

## Determination of Hydrolyzable Tannins (Gallotannins and Ellagitannins) after Reaction with Potassium Iodate

PAUL W. HARTZFELD,<sup>†</sup> REBECCA FORKNER,<sup>‡</sup> MARK D. HUNTER,<sup>‡</sup> AND  
 ANN E. HAGERMAN<sup>\*,†</sup>

Department of Chemistry and Biochemistry, Miami University, Oxford, Ohio 45056 and Institute of Ecology, University of Georgia, Athens, Georgia 30602-2202

A widely used method for analyzing hydrolyzable tannins after reaction with KIO<sub>3</sub> has been modified to include a methanolysis step followed by oxidation with KIO<sub>3</sub>. In the new method, hydrolyzable tannins (gallotannins and ellagitannins) are reacted at 85 °C for 20 h in methanol/sulfuric acid to quantitatively release methyl gallate. Dried plant samples can be methanolized under the same conditions to convert hydrolyzable tannins to methyl gallate. Oxidation of the methyl gallate by KIO<sub>3</sub> at pH 5.5, 30 °C, forms a chromophore with λ<sub>max</sub> 525 nm, which is determined spectrophotometrically. The detection limit of the method is 1.5 μg of methyl gallate, and with plant samples, relative standard deviations of less than 3% were obtained.

**KEYWORDS:** Tannin; polyphenolic compound; hydrolyzable tannin; methyl gallate; galloyl glucose; gallotannin; potassium iodate

### INTRODUCTION

Tannins are high molecular weight polyphenolics found in higher plants including many plants used as foods and feed (1). Tannins ingested with the diet by humans or animals may affect protein utilization by forming insoluble complexes with protein, iron utilization by complexing with iron, and biological anti-oxidant status by participating in redox reactions (2). Tannins may contribute to the chemical defenses that minimize damage to plants by insect and mammalian herbivores (3). Their importance in nutrition and in ecological processes makes qualitative and quantitative analysis of tannins critical to studies of plant nutritional quality and plant ecology (4). Constraints on effective chemical analysis include the variable composition of tannins within plants, which includes variation with species, season, and environmental stresses such as herbivory (5). Because the tannins comprise a structurally complex suite of compounds (1), qualitative as well as quantitative assessment is essential.

Ecological and nutritional studies may require analysis of hundreds of samples, making it impractical to attempt to characterize and quantitate each individual compound. Instead, broad screening methods, based on the similar chemistries of groups of related compounds, are used to provide quantitative information about classes of compounds. This strategy has been employed in many studies of condensed tannins, which are comprised of flavanoid subunits. Under appropriate conditions, virtually all condensed tannins can be degraded to yield

anthocyanidins that can be directly spectrophotometrically determined. This method, known as the acid butanol assay (6), estimates all condensed tannins in terms of a single chemical product that is common to a wide range of parent compounds.

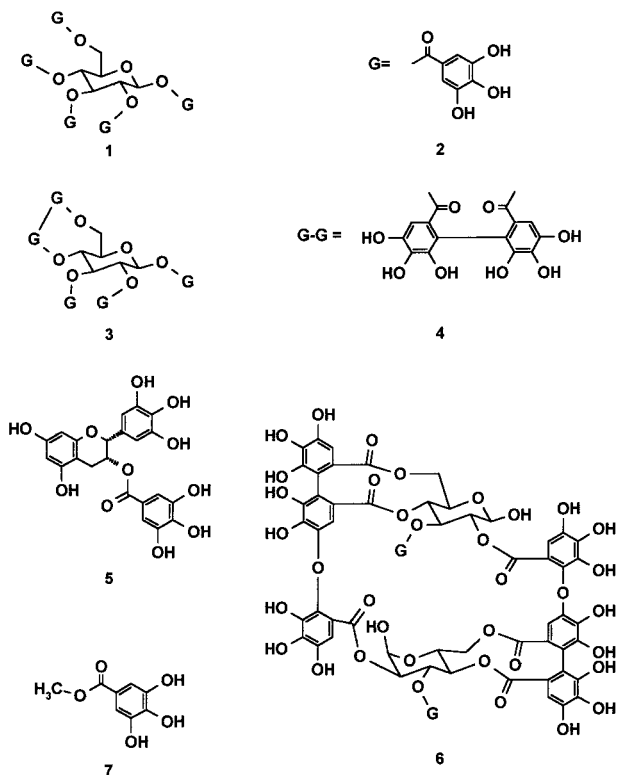
Similar screening methods have not been developed for the hydrolyzable tannins. Hydrolyzable tannins are comprised of a polyol core, often glucose, esterified to gallic acid (Figure 1). The phenolic moieties may be modified by oxidation to hexahydroxydiphenic acid (HHDP) or various macrocyclic polyphenolics (7). The naturally occurring hydrolyzable tannins exhibit almost unlimited variation in the degree of esterification and of cross-linking (7). Individual hydrolyzable tannins can usually be determined by high-performance liquid chromatography (HPLC), but methods for conveniently screening large numbers of samples with variable hydrolyzable tannin compositions have not been developed (8). The rhodanine method (9) for determining gallic acid is preceded by acid hydrolysis of the sample under conditions that must be stringently anaerobic to prevent oxidation of the product. This step is not convenient when large numbers of samples are to be analyzed. The sodium nitrite method (10) for determining ellagic acid, the product of hydrolysis of HHDP esters, requires large quantities of pyridine as a solvent, introducing significant toxicity risk when the method is used for routine analyses of large sample sets. Methanolysis to release methyl gallate and ellagic acid followed by chromatographic analysis has been successfully employed (11) but is inconvenient for screening large numbers of samples and cannot be employed in labs in underdeveloped countries because of the sophisticated instrumentation required.

