Determination of Cannabinoids by Circular Dichroism

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Chloroform extracts from marihuana and other green plant materials have been analyzed spectroscopically using circular dichroism. Marihuana is easily distinguishable from the others by its unique and very intense negative Cotton band with a wavelength maximum at 310 nm. Direct determination of the THC and CBD, without their mutual separation, is possible from the circular dichroism spectrum of the residual nonaqueous solution following extraction of the naturally occurring acid fraction into NaOH.

Numerous methods have been used or are still in use for the identification of cannabinoids in marihuana (*1*). In most circumstances qualitative identifications are sufficient grounds for conviction of an alleged suspect. In these cases rapid qualitative screening is done with any of a number of color spot tests (*2*). When ever a quantitative result is required the method of preference is gas chromatography either alone or in tandem with mass spectrometry (*3*, *4*). Sample prepreparation usually involves solvent extraction, volume reduction, and derivatization of the thermally unstable analogues or metabolites. Other more sensitive methods are employed in the analyses of biological specimens (*5*).

The great majority of the compounds extracted from the manicured leaf of marihuana have been identified. Categories might include cannabinoids, non-cannabinoids, alkaloids, essential oils, etc. The prevailing consensus of opinion is that the primary pharmacologically active ingredient is *trans*-Δ^9-tetrahydrocannabinol (THC). Usually however an analysis encompasses more than just the determination of this one analyte. Phenotype ratios of cannabidiol (CBD) to THC, or CBD to THC plus cannabinol (CBN), have been defined and correlated with both the potency and the geographical region of origin of the native plants (*6*). Plants high in THC content comprise the phenotype I and are considered "drug" type marihuana; those relatively low in THC are phenotype II and referred to as "fiber" marihuana.

From these remarks it would appear that the problems associated with marihuana analyses are satisfactorily resolved but some still exist. For example the clinically accepted EMIT test (*7*) is not without controversy, because of possible false positives, and subsequent confirmation is recommended. Another is that if fewer than five spots are developed in a TLC screening test, confirmation by GC/MS is again required (*8*). One of the objects of this work was to develop a new rapid quantitative screening test for marihuana and cannabinoids in which circular dichroism (CD) is the method of choice. The method was expected to distinguish among different plants after a simple solvent extraction and to determine the amounts of THC and other chiral analytes without the need for a chromatographic separation of any kind. Precedent for this kind of application is found in the recent work on the determination of nicotine in chopped tobacco leaves (*9*). A review of the identities of possible interfering chiral analogues of THC (*10*) suggests that there may be as many as five, namely, THC, CBD, THC acid, cannabichromene, and cannabicyclol. Of those five, GC studies indicate that four may be present in sufficient abundance to complicate the analysis. With these favorable odds, CD would be measurably superior to absorption spectrophotometry if separation is not a prerequisite to determination and more rapid than GC/MS in quantitative work in routine repetitive analyses.

The four chiral substances of particular interest based upon the GC identifications are THC, CBD, and their respective aromatically substituted carboxylic acid derivatives, especially the 4′-substituents, Figure 1, THCA and CBDA. The acids are thermally unstable and must be derivatized before they can be identified as such in GC. Decarboxylation produces the parent THC and CBD molecules. A metabolic pathway believed to occur both in the living plant and on subsequent aging after harvesting is THCA to THC to CBN. CBN is achiral and therefore not amenable to CD detection but at the same time does not interfere with the other determinations. Most often the separate determination of the weight percent of THCA and other acids is excluded in routine GC analyses, and the original amount of THC is estimated by summing the results for THC and CBN.

EXPERIMENTAL SECTION

Marihuana samples were obtained from state agencies. Since the samples had been confiscated, their precise prior history was unknown. The samples varied in age and levels of dryness. Sampling was done by choosing only certain parts of the plants in some cases, e.g., only flowering tops, and in others by simply chopping entire plants.

Solvent extractions were made by shaking the specimens, which weighed anywhere from 20 to 500 mg, in 20-mL aliquots of reagent grade chloroform for 30 min at ambient temperature in an ultrasonic bath. Although other nonpolar solvents have been employed, chloroform is the solvent most frequently recommended for GC studies. Completeness of the extraction was confirmed by comparing the CD spectra for these solutions with extracts obtained after refluxing over a period of hours. Separation of the acid fraction from the remainder was done by extracting twice into equal aliquots of 1.0 M NaOH. The same procedure was followed for the other freshly chopped, green leafy materials used in this study, i.e., marjoram, parsley, oregano, bayleaf, dillweed, sage, cumin, basil, chervil, thyme, hops, and tobacco. Appropriate 2 to 4 times dilution of the extracts were made in order to keep absorptions within the dynamic range of the CD instrument.

