Journal of Chromatography, 253 (1982) 295–303
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,306

Note

Simple high-performance liquid chromatographic method for separating acidic and neutral cannabinoids in *Cannabis sativa* L.

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(Received August 11th, 1982)

Cannabinoids are present in both acidic and neutral form in the *Cannabis* plant and dried plant material. While gas–liquid chromatography (GLC) has been widely used to analyze cannabinoids, cannabinoic acids injected into the gas–liquid chromatograph are converted to the neutral form[1]. Since it is often of chemobotanic, pharmacological, or forensic interest to have detailed information regarding cannabinoid content of plant material[2], several methods have been developed which allow analysis of both acidic and neutral cannabinoids present in a sample. Trimethylsilyl derivatives have been shown to provide a "semiquantitative" means of analyzing cannabinoic acids using GLC[3]. Simply heating a sample in order to decarboxylate cannabinoic acids to produce the neutral form has been reported for GLC[4], and revised for use in high-performance liquid chromatography (HPLC)[5]. An HPLC method alone has also been developed which is capable of separating acidic and neutral cannabinoids[2,6]. However, when analyzing cannabinoids in *Cannabis* plant material and particularly fresh plants, acidic cannabinoids are generally the major form present[1,4]. Using HPLC methods developed so far, without any pretreatment of the sample, the large peak sizes of acidic cannabinoids engulf the smaller peaks of the neutral form. Therefore, it was necessary for our studies to develop an HPLC program, as we report here, that would adequately separate the two forms of cannabinoids.

EXPERIMENTAL

Plant material

Compound leaves, with a 7.5-cm center leaflet, were collected for analysis from vegetative plants of a clone of a drug strain (152) routinely used in our investigations[7–10]. The clone provides a source of genetically stable material on a year-round basis, and is grown in a greenhouse heated or cooled seasonally as required for the Indiana climate. Plants are maintained on a 20-h day to insure vegetative growth.

Sample extraction

All fresh leaf samples were extracted within 1 h of being collected. After samples were weighed, they were placed in glass test tubes and approximately 1 ml of ChromAR grade chloroform (Mallinckrodt) was added to each sample. After 1 h,

0021-9673/82/0000–0000/$02.75

chloroform was removed and filtered. The extraction procedure was repeated twice for a total of three 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 2 internal standards (eicosane and di-*n*-octyl phthalate), each at a concentration of 0.25 mg/ml.

Gas–liquid chromatography

Analyses were performed on a Hewlett-Packard 5710A 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 column using the internal standard method. Glass columns (2.43 m × 2 mm I.D.) were cleaned, treated with 5% dimethyldichlorosilane in toluene, dried, and packed with 3% OV-1 on 80–100 mesh Supelcoport. The inlet and detector temperatures were 250 and 300°C, respectively. A 1-μl aliquot of sample was injected and analyzed with a program of 200–240°C at 2 C/min with an additional 8 min isothermal period at 240°C. Nitrogen was used as the carrier gas with a flow-rate of 20 ml/min.

High-performance liquid chromatography

Analyses were performed on a Hewlett-Packard 1084B liquid chromatograph equipped with a single-wavelength UV detector set at 254 nm. A reversed-phase Altex column (Ultrasil-octyl, 10 μm; 25 cm × 4.6 mm I.D.) 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[11], and then filtered through a Gelman GA-6 0.45-μm filter on a Millipore all-glass filtering system. Samples were filtered with BAS Microfilters equipped with 1-μm regenerated cellulose filters (Bioanalytical Systems). For cannabinoid analysis, the instrument was programmed to pump a gradient starting with 25% acetonitrile at time zero and reaching 85% acetonitrile at 36 min. Flow-rate was 2 ml/min and oven temperature was 40°C. Sample size was generally 20 μl.

Preparation of HPLC peaks for analysis

Peaks appearing during an HPLC program that were to be analyzed further were collected using the HPLC fraction collector controlled by the integrator. A total of ten to twenty repeated injections of the sample, depending on peak sizes, were performed in order to collect an adequate quantity of peaks being analyzed. Each peak sample was freeze-dried and resuspended in 100 μl ethanol, containing internal standards, for analysis by GLC and GLC–mass spectrometry (MS).

