[CONTRIBUTION FROM THE FOREST PRODUCTS LABORATORY, UNIVERSITY OF CALIFORNIA]

EXTRACTIVE COMPONENTS FROM INCENSE-CEDAR HEARTWOOD (*Libocedrus decurrens* Torrey) I. OCCURRENCE OF CARVACROL, HYDROTHYMOQUINONE, AND THYMOQUINONE

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Incense-cedar (*Libocedrus decurrens* Torrey) trees are indigenous to the Pacific Coast region from Oregon to lower California. While the tree finds limited use in the normal wood-using channels, it is presently the world's most important pencil wood, because of its soft texture and ease of cutting. The recovery of usable lumber stock from incense-cedar is considered low, because of the susceptibility and high incidence of pecky rot, *Polyporus amarus*, in the heartwood of mature trees. This fungus attacks only the living tree and becomes dormant when the tree is felled. An excellent review on the heartrot of incense-cedar trees is presented by Boyce (1). Interestingly enough, however, the sound heartwood recovered is prized for fence posts, rails, and other uses requiring resistance to decay. No reason appears to have been made as to why the living tree is so susceptible to pecky rot, while the sound heartwood, when exposed to conditions conducive to decay, is so durable. This reputation for durability has not heretofore occasioned any chemical investigations as to the nature of its heartwood extractive components. The present investigation is the first of a series which deals with the isolation and identification of several of its heartwood extractive constituents, some of which are responsible, in part for the decay resistance of incense-cedar.

On steam-distilling the chloroform-soluble components obtained from the acetone extract of incense-cedar heartwood, a 2.9 per cent yield of volatile oil was obtained (dry wood basis). Approximately 83 per cent of the volatile oil was soluble in dilute sodium hydroxide and, while predominately phenolic, it was found to contain a small amount of acidic components which appears to be tropolonic in nature. The recovered phenolic oil was fractionally distilled, *in vacuo*, of which 34 per cent was identified as carvacrol. In addition, a small amount of hydrothymoquinone, 1.08 per cent, was also found to be present among the fractionated products. The neutral fraction contained 21.7 per cent of thymoquinone.

While carvacrol has been found in a number of plant oils, among trees it has only been found in the *Cupressaceae* family of conifers. Among the eighteen genera of this family, carvacrol, thus far, has been found in one species each of *Tetraclinis* (2), *Chamaecyparis* (3), and *Cupressus* (4). The presence of carvacrol in incense-cedar is of biological interest, for it adds another genus of the *Cupressaceae* family containing this phenolic compound.

The presence of hydrothymoquinone and thymoquinone among the heartwood constituents of incense-cedar is perhaps significant, for these appear to be rare wood components, the only recorded instance of their isolation from wood

