Constituents of *Cannabis sativa* L. VII: Use of Silyl Derivatives in Routine Analysis

CARLTON E. TURNER ˣ, KATHY W. HADLEY, JANIS HENRY, and M. LEONARD MOLE

Abstract □ Naturally occurring cannabinoids previously impossible to separate and analyze were quantitated on a routine basis using silylation. Relative retention times of many silylated cannabinoids are reported for the first time.

Keyphrases □ Cannabinoids—silylation and GLC separation □ *Cannabis sativa* L.—silylation and GLC separation of cannabinoids □ GLC—analysis, *C. sativa* constituents

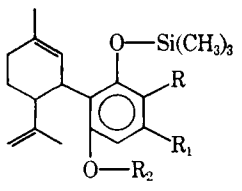
Claussen *et al.* (1) used trimethylsilyl derivatives to separate some cannabinoids from their corresponding carboxylic acid analogs. However, at the time of this report, isolation of cannabinoids with subsequent identification by synthesis and combined GLC–mass spectrometry was in an embryonic state. Thus, Claussen *et al.* (1) were unable to identify many peaks in the chromatogram.

Later, Heaysman *et al.* (2) examined the constituents of *Cannabis* using silyl derivatives, and they reported retention times for the trimethylsilyl ethers of cannabidiol, (−)-Δ^9-*trans*-tetrahydrocannabinol, and cannabinol. Caddy and Fish (3) compared the GLC separation obtained for underivatized cannabidiol, (−)-Δ^9-*trans*-tetrahydrocannabinol, and cannabinol with their trimethylsilyl and trifluoroacetyl derivatives. Other investigators (4, 5) described a trimethylsilyl method for the analysis of cannabidiol, (−)-Δ^9-*trans*-tetrahydrocannabinol, and (−)-Δ^9-*trans*-tetrahydrocannabinolic acid.

Paris and Paris (6) reported the identification of cannabidiolic acid as its trimethylsilyl ester–ether derivative in a sample of hashish, and the use of silyl derivatives to obtain a clear and discrete separation of synthetic cannabidiol and cannabichromene was reported (7); separation of the bis(trimethylsilyl) ether of synthetic cannabidiol from the mono(trimethylsilyl) ether was also obtained. The use of trimethylsilyl derivatives to separate the equatorial and axial isomers of synthetic hexahydrocannabinol was also reported (8).

Therefore, the potential application of trimethylsilyl derivatives in routine separation of naturally occurring cannabinoids not conveniently separated by other means (7) and the need for a routine and practical method of catholic use for assaying acid derivatives of naturally occurring cannabinoids (I–XVIII) with pentyl and propyl side chains prompted this investigation.

The investigation of methyl homologs (9), which also exist in fresh *Cannabis* plant material as their corresponding acid derivatives, and other trace cannabinoids not routinely found in *Cannabis sativa* L. plant material will not be discussed.



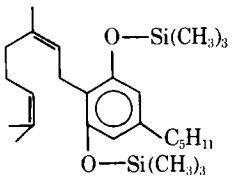
number	cannabinoid	R	R_1	R_2
I	cannabidivarin	H	C_3H_7	$-Si(CH_3)_3$
II	cannabidivarin	H	C_3H_7	H
III	cannabidiol	H	C_5H_{11}	$—Si(CH_3)_3$
VII	cannabidiol	H	C_5H_{11}	H
VIII	cannabidivarinic acid	$CO_2Si(CH_3)_3$	C_3H_7	$—Si(CH_3)_3$
XIV	cannabidiolic acid	$CO_2Si(CH_3)_3$	C_5H_{11}	$—Si(CH_3)_3$

EXPERIMENTAL

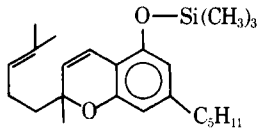
Samples used were grown in Mississippi from seed obtained through the National Institute on Drug Abuse (NIDA), the United Nations, and the U.S. Department of Justice Drug Enforcement Administration (DEA) and from researchers throughout the world. Plant material was grown in 1972[1]. All plant samples were from aboveground plant parts.

