Monomeric Ellagitannins in Oaks and Sweetgum

by

Zhentian Lei

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Richard F. Helm, Chair
Charles E. Frazier
Wolfgang G. Glasser
John L. Hess
David G. I. Kingston

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Abstract

Ellagitannins are plant phenolics characterized by biaryl-coupled gallic acid moieties esterified to a D-glucose core. They are widely distributed through higher plants. In the case of oaks, ellagitannin concentrations in heartwood can reach up to 10% (dry wt. basis). These secondary metabolites are not only important physiologically but also influence the economic value and quality of wood products that contain them.

Efforts were made to develop and validate the methods used to quantify both soluble and insoluble ellagitannins. First, the efficiencies of the two commonly used extraction solvents, aqueous acetone and aqueous methanol were evaluated. The results showed that aqueous acetone is superior to aqueous methanol in obtaining higher vescalagin and castalagin yields. In a separate study, the method used for determining insoluble ellagitannins was found to under-estimate the contents of insoluble ellagitannins in wood products. Anhydrous methanolic HCl was found to be an excellent reagent for releasing insoluble ellagic acid and gallic acid (as methyl gallate) from biomass substrates. Optimization of both the reaction conditions and the gradient HPLC analysis has led to the development of a robust and reliable protocol.

The chemical stability of the two predominant ellagitannins in oaks (vescalagin and castalagin) were evaluated in aqueous methanol and water. It was found that oxygen, pH and higher temperature (60 °C) affect their stability with higher temperature being the most prominent factor. Both vescalagin and castalagin were found unstable in methanolic solutions. Vestalagin, however, is less stable than castalagin.

In the course of finding alternative models for ellagitannin biosynthesis study, both callus tissues and suspension cell cultures of white oak (*Quercus alba*) and sweetgum (*Liquidambar styraciflua*) were investigated for their possible use as models for ellagitannin biosynthesis. It was found that oak callus tissue cultures (*Quercus alba*) are capable of producing ellagitannins, and the production and profile of ellagitannins can be modified by adjusting the media composition. Comparison of extracts from the heartwood of *Quercus alba* with those from callus tissue reveals that they have similar ellagitannin profiles. Through manipulation of the media nitrogen and copper concentrations the callus tissue produced almost 3 times as much castalagin and vescalagin. Suspension cells of *Quercus alba* and *Liquidambar styraciflua* were found to be unsuitable for the study of biosynthesis of ellagitannins. These cells either did not produce any detectable level of ellagitannins or the production was unstable. Although the suspension cells could be elicited to produce ellagic acid with glycanases (Driselase), the levels of ellagic acid were too low for quantitative metabolic studies.

A method using high performance liquid chromatography – mass spectrometry was developed and optimized with purified ellagitannins. Ellagitannins analyzed under the optimal conditions all provide base peaks of $(M\text{-}H)^-$ from which the molecular weights of the ellagitannins can be determined. Mild fragmentation was also achieved to give fragments characteristic of ellagitannins (loss of ellagic acid and gallic acid if present). These characteristic peaks allow for rapid identification of ellagitannins from other secondary metabolites present in the samples. Application of the HPLC/ESI-MS in the identification of monomeric ellagitannins in white oak heartwood extracts revealed that it can unambiguously identify the two monomeric ellagitannins, castalagin and vescalagin,

and their degradation product, ellagic acid. The key fragmentation pathways of the ellagitannins are also described.

Finally, preliminary work using proteomics to study the heartwood formation was conducted. Proteins from transition zone and sapwood were determined and resolved with two-dimensional electrophoresis. It was found that both sapwood and transition woods contain active enzyme(s) capable of catalyzing formation of ellagic acid from pentagalloylglucose. Preliminary results from the 2-D gel separation of sapwood and transition wood proteins showed more protein spots in sapwood than in transition wood, suggesting that sapwood not only had higher protein levels but also a great total number of proteins. The lower complexity of the transition wood proteome suggests that this material may be a good substrate for studying the biaryl-coupling process.

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Chapter One

Literature Review

1. 1. Introduction

The genus *Quercus* (oaks) are the predominant hardwoods found in North America. They are widely distributed throughout the United States, with the southeast region of the United States possessing the largest reserve of accessible oak timber in the world. There are around 70 individual species of oaks native to the United States. About 58 species of them reach tree size with the rest existing in shrub-like form (Harlow et al., 1979). Based on the similarities in botanical features, these different species of oaks can be broadly classified into two groups: *Erythrobalanus* group (red or black oak) and *Leucobalanus* group (white oak) (Harlow et al., 1979). The distinctive anatomical difference between these two groups is the presence of tyloses in the vessels of white oaks and their absence in the red oaks.

Oaks are the most economically important hardwoods in the US, with the annual harvest valued at more than $3 billion. The physical properties and distinctive appearance have made the oaks the most attractive and preferred hardwoods in the US. They are used to manufacture many products such as furniture, flooring, veneer, cabinets and pallets. They can also be used in the production of fine paper, paperboard, cooperage and railroad ties. Clearly, oak-based products are an indispensable sector of the US forest products industry.

Oaks are rich in a class of secondary metabolites called ellagitannins; a type of polyphenol characterized by biaryl-linked galloyl groups esterified to a glucose core. While both the sapwood and heartwood are found to contain ellagitannins, ellagitannins

are mainly found in the heartwood. The concentration of ellagitannins in heartwood can reach up to 10% by weight (dry wt. basis) (Masson et al., 1994 and 1995). Many distinctive properties of oaks, either desirable or undesirable, can be attributed to these ellagitannins. For example, ellagitannins are toxic to microorganisms, preventing the rapid decay of this portion of the wood (Mila et al., 1996; Scalbert, 1991 and 1992). The relative abundance of ellagitannins in oak heartwood endows oak wood with good resistance to fungal degradation (durability). The combined presence of ellagitannins and tyloses in white oak heartwood also allows these woods to be unrivaled for the manufacture of the barrels used to condition and age wines and whiskeys (Mosedale et al., 1999; Quinn and Singleton, 1985; Vivas and Glories, 1996). The deposition of ellagitannins in the heartwood, however, makes the wood darker and this is often objectionable. In addition, because of their high oxidation potential, water solubility and the reactivity with organic and inorganic chemicals, ellagitannins can become a nuisance during wood drying, processing, gluing and pulping. For instance, ellagitannins are blamed for wood staining or brown discoloration during wood processing and drying (Charrier et al., 1995), which makes it difficult to color match different pieces of wood. Ellagitannins can also cause problems in the manufacture of wood composites and panel products due to their interactions with adhesives (Marra, 1992). These problems often result in financial losses to industrial wood dryers and wood product producers.

Ellagitannins are not only limited to oaks. They are also found in many other economically important hardwoods. For example, chestnut (*Castanea* sp.) is found to contain a considerable amount of ellagitannins (Tang and Hancock, 1995; Lampire et al., 1998; Viriot et al., 1994) which is attributed to the well-known durability of this species. Sweetgum (*Liquidambar styraciflua)* also contains ellagitannins (Spencer and Choong, 1997). Ellagitannins are also found in basswood (*Tilia* sp.) (Rowe and Conner, 1979), the

alders (*Alnus* sp.) (Lee et al., 1992) and eucalyptus species (Conde et al., 1995). Although the types and the amounts of ellagitannins present in these hardwoods vary considerably from species to species, they represent a major class of secondary metabolites in many hardwood species.

Due to their importance in the utilization of the hardwoods, ellagitannins are now receiving more and more attention. Efforts have been made by scientists around the world to understand the fundamental chemistry and the biosynthesis of ellagitannins. However, in spite of decades of researches, ellagitannin biosynthesis remains unclear.

1. 2. Structures of Ellagitannins

Ellagitannins are considered a subset of the plant secondary metabolites termed tannins. These secondary metabolites are essentially complex plant polyphenols. They distinguish themselves from other plant phenolic compounds such as lignans and lignin by their ability to bind and precipitate alkaloids, gelatin and other proteins (Okuda, 1999). Indeed, the name "tannins" is derived from the unique ability of these phenolic compounds to tan animal skin into leather, a process that essentially precipitates proteins into a stable solid (i.e. leather). These polyphenols are usually water-soluble and have molecular weights between 500 and 3000 (Haslam and Cai, 1994). Other plant phenolics that fall out of this range may lose this unique protein-binding property and thus are not considered as tannins.

Tannins or plant polyphenols can be classified into condensed tannins (nowadays commonly referred to as proanthocyanidins) and hydrolyzable tannins. This classification is based on their distinctive structures. While the fundamental structure units of condensed tannins are flavan-3-ol units (Hergert, 1988), glucose and gallic acid (3,4,5-

trihydroxybenzoic acid, **1**) make up the whole of the hydrolyzable tannins (Haslam and Cai, 1994). Condensed tannins are formed by flavan-3-ol units that are linked together through carbon-carbon bond (Hergert, 1988). Hydrolyzable tannins which are considered to derive from β-1,2,3,4,6- pentagalloylglucose (PGG, **2**) are, in essence, esters of gallic acid and glucose. The ester bonds can be cleaved under mild hydrolysis conditions, rendering this class of tannins the name "hydrolyzable tannins". Based on the modification of the galloyl groups on the glucose core, hydrolyzable tannins are further divided into gallotannins and ellagitannins. Ellagitannins differ from gallotannins in that some gallic acid moieties or galloyl groups (**3**) are biaryl coupled (Figure 1.1) to each other through carbon-carbon bond to form 6,6'-dicarbonyl-2,2', 3,3', 4,4'-hexahydroxydiphenyl (HHDP) moiety (**4**). Gallotannins have gallic acid groups that are linked exclusively through ester bond (depside linkage **5**) to the gallic acid moieties that are already esterified to the D-glucose core. The degrees of galloyl addition can reach up to as many as 10-12 galloyl units, depending on the source of gallotannins (Nishizawa et al., 1982 and 1983). The HHDP group is the defining structural characteristic of all ellagitannins. They can be cleaved upon hydrolysis from glucose core and spontaneously undergo lactonization to form ellagic acid (**6**). Hydrolysis of ellagitannins may or may not give gallic acid depending on their structures. Gallotannins, however, only release gallic acid (**1**)

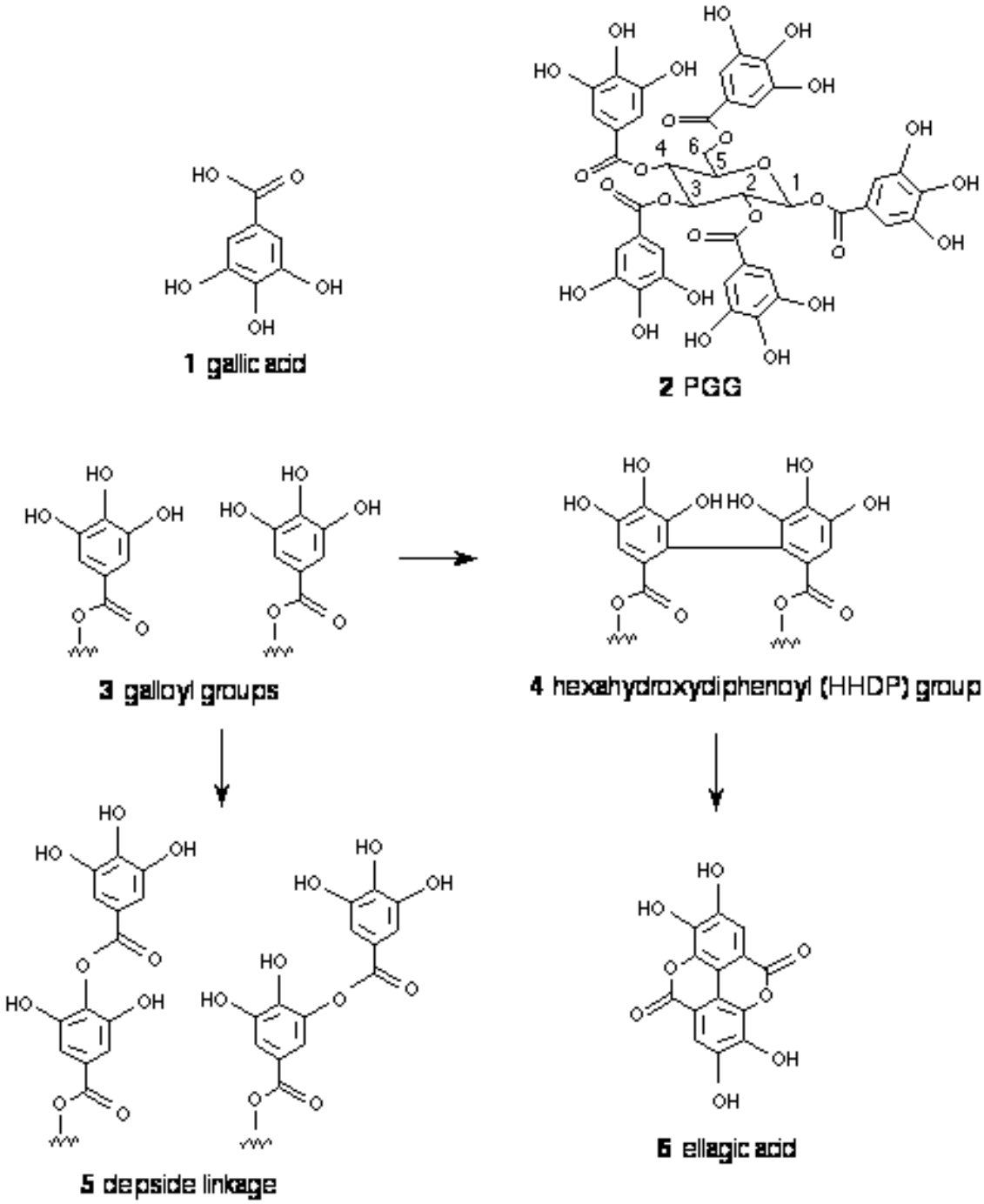


Figure 1.1. Structures of gallic acid (**1**) and PGG (**2**). Oxidative coupling of galloyl groups (**3**) leads to HHDP group (**4**) (ellagitannins) while addition of further galloyl units to PGG through ester bonds results in despside linkage (**5**) (gallotannins)

1. 2. 1. Monomeric Ellagitannins

Monomeric ellagitannins refer to those ellagitannins containing only one glucose core. Intramolecular oxidative C-C couplings between galloyl groups converts these galloyl groups into HHDP groups, resulting in the transformation of PGG (**2**) into monomeric ellagitannins (Haslam and Cai, 1994). The most common and naturally occurring coupling patterns of galloyl groups are 4,6- (coupling between galloyl groups at C4 and C6 of glucose) and 2,3-couplings (coupling between galloyl groups at C2 and C3 of glucose) although coupling between 1,6-, 1,3-, 3,6-, and 2,4-galloyl groups can also be found. These different coupling patterns result in the structural complexity of monomeric ellagitannins, which is further complicated by the differing extent of galloylation, the stereochemistry of HHDP groups, formation of aromatic glycosides and the anomeric stereochemistry of the glucose core (Haslam and Cai, 1994; Quideau and Feldman, 1996).

The most notable ellagitannins in plants are those featuring 4,6-HHDP, 2,3-HHDP or both 4,6-HHDP and 2,3-HHDP couplings (Figure 1.2). Typical monomeric ellagitannins representing those containing 4,6-coupled HHDP are tellimagrandin II (**7**), strictinin (**8**) and tellimagrandin I (**9**). Ellagitannins bearing only 2,3-coupled HHDP groups can be exemplified by pterocaryanin C (**10**), sanguin H5 (**11**) and H4 (**12**). Another subclass of monomeric ellagitannins featuring both 4,6- and 2,3-couplings that are found in woods are casuarictin (**13**), potentillin (**14**) and pedunculagin (**15**).

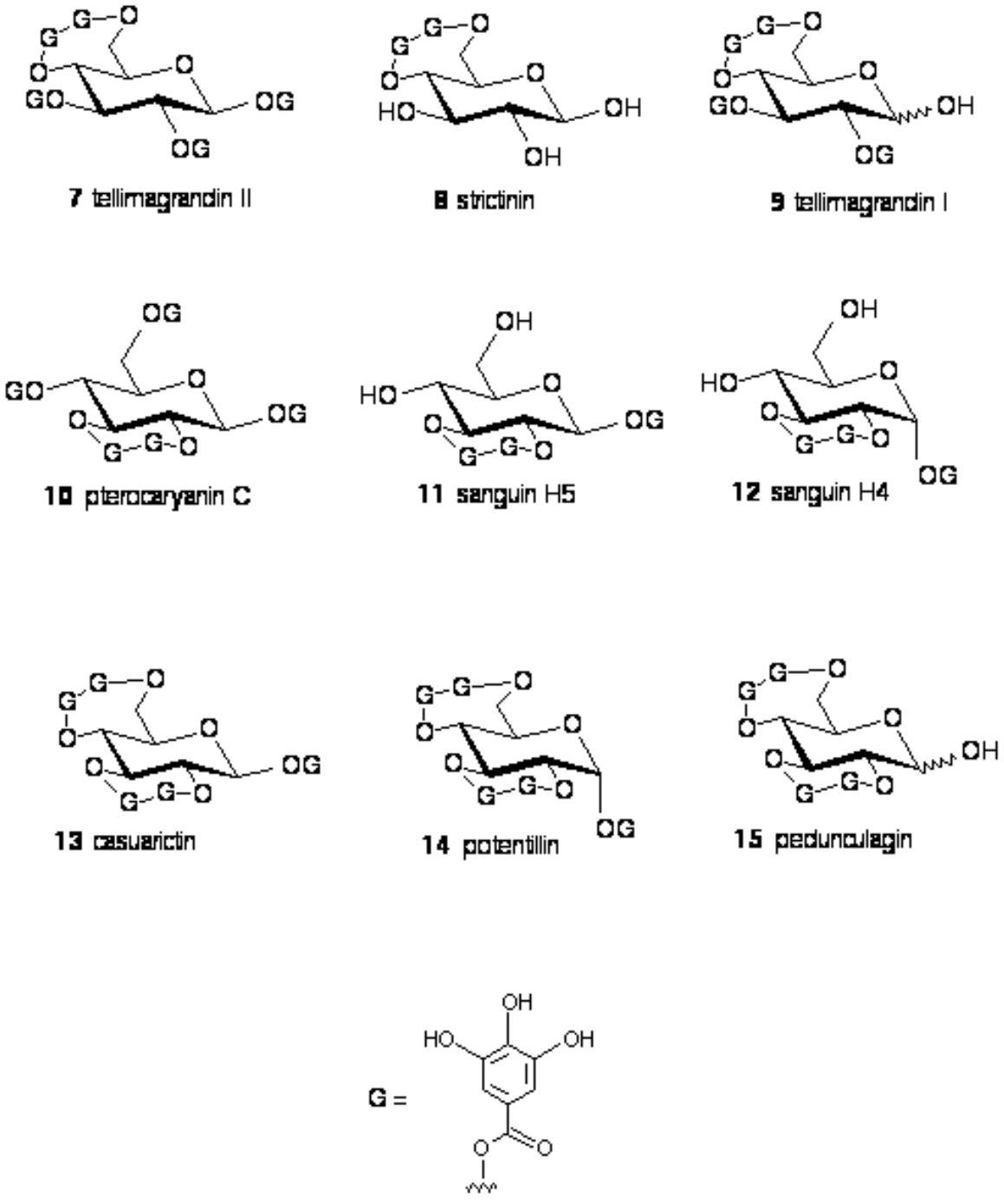
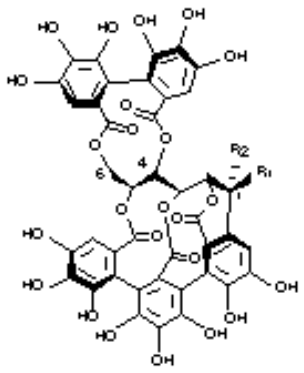
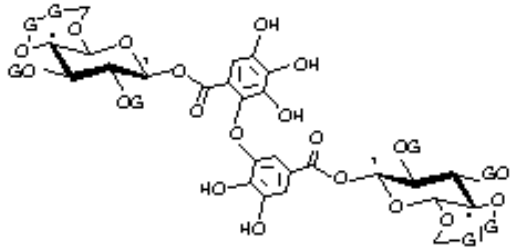


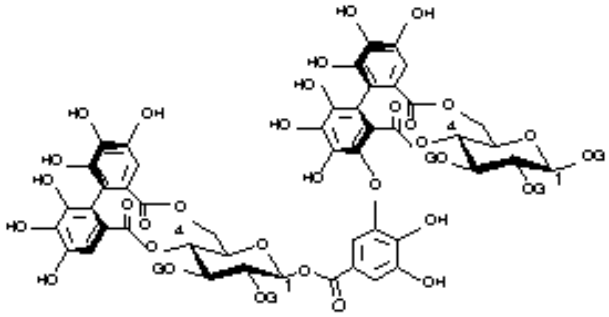
Figure 1.2. Structures of monomeric ellagitannins



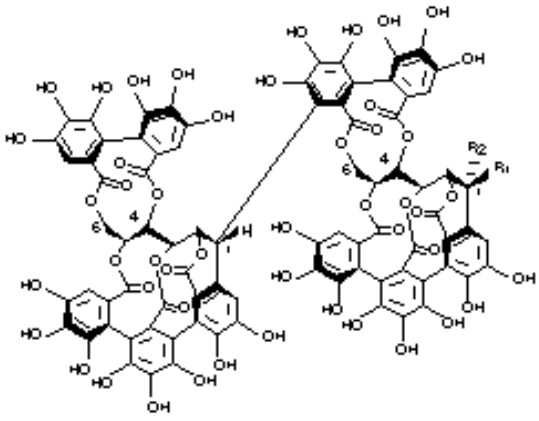
16 R_1=H, R_2=OH, **vescalagin**
17 R_1=OH, R_2=H, **castalagin**



18 **coriariin**



19 **rugosin**



20 R_1=H, R_2=OH, **roburin A**
21 R_1=OH, R_2=H, **roburin D**

Figure 1.3. Structures of Castalagin and Vescalagin and their dimers

Another significant group of monomeric ellagitannins featuring acyclic, aromatic glycosides can be represented by vescalagin (**16**) and castalagin (**17**). These two aromatic glycosides have 4,6- HHDP coupling and a unique flavogallonyl group which are composed of three galloyl groups linked together with C-C bonds. These acyclic monomeric ellagitannins and their polymers are the major ellagitannins found in oaks (Quinn and Singleton, 1985; Viriot et al., 1994).

1. 2. 2. Oligomeric Ellagitannins

Monomeric ellagitannins can be polymerized to form oligomeric or polymeric ellagitannins (Haslam and Cai, 1994) and they are often found in plants along with their monomers. The most prevalent oligomeric ellagitannins are dimeric to tetrameric ellagitannins. The polymerization of monomeric ellagitannins is primarily through the oxidative C-O couplings between galloyl and between galloyl and HHDP moieties and the oxidative C-C couplings between glucose and HHDP groups of appropriate monomeric ellagitannin precursor (Haslam and Cai, 1994; Quideau and Feldman, 1996). The key oligomeric ellagitannins representing oxidative C-O couplings include two tellimagrandin I dimers coriariin A (**18**) (oxidative C-O couplings between galloyl groups) and rugosin D (**19**) (oxidative C-O couplings between galloyl group and HHDP group).

Oligomeric ellagitannins featuring oxidative C-C coupling between the monomeric ellagitannins can be illustrated by vescalagin/castalagin dimers: roburin A (**20**) and roburin D (**21**). Monomeric ellagitannins that undergo the oxidative C-C coupling are typically acyclic aromatic glycosides. Although not as prevalent as C-O couplings, dimers that are derived from acyclic aromatic glycosides through C-C coupling are constituents of oligomeric ellagitannins in oaks.

1. 3. Biosynthesis of Ellagitannins

The fact that ellagitannins are mainly found in the heartwood not in the sapwood leads to the assumption that ellagitannin biosynthesis occurs along the sapwood/heartwood interface, because heartwood is a nonliving part but contains high amount of ellagitannins while although sapwood is a living potion of the tree, it contains very low amount of ellagitannins. Indeed, ellagitannins are found in the highest concentration (Viriot et al., 1994) along the interface where the living sapwood is transformed into the nonliving heartwood. The biosynthesis of ellagitannins is thus considered to be closely associated with the heartwood formation, a process in which non-structural carbohydrates are metabolized to form biocides such as polyphenols (Hauch and Magel, 1998).

Unlike condensed tannins that are derived from the phenylpropanoid pathway and the condensation of malonyl-CoA and 4-coumaroyl-CoA (Hergert, 1988), formation of hydrolyzable tannins operates on a different pathway. 3-Dehydroshikimate, an early intermediate in the shikimate pathway, is now generally accepted as the precursor to gallic acid (**1**). Although it has been previously proposed that gallic acid could be formed from the phenylpropanoid pathway via caffeic acid (Gross, 1992; Lewis, 1988), recent experiments demonstrate that gallic acid is formed predominantly or entirely from the dehydrogenation of 3-dehydroshikimate (Werner et al., 1997).

1. 3. 1. PGG

Clearly, PGG (**2**) is the key intermediate in the biosynthesis of ellagitannins. The biosynthetic pathway leading to this compound has been elucidated by Gross and co-workers (Niemetz et al., 1999). The fact that acyl-CoA thioesters act as the acyl group donors in the biosynthesis of phenolic esters first led researchers to believe that an acyl-CoA thioester analog, galloyl-CoA thioester, may act as a galloyl donor in the galloylation

of glucose (Niemetz et al., 1999). However, studies with cell-free crude enzyme extracts from oak and a synthetic galloyl-CoA thioester showed that galloyl-CoA thioester is not involved in the galloylation of glucose. Instead, free gallic acid, mediated by UDP-glucose dependent galloyltransferase, is directly esterified to UDP-glucose to form β-glucogallin (β-1-galloyl-glucose, **22**) which serves as the principal galloyl donor and acceptor in the subsequent galloylation of glucose, as shown in Figure 1.4. The next galloylation step of β-1-galloyl-glucose leads to β-1,6-digalloylglucose (**23**) with β-1-galloyl-glucose acting as both the galloyl donor and the acceptor. Subsequent galloylation of β-1,6-digalloylglucose, catalyzed by β-glucogallin-dependent gallolytransferases, produces β-1,2,6-trigalloylglucose (**24**), β-1,2,3,6-tetragalloylglucose (**25**) and finally β-1,2,3,4,6-pentagalloylglucose (PGG, **2**). Interestingly, this order of the enzymatic galloylation of glucose corresponds to the chemical esterification of the hydroxyl groups of glucose, which is in the order of 1-OH, 6-OH, 2-OH, 3-OH and 4-OH (Niemetz et al., 1999).

Experiments with both oak and sumac (*Rhus typhina*) leaves revealed that the biosynthetic pathway of PGG is identical in both plants (Niemetz et al., 1999). Enzymes catalyzing the individual galloylation step of this pathway have been isolated from both oak and sumac leaves. They are found to be similar in the general property (Niemetz et al., 1999). For example, they all have pH optima around pH 4-6 and temperature optima between 40-50 °C. They also exhibit a common cold tolerance with residual activities of 10-25% at 0 °C. They are also found to have the unusually high molecular weights between 260,000 and 450,000 dalton with the exception of the UDP-glucose dependent galloyltransferase from oak and the β-glucogallin-dependent 1,6-digalloylglucose-galloyltransferase from sumac, which have molecular weights of 68,000 and 56,000 dalton respectively (Gross, 1992; Niemetz et al., 1999).

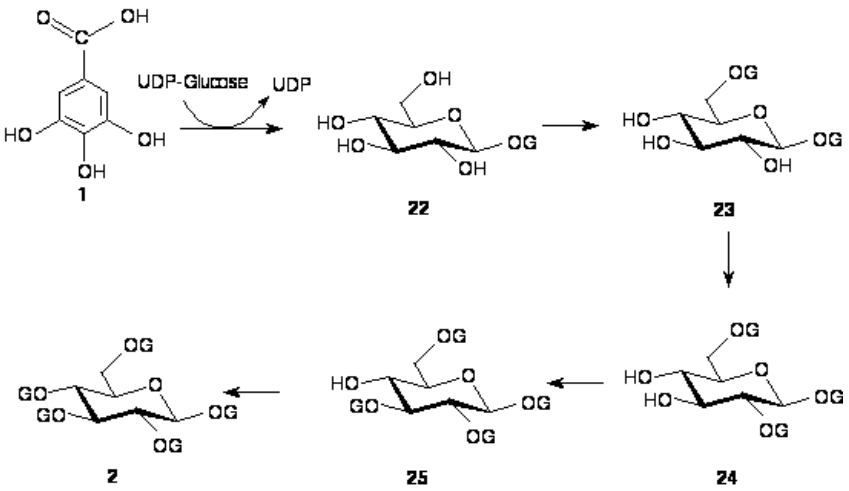


Figure 1.4. Formation of pentagalloylglucose (PGG).

