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LC-DAD/ESI-MS/MS study of phenolic compounds in ash (*Fraxinus excelsior* L. and *F. americana* L.) heartwood. Effect of toasting intensity at cooperage

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The phenolic composition of heartwood extracts from *Fraxinus excelsior* L. and *F. americana* L., both before and after toasting in cooperage, was studied using LC-DAD/ESI-MS/MS. Low-molecular weight (LMW) phenolic compounds, secoiridoids, phenylethanoid glycosides, dilignols and oligolignols compounds were detected, and 48 were identified, or tentatively characterized, on the basis of their retention time, UV/Vis and MS spectra, and MS fragmentation patterns. Some LMW phenolic compounds like protocatechuic acid and aldehyde, hydroxytyrosol and tyrosol, were unlike to those for oak wood, while ellagic and gallic acid were not found. The toasting of wood resulted in a progressive increase in lignin degradation products with regard to toasting intensity. The levels of some of these compounds in medium-toasted ash woods were much higher than those normally detected in toasted oak, highlighting vanillin levels, thus a more pronounced vanilla character can be expected when using toasted ash wood in the aging wines. Moreover, in seasoned wood, we found a great variety of phenolic compounds which had not been found in oak wood, especially oleuropein, ligstroside and olivil, along with verbascoside and isoverbascoside in *F. excelsior*, and oleoside in *F. americana*. Toasting mainly provoked their degradation, thus in medium-toasted wood, only four of them were detected. This resulted in a minor differentiation between toasted ash and oak woods. The absence of tannins in ash wood, which are very important in oak wood, is another peculiar characteristic that should be taken into account when considering its use in cooperage. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: LC-DAD/ESI-MS/MS; *Fraxinus excelsior*; *Fraxinus americana*; heartwood; phenolic compounds; secoiridoids; phenylethanoid glycosides; dilignol; oligolignol

INTRODUCTION

Coopers have produced wooden barrels since at least the time of Imperial Rome, and oak (*Quercus* spp.) heartwood has been the main material used for over 2000 years. In Europe, in recent years, woods other than oak, such as chestnut (*Castanea sativa*), cherry (*Prunus avium*), false acacia (*Robinia pseudoacacia*) and, less commonly, ash (*Fraxinus excelsior* and *Fraxinus vulgaris*) and mulberry (*Morus alba* and *Morus nigra*) have been considered as possible sources of wood, for production of both wines and their derived products, including vinegar, cider or spirits such as brandies.^[1–4] Only oak and chestnut, however, have been approved by International Organisation of Vine and Wine.

The beverages undergo a series of processes that cause significant changes in aroma, colour, taste and astringency during aging due to the extraction of certain compounds present in the wood which are transferred to the beverages, as well as the permeation of oxygen through barrel staves due to wood porosity. The impact that woods other than oak have on chemical composition and sensorial properties of beverages has not to date been fully evaluated. As a step toward this goal, the characteristics of wines and especially vinegars, aged in barrels made from different woods, have very recently been studied,^[2–4] finding in some cases

compounds that could act as markers for the use of a certain wood. However, more information is needed for a more complete evaluation of the impact of these woods, as to their chemical composition and physical-mechanical properties, as well as their evolution during seasoning and toasting at cooperage. An evaluation similar to those carried out over the last 20 years regarding oak wood for cooperage is also needed. In fact, some papers have been recently

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published about the volatile composition of woods other than oak, with a view towards their use in barrel making,^[5–7] and with the same objective, the phenolic composition of chestnut, cherry and acacia seasoned and toasted woods has been studied in our research group.^[8–10]

One of the woods that could be considered as a possible alternative to oak is ash wood. Although no data have been found in regarding its oenological usage, we did find a volatile composition in an early work^[6] that was qualitatively very similar to oak. It did, however, show some differences. European and American ash heartwood, the American specie being the richer of two, are characterized by their high concentrations of hydroxybenzoic and hydroxycinnamic aldehydes in both seasoned and toasted woods. In fact, toasted ash wood shows the highest concentrations of sinapaldehyde compared to other toasted woods (oak, chestnut, acacia and cherry), with levels between 720 and 965 µg/g, in comparison to a range of 240–450 µg/g detected in *Quercus* spp., with the same toasting intensity. When we compare this with other woods, in addition to its richness in aldehydes, toasted ash wood is especially rich in tyrosol (which is only detected in ash wood), syringol, eugenol and *cis* and *trans* isoeugenol, guaiacol, catechol, 3-methylcatechol and benzaldehyde, many of which could contribute to wine aroma with notes such as spicy, smoky, etc.. Moreover, in toasted ash woods, the most significant characteristics are concentrations of cyclotene, 3-ethyl and 3,5-dimethylcyclotene (related to the liquorice aroma in aged wines), as well as those of 4,5-dimethyl-2-ciclohexen-1-one, α -methylcrotonolactone, γ -butyrolactone, solerone, maltol and 2-furanmethanol, together with the low concentration of furfural and their derivatives. Thus, this wood, from a quantitative point of view shows a very different profile of volatile compounds in regard to toasted oak wood, and we could therefore also expect a characteristic sensorial profile.

In relation to phenolic compounds, ash wood has received very little attention compared to published studies on the phenolic composition of leaves and bark,^[11–13] characterized by the presence of coumarins, secoiridoids, phenylethanoid glycosides, lignans, flavonoids and simple phenolic compounds with interesting biological activities. Mammela^[14] found some phenolic compounds in ash (*F. excelsior*) wood dust, but only hexahydroxydiphenol (HHDP)-glucose, tetragalloyl glucose and digalloyl-bis-HHDP-glucose were suggested as possible identifications. On the other hand, Windeisen and Wegener^[15] tentatively identified by GC-MS the lignan syringaresinol in thermally modified *F. excelsior* wood (200 °C for 4 h in a nitrogen atmosphere and under pressure), in order to increase the natural durability of this wood. These limited studies published on the phenolic composition of *F. excelsior* wood point out important differences when is compared with oak wood, since oak heartwood shows high levels of the monomer ellagitannins, castalagin, roburin E, vescalagin, and grandinin, and ellagic and gallic acids.^[16,17]

The objective of this work is to study the phenolic composition of ash (*Fraxinus excelsior* and *F. americana*) heartwood and its possible changes during the toasting process. This work will, thus, enable the completion of the chemical characterization of this wood, with an eye towards its use in cooperage, as well as, while using oak wood as a reference, to discover the effects that it may have on the sensory characteristics of the wines, vinegars and other drinks aged in this wood. This wood, along with others, could be used in many ways: e.g. the manufacture of containers from large vats to barrels, and, in recent years, the production of multi-size pieces (powder, shavings, chips, cubes and staves)

used in cheaper alternative techniques. Oak pieces are normally used, but these other woods could be considered for the purpose of providing a particular identity to these products.

EXPERIMENTAL

Wood samples

Ash (*Fraxinus excelsior* L. and *F. americana* L.) heartwood was provided as staves for making barrels by Tonelería Intona, SL (Navarra, Spain). The wood was naturally seasoned for 24 months, and toasted at two intensities: light (165 °C for 35 min) and medium (185 °C for 45 min), in an industrial kiln specially designed for toasting staves. Samples were taken before and after toasting, using ten staves of each. Several wood pieces were cut out of each stove, and the pieces were ground, sieved and mixed, taking the sawdust ranging from 0.80 to 0.28 mm of size. The number of staves was chosen in that way because our objective was to study the general phenolic profile of this wood both before and after toasting, without going deeply into their natural variation.

Chemicals

Reference compounds were obtained from commercial sources: caffeic acid, 2-methoxycinnamic acid, and protocatechualdehyde (Fluka Chimie AG, Buchs, Switzerland), 4-hydroxypropionic acid, syringaldehyde, and coniferyl aldehyde (Aldrich Chimie, Neu-Ulm, Germany), furfural, 5-methylfurfural, 5-hydroxymethylfurfural, vanillin, protocatechuic acid, and syringic acid (Sigma Chemical, St. Louis, MO), hydroxytyrosol, tyrosol, esculin, esculetin, fraxetin, fraxin, oleuropein, verbascoside, sinapaldehyde, vanillic acid, and ferulic acid (Extrasynthèse, Genay, France), isoverbascoside, conidendrin, secoisolariciresinol and lariciresinol (PhytoLab GmbH & Co, Vestenbergsgreuth Germany), isofraxidin (Chromadex, Irvine, CA). Methanol, diethyl ether, ethyl acetate, anhydrous sodium sulfate, and phosphoric acid were purchased from Panreac (Barcelona, Spain). Methanol HPLC grade was from Scharlab (Barcelona, Spain) and formic acid and ammonium acetate MS spectroscopy from Fluka Chimie AG (Buchs, Switzerland).

Extraction of phenolic compounds

The sawdust (1 g) was extracted with 100 mL of methanol/water (1:1) at room temperature (20 ± 2 °C) and in darkness for 24 h. The extracts were filtered in a Büchner funnel, and the methanol was removed in a rotary evaporator at a temperature below 40 °C. This was extracted three times with 20 mL of diethyl ether and then three times with 20 mL of ethyl acetate. The remaining aqueous solution was freeze-dried. The two organic fractions were dried with 20 g of anhydrous sodium sulfate, evaporated in a rotary evaporator at a temperature below 40 °C, and the residuum re-dissolved in 1 mL of methanol/water 50%. These extracts and an aliquot part of freeze-dried extract re-dissolved in water (30 mg/mL) were used for the HPLC-DAD and LC-DAD/ESI-MS/MS analyses. All the extractions were carried out in duplicate.

HPLC/DAD analysis

Quantification of phenolics was performed by LC-DAD using an Agilent 1100L liquid chromatography system equipped with a diode array detector (DAD), and managed by a Chemstation for LC 3D systems Rev B.03.02 (Agilent Technologies, Palo Alto, CA, USA).