Gas chromatography–mass spectrometry

The instrument used for GLC–MS was a Hewlett-Packard 5990A. A glass GLC column (1.5 m × 2 mm I.D.) was packed with 3% OV-25. Injection port temperature was 250°C and column temperature was 180°C, programmed to 210°C at 1°C/min during analysis. Helium (carrier gas) flow-rate was 30 ml/min. Ion source temperature was 230°C, and electron energy was 70 eV. During analysis, scan speed was 380 a.m.u./sec and mass range 20–400 a.m.u.

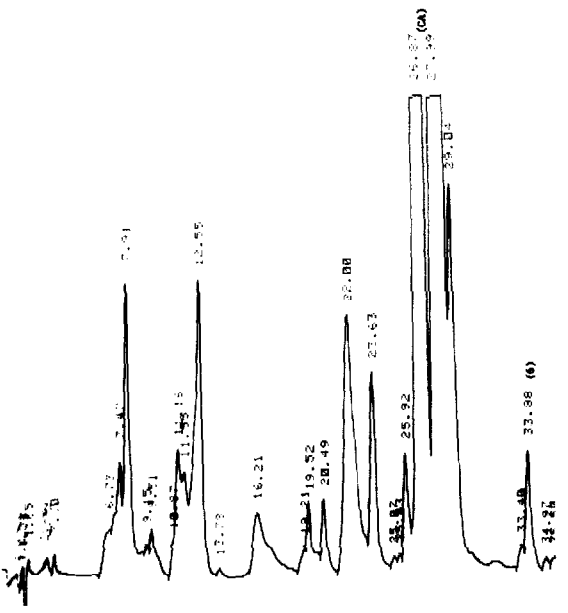

Fig. 1. Chromatograph of fresh plant extract (clone 152). Water solvent at pH 2.7. Peaks: CA = cannabinoic acids; 6 = internal standard. No neutral cannabinoids are apparent. All other peaks are unknowns.

Heated samples

Following analysis of fresh plant extracts by HPLC, samples were evaporated and heated, essentially using the method of Kanter *et al.*[5]. Dried 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.

RESULTS AND DISCUSSION

Solvent pH

For ongoing chemobotanical studies of *Cannabis* in our laboratories, analysis of cannabinoids by HPLC has become desirable (Figs. 1–3). Initially, the method of Wheals and Smith[12] was modified to provide good separation of our mixture of cannabinoid standards (Fig. 3). Modifications involved the use of acetonitrile rather than methanol, sulfuric acid at 0.001 *N* (pH 2.7) instead of 0.02 *N*, and a programmed gradient run. While these conditions separated the neutral cannabinoid standards adequately, the cannabinoid composition of plant extracts was found to be more complex. When extractions from fresh, cloned plant material were analyzed by HPLC for cannabinoids, large peaks appeared in the retention time area where cannabinoid standards chromatographed (Fig. 1). Based on relative retention times published by Smith[6], it was assumed that these large peaks were acidic cannabinoids. When smaller amounts of sample were injected, the large peaks were reduced somewhat, but sample size was probably too small to allow any neutral cannabinoids present to be detected (Fig. 2).

Because acidic cannabinoids are generally the major form of cannabinoids

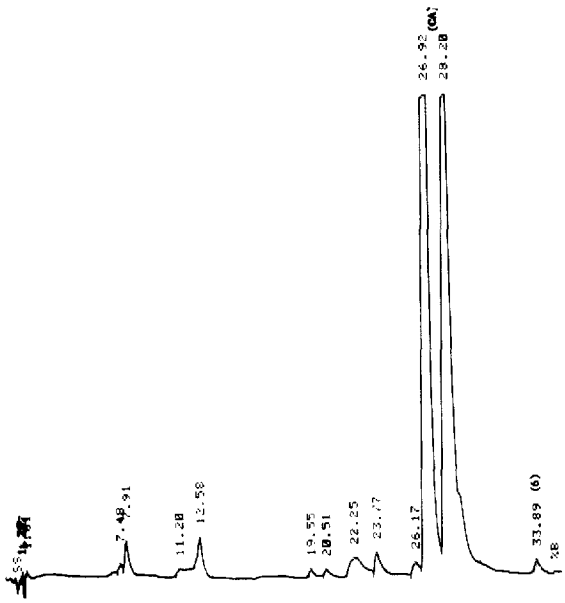

Fig. 2. Same sample analyzed in Fig. 1, but diluted 10 ×. Water solvent at pH 2.7.