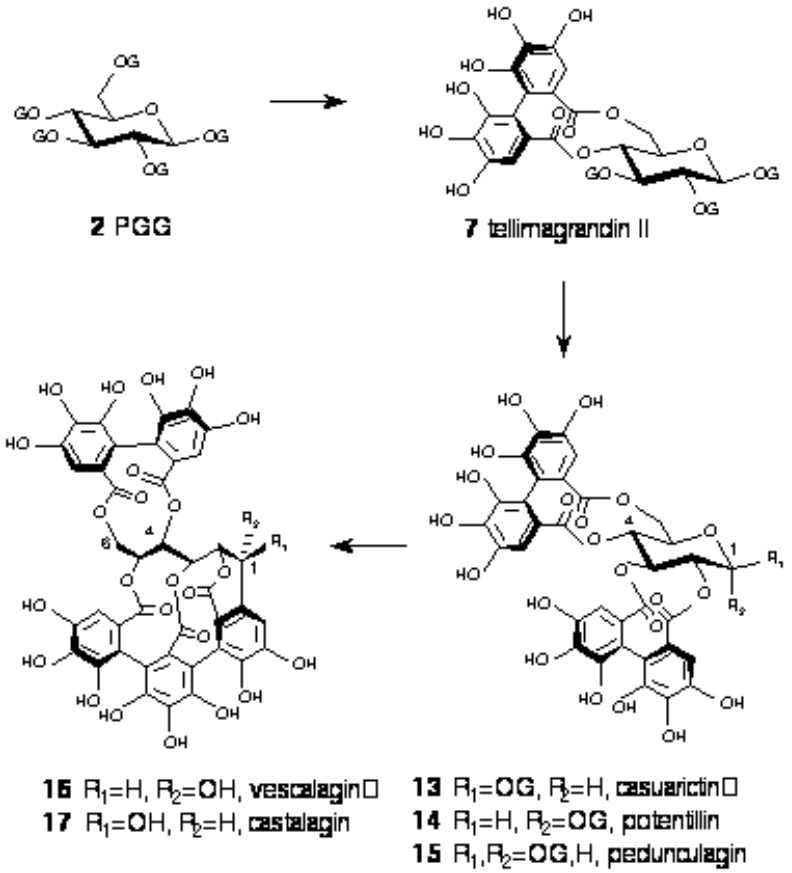


Figure 1.5. Simplified pathway leading to the opening of glucose ring (Helm et al., 1999)

The β-glucogallin-dependent pathway leading to the formation of PGG from gallic acid and UDP-glucose as shown in Figure 1.4 is not exclusive (Gross, 1992). Parallel and apparently β-glucogallin-*independent* reactions have been found in sumac. Enzyme extracts (Kenzel and Gross, 1991) from sumac leaves have been found to be capable of producing 1,2,6-trigalloylglucose (**24**) from 1,6-digalloylglucose in the absence of β-1-galloylglucose (**22**) which was originally thought to be the sole galloyl donor. Similarly, β-1,6-digalloylglucose (**23**) can replace β-1-galloylglucose (**22**) as the galloyl donor in the enzymatic conversion of β-1,2,6-trigalloylglucose (**24**) to β-1,2,3,6-tetragalloylglucose (**25**). Detailed experiments further revealed that in addition to β-glucogallin (**22**) and β-1,6-digalloylglucose (**23**), β-1,2,6-trigalloylglucose (**24**) and β-1,2,3,6-tetragalloylglucose (**25**) might also act as the galloyl donors although with progressively decreasing efficiency. Only 1,2,3,4,6-pentagalloylglucose (PGG, **2**) is completely inactive and does not act as a galloyl donor. It was also found that only the β-1-galloyl groups (not other galloyl groups) of the galloylglucoses serve as the leaving groups when the galloylglucoses act as the galloyl donors. This may explain the observation that many polygalloyl and HHDP esters of D-glucose found in plants possess the unacylated anomeric hydroxyl groups (Haslam and Cai, 1994).

1. 3. 2 Ellagitannins

Compared with the enzymatic synthesis of PGG relatively less is known about the biosynthesis of ellagitannins (Gross, 1992; Haslam and Cai, 1994; Helm et al., 1999). Many proposed biosynthetic pathways are based on the similarity among the chemical structures of ellagitannins and PGG (Haslam and Cai, 1994; Helm et al., 1999). PGG has long been considered as the precursor to ellagitannins although no experimental proof was obtained until recently when a cell-free crude enzyme extract from *Tellima grandiflora* leaves was demonstrated to catalyze the formation of tellimagrandin II (**7**) from PGG (**2**)

(Niemetz et al., 2001). Previous experiments with PGG failed to give detectable level of true ellagitannins but only ellagic acid, a hydrolysis product of ellagitannins (Gross, 1992). This experimental proof is so far the most important evidence that PGG is the precursor to ellagitannins and the oxidative HHDP coupling of PGG leads to ellagitannins. However, it only reveals the first step of ellagitannin biosynthesis, as the process leading to the formation of the more comples ellagitannins (i.e. **13**-**21**) is still unknown.

Based on structural similarity, two distinctive routes leading to monomeric ellagitannins from PGG were proposed in Figure 1.6 (Helm et al., 1999). All ellagitannins are derived from the modification of PGG, the glucose ring of which possesses two distinctive conformations. One conformation of the glucose ring is the $^4C_1$ (a chair conformation of glucose where the hydroxyl groups are in the equatorial position). The other one is the $^1C_4$ conformation (a chair conformation of glucose where hydroxyl groups are in axial positions) where galloyl groups are in the axial positions, which makes the $^1C_4$ conformation thermodynamically less stable due to the repulsive force between the galloyl groups.

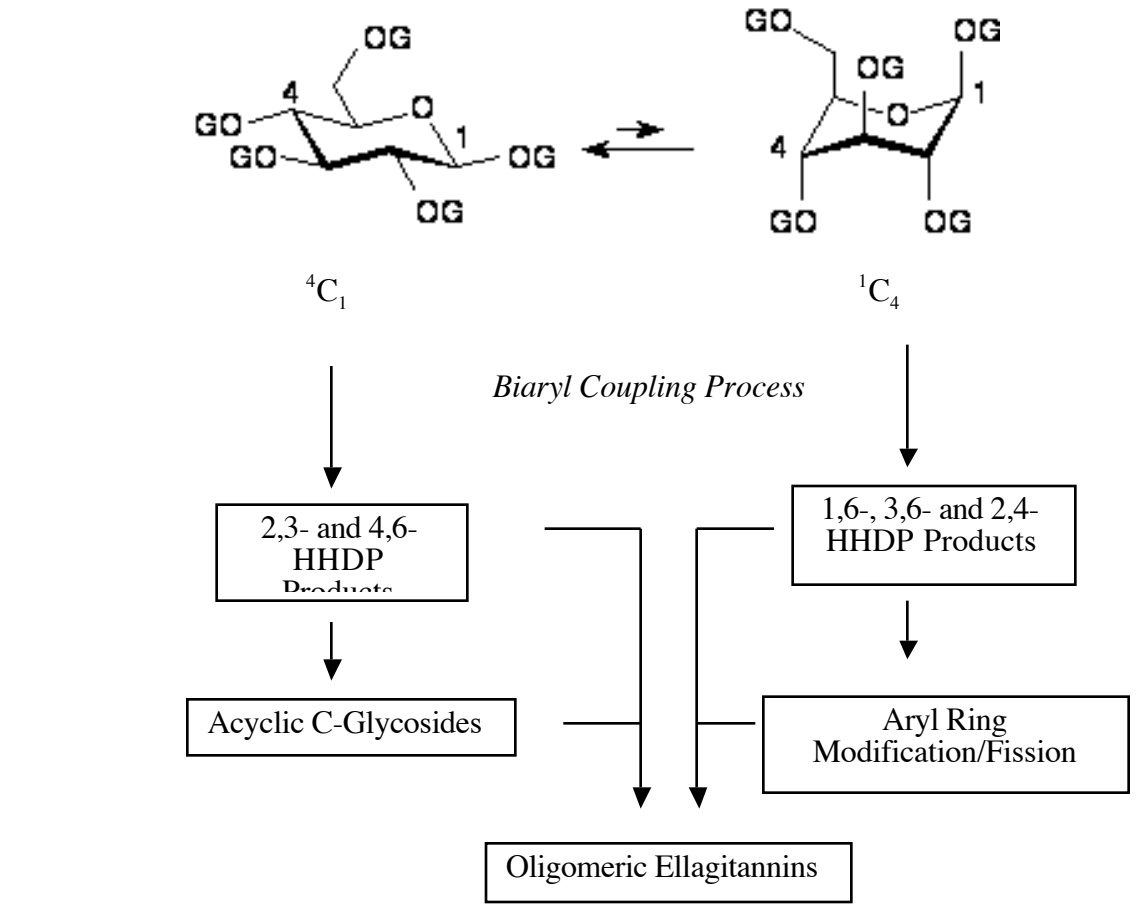


Figure 1.6. Generalized pathway of ellagitannin biosynthesis (Helm et al., 1999)

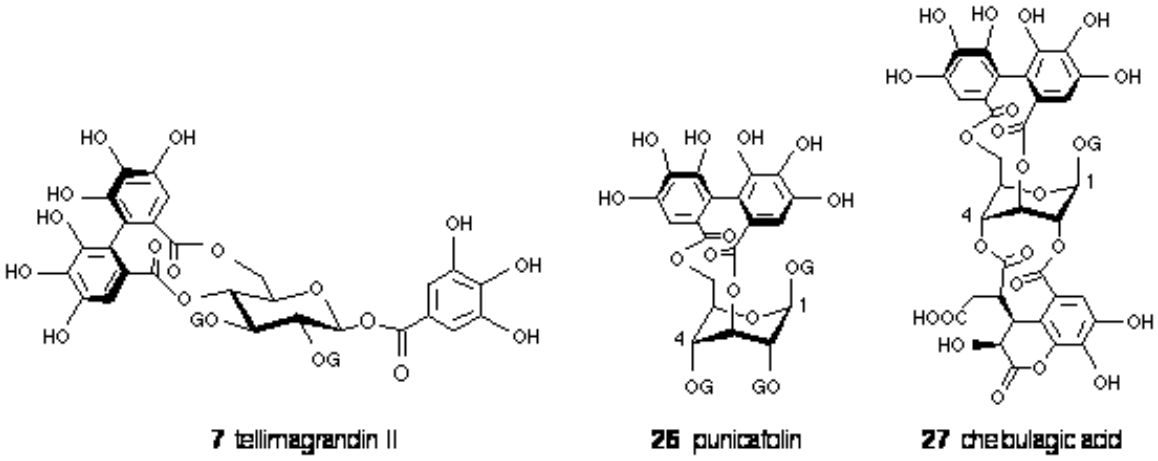


Figure 1.7. Structures of tellimagrandin II (**7**), punicafolin (**26**) and chebulagic acid (**27**)

The glucose conformations are believed to dictate the oxidative coupling patterns of galloyl groups of PGG (the Schmidt-Haslam hypothesis) (Haslam and Cai, 1994). Both 4,6- and 2,3-couplings are thought to originate from PGG possessing $^4C_1$ conformation (a chair conformation of glucose where the hydroxyl groups are in the equatorial position) while couplings between 1,6-, 1,3-, 2,4- and 3,6-galloyl groups are envisioned to derive from $^4C_1$ conformation. Couplings between 4,6- and 2,3-galloyl groups are relatively more favored than bridging between 1,6-, 1,3-, 2,4- and 3,6-galloyl groups due to the thermodynamically favorable $^4C_1$ conformation of the glucose ring. The galloyl groups of PGG are oriented in such a way so as to minimize the repulsive force between them, thus giving rise to HHDP groups of two different configurations (R and S configuration). Two key ellagitannins representing these two different configurations of HHDP units are tellimagrandin II (S-HHDP, **7**) and punicafolin (R-HHDP, **26**). Tellimagrandin II is believed to be the 4,6- HHDP coupling product of PGG possessing $^4C_1$ conformation while punicafolin is derived from the 3,6-HHDP coupling of PGG with the $^1C_4$ conformation (a chair conformation of glucose where hydroxyl groups are in axial positions). While 4,6- and 2,3- couplings generally have a (S)-configuration, 1,6-, 3,6- types of couplings are found in both configurations.

Monomeric ellagitannins derived from both $^4C_1$ and $^1C_4$ conformations can undergo post coupling modification to form more structurally different and complicated ellagitannins. One of the modifications that are associated with $^1C_4$ conformation is the loss of aromaticity in one or more gallate rings due to their fission (Helm et al., 1999). This modification of gallates typically occurs on the 2,4-HHDP units. The key compound that represents this type of ellagitannins is chebulagic acid (**27**) in which the aromaticity of the 2-gallate is destroyed. About 150 ellagitannins of this type have been identified to date and they are found only in just a few plant families (Okuda, 1993). One of the most

important post biaryl coupling modification associated with $^4C_1$ pathway is the opening of the glucose ring to form aromatic C-glycosides. These open ring compounds are the most abundant secondary metabolites found in many economically important hardwoods such as oak and chestnut (Quinn and Singleton, 1985; Tang and Hancock, 1995; Lampire et al., 1998; Viriot et al., 1994). A simplified pathway to the opening of the glucose ring is proposed in Figure 1.5 (Helm et al., 1999). Biaryl coupling between 4,6-galloyl groups is thought to first convert PGG ($^4C_1$) to tellimagrandin II (**7**) which can then be transformed into casuarictin (**13**), potentillin (**14**) and pedunculagin (**15**) through the subsequent 2,3-HHDP coupling. A series of transformation that include glucose ring opening, C-glycosidic bond formation and oxidative C-C coupling between 2,3- HHDP unit and 1-galloyl group to form flavogalloyl groups further transform these ellagitannins into acyclic derivatives such as castalagin (**17**) and vescalagin (**16**). The exact mechanism, however, is still unclear. Haslam has suggested a redox mechanism leading to castalagin (**17**) and vescalagin (**16**) directly from **15** and a stepwise pathway from **16** since compounds **13**-**15** are often found along with **16** and **17** (Haslam and Cai, 1994). More recently, Vivas and co-workers have suggested a different mechanism based on molecular modeling. They proposed that gallate is first transferred from the 1 position to the 5 position, leaving the anomeric position open. Subsequent 4,6- and 2,3- coupling, flavogalloyl synthesis and finally C-glycoside formation then lead to castalagin (**17**) and vescalagin (**16**) (Vivas et al., 1995).

Although the enzymatic conversion of PGG into tellimagrandin II (Niemetz et al., 2001) has now been demonstrated *in vitro*, virtually little is known about the subsequent enzymatic modification of tellimagrandin II (**7**) to many other ellagitannins, let alone the glucose ring opening process and oligomeric ellagitannin formation. In addition, experimental proof demonstrating the formation of punicafolin (**26**) directly from PGG

($^1C_4$ conformation) is still lacking. The slow progress in the elucidation of ellagitannin biosynthesis may be due to the high amount of tannins present in the samples which can bind and inactivate enzymes, as protocols similar to that described previously (Niemetz et al., 2001) failed to produce true ellagitannins but ellagic acid when applied to oak tissues including oak leaves (Gross, 1992).

1. 4. Heartwood Biochemistry

Ellagitannin biosynthesis in hardwoods such as oaks is closely associated with heartwood formation which is a programmed cell death process. While sapwood is a living, lighter and outer part of wood, heartwood is a non-living, darker and inner core surrounded by sapwood. In the temperate zones, heartwood formation occurs in late summer through late fall right before dormancy (Hillis, 1987).

The biological mechanism of heartwood formation is relatively unclear. The transformation of sapwood into heartwood is accompanied by a variety of metabolic changes leading to the formation of secondary metabolites (Hillis, 1987; Magel et al., 1991 and 1997). Studies with oaks revealed that these secondary metabolites associated with the formation of oak heartwood are mainly ellagitannins (Masson et al.,1994 and 1995). This implies higher enzymatic activity in the transition zone, possibly arising from the *de novo* synthesis of enzymes or the activation of enzymes already present in the transition zone. Indeed, elevated peroxidase activity was found in sapwood adjacent to heartwood. Increased enzymatic activities were also found in the transition zone of other species woods (Hauch and Magel, 1998; Hillis, 1987; Magel and Hubner, 1997). Observations of elevated activities of sucrose-metabolizing enzymes (sucrose synthase and neutral invertase) and the consumption of non-structural carbohydrates in the transition zone led to the assumption that carbohydrates are metabolized to provide carbon source for the

biosynthesis of phenolic compounds (Hauch and Magel, 1998; Magel et al., 1991 and 1997; Magel et al., 2001a). More recent studies (Magel et al., 2001b) further revealed that activities of two enzymes involved in oxidative pentose phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) were enhanced. The shift of metabolism towards oxidative pentose phosphate pathway was believed to provide precursors required for polyphenol biosynthesis.

Heartwood formation is thought to occur either abruptly (type I) or gradually (type II) (Magel et al., 2001a; Magel et al., 2001b). Since polyphenols were found in markedly high concentrations in the transition zone (Masson et al.,1994 and 1995), it is reasonable to propose that the heartwood formation in oak is type I rather than type II.

1. 5. A Brief History of Tannins

Research on plant polyphenols can be traced back to about 1770 (for review, see Haslam and Cai, 1994). Carl Wilhelm Scheele, one of the founding fathers of organic chemistry, was considered the pioneer in the chemistry of plant polyphenols. He was the first to obtain a compound related to plant polyphenols. The compound was sediment of the 'essential salt of galls or gall-nut salt' that developed when a solution of gall-nuts had been exposed to air for several weeks. This sediment was later named gallic acid by Henri Braconnot in 1831 (1781-1855), a French professor and the Director of the Botanical Garden in Nancy. The name gallic acid is derived from the French word for the source material: *galle*. Together with Michel Eugene Chevreul, Henri Braconnot first isolated ellagic acid from gall nuts, which is nowadays considered a hydrolysis product of ellagitannins and an indicator of the occurrence of ellagitannins. Knowledge about plant polyphenols was further advanced at the turn of the last century by several chemists including Nobel Laureate Emil Fischer, a German chemist who made great contributions to the chemistry of polyphenols by studying compositions of the gallotannins from

Chinese and Aleppo (Turkish) galls. His work gave the first insights into the compositions of gallotannins and ellagitannins and also stimulated a great deal of interest among organic chemists. The great complexity of plant polyphenols and the lack of isolation and purification techniques at that time, however, soon caused the initial enthusiasm to wane and thwarted progress in this area, so that progress actually came to a halt. By the 1950s, the chemistry of polyphenols had become one of the dark impenetrable areas of organic chemistry.

The renaissance came with the advent of new techniques in the 1960s when Schmidt and Mayer made great contributions to the understanding of both the chemistry and biochemistry of ellagitannins. Mayer and coworkers first described the structures of two important ellagitannins in oaks, vescalagin (**16**) and castalagin (**17**) (Mayer et al., 1969 and 1971). Their work aroused interest again among organic chemists. The interest was further heightened in 1980s when the ellagitannins were reported to have the marked inhibition of hepatitis B virus, tumor, and the HIV virus and anti-carcinogenic activity (Okuda et al., 1989), which makes these compounds potential pharmaceutical products.

One of the outstanding and contemporary investigators in plant polyphenol chemistry that deserves mention here is Dr. Edwin Haslam, the department head of the Chemistry Department at the University of Sheffield and the recipient of the 3rd Tannin Conference Award in 1999. His publications (more than 160) exclusively deal with plant polyphenol chemistry. Several research groups in Japan and France are also very active in the elucidation of the structures of new ellagitannins from a variety of different plants (Viriot et al., 1994; Nonaka et al., 1985; Tanaka et al., 1966; Tanaka et al., 1997a; Tanka et al., 1997b; Okuda et al., 1983).

More recently, plant tannins have become the subject of intense investigations aimed at the elucidation of their chemical structures, properties, chemical synthesis (Feldman and Hunter, 1998; Khanbabaee et al., 1999; Khanbabaee and Lotzerich, 1998) and distribution in the plant kingdom. Owing to the availability of modern NMR techniques and the efforts of many researchers, over 500 ellagitannins have been structurally identified at present. The results of the combined efforts have provided us with a deep insight into the occurrence and the structures of these polyphenols.

1. 6. Ellagitannins in Oak

Oaks are rich in tannins which provide the well-known durability of this genus of trees. While both condensed and hydrolyzable tannins are found in bark, the predominant tannins present in heartwood are ellagitannins. Sapwood also contains ellagitannins but their concentrations are much lower as compared with heartwood. Typically, heartwood contains 7-10% of ellagitannins while sapwood contains 1-3% of ellagitannins (dry wt. basis) (Masson et al., 1994 and 1995).

Mayer and coworkers were the first to isolate and structurally identify ellagitannins from oak heartwood. They first isolated and identified two C-glycosidic ellagitannins castalagin (**17**) and vescalagin (**16**) from oak heartwood (Mayer et al., 1969 and 1997). The structures of both vescalagin and castalagin were revised 20 years later by Nonaka et al. (1990). The revised structures were later confirmed by Tang and Hancock who reported the first full NMR assignment of complete structures of these two ellagitannins (Tang and Hancock, 1995). Nonaka and coworkers also identified a novel C-glycosidic ellagitannin called grandinin (**28**) in oak (Figure 1.8) (Nonaka et al., 1989). Grandinin is a dimer of vescalagin and lyxose, in which lyxose is linked through a carbon-carbon bond to the C-1 position of the C-glucosyl moiety of vescalagin. Herve du Penhoat and co-

workers (Herve du Penhoat et al., 1991) later isolated and identified five dimers called roburins A (**20**), B (**29**), C (**30**), D (**21**), E (**31**) from oaks. Using FAB-MS, they found that roburin E (**31**) has the same molecular weight as that of grandinin (**28**) but has a higher retention time on a reversed phase column. Nuclear magnetic resonance spectroscopy revealed that roburin E (**31**) is also a pentose-vescalagin structure, but in this case the pentose is xylose not lyxose. The other four roburins are dimers of vescalagin and/or castalagin. Roburin D (**21**) is a dimer of vescalagin (**16**) and castalagin (**17**), in which the HHDP group of castalagin is linked to C-1 of the glucosyl residue of vescalagin through C-C bond. Roburin A (**20**) is a vescalagin dimer, with the HHDP unit of one vescalagin unit being linked to C-1 of the glucosyl residue of another vescalagin unit through a C-C bond. Roburins B (**29**) and D (**21**) are also dimers of two vescalagin (**16**) subunits, with the linkages between like two vescalagin (**16**) groups being the same as that in roburin A (**20**), but also contain pentose groups. Roburin B has a xylose moiety attached to C-1 of glucose ring of a vescalagin subunit while roburin C (**30**) has a lyxose moiety.

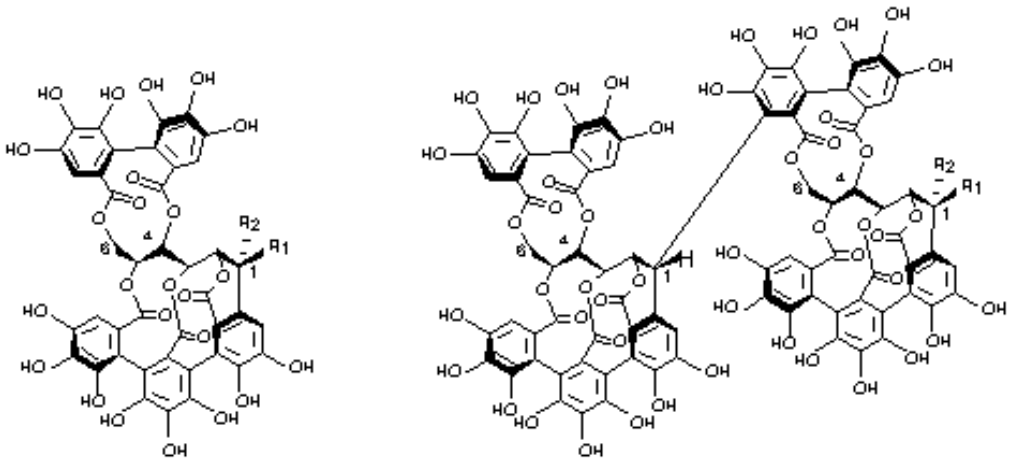


Figure 1.8. Structures of ellagitannin dimers

Although there are more dimers than monomers (6 dimers vs. 2 monomers) in oaks, the monomers represent 40-70 % of the total amount of ellagitannins present (Masson et al., 1995). Obviously monomers are in a unique and important position in that they are not only the subunits of dimers (**20**, **21**, **28**, **29**, **30**, **31**) but also account for half of the ellagitannins found in oaks.

1. 7. Specific Objectives of the Project

Due to the economic importance of oaks and the effects of ellagitannins on their utilization, it is important to understand the fundamental chemistry and biochemistry of ellagitannins. A better understanding of their biosynthesis may eventually lead to the manipulation of these secondary metabolites *in vivo*, allowing us to produce woods according to their applications. One can ensure controlling the process of heartwood formation through genetic modification, allowing for its up-regulation for more durable wood products, or down-regulation for pulp and paper applications. To achieve these long-term goals, one needs to be able to detect and quantify ellagitannins in woods or in other biomass samples so that any changes in their concentrations can be accurately and quantitatively monitored. An appropriate model system capable of producing ellagitannins is also desired in order to study the biosynthesis of these polyphenols. The specific objectives of this project are: (1) develop and validate methods for ellagitannin profiling in oak heartwood; (2) determine the stability of ellagitannins to better understand the wood discoloration problem during wood drying and processing; (3) develop model systems capable of producing ellagitannins; (4) characterize the proteins associated with heartwood formation.

Reference

Charrier, B.; Haluk, J. P. and Metche, M. (1995) Characterization of European Oak Wood Constituents Acting in the Brown Discoloration During Kiln Drying. *Holzforschung*, **49**: 168-172.

Conde, E.; Cadahia, E.; Carcia-Vallejo, M.C. and Desimon, M. B. F. (1995) Polyphenolic Composition of Wood Extracts from *Eucalyptus camaldulensis, Eucalyptus globulus* and *Eucalyptus rudis*. *Holzforschung*, **49**: 411-417.

Feldman, K. S. and Hunter, K. L. (1998) On the basis for Regioselective Oxidation within a Tetragalloylpyranose Substrate. *Tetrahedron Lett.*, **39**: 8943-8946.

Gross, G. G. (1992) Enzymatic Synthesis of Gallotannins and Related Compounds. In *Phenolic Metabolism in Plants*; Satfford, H.A.; Ibrahim, R. K. eds.; Plenum Press, New York, 297-324.

Harlow, W. M.; Harrar, E. S. and White, F. M. (1979) *Textbook of Dendrology*. Sixth Edition. McGraw-Hill Series in Forest Resource, New York, 295.

Haslam, E. and Cai, Y. (1994) Plant Polyphenols (Vegetable Tannins): Gallic Acid Metabolism. *Nat. Prod. Rep.*, **11**: 41-66.

Hauch, S. and Magel, E. (1998) Extractable Activities and Protein Content of Sucrose-Phosphate Synthase, Sucrose Synthase and Neutral Invertase in Trunk Tissues of *Robinia*

Pseudoacacia L. Are Related to Cambial Wood Production and Heartwood Formation. *Planta*, **207**: 262-274.

Helm, F. R.; Lei, Z.; Ranatunga, T.; Jervis, J. and Elder, T. (1999) Toward Understanding Monomeric Ellagitannin Biosynthesis. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology and Ecology;* Gross, G. G.; Hemingway, R. W.; Yoshida, T. eds.; Plenum Press, New York, 83-89.

Hergert, H. L. (1988) Biogenesis of Condensed Tannins - an Overview. In *Chemistry and Significance of Condensed Tannins;* Hemingway, R. W.; Karchesy, J. J. eds.; Plenum Press, New York, 71-79.

Herve du Penhoat, C. L. M.; Michon, V. M. F.; Peng, S.; Viriot, C.; Scalbert, A. and Gage, D. (1991) Structural Elucidation of New Dimeric Ellagitannins from *Quercus robur* L. Roburins A-E. *J. Chem. Soc. Perkin Trans. I*, 1653-1660.

Hillis, W. (1987) Heartwood and Tree Exudates. Berlin, New York, Springer-Verlag.

Khanbabaee, K. and Lötzerich, K. (1998) Synthesis of Enantiomerically Pure Unusual Ellagitannins 1,4,6-Tri-O-Galloyl-2,3-(R)-hexahydroxydiphenoyl-□-D-glucopyranoside and 4,6-Di-O-galloyl-2,3-(R)-hexahydroxydiphenoyl-D-glucoside. The Proposed Chemical Structures for Cercidinin A and B Must Be Revised. *J. Org. Chem.*, **63**: 8723-8728.

Khanbabaee, K.; Lötzerich, K.; Borges, M. and Groer, M. (1999) The First Total Syntheses of Enantiomerically Pure Naturally Occurring Ellagitannins Germin D and Its Regioisomer Hippomanin A. *J. Prakt. Chem.*, **341**: 159-166.

Kenzel, K. and Gross, G. G. (1991) Biosynthesis of Gallotannins. Enzymatic "Disproportionation" of 1,6-Digalloylglucose to 1,2,6-Trigalloylglucose and 6-Galloylglucose by an Acyltransferse from Leaves of *Rhus typhina* L. *Planta*, **184**: 285-289.

Lampire, O.; Mila, I.; Ramionsoa, M.; Michon, V.; Herve Du Penhoat, C.; Facuheur, N.; Laprevote, O. and Scalber, A. (1998) Polyphenols Isolated from the Bark of *Castanea sativea* Mill. Chemical Structures and Auto-Association. *Phytochemistry* , **49**: 623-631.

Lee, M.W.; Tanaka, T.; Nonaka, G. and Nishioka, I. (1992) Tannins and Related Compounds. 110. Hirsunin, An Ellagitannin with a Diarylheptanoid Moiety, from *Alnus hisurta* var. Microphylla. *Phytochemistry*, **31**: 967-970.

Lewis, N. R. and Yamamoto, E. (1988) Tannins-Their Place in Plant Metabolism. In *Chemistry and Significance of Condensed Tannins*; Hemingway, R. W.; Karchesy, J. J. eds.; Plenum Press, New York, 23-46.

Magel, E.; Abdel-Latif, A.; Hampp, R. (2001a) Non-structural Carbohydrates and Catalytic Activities of Sucrose Metabolizing Enzymes in Trunks of two *Juglans* Species and Their Role in Heartwood Formation. *Holzforschung,* **55**:135-145.

Magel, E.; Hillinger, C.; Wagner, T.; Holl, W. (2001b) Oxidative Pentose Phosphate Pathway and Pyridine Nucleotides in Relation to Heartwood Formation in *Robinina pseudoacacia* L. *Phytochemistry*, **57**: 135-145

Magel, E.; Jay-Allemand, C.; Ziegler, H. (1994) Formation of Heartwood Substances in the Stemwood of *Robinia pseudoacacia* L. II. Distribution of Nonstructural Carbohydrates and Wood Extractives across the Trunk. *Trees*, **8**: 165-171.

Marra, A. A. (1992) Technology of Wood Bonding, Principles in Practice, Van Nostrand Reinhold, New York, 150-152.

Masson, G.; Moutounet, M. and Puech, J. -L. (1995) Ellagitannin Content of Oak Wood as a Function of Species and of Sampling Position in the Tree. *Am. J. Enol. Vitic.***46**: 262-268.

Masson, G.; Puech, J. -L.and Moutounet, M. (1994) Localization of the Ellagitannins in the Tissues of *Quercus robur* and *Quercus petraea* Woods. *Phytochemistry*, **37**: 1245-1249.

Mayer, W.; Seitz, H. and Jochims, J. -C. (1969) Die Struktur des Castalagin. *Liebigs Ann. Chem.*, **721**: 186-193.

Mayer, W.; Seitz, H.; Jochims, J. -C.; Schauerte, K. and Schilling, G. (1971) Die Struktur des Vescalagins. *Liebgis Ann. Chem.*, **751**: 60-68.

Mila, I.; Scalbert, A. and Expert, D. (1996) Iron Withholding by Plant Polyphenols and Resistance to Pathogens and Rots. *Phytochemistry*, **42**: 1551-1555.