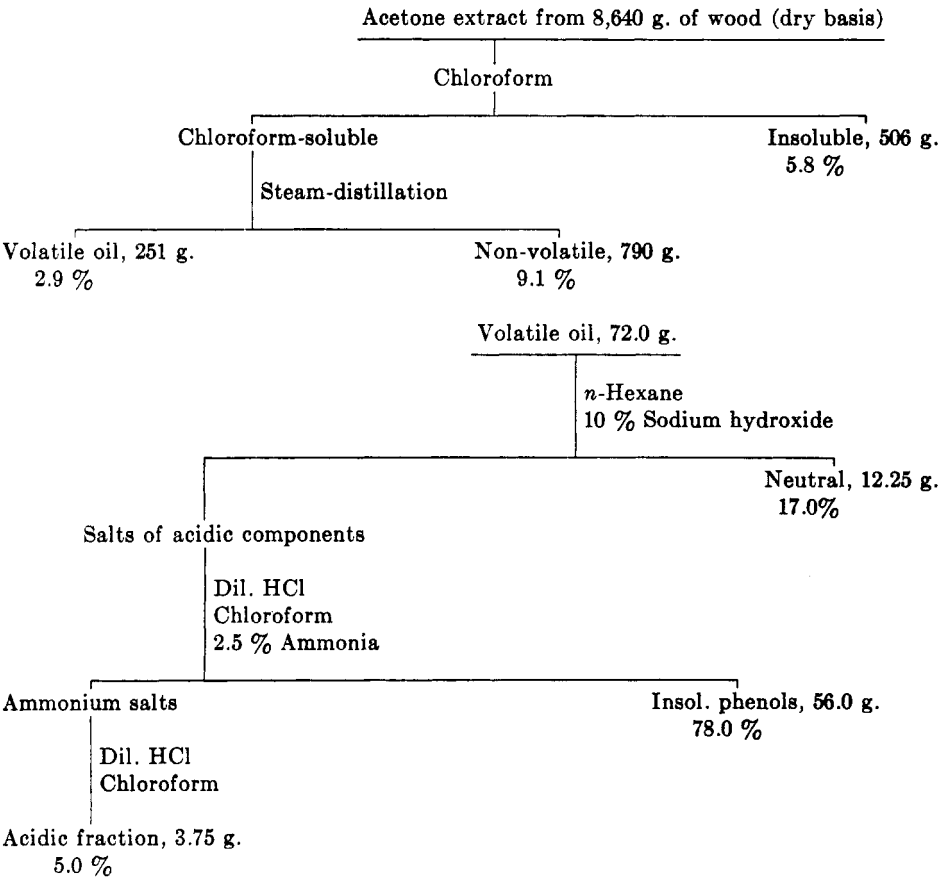

FIGURE I. METHOD OF SEPARATING ACETONE-SOLUBLE EXTRACT

being from *Tetraclinis articulata* (2). While this seems to be only the second instance of the reported isolation of both of these compounds from wood, the question may be raised as to whether hydrothymoquinone and thymoquinone were present, *in situ*, or whether one of the components may have resulted from the other in the process of separation (5). In order to determine their respective behaviors, each of these products was submitted to the same series of steps as employed in the present procedure for isolating and separating the extract into its component fractions as outlined in Figure I. Upon steam-distillation, a portion of the hydrothymoquinone was converted to thymoquinone, while the latter remained unchanged under this treatment. Dilute sodium hydroxide reacted with some of the thymoquinone to form the corresponding hydroquinone, while the latter compound under similar treatment with alkali and in the presence of air partially decomposed to undefinable oxidation products.

It becomes apparent that other procedures had to be employed to ascertain whether thymoquinone or hydrothymoquinone was a secondary product or whether each of these is present in the wood. The existence of hydrothymoquinone

in the wood extract was indicated, for it was readily isolated from non-alkali treated steam-distillate. The co-existence of thymoquinone was likewise indicated by employing chromatography methods.

The occurrence of carvacrol, hydrothymoquinone, and thymoquinone in incense-cedar heartwood is of interest for they have fungicidal properties. The relative toxicity of each of these compounds against various wood-destroying fungi has recently been determined by Erdtman and Rennerfelt (6). Their investigation revealed that hydrothymoquinone is about as active as phenol, while thymoquinone has enhanced toxicity. Carvacrol proved to be a more efficient fungicide than hydrothymoquinone. Hence, the presence of these three compounds in incense-cedar is responsible, in part, for the well-known decay resistance of its heartwood.

EXPERIMENTAL[1]

Extraction of wood. Sound heartwood from the butt log of three incense-cedar trees was ground in a Wiley mill to pass a 6-mm. screen. Air-dried sawdust (9.6 kilograms) was extracted with acetone at room temperature, using 3.5 liters of solvent per kilogram of sawdust. The sawdust was permitted to leach for one week and the acetone was drained. The acetone was recovered from the filtrate by distillation from a water-bath and the recovered solvent was used for further extraction of the sawdust. The procedure was repeated until the extraction liquid contained but a trace of extract.

Component fractionation of extract. The dark red-brown acetone-soluble extracted material was thoroughly triturated with 700 ml. of chloroform. The resulting insoluble material was removed by filtration and washed with chloroform. The brown precipitate was again extracted with 1000 ml. of chloroform, filtered, and washed with the same, leaving 506 g. of brown powdery, largely phlobaphenic material (5.8% dry wood basis). The chloroform filtrates were combined and the solvent was removed by distillation from a water-bath. The residue next was steam-distilled until the distillate no longer gave a green color when shaken with chloroform and copper acetate solution (tropolone test). The non-volatile dark brown semi-solid remaining in the flask was recovered from the aqueous phase, and it weighed 790 g. (9.1% dry wood basis). This residue, which appears to be largely composed of polymeric phenolic compounds, was also set aside. The total steam-distillate was extracted three times with chloroform, and the combined chloroform solution was dried over sodium sulfate, filtered, and the solvent removed by distillation from a water-bath using a 12-inch Vigreaux column. The brown oily residue weighed 251 g. or 2.9% (dry wood basis).