The reaction of potassium iodate (KIO<sub>3</sub>) with hydrolyzable tannins was first described by Haslam (12) and was developed

\* To whom correspondence should be addressed. Tel.: 513-529-2827. Fax: 513-529-5715. E-mail: hagermae@muohio.edu.

<sup>†</sup> Miami University.

<sup>‡</sup> University of Georgia.



**Figure 1.** Structural formulas of representative hydrolyzable tannins from plants. Compound **1** is pentagalloyl glucose, comprised of the gallate (**G**, **2**) esters of glucose. Compound **3** is the simple ellagitannin eugenin, comprised of both the gallate esters and a single HHDP (**G-G**, **4**) ester. Compound **5** is EGCG, the gallate ester of the flavanoid epigallocatechin. Compound **6** is oenothin B, a macrocyclic hydrolyzable tannin. All four of the hydrolyzable tannins react in acidic methanol to yield methyl gallate (**7**).

as an analytical tool by Bate-Smith (*13*). A transient reaction product with maximum absorbance between 500 and 550 nm was obtained when galloyl esters were reacted with  $\text{KIO}_3$  in the presence of either acetone or methanol. Although the original protocol recommended reaction at inconveniently low temperatures, Willis and Allen demonstrated that chilling was not necessary (*14*). However, several practical limitations to the method have not been overcome (*8*). The method requires variable reaction times to achieve maximal color yield with tannins from different sources; the method produces unique chromophores with different spectral properties for different hydrolyzable tannins; and the method yields interfering yellow oxidation products when phenolics other than hydrolyzable tannins are present. In addition, for some plant extracts comprised of mixtures of tannins, precipitates form during the reaction with  $\text{KIO}_3$ . Despite these limitations, the  $\text{KIO}_3$  method has been employed in some studies of oaks and maples, species rich in hydrolyzable tannins, to provide an estimate of those compounds in crude plant extracts (*15, 16*).

The purpose of this work was to refine the  $\text{KIO}_3$  method for analysis of hydrolyzable tannins. The strategy that was employed was similar to that used successfully in the acid butanol method for condensed tannins (*6*). In that method, the formal subunit of the condensed tannins, the flavanoid, is determined after chemical modification to yield a chromophore (the anthocyanidin), providing a convenient colorimetric method for estimating total condensed tannins. We modified the  $\text{KIO}_3$  method so that it could be used to determine the formal monomer of the hydrolyzable tannins, the galloyl group. Our new  $\text{KIO}_3$  method

relies upon conversion of the hydrolyzable tannins to methyl gallate via methanolysis followed by determination of the methyl gallate after reaction with  $\text{KIO}_3$  to yield a chromophore. To facilitate screening plants for hydrolyzable tannins in foods and forages used in subsistence agriculture, we used protocols that can be implemented in simple laboratory settings with limited instrumental capabilities.

