Global fractionation of oak heartwood extractable polymers (lignins, polysaccharides and ellagitannins) by selective precipitations

Marie-Françoise Nonier, Nicolas Vivas, Nathalie Vivas De Gaulejac, Christelle Absalon, Christiane Vitry and Eric Fouquet

1 Tonnellerie Demptos seconded to the CESAMO (Centre d’Etude Structurale et d’Analyse des Molécules Organiques), Université Bordeaux I, 351 Cours de la Libération, F-33405 Talence, France
2 CESAMO, Université Bordeaux I, Talence, France

Abstract: This paper introduces a new fractionation method by selective extractions and precipitations making it possible to collect the three groups of oak heartwood extractable polymers in a single operation. The three families, lignins, polysaccharides and ellagitannins, were obtained and each was characterized by different techniques. We grouped together the techniques used to characterize these different oak wood groups of polymers: IR, GC, GC-MS, LC-MS, 1H NMR, SEC, MALDI-TOF/MS. This work focuses on the qualitative aspect only.

Keywords: oak wood; fractionation; lignins; polysaccharides; ellagitannins; characterization

INTRODUCTION

Various species of oak wood have traditionally been used in cooperage to make barrels. During ageing of wines or spirits in barrels, only some extractive compounds are solubilized and affect the composition and the quality of the products. As a rule, barrels are made with oak heartwood (Quercus robur, Qu. petraea) and sometimes with other species (Qu. alba, Qu. stellata) or with chestnut (Castanea sativa). The largest extractable fractions are mainly polymerized ellagitannins, lignins and lignans, and polysaccharides. While the overall composition of oak heartwoods, of whatever type, has similar values (40% cellulose, 20% hemicellulose, 25% lignins, 10% tannins), the extractable fractions are more variable. Some studies have placed emphasis on the composition of the cell walls using specific and destructive methods (concentrated acid, heat, etc), producing, for each method used, only one group of insoluble polymers, like Brawne lignin. In our case, we tried to develop a global method that would allow us to collect the three families of wood extractable polymers only once by grouping together the different techniques used to characterize each group of macromolecules. This work focuses on the qualitative aspect to characterize each fraction on the one hand and purity estimate on the other.

MATERIALS AND METHODS

Fractionation method

Selective precipitation of polymerized or combined molecules with high molecular mass was developed

Abbreviations

BF₃ etherate
BSTFA N,O-bis-(trimethylsilyl)trifluoroacetamide
G guaiacyl unit
GC/MS gas chromatography/mass spectrometry
HPLC high-performance liquid chromatography
f constant of coupling
LSIMS liquid secondary ion mass spectrometry
Mp peak-average molecular weight
MALDI-TOF/MS matrix-assisted laser desorption ionization time-of-flight/mass spectrometry
(M–H)− deprotonated species of a molecule
NMR nuclear magnetic resonance
S syringyl unit
SEC size-exclusion chromatography
TMCS trimethylchlorosilane
by Glories\textsuperscript{11} in order to fractionate red wine tannins. Applying the same principle, we finalized a fractionation method by selective precipitation of the main groups of oak heartwood macromolecules. The protocol has three successive stages: precipitation of an insoluble fraction in water (fraction A); precipitation of an insoluble fraction in ethanol/water (9:1, v:v) (fraction B); isolation of an insoluble fraction both in water and aqueous ethanol (fraction C). Characterization methods used were IR spectroscopy associated with techniques more specific to each group.

For the preparation of oak extracts, Sawdust (Q petraea) (60 mesh) was extracted with acetone/water (7:3; v:v) at room temperature for 12 h. The extract was filtered, acetone and water being removed under reduced pressure.

**Determination of lignins**

**Thioacidolysis**

The method applied, described in detail by Rolando \textit{et al},\textsuperscript{12} and developed by Lapierre,\textsuperscript{13} consists in a thioacidolysis of the lignin (0.2 M BF$_3$ etherate in dioxane/ethanethiol; 8:75:1). The degradation products, presented in the form of thioethylated derivatives, were analyzed by GC/MS after silylation (BSTFA/TMCS). The amount of reagents stipulated by the authors was modified: 30 mg of fraction A were added to 14 ml of thioacidolysis reagent. The final residue was redissolved in 2 ml of dichloromethane; 1 ml of the thioacidolysis dichloromethane solution was added to 3 ml of Raney nickel aqueous slurry and 15 ml of methanol. The residual was redissolved in 0.5 ml of dichloromethane and silylated using 500 \(\mu\)l of (BSTFA + 1% TMCS) and 50 \(\mu\)l of GC-grade pyridine in a 2-ml reaction vial fitted with a Teflon-lined screwcap placed at ambient temperature; silylation was complete after 4 h.

