SEPARATION OF ACID AND NEUTRAL CANNABINOIDS IN CANNABIS SATIVA L. USING HPLC*

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I. INTRODUCTION

Chemobotanical studies in our laboratory on the site of cannabinoid synthesis in the Cannabis plant required a method for an accurate assessment of the cannabinoid profile. Previous methods primarily involved use of gas-liquid chromatography (GLC) (1,2). Because the majority of cannabinoids are present in the plant are found in the acid form, they cannot be directly detected by GLC due to thermal decarboxylation. High-performance liquid chromatography (HPLC) is able to detect directly both acidic and neutral cannabinoids. However, methods published to date were found to be inadequate for our use (3,4). The abundance of compounds in samples extraced from plant material made their analyses complex, and necessiated development of a more definitive method for determining cannabinoid profiles in plant samples.

II. MATERIALS AND METHODS

A. Plant Material

Compound leaves, with a 7.5 cm center leaflet, were collected for analysis from vegetative plants of a clone of a

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drug strain (152) routinely used in our investigations (5-8). The clone provides a source of genetically stable material on a year-round basis, and is grown in a greenhouse heated and cooled seasonally as required for the Indiana climate. Plants are maintained on a 20 hr day to insure vegetative growth.

B. Cannabinoid Extraction

Fresh leaf samples were extracted within 1 hr of being collected. After leaves had been extracted, they were dried in a 60°C oven for 24 hr and then weighed to determine dry weight. To extract cannabinoids, fresh leaf samples were placed in glass test tubes and approximately 1 ml "ChromAR" grade chloroform (Mallinckrodt) was added to each sample. After 1 hr, the extract was removed and filtered. The extraction procedure was repeated twice for a total of 3 times, and the combined filtrates for each sample were evaporated under a gentle stream of nitrogen. All steps were done at 4°C. Each sample was then resuspended in 100% ethanol containing two internal standards (eicosane and di-n-octyl phthalate), each at a concentration of 0.25 mg/ml.

C. High-performance Liquid Chromatography

Analyses were performed on a Hewlett-Packard 1084B HPLC equipped with a single-wavelength UV detector set at 254 nm. A reverse-phase Altex column (Ultrasil-octyl, 10 micron; 25 cm X 4.6mm ID) was used. The eluting solvents were acetonitrile (Burdick & Jackson, UV grade) and water. Water utilized was deionized, processed through a Lobar RP-8 size B (EM Reagents) column (9), and then filtered through a Gelman GA-6, 0.45 um filter on a Millipore all-glass filtering system. Samples were filtered with BAS Microfilters equipped with 1 mcm regenerated cellulose filters (Bioanalytical Systems, Inc.). For cannabinoid analysis, the instrument was programmed to pump a gradient starting with 25% acetonitrile at time 0 and reaching 85% acetonitrile at 35 min. Flow rate was 2 ml/min and oven temperature was 40°C. Sample size was generally 20 mcl.

D. Gas-liquid Chromatography

Analyses were performed on a Hewlett-Packard 5710A gas chromatograph equipped with a hydrogen flame ionization detector and a Hewlett-Packard 3380A integrator. Cannabinoid standards provided by NIDA were chromatographed and the integrator calibrated the columns using the internal standard method.

Glass columns (2 mm ID X 2.43 m) were cleaned, treated with 8% dimethyldichlorosilane in toluene, dried, and packed with 3% OV-1 or 3% OV-17 on 100/120 mesh Supelcoport. The inlet and detector temperatures were 250°C and 350°C, respectively. Nitrogen was used as the carrier gas with a flow rate of 20 ml/min. Samples injected consisted of 1 lambda aliquots and were analyzed on both the OV-1 and OV-17 columns. For the OV-1 column, a program of 200-240°C at 2°C/min with an additional 8 min isothermal period at 240°C was used. For the OV-17 column, the program was isothermal at 260°C for 15 min.

E. Heated Samples

Following analysis of fresh plant extracts by HPLC and GLC, samples were evaporated and heated, essentially using the method of Kanter et al. (10). Dry samples were placed in an oven at 200°C for 3 min. Samples were then removed, allowed to cool to room temperature and resuspended in ethanol to their original volume.