A portion of the steam-volatile extract was next separated into phenolic, acidic, and neutral fractions. For this purpose, 72.0 g. of the volatile oil was dissolved in 1 liter of *n*-hexane and extracted five times with 450-ml. portions of 10% sodium hydroxide solution, followed by washings with water until neutral to phenolphthalein. The alkaline solution and washings were acidified with slight excess of dilute hydrochloric acid and then extracted four times with 100-ml. portions of chloroform. The combined chloroform solution next was extracted three times with 125 ml. of 2.5% aqueous ammonia, then washed with water. The total chloroform solution was dried over sodium sulfate, filtered, and the chloroform removed by distillation. The rather brown-red phenolic oil fraction weighed 56.0 g. or 78.0% of total steam volatile (2.26% dry wood basis).

The combined ammonia solution was acidified with slight excess of dilute hydrochloric acid and extracted three times with 50 ml. of chloroform, and twice with 100 ml. of ethyl ether. The solvent-extract solution was dried over sodium sulfate, filtered, and the solvent removed as before, leaving 3.75 g. of brown viscous acidic oil, 5.0% of total steam-volatile

[1] All melting points corrected, microanalyses by Microchemical Laboratory, University of California, and ultraviolet spectra run on a Beckman DU spectrophotometer.

(0.14% dry wood basis). This acidic fraction gives a very intense green color with copper acetate and chloroform, suggesting the presence of tropolonic constituents. This fraction is being investigated.

The hexane solution remaining after alkaline extraction was dried over sodium sulfate, filtered, and solvent removed by distillation as before. A non-viscous red-brown neutral material weighing 12.25 g. was recovered, or 17.0% of total volatile oil (0.50% dry wood basis).

Distillation of phenolic fraction. The phenolic oil (56 g.) was fractionally distilled, *in vacuo*, using a chrome wire column (7). The various fractions collected follow:

FRACTIONATION OF PHENOLS

Fraction	Wt., gms.	B.P., °C.	Pressure, mm.	n_D^{25}
1	17.74	91.5–94	2.5–3.5	1.5230
2	2.06	94–119	3.5	1.5238
3	22.53	119–119.7	3.5	1.5264
4	7.00	119–120	3.5	1.5274
5	3.60	120–150	3.5	1.5581
Residue	2.50			

Carvacrol. The physical properties of Fraction 1 are substantially those of carvacrol (8). This fraction (520 mg.) was treated with *p*-nitrobenzoyl chloride in pyridine in the usual manner. The derivative was recrystallized from alcohol, giving 904 mg. of the *p*-nitrobenzoate of carvacrol (90.0%), m.p. 51° (lit. 51°) (9) and the melting point was undepressed on admixture with an authentic sample. Similarly, the aryloxacetic acid and phenylurethan derivatives were prepared, m.p.'s 151° (lit. 151°) (9) and 137° (lit. 138°) (10), respectively, melting points unchanged on admixture with authentic derivatives. Fraction 2 also contained some carvacrol and it was estimated from the physical constants that Fractions 1 and 2 consisted of approximately 19.0 g. of carvacrol, or 34.0% of volatile phenolic fraction (0.77% dry wood basis).

The ultraviolet spectrum of carvacrol was determined in 95% ethyl alcohol. It showed a maximum at 277.5 mμ (log ε 3.262), and a minimum at 244.0 mμ (log ε 2.103). No spectrum of carvacrol has yet been reported.

Intermediate fractions. Fractions 3 and 4 appear to be composed of methoxy-phenolic compounds and are presently under investigation.

Hydrothymoquinone. Fraction 5 (3.60 g.) was triturated with 30 ml. of carbon tetrachloride and cooled in an ice-bath. The crystalline precipitate was filtered, washed with chloroform, and dried. There was recovered 0.6 g. of product, m.p. 142–142.5° (lit. 141.5°) (11) or 1.08% of the phenolic fraction (0.024% dry wood basis). Mixture melting point with authentic hydrothymoquinone remained unchanged. This product was further identified by chromic acid oxidation to thymoquinone, m.p. 43.5–44.5° (lit. 45.5°) (11), which did not depress the melting point of authentic thymoquinone.

The ultraviolet spectrum of hydrothymoquinone was determined in 95% ethyl alcohol. It showed a maximum at 293 mμ (log ε 3.692) and a minimum at 253 mμ (log ε 2.585). No spectrum of hydrothymoquinone appears to have been reported.