## MATERIALS AND METHODS

**Reagents.** Condensed tannins were purified from *Sorghum bicolor* grain (*17*) and *Metrosideros polymorpha* leaves (*18*). The ellagitannin oenothin B was purified from *Epilobium angustifolium* (fireweed) flowers (*19*). The mixture of condensed and hydrolyzable tannins found in *Rubus spectabilis* (salmonberry) leaves was purified from freeze-dried samples collected during a field study (*20*). Samples of *Acer rubrum* (red maple) (*16*) leaves were collected during a field study, dried, and stored in the dark at  $-40^\circ\text{C}$ . Other tannins and phenolics used as standards and controls included tannic acid (Mallinckrodt); gallic acid (Sigma); methyl gallate (Aldrich); and epigallocatechin gallate (EGCG) (Lipton, Doug Ballentine). All other reagents were reagent grade or the best grade available.

**Methanolysis and pH Adjustment.** Approximately 20 mg samples of dried, ground plant tissue were weighed into 16 mm  $\times$  125 mm (20 mL) Pyrex screw top tubes with Teflon cap liners; the top part of the tube acted as a condenser during the methanolysis, so smaller tubes could not be substituted. The exact mass of each sample was recorded before adding 2.0 mL of methanol and 200  $\mu\text{L}$  of concentrated sulfuric acid (18 M). The caps were tightened finger tight so that solvent would not evaporate during heating.

The samples were placed in a heating block previously brought to  $85^\circ\text{C}$  and were allowed to react for 20 h at  $85^\circ\text{C}$ . The samples were then centrifuged briefly in a tabletop centrifuge (3000g), and the supernatant was quantitatively transferred to a 5.0 mL graduated cylinder by washing the methanolysis tube three times with minimum volumes of distilled water and recentrifuging as necessary. The volume was adjusted to 3.0 mL with distilled water, and four 50  $\mu\text{L}$  aliquots of ethanolamine (commercial preparation, 100% ethanolamine) were added with gentle swirling between each addition. The heat of neutralization was quite high so the ethanolamine was added carefully and in small aliquots. To each sample was added 500  $\mu\text{L}$  of 3.7 M ammonium acetate before the pH was adjusted to  $5.5 \pm 0.1$  using a pH meter and small volumes of dilute ethanolamine or dilute sulfuric acid. The pH adjustment was done carefully because the final pH and volume were critical to the success of the method.

After adjusting the pH, each sample was brought to a final volume of 4.0 mL with distilled water, and the sample was mixed thoroughly. Samples were then stored tightly capped at  $4^\circ\text{C}$  for up to 48 h. A precipitate sometimes formed during storage; in that case, the solution was recentrifuged briefly to remove the precipitate before analysis.

**Standards and Controls.** The method was standardized with methyl gallate. To control for any losses during methanolysis, 2.0 mL of 5.00 mg/mL methyl gallate dissolved in methanol was methanolized and pH-adjusted as described above for the plant samples. Tannic acid was used to confirm the methanolysis procedure by treating 2.0 mL of a 1.00 mg/mL solution of tannic acid dissolved in methanol as described above.

A phenolic-free, pH 5.5, blank reagent was prepared by treating a mixture of 2.0 mL of methanol and 200  $\mu\text{L}$  of concentrated sulfuric acid exactly as described above for the samples. This blank solution did not contain any plant sample or standard polyphenolic compound and was used to dilute the standards when preparing the standard curve.

**Analysis.** The pH  $5.5 \pm 0.1$  samples described above were analyzed by reaction with  $\text{KIO}_3$ , which formed a characteristic pigment with  $\lambda_{\text{max}}$  525 nm. Some flavanoids and other plant constituents reacted under these conditions to form brownish pigments that absorbed weakly at 525 nm. To correct background color, each sample was analyzed in parallel with a reagent mixture and with a background mixture at low pH as described below.

For the reactions, up to 100  $\mu\text{L}$  of sample (methanolized and pH-adjusted sample comprised of plant tissue, purified tannin, or standard

methyl gallate) was dispensed into a 2.0 mL microcentrifuge tube. The pH 5.5 blank reagent described above was added as necessary to bring the sample volume to 100  $\mu\text{L}$ . Water (350  $\mu\text{L}$ ) and methanol (1000  $\mu\text{L}$ ) were added, and the samples were vortexed. The tubes were tightly capped and were placed in a 30  $^{\circ}\text{C}$  water bath. For methanolysis products of most plants, a 100  $\mu\text{L}$  aliquot of the methanolysis solution was used. For standards, the volume of pH-adjusted methyl gallate solution that was added to the microfuge tubes was varied from 0 to 100  $\mu\text{L}$ .