The column was a 200 mm × 4 mm i.d., 5 μm, Hypersil ODS C18, maintained at 30 °C and protected with a 4 mm × 4 mm i.d. guard column of the same material (Agilent Technologies). The HPLC profiles were monitored at 255 ± 2, 280 ± 2, 325 ± 75, 340 ± 15 and 525 ± 2 nm, and the UV/Vis spectra were recorded from 190 to 650 nm. The volume injected was 20 μL. With the diethyl ether and ethyl acetate extracts, the elution method involved a multistep linear solvent gradient changing from a starting concentration of 100% phosphoric acid (0.1%) (eluent A) going to 85% (20 min), 75% (30 min), 50% (50 min) and 0% (70 min), using methanol/phosphoric acid 0.1% as eluent B. The total time of analysis was 70 min, equilibration time 10 min and flow rate 1 mL/min. With the same eluents, the elution gradient to analyze the freeze-dried (30 mg) was: 98–50% A (20 min), 50% A (23 min), 50–10% A (30 min), 10% A (33 min), followed by 10 min of re-equilibration of the column (Synergy Polar-reversed phase column (150 × 2.00 mm i.d., particle size 4 μm) (Phenomenex, Torrance, CA, USA)). Quantification was carried out by the external standard method, using peak areas in UV at 280 nm (secoiridoids, dilignols, oligolignols, hydroxytyrosol and tyrosol) or 325 nm (the remainder compounds). The concentration of each substance was measured by comparing it with calibrations made with the pure compound analyzed under the same conditions and linear regression coefficients between 0.9990 and 0.9999 were obtained. In general, more than one linear regression was made for each compound, at different concentration levels. Calibration of a similar compound was used when the pure reference standard was not available. Thus, secoiridoids were quantified with oleuropein calibration, phenylethanoids with that of verbascoside, dilignols and oligolignols as lariciresinol, and peak 18 as esculetin. The total contents of each different chemical family were calculated summing concentrations of individual quantified compounds. The samples were analyzed in duplicate.

LC-DAD/ESI-MS/MS analysis

Analyses were performed using an Agilent 1200 HPLC system consisting of a solvent degasser, a quaternary pump, an auto sampler, a thermostatic column compartment and a DAD (Agilent Technologies, Palo Alto, CA, USA) and coupled to a 3200 QTRAP™ hybrid triple quadrupole/linear ion trap instrument equipped with a TurboV™ ion source (MDS SCIEX, Applied Biosystems, Streetsville, ON, Canada). Ionization and mass spectrometric conditions were optimized by infusing a solution of 16 internal standards (1 μg/mL methanol/water 1:1, containing 0.5% formic acid and 5mM ammonium acetate) at a flow rate of 5 mL/min. The interface was set at the following values: curtain gas 10; ion spray voltage −4.0 kV, source temperature 400°C, nebulizing gas (GS1) 40; heating gas (GS2) 10; interphase heater, on; collisionally activated dissociation gas, high; declustering potential, −30 V; entrance potential, −5 V. The acquisition method consisted of an information-dependent acquisition. Precursors were selected by using an enhanced full scan as the survey scan using the following parameters: scan rate 1000 amu/s, mass range of *m/z* 100.00–900.00, and dynamic fill time. The three most intense ions were analyzed using an enhanced resolution experiment (scan rate 250 amu/s, dynamic fill time) followed by an enhanced product ion scan for the MS/MS data (scan rate 1000 amu/s, mass range *m/z* 100.00–900.00, CE −20 eV, CES 10 V, dynamic fill time). The mass spectrometer was controlled by Analyst 1.4.2 from Applied Biosystems/MDS Sciex.

The chromatographic separation was achieved at 40 °C using the same columns and multistep linear solvent gradients, but the eluents used were 0.5% formic acid, 5 mM ammonium acetate as eluent A and methanol/formic acid 0.5% as eluent B. For the diethyl ether and ethyl acetate extracts, the volume injected was 40 μL, and the flow rate was set at 1 mL/min and reduced by splitting (1:2) before transferring into the mass spectrometer. To analyze the freeze-dried extracts, the volume injected was 20 μL and the flow rate was set at 250 μL/min.

Statistical analysis

The obtained data were analyzed by ANOVA and multivariate canonical discriminant analysis using the SAS program (version 9.1; SAS Institute, Cary, NC). When significant differences were revealed ($p < 0.05$), means were compared applying the Student Newman–Keuls multiple range test.

RESULTS AND DISCUSSION

Identification of phenolic compounds

Figures 1–3 illustrate the HPLC-DAD chromatograms of the phenolic compounds from seasoned (Fig. 1 and 2) and toasted (Fig. 3) ash heartwood. The diethyl ether chromatogram of seasoned *F. excelsior* is omitted in Fig. 1 due to the presence of few minor peaks, already detected in the other extracts. Similarly, for medium-toasted heartwood, only the diethyl ether extract chromatogram of *Fraxinus excelsior* is shown because the absence of peaks in freeze-dried extracts, and the similarity of the chromatograms displayed in both species and with the ethyl acetate extracts.

It highlighted the qualitative and quantitative differences in the chromatograms related to three factors: the condition of the wood (seasoned or toasted), the ash species and the efficiency of extraction of phenolic compounds depending on the solvent used, especially for seasoned wood, according to data found in the literature.^[16,18] LC-DAD/ESI-MS/MS data for the different peaks are summarized in Table 1, as well as the characteristic fragmentations and their structure attributions observed in the ESI-MS/MS analysis.

A total of 57 individual compounds were detected in the two ash species studied, thus revealing a wide variety of polyphenols which belong to very different chemical families, including low-molecular weight (LMW) phenolic compounds, secoiridoids, phenylethanoid glycosides, dilignols and oligolignols, but not hydroxycoumarins or flavonoids. The chemical structures of some of these compounds are displayed in Fig. 4. The presence of some flavonoids and hydroxycoumarins has been described in bark and leaves of *Fraxinus* spp, but not in wood.^[11,19] Among detected compounds, 48 were identified, or tentatively characterized, based on their retention times, UV/Vis spectra and MS fragmentation patterns, whereas nine peaks remained unidentified.

LMW phenolic compounds

The hydroxybenzoic acids, protocatechuic (**peak 2**), vanillic (**5**) and syringic (**8**), the hydroxybenzoic aldehydes protocatechuic (**3**), vanillic (**7**) and syringic (**10**), the hydroxycinnamic acid ferulic (**12**), the hydroxycinnamic aldehydes coniferyl (**14**), and sinapic (**17**), as well as hydroxytyrosol (**1**) and tyrosol (**4**) were identified by comparison of their retention times, as well as comparing UV/Vis and mass spectra with those of pure commercial standards. These compounds were frequently found in phenolic extracts from

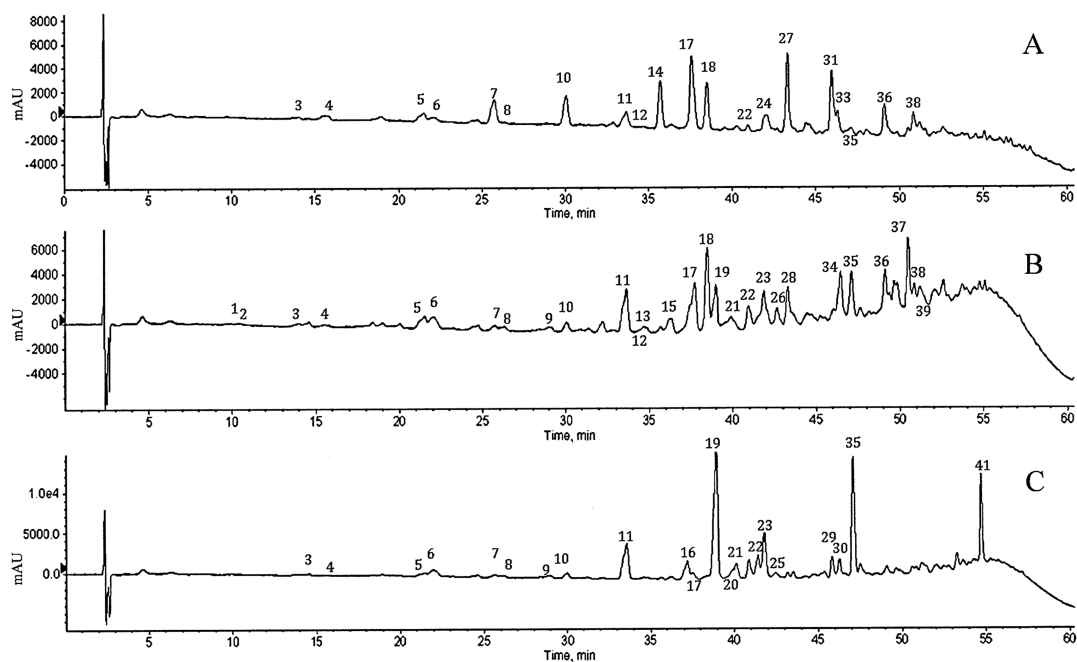


Figure 1. HPLC-DAD chromatograms of *Fraxinus* spp. seasoned heartwood extracts, monitored at 325 ± 75 nm. A = Ethyl acetate extract of *F. americana*. B = Diethyl ether extract of *F. americana*. C = Ethyl acetate extract of *F. excelsior*. Peak numbers shown in Tables 1, 2.

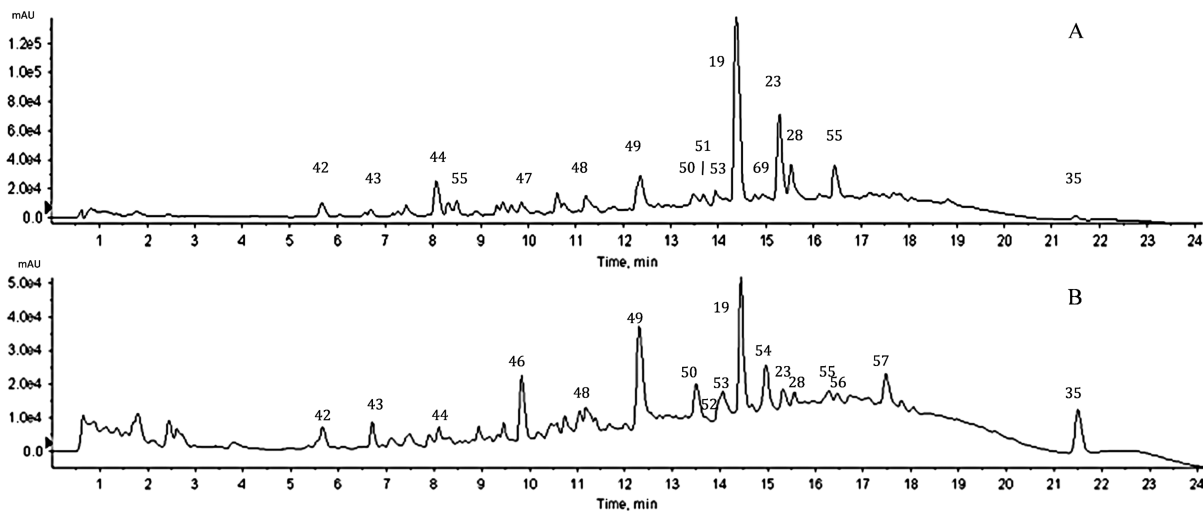


Figure 2. HPLC-DAD chromatograms of freeze-dried aqueous fraction *Fraxinus* spp. seasoned heartwood extracts, monitored at 325 ± 75 nm. A = *F. americana*; B = *F. excelsior*. Peak numbers shown in Tables 1, 2.

