Quantitative Determination of Ellagic Acid†

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A new spectrophotometric method for determining ellagic acid is based on the formation of the quinone oxime of the ellagic acid nitrosylation product. The detection limit of the method is 1 μg of ellagic acid, and the relative standard deviation of the method is 0.8%. The method is selective, with positive reaction from ellagic acid but not from a variety of other common plant phenolics including ellagitannins, gallotannins, proanthocyanidins, phenylpropanoids, flavonoids, and gallic acid. Preparation of hydrolysates of plant samples for analysis of the ellagic acid released from ellagitannins is simple.

INTRODUCTION

Ellagitannins (Figure 1), which are hydrolyzable tannins containing hexahydroxydiphenic acid, occur in a variety of dicots (Haslam, 1981). There is little quantitative information on the occurrence of ellagitannins, although they are thought to be more common than the gallotannins (Haslam, 1981). Methods for determining condensed tannins (proanthocyanidins) (Porter et al., 1986) and gallotannins (Inoue and Hagerman, 1988) are available, but an adequate quantitative method for ellagitannins has not been described. The Bate-Smith method for ellagitannin determination (Bate-Smith, 1972) is subject to interference from gallic acid (Scalbert et al., 1988) and is unreliable (Mole and Waterman, 1987). Chromatographic methods (Klocke et al., 1986; Scalbert et al., 1988) are not always appropriate for complex mixtures such as plant extracts.

Like dietary gallotannins and condensed tannins, dietary ellagitannins have adverse nutritional effects on some animals (Mehansho et al., 1987; Robbins and Hagerman, unpublished observation). Ellagic acid may be useful in preventing cancer (Mandal et al., 1986; Barch and Fox, 1988) and has a variety of other effects on animal and plant physiology (Klocke et al., 1986). Further investigation of the effects of ellagitannins and ellagic acid would be facilitated by improved analytical methods. The method described here for determining ellagic acid in plant samples is a spectrophotometric method, and common plant phenolics do not interfere with the assay.

MATERIALS AND METHODS

Ellagic acid was obtained from Sigma (St. Louis, MO). Chestnut tannin was obtained from Pilar River Plate Corp. (Newark, NJ). All other chemicals were of reagent grade or the best grade available. Samples were filtered with Gelman GA-8S modified polysulfone membrane filters (2-μm pore) or Gelman Type E glass fiber filters (Gelman Instruments, Chelsea, MI).

Statistical comparisons were done with Student's t-test.

Safety. Pyridine should be used in a chemical fume hood because of its irritating fumes. Pyridine can be distilled for reuse to minimize disposal.

Determination of Ellagic Acid. A sample of ellagic acid in pyridine was added to pyridine to give a final volume of 2.10 mL in a new, dry test tube. After adding 0.100 mL of concentrated HCl and mixing, the sample was brought to 30 °C. The sample was quickly mixed after 0.100 mL of 1% (w/v) $NaNO_2$ in H_2O was added, and the absorbance at 538 nm was immediately recorded. After the sample was incubated for 36 min at 30 °C, the absorbance was again recorded. The difference between the initial absorbance and the absorbance at 36 min (ΔA_{538}) was proportional to the ellagic acid concentration. Quartz or glass cuvettes were used because plastic cuvettes dissolved in pyridine. New glass test tubes were used because a residue from routine glass washing procedures inhibited the reaction.

Sample Preparation for Ellagic Acid Determination. Leaf samples were collected on the Miami University campus during July 1987, and voucher specimens were deposited at the Willard Sherman Turrell Herbarium at Miami University, Oxford, OH. The largest veins were removed, and the remainder of the leaves was cut into ~1-cm^2 pieces. The samples were frozen with liquid N_2, ground in a mortar and pestle, and lyophilized.

For hydrolysis, samples were put into constricted test tubes with 2 N H_2SO_4 (5–10 mg of sample/mL of acid), frozen in a dry ice/2-propanol bath, evacuated and sealed, and heated for appropriate times at 100 °C. After hydrolysis, the samples were cooled to room temperature, opened, and cooled in an ice bath for 10 min. Samples either were vacuum-filtered through a membrane filter (method 1) or were centrifuged at 2000g before the supernatants were siphoned off (method 2).

