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Effect of Toasting Intensity at Cooperage on Phenolic Compounds in Acacia (*Robinia pseudoacacia*) Heartwood

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ABSTRACT: The phenolic composition of heartwood from *Robinia pseudoacacia,* commonly known as false acacia, before and after toasting in cooperage was studied by HPLC-DAD and HPLC-DAD/ESI-MS/MS. A total of 41 flavonoid and nonflavonoid compounds were identified, some tentatively, and quantified. Seasoned acacia wood showed high concentrations of flavonoid and low levels of nonflavonoid compounds, the main compounds being the dihydroflavonols dihydrorobinetin, fustin, tetrahydroxy, and trihydroxymethoxy dihydroflavonol, the flavonol robinetin, the flavanones robtin and butin, and a leucorobinetinidin, none of which are found in oak wood. The low molecular weight (LMW) phenolic compounds present also differed from those found in oak, since compounds with a β-resorcylic structure, gallic related compounds, protocatechuic aldehyde, and some hydroxycinnamic compounds are included, but only a little gallic and ellagic acid. Toasting changed the chromatographic profiles of extracts spectacularly. Thus, the toasted acacia wood contributed flavonoids and condensed tannins (prorobinetin type) in inverse proportion to toasting intensity, while LMW phenolic compounds were directly proportional to toasting intensity, except for gallic and ellagic acid and related compounds. Even though toasting reduced differences between oak and acacia, particular characteristics of this wood must be taken into account when considering its use in cooperage: the presence of flavonoids and compounds with β-resorcylic structure and the absence of hydrolyzable tannins.

KEYWORDS: *Robinia pseudoacacia*, false acacia, heartwood, tannins, phenolic compounds, toasting

INTRODUCTION

The use of wood during the fermentation and aging processes of wine and derivatives such as vinegar and spirits, or other drinks such as cider, develops important changes in their chemical composition and organoleptic properties. Regarding wine aging in oak barrels, these changes are attributed to physical, chemical and biochemical complex processes in which tannins and phenolic compounds are especially involved, not only those characteristic of wine (anthocyanins, flavonoids and tartaric esters of hydroxycinnamic acids) but also those supplied by wood (ellagitannins and polyphenols in oak wood). The simple extraction of aromatic compounds (volatiles and polyphenols), and tannins from wood can add richness and complexity to the aroma and taste.[1] Polyphenolic compounds from wood can also contribute directly or indirectly to color evolution and stability, influencing the formation of anthocyanin derivatives that takes place during wine aging.[2,3] The ellagitannins of oak wood also play an important role in color and astringency attributes. They behave as antioxidants due to their ability to consume high quantities of oxygen, regulating oxidation reactions. They are involved in numerous chemical reactions, for example, in condensation processes of wine anthocyanins and tannins with acetaldehyde[4] or reactions with flavanols.[5]

Oak wood (*Quercus* spp.) is the material par excellence used in cooperages to make containers for fermentating and aging wines. However, other species such as acacia (*Robinia pseudoacacia*), chestnut (*Castanea sativa*), cherry (*Prunus avium*), and ash (*Fraxinus excelsior* and *Fraxinus americana*) are being considered as possible sources of wood for the production of wines and their derived products, like spirits, and especially vinegars, in order to give them a special personality. The very little information reported in the literature on the effects of these woods compared to oak shows a different evolution of the phenolic and volatile composition, and organoleptic properties in wines and vinegars produced with them.[6−9] The use of acacia wood for aging vinegars is increasing due to the air transfer efficiency that favors a good acetification rate[10] and to its effects on the phenolic composition evolution and sensory quality. In addition, it has been observed that vinegars aged in acacia wood contain a characteristic compound, (+)-dihydrorobinetin, which increases

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during the aging process and can be used for authenticity purposes.[6,11]

Oak heartwood shows high levels of polyphenols, ellagitannins and volatile compounds that can vary greatly in relation to the species and geographical origin, as well as its processing in cooperage.[12,13] The most abundant polyphenols are the monomer ellagitannins, castalagin, roburin E, vescalagin, and grandinin, and low molecular weight phenolic compounds, ellagic and gallic acids, and lignin constituents such as benzoic and cinnamic acids, and their respective aldehydes, especially vanillin. It also provides a lot of volatile compounds to the aroma and flavor of aged wines, the *cis* and *trans* isomers of β-methyl-γ-octalactone being the most characteristic, turning out to be a balanced wood, since it can make these contributions without masking the wine primary and secondary aromas.[14]

The few published works about the chemical composition of *Robinia pseudoacacia* wood, commonly known as false acacia, locust tree, or black locust, point out important chemical differences in relation to oak woods that should be taken into account when considering using it for cooperage. Roux and Paulus[15] identified 14 flavonoids from the methanolic extracts of heartwood using paper chromatography, the flavanonol dihydrorobinetin, and the flavonol robinetin being the most prominent ones. Both compounds were later described together with a hydroxycinnamic acid derivative as the three main extractives,[16] and (+)-dihydrorobinetin has been recently reported as a chemical marker of vinegars aged in acacia wood.[11] Some oligomeric robinetinidins have also been identified in aqueous acetone extracts.[17]

Seasoned acacia wood has also shown a high variety of volatile compounds, lignin, lipid and carbohydrate derivatives.[18−20] In hydroalcoholic extracts, sinapaldehyde was found to be the most abundant compound, followed by coniferyl alcohol, 2,4-dihydroxybenzaldehyde, coniferaldehyde, syringaldehyde, and 3,4,5-trimethoxy phenol, and although this wood is poorer in most of them compared to oak, after toasting it shows a very high richness in volatiles in relation to toasted oak woods.[19]

The objective of this work is to know the tannic and polyphenolic composition of acacia (*Robinia pseudoacacia*) heartwood and its possible changes during the toasting process, with the purpose of completing its chemical characterization in regards to considering its use in cooperage, and to find out what effects it may have on the characteristics of wines, vinegars, and other drinks aged in this wood, always using oak wood as a reference. This and other woods could be used in many ways: for manufacturing containers, from large vats to barrels, and in recent years, for making pieces of many sizes (powder, shavings, chips, cubes, and staves) used as cheaper alternative techniques. Usually, oak is used, but these other woods could also be used in order to give a special personality to these products.

MATERIALS AND METHODS

Wood Samples. Acacia (*Robinia pseudoacacia*) 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 three intensities: 165 °C for 20 min (light) and 35 min (medium) and 185 °C for 45 min (medium plus), in an industrial kiln specially designed for toasting staves. Samples were taken before and after toasting, ten staves of each. Several wood pieces were cut out of each stave 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: 2,4−dihydroxybenzoic aldehyde, resorcinol, caffeic acid, gallic acid, methyl gallate and protocatechualdehyde (Fluka Chimie AG, Buchs, Switzerland), 2,4-dihydroxybenzoic acid, dihydrocaffeic acid, dihydroferulic acid, syringaldehyde, and coniferyl aldehyde (Aldrich Chimie, Neu-Ulm, Germany), ellagic acid and robinetinidin chloride (Apin, Oxon, UK), (+)-catechin, furfural, 5-methylfurfural, 5-hydroxymethylfurfural, vanillin, and syringic acid (Sigma Chemical, St. Louis, MO), dihydrorobinetin, fustin, isoliquiritigenin, robinetin, liquiritigenin, fisetin, cyanidin chloride, sinapaldehyde, vanillic acid, B2 procyanidin, isorhamnetin, ferulic acid, and butein (Extrasynthèse, Genay, France), and robtein (Transmit, Marburg, Germany). 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 redissolved in 1 mL of methanol/water 50%. These extracts and an aliquot part of freeze-dried extract redissolved in water (30 mg/mL) were used for the HPLC-DAD and LC-DAD/ESI-MS/MS analyses. Moreover, the ethyl acetate and freeze-dried extracts were used for the characterization of tannins. In the diethyl ether extract tannins were not detected. All the extractions were carried out in duplicate.

