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EFFECTS OF SAMPLE TREATMENT ON CHROMATOGRAPHIC ANALYSIS OF CANNABINOIDS IN *CANNABIS SATIVA* L. (CANNABACEAE)

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SUMMARY

Gas–liquid chromatographic and high-performance liquid chromatographic analyses on the effects of leaf treatment as well as the conditions for cannabinoid extraction were examined in two clones of *Cannabis sativa* L. Cannabinoid extracts of dried leaves, when analyzed by gas–liquid chromatography, showed no significant quantitative or qualitative differences regardless of drying procedure or temperature and duration of extraction investigated. Comparable high-performance liquid chromatographic analyses, however, indicated that while extraction temperature did not influence the cannabinoid profile, drying conditions had a significant effect. High ratios of acid to neutral forms were derived only from extracts of leaves dried at 37°C as compared to 60°C. Fresh, non-dried leaf material also yielded high ratios of acid to neutral forms, but the duration of extraction was found to affect cannabinoid yield significantly. Longer extractions of fresh leaves resulted in lower amounts of cannabinoids extracted. This study determined optimal procedures for analyzing fresh plant materials.

INTRODUCTION

Studies in our laboratory[1–4] on cannabinoid biosynthesis and localization in *Cannabis sativa* L. require accurate identification of the cannabinoid profile in fresh plant tissues. Analysis of cannabinoids had been done routinely with gas–liquid chromatography (GLC). Because cannabinoid acids, the predominant form of cannabinoids present in living plants[5–7], are converted by high temperature to neutral cannabinoids in the chromatograph[6], a derivatization of the cannabinoid acids had been recognized as the only way to detect these compounds by GLC[6,8]. High-performance liquid chromatography (HPLC) has made it possible to detect cannabinoids in both the acid and neutral forms. Because HPLC is done essentially at room temperature, samples can be chromatographed without the need for pretreatment such as heating[5,9] or derivatization[8]. Thus, HPLC can provide a more accurate assessment of the cannabinoid profile in plant material, and has become the method of choice in our laboratory. Although methods have been published for analyzing cannabinoids by means of HPLC, these techniques generally have been for plant resins, street marijuana, or human fluids.

The purpose of the current study was to optimize procedures for analyzing can-

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nabinoids in fresh plant materials. We have found that reported HPLC procedures[10,11] were not readily applicable to fresh materials[7]. In order to detect cannabinoid profiles in extracts of fresh materials with accuracy, some modifications of HPLC methods currently in use were necessary[7]. The standard procedure used to prepare plant material for analysis by GLC also required investigation to determine suitability of preparative procedures for HPLC application. The current study, therefore, evaluates various aspects of sample preparation on quantities of cannabinoids detected both by GLC and HPLC.

EXPERIMENTAL

Plant material

Compound leaves, with a 7.5-cm center leaflet, were collected from vegetative plants of two clones of *Cannabis sativa* L. routinely used in our investigations[1–4]. Clones 87 (a fiber type) and 152 (a drug type) were employed in this study. Leaf samples were always collected in triplicate, and data presented in tables represent the mean values. The plants were maintained in a vegetative state in an Indiana University greenhouse under a 20-h long-day condition. Sunlight was supplemented as necessary with incandescent light to insure active vegetative growth. Clones were grown on a year-round basis.

Sample preparation

Fresh leaf samples were extracted within 1 h of being collected. After extraction, fresh leaf samples were placed in a 60°C oven for 24 h and then weighed to determine dry weights. Leaf samples that were to be oven-dried were collected, and within 1 h placed in a 60°C oven in open glass petri dishes for 12–24 h. Samples were then weighed to determine dry weights and immediately extracted. Air-dried samples were placed in open glass petri dishes in a work-free standard laboratory hood for two weeks. Samples were then weighed and extracted. In most cases, sample dry weights were *ca.* 100 mg.

Cannabinoid extraction

Samples were weighed, placed in glass test tubes, and *ca.* 1 ml of ChromAR grade chloroform (Mallinckrodt) was added to each sample. After 1 h, the extract 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. Except for experiments on extraction temperatures, all samples were extracted in the cold (4°C). Each sample was then resuspended in 1 ml 100% ethanol containing two 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. Glass columns (2.43 m × 2 mm I.D.) 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 at a flow-rate of 20 ml/min. Samples injected con-

sisted of 1 lambda aliquots and were analyzed separately 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. Cannabinoid standards, provided by the National Institute of Drug Abuse, and eicosane, as an internal standard, were used for column calibration.

High-performance liquid chromatography

Analyses were performed on a Hewlett-Packard 1084B liquid chromatograph equipped with a UV detector set at 254 nm. A reversed-phase Altex column (Ultrasil-Octyl, 10 μm; 25 cm × 4.6 mm I.D.) was used. Column calibration was done using cannabinoid standards and di-*n*-octyl phthalate as an internal standard. The eluting solvents were acetonitrile (Burdick & Jackson, UV grade) and water (pH 5.0). Water utilized was deionized, processed through a Lobar RP-8 size B (EM Reagents) column[12], 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 0 and reaching 85% acetonitrile at 36 min. Flow-rate was 2 ml/min and oven temperature was 40°C. Sample size was 20 μl.