Preparation of p-nitrobenzoyl derivative of hydrothymoquinone. Hydrothymoquinone (331 mg.) was added to a mixture of 4 ml. of pyridine and 900 mg. of *p*-nitrobenzoyl chloride and heated on a water-bath for one hour. The mixture was cooled, and diluted with 40 ml. of water plus 10 ml. of a saturated solution of sodium bicarbonate. The crystals, which separated, were filtered and recrystallized from 25 ml. of glacial acetic acid, yielding 812 mg. of faintly yellow needles, m.p. 224.7–225.3°, the di-*p*-nitrobenzoate of hydrothymoquinone (88% yield).

Anal. Calc'd for $C_{24}H_{20}N_2O_8$: C, 62.06; H, 4.34; N, 6.03.
Found: C, 62.29; H, 4.41; N, 6.00.

This convenient derivative does not appear to have been previously reported.

Alternate isolation of hydrothymoquinone. Since small amounts of hydrothymoquinone may be formed when thymoquinone is treated with dilute sodium hydroxide solution, it became necessary to demonstrate the presence of the hydroquinone by an alternate method. For this purpose, 68 g. of the aforementioned steam-volatile oil, which had not been treated with alkali, was fractionally distilled, *in vacuo*, into 5 fractions under the same conditions as previously outlined for the fractionation of the phenolic oil. The final fraction (*i.e.*, fraction 5) weighing 3.2 g. was heated with 15 ml. of *n*-hexane, cooled, filtered, and the crystals were washed with 5 ml. of *n*-hexane. The dried product of 0.520 g. of hydrothymoquinone, with m.p. 142–142.5° alone and mixed, corresponds to 0.76% of total volatile oil (0.0222 dry wood basis). This recovery is slightly less than that obtained from the alkali treated extract.

Thymoquinone. The neutral fraction (12.25 g.) was distilled, *in vacuo*, and five fractions were collected as follows:

Fraction	Wt., gms.	B.P., °C.	Pressure, mm.	n_D^{20}
1	0.73	88–98	30	1.4746
2	1.63	98–108	30	1.4833
3	3.46	108–114	30	1.5012
4	1.22	114–134	29	1.5046
5	1.71	134–160	28	1.5199
Residue	3.50			

Fraction 3 (253 mg.) was dissolved in 5 ml. of petroleum ether and cooled in a Dry Ice-acetone bath for 10 minutes. A yellow crystalline product separated which, upon filtering and drying, weighed 70 mg. The filtrate was reduced to a volume of 2 ml. and cooled as before, yielding 18 mg. more of the same product. Subsequent attempts to form additional crystals from the mother liquor failed. A portion of the crystals was purified by sublimation to give a product with m.p. 44.2–44.7° (lit. 45.5°) (11) and showed no depression when admixed with authentic sample of thymoquinone. The compound readily formed hydrothymoquinone, m.p. 142–143°, when reduced with stannous chloride and hydrochloric acid in the usual manner. It was further identified as thymoquinone, for it readily formed the benzenesulfinic acid adduct with m.p. 137–138° (lit. 136°) (12).

In order to estimate the amount of thymoquinone present in the neutral oil, 150-mg. portions of fractions 2, 3, and 4 were titrimetrically analyzed for the amount of quinone present by using the iodometric procedure developed by Willstatter and Majima (13), which consists of determining the amount of iodine liberated from the oxidation of hydrogen iodide in ether solution. In the controls, these fractions absorbed only negligible amounts of iodine, hence the liberated iodine is indicative of the amount of quinone present. This method indicated that fractions 2, 3, and 4 contained 18.5, 56.5, and 35.5% of thymoquinone, respectively, or a total of 2.68 g. (21.7%) in the neutral oil or 0.10% (dry wood basis).

The ultraviolet spectrum of thymoquinone in *n*-heptane showed maxima of 250–251 mμ (log ε 4.310), 258 mμ (log ε 4.260); and 306 mμ (log ε 2.467), with minima of 256 mμ (log ε 4.256) and 278 mμ (log ε 2.216). These values agree favorably with those of Braude (14).

Chromatographic detection of thymoquinone. The existence of thymoquinone in the wood was further demonstrated by means of paper partition chromatography. For this purpose, freshly prepared incense-cedar heartwood sawdust (110 g.) was extracted with petroleum ether for 8 hours in a Soxhlet extractor. The petroleum ether was distilled from a water-bath leaving 2.9 g. of extract.