Tannin Characterization. In the ethyl acetate and freeze-dried extracts, the characterization of condensed tannins was carried out by HPLC analysis of anthocyanidins released after acid butanolysis.[21] All determinations were carried out in duplicate.

HPLC/DAD Analysis. Quantification of phenolics was performed by LC-DAD using an Agilent 1100 L 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 × 4 mm i.d., 5 μm, Hypersil ODS C18, maintained at 30 °C and protected with a 4 × 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 extract (30 mg) was: from 100% of A to 95% in 50 min, going to 70% (85 min), and 0% (105 min), with 10 min as equilibration time. Quantification was carried out by the external standard method, using peak areas in UV at 325 or 280 nm (tannins). 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.

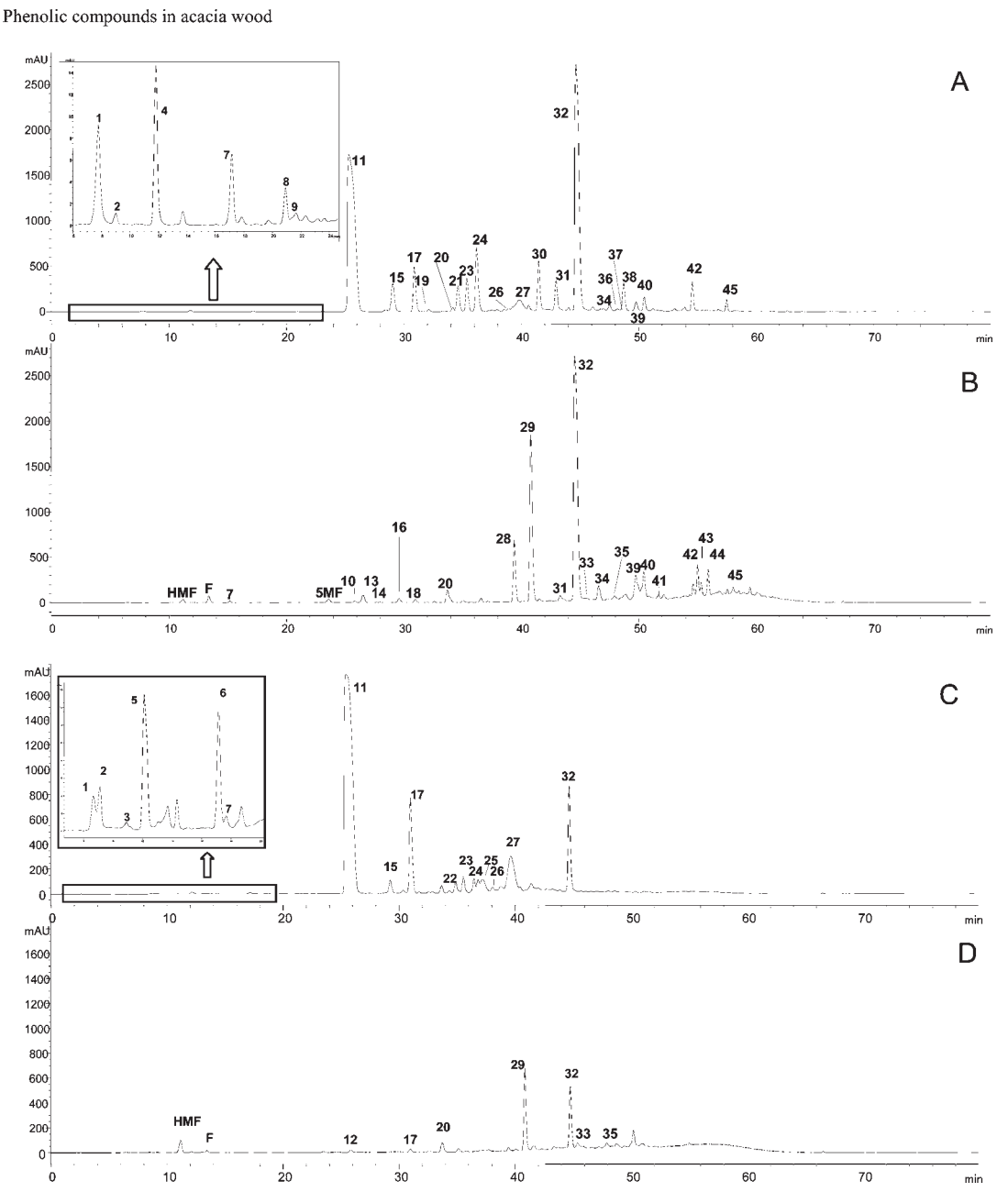

Figure 1. - HPLC-DAD chromatograms of *Robinia pseudoacacia* heartwood extracts, monitored at 325 ± 75 nm. A-Diethyl ether extract of seasoned heartwood. B- Diethyl ether extract of medium plus toasted heartwood. C- Ethyl acetate extract of seasoned heartwood. D- Ethyl acetate extract of medium plus toasted heartwood. Peak numbers as in Tables 1 and 2.

Thus, gallic aldehyde was quantified with syringaldehyde calibration, and peaks 27 and 30 with ferulic acid. Unidentified flavonoids were quantified as dihydrorobinetin (peaks 15, 17, 19, 21, 23, 24, 26, and 31), robinetin (peak 38 and 40) or butein (peak 37). Unidentified tannins (peaks 3, 5, 6, 9, 25, 46, and 47) were quantified as procyanidin B2, in agreement with their UV/vis profile (99.8% matching those of commercial standard). 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 diode array detector (DAD) (Agilent Technologies, Palo Alto, CA) 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 sixteen internal standards (1 μg/mL methanol/water 1:1, containing 0.5% formic acid and 5 mM 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 IDA (information-dependent acquisition). Precursors were selected by using an enhanced full scan (EMS) 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 more intense ions were analyzed using an enhanced