Mosedale, J. R.; Puech J. -L. and Feuillat, F. (1999) The Influence on Wine Flavor of the Oak Species and Natural Variation of Heartwood Components. *Am. J. Enol. Vitic.***50**: 503-512.

Niemetz, R.; Niehaus, J. U. and Gross, G. G. (1999) Biosynthesis and Biodegradation of Complex Gallotannins. In *Plant polyphenols 2: Chemistry, Biology, Pharmacology and Ecology;* Gross, G. G.; Hemingway, R. W.; Yoshida, T. eds.; Plenum Press, New York, 63-82.

Niemetz, R.; Schilling, G. and Gross, G. G. (2001) Ellagitannin Biosynthesis: Oxidation of Pentagalloylglucose to Tellimagrandin II by an Enzyme from *Tellima grandiflora* Leaves. *Chem. Commun.*, 35-36.

Nishizawa, M.; Yamagishi, T.; Nonaka, G. and Nishioka, I. (1983) Tannins and Related Compounds Part 9. Characterization of Polygalloylglucoses from Turkish Galls. *J. Chem. Soc. Perkin Trans. I:*, 961-965.

Nishizawa, M.; Yamagishi, T.; Nonaka, G. and Nishioka, I. (1982) Tannins and Related Compounds Part 5. Isolation and Characterization of Polygalloylglucoses from Chinese Gallotannin. *J. Chem. Soc. Perkin Trans. I*, 2963-2968.

Nonaka, G.; Ishimaru, K.; Azuma, R.; Ishimatsu, M. and Nishioka, I. (1989) Tannins and Related-Compounds. 85. Structures of Novel C-Glycosidic Ellagitannins, Grandinin and Pterocarinin-A and Pterocarinin-B. *Chem. Pharm. Bull.*, **37**: 2071-2077.

Nonaka, G.; Nishimura, H. and Nishioka, I. (1985) Tannins and Related Compounds. Part 26. Isolation and Structures of Senolphyllanins A, B and C, Novel Tannins from *Quercus stenophylla*. *J. Chem. Soc. Perkin Trans. I*, 163-172.

Nonaka, G.; Sakai, T.; Tanaka, T.; Mihashi, K. and Nishioka, I. (1990) Tannins and related Compounds. XCVII[1)]. Structure Revision of C-Glycosidic Ellagitannins, Castalagin, Vescalagin, Casuarinin and Stachyurin, and Related Hydrolyzable Tannins. *Chem. Pharm. Bull.*, **38**: 2151-2156.

Okuda, T. (1999) Novel Aspects of Tannins – Renewed Concepts and Structure-Activity Relationships. *Curr. Org. Chem.*, **3**: 609-622.

Okuda, T.; Yoshida, T. and Hatano, T. (1993) Classification of Oligomeric Hydrolyzable Tannins and Specificity of Their Occurrence in Plants. *Phytochemistry*, **32**: 507.

Okuda, T.; Yoshida, T.; Ashida, M. and Yazaki, K. (1983) Tannins of *Casuarina* and *Stachyurus* Species. Part 1. Structures of Pendunculagin, Casuarictin, Strictinin, Casuarinin, Casuariin, and Stachyurin. *J. Chem. Soc. Perkin Trans. I*, 1765-1772.

Okuda, T.; Yoshida, T. and Hatano, T. (1989) Ellagitannins as Active Constituents of Medicinal Plants. *Planta Medica* , **55**: 117-121.

Quideau, S. and Feldman, K. S. (1996) Ellagitannin Chemistry. *Chem. Rev.*, **96**: 475-503.

Quinn, M. K. and Singleton, V. L. (1985) Isolation and Identification of Ellagitannins from White Oak Wood and an Estimation of Their Roles in Wine. *Am. J. Enol. Vitiv.***36**: 148-155.

Rowe, J. W. and Conner, A. H. (1979) Extractives in Eastern Hardwoods – A Review. In *FPL General Technical Report 18.* USDA Forest Products Laboratory, Madison, WI, 16.

Scalbert, A. (1992) Tannins in Woods and Their Contribution to Microbial Decay Prevention. In *Plant Polyphenols*: *Synthesis, Properties, Significance;* Hemingway, R.W.; Laks, P. E. eds.; Plenum Press, New York, 935-951.

Scalbert, A. (1991) Antimicrobial Properties of Tannins. *Phytochemistry*, **30**: 3875-3883.

Spencer, R. W. and Choong, E. T. (1997) Isolation and Characterization of Several Extractives in the Bark and Wood of Sweetgum. *Holzforschung*, **31**: 25-31.

Tanaka, T.; Jiang, Z. H. and Kouno, I. (1997) Structures and Biogenesis of Rhoipteleanins, Ellagitannins Formed by Stereospecific Intermolecular C-C Oxidative Coupling, Isolated from *Rhoiptelea chiliantha*. *Chem. Pharm. Bull.*, **45**: 1915-1921.

Tanaka, T.; Ueda, N.; Shinohare, H.; Nonaka, G.; Fujioka, T.; Mihashi, K. and Kouno, I. (1996) C-Glycosidic Ellagitannin Metabolites in the Heartwood of Japanese Chestnut Tree (*Castanea crenata* SIEB. *et* Zucc.). *Chem. Pharm. Bull.*, **44**: 2236-2242.

Tanaka, T.; Ueda, N.; Shinhara, H.; Nonaka, G. and Kouno, I. (1997) Four New C-Glycosidic Ellagitannins, Castacrenins D-G, from Japanese Chestnut Wood (*Castanea crenata* SIEB. *et* ZUCC.) *Chem. Pharm. Bull.*, **45**: 1751-1755.

Tang, H. R. and Hancock, R. A. (1995) Studies of Vegetable Tannins. Complete Structural Determination of Two Chestnut Tannins-Vescalagin and Castalagin Using Nuclear Magnetic Resonance Spectroscopic Methods. *J. Soc. Leather Technol. Chem.***79**: 181-187.

Viriot, C.; Scabert, A.; Herve Du Penhoat, C. L. M. and Moutounet, M. (1994) Ellagitannins in Woods of Sessile Oak and Sweet Chestnut Dimerization and Hydrolysis During Wood Aging. *Phytochemistry*, **36**: 1253-1260.

Vivas, N. and Glories, Y. (1996) Role of Oak Wood Ellagitannins in the Oxidation Process of Red Wines During Aging. *Am. J. Enol. Vitic.***47**: 103-107.

Vivas, N.; Laguerre, M.; Glories, Y.; Bourgeois, G. and Vitry, C. (1995) Structure Simulation of Two Ellagitannins from *Quercus robur* L. *Phytochemistry*, **39**: 1915-1199.

Werner, I.; Bacher, A. and Eisenreich, W. (1997) Retrobiosynthetic NMR Studies with ^{13}C-Labeled Glucose: Formation of Gallic Acid in Plants and Fungi. *J. Biol. Chem.*, **272**: 25474-25482.

Chapter Two

Improved Methods for Extracting Soluble Ellagitannins and Quantifying Insoluble Ellagitannins in Biomass Samples and its Application in the Quantification of Ellagitannins in Wood and Food Samples

Abstract: Efforts were made to fully evaluate the performances of the two commonly used solvents, aqueous acetone (acetone:water=7:3) and aqueous methanol (MeOH:water=7:3), on extracting vescalagin and castalagin from freshly harvested white oak (*Quercus alba*). The results show that aqueous acetone is superior to aqueous MeOH in obtaining higher vescalagin and castalagin yields with lower total "insoluble ellagitannins" remaining in the samples. The data indicate that an acetone: water mixture should be used in the quantitative analysis of ellagitannins. In working with insoluble ellagitannins, anhydrous methanolic HCl was found to be an excellent reagent for releasing ellagic acid and gallic acid (as methyl gallate) from biomass substrates. Optimization of both the reaction conditions and the gradient HPLC analysis has led to the development of robust and reliable protocol. The method provides ellagic acid yields significantly higher than those obtained previously, indicating total ellagic acid contents of substrates have previously been underestimated.

2. 1. Introduction

Key to any study on ellagitannins is the availability of quantitative protocols for ascertaining the concentration of ellagitannins in the individual samples. A robust and accurate protocol is necessary for monitoring the levels and changes of ellagitannin contents arising from external and internal stimuli. Currently there are a number of assays to quantify tannins (Hagerman and Bulter, 1980; Inour and Hagerman, 1988; Kilkowski and Gross, 1999; Mueller-Harvey, 2001; Scalbert, 1992; Scalbert et al., 1993; Schultz et al., 1981; Willis and Allen, 1998). These assays can be classified into three types based on the techniques employed: (1). Protein precipitation; (2). Colorimetric quantification after chemical reaction with phenols; and (3). Chromatographic separation and quantification.

Assays of the first type are based on the interaction of tannins with proteins, which is a unique property of tannins. Several variants of this type of method have been developed over the past 30 years (Scalbert, 1992; Scalbert et al., 1993), and they differ in the choice of proteins such as bovine serum albumin (BSA) (Scalbert, 1992) and hemoglobin (Schultz et al., 1981), as well as other parameters (temperature, pH, duration of precipitation and solvents). Proteins in the precipitate are determined after the precipitate is redissolved in hot aqueous NaOH solutions (Hagerman and Bulter, 1980) and the results of tannin contents are usually expressed as the amount of protein precipitated. Although relatively simple and popular among ecologists, this method is not considered accurate or robust. First, the results are dependent on the nature of tannins and proteins. The same amount of two different tannins may give remarkably different results due to their different affinity with proteins. Secondly, It can not be used to quantify any specific individual tannin and not even a specific type of tannin because other tannins, either ellagitannins or gallotannins or other condensed tannins, will undoubtedly interfere with the precipitation.

Thirdly, this method actually measures how efficiently the tannin samples interact with proteins, not the amount of tannins.

The second method, which is based on the reactivity of phenolic rings in tannin molecules, is a spectrophotometric method that is more sophisticated than the first method. By using different reagents such as KIO_3 (Willis and Allen, 1998), rhodanine (Inour and Hagerman, 1988) and $NaNO_2$ (Wilson and Hagerman, 1990), one can at least differentiate hydrolyzable tannins from condensed tannins. Rhodanine was found to react specifically with gallic acid, and it is inactive toward galloyl esters, ellagic acid, ellagitannins and other phenolics (Inour and Hagerman, 1988). Thus it can be used to measure gallotannins by quantifying gallic acid before and after hydrolysis of tannins. However, it can not provide an absolute amount of gallotannins as the number of galloyl groups differs from one gallotannin to another. The assay is further complicated by the interference of ellagitannins which may also have galloyl groups. Hydrolysis of these ellagitannins will also produce gallic acid, leading to the assumption that the moiety was derived from a gallotannin. In addition, the assay needs to be performed in the absence of oxygen, making it difficult to use. Ellagitannins can be specifically assayed using a different reagent. Wilson and Hagerman (Wilson and Hagerman, 1990) reported a modified $NaNO_2$ assay where $NaNO_2$ reacts selectively with ellagic acid. It was found not to react with gallic acid, ellagitannins, gallotannins, condensed tannins or other phenolics. By quantifying ellagic acid contents before and after hydrolysis, one thus can estimate the ellagitannin contents. The assay suffers the same problem as the rhodanine assay, in that the method can not provide the absolute amount of ellagitannins and needs to be carried out in the absence of oxygen. Moreover, brand new test tubes are strongly recommended by the authors because residue from glass washing in the test tubes will inhibit the reaction, which makes

the assay inconvenient. Obviously, this method needs further improvements in order to be robust.

Unlike the first and the second methods, the third method uses neither chemicals nor proteins to interact with tannins. It employs chromatographic techniques to separate and quantify individual tannins. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) are the two most common techniques in the separation of tannin mixtures. Although CE (capillary electrophoresis) has been reported effective in the separation of plant phenolics (Andrade et al., 1997; Ferreres et al., 1994), application of CE in the separation of ellagitannins has not been successful [Lei and Helm, unpublished data]. The lower resolution and relatively poor reproducibility of CE also make it less favorable. Although CE may offer some advantages such as shorter running time, HPLC is still considered the superior method of analysis for plant polyphenols (Tomas-Barberan and Garcia-Viguera, 1997). Although cheap and fast, TLC is generally not considered as a quantitative method and is now only used for screening plant extracts for the occurrence of different types of tannins. Compared with TLC, HPLC is a powerful tool and has been successfully used in separating and quantifying ellagitannins (Okuda et al., 1986; Daniel et al., 1989; Scalbert et al., 1990; Charrier et al., 1992; Bianco et al., 1998; Häkkinen et al., 1998; Peng et al., 1991).

Accurate quantitative analysis of individual ellagitannin contents with HPLC, however, hinges on the solubilization or extraction of ellagitannins from biomass samples. Although there are quite a few reports addressing ellagitannin quantification with HPLC, they generally focus on optimization of separation conditions such as the choice of mobile phases and gradient conditions (Bianco et al., 1998; Charrier et al., 1992; Daniel et al., 1989; Häkkinen et al., 1998; Okuda et al., 1986; Peng et al., 1991; Scalbert et al., 1990).

Few of them have directly addressed the extraction protocol and how it modifies ellagitannin yields (Peng et al., 1991; Scalbert et al., 1993). In our work with ellagitannins, the two most commonly employed solvents for wood extraction (acetone: water and MeOH:water) were compared for the release of vescalagin and castalagin as well as the insoluble ellagitannins remaining in the wood meal.

Not all ellagitannins can be removed from wood by solvent extraction (Peng et al., 1991). While soluble ellagitannins are free and can be extracted with solvents, insoluble ellagitannins are considered bound to cell walls (Helm et al., 1997) and therefore can not be solvent extracted. Experiments with synthetic PGG and lignin monomer model molecules (Helm et al., 1997) revealed that dehydrogenative co-polymerization occurs between PGG and coniferyl and sinapyl alcohol, suggesting that the interaction between ellagitannins and lignin is the primary cause for insoluble ellagitannins. The dehydrogenative co-polymerization is thought to occur through radical reactions (Helm et al., 1997). Insoluble ellagitannins are an important issue considering that a large portion of the total ellagitannins in wood can become irreversibly bound to the woody cell wall (Klumpers et al., 1994; Peng et al., 1991; Viriot et al., 1994). Studies concerned with heartwood formation in trees have determined that the insoluble ellagitannin concentration increases with age. In the case of sweet chestnut (*Castanea sativa*), about 50% of the ellagitannins present near the tree center (oldest wood) were insoluble by standard solvent extraction protocols (Peng et al., 1991; Viriot et al., 1994). These insoluble ellagitannins can potentially have a significant impact on the end use of the wood (i.e. barrel manufacture) (Mosedale et al., 1999; Quinn and Singleton, 1985; Vivas and Glories, 1996).

In this work, we evaluated the performance of two most commonly used solvents, aqueous acetone (acetone:water=7:3) and aqueous methanol (MeOH: water =7:3), on extracting vescalagin and castalagin from freshly harvested white oak (*Quercus alba*). Method used to assay insoluble ellagitannins was also optimized.

2. 2. Materials and Methods

All solvents were HPLC grade. Evaporations were performed under reduced pressure at temperatures not exceeding 40 °C. Acetyl chloride (98%) was used as received; ellagic acid was crystallized from pyridine before use. Extract-free heartwood and callus samples for the optimization of insoluble ellagitannin determination protocol were prepared by extracting heartwood and callus powder as described below. Heartwood and callus powder were extracted with Several oak wood samples were kindly provided by Cal Craik, Okanagan Barrel Works (Oliver BC, Canada). Blackberry fruits and stems were wild-type cultivars collected from the Washington and Jefferson National Forests (Montgomery County, Virginia). The whiskey analyzed was from a major commercial manufacturer and purchased locally.

2. 2. 1. Isolation and Purification of Vescalagin and Castalagin.

Ellagitannin standards are needed in order to use HPLC to quantify each individual ellagitannin. Since they are not available commercially, they first need to be isolated and purified from oak heartwood extracts. 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. Callus powder was obtained by grinding freeze dried callus in a mortar with a pestle. The powders were

then extracted with acetone:water (7:3) for 24 hours in dark with magnetic stirring. Extracts were then filtered through a 0.2 μm membrane filter under vacuum and collected. Extraction was repeated twice and the extracts were combined and freeze dried. The extracted heartwood and callus powders were air dried and used as extract-free samples in the optimization of insoluble ellagitannin determination. The purification of vescalagin and castalagin was conducted on a low-pressure reverse-phase C-18 column (15 mm x 335 mm). Successive elution with water, 5% MeOH, and MeOH 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 sample was further purified on a Toyopearl HW40-F column (I. D. 15 mm x 335 mm) with the elution of MeOH: 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 possible degradation of vescalagin in MeOH. Fractions were combined according to TLC results, evaporated and freeze-dried. The structures of both vescalagin and castalagin were confirmed by the ^{1}H NMR spectra obtained from Bruker AM 360 located in the Chemistry Department and compared with the published ^{1}H NMR spectra (Tang and Hancock, 1995).

2. 2. 2. Solvent Extraction of Ellagitannins

Sample powder was extracted in the dark and under nitrogen with either acetone: water (7:3) or MeOH: water (7:3) at 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 acetone or MeOH) at temperature less than 40 $^{\circ}$C and then freeze dried. All yields are reported on a dry weight basis. Total soluble polyphenols were determined with Folin-Ciocalteu spectrophotometric method (Scalbert, 1992). The Folin Ciocalteu reagent was obtained commercially and used as

received. Determination of vescalagin and castalagin was performed on a Gilson HPLC apparatus operating in the gradient mode using a Merck Lichrospher RP-18 (endcapped 5 μm) column (250 mm X 4 mm I. D.). The solvents were MeOH and aqueous 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 with aqueous 0.2% TFA. Gallic acid was used as the standard and the total phenols in the samples were expressed as gallic acid equivalents (GAE). 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 aqueous HCl (6M, 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. The results were expressed as castalagin equivalents (CE) provided that 1 mol of castalagin produced 1 mol of ellagic acid.

2. 2. 3. Methanolysis of Biomass Samples

Anhydrous methanolic HCl was prepared by the slow addition of acetyl chloride to a well-stirred cold (ca -20 °C) solution of anhydrous MeOH (final component ratio: 190 μL acetyl chloride per ml MeOH). Dry, extractive-free wood meal (100 mg) or non-extracted samples (ca 20 mg) were placed in teflon-lined screw cap tubes (15 ml) containing a small magnetic stir bar. After anhydrous methanolic HCl (5 ml) was added, the tubes were sealed tightly and placed in a Reacti-Therm system (Pierce Chemical Co.) with stirring at 100 °C for 60 min. After completion, the solutions were cooled to room temperature and filtered (0.2 μm). The filtrates were evaporated to dryness and subsequently re-dissolved in MeOH before being analyzed by HPLC. In the case of an aqueous substrate (*i.e.*, whiskey), the sample (20 ml) was evaporated to remove any volatile components, and then

freeze-dried. HPLC was performed on the Gilson HPLC apparatus with a Merck Lichrospher RP-18 (end-capped 5 μm) column (250 mm x 4 mm I.D.) using an external standard analysis. The solvents were MeOH (solvent A) and 0.2% trifluoroacetic acid (solvent B). The flow rate was set at 0.75 ml/min. Gradient conditions: linear gradient from 0 to 100% of solvent A over a 40 min period. All samples were filtered with 0.2 μm syringe filter before injection. Dual wavelengths (252 nm and 280 nm) were used to detect ellagic acid and methyl gallate, with the 252 nm wavelength used for external standard analysis (Charrier et al., 1992; Bianco et al., 1998). Linear ellagic acid and methyl gallate calibration curves were generated using ellagic acid and methyl gallate in the concentration range of 5 –50 μg/ ml (R^2 = 0.995).

2. 3 Results and Discussion

Aqueous MeOH (MeOH:water =7:3) and aqueous acetone (acetone:water = 7:3) were used to extract heartwood powder containing an internal standard, 4-hydroxybenzoic acid. The amount of vescalagin and castalagin solubilized relative to the internal standard over a 3 day time period was determined, and the data for castalagin are shown in Figure 2.1. It is apparent from the Figure 2.1 and Table 2.1 that aqueous acetone is better at extracting castalagin and vescalagin from the wood meal, strongly suggesting that this solvent is a better choice for the extraction and quantification of ellagitannins in woods. Very similar results have been reported by Scalbert's group (Peng et al., 1991) for 16 hr extractions. They have also reported that hot aqueous acetone and cold aqueous MeOH performed equally well in extracting ellagitannins (Scalbert et al., 1993). One of the possible reasons that hot aqueous acetone and cold aqueous MeOH produced similar yields is that the higher temperature of the hot aqueous acetone may have degraded some ellagitannins during the extraction process, decreasing the overall recovery. Indeed, higher temperature has been found to accelerate the degradation of ellagitannins (Charrier et al., 1995).

Alcoholic solvents have been proven to be detrimental to ellagitannins (Viriot et al., 1993; Puech, et al., 1999). Ellagitannins were found to undergo hydrolysis to give ellagic acid in aqueous ethanol solution (Viriot et al., 1993), while Puech and coworkers demonstrated that the hydrolysis of ellagitannins increases with the ethanol concentration (Puech et al., 1999). They also found that hydrolysis is not the only degradation process associated with ellagitannins in aqueous ethanol solutions. Oxidation also occurs, giving ellagitannin derivatives containing ethoxy groups (Puech et al., 1999). Methanol was also found to cleave depside bonds (ester bonds) in gallotannins at room temperature through methanolysis (Mueller-Harvey, 2001), suggesting that MeOH is not a suitable solvent for extracting ellagitannins. The figures also address the concern that aqueous MeOH might give the same results if the extraction time is longer. Clearly, prolonged extraction time does not increase the yields.

In general, ellagitannins are labile both in solution and in the field, undergoing hydrolysis and polymerization reactions (Viriot et al., 1994; Klumpers et al., 1994). Hydrolysis (or methanolysis) of ellagitannins containing the HHDP groups produces ellagic acid via spontaneous lactonization. In the case of tellimagrandin II, complete hydrolysis would yield one mole of ellagic acid and 3 moles of gallic acid per mole of tellimagrandin II. Castalagin, on the other hand, would release one mole of ellagic acid per mole of castalagin. The polymerization process would lead to insolubilization of ellagitannins and/or covalent attachment to the cell wall components (Helm et al., 1997; Klumpers et al., 1994). To further investigate the effects of these two solvents on the insoluble ellagitannin contents, heartwood powders were extracted with aqueous acetone and aqueous MeOH for 48 hours. The extracted vescalagin and castalagin contents were determined by HPLC and the dried extracted wood meals were then subjected to an insoluble ellagitannin determination according to the method reported by Scalbert's group

(Peng et al., 1991). The results are shown in Table 2.1. Again the data confirmed that aqueous acetone gives higher yields of both vescalagin and castalagin than aqueous methanol.

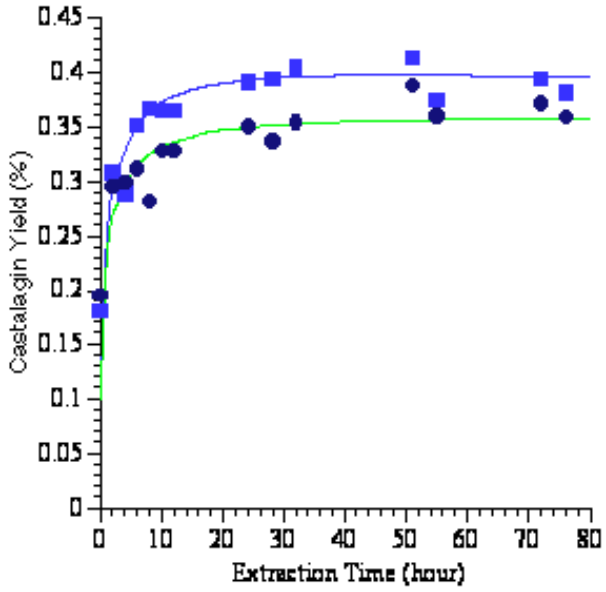


Figure 2.1. Effect of acetone:water (7:3; closed squares) and MeOH:water (7:3; closed circles) on the yields of castalagin (expressed as percentage relative to the dry wt of samples)

Table 2.1 Effect of solvents 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	0.3	0.4	1.08	3.12
Increase by	27%	50%	38%	51% less	17%

[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.

Total yield from heartwood increased by 27%, while the two major ellagitannins, vescalagin and castalagin, increased by at least one third. Total phenolics also increased significantly. The better extracting ability of aqueous acetone can also be exemplified by the relatively lower amount of insoluble ellagitannins present in the extracted wood meal. Higher insoluble ellagitannin content present in aqueous MeOH extracted wood meal indicate that aqueous MeOH can not remove as much ellagitannins as aqueous acetone. Clearly, if one wishes to perform quantitative analyses for wood ellagitannin contents, the use of aqueous acetone is warranted and insoluble ellagitannin determinations should be performed to determine the amount of insoluble ellagitannins.

Because the quantification of insoluble ellagitannins is based on ellagic acid released during acidic hydrolysis, a method that can efficiently cleave all hexahydroxydiphenoyl groups is obviously important. The previously reported method (Peng et al., 1991) involves the use of a mixture of MeOH and 6M HCl (MeOH: 6M HCl; 9: 1). The optimal conditions were found to be 120 ^{0}C and 160 min for the release of ellagic acid. In our hands, this method was found to be quite suitable and very reproducible, although a relatively high percentage of reactions failed due to the loss of the hydrolysis reagent due to the vial leakage brought about by the high pressure generated by the high temperature employed (160 °C). In a preliminary search for an alternative lower temperature reaction, we found that anhydrous methanolic HCl was much more effective in releasing ellagic acid from insoluble ellagitannins, at both lower temperatures and shorter reaction times. The reaction (reaction time and temperature) was then optimized so that the maximum ellagic acid was obtained.

Reaction optimization. In order to optimize the method, extractive-free wood meals (Lei et al., 1999) were subjected to methanolysis in anhydrous methanolic HCl solution in

the range of 80-120°C and the resulting ellagic acid yields were determined as shown in Figure 2.2. Maximum ellagic acid recovery was dependent on the reaction time and temperature. At 80 °C, the maximum recovery was reached in 150 minutes, and in 30 minutes for reactions performed at 120 °C. For practical purposes, we chose 100 ^{0}C/60 min. At higher temperatures, loss of reagents due to leakage out of the vials was more prevalent. Furthermore, prolonged reactions led to a decrease in ellagic acid yields. The lower temperatures were not chosen due to the longer reaction times required. The observation that ellagic acid yields decreased over time was noted previously (Peng et al., 1991). The reason for the decreasing yields under acidic conditions, however, has not been suggested, although ellagic acid has long been known to undergo extensive decarboxylation under alkaline conditions (Hemingway and Hillis, 1971). In order to provide some insight into the ellagic acid degradation pathway, preliminary work was done by subjecting pure ellagic acid to methanolysis at 100 ^{0}C. Surprisingly, for up to 150 minutes, no decrease in ellagic acid content was observed. This indicates that ellagic acid itself is quite stable in methanolic HCl, and the observed decrease with biomass substrates is probably due to condensation reactions with other degradation products present in the hydrolyzate.

Both the optimized methanolysis protocol and the previously reported method (Peng et al., 1991) were applied to extractive-free wood samples for comparative purposes, and the data are shown in Table 2.2. For both wood samples, yields obtained from anhydrous methanolic HCl were twice that from the MeOH: 6M HCl method. A significantly higher amount of ellagic acid was also recovered from the callus tissues. Interestingly, the milled wood lignin (MWL) sample gave identical results. These results clearly indicate that methanolysis releases more ellagic acid than acidic hydrolysis (MeOH:6M HCl).

Table 2.2 Comparison of the original and modified methods for recovery of ellagic acid (%, dry weight basis).		
Sample	Methanolic HCl[1]	MeOH:6M HCl[2]
Quercus alba heartwood	0.23	0.12
Quercus prinus heartwood	0.12	0.06
Quercus alba callus	0.37	0.28
Castanea dentata MWL	1.04	1.04
[1]This work. [2]Peng and Scalbert (1991).		

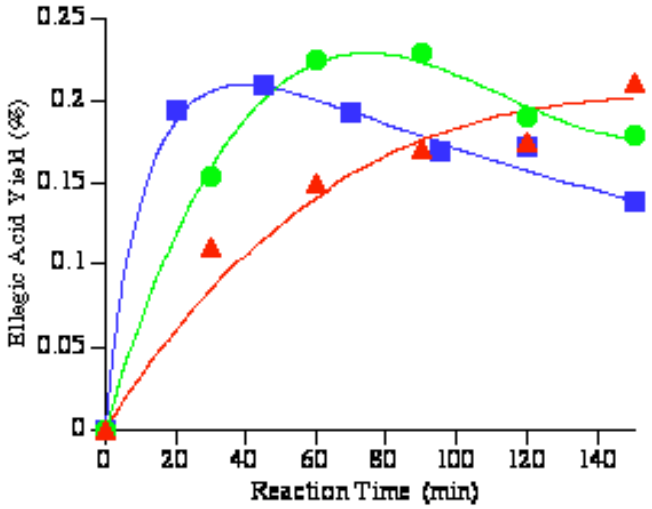


Figure 2.2. Effect of temperature on the ellagic acid yield during methanolysis (80 ºC, closed triangle; 100 ºC, closed circle; 120 ºC, closed square)

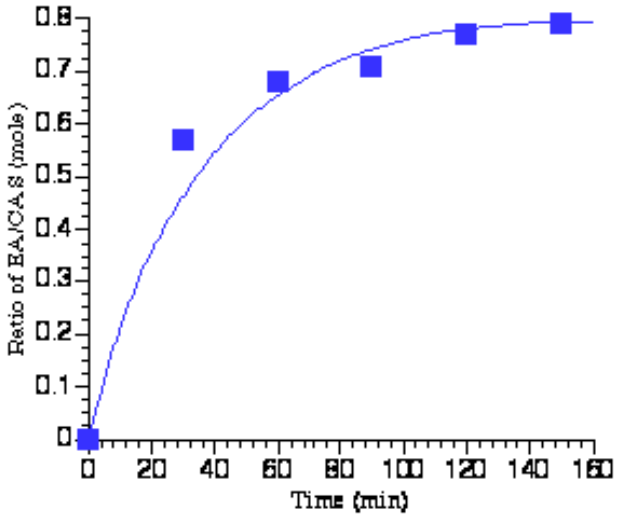


Figure 2.3. Time course production of ellagic acid from the methanolysis of castalagin

In a brief study of molar response factors, castalagin (purity *ca*. 92% according to NMR) was submitted to methanolysis at 100 °C and the molar amount of ellagic acid released was determined. Ellagic acid released from castalagin during methanolysis as shown in Figure 2.3. That the molar ratio of ellagic acid to castalagin did not exceed 1 indicates that the ellagic acid yield is not overestimated by breakdown of the flavogallonyl group. Indeed, the flavogallonyl group has been reported to form flavogallonic acid and not ellagic acid during acid hydrolysis (Mayer et al., 1971).

