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A Comparative Analysis of *Cannabis* Material

It is an offense under the Narcotic Control Act of Canada for a person to have in his possession *Cannabis sativa* (marihuana), *Cannabis* resin (hashish), or any of the cannabinoid constituents of *C. sativa*, including cannabinol (CBN), cannabidiol (CBD), and tetrahydrocannabinol (THC), unless such possession is legally authorized. Each year, thousands of Canadians are convicted under this Act; the figure for 1976 was in excess of 33 000 [1].

In Canada the forensic science identification of *Cannabis* materials is performed by two federal agencies: Health and Welfare (Health Protection Branch) and Royal Canadian Mounted Police (RCMP) Crime Detection Laboratories. The number of samples analyzed is obviously considerably greater than the number of convictions. For such a large number of samples to be analyzed, it is essential that the methods used to identify *Cannabis* materials are not only specific but also as rapid as possible.

Forensic science laboratories employ three tests to identify marihuana and hashish: (1) a microscopic examination of the material, (2) a modified Duquenois-Levine [2] color test on extracts of plant materials, and (3) a thin-layer chromatographic examination of extracts of plant materials. Marihuana leaves possess short, cystolith hairs ("retort hairs") on the top surface of the leaf and numerous long cystolith hairs on the bottom surface which are easily recognized under magnification [3,4]. If the sample is plant material but lacks cystolith hairs, it cannot be marihuana. If the substance is not plant material but is a resinous substance, it may be hashish.

In the Duquenois-Levine test [2], a hexane extract of the plant or resinous material is evaporated to dryness and the residue is dissolved in an ethanolic solution of vanillin and acetaldehyde. The solution is acidified with hydrochloric acid and examined for a sequence of color changes. An extract of *Cannabis* material changes gradually from being virtually colorless to pale green and through green blue, blue, blue violet, finally to violet. When the final solution is extracted with chloroform, most of the violet color enters the chloroform layer. Few, if any, other plant products react identically in the Duquenois-Levine test [5]. A positive test is strongly suggestive of the presence of cannabinoids in the sample and, since cannabinoids are a group of compounds present only in *C. sativa* [6, p. 2], strongly indicative that the sample is a *Cannabis* material.

The hexane extract of the *Cannabis* material is also examined by thin-layer chromatography (TLC) on silica gel G plates previously soaked in diethylamine and air-dried. The toluene-developed plates are dried and sprayed with a solution of the dye Fast Blue 2B. Up to five cannabinoid spots (resulting from the presence of (-)-*trans*-Δ^9-tetrahydrocannabinol [Δ^9-THC], CBD, CBN, cannabichromene [CBC], and cannabigerol [CBG]), each with a different R_f value and a distinctive color, can be observed, as can other unidentified materials [6, p. 144]. The TLC behavior is compared to that of a standard *Cannabis* resin sample, treated similarly. Whether or not all five cannabinoids are observed on the plate depends on the source and age of the *Cannabis* sample.

No other plant extract is known to behave in identical fashion when subjected to the Duquenois-Levine test and the TLC analysis just described. Most forensic science laboratories hold the view that the three tests employed by them are scientifically correct and permit the qualitative identification of marihuana and hashish without a reasonable doubt. However, the specificity of this "three parameters approach" to identify *Cannabis* material has been under severe scrutiny by several defense councils (for example, see Ref 7) and has resulted in lengthy dialogue in the cross-examination of expert witnesses. It is often suggested that more sophisticated methods of analysis are available (such as infrared [IR] spectroscopy and combined gas chromatography-mass spectrometry [GC-MS]) and should be used to identify *Cannabis* materials. It is undoubtedly true that IR and GC-MS techniques can be powerful and specific methods of identifying organic compounds. It would be very tedious and costly, however, to have to separate all the components of a plant material by TLC or GC and record an IR spectrum, or a mass spectrum, or both, of each component. Such a sophisticated approach to identify marihuana or hashish would be warranted only if it could be shown unequivocally that it was more specific for *Cannabis* analysis than the three parameters approach currently employed by most forensic science laboratories.