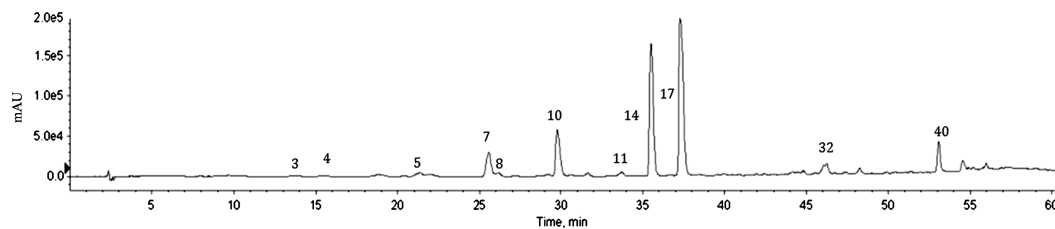


Figure 3. HPLC-DAD chromatogram of diethyl ether extract of *Fraxinus excelsior* medium-toasted heartwood monitored at 325 ± 75 nm. Peak numbers shown in Tables 1, 2.

Table 1. Spectroscopic and spectrometric data of peaks in HPLC chromatograms of seasoned and toasted *Fraxinus americana* and *F. excelsior* extracts

Peak	R _t (min)	Compound	λ _{max} (nm)	[M-H] ⁻ m/z	MS/MS m/z (%) [attribution] ^a
LMW phenolic compounds					
1	10.2	hydroxytyrosol	256, 286	153	153 (100) [M-H] ⁻ ; 123 (31) [M-H-CH ₂ OH] ⁻
2	10.4	protocatechuic acid	258, 297	153	153 (100) [M-H] ⁻ ; 109 (43) [M-H-CO ₂] ⁻
3	14.0	protocatechualdehyde	280, 310	137	137 (100) [M-H] ⁻
4	15.5	tyrosol	275	137	137 (100) [M-H] ⁻ ; 93 (77) [M-H-CO ₂] ⁻
5	21.3	vanillic acid	260, 290	167	167 (100) [M-H] ⁻ ; 152 (20) [M-H-CH ₃] ⁻ ; 123 (5) [M-H-CO ₂] ⁻ ; 108 (15) [M-H-CH ₃ -CO ₂] ⁻
7	25.6	vanillin	280, 312	151	151 (32) [M-H] ⁻ ; 136 (100) [M-H-CH ₃] ⁻
8	26.3	syringic acid	274	197	197 (100) [M-H] ⁻ ; 182 (53) [M-H-CH ₃] ⁻ ; 167 [M-H-2CH ₃] ⁻ ; 153 (13) [M-H-CO ₂] ⁻
10	30.0	syringaldehyde	232sh, 308	181	181 (100) [M-H] ⁻ ; 166 (13) [M-H-CH ₃] ⁻ ; 151 (31) [M-H-2CH ₃] ⁻
12	34.5	ferulic acid	238, 290sh*, 322	193	193 (100) [M-H] ⁻ ; 178 (25) [M-H-CH ₃] ⁻
14	35.5	coniferaldehyde	290sh, 322	177	177 (84) [M-H] ⁻ ; 162 (100) [M-H-CH ₃] ⁻
17	37.5	sinapaldehyde	300sh, 338	207	207 (100) [M-H] ⁻ ; 192 (77) [M-H-CH ₃] ⁻ ; 177 (77) [M-H-2CH ₃] ⁻
18	38.5	unidentified cinnamic	308sh, 340		177 (52)[M-H] ⁻ ; 162 (100) [M-H-CH ₃] ⁻ ; 133 (4) [M-H-CO ₂] ⁻
Secoiridoids					
21	40.1	demethyl ligstroside	278	509	509 (100) [M-H] ⁻ ; 347 (15) [M-H-glc] ⁻ ; 277 (11) [M-H-glc-C ₄ H ₆ O] ⁻ ; 233 (8) [M-H-glc-C ₄ H ₆ O-CO ₂] ⁻ ; 165 (3) [M-H-glc-CH ₂ CHPhOH-H ₂ O-CO ₂] ⁻ ; 121 (4) [M-H-glc-CH ₂ CHPhOH-H ₂ O-2CO ₂] ⁻
28	43.4	oleuropein	238, 282	539	539 (100) [M-H] ⁻ ; 377 (8) [M-H-glc] ⁻ ; 307 (33) [M-H-glc-C ₄ H ₆ O] ⁻ ; 275 (28) [M-H-glc-C ₄ H ₆ O-CH ₃ OH] ⁻ ; 223 (5) [M-H-glc-CH ₂ CHPh(OH) ₂ -H ₂ O] ⁻ ; 149 (11) [M-H-glc-CH ₂ CHPh(OH) ₂ -CH ₃ COOH-CH ₃ OH] ⁻ ; 139 (5) [M-H-glc-C ₄ H ₆ O-CH ₃ OH-CH ₂ CHPh(OH) ₂] ⁻
35	47.1	ligstroside	240, 278	523	523 (100) [M-H] ⁻ ; 361 (25) [M-H-glc] ⁻ ; 291 (68) [M-H-glc-C ₄ H ₆ O] ⁻ ; 259 (35) [M-H-glc-C ₄ H ₆ O-CH ₃ OH] ⁻ ; 223 (6) [M-H-glc-CH ₂ CHPhOH-H ₂ O] ⁻ ; 139 (4) [M-H-glc-C ₄ H ₆ O-CH ₃ OH-CH ₂ CHPhOH] ⁻ ; 101 (7)
41	54.7	ligstroside isomer 1	240, 279	523	523 (63) [M-H] ⁻ ; 291 (100) [M-H-glc-C ₄ H ₆ O] ⁻ ; 259 (63) [M-H-glc-C ₄ H ₆ O-CH ₃ OH] ⁻
47	9.9 ^b	oleoside	220, 266	389	389 (100) [M-H] ⁻ ; 345 (9) [M-H-CO ₂] ⁻ ; 209 (2) [M-H-glc-H ₂ O] ⁻ ; 165 (3) [M-H-glc-H ₂ O-CO ₂] ⁻ ; 139 (4) [M-H-glc-2CO ₂] ⁻ ; 121 (5) [M-H-glc-H ₂ O-2CO ₂] ⁻
56	16.5 ^b	ligstroside hexoside	222, 278	685	523 (100) [M-H-glc] ⁻ ; 361 (21) [M-H-2glc] ⁻ ; 291 (63) [M-H-2glc-C ₄ H ₆ O] ⁻ ; 223 (4) [M-H-2glc-CH ₂ CHPhOH-H ₂ O] ⁻ ; 139 (6) [M-H-2glc-C ₄ H ₆ O-CH ₃ OH-CH ₂ CHPhOH] ⁻ ; 101 (4)
57	18.0 ^b	ligstroside isomer 2	240, 278	523	523 (100) [M-H] ⁻ ; 361 (21) [M-H-glc] ⁻ ; 291 (63) [M-H-glc-C ₄ H ₆ O] ⁻ ; 223 (4) [M-H-glc-CH ₂ CHPhOH-H ₂ O] ⁻ ; 139 (4) [M-H-glc-C ₄ H ₆ O-CH ₃ OH-CH ₂ CHPhOH] ⁻ ; 101 (4)
Phenylethanoid glycosides					
16	37.1	calcelarioside A	250, 288sh, 326	477	477 (100) [M-H] ⁻ ; 315 (3) [M-H-caffeoyl] ⁻ ; 179 (6) [caffeic acid-H] ⁻ ; 161 (30) [gluc-H-H ₂ O] ⁻ ; 135 (4) [caffeic acid-H-CO ₂] ⁻
19	39.0	verbascoside	246, 286sh, 330	623	623 (100) [M-H] ⁻ ; 461 (9) [M-H-caffeoyl] ⁻ ; 161 (40) [179-H ₂ O] ⁻
20	39.9	calcelarioside B	250, 288sh, 326	477	477 (100) [M-H] ⁻ ; 315 (4) [M-H-caffeoyl] ⁻ ; 161 (36) [gluc-H-H ₂ O] ⁻ ; 135 (10) [M-H-caffeoyl-glc-H ₂ O] ⁻
23	41.9	isoverbascoside	286sh, 328	623	623 (100) [M-H] ⁻ ; 461 (4) [M-H-caffeoyl] ⁻ ; 161 (12) [179-H ₂ O] ⁻
25	42.5	eukovoside	252, 284, 328	637	637 (100) [M-H] ⁻ ; 461 (9) [M-H-feruloyl] ⁻ ; 193 (3) [ferulic acid-H] ⁻ ; 175 (9) [ferulic acid-H ₂ O] ⁻ ; 161 (7) [ferulic acid-CH ₃ OH] ⁻
44	8.2 ^b	verbascoside	222, 280	461	461 (100) [M-H] ⁻ ; 315 (9) [M-H-rham] ⁻ ; 297 (5) [M-H-rham-H ₂ O] ⁻ ; 135 (10) [M-H-rham-glc] ⁻
45	8.5 ^b	cistanoside F	298sh, 328	487	487 (100) [M-H] ⁻ ; 179 (45) [M-H-caffeoyl-rham] ⁻ ; 161 (2) [M-H-caffeoyl-rham-H ₂ O] ⁻ ; 135 (28) [caffeic acid-CO ₂] ⁻
49	12.4 ^b	β-hydroxyverbascoside isomer 1	220, 286sh, 328	639	639 (14) [M-H] ⁻ ; 621 (19) [M-H-H ₂ O] ⁻ ; 477 (40) [M-H-caffeoyl] ⁻ ; 459 (12) [M-H-H ₂ O-caffeoyl] ⁻ ; 179 (43) [caffeoyl-H] ⁻ ; 161 (100) [glc-H-H ₂ O] ⁻ ; 145 (41) [rham-H-H ₂ O] ⁻ ; 135 (22) [caffeoyl-H-CO ₂] ⁻
51	13.7 ^b	β-hydroxyverbascoside isomer 2	228, 278, 330sh	639	639 (27) [M-H] ⁻ ; 621 (50) [M-H-H ₂ O] ⁻ ; 459 (11) [M-H-H ₂ O-caffeoyl] ⁻ ; 179 (43) [caffeoyl-H] ⁻ ; 161 (100) [glc-H-H ₂ O] ⁻ ; 135 (44) [caffeoyl-H-CO ₂] ⁻
53	14.1 ^b	β-methoxyverbascoside	278, 324sh	653	653 (14) [M-H] ⁻ ; 621 (30) [M-H-CH ₃ OH] ⁻ ; 491 (10) [M-H-caffeoyl] ⁻ ; 179 (98) [caffeoyl-H] ⁻ ; 161 (100) [glc-H-H ₂ O] ⁻