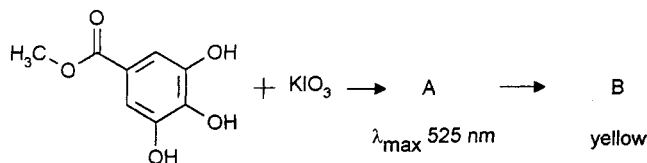
A background sample was prepared for each sample. The background samples were exactly like the reaction samples except that the 350  $\mu\text{L}$  of water was replaced by 350  $\mu\text{L}$  of 0.3 N HCl.

After all of the samples and backgrounds were dispensed and diluted with water or HCl and methanol, the  $\text{KIO}_3$  was added at timed intervals. Exactly 40  $\mu\text{L}$  of 5% (w/v)  $\text{KIO}_3$  was added to each sample; the sample was capped, vortexed, and returned to the 30  $^{\circ}\text{C}$  water bath. Exactly 50 min after the  $\text{KIO}_3$  was added to the sample, its absorbance at 525 nm was recorded (vs water).

**HPLC Analysis of Methanolysis Products.** The products of methanolysis were also analyzed directly by HPLC. A 200  $\mu\text{L}$  aliquot of the methanolized sample was mixed with 20  $\mu\text{L}$  of 12% aqueous sodium dodecyl sulfate (SDS), and 10  $\mu\text{L}$  of that solution was analyzed by reversed-phase (RP)-HPLC. Separation was on a 100 mm  $\times$  4.6 mm C-18 column with the flow rate set at 1.0 mL/min, with a solvent gradient as follows: 20–40% acetonitrile over 7 min, returning to the starting conditions over 3 min, followed by 5 min isocratic to prepare for the next sample. Both acetonitrile and water were modified with trifluoroacetic acid at 0.04%. Detection was at 220 nm. Methyl gallate had a retention time of about 2.5 min, and galloyl esters had longer retention times. Condensed tannins could not be chromatographed with this system.

## RESULTS AND DISCUSSION

The reaction between galloyl esters and  $\text{KIO}_3$  is an oxidation (21), with a somewhat stable red-colored product (A) formed in the initial step of the reaction (reaction 1). A second, slower reaction (reaction 2) further oxidizes A to yield a yellow product (B).



We found that other oxidizing agents such as hypochlorite could be substituted for  $\text{KIO}_3$ , but the lifetime of A was shorter with stronger oxidizing agents. Potassium periodate ( $\text{KIO}_4$ ) did not yield any detectable red product presumably because it very rapidly oxidized the galloyl groups past the red intermediate to the final product (22). Other polyphenolics including gallic acid, ellagic acid, condensed tannin, and various flavonoids did not form red-colored products when reacted with  $\text{KIO}_3$ .

In our preliminary studies using the published method for the  $\text{KIO}_3$  reaction (15) with several purified hydrolyzable tannins, we obtained different color yields for structurally different gallotannins (e.g., **Figure 1**), whether the results were calculated on a mass or molar basis. These differences were at least in part a result of the large differences in rates of reaction for these chemically different species (14). Differences in color yield meant that conversion of the absorbance to a meaningful analytical value was impossible for typical plant extracts containing mixtures of hydrolyzable tannins.

We modified the method to include a first step in which the hydrolyzable tannins in the sample are converted to a single chemical species, methyl gallate. Hydrolyzable tannin levels are expressed in terms of a commercially available standard