Routine GLC Analyses—Three 1-g samples were extracted simultaneously with 40 ml of spectrograde chloroform. Resulting solutions were allowed to stand at room temperature for 1 hr. During the hour, each sample was shaken for approximately 15 sec at 20-min intervals. The plant material was then removed by filtration and the mother liquor was concentrated *in vacuo* at ambient temperature to a greenish paste void of solvent.

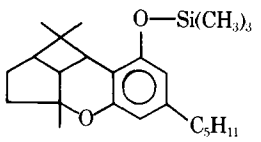
At this point, 1.5 ml of an ethanolic solution containing 10 mg/ml of androst-4-ene-3,17-dione was added as the internal standard. Continuous vibration from an ultrasonic vibrator was then applied until all resin was in solution. Usually this 15 mg of solution of the internal standard was adequate. Routinely, 0.2 μl of the resulting solution was injected with 0.2 μl of ethanol as a flush solvent. In these laboratories, this method provides excellent results.



XIII: bis(trimethylsilyl)ether of cannabigerol

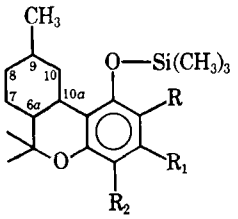


VI: trimethylsilyl ether of cannabichromene



X: trimethylsilyl ether of cannabicyclol

[1] *Cannabis* herbarium specimens are stored in the Herbarium, Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677



number	cannabinoid	other	R	R_1	R_2
IV	(−)-Δ^9-*trans*-tetrahydrocannabivarin	$\Delta^{9,10}$	H	C_3H_7	H
XVI	(−)-Δ^9-*trans*-tetrahydrocannabivarinic acid	$\Delta^{9,10}$	$CO_2Si(CH_3)_3$	C_3H_7	H
IX	(−)-Δ^8-*trans*-tetrahydrocannabinol	$\Delta^{8,9}$	H	C_5H_{11}	H
XI	(−)-Δ^9-*trans*-tetrahydrocannabinol	$\Delta^{9,10}$	H	C_5H_{11}	H
XVII	(−)-Δ^9-*trans*-tetrahydrocannabinolic acid A	$\Delta^{9,10}$	$CO_2Si(CH_3)_3$	C_5H_{11}	H
XVIII	(−)-Δ^9-*trans*-tetrahydrocannabinolic acid B	$\Delta^{9,10}$	H	C_5H_{11}	$CO_2Si(CH_3)_3$
V	hexahydrocannabinol (C_9 methyl axial)	—	H	C_5H_{11}	H
XII	hexahydrocannabinol (C_9 methyl equatorial)	—	H	C_5H_{11}	H
XV	cannabinol	$\Delta^{6a,7}$ $\Delta^{8,9}$ $\Delta^{10,10a}$	H	C_5H_{11}	H

Analyses were performed using gas chromatographs[2] equipped with hydrogen flame-ionization detectors and operated isothermally at 210°. Inlet and detector temperatures were 240 and 260°, respectively. Glass columns, 0.64 cm (0.25 in.) o.d. and 2 mm i.d. × 2.43 m (8 ft), were packed with 2% OV-17 (high purity polar methyl silicone; approximately 30,000 mol. wt.) on 100–120-mesh Chromosorb WHP. Nitrogen was used as the carrier gas at a flow rate of 10–30 ml/min, depending upon separation and instrument requirements. Usually the head pressure was between 26 and 40 psi.

Peak area measurements were made with a computer[3]. The peak area, measured in millivolts, was compared with the peak area of the internal standard. Relative response factors obtained from synthetic and natural cannabinoids were prerequisites for reproducibility and accuracy.

Silylation of Plant Extract—Plant material was processed as described for the routine GLC analyses. After the greenish paste void of solvent was obtained, 1.5 ml of anhydrous pyridine containing 10 mg/ml of androst-4-ene-3,17-dione was added. Continuous vibration from an ultrasonic vibrator was then carried out until all resin was in solution. At this point, 0.5 ml of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane[4] was added.

The reaction mixture was heated, using a heating mantle, for approximately 10 min at 80°. Then 0.2 μl of the reaction mixture was routinely injected. Decarboxylation of cannabinoid acids was accomplished by heating the plant material extracts at 110° for 1.5 hr prior to silylation. A positive nitrogen head pressure can be used to prevent oxidation of certain cannabinoids. However, the nitrogen head pressure is not mandatory for good results when decarboxylating extracts, but it is recommended if plant material is heated prior to extraction.