For GC-MS analysis a DBXLB (J&W Scientific, USA) column (30 m × 0.25 mm id × 0.25 \(\mu\)m) was used. The temperature was programmed from 80°C (1 min) to 120°C at a rate of 50°C min\(^{-1}\) to 260°C at a rate of 4°C min\(^{-1}\) and to 320°C at a rate of 8°C min\(^{-1}\). The injector temperature was 240°C, and 1-\(\mu\)l aliquots were injected in splitless mode. Detection was accomplished with a magnetic spectrometer: VG-Autospec EQ (Micromass, Manchester, UK), operating in electron mode with an ionization energy of 70 eV and temperature of transfer line at 240°C.

**Desulfuration**

The method applied is described in detail by Lapierre \textit{et al}.
\textsuperscript{14} It consists of thioacidolysis of the lignin (0.2 M BF$_3$ etherate in dioxane/ethanethiol; 8:75:1) followed by desulfuration of the thioacidolysate. The amounts of reagents stipulated by the authors were modified: 1 ml of the thioacidolysis dichloromethane solution was added to 3 ml of Raney nickel aqueous slurry and 15 ml of methanol. The final residue was redissolved in 0.5 ml of dichloromethane and silylated using 500 \(\mu\)l of (BSTFA + 1% TMCS) and 50 \(\mu\)l of GC-grade pyridine in a 200-\(\mu\)l reaction vial fitted with a Teflon-lined screwcap placed at room temperature for 1 h.

The GC-MS was performed on a DBXLB column (30 m × 0.25 mm id × 0.25 \(\mu\)m). The temperature was programmed from 80°C (1 min) to 120°C at a rate of 50°C min\(^{-1}\), to 260°C at a rate of 4°C min\(^{-1}\) and to 320°C at a rate of 8°C min\(^{-1}\) (10 min). The injector temperature was 240°C and 2-\(\mu\)l aliquots were injected in splitless mode. Detection was accomplished with a magnetic spectrometer: VG-Autospec EQ, operating in electron mode with an ionization energy of 70 eV and temperature of transfer line at 240°C.

**Pyrolysis**

A Pyrojector II instrument was used in conjunction with a Varian (Les Ulis, France) 3400 CX model gas chromatograph equipped with a Varian Saturn 4D ion-trap detector. The fractions were located in the pyrolyzer and duplicate pyrolysis experiments were carried out at 450°C. A fused-silica capillary column of RTX-20 WCOT (30 m × 0.25 mm id × 1 \(\mu\)m phase thickness) (Restek, Bellefonte, USA) was used to separate the pyrolysis products. Helium was the carrier gas. The inlet mode was splitless. The gas chromatograph was programmed from 50°C (10 min) to 280°C at a rate of 6°C min\(^{-1}\). The mass spectrometer was set at 70 eV. Spectra were acquired by a ChemStation software package (PL Laboratories, Marseille, France). Identification was achieved by mass fragmentometry, a library search (NIST) and comparison with literature data. When possible, identifications were made by comparison with authentic standards.
Analysis by IR spectroscopy
Infrared spectra were realized on pastilles of fraction A and KBr, between 4000 and 700 cm$^{-1}$. The IR spectrometer was a Perkin-Elmer (Courtabœuf, France) instrument. Attribution of the IR bands was performed according to correlations by Puech,\textsuperscript{15} Faix\textsuperscript{16} and Collier et al.\textsuperscript{17}

$^1$H NMR analysis
The NMR spectra were obtained after acetylation (see above for the protocol). They were recorded on a Bruker (Wissembourg, France) DPX 400-MHz instrument with deuterated chloroform as the solvent. Sixty-four scans were accumulated with spectral windows of 4789 Hz. The resolution was 1247 Hz/plot.

Determination of polysaccharides
Acid hydrolysis
A known quantity (2 mg) of fraction B was lyophilized. The lyophilizate was hydrolyzed with 4 M trifluoroacetic acid (2 ml) at 100°C for 4 h in a tube fitted with a Teflon-lined screwcap. The hydrolyzate obtained was dried by evaporation and silylated with 20 µl of anhydrous pyridine, 70 µl of (BSTFA + 1%TMCS) and 10 µl of trifluoroacetic acid in a tube fitted with a Teflon-lined screwcap. The tube was placed at 60°C for 30 min to facilitate the reaction and dissolve the two phases (which can appear when the reagents are added).