The modified protocol was applied to several ellagitannin-containing substrates including oak wood (unextracted), whiskey, and blackberry fruits and stems. With respect to ellagitannin-containing food products, free and bound ellagic acid contents of various fruits and nuts will undoubtedly differ with respect to maturity, cultivar, and growing conditions (Daniel et al., 1989; Häkkinen et al., 1998; Maas et al., 1991). In order to accurately monitor ellagitannin levels without resorting to isolation and quantification of each individual component, one can determine the total ellagic acid content of a sample by hydrolyzing all bound HHDP units. We applied the method to several samples and the results are shown in Table 2.3. Both extractable and insoluble ellagitannins in the samples contributed to ellagic acid yields since the samples had not been extracted previously. Thus the results represent the total ellagic acid contents of these substrates. As would be expected, oak barrel-aged whiskey was found to contain ellagic acid (Clifford and Scalbert, 2000).

The yield of methyl gallate, the methanolysis product of uncondensed galloyl groups is also reported in Table 2.3. Having both the ellagic acid and methyl gallate concentrations allows for the determination of a "degree of biaryl coupling" for any substrate; *i.e*., the ratio of free plus esterified gallic acid to free ellagic acid plus HHDP-

linked moieties. In comparing the different white oak samples, one can see a fairly tight range of values. The concentrations of ellagic acid and methyl gallate in the Oregon White Oak suggest that it

Table 2.3 Ellagic and gallic acid contents (%, dry wt. basis) of several biomass samples.

Sample	Ellagic acid	Methyl gallate	Ratio[1]
White Oak (*Quercus alba*, Missouri)	0.79	0.15	6.42
White Oak (*Quercus alba*, Virginia)	1.08	0.31	4.25
Oregon White Oak (*Quercus garryana*)	1.54	trace	---
Sessile Oak (*Quercus petraea*, France)	1.18	0.25	5.75
Sessile Oak (*Quercus petraea*, Czech Republic)	2.55	trace	---
Chestnut Oak (*Quercus prinus*, Virginia)	0.63	0.17	4.52
American Chestnut MWL (*Castanea dentata*)	1.04	0.35	3.62
White Oak Callus	1.15	1.98	0.71
Blackberry (*Rubus fructicosa*) Fruits	1.09	trace	---
Blackberry Stems	n.d.	n.d.	---
Whiskey (Sour Mash) [2]	23.852	13.042	2.23

[1]Molar ratio of ellagic acid to methyl gallate. [2]Reported in mg/ml

may be a useful wood for aging spirits/wine. We interpret the white oak callus tissue data (*i.e.*, a low ellagic acid to methyl gallate ratio) to indicate that while the precursors to ellagitannins are present, complete conversion to the final metabolites is not occurring as efficiently as it does in native wood. The data we have obtained for blackberries is much higher than that reported previously (Maas et al., 1991). This could be due to the cultivar

chosen (wild type in our case), or the fact that previous methods have not released all ellagic acid present.

Use of Dual Wavelength Detection. The dual wavelengths chosen (252 and 280 nm) are very useful in confirming whether the peak is ellagic acid or not. This is of importance as during evaluation of model compounds and gradient conditions, it was determined that ellagic acid can potentially co-elute with the methanolysis product of catechin. However, the ratio of 252 nm/280 nm for ellagic acid is 3.9, whereas it is 2.1 for the methanolysis product of catechin. This difference allows one to distinguish ellagic acid from other potential co-eluting methanolysis products (as would a photodiode array scan of the peak itself). If one suspects co-elution, it may be possible to modify the gradient. It is also worth mentioning that phenyl-modified silica columns for phenolic analyses were found to be excellent replacements for the C18 HPLC media (Lei and Helm, unpublished results).

2. 4. Conclusion

Aqueous acetone is found to be a better solvent than aqueous methanol at extracting ellagitannins from wood samples. It provides higher yields than aqueous methanol which has been found to degrade ellagitannins (See Chapter 3). The better extracting ability of aqueous acetone is also exemplified by the reduced insoluble ellagitannin level for wood samples extracted with aqueous acetone. This finding is of significance considering that aqueous methanol has been used frequently to extract ellagitannins. For quantitative analyses, aqueous acetone, not aqueous methanol, should be used.

Anhydrous methanolic HCl is much more effective in releasing ellagic acid from solid biomass samples than methanol: 6M HCl. The new protocol provides a reliable quantification of insoluble ellagitannins from woods as well as fruits and aged spirits. It has also been demonstrated that ellagic acid is stable in the anhydrous methanolic HCl

under the reaction conditions employed, suggesting that the observed decrease of ellagic acid during the acidic methanolysis is probably due to condensation reactions with other compounds present in the hydrolyzate.

Reference

Andrade, P.; Ferrers, F.; Gil, M. I. and Tomas-Barberan, F. A. (1997) Determination of Phenolic Compounds in Honeys with Different Floral Origin by Capillary Zone Electrophoresis. *Food Chem.*, 60: 79-84**.**

Bianco, M.-A.; Handaji, A. and Savolanien, H. (1998) Quantitative Analysis of Ellagic Acid in Hardwood Samples. *Sci. Total Environ.*, **222**: 123-126.

Charrier, B.; Haluk, J. P. and Metche, M. (1995) Characterization of European Oak Wood Constituents Acting in the Brown Discoloration During Kiln Drying. *Holzforschung*, **49**: 168-172.

Charrier, B.; Marques, M. and Haluk, J. P. (1992) HPLC Analysis of Gallic and Ellagic Acids in European Oakwood (*Quercus robur* L.) and Eucalyptus (*Eucalyptus glubulus*). *Holzforschung,* **46**: 87-89.

Clifford, M. N.; Scalbert, A. (2000) Ellagitannins - Nature, Occurrence and Dietary Burden. *J. Sci. Food Agric.*, **80**: 1118-1125.

Daniel, E. M.; Krupnick, A. A.; Heur, Y. -H.; Blinzler, J. A.; Nims, R. W. and Stoner, G. D. (1989) Extraction, Stability and Quantitation of Ellagic Acid in Various Fruits and Nuts. *J. Food Comp. Anal.*, **2**: 338-349.

Ferreres, F.; Blazquea, M. A.; Gil, M. I. and Tomas-Barberan, F. A. (1994) Separation of Honey Flavonoids by Micellar Electrokinetic Capillary Chromatography. *J. Chromatogr. A,* 669: 268-274.

Hagerman, A. E. and Bulter, L. G. (1980) Determination of Protein in Tannin-Protein Precipitates. *J. Agric. Food Chem.*, **28**: 944-947.

Häkkinen, S. H.; Kärenlampi, S. O.; Heinonen, I. M.; Mykkänen, H. M. and Törrönen, A. R. (1998) HPLC Method for Screening of Flavonoids and Phenolic Acids in Berries. *J. Sci. Food Agric.*, **22**: 543-551.

Helm, R. F.; Ranatunga, T. D. and Chandra, M. (1997) Lignin-Hydrolyzable Tannin Interactions in Wood. *J. Agric. Food Chem.*, **45**: 3100-3106.

Hemingway, R. W. and Hillis, W. E. (1971) Behavior of Ellagitannins, Gallic Acid and Ellagic Acid under Alkaline Conditions. *Tappi.*, **54**: 933-936.

Inour, K. H. and Hagerman, A. E. (1988) Determination of Gallotannins with Rhodanine, *Anal. Biochem.*, **169**: 363-369.

Kilkowski, W. J. and Gross, G. G. (1999) Color Reaction of Hydrolyzable Tannins with Bradford Reagent, Coomassie Brilliant Blue. *Phytochemistry*, **51**: 363-366.

Klumpers, J.; Scalbert, A. and Janin, G. (1994) Ellagitannins in European Oak Wood: Polymerization during Wood Aging. *Phytochemistry*, **36**: 1249-1252.

Lei, Z.; Jervis, J. and Helm, R. F. (1999) C-Glycosidic Ellagitannins from White Oak Heartwood and Callus Tissues. *Phytochemistry*, **51**: 751-756.

Maas, J. L.; Wang, S. Y. and Galletta, G. J. (1991) Evaluation of Strawberry Cultivars for Ellagic Acid Content. *HortSci*, **26**: 66-68.

Mayer, W., Kuhlmann, F. and Schilling, G. (1971) On Tanning Compounds from the Wood of Chestnut and Oak. V. The Structure of Vescalin. *Liebigs Ann. Chem.*, **747**: 51-59.

Mosedale, J. R.; Puech J. -L. and Feuillat, F. (1999) The Influence on Wine Flavor of the Oak Species and Natural Variation of Heartwood Components. *Am. J. Enol. Vitic.*, **50**: 503-512.

Mueller-Harvey, I. (2001) Analysis of Hydrolysable Tannins. *Animal Feed Sci. and Technol.*, **91**: 3-20.

Okuda, T.; Yoshida, T.; Hatano, T.; Yazaki, K.; Kira, R. and Ikeda, Y. (1986) Chromatography of Tannins. II. Preparative Fractionation of Hydrolyzable Tannins by Centrifugal Partition Chromatography. *J. Chromatogr.*, **362**: 375-381.

Peng, S.; Scalbert, A. and Monties, B. (1991) Insoluble Ellagitannins in *Castanea sativa* and *Quercus petraea* Woods. *Phytochemistry*, **30**: 775-778.

Puech, J. – L.; Mertz, C.; Michon, C.; Guerneve, C. L.; Doco, T. and Herve du Penhoat, C. (1999) Evolution of Castalagin and Vescalagin in Ethanol Solutions. Identification of New Derivatives. *J. Agric. Food Chem.*, **47**: 2060-2066.

Quinn, M. K. and Singleton, V. L. (1985) Isolation and Identification of Ellagitannins from White Oak Wood and an Estimation of Their Roles in Wine. *Am. J. Enol. Vitiv.*, **36**: 148-155.

Scalbert, A. (1992) Quantitative Methods for the Estimation of Tannins in Plant Tissues. In *Plant Polyphenols;* Hemingway, R. W. and Laks, P. E. eds.; Plenum Press, New York, 259-280.

Scalbet, A.; Duval, L.; Peng, S. and Monties, B. (1990) Polyphenols of *Quercus robur* L. II. Preparative Isolation by Low-Pressure and High-Pressure Liquid Chromatography of Heartwood Ellagitannin. *J. Chromatogr.*, **502**: 107-119.

Scalbert, A.; Monties, B. and Janin, G. (1993) Tannins in Wood: Comparison of Different Estimation Methods. *J. Agric. Food Chem.*, **37**: 1324–1329.

Schultz, J. C.; Baldwin, T. T. and Nothnagle, P. J. (1981) Hemoglobin as a Binding Substrate in the Quantitative Analysis of Plant Tannins. *J. Agric. Food Chem.*, **29**: 823.

Tang, H. R. and Hancock, R. A. (1995) Studies of Vegetable Tannins. Complete Structural Determination of Two Chestnut Tannins-Vescalagin and Castalagin Using Nuclear Magnetic Resonance Spectroscopic Methods. *J. Soc. Leather Technol. Chem.,* **79**: 181-187.

Tomas-Barberan, F. A. and Garcia-Viguera, C. (1997) Capillary Electrophoresis versus HPLC in Plant Polyphenol Analysis. *Analusis Magazine* , **25**: 23-25.

Viriot, C.; Scalbert, A.; Herve du Penhoat, C. L. M. and Moutounet, M. (1994) Ellagitannins in Woods of Sessile Oak and Sweet Chestnut Dimerization and Hydrolysis During Wood Aging. *Phytochemistry*, **36**: 1253-1260.

Viriot, C.; Scalbert, A.; Lapierre, C. and Moutounet, M. (1993) Ellagitannin and Lignins in Aging of Spirits in Oak Barrels. *J. Agric. Food Chem.*, **41**: 1872-1879.

Vivas, N. and Glories, Y. (1996) Role of Oak Wood Ellagitannins in the Oxidation Process of Red Wines During Aging. *Am. J. Enol. Vitic.*, **47**: 103-107.

Willis, R. B. and Allen, P. R. (1998) Improved Method for Measuring Hydrolyzable Tannins Using Potassium Iodate. *Analyst*, **123**: 435-439.

Wilson, T. C. and Hagerman, A. E. (1990) Quantitative Determination of Ellagic Acid. *J. Agric. Food Chem.*, **38**: 1678-1683.

Chapter Three

Stability of Castalagin and Vescalagin in Aqueous Solutions

Abstract: The degradation of ellagitannins in oak woods results in the discoloration of oak heartwood, and the variability of this discoloration causes considerable economic loss to the US forest products industry each year. In this study, the stability of the major ellagitannins in oaks, vescalagin and castalagin were determined in aqueous solutions under different conditions (at 23 °C or 60 °C, in the presence of O_2 or N_2). Their degradation kinetics in aqueous solutions were obtained for the first time and used to describe their stability quantitatively. It was found that both vescalagin and castalagin are unstable in aqueous solutions, even at room temperature and in the absence of oxygen. Castalagin, however, is relatively more stable than vescalagin. Oxygen, higher temperature and pH are found to increase the rate of degradation with higher temperature being the most prominent factor. The yellowing of aqueous solutions was found to be associated with the decrease of vescalagin and castalagin, strongly suggesting that the brown discoloration of oak heartwood during kiln drying is caused by the chemical transformation of ellagitannins. The presence of ellagic acid in the solutions revealed by HPLC indicated that hydrolysis was one of the degradation processes that castalagin and vescalagin underwent in aqueous solutions. The stability of vescalagin and castalagin in aqueous methanol was also investigated and found to relate strongly to the concentration of methanol.

3. 1. Introduction

Yellow and brown discoloration in timber and lumber during processing (kiln drying) is a major and serious defect in wood, resulting in substantial economic loss to the US forest products industry. The discoloration appears mainly as sticker stain or longitudinally oriented yellow or brown streaks during air- and kiln drying. Many efforts have been made to understand and minimize the brown discoloration by optimizing kiln drying operations, and/or employing quick kiln drying, pre drying or vacuum drying (Bauch et al., 1991; Charrier et al., 1992). It is generally agreed that the discoloration can be divided into two color groups: yellow and brown discoloration. While yellow discoloration during kiln drying is caused by fungi, brown discoloration is induced by chemical reactions associated with extractives (Bauch et al., 1991; Charrier et al., 1992). Chromophoric pigments in the hyphae of fungi are thought to lead to wood yellow discoloration, but the discoloration associated with fungi is usually limited to sapwood of green woods (Bauch et al., 1991). Indeed, Ward and Groom (Ward and Groom, 1983) found a fungus, *Paecilomyces variotii* Bain., in the heartwood of oak (*Quercus rubrae* L.). Although the fungus had been previously identified as one of the causes of yellow discoloration, the discoloration did not develop in the heartwood of *Quercus rubrae* during kiln drying (Ward and Groom, 1983). Bauch *et al* (1991) later reported the same result but also observed the discoloration in the heartwood of *Quercus robur* and *Castanea sativa* Mill. caused by the fungus. Subsequent extraction of discolored heartwood led to the separation of yellow compounds I and II (Bauch et al., 1991). These yellowing compounds are structurally so similar to ellagic acid that they are believed to be the derivatives of ellagic acid (Bauch et al., 1991; Charrier et al., 1995). This finding suggests that chemical reactions involving ellagitannins caused by the growth of fungus might be responsible for the yellow discoloration in heartwood, which had been previously thought to be caused only by pigments in fungal hyphae.

Brown discoloration in heartwood is believed to result mainly from chemical reactions of extractives in the heartwood (Wegener and Fengel, 1987). Oaks, being the most important hardwoods in the USA, suffer severely from brown discoloration during kiln drying due to their relatively high extractive contents. While contributing to natural wood durability (Scalbert, 1992), extractives are blamed for the brown discoloration in woods, a defect only second to fungi decay in causing the reduction in the values of wood products. As the predominant extractives found in oak heartwood are ellagitannins that can reach up to 10% of wood dry weight (dry wt. basis), the brown discoloration in heartwood is believed to be closely associated with ellagitannins. Indeed, extraction of the discolored and non-discolored portions of heartwood revealed that ellagitannin contents, particularly vescalagin and castalagin, are lower in the discolored areas than in the non discolored areas, indicating that the discoloration is correlated to the changes of ellagitannins (Charrier et al., 1995). After ultrafiltration of extracts from both the discolored and the non-discolored areas for three days, extracts from discolored areas were found to contain five to six times more compounds of higher molecular weights (Charrier et al., 1995). This indicates that oxidative polymerization of ellagitannins might be one of the causes of brown discoloration. Solutions of crude extracts and pure ellagitannins subject to different temperatures were also found to produce ellagic acid and cause the yellowing of the solutions, suggesting that discoloration in heartwood may also be induced by hydrolysis. Similar results were also obtained from five different species of oak woods during their toasting (Cadahia et al., 2001). Both hydrolysis and polymerization of ellagitannins were found to occur when oak woods were heated or toasted in preparation for stave assembly. These data indicate that chemical changes of ellagitannins are responsible for discoloration in woods.

It is now generally accepted that ellagitannins are unstable (Viriot et al., 1994; Klumpers et al., 1994) and result in wood discoloration. In order to better understand the chemical changes of ellagitannins during wood drying, studies addressing the stability of ellagitannins are obviously important. Since a quantitative study for determining the stability of ellagitannins has not been reported, we investigated the stability of vescalagin and castalagin in aqueous solutions, and the effects of oxygen, temperature and pH.

3. 2 Materials and Methods

A white oak (*Quercus alba)*, approximately 80 years old, was harvested in early December 1997 in Giles County, Virginia. Wood slices of approximately 2.5-4 cm thick were taken from the oak with a chain saw. After freeze-drying, heartwood and sapwood were separated and ground to a sample powder (1 mm size) for extraction. All solvents were HPLC grade. 4-Hydroxybenzoic acid (Fluka) was crystallized three times before use. Evaporation was performed under reduced pressure at temperatures less than 40 ^{o} C.

3. 2. 1. HPLC

HPLC was performed on the Gilson HPLC apparatus using a Merck Lichrospher RP-18 (endcapped 5 μm) column (250 mm X 4 mm I. D.). The solvents were MeOH and 0.2% trifluoroacetic acid (TFA). The flow rate was set at 0.75 ml/min. Gradient condition:

Time (min)	0	40	45	50	70
0.2% TFA	100%	95%	0%	0%	100%
MeOH	0%	5%	100%	100%	0%

3. 2. 2 Isolation and Purification of Vescalagin and Castalagin.

Pure ellagitannins used for the stability study were isolated and purified from oak heartwood extracts. The extracts were prepared by extracting the oak heartwood powder

with acetone: water (7:3) for 24 hours. Extraction was repeated twice and the extracts were combined and freeze dried. The extracts (500 mg) were then dissolved in 50 ml of methanol and centrifuged for 20 minutes (8000 g). The supernatant was collected and evaporated to remove methanol. The sample was then separated on a reverse-phase C-18 column (I. D. 25 mm x 335 mm) with elution of 0.2% TFA (250 ml), 5% of methanol: 0.2% TFA (400 ml) and methanol (500 ml). Flow rate was maintained at 1 ml/min. Fractions were combined according to TLC results (Merck F254 cellulose plate, elution system: MeOH: H2O: HOAc; 1:89:10), evaporated and freeze-dried. The crude vescalagin sample was further purified on a Toyopearl HW40-F column (I. D. 15 mm x 335 mm) with the elution of MeOH: 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 possible degradation of vescalagin in MeOH. Fractions were combined according to TLC results, evaporated and freeze-dried. The structures of both vescalagin and castalagin (purity about 92%) were confirmed by the ^{1}H NMR spectra obtained from Bruker AM 360 located in the Chemistry Department and compared with the published ^{1}H NMR spectra (Tang and Hancock, 1995).

3. 2. 3 Degradation in Aqueous Solutions

Degradation experiments were performed by dissolving 3.3 mg of vescalagin and 3.0 mg of castalagin respectively into 10 ml of buffer solutions saturated with oxygen or nitrogen, making the initial vescalagin concentration of 330 μg/ml and castalagin concentration of 300 μg/ml. 4-Hydroxybenzoic acid was also added into the solution as the internal standard (internal standard con. = 80 μg/ml). The buffer solutions (50 ml) were prepared by adjusting sodium acetate solution (100 mM) to pH 4 and pH 7 with acetic acid (100 mM) and bubbled with either oxygen or nitrogen for 4 hours and kept under oxygen or nitrogen atmosphere. A blank experiment was also conducted using

deionized water. The solutions were divided into two parts. One part was placed into a heating module preset at 60 °C, while the others were left at the room temperature (23 °C). Concentrations of vescalagin and castalagin in the buffer solutions were determined every 24 hours by HPLC and the UV-Vis absorption patterns of the buffer solutions were monitored with the spectrophotometer.

Degradation of vescalagin and castalagin in aqueous methanol was performed by dissolving the ellagitannins in a series of solutions of different methanol concentrations. Stock solutions were prepared by dissolving samples of vescalagin and castalagin in HPLC grade water (2 ml). The effect of methanol concentration on degradation was studied by adding the same amount of the stock solutions to amber vials containing 10%, 20%, 30%, 40%, 50%, 60% and 70% (v/v) aqueous methanol. This gave 7 $MeOH/H_2O$ solutions with a sample of vescalagin + castalagin for each solution (14 samples totally). Samples were analyzed for vescalagin and castalagin contents after 72 and 144 hours (3 and 6 days).

3. 3. Results and Discussion

Ellagitannins have previously been demonstrated to be unstable in both aqueous solution and in woody tissues (Charrier et al., 1995; Klumpers et al., 1994; Viriot and Scalbert, 1994) They can undergo hydrolysis to produce ellagic acid and polymerization to form oligomeric ellagitannins. These changes may explain the previous reports (Klumpers et al., 1994; Peng et al., 1991; Viriot and Scalbert, 1994) that ellagitannin concentrations in the oak (*Quercus robur* and *Quercus petraea*) heartwood decrease with the increase of free ellagic acid, dimers and insoluble ellagitannins as the oaks age. The instability of ellagitannins can be exemplified by the finding that all eight ellagitannins, castalagin, vescalagin, grandinin and roburin A-E, decreased during natural seasoning of oaks, a

process of air-drying of oak woods at the ambient temperature (Cadahia et al., 2001), and were found to decrease at a faster rate at elevated temperature such as during oak wood toasting (Cadahia et al., 2001).

Degradation of ellagitannins has been demonstrated to cause wood discoloration. Charrier *et. al*. (1995) reported that model solutions of vescalagin and castalagin turned yellow and produced ellagic acid after a few days (Charrier et al., 1995). Indeed, during kiln drying, maximum discoloration occurs only in woods with moisture content above 30% (Charrier et al., 1992), confirming that ellagitannins are not stable in aqueous solution. Temperature was found to increase ellagic acid production and high molecular weight compounds (Charrier et al., 1995). However, quantitative information on ellagitannin stability is lacking, making it impossible to compare the stabilities of castalagin and vescalagin under different conditions. In addition, data concerning the effects of oxygen and pH on their stability are not available. A better understanding of ellagitannin stability under different conditions will give us an insight into the wood discoloration problem, possibly allowing the rational design of procedures which minimize the discoloration.

To study the stability of ellagitannins, vescalagin and castalagin were used as representative structures. Purified vescalagin (**16**) and castalagin (**17**) were dissolved in sodium acetate buffer solutions (100 mM). Two different pH values (pH 4 and pH 7) were used in the experiment in order to find out the pH effects on the degradation of ellagitannins. Concentrations of vescalagin and castalagin in the buffer solutions were determined every 24 hours on the HPLC using 4-hydroxybenzoic acid as the internal standard. The temperatures (23 ^{o}C and 60 ^{o}C) were chosen to allow the comparisons of the behaviors of ellagitannins in kiln drying (60 ^{o}C) and at the room temperature (23 ^{o}C).

The effect of temperature on vescalagin and castalagin is shown in Figure 3.1 (vescalagin) and Figure 3.2 (castalagin). Both oxygen/nitrogen and pH were also found to affect the stability of ellagitannins. Their effects on vescalagin can be seen in Figures 3.3 and 3.4.

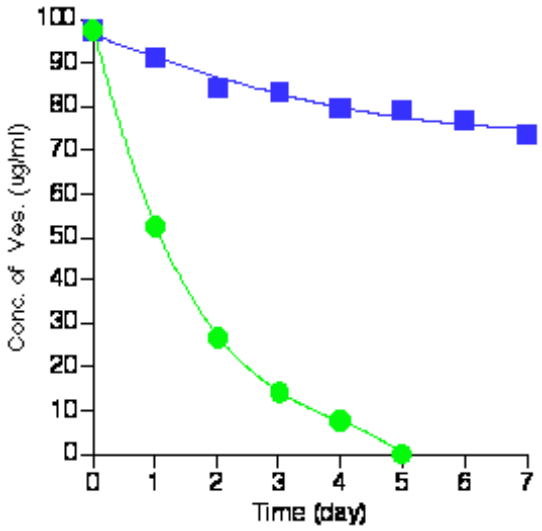


Figure 3.1. Vescalagin in pH 4 solutions saturated with nitrogen at 23 ºC (closed square) and 60 ºC (closed circle).

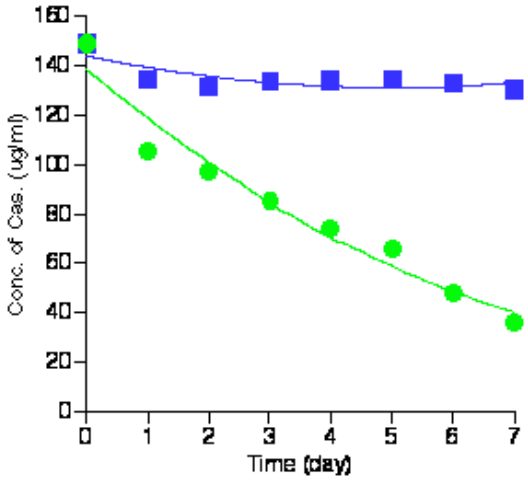


Figure 3.2. Castalagin in pH 4 solutions saturated with nitrogen at 23 ºC (closed square) and 60 ºC (closed circle).

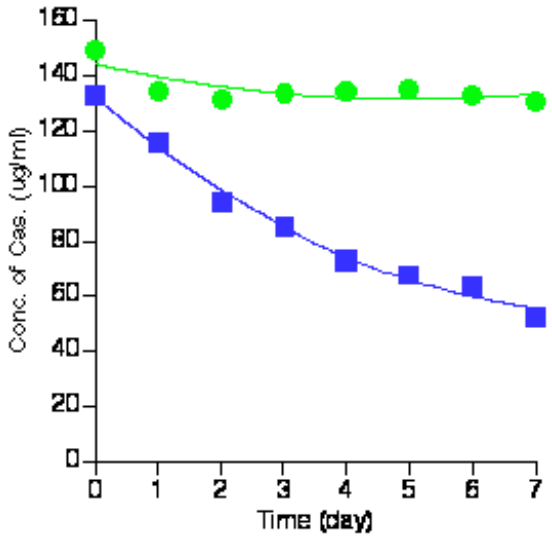


Figure 3.3. Effect of pH (pH4, closed circle) and pH 7 (closed square) on castalagin in nitrogen saturated solution at 23 ºC

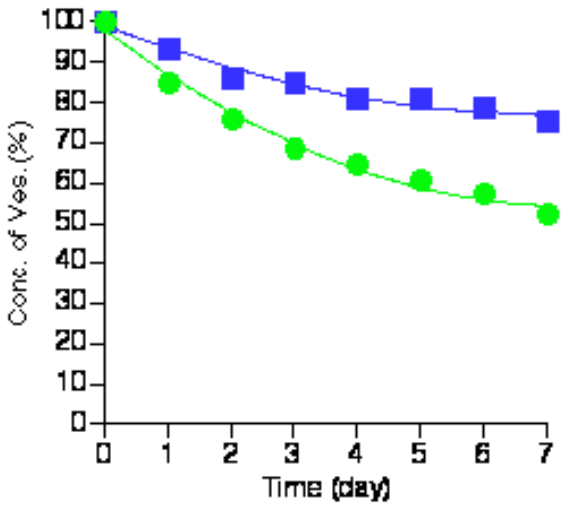


Figure 3.4. Effects of nitrogen (closed square) and oxygen (closed circle) on vescalagin in pH4 solution at 23 ºC

It is obvious from the above figures that both vescalagin and castalagin are not stable in aqueous solution, even in the presence of nitrogen and at room temperature. This work is consistent with the statement (Charrier et al., 1995) that "…ellagitannins in aqueous solutions are unstable even at low temperature…". Their instability is affected by temperature, pH and oxygen. These data (Figures 3.1 and 3.2) also confirm a previous report (Charrier et al., 1995) that the decrease of ellagitannins in solution relates strongly to temperature increase. Oxygen effect is shown in Figure 3.4. The oxygen effect is less predominate as compared with temperature and pH. However, its effect can still be seen from Figure 3.4. Vescalagin degraded a little bit faster in the presence of oxygen than in the presence of nitrogen. Although the exact mechanism involving oxygen is unclear, the effect of oxygen may explain the observation (Charrier et al., 1992) that kiln drying under vacuum or using air flow that is low in oxygen can minimize wood discoloration.