We wished to examine, by GC and GC-MS, *Cannabis* samples that had previously been analyzed by analysts at an RCMP Crime Detection Laboratory, to establish whether they were correct in their assertion that the three parameter approach was sufficient to identify *Cannabis* samples unequivocally. We requested and obtained from the RCMP extracts of 100 suspected *Cannabis* samples that were identified by numbers only; no analytical results were supplied. The present study describes our independent investigation of these extracts.

Experimental Procedure

Reference Compounds

The following materials were supplied by the Department of Health and Welfare, Health Protection Branch, Ottawa: cannabicyclol (CBL), CBC, CBN, CBD, (-)-*trans*-Δ^8-tetrahydrocannabinol (Δ^8-THC), Δ^9-THC, CBG, hashish resin (39% Δ^9-THC, 35% CBD, 18% CBN), two authenticated samples of U.S. marihuana,[2] and one of Canadian marihuana.

Instrumentation

Gas chromatography was performed on a Hewlett-Packard Model 5700A instrument (dual column), incorporating a flame ionization detector using two GC systems: System A1—3% PC-3210[3] coated on Chromosorb W HP, 80-100 mesh, packed in a 1.7-m glass column, 4-mm inside diameter; and System B—Ultrabond,[4] 100-120 mesh packed in a 1.3-m glass column, 3-mm inside diameter. The operating conditions were the same for both columns: helium was the carrier gas (60 ml/min), the oven temperature was 230°C, and the injection port and detector temperatures were 300°C. The GC peak areas and retention times were recorded (Table 1) with a Hewlett-Packard recording integrator, Model 3380A.

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[2]Originally supplied and analyzed by Dr. C. E. Turner, Research Institute of Pharmaceutical Sciences, School of Pharmacy, Mississippi.

[3]PC-3210 is a commercial, pure methyl silicone (Pierce Chemical Co.).

[4]Ultrabond is a commercial preparation of a heat-treated ultra-thin coating of Carbowax 20M on Chromosorb W HP (Alltech Associates).

TABLE 1—*GC and TLC data on the principal cannabinoids.*

	GC Retention Time, min		TLC	
Cannabinoid	System A1	System B	R_f Value	Color
CBC	3.11	2.75	0.16	purple[a]
CBD	3.07	2.49	0.41	yellow/orange
Δ^8-THC	3.62	2.66	ND[b]	...
Δ^9-THC	3.84	2.94	0.36	red
CBN	4.69	4.98	0.27	purple
CBG	4.48	6.41	0.23	orange

[a] Changes to orange after about 30 min.
[b] ND = not determined.

Gas chromatography-mass spectrometry was carried out by using a Hewlett-Packard Model 5710A GC connected to a Model 5981A mass spectrometer, using two GC systems: System A2—identical to GC System A1 except a 1.3-m column was used and the oven temperature was 200°C; and System B—identical to the GC System B described above. Helium was the carrier gas (60 ml/min), the ionizing energy was 70 eV, and the ion source and analyzer temperatures were 180 and 200°C, respectively. Mass spectra of authentic samples of CBC, CBD, Δ^9-THC, CBN, and CBL are shown in Fig. 1.

Thin-Layer Chromatography

Extracts of suspected *Cannabis* preparations were spotted onto silica gel plates ($60F_{254}$, spread to a thickness of 0.25 mm, Merck). The plates were presoaked in diethylamine for 5 s, air-dried for 2 min, and then run in toluene. The spots were visualized with Fast Blue base 2B (azoic diazo component 20; Matheson Coleman and Bell; 0.5% in 50% ethanol). See Table 1 for chromatographic data.

Samples

Samples were supplied by the RCMP Crime Detection Laboratory, Edmonton, as dried hexane extracts of confiscated material (case samples) suspected to be marihuana or hashish (resin or liquid). Approximately 200 to 500 mg of the plant material or a smaller amount of hashish was extracted with hexane (5 ml) by RCMP personnel. Pipes suspected to have been used for smoking material containing cannabinoids were also extracted with hexane. The extracts were supplied labeled with a code known only to the RCMP and the results compared when both analyses were complete.

Of the 100 samples supplied by the RCMP, 68 were classified by them as extracts of marihuana, 20 of hashish (liquid or resin), 6 of pipe extracts, and one of an "unidentified green material." Five extracts were of plant materials not containing cannabinoid (all this information was conveyed to us after our analyses were completed).

