% and 11% of European Union laboratories reported the use of HPLC for identification of drugs in urine. Many laboratories (13%) reported HPLC as a method used for quantification (Badia et al., 1998a). Methods using UV detection (ElSohly et al., 1983; Posey and Kimble, 1984; Karlsson and Roos, 1984; Johansson and Halldin, 1989; Ferrara et al., 1992), electrochemical detection (Bourquin and Brenneisen, 1987; Craft et al., 1989; Fisher et al., 1996), and RIA (Law et al., 1984b) have been published. Breindahl and Andreasen (1999) developed an LC method using atmospheric pressure ionization electrospray mass spectrometry (API-ES-MS) for detection. This method overcomes the lack of specificity of the aforementioned methods and allows for isotope dilution for quantitative methods using THC-COOH-d_3 as the internal standard. A gradient elution varying the concentration of acetonitrile in a constant 4 mM formic acid solution through a 150 × 3.0-mm C_8 column was used. The instrument was used in the positive ion mode. A LOD of 15 ng/mL was obtained using the authors' acceptance criteria, which included the m/z 345 ion (THC-COOH-H^+) and the m/z 327 and 299 ions created by up-front collision-induced dissociation. These two ions must have ion ratios within ±20% of standards. Using the m/z 345 ion alone gave a LOD of 2 ng/mL. Tai and Welch (2000) used a C_{18} column and an isocratic mobile phase (0.05 M ammonium acetate in methanol–water, 75:25) in a LCESMS method to measure THC-COOH in SRM 1507b. The negative ion mode was used, and m/z 343 and 346 for THC-COOH and THC-COOH-d_3 were monitored. A LOD of 5 pg/mL is reported for this method on spiked urine. No hydrolysis was needed, and no qualifying ions were used since identification was not the goal of the analysis. A summary of HPLC methods is shown in Table 3.4.

Table 3.4
HPLC methods for THC-COOH identification and quantitation

Source	Column	Mobile Phase	Internal Standard	Detector	Run Time (min)	Limit of Detection (ng/mL)	Limit of Quantification (ng/mL)
ElSohly et al. (1983)	2.5 cm × 4.6-mm C-8	65% acetonitrile, 35% 50 mM H_3PO_4	CBN-COOH	UV 214 nm	6	25	
Posey and Kimble (1984)	30 cm × 3.9-mm C-18	45% ACN, 55% phosphate buffer pH 6.0	Δ^8-THC-COOH	UV 205 nm	8		20
Karlsson and Roos (1984)	125 × 4-mm C-8	50% acetonitrile, 50% 0.05 M $(NH_4)H_2PO_4$	Δ^8-THC-COOH	UV 220–225 nm, quant. by GC of eluate	15		20
Law et al. (1984a)	160 × 5-mm C-18	82.5% v/v MeOH in pH 1.95 buffer	None	UV 220 nm, monitor RIA	15	3.3 total cannabinoids	
Bourquin and Brenneisen (1987)	150 × 4.6-mm C-18	MeOH/5% HOAc (76:24)	Cannabinol	EC	16	5	
Johansson and Halldin (1989)	250 × 4.6-mm C-18	MeOH:50 mM H_3PO_4 3:1 (pH 3.2)	11-nor-Cannabinol-9-carboxylic acid	UV/EC	12	7	
Craft et al. (1989)	250 × 4.6-mm	Gradient	11-nor-11-Hydroxy-Δ^9-tetrahydro-cannabinol (not suitable in casework)	EC	25	Not reported	
Ferrara et al. (1992)	250 × 4-mm C-8	0.05 M H_3PO_4: acetonitrile 35:65 v/v		UV	13	50	
Breindahl and Andreasen (1999)	150 × 3-mm C-18	Gradient	THC-COOH-d_3	APIESMS positive ion	6	15	
Tai and Welch (2000)	C-18	0.05 M ammonium acetate in MeOH/H_2O (75:25)	THC-COOH-d_3	ESMS negative ion	7	0.005	