Drug standards for *trans*-Δ^8- and *trans*-Δ^9-THC and CBD were obtained from Sigma Chemical Co. The THC derivatives are available only in an alcoholic solution (100 mg/mL); CBD was obtained as a pure solid. Standards for the carboxylic acid analogues and for the more recently reported *cis*-Δ^9-THC are unavailable in sufficient quantities for spectral calibration purposes at this time. The quantitative segment of the present work is limited therefore to a study of only THC and CBD.

CD spectra were obtained on a JASCO-500A automatic recording spectropolarimeter fitted with the DP-500N data processor. The instrument was calibrated daily using a standard solution of androsterone in dioxan as recommended. Scan rates, sensitivities, and repeat functions were selected to give optimum signal to noise ratio.

RESULTS AND DISCUSSION

The molecular structures for *trans*-Δ^9-THC and CBD are shown in Figure 1. The acid analogues are substituted with –COOH functional groups, in position 4′.

UV spectra for both compounds show relatively weak absorptions with maxima at approximately 273 and 282 nm. Other analogues absorb similarly in the same wavelength

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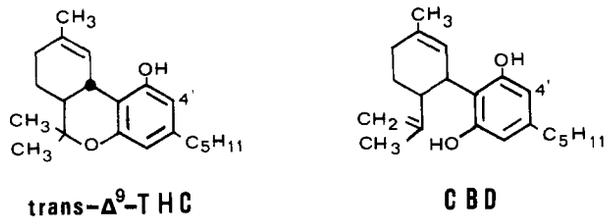

Figure 1. Molecular structures of *trans*-Δ^9-THC and CBD.

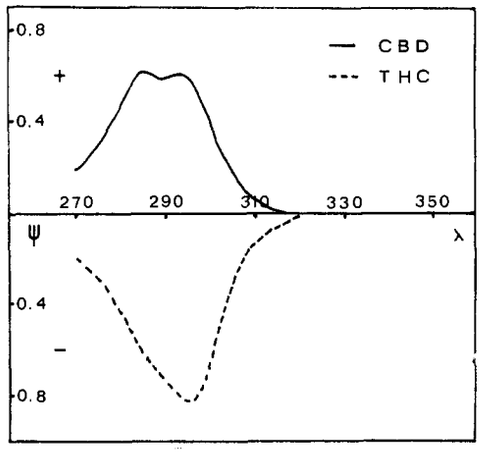

Figure 2. Standard CD spectra of THC (6.36×10^{-5} M) and CBD (1.05×10^{-4} M) in a 50:50 mixture of chloroform and 0.05 M KOH in methanol.

range. A second strong absorption band is observed around 200–220 nm. All of these absorptions are typical of the aromatic chromophore. The plethora of compounds with this chromophore which must be extracted into chloroform from the leaf, makes a UV determination of any analyte impossible without first separating the mixture.

While the CD spectrum of CBD in chloroform is of substantial intensity for its determination, THC shows no measurable CD spectrum at the same sensitivity scale of 2 mdeg/cm. The addition of an equal aliquot of 0.05 M KOH–methanol enhances the CD signals for both compounds. A conceivable interpretation for the red shift and increased ellipticity in basic medium is that the phenolic group at position 5 is fully ionized. Analogous spectral changes are observed for morphine (*11*) and other phenolic opiates. The interpretation is supported by the observation that the spectral change is reversible on acidification and back extraction into chloroform. The spectra shown in Figure 2 are for the *trans*-Δ^9-THC and CBD standards; the spectra for the Δ^8- and Δ^9-THC analogues are equivalent and these would therefore be indistinguishable in a possible CD determination experiment. Cotton bands occur in the spectral range of the aromatic absorptions. These are of opposite sign, negative for THC and positive for CBD. The molar ellipticities at the appropriate wavelength maxima, measured from the slopes of the calibration curves, are −129.3 (293 nm) and −89.5 (282.5 nm) and +56.9 (293 nm) and +57.9 (283.5 nm) respectively.