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Table 1. Spectroscopic and Spectrometric Data of Peaks in HPLC Chromatograms of Seasoned and Toasted *Robinia pseudoacacia* Heartwood Extracts[a]

peak	R_t (min)	comp.	λ_{max} (nm)	$[M-H]^- m/z$	MS/MS m/z (%) [attribution][a]
			LMW Phenolic Compounds		
2	9.0	gallic acid	272	169	**169** (100) $[M-H]^-$; 125 (60) $[M-H-CO_2]^-$
4	11.8	gallic aldehyde	302	153	**153** (100) $[M-H]^-$
7	17.1	protocatechualdehyde	280, 310	137	**137** (100) $[M-H]^-$
8	20.9	methyl gallate	272	183	**183** (100) $[M-H]^-$; 124 (35) $[M-H-CO_2-CH_3]^-$
10	25.8	β-resorcilyc acid	256, 296	153	**153** (100) [M-H]; 109 (25) $[M-H-CO_2]^-$
12	26.5	vanillic acid	260, 290	167	**167** (100) $[M-H]^-$; 152 (20) $[M-H-CH_3]^-$; 123 (5) $[M-H-CO_2]^-$; 108 (15) $[M-H-CH_3-CO_2]^-$
13	26.5	β-resorcilyc aldehyde	278, 316sh	137	**137** (100) $[M-H]^-$
14	28.6	caffeic acid	294sh, 322	179	**179** (100) $[M-H]^-$; 135 (25) $[M-H-CO_2]^-$; 109 (15)
16	29.6	vanillin	280, 312	151	**151** (32) $[M-H]^-$; 136 (100) $[M-H-CH_3]^-$
18	31.0	syringic acid	274	197	**197** (100) $[M-H]^-$; 182 (53) $[M-H-CH_3]^-$; 167 $[M-H-2CH_3]^-$153 (13) $[M-H-CO_2]^-$
20	33.7	syringaldehyde	232sh, 308	181	**181** (100) $[M-H]^-$; 166 (61) $[M-H-CH_3]^-$; 151 (31) $[M-H-CH_3]^-$
27	39.3	hydroxycinnamic compound	290sh, 320		**195** (97); 163 (21); 135 (100); 91 (48)
28	39.4	coniferaldehyde	290sh, 322	177	**177** (84) $[M-H]^-$;162 (100) $[M-H-CH_3]^-$
29	40.8	sinapaldehyde	300sh, 338	207	**207** (100) $[M-H]^-$; 192 (77) $[M-H-CH_3]^-$; 177 (77) $[M-H-2CH_3]^-$
30	41.5	hydroxycinnamic compound	305sh, 322		**545** (100); 527 (38); 417 (6); 389 (5); 243 (8)
36	48.0	ellagic acid	254, 368	301	**301** (100) $[M-H]^-$
			Flavonoids		
			Dihydroflavonols (or Flavanonols)		
11	25.3	dihydrorobinetin	276, 312sh	303[c]	**303** (37) $[M-H]^-$; 285 (100) $[M-H-H_2O]^-$; 135 (27) $[^{1,3}A]^-$; 167 (10) $[^{1,3}B]^-$; 109 (28) $[^{1,4}A]^-$; 193 (7) $[^{1,4}B]^-$; 163 (21) $[^{1,2}A]^-$; 91 (8) $[^{1,2}A\text{-}CO-CO_2]^-$; 139 (10) $[^{1,2}B]^-$ **285** (100) $[M-H-H_2O]^-$; 257 (20) $[M-H-CO]^-$; 241 (35) $[M-H-CO_2]^-$; 213 (34) $[M-H-CO_2-CO]^-$; 109 (4) $[^{1,4}A]^-$; 175 (56) $[^{1,4}B]^-$
15	29.0	pentahydroxydihydroflavonol	290, 312sh	319[c]	**319** (4) $[M-H]^-$; 301 (129) $[M-H-H_2O]^-$; 233 (2) $[M-H-C_2H_2O-CO_2]$ 193 (100) $[^{1,4}B]^-$; 179 (7) $[^{1,2}A]^-$ **301** (100) $[M-H-H_2O]^-$; 257 (24) $[M-H-CO_2]^-$; 125 (11) $[^{1,4}A]^-$; 175 (73) $[^{1,4}B]^-$
17	30.9	tetrahydroxydihydroflavonol	280, 316sh	303[c]	**303** (10) $[M-H]^-$; 285 (100) $[M-H-H_2O]^-$; 135 (20) $[^{1,3}A]^-$; 167 (14) $[^{1,3}B]^-$; 109 (10) $[^{1,4}A]^-$; 193 (10) $[^{1,4}B]^-$; 163 (21) $[^{1,2}A]^-$ **285** (100) $[M-H-H_2O]^-$; 241 (10) $[M-H-CO_2]^-$; 109 (2) $[^{1,4}A]^-$
19	32.4	trihydroxydihydroflavonol	282, 326sh	287[c]	**287** (89) $[M-H]^-$; 269 (100) $[M-H-H_2O]^-$; 259 (29) $[M-H-CO]^-$; 135 (21) $[^{1,3}A]^-$; 151 (7) $[^{1,3}B]^-$; 109 (25) $[^{1,4}A]^-$; 163 (27) $[^{1,2}A]^-$; 91 (3) $[^{1,2}A\text{-}CO-CO_2]^-$; 123 (8) $[^{1,2}B]^-$ **269** (100) $[M-H-H_2O]^-$; 225 (35) $[M-H-CO_2]^-$
21	34.2	trihydroxymethoxydihydroflavonol	292, 334sh	317[c]	**317** (100) $[M-H]^-$; 299(100) $[M-H-H_2O]^-$; 289 (8) $[M-H-CO]^-$; 284 (23) $[M-H-CH_3-H_2O]^-$; 274 (6) $[M-H-CH_3-CO]^-$; 135 (14) $[^{1,3}A]^-$; 109 (4) $[^{1,4}A]^-$; 163 (16) $[^{1,2}A]^-$ **299** (100) $[M-H-H_2O]^-$; 284 (46) $[M-H-CH_3]$
22	34.6	fustin	280, 314sh	287[c]	**287** (86) $[M-H]^-$; 269 (100) $[M-H-H_2O]^-$; 259 (31) $[M-H-CO]^-$; 243 (3) $[M-H-C_2H_2O]^-$; 135 (56) $[^{1,3}A]^-$; 151 (8) $[^{1,3}B]^-$; 109 (43) $[^{1,4}A]^-$; 177 (3) $[^{1,4}B]^-$; 163 (32) $[^{1,2}A]^-$; 91 (6) $[^{1,2}A\text{-}CO-CO_2]^-$; 123 (13) $[^{1,2}B]^-$ **269** (100) $[M-H-H_2O]^-$; 225 (35) $[M-H-CO_2]^-$; 197 (5) $[M-H-CO_2-CO]^-$
23	35.4	trihydroxymethoxy dihydroflavonol	278, 312sh	317[c]	**317** (100) $[M-H]^-$; 299 (31) $[M-H-H_2O]^-$; 289 (11) $[M-H-CO]^-$; 284 (40) $[M-H-CH_3-H_2O]^-$; 274 (24) $[M-H-CH_3-CO]^-$; 258 (9) $[M-H-CH_3-CO_2]^-$; 207 (4) $[^{1,4}B]^-$; 181 (4) $[^{1,3}B]^-$; 163 (31) $[^{1,2}A]^-$; 135 (65) $[^{1,3}A]^-$; 109 (12) $[^{1,4}A]^-$; 91 (7) $[^{1,2}A\text{-}CO-CO_2]^-$
26	38.7	trihydroxymethoxy dihydroflavonol	280, 316sh	317[c]	**317** (100) $[M-H]^-$; 299 (9) $[M-H-H_2O]^-$; 289 (1) $[M-H-CO]^-$; 284 (10) $[M-H-CH_3-H_2O]^-$; 163 (16) $[^{1,2}A]^-$; 135 (9) $[^{1,3}A]^-$; 91 (2) $[^{1,2}A\text{-}CO-CO_2]^-$