Sample Analysis

In the present study, each of the 100 hexane extracts was reconstituted with 0.1 to 2.0 ml pentane and 1 to 3 μl analyzed by GC (Systems A1 and B) and GC-MS (System A2). In addition, hexane extracts of TLC spots corresponding to the main cannabinoid components (CBC, CBD, Δ^9-THC, and CBN) in 20 of the original case sample extracts were analyzed by GC-MS (System A2).
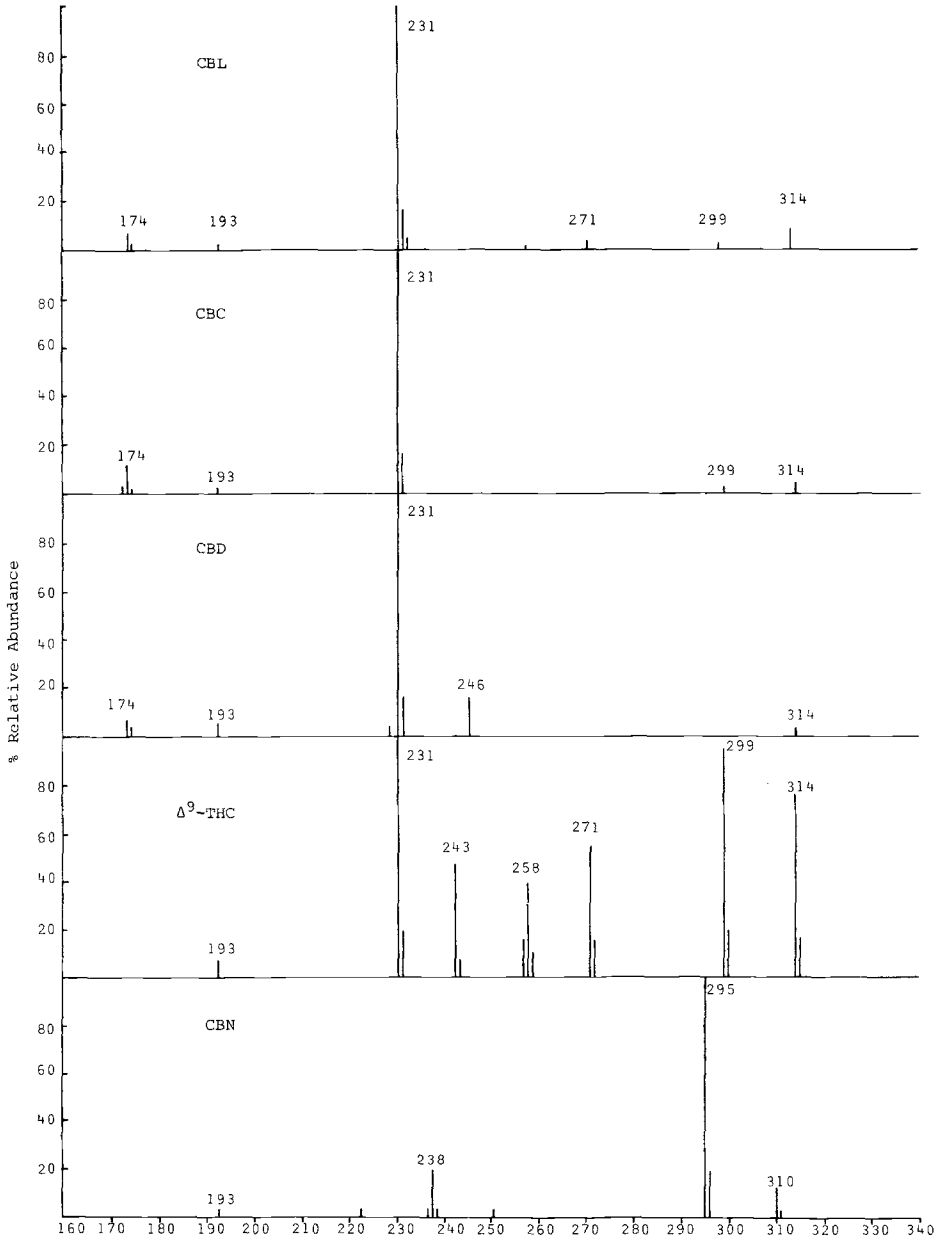
FIG. 1—*GC mass spectra of authentic samples of CBL, CBC, CBD, Δ⁹-THC, and CBN.*

Each cannabinoid was deemed to be positively identified if its retention times on GC Systems A1 and B and on GC-MS System A2 were the same as those of the authentic standard (± 0.05 min) and if its mass spectrum was also identical to that of the authentic standard.