A CD spectrum, typical of the many unseparated chloroform extracts of marihuana investigated, is shown in Figure 3 (curve A). All four spectra in this figure were obtained for the same sample of marihuana in order to exactly compare the solvent effects. The spectrum for the original chloroform extract (A), theoretically includes contributions for all four principal analytes, but the contribution for THC is negligible. Curve D shows the red shift and enhancement effects produced by adding methanolic KOH. Curve B is the spectrum for the aqueous 1.0 M NaOH extraction from chloroform believed to contain only the fully ionized carboxylic acid derivatives since THC and CBD are essentially insoluble in

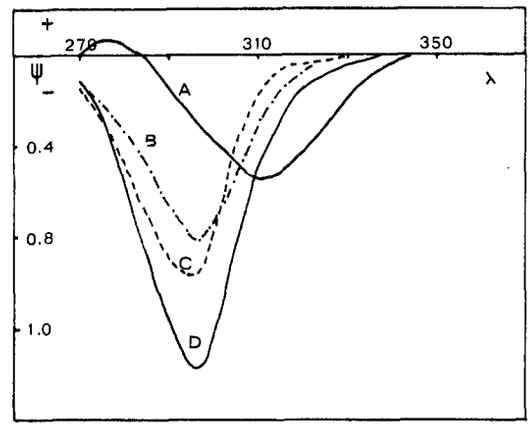

Figure 3. CD spectra of marihuana extracts: (A) THC, CBD, THCA, and CBDA in chloroform; (B) THC and CBD in chloroform and methanolic KOH after extraction of THCA and CBDA; (C) THCA and CBDA in aqueous 1.0 M NaOH; (D) THC, CBD, THCA, and CBDA in a 50:50 mixture of chloroform and methanolic KOH.

aqueous solution. Curve C is the CD spectrum for the residual chloroform extract to which an equal aliquot of methanolic KOH has been added. Data from the spectra for these solutions were analyzed as binary mixtures of THC and CBD.

Compared to marihuana, the CD spectra of the other leafy materials are relatively uninteresting (Figure 4). Only those five plants which are CD active under the extraction conditions used are shown. The spectrum for nicotine extracted from a fresh green tobacco leaf is almost exactly that already reported for extracts from commercial cigarette and smokeless tobaccos (*9*). The differences between these spectra and that for marihuana are so great that confirmation of the latter would be elementary. Hops were included in the study because of the reputed morphological similarity to marihuana. Extracts from fresh leaves yielded no CD spectrum. The spectrum in Figure 4 was obtained for the extract from the berries. Only sage shows any possibility of being mistaken for marihuana. The first objective of the study is satisfied.

Without satisfactory information to the contrary, we have proceeded with the quantitative work on the assumption that four cannabinoid analytes are extracted, and that only THC and CBD remain in the chloroform phase after NaOH extraction. In the mathematical procedure used for multicomponent analyses, ellipticity data were taken at every nanometer over the absorption range in the spectra for both the standard solutions and for the extracts. A least-squares fit of the spectra for the unknowns to a weighted algebraic sum of the spectra of the standards was made in a simple microcomputer program which minimized the coefficients in the solution of a set of linear equations. Standard spectra, normalized to 1.0 M, are stored on a library disk and recalled by way of a search program when required. Each library entry is typically the average of almost 20 independent measurements. The simultaneous determination was first tested on two prepared mixtures of known composition, where the ratios of THC to CBD were 0.27 and 2.7, respectively. When analyzed as binary mixtures, relative errors were less than 1% for both components. When treated as either CBD alone or as THC alone, these being the analytes in excess, relative errors were 54% and 18%, respectively. Confidence in the mathematical procedure is obtained from this very good correspondence between the experimental and calculated results for the mixtures. Results for seven marihuana specimens are given in Table I. Variations among the results from the same specimens reflect the uncertainty in withdrawing uniform representative samples.

Since the prior history of the samples is unknown, an exact comparison with earlier results from other methods is not