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Table 1. Continued

peak	R_t (min)	comp.	λ_{max} (nm)	$[M-H]^- m/z$	MS/MS m/z (%) [attribution][a]
			Dihydroflavones (or Flavanones)		
24	36.2	robtin	278, 314sh	287[c]	**287** (90) $[M-H]^-$;177 (4) $[^{1,4}B]^-$; 151 (100) $[^{1,3}B]^-$; 135 (62) $[^{1,3}A]^-$; 91 (16) $[^{1,3}A\text{-}CO_2]^-$
31	43.0	butin	278, 312sh	271[c]	**271** (57) $[M-H]^-$; 161 (5) $[^{1,4}B]^-$; 135 (100) $[^{1,3}A]^-$; 91 (11) $[^{1,3}A\text{-}CO_2]^-$; 135 (100) $[^{1,3}B]^-$
34	47.5	liquiritigenin	276, 318sh	255[c]	**255** (100) $[M-H]^-$; 135 (48) $[^{1,3}A]^-$; 119 (68) $[^{1,3}B]^-$; 91 (18) $[^{1,3}A\text{-}CO_2]^-$
			Flavonols		
32	44.6	robinetin	254, 364	301[c]	**301** (79) $[M-H]^-$; 273 (8) $[M-H-CO]^-$; 245 (6) $[M-H-2CO]^-$; 229 (4) $[M-H-CO_2-CO]^-$; 135 (54) $[^{1,3}A]^-$; 91 (9) $[^{1,3}A\text{-}CO]^-$
39	49.8	fisetin	248, 360	285[c]	**285** (100) $[M-H]^-$; 257 (3) $[M-H-CO]^-$; 241 (4) $[M-H-CO_2]^-$; 229 (5) $[M-H-2CO]^-$; 213 (1) $[M-H-CO_2-CO]^-$; 135 (14) $[^{1,3}A]^-$; 91 (3) $[^{1,3}A\text{-}CO]^-$
40	50.5	trihydroxymethoxy flavonol	256, 352	315[c]	**315** (80) $[M-H]^-$; 300 (100) $[M-H-CH_3]^-$; 271 (3) $[M-H-CO]^-$; 255 (7) $[M-H-CO_2]^-$; 243 (2) $[M-H-CO_2-CO]^-$;188 (4) $[^{1,4}B\text{-}2H]^-$; 164 (9) $[^{1,3}B]^-$; 135 (5) $[^{1,3}A]^-$
			Chalcones		
37	48.3	robtein	256, 386	287[c]	**287** (100) [$[M-H]^-$; 269 (4) $[M-H-H_2O]^-$; 177 (3) $[^oB]^-$; 151 (72) $[^1B]^-$; 135 (20) $[^1A]^-$;
42	54.6	butein	260, 382	271[c]	**271** (57) $[M-H]^-$; 253 (9) $[M-H-H_2O]^-$; 161 (5) $[^oB]^-$; 135 (100) $[^1B]^-$; 135 (100) $[^1A]^-$; 91 (29) $[^2A\text{-}CO_2]^-$;
45	57.5	isoliquiritigenin	238, 375	255[c]	**255** (100) $[M-H]^-$; 135 (20) $[^1A]^-$; 119 (65) $[^1B]^-$; 91 (16) $[^3B]^-$
			Aurones		
38	48.7	tetrahydroxyaurone	262, 400	285[c]	**285** (100) $[M-H]^-$; 149 (60) $[^{1,2}B]^-$; 135 (6) $[^{1,2}A]^-$; 109(3)$[^{1,3}A]^-$
			Unidentified Tannins		
3	10.4	leucorobinetinidin	280	305[c]	**305** (0) $[M-H]^-$; 287 (7) $[M-H-H_2O]^-$; 167 (23) $[^{1,3}B]^-$; 137 (100) $[^{1,3}A]^-$
5	12.1	leucorobinetinidin	278	305[c]	**305** (0) $[M-H]^-$; 287 (21) $[M-H-H_2O]^-$; 167 (23) $[^{1,3}B]^-$; 137 (100) $[^{1,3}A]^-$
6	17.1	leucorobinetinidin	278	305[c]	**305** (0) $[M-H]^-$; 167 (68) $[^{1,3}B]^-$; 137 (100) $[^{1,3}A]^-$
9	21.2	prorobinetinidin	279	289	**289** (100) $[M-H]^-$; 245 (42) $[M-C_2H_2O]^-$; 227 (6) $[M-C_2H_2O-H_2O]^-$
25	37.2	dimeric prorobinetinidin	280	591	**591** (100) $[M-H]^-$; 573 (29)$[M-H-H_2O]^-$; 285 (9)
46	18.0[b]	dimeric prorobinetinidin	280	589	**589** (100) $[M-H]^-$; 421 (19) $[M-C_8H_8O_4]^-$; 301 (20) $[QM_B]^-$; 287 (9) $[QM_T]^-$
47	20.1[b]	dimeric prorobinetinidin	279	589	**589** (100) $[M-H]^-$; 421 (13) $[M-C_8H_8O_4]^-$; 301 (29) $[QM_B]^-$; 287 (9) $[QM_T]^-$
			Unknown Compounds		
1	7.8	unkown compound	282, 318sh		**181** (45); 137 (47); 109 (100)
33	45.4	unkown compound	284sh, 308		**313** (100); 177 (13)
35	47.8	unkown compound	316, 376		**497** (100); 451 (100)
41	51.3	unkown compound	272		**643** (100); 595 (56); 417 (31); 387 (31); 225 (25); 195 (44); 165 (10)
43	55.8	unkown compound	330		**493** (100); 313 (36); 301 (13); 285 (15); 177 (9); 163 (14); 149 (6); 135 (10)
44	55.9	unkown compound	312, 340sh		**313** (100); 298 (87); 283 (75); 255 (21); 227 (12)

[a] Fragment ions displayed were obtained from the EPI spectrum of the m/z value shown in bold. [b] R_t referred to the aqueous extracts chromatogram. [c] EMS spectrum indicated the presence of $[2M-H]^-$ ion in addition to $[M-H]^-$.

resolution (ER) experiment (scan rate 250 amu/s, dynamic fill time) followed by an enhanced product ion (EPI) 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.

For the diethyl ether and ethyl acetate extracts analyses, the chromatographic separation was achieved at 40 °C on a Hypersil ODS C18 reversed phase column (200 × 4 mm i.d., particle size 5 μm), protected with a 4 × 4 mm i.d. guard column of the same material (Cheshire, UK). The elution method involved a multistep linear solvent gradient changing from a starting concentration of 0.5% formic acid, 5 mM ammonium acetate (eluent A) going to 85% (20 min); 75% (30 min); 50% (50 min); and 0% (70 min), using methanol/formic acid 0.5% as eluent B. The total time of analysis was 70 min, equilibration time 10 min. 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 separation was carried out at 40 °C on a Synergy Polar-reversed phase column (150 × 2.00 mm i.d., particle size 4 μm) (Phenomenex, Torrance, CA). With the same eluents described above the gradient was as follows: 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. The volume injected was 20 μL and the flow rate was set at 250 μL/min. DAD detection was performed between 190 and 650 nm.

Statistical Analysis. The obtained data were analyzed by ANOVA using the program SAS (version 9.1; SAS Institute, Cary, NC). When

significant differences were revealed ($p < 0.05$), means were compared applying the Student−Newman−Keuls (SNK) multiple range test.