GC/MS analysis of the products was carried out under the following conditions: CPSil 5 CB column (25 m × 0.25 mm id × 0.12 µm) (Chrompack, The Netherlands), temperature of injector 280°C, temperature programming 120°C to 320°C at a rate of 2°C min$^{-1}$, injection volume of 2 µl in split mode. The mass spectrometer was a VG-Autospec EQ operating in electron mode with an ionization energy of 70 eV and temperature of transfer line at 220°C.

Pyrolysis
See section above for analysis conditions.

Determination of ellagitannins
Nitrous acid oxidation reaction
According to this method proposed by Bate-Smith,\textsuperscript{18} the esters from the hexahydroxydiphenic acid and the glucose are oxidized by nitrous acid under nitrogen. The reaction produces a blue colouration. One millilitre of the dosing solution was mixed with 1 ml of methanol and 160 µl of 6% acetic acid, after which the oxygen was expelled by nitrogen sparging for 10 min of; finally 160 µl of 6% sodium nitrite were added followed by a brief sparging (1 min). The tube was hermetically sealed and the reaction developed within 60 min in a water-bath at 30°C. The intensity of the colour that developed was measured at 600 nm.

Acid degradation
The proposed method was adapted from the one perfected by Peng et al.\textsuperscript{19} It is based on acid hydrolysis of the ellagitannins in a water-bath, followed by an HPLC quantitative analysis of the free ellagic acid. A subsample of 2.5 mg of fraction C (dried) was introduced into a hydrolysis tube fitted with a Teflon seal and the contents was solubilized by 5 ml of methanol/2 M hydrochloric acid. The ellagic acid present, and then the liberated ellagic acid, after 2 h of acid hydrolysis (oil bath at 100°C), were measured. The difference between the two values corresponds to the ellagic acid released by the ellagitannins. The HPLC quantitative determination was made on a Waters (Saint-Quentin Yvelines, France) HPLC using a reverse-phase column of Interchrom\textsuperscript{18} C18 10 µm (250 × 4.6 mm). The injection volume was 20 µl. The elution programme was performed at a constant flow of 1 ml min$^{-1}$, passing from 0 to 30% of B in 3 min, and then rising to 50% of B in 2 min, to 70% of B in 5 min, to 80% of B in 5 min, and finally to 100% of B in 2 min (solvent A: H$_2$O/H$_3$PO$_4$ pH adjusted to 2.4; solvent B : MeOH/H$_3$PO$_4$ pH adjusted to 2.4). Detection was performed at 370 nm.

HPLC/liquid secondary ion mass spectrometry (LSIMS)
A subsample of 1 mg of fraction C was solubilized with a minimum of methanol:water (6:4) for HPLC analysis coupled, through a flux divider, to an UV detector and a ‘LSIM’ mass spectrometer using the device perfected by Vivas et al.\textsuperscript{20} The HPLC separation method is described in the next paragraph. Some modifications were made, however: first, to ensure LSIMS detection, 2% of glycerol, which acts as a matrix, had to be added to the elution solvents and, second, the injected volume was increased (to 100 µl) to improve the quality of the recorded spectra. The spectra were obtained with a VG-Autospec EQ with a caesium gun, in negative mode, under the following conditions: calibration: 200–1500 Da caesium iodate salt; matrix: thioglycerol; bombardment energy: 35 KeV; 2 µA; temperature <40°C; sample solvent: methanol.

HPLC qualitative determination
The chromatographic separation technique complied with the method perfected by Scalbert et al.\textsuperscript{21} The extract was performed with the equipment described below. The injection volume was 20 µl. The elution programme was performed at a constant flow of 1 ml min$^{-1}$, passing from 0 to 10% of B in 40 min., and then rising to 100% of B in 25 min (solvent A: H$_2$O/H$_3$PO$_4$ 999:1, solvent B : MeOH/H$_3$PO$_4$ 999:1). Detection was performed at 280 nm.

Analysis by size-exclusion chromatography (SEC)
The study of the $M_0$ distribution of fraction C was performed using the acetyl derivatives. Samples (10 mg) of freeze-dried material were acetylated with pyridine–acetic anhydride (1:1; v/v) for 3 days at room temperature. The precipitate obtained by pouring the mixture into cooled water was recovered by centrifugation. This precipitate was then washed with

\textsuperscript{1} J Sci Food Agric 85:343–353 (2005)
distilled water, methanol and finally chloroform. It was dried and dissolved in 0.5 ml of THF and filtered before analysis by SEC.