III. RESULTS AND DISCUSSION

A. HPLC Program

Initially, the method of Wheals and Smith was used to determine cannabinoid profiles in plant samples (3). Their HPLC method involved the use of methanol and 0.02 N sulfuric acid (80:20) at a flow rate of 2 ml/min. While this was quite adequate for separating cannabinoid standards (all neutral), the presence of additional extracted compounds made plant material more difficult to chromatograph. The Wheals and Smith isocratic method provided separation of cannabinoids in approximately 5 min, but plant samples contained a number of non-cannabinoid compounds that also chromatographed in the same region as the cannabinoids. Since it is desirable for our studies to analyze samples efficiently with little, if any, pretreatment, we therefore investigated other HPLC programs. We found that use of a gradient solvent system over a longer period of time could efficiently separate neutral cannabinoid standards (Fig. 1). At the same time, the gradient program allowed other plant compounds to chromatograph at retention times different from those of the cannabinoids, thus effectively separating cannabinoids from other plant compounds (Fig. 2). Therefore, our current method uses water and acetonitrile, beginning with 25% acetonitrile and progressing to 85% acetonitrile by 35 min.

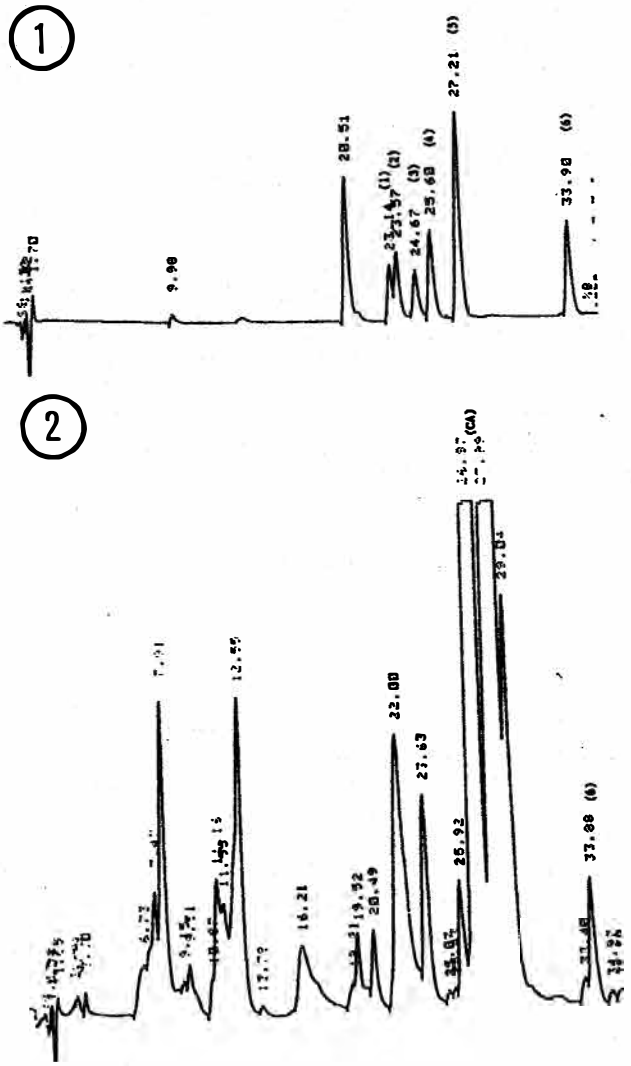


FIGURE 1. Chromatogram of a mixture of neutral cannabinoid standards. Water solvent at pH 2.7. Peaks: (1) CBD; (2) CBG; (3) CBN; (4) THC; (5) CBC; and (6) di-n-octyl phthalate (internal standard, IS). The peak at RT 20.51 is a second IS.

FIGURE2. Chromatogram of plant extract (clone 152). Water solvent at pH 2.7. (CA) cannabinoid acids; (6) IS. No neutral cannabinoids were detected.

