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ELLAGITANNINS IN WOODS OF SESSILE OAK AND SWEET CHESTNUT DIMERIZATION AND HYDROLYSIS DURING WOOD AGEING

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IN HONOUR OF PROFESSOR RAGAI K. IBRAHIM'S SIXTY-FIFTH BIRTHDAY

Key Word Index—*Quercus petraea*; *Castanea sativa*; Fagaceae; oak; chestnut; wood; ageing; ellagitannins; dimers; dimerization; hydrolysis.

Abstract Monomeric and dimeric *C*-glucosidic ellagitannins and phenolic acids were studied by reverse-phase HPLC and gel permeation chromatography in various sapwood and heartwood samples of sessile oak (*Quercus petraea*) and sweet chestnut (*Castanea sativa*). Sessile oak differs from sweet chestnut by the presence in heartwood of pentose-substituted ellagitannins. Analysis of ellagitannin concentrations and a model experiment show that vescalagin is a common precursor of all dimers which are likely formed by a non-enzymatic reaction. Ellagic acid and gallic acid in oak and chestnut woods result, respectively, from the hydrolysis of ellagitannins and some unknown galloyl esters. Both dimerization and hydrolysis occur at the sapwood–heartwood transition and in the dead heartwood.

INTRODUCTION

Heartwoods of various chestnut, oak and eucalypt species contain high amounts of ellagitannins [1–3] which protect them against decay by fungi or bacteria [2, 4, 5]. The woods of three of such species have been used in Europe for the commercial production of tannin extracts. Several thousand tons of sweet chestnut (*Castanea sativa*) tannins are produced every year in France, Italy and Slovenia, principally for the leather industry. Small amounts of pendunculate oak (*Quercus robur*) or sessile oak (*Q. petraea*) tannins are also used in the wine industry.

The first ellagitannins to be purified from these woods were vescalagin (**1**) and castalagin (**2**) [6]. These two molecules were the first known *C*-glucosidic ellagitannins (they contain a carbon–carbon linkage between an aromatic carbon and C-1 of the glucose backbone). Their structures were elucidated by Mayer *et al.* [7, 8], although the exact configuration of the asymmetric carbon of the aliphatic chain was determined only recently [9, 10] (all ellagitannin structures shown in this paper have been corrected accordingly). The application of low pressure and high pressure chromatography to extracts of *Q. robur* wood, allowed the isolation of six other ellagitannins, namely gradinin (**3**) and the newly described roburins A, B, C, D and E (**5**, **7**, **8**, **6** and **4**, respectively) [11, 12]. Roburin A, B, C and E were the first *C*-glucosidic dimers to be identified. The same ellagitannins in similar amounts and proportions are found in *Q. petraea* (Scalbert, unpublished results and [13]).

No comparable study on wood of *C. sativa* has so far been published. In this paper, the main ellagitannins of sweet chestnut heartwood are identified and routes leading to the formation of *C*-glucosidic ellagitannin dimers in both oak and chestnut heartwood are proposed. Evidence for ellagitannin dimerization and hydrolysis during heartwood ageing is presented.

RESULTS

Ellagitannin monomers and dimers in oak and chestnut woods

Chestnut heartwood, just as oak heartwood, contains large amounts of extractives, easily solubilized in methanol–water or acetone–water mixtures [14]. The large majority of these extractives are water-soluble polyphenols [1]. The compounds corresponding to the four major peaks observed by reverse-phase HPLC

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(Fig. 1) were purified by chromatography on Sephadex LH 20 and semi-preparative reverse-phase LH 20. The two major compounds were vescalagin (**1**) and castalagin (**2**). The two others were shown to be identical (R_fs, UV and ^{1}H NMR spectra) to roburin A (**5**) and roburin D (**6**),