The SEC analysis was performed using a Thermo Quest (Courtaboeuf, France) instrument equipped with three columns (300 × 7.8 mm): TSK Gel G 1000 HXL, TSK Gel G 2000 HXL, TSK Gel G 2500 HXL, in series, protected with a guard column of the same material. The analysis conditions were: THF as the eluent; flow-rate: 1 ml min⁻¹; injection volume: 20 µl and analysis time of 45 min. The calibration curve was obtained with polystyrene, and peracetylated elagic acid was used as standard. Detection was made at 280 nm with a UV detector (Spectra Series UV-150) (Thermo Quest, Courtaboeuf, France) and a refractometric detector (Spectra Series RI-150). PL Caliber software (PL Laboratories, Marseille, France) was used for data acquisition.

**Analysis by matrix-assisted laser desorption time-of-flight MS (MALDI-TOF/MS)**

MALDI spectra were recorded on a Tofspec E instrument (Micromass, Manchester, UK). The irradiation source was a pulsed nitrogen laser with a wavelength of 337 nm. The laser pulse duration was 3 ns pulse⁻¹. Analysis was performed in reflectron mode, positive polarity and the acceleration voltage was 20 kV.

**MALDI-TOF sample preparation**

The sample was dissolved in methanol (10 mg ml⁻¹). The sample solutions were mixed with a methanol solution (10 mg ml⁻¹) of the matrix, for which 2,5-dihydroxybenzoic acid (more commonly called DHB) was used. To enhance ion formation, sodium chloride was added. The solutions of the sample, the matrix and the salt were mixed: 10:1:1 (v:v:v) respectively, and 0.5–1 µl of the resulting solution were placed on the MALDI target. After evaporation of the solvent, the MALDI target was introduced into the spectrometer.

**RESULTS AND DISCUSSION**

**Characterization of fraction A**

**Characterization by IR spectroscopy**

IR spectra of fraction A (underivatized) presents a first series of bands identical to many phenolic structures: 3420–3380 (O–H elongation in the alcohol and phenol functions), 3000–2800 cm⁻¹ (C–H aliphatic and aromatic elongation in the methyl and methylene groups), a second series of bands characteristic of lignins: 1515–1503 (vibration of aromatic skeleton and C=O elongation), 1465–1458 (deformation of C–H in the –CH₂ and –CH₃ groups), 1430–1422 cm⁻¹ (deformation of C–H in the –OCH₃ group; alcoholic and phenolic –OH), and a third series of bands: 1328–1320 (O–H and C–O elongation in tertiary alcohols), 1122–1118 (aryl–alkyl ethers and C=O elongation), 1045–1038 cm⁻¹ (aryl–aryl ethers; C–O deformation in primary alcohols and deformation of non-conjugated C=O).

**Thioacidolysis and GC/MS of degradation products**

Thioacidolysis is an acid-catalyzed reaction which results in the depolymerization of lignins. This is a recently developed method. The alkyl- and arylether bonds are split into dioxan in the presence of a strong acid (Et₂O-BF₃) and a weak nucleophile (EtSH: ethanethiol). The cleavage of ether bonds is equally specific, but more complete than other methods like thioacetolysis. The detection of C₆C₃ trithioethyl phenylpropane compounds provides unambiguous evidence of the occurrence of aryl ether structures, the most characteristic structures in lignin. On this basis, thioacidolysis is considered the best method to characterize lignins.

Figure 2 shows the GC analysis of the products recovered from fraction A after thioacidolysis. The mass fragmentations of their trimethylsilylated derivatives have been compared with those published in the literature so as to identify the different peaks. The structures of the compounds represented by the main peaks are shown in Fig 2. Peaks 1 and 2 were attributed to xylan thioacidolysis products. These essentially come from the xylose units of these polysaccharides and are considered to be impurities. Guaiacyl and syringyl units are found exclusively in this fraction. On the chromatogram, products 4, 5 and 7, 8 are the most important and correspond to the main thioacidolysis products; they are attributed, respectively, to erythro and threo forms of the guaiacyl and syringyl monomers of lignins.12

The identification of these different products of mono- and dimethoxylated thioacidolysis indicates that fraction A represents the lignin fraction. This is confirmed by the second reaction.

