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Note

Preparative separation by high-performance liquid chromatography of an extract of oak wood and determination of the composition of each fraction

J.-L. PUECH*, P. RABIER and M. MOUTOUNET
Laboratoire des Polymères et des Techniques Physico-chimiques, Institut des Produits de la Vigne, Institut National de la Recherche Agronomique, 9 Place Viala, 34060 Montpellier Cedex (France)
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Oak wood is used to age spirits both because of its mechanical properties, as it provides a good seal, and because of its effect on the organoleptic properties of the spirits. Among the major constituents of this type of wood, lignin and tannins play a major role in the sensory aspects of spirits[1–5]. In this context, interest is centred on extractables in the oak wood. Low-molecular-weight substances derived from oak wood tannins include gallic and ellagic acids, which are found in methanol-containing[6] or acetone–water extracts[7,8] and in ethyl acetate extracts of commercial tannins[9]. Chen[7] and Seikel *et al.*[8] noted the presence of gallotannins and ellagitannins. Monties[10] estimated that the ellagic tannin content in heartwood was 75.4 mg/g after extraction with water–methanol (20:80). Mayer and co-workers[11–14] determined the structure of these ellagitannins in *Quercus sessiliflora* and reported that they were castalin, castalagin, vescalin and vescalagin. Hydrolysis of castalagin gives ellagic acid and castalin and that of vescalagin gives ellagic acid and vescalin. Castalagin and vescalagin are isomers; isomerization involves an epimerization at the C-1 position of the sugar. In more recent work using high-performance liquid chromatography (HPLC) tannins were separated from oak wood[15].

Simple phenols connected with the biosynthesis of lignin and present in oak wood include vanillin, syringaldehyde, coniferaldehyde and sinapaldehyde[8,16–18]. A more complex group of substances referred to as lignan is found in wood; the compound identified in oak wood is lyoniresinol[8,19]. Scopoletin is dominant among the coumarins[20,21] in comparison with umbelliferone and methylumbelliferone. The use of preparative or semi-preparative HPLC has made it possible to separate the phenolic compounds found in various plants[20–25].

The purpose of the work described here was to separate oak wood extractables which were soluble in water–ethanol. The content by weight was determined in each fraction, together with the amount of methoxy groups representing the soluble lignin fraction and finally the total phenolics content. In addition, a direct injection analytical HPLC technique was used to identify and especially to determine certain phenolic compounds previously reported in the literature.

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EXPERIMENTAL

Sample

Chips obtained from Limousin oak wood dried in air for 3 years were macerated for 24 h in water–ethanol (45:55, v/v) and adjusted to pH 4.25 with acetic acid. The solution was concentrated by evaporation and then freeze-dried to obtain a powder.

Determination of methoxy groups

The methoxy groups were separated by boiling in the presence of hydriodic acid, leading to the formation of alkyl iodides. These compounds were removed by a flow of carbon dioxide and trapped in toluene[26]. Determination was carried out using gas chromatograpy to separate methyl and ethyl iodides[27].

Total phenolic compounds

Folin–Ciocalteu reagent[28] was used with gallic acid as the standard. The results are expressed in milligrams gallic acid equivalent.

Preparative liquid chromatography

A Modulprep (Jobin-Yvon) system apparatus was used, fitted with a stainless-steel column (500 mm × 40 mm I.D.). The lower part of the column was fitted with a piston to permit axial compression of the stationary phase [LiChroprep RP-18 (15–25 μm) bonded silica] at 10^6 Pa. A 1-g amount of freeze-dried preparation was placed at the top of the column for each analysis. Detection was carried out at 280 nm. Elution was carried out using a step gradient with methanol–water mixtures from 10:90, increased in 10% (v/v) steps to reach absolute methanol at a flow-rate of 40 ml/min; this technique gave ten fractions (Fig. 1).

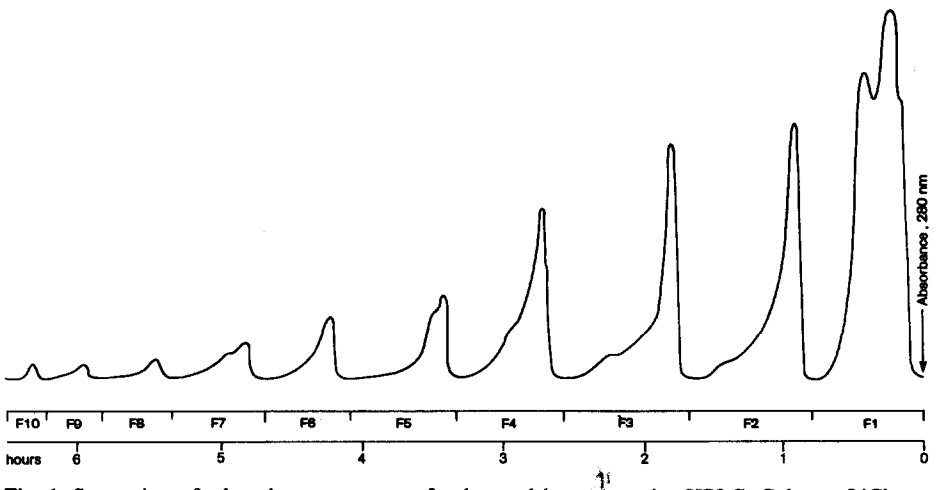

Fig. 1. Separation of ethanol–water extract of oak wood by preparative HPLC. Column, LiChroprep RP-18 (15–25 μm) (50 cm × 40 mm I.D.); mobile phase, methanol–water (10:90) for F_1, methanol–water (20:80) for F_2, etc.; Flow-rate, 40 ml min^{-1}; UV detection, 280 nm.