RESULTS AND DISCUSSION

Identification of Phenolic Compounds. The analysis of the extracts of *Robinia pseudoacacia* heartwood revealed the presence of a wide variety of polyphenols which belong to very different chemical families. Figure 1 shows an example of HPLC-DAD chromatograms obtained from diethyl ether and ethyl acetate extracts of seasoned and toasted wood. The four chromatograms show qualitative and especially quantitative differences related to both the efficiency of extraction of phenolic compounds depending on the solvent used, especially for seasoned wood, and the condition of the wood (seasoned or toasted). Thus, almost all low molecular weight phenolic compounds were extracted in the diethyl ether, and some unidentified tannins in the ethyl acetate extract, with the remaining flavonoid compounds in a 3:1 ratio in the two extracts as previously reported in the literature.[12,22] Chromatograms of the freeze-dried extracts were not shown because of the presence of only two main peaks (1 and 11) in seasoned wood, and the absence of peaks, except a minor peak 1, in toasted wood. Peak 1 was also found in diethyl ether and ethyl acetate extracts, but showed the highest solubility in freeze-dried extract, revealing its high polarity. Peak 11 was found in considerable quantity in both diethyl ether and ethyl acetate extracts.

A total of 22 flavonoid and nonflavonoid compounds were identified by comparing their retention times and UV/vis and mass spectra with those of the standards. In addition, 19 peaks were tentatively identified, or a possible identity was suggested, taking into account these data and those in literature. Moreover, 5 peaks remain unidentified. LC-DAD/ESI-MS/MS data of the different peaks are summarized in Table 1.

Some nonflavonoid compounds, phenolic acids and aldehydes, were identified by comparing their retention times and UV/vis and mass spectra with those of pure standards: the phenolic acids, gallic, 2,4-dihydroxybenzoic or β-resorcylic, vanillic, caffeic, syringic and ellagic (peaks **2**, **10**, **12**, **14**, **18**, and **36**), the phenolic aldehydes, protocatechuic, β-resorcylic, vanillic, syringic, coniferylic, and sinapic (peaks **7**, **13**, **16**, **20**, **28**, and **29**), and the gallic acid methyl ester (peak **8**). In seasoned wood extracts, these compounds showed only minor peaks while in toasted wood extracts some of them produced major peaks. With the exception of caffeic acid, and β-resorcylic acid and aldehyde, these compounds have been described in a lot of woods from different species, some of them with a view to their use in cooperage, such as cherry, chestnut or oak.[12,23−25] β-resorcylic acid and its methyl ester, as well as the aldehyde, were previously described in false acacia heartwood.[19,26]

Table 1 also shows spectroscopic and spectrometric data for other peaks, which are also considered nonflavonoid (**4**, **27**, and **30**). Among them, peak **4**, which eluted after gallic acid at 11.8 min, was tentatively identified as the gallic aldehyde according to the literature data.[27] It had an UV/vis spectrum with a single absorption peak at 302 nm, characteristic of symmetrical chemical structures,[28] and mass spectra data that showed a quasi-molecular ion peak at m/z 153 consistent with its molecular weight. The UV spectrum of peak **27** was suggestive of a hydroxycinnamoyl type compound. The full scan mass spectrum of this peak gave a prominent ion at m/z 195 and its product ion spectrum showed neutral losses of 60 and 28 Da (m/z at 135 and 163). The deprotonated molecular ion could accommodate to dihydroferulic acid, however no fragment assignment that supported this hypothesis was possible, and R_t and λ_{max} were different from the dihydroferulic standard (32.2 min and 280 nm, respectively). Peak **30** showed retention time and UV/vis spectrum very similar to those of ferulic acid (R_t = 38.3 min; λ_{max} = 290 sh, 322 nm), but their MS spectra were very different (ferulic acid standard m/z 193 (100%) $[M\text{-}H]^-$, 178 (25%) $[M\text{-}H\text{-}CH_3]^-$), so peak **30** was only tentatively identified as a hydroxycinnamic compound. Magel et al.[16] also found a hydroxycinnamic compound, with the same UV/vis spectrum, as an important component in the heartwood of recently felled *Robinia pseudoacacia* trees.

The spectroscopic and spectrometric data of flavonoid compounds are also shown in Table 1. Along with the discussion of their mass fragmentation, the nomenclature proposed by Ma et al.[29] will be used to describe the resulting fragment ions. The $[^{i,j}A]^-$ and $[^{I,j}B]^-$ represent product ions containing intact A and B rings of the flavonoid skeleton; superscripts i and j indicate the C-ring bonds that were broken. The most abundant peaks in the HPLC-DAD chromatograms of seasoned acacia wood extracts eluted between 20 and 60 min (Figure1A, C) and showed the characteristic UV/vis spectra of flavonoids, with A and B bands (276−290 and 312−318 nm range) related to the A and B rings of the flavonoid skeleton. The UV/vis spectra of peaks **11**, **15**, **17**, **19**, **21**−**24**, **26**, **31**, and **34** were particularly characteristic of dihydroflavanones or dihydroflavonols in which B band is reduced to little more than a shoulder (312−318 nm); those of peaks **32**, **39**, and **40** were like flavonols (B band at 352−364 nm); those of **37**, **42**, and **45**, like chalcones, were characterized by a very prominent B band around 340−390 nm, with another peak of much lower intensity (A band in 220−270 nm range); and the UV profile of peak **38** showed an intense B band at 390−420 nm that suggested an aurone structure.

The final structural assignment of dihydrorobinetin, fustin, liquiritigenin, robinetin fisetin, robtein, butein, and isoliquiritigenin were confirmed using the available commercial standards. In addition they were previously described in heartwood of this same species (*Robinia pseudoacacia*),[15,16,26,30] but their description in this wood by MS/MS spectrometry was first reported here.

Eight other peaks with UV spectrum characteristic of dihydroflavonol or flavanone structure were detected (Figure 1, Table 1). Among them, peaks **15**, **17**, **19**, **21**, **23**, and **26** were assigned as dihydroflavonols based on their MS fragmentation behavior. All of them exhibited an important loss of H_2O, a characteristic commonly observed in dihydroflavonol standards, which yielded the dehydrated dihydroflavonol moiety (flavone structure). Product ions of the deprotonated molecular ion $[M\text{-}H]^-$ and of the $[M\text{-}H\text{-}H_2O]^-$ (corresponding to flavone structure) supported these assignments.

Peak **15** provided $[M\text{-}H]^-$ at m/z 319 and fragment ions corresponding to losses of H_2O and C_2H_2O and CO_2 in the two former fragments. The most intense fragment, at m/z 193, and the fragment at m/z 179 could be attributed to the C-ring fission fragments $^{1,4}B^-$ and $^{1,2}A^-$. Fragmentation of the $[M\text{-}H\text{-}H_2O]^-$ ion yielded the fragments $[^{1,4}A]^-$ and $[^{1,4}B]^-$ at m/z 125 and 175. These *retro*-Diels−Alder (RDA) fragments indicated the dihydroxy substitution of the A-ring and the trihydroxy substitution of B-ring in the dihydroflavanonol skeleton of this compound. Peak **17** was found to have a deprotonated molecular ion and product ion

Table 2. HPLC-DAD Quantitative Evaluation of Phenolic Compounds in Seasoned and Toasted Acacia Heartwood and F-values from the Analysis of Variance (ANOVA)[a]

