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C-Glycosidic ellagitannins from white oak heartwood and callus tissues

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Abstract

Efforts were made to fully evaluate the performances of the two commonly used solvents, aq. Me_2CO and aq. MeOH, on extraction of vescalagin and castalagin from freshly harvested white oak (*Quercus alba*). The results show that aq. Me_2CO is superior to aq. MeOH in obtaining higher vescalagin and castalagin yields with lower total insoluble ellagitannins. A comparison of aq. Me_2CO extracts from *Q. alba* heartwood and callus tissues reveals similar ellagitannin profiles with callus tissues producing almost 3 times as much castalagin and vescalagin via manipulation of the media nitrogen and copper concentrations.

Keywords: *Quercus alba*; Callus; Castalagin; Ellagitannin; Heartwood; Polyphenol; Vescalagin

1. Introduction

The genus *Quercus* is represented by some 58 individual species in North America that reach tree size, with several others existing in shrub-like forms. The oaks are the most important commercial hardwoods in North America, with the eastern regions of the United States possessing the largest reserve of accessible oak timber in the world. Oak is used commercially in the manufacture of items such as furniture, flooring, veneer, cabinets and pallets and can also be used in the production of fine paper and paperboard.

The heartwood of oaks can contain up to 10% of ellagitannins by weight (Masson, Puech, & Moutounet, 1994; Masson, Moutounet, & Puech, 1995). These polyphenols are toxic to microorganisms, thereby preventing the rapid decay of this portion of the tree (Scalbert, 1992; Mila, Scalbert, & Expert, 1996). Due to their high oxidation potential and water solubility, ellagitannins can become a significant problem during wood drying, gluing and pulping, often resulting in the loss of value for the wood products produced (Charrier, Haluk, & Metche, 1995). Ellagitannins are also partially responsible for the quality and taste of the wines and spirits conditioned (aged) in oak barrels (Quinn & Singleton, 1985; Viriot, Scalbert, Lapierre, & Moutounet, 1993; Vivas & Glories, 1996; Feuillat et al., 1997). In addition, ellagitannins have long been known to have antiviral activity, making these compounds potentially useful as pharmaceuticals (Okuda, Yoshida, & Hatano, 1989; Haslam, 1996).

The predominant ellagitannins found in oak heartwood are castalagin (**1**) and vescalagin (**2**) (Scalbert, Monties, & Favre, 1988; Herve du Penhoat et al., 1991). The biosynthetic pathway for these compounds as well as the signals involved in initiating their biosynthesis are not known (Schmidt, 1956; Schmidt & Mayer, 1956; Gross, 1992; Haslam & Cai, 1994). Identification of the biosynthetic pathway (enzymes and genes) may provide the needed insight into the application of biotechnological methods suitable to increase or decrease the overall concentration of ellagitannins in heartwood, leading to 'tailor-made' wood products. Woods high in tannin content would be suit-

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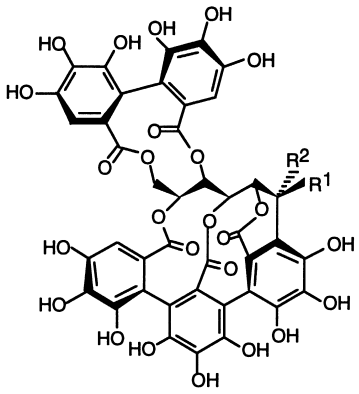
able for situations where high durability is required, while woods with low tannin content would be preferable for items such as furniture and pulpwood.



1 castalagin $R^1 = H, R^2 = OH$
2 vescalagin $R^1 = OH, R^2 = H$

Key to any study on ellagitannins is the availability of quantitative protocols for ascertaining their concentrations in any given species. This is an important issue considering that a large portion of the total ellagitannins in wood can become irreversibly bound to the woody cell wall (Peng, Scalbert, & Monties, 1991; Klumpers, Scalbert, & Janin, 1994; Viriot, Scalbert, Herve du Penhoat, & Moutounet, 1994). Quantitative analysis of individual ellagitannin contents is based on wood meal preparation and subsequent solvent extraction. Only a few reports have directly addressed the extraction protocol and how it moderates ellagitannin yields (Scalbert, Monties, & Janin, 1989; Peng et al., 1991).

