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Polyphenols of *Quercus robur* L.

II[a]. Preparative isolation by low-pressure and high-pressure liquid chromatography of heartwood ellagitannins

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SUMMARY

Heartwood of *Quercus robur* L. contains about 10% (w/w), of hexahydroxydiphenoyl esters (ellagitannins). Five of the most abundant hexahydroxydiphenoyl esters were purified by a combination of chromatography on Sephadex LH-20 and high-performance reversed-phase liquid chromatography. Two of them were identified as castalagin and vescalagin.

INTRODUCTION

Heartwoord of *Quercus robur* L. (pedonculate oak) is known to contain about 10% (w/w) of hexahydroxydiphenoyl esters (HHDP esters)[1]. These HHDP esters, also known as ellagitannins, are responsible for the high durability of this wood[2]. They also contribute to the taste and the colour of brandies and wines aged in oak barrels[3–5].

The two main HHDP esters of *Q. robur* heartwood, castalagin and vescalagin (Fig. 1), were characterized by Mayer *et al.*[6] more than 20 years ago. However, the presence of at least six other HHDP esters in this wood was reported recently[1]. In order to determine their structures, their purification was undertaken.

Several methods have previously been used for the purification of hydrolysable tannins; the most widely used technique has been chromatography on Sephadex LH-20. High-performance liquid chromatography (HPLC) has also been used for the separation of hydrolysable tannins[1,7,8], although seldom on a preparative scale[7,9]. We have shown previously that HHDP esters from oak wood extracts were well

[a] For Part I, see ref. 1

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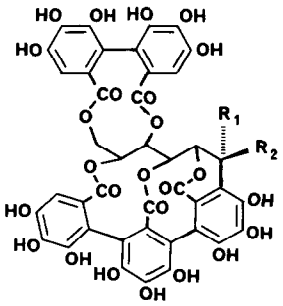

Fig. 1. Structures of castalagin (R_1 = H; R_2 = OH) and vescalagin (R_1 = OH; R_2 = H).

separated by reversed-phased HPLC, although they were poorly resolved by silica chromatography or paper chromatography[1]. For this reason, we undertook their purification by a combination of preparative reversed-phase HPLC and Sephadex LH-20 chromatography. The influence of some parameters on the quality of the separation is detailed.

EXPERIMENTAL

Materials

Heartwood was obtained from a branch 25 cm in diameter, cut from a 100-year-old, freshly felled pedonculate oak (*Q. robur*). The wood was dried at room temperature and ground in a Retch SM 1 rotating-knife grinder (particle size less than 60 mesh).

The water used for chromatography was deionized [Milli-Q (Millipore)]. Methanol was of RPE grade (Carlo Erba) and all other solvents of R. P. Normapur grade (Prolabo); acetone was redistilled before use. For HPLC, all solvents were ultrafiltered (modified cellulose, 0.2 μm; Sartorius) and degassed daily under vacuum.

Solvents were eliminated under reduced pressure in a rotary evaporator at 40°C. Water in concentrated solutions was eliminated by lyophilization, yielding easily recovered powders.

Extraction of HHDP esters

Wood powder (1 kg) was extracted with methanol–water (4:1)[10] (2 l for 8 h, then 1 l for 18 h) with magnetic stirring at room temperature.

The mixture was filtered on a Büchner funnel and methanol was eliminated under reduced pressure. The residual solution was successively extracted with light petroleum (b.p. 35–60°C) (7 × 250 ml), diethyl ether (7 × 250 ml) and ethyl acetate (7 × 250 ml). All the extracts were dried. Yields were typically 0.5, 7.4, 11.9 and 98.3 g for light petroleum, diethyl ether, ethyl acetate and water, respectively. HHDP esters (positive reaction with nitrous acid[11]) were present mainly in the water extract and to a lesser extent in the ethyl acetate extract.

Two-dimensional paper chromatography

Samples were eluted on Whatman No. 1 paper (12.5 × 12.5 cm^2) with 6% acetic acid for the first dimension and *sec.*-butanol–acetic acid–water (14:1:5) for the

second dimension. Polyphenols (*o*-dihydric phenols) were revealed by spraying with $FeCl_3$–$K_3Fe(CN)_6$ reagent[12].

