Analysis of the Fixed Oil of *Clitocybe illudens* Schw.

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The fixed oil of the poisonous Jack O'Lantern mushroom has been analyzed, and the usual physical and chemical constants for fats have been determined. The fatty acids, as methyl esters, were resolved by gas chromatography using a liquid phase of 25 per cent diethyleneglycolsuccinate polyester and 2 per cent phosphoric acid on Chromosorb W and a thermal conductivity detector. The fatty acids present in the oil include myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, and linoleic acids. The unsaponifiable fraction was analyzed by gas chromatography using a liquid phase of 0.4 per cent silicone gum rubber on Chromosorb W and a hydrogen flame ionization detector. Ergosterol accounted for 76.2 per cent of this fraction.

CLITOCYBE ILLUDENS, known as the "Jack O' Lantern" because of its phosphorescent glow, has been classified as a poisonous mushroom which causes nausea, vomiting, diarrhea, and prostration (1). Ford (2, 3) found no hemolysins in this species, but reports that acute intoxication in guinea pigs is fatal in 1 to 7 days. Clark and Smith (4) claim that muscarine or a muscarine-like compound is present in extracts of the plant based on atropine-reversal of the effect on frog heart. Though still classed as a poisonous mushroom (5, 6), no chemical investigation of the plant has been reported.

These investigations report results of analysis of the petroleum ether extract of *C. illudens*. Studies of other fractions are currently under investigation.

EXPERIMENTAL

The plants were collected from a single location in Ann Arbor, dried at 45°, and reduced to a 20-mesh powder in a Wiley mill. The milled plant (1.865 Kg.) was extracted with petroleum ether (b.p. 30–60°) for 5 days in a Soxhlet apparatus. The extract was transferred to a weighed flask, and the solvent was removed by distillation. The oil was freed from traces of solvent and moisture by heating the flask at 110° under reduced pressure. A total yield of 51.6 Gm. of oil, or 2.8%, was obtained; it was stored under nitrogen at refrigerator temperature.

Physical Constants.—The specific gravity was determined by the pycnometer method and the refractive index, using an Abbe refractometer.

Sp. gr. $\frac{20°}{20°}$ = 0.9611; $n^{20.8}_{D}$ = 1.4634 (average of 5). The oil showed no optical rotation.

Chemical Constants.—Except for the iodine value, chemical constants were determined by standard procedures (7). Because of the limited amount of oil available, the micro method of Toms (8) was used to determine the iodine value. Table I lists the chemical constants determined for the oil.

Isolation of the Fatty Acids.—Two grams of the oil was saponified in 25 ml. of 0.5 *N* alcoholic KOH by refluxing on a steam bath for 2 hours. The soap solution was separated from the unsaponifiable material, concentrated to approximately half its volume, diluted with 10 ml. of water, and the solution washed with three 20-ml. portions of ether. The free acids were regenerated with HCl solution and extracted with ether until the aqueous layer was neutral to Congo red paper. The ether extracts were combined, dried over anhydrous sodium sulfate, filtered, and the solvent removed under reduced pressure at 45°. The fatty acids were freed of traces of solvent by bubbling nitrogen through the liquid. The mixture was stored under nitrogen at refrigerator temperature.

TABLE I.—CHEMICAL CONSTANTS OF THE FIXED OIL

Sample	I	II
Saponification value	149.7	149.8
Unsaponifiable matter, %	11.4	11.3
Acid value	50.3	51.1
Iodine value	138.4	138.2
Reichert value	1.0	1.0
Polenske value	5.1	5.2
Kirschner value	0.5	0.5

TABLE II.—RETENTION TIMES OF KNOWN METHYL ESTERS OF FATTY ACIDS

Methyl Ester	Retention Time, min.
Caproate	1.62
Caprylate	2.22
Caprate	3.24
Laurate	4.68
Myristate	7.08
Myristoleate	8.76
Palmitate	11.04
Palmitoleate	13.32
Stearate	17.40
Oleate	20.40
Linoleate	26.05
Arachidate	27.79

Gas Chromatography of the Fatty Acids.—The fatty acids were converted to their methyl esters by the acid catalyzed esterification method (9). Twelve known fatty acids[1] were esterified in like manner.

The apparatus used was the F&M Scientific Corp. model 300 gas chromatograph equipped with a thermal conductivity cell. The column, 10-ft. × 1/4-in. (O.D.) stainless steel, was packed with 25% diethyleneglycolsuccinate polyester and 2% phosphoric acid on Chromosorb W, 60–80 mesh.

Helium was used as the carrier gas at a flow rate of 60 ml./minute with an inlet pressure of 50 p.s.i. The column temperature was maintained at 205°, the injection port at 250°, and the detector block at 275°. The bridge current was maintained at 150 ma., the attenuation at a setting of 2; the recorder chart was driven at a speed of 20 in./hour.