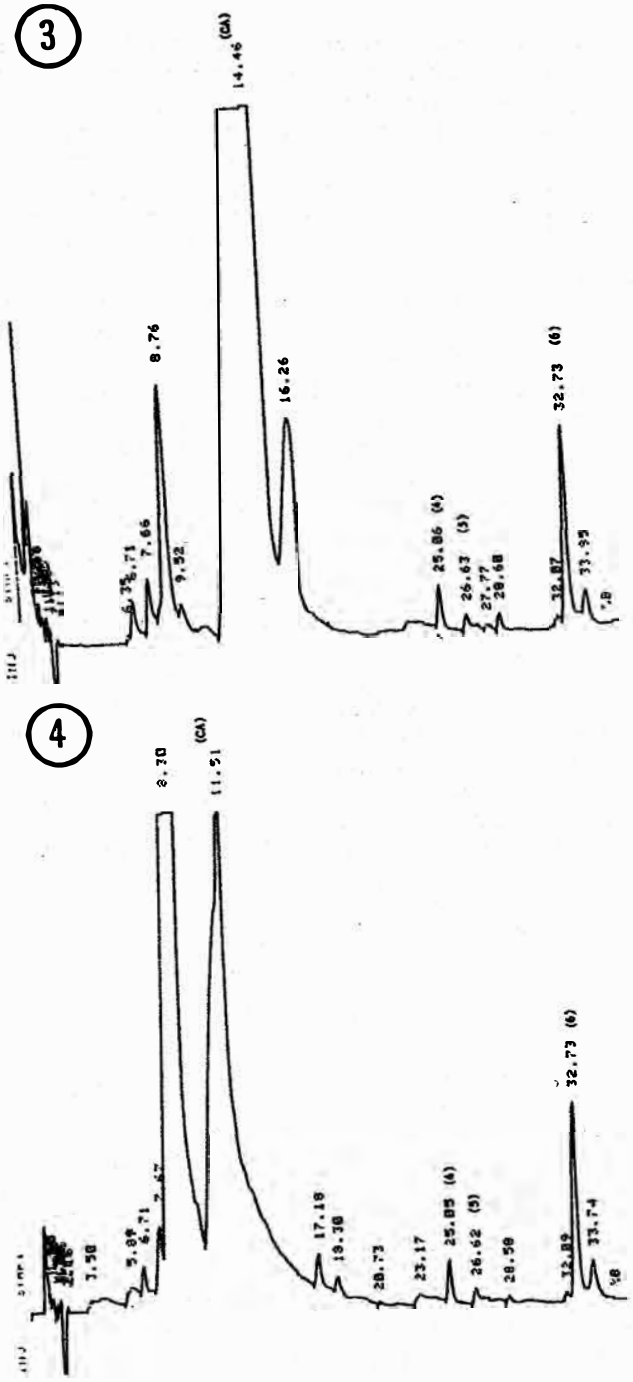
B. Solvent pH

The cannabinoids were chromatographed by the solvent gradient at unique retention times as compared to other plant components. Since acidic cannabinoids are the predominate form in living Cannabis plants, the separation of acid from neutral cannabinoids in plant material presented an additional problem. Large peaks of acidic cannabinoids occurred in the retention time region of the neutral forms, as determined from standards, and engulfed the small peaks of any neutral cannabinoids present (Fig. 2). Previous HPLC studies on marijuana had not encountered this problem since normal drying procedures had decarboxylated many of the acidic cannabinoids to the neutral form.

In order to detect and distinguish between acid and neutral cannabinoids, we changed the pH of the water solvent. It was found that an increase in water solvent pH resulted in a decrease in the retention time of cannabinoid acids (Figs. 3,4). To confirm the identity of the more rapidly eluted peaks as cannabinoid acids, the large peak area was collected from the HPLC using a fraction collector. Compounds present in the collected material were analyzed both by GLC and gas chromatography-mass spectrometry (GC/MS) and the presence of cannabinoids was confirmed [11]. Cannabinoid acids cannot be directly detected by GLC or GC/MS due to thermal decarboxylation. It was assumed that the large moveable peaks were indeed acidic cannabinoids since cannabinoids were also confirmed in peaks at retention times corresponding to those found for neutral cannabinoid standards. Neutral cannabinoids were found to have stable retention times with regard to changes in solvent pH (Figs. 3,4). The other unknown peaks in the sample, also stable with regard to changes in solvent pH, were collected as separate fractions and found to contain no cannabinoids.