Thin-layer chromatography on silica gel

Silica layers on aluminium foils (60 F_{254}, 7 × 7 cm^2; Merck) were eluted with ethyl acetate–formic acid–water (7:2:1, v/v/v). To reduce streaking, the layer was preconditioned, before application of samples and development, by spraying the eluent and rapid drying with a hair dryer.

Analytical HPLC

A Gilson apparatus composed of two pumps (303 and 305), equiped with 5 SC pumps heads, an 811 dynamic mixer, an 803C manometric module and a Holochrome detector (280 nm; cell volume 11 μl; path length 10 mm) was used with a Merck LiChrospher RP-18e (5-μm) column (25 cm × 4 mm I.D.). The elution conditions were as follows: linear gradient, 0–10% solvent B from 0 to 40 min, solvent A = water–phosphoric acid (990:1, v/v), solvent B = methanol–phosphoric acid (990:1, v/v); flow-rate, 1 ml/min. UV spectra (240–400 nm) were obtained by on-line detection with a Hewlett-Packard 1040A diode-array detector.

Chromatography on Sephadex LH-20

Aqueous extracts (10 or 100 g dissolved in a minimum volume of water, *i.e.*, 20–50 ml) were loaded on an 80 cm × 25 mm I.D. Sephadex LH-20 (100 μm; Pharmacia) column. A peristaltic pump delivered a water–methanol mixture at a flow-rate of 5 ml/min. The concentration of methanol was increased stepwise (20% increments). Ascorbic acid (1 g per litre of water) was added to limit the oxidation of polyphenols. Fractions of 50–500 ml were collected. Each fraction was analysed by high-performance thin-layer chromatography on cellulose (Merck). The same eluents as described for two-dimensional paper chromatography were used. Chromatography was run in a linear development chamber (Camag). Similar fractions were collected, dried under reduced pressure and lyophilized. Their degree of purification was controlled by analytical HPLC and two-dimensional paper chromatography.

Preparative HPLC

The same Gilson equipment was used with 25 SC pump heads and a preparative cell (volume 2 μl; path length 5 mm). The solvents were the same as for analytical HPLC. A Hibar LiChrospher RP-18, 7-μm column (25 cm × 25 mm I.D.) (Merck) was used with a precolumn 3 cm × 4 mm I.D. filled with LiChrospher RP-18, 10-μm (Merck). Samples were dissolved in water and filtered on a Millex SR 0.5-μm filter (Millipore). According to the mixture to be purified, up to 400 mg can be introduced into the 2-ml loop. Elution was conducted in the isocratic mode with 4 or 6% B, depending on the retention time of the compounds on the column. The flow-rate was 20 ml/min. The rising, top and decreasing parts of the peaks were collected separately and their purity was monitored by analytical HPLC.

The fractions obtained were concentrated under reduced pressure to eliminate the methanol, and adsorbed on a small Sephadex LH-20 column (5 cm × 1.5 cm I.D.) to eliminate phosphoric acid which was eluted with water. As soon as the pH was neutral (monitored with pH paper), the adsorbed HHDP esters were eluted with

methanol. The presence of the HHDP esters in the eluent was followed by reaction with $FeCl_3$–$K_3Fe(CN)_6$ reagent. Solutions were dried under reduced pressure and residual water was eliminated through repeated washings with acetone. Samples were solubilized in [$^2H_6$]acetone or, if not soluble enough, in [$^2H_4$]methanol, for ^1H NMR spectroscopic analysis (Cameca 250-MHz or Bruker AM 400-MHz spectrometer).

RESULTS AND DISCUSSION

Analytical HPLC

Compared with other HHDP esters, oak wood tannins are highly polar compounds which cannot be properly separated on silica or cellulose. On the other hand, reversed-phase HPLC affords an easy separation, although several parameters had to be determined in order to maximize the retention on the column, thus allowing a better separation.