Analytical liquid chromatography

The fractions obtained were analysed with an apparatus consisting of a Gilson M231 automatic injector, Waters (Millipore, Bedford, MA, U.S.A.) M6000A and M510 pumps, a Waters M680 gradient programmer, a Waters M490 detector operating at two wavelengths (280 and 320 nm), a Shimadzu RF530 fluoroescence detector and an Enica 21 (Delsi) recorder–integrator. A 250 mm × 4 mm I.D. Knauer Vertex column was used packed with LiChrospher RP-18 (5 μm); it was maintained at 40°C in a Waters Model CX 4-2 oven. Solvent A was water–formic acid (98:2) and solvent B consisted of 700 ml of methanol containing 2% formic acid made up to 1000 ml with solvent A. Samples were filtered through a Millipore membrane (0.45 μm) and degassed in an ultrasonic bath; the flow-rate was 1 ml/min. Initially solvent A was used; after 3 min the proportion of B was increased to 100% over a period of 72 min using a linear gradient.

Solutes

Different samples of gallic acid, vanillic acid, syringic acid, ellagic acid, vanillin, syringaldehyde and scopoletin were purchased from Fluka (Buchs, Switzerland). Coniferaldehyde and sinapaldehyde were isolated using the method proposed by Alibert and Puech[29]. Lyoniresinol was a generous gift from Dr. Nabeta (University of Agriculture and Veterinary Medicine, Hokkaido, Japan) and castalin, castalagin and vescalagin were a generous gift from Dr. Mayer (University of Heidelberg, Heidelberg, F.R.G.).

Solvents

HPLC-grade solvents were used.

RESULTS AND DISCUSSION

Weight distribution of fractions and chemical characteristics

Preparative chromatography was used to separate into ten fractions all the

TABLE I

AMOUNTS OBTAINED FROM 5 g OF A FREEZE-DRIED PREPARATION OF ETHANOL–WATER EXTRACT OF OAK WOOD: DISTRIBUTION OF METHOXY GROUPS AND TOTAL PHENOLIC COMPOUNDS IN THE VARIOUS FRACTIONS

Fraction	*Amount in each fraction (mg)*	*Percentage*	*OCH_3 in each fraction (mg)*	*Percentage of OCH_3*	*Total phenolics in each fraction (mg)*	*Percentage of total phenolics*
F_1	2578	53.6	6.0	4.7	1425.0	64.5
F_2	543	11.3	14.1	10.9	308.9	14.0
F_3	436	9.1	30.0	23.3	199.7	9.0
F_4	324	6.8	25.2	19.6	158.0	7.1
F_5	279	5.8	23.5	18.2	90.6	4.1
F_6	263	5.4	18.7	14.6	19.5	0.5
F_7	261	5.4	9.1	7.0	7.0	0.3
F_8	53	1.1	1.8	1.4	1.7	0.07
F_9	30	0.6	0.3	0.2	0.7	0.03
F_{10}	45	0.9	0.2	0.1	1.0	0.04