GLC–mass spectrometry and TLC were used as supportive tools for identification and assignment of previously unknown peaks (10). Fragmentation patterns of silyl ester–ether derivatives of cannabinoids and electrovoltage–mass fragment intensity graphs (9) will be presented elsewhere.

RESULTS AND DISCUSSION

Betts and Holloway (11) reported the formation of trimethylsilyl ethers of cannabinoids by an overnight reaction of hexamethyldisilazane and a drop of trimethylchlorosilane. They observed that cannabidiol reacted more slowly than the other cannabinoids; thus, the long reaction time reported by this research group allowed sufficient time for cannabidiol to react and for trace amounts of ammonium chloride formed as a by-product to precipitate.

Claussen *et al.* (1) also preferred overnight reactions, whereas Makita and Wells (12) and Heaysman *et al.* (2) used a shorter reaction time. These investigators employed hexamethyldisilazane and trimethylchlorosilane with a solvent such as anhydrous pyridine or anhydrous isopropylamine. Claussen *et al.* (1) used the trimethylsilyl derivative of cannabidiol as an internal standard, whereas Heaysman *et al.* (2) used anthracene, which has a retention time between silylated cannabidiol and Δ^9-tetrahydrocannabinol. Betts and Holloway (11) used the hydrocarbon *n*-eicosane as an internal standard.

Davis *et al.* (5) first reported the use of androst-4-ene-3,17-

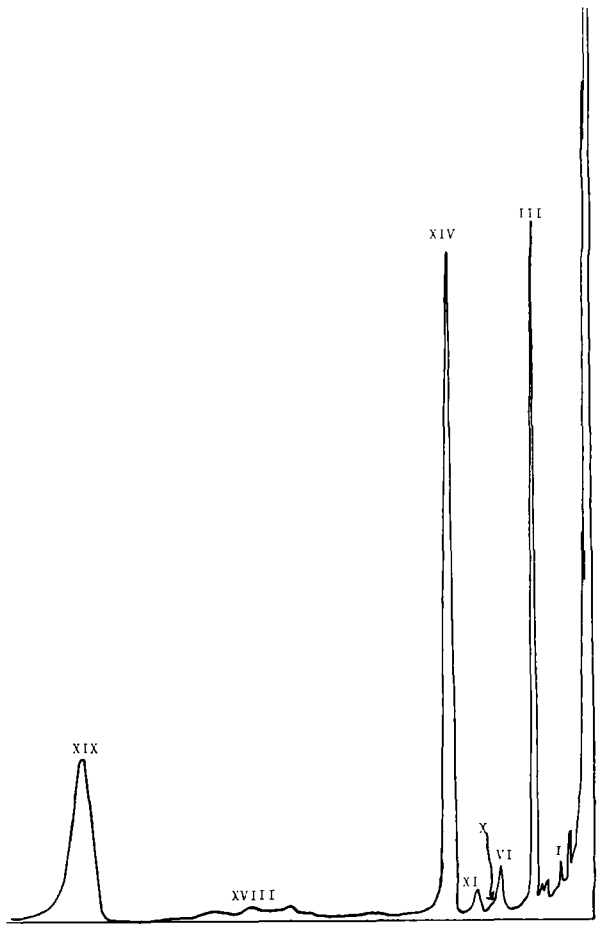


Figure 1—*Gas chromatogram of silylated male Turkish (TU-A) C. sativa L. showing cannabidivarin disilylated (I), cannabidiol disilylated (III), cannabichromene (VI), cannabicyclol (X), (−)-Δ^9-trans-tetrahydrocannabinol (XI), cannabidiolic acid trisilylated ester–ether (XIV), (−)-Δ^9-trans-tetrahydrocannabinolic acid B disilylated ester–ether (XVIII), and androst-4-ene-3,17-dione (XIX), the internal standard.*

[2] Beckman GC-45, GC-72-5, and GC-65.
[3] Digital, PDP-8.
[4] BSTFA with 1% TMCS, Pierce Chemical Co. or Regis Chemical Co.