**Desulfuration and GC/MS of degradation products**

The above method was extended to the detection of condensed lignin structures. The desulfuration of the thioacidolysis components, dimers which represent condensed lignin structures, makes it possible to separate these components by gas chromatography. The best characterization of lignin (qualitative aspect) is achieved with the dilignols that are recovered after these two consecutive thioacidolysis and desulfuration steps.

Figure 3 shows the GC analysis of the products recovered from fraction A. The structures of the compounds represented by the main peaks are shown in the same figure. These assignments were made on the basis of the mass fragmentation patterns of their trimethylsilylated derivatives compared with those published in the literature.14 Thus, each peak was identified.

Fraction A is characterized by:

- Monomeric degradation products: 4-propylguaiacol (peak 1) essentially originates in the desulfuration...
Fractionation of oak hardwood extractable polymers

Structures of the syringyl derivatives

\[
\text{EtS-CH-SEt} \quad \text{EtS-CH-SEt} \\
\text{H}_2\text{CO} \quad \text{H}_2\text{CO} \\
\text{OH} \quad \text{OH} \\
\text{OCH}_3 \quad \text{OCH}_3 \\
3: \text{SCH}_2\text{CHR}_2 \quad 4 - 5: \text{SCHR-CHR-CH}_2\text{R (erythro/threo)}
\]

Structures of the guaiacyl derivatives

\[
\text{EtS-CH-SEt} \quad \text{EtS-CH-SEt} \\
\text{H}_2\text{CO} \quad \text{H}_2\text{CO} \\
\text{OH} \quad \text{OH} \\
\text{OCH}_3 \quad \text{OCH}_3 \\
6: \text{GCH}_2\text{CHR}_2 \quad 7 - 8: \text{GCHR-CHR-CH}_2\text{R (erythro/threo)}
\]

Figure 2. GC chromatogram of trimethylsilylated products from fraction A after thioacidolysis and structures of the products of thioacidolysis identified in lignins. (EtS: ethanethiol; S: syringyl units; G: guaiacyl units; R: EtS).

- Dimeric degradation products: the 5-5 biphenyl bonding pattern is illustrated by dilignols 3–5. Peak 5 is 2,2′-dihydro-3,3′-dimethoxy-5,5′-dipropylbiphenyl. Dimers 3 and 4 come from 5-5-biphenyl structures which have lost Cγ groups during thioacidolysis. The 4-O-5-biphenyl ether linkage is represented by the dilignol 6. Peak 7 corresponds to TMS-derivative of diguaiacylethane; the formation of this dilignol is closely associated with that of dilignol 9. The β-5-bonding pattern occurring in the phenylcoumaran structures is represented by dilignols 8, 10 and 11.

Products obtained after pyrolysis

Pyrolysis/gas chromatography/mass spectrometry (PY/GC/MS) is an useful tool for the characterization of lignins and polysaccharides. PY/GC/MS is based on the depolymerization of macromolecules by heat. For the subunits of the polymers which are not or only slightly volatile, the increase in temperature leads to their thermal degradation and then to the identification of the products formed. The pool of pyrolysis products, separated by gas chromatography and identified by mass spectrometry, provides a diagnostic of the starting material, which itself would be unsuitable for GC/MS analysis due to its high molecular weight. Figure 4 shows the total ion chromatogram of the thermal degradative products obtained after pyrolysis of fraction A. A number of peaks were identified (Table 1). Essentially, one class of compounds is recognizable, ie phenolics with various methoxy and alkyl substituents. This group can be subdivided into molecules with 2-methoxy-p-hydroxyphenyl (guaiacyl or G units) (peaks: 2, 3, 4, 6, 8, 9, 11, 12), and 2,6-dimethoxy-p-hydroxyphenyl (syringyl or S units).

\[\text{OH} \quad \text{OCH}_3 \quad \text{H}_3\text{CO} \quad \text{EtS-CH-SEt} \quad \text{EtS-CH-SEt} \quad \text{EtS-CH} \quad \text{EtS-CH} \]

of the main thioacidolysis monomers and is thus the final product from the uncondensed β-O-4 linked guaiacyl-propane units; peak 2 corresponds to dihydroconiferyl alcohol.
Figure 3. GC chromatogram of trimethylsilylated degradation products from fraction A after thioacidolysis and desulfuration over Raney nickel, and structures of thioacidolysis and Raney nickel desulfuration products identified in lignins.

