J Forensic Sci, Oct. 1972, Vol. 17, No. 4

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The Specificity of the Duquenois Color Test for Marihuana and Hashish

The Duquenois color test [*1,2*], coupled when possible with botanical examination [*3*], is regarded as one of the most reliable indications of the trace presence of cannabinoids, the physiologically active constituents of marihuana and hashish [*4*]. Consequently, this color test is used widely in forensic laboratories in the United States. Analysis of extracts from a substantial number of plant families has failed as yet to discover any exception to the specificity of the test [*3,5*]. However, it is clear that an understanding of the chemical basis of the test would allow a better appreciation of the molecular features which are necessary for a positive color test, and an increased confidence in its specificity. With this end in mind, we have studied the minimum structural features of the cannabinoid skeleton which are necessary for a positive test.

Experimental Procedures

The Duquenois reagent was prepared by dissolving vanillin (8 g) and acetaldehyde (1 ml) in 95 percent aqueous ethanol (400 ml). Samples (2 mg) of compounds under investigation were dissolved in this reagent (5 ml) and 37 percent aqueous hydrochloric acid (5 ml) was then added. After 20 min the absorption spectrum was obtained by diluting an aliquot of this solution in ethanol and scanning from 350 to 800 nm using a Cary 14 spectrophotometer. The intensities recorded in Table 1 represent the dilution of the initial 10 ml of test solution which was necessary to obtain an absorbance of 1.0 using a 1-cm cell pathlength. For example, dilution of the 10 ml to a volume of 15 ml represents an intensity of 1.5 units. It was necessary to calculate intensities in this manner because neither the extinction coefficients of the maxima nor the weights of the compounds responsible for the maxima are accessible.

The monocyclic phenols listed in Table 1 were obtained from commercial sources. The chromane derivatives were synthesized by procedures in the literature [*6,7*]. Tetrahydrocannabidiol was prepared by catalytic hydrogenation of cannabidiol [*8*] and purified by elution from a silver nitrate-silica gel column. The dimethyl ether of cannabidiol was prepared by the reaction of cannabidiol with methyl iodide and potassium carbonate in dimethylformamide [*9*]. The benzyl ether of Δ^8-THC was prepared by the same procedure, using benzyl bromide as the alkylating agent. The structures of these derivatives were confirmed by NMR (nuclear magnetic resonance) and high resolution mass spectrometric analysis.

Received for publication 15 Feb. 1972; accepted for publication 5 May 1972.

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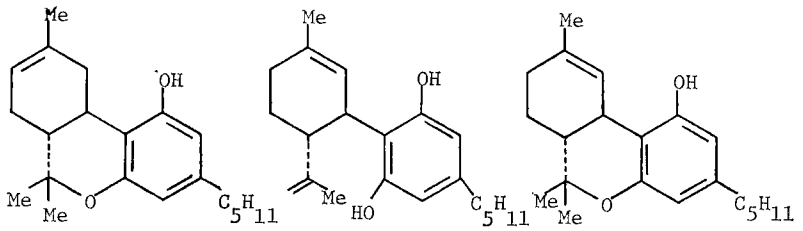
TABLE 1—*Visible absorption spectra of D reaction with cannabinoid analogs.*

Substrate	Absorption Maxima and Unit of Intensity[a]			Visual Color
Duquenois Reagent	413 (1.4)	[b]	605 (0.1)	Yellow-green
Phenol	412.5 (1.4)	[b]	590.5 (0.4)	Pale green
1,2-dihydroxybenzene	415 (1.0)	[b]	614.5 (0.2)	Pale green
1,3-dihydroxybenzene	424 (1.6)	501 (1.8)	591.5 (1.9)	Red-blue
1,4-dihydroxybenzene	414 (1.0)	[b]	625 (0.15)	Pale-green
2-methyl-1,3-dihydroxybenzene	422.5 (1.4)	496 (2.0)	595 (0.8)	Red-purple
5-methyl-1,3-dihydroxybenzene	427 (1.4)	503 (2.6)	599 (3.1)	Blue-purple
2,2-dimethylchroman-5-ol	417.5 (1.2)	502 (0.9)	605 (1.1)	Blue-purple
2,2-dimethylchroman-7-ol	418.5 (1.2)	494 (0.8)	605 (2.4)	Blue-purple
2,2,7-trimethylchroman-5-ol	416.5 (1.0)	[b]	592.5 (9.3)	Deep blue-violet
2,2,5-trimethylchroman-7-ol	421 (1.4)	510.5 (2.5)	609.5 (5.7)	Deep blue-violet
Cannabidiol	415.5 (1.4)	[b]	592.5 (14.4)	Deep blue-violet
Tetrahydrocannabidiol	419 (1.2)	507 (2.0)	597.5 (1.5)	Blue-purple
Cannabidiol dimethyl ether	414.5 (1.0)	[b]	598.5 (1.0)	Blue
Cannabinol	413.5 (0.9)	[b]	602 (1.6)	Blue
Δ^8-THC	418.5 (0.6)	[b]	594 (12.2)	Deep blue-violet
Δ^8-THC benzyl ether	413.5 (1.1)	[b]	602 (0.9)	Green-blue
Δ^9-THC	412.5 (1.1)	[b]	592 (10.3)	Deep blue-violet