The purpose of the work described here was twofold. First, the two most commonly employed solvents for wood extraction (Me_2CO:water and MeOH:water) were compared for the release of **1** and **2**, as well as insoluble ellagitannins remaining in the wood meal. The best overall protocol was then employed to extract white oak callus tissues grown on different media in order to determine if such tissues can serve as suitable models for the analysis of the ellagitannin biosynthetic pathway.

2. Results and discussion

2.1. Extraction protocols

Aqueous MeOH (MeOH:water; 7:3) and aq. Me_2CO (Me_2CO:water; 7:3) were used to extract heartwood powder containing an internal standard, 4-hydroxybenzoic acid. The amount of **1** and **2** solubilized relative to the internal standard over a 3 day time period was determined and the data for **2** are shown in Fig. 1. Aqueous Me_2CO was more efficacious at solubilising **2** from the wood meal, strongly suggesting that this solvent system is a better choice for the extraction and quantification of ellagitannins in woods. Very similar results have previously been reported by Scalbert's group (Peng et al., 1991) for 16 h extractions. They also reported that hot aq. Me_2CO and cold aq. MeOH performed equally well in extracting the ellagitannins (Scalbert et al., 1989). One of the possible reasons that hot aq. Me_2CO and cold aq. MeOH produced similar yields is that the higher temperature of the hot aq. Me_2CO may have degraded some ellagitannins during the extraction process, decreasing the overall recovery.

To further investigate the effects of both solvent systems on the insoluble ellagitannin contents, heartwood powders were extracted with aqueous acetone and aq. MeOH for 48 h. The extracted vescalagin and castalagin contents were determined by HPLC and the dried extracted wood meals were then subjected to an insoluble ellagitannin determination. The results are shown in Table 1. Aqueous Me_2CO gives higher yields of both **1** and **2** and the wood meal contains significantly less insoluble ellagitannins. Clearly, the use of aq. Me_2CO is more effective.

2.2. Studies with callus tissues

Callus tissues were generated from fresh stems using Murishage-Skoog media supplemented with sucrose, naphthaleneacetic acid, benzyladenine and Linsmaier/Skoog vitamins (Tanaka, Shimomura, & Ishimaru, 1995). We subsequently confirmed by both HPLC and

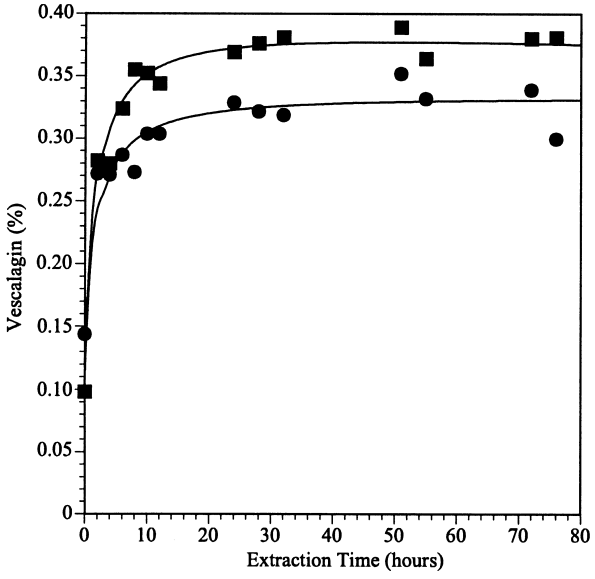


Fig. 1. A comparison of the extraction time profiles for *Quercus alba* wood meal extracted with Me_2CO:water (7:3; closed squares) and MeOH:water (7:3; closed circles).