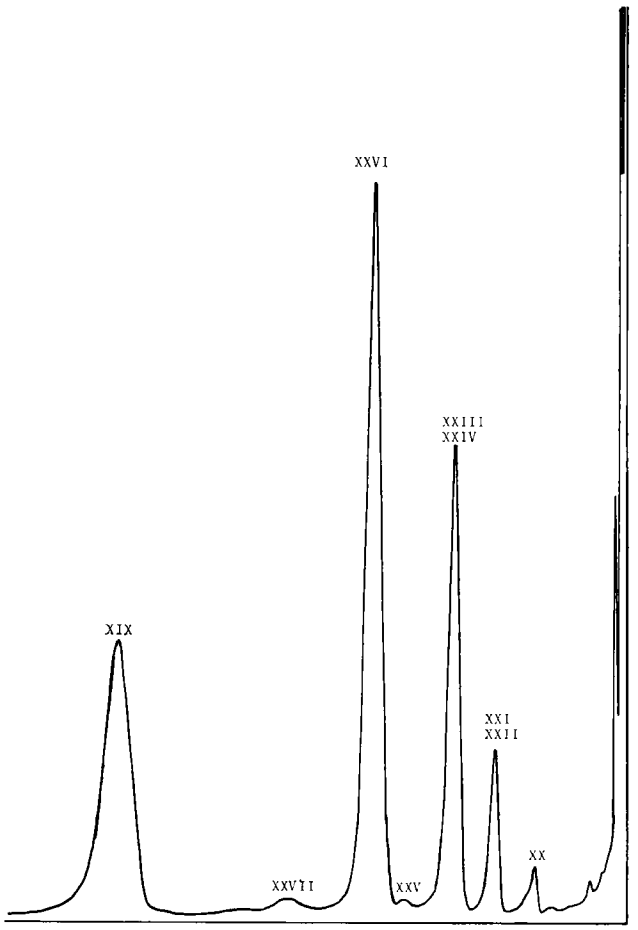


Figure 2—*An OV-17 chromatogram of underivatized female Afghanistan* Cannabis *[AF-B(1)/C-71] showing cannabidivarin (XX), (−)-Δ⁹-trans-tetrahydrocannabivarin (XXI), cannabicyclol (XXII), cannabichromene (XXIII), cannabidiol (XXIV), (−)-Δ⁸-trans-tetrahydrocannabinol (XXV), (−)-Δ⁹-trans-tetrahydrocannabinol (XXVI), cannabinol (XXVII), and androst-4-ene-3,17-dione (XIX).*

dione as an internal standard. Subsequently, this standard has gained in popularity since it can be used in routine quantitation of cannabinoids as well as quantitation provided by the trimethylsilyl method. Other investigators (4, 5) also used androst-4-ene-3,17-dione. Silylation was accomplished by these groups with bis-*N,O*-(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane in anhydrous pyridine.

When using this method to quantitate cannabidiol and cannabichromene, it was found that cannabichromene was present in all variants examined and that, as reported (7), cannabidiol was absent in some variants. For example, Fig. 1 of Turkish (TU-A) *Cannabis* shows a peak (VI) for cannabichromene. Although Fig. 1 is a chromatogram of silylated fresh plant material, when decarboxylation was carried out and assignments of the peaks were made using instrumentation as described in the *Experimental* section, cannabichromene accounted for 4.8% of the peak classically labeled cannabidiol when a routine analysis was performed using an OV-17 column.

Figure 2, a classical chromatogram of Afghanistan *Cannabis* AF-B(1)/C-71, shows the separation obtained using OV-17 columns with underivatized cannabinoids. Cannabidiol and cannabichromene are under one peak and are routinely reported as cannabidiol. In this plant sample [AF-B(1)/C-71], when using decarboxylated and silylated plant material, it was ascertained that the peak labeled cannabichromene and cannabidiol in Fig. 2 contains 81.2% cannabichromene and 18.8% cannabidiol. Figure 3 of decarboxylated and silylated AF-B(1)/C-71 also shows the presence of hexahydrocannabinol (C_9 methyl equatorial) (XII) and a slight amount of the C_9 axial isomer; the latter is not labeled but it is the shoulder preceding cannabichromene (VI).