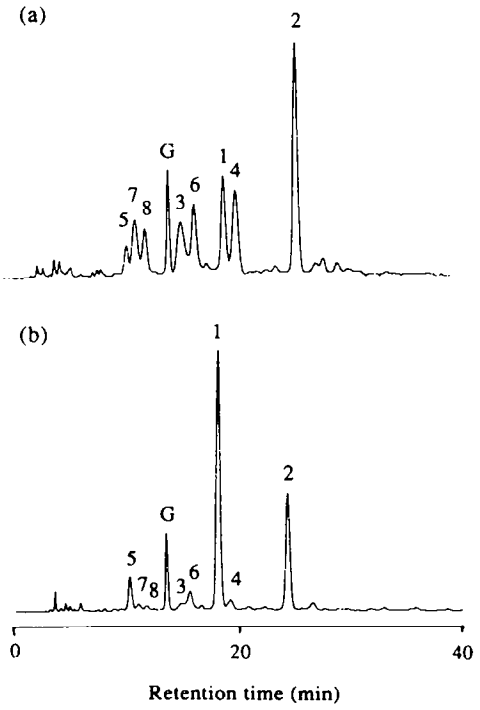


Fig. 1. Reverse-phase HPLC chromatograms of acetone–water extracts from sessile oak heartwood (a) and sweet chestnut heartwood (b). G, Gallic acid; numbers refer to the structures given in the text.

two ellagitannin dimers previously identified in oak heartwood. Some minor peaks on the chestnut chromatogram have identical retention times to those of grandinin (**3**), roburin E (**4**), roburin B (**7**) and roburin C (**8**) found in oak heartwood. Their low concentration precluded their purification and confirmation of their identity.

Only **1** and **2** could be detected by reverse-phase HPLC in sapwoods of both oak and chestnut. Typical concentrations of each ellagitannin for both sapwood and heartwood of oak and chestnut are given in Table 1.

Ellagitannins and wood ageing

As expected, the content of all identified ellagitannins increases sharply at the sapwood heartwood boundary and decreases regularly as heartwood ages for both chestnut and oak (Fig. 2). This decrease does not affect similarly all the ellagitannins. In oak, **2** is the major ellagitannin, but the more pronounced decrease affects **1**, whereas the relative concentrations of most dimers [the structures of which contain at least one vescalagin (**1**) residue] increase, particularly during the first years of ageing (Fig. 3a).

In chestnut, the major ellagitannin is **1**, but here again, its concentration decreases more than that of **2** during ageing (Fig. 3b).

Together with this decrease of ellagitannin content, an increase in free ellagic acid (**9**) and gallic acid (**10**) is observed (Fig. 4). It is particularly noticeable for oak.

Wood extracts were analysed by gel permeation chromatography after acetylation. Ellagitannin monomers, dimers and eventually higher M_r polymers were well separated from each other and from low M_r phenols (Fig. 5). The peak heights were used to determine the ratios of dimers to monomers (D/M) in the various heartwood samples. All results were in good agreement

Table 1. Polyphenol contents (mg g^{-1} wood) of sessile oak and sweet chestnut heartwoods

		Oak		Chestnut	
	Structure*	Sapwood	Heartwood†	Sapwood	Heartwood†
Ellagitannins					
Monomers					
1	V	1.0	14.1	3.3	43.2
2	C	1.0	19.3	2.0	20.0
3	Vl	0	7.7	0	2.0‡
4	Vx	0	9.0	0	2.0‡
Dimers					
5	VV	0	2.0	0	4.5
6	VC	0	5.0	0	3.3
7	VVl	0	2.6	0	0.7‡
8	VVx	0	2.3	0	0.6‡
Phenolic acids					
9		0.1	2.4	0.1	1.7
10		0.1	1.0	0.1	1.8

*V, vescalagin; C, castalagin; l, lyxose; x, xylose.
†Outermost sample in the wood section.
‡Tentative identification.

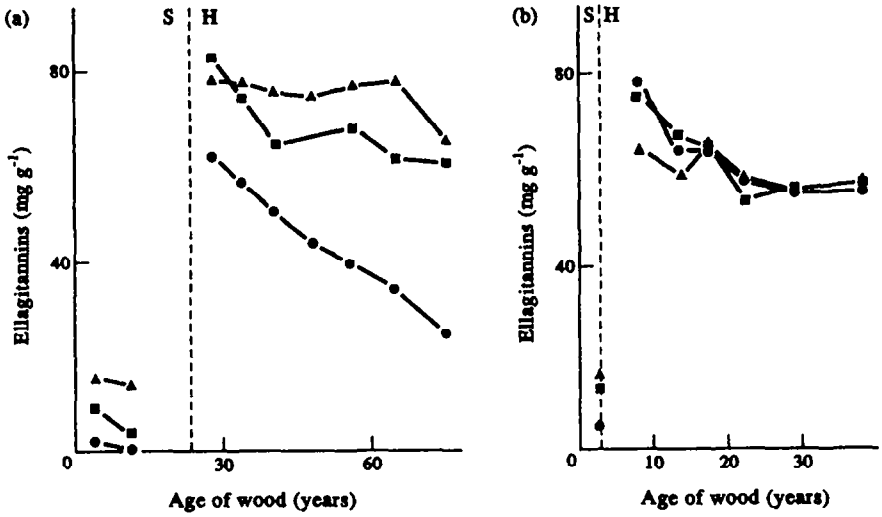


Fig. 2. Total ellagitannin contents in wood radial sections of sessile oak (a) and sweet chestnut (b) estimated by reverse-phase HPLC (●), gel permeation chromatography (▲) and acid degradation and ellagic acid determination (■). S: sapwood. H: heartwood.

