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Forensic Chemical Study on Marihuana. I. A Detection Method of the Principal Constituents by Thin-layer and Gas Chromatographies

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A new solvent system benzene–*n*–hexane–diethylamine (25:10:1), was found to show a good separation of cannabidiol, tetrahydrocannabinol and cannabinol in hemp resin by thin-layer chromatography, in which *Rf*–values of three constituents were 0.45, 0.35 and 0.25, respectively.

Furthermore, the same solvent system was successfully applied to silica gel column chromatography for isolation of three constituents of hemp resin.

Using cocaine hydrochloride as an internal standard of gas chromatography, relative retention times of cannabidiol, tetrahydrocannabinol and cannabinol were calculated to be 1.76, 2.34 and 2.88, respectively.

Six kinds of hemps grown in India, U.S.A. and Japan, were quantitatively analyzed using gas chromatography, and against a common opinion, Japanese hemps were found to contain considerable amounts of tetrahydrocannabinol, a physiologically active constituent.

Recently in Japan the cases of illegal use of marihuana have been increasing, while the reliable detection methods have not been available, except the unsatisfactory color reactions named Beam, Duquenois and Ghamrawy. These reactions lack in adequate specificity, because the similar color will develop also in the extracts of some other plants or materials different from true marihuana.

In 1964, Korte and Sieper[2,3] reported the thin–layer chromatographic method using silica gel plate impregnated with dimethylformamide. Their method affords to separate the three constituents of marihuana, but is inconvenient.

Present paper shows the simple method in which the three constituents of marihuana can be separated successfully by the new solvent system of thin–layer chromatography without impregnating procedure.

By adopting this solvent system to silica gel column chromatography, cannabidiol, tetrahydrocannabinol and cannabinol can be readily isolated from the extract of hemps, and be easily utilized as the standard substances. Using these standard substances, gas chromatographic method of marihuana is investigated, and the method can afford to analyze the three constituents in marihuana resin qualitatively and quantitatively. The three constituents in Japanese, Indian and American hemps are quantitatively analyzed by this method and the amounts of them are compared with each other.

Materials

Japanese Hemp I——A wild female hemp grown in Saga Prefecture was harvested in bloom, and was stored one year before analysis.

Japanese Hemp II——A cultivated hemp in the Experimental Farm of Tochigi Prefecture was stored two years before analysis.

1) *Katakasu, Fukuoka.*
2) F. Korte and H. Sieper, *J. Chromatog.*, 13, 90 (1964).
3) F. Korte and H. Sieper, *J. Chromatog.*, 14, 178 (1964).

Japanese Hemp III——A female hemp cultivated in a pot by us. The seed was obtained from Saga Prefecture. It was harvested and stored three months before analysis. Comparing with I and II, it was very small and slender.

American Hemp——A powder. It was unknown whether male or female, and when harvested.

Ganja——A kind of crude drugs produced in India. It was a mixture of dried branches, leaves and seeds, and contained much resin.

Charas——A crude drug of Indian hemp. It was a rod which was about 15 cm length and about 1 cm of its dameter.

Synthetic Cannabinol——It was synthesized by Adams' method.[4-6] The product was distilled *in vacuo*. The further purification was done by converting it to *p*-nitrobenzoate, and was again hydrolyzed to cannabinol with alcoholic potassium hydroxide.

The purified sample was very viscous oil and has not crystallized yet. The physical constants of the product and its derivative corresponded to those of the references.[4-7]

Methods

Preparation of Samples——Six kinds of hemps described in "Materials," were dried in desiccator for two days and tops of branches which were considered to contain much resin, were powdered. The powdered samples which were exactly weighed to be about 1.0 g, were allowed to stand in covered vessels containing 10 ml of EtOH at room temperature for two days. The extracts were evaporated to dryness and were again dissolved in a small amount of EtOH. Insoluble white crystals were filtered and recrystallized from acetone to white wax,[8] mp 53°. The filtrates were exactly diluted with EtOH to 10 ml. These samples were submitted to thin-layer and gas chromatographic analyses.

Thin-layer Chromatography——The samples were applied to a silica gel plate (0.25 mm in thickness, activated at 105° for 1 hour), and were developed with the solvent system of benzene–*n*-hexane–diethylamine (25:10:1). The plate was dried and sprayed with the following color reagents.