Several columns were compared. Retention was higher on RP-18 columns than on RP-8 columns (Fig. 2a and b). Very different performances were obtained depending on the commercial origin of the columns. Lichrospher columns provided the best separations; columns with 5-μm particle size material gave a higher number of peaks

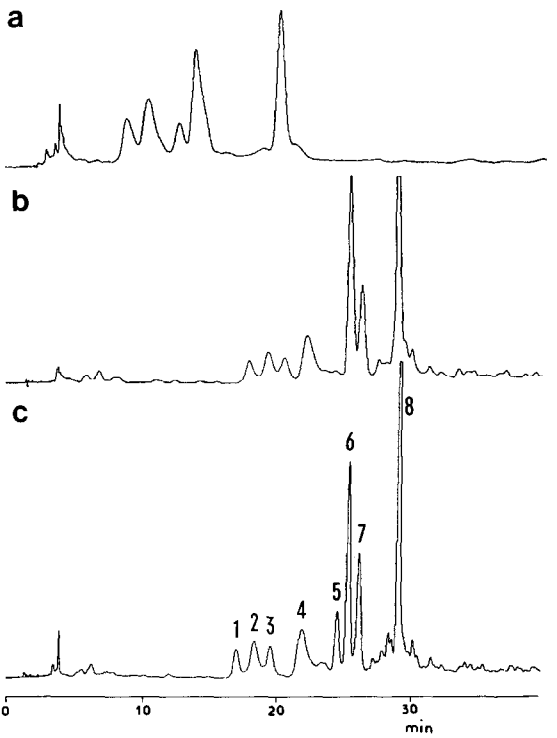

Fig. 2. HPLC of an aqueous extract of *Quercus robur* L. heartwood on different reversed-phase columns (25 cm × 4 mm I.D.). (a) LiChrospher 100-CH8, 10 μm; (b) LiChrospher RP-18, 10 μm; (c) LiChrospher RP-18, 5 μm. See text for elution conditions. Numbers 1–8 refer to the different HHDP esters identified in the extract.

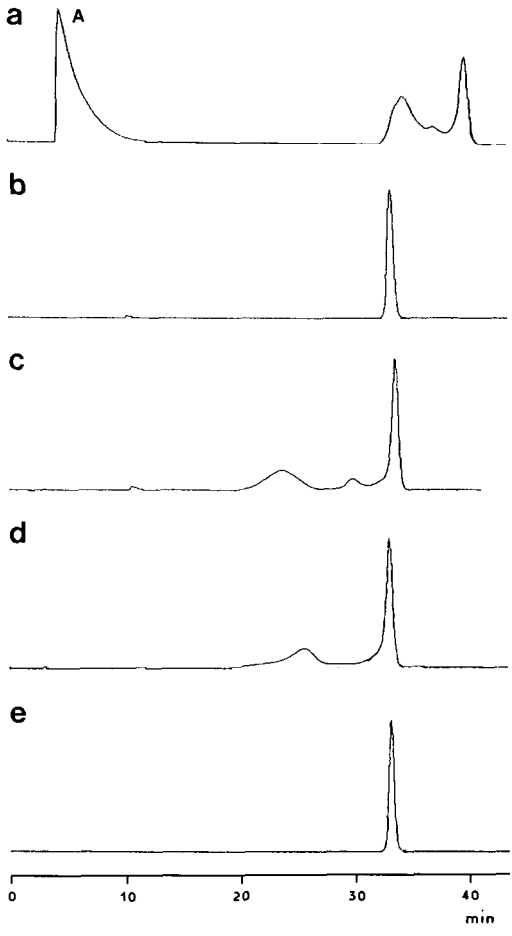

Fig. 3. HPLC of castalagin solubilized in different solvents before injection. (a) Acetone; (b) same sample as (a) dried and resolubilized in water; (c) methanol; (d) same sample as (c) to which is added water (one tenth of its volume); (e) same sample as (c) dried and resolubilized in water. See text for elution conditions. Peak A = acetone.

than 10-μm particle size material, regardless of flow-rate (Fig. 2b and c). The nature of the acid in the eluent also affected the retention times. Phosphoric acid (1%, v/v) doubled the retention times compared with acetic acid (1%, v/v).

The solvent used to solubilize the sample also had a strong influence on the chromatographic profile. When 10 μl of a solution of a pure HHDP ester in acetone (Fig. 3a) or methanol (Fig. 3c) was injected, several peaks were observed. The relative area of the extra peaks was reduced on addition of water to the sample solution (Fig. 3d). If the organic solvents were completely removed by evaporation, and the same sample was then resolubilized in water, a single peak was observed (Fig. 3b and e). Therefore, it was necessary to dissolved these HHDP esters in water and not in organic solvents, in order to obtain an optimum analysis with the present system. These observations could be due to reversible autoassociation of these very polar

tannins, whose solubility in organic solvents is limited. Solvation by water would induce the dissociation of these soluble complexes[13].