[a] Refer to "Experimental Procedures" in test.
[b] 500-nm band obscured by strong neighboring absorption bands.

Results and Discussion

The Duquenois test involves treating the sample under investigation with an aliquot (for example, 1–5 ml) of a standard solution consisting of vanillin (x g) and acetaldehyde (10 x drops) in 95 percent ethanol (50 x ml), followed by an equal aliquot of concentrated hydrochloric acid. If the cannabinoids cannabidiol, Δ^8- or Δ^9-tetrahydrocannabinol (formula 1) are present in the test sample, an intense blue-violet color develops immediately. On shaking with chloroform the blue-violet color is extracted into the organic phase.



Δ^8-THC Cannabidiol Δ^9-THC (1)

Using both this visual color and the absorption spectrum of the ethanol solution as criteria of the specificity of the test, we have examined a number of compounds which possess, to varying degrees, the structural features of the cannabinoid skeleton. The absorption spectrum derived from Δ^9-THC is shown in Fig. 1, and demonstrates that the chromophore which is primarily responsible for the blue color of the test has a maximum at 592 nm. There are also weaker, partially obscured bands at approximately 650, 500, and 425 nm. Interestingly, the Duquenois reagent itself, when mixed with hydrochloric acid, exhibits absorption at 600 nm, but this band is very weak (<10 percent) relative to a much stronger band at 412 nm and a yellow-green color is observed. The results for the other compounds studied are summarized in Table 1.

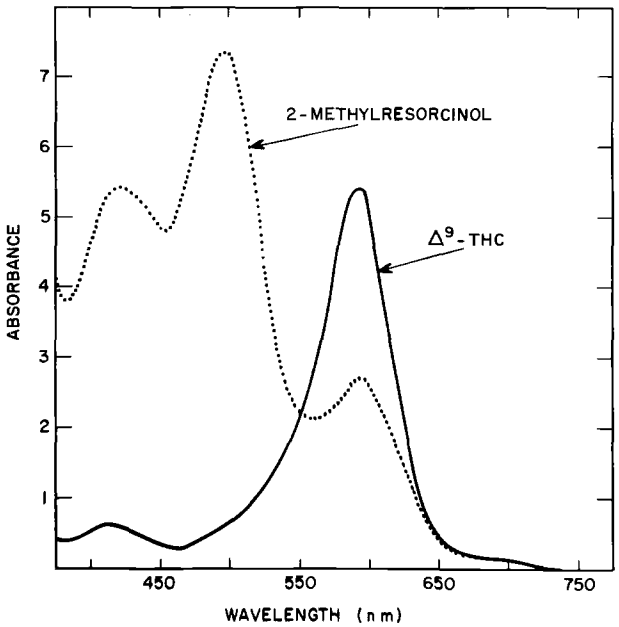


FIG. 1—*Visible absorption spectra of* Δ^9*-THC AND 2-methylresorcinol.*

With the possible exception of 1,2- and 1,4-dihydroxybenzene, all of the compounds in Table 1 exhibit a band in the region of 590 nm which is stronger (relative to the 412 nm band) than that observed for the Duquenois reagent itself. There is a slight variation (<10 nm) in the actual maximum of the 590-nm band, but this is attributed to minor substituent effects rather than a change in the basic chromophore; the overlap of other bands will also contribute to this slight variation. Furthermore, the relative intensities of the bands change with time, and at different rates with different compounds. To compensate for this, all of the data in Table 1 were obtained after a fixed time of 20 min.