Diazotized benzidine: A solution of benzidine (5 g) in conc. HCl (14 ml) is diluted to 1 liter with distilled water, and a desired volume of this solution is mixed with an equal volume of 10% sodium nitrite solution.

Diazotized sulfanilic acid: Ten milligrams of crystalline diazotized sulfanilic acid is dissolved in 20 ml of 5% sodium carbonate. The solution should be freshly prepared before use.

Echt Blau Salz B Merck[2]: Fifteen milligrams of Echt Blau Salz B Merck[9] is dissolved in 20 ml of 0.1 N NaOH. The solution should be freshly prepared before use.

Beam reagent——Five percent of alcoholic potassium hydroxide. The reagent slowly develops the color for only cannabidiol.[10] It takes about a half day in room temperature, but only 5 min, when kept in an oven at 105°.

Duquenois reagent: To a solution of vanilline (0.5 g) in 20 ml of EtOH, a few drops of paraldehyde are added. After being sprayed with this solution and dried, the chromatogram was sprayed with conc. HCl and was heated at 105° for 5 min.

Extraction and Isolation of Cannabidiol, Tetrahydrocannabinol and Cannabinol from Ganja by Column Chromatography——Four hundreds grams of Ganja was percolated with EtOH. The crude extract (53 g) was chromatographed through 500 g of Florisil column using benzene as the effluent solvent. The eluate was fractionated into 150 fractions, and every fraction contained 10 ml. They were examined by spot test with diazotized benzidine, and the positive fractions which were from No. 66 to No. 111, were combined and evaporated under reduced pressure to leave 15.9 g of very viscous, slightly brown oil.

The oil was further submitted to column chromatography on silica gel, employing the new solvent system of benzene–*n*-hexane–diethylamine (25:10:1) which was found to separate the components fairly well in the thin-layer chromatography. The details are as follows.

A slurry which was made by mixing 40 g of silica gel (100—200 mesh) and the solvent (benzene–*n*-hexane–diethylamine), was poured into a column, 2.5 × 27 cm. One gram of partially purified Ganja extract which was dissolved in the same solvent as little as possible, was applied to the column. The effluent was fractionated into every 2 ml and the constituents in each fraction were checked by the thin-layer chromatography. The first several fractions which contained none of the constituents were discarded, and the following

4) R. Adams, D.C. Pease, and J.H. Clark, *J. Am. Chem. Soc.*, **62**, 2194 (1940).
5) R. Adams, D.C. Pease, and J.H. Clark, *J. Am. Chem. Soc.*, **62**, 2204 (1940).
6) R. Adams, D.C. Pease, and J.H. Clark, *J. Am. Chem. Soc.*, **62**, 2401 (1940).
7) F. Korte and H. Sieper, *Ann.*, **630**, 83 (1960).
8) This was negative for the phenol reagents. By the gas chromatography described in "Methods," it appeared as the very broad two peaks possessing the retention times about 15 and 30 min, respectively.
9) Di-*o*-anisidinetetrazolium chloride.
10) R. Adams, D.C. Pease, and J.H. Clark, *J. Am. Chem. Soc.*, **62**, 2402 (1940).

fractions were divided into five groups according to their chromatographic patterns; the first group containing cannabidiol alone, the second group containing the mixture of cannabidiol and tetrahydrocannabinol, the third group containing the mixture of cannabidiol, tetrahydrocannabinol and cannabinol, the fourth group containing the mixture of tetrahydrocannabinol and cannabinol, and the last group containing cannabinol alone. The elution of these components finishes usually after taking 30 to 40 fractions and it takes about 90 min for the whole fractionation. The groups which contained the mixture of two or three components were rechromatographed in the same manner.

Preparation of the Standard Solution——Cannabidiol, tetrahydrocannabinol and cannabinol which were isolated from Ganja, were exactly weighed and dissolved to contain about 1 μg in 1 μl of MeOH, respectively. These solution were utilized as the standards in the whole research.

Gas Chromatography——The instrument used for this work was a Shimadzu Model GC–IB Type Gas Chromatograph equipped with a hydrogen flame ionization detector (dual column and differential flame). The column was a stainless steel U-tube, 2.25 m×4 mm. The column packing was 1.5% SE–30 on Chromosorb W (60—80 mesh), which was treated with hexamethyldisilazane. The column temperature was maintained at 220°, the sample chamber temperature was 290°, and detector cell temperature was 230°. The flow rate of N_2 was 35 ml/min.