(Continues)

Table 1. (Continued)

Peak	R _t (min)	Compound	λ _{max} (nm)	[M-H] ⁺ m/z	MS/MS m/z (%) [attribution] ^a
Dilignols and Oligolignols					
9	29.0	cyclooolivil	282	375	375 (100) [M-H] ⁺ ; 360 (15) [M-H-CH ₃] ⁺ ; 345 (5) [M-H-CH ₂ O] ⁺ ; 327 (5) [M-H-H ₂ O-CH ₂ O] ⁺
11	33.6	olivil	279	375	375 (94) [M-H] ⁺ ; 360 (6) [M-H-CH ₃] ⁺ ; 345 (10) [M-H-CH ₂ O] ⁺ ; 327 (13) [M-H-H ₂ O-CH ₂ O] ⁺ ; 195 (66) [A] ⁺ ; 179 (100) [B] ⁺ ; 164 (17) [B-CH ₃] ⁺ ; 146 (22) [B-H ₂ O-CH ₃] ⁺
13	35.1	S(t8-O-4)G	278	405	405 (100) [M-H] ⁺ ; 390 (6) [M-H-CH ₃] ⁺ ; 375 (6) [M-H-CH ₂ O] ⁺ ; 357 (6) [M-H-H ₂ O-CH ₂ O] ⁺ ; 225 (32) [A] ⁺ ; 209 (9) [A-CH ₃] ⁺ ; 195 (4) [A-CH ₂ O] ⁺ ; 179 (19) [B] ⁺ ; 164 (6) [B-CH ₃] ⁺ ; 161 (7) [B-H ₂ O] ⁺
15	36.3	G(t8-O-4)G	270, 306sh	375	375 (50) [M-H] ⁺ ; 345 (17) [M-H-CH ₂ O] ⁺ ; 327 (100) [M-H-H ₂ O-CH ₂ O] ⁺ ; 165 (37) [A-CH ₂ O] ⁺
22	40.8	S(t8-O-4)G'	276	403	403 (90) [M-H] ⁺ ; 373 (100) [M-H-CH ₂ O] ⁺ ; 358 (9) [M-H-CH ₂ O-CH ₃] ⁺ ; 343 (17) [M-H-2CH ₂ O] ⁺ ; 315 (12); 299 (16); 223 (8) [A] ⁺ ; 211 (5); 179 (7) [B] ⁺ ; 149 (4) [B-CH ₂ O] ⁺
26	42.7	secoisolariciresinol	252, 284sh, 352	361	361 (100) [M-H] ⁺ ; 346 (8) [M-H-CH ₃] ⁺ ; 331 (2) [M-H-CH ₂ O] ⁺ ; 179 (4) [X ³ X] ⁺ ; 165 (8) [X-CH ₃] ⁺
27	43.2	lariciresinol	238, 282	359	359 (4) [M-H] ⁺ ; 329 (100) [M-H-CH ₂ O] ⁺ ; 314 (43) [M-H-CH ₂ O-CH ₃] ⁺ ; 299 (13) [M-H-2CH ₂ O] ⁺ ; 269 (22) [M-H-2CH ₂ O-2CH ₃] ⁺
32	46.1	syringaresinol	240, 272	417	417 (100) [M-H] ⁺ ; 387 (12) [M-H-CH ₂ O] ⁺ ; 371 (14) [M-H-HCOOH] ⁺ ; 327 (7) [M-H-CH ₂ O-4CH ₃] ⁺ ; 181 (18) [^{2,5} X] ⁺ ; 166 (4) [^{2,5} X-CH ₃] ⁺ ; 151 (7) [^{2,5} X-2CH ₃] ⁺
33	46.3	condendrin	244, 272	355	355 (100) [M-H] ⁺ ; 340 (25) [M-H-CH ₃] ⁺ ; 337 (100) [M-H-H ₂ O] ⁺ ; 322 (14) [M-H-H ₂ O-CH ₃] ⁺ ; 310 (13) [M-H-CH ₂ O-CH ₃] ⁺ ; 295 (18) [M-H-2CH ₂ O] ⁺ / [M-H-CH ₂ O-2CH ₃] ⁺
34	46.4	unidentified trilignol	248, 266sh, 298sh, 340sh	583	583 (61) [M-H] ⁺ ; 535 (1) [M-H-H ₂ O-CH ₂ O] ⁺ ; 421 (2); 415 (4); 329 (5); 209 (10); 185 (34); 167 (100); 149 (19); 123 (5); 108 (4)
36	49.1	G(t8-O-4)S(8-8)G / G(8-O-4)G(8-8)S	242, 280, 340	583	583 (100) [M-H] ⁺ ; 535 (74) [M-H-H ₂ O-CH ₂ O] ⁺ ; 519 (49) [M-H-H ₂ O-HCOOH] ⁺ ; 507 (18) [M-H-H ₂ O-CH ₂ O-CO] ⁺ ; 489 (18) [M-H-H ₂ O-CH ₂ O-HCOOH] ⁺ ; 477 (11); 387 (40) [AE, II, ⁸ B] ⁺ ; 357 (10) [AE, II, ⁸ B-CH ₂ O] ⁺ ; 195 (27) [AE, II, ⁸ A] ⁺ ; 165 (44) [AE, II, ⁸ A-CH ₂ O] ⁺ ; 150 (28) [AE, II, ⁸ A-CH ₂ O-CH ₃] ⁺
38	50.8	G(8-O-4)S(8-5)G'	250, 282sh, 342	581	581 (100) [M-H] ⁺ ; 551 (9) [M-H-CH ₂ O] ⁺ ; 535 (42) [M-H-H ₂ O-CO] ⁺ ; 517 (78) [M-H-2H ₂ O-CO] ⁺ ; 505 (38) [M-H-CH ₂ O-H ₂ O-CO] ⁺ ; 367 (33) [PC, I] 355 (16) [PC, II]; 195 (19) [AE, II, ⁸ A] ⁺ ; 165 (29) [AE, II, ⁸ A-CH ₂ O] ⁺ ; 150 (13) [AE, II, ⁸ A-CH ₂ O-CH ₃] ⁺
39	51.1	G(8-O-4)S(t8-O-4)G(8-8)S	242, 280	809	809 (100) [M-H] ⁺ ; 791 (12) [M-H-H ₂ O] ⁺ ; 763 (26) [M-H-HCOOH] ⁺ ; 745 (9) [M-H-2H ₂ O-HCOOH] ⁺ ; 733 (12) [M-H-HCOOH-CH ₂ O] ⁺ ; 613 (9) [AE, II, ⁸ B] ⁺ ; 565 (12) [AE ^{2B} , I] ⁺ ; 421 (12) [AE ^{2B} , II, ⁸ A] ⁺ ; 387 (13) [AE ^{2B} , II, ⁸ B] ⁺ ; 195 (12) [AE, II, ⁸ A] ⁺ ; 165 (12) [AE, II, ⁸ A-CH ₂ O] ⁺
46	9.9 ^b	hydroxyariciresinol hexoside 1	220, 266	537	537 (0); 375 (13) [M-H-glc] ⁺ ; 360 (6) [M-H-glc-CH ₃] ⁺ ; 345 (2) [M-H-glc-CH ₂ O] ⁺ ; 327 (39) [M-H-H ₂ O-CH ₂ O] ⁺ ; 312 (12) [M-H-H ₂ O-CH ₂ O-CH ₃] ⁺ ; 297 (7) [M-H-H ₂ O-CH ₂ O-2CH ₃] ⁺ ; 195 (35) [A] ⁺ ; 191 (42); 179 (100) [B] ⁺ ; 164 (40) [B-CH ₃] ⁺ ; 161 (24) [B-H ₂ O] ⁺ ; 150 (17) [A-CH ₂ O-CH ₃] ⁺ ; 146 (51) [B-H ₂ O-CH ₃] ⁺
48	11.1 ^b	hydroxyariciresinol hexoside 2	230sh, 280	537	537 (0); 375 (23) [M-H-glc] ⁺ ; 360 (9) [M-H-glc-CH ₃] ⁺ ; 345 (2) [M-H-glc-CH ₂ O] ⁺ ; 327 (25) [M-H-H ₂ O-CH ₂ O] ⁺ ; 312 (15) [M-H-H ₂ O-CH ₂ O-CH ₃] ⁺ ; 195 (42) [A] ⁺ ; 179 (100) [B] ⁺ ; 164 (50) [B-CH ₃] ⁺ ; 161 (29) [B-H ₂ O] ⁺ ; 150 (17) [A-CH ₂ O-CH ₃] ⁺ ; 146 (46) [B-H ₂ O-CH ₃] ⁺
50	13.5 ^b	syringaresinol hexoside 1	278	579	579 (3) [M-H] ⁺ ; 417 (83) [M-H-glc] ⁺ ; 402 (33) [M-H-CH ₄] ⁺ ; 387 (29) [M-H-CO ₂] ⁺ ; 181 (100) [^{2,5} X] ⁺ ; 166 (53) [^{2,5} X-CH ₃] ⁺ ; 151 (15) [^{2,5} X-2CH ₃] ⁺ 417 (8) [M-H] ⁺ ; 402 (9) [M-H-CH ₃] ⁺ ; 387 (58) [M-H-CH ₂ O] ⁺ ; 181 (65) [^{2,5} X] ⁺ ; 166 (100) [^{2,5} X-CH ₃] ⁺ ; 151 (58) [^{2,5} X-2CH ₃] ⁺
52	13.9 ^b	fraxiresinol hexoside	nd	565	565 (0); 403 (19) [M-H-glc] ⁺ ; 373 (57) [M-H-glc-CH ₂ O] ⁺ ; 343 (19) [M-H-glc-2CH ₂ O] ⁺ ; 299 (26) [M-H-glc-2CH ₂ O-CO ₂] ⁺ ; 181 (100) [^{2,5} X] ⁺ ; 166 (87) [^{2,5} X-CH ₃] ⁺
54	15.0 ^b	syringaresinol hexoside 2	276	579	579 (15) [M-H] ⁺ ; 417 (100) [M-H-glc] ⁺ ; 402 (3) [M-H-CH ₄] ⁺ ; 387 (2) [M-H-CO ₂] ⁺ ; 181 (12) [^{2,5} X] ⁺ ; 166 (4) [^{2,5} X-CH ₃] ⁺ ; 151 (2) [^{2,5} X-2CH ₃] ⁺