		(μg/g wood)				
peak	compound	seasoned	light toasted	medium toasted	medium+ toasted	F-values
		LMW Phenolic Compounds				
2	gallic acid	27.09 ± 10.31 bc	43.0 ± 17.7 b	83.3 ± 22.7 a	6.92 ± 0.65 c	20.8***
10	β-resorcilyc acid	nd c	nd c	137 ± 29.1 b	174 ± 16.3 a	30.9***
12	vanillic acid	nd b	nd b	nd b	6.52 ± 1.02 a	120***
14	caffeic acid	nd c	nd c	23.8 ± 7.15 b	46.5 ± 11.2 a	113***
18	syringic acid	nd c	nd c	51.8 ± 5.49 b	120 ± 6.21 a	107***
36	ellagic acid	14.2 ± 7.58 a	2.76 ± 1.44 b	1.01 ± 0.58 b	nd c	9.16**
4	gallic aldehyde	108.2 ± 43.9 b	137 ± 11.3 b	245 ± 87.7 a	21.1 ± 0.99 b	10.3**
7	protocatechualdehyde	29.67 ± 9.90 a	25.2 ± 2.18 a	44.1 ± 17.5 a	20.4 ± 0.86 a	3.15
13	β-resorcilyc aldehyde	48.49 ± 10.14 c	205 ± 100 b	357 ± 8.67 a	353 ± 2.65 a	99.8***
16	vanillin	nd c	8.29 ± 3.02 c	46.0 ± 21.6 b	71 ± 0.37 a	36.6***
20	syringaldehyde	nd d	19.8 ± 4.28 c	88.3 ± 12.8 b	326 ± 22.3 a	355***
28	coniferaldehyde	nd c	69.5 ± 18.5 b	276 ± 39.5 a	300 ± 26.8 a	291***
29	sinapaldehyde	nd d	57.0 ± 9.52 c	239 ± 114 b	1666 ± 6.35 a	386***
8	methyl gallate	8.09 ± 3.52 a	4.25 ± 0.65 a	2.06 ± 0.90 a	nd a	1.28
27	hydroxycinnamic derivative	2506 ± 1003 a	748 ± 124 b	419 ± 111 b	nd c	13.4***
30	hydroxycinnamic derivative	437 ± 136 a	331 ± 63.6 a	255 ± 62.4 a	nd b	7.27*
	Σ *LMW phenolic compounds*	*3179 ± 1079*	*1653 ± 33.0*	*2337 ± 373*	*3011 ± 72.9*	
		Flavonoids				
11	dihydrorobinetin	32265 ± 7391 a	23662 ± 663 a	7729 ± 560 b	nd c	30.6***
15	trihydroxymethoxydihydroflavonol	1288 ± 636 a	865 ± 86.4 ab	313 ± 120 ab	nd c	6.47*
17	tetrahydroxydihydroflavonol	4142 ± 1334 a	3196 ± 198 a	848 ± 48.6 b	nd c	16.6***
19	trihydroxydihydroflavonol	364 ± 118 a	155 ± 32.7 b	130 ± 18.3 b	nd c	13.7***
21	trihydroxymethoxydihydroflavonol	183 ± 57.7 a	74.2 ± 3.02 b	53.0 ± 17.4 b	nd c	16.4***
22	fustin	3986 ± 1907 a	2261 ± 67.2 ab	1079 ± 367 ab	nd b	6.77*
23	trihydroxymethoxydihydroflavonol	841 ± 299 a	859 ± 25.1 a	352 ± 77.8 ab	nd c	9.34**
26	trihydroxymethoxydihydroflavonol	325 ± 77.2 a	148 ± 14.7 b	82.4 ± 30.1 bc	nd c	28.6***
24	robtin	1757 ± 559 a	1835 ± 190 a	869 ± 112 ab	157 ± 23.2 b	9.20**
31	butin	1244 ± 283 a	647 ± 89.3 b	308 ± 34.3 b	297 ± 7.25 b	18.4***
34	liquiritigenin	253 ± 153 a	135 ± 14.5 a	61.6 ± 10.5 a	63.6 ± 3.33 a	4.14
32	robinetin	7870 ± 1404 a	6598 ± 382 a	7461 ± 700 a	6988 ± 43.1 a	1.33
39	fisetin	706 ± 400 a	1140 ± 411 a	1335 ± 334 a	719 ± 41.3 a	6.83*
40	trihydroxymethoxy flavonol	166 ± 53.6 a	179 ± 26.1 a	170 ± 21.3 a	193 ± 13.2 a	0.19
37	robtein	38.5 ± 12.9 a	20.2 ± 3.69 ab	9.04 ± 7.16 b	nd b	13.3***
42	butein	238 ± 158 a	220 ± 33.3 a	279 ± 87.3 a	205 ± 6.81 a	0.23
45	isoliquiritigenin	100 ± 56.6 a	52.3 ± 1.49 a	66.0 ± 19.4 a	67.1 ± 3.22 a	1.60
38	tetrahydroxyaurone	184 ± 64.0 ab	248 ± 58.3 a	94.3 ± 12.8 bc	nd c	9.85**
	Σ *Other flavonoids*	*55959 ± 12612*	*42303 ± 398*	*21444 ± 1148*	*8690 ± 722*	
		Unidentified Tannins				
3	leucorobinetinidin	674 ± 299 a	365 ± 89.3 ab	164 ± 23.3 ab	nd c	8.26**
5	leucorobinetinidin	1513 ± 521a	654 ± 187 b	62.6 ± 29.6 b	nd c	19.8***
6	leucorobinetinidin	315 ± 162 a	383 ± 156 a	33.4 ± 21.8 b	nd c	7.77*
9	prorobinetinidin	657 ± 129 a	483 ± 35.5 a	243 ± 89.8 b	73.7 ± 3.82 c	26.4***
25	dimeric prorobinetinidin	44.5 ± 19.8 a	21.5 ± 2.83 ab	23.1 ± 13.4 ab	nd b	4.70
46	dimeric prorobinetinidin	73.8 ± 34.7 b	622 ± 214 a	463 ± 327 a	nd b	13.5***
47	dimeric prorobinetinidin	446 ± 127 b	677 ± 48.8 a	147 ± 21.8 c	nd c	14.4***
	Σ *Unidentified tannins*	*3725 ± 857*	*3209 ± 125*	*1137 ± 552*	*73.7 ± 3.82*	

[a] Different letters in the same row denote a statistical difference with 95% confidence level (Student Newman-Keuls multiple range test). *, **, and *** indicate significance at $p < 0.05$, 0.01, and 0.001, respectively.

spectrum similar to that of peak **11**. Moreover the UV/vis spectra completely matched in both cases which suggest that this dihydroflavonol could be an enantiomer or a positional isomer of the (+)-dihydrorobinetin (although further NMR

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experiments would be necessary to elucidate the exact position of the substituent). Similarly, peak **19** was assumed to be a dihydroflavonol isomer of peak **22**, fustin. Three peaks (**21**, **23**, and **26**) gave a molecular ion at m/z 317. The product ion spectrum obtained from the deprotonated molecular ion yielded among others fragments characteristic of the loss of CH_3 and H_2O, CH_3 and CO, and CH_3 and CO_2, indicating methoxylated compounds. The different A- and B-type fragments established the monohydroxy-substitution of the A-ring, and the presence of the $[M\text{-}H\text{-}H_2O]^-$ ion in the MS/MS spectra of the molecular ion, and the posterior loss of CH_3 in the EPI spectra of the $[M\text{-}H\text{-}H_2O]^-$ ion suggested no methoxylation in the C-3 position. Consequently, these results established the B-rings dihydroxylation and monomethoxylation.