3. 3. 1. Chemical Kinetics

Plots of the natural logarithm of the ratio of concentration to initial concentration (ln C/C_o) versus time for all data from the experiments were prepared in order to investigate the chemical kinetics of vescalagin and castalagin degradation. Figures 3.5 and 3.6 show two plots for vescalagin and castalagin in pH 4 buffer solutions saturated with oxygen or nitrogen at different temperatures. A linear relationship between ln (C/C_o) and time indicates that the degradation of both vescalagin and castalagin is first order in aqueous solutions. The rate constants (k) for vescalagin and castalagin degradation under these conditions are shown in Table 3.1. Comparisons of the rate constants will reveal to what extent temperature, oxygen, and pH affect the degradation rates. For the purpose of comparison, data concerning ellagitannin degradation in aqueous ethanol solutions were also processed and listed in Table 3.1. These data were kindly provided by Dr. Jean-Louis

Puech, Laboratoire des Polymeres et des Techniques Physico-chimiques, France, who identified ethoxy derivatives from vescalagin/castalagin in aqueous ethanol solutions

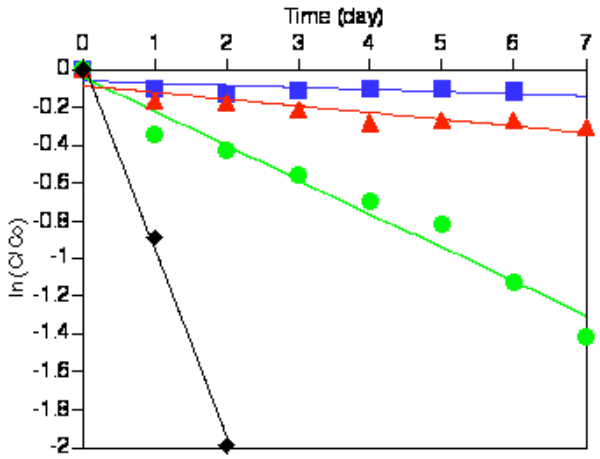


Figure 3.5. Degradation of castalagin in pH4 solution under different conditions
1. 23 ºC and N_2, ■. 2. 23 ºC and O_2, ▲
3. 60 ºC and N_2, ●. 4. 60 ºC and O_2, ◆.
C: concentration (□g/ml), Co: initial concentration (□g/ml)

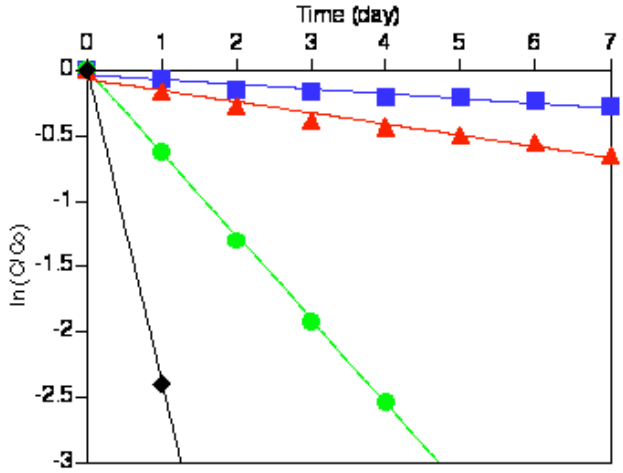


Figure 3.6. Degradation of vescalagin in pH4 solution under different conditions
1. 23 ºC and N_2, ■. 2. 23 ºC and O_2, ▲
3. 60 ºC and N_2, ●. 4. 60 ºC and O_2, ◆.
C: concentration (□g/ml), Co: initial concentration (□g/ml)

Table 3.1. Table of k Values (rate constants) under different conditions						
Sample	Conditions			k (day^{-1})	K_{rel} [2]	R-square
	N_2 / O_2	Temp.	pH			
Vescalagin	N_2	23 °C,	pH3.7[4]	-0.025	1.0	0.94
	N_2	23 °C,	pH 4	-0.036	1.4	0.93
	N_2	23 °C,	pH 7	-0.24	9.6	0.96
	N_2	60 °C,	pH3.7[4]	-0.20	8.0	0.91
	N_2	60 °C,	pH 4	-0.64	25	0.99
	N_2	60 °C,	pH 7	----- [1]		
	O_2	23 °C,	pH3.7[4]	-0.10	4.1	0.99
	O_2	23 °C,	pH 4	-0.085	3.4	0.96
	O_2	23 °C,	pH 7	----- [1]		
	O_2	60 °C,	pH3.7[4]	-0.78	31	0.95
	O_2	60 °C,	pH 4	-2.41	96	1.00
	O_2	60 °C,	pH 7	----- [1]		
	40% EtOH			-4.92[3]	196	0.95
	70% EtOH			-5.07[3]		0.91
Castalagin	N_2	23 °C,	pH4.6[5]	-0.023	1.0	0.76
	N_2	23 °C,	pH 4	-0.019	0.8	0.41
	N_2	23 °C,	pH 7	-0.13	5.5	0.98
	N_2	60 °C,	pH4.6[5]	-0.31	13	0.95
	N_2	60 °C,	pH 4	-0.18	7.9	0.96
	N_2	60 °C,	pH 7	----- [1]		
	O_2	23 °C,	pH4.6[5]	-0.10	4.4	0.92
	O_2	23 °C,	pH 4	-0.36	15	0.78
	O_2	23 °C,	pH 7	----- [1]		
	O_2	60 °C,	pH4.6[5]	-0.71	30	0.99
	O_2	60 °C,	pH 4	----- [1]		
	O_2	60 °C,	pH 7	----- [1]		
	40% EtOH			-2.63[3]	114	0.99
	70% EtOH			-3.07[3]	135	0.98

(1). The rate of degradation was too fast to allow for acquiring enough data points for accurate rate determination. It should be greater than the largest number in the table.

(2). $K_{rel} = k/k_{(N2,\ 23\ 0C,\ blank)}$

(3). Experimental data were kindly provided by Dr. Jean-Louis Puech, Laboratoire des Polymeres et des Techniques Physico-chimiques, France.

(4). Vescalagin was dissolved in water as a control. (5). Castalagin was dissolved in water as a control.

(Puech et al., 1999) but did not process the data to obtain their degradation constants. The degradation of vescalagin and castalagin is also the first order but the rates are much faster in aqueous solutions than in ethanol solutions.

3. 3. 2. Effects of Temperature, pH and Oxygen

As expected, both temperature and oxygen can accelerate the degradation of vescalagin and castalagin, with the temperature effect being the most predominant. Effects of temperature and oxygen on the stability of vescalagin and castalagin can be clearly seen from Table 3.1. The rate constant for vescalagin at 23 °C in the presence of nitrogen is –0.025 day $^{-1}$ while in the presence of oxygen the rate constant increases to -0.10 day $^{-1}$, almost 5 times higher. At 60 °C, vescalagin degraded 4 times faster in the presence of oxygen than in the presence of nitrogen. Castalagin showed similar trend. In the presence of oxygen, the rate was about 5 times faster at room temperature and 2 times faster at 60 °C. Effect of temperature on vescalagin and castalagin can also be revealed by comparing the rate constants at different temperatures. For example, vescalagin degraded 8 times faster at 60 °C than at 23 °C in nitrogen saturated deionzied water. Castalagin was also found to degrade faster at the higher temperature. A comparison of rate under different conditions reveals that the effect of higher temperature outweighs the effect of oxygen. As shown in Figures 3.1 and 3.2 and Table 3.1, vescalagin decreased almost 18 times faster at a higher temperature (60 °C) than at the room temperature in nitrogen saturated solutions. However, when the solutions were saturated with oxygen at room temperature, the degradation was only about 2.5 times faster. The data demonstrate that when processing wood samples (grinding) and extracting polyphenols from wood samples, higher temperature should be avoided in order to obtain the "actual" contents of ellagitannins. The results also suggest that during operations such as kiln drying of oak wood,

degradation is unavoidable but could be reduced under the lower temperature conditions used during vacuum drying.

Friedman *et al*. (2000) studied the behavior of eight phenolic compounds in solutions of different pH (pH 3-11). Higher pH was found to destabilize some plant phenolic compounds but did not affect others. Furthermore, for those compounds that were destabilized, the changes were found to be irreversible (Friedman et al., 2000). In this work, we found that changing pH from 4 to 7 increases the degradation rate constants significantly for both vescalagin and castalagin. For example, at 23 °C, the rate constant for vescalagin in nitrogen saturated buffer was –0.036 day $^{-1}$ at pH 4 and –0.24 day $^{-1}$ at pH 7, indicating that vescalagin is about 7 times more stable at pH 4 than at pH 7. A large difference (also about 7 times difference) between k values for vescalagin at pH 4 (-0.019 day $^{-1}$) and at pH 7 (-0.13 day $^{-1}$) was also observed for castalagin.

An interesting observation was found with vescalagin and castalagin dissolved in deionized water which has a pH of 6.9. Vescalagin/castalagin are more stable in water than in pH 7 buffer solutions. For example, at 23 °C, vescalagin is 10 times more stable in denoized water saturated with nitrogen (-0.025 day $^{-1}$) than in pH 7 sodium acetate buffer solution saturated with nitrogen (-0.24 day $^{-1}$). This huge difference can not be explained by the pH difference since both solutions have similar pH values. While the exact reason for the difference is unclear, it is proposed that ionic strengths were responsible for the faster decrease of vescalagin and castalagin in pH 7 buffer solutions because pH 7 buffer solutions had much higher ion concentrations than the blank solutions.

A comparison of the rate constants for vescalagin (**16**) and castalagin (**17**) under the same conditions revealed that vescalagin is less stable than castalagin. For example, at 23

°C vescalagin degraded at the rate of –0.036 day $^{-1}$ in pH 4 buffer solutions saturated with nitrogen while the degradation rate constant for castalagin was –0.019 day $^{-1}$. The difference in the rate constants indicate that castalagin is 2 times more stable than vescalagin under the above conditions. Computer simulation of vescalagin and castalagin structures (Vivas et al., 1995) showed that vescalagin (**16**) is a little less stable than castalagin (**17**) (473 kJ mol $^{-1}$ *vs* 471 kJ mol $^{-1}$). This energy or stability difference was the result of an additional hydrogen bond of hydroxyl group at C-1 position in castalagin. The hydrogen bond was thought to occur between C-1 hydroxyl group and hydroxyl group on the galloyl group at C-1 position via a six member ring. This additional hydrogen bond is not possible in vescalagin in which the C-1 hydroxyl group is pointing away from galloyl group at C-1 position.

HPLC analyses of the degradation reactions indicated that ellagic acid was formed. The presence of ellagic acid strongly suggests that hydrolysis was one of the changes vescalagin and castalagin underwent. It was also found that there was a correlation between the decrease of vescalagin/castalagin and the yellowing of solutions. This observation is consistent with previous work in which it was reported that vescalagin and castalagin contents were lower in colored areas of European oakwood than in non-colored areas (Charrier et al., 1995). The color of the aqueous solutions after diluted with water by 10 times was monitored every 24 hours. Figure 3.7 shows the UV-Vis absorption of vescalagin solutions at the time of T=0, 3 and 7 days (200 - 500 nm). An increase in absorbency at 366 nm clearly indicated that the solution became more and more brown-yellow as the degradation continued.

Degradation in Aqueous Methanol. Aqueous ethanol has been found to affect the stability of ellagitannins. Vescalagin and castalagin are found to degrade through

ethanolysis to produce ellagic acid and other derivatives containing ethoxy group (Puech et al., 1999). This suggests that ellagitannins may also be unstable in aqueous methanol. Although methanol has been found to cleave the depside bond in gallotannins, its effect on ellagitannins has not been reported. In order to understand the effect of aqueous methanol on the stability of vescalagin and castalagin, a quantitative chromatographic study was performed. The data are shown in Table 3.2 and Figure 3.8.

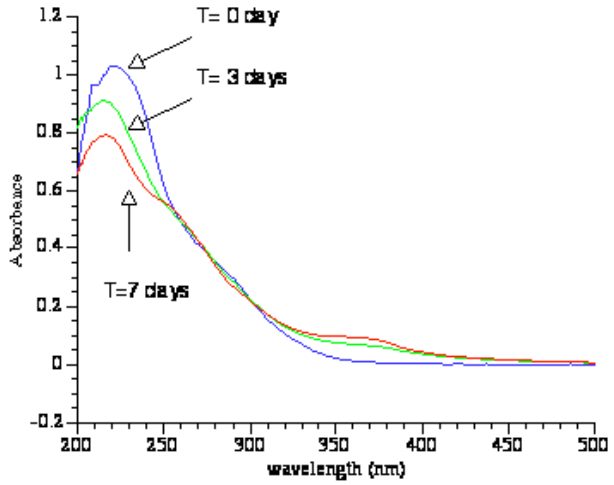


Figure 3.7. UV- Vis spectra of vescalagin in pH4 solution saturated with nitrogen at 60 ºC

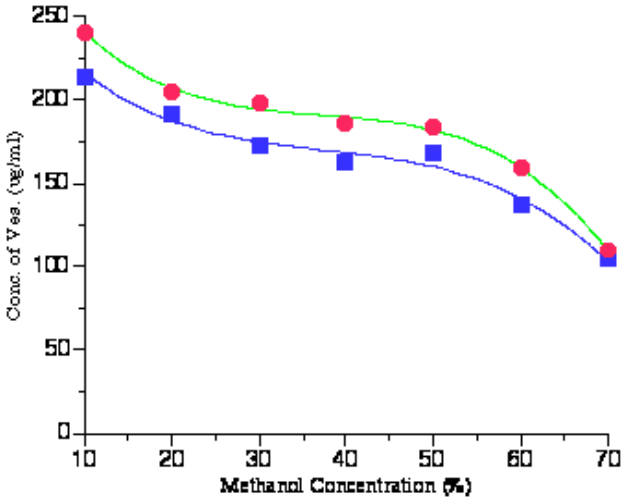


Figure 3.8. Effect of methanol concentration on the stability of vescalagin at 3 days (closed circle) and 6 days (closed square)

Table 3.2 Effect of MeOH content on vescalagin and castalagin unit: μg/ml					
Condition	MeOH%	Vescalagin Content		Castalagin Content	
		3 days	6 days	3 days	6 days
1	10%	240	214	303	231
2	20%	205	192	290	256
3	30%	198	173	305	230
4	40%	186	163	307	227
5	50%	183	168	305	235
6	60%	159	137	299	222
7	70%	110	104	294	220
* Each condition was comprised of two samples: one for vesclagin and one for castalagin					

Vescalagin and castalagin concentrations in aqueous methanol solutions were found to decrease. The effect of the aqueous methanol is proportional to the methanol concentration, with higher methanol concentrations leading to increased rates of degradation. The decrease in vescalagin and castalagin concentrations with the increase of methanol concentration indicates that aqueous methanol is not a good solvent for ellagitannins. This again demonstrates that one should not use methanol:water to extract ellagitannins, an issue addressed in Chapter 2.

3. 4. Conclusion

Vescalagin and castalagin are not stable in aqueous solutions. They can undergo hydrolysis plus non-specific reactions that lead to brownish colored solutions. Oxygen, ionic strength and higher temperature (60 °C) were demonstrated to increase their instability with higher temperature and pH being the two most prominent factors. This finding indicates that when processing samples for the analyses of ellagitannins, high temperature should be avoided.

Both vescalagin and castalagin are also found to degrade in aqueous methanol although the degradation mechanisms are unclear. The extent of degradation depends on the concentration of methanol. The higher the methanol concentration, the greater the degradation. More work needs to be done to investigate the mechanisms. Considering the effect of methanol on vescalagin and castalagin, any attempt to use aqueous methanol to isolate, purify and quantify ellagitannins should be cautioned against.

Reference

Bauch, J.; Hundt, H. V.; Wei□mann, G. Lange, W. and Kubel, H. (1991) On the Cause of Yellow Discolorations of Oak Heartwood (*Quercus* Sect. *Robur*) during Drying. *Holzforschung,* **45**: 79-85.

Cadahia, E.; Varea, S.; Munoz, L; Fernandez de Simon, B. and Garcia-Vallejo, M. C. (2001) Evolution of Ellagitannins in Spanish, French, and American Oak Woods during Natural Seasoning and Toasting. *J. Agric. Food Chem.*, **49**: 3677-3684.

Charrier, B.; Haluk, J. P. and Janin, G. (1992) Prevention of Brown Discoloration in European Oakwood Occurring during Kiln Drying by a Vacuum Process: Colorimetric Comparative Study with a Traditional Process. *Holz als Roh- Und Werkstoff*, **50**: 433-437.

Charrier, B.; Haluk, J. P. and Metche, M. (1995) Characterization of European Oakwood Constituents Acting in the Brown Discoloration during Kilin Drying. *Holzforschung*, **49**: 168-172.

Friedman, M. and Juragens, H. S. (2000) Effect of pH on the Stability of Plant Phenolic Compounds. *J. Agric. Food Chem.*, **48**: 2101-2110.

Klumpers, J.; Scalbert, A. and Janin, G. (1994) Ellagitannins in European Oak Wood: Polymerization during Wood Aging. *Phytochemistry*, **36**: 1249-1252.

Peng, S.; Scalbert, A. and Monties, B. (1991) Insoluble Ellagitannins in *Castanea sativa* and *Quercus petraea* Woods. *Phytochemistry*, **30**: 775-778.

Puech, J. –L.; Mertz, C.; Michon, C.; Guerneve, C. L.; Doco, T. and Herve du Penhoat, C. (1999) Evolution of Castalagin and Vescalagin in Ethanol Solutions. Identification of New Derivatives. *J. Agric. Food Chem.*, **47**: 2060-2066.

Scalbert, A. (1992) Tannins in Woods and Their Contribution to Microbial Decay Prevention. In *Plant Polyphenols*; Hemingway, R. W.; Laks, P. E. eds., Plenum Press, New York, 935-951.

Tang, H. R. and Hancock, R. A. (1995) Studies of Vegetable Tannins. Complete Structural Determination of Two Chestnut Tannins-Vescalagin and Castalagin Using Nuclear Magnetic Resonance Spectroscopic Methods. *J. Soc. Leather Technol. Chem.*, **79**: 181-187.

Viriot, C.; Scalbert, A.; Herve du Penhoat, C. L. M. and Moutounet, M. (1994) Ellagitannins in Woods of Sessile Oak and Sweet Chestnut Dimerization and Hydrolysis During Wood Aging. *Phytochemistry*, **36**: 1253-1260.

Vivas, N.; Laguerre, M.; Glories, Y.; Bourgeois, G. and Vitry, C. (1995) Structure Simulation of Two Ellagitannins from *Quercus Robur* L. *Phytochemistry*, **39**: 1193-1199.

Ward, J. C. and Groom, D. A. (1983) Bacterial Oak: Drying Problems. *Forest Product. J.*, **33**: 57-65.

Wegener, G. and Fengel, F. (1987) Investigation on Color Changes Resulting from Drying of European Oakwood. Fourth International Symposium on Wood and Pulping Chemistry, 1, 121-123.

Chapter 4

Callus Tissue and Suspension Cell Cultures as Models for Ellagitannin Biosynthesis Study

Abstract: Both callus tissue cultures of *Quercus alba* and suspension cell cultures of *Quercus alba* and *Liquidambar styraciflua* were investigated for their possible use as models for ellagitannin biosynthesis. It was found that oak callus tissue cultures (*Quercus alba*) are capable of producing ellagitannins, and the production and profile of ellagitannins can be modified by adjusting the media composition. Comparison of extracts from the heartwood of *Quercus alba* with those from callus tissue reveals that they have similar ellagitannin profiles Through manipulation of the media nitrogen and copper concentrations the callus tissue produced almost 3 times as much castalagin and vescalagin. Suspension cells of *Quercus alba* and *Liquidambar styraciflua* were found to be unsuitable for the study of biosynthesis of ellagitannins. These cells either did not produce any detectable level of ellagitannins or the production was unstable. Although the suspension cells could be elicited to produce ellagic acid with glycanases (Driselase), the levels of ellagic acid were too low for quantitative metabolic studies.

4. 1. Introduction

The importance of ellagitannins in many economically important hardwoods such as oaks has long been recognized (Mila et al., 1996; Mosedale et al., 1999; Quinn and Singleton, 1985; Scalbert, 1992 and 1991; Vivas and Glories, 1996). While these secondary metabolites contribute to wood durability (Mila et al., 1996; Scalbert, 1992 and 1991), they are often a nuisance in wood processing and drying (Charrier et al., 1995). Oxidation and hydrolysis of ellagitannins lead to wood discoloration during kiln drying and they can also affect the pulping and the bleaching of these woods as ellagitannins consume pulping and bleaching reagents. Ellagitannins also cause problems in the manufacture of wood composites due to their reactivity towards adhesives (Marra, 1992). However, from the standpoint of wine and whiskey manufacturing, ellagitannins in oak barrels are desirable, as they are important and indispensable in the conditioning and aging of alcoholic beverages (Mosedale et al., 1999; Quinn and Singleton, 1985; Vivas and Glories, 1996). Considering the dual effects of ellagitannins, tailored production of ellagitannin *in vivo* is obviously important and would have a tremendous effect on wood products industry. For example, oaks with reduced ellagitannin contents would alleviate the discoloration and other ellagitannins-related problems encountered in wood drying and processing. On the other hand, ellagitannin contents could be up regulated to meet the need for long durability as well as alcoholic beverage conditioning.

The key to the manipulation of ellagitannins centers on our understanding of ellagitannin biosynthesis. However, despite decades of research, the enzymology of ellagitannin biosynthesis is still poorly understood (Helm et al., 1999). This lack of fundamental understanding is at least in part due to the seasonal availability of appropriate plant materials (Gross, 1983; Krajci and Gross, 1987). While it is convenient to collect leaf samples from oak trees in fields during the growing season, winter leaves us with

virtually no fresh samples to work with. The problem is further complicated by the presence of high amounts of other metabolites in oak leaves. The presence of these contaminants makes it extremely difficult to purify ellagitannin-related enzymes using standard techniques, often leading to significantly varying and irreproducible results (Krajci and Gross, 1987). In fact, many efforts to isolate enzymes form oak leaves have failed (Gross, 1992). Obviously, appropriate samples are crucial to elucidating the biosynthetic pathway of ellagitannins.

The first demonstration of ellagitannin formation from PGG *in vitro* was reported by Gross' group (Niemetz et al., 2001). Using leaves from a weed (*Tellima grandiflora*) which is known to contain an ellagitannin, tellimagrandinin II, they recently isolated an enzyme that can catalyze the formation of tellimagrandinin II directly from PGG. This is the first and the only report of ellagitannin formation *in vitro*. However, the yield was low (1.7%, based on substrate weight) and the product was contaminated.

In search for alternative models for the study of ellagitannin biosynthesis, we investigated the use of tissue cultures (Ishimaru and Shimomura, 1991; Neera and Ishmaru, 1992; Neera et al., 1993; Scalbert et al., 1988; Tanaka et al., 1995). The objective of this work was to generate and evaluate callus tissue and suspension cells cultures for the production of ellagitannins and optimize the growth conditions for ellagitannin biosynthesis.

4. 2. Materials and Methods

4. 2. 1. 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 and 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 x 15 mm plastic petri dishes charged with Murashige-Skoog (MS) (Murashige and Skoog, 1962) (20 ml) solid medium supplemented with sucrose (40 g/L), napthaleneacetic acid (NAA, 2 mg/L), benzyladenine (BA, 0.1 mg/L) and Linsmaier/Skoog vitamins (Tanaka et al., 1995). The pH was adjusted to 5.7 before autoclaving at 120 $^{\circ}$C for 20 minutes. The prepared dishes were sealed with parafilm and placed in a dark incubator preset to 23 $^{\circ}$C. Callus induction took approximately two 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.

4. 2. 2. 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 ($CuSO_4 \cdot 5\ H_2O$) is 0.025 mg/L. An identical set of media was prepared using the MS media and supplemental copper but without ammonium nitrate.

4. 2. 3. Suspension Cells Cultures

Oak suspension cell cultures were initiated by transferring oak callus into liquid medium of the same ingredients. The oak cells were shaken in the dark and subcultured weekly. Sweetgum suspension cell cultures were initiated from cells of *Liquidambar styraciflua* grown on solid media agar. The cells were kindly provided by Dr. Merkle at the University of Georgia (Merkle et al., 1998). The cells were suspended in liquid medium and shaken in the dark. The medium was regular MS medium supplemented with NAA (0.5 mg/L)+BA (0.5 mg/L) + kinetin (1 mg/L). Every week, the sweetgum cells were subcultured into fresh media.

4. 2. 4. Driselase Induction

For sweetgum suspension cells, driselase (10 mg, a commercial hemicellulase preparation, Sigma) was dissolved in water (2 ml) and sterilized by pushing through a sterile filter (0.2 μm) with a sterile syringe. The sterilized driselase solution was then added to liquid media (50 ml media in 250 ml flask) containing suspension cells. Driselase was added daily for three days. Each day, before the addition of the driselase solution, cells were sampled and analyzed. For oak suspension cells, the concentration of driselase in media (25 ml media in 125 ml flask) was 100 μg/ml. Each week, cells were sampled and analyzed before they were subcultured into fresh media of the same composition and driselase concentration.

4. 2. 5. Ellagitannins and Ellagic Acid Determinations

Ellagitannin concentrations were determined by first freeze drying the oak callus tissues and determining their dry weight. The callus were subsequently pulverized into a fine powder with a mortar and pestle and suspended in acetone: water (7:3) for 24 hours. Extracts were then analyzed by HPLC for vescalagin and castalagin as described in

Chapter 2. Ellagic acid was determined by hydrolyzing the samples as previously reported (Peng et al., 1991).

4. 3. Results and Discussion

4. 3. 1. Study with Callus Tissues

Tanaka and coworkers (Tanaka et al., 1995) have previously demonstrated that although callus of *Quercus acutissima* grew most slowly in medium containing the plant hormone naphthaleneacetic acid (NAA) and benzyladenine (BA), its tannin productions were higher than those grown in media supplemented with other plant hormones such as either 2,4-dichlorophenoxyacetic acid (2,4-D) plus benzyladenine (BA) or indole-3-acetic acid (IAA) plus kinetin. Therefore in this experiment, *Quercus alba* callus was grown in MS media containing NAA and BA. Callus tissues (Figure 4.1) were generated from fresh stems using MS media supplemented with sucrose, napthaleneacetic acid (2 mg/l), benzyladenine (0.1 mg/l) and Linsmaier/Skoog vitamin (Tanaka, 1995). After 5 weeks of growth, the calli were extracted with acetone: water (7:3) and analyzed with HPLC. Both vescalagin and castalagin were found in the extracts, indicating that oak callus tissues are capable of producing ellagitannins (Figure 4.2). In order to optimize our growth conditions for ellagitannin production, we then began to manipulate the copper and nitrogen levels of the agar media.

Nitrogen has been reported to play an important role in the biosynthesis of tannins in plant tissue cultures (Tanaka, 1995). To evaluate the effect of nitrogen on the production of vescalagin and castalagin by *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 et al., 1986) prompted us to investigate the effect of copper on polyphenol production, since the redox potentials of laccases relative

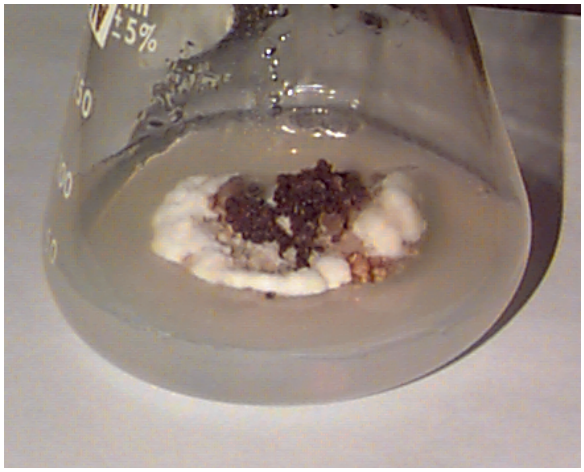


Figure 4.1. Callus of *Quercus alba*

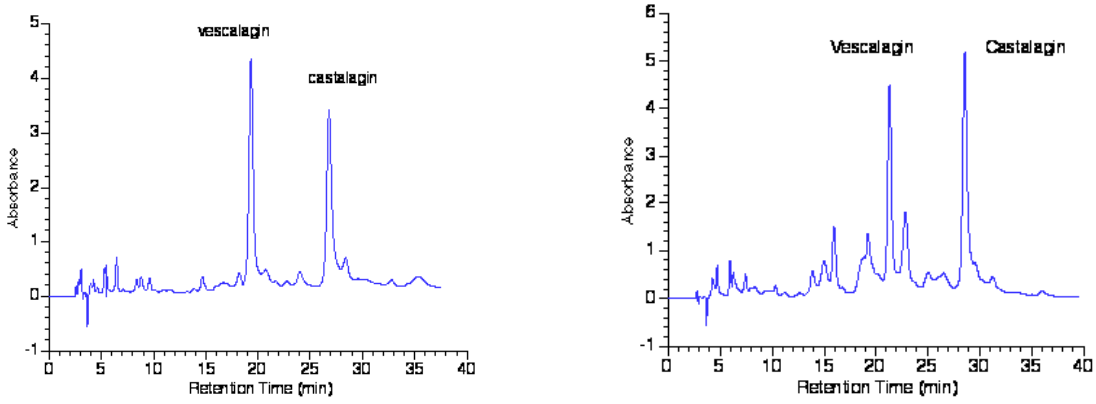


Figure 4.2. HPLC chromatograms of extracts from oak callus (left) and heartwood (right) of *Quercus alba*

Table 4.1 Ellagitannin contents of *Quercus alba* callus tissues grown in modified media preparations[a].				
Copper (mg/L)	Medium	Vescalagin	Castalagin	Insoluble ellagitannins
25	Regular MS	0.75%	0.7%	0.48%
25	MS - NH_4NO_3	0.91%	0.94%	0.5%
62.5	Regular MS	1.13%	0.87%	0.37%
62.5	MS - NH_4NO_3	>0.2%	>0.2%	0.48%
125	Regular MS	0.97%	0.87%	0.42%
125	MS - NH_4NO_3	1.22%	1.01%	0.37%
190	Regular MS	1.18%	0.89%	0.44%
190	MS - NH_4NO_3	1.63%	1.75%	0.51%
250	Regular MS	1.46%	1.25%	0.7%
250	MS - NH_4NO_3	1.2%	1.09%	0.66%

[a]Standard Murishage-Skoog media supplemented with cupric sulfate (mg/L), in the presence (regular MS) or absence (MS - NH_4NO_3) of supplemental nitrogen.

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 calli were harvested and subjected to tannin analyses. As can be seen in Table 4.1, calli grown with additional copper (cupric sulfate) and in the absence of nitrogen produced more vescalagin and castalagin than on the media containing ammonium nitrate. The relatively higher yield of vescalagin and castalagin from the calli grown on nitrogen-deficient media suggests that nitrogen levels may be important in controlling polyphenol biosynthesis in *Quercus alba*. Although the exact cause is unclear, it can be envisioned that media pH changes, cell stress and death arising from nitrogen deficiency may contribute to the increased production of vescalagin and castalagin. The reason for the absence of vescalagin and castalagin in the nitrogen deficient callus tissue grown with a copper content of 62.5 □g/L is unclear.