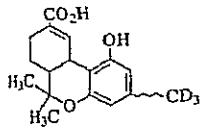
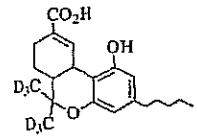
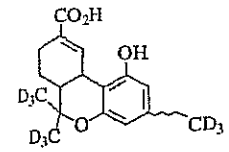
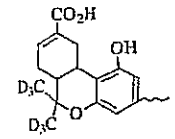
3.4.1.4.3 Gas Chromatography and Gas Chromatography–Mass Spectrometry

Many of the methods of TLC and HPLC already described lack the specificity required to meet the demands of modern forensic or regulated drug-testing laboratories (Federal Register 59, 299908-2993, 1994). In 1998 over 50% of the laboratories in the European Union (Badia et al., 1998b) and all of the federally regulated laboratories in the United States used GC/MS methods to confirm THC-COOH. In the European Union 14% of the laboratories use GC without MS to identify THC-COOH (Badia et al., 1998b). Several excellent reviews of analytical methodology related to cannabinoid analysis in biological samples have been published within the past 10 years. Cody and Foltz (1995) and Goldberger and Cone (1994) have published excellent reviews of GC/MS of drugs of abuse in body fluids. Bronner and Xu (1992) extensively covered the literature of GC/MS analysis of THC-COOH through the middle of 1991. Staub (1999) has reviewed chromatographic procedures for determination of cannabinoids in matrices other than urine.

Internal Standards

Most, if not all, GC methods for THC-COOH and other cannabinoids utilize an internal standard (IS). Ideally the IS is added to the specimen at the beginning of the analysis, i.e., before the extraction of the specimen. The standard should be chemically similar to the analyte. If the IS is chosen well, it will serve as a quantitative reference, as a monitor of the extraction and derivatization procedure, and as a means of compensating for analytical variables such as extraction efficiency, efficiency of derivative formation, and minor changes in gas chromatographic parameters. If GC is being used without MS, the IS must be chromatographically separable from the analyte. In the case of THC-COOH the IS should contain a carboxyl function and a phenolic function. Frederick et al. (1985) used Δ^8-THC-COOH as an internal standard, which has extraction characteristics identical to those of Δ^9-THC-COOH and which is separated by GC. Bronner and Xu (1992) cite numerous examples of internal standards that are inadequate. For analysis by GCMS, isotopically labeled THC and THC-COOH are available as internal standards for these analytes. Isotope dilution with single-ion monitoring is the preferred method for THC-COOH. When using multiple- or single-ion monitoring, these are the ideal internal standards. If full-scan spectra are being used, an IS that is chromatographically separable must be used. Common deuterated internal standards for THC-COOH analysis are shown in Table 3.5 along with the common ions observed in EIMS with various derivatives. The most commonly referenced IS is 5′-($^2H_3$)-11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH-d_3) (Cerilliant, Sigma, Research Triangle Institute). This IS is still widely used as its methyl ester–methyl ether, in spite of a minor m/z 316 ion in the MS of the analyte.

Table 3.5
Internal standards commonly used in mass spectrometry of THC-COOH and the major ions of common derivatives

	Derivative	Ions D(H)	References
 5'($^2H_3$)-11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid THC-COOH-d_3	—CH_3, —CH_3	316, 360, 375 (313, 357, 372)	Paul et al. (1987)
	—C_3H_7, —C_3H_7	344, 388, 431 (341, 385, 428)	Mulé and Casella (1988)
	TMS, TMS	374, 476, 491 (371, 473, 488)	
	TBDMS, TBDMS	416, 518, 560, 575 (413, 515, 557, 572)	Clouette et al. (1993)
	—$CH(CF_3)_2$ / —C(=O)—CF_2CF_3	605 (432, 474, 602, 622)	Kintz et al. (1995a, 1995b)
	—$CH_2CF_2CF_3$ / —C(=O)—CF_2CF_3	610, 625 (445, 459, 607, 622)	Joern (1987)
 THC-COOH-d_6	—CH_3, —CH_3	319, 360, 378 (313, 357, 372)	ElSohly et al. (1992)
	TMS, TMS	377, 494 (371, 473, 488)	ElSohly and Feng (1998)
 THC-COOH-d_9	TBDMS, TBDMS	422, 524, 563, 581 (413, 515, 557, 572)	Clouette et al. (1993)
	—$CH_2CF_2CF_3$ / —C(=O)—CF_2CF_3	454, 468 (445, 459, 489, 622)	Stout et al. (2001)
 Δ^8-THC-COOH-d_6	—CH_3, CH_3	248, 322, 378 (313, 357, 372)	ElSohly et al. (1988)
	TMS, TMS	306, 438, 494 (371, 473, 488)	ElSohly et al. (1988)