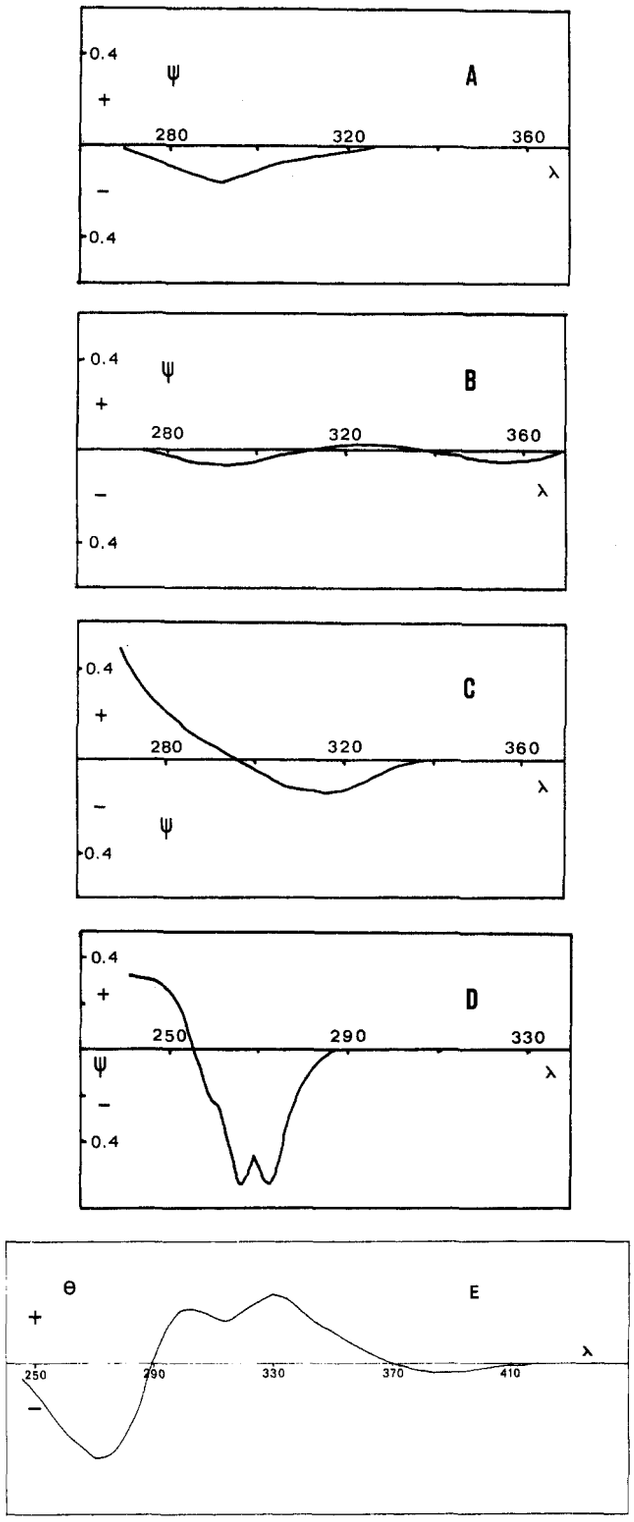

Figure 4. CD spectra for leafy extracts (A) oregano (0.1123 g); (B) dillweed (0.0926 g); (C) sage (0.1061 g); (D) tobacco (0.3615 g); and (E) hops (0.0492 g), after extraction into chloroform and the addition of an equal volume aliquot of 0.05 M KOH in methanol.

possible. Figures around a few percent for both constituents are however consistent with previous results. In spite of this apparently good comparison, it should be emphasized that whereas the GC results often report a total THC and CBD, which includes that produced by thermal decarboxylation of the acid analogues, the CD results do not include these analytes. The almost equivalent experimental ellipticity values observed for the chloroform–methanolic KOH and the aqueous extracts, Figure 3, implies a total weight percentage for the two acids somewhat similar to that for THCA and CBDA. However, it should be borne in mind that the experimental ellipticity is the product of the molar ellipticity coefficient and the analyte concentration, which separately are not necessarily equivalent to the values for THC and CBD. Furthermore it assumes that the Cotton effects are of opposite sign for THCA and CBDA, which may not be the case.

Table I. Determinations of Cannabinoids in Marihuana

specimen	organ	amt of THC, wt %	amt of CBD, wt %
plant 1	leaves	0.67	0.75
	(chopped)	1.35	0.49
		1.48	0.42
plant 2	leaves	0.59	0.40
	(chopped)	0.89	0.12
		2.18	1.06
plant 3	seeds	~0	~0
plant 4	flowering tops	2.48	2.90
		2.00	0.83
		2.10	2.32
plant 5	flowering tops	2.31	0.94
		2.01	0.25
		1.27	0.05
plant 6	whole plant	1.54	~0
	(chopped)	3.98	~0
		1.07	~0
rolled cigarette	whole plant (chopped)	1.28	~0
		1.27	~0

Results from gas chromatography have indicated lower total weight percentages for THCA and CBDA compared to THC and CBD which, if true for the present specimens, would imply larger values for the molar ellipticity coefficients for the acid forms. Assuming an arbitrary 1% total weight for the acids in the original leaf, and using the experimental ellipticity maximum observed for an aqueous NaOH extract, the estimated molar ellipticity for the combined acid forms is −839. The calculation assumes negative ellipticities for both THCA and CBDA. In actual fact then a positive confirmatory test for marihuana, which might use CD, is the recognition of the presence of THCA and CBDA. Using the given, very approximate, figure for the molar ellipticity for these acids, one can calculate a minimum detectable quantity on the order of 25 ng/mL, which may well be in the range of detection for marihuana in a saliva swab or perhaps even in the breath of a recent user. No interferences are to be expected from proteins which would normally show Cotton bands at wavelengths less than 235 nm.