The presence of hexahydrocannabinol in a plant sample stored at 50° for 2 years was postulated by Turner *et al.* (8). Since this particular sample was heated at 110° for longer than 1.5 hr and since no hexahydrocannabinol is observed in Fig. 2, which is a routine analysis of AF-B(1)/C-71, these data support the previous postulation (8) that hexahydrocannabinol can be formed in plant material by heating. Hexahydrocannabinol (C_9 methyl equatorial) probably would not be detected by an untrained individual, since its relative retention time is near that of silylated cannabigerol (XIII). However, by observing the routine chromatogram of AF-B(1)/C-71 (Fig. 2) and then Fig. 4, it is evident that cannabigerol is not present in this plant sample.

Figure 5, a chromatogram of an Indian variant (IN-F) which was decarboxylated and silylated, shows a large cannabicyclol peak (X). This was common in certain samples containing significant amounts of cannabichromene. Rather uniquely, however, some samples containing large amounts of cannabichromene do not, when silylated, show significant amounts of cannabicyclol. Cannabicyclol, on the other hand, does always appear under the peak routinely labeled (−)-Δ⁹-*trans*-tetrahydrocannabivarin in underivatized analyses of plant materials containing cannabichromene (Fig. 2). Moreover, it is always present when synthetic cannabichromene is subjected to GLC analyses. This points to an unex-

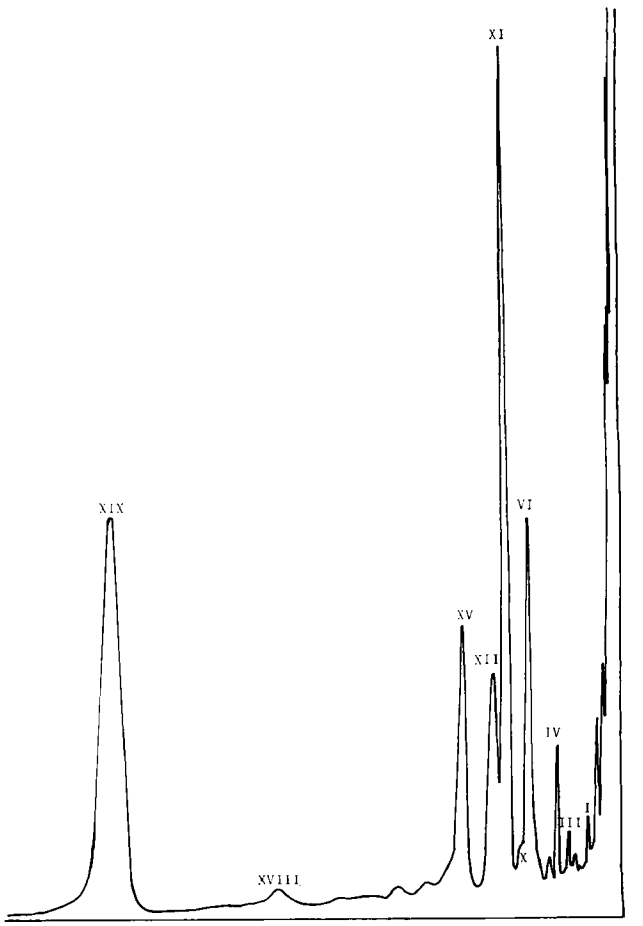


Figure 3—*Chromatogram of decarboxylated and silylated female Afghanistan* Cannabis *[AF-B(1)/C-71] showing cannabidivarin disilylated (I), cannabidiol disilylated (III), (−)-Δ⁹-trans-tetrahydrocannabivarin (IV), cannabichromene (VI), cannabicyclol (X), (−)-Δ⁹-trans-tetrahydrocannabinol (XI), hexahydrocannabinol (C_9 methyl equatorial) (XII), cannabinol (XV), (−)-Δ⁹-trans-tetrahydrocannabinolic acid B disilylated ester–ether (XVIII), and the internal standard (XIX). (−)-Δ⁹-trans-Tetrahydrocannabinolic acid B (XVIII), not as readily decarboxylated as is acid A, is observed here; it is under acid A (XVII) in Fig. 4.*