The effect of copper on the production of vescalagin and castalagin 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 culture (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 et al., 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 the sole criterion for assigning its role to that of lignin

formation. More recently, Tanaka and coworkers (Tanaka et al., 2001) grew *Cornus captitata* adventitious roots in media of different copper concentrations and then incubated PGG with enzyme extracts from the roots. They found that the ability of the crude enzyme extracts to transform PGG into ellagic acid was dependent on the media copper concentrations. Crude enzyme extracts from adventitious roots grown in higher copper concentration media produce almost ten times higher ellagic acid than those from lower copper concentration media. This confirms our experiment that ellagitannin productions are related to copper concentration in media.

Comparison of the polyphenol yields/HPLC profiles of the oak heartwood with those of callus reveals that callus produces significantly more vescalagin and castalagin (vescalagin: 0.45% in heartwood and 1.46% in callus, castalagin 0.55% in heartwood and 1.25% in callus; based on dry wt.). This demonstrates that it is possible to regulate the biosynthesis of polyphenols in callus by changing the composition and contents of media. The ratio of vescalagin and castalagin differs between heartwood and callus, with more vescalagin present in the callus tissues. This can be explained by both the relative instability of vescalagin as well as the fact that most of the C-glycosidic ellagitannin dimers found in oak are derived from vescalagin; these compounds are not present in high concentrations in the callus tissues. The presence of ellagitannins and ellagic acid indicates the presence of biaryl coupling enzymes. To confirm this and see if they are readily extracted from callus, oak callus tissue were ground in nitrogen and extracted with Tris-acetate buffers (50 mM, pH 6). After ammonium sulfate precipitation (30-60% of saturation point), dialysis and subsequent concentration with molecular weight cutoff 30 kD, the crude enzyme extracts were incubated with synthetic PGG. The control was performed by incubating PGG with the crude enzyme solutions denatured at 100 °C for 5

min. The HPLC revealed the formation of ellagic acid in the solution as shown in Figure 4.3.

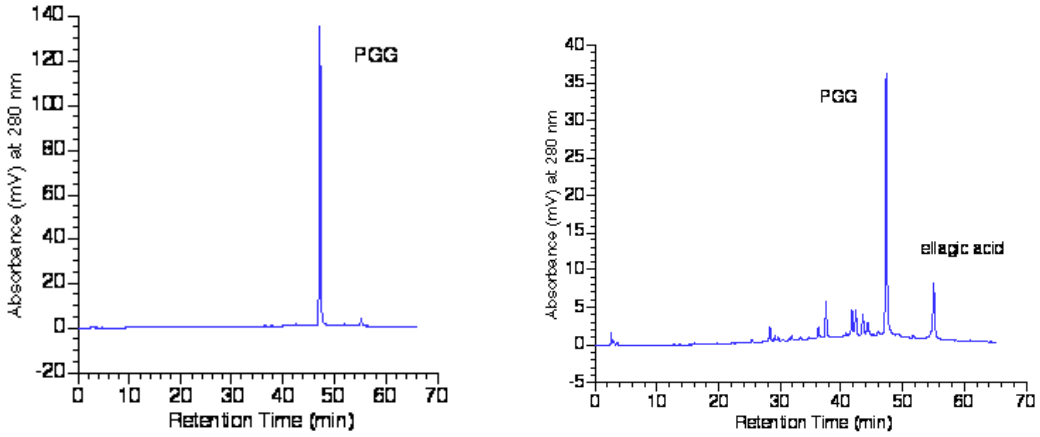


Figure 4.3. HPLC chromatograms of oak (*Quercus alba*) callus crude enzyme extracts incubated with PGG at time zero (left) and after 3 days (right).

Although no ellagitannins were found, the HPLC chromatogram (Figure 4.3) revealed the presence of the ellagitannin degradation product, ellagic acid. The HPLC analyses indicated that PGG decreased by 74.3%, ellagic acid increased by 58.9% and no significant gallic acid peak was found. In the control, no decrease of PGG was observed. This finding indicates that active enzyme was present in crude enzyme extracts. The presence of both vescalagin/castalagin and active enzyme suggest that the oak callus tissue culture is an appropriate model for the study of the biosynthesis of vescalagin and castalagin *in vitro*. Similar results were also obtained with *Cornus capitata* adventitious roots grown in MS media supplemented with different amounts of with $CuSO_4$ (Tanaka et al., 2001). Enzyme extracts from the roots was also found to convert PGG into ellagic acid. But no ellagitannins were found.

Although the oak callus produces ellagitannin, it grows very slowly. The slow growth of callus limits the availability of enough sample for enzyme purification and characterization. To solve this problem, we turned to suspension cell cultures. Suspension cell cultures have been demonstrated to grow much faster than callus tissue cultures (Neera and Ishmaru, 1992; Neera et al., 1993). In the case of sweetgum suspension cell cultures, cell growth is about 25 times that of callus tissue (Neera et al., 1993), suggesting that suspension cells might be a good alternative system. Oak suspension cells were first initiated by transferring oak callus into liquid medium containing the same ingredients as in the solid medium. Four weeks after the induction of oak suspension cells, cells were tested for ellagic acid. However, no ellagic acid was found in the oak suspension cells. The lack of ellagic acid indicates that no ellagitannins are produced in oak suspension cells. While the exact reason for the absence of ellagitannins is unclear, the environment in suspension cell cultures is certainly different from that in calli and trees. Indeed, although ellagitannin production has been reported in oak callus tissues (Scalbert et al., 1988; Tanaka et al., 1995), it has never been reported in oak suspension cells.

Considering that the ellagitannins are defense compounds whose synthesis responds to external stress, we used driselase to elicit the oak cells. Driselase is a commercial hemicellulase preparation that can mediate the breakdown of carbohydrates in cell walls. This would mimic the attack of fungi that also secrete enzymes to break down cell wall components to simple carbohydrates. Driselase stock solution was prepared by dissolving driselase into water and sterilized using sterile 0.2 □m filters. The sterile driselase solution was then added to the oak suspension cell cultures to make the final concentration of driselase of 100 □g/ml. Every week, the oak cells were subcultured in fresh media of the same compositions and PGG concentration after the cells were sampled.

In order to assure the effectiveness of the driselase treatment, synthetic PGG was also used in the experiment. The final concentration of PGG in the oak suspension cell cultures was 65 □g/ml. Cells were analyzed for ellagic acid two and four weeks after the addition of driselase and driselase plus PGG. Controls were performed without the addition of driselase. For the purpose of comparison, oak callus grown on solid medium of the same compositions were also analyzed for ellagic acid and also shown in Table 4.2.

It is evident that oak suspension cells can be elicited to produce ellagitannins by the addition of driselase and PGG. Sensing the attack, oak suspension cells trigger a series of biochemical events leading to the synthesis of biaryl coupling enzymes as evidenced by

Table 4.2 Ellagitannin production in oak suspension cells and callus

Time (week)	Treatment	Ellagic acid	Methyl gallate	EA/MG ratio
2	control	n. d.	n. d.	N/A
2	Driselase	n. d.	n. d.	N/A
2	Driselase + PGG	0.05%	0.12%	0.55
4	control	n. d.	n. d.	N/A
4	Driselase	n. d.	n. d.	N/A
4	Driselase +PGG	0.07%	0.15%	0.64
Oak callus		0.58%	0.19%	3.7

EA/GA is the molar ratio of biaryl-linked gallic acid to non-biaryl linked gallic acid. Higher EA/GA ratio means that more galloyl groups are linked together via biaryl linkage.

the presence of ellagic acid in oak suspension cells treated with both driselase and PGG. The lack of ellagitannins in oak suspension cells treated with driselase but without PGG suggests that the formation of PGG is not occurring in oak suspension cells. Comparison

of oak callus and oak suspension cells reveals the low concentrations of ellagic acid or ellagitannins present in these suspension cells. A lower EA/MG value also demonstrates that although substrate PGG was supplied, oak suspension cells could not efficiently convert it into ellagitannins. This work indicates that while oak callus tissues may be used as models for biosynthesis study, under our conditions oak suspension cells cultures may not.

4. 3. 2. Study with Sweetgum Suspension Cell Cultures

Although the oak callus appears to be an appropriate model for ellagitannin biosynthesis study, it grows too slowly. As described above, oak suspension cell cultures are not suitable for the biosynthetic studies either. Because of this we then turned to sweetgum suspension cell cultures. Although oaks and sweetgum belong to different families (oaks belong to the family of Fagaceae while sweetgum belongs to the family of Hamamelidaceae), sweetgum has been demonstrated to produce ellagitannins and grow much faster than callus tissue cultures (Neera et al., 1993). In addition, sweetgum cells are readily available from Dr Scott Merkle's laboratory at the Department of Forestry in the University of Georiga. The sweetgum suspension cell cultures were initiated by transferring 4 different sweetgum cell lines (Merkle etal., 1998) into liquid media containing 2,4-D and BA as described previously (Neera et al., 1993). The cells were shaken in the dark and subcultured into fresh media weekly.

Six weeks after the induction of suspension cell cultures, all four cell lines were analyzed for ellagic acid (Peng et al., 1991), which confirmed the presence of ellagic acid in the samples. The presence of ellagic acid demonstrates that sweetgum suspension cells are capable of producing ellagitannins. Subsequent extraction of enzymes from sweetgum cells and the incubation of substrate PGG with the crude enzyme extracts revealed the

presence of active enzymes in the crude extracts. The control with boiled crude enzyme solution provided little ellagic acid, and no PGG was consumed. Incubation of the crude enzyme extracts with gallic acid revealed that ellagic acid was not formed, suggesting that PGG is the substrate. If PGG was hydrolyzed first to give gallic acid (which was then oxidized to ellagic acid), incubation with gallic acid would have produced ellagic acid. In a more recent report (Tanaka et al., 2001), crude enzyme preparations from *C. capitata* roots were found to convert PGG into ellagic acid but not gallic acid into ellagic acid. This finding also confirmed our data that PGG is the substrate in the formation of ellagic acid.

Unfortunately, the capability of sweetgum cells to produce ellagitannins and their crude enzyme extracts to convert PGG into ellagic acid was unstable. During attempts to purify enzymes from sweetgum cells over a 4 month period, sweetgum cells were found to gradually lose the capability to produce ellagitannins, and their crude enzyme extracts could not convert PGG into ellagic acid any more. This phenomenon reflects the instability of suspension cells in the production of secondary metabolites (Holden et al., 1988b). Indeed, temporal and spatial variations in suspension cell cultures have often been observed. Although the variation may give rise to higher yield of secondary metabolites, it often results in the decrease in the production of secondary metabolites by suspension cells (Holden et al., 1988b). And this instability of cell cultures often makes it extremely difficult to rely on suspension cells cultures for the production of secondary metabolites (Fowler, 1986). Although the variation in cell cultures is a common phenomenon, the exact causes in this case are unclear as many factors could contribute to the variation and instability (Fowler, 1986). The genomic instability in cell cultures may also be the cause of the variation in cell cultures (Fowler, 1986; Bayliss, 1973 and 1980).

Cell cultures are known to be elicited to produce secondary metabolites (Holden et al., 1988a). In an attempt to stress sweetgum suspension cells to produce ellagitannins, driselase was used to elicit the cells. Two treatments were used: (1) driselase, and (2) driselase plus PGG. A control was also performed without addition of driselase and PGG. No ellagic acid was found in both the control and the driselase treated cells. An ellagic acid peak was found in the cells treated with driselase and PGG. But the ellagic acid peak was too small to be quantified, indicating that ellagitannin contents were very low in the cells.

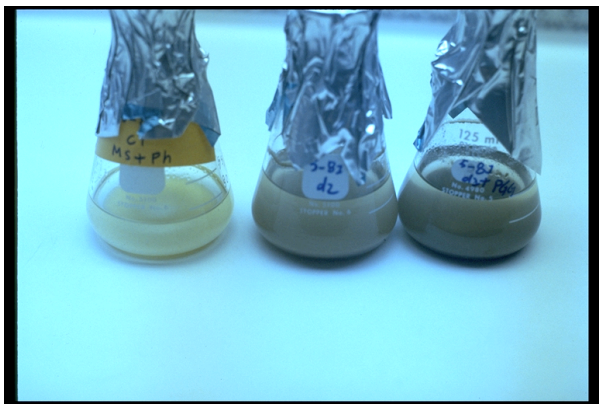


Figure 4.4. Suspension cells cultures of *Liquidambar styraciflua*
left: control; middle: treated with driselase; right: driselase + PGG

4. 4. Conclusion

Oak callus tissues have been demonstrated to be capable of producing ellagitannins. The ellagitannin production and profile are similar to those found in white oak heartwood. This is the first report of a white oak (*Quercus alba*) callus that can produce ellagitannins similar to the oak heartwood. Furthermore, the production and profile of ellagitannins can be modified by adjusting the media compositions. Comparison of extracts from the heartwood of *Quercus alba* with those from callus tissue reveals callus tissues can produce almost 3 times as much castalagin and vescalagin, through manipulation of the

media nitrogen and copper concentrations. Suspension cells of *Quercus alba* and *Liquidambar styraciflua* were found unsuitable for the study of biosynthesis of ellagitannins. Production of ellagitannins in sweetgum cell cultures were unstable and no ellagitannins were found in oak suspension cells cultures. Although they can be elicited to produce ellagic acid with driselase, the levels of ellagic acid was too low for metabolic study.

REFERENCES

Bayliss, M. W. (1980) Chromosomal Variation in Plant Tissues in Culture. *Int. Rev. Cytol.* Suppl., **11A**: 113-144.

Bayliss, M. W. (1973) Origin of Chromosome Number Variation in Cultured Plant Cells. *Nature*, **246**: 529-530.

Bligny, R.; Gaillard, J. and Douce, R. (1986) Extraction of Laccase by Sycamore (*Acer pseudoplatanus* L.) Cells: Effects of a Copper Deficiency. *Biochem. J.*, **237**: 583-588.

Charrier, B.; Haluk, J. P. and Metche, M. (1995) Characterization of European Oak Wood Constituents Acting in the Brown Discoloration During Kiln Drying. *Holzforschung*, **49**: 168-172.

Fowler, M. W. (1986) Industrial Applications of Plant Cell Culture. In *Plant Cell Culture Technology*. Yeoman, M. M. eds.; Blackwell Scientific Publication, 202-227.

Gross, G. G. (1992) Enzymatic Synthesis of Gallotannins and Related Compounds. In *Phenolic Metabolism in Plants*; Satfford, H.A.; Ibrahim, R. K. eds.; Plenum Press, New York, 297-324.

Gross, G. G. (1983) Partial Purification and Properties of UDP-Glucose: Vanillate 1-O-Glucosyl Transferase from Oak Leaves. *Phytochemistry*, **22**: 2179-2182.

Helm, F. R.; Lei, Z.; Ranatunga, T.; Jervis, J. and Elder, T. (1999) Toward Understanding Monomeric Ellagitannin Biosynthesis. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology and Ecology;* Gross, G. G.; Hemingway, R. W.; Yoshida, T. eds.; Plenum Press, New York, 83-89.

Holden, M. A.; Holden, P. R. and Yeoman, M. M. (1988a) Elicitation of Cell Cultures. In *Manipulating Secondary Metabolism in Culture*. Robins, R. J.; Rhodes, M. J. C. eds.; Cambridge University Press, 57-65.

Holden, P. R.; Holden, M. A. and Yeoman, M. M. (1988b) Genetic and Biochemical Variation in Culture. In *Manipulating Secondary Metabolism in Culture*. Robins, R. J.; Rhodes, M. J. C. eds.; Cambridge University Press, 15-29.

Ishimaru, K. and Shimomura, K. (1991) Tannin Production in Hairy Root Culture of *Geranium thunbergii*. *Phytochemistry*, **30**: 825-828.

Krajci, I. and Gross, G. G. (1987) Formation of Gallotannins in Callus Cultures from Oak (*Quercus robur*). *Phytochemistry*, **26**: 141-143.

Marra, A. A. (1992) Technology of Wood Bonding, Principles in Practice, Van Nostrand Reinhold, New York, 150-152.

Merkle, S. A.; Neu, K. A.; Battle, P. J. and Bailey, R. L. (1998) Somatic Embryogenesis and Plantlet Regeneration from Immature and Mature Tissues of Sweetgum (*Liquidambar styraciflua*). *Plant Science*, **132**: 169-178.

Mila, I.; Scalbert, A. and Expert, D. (1996) Iron Withholding by Plant Polyphenols and Resistance to Pathogens and Rots. *Phytochemistry*, **42**: 1551-1555.

Mosedale, J. R.; Puech J. -L. and Feuillat, F. (1999) The Influence on Wine Flavor of the Oak Species and Natural Variation of Heartwood Components. *Am. J. Enol. Vitic.,* **50**: 503-512.

Murashige, T. and Skoog, F. A. (1962) Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum* , **15**: 473-497.

Neera, S.; Arakawa, H. and Ishimaru, K. (1993) Tannin Production in Cell Cultures of *Liquidambar styraciflua*. *Phytochemistry*, **32**: 921- 924.

Neera, S. and Ishmaru, K. (1992) Tannin Production in Cell Cultures of *Sapium sebiferum*. *Phytochemistry*, **31**: 833-836.

Niemetz, R.; Schilling, G. and Gross, G. G. (2001) Ellagitannin Biosynthesis: Oxidation of Pentagalloylglucose to Tellimagrandin II by an Enzyme from *Tellima Grandiflora* Leaves. *Chem. Commun.*, 35-36.

Peng, S.; Scalbert, A. and Monties, B. (1991) Insoluble Ellagitannins in *Castanea sativa* and *Quercus petraea* Woods. *Phytochemistry*, **30**: 775-778.

Quinn, M. K. and Singleton, V. L. (1985) Isolation and Identification of Ellagitannins from White Oak Wood and an Estimation of Their Roles in Wine. *Am. J. Enol. Vitiv.*, **36**: 148-155.

Scalbert, A. (1992) Tannins in Woods and Their Contribution to Microbial Decay Prevention. In *Plant Polyphenols*: *Synthesis, Properties, Significance;* Hemingway, R.W.; Laks, P. E. eds.; Plenum Press, New York, 935-951.

Scalbert, A. (1991) Antimicrobial Properties of Tannins. *Phytochemistry*, **30**: 3875-3883.

Scalbet, A.; Monties, B. and Favre, J. –M. (1988) Polyphenols of *Quercus Robur*: Adult Tree and *in vitro* Grown Calli and Shoots. *Phytochemistry*, **27**: 3483-3484.

Sterjiades, R.; Dean, J. F. D. and Eriksson, K. -E. L. (1992) Laccase from Sycamore Maple (*Acer pseudoplatanus*) Polymerizes Monolignols. *Plant Physiol*., **99**: 1162-1168.

Tanaka, N.; Shimomura, K. and Ishimaru, K. (2001) Ellagic Acid Formation from Galloylglucose by a Crude Enzyme of *Cornus capitata* Adventitious Roots. *Biosci. Biotechnol. Biochem.*, **65**: 1869-1871.

Vivas, N. and Glories, Y. (1996) Role of Oak Wood Ellagitannins in the Oxidation Process of Red Wines During Aging. *Am. J. Enol. Vitic*., **47**: 103-107.

Tanaka, N.; Shimomura, K. and Ishimaru, K. (1995) Tannin Production in Callus Cultures of *Quercus acutissima*. *Phytochemistry*, **40**: 1151-1154.

Xu, F.; Shin, W.; Brown, S.H.; Wahleithner, J.A.; Sundaram, U.M. and Solomon, E.I. (1996) A Study of a Series of Recombinant Fungal Laccases and Bilirubin Oxidase that

Exhibit Significant differences in Redox Potential, Substrate Specificity, and Stability. *Biochim. Biophys. Acta*, **1292**: 303-311.

Chapter Five

Use of High Performance Liquid Chromatography – Mass Spectrometry in the Identification of Ellagitannins

Abstract: A method using high performance liquid chromatography – mass spectrometry was developed and optimized with purified ellagitannins. Ellagitannins analyzed under the optimal conditions all provide base peaks of $(M-H)^-$ from which the molecular weights of the ellagitannins can be determined. Mild fragmentation was also achieved to give fragments characteristic of ellagitannins (loss of ellagic acid and gallic acid if present). These characteristic peaks allow for rapid identification of ellagitannins from other secondary metabolites present in the samples. Application of the HPLC/ESI-MS in the identification of monomeric ellagitannins in white oak heartwood extracts revealed that it can unambiguously identify the two monomeric ellagitannins, castalagin and vescalagin, and their degradation product, ellagic acid. The key fragmentation pathways of the ellagitannins are also described.

5. 1. Introduction

Reliable analytical methods play important roles in the study of secondary metabolism either *in vivo* or *in vitro*. Although simple and popular, colorimetric assays involving chemical reagents such as KIO_3, rhodanine and $NaNO_2$ are not suitable for the identification of individual ellagitannins (Mueller-Harvey, 2001; Scalbert, 1992). To ascertain the amount or presence/absence of a particular secondary metabolite in samples or a product of an enzyme mediated reaction *in vitro*, one needs to separate it from other compounds, and subsequently identify and quantify the compound. High performance liquid chromatography (HPLC) has been proved to be very useful in the separation of secondary metabolites (Charrier et al., 1992; Häkkinen et al., 1998; Scalbert et al., 1990). However, the complexity and the broad range of secondary metabolites in plant extracts have made it almost impossible to rely solely on HPLC to rapidly identify compounds in the samples. It is a secondary method based on similar retention times of standard and unknown. The lack of readily available ellagitannin standards makes identification by this method difficult. Even with appropriate standards at hand, rapid and unambiguous identification of compounds is sometimes not straightforward as compounds may still co-elute.

HPLC coupled with mass spectrometry is a more robust method for identifying plant secondary metabolites. Combining the separation power of HPLC with the high sensitivity of mass spectrometry, HPLC-MS has found many uses in biological science including drug metabolism (Iwabuchi et al., 1994) and secondary metabolism (Nawwar et al., 1997; Salminen et al., 1999). However, HPLC/MS has a characteristic problem, i. e. matching the interface of HPLC/MS to the nature of analytes. The interface is very important as an appropriate interface will allow analytes to be ionized and subsequently detected by mass spectrometers. Several interfaces such as atmospheric pressure chemical ionization

(APCI), thermospray (TSP) and electrospray ionization (ESI) are now available for the analyses of non-volatile and polar organic compounds. Successful applications of HPLC/MS in the identification of phenolic compounds have previously been reported (Nawwar et al., 1997; Salminen et al., 1999). But only a few reports concerned ellagitannins (Barry, 2001; Salminen et al., 1999). In this work, we reported the development and use of a HPLC/ESI-MS method in the identification of ellagitannins. Purified ellagitannin were analyzed in order to establish the fragmentation patterns of ellagitannins in ESI mode. These data may serve as database for rapid screening of ellagitannins in samples. The availability of the sensitive and fast analytical HPLC/MS method should be valuable and aid the elucidation of ellagitannin biosynthesis.

5. 2. Materials and Methods

Six ellagitannins, pedunculagin, casuarictin, potentillin, tellimagrandin I, tellimagrandin II and casuarinin were kindly provided by Prof. Takashi Yoshida at the Faculty of Pharmaceutical Science, Okayama University, and Prof. Takashi Tanaka at the School of Pharmaceutical Sciences, Nagasaki Univerisity, Japan. Vescalagin and castalagin were isolated from extracts of white oak (*Quercus alba*) (Lei et al., 1999) and confirmed by the ^{1}H NMR spectra obtained from Bruker AM 360 located in the Chemistry Department and compared with the published ^{1}H NMR spectra (Tang and Hancock, 1995).

HPLC/UV/ESI-MS analysis: HPLC separations were carried out on a Shimadzu unit using a Phenyl-3 column (250mm x 4.6mm). The mobile phase was composed of acetonitrile (solvent A) and 10 mM formic acid (solvent B). Gradient condition: solvent B decreased from 100 to 50% over 45 min, from 50% to 0% in 5 min, maintained at 0% for 5 minutes, then increased to 100% over 5 minute and maintained at 100% for 5 minutes to equilibrate the column. The flow rate was 0.75 ml/min. Compounds were detected with

UV dual wavelength monitoring at 240 and 280 nm. Mass spectrometry was carried out on a Shimadzu LCMS-QP8000 with an electrospray ion source, using the Class-8000 version software. The instrument was operated in the negative ionization mode, scanning from *m/z* 150-1500. Operating conditions: negative ionization ESI probe voltage: -4.5 KV, CDL (curved dissociation line) voltage: 90 V and temp: 230 °C, all four deflector voltage: 100V, detector voltage: 2.5 KV, and nebulizing gas (N_2): 4.5 ml/min.

5. 3. Results and Discussion

5. 3. 1. ESI Optimization

Separation of ellagitannins in plant extracts with HPLC has been well-established (Charrier et al., 1992; Häkkinen et al., 1998; Scalbert et al., 1990). But the gradient conditions developed for the HPLC separation may not be optimal for the HPLC/MS analyses as mobile phases affect the ionization of analytes in mass analyzer (Salminen et al., 1999). While methanol/water has been found to be excellent mobile phases in the reverse phase HPLC separation of ellagitannins, methanol was found to suppress the ionization of ellagitannin standards in the negative ESI mode. In the negative ESI mode, analytes are deprotonated under the influence of electrical field to give ions $(M-H)^-$. Methanol is thought to compete for the deprotonation and result in the poor ionization of ellagitannins. Therefore in this experiment, acetonitrile / 10 mM formic acid was used. The purpose of dilute acid (10 mM formic acid) in the mobile phase is to ensure a satisfactory separation of plant phenolics as trace acid in mobile phase can sharpen phenolics peaks, reduce peak tailing and increase resolutions. However, higher concentrations of formic acid should be avoided. Like any other carboxylic acid that can easily be deprotonated, higher concentration of formic acid can suppress the ionization of compounds in the negative ionization mode.

The Shimadzu LC/MS-8000 system is equipped with two ionization probes, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) probes. Comparison of three interfaces (APCI, TSP and ESI) (Iwabuchi et al., 1994) revealed ESI is much more sensitive than both APCI and TSP ionization methods. In the case of analyzing drugs (Iwabuchi et al., 1994), ESI ionization method was found to be 100 times more sensitive than APCI ionization. Successful application of ESI-MS in the analyses of hydrolyzable tannins has also been reported (Barry, 2001; Salminen et al., 1999). Therefore, in this experiment, the analyses are performed in negative ESI mode.

After eluting from the HPLC column, compounds are introduced into the ESI probe, the tip of which is applied a high voltage. As the compounds are sprayed from the tip, they are ionized under the influence of the high electrical field. Electrospray ionization is a soft or gentle ionization method in mass spectrometry, which can generate multiply charged molecular ions $(M +/-n\ H)^{+/-n}$. Because of its ability to generate multiple charged ions, ESI was originally intended for the identification of large molecules such as proteins and peptides (Heath and Giordani, 1993; Mock et al., 1993). While the molecular ion $(M-H)^-$ provides information about analytes' molecular weight, it does not give any insights into the structure. Information about the structures can be gathered from fragmentation of the molecules. Fragmentation of molecular ions can be achieved by collision induced dissociation (CID). This can be done by adjusting the voltages of the curved dissociation line (CDL) and the deflectors. It is recommended by the manufacturer that for compounds to fragment, voltages for CDL should be above 70V and the voltage for deflectors should be above 80V. Using pentagalloylglucose (PGG), ESI conditions were optimized in such a way that the molecular ion is the base peak and yet fragmentation also occurs to give fragments. The optimal conditions are as followings: negative ionization, the probe tip

voltage: -4.5 KV, CDL voltage: 90 V and temp: 230 °C; all four deflector voltages: -100V. detector voltage: 2.5 KV; nebulizing gas (N_2): 4.5 ml/min. Contrary to the previous report (Salminen et al., 1999) that "the fragmentation pattern of ellagitannins was less clear", we found that the fragmentation of ellagitannins follows a certain pattern characteristic of ellagitannins, i. e. loss of ellagic acid. Under these optimal conditions, ellagitannins undergo mild fragmentation to give fragment ions but still give the base peaks of $(M-H)^-$. As shown in Table 5.1, all the monomeric ellagitannins analyzed (Figure 4.1) under the optimal conditions give the base peaks of $(M-H)^-$. Molecular weights of ellagitannins thus can be derived easily from the base peaks. Another peak is a peak with the *m/z* value of 301. This is a diagnostic peak of ellagitannins derived from the loss of ellagic acid and was found in all ellagitannin investigated. Other major peaks are simply fragments of ellagitannins losing the ellagate moiety or galloyl groups.