present in fresh plant material, it was probable that the neutral cannabinoids were being engulfed by the large peaks of the acidic cannabinoids. It was necessary, therefore, to have a means for separating more completely the acidic and neutral cannabinoids; and it was contemplated that a change in solvent pH might be effective. Since the initial runs were done with the water solvent acidified to pH 2.7, plant samples were then run with the water solvent at pH 4.0 (Fig. 4) and subsequently at pH 6.0 (Fig. 5). The results showed the large peaks, later verified as acidic cannabinoids, to chromatograph at progressively earlier retention times as the pH increased. Cannabinoid standards (all neutral) were run as a control at pH 6.0 (Fig. 6), and found to chromatograph essentially at the same retention time as the standards

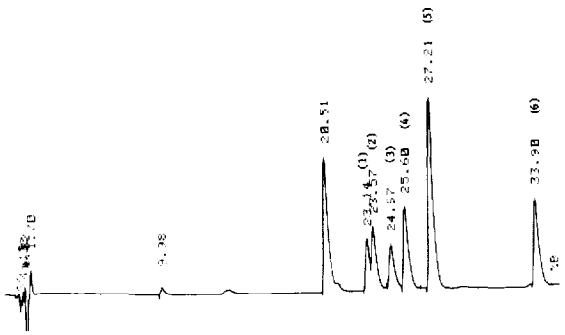

Fig. 3. Chromatograph of a mixture of neutral cannabinoid standards. Water solvent at pH 2.7. Peaks: 1 = cannabidiol (CBD); 2 = cannabigerol (CBG); 3 = cannabinol (CBN); 4 = Δ^9-tetrahydrocannabinol (Δ^9-THC); 5 = cannabichromene (CBC); 6 = internal standard (IS) (di-*n*-octyl phthalate). The peak at retention time 20.51 is a second internal standard.

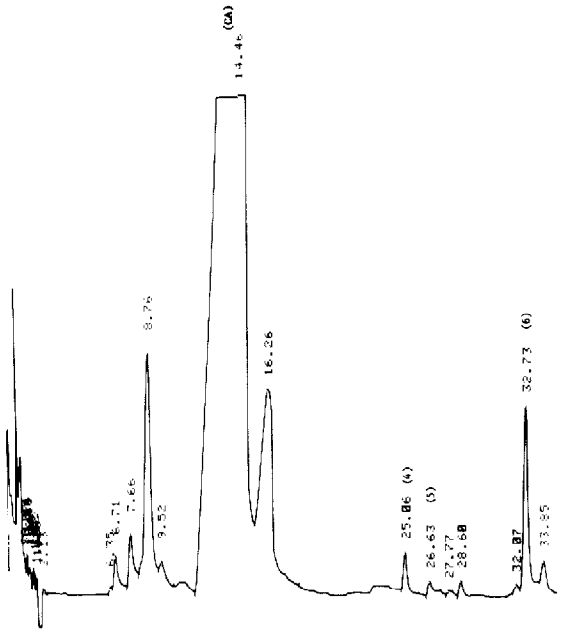

Fig. 4. Chromatograph of fresh plant extract (clone 152). Water solvent at pH 4.0. Peaks: CA = cannabinoic acids; 4 = Δ^9-THC; 5 = CBC; 6 = IS.

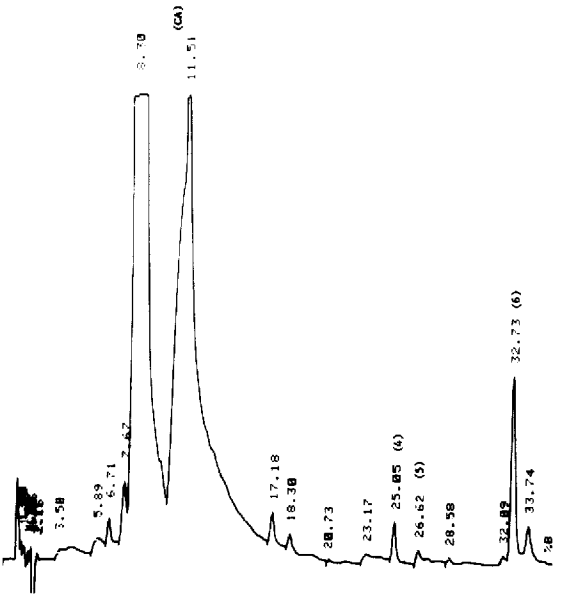

Fig. 5. Same sample analyzed in Fig. 4, but water solvent is at pH 6.0.