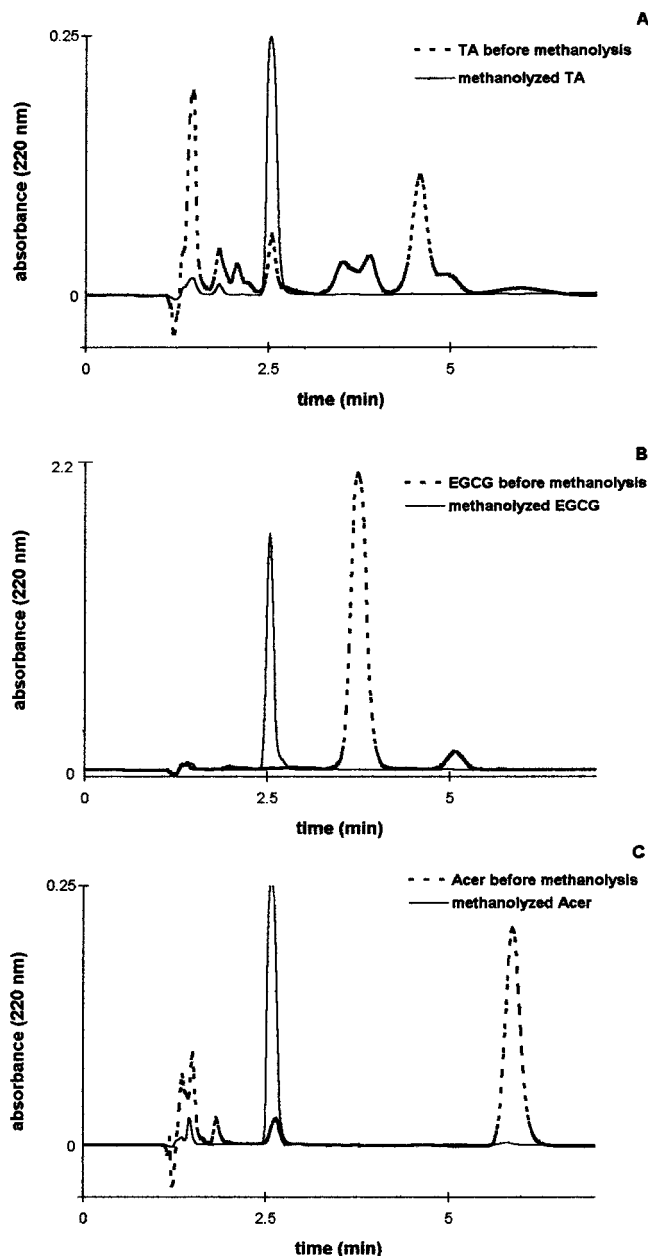
compound rather than in terms of “tannins” that have variable reactivities and color yields. Furthermore, we have improved the utility of the method by finding convenient conditions in which the chromophore is stable and which eliminate problems with precipitates and interferences (8).

To achieve consistent results with structurally diverse hydrolyzable tannins, it was necessary to generate a common structural element from the various potential analytes. The common structural moiety for most of the hydrolyzable tannins is gallic acid. In the ellagitannins, some of the galloyl groups have been oxidatively coupled to HHDP and other complex products, but in all but a few ellagitannins (e.g., pedunculagin and castalgin), at least some simple galloyl esters remain. Thus, gallic acid is produced by acid hydrolysis of most gallotannins and ellagitannins (**Figure 1**). However, gallic acid is oxidized by  $\text{KIO}_3$  to form a yellow compound that has spectral properties similar to the brownish oxidation products formed by flavanoids and other phenolics. These yellow/brown products are not useful for selective determination, so conversion of hydrolyzable tannins to gallic acid was not a useful strategy for improving the  $\text{KIO}_3$  method. We found that methyl gallate reacts with  $\text{KIO}_3$  to form a red chromophore (A in reaction 1), so conversion of hydrolyzable tannins to methyl gallate was optimized.

Methyl gallate is produced from gallate esters including hydrolyzable tannins reacted in anhydrous methanol containing strong mineral acid (11). Conditions for methanolysis were established using HPLC to assess acid strength, temperature, and time required to maximize yield of methyl gallate from several different purified compounds and from the polyphenolics in *Acer* leaves (**Figure 2**). Acid strength and concentration were critical to efficient conversion. Hydrochloric or trifluoroacetic acid at several concentrations gave poor yields of methyl gallate, but sulfuric acid diluted with methanol to a final concentration of about 1.6 M effectively released all of the methyl gallate from various hydrolyzable tannins. The temperature for methanolysis was fixed at 85  $^{\circ}\text{C}$  to avoid the disappearance of some methyl gallate and formation of unidentified phenolic products observed at higher temperatures, especially in the crude plant extracts. After 6 h of methanolysis at 85  $^{\circ}\text{C}$ , about 80% of the complex polyphenolic esters had been converted to methyl gallate. Maximum conversion was achieved in about 11 h of reaction, with no further change in the amount of methyl gallate produced during an additional 10 h of methanolysis. For convenience, 20 h of methanolysis was used routinely. Under these methanolysis conditions, commercial methyl gallate was quantitatively recovered. When commercial gallic acid was treated in the same fashion, it was quantitatively converted to methyl gallate.