Table 1. Principal pyrolysis products and their fragmentations of the fraction A

<table>
<thead>
<tr>
<th>Peak</th>
<th>Products</th>
<th>Important MS ion (70 eV), m/z (relative intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetic acid</td>
<td>61 (18) 44 (20) 43 (100)</td>
</tr>
<tr>
<td>2</td>
<td>Guaiacol</td>
<td>124 (100) 109 (79) 95 (4) 81 (55)</td>
</tr>
<tr>
<td>3</td>
<td>p-Methyl guaiacol</td>
<td>138 (100) 123 (72) 110 (45) 95 (43) 81 (17) 67 (53) 44 (69)</td>
</tr>
<tr>
<td>4</td>
<td>p-Ethyl guaiacol</td>
<td>152 (49) 137 (100) 122 (17) 109 (7) 81 (9) 91 (14) 79 (11) 69 (10) 44 (80)</td>
</tr>
<tr>
<td>5</td>
<td>4-Hydroxy-2-methylacetophenone</td>
<td>150 (100) 135 (40) 107 (36) 79 (12) 77 (13)</td>
</tr>
<tr>
<td>6</td>
<td>Eugenol</td>
<td>164 (100) 149 (48) 137 (23) 131 (25) 121 (22) 103 (27) 91 (20) 77 (32) 65 (6)</td>
</tr>
<tr>
<td>7</td>
<td>Syringol</td>
<td>154 (100) 139 (20) 125 (2) 111 (16) 93 (15) 81 (2) 79 (3) 39 (14)</td>
</tr>
<tr>
<td>8</td>
<td>Vanillin</td>
<td>152 (100) 137 (6) 122 (23) 109 (32) 92 (5) 81 (40) 76 (10) 53 (22)</td>
</tr>
<tr>
<td>9</td>
<td>Iso-eugenol</td>
<td>164 (100) 149 (33) 137 (7) 131 (20) 121 (22) 103 (32) 91 (27) 77 (26) 65 (9)</td>
</tr>
<tr>
<td>10</td>
<td>Vanillic acid</td>
<td>168 (100) 153 (48) 137 (2) 125 (34) 108 (2) 97 (14) 85 (10) 79 (18) 65 (11) 53 (16)</td>
</tr>
<tr>
<td>11</td>
<td>Guaiacylpropene</td>
<td>166 (30) 137 (100) 122 (25) 94 (12)</td>
</tr>
<tr>
<td>12</td>
<td>Acetovanilione</td>
<td>166 (46) 151 (100) 136 (3) 123 (31) 108 (9) 79 (5) 77 (9) 67 (9) 65 (10) 52 (10)</td>
</tr>
<tr>
<td>13</td>
<td>Coniferaldehyde</td>
<td>182 (52) 167 (100) 151 (11) 139 (6)</td>
</tr>
<tr>
<td>14</td>
<td>Coniferyl alcohol</td>
<td>180 (50) 151 (30) 124 (42) 109 (23) 91 (38) 77 (30) 65 (28) 51 (36)</td>
</tr>
<tr>
<td>15</td>
<td>Allyl-4-syringol</td>
<td>194 (30) 153 (100) 125 (20) 110 (20) 93 (18)</td>
</tr>
<tr>
<td>16</td>
<td>Methoxyeugenol</td>
<td>194 (100) 179 (15) 167 (15) 151 (14) 147 (14) 131 (20) 119 (25) 91 (32)</td>
</tr>
<tr>
<td>17</td>
<td>Acetosyringone</td>
<td>196 (12) 181 (63) 153 (9) 123 (6) 109 (5) 107 (5) 93 (8) 91 (12) 85 (10) 83 (34)</td>
</tr>
</tbody>
</table>

Fractionation of oak hardwood extractable polymers

Figure 4. Pyrolysis chromatogram of fraction A ($T = 450 \, ^\circ C$).

aromatic moieties (peaks: 7, 15). The main distinctive compound identified in the pyrogram is peak 7: syringol. Peak 14, corresponding to coniferyl alcohol, is largely present in the fraction A pyrolyzate. All these products are essentially contained in lignin pyrolyzates.

Characterization by $^1H$ NMR
Using data from the literature, we attributed the signals on the 1D spectrum of the $^1H$ of the syringyl unit: $\delta$ 6.6 ppm and guaiacyl unit: $\delta$ 6.9 – 7.05 ppm. The analysis of this spectrum confirmed that fraction A contained mainly monomethoxylated (guaiacyl) and dimethoxylated (syringyl) phenolic units.