C. Heated Samples

Since cannabinoid acids decarboxylate upon being heated, this property was used to evaluate further the components of the peak determined to contain cannabinoid acids. Prior to heating, plant samples were anlayzed and the large cannabinoid acid peak was present (Fig. 5), while neutral cannabinoids were not detected. Acid cannabinoids represent the form primarily present in the living plant. The presence of neutral cannabinoids in plant samples analyzed by HPLC was found to reflect the method of sample preparation (12). A sample drying temperature of 60°C or prolonged drying at room temperature prior to extraction can result in decarboxylation of


3
14.46 (BA)
8.76
16.26
32.73 (6)
25.86 (4)
26.63 (5)
27.77
28.68
6.71
7.66
9.52
4
8.30
11.51 (BA)
17.18
19.38
20.73
23.17
25.85 (4)
26.62 (5)
28.50
32.73 (6)
33.74
6.71
7.58

acid forms resulting in detectable levels of neutral cannabinoids in HPLC analyses (Figs. 3,4). After analyzing the unheated plant extract, it was then heated and reanalyzed. The large cannabinoid acid peak disappeared and significant amount of neutral cannabinoids were detected (Fig. 6). The fact that the large peaks previously determined to be cannabinoid acids disappeared after sample heating and neutral cannabinoid peaks appeared provides additional confirmation that the peaks were indeed cannabinoid acids. It also indicates that heating plant extracts to transform acid to neutral forms can be used as a procedure to quantify cannabinoids.

D. GLC Analyses

Plant samples were analyzed both by GLC and HPLC prior to heating the sample, and then again after the sample had been heated. Table I shows the quantities of cannabinoids detected. Using GLC, the amount of neutral cannabinoids detected in plant extracts and heated extracts was essentially the same. For GLC analyses, we routinely chromatograph samples on each of 2 columns. The OV-1 column separates cannabidiol (CBD) and cannabichromene (CBC) relatively well, provides excellent separation of delta-8-tetrahydrocannabinol (8-THC) and delta-9-THC (9-THC) and co-chromatographs cannabigerol (CBG) and cannabinol (CBN). The OV-17 column separates CBG distinctly from CBN, provides excellent separation of 8-THC and 9-THC, and separates CBD and CBC rather poorly. HPLC analysis in parallel with GLC reconfirms the presence of the above compounds in any given sample since CBD, CBN, and CBC are well separated. CBG chromatographs close to CBD and 8-THC cochromatographs with 9-THC. Since neutral cannabinoids may not be present in the living plant, plant extracts must be heated in order to quantitate the cannabinoid acids as decarboxylated neutral forms. While quantities compare well to those found for GLC analyses of the same sample (Table I), it is still unclear whether each cannabinoid acid, when decarboxylated by thermal treatment, is actually converted to its neutral form.

FIGURE 3. Chromatogram of plant extract (clone 152) dried at 60°C for 24 hr prior to extraction. Water solvent at pH 4.0. (CA) cannabinoid acids; (4) THC; (5) CBC; (6) IS.

FIGURE 4. Same sample analyzed in Fig. 3, but water solvent was at pH 6.0.