Results

Results of our analyses are summarized in Fig. 2 and Table 2. Of the 100 extracts examined, 7 were classified as negative, that is, no cannabinoids could be detected in these samples. A subsequent comparison of our findings with those of the RCMP revealed complete agreement with this result. Five of these seven samples were "placebo" extracts made from plant material other than marihuana, and the other two were extracts from confiscated pipes thought to have been used for smoking marihuana. The remaining 93 extracts were found, by us, to contain at least two and usually three commonly encountered cannabinoids (CBC, CBD, Δ^9-THC, or CBN). For comparison purposes, each of these sample extracts was classified according to the relative amounts of each cannabinoid it contained (see Fig. 2). The largest group, A, comprised those samples where the area of the GC peak for Δ^9-THC was greater than 50% of the total area of the four cannabinoid peaks (CBC, CBD, Δ^9-THC, CBN). Sixty-four of the 93 samples containing cannabinoids were allocated to this group, which was further subdivided arbitrarily according to the amount of CBN present. These groups (A1–A3) were further characterized by the relatively small amounts of CBD and CBC they contained; this finding is discussed later. Another subgroup, A4, is composed of just one sample, unique in the 93 positive samples because it contained a fairly high ($\sim 20\%$) relative amount of CBC; samples of marihuana containing relatively large amounts of CBC have been reported [8].

The major cannabinoid in the Group B samples was CBD, and CBN was the major cannabinoid in the Group D samples; in Group C, quantities of the cannabinoids CBD, Δ^9-THC, and CBN were more evenly distributed.

It was subsequently revealed to us by the RCMP that of the 63 samples allocated by us to Groups A1 to A3, all but 5 had been identified as marihuana by that agency; those 5 exceptions were all green liquid hashish (4 in Group A1 and one in Group A2). Of the 24 samples in Groups B, C, and D, 14 were identified in RCMP records as being either extracts of hashish resin or brown liquid hashish and 9 of the remaining 10 as being marihuana. Interestingly, 10 of the 12 samples in Group C (CBD, Δ^9-THC, and CBN were of roughly equal proportion) were extracts of hashish (resin or brown liquid), whereas 6 of the 7 samples in Group D (CBN was the main cannabinoid) were extracts of marihuana. Five samples were not allocated by us to Groups A to D. In each case, they were found to contain two or more cannabinoids, but in too small an amount relative to other extraneous material for a classification to be meaningful; 3 of the 5 were "pipe rinses," one was "unidentified green material," and the fifth was a weak extract of brown liquid hashish. The difficulty in estimating the proportions of cannabinoids in these samples was compounded by the presence of noncannabinoid materials interfering with the GC analysis.