A Hamilton 10-μl. capacity syringe was used for injection of samples. One-microliter quantities of the known methyl esters and both 2-μl. and 3-μl. quantities of the unknown mixture of methyl esters were used without dilution.

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[1] Purchased from K & K Laboratories, Inc., Jamaica, N. Y.

TABLE III.—GAS CHROMATOGRAPHIC RESULTS OF THE ANALYSIS OF THE ESTERIFIED FATTY ACID FRACTION OF *C. illudens* OIL

Peak No.	Retention[a] Time, min.	Confirmed by Enrichment as	Methyl Ester, %[a]	% Expressed as Free Acid
1	7.15 ± 0.02	Myristate	0.4 ± 0.1	0.4
2	8.84 ± 0.12	Myristoleate	0.6 ± 0.2	0.6
3	11.08 ± 0.19	Palmitate	17.2 ± 1.1	17.2
4	13.40 ± 0.21	Palmitoleate	1.1 ± 0.2	1.1
5	17.64 ± 0.27	Stearate	3.9 ± 0.2	3.9
6	20.81 ± 0.32	Oleate	36.1 ± 1.7	36.2
7	26.24 ± 1.27	Linoleate	40.7 ± 0.8	40.7

[a] Average of eight determinations.

Comparison of the retention times of the unknown compound with those of the known compounds was used for identification. Each unknown peak was confirmed by the enrichment method. Table II lists the retention times of the known methyl esters.

Quantitative estimates of the proportion of each compound present in the unknown mixture were based on calculation of the area under each peak. Table III lists the averages of eight gas chromatographic determinations of the unknown mixture of methyl esters. Standard deviations were calculated in the usual manner. The percentages of free acids were calculated from the molecular weights of the acids compared with their methyl esters and the proportion of each acid then calculated as per cent.

Gas Chromatography of the Unsaponifiable Matter.—The fraction used for the determination of unsaponifiable matter was analyzed for steroid content. The method of Horning, *et al.* (10, 11), was utilized in these investigations.

The F & M Scientific Corp. model 609 gas chromatograph, equipped with a flame ionization detector, was used. A 6-ft. × 1/4-in. (O.D.) stainless steel column was packed with 0.4% silicone rubber (SE-30) on Chromosorb W, 60–80 mesh. The operating conditions were—flow rates as indicated on the rotometer—helium, 8.5; hydrogen, 9.0; air, 8.5; column temperature, 222°; injection port temperature, 302°; detector block temperature, 350°; chart speed, 20 in./hour, attenuation, 8; range, 1000.

A saturated solution of ergosterol (<2%) and 2% solutions of β-sitosterol and stigmasterol in benzene were used as controls.[2] Samples were injected in 5 or 7-μl. quantities using a 10-μl. capacity Hamilton syringe; a 2% solution of the unsaponifiable matter in benzene was analyzed in the same manner. Comparison of the retention times and confirmation by the enrichment method were used for identification.

Two peaks appeared in chromatograms of the unsaponifiable fraction. Peak 1, with an average retention time of 9.0 minutes, was broad and did not compare with tested or reported steroids. Peak 2, with an average retention time of 17.62 minutes, compared with ergosterol (retention time, 17.46 minutes). This was confirmed by the enrichment method.

On prolonged storage of solutions of the unsaponifiable matter, peak 1 appeared larger on chromatograms at the expense of peak 2. Cochromatography of the unsaponifiable matter with calciferol and viosterol[3] indicated that peak 1 could be attributed to calciferol. The broadness of the peaks, the impurity of these latter two compounds, and apparent decomposition of them during chromatography prevented confirmation.

The quantitative estimates were determined by calculation of the area under the peaks. The average of four determinations indicated that a quantity of 76.2 ± 1.5% ergosterol occurred in the unsaponifiable fraction.

SUMMARY

The petroleum ether extract of *C. illudens* has been analyzed. The plant contains 2.8% fixed oil on a dry weight basis.

Specific gravity, refractive index, per cent unsaponifiable matter, acid, iodine, Reichert, Polenske, and Kirschner values are reported.

The methylated fatty acid fraction, analyzed by gas chromatography, contained myristic acid, 0.4%; myristoleic acid, 0.6%; palmitic acid, 17.2%; palmitoleic acid, 1.1%, stearic acid, 3.9%; oleic acid, 36.2%; and linoleic acid, 40.7%.

The unsaponifiable fraction, analyzed by gas chromatography, contains 76.2% ergosterol; the remainder appears to be calciferol, but this has not been confirmed.

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[2] Purchased from California Corp. for Biochemical Research, Los Angeles.

[3] Purchased from General Biochemicals, Chagrin Falls Ohio.