It was essential to use anhydrous methanol for the methanolysis, because even traces of water caused production of a mixture of gallic acid and methyl gallate (11). Gallic acid is susceptible to oxidative destruction at high temperatures in the presence of oxygen (9), so production of gallic acid must be avoided to optimize yields. If the methanolysis tubes were not tightly capped during methanolysis, significant evaporation of the methanol during the 20 h reaction caused problems in quantitation. Evaporation and other irreproducible results during methanolysis were minimized by using a heating block that held the temperature constant for the entire period with minimal cycling to higher or lower temperatures.

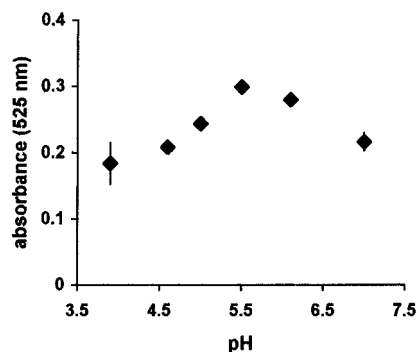
The red product (A in reaction 1) produced by oxidation of methyl gallate with  $\text{KIO}_3$  is not stable, and its accumulation is dependent upon reaction solvent, pH, and temperature. Extensive efforts were made to optimize conditions so that reproducible



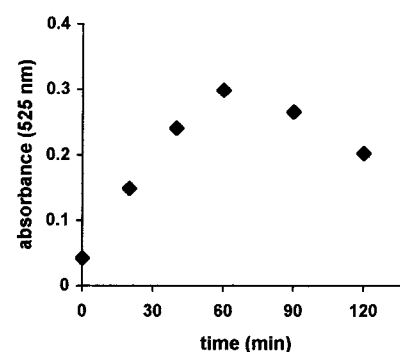
**Figure 2.** Conversion of hydrolyzable tannins to methyl gallate by 20 h of methanolysis at 85 °C. Methanolysis samples (—) of tannic acid (A), EGCG (B), or *Acer* leaf powder (C) were reacted with methanol/sulfuric acid at 85 °C for 20 h. Control samples (- - -) were mixed with methanol/sulfuric acid immediately before analysis. The reaction mixtures were mixed with SDS and chromatographed on a C-18 RP column with an acetonitrile gradient as described in the text. Methyl gallate elutes at 2.5 min, and galloyl glucoses at longer retention times. In the figure, the chromatograms of the methanolized samples in A and C are scaled by 0.1.

color yields could be achieved for plant samples as well as standards of methyl gallate. Methanol or acetone stabilized the chromophore A, while water accelerated its decomposition. However,  $\text{KIO}_3$  has limited solubility in nonaqueous solvents, and thus, reaction could not be done in pure organic solvent. Using a mixed solvent containing methanol plus water achieved reasonable stability of the product A while maintaining solubility of the inorganic salts and was convenient for samples that had undergone methanolysis.

It was critical to control the pH of the reaction (Figure 3). The pH dependence of oxidation of phenolics has been well-documented for a variety of conditions (23), and similar trends



**Figure 3.** Reaction of  $\text{KIO}_3$  with methyl gallate as a function of pH. Methyl gallate solutions dissolved in methanol were diluted with a series of solutions comprised of ethanolamine and ammonium acetate buffer prepared at various pH values between 4.5 and 7.0. These samples were reacted with  $\text{KIO}_3$  as described in the text. Points represent the mean of triplicate values, and error bars represent the standard deviation.



**Figure 4.** Reaction of  $\text{KIO}_3$  with methyl gallate as a function of time. Methyl gallate solutions were adjusted to pH 5.5, and the reaction with  $\text{KIO}_3$  was conducted as described in the text. Samples were kept at 30 °C until the indicated time, when the absorbance was read. Points represent the means of triplicate values; standard deviations are smaller than the symbols used in the graph.

were observed for the  $\text{KIO}_3$  oxidation. If the reaction was run under conditions that were too basic, oxidation was accelerated and only the yellow end product of the reaction was observed. When the reaction mixture was too acidic, the chromophore A did not accumulate, presumably because the rate of oxidation was retarded. To obtain reproducible results, the pH of the sample must be  $\text{pH } 5.5 \pm 0.2$ .

Methanolized samples were extremely acidic and in nonaqueous solvent, so adjusting the pH for the  $\text{KIO}_3$  reaction was not easy. The samples were neutralized with ethanolamine instead of an inorganic base such as KOH to minimize addition of water to the reaction mixture. Ammonium acetate was added to buffer the final reaction mixture at the desired pH. Other acetate salts were not soluble in the nonaqueous methanolysis mixture. It was necessary to adjust the final pH of each sample using the pH meter to ensure that the pH was as close as possible to 5.5 before starting the analysis.