Sample residues prepared by method 1 were washed with several volumes of ice-cold wash solvent [acetone/H_2O/concentrated HCl (70/30/1 v/v/v)] and air-dried. When the residue was dry, both sample and filter were transferred to a test tube and were dissolved in 10 mL of pyridine. Undissolved material was removed by a second filtration.

Sample residues prepared by method 2 were washed twice with 5 mL of ice-cold wash solvent, dried under N_2, and dissolved in 10 mL of pyridine. Insoluble material was removed by filtration.

Samples prepared by either method were filtered through a glass fiber filter supported on a fine sintered glass filter to remove insolubles including undissolved membrane filter from method 1 and undissolved plant material. The final filtrate was assayed for ellagic acid.

Chestnut Tannin Purification. Chestnut tannin (800 mg) was suspended in 50 mL of 99% methanol [methanol/H_2O (99/1 v/v)] and centrifuged 10 min at 12000g at 4 °C. The supernatant was mixed with about 50 g of Sephadex LH-20 equilibrated in 99% methanol (Strumeyer and Malin, 1975). The Sephadex was washed with 99% methanol until the eluate was colorless. Tannins, which were detected with the Prussian blue test (Price and Butler, 1977), were then eluted from the Sephadex LH-20 with acetone/H_2O (7/3 v/v). After evaporation under reduced pressure to remove the acetone, the eluate was lyophilized to yield 80 mg of white powder.

Bate-Smith Method for Ellagitannin Determination. A

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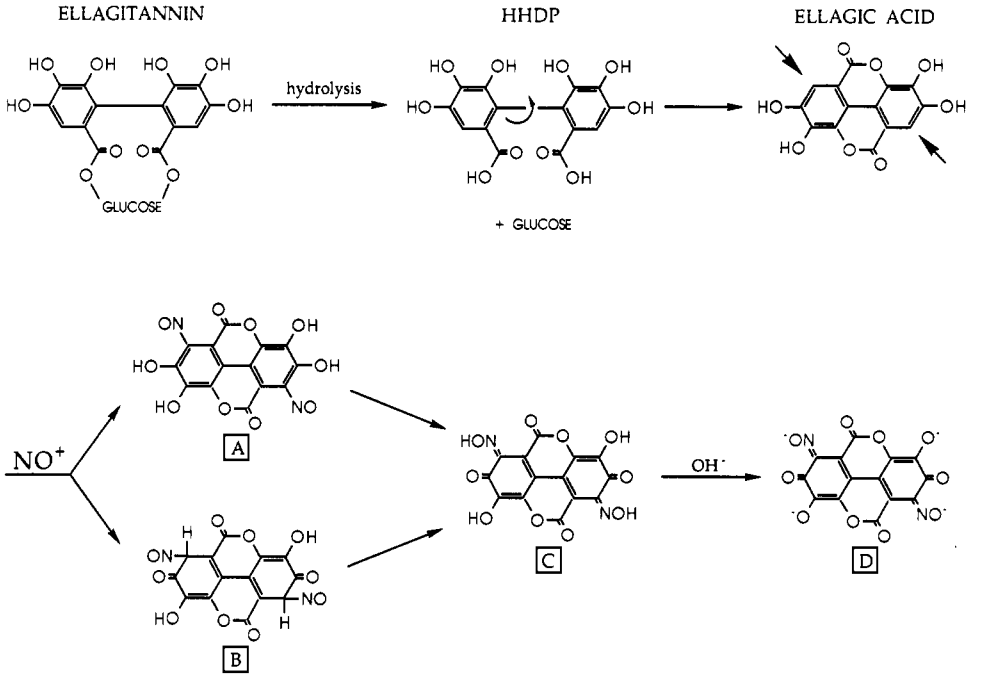