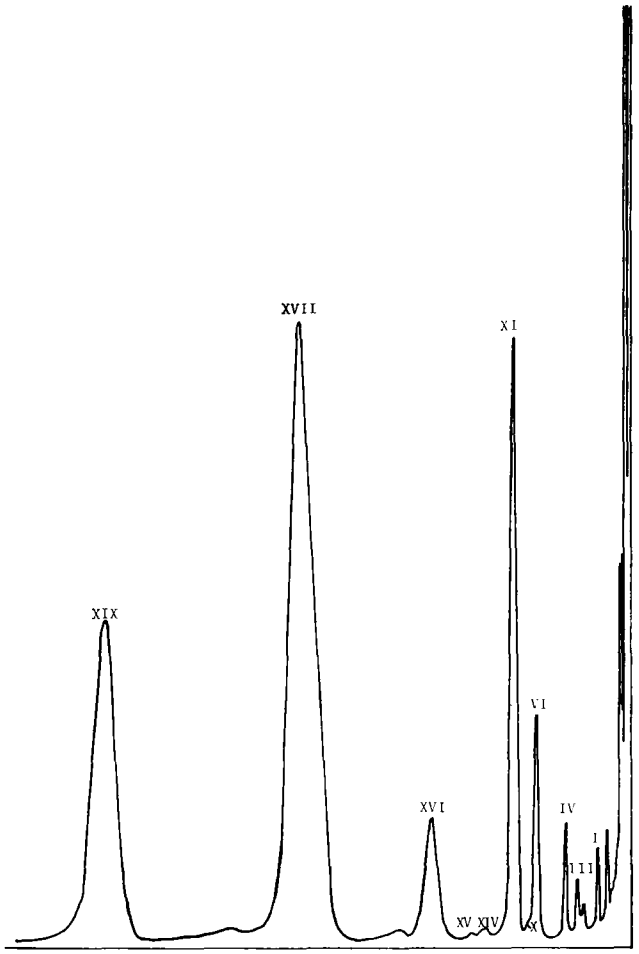


Figure 4—*Chromatogram of silylated female Afghanistan* Cannabis [*AF-B(1)/C-71*] *showing cannabidivarin disilylated* (*I*), *cannabidiol disilylated* (*III*), (−)-Δ^9-trans-*tetrahydrocannabivarin* (*IV*), *cannabichromene* (*VI*), *cannabicyclol* (*X*), (−)-Δ^9-trans-*tetrahydrocannabinol* (*XI*), *cannabidiolic acid* (*XIV*), *cannabinol* (*XV*), (−)-Δ^9-trans-*tetrahydrocannabinolic acid A* (*XVII*), *and androst-4-ene-3,17-dione* (*XIX*).

plained phenomenon: certain plant samples containing a high cannabichromene content show more cannabicyclol than others.

For example, the plant material in Fig. 3, AF-B(1)/C-71, contains only a relatively small amount of cannabicyclol, even after being heated to such extremes that hexahydrocannabinol was formed. The plant material in Fig. 5, IN-F, contains more cannabicyclol. When a normalization analysis was run on AF-B(1)/C-71 and IN-F, the percent of cannabichromene in regard to all cannabinoids present was 12.87 and 18.48%, respectively. Subsequently, cannabicyclol for AF-B(1)/C-71 and IN-F was 4.28 and 2.92%, respectively. This phenomenon was observed in many other variants.

Cyclization of cannabichromene in the injection port of the gas chromatograph is known (7). Silylation prevents any observable cyclization since a TLC-purified sample of cannabichromene can be silylated and analyzed without any cannabicyclol being formed. Thus, it seems that other undefined physical parameters must be specific in *Cannabis* of different variants. Since *C. sativa* L. from different geographical locations (13, 14) exhibits pronounced chemical and morphological differences, it is possible for many other factors to influence this observed phenomenon.

Cannabicyclol and (−)-Δ^9-*trans*-tetrahydrocannabivarin are not separated in routine underivatized analyses (Fig. 2) but can be separated using the silyl method (Figs. 4 and 5). And, as previously reported (4–6), acid derivatives of cannabinoids can be semiquantitated using a silyl procedure. In these laboratories, using the procedure described, it is possible to analyze routinely for (−)-Δ^9-*trans*-tetrahydrocannabivarinic acid and (−)-Δ^9-*trans*-tetrahydrocannabinolic acids A and B. Acid B has only been isolated and

Table I—Relative Retention Times of Trimethylsilyl Derivatives of Cannabinoids[a]