(Continues)

Peak	R _t (min)	Compound	λ _{max} (nm)	[M-H] ⁻ m/z	MS/MS m/z (%) [attribution] ^a
55	16.3 ^b	G(t8-O-4)S(8-8)G hexoside	276	745	745 (30) [M-H] ⁻ ; 583 (23) [M-H-glc] ⁻ ; 535 (100) [M-H-glc-H ₂ O-CH ₂ O] ⁻ ; 505 (12); 387 (93) [AE, II, ⁸ B] ⁻ ; 372 (13) [AE, II, ⁸ B-CH ₃] ⁻ ; 357 (26) [AE, II, ⁸ B-CH ₂ O] ⁻ ; 329 (11) [AE, II, ⁸ A] ⁻ ; 195 (29) [AE, II, ⁸ A] ⁻ ; 165 (81) [AE, II, ⁸ A-CH ₂ O] ⁻ ; 150 (57) [AE, II, ⁸ A-CH ₂ O-CH ₃] ⁻
Unknown compounds					
6	21.8	unknown compound	288		181 (56) [M-H] ⁻ ; 163 (17) [M-H-H ₂ O] ⁻ ; 137 (100) [M-H-CO ₂] ⁻ ; 122 [M-H-CO ₂ -CH ₃] ⁻
24	42.0	unknown compound	248, 284, 316sh		329 (26) [M-H] ⁻ ; 311 (95) [M-H-H ₂ O] ⁻ ; 299 (100) [M-H-2CH ₂ O/2CH ₃] ⁻ ; 296 (32); 284 (27); 281 (32); 269 (26); 265 (9); 253 (10); 159 (4)
29	45.8	unknown compound	274, 312sh		517 (100); 307 (47); 223 (14); 145 (15); 137 (10)
30	46.0	unknown compound	248, 278, 326sh		637 (79); 523 (8) [M-H-114] ⁻ ; 461 (8) [M-H-feruloyl] ⁻ ; 113 (100) [114-H] ⁻ ; 69 (10)
31	46.0	unknown compound	248, 284, 344		367 (100); 352 (22); 336 (41); 321 (41); 238 (9)
37	50.5	unknown compound	242, 286sh, 326		609 (100) [M-H] ⁻ ; 447 (3) [M-H-glc] ⁻ ; 415 (2) [M-H-feruloyl-H ₂ O] ⁻ ; 345 (14); 253 (3) [M-H-glc-feruloyl-H ₂ O] ⁻ ; 209 (9); 193 (73) [ferulic acid-H] ⁻ ; 185 (27); 175 (7) [ferulic acid-H ₂ O] ⁻ ; 149 (22) [ferulic acid-CO ₂] ⁻ ; 134 (12)
40	53.1	unknown compound	302sh, 330		271 (100); 256 (50); 241 (54); 213 (15); 185 (3)
42	5.6 ^b	unknown compound	280		433 (100); 124 (14)
43	6.7 ^b	unknown compound	220sh, 278		463 (100); 169

* sh = shoulder; ^a Fragment ions displayed were obtained from the EPI spectrum of the *m/z* value in bold. ^bR_t from the aqueous extracts chromatogram.

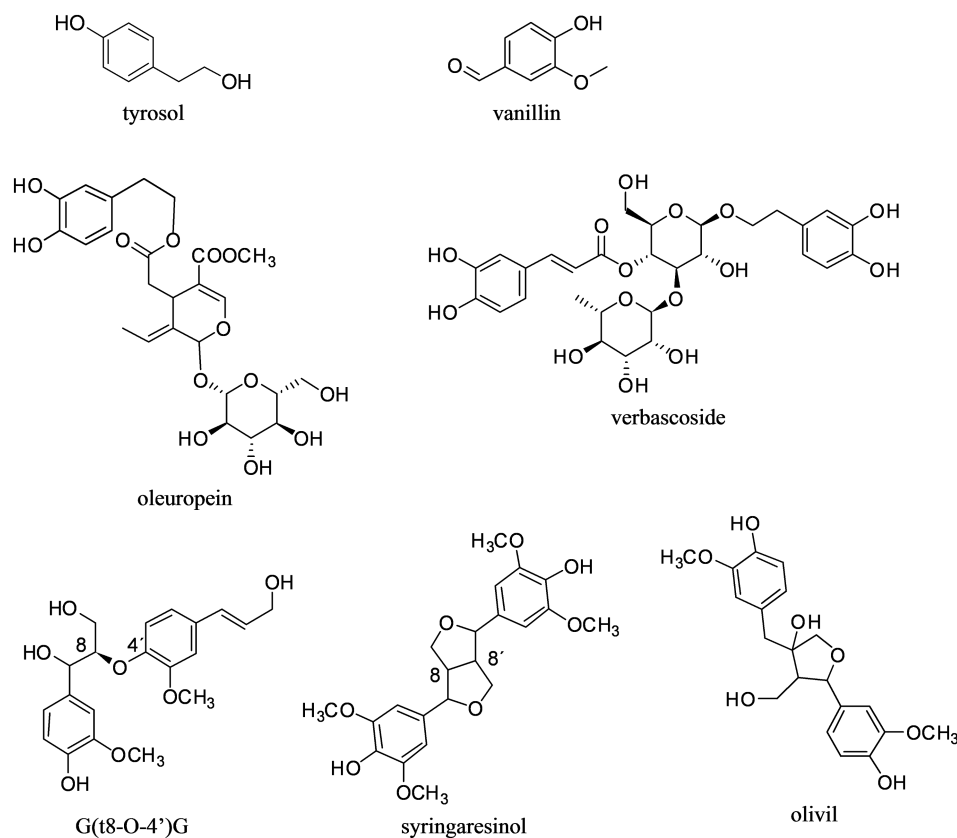


Figure 4. Chemical structures of some identified compounds.

other woods for cooperage,^[8-10,16] with the exception of hydroxytyrosol and tyrosol. These last two compounds were isolated in *F. americana* leaves,^[20] and tyrosol was also identified in

F. americana and *F. excelsior* heartwood by GC-MS.^[6] Peak **18** showed a UV/Vis spectrum suggestive of cinnamic compound or the hydroxycoumarin esuletin, and a mass spectrum consistent

with the elimination of methyl and CO₂ moieties. Identification as 2-methoxycinnamic acid, a compound already found in *Fraxinus* spp.,^[11] was ruled out with the commercial standard, so peak **18** was only tentatively identified as a cinnamic compound.

Secoiridoids

Mass spectrum and fragmentation pattern, together with literature data, allowed the identification of compounds **21**, **28**, **35**, **41**, **47**, **56** and **57** as oleoside-type secoiridoids. Peak **28** was identified as oleuropein using the available commercial standard. Mass and UV/Vis spectra of peak **35** and **47** matched literature data for ligstroside and oleoside, respectively.^[12,21,22] Compound **35** showed characteristic ions due to the cleavage of the glycosyl bond and the consecutive loss of C₄H₆O and CH₃OH fragments,^[23] and those relating to rearrangements fragments.^[23,24] The mass spectrum of peak **47** revealed the ion of deprotonated oleoside molecule. The fragmentation profile was characterized by the fragments due to the losses of CO₂ and hexose, commonly described for this secoiridoid.^[21,25] Both compounds have been previously described in *Fraxinus* species.^[12] Peak **56** showed a [M-H]⁻ molecular ion 162 Da higher than ligstroside and product ion spectrum similar to this compound. This suggested that compound **56** was a hexoside derivative of ligstroside and it was in agreement with that described previously.^[12] Compound **21** produced a deprotonated molecular ion 14 Da less than the ligstroside. Further fragmentation of the deprotonated molecular ion produced main ions consistent with the reported fragmentation pattern for ligstroside after loss of a CH₂ group. Presence of demethyligstroside has already been reported in *Fraxinus americana*.^[11]

Compounds **41** and **57** have been identified as possible ligstroside isomers. Both compounds exhibited a deprotonated molecular ion at *m/z* 523, and in the second generation mass spectrum, the same fragmentation pattern observed for ligstroside. Isoligstroside was also identified in leaves of *Fraxinus griffithii*^[13] and *F. malacophylla*.^[26]

Phenylethanoid glycosides

Ten compounds (**16**, **19**, **20**, **23**, **25**, **44**, **45**, **49**, **51** and **53**) have been identified as phenylethanoid glycosides. Among them, peaks **19** and **23** were identified as verbascoside and isoverbascoside, respectively, by comparing them with the reference standard.