Table 1
Effect of different solvent systems on ellagitannin yields

	Total yield (%)[a]	Vescalagin yield (%)	Castalagin yield (%)	Insoluble ellagitannins (%)[b]	Total phenols[c]
Acetone:water	6.72	0.45	0.55	0.53	3.65
Methanol:water	5.28	0.30	0.40	1.08	3.12

[a] Gravimetric yield (dry wt. basis).
[b] Expressed as castalagin equivalents (CE) provided that 1 mol of castalagin produced 1 mol of ellagic acid.
[c] Folin-Ciocalteau assay, expressed as gallic acid equivalents.

NMR analyses that the cultures produced both **1** and **2** (Fig. 2). In order to optimize our growth conditions for ellagitannin production, we then began to manipulate the copper and nitrogen levels of the agar solutions.

Nitrogen has been reported to have an important role in the biosynthesis of tannins in plant tissue cultures (Ishimaru & Shimomura, 1991; Neera, Arakawa, & Ishimaru, 1992; Tanaka et al., 1995). To evaluate the effect of nitrogen on the accumulation/formation of **1** and **2** in the *Quercus alba* callus, growth media with and without ammonium nitrate were used. The fact that the copper level in media increases the production and activity of laccase (Bligny, Gaillard, & Douce, 1986) prompted us to investigate the effect of copper on polyphenol production, since the redox potentials of laccases relative to peroxidases make them appropriate candidates for a galloyl biaryl coupling reaction (Xu et al., 1996). This was done by adding additional cupric sulfate to standard MS media preparations. After 3 months, the various calli were harvested and subjected to tannin analyses. As can be seen in Table 2, calli grown with additional copper (cupric sulfate) and in the absence of nitrogen produced more **1** and **2** than on the media containing ammonium nitrate. The slightly higher yields of **1** and **2** on average from the calli grown on a nitrogen-decreased media suggests that nitrogen levels may be important in controlling polyphenol biosynthesis in *Quercus alba*. The reason for the absence of **1** and **2** in the nitrogen deficient callus tissue grown with a copper content of 62.5 μg/l is unclear.

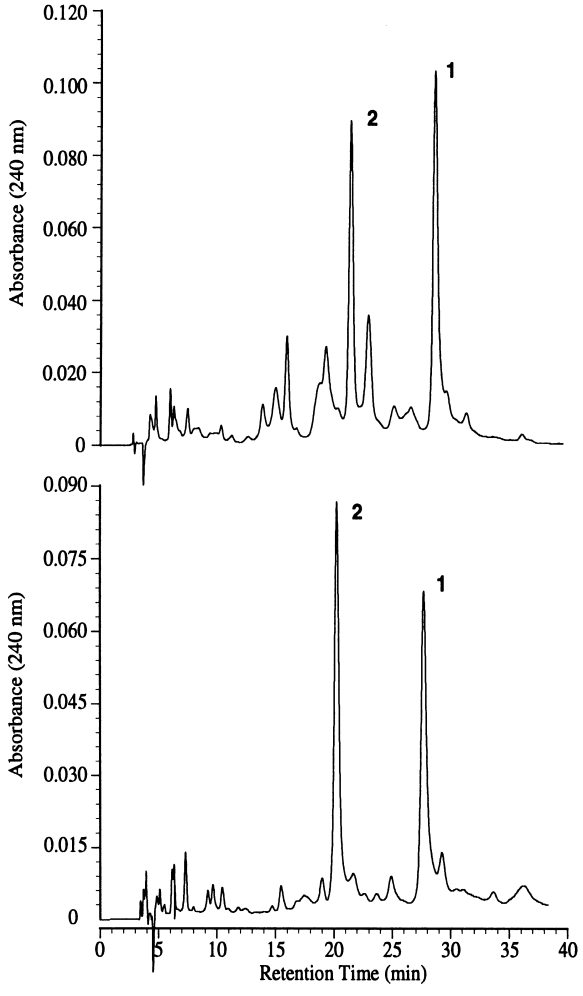


Fig. 2. HPLC elution profiles of the Me_2CO:water extracts of *Quercus alba* heartwood (top) and callus tissue (bottom). Compound identification was accomplished by both spiking the chromatograms as well as through the isolation and NMR characterization of **1** and **2** from both the heartwood and callus samples.