RESULTS AND DISCUSSION

Extraction temperature

Extraction temperature was found to have no significant influence on amounts of cannabinoids extracted. Fresh and oven-dried leaves from both the fiber and drug

TABLE I

EFFECT OF EXTRACTION TEMPERATURE ON CANNABINOID AMOUNTS

Leaf treatment and clone	*Temperature (°C)*	*Total cannabinoids (mg/100 mg DW)*		
		GLC	*HPLC*	
		Neutral	*Acid*	*Neutral*
Fresh				
Fiber	4	0.57	D*	ND**
	RT***	0.67	D	ND
Drug	4	2.55	D	ND
	RT	1.72	D	ND
Oven-dried				
Fiber	4	1.00	D	0.25
	RT	1.04	D	0.20
Drug	4	3.09	D	0.37
	RT	3.20	D	0.49

* Cannabinoid acids detected.
** No neutral cannabinoids detected.
*** Ambient room temperature.

clones were extracted in the cold or at room temperature, and the results are shown in Table I. Each sample was analyzed both by GLC and HPLC. The results show little if any difference between the two extraction temperatures, regardless of which clone or sample treatment was used. As expected in the GLC analyses, there are quantitative differences between the clones and types of leaves extracted. Minor quantitative variations seen within experimental pairs, particularly in fresh leaves, are representative of variability within the clones. Each quantity listed in the table is the mean of triplicate samples, and the experimental pairs were statistically analyzed using the Student's t-ratio. In either GLC or HPLC analyses, no significant difference between the means was found for any of the four pairs. For all statistical analyses, a significance level of 0.01 was utilized.

It can be concluded that cold temperatures do not reduce the efficiency of cannabinoid extraction in any apparent way. In addition, during the 3-h extraction period, room temperature does not appear to contribute to thermal decarboxylation of cannabinoids. Our extractions are routinely done in the cold, and samples are then maintained in the cold primarily to prevent quantitative changes resulting from solvent evaporation.

Oven-drying

For GLC analyses, our standard procedure involves drying leaves at 60°C prior to extraction. Within the experimental conditions, the length of time leaves were dried at this temperature did not contribute to quantitative differences for extracted cannabinoids. Samples were dried in a 60°C oven for different lengths of time, and each sample was analyzed by GLC as well as HPLC. As shown in Table II, samples were dried for 12, 18 or 24 h. Statistically, using the F-ratio, there was no significant difference among the means of samples analyzed by GLC or among those analyzed by HPLC. Visually, with longer drying times, there appeared to be a trend toward an increased

TABLE II

EFFECT OF LEAF DRYING TIME AND TEMPERATURE ON CANNABINOID AMOUNTS

Cannabinoids were extracted at 4°C from leaves of fiber clone 87. Separate leaf collections were made for the time and temperature experiments.

Drying condition	*Total cannabinoids (mg/100 mg DW)*		
	GLC	*HPLC*	
	Neutral	*Acid*	*Neutral*
Time (h)*			
12	1.06	D**	0.38
18	1.22	D	0.32
24	1.37	D	0.52
Temperature (°C)***			
37	1.61	D	ND§
60	1.60	D	0.70

* Samples were dried at 60°C.
** Cannabinoid acids detected.
*** Samples were dried for 24 h.
§ No neutral cannabinoids detected.

amount of cannabinoids detected by both GLC and HPLC. However, variation within the samples negated these differences. Our current procedure is to dry plant tissue for 12–24 h as convenient.

The effect of drying temperature was also investigated with both GLC and HPLC (Table II). Samples were dried for 24 h at 37°C or 60°C. When analyzed by GLC, no significant difference was found between amounts of cannabinoids extracted from samples dried at each of the two temperatures. However, analyses of the same samples by HPLC did reveal a significant difference. Samples dried at 60°C were found to contain both acid and neutral cannabinoids, while only cannabinoid acids were found in samples dried at 37°C. Although no neutral cannabinoids were detected during HPLC analyses of samples dried at 37°C, it remains unclear whether neutral forms occur in living tissue. However, if dried plant material is to be analyzed using HPLC, the effect of drying temperature should be considered. At temperatures higher than 37°C, the presence of cannabinoids in the neutral form may be conversion products from naturally occurring acids.

Leaf preparation

Three methods of preparing leaves to be extracted were compared. Cannabinoids were extracted from fresh leaves, from leaves dried at 60°C for 12 h, and from leaves air-dried for two weeks. Results are presented in Table III, and indicate that drying, either a long air-drying period or a short period of 60°C oven-drying, converts a percentage of cannabinoid acids to the neutral form. Statistically, no significant difference was found between the oven-dried and air-dried samples when analyzed either by GLC or HPLC. Quantitative differences listed in the table reflect variation within samples. However, the results from extractions of fresh leaves differed from those of dried tissues. HPLC analyses of fresh leaf samples detected only cannabinoid acids, while both acid and neutral cannabinoids were detected in dried leaves (Tables I and III). GLC analyses of fresh leaves routinely detected lower amounts of cannabinoids than detected in dried leaves (Tables I and III). Quantities of cannabinoids extracted from fresh leaves yielded statistically significant amounts as low as one-half that derived from dried leaves. Further experiments were done to try to explain the quantitative differences between extracts of fresh and dried leaves.