Two compounds, peaks **16** and **20**, exhibited the same deprotonated molecule, UV spectrum and fragmentation patterns. Their molecular mass (146 Da less than the verbascoside) revealed the absence of rhamnose attached to the molecule. In the second generation mass spectrum of the deprotonated molecule, the ions observed matched to typical fragmentation pattern of the phenylethanoid glycosides. By examining the literature, these compounds were reasonably identified as calceolareoside A and B, respectively, phenylethanoid glycosides previously described in the phloem of ash species.^[12] Compound **25** was tentatively identified as a verbascoside derivative showing a deprotonated molecular ion 14 Da higher than the verbascoside, indicating the presence of a methyl group in the molecule. The MS² experiment revealed a fragment that can be attributed to the loss of feruloyl unit. The presence of a feruloyl moiety was also supported by the detection of fragment ions at *m/z* 193 and 175. The first expulsion of the feruloyl unit was in agreement with the position of the feruloyl moiety at the terminal rhamnose unit. These results are in accordance with the fragmentation pattern described in the literature^[27] for eukovoside, reported in solid olive residue. Phenylethanoids detected in

leaves, bark and phloem of *Fraxinus* showed as caffeoyl esters,^[11] and to the best of our knowledge, this is the first report on the occurrence of ferulic acid derivatives. Peak **44** gave a [M-H]⁻ ion at *m/z* 461 and fragmentation ions corresponding to the successive losses of pentose, pentose and water, and pentose and hexose, indicating the ion *m/z* 135 the hydroxytyrosol moiety after the loss of water.^[28] Its molecular mass and the absence of ions at *m/z* 299, 179 and 161 indicated the absence of a caffeoyl moiety in its structure. Having referred to the literature, compound **44** was tentatively characterized as verbascoside or decaffeoylverbascoside.^[28] Peak **45** showed a [M-H]⁻ at *m/z* 487 and fragments suggesting the elimination of caffeoyl and pentosyl units, as well as the further loss of water, respectively. Cistanoside F was proposed as its structure taking into account the presence of caffeic acid and literature data.^[28]

Peaks **49** and **51** were tentatively identified as isomers of β -hydroxyverbascoside. Both compounds gave a [M-H]⁻ 16 Da higher than the verbascoside, indicating the presence of a hydroxyl group in their structures and yielded very similar MS/MS spectra. Product ion spectra of these peaks are in agreement with the loss of a water molecule, and the presence of a caffeic acid moiety and its decarboxylated product, and the presence of a hexose molecule, in accordance with the literature data.^[27,29]

The molecular mass of peak **53** was 30 Da higher than the verbascoside, suggesting the presence of a methoxyl group. Ions observed in the MS² analysis of the deprotonated molecule showed typical fragmentation pathways with a methoxyl group substituted in the β position.^[30] With reference to its demethoxyl analogue, and to the absence of other kinds of phenylethanoid glycosides, compound **53** was tentatively identified as β -methoxylverbascoside.

Dilignols and oligolignols

Peaks **9**, **11**, **13**, **15**, **22**, **26**, **27**, **32**, **33**, **34**, **36**, **38**, **39**, **46**, **48**, **50**, **52**, **54** and **55** were identified as dilignols or oligolignols, using literature data for UV/Vis absorbance and mass spectra when standards were not available. Among them, different class of dilignols and oligolignols were identified. When a common name is not usual, they were named according to linkage (8-8', 8-5' or 8-O-4') and unit (G = guaiacyl, S = syringyl) type, as it was described previously.^[31]

Compounds **26**, **27** and **33** were identified as secoisolariciresinol, lariciresinol and conidendrin by comparing them with the commercial available standards. Another dilignol belonging to the 8-8' linkage group is compound **32**, tentatively identified as syringaresinol since their [M-H]⁻ and fragmentation ions were consistent with data previously reported in the literature.^[31,32] Syringaresinol was also detected in ash thermal treated wood (200 °C, 4 h) using GC-MS¹⁵, and in the phloem of *F. americana* by LC-MS.^[12]

On the basis of the fragmentation patterns, compounds **13**, **15** and **22** were characterized as β -aryl ether dilignols.^[31] Consecutive losses of formaldehyde and water and A⁻ and B⁻ products ions allowed the characterization of the bonding structure and the aromatic units involved in these dimers (*m/z* 195 or *m/z* 179 for guaiacyl A or B ions, respectively; *m/z* 225 for syringyl A⁻ ion, and *m/z* 223 for unit derived for sinapaldehyde). Accordingly, they were tentatively identified as S(t8-O-4)G, G(t8-O-4)G and S(t8-O-4)G', respectively.

Peaks **9** and **11** displayed the same [M-H]⁻ ion at *m/z* 375, such as the β -aryl ether dimer G(t8-O-4)G (peak **15**), but they presented a characteristic UV/Vis spectrum of furano lignans.^[33] The MS² spectrum of these compounds showed characteristic

ions associated to dilignols. By examining the lignans described in *Fraxinus*, two isomers matched data spectra of compounds **9** and **10**, cycloolivil and olivil or 8-hydroxyariciresinol.^[34] Olivil was proposed as the structure for compound **11** taking into account its molecular weight, their UV bands matching to literature data, and the provided ion fragments, which are consistent with the opening and cleavage of the tetrahydrofuran ring and further losses of a methyl group and a water molecule.^[35]

Base peak at m/z 583 was attributed to two compounds, **34** and **36**. However, apart from the characteristic loss of CH_2O and water moieties, these isomers yielded different spectra in MS/MS analysis. The MS^2 spectrum of compound **36** was very similar to those previously reported for other trilignols^[36] and suggested the presence of β -aryl ether and resinol linkages (neutral losses of H_2O , CH_2O and HCOOH moieties). Cleavage of the β -aryl ether linkage provided the A and B series ions and evidenced the dimeric substructure and the G unit 8-O-4' linked on the end position. Hence, two structures may be inferred from the MS spectrum, G(8-O-4)S(8-8)G or G(8-O-4)G(8-8)S. Accordingly, compound **38** was tentatively identified as G(8-O-4)S(8-5)G'.^[36] No tentative identification could be proposed for compound **34** since no assignment for the obtained ion fragments was possible.

Compound **39** showed a deprotonated molecular ion at m/z 809, in the range of those observed for tetramers.^[34] MS^2 spectrum yield peaks from the trimeric and monomeric substructures and peaks resulting from the cleavage of the central linkage. These data suggested the central 8-O-4 linkage position, the G(8-O-4)S and G(8-8)S as dimeric substructures and a G unit β -aryl ether linked to the trimeric moiety. Furthermore, neutral losses of two H_2O and HCOOH evidenced the type of linkages involved.^[37] Hence, two structures could be assigned based on these MS data, G(8-O-4)S(8-O-4)G(8-8)S or G(8-O-4)S(8-O-4)S(8-8)G.

Compounds related to peaks **50** and **54** were identified as a pair of isomers. Both exhibited a deprotonated molecular ion at m/z 579 and an intense fragment at m/z 417, attributed to the loss of a hexoside moiety. Further, MS analysis of the m/z 417 ion produced identical product ions as tentatively identified as syringaresinol. Hence, they were identified as syringaresinol hexoside derivatives. Similarly, peak **52** was tentatively characterized as a fraxiresinol hexoside, since the MS/MS spectrum of the aglycone ion yielded fragments that suggested syringyl and guaiacyl units linked by a resinol bonding structure. By examining the literature, fraxiresinol-8-O- β -D-glucopyranoside was described in *Fraxinus* spp.^[11] Furthermore, spectrometric data also allowed the tentative identification of compounds **46** and **48** as lignan glycosides. Different UV spectra were observed for these structures, while displayed MS spectra were quite similar. A typical neutral loss of 162 Da was observed, suggesting a hexose residue bounded to the phenolic oxygen atom in the lignan moiety.^[35] Glycosylation at the A or B ring cannot be inferred from the MS analysis since it does not influence the sugar loss. MS/MS spectra of these compounds produced the ions as the fragmentation of the 8-hydroxyariciresinol.^[35] Based on the above data, an assignment as hydroxyariciresinol hexoside isomers is possible. Similarly, compound **55** was tentatively identified as the hexoside derivative of the trilignol **38**.

Phenolic compounds in seasoned and toasted ash wood. The HPLC-DAD quantitative evaluations of phenolic compounds extracted from seasoned and toasted woods are included in Table 2. The total contents of different chemical families, calculated by adding concentrations of individual compounds, are also

shown. In seasoned and toasted ash wood, tannins were not detected, neither hydrolysable nor condensed tannins, thus establishing a very important qualitative difference in relation to the chemical composition of oak woods used in cooperage, as oak woods are characterized by their significant concentrations of ellagitannins.^[16,17] Moreover, other woods that can be used in cooperage, such as chestnut, acacia or cherry, also mainly contain hydrolysable or condensed tannins, the levels of which being especially high in the case of chestnut.^[8-10] The second important qualitative difference is the presence of secoiridoids, phenylethanoid glycosides, di and oligolignols, which are undetected in oak or the other woods. However, although these compounds were found at high concentrations in seasoned ash wood, the toasting provoked their degradation, and only four of them were detected at low levels in the most toasted ash wood, resulting in a minor differentiation between toasted ash and oak woods. Thirdly, in relation to LMW phenolic compounds, some of those detected in ash wood – such as tyrosol, protocatechuic aldehyde and acid, and hydroxytyrosol –, have never been found in oak wood, although the last two disappeared during toasting. Contrary, gallic and ellagic acids, which are very important in oak wood, were not found in ash wood.

Regarding LMW phenolic compounds from a quantitative point of view, the levels in toasted ash woods of some hydroxybenzoic and hydroxycinnamic acids and aldehydes were much higher than those detected in toasted oak and in other woods that can be used in cooperage, highlighting those of vanillin which reached 404 $\mu\text{g/g}$ in *F. excelsior* and 329 $\mu\text{g/g}$ in *F. americana*. Vanillin is the most important compound from an organoleptic point of view, in relation to the aging of wines, since it is an impact molecule with a vanilla smell. In fact, in the market for alternatives to barrel oak products, some makers modify their production system with the objective of obtaining the largest possible quantity of this compound, resulting in a very wide range of concentrations detected, with 456 $\mu\text{g/g}$ the highest when 232 toasted samples were analyzed, while only ten of them showing values higher than 300 $\mu\text{g/g}$.^[38] A more pronounced vanilla character can therefore be expected when using toasted ash wood in aging wines.

In seasoned wood, a great variety of phenolic compounds were detected with an average concentration range from 1 to more than 2700 $\mu\text{g/g}$ of wood, with oleuropein, ligstroside and olivil being the most prominent, together with verbascoside and isoverbacoside in *F. excelsior*, and oleoside in *F. americana*. All having average concentrations higher than 300 $\mu\text{g/g}$ of wood. In Table 2, it can be seen that the two species of seasoned woods showed qualitatively different phenolic composition, since 19 of the 36 compounds identified among secoiridoids, phenylethanoids and lignols were detected in only one specie, in addition to hydroxytyrosol, protocatechuic acid and the unidentified cinnamic, thus highlighting the concentration of oleoside in *F. americana*.