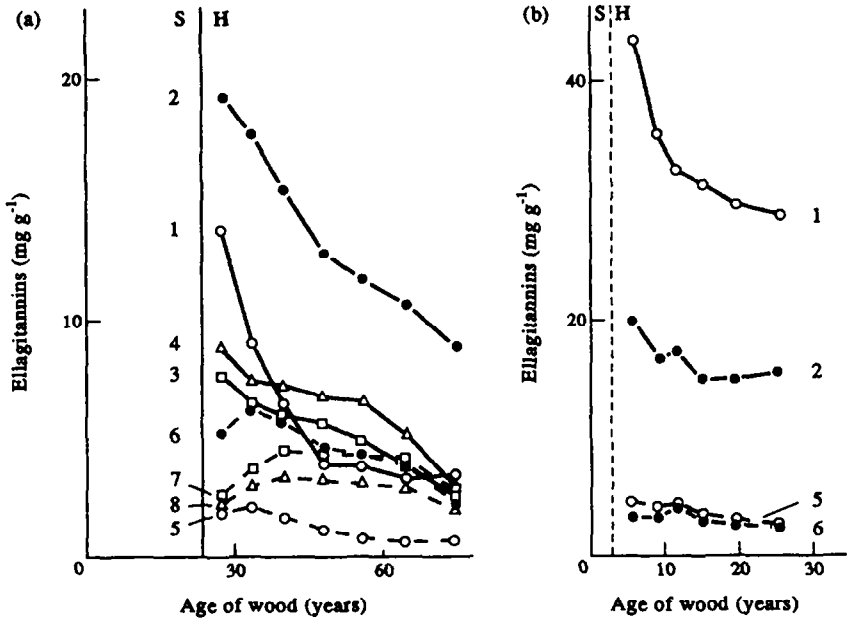
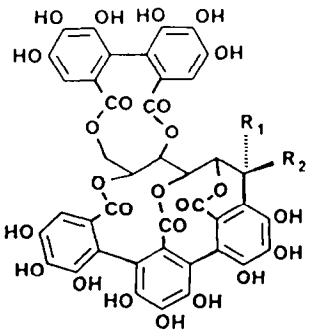


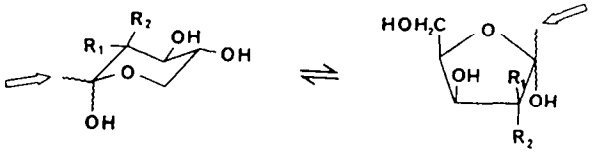
Fig. 3. Monomeric and dimeric ellagitannin contents in wood radial sections of sessile oak (a) and sweet chestnut (b). Plain line, monomers; dotted line, dimers. Numbers refer to structures given in the text. S: sapwood. H: heartwood.

with reverse-phase HPLC analyses, the proportion of (D/M = 0.38–0.54) as compared to chestnut (D/M = 0.24–0.30). In oak, the proportion of dimers increased quickly during the first five years of ageing (D/M increases from 0.38 to 0.49) followed by a smaller but regular increase over the next years (D/M = 0.54 after 35 years of ageing). On the other hand, no change in the proportion of monomers and dimers was observed in chestnut.