The Extracts of Cannabinoids

Identification by the RCMP of individual cannabinoids relies greatly on TLC R_f values and on distinctive colors produced by reaction with Fast Blue 2B dye. It was essential to this present study to confirm that the TLC-separated spots were correctly identified by this procedure. A random selection was made of 20 of the extracts concluded by the RCMP to contain CBC, Δ^9-THC, CBN, and sometimes CBD, and TLC separations were repeated. Each separated spot (untreated with Fast Blue 2B dye) was eluted with hexane and each concentrated eluate was analyzed by GC-MS (System A2). In each case the material
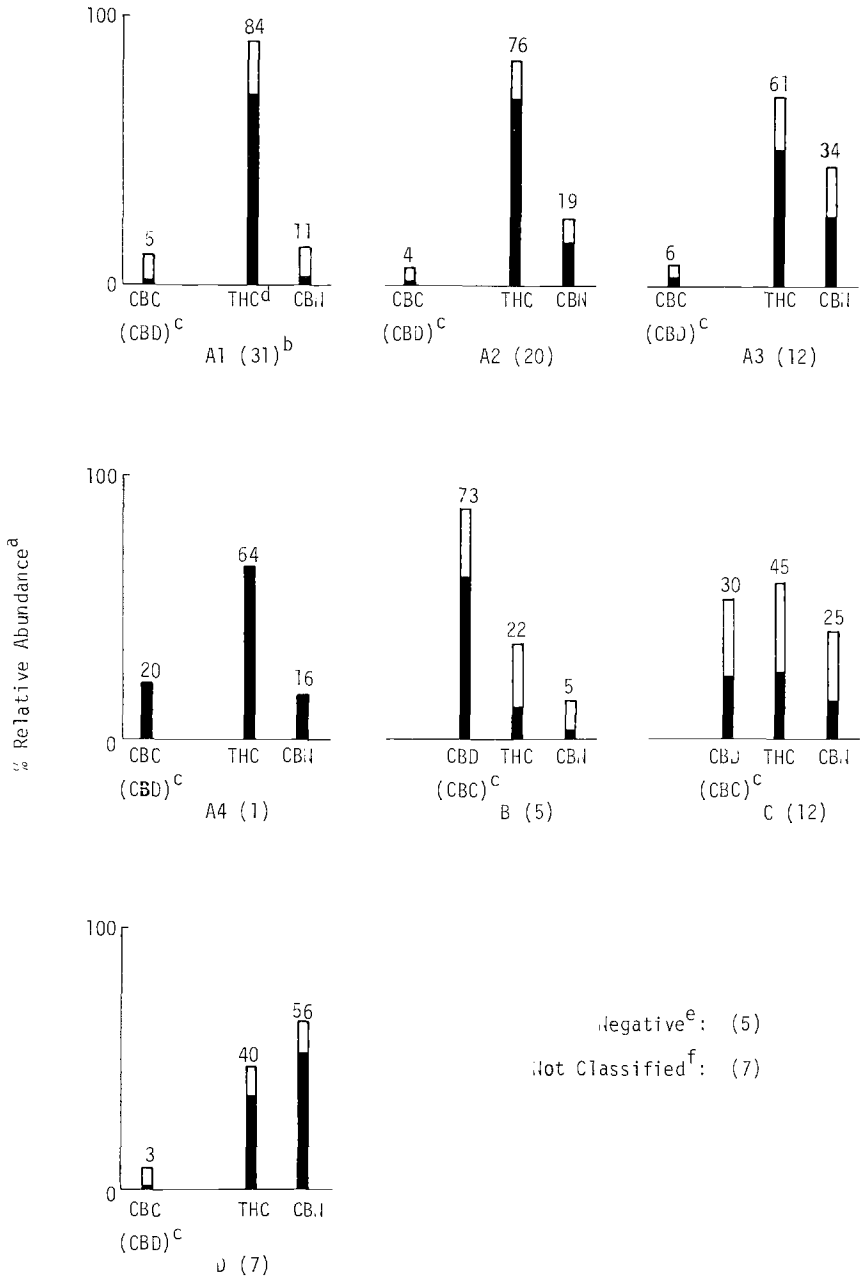
FIG. 2—*Results of the analyses of* Cannabis *samples by GC.* (a) *GC peak areas of the main cannabinoids expressed as a percentage of the total area for those peaks. The open area of the bars represents the range and the figures above the bars the weighted means of the ranges.* (b) *The arbitrary group classification and the number of samples in that group (in parentheses).* (c) *Where the percentage of CBD is small relative to CBC, or vice versa, the total percentage of both is given over the heading of the major component.* (d) *THC* = Δ^9-*THC.* (e) *The number of samples where no cannabinoids were identified by GC and GC-MS (including five deliberate blanks).* (f) *These samples were too weak for a classification in the above groups to be meaningful (usually pipe extracts).*

TABLE 2—*Distribution of sample identity according to cannabinoid content.*

Classification[a]	Marihuana	Hashish				Blank (Placebo)	Unidentified (Green Plant)	Total Samples
		Green Liquid	Brown Liquid	Resin	Pipe			
A1	27	4	...	...	...	...	...	31
A2	19	1	...	...	...	...	...	20
A3	12	...	...	...	...	...	...	12
A4	1	...	...	...	...	...	...	1
B	2	...	1	2	...	...	...	5
C	1	...	1	9	1	...	...	12
D	6	...	1	1	...	...	1	7
NC[b]	0	...	...	...	3	...	...	5
Negative	0	...	...	...	2	5	...	7

[a] See Fig. 2.
[b] NC = positive, but not classified in the above groups.

extracted from the TLC plate gave a GC peak of appropriate retention time and a mass spectrum consistent with those of the corresponding authentic standard.

