Use of Methanolysis for the Determination of Total Ellagic and Gallic Acid Contents of Wood and Food Products

Zhentian Lei, Judith Jervis, and Richard F. Helm*

Department of Wood Science and Forest Products, Fralin Biotechnology Center, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0346

Anhydrous methanolic HCl has been 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 a new protocol. The method provides ellagic acid yields significantly higher than those obtained previously, indicating total ellagic acid contents of several substrates have previously been underestimated.

Keywords: *Ellagic acid; gallic acid; HPLC; ellagitannins; methanolysis; methyl gallate*

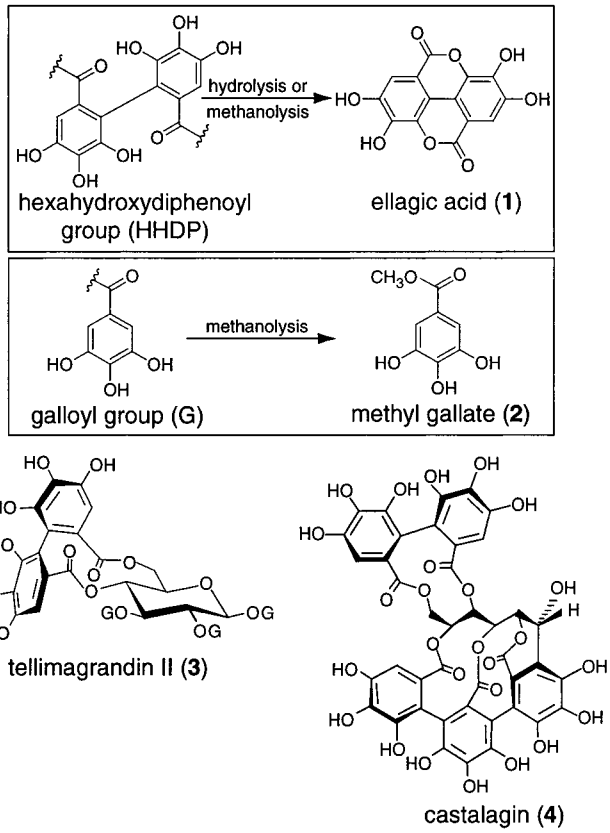
INTRODUCTION

Ellagitannins are a complex class of polyphenols characterized by biaryl-coupled gallic acid moieties esterified to a D-glucose core (*1*, *2*). They are found in many economically important trees (oaks, eucalypts), fruits (strawberries, blackberries, raspberries), and edible nuts (walnuts). Although the exact biochemical pathway to these compounds is unknown (*3*), ellagitannins are generally considered to be physiologically important to both plants (protection from pathogens, *4*) and humans (*5*, *6*).

In general, ellagitannins are labile both in solution and in the field, undergoing hydrolysis and polymerization reactions (*7*, *8*). Hydrolysis (or methanolysis) of ellagitannins containing the hexahydroxydiphenoyl (HHDP) group produces ellagic acid (**1**) via spontaneous lactonization. In the case of tellimagrandin II (**3**), complete hydrolysis would yield 1 mol of ellagic acid and 3 mol of gallic acid per mole of **3**. Castalagin (**4**), on the other hand, would release 1 mol of **1**/mol of **4**.

The polymerization process would lead to insolubilization of ellagitannins and/or covalent attachment to the cell wall components (*9*). The hydrolysis reaction in native materials accounts for the fact that ellagitannins are typically found in the presence of free gallic and ellagic acids. 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*), 40% of the ellagitannins present near the tree center (oldest wood) were insoluble by standard solvent extraction protocols (*10*). These insoluble ellagitannins can potentially have a significant impact on such end use properties as barrel manufacture (*11*). With respect to ellagitannin-containing food products, free and bound ellagic acid contents of various fruits and nuts will differ with respect to maturity, cultivar, growing conditions, and storage (*12*–*15*).

To accurately monitor ellagitannin levels without resorting to isolation and quantification of each individual component, one can determine the total ellagic and gallic acid contents of an extracted or unextracted sample by reacting all bound and/or free HHDP and galloyl units. During our studies on heartwood formation in oak, we discovered that the method typically used for insoluble ellagitannin determination (*10*) significantly underestimated the total amount amount of ellagic acid present. We have therefore developed a new protocol, based on methanolysis, and the specifics of the method are reported below.