One gram of the petroleum ether-soluble extract was dissolved in 4 ml. of 75% alcohol and treated with 300 mg. of semicarbazide hydrochloride and 3 drops of conc'd hydrochloric acid for 2 hours and 20 minutes. The reaction mixture then was diluted with 25 ml. of

water and extracted with ethyl ether. The ether extracts were combined and extracted with 25 ml. of 10% sodium hydroxide, followed by 25 ml. of water. The alkaline extract was acidified and extracted twice with 20-ml. portions of ether. The combined ether extracts were dried, evaporated to dryness, and diluted to 50 ml. with alcohol.

The descending chromatography procedure used was similar to that described by Lindstedt (15). The developing solvent consisted of the organic phase of the mixture containing benzene (50 vol.), methanol (1 vol.), isoöctane (50 vol.), and water (50 vol.). The chromagenic agent was a 50% saturated solution of barium hydroxide, which when in contact with the semicarbazone produced an intense yellow color. As little as 1×10^{-7} g. of thymoquinone semicarbazone could be detected. The most satisfactory amount of semicarbazone for obtaining spots with no trailing and giving reproducible R_f values was found to be 4–8 $\times$ 10^{-7} g. (corresponding to the application of 4 μl. of a 0.01–0.02% solution of the compound).

The appropriate amount of the above semicarbazide reacted alcoholic solution (4 μl.) was brought to the paper (15.5 x 56 cm., Whatman No. 1) along with the reference sample of an 0.01% alcoholic solution of thymoquinone semicarbazone. The chromatogram was allowed to run 6 to 8 hours, until the solvent front had reached within 10 cm. of the bottom of the paper. The paper was removed, air-dried, and then sprayed with the barium hydroxide solution. The R_f values of the reference material, thymoquinone semicarbazone, were 0.09–0.11 and corresponding spots were readily detected in the sample derived as indicated from the petroleum ether extract.

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SUMMARY

Carvacrol, hydrothymoquinone, and thymoquinone were identified among the extractive components isolated from incense-cedar heartwood (*Libocedrus decurrens* Torrey). The amounts of the latter two compounds obtained do not necessarily represent the precise quantity, existing *in situ*, because the isolation methods employed were found to change, in part, the relative quantities of each. Other procedures, including filter paper chromatography, were developed to indicate that each of these products does occur in the wood as a primary constituent and is not the result of their inter-conversion. Since these three extractive constituents have fungicidal properties, they appear to be partially responsible for the well-known decay resistance of incense-cedar heartwood.

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LITERATURE REFERENCES

(1) BOYCE, *The Dry-Rot of Incense-Cedar*, U. S. Department of Agriculture, Bulletin No. 871, pp. 1–58. November 10, 1920, Washington, D. C.
(2) GRIMAL, *Compt. rend.*, **139**, 927 (1904).
(3) CARLSSON, ERDTMAN, FRANK, AND HARVEY, *Acta Chem. Scand.*, **6**, 690–696 (1952).
(4) CORBETT AND WRIGHT, *Chem. and Ind.*, **47**, 1258–1259 (1953).
(5) ERDTMAN, in *Progress in Organic Chemistry* Vol. I, ed. by J. W. Cook, Academic Press, Inc., New York, N. Y., 1952, p. 43.
(6) ERDTMAN AND RENNERFELT, *Acta Chem. Scand.*, **3**, 906–911 (1949).
(7) BOWER AND COOKE, *Anal. Chem.*, **15**, 290 (1943).
(8) GUENTHER, *The Essential Oils*, Vol. II, D. Van Nostrand Co., New York, N. Y., 1949, p. 504.

(9) McElvain, *The Characterization of Organic Compounds*, The MacMillan Co., New York, N. Y., 1947, p. 250.
(10) Sherk, *Am. J. Pharm.*, **93**, 115 (1921).
(11) Guenther, *The Essential Oils*, Vol. II. D. Van Nostrand Co., New York, N. Y., 1949, p. 553.
(12) Hinsberg, *Ber.*, **28**, 1315 (1895).
(13) Willstatter and Majima, *Ber.*, **41**, 1171 (1910).
(14) Braude, *J. Chem. Soc.*, 490 (1945).
(15) Lindstedt, *Acta. Chem. Scand.*, **4**, 448–455 (1950).