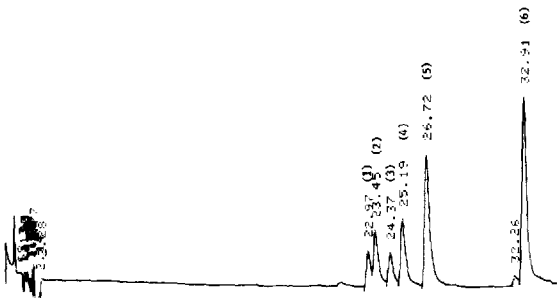

Fig. 6. Chromatograph of a mixture of neutral cannabinoid standards. Water solvent at pH 6.0. Peaks: 1 = CBD; 2 = CBG; 3 = CBN; 4 = Δ^9-THC; 5 = CBC; 6 = IS.

analyzed using a solvent pH of 2.7. The slight difference seen in retention times for the standards is probably not a factor of solvent pH, but instead is normal fluctuation of the column that occurs throughout the day and from day to day since the HPLC is used for analyzing other compounds in addition to cannabinoids. In a comparison of Fig. 1 with Figs. 4 and 5, the observed quantitative difference is due to different collections of the cloned plant material. However, with these differences aside, it is apparent that an increase in the pH of the water solvent decreases the retention time of peaks identified as acidic cannabinoids.

Peak identification

The identity of the neutral cannabinoids present in plant samples was presumed by comparison with retention times of cannabinoid standards. To confirm peak identity, each peak was collected and subsequently analyzed by GLC. Cannabidiol (CBD), Δ^9-tetrahydrocannabinol (Δ^9-THC), and cannabichromene (CBC) were confirmed for these respective peaks according to our initial presumptions. There was an indication that cannabigerol (CBG) also was present, but the OV-1 column used for GLC analysis does not separate CBG from cannabinol (CBN) so the

TABLE I

GLC AND HPLC ANALYSIS OF CANNABINOIDS PRESENT IN FRESH AND SUBSEQUENTLY HEATED PLANT EXTRACTS

ND = Not detected.

Extract	*Method of analysis*	*Total Cannabinoids (mg/100 mg dry weight)*	
		Neutral	*Acid*
Fresh	GLC	0.92	–*
	HPLC	ND**	CA***
Heated	GLC	0.88	–
	HPLC	0.82	ND

* Cannabinoic acids are not detected by GLC.

** No neutral cannabinoids detected.

*** Cannabinoic acids detected.

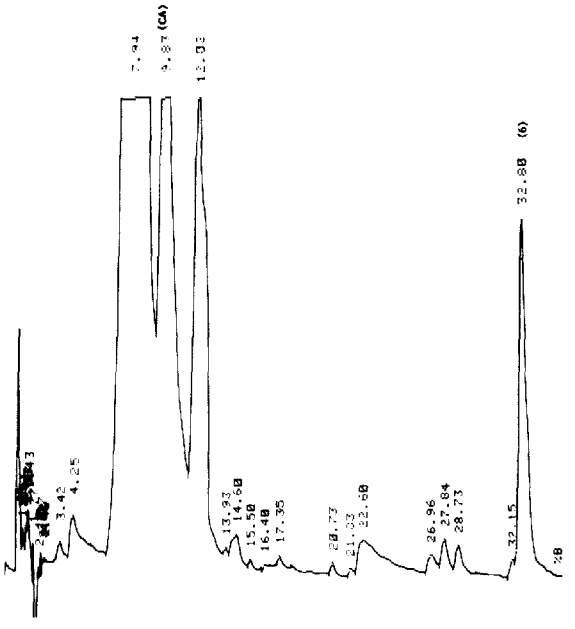

Fig. 7. Chromatograph of fresh plant extract (clone 152). Water solvent at pH 5.0. Peaks: CA = cannabinoic acids; 6 = IS.

identity of CBG could not be absolutely confirmed. We know from related, more recent analyses, however, that both CBG and CBN may be present in this clone[13].