In vitro *dimerization of vescalagin*

Vescalagin (1) in the presence of trifluoroacetic acid reacted with itself to form the dimer roburin A (5). After 11 days, 38% of the vescalagin had reacted; 10% of the original substrate had dimerized. Another fraction was probably hydrolysed as suggested by the formation of a precipitate, likely assigned to ellagic acid. The substrate concentration in this experiment (100 gl^{-1}) was critical as





L $R_1 = H, R_2 = OH$

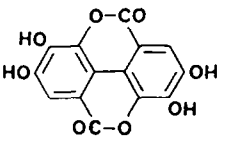
X $R_1 = OH, R_2 = H$

1 $R_1 = H, R_2 = OH$

2 $R_1 = OH, R_2 = H$

3 $R_1 = H, R_2 = L$

4 $R_1 = H, R_2 = X$



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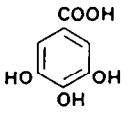

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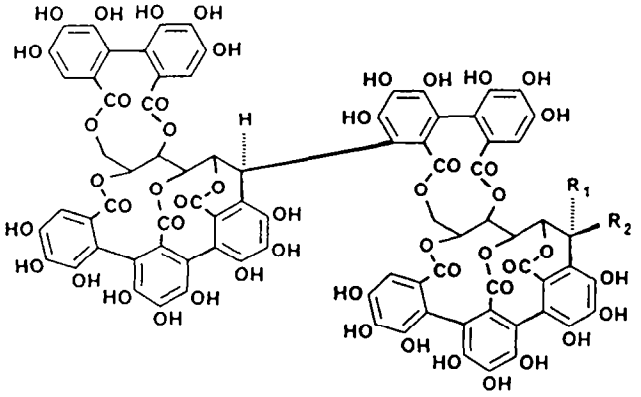
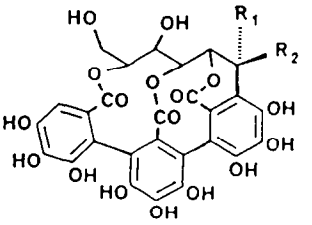


5 $R_1 = H, R_2 = OH$

6 $R_1 = OH, R_2 = H$

7 $R_1 = H, R_2 = L$

8 $R_1 = H, R_2 = X$



11 $R_1 = H, R_2 = OH$

12 $R_1 = OH, R_2 = H$

no dimerization could be observed at a lower concentration (2 $g l^{-1}$). Roburin A was also formed without addition of an acid, although in smaller yield (2% of the original substrate).

DISCUSSION

Ellagitannin dimerization

Heartwoods in sessile oak and sweet chestnut contain ellagitannins, *ca* 10% DM with large variations according to the sample analysed [13]. Eight different molecules, four monomers and four dimers, account for most of these ellagitannins (Fig. 1 and Table 1). Dimers represent 12% (chestnut) to 19% (oak) of the total. A major difference is observed between sessile oak and sweet chestnut. In oak, four of these ellagitannins are substituted by a pentose residue, lyxose or xylose [grandinin (**3**), roburin E (**4**), roburin B (**7**) and roburin C (**8**)]. In chestnut wood, these four ellagitannins, if their presence is confirmed, represent only a minor proportion of the total tannins.

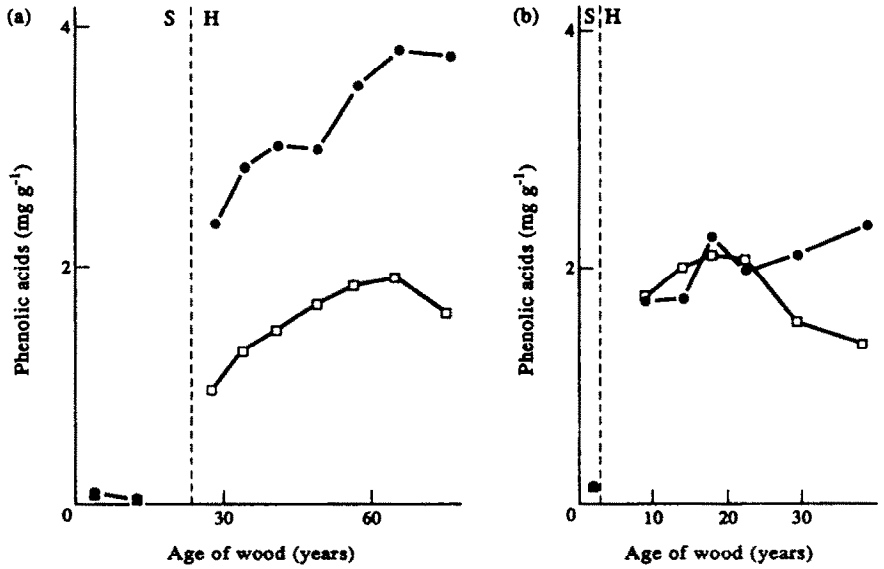


Fig. 4. Ellagic acid (●) and gallic acid (□) contents in wood radial sections of sessile oak (a) and sweet chestnut (b). S: sapwood. H: heartwood.