Figure 1. Formation of ellagic acid from an ellagitannin and reaction of ellagic acid with the electrophile NO^+. Hydrolysis of the ellagitannin produces hexahydroxydiphenic acid (HHDP), which spontaneously lactonizes to ellagic acid. The unsubstituted carbons of ellagic acid, susceptible to electrophilic attack, are indicated by arrows. Two possible primary products are A, the simple substitution product, and B, the nitrosyl dienone. The same decay product, a quinone oxime (C), and ionized decay product (D) are produced from either A or B.

mixture of 2.0 mL of chestnut tannin [0.02% (w/v) in methanol/H_2O (1/1 v/v)] and 0.16 mL of 6% acetic acid [glacial acetic acid/H_2O (6/94 v/v)] in a cuvette was flushed with N_2 for 15 min. After 0.16 mL of 6% (w/v) $NaNO_2$ in H_2O was added, the solution was flushed an additional 15 s and the cuvette was sealed with a Teflon stopper. After 1 h, the absorbance at 600 nm was recorded, and the extinction coefficient for ellagitannins containing one hexahydroxydiphenic acid group was used to calculate ellagitannin content (Bate-Smith, 1972).

Determination of Gallic Acid and Condensed Tannins. Leaf samples were exhaustively extracted with acetone/H_2O (7/3 v/v). Free gallic acid and esterified gallic acid in the extract were determined with the rhodanine assay (Inoue and Hagerman, 1988). Condensed tannins in the extracts were assayed with the acid butanol assay (Porter et al., 1986).

RESULTS

Ellagic acid, which does not absorb in the visible wavelengths (Figure 2a), forms a red nitrosylated product (λ_{max} = 538 nm in pyridine, Figure 2b). The reaction product (primary product) eventually decays to a yellow compound (decay product) (Figure 2c), which becomes orange in basic solution. The spectra of the primary product and the decay product are identical at wavelengths below 400 nm (Figure 2b,c).

Gallic acid is structurally similar to ellagic acid, but the reaction of gallic acid with $NaNO_2$ gives a yellow-brown product (Figure 2e). The following phenolics, which have a variety of substitution patterns, also react to form yellow or orange nitrosylated products rather than the red product characteristic of ellagic acid: phenol, phloroglucinol, catechol, gallic acid, 2,5-dimethoxycinnamic acid, ferulic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, 4-hydroxy-3-methoxybenzyl alcohol, 3,4-dimethoxybenzoic acid, catechin, and quercetin. Tannins including *Sorghum* tannin (a condensed tannin), tannic acid (a gallotannin), and chestnut tannin (an ellagitannin) do not form the primary reaction product.

Yellow products were formed when diethylamine or aniline was reacted with $NaNO_2$ under the conditions of this assay (Boyer, 1969; Rao and Bhaskar, 1969), but amines are soluble in strong acid and are separated from ellagic acid during sample hydrolysis. Therefore, amines in plant samples will not interfere with ellagic acid determination.

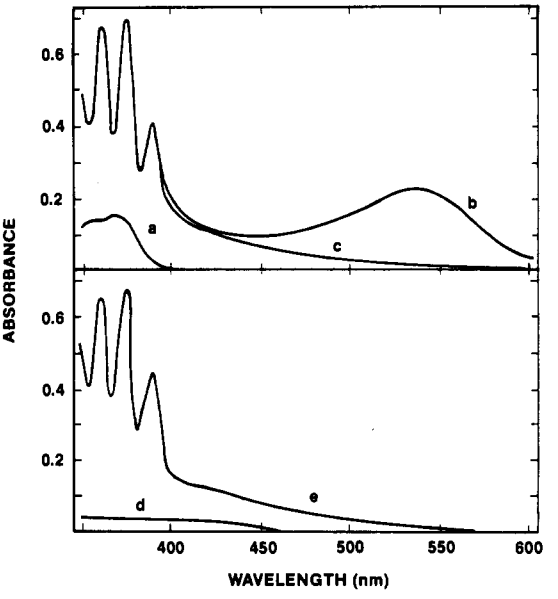

Figure 2. Absorbance spectra. Ellagic acid (10 μg) or gallic acid (10 μg) was nitrosylated under the optimized conditions described in the text. Spectra were recorded with a double-beam spectrophotometer using a reference solution containing pyridine, concentrated HCl, and $NaNO_2$. (a) Ellagic acid in pyridine. (b) The primary product of the reaction of ellagic acid with $NaNO_2$ in pyridine. (c) The decay product. (d) Gallic acid in pyridine. (e) The product of the reaction of gallic acid with $NaNO_2$ in pyridine.