Discussion

Separation of the Cannabinoids by GC

An appropriate choice of GC systems was essential for reliable qualitative identification of the cannabinoids. Two systems were required, each having markedly different retention properties, which would separate all the major cannabinoids. Chromatography of the underivatized cannabinoids, rather than their trimethylsilyl derivatives, was preferred to obviate possible problems of incomplete derivatization and of the potential instability of such derivatives [9].

Turner and others [9,10] have devoted considerable effort towards finding suitable GC systems for *Cannabis* analysis. They described the use of a phenylmethyl silicone column (2% OV-17) that gave excellent separation of Δ^9-THC and CBN from CBD, but on which CBD and CBC were unresolved. A methyl silicone column (6% OV-1) was also described; it partially resolved CBD and CBC ($\sim$50% valley resolution in Ref *10*), but CBN and CBG were incompletely resolved, even at a 30-min retention time. A compromise column with a 4% coating of a silicone oil stationary phase (6.5% phenyl, 92.5% methyl substitution), used by Turner, gave excellent separation of Δ^9-THC from CBD and CBN but only poorly separated CBD from CBC and CBG from CBN [10].

In earlier studies by others a complete separation of CBC from CBD on similar systems was claimed. However, as stated by Turner [8,9], it is likely that these workers [6,11,12] mistakenly identified CBL for CBC, since the former cannabinoid has a mass spectrum similar to that of CBC [9,13,14] although it has a shorter retention time on most GC silicone columns; CBC usually overlaps CBD [10]. Numerous other workers have described alternative GC systems for cannabinoid analysis but none is superior to those used by Turner.

Bearing in mind these factors, we eventually chose a slightly polar methyl silicone column (3% PC-3210) and a polyethylene glycol column (a commercial heat-treated ultrathin coating of Carbowax 20M on Chromosorb W-Ultrabond) for our GC analyses. As with Turner's systems [10], the choices were a compromise. The PC-3210 column gave baseline separation of Δ^9-THC, CBD (or CBC), and CBN in less than 5 min although CBG partially overlapped with CBN, and CBC was virtually unresolved from CBD (3.11 and 3.07 min, respectively). The Ultrabond column gave about 90% valley resolution of CBD from CBC and of CBD from Δ^9-THC. However, the CBC and Δ^9-THC GC peaks overlapped extensively although they were sufficiently well separated to be distinguished (2.75 and 2.94 min, respectively). On the Ultrabond column, CBN was well separated from Δ^9-THC (see Fig. 3).

Samples were analyzed on both systems alternately in a dual column GC with a constant oven temperature. Retention times of the cannabinoids, recorded automatically by the integrator-recorder, were usually reproducible within $\pm$2 s of the average values obtained for authentic standards.