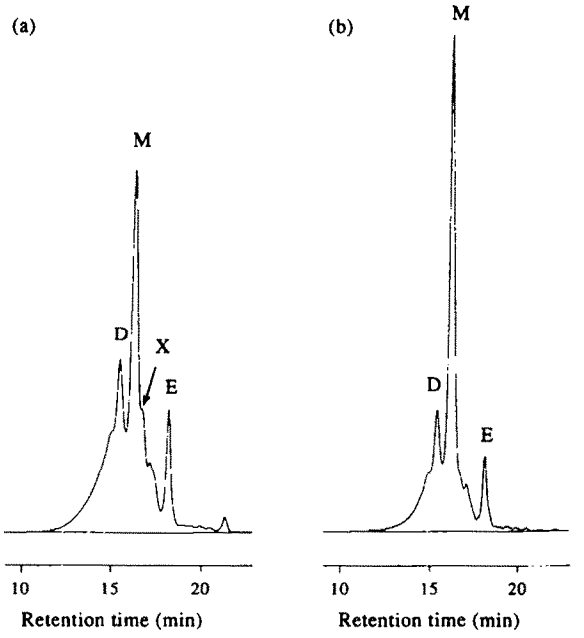


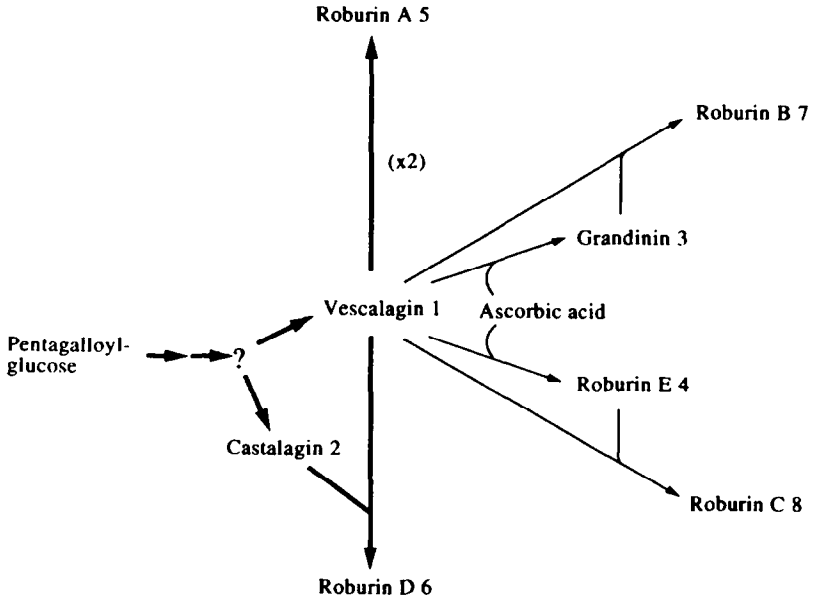
Fig. 5. Gel permeation chromatograms of acetylated extracts from sessile oak (a) and sweet chestnut (b) heartwoods. D, dimers; M, monomers; E, ellagic acid; X, tentatively assigned to vescalin and castalin.

Chestnut wood also differs from sessile oak wood by its low castalagin to vescalagin ratio (0.5 and 1.4 for chestnut and oak, respectively). This difference can be explained by the more important synthesis in oak, of the various dimers and pentose-substituted monomers, all derived from vescalagin. Indeed, if one calculates the total quantities of vescalagin and castalagin, both free and part of a dimeric or pentose-substituted ellagitannin, the castalagin to vescalagin ratio becomes nearly identical for both species (0.4 and 0.6 for chestnut and oak, respectively). The biosynthetic control of the glucose C-1 configuration thus appears to be similar in both species.

The variations in the proportions of the various ellagitannins with wood ageing, suggest a post-mortem non-enzymatic synthesis for dimers. The dominant pattern of the evolution of ellagitannin content is a general decrease of the concentration of all ellagitannins during wood ageing (Fig. 2). However, in oak, a transient increase of the content of all dimers is observed during the first 20 years of heartwood ageing (Fig. 3a). The rapid decrease of the vescalagin content during the same period accounts for the formation of these dimers.