Reaction Conditions. Color formation was maximized when ellagic acid and $NaNO_2$ were reacted in pyridine. Although ellagic acid and $NaNO_2$ react in aqueous tetrahydrofuran, in *N*,*N*-dimethylacetamide, or in dime-

Table I. Reaction of Ellagic Acid with $NaNO_2$ with Various Acid Catalysts

acid	molarity	absorbance	time, min
	Reaction A[a]		
HCl	0.054	0.820	6.0
HNO_3	0.072	1.100	2.5
$HClO_4$	0.045	0.600	24
CH_3CO_2H	2.04	0.780	4.0
H_3PO_4	0.067	turbid	
H_2SO_4	0.081	turbid	
	Reaction B[b]		
HCl	0.52	0.479 ± 0.002	36
HNO_3	0.70	0.482 ± 0.008	36

[a] Reaction mixtures contained 50 μg of ellagic acid in 2.1 mL of pyridine. $NaNO_2$ (100 μL of a 10% solution) was added, and the absorbance was monitored for 30 min. The maximum absorbance at 538 nm and the time required to reach that absorbance were recorded. [b] HCl and HNO_3 were compared under the optimized assay conditions in a reaction containing 21 μg of ellagic acid. Data are the average of triplicate determinations.

thyl sulfoxide, the absorbance of the product in pyridine is 5 times the absorbance in other solvents.

A precipitate formed when the reaction was carried out in absolute pyridine, but not when a small amount of H_2O was incorporated into the mixture by using an aqueous $NaNO_2$ solution. The final concentration of H_2O in the assay mixture is about 4% (by volume).

Several acid catalysts were tested in an attempt to maximize color yield (Table I, reaction A). Phosphoric acid and sulfuric acid yielded turbid reaction mixtures in pyridine, and perchloric acid and acetic acid produced less color than HCl or HNO_3. The concentration of HNO_3 had to be about 33% greater than the concentration of HCl to give a similar response (Table I, reaction B). In preliminary experiments conducted in tetrahydrofuran/H_2O (1/1 v/v), ellagic acid was reacted with $NaNO_2$ in solutions containing from 0.06 to 0.28 M HCl. When small amounts of acid were used, only the decay product was observed. When the amount of acid was increased, the primary reaction product accumulated.

Waite and Tanzer (1981) suggested that solutions of $NaNO_2$ are not stable, so a solution of 1% (w/v) aqueous $NaNO_2$ was tested when first prepared and after 6 weeks of storage at room temperature. The absorbance obtained with the aged solution was not statistically different from the value obtained with the fresh solution ($P > 0.05$, $n = 3$).

The primary product of nitrosylation of ellagic acid was transient (Figure 2b,c), so control of temperature and time was critical. A convenient temperature for the assay was 30 °C. There is a delay at the beginning of the reaction, followed by rapid formation and gradual decay of the primary reaction product (Figure 3). Increasing the concentration of $NaNO_2$ in the reaction mixture increases the rate of formation of the primary product (data not shown). It takes longer to reach the maximum absorbance as the amount of ellagic acid is increased (Figure 4), and the decay reaction is faster when the ellagic acid concentration is higher (data not shown). Maximum absorbances at 538 nm were reached by 36 min for reaction mixtures containing 5–50 mg of ellagic acid (Figure 4), so in the optimized procedure absorbances were read 36 min after the reaction was started.