Chromatography on Sephadex LH-20

Sephadex LH-20 has been widely used to purify tannins. Ethanol and methanol, usually in mixtures, are the most widely used eluents. However, oak heartwood tannins are not sufficiently soluble in alcohols, and water followed by methanol–water mixtures were preferred. Similar gradients of methanol in water have been used previously for ellagitannins[14] or proanthocyanidins[15]. We encountered difficulties with this purification step, owing to extensive oxidation of the HHDP esters, as the chromatography lasts several weeks and the volume of eluents occasionally exceeded

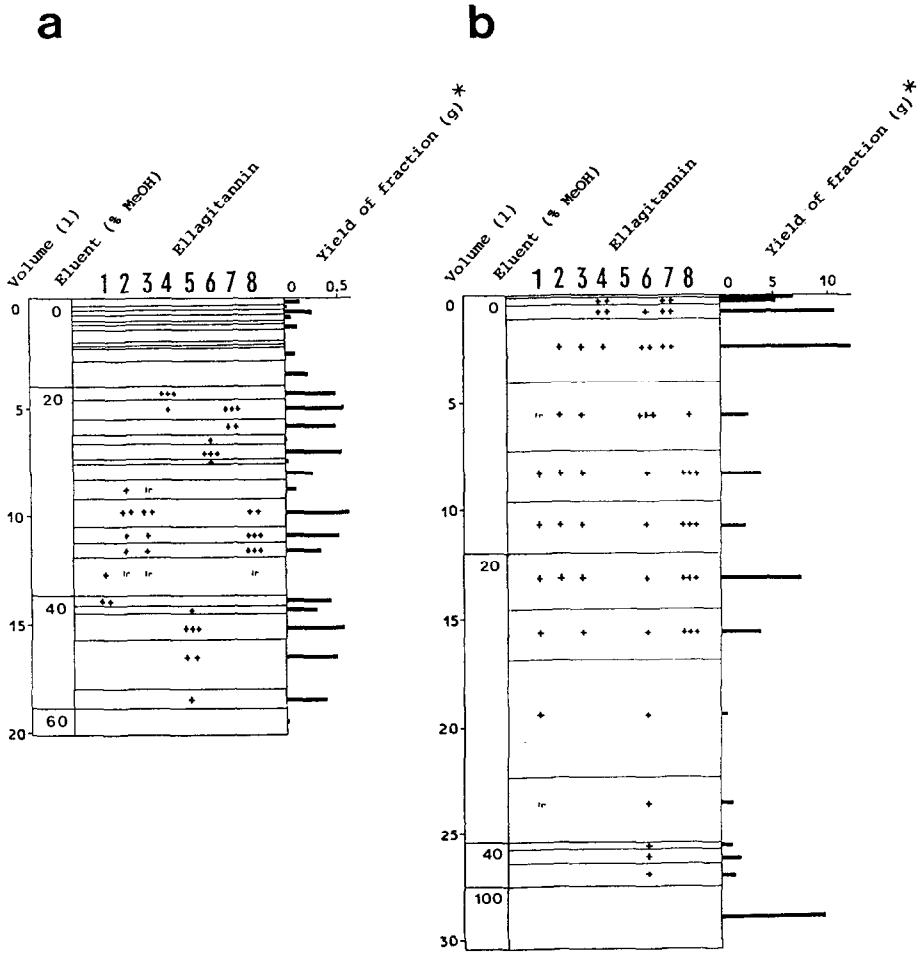

Fig. 4. Chromatography on Sephadex LH-20 of an aqueous extract of *Quercus robur* L. heartwood: (a) 10 g and (b) 100 g of extract loaded on the column. tr (traces), +, + + and + + + refer to the relative abundances of the eight main compounds (1–8, Fig. 2c) as observed by HPLC. (*) Mass of the ascorbic acid in the eluent has been deduced. MeOH = Methanol.

25 l. The addition of ascorbic acid as an antioxidant in the eluent[16] improved the purification yields substantially. Ascorbic acid has the advantage of being easily eliminated by preparative HPLC in the next step of purification, as it is not retained on the reversed-phase column. Further, its presence in the fraction is easily monitored by UV detection (see following section).