All dimers can be considered to be formed by nucleophilic attack by one of the hydrogen-substituted aromatic carbons in any of the four monomers **1**–**4** on to C-1 of **1** followed by elimination of the hydroxyl group attached to this carbon. A similar nucleophilic attack of C-1 of *C*-glucosidic tannins by (+)-catechin and ascorbic acid has been shown to be involved in the synthesis of catechin-substituted ellagitannins (e.g. stenophyllanin A [15] and acutissimin A [16]) and pentose-substituted ellagitannins [17] (e.g. grandinin or pterocarinin A). Probably for steric reasons, the H-substituted aromatic carbon involved in the nucleophilic attack on to vescalagin is for all four ellagitannin monomers the one in the δ-position relative to C-6 of the glucose chain.

Methanol was shown to attack C-1 of some *C*-glucosidic tannins, such as vescalagin or stachyurin, to form a methanol adduct in high yields [10]. It is remarkable that this reaction does not affect castalagin and casuarinin, C-1 epimers of vescalagin and stachyurin, respectively. It was explained by either an increased steric hindrance of



Scheme 1. Ellagitannin formation in sessile oak (bold and plain arrows) and sweet chestnut (bold arrows).

C-1 or by stabilization of the C-1 α-hydroxyl group by hydrogen-bonding in castalagin and casuarinin. Very likely, the formation of dimers in oak and chestnut woods is governed by the same parameters. The higher reactivity of vescalagin as compared to castalagin explains the structure of the dimers and the differences in the castalagin to vescalagin ratios.

Scheme 1 for ellagitannin formation in oak and chestnut woods can thus be proposed. At the sapwood–heartwood boundary, **1** and **2** are synthesized in a proportion of 2:1 from β-penta-*O*-galloyl-D-glucose through successive enzymatically controlled and still unknown reactions (for a discussion see [18 22]). Vescalagin (**1**) would then be attacked by the main nucleophiles present in the tissues. The monomeric grandinin (**3**) and roburin E (**4**) would be formed by reaction of **1** with ascorbic acid [17]. These reactions which only occur in oak could be controlled by the availability of ascorbic acid. The various monomers **1**–**4** would react with **1** to form the dimers **5**–**8**, respectively. Dimerization could occur both in living cells at the sapwood–heartwood transition and in the dead heartwood.

Ellagitannin hydrolysis

Other post-mortem reactions occur during wood ageing. All ellagic acid (**9**) should originate from the hydrolysis of hexahydroxydiphenoyl units in ellagitannins. It can be calculated that the concentration of ellagic acid at the sapwood–heartwood transition (Fig. 4) represents hydrolysis of *ca* 8% of the total ellagitannins formed for both oak and chestnut. Hydrolysis then goes on at a slow rate during wood ageing. About 1% of the total ellagitannins are hydrolysed into ellagic acid every 10 years.

Mayer reported the presence of vescalin (**11**) [23] and castalin (**12**) [24] in oak and chestnut woods, which derive from **1** and **2**, respectively, through hydrolysis of their hexahydroxydiphenoyl unit. Although vescalin and castalin were not recovered from the oak [25] and chestnut wood samples which we used for tannin purification, their presence is suggested in gel permeation chromatograms by a small peak or shoulder having a retention time slightly longer (16.7 min) than that of vescalagin or castalagin (Fig. 5). The height of this peak, relative to the main peak of monomers, increases with the age of the heartwood samples (data not shown), in agreement with hydrolysis of ellagitannins as discussed above.

The origin of gallic acid (**10**), which is with ellagic acid (**9**) one of the two main low M_r phenols in oak and chestnut woods, is still unclear. The increase of its content during wood ageing (Fig. 4a) suggests that it is formed by hydrolysis of some galloyl esters. So far, no such esters have been identified in oak or chestnut wood extracts. These putative esters are possibly attached to some cell wall components.

Other reactions involved in wood ageing

Hydrolysis alone does not account for the overall decrease of ellagitannin content during heartwood ageing. A significant fraction of ellagitannins is also insolubilized [14]. Hydrolysis and insolubilization account, respectively, for 10 and 84% of the loss of ellagitannins in the oak section and 31 and 40% in the chestnut section (the values used for this calculation are those obtained by determination of ellagitannins through acid degradation). The remaining loss likely results from the polymerization of ellagitannins into larger soluble polyphenols of indefinite structure [26].