TABLE III

EFFECT OF SAMPLE TREATMENT ON CANNABINOID AMOUNTS

Cannabinoids were extracted from fiber clone 87.

Leaf treatment	*Total cannabinoids (mg/100 mg DW)*		
	GLC	*HPLC*	
	Neutral	*Acid*	*Neutral*
Fresh	0.60	D*	ND**
Oven-dried***	1.20	D	0.02
Air-dried§	1.26	D	0.04

* Cannabinoid acids detected.

** No neutral cannabinoids detected.

*** Leaves were dried for 12 h at 60°C.

§ Leaves were dried for 2 weeks at room temperature.

TABLE IV

EFFECT OF EXTRACTION TIME ON CANNABINOID AMOUNTS*

Cannabinoids were extracted from clone 152.

Leaf treatment and sample	*Total cannabinoids (mg/100 mg DW)*		
	Extraction time		
	1.5 h	*3.0 h*	*10.0 h*
Fresh			
1	2.96	2.83	2.20
2	4.42	3.31	—*
3**	—	1.54	0.88
Dried			
3**	—	1.43	1.47
4	—	3.01	2.99
5	1.85	—	1.86

* No sample.

** Samples collected simultaneously.

Fresh leaf extractions

The total length of the extraction period was investigated and found to be the apparent cause of the variability of quantities of cannabinoids extracted from fresh leaves. Our standard extraction procedure involved three 1-h extraction periods for a total extraction time of 3 h. Total extraction times of 1.5 h (three 0.5-h periods) as well as extended extractions (up to 10 h: three 3-h or longer periods) were done and the results showed no significant difference when dried leaves were extracted (Table IV). When fresh leaves were extracted, longer extraction periods resulted in decreased amounts of cannabinoids (Table IV). Differences in amounts of cannabinoids found were statistically significant except between samples extracted for 1.5 and 3 h in sample 1.

It was possible that a 3-h total extraction time was marginal for extraction efficiency. While adequate for some samples, it might have been too long for others. This would explain why some extractions of fresh leaves were comparable to extracts of dried leaves, and other extractions varied to as low as half of the amount extracted from dried leaves. Since too short an extraction time might be inefficient, an extraction procedure involving three 45-min periods (total time 2.25 h) was tried. The results from two experiments indicated comparable extraction efficiency for both fresh and dried leaves. In the first experiment, 3.35 and 3.36 mg total cannabinoids/100 mg dry weight (DW) were found for fresh and dried leaves, respectively. For the second experiment, the results respectively were 2.83 and 2.78 mg total cannabinoids/100 mg DW.

The reasons for a decrease in the amount of cannabinoids extracted from fresh material with longer extraction times are unclear. Stability of the extracted cannabinoids in the extracting solvent was considered, although, based on previous publications[13,14], chromatography grade chloroform is the preferred solvent for these extraction periods. To confirm this, fresh leaves were extracted for three 0.5-h periods. Extracts were then either evaporated immediately or stored in the extracting solvent in the refrigerator for 24 h before being evaporated. The results of two experiments

indicated no significant difference between the two regimes. In the first experiment, the samples evaporated immediately averaged 1.69 mg total cannabinoids/100 mg DW while those evaporated after 24 h averaged 2.01 mg total cannabinoids/100 mg DW. The second experiment yielded results of 2.79 and 2.48 mg total cannabinoids/100 mg DW, respectively. We concluded that a decrease in cannabinoid amounts required prolonged contact between chloroform and leaves rather than just with extracted cannabinoids. The specific cause of the decrease is unknown, but it is clear that the total length of the extraction period is critical for fresh leaves. We now routinely use three 45-min periods for a total extraction time of 2.25 h.

CONCLUSIONS

Our studies have shown that sample treatment prior to extraction is critical for producing an accurate profile of acid and neutral cannabinoids in plant tissue analyzed using HPLC. Since both heat and time can decarboxylate cannabinoid acids, cannabinoid profiles of dried plant material must be interpreted with drying procedures in mind. For extractions of fresh plant material, cannabinoids can only be quantitated if total extraction time is known to be long enough but not too long. HPLC is currently the most useful procedure for accurately determining the ratio of acid to neutral forms, but results can only be produced in direct relationship to the quality of the sample under investigation. Procedures routinely used to prepare samples for GLC analysis may not be appropriate for preparation of samples to be analyzed using HPLC. Therefore, when analyzing *Cannabis* by HPLC for the presence of cannabinoids, special attention must be directed to treatment of the sample.

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