Characterization of Fraction B
Acid hydrolysis and GC/MS of degradation products
The mass fragmentations of the trimethylsilylated derivatives compared with those found in libraries, and the retention times compared with those obtained with some standards, confirmed that fraction B was composed exclusively of sugars. L-arabinose, D-galactose, D-xylose, D-glucose, D-mannose and L-rhamnose are the main monosaccharides identified. We know that hemicellulose units are easily hydrolyzed by the acids in these monomers and by the following uronic acids: D-glucuronic acid, 4-O-methylglucuronic acid, D-galacturonic acid. A number of other sugars were observed with the same fragmentations and only the relative intensities were different; that is why their identification is difficult. Thus fraction B represents the polysaccharidic fraction of oak heartwood extract.

Products obtained after pyrolysis
Essentially two classes of compounds are recognizable, ie furanoic and pyranoic derivatives. These are the products of multiple dehydrations and rearrangements.
due to heating and can be considered to be pyrolysis markers of the structural polysaccharides. The main compounds identified are listed in Table 2.

The pyrolysis of lignins results in more abundant and more easily identifiable products than the pyrolysis of polysaccharides. This is due to the stability of the aromatic moieties of lignins under the thermal energy supplied by the pyrolyzer and under the electron impact of the mass spectrometer. By contrast, polysaccharides undergo multiple dehydrations under pyrolysis and yield various positional isomers whose mass spectra can be difficult to interpret because of the low level or absence of a molecular ion.

**Characterization of fraction C**

Oak heartwood contains large amounts of phenolic compounds, chiefly ellagitannins. Two ellagitannins are predominant in oak, and two isomers in particular: vescalagin and castalagin.20,25 More recently, the dimer ellagitannins and pentosylated forms have been described:26–28 roburins A, B, C, D, E and grandinin. Their structure is shown in Fig 5.

*The nitrous acid oxidation reaction: Bate–Smith method*18

This reaction was applied to fraction C. After 60 min in a water-bath at 30°C, the solution turns blue (characteristic of the presence of ellagitannins) and the colour intensity was measured at 600 nm. This method is used to characterize a typical group of tannins: ellagitannins.

**Acid degradation**

This reaction was applied to the fraction C at different concentrations. The results are grouped in Fig 6. Ellagic acid was formed during the reaction, indicating that fraction C contains ellagitannins.29 We noted that the more fraction C concentration increased the more ellagic acid was released.

**Figure 5.** Structures of ellagitannins identified in oak heartwood extracts.

**Figure 6.** Acid degradation of fraction C.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Products</th>
<th>Important MS ion (70 eV), m/z (relative intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetic acid</td>
<td>61 (18) 44 (20) 43 (100)</td>
</tr>
<tr>
<td>2</td>
<td>4-Methyl-2,3-dihydrofuran</td>
<td>84 (100) 83 (50) 69 (15) 55 (30)</td>
</tr>
<tr>
<td>3</td>
<td>3-Furanmethanol</td>
<td>98 (100) 81 (6) 69 (20) 53 (14) 41 (26)</td>
</tr>
<tr>
<td>4</td>
<td>Furfural</td>
<td>96 (36) 95 (85) 67 (8) 39 (100)</td>
</tr>
<tr>
<td>5</td>
<td>2-Methylfuran</td>
<td>82 (29) 81 (17) 53 (14) 43 (100)</td>
</tr>
<tr>
<td>6</td>
<td>2-propylfuran</td>
<td>110 (31) 81 (100) 53 (97) 39 (24)</td>
</tr>
<tr>
<td>7</td>
<td>5-Methyl-2(3H)-furanone</td>
<td>98 (11) 55 (21) 43 (100)</td>
</tr>
<tr>
<td>8</td>
<td>2,4-Dimethylfuran</td>
<td>96 (98) 95 (70) 81 (10) 67 (95) 53 (50) 41 (52) 39 (48)</td>
</tr>
<tr>
<td>9</td>
<td>1-(2-Furanyldimethyl)</td>
<td>110 (28) 95 (85) 68 (14) 67 (12) 44 (100) 43 (38) 39 (52)</td>
</tr>
<tr>
<td>10</td>
<td>Cyclohexanone</td>
<td>98 (100) 83 (11) 70 (20) 69 (25) 55 (98) 42 (78)</td>
</tr>
<tr>
<td>11</td>
<td>2-(5H)-Furanone</td>
<td>84 (52) 55 (100) 39 (38) 27 (50)</td>
</tr>
<tr>
<td>12</td>
<td>Phenol</td>
<td>94 (100) 66 (40) 65 (38) 39 (30)</td>
</tr>
<tr>
<td>13</td>
<td>5-Methyl-2-furfural</td>
<td>110 (79) 109 (91) 81 (17) 66 (11) 65 (11) 55 (35) 53 (100)</td>
</tr>
<tr>
<td>14</td>
<td>3-Methyl-2,5-furandione</td>
<td>112 (2) 68 (40) 53 (11) 40 (40) 39 (100)</td>
</tr>
<tr>
<td>15</td>
<td>5-(Hydroxymethyl)-2-furfural</td>
<td>126 (45) 109 (38) 97 (76) 69 (41) 53 (19) 41 (100) 39 (84)</td>
</tr>
</tbody>
</table>
Fractionation of oak hardwood extractable polymers