Some discrepancies in the estimation of ellagitannins by various assays remain unexplained. In chestnut, the

ellagitannin contents over the whole period of heartwood ageing are very much the same whatever the method used, reverse-phase HPLC, gel permeation chromatography or determination of hexahydroxydiphenoyl units (Fig. 2b). In oak, the decrease of ellagitannin content is much larger when determined by reverse-phase HPLC (Fig. 2a). A possible explanation is that some non-tannin compounds are released in oak wood as it ages, and interfere with HPLC analyses. Reverse-phase HPLC analysis of *C*-glucosidic ellagitannins was shown to be very sensitive to the presence of extraneous compounds which might favour tannin complexation [25].

Some non-tannin compounds present in oak wood and not in chestnut wood may also explain why an oak extract reduces iodate into iodine whereas a chestnut extract does not [27] (it was checked that all ellagitannins present in oak and chestnut wood form iodine from iodate). Further insight of the nature of non-tannin compounds in oak and chestnut woods should help to understand why extracts of both woods differ in some of their properties and how these properties are affected by wood ageing.

EXPERIMENTAL

General. Gallic and ellagic acids were obtained from Fluka. Vescalagin, castalagin, grandinin and roburin A–E have been previously purified from pedunculate oak (*Q. robur*) [25]. ^{1}H NMR: 400 MHz; solvent: pyridine-d_5 or DMSO-d_6; int. signal: δ 8.70 for the low field pyridine signal; the digital resolution of the ^{1}H spectra was 0.5 Hz pt^{-1} and the acquisition time 2.01 sec.

Plant material. Sessile oak (*Q. petraea* Liebl.) and sweet chestnut (*C. sativa* Mill.) trees were the same as those used in a previous study [14]. Trees were 90- and 50-years-old, respectively; samples were collected two months after the trees were felled, at the base of the trunk for chestnut and 6 m above ground for oak. Samples were obtained from successive and continuous zones delimited over a radius of a tree section. Radii were 12.5 cm long for both sections. Areas between rings 15–25 (numbered from the cambium) in the oak section and 3–5 in the chestnut section include the sapwood–heartwood boundary (around ring 23 and 3 for oak and chestnut, respectively) and were not analysed. Samples were air-dried and ground in a Retsch mill SM1 (particle-size less than 60 mesh).

Polyphenol extraction. Wood powders (100 mg) were extracted in duplicates with 5 ml H_2O–Me_2CO (3:7) at room temp. under magnetic stirring for 160 min. Ellagic acid was directly estimated from the filtrate. In order to determine gallic acid and ellagitannins, a 2 ml aliquot from the filtrate was concd under a N_2 stream to remove Me_2CO and its vol. made to 3 ml with H_2O. Me_2CO must be completely removed, otherwise each ellagitannin will give multiple peaks which will considerably alter the resolution of the reverse-phase HPLC separation [25].

Analytical HPLC. The solns were analysed on a Merck LiChrospher RP-18e (5 μm) column (25 cm × 4 mm i.d.) with the following eluents: solvent A, H_2O–H_3PO_4 (990:1), solvent B, MeOH. For ellagic acid determination: linear gradient from 0 to 90% solvent B; gradient duration: 30 min; speed flow: 1 ml min^{-1}; detection: 370 nm. For gallic acid and ellagitannin determination: linear gradient from 0 to 10% solvent B; gradient duration: 30 min; speed flow: 1 ml min^{-1}; detection: 280 nm. Identity of the products was checked by co-chromatography with standards of known structure and by comparison of their UV spectra with those of the same references. The 240–400 nm spectra were obtained by on-line detection with a diode-array detector (absorption maxima for gallic acid and ellagic acid: 272 and 254 nm, respectively; ellagitannins: no maximum but a shoulder around 280 nm).

Gel permeation chromatography [28]. A 1.5 ml aliquot of the filtrates was freeze-dried and acetylated in Ac_2O–pyridine (1:1). Reagents were removed under vacuum (Speedvac). THF (1 ml) was added to solubilize the acetylated polyphenols. A small fr. eventually remained insoluble but it never exceeded 3.5% of the initial sample. Acetylated extracts were analysed on two Ultrastyragel columns, 500 and 1000 Å, 300 × 7.8 mm i.d. (Waters) used in series. THF was delivered at 1 ml min^{-1}. Detection was at 280 nm. Various polystyrenes, acetylated monomeric and dimeric ellagitannins and ellagic acid were used as standards.