Since the precise amount of material originally extracted was not known to us, quantitation of the cannabinoids present in the extracts supplied by the RCMP was not possible. However, as a guide to the *relative proportions* of the main cannabinoids present in the samples, the GC peaks obtained for CBC and CBD (combined), for Δ^9-THC, and for CBN were summed and the area for each cannabinoid expressed as a relative percentage of the total area. Detector responses for each of the cannabinoids CBD, Δ^9-THC, and CBN were confirmed to be approximately equivalent by using a standardized hashish extract.
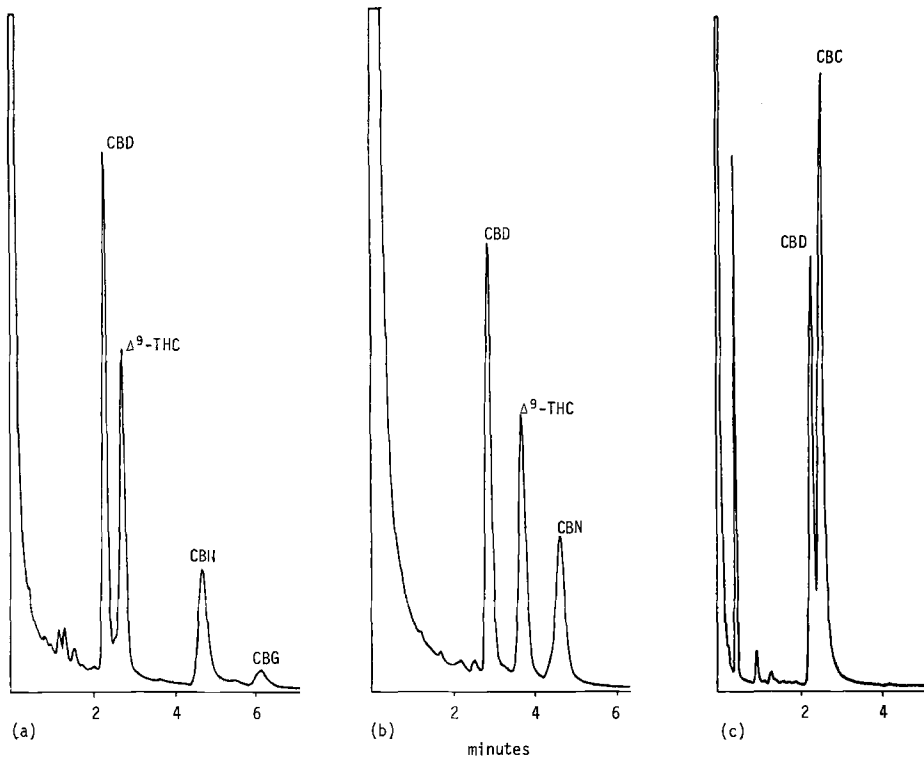
FIG. 3—*GC traces of a standardized hashish extract* (a) *on Ultrabond,* (b) *on a PC-3210 column,* *and* (c) *on Ultrabond of authentic CBD and CBC.*

Absence of CBD in Some Samples

No single system could separate CBC from CBD and at the same time completely separate CBC from Δ^9-THC. However, by careful comparison of the results obtained from both the PC-3210 and Ultrabond GC columns, and from a GC-MS of the combined CBC/CBD peak using the PC-3210 column, it was possible to distinguish CBC from CBD and estimate the approximate proportion of each present. The mass spectrum of CBD contained a fragment ion of m/e 246 (10 to 12% relative abundance) that was completely absent from the spectrum of CBC; the base peak, m/e 231, was common to the mass spectra of both cannabinoids (see Fig. 1).

A high proportion of samples analyzed by GC and GC-MS, particularly of marihuana, contained only very small amounts of CBD; in many samples CBD could not be detected. These findings reflect the TLC results obtained by the RCMP for the same samples and concur with recent literature on the analyses of marihuana samples from various origins. Turner and others [8] analyzed over 100 marihuana samples grown from seeds of various known geographical origins by methods which distinguished between CBC, CBD, and CBL. They found that CBD was undetectable in some samples or only present in trace quantities, whereas some other samples contained relatively large quantities of CBD. The amount of CBC was also variable, and with a few notable exceptions was often present in greater quantities than CBD. De Faubert Maunder [15], using various TLC systems, also showed that CBD was absent from some samples of marihuana.

In the older literature, Δ^9-THC, CBD, and CBN were considered the major cannabinoids in marihuana and hashish samples; this is often the case. A recent Canadian decision [7]

was partly based on failure by an RCMP laboratory to detect CBD in a marihuana sample. This failure contributed to the conclusion that there was "reasonable doubt" as to the identity of the sample, even though Δ^9-THC and CBN were identified. The present work and work of other laboratories clearly demonstrates that CBD may indeed be absent from some samples of marihuana and present in very small quantities in many others.

It is likely that many previous analytical procedures based on GC have not distinguished between CBD and CBC. On most systems the two cannabinoids have close or completely overlapping retention times. Fairburn and Liebman [16] quantitated the CBD present in different varieties of marihuana. However, the GC method did not distinguish between CBC and CBD; therefore, their results for CBD may be erroneously high. They confirmed the presence of CBD in those samples by a selective TLC method which, at the same time, showed CBD was absent from many other marihuana samples although the authors state that a GC peak was observed "corresponding to CBD" (probably CBC).