As is expected for a transient species, the color yield of product A was temperature-dependent. When color development was done at room temperature, there were significant day-to-day variations due to changes in room temperature in the lab. To overcome this problem, the reaction was routinely run at 30 °C, a temperature easily maintained with a water bath in almost any lab. At that temperature, color development reached a maximum 50–60 min after the  $\text{KIO}_3$  was added to the reaction mixture (Figure 4). Because color development is very time-

**Table 1.** Theoretical Production of Methyl Gallate from Hydrolyzable Tannins

hydrolyzable tannin	yield of methyl gallate <sup>a</sup>
monogalloyl glucose	0.55
digalloyl glucose	0.76
trigalloyl glucose	0.87
tetragalloyl glucose	0.93
pentagalloyl glucose	0.98
hexagalloyl glucose	1.01
heptagalloyl glucose	1.04
octagalloyl glucose	1.05
oenothein B	0.23

<sup>a</sup>Yield of methyl gallate is calculated from molecular weights of tannin and methyl gallate and is expressed in grams of methyl gallate per gram of tannin.

dependent, absorbances were routinely determined at exactly 50 min after the reaction was started by the addition of the KIO<sub>3</sub>.

Under these conditions, a linear relationship between methyl gallate and absorbance at 525 nm was obtained

$$\text{Abs} = 0.0132 \times \mu\text{g} + 0.0701$$

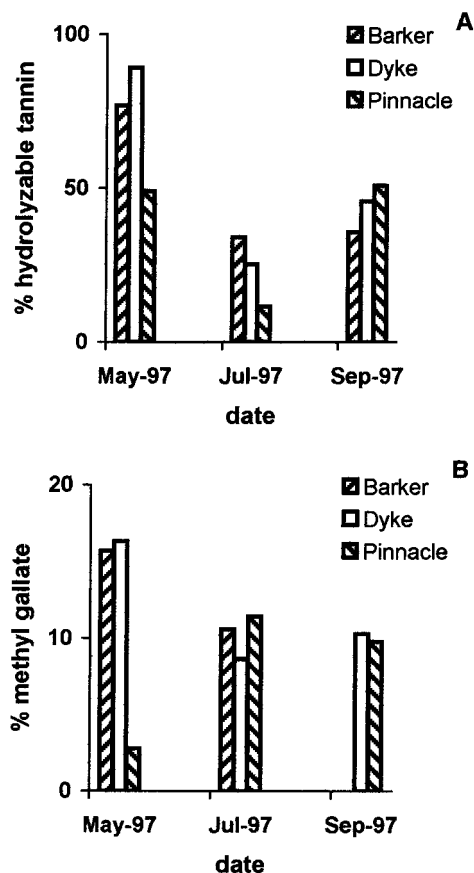
where  $R^2 = 0.996$ , standard error of  $Y = 0.0303$ , and standard error of slope = 0.000 19.

The limits of detection by this method were 1.5  $\mu\text{g}$  of methyl gallate, and the method was linear through at least 120  $\mu\text{g}$ . There were no interferences when the method was used to determine methyl gallate after methanolysis of purified hydrolyzable tannins including tannic acid, EGCG, and oenothein B.

When the method was used with samples of pure condensed tannin such as the procyanidins from *Sorghum* or *Metrosideros*, there was no development of the pink oxidation product typical of the reaction between KIO<sub>3</sub> and gallate esters. However, the KIO<sub>3</sub> did slowly oxidize the procyanidins to nonspecific brown products. Although the absorbance of these products at 525 nm was low, it was desirable to eliminate this potential interference. This was achieved by running "background" reaction mixtures at low pH. At this pH, methyl gallate does not react with KIO<sub>3</sub> so there is no development of the specific pink product of analysis, but the nonspecific oxidation reaction of condensed tannins proceeds to form the brown interfering pigments. The absorbance of the background reaction mixture is subtracted from the absorbance of the actual reaction mixture before calculating methyl gallate. The absorbance of the background mixture was undetectable for up to 100  $\mu\text{g}$  of methanolized tannic acid, as expected. The absorbance of the background mixture was 0.08 for 100  $\mu\text{g}$  of *Sorghum* condensed tannin and 0.11 for 100  $\mu\text{g}$  of *Metrosideros* condensed tannin.