Analysis by HPLC/LSIMS

HPLC/LSIMS coupling made it possible to confirm peak allocation, a step usually performed by comparing UV spectra and the retention time of the identifiable molecules with reference materials/products. The chromatogram of fraction C and the LSIMS spectra recorded at the apex of some peaks are grouped in Fig 7.
In the sample, we found ellagic acid indicating that fraction C contained ellagitannins; the presence of this acid is easily explained by the hydrolysis of numerous wood ellagitannins. We also found vescalin and castalin identified by LSIMS, \( m/z: 631 (M–H)\)−; these are considered products of vescalagin and castalagin hydrolysis. The presence of these molecules in the \( Q \) robur heartwood extracts has already been reported by Mayer et al.\(^{19,30,31}\) Four monomers were identified: vescalagin/castalagin and grandinin/roburin E characterized, respectively, for the first pair by \( m/z: 933 (M–H)\)− and for the second by \( m/z: 1065 (M–H)\)−. The HPLC/LSIMS coupling also enabled us to identify two dimers: roburin A/roburin D (\( m/z: 1849 (M–H)\)−). Vescalagin and castalagin appear as the principal ellagitannins in the fraction.

**SEC Analysis**

Fraction C was acetylated with pyridine–acetic anhydride; peracetates were separated by SEC, using TSK Gel G columns with only THF as eluent. In the SEC analysis, fraction C was eluted with an UV detector into three chromatographic peaks. The calibration curve was obtained with polystyrene standards. Thus the \( M_p \) distribution obtained is expressed in polystyrene equivalents. It is difficult to find standards for vescalagin, because they are not products on the market; nevertheless, we used ellagic acid as standard. The three peaks obtained correspond to ellagic acid (\( t_R = 22.17 \) min), dimers (\( t_R = 18.27 \) min) and monomers (\( t_R = 17.48 \) min) according to their retention times and their \( M_p \) values. Almost all the tannic polymers were included in these three peaks. The peak corresponding to monomers was the highest: 59.5%, followed by that of dimers.\(^{32–34}\)

**Analysis by MALDI-TOF/MS**

The principle consists in diluting the sample in a matrix of small organic molecules absorbing very strongly at the laser wavelength (370 nm). The mixture is irradiated with a laser beam; the relaxation of the absorbed energy rapidly leads to the volatilization of the solids. In these conditions, the unbroken molecules are absorbed and ionized. The MALDI-TOF spectrum of fraction C had eight easily identifiable peaks: peak 1: vescalin/castalin; peak 3, monomers: vescalagin/castalagin; peak 5, dimers: roburin A/roburin D; peak 7, trimers; and peaks 2, 4, 6, 8 their perosylated forms (\( m/z \) of the native form + \( m/z = 132 \)) Da: grandinin/roburin E (respectively for vescalagin/castalagin monomers), roburin B/roburin C (respectively for roburin A/roburin D dimers) and finally the perosylated form of trimers (Fig 8).

The \( m/z = 655 \) peak was obtained from the 957 peak by loss of an ellagic acid structure. However, we observed that it was the monomer forms that yielded the best response in MALDI-TOF (the major peak at 957 was the 934 Da castalagin or vescalagin), and this is one reason why there is a low degree of polymerization.

**REFERENCES**

Fractionation of oak hardwood extractable polymers


9 Vivas N, Recherches sur la qualité du chêne français de tonnellerie (Quercus petraea Liebl, Q. robur L.) et sur les mécanismes d’oxydo-réduction des vins rouges au cours de leur élevage en barriques, Thèse, Université de Bordeaux 2, France (1997).


22 Vitiello D, Etude analytique des produits de pyrolyse et d’extraction de bois tropicaux, Thèse docteur-ingénieur, Université de Bordeaux I, France (1982).