The ratio of the m/z 313 ion in the analyte to the m/z 316 ion in the IS is used for quantitative analysis, and the presence of a minor m/z 316 ion in the analyte yields nonlinearity above 800 ng/mL.

ElSohly et al. (1988) developed $^2H_6$-11-nor-Δ^8-tetrahydrocannabinol-9-carboxylic acid (Δ^8-THC-COOH-d_6) (ElSohly Labs, Meridian, MS) as an IS that has the same extraction properties as THC-COOH, can be separated chro-

matographically from THC-COOH, and does not suffer from the interference noted for THC-COOH-d_3 when used as the methyl ester–methyl ether derivative. Joern (1992a) successfully used this IS in the procedure of Paul et al. (1987).

ElSohly et al. (1992) reported $^2H_6$-11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid as an IS and its use as the methyl ester–methyl ether derivative. With this IS the analysis was linear over a wide range. ElSohly and Feng (1998) reported the use of this IS as the TMS derivative.

Clouette et al. (1993) reported the use of 5′-$^2H_3$-11-nor-Δ^9-tetrahydro-6, 6-di(methyl-$^2H_3$)-cannabinol-9-carboxylic acid (THC-COOH-d_9) (Cerilliant, Austin, TX) and THC-COOH-d_3 in a study of the mechanism of fragmentation of the *t*-butyldimethylsilyl derivative of THC-COOH. Szirmai et al. (1996) reported the use of THC-COOH-d_{10}, with no apparent advantages over existing standards.

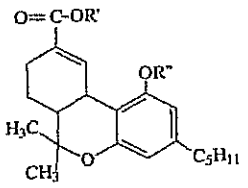
Stout et al. (2001) have developed a method, suitable for high-volume laboratories, using pentafluoproprionic acid and pentafluropropanol as derivitizing agents and THC-COOH-d_9 as the IS. An anion exchange SPE was used to give 95% recovery, 0.875 ng/ml LOD with 3-mL samples, and linearity to 900 ng/mL. With THC-COOH-d_9 there is negligible contribution from IS to very weak samples.

Derivatization

Analysis of THC-COOH by GC universally involves derivatizing the carboxyl and phenol functions of the molecule. A wide variety of approaches have been utilized (Bronner and Xu, 1992). The choice of derivatives will depend on several factors, including GC detectors, MS methods, number of samples, sensitivity, and stability required. A summary of published derivatives is shown in Table 3.6. These derivatives can be divided into four groups based on the chemistry utilized in preparing them.

Alkyl Ester–Alkyl Ether Derivatives The most commonly reported method (since its first use and currently) is the esterification of the carboxyl function and alkylation of the phenolic group to give an alkyl ester–alkyl ether (Whiting and Manders, 1982). In this method the extract is treated with tetramethylammoniumhydroxide followed by iodomethane to yield the methyl ester–methyl ether. Ethyl, propyl, and butyl derivatives have been evaluated (McCurdy et al., 1986), and the propyl derivative has been widely used in the method of Mulé and Casella (1988). Baker et al. (1984) and Nakahara et al. (1995) have compared the methyl ester–methyl ether with TMS derivatives. Dimethyl sulfate has been used to methylate THC-COOH (Wall et al., 1979), and THC-COOH has been methylated on column using dimethylformamide dimethylacetal

Table 3.6
Commonly used derivatives of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid



R'	R"	m/z of Abundant Ions	References
$-CH_3$	$-CH_3$	313, 357, 372	Whiting and Mauders (1983)
$-C_3H_7$	$-C_3H_7$	341, 385, 413, 428	McCurdy et al. (1986)
$-Si(CH_3)_2-CH_3$	$-Si(CH_3)_2-CH_3$	371, 473, 488	Harzer and Kächele (1983) McCurdy et al. (1986)
$-Si(CH_3)_2$-tbutyl	$-Si(CH_3)_2$-tbutyl	413, 515, 572	Bourquin and Brenneisen (1987) Clouette et al. (1993)
$-CH(CF_3)_2$	$-C(=O)-CF_2CF_3$	429, 477, 489, 640	O'Connor and Rejent (1981)
$-CH(CF_3)_2$	$-C(=O)-CF_2CF_2CF_3$	344, 492 (daughter ions), 670 (parent ion) (NCI, MS-MS)	Baumgartner et al. (1995)
$-CH_2CF_2CF_3$	$-C(=O)-CF_2CF_3$	445, 459, 607, 622	Joern (1987)
$-CH_3$	$-C(=O)-CF_3$	454 (NCI)	Foltz et al. (1983)

(Björkman, 1982) or 4:1 methanol–10% tetramethylammonium hydroxide in methanol (Nakamura et al., 1985). The methyl ester–methyl ether is widely used in part due to its stability, ease of preparation, and lack of adverse effects on columns and MS sources (Paul et al., 1987; Mulé and Cassella, 1988). According to Cody and Foltz (1995), when using EIMS the alkyl derivatives provide maximum stability and sensitivity. Studies utilizing alkyl ester–alkyl ether derivatives are listed in Table 3.7.

Table 3.7
Publications demonstrating the use of common derivatizing agents in urine analysis

References	Reagent
Selected Studies Using Silyl Ester–Silyl Ether Derivatives	
Fredrick et al. (1985)	MSTFA
Craft et al. (1989)	BSA
Parry et al. (1990)	BSTFA
Clouette et al. (1993)	MTBSTFA
Kintz et al. (1995c)	BSTFA
Singh and Johnson (1997)	MSTFA
O'Dell et al. (1997)	BSTFA
Whitter et al. (1999)	MTBSTFA
Langen et al. (2000)	MTBSTFA
Selected Studies Using Alkyl Ester–Alkyl Ether Derivatives	
Whiting and Manders (1982, 1983)	TMAH/CH_3I
ElSohly et al. (1984)	PFBBr/BTMAH
McCurdy et al. (1986)	TMAH/C_3H_7I
Paul et al. (1987)	TMAH/CH_3I
Cone et al. (1987)	TMAH/CH_3I
Mulé and Casella (1988)	TMAH/C_3H_7I
Rosenfeld et al. (1989)	PFBBr (XAD-2)
Lisi et al. (1993)	THAH/CH_3I
Cone et al. (1993)	TMAH/CH_3I
Liu et al. (1994)	TMAH/CH_3I
Huestis et al. (1995)	TMAH/CH_3I
Jenkins et al. (1995)	TMAH/CH_3I
Huestis et al. (1996)	TMAH/CH_3I
Huestis and Cone (1998b)	TMAH/CH_3I
Fraser and Worth (1999)	TMAH/C_3H_7I
Selected Studies Using Alkyl Ester–Alkyl Ester Derivatives	
O'Conner and Rejent (1981)	PFPA/PFIP
Karlsson and Roos (1984)	PFPA/PFIP
Joern (1987)	PFPA/PFPOH
Stout et al. (2001)	PFPA/PFPOH

Silylester–Silyl Ether Derivatives Silyl ester–silyl ether derivatives of THC-COOH have been widely used. The trimethylsilyl (TMS) derivative is the most common and is readily prepared using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), *N,O*-bis(trimethylsilyl)acetamide (BSA), or bis(trimethylsilyl)trifluoroacetamide (BSTFA), with or without 1% trimethylchlorosilane as a catalyst