The validity of the background correction was evaluated by spiking samples of salmonberry tannin with known amounts of tannic acid. The salmonberry tannin, which is a mixture of condensed and hydrolyzable tannins (24), and the tannic acid were methanolized separately and were mixed after the pH adjustment step. The average recovery of tannic acid in the spiked samples was 116% when the background correction was used but was 126% when the correction was not applied. This background procedure was routinely run for all plant samples to account for the presence of condensed tannins, flavanoids, or other phenolics that could produce nonspecific oxidation products with KIO<sub>3</sub>.

Simple gallotannins theoretically yield about 1  $\mu\text{g}$  of methyl gallate per 1  $\mu\text{g}$  of galloyl glucose (Table 1), so these compounds can be estimated quite reliably by quantitating methyl gallate after methanolysis. Some hydrolyzable tannins are comprised largely of dimerized and oxidized galloyl groups



**Figure 5.** Hydrolyzable tannins in samples of *Acer* collected at three sites (Barker, Dyke, and Pinnacle) on three dates (May, July, and September). Analysis was done either with the original KIO<sub>3</sub> method (A) standardized with crude plant polyphenolics and expressed as percent of leaf mass as hydrolyzable tannin (15) or with the new method described here (B) standardized with methyl gallate and expressed as percent of leaf mass as methyl gallate. For the original KIO<sub>3</sub> method, values shown are single determinations on the plant sample. For the modified KIO<sub>3</sub> method, the values shown are the means of three determinations from a single plant sample, with an average standard deviation of 2.4% of the mean. The data for Barker, September, with the new KIO<sub>3</sub> method is missing.

and yield far less methyl gallate than the simple gallotannins. For example, oenothein B only has two galloyl groups per molecule of tannin (Figure 1) and thus produces rather little methyl gallate (Table 1). The new method provides a good estimate of simple gallotannins but underestimates ellagitannins. This is similar to the acid butanol method, which provides a good estimate of simple procyanidins but underestimates 5-deoxy proanthocyanidins because of their low color yield (25).

Results of the KIO<sub>3</sub> analysis and the calculated theoretical yields of methyl gallate (Table 1) can be used to obtain limited structural information about simple gallotannins such as those found in tannic acid. Our KIO<sub>3</sub> analysis of Mallinckrodt tannic acid indicated that the material contained 0.77  $\mu\text{g}$  of methyl gallate per 1  $\mu\text{g}$  of tannic acid, characteristic of a galloyl ester with an average degree of esterification of about two (Table 1). HPLC analysis of this preparation of tannic acid confirmed that at least 70% of the esters in that preparation were smaller than pentagalloyl glucose (26).

We tested our method with samples of red maple leaves collected for a large ecological study (16). In that study, hydrolyzable tannins were analyzed in the dried leaf samples using the original Bate-Smith KIO<sub>3</sub> method (15). The method was standardized with a freeze-dried polyphenol extract of a

bulk sample of the leaves, and the results were expressed as "gallotannins as percent dry mass" (16). The analyses indicated that the average composition of the leaves was about 40% gallotannin and that some individual samples contained 90% hydrolyzable tannins by weight (16).

A subset of samples from the ecological study was selected for analysis with the new KIO<sub>3</sub> method standardized with methyl gallate. This analysis indicated that upon methanolysis, the leaves produced up to 20% by mass methyl gallate. The gallotannin in maple leaves is principally acertannin, a simple digalloyl glucose (1), so on the basis of the mass ratio for conversion of digalloyl glucose to methyl gallate (Table 1), it is reasonable to conclude that these leaves contain about 20% by mass tannin. It is likely that the moderate values obtained with the modified KIO<sub>3</sub> method reflect the tannin content of *Acer* leaves more reliably than the extraordinarily high levels reported by Hunter and Forkner (16).

In the ecological study (16), maple samples were collected at various times throughout a growing season. Representative samples were analyzed with the modified KIO<sub>3</sub> method, and the results were compared to data obtained in the original study with the original KIO<sub>3</sub> method (Figure 5). The graph clearly shows the extremely high values provided by the original KIO<sub>3</sub> method and the more reasonable values from the new method. Despite these major quantitative differences, qualitatively the two methods document similar trends in hydrolyzable tannin levels. This similarity is expected since both methods detect galloyl esters. These data suggest that the new KIO<sub>3</sub> method will be useful for examining hydrolyzable tannin in ecological, physiological, and nutritional studies of plants.

#### ABBREVIATIONS USED

EGCG, epigallocatechin gallate; HHDP, hexahydroxydiphenic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate.

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