Without any quantitative information on the achiral analyte CBN available from CD determinations, phenotype ratios, as presently defined, cannot be obtained from this work. The value of this ratio is open to question however in that the GC procedure converts underivatized THCA and CBDA to THC and CBD, so the ratio does not accurately describe the composition that exists in the plant. Smoking however may effect the same conversion. THCA and CBDA can be determined by GC after derivatization if a standard is available, but not as rapidly as it could be done by CD. Attempts are being made in our laboratory to isolate and separate sufficient quantities of the acids. The ratios of THC to CBD that we have observed suggest that the plants are "drug" quality and perhaps grown south of the arbitrary 30°N latitude. The CD results also agree with earlier data on the cannabinoid distribution in the various plant organs, showing flowering tops to be the most abundant reserve.

This work is another illustration of the selectivity potential of CD to identify particular chiral analytes in very complex mixtures without the need for elaborate separation and standardization procedures. One could expect that a multitude of CD active, naturally occurring substances are extracted

from a plant, but the high relative abundance, and high relative molar ellipticity coefficients, of nicotine in tobacco (9), and of the cannabinoids in this case, produce the necessary signal intensity for analytical determinations.

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Registry No. *trans*-Δ^9-THC, 1972-08-3; CBD, 13956-29-1; 4-CO_2H-*trans*-Δ^9-THC, 23978-84-9; 4-CO_2H-CBD, 1244-58-2.

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Immobilized Fluorophores in Dynamic Chemiluminescence Detection of Hydrogen Peroxide

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It is shown that the peroxy oxalate chemiluminescence system for the detection of H_2O_2 can be greatly simplified by using immobilized fluorophores. Various immobilization procedures for 3-aminofluoranthene are discussed. The chemiluminescence properties of materials based on cellulose, silica gel, and glass beads are compared. Controlled pore glass (CPG-10) was found to be the most suitable carrier. The material, packed in a quartz cell, is applied in a flow injection system in combination with a bed reactor containing bis(2,4,6-triphenyl) oxalate (TCPO) in solid form. For the determination of hydrogen peroxide in rain water samples, detection limits of $\sim 1 \times 10^{-8}$ M H_2O_2 (0.3 ppb) were obtained using a homemade luminescence detector. Linear calibration curves up to 10^{-5} M were observed.

The immobilization of fluorophores has drawn much attention in recent years. Seitz (*1*) reported the use of chemical sensors based on immobilized fluorophores in a recent review. A simple pH sensor was obtained by immobilizing fluoresceinamine on cellulose (*2*). The principle of dynamic fluorescence quenching of immobilized fluorophores was used for the development of sensors for O_2 (*3*, *4*) and chloride (*5*). In view of their relatively long lifetimes, immobilized phosphors can also have potential for a promising detection technique based on phosphorescence quenching (*6*). Fluorescence studies of a ligand–metal ion complex immobilized on silica gel have been described by Ditzler et al. (*7*). Lochmüller et al. immobilized pyrene on silica gel for studies of the distribution of silanol groups on the surface (*8*, *9*).

The application of immobilized fluorophores for chemiluminescence measurements has not yet been introduced. Chemiluminescence, used with the peroxy oxalate system, represents a simple and sensitive approach for the determination of H_2O_2 (*10–13*). However, the instrumental setup used in such a system is relatively complex equipment. Two additional pumps are needed for delivering the oxalate and the fluorophore. Mixing problems may also occur in such an arrangement. A simplification has already been introduced in a flow injection system by using the bis(2,4,6-trichlorophenyl) oxalate (TCPO) packed in solid form in a flow-through reactor and adding the fluorophore (perylene) to the mobile carrier phase (*14*). It is thus possible to eliminate the reagent pumping and mixing systems. Nevertheless, having to add the fluorophore to the carrier phase still imposes certain complications and restrictions such as solubility limitations, toxicity, cost, compatibility with the mobile phase, and flow dependence of the signal (requiring a nonpulsating solvent delivery system).

The idea of using immobilized fluorophores seems therefore promising for a further simplification of the instrumental setup and for an improvement of the detection properties through choice of more efficient fluorophores and with the reaction taking place directly in the detector cell in front of the photomultiplier.

In this paper the immobilization of 3-aminofluoranthene to solid carriers like cellulose, silica gel and glass beads is described. This highly efficient fluorophore cannot easily be used in a homogeneous system (*14*) for solubility and also

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