Part of the extract (7–9%) was not retained on the column, and was eluted with water before the main HHDP esters (Fig. 4). These fractions were also poorly retained on the reversed-phase HPLC column. They consist of a complex mixture of products and were not further purified.

Most of the extract (60%) was eluted after these compounds and corresponded to the various peaks (1–8, Fig. 2) observed in HPLC (some of the fractions were selected for further purification by preparative HPLC). The quality of the separation of these products was strongly affected by the amount of extract injected (Fig. 4). When 100 g of extract, instead of only 10 g, were loaded on the column, although the order of elution was not modified, the products were more easily eluted with lower concentrations of methanol in the eluent. However, in most instances, the resolution was so low that it became very difficult to obtain pure products from these fractions by preparative HPLC.

Other HHDP esters (3–4% of the extract) were eluted from the gel with concentrations of methanol in the eluent of 60% or higher. These HHDP esters were not resolved by HPLC and appeared as a streak on two-dimensional paper chroma-

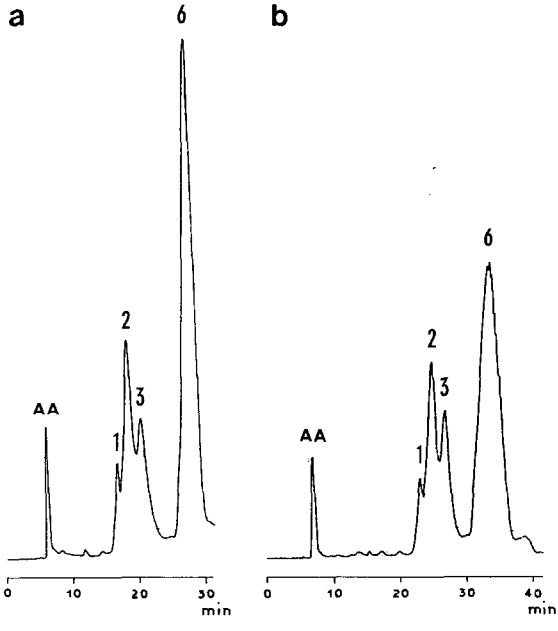

Fig. 5. Preparative HPLC of a fraction obtained after chromatography on Sephadex LH-20. (a) Isocratic elution, 2% B from 0 to 20.4 min, 5% B from 20.4 min; (b) gradient elution, 0 to 5% B from 0 to 40 min. Amount of sample injected, 200 mg. See text for other elution conditions. Numbers refer to the components of the aqueous extract of *Quercus robur* L. heartwood (Fig. 2c). AA = Ascorbic acid.

tograms. These fractions, which were more intensely coloured than the previous ones, probably correspond to partially oxidized (and polymerized) HHDP esters.

The total amount of products recovered in the fractions never exceeded 70% of the extract (Fig. 4). Most of what remained bound to the gel was removed by elution with 0.1 *M* sodium hydroxide solution.

Preparative HPLC

As analytical HPLC with reversed-phase columns afforded good separations of HHDP esters, it was tempting to use this technique on a preparative scale. The same solvents were used and different elution conditions were compared. In difficult separations, no better resolution was obtained with gradient elution than with isocratic