EXPERIMENTAL PROCEDURES

All solvents were of HPLC grade. Evaporations were performed under reduced pressure at temperatures not exceeding 40 °C. Acetyl chloride (98%) was used as received.

* Author to whom correspondence should be addressed [fax (540) 231-7126; telephone (540) 231-4088; e-mail helmrf@vt.edu].

10.1021/jf000974a CCC: $20.00
Published on Web 02/21/2001

Ellagic acid was crystallized from pyridine before use, and castalagin was purified as described (*16*). Heartwood and callus samples for protocol optimization were extracted with acetone/water (*16*). Several oak wood samples were kindly provided by Cal Craik, Okanagan Barrel Works (Oliver, BC, Canada). Blackberry fruits and stems were from wild-type cultivars collected from the Washington and Jefferson National Forest (Montgomery County, Virginia). The whiskey analyzed was from a major commercial manufacturer and purchased locally.

Methanolysis Procedure. Anhydrous methanolic HCl is prepared by the slow addition of acetyl chloride to a well-stirred cold (~ −20 °C) solution of anhydrous methanol (final component ratio = 190 μL of acetyl chloride/mL of MeOH, *17*). Dry, extractive-free wood meal (100 mg) or nonextracted samples (~20 mg) are placed in a Teflon-lined screw-cap tube (15 mL) containing a small magnetic stir bar. After anhydrous methanolic HCl (5 mL) is added, the tubes are sealed tightly and placed in a Reacti-Therm system (Pierce Chemical Co.) with stirring at 100 °C for 60 min. After completion, the solutions are cooled to room temperature and filtered (0.2 μm). The filtrates are evaporated to dryness and subsequently redissolved in methanol 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 Analysis. HPLC was performed on the Gilson HPLC apparatus using a Merck Lichrospher RP-18 (end-capped 5 μm) column (250 mm × 4 mm i.d.) using an external standard analysis. The solvents were methanol (solvent A) and 0.2% aqueous trifluoroacetic acid (solvent B). The flow rate was set at 0.75 mL/min. Gradient conditions were as follows: linear gradient from 0 to 100% solvent A; gradient duration, 40 min. All samples were filtered with a 0.2 μm syringe filter before injection. Dual wavelengths (252 and 280 nm) were used to detect ellagic acid and methyl gallate, with the 252 nm wavelength used for external standard analysis of **1** (*18*) and 280 nm used for **2**. Linear calibration curves were generated for both compounds in the concentration range of 5−50 μg/mL ($R^2 = 0.995$).

RESULTS AND DISCUSSION

Because the quantification of insoluble ellagitannins is based on ellagic acid released during acidic hydrolysis, a method that can efficiently cleave all HHDP and galloyl groups is obviously important. The method developed by Peng et al. (*10*) involves the use of a mixture of MeOH and 6 M HCl (MeOH/6 M HCl, 9:1). The optimal conditions were found to be 120 °C and 160 min for the release of ellagic acid. In our hands, this method was found to be very reproducible, although a percentage of reactions failed due to loss of reagent (vial leakage) brought about by the high pressure generated during the reaction. Furthermore, both free and methyl-esterified gallic acid were formed (gallic acid/methyl gallate, 1:7), making quantitation of **2** difficult. In a search for alternative, lower temperature reactions, we found that anhydrous methanolic HCl was much more effective in releasing ellagic acid from biomass samples, at both lower temperatures and shorter reaction times. In addition, gallic acid was completely converted to **2**.

Reaction Optimization. To optimize the method, extractive-free wood meals (*16*) 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 1. Maximum ellagic acid recovery was dependent on the reaction time and temperature. At 80 °C, the maximum recovery was reached in 150 min and in 30 min for reactions performed at 120 °C. For practical purposes, we chose 100 °C and 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.