However, when one of these compounds was detected in the two species, there was no statistical difference in their average concentrations because of the intra-specific variability, as occurred, for example, for verbascoside, isoverbacoside, oleuropein or ligstroside. We only found statistically significant differences in seasoned wood for average concentrations of seven compounds: vanillic acid, verbascoside, cycloolivil, hydroxyariciresinol hexoside 2, syringaresinol hexoside 1, the unidentified trilignol, and G(8-O-4)S(8-8)G hexoside, with higher concentrations in *F. excelsior* except for the vanillic acid and trilignol (Table 2). However, if we only take into account data related to species but not those related to toasting level (Table 3)

Table 2. HPLC-DAD quantitative evaluation of phenolic compounds in seasoned and toasted *Fraxinus americana* and *F. excelsior* extracts

Peak	compound	(µg/g wood)					
		<i>Fraxinus americana</i>			<i>Fraxinus excelsior</i>		
		seasoned	light toasted	medium toasted	seasoned	light toasted	medium toasted
LMW Phenolic Compounds							
1	hydroxytyrosol	4.08 ± 1.80a	nd b	nd b	nd b	nd b	nd b
4	tyrosol	139 ± 49.7a	108 ± 1.08ab	35.5 ± 3.97c	100 ± 51.9abc	79.9 ± 11.3abc	53.9 ± 1.64bc
2	protocatechuic acid	1.49 ± 1.55a	nd a	nd a	nd a	nd a	nd a
5	vanillic acid	16.5 ± 6.93c	71.6 ± 8.98b	99.4 ± 3.74a	6.04 ± 2.28d	66.6 ± 8.77b	97.3 ± 5.44a
8	syringic acid	4.11 ± 2.14e	61.0 ± 5.61d	122 ± 10.0c	2.44 ± 1.61e	161 ± 5.61b	220 ± 23.14a
12	ferulic acid	4.02 ± 2.51e	17.2 ± 3.21d	28.7 ± 0.56c	2.89 ± 0.91e	34.7 ± 4.10b	46.2 ± 2.45a
3	protocatechualdehyde	14.9 ± 7.70ab	21.0 ± 2.74a	20.6 ± 0.39a	9.48 ± 2.81b	21.7 ± 3.16a	20.4 ± 0.32a
7	vanillin	23.4 ± 11.1d	245 ± 21.1c	329 ± 8.80b	12.0 ± 4.92d	313 ± 18.7b	404 ± 12.9a
10	syringaldehyde	20.6 ± 5.52e	260 ± 3.01d	461 ± 7.69c	13.8 ± 7.63e	560 ± 3.01b	902 ± 14.9a
14	coniferaldehyde	10.6 ± 4.13e	588 ± 9.51b	826 ± 5.0a	6.01 ± 1.66e	388 ± 9.51d	557 ± 1.72c
17	sinapaldehyde	18.6 ± 8.37e	672 ± 14.5d	1196 ± 0.09b	9.94 ± 2.43e	773 ± 14.5c	1358 ± 2.45a
18	unknown (<i>m/z</i> 177)	32.6 ± 16.1a	nd b	nd b	nd b	nd b	nd b
	∑ LMW phenolic compounds	290 ± 40.1	2044 ± 38.6	3117 ± 24.7	163 ± 52.6	2396 ± 31.7	3660 ± 56.7
Secoiridoids							
28	oleuropein	552 ± 170a	121 ± 12.0c	nd d	909 ± 359a	296 ± 85.0b	nd d
47	oleoside	475 ± 300a	276 ± 57.1ab	nd b	nd b	nd b	nd b
35	ligstroside	365 ± 106a	nd b	nd b	600 ± 230a	nd b	nd b
41	ligstroside isomer 1	nd b	nd b	nd b	144 ± 59.6a	nd b	nd b
57	ligstroside isomer 2	nd b	nd b	nd b	275 ± 129a	nd b	nd b
56	ligstroside hexoside	nd b	nd b	nd b	69.2 ± 10.8a	nd b	nd b
21	demethyl ligstroside	133 ± 103ab	nd b	nd b	262 ± 168a	nd b	nd b
	∑ secoiridoids	1527 ± 463	396 ± 69.1	nd	2260 ± 385	296 ± 85.0	nd
Phenylethanoid glycosides							
16	calcelarioside A	nd b	nd b	nd b	45.8 ± 25.8a	nd b	nd b
20	calcelarioside B	nd b	nd b	nd b	72.8 ± 46.0a	nd b	nd b
44	verbascoside	27.5 ± 22.5b	12.2 ± 4.55b	nd b	114 ± 86.0a	59.7 ± 4.41ab	nd b
45	cistanoside F	26.8 ± 20.7a	8.88 ± 2.35b	nd b	nd b	nd b	nd b
19	verbascoside	217 ± 155a	17.7 ± 1.11a	31.2 ± 6.04a	2716 ± 1877a	495 ± 110a	26.0 ± 0.49a
23	isoverbascoside	68.0 ± 59.7ab	5.65 ± 3.76b	nd b	510 ± 342a	59.2 ± 6.21b	nd
25	eukovoside	nd b	nd b	nd b	7.95 ± 3.18a	nd b	nd b
49	β-hydroxyverbascoside 1	100 ± 60.0ab	10.7 ± 1.87 b	nd b	136 ± 89.6a	16.7 ± 4.26 b	nd b
51	β-hydroxyverbascoside 2	7.65 ± 5.45a	nd b	nd b	nd b	nd b	nd b
53	β-methoxyverbascoside	23.0 ± 5.73ab	nd b	nd b	42.5 ± 35.4a	nd b	nd b
	∑ phenylethanoids	470 ± 223	55.1 ± 13.6	31.2 ± 6.04	3645 ± 2451	630 ± 112	26.0 ± 0.49
Di and Oligolignols							
9	cyclooolivil	21.9 ± 5.19b	24.1 ± 0.61b	62.4 ± 2.81a	14.5 ± 3.23c	26.0 ± 1.26b	65.9 ± 1.26a
11	Olivil	396 ± 124a	107 ± 4.10b	118 ± 19.8b	306 ± 212ab	208 ± 59.1ab	158 ± 1.36b
13	S(8-O-4)G	37.4 ± 13.2a	nd b	nd b	nd b	nd b	nd b
15	G(8-O-4)G	54.2 ± 30.6a	nd b	nd b	nd b	nd b	nd b
22	S(8-O-4)G'	40.9 ± 10.4a	nd b	nd b	34.6 ± 26.6a	nd b	nd b
33	conidendrin	5.83 ± 1.19a	nd b	nd b	nd b	nd b	nd b
26	secoisolaricresinol	27.9 ± 11.4a	nd b	nd b	nd b	nd b	nd b
27	laricresinol	178 ± 84.1a	nd b	nd b	nd b	nd b	nd b
46	hydroxylaricresinol hexoside 1	nd c	nd c	nd c	154 ± 75.54a	41.4 ± 4.70b	nd c
48	hydroxylaricresinol hexoside 2	22.5 ± 5.89c	26.4 ± 2.21c	nd c	124 ± 45.6a	66.4 ± 25.3b	nd c
32	syringaresinol	nd e	74.3 ± 9.33c	157 ± 1.09a	nd e	64.3 ± 2.22d	147 ± 2.22b
50	syringaresinol hexoside isomer 1	36.3 ± 26.6b	13.1 ± 1.73bc	nd c	60.4 ± 17.9a	25.8 ± 4.76bc	nd c
54	syringaresinol hexoside isomer 2	66.7 ± 18.5a	25.6 ± 4.36bc	nd c	90.9 ± 43.3a	55.3 ± 3.84ab	nd c
52	fraxiresinol hexoside	nd c	nd c	nd c	124 ± 31.6a	64.0 ± 2.02b	nd c
34	trilignol	41.8 ± 16.8a	nd b	nd b	12.9 ± 9.84b	nd b	nd b
36	G(8-O-4)S(8-8)G	79.4 ± 29.8a	nd b	nd b	ndb	nd b	nd b

(Continues)

Table 2. (Continued)

Peak	compound	(µg/g wood)					
		<i>Fraxinus americana</i>			<i>Fraxinus excelsior</i>		
		seasoned	light toasted	medium toasted	seasoned	light toasted	medium toasted
38	G(8-O-4)S(8-5)G'	80.0 ± 31.8 ^a	nd b	nd b	nd b	nd b	nd b
55	G(8-O-4)S(8-8)G hexoside	14.0 ± 6.04 ^b	nd c	nd c	37.6 ± 12.4 ^a	nd c	nd c
39	G(8-O-4)S(8-O-4)G(8-8)S	29.6 ± 9.41 ^a	nd b	nd b	nd b	nd b	nd b
	∑ di and oligolignols	1132 ± 251	271 ± 21.1	338 ± 21.5	960 ± 401	551 ± 49.5	371 ± 2.33

Different letters in the same row denote a statistical difference with 95% confidence level (Student Newman–Keuls multiple range test).

Table 3. F-values from the analysis of variance (ANOVA) of quantitative evaluation of phenolic compounds in seasoned and toasted *Fraxinus americana* and *F. excelsior* extracts^a

Compound	specie	toasting level ^b	specie × toasting level
LMW phenolic compounds			
hydroxytyrosol	11.2*	5.41	22.8***
tyrosol	1.60	9.70**(a a b)	5.43*
protocatechuic acid	5.19	2.51	4.13*
vanillic acid	0.25	448***(c b a)	245***
syringic acid	3.03	57.8***(c b a)	497***
ferulic acid	2.07	72.8***(c b a)	282***
protocatechualdehyde	1.32	13.8***(b a a)	7.26**
vanillin	0.27	447***(c b a)	996***
syringaldehyde	2.80	64.1*** (c b a)	10 652***
coniferaldehyde	1.15	185*** (c b a)	21 004***
sinapaldehyde	0.10	1742*** (c b a)	25 937***
unknown (m/z 177)	10.36*	5.01	35.7***
secoiridoids			
oleuropein	2.60	9.07**(a b b)	7.12**
oleoside	18.1**	2.60	11.05***
ligstroside	0.05	29.7*** (a b b)	12.6***
ligstroside isomer 1	11.5*	5.58*(a b b)	26.1***
ligstroside isomer 2	10.8*	5.23	20.1***
ligstroside hexoside	14.4**	6.95*(a b b)	183***
demethyl ligstroside	1.62	13.3*** (a b b)	7.21**
phenylethanoid glycosides			
calcelarioside A	9.65*	4.66	14.0***
calcelarioside B	8.83*	4.27	11.2***
verbascoside	7.82*	4.42	5.78**
cistanoside F	11.3*	2.58	7.06**
verbascoside	0.07	7.75*(a b b)	3.25
isoverbascoside	2.40	6.38*(a b b)	4.07*
eukovoside	11.7*	5.66*(a b b)	27.8***
β-hydroxyverbascoside 1	0.50	16.7*** (a b b)	6.70**
β-hydroxyverbascoside 2	7.94*	3.84	8.77***
β-methoxyverbascoside	1.24	11.8** (a b b)	5.82*
di/oligolignols			
cyclooolivil	0.11	310*** (c b a)	210***
oolivil	0.03	9.82** (a b b)	4.63*
S(8-O-4)G	12.3*	5.95*(a b b)	35.7***
G(8-O-4)G	9.63*	4.66	13.9***
S(8-O-4)G'	0.14	28.2*** (a b b)	10.5***
conidendrin	13.98**	6.76*(a b b)	107***
secoisolaricresinol	11.6*	5.61*(a b b)	26.8***
laricresinol	10.8*	5.20	19.8***
hydroxylaricresinol hexoside 1	16.0**	3.90	17.6***

(Continues)

Table 3. (Continued)

Compound	specie	toasting level ^b	specie × toasting level
hydroxylariciresinol hexoside 2	14.7**	6.80*(a a b)	22.7***
syringaresinol	0.05	2598***(c b a)	2230***
syringaresinol hexoside isomer 1	2.58	19.7***(a b c)	11.5***
syringaresinol hexoside isomer 2	1.80	25.1***(a b c)	12.8***
fraxiresinol hexoside	29.8***	3.98	67.5***
trilignol	4.84	14.5***(a b b)	18.2***
G(8-O-4)S(8-8)G	12.0*	5.82*(a b b)	31.7***
G(8-O-4)S(8-5)G'	11.7*	5.67*(a b b)	28.1***
G(8-O-4)S(8-8)G hexoside	4.29	21.4***(a b b)	28.7***
G(8-O-4)S(8-O-4)G(8-8)S	12.7*	6.16*(a b b)	44.1***

^a*, ** and *** indicate significance at $p < 0.01$, 0.001 and 0.0001, respectively.