Small and Beckstead [12] studied the cannabinoid content in 350 stocks of *Cannabis*, with particular emphasis on the CBD and THC content. The authors recognized that on some GC systems CBC could be mistakenly identified as CBD but claimed that their system separated these cannabinoids. However, it is likely that the GC peak they identified as CBC was in fact CBL, a cannabinoid with a GC-MS similar to CBC (see Fig. 3) and stated by Turner [9] to be evident whenever CBC is chromatographed (GC); CBC probably overlaps CBD on their system (OV-7). On similar systems (OV-101, OV-3, OV-7, OV-17) examined in our laboratories and those of Turner [9,10], CBD is virtually unresolved from CBC. The quantitation of CBD in some of the samples reported by Small and Beckstead [12] may therefore be erroneously high although their results clearly demonstrate the large variations that can occur in cannabinoid content of marihuana samples.

Conclusions

All 91 samples identified as *Cannabis* material (marihuana or hashish) by the RCMP methods of analysis (microscopic examination for marihuana, a modified Duquenois-Levine color test, and TLC analysis followed by a selective spray reagent) are confirmed to contain cannabinoids by GC and GC-MS analyses. In addition, 2 samples concluded to be negative for cannabinoids by the RCMP because of an inconclusive Duquenois-Levine test (although the TLC test was positive for Δ^9-THC and CBN) are concluded by us to contain at least Δ^9-THC and CBN; both extracts (one pipe extract and one unidentified) were low in cannabinoid content. Two other samples deduced by the RCMP *not* to be *Cannabis* materials and 5 blank extracts supplied by the RCMP (of non-*Cannabis* plants) were confirmed to be devoid of cannabinoids by GC and GC-MS.

We wish to emphasize that the majority of *Cannabis* samples examined contained very low levels of CBD. In some instances CBD was absent or not distinguishable from the greater quantities of CBC present. The latter cannabinoid was identified by GC and GC-MS in all *Cannabis* samples examined except those in which a greater quantity of CBD masked its presence. Although CBD and CBC are difficult to separate on GC, they are efficiently separated on TLC.

Extracts of individual TLC-separated compounds, concluded by the RCMP to be cannabinoids present in 20 confiscated materials, were made and supplied to us by that agency. Our GC-MS analyses of these extracts have further confirmed, in every case, the accuracy of the identification of CBC, CBD, Δ^9-THC, and CBN by RCMP procedures.

We believe that our study has shown conclusively that the three parameters approach used by the RCMP to identify *Cannabis* material is specific and unequivocal. We also conclude that TLC identification of only two cannabinoids (Δ^9-THC and one other) in an extract is sufficient evidence that the source of the extract was *Cannabis* material (marihuana, hashish, or a preparation containing the two cannabinoids). It is neither necessary

nor wise to specify which other cannabinoid should be identified since the proportions of cannabinoids will vary, depending on the origin of the marihuana or hashish sample (such as growth conditions, strain, or storage). Both Δ^9-THC and CBN were detected in all the *Cannabis* preparations examined by us, although in fresh or young samples of marihuana CBN may be undetectable [16]; the CBN content of marihuana preparations increases with age [15]. In addition, identification of marihuana or its preparations should not rely on the detection of CBD, since this cannabinoid is often absent or undetectable. Cannabichromene is now becoming recognized as one of the main cannabinoids and is present in most *Cannabis* preparations [17]. Its presence, as well as Δ^9-THC, in an extract is in our opinion conclusive evidence that the material in the extract originated from a *Cannabis* preparation.

Summary

Extracts of 100 plant-like or resinous materials were analyzed for CBD, CBC, Δ^9-THC, and CBN by GC using two different column packings and by GC-MS. Our independent identification of these cannabinoids confirmed those of other forensic science analysts who used microscopic examination, the Duquenois-Levine color test, and TLC for their analyses of the same samples. The identifications of cannabinoids by forensic science analysts using TLC were corroborated by GC-MS analysis of hexane extracts of appropriate chromatogram spots.

Acknowledgments

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