5. 3. 2 Application of HPLC/ESI-MS in the Identification of ellagitannins

Use of HPLC/ESI-MS in the identification of ellagitannins has been reported. Salminen and coworkers (Salminen et al., 1999) reported a base peak of *m/z* 939 in the ESI mass spectrum of pentagalloylglucose (PGG) and several other peaks (*m/z*: 769, 469, 393) but did not report a peak of *m/z* 169. In our mass spectrum of PGG, three major peaks (*m/z*: 939, 769, and 169) were observed as shown in Table 5.1 and Figure 5.2. Peaks of *m/z* 469 and 313 were also observed but at very low abundance. These three peaks suffice the identification of PGG in the negative ESI mode. The peak of *m/z* 169 is very important, as it is the characteristic peak of galloylglucose and any ellagitannins bearing free galloyl groups. Indeed, vescalagin/castalagin which do not have free galloyl do not show a peak

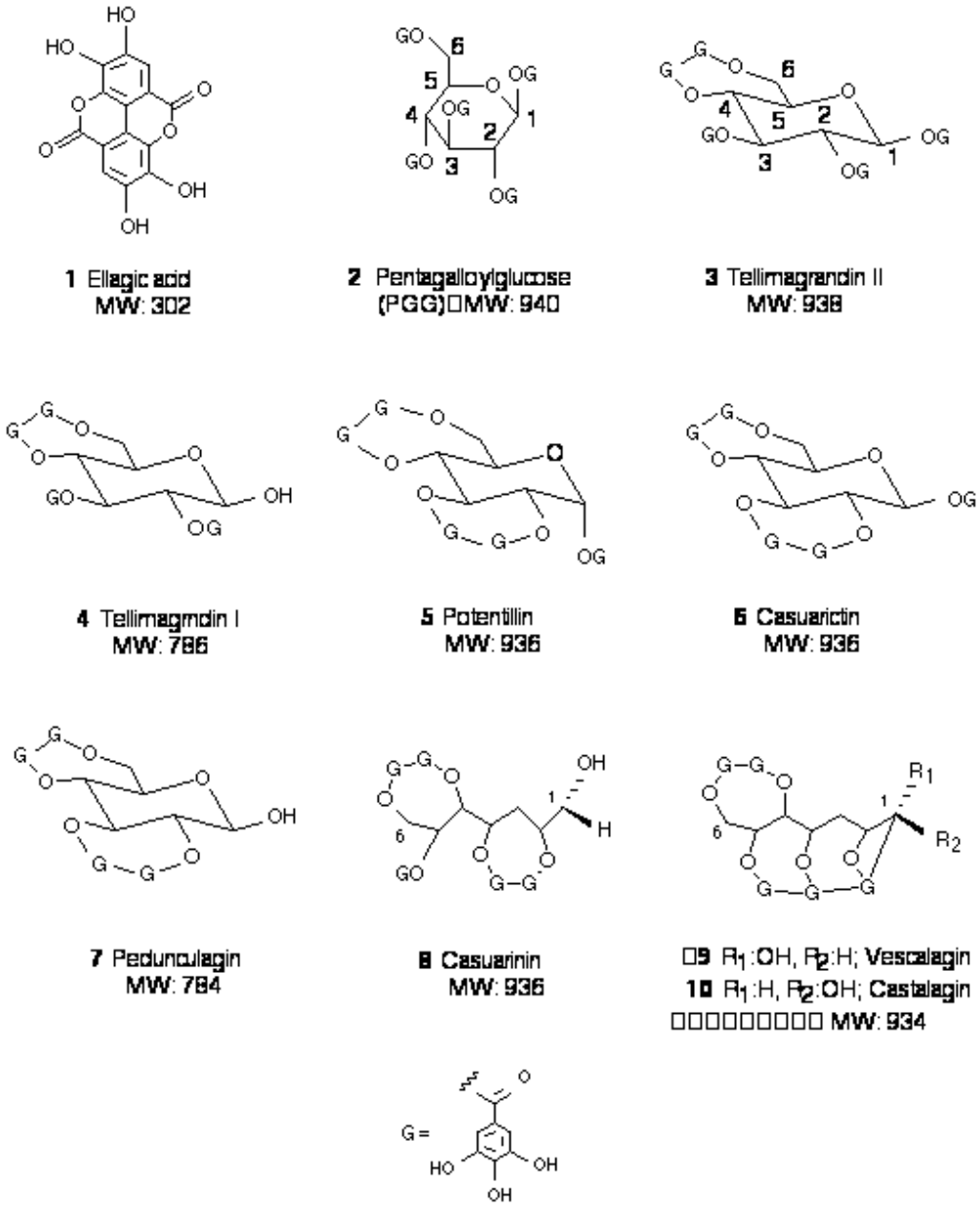


Figure 5.1. Simplified structures and molecular weights of monomeric ellagitannins

Table 5.1 Characteristic ellagitannin HPLC/ESI-MS data						
Compound		MW	(M-H)-	Ret. Time (min)	Major Ions (*m/z*)	
					Base Peak	Other
1	Ellagic acid	302	301	30.8	301	
2	PGG	940	939	32.5	939	169, 769
3	Tellimagrandin II	938	937	31.1	937	169, 301, 767
4	Tellimagrandin I	786	785	24.9	785	169, 301, 615
5	Potentillin	936	935	30.6	935	169, 301, 633
6	Casuarictin	936	935	29.8	935	169, 301, 633
7	Pedunculagin	784	783	21.1	783	169, 301, 481
8	Casuarinin	936	935	25.1	935	301, 631
9	Vescalagin	934	933	15.8	933	301, 631
10	Castalagin	934	933	19.3	933	301, 631

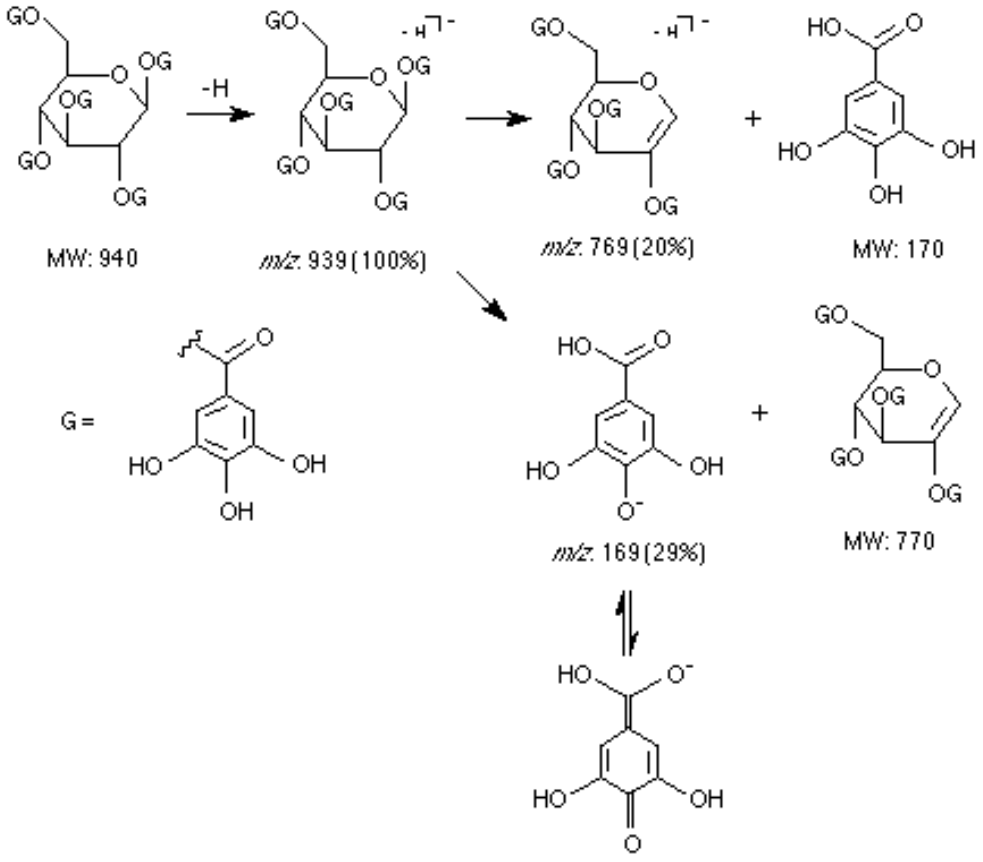


Figure 5.5. Proposed fragmentation of PGG (MW: 940) and loss of galloyl group in negative HPLC/ESI-MS. Numbers in parenthesis are relative abundance.

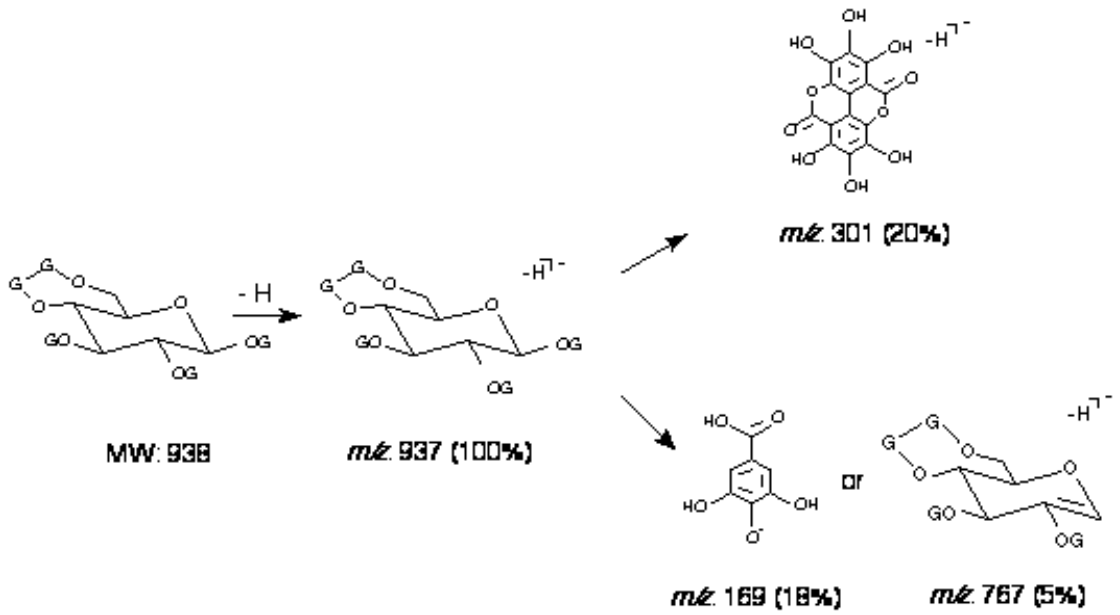


Figure 5.6. Proposed loss of HHDP groups from tellimagradin II (MW: 938) in negative HPLC/ESI-MS. Numbers in parenthesis are relative abundance.

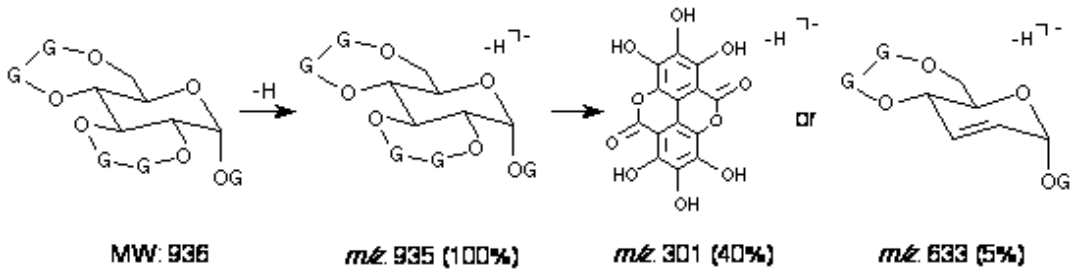


Figure 5.7. Proposed loss of HHDP groups from potentillin (MW: 936) in the negative HPLC/ESI-MS. Numbers in parenthesis are relative abundance

of *m/z* 169 in the mass spectrum (Table 5.1, Figure 5.3). This can be further exemplified by the mass *m/z* ions of potentillin (Figure 5.4) which has two HHDP groups (2,3- and 4,6-HHDP) and one galloyl group (1-β-G). The spectrum (Figure 5.4, Table 5.1) shows several peaks including a base peak of *m/z* 935 corresponding to $(M-H)^-$, a peak of *m/z* 301, and a peak of *m/z* 169. While the peak of *m/z* 169 indicates the presence of free galloyl group in the molecule, the peak of *m/z* 301 is the evidence of the occurrence of HHDP groups (ellagic acid – $H)^-$. This peak (*m/z* 301) like one of the *m/z* 169, however, was not looked at and reported by Salminen and coworkers (Salminen et al., 1999) who only reported peaks above *m/z* 330 (the smallest galloylglucose: 1-β-galloylglucose has the molecular weight of 332) and relied on the molecular weights for the identification of hydrolyzable ellagitannins. We feel it inadequate for unambiguous identification of both gallotannins and ellagitannins as molecular weights alone do not provide structural information about the analytes. We strongly recommend looking for peaks of galloyl and HHDP groups in the lower *m/z* ranges for the evidence of ellagitannins and gallotannins when identifying ellagitannins. It is interesting to see that under the optimized conditions, both tellimagradnin I and II do not fragment to give significant *m/z* values of and 483 and 635 (M-ellagic acid-$H)^-$. Their relative abundance was only about 1%, suggesting the loss of deprotonated ellagic acid is predominant (Figure 5.6). The fragmentation of PGG, tellimagradin II and potentillin are proposed to illustrate the loss of galloyl and HHDP groups (Figures 5.5, 5.6 and 5.7).

5. 3. 3. Application of HPLC/ESI-MS in the Screening of Oak heartwood Extracts for Monomeric Ellagitannins

Oak heartwood extracts have been known to contain two monomeric ellagitannins together with ellagic acid and six oligomeric ellagitannins (Herve du Penhoat, 1991; Mayer et al., 1969; Mayer et al., 1990; Nonaka et al., 1989; Nonaka et al., 1989). The optimized

LC/MS protocol was applied to the extracts which were prepared from white oak heartwood previously (Barry, 2001) and the extracts were analyzed on a Shimadzu LC/MS unit using a Phenyl-3 column (250mm x 4.6mm). Compounds were detected with UV dual wavelength monitoring at 240 and 280 nm. Mass spectrometry was carried out on a Shimadzu LCMS-QP8000 with an electrospray ionization source, using the Class-8000 version software. The instrument was operated in the negative ion mode, scanning from *m/z* 150-1500. The HPLC chromatogram is shown in Figure 5.8. Mass spectrum of each peak reveals that ellagitannins elute between 10 to 20 min as evidence by the presence of *m/z* 301 peak. Peaks with retention time of 15.9 min and 19.4 min have base peaks of *m/z*: 933 $(M\text{-}H)^-$, the characteristic peak (*m/z*: 301) of ellagitannins and a peak at *m/z*: 631. $(M\text{-}H$ - ellagic acid$)^-$. MS spectrum demonstrated these two compounds are vescalagin and castalagin respectively. Compound with retention time of 30.7 min is ellagic acid as its mass spectrum shows only one major peak which is also a base peak (*m/z*: 301). Compounds eluting at 14.2 min and 15.2 min show *m/z* values at 1065 $(M\text{-}H)^-$ and 301. These two compounds are grandinin / roubrin (MW: 1066). The other four peaks between 10 and 20 min are the 4 dimers of vescalagin and castalagin. They all have the base *m/z* values of 301 (ellagic acid – $H)^-$ and other *m/z* values such as 933 (M-castalagin/vescalagin–$H)^-$. The fact that *m/z* value of 301 is the base peak indicates that molecular ions $(M\text{-}H)^-$ of these dimers were not observed, as based upon the results of the monomeric ellagitannins described above, the base peaks should be the molecular ions. The missing molecular ions are due to the lower detection range of this mass spectrometer (maximum limit: *m/z* 1500) while the molecular weights of ellagitannin dimers are above 1800. However, since the molecular weights of monomeric ellagitannins are less than 1000, the system is suitable for monitoring the formation of these ellagitannins.

Reference

Barry, K. M. (2001) Antimicrobial Defense in the Sapwood of *Eucalyptus nitens: Studies of the Reaction Zone*. Ph. D thesis, School of Agricultural Science, University of Tasmania, Australia.

Charrier, B.; Marques, M. and Haluk, J. P. (1992) HPLC Analysis of Gallic and Ellagic Acids in European Oakwood (*Quercus robur* L.) and Eucalyptus (*Eucalyptus globulus*). *Holzforschung*, **46**: 87-89.

Häkkinen, S. H.; Karenlampi, S. O.; Heionen, M. I. (1998) HPLC Method for Screening of Flavonoids and Phenolic Acids in Berries. *J. Sci. Food Agric.*, **77**: 543-551.

Heath, T. G. and Giordani, A. B. (1993) Reversed Phase Capillary High Performance Liquid Chromatography with On-Line UV, Fluorescence and Electrospray Ionization Mass Spectrometric Detection in the Analysis of Peptides and Proteins. *J. Chromatogr.*, **638**: 9-19.

Herve du Penhoat, C. L. M.; Michon, V. M. F.; Peng, S.; Viriot, C.; Scalbert, A. and Gage, D. (1991) Structural Elucidation of New Dimeric Ellagitannins from *Quercus robur* L. Roburins A-E. *J. Chem. Soc. Perkin Trans. I.*, 1653-1660.

Iwabuchi, H.; Kitazawa, E.; Kobayashi, N.; Watanabe, H.; Kanai, M. and Nakamura, K. I. (1994) Studies on Drug Metabolism Using Liquid Chromatography / Mass Spectrometry: Comparison of Three Liquid Chromatographic / Mass Spectrometric Interfaces. *Biol. Mass Spectrom.*, **23**: 540–546.

Lei, Z.; Jervis, J. and Helm, R. F. (1999) C-Glycosidic Ellagitannins from White Oak Heartwood and Callus Tissues. *Phytochemistry*, **51**: 751-756.

Mayer, W.; Seitz, H. and Jochims, J. -C. (1969) Die Struktur des Castalagin. *Liebigs Ann. Chem.*, **721**: 186-193.

Mayer, W.; Seitz, H.; Jochims, J. -C.; Schauerte, K. and Schilling, G. (1971) Die Struktur des Vescalagins. *Liebigs Ann. Chem.*, **751**: 6-68.

Mock, K.; Hail, M; Mylchreest, I.; Zhou, J.; Johnson, K. and Jardine, I. (1993) Rapid High Sensitivity Peptide Mapping by Liquid Chromatography-Mass Spectrometry. *J. Chromatogr.*, **646**: 169-174.

Mueller-Harvey, I. Analysis of Hydrolysable Tannins. (2001) *Animal Feed Science and Technology*, **91**: 3-20.

Nawwar, M. A. M.; Marzouk, M. S.; Nigge, W. and Linscheid, M. (1997) High Performance Liquid Chromatographic / Electrospray Ionization Mass Spectrometric Screening for Polyphenolic Compounds of *Epilobium hirsutum* - The Structure of the Unique Ellagitannin Epilobamide –A. *J. Mass Spectrom.*, **32**: 645-654.

Nonaka, G.; Ishimaru, K.; Azuma, R.; Ishimatsu, M. and Nishioka, I. (1989) Tannins and Related-Compounds. 85. Structures of Novel C-Glycosidic Ellagitannins, Grandinin and Pterocarinin-A and Pterocarinin-B. *Chem. Pharm. Bull.*, **37**: 2071-2077.

Nonaka, G.; Sakai, T.; Tanaka, T.; Mihashi, K. and Nishioka, I. (1990) Tannins and related Compounds. XCVII[1]. Structure Revision of C-Glycosidic Ellagitannins, Castalagin, Vescalagin, Casuarinin and Stachyurin, and Related Hydrolyzable Tannins. *Chem. Pharm. Bull.*, **38**: 2151-2156.

Salminen, J. –P.; Ossipov, V.; Loponen, J.; Haukioja, E. and Pihlaja, K. (1999) Characterization of Hydrolyzable Tannins from Leaves of *Betula pubescens* by High-Performance Liquid Chromatography -Mass Spectrometry. *J. Chromatogr.*, **864**: 283-291.

Scalbert, A. (1992) Quantitative Methods for the Estimation of Tannins in Plant Tissues. In *Plant Polyphenols*. Hemingway, R. W.; Laks, P. E. eds.; Plenum Press, New York, 259-280.

Scalbert, A.; Duval, L.; Peng, S.; Monties, B. and Penhoat, C. D. (1990) Polyphenols of *Quercus robur* L. II. Preparative Isolation by Low-Pressure and High-Pressure Liquid Chromatography of Heartwood Ellagitannins. *J. Chromatogr.*, **52**: 107-119.

Tang, H. R. and Hancock, R. A. (1995) Studies of Vegetable Tannins. Complete Structural Determination of Two Chestnut Tannins-Vescalagin and Castalagin Using Nuclear Magnetic Resonance Spectroscopic Methods. *J. Soc. Leather Technol. Chem.*, **79**: 181-187.

Chapter Six

Use of Proteomics in the Study of Heartwood Formation and Ellagitannin Biosynthesis – A Preliminary Study

Abstract. Proteins in both sapwood and transition wood were extracted and subjected to two-dimensional polyacrylamide gel electrophoresis, activity assays and concentration determinations. It was found that protein concentration in sapwood was much higher than in transition wood. In the case of scarlet oak, the protein concentration in sapwood is about ten times higher than in transition wood. Activity assay indicated that both sapwood and transition wood contained the active enzyme(s) responsible for the biaryl coupling process as evidenced by the formation of ellagic acid from the ellagitannin precursor pentagalloylglucose (PGG). Preliminary results from the 2-D gel separation of sapwood and transition wood proteins showed more protein spots in sapwood than in transition wood, suggesting that sapwood not only had higher protein levels but also a greater total number of proteins. The lower complexity of the transition wood proteome suggests that this material may be a good substrate for studying the biaryl-coupling process.

6. 1. Introduction

Wood has two different regions, sapwood and heartwood (Figure 5.1). While sapwood is a lighter, living, outer part of wood, heartwood is a darker, non-living, central core surrounded by sapwood. Heartwood functions primarily as mechanical support. Whereas sapwood conducts water and minerals from root to all parts of the tree and serves as a reservoir of water, minerals and energy. The formation of heartwood from sapwood is a programmed cell senescence and death process in which a wide range of secondary metabolites are synthesized and deposited in the heartwood (Han, 2001). While the type and the amount of these secondary metabolites vary among different species of trees, they are mainly plant phenolics. These secondary metabolites not only influence the economic value and quality of wood products but also affect wood processing. Understanding the heartwood formation will enable us to control and regulate the process and ultimately improve wood quality.

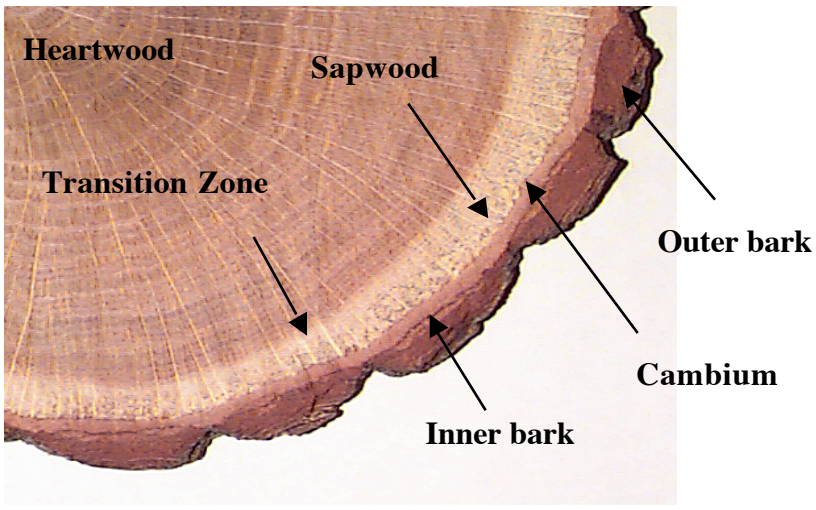


Figure 6.1. Cross section of a white oak (*Quercus alba*)

Transformation of sapwood into heartwood is thought to occur in the late summer and the early autumn, shortly before the beginning of dormancy (Han, 2001; Hauch and Magel, 1998; Magel et al., 2001). During this time, the transition zone or the sapwood/heartwood interface shows a marked increase in metabolic activity (Hauch and Magel, 1998; Magel et al., 2001; Magel et al., 1994). Holl and coworkers observed (Holl and Lendzian, 1973) a maximum consumption of oxygen in the transition zone of *Robinia pseudoacacia*, indicating higher metabolic activities in the transition zone relative to sapwood and heartwood regions. Higher metabolic activities were also found in the transition zone in other heartwood forming species such as oak (Ebermann and Stich, 1985), acacia (Baqui and Shah, 1985), mahogany (Baqui et al., 1979), and black locust (Magel et al., 1994). Enhanced activities of sucrose-metabolizing enzymes such as sucrose synthase (SuSy; EC 2.4.1.13) and neutral invertase (NI; EC 3.2.1.26) were reported in the transition zone in autumn (Hillis and Yazaki, 1973). An increase in the SuSy and NI activities (Hauch and Magel, 1998), glycolysis and respiration (Holl and Lendzian, 1973) indicated that carbohydrates such as sucrose are metabolized to supply carbon atom necessary for the formation of these phenolics (Hauch and Magel, 1998). The higher metabolic activity is also represented by the increased activities of several enzymes. Hillis and co workers (Hillis and Yazaki, 1973) reported the elevated levels of phenol-oxidizing enzymes in the transition zone of *Eucalyptus polyanthemos*. Increased peroxidase activity was also found in the transition zone of *Eucalyptus elaeophora* (Wardrop and Cronshaw, 1962) and *Fagus sylvatica* (Dietrichs, 1964). In addition, other enzymes such as catechol oxidase, malic dehydrogenase, amylase and glucose-6-phosphate dehydrogenase were also found to be active in the transition zone (Hillis, 1987). Two enzymes in the transition zone of black locust which are involved in phenylpropanoid pathway (phenylalanine ammonia-lyase, PAL) and flavonoid biosynthesis (chalcone synthase, CHS) have been studied by Magel and coworkers (Magel et al., 1994). Investigation of their seasonal activities

revealed that PAL was active in the youngest wood near the cambium in April and September but it was active in all seasons in the transition zone. CHS activity was not found in the youngest wood. Based on these results, it was suggested that PAL in youngest wood is involved in lignin biosynthesis, while its role in transition zone is related to the biosynthesis of plant phenolics. It is clear that heartwood formation from sapwood is accompanied by the synthesis of plant phenolics.

The type and amount of these phenolics depend on the species of trees. In the case of oaks, ellagitannins constitute the major phenolics in oaks (Masson et al., 1994 and 1995). Although ellagitannins can be found in sapwood, the highest concentrations of ellagitannins are only found in heartwood with the transition zone containing the highest amount. This is in accord with the black locust results – that ellagitannins biosynthesis occurs at the transition zone.

Heartwood formation is a complex process involving a series of biochemical events leading to the formation of plant phenolics. However, the exact mechanism is still unclear. Although there are several reasons (Han, 2001) for the lack of the knowledge about this important process, lack of appropriate techniques is a major reason. The objective of this work is to evaluate the use of proteomics (analysis of proteins present in transition zone) in the study of heartwood formation.

6. 2. Materials and Methods.

A scarlet oak tree (40 years old) was felled in mid October 2001 with a saw chain. Bolts were cut from the oak trunk and sampled with a wood boring drill (Figure 6.2). Samples (wood dust) were collected with a hand-held vacuum, transferred to a plastic

Figure 6.2. Sampling of sapwood

bag, frozen in liquid nitrogen, and stored in foam boxes containing dry ice. Upon return to the laboratory, the sample bags were transferred into a freezer (-70 °C) for storage. Two bags containing sapwood and transition samples were thawed with running water and were subject to enzyme extraction.

6. 2. 1. Enzyme Extraction and Assay.

Wood dust (about 10 g, wet wt.) was mixed with insoluble PVPP (polyvinylpyrrolidone, 50% wet basis) and transferred to centrifuge bottles with lids. Extraction was performed by vortexing the wood dust in Tris/NaB_4O_7/□-mercaptoethanol buffer (100 / 300 / 1 mM, pH7, 70 ml) (Hauch and Magel, 1998) for 30 min and squeezing the extracts through a 20 ml syringe packed with 4 layers of Miracloth or chessecloth. The filtrate was then centrifuged at 18,000 g for 10 min. The supernatant (enzyme extract) was then precipitated with ammonium sulfate (30-80%, saturation point of ammonium sulfate) and spun at 16,000 g for 30 min. Pellets were collected and redissolved in a minimum amount of Tris-acetate buffer (25 mM, pH 5.7) and desalted on a column (Bio-Rad P-6, DG desalting gel). The UV-active fraction was collected and concentrated with a centrifugal concentrator (molecular weight cutoff: 30 kD). The protein

concentration of both sapwood and transition wood were assayed as previously described (Zuo and Lundhal, 2000). Enzyme assays were performed by incubating 5 μl of PGG stock (26 μg/μl) solution with 150 μl of the concentrated enzyme extracts overnight. The products were analyzed with HPLC.

HPLC was performed on the Gilson HPLC apparatus with a Phenyl-3 column (250mm x 4.6mm) using an external standard analysis. The solvents were MeOH (solvent A) and 0.2% trifluoroacetic acid (solvent B). The flow rate was set at 0.75 ml/min. Gradient conditions: solvent A increased from 0 to 40% over 5 min, followed by a linear increase from 40% to 50% in a 20 min period. All samples were filtered with 0.2 μm syringe filter before injection. Dual wavelengths (240 nm and 280 nm) were used to monitor elution.

6. 2. 2. Two-dimensional polyacrylamide gel electrophoresis

Concentrated enzyme solutions were precipitated with TCA (trichloroacetic acid) (final TCA concentration: 20%) overnight on ice. Pellet was collected by centrifuging the solution at 10,500 g for 15 min. The pellet was then washed with acetone (3 ml x 2) and re-suspended in rehydration buffer adapted from (Görg et al., 2000; Herbert, 1999) (2 M Thiourea, 7 M Urea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.2 M NDSB-256 (benzyldimethylamminopropane sulfonate, Rabidilloud et al., 1997) and 10 % Glycerol).

Two dimensional gel electrophoresis of proteins was adapted from the previously described protocol (Görg et al., 2000; Herbert, 1999). Immobilized pH gradient (IPG) strips (7 and 18 cm) and IPG buffer (pH 3-10) were from Amersham. To each 100 μl of re-suspended protein sample (sapwood and transition wood), added IPG buffer (pH 3-10,

10 µl), ampholites (10 µl), rehydration buffer (400 µl) and 200 mM Tributylphosphine (TBP) solution (10 µl, 500 µl of 97% TBP in 4500 µl anhydrous isopropanol). The samples (total 530 µl) were then shaken for 30 minutes to ensure all the proteins were reduced. Out of the total 530 µl solution, 400 µl was used for the 18 cm IPG strips and the rest of about 125 µl was used in the 7 cm IPG strips. After rehydration for 7 hours, the strips were subject to isoelectric focusing. First dimension isoelectric focusing was carried out as follows: voltage maintained at 1000V for 4 hr, linear increase from 1000 to 8000V in 2 hr and held at 8000V for 13 hr. The second dimension SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) was conducted by sealing the IPG strip on the top of a pre-made 12% total acrylamide gel with agarose (0.5% in SDS electrophoresis buffer). Before being sealed with the second dimensional gel, the IPG strip was equilibrated in 5 ml of equilibration buffer for 30 min to saturate the strip with buffer needed to run the second dimensional gel. The equilibration buffer consisted of 6 M Urea, 30% Glycerol, 2% SDS in 0.05% M Tris-HCl (pH 8.8) and tributylphophine (200mM). After solidification of the agarose, the gel was run at the following conditions: 25 V and 15 mA per gel for the first hour, then voltage increased to 120 V and 15 mA per gel and left over night or until the dye migrated out of the gel. The gel was then placed in fixing solution (45 % Methanol, 10% acetic acid) overnight and then stained with coomassie blue.

The gels were placed on a Bio-Rad scanner (GS-800 calibrated densitometer) and the acquisition parameter was set for coomassie blue gel. The software used to scan the gels was the MagicScan (UMAX Data System, Inc.). After the images were obtained, they were than processed with Melanie 3 software (Genebio, Geneva, Switzland).

6. 3. Result and Discussion.