For acidic cannabinoids, the large peak was collected from the HPLC and subsequently analyzed by GLC. Using an OV-1 column, CBC, Δ^9-THC and CBN were identified as being present. For more specific confirmation, the peak sample was also analyzed using MS. The results indicated the presence of Δ^9-THC, and probably CBC and CBN. The peak chromatographing on GLC–MS with a retention time comparable to Δ^9-THC had a mass spectrum of 314 (86% relative intensity), 299 (100), 271 (49), 258 (33), 231 (84), which confirmed Δ^9-THC. Two additional peaks

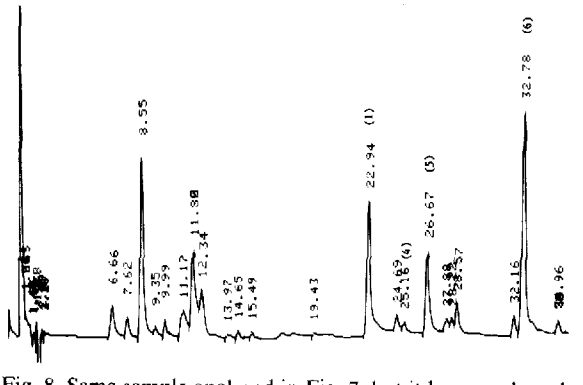

Fig. 8. Same sample analyzed in Fig. 7, but it has now been heated. Water solvent at pH 5.0. Peaks: 1 = CBD; 4 = Δ^9-THC; 5 = CBC; 6 = IS.

chromatographed by GLC–MS were tentatively identified as CBC and CBN; however, peak size was too small in both cases to provide a mass spectrum sufficiently complete for good identification of the compounds. There was no indication, using either GLC or GLC–MS, that CBD and CBG were present; however, it is possible that they were present (as cannabinoic acids) in the plant extract but were not included in the fraction collected from the HPLC.

An additional step was taken to determine that acidic cannabinoids were present in the large HPLC peak, since GLC and GLC–MS thermally convert acidic cannabinoids to the neutral forms. Portions of the samples extracted from fresh plant material were analyzed for cannabinoids by both GLC and HPLC (Table I), while the remainder was heated and subsequently analyzed both by HPLC and GLC (Table I). Using HPLC, the large acidic cannabinoid peak is present in the fresh plant extract (Fig. 7), but is no longer detectable in the heated extract (Fig. 8). As expected, after the sample was heated, neutral cannabinoid peaks were detected on the chromatogram and were present in quantities similar to those determined by GLC (Table I). In an attempt to detect the presence of even low levels of neutral cannabinoids in the fresh plant extract (Fig. 7), twice the normal quantity of sample was injected onto the column. None were detectable; but tailing on some peaks indicated that high levels of compounds were being injected into the instrument. In Fig. 8, CBD is seen to have a larger peak area than Δ^9-THC, which is not expected in a drug clone. However, the detector wavelength (254 nm) is not optimal for Δ^9-THC. Peak areas cannot be directly compared from compound to compound to determine quantity with a single-wavelength detector.

COMMENTS AND CONCLUSIONS

The ability to separate acidic and neutral cannabinoids is essential in order to determine accurately which cannabinoids are present in various types of plant material. Previous methods developed for such analyses were adequate for plant resins, street marijuana, or dried plant material, but the prevalence of acidic cannabinoids in fresh plant material preclude their use. The method described here, which involves simply varying solvent pH, provided excellent separation of the two forms of cannabinoids. Also, our method, in contrast to others published[1,3–5], does not require pretreatment of the samples. Fresh plant material can be collected, extracted, and analyzed efficiently without producing potential artifacts.

Acidic cannabinoids respond to changes in solvent pH while neutral cannabinoids do not, and therefore our procedure provides flexibility in manipulating the retention times of these compounds. With a change in solvent pH, one can place acidic cannabinoids where convenient (within obvious limits) in the chromatogram as desired for a particular analytical study of these and other compounds. Duplicate runs can also be done with a different solvent pH each time to move the cannabinoic acid peaks and determine if peaks of other compounds had been hidden. Numerous unknowns remain in the *Cannabis* plant extract and are evident on the chromatogram, but they appear stable with respect to solvent pH. Further work is needed to identify retention times for specific cannabinoic acids as well as to quantify them. The method described here can accurately indicate the presence of acid and/or neutral cannabinoids in plant tissue extracts, and provides a basis for further experimen-

tation now in progress on the identity of specific cannabinoids derived from various fresh tissues.

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