The lack of an important loss of H_2O in the EPI spectrum of the deprotonated molecular ion of peaks **24** and **31** suggested a dihydroflavone moiety for these compounds, discarding the dihydroflavonol structure. Peak **24** exhibited a $[M\text{-}H]^-$ ion at m/z 287. Its corresponding product ion spectrum yielded A- and B-ring fragments which could be attributed at $[^{1,4}B]^-$, $[^{1,3}B]^-$, $[^{1,3}A]^-$ and $[^{1,3}A\text{-}CO_2]^-$. These results allowed us to establish it as the A-monohydroxy- B-dihydroxy substituted dihydroflavone. The MS spectrometry data for this peak is as expected for the flavanone robtin, reported to be the fourth prominent compound in the heartwood of *Robinia pseudoacacia*.[30] The UV/vis spectrum was also consistent with that reported by Roux and Paulus.[30] No other peak brings together the characteristic of robtin, thus, this compound was tentatively identified as robtin. Peak **31** displayed a $[M\text{-}H]^-$ ion at m/z 271 and a product ion spectrum consistent with the monohydroxy and dihydroxy substitution in A- and B-rings flavanone moiety respectively. Roux and Paulus[30] isolated the flavanone butin from the heartwood of *Robinia pseudoacacia*, which pattern of substitution and UV were consistent with compound 31. Nevertheless, further analysis would be necessary to confirm these assignments.

Peak **40** gave a $[M\text{-}H]^-$ at m/z 315 and an UV spectrum typical of flavonols. The product ion spectrum of the deprotonated molecular ion showed a base peak corresponding to the loss of 15 units, suggesting a methoxylated flavonol. Fragments at m/z 164 and 188 ($[^{1,3}B]^-$ and $[^{1,4}B\text{-}2H]^-$) established the monohydroxy substitution of the A-ring and the trihydroxy substitution of the B-ring. This suggested isorhamnetin as a possible identification. However, its retention time (using commercial standard) in our chromatographic conditions was higher (57.8 min), showing different λ_{max} (372 nm) for its UV/vis spectrum. Thus, the methyl group position could not be elucidated.

The UV profile of peak **38** showed an intense A Band in the 390−420 nm range suggesting an aurone skeleton. The product ion spectrum of the $[M\text{-}H]^-$ showed ions which could be attributed at the A-monohydroxysubstituted and B-trisubstituted aurone.

Moreover, seven compounds with proanthocyanidin type UV spectra were detected in the ethyl acetate and freeze-dried seasoned wood extracts. The presence of prorobinetinidins in *Robinia pseudoacacia* have been previously reported in literature,[15,30] and Coetzee et al.[17] showed that the range of naturally occurring prorobinetinidins is extensive, characterizing structures based on robinetinidol-leucorobinetinidin, robinetinidol-dihydrorobinetin, a robinetinidol-robinetin and its analogue robinetinidol-flavone. This led us to carry out oxidative ruptures on the extracts with the objective of analyzing the generated compounds. Thus, this oxidative rupture in *n*-butanol-HCl carried out on ethyl acetate and freeze-dried aqueous extracts generated only one anthocyanic pigment identified as robinetinidin (λmax at 520 nm, $[M\text{-}H]^-$ at m/z 321, using commercial standard), suggesting the presence of condensed tannins only as prorobinetinidins. Under the conditions of the oxidative reaction with *n*-butanol-HCl all these units could generate the most oxidized state, the anthocyanic pigment robinetinidin. In this regard, peaks **3**, **5**, and **6** were tentatively identified as leucorobinetinidin isomers. The three compounds gave a deprotonated molecular ion at m/z 305 and exhibited similar product ion spectrum. The fragment at m/z 287 could have been caused by the loss of water and the major fragment at m/z 137 was consistent with the C-ring cleavage through a RDA reaction ($[^{1,3}A]^-$). The fragments at m/z 167 may be caused by the heterocyclic ring fission of the C-ring ($[^{1,3}B]^-$). These structures would be in agreement with the leucorobinetinidin (+)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol isolated from the methanolic extracts of *Robinia pseudoacacia* heartwood by Roux and Paulus.[15] Similarly, peak **9** was tentatively identified as a flavan-3-ol or flavan-3,4-diol compound. The deprotonated molecular ion at m/z 289 yielded a major product ion at m/z 245 that would correspond to the loss of C-ring fragment $[M\text{-}C_2H_2O]^-$. Roux and Paulus[15] isolated two compounds compatible with these characteristics, the 7,3′,4′,5′-tetrahydroxyflavan-3-ol (robinetinidol) and the 7,3′,4′-trihydroxyflavan-3,4-diol. Peaks **46** and **47** gave a deprotonated molecule at m/z 589 and fragment ions at m/z 421, 301, and 287. The fragment ions at m/z 301 and 287 could correspond to the upper and lower monomers comprising this compound (robinetin and robinetinidol or other isomers). The fragment at m/z 421 (neutral loss of 168 amu) could be due to the RDA fission in the C-ring of the flavonoid skeleton of prorobinetinidin dimers $[M\text{-}C_8H_8O_4]^-$.[31] Coetzee et al.[17] characterized a prorobinetinidin structure based on robinetinidol-robinetinidin moieties. However further analysis would be necessary to support this assignment. Although the deprotonated molecular ion of peak **25** at m/z 591 could accommodate a dimeric prorobinetinidin (robinetinidol-dihydrorobinetinidin) as noted by Coetzee et al.,[17] no identification was possible.

Lastly peaks **1**, **33**, **35**, **41**, **43**, and **44** could not be elucidated by their fragmentation patterns and remain unidentified.

In Figure 1 three peaks named HMF, F, and 5MF were also identified using commercial standards of 5-hydroxymethylfurfural, furfural and 5-methylfurfural, on the basis of their retention times and UV spectra. Since they are not phenolic compounds no further consideration is given to them here.

Phenolic Compounds in Seasoned and Toasted Acacia Wood. The HPLC-DAD quantitative evaluations of flavonoid and nonflavonoid compounds extracted from seasoned and toasted woods are included in Table 2. The total contents of different chemical families, calculated adding the concentrations of individual compounds, are also shown. Seasoned acacia wood showed very high concentrations of flavonoids and low levels of nonflavonoid compounds. These results first establish important differences in relation to the chemical composition of oak woods used in cooperage, since oak woods are characterized by their significant concentrations of ellagitannins, as well as of phenolic acids and aldehydes, all of them nonflavonoid compounds, being almost undetectable the levels of flavonoids and proanthocyanidins.[12,13]

Regarding low molecular weight (LMW) phenolic compounds, as in oak wood,[12,13] the toasting of acacia wood results

in the progressive increase in lignin constituents, especially phenolic aldehydes, with regard to toasting intensity. Some phenolic acids and aldehydes were not possible to quantify in the seasoned wood because of interference by flavonoid compounds which were very abundant and appear at very similar retention times in the HPLC analysis. Since most of them, particularly the aldehydes, have already been quantified by GC-MS,[19] their concentrations in seasoned wood are only a small percentage compared to those of flavonoid, and are insignificant when contrasted with those of toasted wood. The concentration increase was particularly important in sinapaldehyde which reached 1666 μg/g, followed by syringaldehyde and coniferaldehyde, with 326 and 300 μg/g, and vanillin, with a significantly lower concentration, 71.3 μg/g. During wood toasting, lignin depolymerization takes place, producing hydroxycinnamic aldehydes in a first step and hydroxybenzoic aldehydes in a second one, the final concentration of these molecules in toasted woods being related to the lignin structure of each wood. The higher values obtained for sinapaldehyde in acacia (1666 μg/g) than in oak (212−590 μg/g)[12] toasted wood can be explained by a higher presence of dimethoxyphenyl final units in the acacia lignin structure, which are more easily thermodepolymerized than the monomethoxyphenyl ones.[32] On the other hand, although more heat is able to generate higher quantities of lignin constituents, at very intense toasting the aldehydes are degraded and other compounds, such as volatile phenols, are generated. Thus, the concentrations of some of these nonflavonoid compounds, like gallic and protocatechuic aldehydes, increase at light and medium toasting but decrease at higher toasting intensity. Other LMW phenolic compounds were also sensitive to heat treatment and its content decreases in the wood with the duration of toasting, as gallic and ellagic acid, methyl gallate and hydroxycinnamic derivatives.