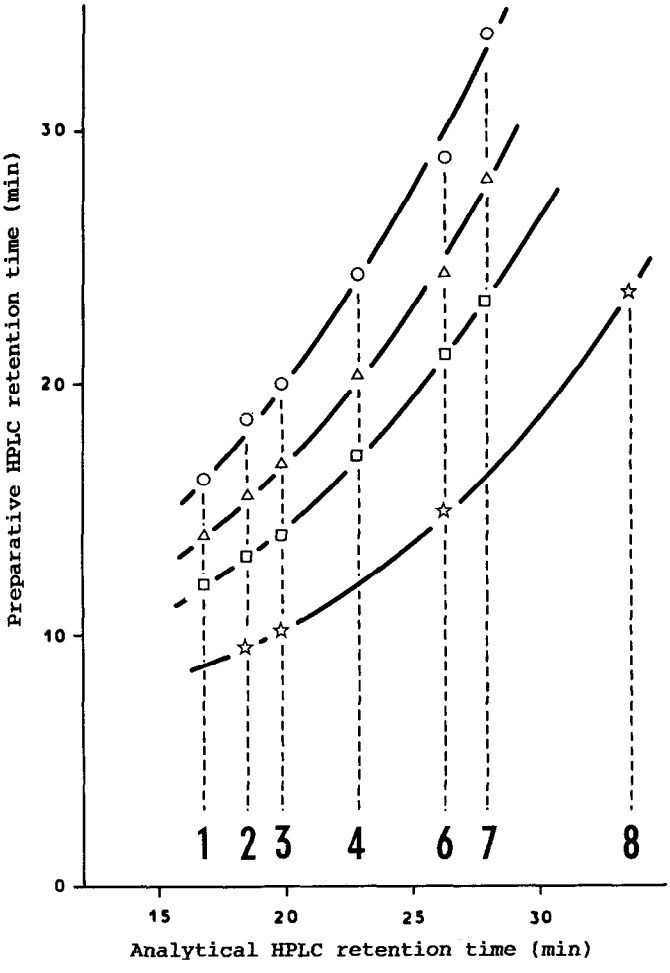

Fig. 6. Dependence of the preparative HPLC retention times on the analytical HPLC retention times for various compositions of the eluent used in preparative HPLC: (○) 2% B; (△) 3% B; (□) 4% B; (☆) 6% B. Amounts of sample injected into the preparative column, 1–2 mg. Numbers refer to the main components of aqueous extract of *Quercus robur* L. heartwood (Fig. 2c). See text for elution conditions.

elution (Fig. 5). Here, gradient elution cannot improve the resolution as it does for analytical HPLC, probably because of the high loading of the column.

Isocratic elution was therefore used and the concentration of methanol in the eluent was chosen so as to obtain a satisfactory separation without increasing the volume of the fractions collected too much. A relationship could be established between the retention times observed by analytical HPLC and preparative HPLC (Fig. 6). The concentration of methanol in the eluent was varied between 4 and 6% in order to obtain retention times that did not exceed 25 min.

As with Sephadex LH-20, the retention times were affected by the amounts of sample loaded on the column. The retention times were reduced by about 20% when