Derivative	Relative Retention Time
Cannabidivarin disilylated	0.07
Cannabidivarin monosilylated	0.11
Cannabidiol disilylated	0.11
(−)-Δ^9-*trans*-Tetrahydrocannabivarin	0.12
Hexahydrocannabinol (C_9 methyl axial)	0.16
Cannabichromene	0.17
Cannabidiol monosilylated	0.18
Cannabidivarinic acid	0.18
(−)-Δ^8-*trans*-Tetrahydrocannabinol	0.20
Cannabicyclol	0.21
(−)-Δ^9-*trans*-Tetrahydrocannabinol	0.22
Hexahydrocannabinol (C_9 methyl equatorial)	0.22
Cannabigerol disilylated	0.26
Cannabidiolic acid	0.28
Cannabinol	0.31
(−)-Δ^9-*trans*-Tetrahydrocannabivarinic acid	0.38
(−)-Δ^9-*trans*-Tetrahydrocannabinolic acid A	0.64
(−)-Δ^9-*trans*-Tetrahydrocannabinolic acid B	0.68
Androst-4-ene-3,17-dione	1.00

[a] Relative retention times were obtained from synthetic cannabinoids supplied by the National Institute on Drug Abuse. Cannabinoids not available were isolated from *Cannabis sativa* L. Peak assignments were based on data from synthetic and natural cannabinoids. A GLC–mass spectrometry system was used to confirm assignment of peaks; see Refs. 9, 10, 13, and 14 for methods and additional data.

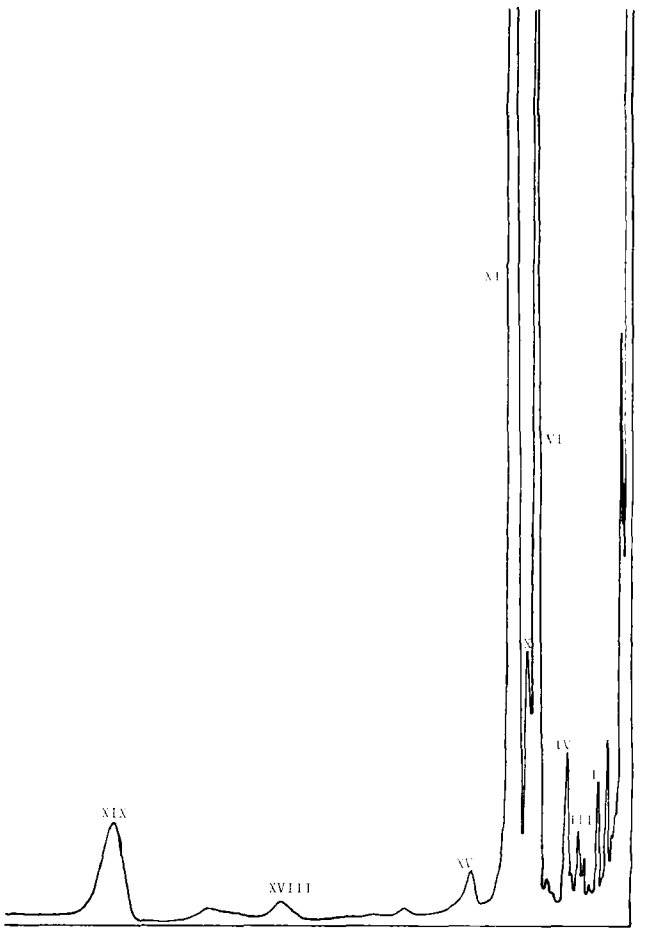


Figure 5—*Gas chromatogram of an Indian variant of* Cannabis (*IN-F*). *This decarboxylated and silylated sample shows cannabidivarin disilylated* (*I*), *cannabidiol disilylated* (*III*), (−)-Δ^9-trans-*tetrahydrocannabivarin* (*IV*), *cannabichromene* (*VI*), *cannabicyclol* (*X*), (−)-Δ^9-trans-*tetrahydrocannabinol* (*XI*), *cannabinol* (*XV*), (−)-Δ^9-trans-*tetrahydrocannabinolic acid B* (*XVIII*), *and androst-4-ene-3,17-dione* (*XIX*).