The usual sample size was 2 to 8 μl corresponding to 2—10 μg of each constituent in hemp. The amount of three constituents in hemp was calculated by measuring their peak areas. The calibration curves were plotted by running the same procedure for the three purified standard constituents.

Results and Discussion

Thin-layer Chromatography and Its Color Reactions on Chromatogram——Korte's method which involves impregnating procedure with N,N'–dimethylformamide in CCl_4, was found that the procedure itself seemed rather troublesome and *Rf*–values of constituents were greatly affected by grade of dryness of impregnating solvent. It will be more preferable to find any other solvent systems which could separate three constituents of marihuana without impregnation. In such investigation it was found that benzene alone as a developing solvent could slightly separate three constituents in the samples, and that addition of an alkaline solvent to benzene was more effective.

The new solvent system of benzene–*n*–hexane–diethylamine (25:10:1) was thus found to be most satisfactory. *n*–Hexane in this system has a lowering effect for the *Rf*–values of the spots. The representative chromatograms are shown in Fig. 1.

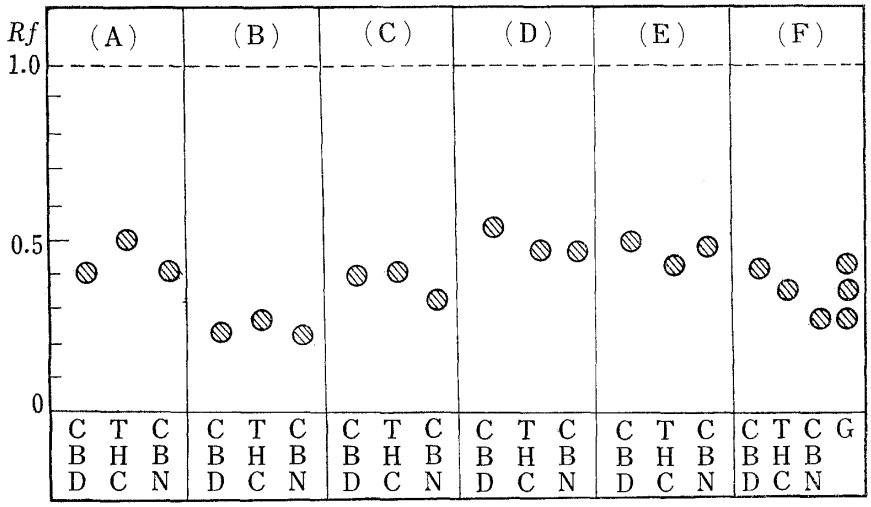

Fig. 1. Thin–layer Chromatograms with Various Solvent Systems

Solvent System
(A) MeOH: *n*–Hexane (1:15)
(B) Me_2CO: *n*–Hexane (1:15)
(C) AcOEt: *n*–Hexane (1:10)
(D) $CHCl_3$: *n*–Hexane (1:2)
(E) Benzene: *n*–Hexane (5:1)
(F) Benzene: *n*–Hexane: Et_2NH (25:10:1)
CBD: Cannabidiol, THC: Tetrahydrocannabinol,
CBN: Cannabinol, G: Crude Extract of Ganja.

By developing with this system and spraying with the color reagents described in "Method," cannabidiol, tetrahydrocannabinol and cannabinol could be identified as well-separated and differently colored spots at *Rf* 0.45, 0.35 and 0.25 respectively.

The coloration of the spots on the chromatogram with above reagents and their limits of identification are summarized in Table I.

Table I. Coloration of Marihuana Constituents on TLC

Constituents	Diazotized Benzidine	Diazotized Sulfanilic acid	Echt Blau Salz B Merck	Beam Reagent	Duquenois Reagent
Cannabidiol	yellow orange (0.1)	slightly yellow (0.3)	yellow pink (0.1)	violet (−)	blue (5)
Tetrahydrocannabinol	red orange (0.1)	bright yellow (0.1)	violet pink (0.1)	——	violet blue (3)
Cannabinol	red brown (0.1)	yellow (0.1)	violet red (0.1)	——	violet blue (5)

The figures in parentheses indicate the limit of identification (μg).