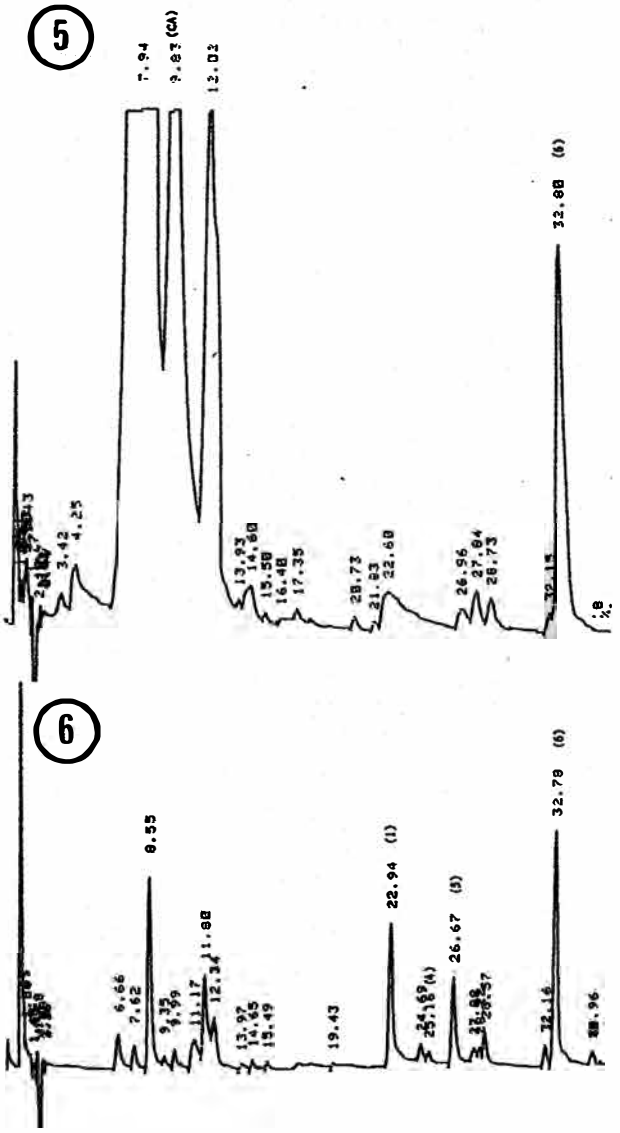


FIGURE 5. Chromatogram of plant extract (clone 152). Water solvent at pH 5.0. (CA) cannabinoid acids; (6) IS.

FIGURE 6. Same sample analyzed in Fig. 5, except that it was heated. (1) CBD; (4) THC; (5) CBC; (6) IS.

IV. COMMENTS AND CONCLUSIONS

Previously, accurate cannabinoid profiles of plant extracts only could be obtained by heating (10,13) or derivatization (2). The HPLC method we have developed provides efficient evaluaton of a plant sample with no pretreatment other than the actual extraction. We can definitively determine the presence of both acid and neutral cannabinoids, although individual cannabinoid acids have yet to be identified in our program. Also, while neutral cannabinoids are easily quantitated both GLC and HPLC, quantitation is not yet applicable with acidic cannabinoids. If the assumption is made that each cannabinoid acid decarboxylates directly to its neutral form on a one to one basis, then heating plant extracts would provide an easy method for quantification. However, the correctness of this assumption has yet to be demonstrated.

The ability to change cannabinoid acid retention times by adjusting solvent pH while neutral cannabinoid peaks remain stable has additional merits. For one, the peaks can be manipulated so as not to interfere with other compounds being chromatographed. Also, by rechromatographing the same sample but using a different solvent pH each time, cannabinoid acid peaks can be moved to reveal previously hidden peaks. In addition, the fact that the peak moves provides an indication of its identity as an acid cannabinoid. All other compounds found so far in chromatograms of Cannabis extracts are stable with changes in solvent pH.

TABLE I. GLC and HPLC Analyses of Cannabinoids Present in Fresh and Subsequently Heated Plant Extracts

		Total Cannabinoids (mg/100 mg dw)	
		Neutral	Acid
Fresh extract:	GLC	0.92	
	HPLC	ND[b]	
Heated extract:	GLC	0.88	
	HPLC	0.82	ND

[a]Cannabinoid acids are not detected by GLC.
[b]No neutral cannabinoids detected.
[c]Cannabinoid acids detected.

In summary, the acidic cannabinoids can be identified and isolated from plant materials by HPLC, as verified by GLC and GC-MS. A solvent gradient program, using the water solvent at pH 5.0, is now in routine use in our laboratory. The mobility of acid cannabinoids in contrast to neutral forms and other compounds was used to advantage to separate acid cannabinoids. Further studies are needed to identify individual cannabinoid acids, as well as to determine definitively whether cannabinoid acids are the only form present in living plants.

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