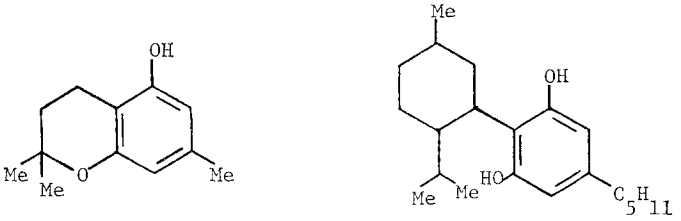
It is seen in Table 1 that the basic functionality responsible for the 590 nm absorption band is the oxygenated benzene ring, and this band is weakly observed even in the simplest case of phenol. However, despite the presence of this band in the case of phenol and 1,3-dihydroxybenzene (resorcinol) and their monoalkyl derivatives such as 2-methyl- and 5-methyl-resorcinol, the visual color ranges from pale green to at best a blue-purple hue. This is because the human eye detects only the resultant of the three bands at 590, 500, and 420 nm, and the greater intensity of the latter two bands relative to the 590-nm band (see Table 1) produces visual colors which differ from that when the 590-nm band is dominant.

Since the intensities were all measured after a 20-min time period, they reflect the relative efficiency (rate + yield) with which the substrates are converted to the 590-nm chromophore. Table 1 shows that the absolute intensity of the 590-nm band varies from ca. 0.3 in the case of phenol, to 14 in the case of cannabidiol which is the most sensitive substrate. This corresponds to a sensitivity factor of ca. 50 on a weight basis, and 150 on a molar basis. On the other hand the intensity of the 420-nm band is essentially constant (ca. 1) and must originate primarily from the Duquenois reagent itself. The 500-nm band

also remains fairly constant in intensity (<2.6), and in many cases is obscured by the neighboring bands. However, the 500-nm band does play an important role in determining the visual color when the absolute intensity of the 590-nm band decreases, and is responsible for the reddish hue observed for the simpler 1,3-dihydroxybenzenes and chromanols.

The rate of the formation of the 592-nm chromophore relative to other chromophores is important. For example, 1,3,5-trihydroxybenzene immediately gives a bright red color when treated with the Duquenois reagent. However, on standing for some time (>30 min) the color changes to the deep blue associated with a positive test. A similar change to a positive test is observed with phenol and 1,2-dihydroxybenzene, where the initial color is yellow-green, and the change to blue is over a number of hours.

The simplest molecule of those tested which gives an immediate positive visual test is 2,2,7-trimethylchroman-4-ol (formula 2). The positive response of this compound is not surprising since it possesses two of the three rings of the cannabinoid skeleton, and Table 1 also shows that alkylation of resorcinol in the 2 or 5 positions enhances sensitivity.

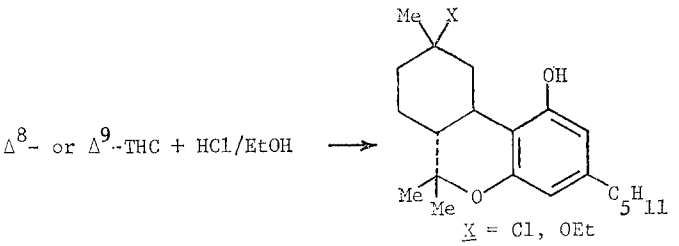


(2)

2,2,7-Trimethylchroman-5-ol Tetrahydrocannabidiol

However, the fact that tetrahydrocannabidiol (formula 2) gives only a weakly positive test does suggest that 2,5-dialkylation of resorcinol is not the optimum structural requirement for optimum sensitivity. Possibly formation of the heterocyclic chromane ring reduces the steric effects of the 2- alkyl substituent and enhances the reaction with the Duquenois reagent.

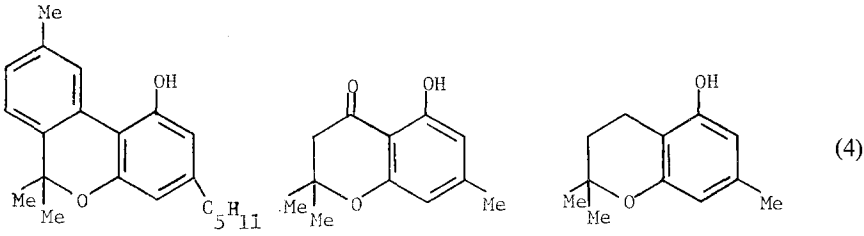
Cannabidiol, Δ^8- and Δ^9-THC all give positive tests and similar absorption spectra when treated with the Duquenois reagent. Since cannabidiol is at least partially converted to Δ^8- and Δ^9-THC under the acidic conditions of the test (approximately 25 percent conversion after 1 min), and both Δ^8- and Δ^9-THC are converted to the same C-9 derivatives (formula 3) [4], this result is to be expected.