The limit of identification of Beam reagent was not clear, because the spot of cannabidiol already colored violet before being sprayed with the reagent when it was completely dried with a dryer, and the original coloration of Beam reagent for cannabidiol is violet. Among the color reagents, diazotized benzidine,as well as Echt Blau Salz B Merck, was most sensitive. This thin-layer chromatography is useful enough as the detection method of marihuana for the forensic chemical purpose.

Isolation of Standard Cannabidiol, Tetrahydrocannabinol and Cannabinol by Column Chromatography——One gram of Ganja extract partially purified through Florisil could be separated into the three standard constituents by the six times-rechromatographies, and yields of cannabidiol, tetrahydrocannabinol and cannabinol were 0.117 g, 0.231 g and 0.278 g, respectively. These samples were identified by comparision of their physical constants with those of the references.[4-7,11] The derivatives of them were also synthesized according to the usual methods and were utilized for identification.

Cannabidiol: A reddish brown very viscous oil was crystallized by being dissolved in a small amount of *n*-hexane and being kept in an ice box for two or three days to colorless rods.

Tetrahydrocannabinol: Almost colorless, but a little greenish fluorescent oil. It was repeatedly tried to crystallize with *n*-hexane, but without success.

Cannabinol: A slightly yellow and very viscous oil. Using a micro instrument of sublimation, it was distilled at 180° (temperature of oil bath), 0.05 mmHg. The physical constants were completely coincident with those of synthetic cannabinol.

The Quantitative Analysis of the Three Constituents of Japanese, American and Indian Hemps by Means of Gas Chromatography——In the gas chromatograms of the three standards, the peaks were quite sharp, and their retention times were shown to be 4.92 for cannabidiol, 6.58 for tetrahydrocannabinol and 8.17 minutes for cannabinol.[12] Comparing with the retention times of the standards, those of the hemp extracts were definitely recognized, although a few small other peaks coexisted. Using cocaine hydrochloride as an internal standard, the relative retention times of cannabidiol, tetrahydrocannabinol and cannabinol were calculated to be 1.76, 2.34 and 2.88, respectively. By the gas chromatographic method, the three constituents contained in six kinds of samples were quantitatively analyzed. The results are summarized in Table II.

11) R. Adams, *J. Am. Chem. Soc.*, **62**, 196 (1940).
12) *cf.* T.W.M. Davis and C.G. Farmilo, *Anal. Chem.*, **35**, 751 (1963).

Table II. Result of Quantitative Analyses

Components	Japanese			American Hemp	Indian	
	Hemp I	Hemp II	Hemp III		Ganja	Charas
Cannabidiol	1.05	0.41	0.20	0.14	0.57	none
Tetrahydrocannabinol	1.17	1.68	0.63	0.81	1.42	0.37
Cannabinol	0.06	0.15	trace	0.54	1.49	4.45
Total	2.28	2.24	0.83	1.49	3.48	4.82

Figure shows percentage of the components in dried and powdered materials.

As can be seen in Table II, total amount of three constituents are much more in Indian hemps than in hemps yielded in other countries. Content in Japanese hemp III cultivated in a pot is exceptionally low level, probably owing to insufficient nourishment. The percentages of each constituent in different hemps vary considerably. Japanese hemps contain cannabidiol comparatively more than those in Indian and American hemps. Indian hemps contain mostly cannabinol, whereas Japanese hemps do not contain appreciable amount of it, but much larger quantity of tetrahydrocannabinol, the physiologically active constituent in hemp resin. Against a common opinion that Japanese hemps have very low psychosomatic effect, the present study has strongly suggested that Japanese hemps have contained the active constituent considerably.

It has been widely believed that cannabidiol was isomerized to tetrahydrocannabinol, which was then oxidized to cannabinol biogenetically or spontaneously.[13,14] It must be, therefore, noticed that the total amount of these constituents and ratio of each constituent in hemp resin, might be changeable according to when the hemps were harvested, how long they passed since harvest and how to store them.

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13) O.E. Schultz, and G. Haffner, *Arch. Pharm.*, **293**, 1 (1960).
14) R. Mechoulam and Y. Gaoni, *Tetrahedron*, **21**, 1223 (1965).