In our previous attempt to isolate and purify enzymes involved in the biosynthesis of

ellagitannins from oak callus and suspension cell cultures, we encountered one major problem. The productions of ellagitannins in suspension cells are low and unstable. And although the callus tissues can produce ellagitannins similar to those in oak heartwood, the slow growth of callus makes it extremely difficult to generate enough callus tissue for the study of ellagitannin biosynthesis. While oak and sweetgum leaves are abundant, they are also rich in other secondary metabolites which make them virtually unsuitable for isolating and purifying enzymes involved in ellagitannin biosynthesis (Krajci and Gross, 1987). Although the first report of *in vitro* transformation of PGG into tellimagrandin II was catalyzed from an enzyme prepared from leaves of a weed *Tellima Grandiflora* (Niemetz et al., 20001), study of those ellagitannins present in oaks but not in weeds has to rely on oaks.

Masson and coworkers (Masson et al., 1995) observed a sharp increase and the highest concentration of ellagitannins in the transition zone in oaks. This finding indicates that the metabolic activity leading to ellagitannin biosynthesis is the highest along the sapwood/heartwood interface in oak, suggesting the presence of higher amount or elevated activity of enzymes in the transition zone. By looking at all the proteins in the transition zone at different times, i. e. before and at the time of heartwood formation, one should be able to provide a global picture of proteins involved in the heartwood formation and ellagitannins synthesis in oaks. The advent of proteomics based on HPLC/MS and two-dimensional polyacrylamide gel electrophoresis (2-D gel) has made this task possible. While a 2-D gel is capable of resolving thousands of proteins in cells or tissues, the sensitivity of mass spectrometry allows one to identify and characterize each protein spot that can be visualized on a coomassie-stained 2-D gel.

Both sapwood and heartwood were extracted for enzymes with Tris/NaB_4O_7/□-

mercaptoethanol buffer (100 / 300 / 1 mM, pH7) (Hauch and Magel, 1998). Proteins were precipitated with $(NH_4)_2SO_4$ (30-80%), desalted on a Bio-Rad P-6, DG desalting gel column, and concentrated with a centrifugal concentrator (molecular weight cut off: 30 KD). For 2-D gel analyses and protein determination, the concentrated enzyme solution was subject to TCA (trichloroacetic acid, 20%) precipitation. The TCA precipitate was then used in protein concentration determination (Zuo and Lundahl, 2000) and 2-D gel analyses (Görg et al., 2000; Herbert, 1999). For activity assay, the concentrated enzyme preparations were incubated with synthetic PGG for 18 hours (overnight) and analyzed by HPLC.

Protein determinations indicated that the sapwood contained much more protein than transition wood. The protein content for sapwood was at the level of 100 μg/ g wood (wet wt. basis) and 12 μg/ g for the transition wood, about 10 times higher. The lower amount of proteins in the transition zone can be explained by the fact the heartwood formation is a programmed cell death process in which proteases are usually active. However, the lower protein content is also a sharp contrast with the higher metabolic activity and the largest contents of ellagitannins in the transition zone, suggesting that while other proteins are degraded during the transformation of sapwood to heartwood, enzymes involved in the secondary metabolism (ellagitannin biosynthesis in the case of oak) are up regulated. HPLC analyses of the incubation of PGG with crude enzyme preparations from both sapwood and transition wood revealed the formation of ellagic acid from ellagitannin precursor pentagalloylglucose (PGG) as shown in Figures 6.3 and 6.4, confirming the presence of active enzymes in both the sapwood and transition zones. However, no detectable levels of ellagitannins could be identified in the incubation solutions. This again confirms previous statements that “It is evident that sophisticated techniques and unconventional new strategies will be required for the eventual clarification of this

challenging question" (Gross, 1992) and "ellagitannin biosynthesis is an area where much fundamental work still needs to be done" (Helm et al., 1999).

Proteomics based on 2-D gel and mass spectrometry has been extensively used in functional genomics study. Successful use of proteomics in research related to wood has also been reported. Costa and coworkers (Costa et al., 1999) identified several different types of proteins in needles and xylem of maritime pine. Vander-Mijinsbrugge *et al* (Vander-Mijnsbrugge et al., 2000) later applied proteomics in the study of wood formation in poplar. By using comparative 2-D gel and mass spectrometry to look at proteins expressed differentiating in xylem and bark, they identified fifteen out of seventeen proteins expressed preferentially in xylem. However, use of this technique in the identification of proteins involved in the transformation of sapwood into heartwood has not been reported. Preliminary 2-D gel separation of proteins was performed in this work to explore the use of proteomics in the study of biaryl coupling process. Proteins extracted from both sapwood and transition wood samples were then subject to the 2-D gel separation with the 7 and 18 cm IPG strips. As shown in Figures 6.5 and 6.6, more protein spots were present in sapwood than in the transition wood. This suggested that sapwood has more proteins not only in the quantity but also in the number of proteins. The relative fewer protein number in the transition woods may imply the relative ease to isolate enzymes from transition woods, as many other compounds present including proteins can be envisioned to implicate the protein isolation. Separation of proteins with larger 2-D gels (18 cm) also provided similar results (Figure 6.7). But the proteins in the 16 cm 2-D gel were less visible and the protein spots of transition wood samples were too faint to be imaged. This could be the result of protein diffusion during longer run of second dimension (18 hours as opposed to 3 hours in smaller gels) or relatively small load of proteins. Efforts to optimize the 2-D gel separation of proteins are still underway.

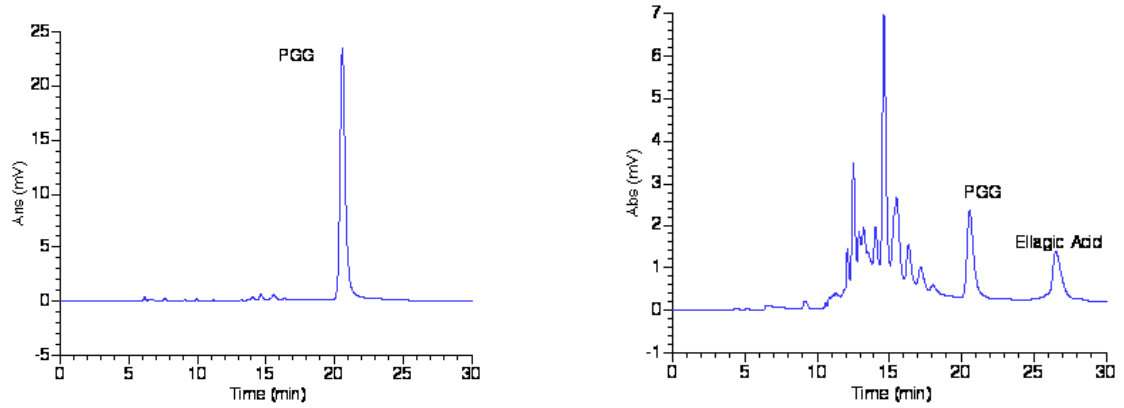


Figure 6.3. HPLC chromatograms (280 nm) of incubation of PGG with crude enzyme preparation from sapwood at t= 0 (left) and t = 18 hours (right)

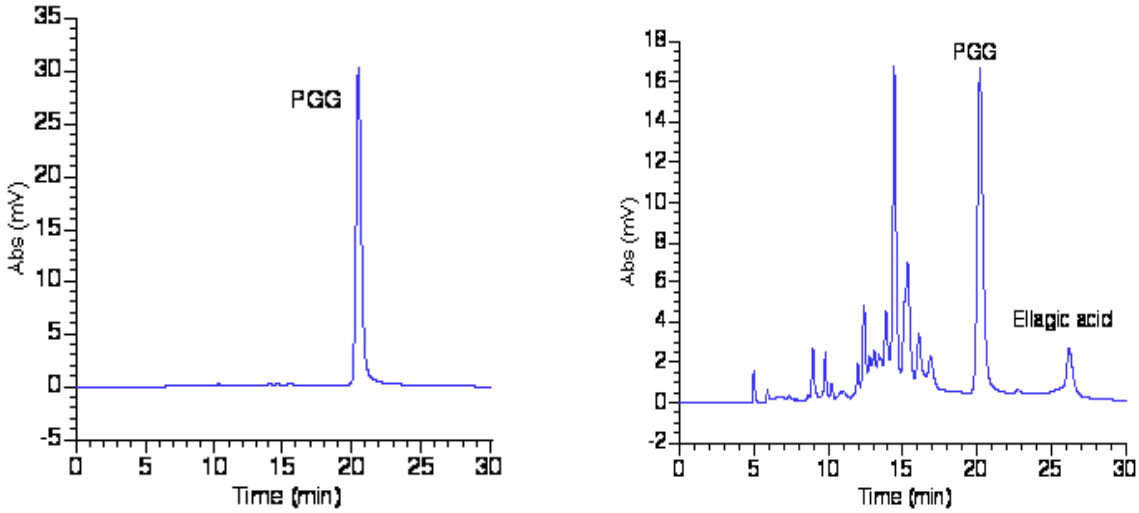


Figure 6.4. HPLC chromatograms (280nm) of incubation of PGG with crude enzyme preparation from transition wood at t= 0 (left) and t = 18 hours (right)

6. 4. Conclusion

This investigation revealed that both sapwood and transition wood contained active enzyme(s) that catalyze the biaryl coupling process, suggesting both may be used in the study of ellagitannin biosynthesis. It was further determined that sapwood contains more total protein than transition wood. In the case of scarlet oak, the protein concentration in sapwood is about ten times higher than in transition wood. 2-D gel separation of proteins from both sapwood and transition wood further demonstrated that transition wood contains fewer total number of proteins. The lower complexity of the transition wood proteome suggests that transition wood may be a good substrate for studying the biaryl coupling process.

Acknowledgement

The author would like to thank Karsten Klage and Brian Jordan for their assistance in the two dimensional electrophoresis.

pH3 pH10

Figure 6.5. Two–dimensional polyacrylamide gel electrophoresis (7 cm) of sapwood.

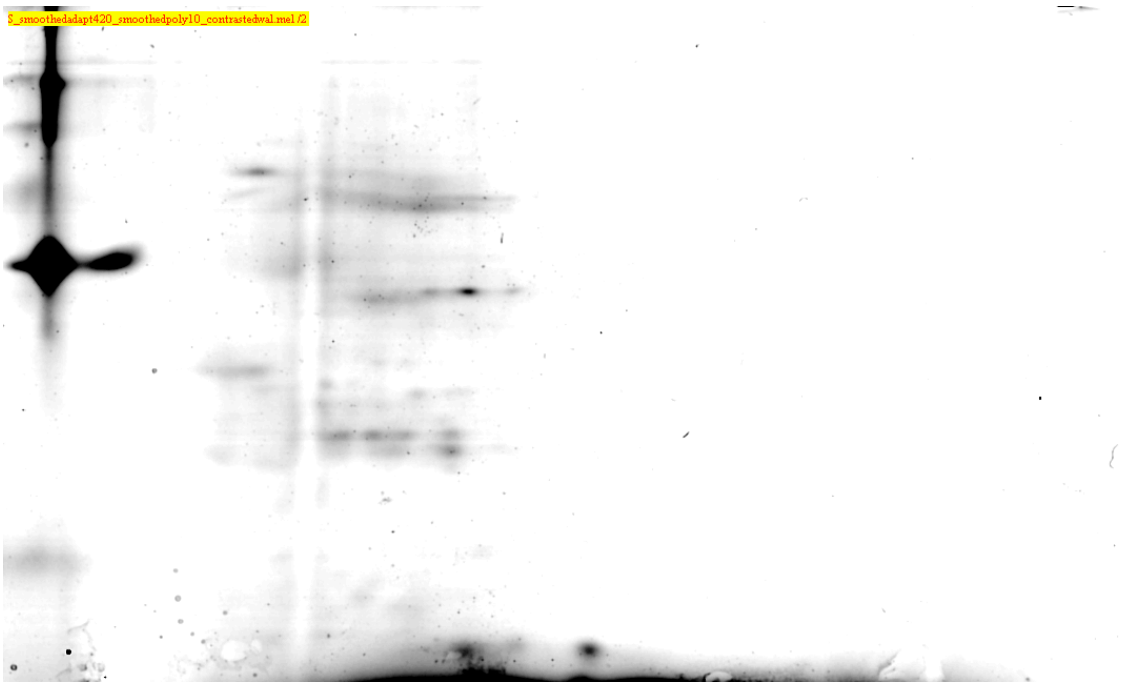


Figure 6.6. Two–dimensional polyacrylamide gel electrophoresis (7 cm) transition wood proteins.

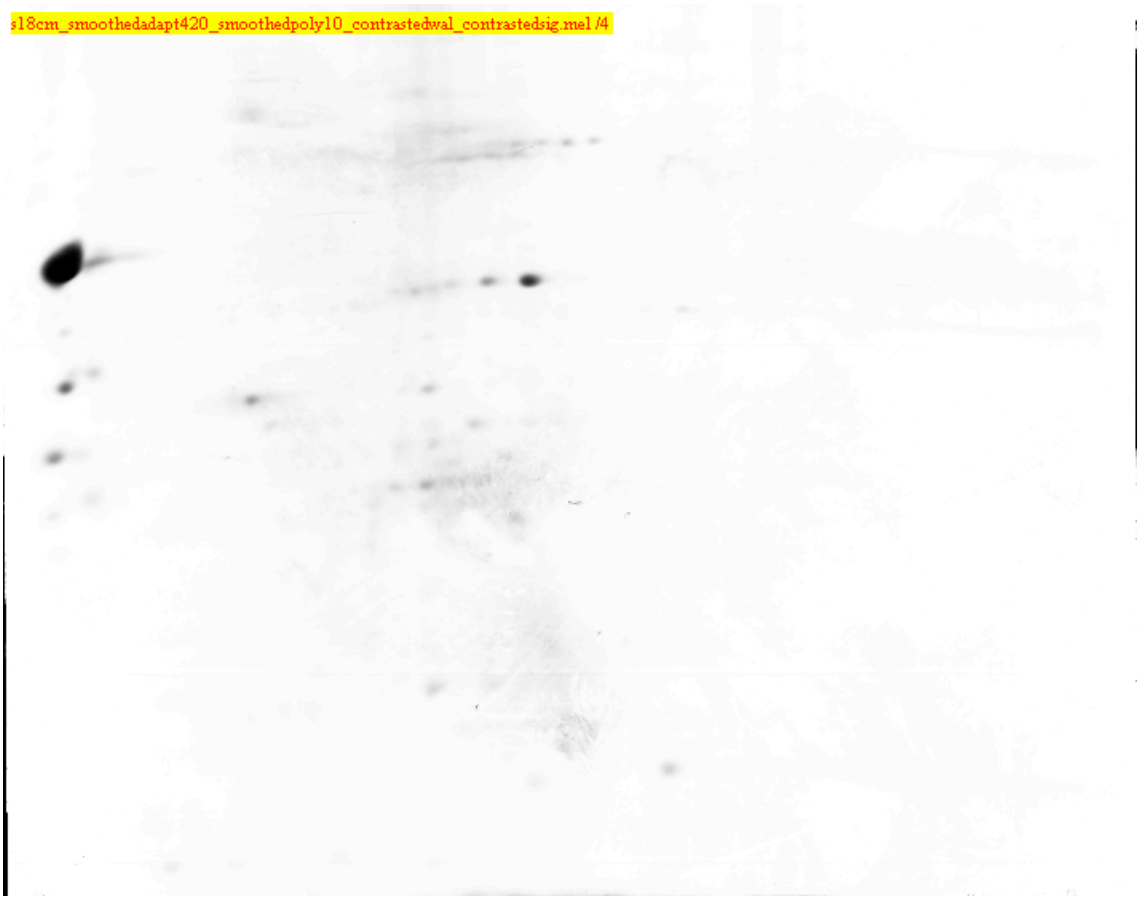


Figure 6.7. Two–dimensional polyacrylamide gel electrophoresis (18 cm) sapwood proteins.

Reference

Baqui, S. and Shah, J. (1985) Histoenzymatic Studies in Wood of *Acacia auriculiformis* Cunn. during Heartwood Formation. *Holzforschung*, **39**: 311-320.

Baqui, S.; Shah, J.; Pandalai, R. and Kothari, I. (1979) Histochemical Changes during Transition from Sapwood to Heartwood in *Melia azedarach*. *Indian J. Exp. Biol.*, **17**: 1032-1037.

Costa, P.; Pionneau, C.; Bauw, G.; Dubos, C.; Bahrmann, N.; Kremer, A.; Frigerio, J. –M. and Plomion, C. (1999) Separation and Characterization of Needle and Xylem Maritime Pine Proteins. *Electrophoresis*, **20**: 1098-1108.

Dietrichs, H. (1964) Das Verhalten von Kohlenhydraten bei der. *Holzforschung*, **18**: 14-24.

Ebermann, R. and Stich, K. (1985) Distribution and Seasonal Variation of Wood Peroxidase Activity in Oak (*Quercus rubur*). *Wood Fiber Sci.*, **17**: 391-396.

Görg, A.; Obermaier, C.; Boguth, G.; Harder, A.; Scheibe, B.; Wildgruber, R. and Weiss, W. (2000) The Current State of Two -Dimensional Electrophoresis with Immobilized pH Gradients. *Electrophoresis*, **21**: 1037-1053.

Gross, G. G. (1992) Enzymatic Synthesis of Gallotannins and Related Compounds. In *Phenolic Metabolism in Plants*; Satfford, H.A.; Ibrahim, R. K. eds.; Plenum Press, New York, 297-324.

Han, K. –H. (20001) Molecular Biology of Secondary Growth. *J. Plant Biotechnol.*, **3**: 45-57.

Hauch, S. and Magel, E. (1998) Extractable Activities and Protein Content of Sucrose-Phosphate Synthase, Sucrose Synthase and Neutral Invertase in Trunk Tissues of *Robinia pseudoacacia* L. Are Related to Cambial Wood Production and Heartwood Formation. *Planta*, **207**: 266-274.

Helm, R. F.; Lei, Z.; Ranatunga, T.; Jervis, J. and Elder, T. (1999) Toward Understanding Monomeric Ellagitannin Biosynthesis. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology,* Gross et al. Eds.; Kluwer Academic/Plenum Publishers, New York, 83-99.

Herbert, B. (1999) Advances in Protein Solubilization for Two-Dimensional Electrophoresis. *Electrophoresis*, **20**: 660-663.

Hillis, W. (1987) Heartwood and Tree Exudates. Berlin, New York, Springer-Verlag.

Hillis, W. and Yazaki, Y. (1973) Wood Polyphenols of *Eucalyptus polyanthemos*. *Phytochemistry*, **12**: 2967-2977.

Holl, W. and Lendzian, K. (1973) Respiration in the Sapwood and Heartwood of *Robinia pseudoacacia*. *Can. J. Bot.*, **52**: 727-734.

Krajci, I. and Gross, G. G. (1987) Formation of Gallotannins in Callus Cultures from Oak (*Quercus robur*). *Phytochemistry*, **26**: 141-143.

Magel, E.; Abdel-Latif, A. and Hampp, R. (1994) Non-Structural Carbohydrates and Catalytic Activities of Sucrose Metabolizing Enzymes in Trunks of Two Juglans Species and their Role in Heartwood Formation. *Holzforschung*, **55**: 135-145.

Magel, E.; Drouet, A.; Claudot, A. and Ziegler, H. (1994) Formation of Heartwood Substances in the Stemwood of *Robinia pseudoacacia* L. I. Distribution of Phenylalanine Ammonium-Lyase and Chalcone Synthase across the Trunk. *Trees*, **8**: 165-171.

Magel, E.; Hillinger, C.; Wagner, T. and Holl, W. (2001) Oxidative Pentose Phosphate Pathway and Pyridine Nucleotides in Relation to Heartwood Formation in *Robinia pseudoacacia* L. *Phytochemistry*, **57**: 1061-1068.

Masson, G.; Moutounet, M. and Puech, J. L. (1995) Ellagitannin Content of Oak Wood as a Function of Species and of Sampling Position in the Tree. *Am. J. Enol. Vitic.*, **46**: 262-268.

Masson, G.; Puech, J. L. and Moutounet, M. (1994) Localization of the Ellagitannins in the Tissues of *Quercus robur* and *Quercus petraea* Woods. *Phytochemistry*, **37**: 1245-1249.

Niemetz, R.; Schilling, G. and Gross, G. G. (20001) Ellagitannin Biosynthesis: Oxidation of Pentagalloylglucose to Tellimagrandin II by an Enzyme from *Tellima Grandiflora* Leaves. *Chem. Commun.*, 35-36.

Rabilloud, T.; Adessi, C.; Giraudel, A. and Lunardi, J. (1997) Improvement of the Solubilization of Proteins in Two-Dimensional Electrophoresis with Immobilized pH Gradients. *Electrophoresis,* **18**: 307-316.

Vander-Mijnsbrugge, K.; Meyermans, H.; Montagu, M. V.; Bauw, G. and Boerjan, W. (2000) Wood Formation in Poplar: Identification, Characterization, and Seasonal Variation of Xylem Proteins. *Planta*, **210**: 589-598.

Wardrop, A. and Cronshaw, J. (1962) Formation of Phenolic Substances in Ray Paranchyma of Angiosperms. *Nature*, **193**: 90-92.

Zuo, S.-S. and Lundahl, P. A. (2000) Micro-Bradford Membrane Protein Assay. *Anal. Biochem.,* **284**: 162-164.

Chapter Seven

Summary

Ellagitannins are plant polyphenols characterized by hexahydroxydiphenoyl groups esterified to a glucose ring (Haslam et al., 1994). They provide passive defense to fungi attack (Scalbert, 1991), contribute to wood durability (Scalbert, 1992a) and affect wood utilization in many ways.

Ellagitannins in woods can be soluble or insoluble (Klumpers et al., 1994; Peng et al., 1991). Soluble ellagitannins are those that can be extracted with organic solvents whereas insoluble ellagitannins are the extractives that can only be removed by chemical degradation. The most commonly used organic solvents for soluble ellagitannins are aqueous methanol and acetone (7:3, organic:water) (Scalbert et al., 1989 and Scalbert, 1992b). These two solvents were found to be more effective than pure water, neat methanol or neat acetone. Variation of water contents in aqueous methanol and acetone within the range of 20–70 % was found to have little effect on total phenol yields. For the convenience of removing extraction solvents, 70% of methanol and acetone have been used. They have been found to provide similar amounts of total phenols and have been used interchangeably in the extraction of tannins. However, the amount of total phenols present in a sample is not equivalent to the amount of ellagitannin. In our method development and validation, the ellagitannin extraction ability of these two solvents were compared. Vescalagin yield increased by 50% and castalagin increased by 38%. The better extraction ability of aqueous acetone is also exemplified by the lower insoluble ellagitannin level remaining in the wood samples after extraction with aqueous acetone.

Insoluble ellagitannins are bound to cell wall components and cannot be extracted with organic solvents (Helm et al., 1997; Klumpers et al., 1994 and Peng et al., 1991). The determination of insoluble ellagitannins therefore relies on the cleavage of HHDP groups from insoluble ellagitannins to form ellagic acid. The first method to quantify insoluble ellagitannins was published in 1991 (Peng et al.). The method uses a mixture of MeOH and 6 M HCl (9:1 v/v) to hydrolyze insoluble ellagitannins. The optimal hydrolysis was found to be at 120 °C for 160 min. The resulting ellagic acid is then quantified with HPLC and insoluble ellagitannin contents can be calculated as the castalagin equivalent providing that one mole of castalagin gives one mole of ellagic acid. In our work with insoluble ellagitannins, a mixture of MeOH and acetyl chloride (0.19 ml acetyl chloride per ml MeOH) was found to be more effective in releasing ellagic acid from insoluble ellagitannins. The methanolysis conditions were optimized as 100 °C for 60 min. The improved insoluble ellagitannin determination method (methanolysis) gives two times the ellagic acid yields obtained with previously reported method (acidic hydrolysis). The data indicate that insoluble ellagitannin contents have previously been underestimated significantly. The improved method not only provides higher yields but also requires shorter reaction time and lower reaction temperature.

Ellagitannins are unstable and are blamed for wood discoloration. Previous work (Charrier et al., 1995) demonstrated that they underwent both polymerization and hydrolysis during wood drying. Our work with purified vescalagin and castalagin further revealed that their degradations were first order and the temperature, pH and oxygen affected their stability, with temperature being the most predominant factor. Increasing temperature from room temperature (23 °C) to 60 °C increased vescalagin degradation rate by 18 times and castalagin by 17 times in the presence of nitrogen at pH 4. Change of pH from pH 4 to pH 7 at room temp. increased degradation rates of both vescalagin and

castalagin by about 7 times while oxygen accelerated the rates only two fold. UV-Vis absorption of both the aqueous vescalagin and castalagin solutions showed the development of yellow-brown compounds over time and HPLC analyses revealed that ellagitannin hydrolysis product, ellagic acid was present. The experiments demonstrated that ellagitannin degradations are correlated to wood discoloration.

For the rapid and reliable detection of ellagitannins, a method using negative HPLC/ESI-MS was developed and optimized. Ellagitannins analyzed under the optimal conditions all provide base peaks of $(M-H)^-$ from which the molecular weights of ellagitannins can be determined. Contrary to previous report (Salminen, 1999), we found that fragmentation of ellagitannins follows a certain pattern, i.e. the loss of HHDP groups to give a fragment of *m/z* 301 which is the characteristic of ellagitannins and the loss of free galloyl groups to give *m/z* 169 if the ellagitannins bear the free galloyl groups.

In the search for models for ellagitannin biosynthesis study, oak callus and suspension cell cultures and sweetgum suspension cell cultures were investigated. White oak (*Quercus alba*) callus was found to be able to produce about three times the amounts of vescalagin and castalagin that oak heartwood produced via the manipulation of nitrogen and copper concentrations in the media. Removing nitrogen (ammonium nitrate) from the media increased the production of ellagitannins, suggesting that nitrogen might be important in regulating the synthesis of ellagitannins. Higher copper concentrations induced the production of ellagitannins. This suggests that laccase(s) be involved in the biosynthesis of ellagitannins as the activity of laccase(s) was previously found to be dependent on copper concentrations in the media (Bligny et al., 1986). Oak and sweetgum suspension cell cultures were also studied as oak callus grows too slowly. Both oak and sweetgum suspension cell cultures were found to be unstable in the production of

ellagitannins. Although they can be induced to produce ellagitannins by the addition of glycan hydrolases (Driselase), the level of ellagic acid was too low for metabolic study.

The slow growth of oak callus and the instability oak and sweetgum suspension cell cultures in the production of ellagitannins prompted us to look at sapwood and sapwood/heartwood interface in oaks where ellagitannins are produced. Both sapwood and transition wood were found to contain enzyme(s) that can catalyze the formation of ellagic acid from PGG. Preliminary 2D gel experiments showed that proteome in the transition zone is less complex, suggesting that transition zone might be a good substrate for biaryl coupling study.

The work described in this dissertation has established standard protocols for ellagitannin analysis and quantification. The tissue culture work also provides the first reported cell suspension system for oak. Although the system was not useful for investigating ellagitannin biosynthesis, they may be of use for those scientists interested in somatic embryogenesis of oak. The final aspect of this work, analysis of the sapwood and heartwood proteome, provides data which strongly supports further experimentation using this approach. There are clear differences between that sapwood and heartwood proteome and utilization of mass spectrometric techniques will aid in identifying the major proteins present in the samples. Furthermore, the oak callus tissues should be re-evaluated using similar approaches. While they are slow-growing, they produce high levels of ellagitannins. It may be that a proteomic analysis of these tissues will help unravel the monomeric ellagitannin biosynthetic process.

Reference

Bligny, R.; Gaillard, J. and Douce, R. (1986) Extraction of Laccase by Sycamore (*Acer pseudoplatanus* L.) Cells: Effects of a Copper Deficiency. *Biochem. J*., **237**: 583-588.

Charrier, B.; Haluk, J. P. and Metche, M. (1995) Characterization of European Oakwood Constituents Acting in the Brown Discoloration during Kilin Drying. *Holzforschung*, **49**: 168-172.

Haslam, E. and Cai, Y. (1994) Plant Polyphenols (Vegetable Tannins): Gallic Acid Metabolism. *Nat. Prod. Rep.,* **11**: 41-66.

Helm, R. F.; Ranatunga, T. D. and Chandra, M. (1997) Lignin-Hydrolyzable Tannin Interactions in Wood. *J. Agric. Food Chem*., **45**: 3100-3106.

Klumpers, J.; Scalbert, A. and Janin, G. (1994) Ellagitannins in European Oak Wood: Polymerization during Wood Ageing. *Phytochemistry*, **36**:1249-1252.

Peng, S.; Scalbert, A. and Monties, B. (1991) Insoluble Ellagitannins in *Castanea sativa* and *Quercus petraea* Woods. *Phytochemistry*, **30**: 775-778.

Salminen, J. –P.; Ossipov, V.; Loponen, J.; Haukioja, E. and Pihlaja, K. (1999) Characterization of Hydrolyzable Tannins from Leaves of *Betula pubescens* by High-Performance Liquid Chromatography -Mass Spectrometry. *J. Chromatogr*., **864**: 283-291.

Scalbert, A. (1992a) Tannins in Woods and Their Contribution to Microbial Decay Prevention. In *Plant Polyphenols*: *Synthesis, Properties, Significance;* Hemingway, R.W.; Laks, P. E. eds.; Plenum Press, New York, 935-951.

Scalbert, A. (1992b) Quantitative Methods for the Estimation of Tannins in Plant Tissues. In *Plant Polyphenols*, Hemingway, R.W.; Laks, P. E. eds.; Plenum Press, New York, 259-280.

Scalbert, A. (1991) Antimicrobial Properties of Tannins. *Phytochemistry*, **30**: 3875-3883.

Scalbert, A.; Monties, B. and Janin, G. (1989) Tannins in Wood: Comparison of Different Estimation Methods. *J. Agric. Food Chem.*, **37**: 1324-1329.

Vita

Zhentian Lei was born in Yongning County, Guangxi Province, China on December 26, 1964, the third of four children of Shijian Lei and Meijing Zeng. He obtained his Bachelor of Science in Forest Products Chemistry from Nanjing Forestry University, Chemical Engineering Department in 1986.

He worked as an assistant wood chemist from 1986-1988 and then as an associate wood chemist from 1988-1997 at the Research Institute of Chemical Processing and Utilization of Forest Products, Chinese Academy of Forestry. He then decided to pursue his Ph. D. In 1997, he was awarded an assistantship from the Department of Wood Science and Forest Products, Virginia Tech to study for his Ph. D. During his stay at Virginia Tech, He, together with others, published three papers and gave a presentation at the 219th American Chemical Meeting in San Francisco, CA. March 26 -30, 2000. He is also a member of American Chemical Society.

Zhentian Lei is married to Hua Wang and they have a daughter, Ying-ying.