(3)

Derivatization of the phenolic hydroxyl group appears to retard the Duquenois reaction. Thus the intensities of the 590-nm band in the spectra of Δ^8-THC O-benzyl ether and cannabidiol O-dimethyl ether are substantially reduced (Table 1), and the visual color of the former is green-blue.

While cannabinol (formula 4) gives a positive visual color, the intensity of the absorption spectrum is relatively weak and comparable to that obtained for the alkylated resorcinols. The reason that cannabinol gives a positive visual test, whereas the resorcinols do not, is the absence of the 500-nm band in the former. The reduced sensitivity of cannabinol, relative to Δ^8- and Δ^9-THC, must be related to the presence of the second aromatic ring which is in conjugation with the phenolic ring. A similar reduction in sensitivity is observed in the chromane series when a group in conjugation with the aromatic ring is present. For example, 2,2,7-trimethylchromane-5-ol-4-one (formula 4) fails to give a positive visual test, in contrast to the saturated analog, 2,2,7-trimethylchromane-5-ol.



(4)

Cannabinol | 2,2,7-trimethylchroman-5-ol-4-one | 2,2,7-trimethyl-chroman-5-ol

Chloroform Solubility

One of the requirements of a positive Duquenois test, first introduced by Levine [*2*], is that on shaking the test solution with chloroform, the blue color is transferred to the organic phase. Of the compounds shown in Table 1, only the monocyclic compounds fail to meet this requirement. This indicates that the compound which is responsible for the 590-nm band is relatively polar and that chloroform solubility will only be observed when there are sufficient lipophilic substituent groups in the molecule. Building up the hydrocarbon side chains and the ring skeleton will clearly increase the lipophilicity and so chloroform solubility is not a very exacting requirement. However, chloroform solubility will serve to exclude phenols which occur in plants as polyhydroxylated derivatives. Carrying out the Duquenois test on an evaporated petroleum extract of the substrate under investigation achieves a similar purpose and, in addition, excludes naturally occurring plant materials which are strongly intracellularly bound.

The Chemical Role of Acetaldehyde, Vanillin and Hydrochloric Acid

As a first step toward the determination of the products of the Duquenois color test, the chemical roles of the reagents employed in the test were studied. It was found that the positive blue color does appear even if acetaldehyde is omitted from the Duquenois reagent, albeit more slowly, and the same absorption maximum at 590 nm is obtained. This suggests that acetaldehyde may be functioning as an oxidizing reagent. On the other hand mass spectroscopic analysis of the reaction products indicates that acetaldehyde is incorporated into the molecular structure of the products in some manner. Furthermore, formaldehyde, propionaldehyde and butyraldehyde cannot be substituted for acetaldehyde [*1*,*2*]. Also when the test is carried out in the absence of acetaldehyde, TLC analysis shows the product mixture is substantially more complex, despite the positive coloration obtained. Acetaldehyde must therefore be considered unique.

[2] Metaldehyde, $(C_2H_4O)_n$, a polymeric form of acetaldehyde may be substituted for acetaldehyde [*5*]. However, under the acidic conditions of the test, it is expected to behave in a chemically similar manner.

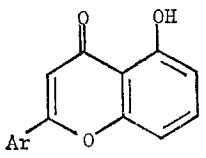
Using benzaldehyde in place of vanillin produces a deep red solution which slowly changes to brown; *p*-hydroxybenzaldehyde affords a dark red-blue solution; 2,4-dimethoxy- and 3,4-dimethoxybenzaldehyde both produce deep blue solutions. These results with the latter two compounds indicate that the hydroxyl group of vanillin is not derivatized in the Duquenois reaction, but probably functions both as an auxochrome and to activate the aldehydic carbon toward electrophilic or oxidative or both attack.

The role of hydrochloric acid in the Duquenois color test is two-fold. First, it catalyzes the reaction of vanillin or acetaldehyde or both, with the phenolic ring of the cannabinoid. Second, the absorption maxima of the reaction products are pH dependent. In neutral solution (pH 7) the products absorb at 470 nm and are caramel colored, but under the acidic conditions of the test the absorption band is shifted to 590 nm and the characteristic blue coloration is obtained.