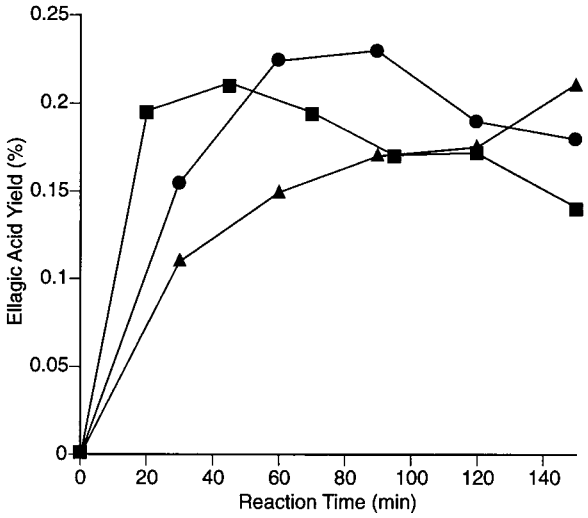


Figure 1. Effects of time and temperature on the yield of ellagic acid from extractive-free white oak heartwood: ▲, 80 °C; ●, 100 °C; ■, 110 °C.

Table 1. Comparison of the Two Ellagitannin Hydrolysis Methods for Recovery of Ellagic Acid (Percent, Dry Weight Basis)

sample (extractive-free)	methanolic HCl[a]	MeOH/6 M HCl[b]
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[c]

[a] This work. [b] Reference *10*. [c] Reference *9*.

The observation that ellagic acid yields decreased over time was noted previously (*10*). 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 (*19*). To provide some insight into the ellagic acid degradation pathway, ellagic acid was subjected to methanolysis at 100 °C. Surprisingly, for up to 150 min, 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 hydrolysate.

Both the optimized methanolysis protocol and the previously reported method (*10*) were applied to extractive-free wood samples for comparative purposes, and the data are shown in Table 1. For both wood samples, yields obtained from anhydrous methanolic HCl were twice that from the MeOH/6 M HCl method. A significanttly higher amount of ellagic acid was also recovered from the callus tissus. Interestingly, the milled wood lignin (MWL) sample gave identical results. These results clearly indicate that methanolysis releases more insoluble ellagic acid than MeOH/6 M HCl.

In a study of molar response factors, castalagin (**4**) (purified from oak heartwood, purity ~92% based on NMR, *16*) was submitted to methanolysis at 100 °C, and the molar amount of ellagic acid released was determined. Ellagic acid was released from castalagin during methanolysis as shown in Figure 2. That the molar ratio

Table 2. Total Ellagic and Gallic Acid Contents (Percent, Dry Weight Basis) of Several Biomass Samples[a]

sample	ellagic acid	methyl gallate	degree of coupling[b]
white oak (*Quercus alba*, Missouri)	0.79 ± 0.02	0.15 ± 0.00	6.42
white oak (*Quercus alba*, Virginia)	1.08 ± 0.06	0.31 ± 0.01	4.25
Oregon white oak (*Quercus garryana*)	1.54 ± 0.00	trace	
sessile oak (*Quercus petraea*, France)	1.18 ± 0.01	0.25 ± 0.00	5.75
sessile oak (*Quercus petraea*, Czech Republic)	2.55 ± 0.07	trace	
chestnut oak (*Quercus prinus*, Virginia)	0.63 ± 0.03	0.17 ± 0.00	4.52
American chestnut MWL (*Castanea dentata*)	1.04 ± 0.00	0.35 ± 0.08	3.62
white oak callus	1.15 ± 0.01	1.98 ± 0.75	0.71
blackberry (*Rubus fruticosus*) fruits	1.09 ± 0.04	trace	
blackberry stems	nd	nd	
whiskey (sour mash)	23.85 ± 1.56[c]	13.04 ± 2.75[c]	2.23

[a] Average of duplicate analyses. [b] Degree of coupling = 2(mol of ellagic acid)/(mol of methyl gallate). [c] Reported in μg/mL.