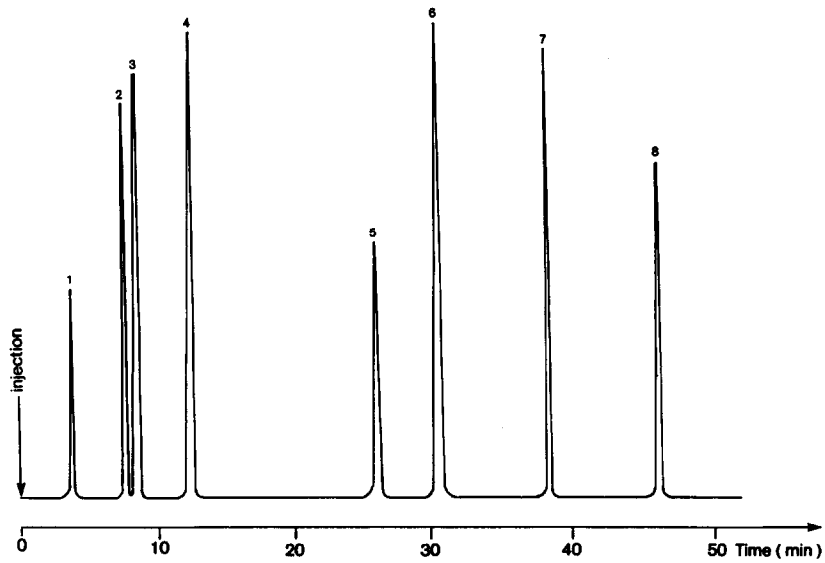

Fig. 2. HPLC resolution of a standard mixture of phenolic compounds. Column, 250 mm × 4 mm I.D.; stationary phase, LiChrospher RP-18 (5 μm); mobile phase, (A) water–formic acid (98:2) (B) 70% methanol–formic acid (98:2) + 30% A, programmed from A (3 min) to B at 75 min; flow-rate, 1 ml min^{-1}; UV detection, 280 nm. Solutes: 1 = castalin; 2 = gallic acid; 3 = vescalagin; 4 = castalagin; 5 = vanillic acid; 6 = syringic acid; 7 = lyoniresinol; 8 = ellagic acid.

compounds present in the freeze-dried preparation obtained after maceration of oak wood in ethanol–water solution. The recovery was 96.3% (Table I). Fraction F_1, the largest by weight, comprised 53.6%; the amounts in the other fractions decreased with increased methanol content in the eluent. The polarity of the substances was thus very widely distributed with eluents containing from 10 to 70% of methanol. The abundance of fraction F_1 demonstrated the predominance of polar substances.

Determination of the methoxy groups was used to assess the lignin content of the various fractions. Fraction F_3 contained the highest methoxy group content (Table I); 75% of these groups were present in the fractions obtained using solvents containing 30–60% of methanol.

With regard to total phenolic compounds, these predominated in fraction F_1; this fraction alone represented 64.5% of the phenolics (Table I). The phenolics contents of the other fractions decreased as the methanol content in the eluent increased.

All these results show that the analysis of each fraction is necessary in order to judge the chemical composition.

Phenolic compounds in the various fractions

The analytical separation of a standard mixture containing castalin, gallic acid, vescalagin, castalagin, vanillic acid, syringic acid, lyoniresinol and ellagic acid with detection at 280 nm is shown in Fig. 2. Aromatic aldehydes (vanillin, syringaldehyde, coniferaldehyde and sinapaldehyde) were detected at 320 nm. For the detection of scopoletin the excitation wavelength was 325 nm and the emission wavelength 454 nm.

TABLE II
PHENOLICS CONTENTS OF THE VARIOUS FRACTIONS OF THE ETHANOL–WATER EXTRACT

Compound	*Fraction*	*Content (mg)*
Castalin	F_1	94.1
Gallic acid	F_1	38.26
Vescalagin	F_1	799.18
Castalagin	F_1	488.78
Vanillic acid	F_2	0.85
Syringic acid	F_2	0.77
Vanillin	F_3	0.31
Syringaldehyde	F_3	0.71
Lyoniresinol	$F_3 + F_4$	23.9
Scopoletin	$F_3 + F_4$	0.091
Coniferaldehyde	F_4	0.29
Sinapaldehyde	F_4	0.51
Ellagic acid	$F_4 + F_5$	41.8

Each fraction place in ethanol–water (55:45) solution was analysed by HPLC. Fraction F_1 was found to contain mainly ellagitannins, confirming the predominance of total phenolic compounds in this fraction. Castalin, castalagin and vescalagin were identified. The vescalagin and castalagin contents in the extract were 799 and 488 mg, respectively (Table II). The castalin content was lower. In addition to these compounds, gallic acid was identified in this fraction. Subsequent fractions contained lignin derivatives consisting of phenolic acids on the one hand and aromatic aldehydes on the other. Fraction F_2 contained low concentrations of vanillic and syringic acid. The amount extracted depended on the ethanol content of the maceration solvent and also on the acidity of the medium[30]; in addition, these compounds can be found in wood in ester form [8]. The benzoic-type phenolic aldehydes vanillin and syringaldehyde were found in fraction F_3. These compounds were also identified in oak heartwood and in sapwood[8].

Both fractions F_3 and F_4 contained the lignan lyoniresinol; this was found at 23.9 mg in the ethanol–water extract of oak wood. Scopoletin were also present in these two fractions but in small amounts, necessitating spectrofluorimetric measurement. This substance was present in larger amounts in American oak wood (*Quercus alba*)[21] than in European woods (*Quercus robur and Quercus petraea*). Fraction F_4 consisted of the cinnamic-type phenolic aldehydes coniferaldehyde and sinapaldehyde identified in oak wood[8,16] and in methanol–water solutions[30]. Fractions F_4 and F_5 contained free ellagic acid[7,8,20], which is a constituent of ellagitannins. None of the substances studied was found in fractions F_6–F_{10}. Work on the identification of these compounds should therefore be undertaken.

CONCLUSION

Use of preparative HPLC made it possible to separate an ethanol–water extract of oak wood and to determine the amounts of methoxy groups in each fraction and total

phenolic compounds where then determined. In addition, new compounds were identified and quantified in these fractions, in particular ellagitannins and a lignan. This methodology makes it possible to obtain sufficient amounts for the identification and assay of a large number of phenolic compounds in oak wood extracts.

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