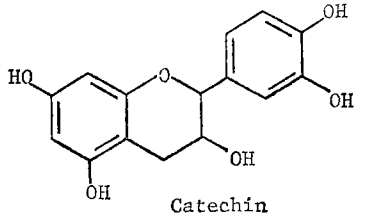
The compound(s) responsible for the 590-nm absorption band are formed in very low yield (< 1 percent) and have not yet been obtained in sufficient amounts to allow complete identification. However, mass spectroscopic and combustion analyses indicate the empirical formula of the major product from Δ^8-THC is $C_{33}H_{40}O_4$, corresponding to [vanillin + Δ^8-THC + 2 acetaldehyde − 3 water]. Based on the findings that (a) the 590-nm chromophore is produced even in the case of phenol, (b) the reaction is facilitated by additional hydroxyl substituents on the aromatic ring, and (c) the reaction is acid catalyzed, it is likely that the Duquenois reaction proceeds via electrophilic substitution of the aromatic ring of the substrate by the protonated aldehydic group of vanillin or acetaldehyde or both.

Conclusions Concerning the Specificity of the Duquenois Color Test

This study has demonstrated that the Duquenois color test for cannabinoids is chemically based primarily on the presence of the 1,3-dioxybenzene (resorcinol) partial structure. A survey of the chemical literature on phenolic plant materials shows that there are a substantial number of naturally occurring compounds, for example the flavonoids [*10*] which contain this partial structure. Fortunately, however, the great majority of such compounds also contain other structural features, for example, conjugating groups, which retard the Duquenois reaction. For example, the isoflavones possess a ketonic group in the 4-position (formula 5) which in the chromane series completely suppresses the

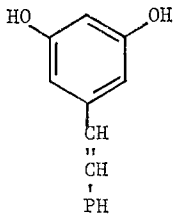


5-Hydroxyflavones

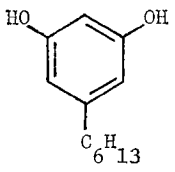


Catechin (5)

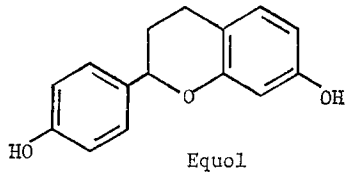
Duquenois color reaction. Also the flavonoids are generally alkylated in only one position (C-2) of the resorcinol nucleus, whereas the optimum sensitivity is observed with dialkylated (C-2, C-5) resorcinols. The catechins (formula 5) are derivatives of 1,3,5-trihydroxybenzene, a compound which gives an initial red coloration, but which changes to a blue-violet coloration on standing a short time. Pinosylvin [*11*] (which occurs in pine heart wood), 4-hexylresorcinol (sold commercially as Sucrets), and equol (which is present in horse urine) [*12*] are other examples of resorcinols which contain at least part of the structural features (formula 6) required for a positive Duquenois test. As expected for a



Pinosylvin



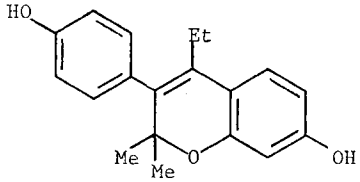
Sucrets



Equol

(6)

5-alkylresorcinol, the color reaction of Sucrets is initially red, slowly developing a violet coloration. The *synthetic* isoflavene (formula 7), which was prepared [*13*] for contraceptive evaluation slowly generates a green-blue color and when shaken with chloroform the organic phase develops a blue coloration.



(7)

In conclusion, it is believed that if the criteria [*1*,*2*] for a positive Duquenois test are rigorously adhered to, and botanical evidence is also available, then the Duquenois color test is a reliable screen for cannabinoids. However, if botanical evidence is not available, the ubiquitousness of phenols in nature and their diversity of structure makes it mandatory to supplement the colorimetric test with chromatographic evidence. This conclusion is substantiated by the recent report [*14*] that certain commercial brands of coffee give a positive Duquenois-Levine color test.

Summary

The specificity of the Duquenois color test for cannabinoids, the physiologically active constituents of marihuana and hashish, has been evaluated by determining the minimum structural features necessary for a positive test. Using both the visual color and the absorption spectrum as a criterion of specificity, it has been shown that the resorcinol part structure is a necessary but not sole prerequisite. The validity of the Duquenois color test is discussed.

Acknowledgments

Financial support of this work by the Law Enforcement Association Agency and the State of North Carolina is gratefully acknowledged. Mass spectroscopic analyses were obtained at the Research Triangle Center for Mass Spectrometry under NIH Grant No. P07 RR 00330-03 MCHO. We are indebted to the members of the North Carolina State Bureau of Investigation for many helpful discussions.

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