Ellagitannin estimation. (i) By reverse-phase HPLC. Purified vescalagin was used as a standard. Weight absorbancies of the various ellagitannin monomers and dimers were considered to be identical to that of vescalagin. (ii) By gel permeation chromatography. Acetylated vescalagin was used as a standard to estimate the sum of monomers, dimers and polymers. (iii) By acid degradation and ellagic acid determination. The values previously obtained [14] have been corrected for the presence of free ellagic acid in the extracts.

Isolation of chestnut ellagitannins. Methods previously applied to oak heartwood have been described in detail [25] and are summarized here. Sweet chestnut heartwood powder (1 kg) was extracted at room temp. with MeOH–H_2O (4:1). The extract, after removal of MeOH in a rotary evaporator, was fractionated into Et_2O (3.3 g), EtOAc (8.9 g) and H_2O (62.4 g) soluble frs. Et_2O and EtOAc removed most of the gallic and ellagic acids. The aq. fr. (20 g) was chromatographed on a 80 cm × 25 mm i.d. Sephadex LH 20 (Pharmacia) column, and successively eluted by 8 l H_2O, 10 l H_2O–MeOH (4:1) and 10 l H_2O–MeOH (3:2). Ascorbic acid (1 g l^{-1} H_2O) was added to avoid oxidation of polyphenols. Four frs containing the main ellagitannin peaks observed on analytical HPLC chromatograms were selected for further purification: 0–0.8 l H_2O–MeOH (4:1) for vescalagin, 4–10 l H_2O–MeOH (4:1) for castalagin, 0.6–2 l H_2O–MeOH (3:2) for roburin A and 2.6–3.6 l H_2O–MeOH (3:2) for roburin D. The 4 frs were further purified by prep. HPLC on a Hibar LiChrospher RP-18 column (25 cm × 25 mm i.d.) (Merck). Eluents were H_2O–MeOH–H_3PO_4 (980:20:1) (vescalagin, roburin A), (960:40:1) (roburin D) or H_2O–MeOH–H_3PO_4 (940:60:1) (castalagin). MeOH was removed in a rotary

evaporator from the collected frs, which were then chromatographed on a 5 × 1.5 cm i.d. Sephadex LH 20 column to remove H_3PO_4. Ellagitannins were eluted with MeOH and dried to afford white or creamy powders: 1.0 g vescalagin (**1**), 0.60 g castalagin (**2**), 236 mg roburin A (**5**) and 107 mg roburin D (**6**). Purity was checked by HPLC, bidimensional PC and NMR spectroscopy.

Vescalagin, castalagin, roburin A and roburin D. ^{1}H NMR spectra of vescalagin, castalagin and roburin A in DMSO-d_6 [11] and of roburin D in pyridine-d_5 [12] were identical to those previously published. Roburin A: ^{1}H NMR (pyridine-d_5): δ Glc1: 6.22 (*br d*, AB, $J = 6$ Hz, H-5), 6.18 (*t*, AB, $J = 6.5$ Hz, H-4), 5.55 (*br s*, H-2), 5.29 (*d*, $J = 7$ Hz, H-3), 5.23 (*br d*, nd, H-6), 4.79 (*br s*, H-1), 4.18 (*br d*, $J = 12.2$ Hz, H-6'); Glc2: 5.94 (*t*, AB, $J = 6.5$ Hz, H-4), 5.88 (*br d*, AB, $J = 6.3$ Hz, H-5), 5.85 (*br s*, H-2), 5.48 (*br s*, H-1), 5.25 (*d*, $J = 6$ Hz, H-3), 3.75 (*br d*, $J = 12.4$ Hz, H-6), 2.65 (*br d*, $J = 12.4$ Hz, H-6'). ^{13}C NMR (pyridine-d_5): δ 42.1 (C-1 of Glc1), 64.9, 66.3, 68.8, 69.7, 70.7, 71.1, 71.6, 72.0, 72.6, 78.0, 79.3 (Glc carbons), 108.0, 108.8, 109.7, 111.1, 111.9 (H-substituted aromatic carbons), 114.4–119.1 (15C), 123.7–128.9 (>10C), 136–140.2 (>9C), 145.4 149.4 (~20C) (quaternary carbons), 165.5, 165.7, 167.0, 167.0, 167.2, 168.1, 168.2, 168.4, 168.5, 170.4 (carbonyl carbons).

In vitro dimerization of vescalagin. Vescalagin (20 mg) in H_2O–TFA 100:1 (0.2 ml) was kept standing at 37° during 11 days. The mixt. was analysed by reverse-phase HPLC as described above.

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