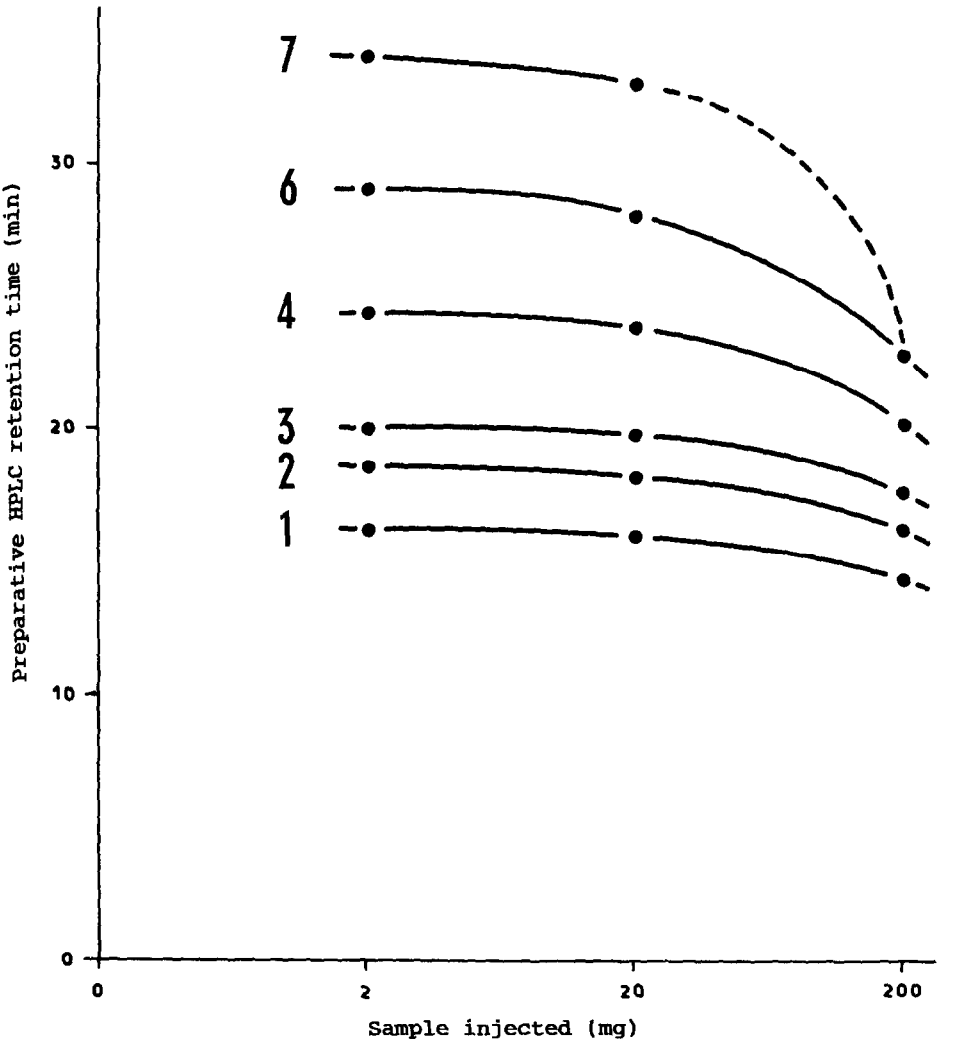

Fig. 7. Dependence of the preparative HPLC retention times on the amount of sample injected. The sample consists of a Sephadex fraction containing compounds 1, 2, 3, 4, 6 and 7 of the aqueous extract of *Quercus robur* L. heartwood (Fig. 2c). Eluent: 2% B.

the sample size was increased from 2 to 200 mg (Fig. 7). In some instances the resolution was severely affected by too high a loading of the column (in the example shown in Fig. 7, peak 7 is no longer resolved from the major peak 6 when the amount of sample injected exceeds 20 mg). Depending on the complexity of the mixture and the ease of separation, between 100 and 400 mg could usually be chromatographed in one run.

As with analytical HPLC, the solvent used to dissolve the sample before injection had a strong influence on the chromatographic profile. The sample had to be introduced in water in order to avoid the formation of multiple peaks.

Addition of an acid to the eluent is recommended in order to avoid ionization of the phenols, unless the peaks are considerably flattened and no reproducible retention times are obtained. The acid in the fractions collected must then be removed before concentration of the sample. Otherwise, the HHDP esters are rapidly hydrolysed and a precipitate of ellagic acid is observed. In order to remove phosphoric acid, HHDP esters were adsorbed on a column. Two different gels were examined: Sephadex LH-20 and Sep-Pak C_{18} (Millipore). The column was washed with water until neutrality was obtained and tannins were desorbed with methanol. HHDP esters were not retained strongly enough on Sep-Pak C_{18} and were partially eluted with water before reaching neutrality (Fig. 8). On the other hand, chromatography on Sephadex LH-20 allowed the total recovery of HHDP esters in methanol.

Purity of the fractions obtained

The purity of the different fractions obtained after preparative HPLC was determined by different chromatographic methods: two-dimensional paper chromatography, thin-layer chromatography on silica and HPLC. After separation, only one spot or one peak was observed for each of the eight main peaks on the HPLC trace of the raw extract (Fig. 9, Table I). The products corresponding to the eight peaks had

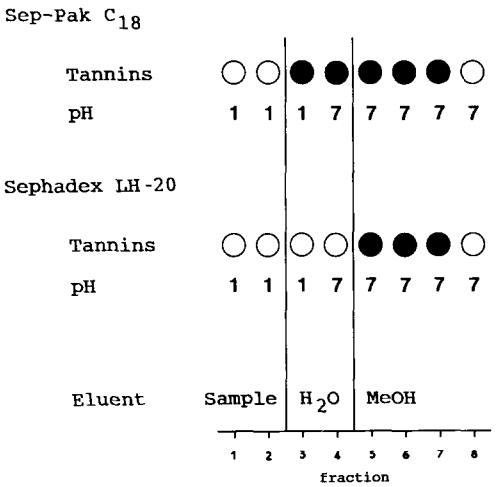

Fig. 8. Adsorption of HHDP esters on Sep-Pak C_{18} and Sephadex LH-20 for elimination of phosphoric acid. The presence (●) or absence (○) of HHDP esters is determined by their reaction with $FeCl_3$–$K_3Fe(CN)_6$ reagent. Volume of fractions, 20 ml. MeOH = Methanol.