Table II—Relative Retention Times of Underivatized Cannabinoids and Other Components Found in *Cannabis*

Component	Relative Retention Time
Olivetol	0.04
Cannabidivarin	0.18
Tetrahydrocannabivarin	0.26
Cannabicyclol	0.26
Cannabichromene	0.34
Cannabivarin	0.34
Cannabidiol	0.34
Hexahydrocannabinol	0.37
Cannabigerol monomethyl ether	0.38
$\Delta^{9,11}$-Tetrahydrocannabinol (exocyclic)	0.41
(−)-Δ^{8}-*trans*-Tetrahydrocannabinol	0.44
Cannabielsoin	0.48
(−)-Δ^{9}-*trans*-Tetrahydrocannabinol	0.49
Cannabigerol	0.57
Cannabinol	0.63
C_{29}-Hydrocarbon	0.67
Androst-4-ene-3,17-dione (Δ^{4}-dione)	1.00

identified once from hashish (15). Recent work in these laboratories, however, has resulted in acid B being isolated from fresh *Cannabis* (16). These findings will be the subject of a forthcoming report. Additionally, it is possible to analyze for cannabidivarinic acid and cannabidiolic acid. Cannabichromene acid is not consistently observed in *Cannabis*, even when a large content of cannabichromene is confirmed by TLC, mass spectroscopy, and the silyl GLC method.

In these laboratories, quantitation of cannabinoids using the trimethylsilyl method was slightly less accurate than the underivatized procedure, since response factors of silyl cannabinoids are difficult to obtain and may vary greatly. Thus, these authors prefer to use "semiquantitative" when referring to data obtained by the silyl method. Underivatized cannabinoids in these laboratories, using good analytical techniques and proper response factors (see Table III), can be quantitated with better than 98% accuracy. Accuracy using the silyl procedure is from 93 to 95%[5].

Although the silyl method used may be semiquantitative, it affords better results than methods employing no internal standard or an external standard. Thus, for laboratories wishing to separate cannabinoids not routinely separated by conventional GLC, the silyl method is sufficient unless accuracy greater than 95% is required.

Table I shows the relative retention times of all cannabinoids routinely analyzed by these laboratories as their trimethylsilyl ethers or ester–ethers; Table II gives the relative retention times of cannabinoids as their free phenols. All relative retention times are based on the same internal standard and chromatographic conditions (7). By using these retention times, it is possible, in most cases, to identify most cannabinoids. For best results, an all-glass system is preferred. Stainless steel columns consistently give results 20–30% below the glass system in these laboratories. Presilylated stainless steel columns reduce this apparent adsorption to approximately 15%.

De Zeeuw *et al.* (17) showed that hydrocarbons are present in *Cannabis* and can possibly distort analytical data. Extraction of *Cannabis* with chloroform to date has not indicated the presence of significant amounts of hydrocarbons when the extract is silylated. A major source of error, however, in preparing silyl derivatives is exposure of the reaction mixture to rubber septums, which produces many peaks that appear to be cannabinoids. This disadvantage can be negated by proper laboratory procedures.

[5] The accuracy was obtained after correlating data from over 6500 samples analyzed in these laboratories during the calendar year of 1973. These samples included synthetic, herbal, hash, cookies, and other samples prepared by outside sources. Data obtained from silylated cannabinoids were consistently 3–5% lower than from unsilylated cannabinoids. Additionally, the 3–5% range was observed whether peaks were calculated by peak height times width at half-height or by computer. Response factors were used in all analyses.

Table III—Relative Response Factors of Silylated and Unsilylated Synthetic Cannabinoids[a]

Cannabinoid	Unsilylated	Silylated
Cannabidiol	1.07	0.68
(−)-Δ^{9}-*trans*-Tetrahydrocannabinol	1.05	0.80
Cannabinol	0.97	0.72
Androst-4-ene-3,17-dione (internal standard)	1.00	1.00

[a] Volume injected was 0.25 μl. Response factors vary with each instrument in these laboratories.

SUMMARY

The silyl procedure described can be used as a routine method for quantitating cannabinoids not quantitated by other means. Accuracy is between 93 and 95%. Cannabinoids, whether free phenols or the carboxylic acid derivatives, can be quantitated by this method.

Data generated by this method indicate that cannabichromene, previously thought to be a minor component in *Cannabis*, is more abundant than cannabidiol in many variants.

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