Comparing the LMW phenolic composition of acacia heartwood with that of oak wood, seasoned and toasted, acacia shows some clear differences, such as the presence of compounds with a β-resorcylic structure (acid and aldehyde), some gallic (aldehyde and methyl ester), and hydroxycinnamic (caffeic acid and derivatives) related compounds, and protocatechuic aldehyde. The concentrations of some of these compounds were particularly important, for instance those of β-resorcylic acid and aldehyde in toasted samples, and hydroxycinnamic derivatives, the most abundant nonflavonoid in seasoned wood, which are sensitive to toasting. The effect of these compounds on the organoleptic characteristics of the beverages in contact with acacia wood during their aging, acetification, fermentation or other process is not known. On the other hand, the levels of phenolic acids were very different to those of oak: very little gallic acid, particularly at intense toasting, and ellagic acid in seasoned and toasted samples, and high levels of syringic acid were present after intense toasting. Recently, in red wine, the hydroxybenzoic and hydroxycinnamic acids were related to an astringent mouth feel, and their ethyl esters also contribute to astringency, at low taste thresholds.[33] Thus, a different contribution to this sensation would be expected when red wines were aged in acacia barrels. Among LMW phenolic compounds, 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. Its concentrations, in both seasoned and toasted acacia wood, were similar to that of other woods used in cooperage, and within the range of concentrations that can be expected for this compound.[12,25] In fact, the concentrations detected in wines or vinegars aged in acacia and oak barrels were very similar.[6,7] However, it is found in the literature that when the same red wine is aged in oak and acacia barrels, the last ones had a more pronounced vanilla character.[8]

In seasoned wood a great variety of flavonoid compounds were detected with an average concentration range from 38 to more than 30 000 μg/g of wood, mainly due to the dihydroflavonols dihydrorobinetin, fustin, tetrahydroxy, and trihydroxymethoxy dihydroflavonol, the flavonol robinetin, the flavanones robtin and butin, and a leucorobinetinidin, all with average concentrations higher than 1000 μg/g of wood. Data in the literature also showed dihydrorobinetin and robinetin as the most abundant flavonoids in acacia heartwood,[15,16,26] and Magel et al.[16] found concentrations of up 100 μmol/g in heartwood, equivalent to more than 30 000 μg/g, very similar to that obtained here. The average concentrations of flavonoid compounds decreased with toasting, in relation to toasting intensity. This decrease is especially important in dihydrorobinetin, the main compound in seasoned wood, found in diethyl ether, ethyl acetate, and freeze-dried aqueous extracts, but not found in any extract after the medium plus toasting of heartwood, with the average concentrations decreasing up to 25 and 75% at light and medium toasting, respectively. The decrease of the other dihydroflavonols, robtein, tetrahydroxy aurone, butin, and robtin was also very significant; most of them not detected in medium plus toasted wood. In a similar way, heat also causes the degradation of the prorobinetinidins, their average concentrations decreasing in relation to toasting intensity, and they were not found in medium plus toasted wood, with the exception of one of them. The heat-induced degradation of flavonoid compounds has been previously described. Thus, a decrease in procyanidin levels, caused by heat, was detected in peanut skins after roasting (175 °C, 5 min.), as they are the most heat-sensitive monomers,[34] and this has happened in the toasting of cherry wood as well.[23] Moreover, thermal processes such as boiling, frying and microwave cooking reduce the flavonol content of vegetables,[35] in both oxidant and autoxidant conditions.[36] In a similar way, toasting of cherry wood also provokes the degradation of flavononols, flavanones, flavonols, flavones, and chalcones.[23] However, in our toasting conditions some compounds such as robinetin, trihydroxymethoxy flavonol, butein, isoliquiritigenin, and fisetin, appear to be quite insensitive to heat, although in this last compounds some statistically no significant variations were detected. Thus, in medium plus toasted wood only nine flavonoids were quantified, and their average concentrations were low, between 60 and 720 μg/g, with the exception of robinetin which was the most abundant phenolic compound in medium plus toasted samples. Consequently flavonoids were the major phenolic compounds in seasoned and toasted acacia heartwood, in spite of the decrease in their concentrations with toasting, and the increase in lignin constituents.

Comparing these data with those of oak and other species, like chestnut or cherry, in consideration of their use in cooperage, only acacia and cherry heartwood showed flavonoids among their phenolic constituents, but with different profile since cherry includes (+)-catechin, (−)-epicatechin, B-type procyanidin dimers and trimers, and many other flavonoids such as naringenin, aromadendrin, isosakuranetin, or taxifolin, none of which are found in acacia,[12,23,24] but which also show high sensitivity to heat. Taking oak wood as a reference, in the interaction between acacia wood and the different kinds of beverages that may come in contact with it (wines, spirits, vinegars, ciders, etc.), some aspects of its phenolic composition should be kept in mind. If

either toasted or untoasted barrels are used, acacia wood will not provide hydrolyzable tannins while oak will, and therefore, the chemical reactions in which oak ellagitannins usually participate during aging [4,5,37] will not take place. In untoasted barrels the provided tannins will be condensed tannins of the prorobinetinidin type, never found in oak and different to those we can found in wine; thus the beverages will increase their tannin concentrations, resulting in the possible formation of new compounds during aging,[3] as well as an increase in their antioxidant capacity,[38] but the resulting organoleptic contribution is unknown. The antioxidant capacity will also be increased by other flavonoids such as dihydrorobinetin, robinetin, and other dihydroflavonols, flavanones, flavonols, and chalcones, especially in the case of untoasted acacia wood, but also in light or medium toasted acacia wood. These flavonoids have never been detected in these beverages after aging, and therefore the implications in the chemical modifications that take place during beverage aging, as well as in their organoleptic characteristics, are unknown. On the other hand, untoasted acacia wood contributed low quantities of LMW phenolic compounds in a way quite different to oak, since compounds with a β-resorcylic structure, some gallic related compounds, protocatechuic aldehyde, and some hydroxycinnamic compounds are included, but only a little gallic and ellagic acid. If toasted barrels are used, the acacia wood will contribute condensed tannins as well as the other flavonoids, in inverse proportion to toasting intensity, whereas LMW phenolic compounds will be directly proportional to toasting intensity, except gallic and ellagic acid and related compounds, as well as hydroxycinnamic derivatives. The phenolic aldehydes were within the range of concentrations that can be expected for these compounds in toasted oak wood, apart from sinapaldehyde, which was higher. On the other hand, the compounds with the β-resorcylic structure detected in untoasted acacia wood were also detected in toasted wood, and in increasing concentrations correlated to toasting intensity. A detailed study about how acacia wood affects the phenolic composition during the aging of different beverages would be of interest for a more complete evaluation of the impact of this wood, taking into account that the different beverages have varying capacities of removing phenolic compounds from wood.

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