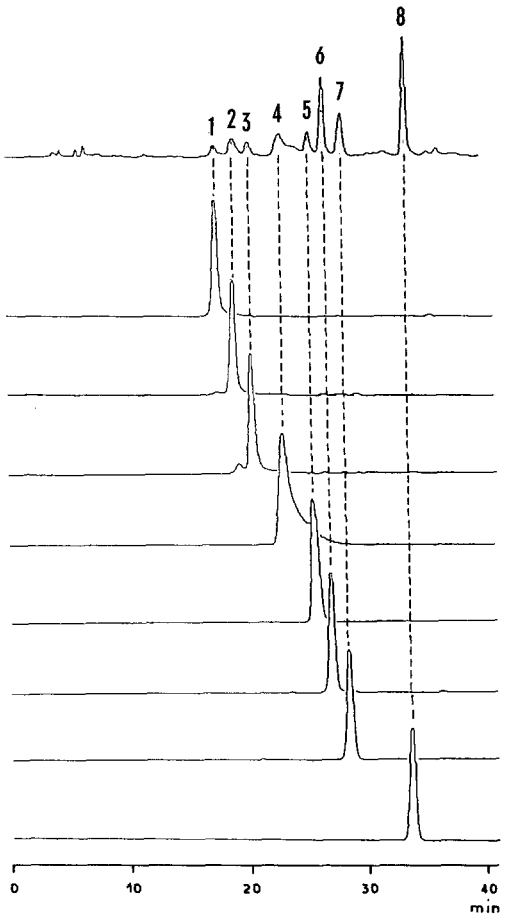

Fig. 9. HPLC of the fractions obtained from aqueous extracts of *Quercus robur* L. heartwood after chromatography on Sephadex LH-20 and preparative HPLC. The corresponding chromatogram of the raw aqueous extract is shown for reference.

TABLE I

YIELDS AND R_F VALUES OF THE COMPOUNDS PURIFIED FROM *Q. ROBUR* HEARTWOOD

Parameter	*Peak No.*[a]							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Yield (g/kg)	0.4	0.3	0.4	3.0	1.3	3.1	1.7	1.1
Paper chromatography, R_F:								
Acetic acid (6%)	0.38	0.45	0.55	0.56	0.37	0.57	0.60	0.43
sec.-Butanol–acetic acid–water (14:1:5)	0.00	0.00	0.00	0.03	0.00	0.06	0.03	0.06
Silica thin-layer chromatography, R_F	0.31	0.18	0.18	0.45	0.37	0.65	0.46	0.65

[a] See Fig. 2c.

identical UV spectra, showing no maxima between 240 and 400 nm, but a shoulder at 280 nm. They all gave the characteristic colour of HHDP esters when sprayed with nitrous acid.

Each fraction was analysed by ^{1}H NMR spectroscopy. Spectra were run in deuterated acetone, or in deuterated methanol if the sample was not soluble enough in acetone (peaks 2 and 3, Fig. 9). Compounds were considered pure when all the integrals of the different protons were similar. This was the case for five of the eight peaks (1, 3, 5, 6 and 8, Fig. 9). Among the pure compounds obtained, two (peaks 6 and 8) had spectra identical with those reported by Mayer *et al.*[6] and could be identified as vescalagin and castalagin, respectively. The other compounds, whose structures have not yet been reported, are in the process of being characterized. The other three peaks (2, 4 and 7) may require further purification as the integrals were not all similar. Chromatography on Fractogel (Merck) or speed flow counter-current chromatography, techniques already used by other workers[17,18], may complete the purification.

The yields calculated for 1 kg of wood extracted are given in Table I. The lower yields (peaks 1, 2 and 3) correspond to the minor products in HPLC trace of the raw extract. Vescalagin (peak 6), which was one of the two most important peaks in the HPLC trace of the raw extract, gave the best yield. However, the yields depended not only on the initial concentration of the compounds in the raw material, but also on the dispersion of each compound in one or several fractions after each purification step. This explains why the yields of some compounds are relatively small compared with what was expected.

CONCLUSIONS

Chromatography on Sephadex LH-20 and preparative HPLC have been shown to be complementary methods for the purification of HHDP esters, even for compounds with similar polarities. Purification of the HHDP esters is no longer limited to the one or two main compounds present in plant materials.

It is now widely accepted that the properties of HHDP ester, notably their astringency, are strongly dependent on their structure[13]. Hence the modern purification methods now available to the biologist should lead to a better understanding of the biological and physico-chemical properties of plant materials rich in HHDP esters which are not necessarily determined by the most abundant compounds.

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