The effect of copper on the production of **1** and **2** is noteworthy. Supplemental copper can increase the production of these two C-glycosidic ellagitannins in both the presence and absence of available nitrogen. This is comparable to the results obtained with *Acer pseudoplatanus* cell suspension cultures (Bligny et al., 1986) where it was found that laccase activity was closely related to the copper level of the media. This similarity (i.e. both tannin production and laccase activity are associated with copper levels in media) suggests that a laccase or laccases may be involved in the biosynthesis of galloyl biaryl linkages in white oak callus tissue. It deserves mention that *Acer pseudoplatanus* produces gallic acid derivatives along with condensed tannins. It may be possible that although the isolated laccase was shown to oxidize monolignols (Sterjiades, Dean, & Eriksson, 1992), its actual role may be to oxidize tannin-based secondary metabolites. Therefore, when characterizing an isolated oxidase, data which shows that the enzyme can oxidize monolignols should not be

Table 2
Ellagitannin contents of *Quercus alba* callus tissues grown on modified media preparations

Copper (μg/l)	Medium[a]	Vescalagin (**2**) (%)	Castalagin (**1**) (%)	Insoluble ellagitannins (%)	Total (%)
25	MS	0.75	0.70	0.48	1.83
25	MS–NH_4NO_3	0.91	0.94	0.50	2.35
62.5	MS	1.13	0.87	0.37	2.37
62.5	MS–NH_4NO_3	< 0.2	< 0.2	0.48	0.48
125	MS	0.97	0.87	0.42	2.26
125	MS–NH_4NO_3	1.22	1.01	0.37	1.60
190	MS	1.18	0.89	0.44	2.51
190	MS–NH_4NO_3	1.63	1.75	0.51	3.89
250	MS	1.46	1.25	0.70	3.41
250	MS–NH_4NO_3	1.20	1.09	0.66	2.95

[a] Murishage-Skoog media supplemented with cupric sulfate (μg/l), in the presence (MS) or absence (MS–NH_4NO_3) of supplemented nitrogen. Yields are as described in Table 1.

the sole criterion for assigning its role to that of lignin formation.

Comparison of the polyphenol yields/HPLC profiles of the oak heartwood with those of callus reveals that callus produces significantly more **1** and **2**, demonstrating that it is possible to regulate the biosynthesis of polyphenols in callus by changing the compositions and contents of media. The ratio of **1** to **2** differs between heartwood and callus, with more **2** present in the callus tissues. This can be explained by both the relative instability of **2** as well as the fact that most of the C-glycosidic ellagitannin dimers found in oak are derived from **2**; these compounds are not present in high concentrations in the callus tissues. This system appears to be an appropriate model for the study of the biosynthesis of **1** and **2** in vivo.

3. Experimental

3.1. General

An 80-year old white oak (*Quercus alba*) was felled in early December 1997 from National Forest lands in Giles County, Virginia. Wood slices of approximately 2.5–4 cm thick were taken from the oak log with a chain saw. After freeze drying the slices, the heartwood and sapwood were separated, broken up into small chips and ground in a Wiley mill so as to pass a 1 mm screen. The white oak callus tissues arise from stem explants removed from a white oak growing in Craig County, Virginia. All solvents and water were HPLC grade. The Folin Ciocalteu reagent, ellagic acid and gallic acid were obtained commercially and used without further purification. Evaporation was performed under reduced pressure at temperature less than 40°C. HPLC was performed on Gilson HPLC apparatus operating in the gradient mode using a Merck Lichrospher RP-18 (endcapped 5 μm) column (250 × 4 mm I.D.). The solvents were MeOH and aq. 0.2% trifluoroacetic acid (TFA) and the flow rate was set at 0.75 ml/min. Gradient conditions: MeOH increased from 0 to 10% over a 40 min time period. This was followed by a 5 min gradient to 100% MeOH, 5 min at 100% MeOH, a 10 min gradient to 0% MeOH and, finally, a 5 min re-equilibration.