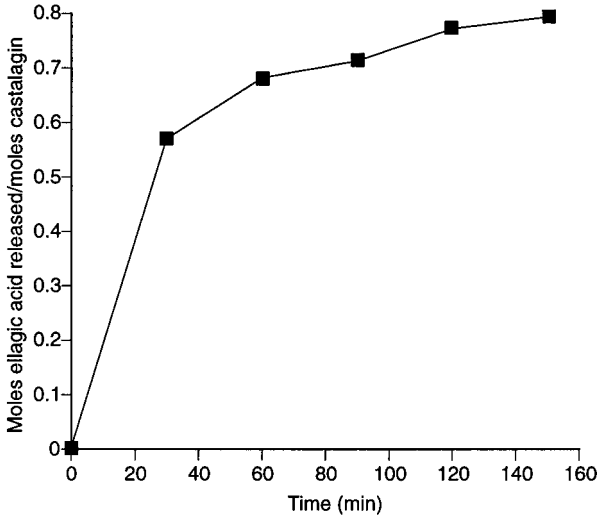


Figure 2. Molar yield of ellagic acid from castalagin (**4**) by methanolysis as a function of time.

of ellagic acid to castalagin did not exceed 1 indicates that the ellagic acid yield is not being 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 (*20*).

The amount of ellagic acid obtained from castalagin is very similar to that previously obtained using aqueous HCl/MeOH (*10*). This suggests that the two methods provide equivalent conversions with soluble ellagitannins, but methanolysis is more efficient at releasing bound HHDP units. In experiments in which known amounts of ellagic acid were added to extracted biomass samples for which the ellagic acid content had been previously determined, 90% of the total ellagic acid was recovered. Thus, the protocol still underestimates the true ellagic acid content, but the method provides significantly higher yields than previously possible. To minimize the effect of ellagic acid condensation on the ellagic acid yield, one can extract and quantify the soluble ellagitannins and free ellagic acid with HPLC, followed by the methanolysis of the extracted samples to determine insoluble ellagitannin content. The sum of these two would be closer to the "true" ellagic acid content.

The modified protocol was applied to several ellagitannin-containing substrates including oak wood (unextracted), whiskey, and blackberry fruits and stems. The results are shown in Table 2. Both free and insoluble ellagitannins contributed to the ellagic and gallic acid yields because 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 (*21*).

Also reported is the yield of **2**, the methanolysis product of uncondensed galloyl groups and free gallic acid. Having both the ellagic acid and methyl gallate concentrations allows for the determination of a "degree of biaryl coupling" for any substrate. This is calculated on a gallate equivalents basis (i.e., ellagic acid contains two gallic acid equivalents per mole) and is ratio of free ellagic acid plus HHDP-linked moieties to free plus esterified gallic acid. This value is useful for ascertaining the pool of gallate converted to biaryl-coupled compounds. In comparing the different white oak samples, one can see that species as well as tree location can lead to different values. The amounts of ellagic acid and methyl gallate found by methanolysis of Oregon white oak suggest that further research on the applicability of this species for spirit aging may be useful. We interpret the white oak callus tissue data (i.e., a low ellagic acid to methyl gallate ratio) to indicate that although 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 are much higher than those reported previously (*6*). 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 the evaluation of model compounds and gradient conditions, it was determined that ellagic acid can potentially coelute with the methanolysis product of catechin. However, the ratio of 252 nm/280 nm for ellagic acid is 3.9 and is 2.1 for the methanolysis product of catechin. This difference allows one to distinguish ellagic acid from other potential coeluting methanolysis products (as would a photodiode array scan of the peak itself). If one suspects coelution, 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 medium (Z. Lei and R. F. Helm, unpublished results).

Conclusion. Anhydrous methanolic HCl is much more effective in releasing ellagic acid from solid biomass samples than methanol/6 M 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 hydrolysate.

ACKNOWLEDGMENT

We thank Cal Carik, Okanagan Barrel Works, Oliver BC, Canada, for several oak wood samples.