^bLetters between parentheses show the differences among toasting level, in the order seasoned, light toasting and medium toasting. Different letters denote a statistical difference with 95% confidence level (Student Newman–Keuls multiple range test), with *a* being the highest concentration.

fraxiresinol hexoside showed the highest *F*-value and significance at $p < 0.0001$ level, followed by oleoside, hydroxylariciresinol hexosides 1 and 2, ligstroside hexoside, and conidendrin, all showing $p < 0.001$.

As in other woods, the toasting of ash generate a progressive increase of lignin constituents, with regard to toasting intensity, since a depolymerization takes place, thus producing hydroxycinnamic aldehydes at the first step, hydroxybenzoic aldehydes at the second, and finally the acids, causing the final concentration of these molecules in toasted woods related to the lignin structure of each wood. The increase was particularly important in sinapaldehyde for the two species, followed by syringaldehyde in *F. excelsior*, and coniferaldehyde in *F. americana*. The differences between species at light and medium toasting were significant for the four aldehydes, but also for syringic and ferulic acid, with higher concentrations in *F. excelsior*, with the exception of coniferaldehyde.

Other LMW phenolic compounds – such as hydroxytyrosol, tyrosol, protocatechuic acid and the unidentified cinnamic – were also sensitive to heat treatment, but in the opposite way, since its content decreased in the wood with the duration of toasting. The same happened for concentrations of secoiridoids, phenylethanoids, oligolignols and dilignols. The exceptions were cycloolivil and syringaresinol which significantly increased their concentrations during toasting, without differences between the two

species and syringaresinol being the compound that showed the highest *F*-valued related to toasting level. This dilignol was the major component in the acetone extract from thermally modified *F. excelsior* wood,^[15] after 4 h at 200 °C in a nitrogen atmosphere and under pressure. It can therefore be considered thermally very stable, which facilitate its use as a food additive, a nutraceutical or a drug, since these families of compounds have been the subject of many papers in recent years,^[21–37] due to their important biological activity.^[39] Unfortunately, we found very little data in regard to their response to thermal treatments that could be used in the food or pharmaceutical industry. In a recent work,^[40] oleuropein was the only compound different from the simple phenols detected in the HPLC analysis, when hot water and steam explosion treatments were used to upgrade the extraction of these compounds from olive tree pruning residues, and the reducing power and radical scavenging capacities were maximal at the lowest temperatures used (170°C – 10 min. or 200°C – 5 min.). In our samples, we found significant differences between the average values of seasoned and light-toasted woods for almost all studied compounds. However, only three of these compounds that decrease during toasting (hydroxylariciresinol hexoside 2, and the two syringaresinol hexoside isomers) allowed distinguishing between light and medium-toasted samples (Table 3). Thus, the effect of temperature on these compounds was very important even when applying light toasting.

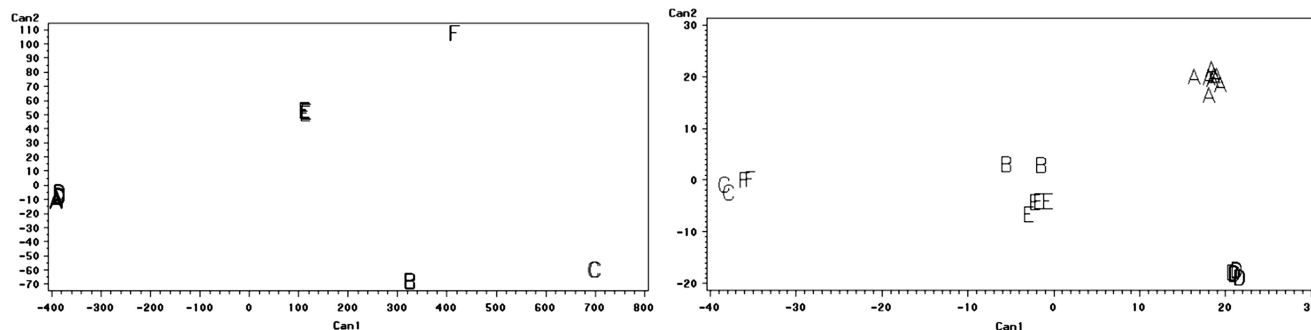


Figure 5. Canonical discriminant analysis of LMW phenolic compounds (left) and secoiridoids, phenylethanoid glycosides and lignols (right) in *Fraxinus* spp. heartwood. A = seasoned *F. americana*; B = light toasted *F. americana*; C = medium-toasted *F. americana*; D = seasoned *F. excelsior*; E = light toasted *F. excelsior*; F = medium-toasted *F. excelsior*. $n = 60$. Left: 99.98% of dispersion (98.43% Can1 and 1.55% Can2); Canonical correlation of 0.9999 and 0.9998, for Can 1 and Can 2, respectively. Right: 98.85% of dispersion (73.96% Can1 and 24.89% Can2); Canonical correlation of 0.9992 and 0.9977, for Can 1 and Can 2, respectively.

With the purpose of having an overall view of the influence that botanical wood species and toasting intensity have on phenolic composition, we carried out two multivariate data analyses, those LMW and the remainder of phenolics separately. The graphic representation of the samples, in the space defined by the two main canonical functions obtained in each analysis, shows a distribution of samples that only allow clear distinctions among some groups. In Fig. 5-left, related to LMW phenolic compounds, we can see that canonical function 1 (Can 1) allows us to distinguish seasoned wood (A and D in figure) from light (B and E) and medium (C and F) toasted wood of both ash species. This function explains 98.43% of variance, and the more correlated variables were coniferaldehyde, sinapaldehyde, vanillic acid and aldehyde, all of them with negative coefficients higher than 0.9, according to total canonical structure. The distances among samples throughout Can 2 were statistically negligible, since this function explains only 1.55% of variance. For the remaining phenolic compounds (Fig. 5-right), we can see that the canonical function 1 (73.96% of variance) allows us to distinguish the samples only on the basis of their toasting level, while the canonical function 2 (24.89%) allows us to do only on the basis of the specie, although the statistical distances among species gradually decrease, and it is not possible to distinguish the woods with medium toasting. Cyclooolivil and syringaresinol, both with negative coefficients, along with the syringaresinol glycosides, with positive coefficients, were the variables that correlated closest to Can 1, while oleoside, with positive coefficient, and hydroxylariciresinol hexoside 1 and fraxiresinol hexoside, with negative coefficient, correlated closest to Can 2, according to total canonical structure.

Syringaresinol, as well as other furofuran lignans have been reported to exhibit various biological activities, including antifungal, anti-inflammatory, antimalarial activities, inhibition of cyclic AMP phosphodiesterase, inhibition of platelet aggregation, anti-leukemic, antioxidation and cytotoxic activities, DNA cleavage effect, etc., and some methods have been proposed for its synthesis,^[39,41] in addition to its use in natural extracts. Other than being an effective radical scavenger, (+)-cyclooolivil possesses antiplatelet aggregation properties and inhibits protein tyrosine phosphorylation, suggesting that it may prevent diseases associated with platelet hyper-aggregability.^[42] Its presence in most toasted woods, together with verbascoside, olivil and syringaresinol, all biologically active compounds,^[39] could contribute to producing healthier wines when using seasoned or toasted ash wood in the wine aging.

In regard to their organoleptic qualities, although they have already been studied, the exact relationship of the individual compounds and their sensory characteristics has not completed to date. Oleuropein is known to be responsible for bitterness and astringency in olives, but it is not found in significant quantities in the oil, although its aglycone, along with some isomers, has been found. Thus, the proposed reduction of oil bitterness by way of the heat treatment of olives was based on a significant correlation between oil bitterness and hydroxytyrosol derivatives secoiridoids content.^[43] This has therefore led to the study of aldehydic and dialdehydic forms of oleuropein and ligstroside,^[44,45] as well as the study of two other derivatives from oleuropein and methyloleuropein, as causes of bitterness.^[43] These compounds have also been associated with spicy and pungency character of olive oil,^[44,46] showing a relationship between bitterness and spicy sensory properties and ligstroside derivatives^[47] as well as aldehyde form of oleuropein aglycone contents.^[45]

Andrewes *et al.*^[48] suggest that the pungency of olive oil is due to the demethylcarboxylic form of ligstroside aglycone. These authors also isolated some of these compounds and estimated their individual detection thresholds and sensory properties.

Despite these data, we cannot expect a significant contribution to the bitter and astringent character of wines, or other beverages aged in ash barrels, since, although the concentrations can be considered high in seasoned wood, the decrease caused by toasting is important even at light toasting. Therefore, after medium toasting, only the organoleptic effects produced by syringaresinol and olivil should be taken into account, although they are not known. From our point of view, there are two polyphenolic characteristics of these woods that may have important organoleptic consequences. The first being the high vanillin content in relation to other woods used for cooperage, which should imply a more pronounced vanilla character in aged wines, while the second being the absence of tannins, which could affect the evolution of the redox potential during the aging wine in ash barrels.

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