Purification of vescalagin (**2**) and castalagin (**1**) was conducted on a low pressure reverse-phase C-18 column (15 × 335 mm). Successive elution with water, 5% methanol and methanol at 1 ml/min provided fractions that were combined according to tlc results (Merck F254 cellulose plate, elution system: MeOH:H_2O:HOAc; 1:89:10), evaporated and freeze-dried. The vescalagin (**2**) sample was further purified on Toyopearl HW40-F column (I.D. 15 × 335 mm) with the elution of methanol:water (8:2), MeOH, MeOH:acetone:water (7:1:2) and acetone:water (1:1). A high flow rate (2 ml/min) was adopted to minimize the degradation of vescalagin (**2**) in MeOH. Fractions were combined, evaporated and freeze-dried. The ^1H NMR spectra matched those reported for vescalagin (**2**) and castalagin (**1**).

3.2. Solvent extraction

Sample powder was extracted in the dark and under nitrogen with either Me_2CO:water (7:3) or MeOH:water (7:3) at the room temperature with magnetic stirring. For the quantitative evaluation of the two solvents, an internal standard (4-hydroxybenzoic acid) was added as well. The solvent to wood meal ratio was kept at 10 ml/g. The extracts were filtered, evaporated under reduced pressure (to remove Me_2CO or MeOH) and then freeze dried. All yields are reported on a dry weight basis.

3.3. Determination of polyphenols

Total soluble polyphenols were determined with Folin-Ciocalteu spectrophotometric method [21]. Gallic acid was used as the standard and the total phenols in the samples were expressed as gallic acid equivalent (GAE). Determination of vescalagin (**2**) and castalagin (**1**) concentrations was carried out by HPLC using internal analyses.

Insoluble polyphenols were determined according to the protocol of Peng and Scalbert (Peng et al., 1991). Samples (200–300 mg) were placed in Teflon-lined screw cap test tubes containing MeOH (4.5 ml) and aq. HCl (6 M, 0.5 ml). The tubes were heated for 160 min at 120°C and the ellagic acid contents were subsequently determined by HPLC using an external standard analysis (Helm, Ranatunga, & Chandra, 1997). The results are expressed as castalagin equivalents (CE) provided that 1 mol of castalagin produced 1 mol of ellagic acid.

3.4. Callus tissues

Stem explant pegs, 10–20 mm long, were cut from unblemished first year growth between leaf buds. The bark and underlying cambium was stripped off and the explant was surface-sterilized in a 4-stage procedure of 70% ethanol for 5 min, 20% bleach for 5 min and two washings with sterile distilled water. Alternatively, the explants were surface-sterilized with 15% bleach for 5 min, the ends were recut then the explants were placed in fresh 15% bleach for 5 min followed by five washings with sterile distilled water. Both sterilization methods gave approximately the same percentage of contaminant-free callus tissue.

Surface sterilized explants were placed on 100 × 15 mm plastic petri dishes charged with MS (20 m) solid medium supplemented with sucrose (40 g/), naphthaleneacetic acid (NAA, 2 mg/l), benzyladenine (BA, 0.1 mg/l) and Linsmaier/Skoog vitamins. The pH was adjusted to 5.7 before autoclaving at 120°C for 20 min. The prepared dishes were sealed with parafilm and placed in a dark incubator preset to 23°C. Callus induction took approximately 2 weeks and after 5–6 weeks, the induced white/yellow calli were sectioned into 5-mm squares and subcultured onto fresh media. Subsequently, the calli were subcultured monthly.

Ellagitannin concentrations were determined by first freeze drying the callus tissues and determining their dry weight. The cells were subsequently pulverized into a fine powder with a mortar and pestle and suspended in Me_2CO:water for 24 h. Further processing was performed as described for the heartwood samples. Yields were determined on a dry weight basis.

3.5. Callus induction

Five different media preparations were evaluated for their abilities to increase ellagitannin production. Four of the preparations were based on the MS media described above, but supplemented with cupric sulfate to bring the final concentrations to 0.25, 0.19, 0.125 and 0.0625 mg/l. MS media concentration of cupric sulfate ($Cu_2SO_4{\cdot}5H_2O$) is 0.025 mg/l. An identical set of media was prepared using the MS media and supplemental copper but without ammonium nitrate.

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