LITERATURE CITED

(1) Haslam, E.; Cai, Y. Plant polyphenols (vegetable tannins): Gallic acid metabolism. *Nat. Prod. Rep*. **1994**, *11*, 41−66.
(2) Quideau, S.; Feldman, K. S. Ellagitannin chemistry. *Chem. Rev*. **1996**, *96*, 475−503.
(3) Helm, R. F.; Zhentian, L.; Ranatunga, T.; Jervis, J.; Elder, T. Toward understanding monomeric ellagitannin biosynthesis. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*; Gross, G. G., Hemingway, R. W., Yoshida, T., Eds.; Plenum Press: New York, 1999; pp 83−99.
(4) Scalbert, A. Tannins in woods and their contribution to microbial decay prevention. In *Plant Polyphenols*; Hemingway, R. W., Laks, P. E., Eds.; Plenum Press: New York, 1992; pp 935−952.
(5) Yang, L.-L.; Wang, C.-C.; Yen, K.-Y.; Yoshida, T.; Hatano, T.; Okuda, T. Antitumor activities of ellagitannins on tumor cell lines. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*; Gross, G. G., Hemingway, R. W., Yoshida, T., Eds.; Plenum Press: New York, 1999; pp 615−628.
(6) Clifford, M. N.; Scalbert, A. Ellagitannins—nature, occurrence and dietary burden. *J. Sci. Food Agric*. **2000**, *80*, 1118−1125.
(7) Klumpers, J.; Scalbert, A.; Janin, G. Ellagitannins in european oak wood: Polymerization during wood aging. *Phytochemistry* **1994**, *36*, 1249−1252.
(8) Viriot, C.; Scalbert, A.; Herve du Penhoat, C. L. M.; Moutounet, M. Ellagitannins in woods of sessile oak and sweet chestnut dimerization and hydrolysis during wood aging. *Phytochemistry* **1994**, *36*, 1253−1260.
(9) Helm, R. F.; Ranatunga, T. D.; Chandra, M. Lignin-hydrolyzable tannin interactions in wood. *J. Agric. Food Chem*. **1997**, *45*, 3100−3106.
(10) Peng, S.; Scalbert, A.; Monties, B. Insoluble ellagitannins in *Castanea sativa* and *Quercus petraea* woods. *Phytochemistry* **1991**, *30*, 775−778.
(11) Matricardi, L.; Waterhouse, A. L. Influence of toasting technique on color and ellagitannins of oak wood in barrel making. *Am. J. Enol. Vitic*. **1999**, *50*, 519−526.
(12) Daniel, E. M.; Krupnick, A. A.; Heur, Y.-H.; Blinzler, J. A.; Nims, R. W.; Stoner, G. D. Extraction, stability and quantitation of ellagic acid in various fruits and nuts. *J. Food Compos. Anal*. **1989**, *2*, 338−349.
(13) Maas, J. L.; Wang, S. Y.; Galletta, G. J. Evaluation of strawberry cultivars for ellagic acid content. *HortScience* **1991**, *26*, 66−68.
(14) Häkkinen, S. H.; Kärenlampi, S. O.; Heinonen, I. M.; Mykkänen, H. M.; Törrönen, A. R. HPLC method for screening of flavonoids and phenolic acids in berries. *J. Sci. Food Agric*. **1998**, *22*, 543−551.
(15) Ancos de, B.; González, E. M.; Cano, M. P. Ellagic acid, vitamin C and total phenolic contents and radical scavenging capacity affected by freezing and frozen storage in raspberry fruit. *J. Agric. Food Chem*. **2000**, *48*, 4565−4570.
(16) Lei, Z.; Jervis, J.; Helm, R. F. C-Glycosidic ellagitannins from white oak heartwood and callus tissues. *Phytochemistry* **1999**, *51*, 751−756.
(17) Fieser, L. F.; Fieser, M. *Reagents for Organic Synthesis*; Wiley: New York, 1967; Vol. 1, p 668.
(18) Bianco, M.-A.; Handaji, A.; Savolanien, H. Quantitative analysis of ellagic acid in hardwood samples. *Sci. Total Environ*. **1998**, *222*, 123−126.
(19) Hemingway, R. W.; Hillis, W. E. Behavior of ellagitannins, gallic acid and ellagic acid under alkaline conditions. *Tappi* **1971**, *54*, 933−936.
(20) Mayer, W.; Kuhlmann, F.; Schilling, G. On tanning compounds from the wood of chestnut and oak, V. The structure of vescalin. *Liebigs Ann. Chem*. **1971**, *747*, 51−59.
(21) Viriot, C.; Scalbert, A.; Lapierre, C.; Moutounet, M. Ellagitannin and lignins in aging of spirits in oak barrels. *J. Agric. Food Chem*. **1993**, *41*, 1872−1879.

Received for review August 2, 2000. Revised manuscript received December 28, 2000. Accepted January 4, 2001. This work was supported by grants from the USDA National Research Initiative Competitive Grants Program (96-35103-3833 and 99-35103-8070). Portions of this work were presented at 219th National Meeting of the American Chemical Society (San Francisco, CA, March 2000, Paper CELL-140).

JF000974A