The method was standardized under the optimized reaction conditions with commercial ellagic acid. The equation of a typical linear calibration curve was $A_{538} = 22.2$ (mg of ellagic acid) + 0.007, $R^2 = 0.99$ (triplicate values, each of six concentrations of ellagic acid). The response became nonlinear at absorbances above 1.1. The

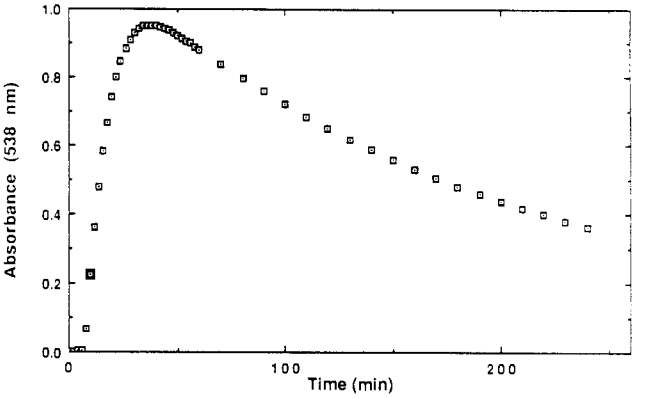

Figure 3. Kinetics of the reaction of ellagic acid with $NaNO_2$ in pyridine. Ellagic acid (39 μg) was reacted with $NaNO_2$ in pyridine under the optimized conditions described in the text, and the absorbance at 538 nm was recorded as a function of time.

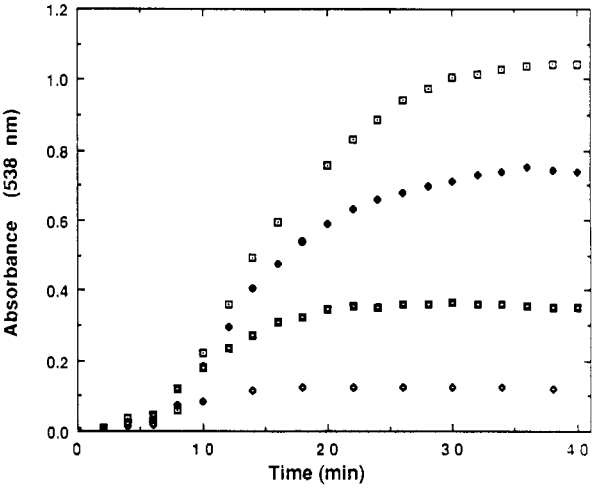

Figure 4. Kinetics of the reaction of various concentrations of ellagic acid with $NaNO_2$. Ellagic acid was reacted with $NaNO_2$ in pyridine under the optimized conditions described in the text, and the absorbance at 538 nm was recorded as a function of time. (White square, black dot) 46 μg ellagic acid. (Black diamond) 31 μg ellagic acid. (Black square, white dot) 16 μg ellagic acid. (Black diamond, white dot) 5.2 μg ellagic acid.

relative standard deviation was 0.85%. The smallest amount of ellagic acid detectable was 1 μg.

Fresh stock solutions of ellagic acid in pyridine were yellow, and solutions stored 6 weeks at room temperature were brown. The absorbance obtained with a fresh solution of ellagic acid was not statistically different from that obtained with a 6-week-old solution ($P > 0.05$, $n = 3$), indicating that the color change does not affect the determination of ellagic acid.

Sample Analysis. To use this reaction for ellagitannin determination, the tannins must first be hydrolyzed. Several conditions for hydrolysis of ellagitannins to produce ellagic acid were tested, including heating in 1 N trifluoroacetic acid (G. Stoner, personal communication), heating in 1 N hydrochloric acid (Scalbert et al., 1988), or heating in 2 N sulfuric acid (Inoue and Hagerman, 1988). The amount of ellagic acid released from ellagitannin was the same when the hydrolysis was carried out at 100 °C in sulfuric or hydrochloric acid, but much less ellagic acid was released by hydrolysis at 68 °C in trifluoroacetic acid. Sulfuric acid was routinely used for hydrolysis.

The release of ellagic acid from chestnut tannin is complete within 10 h. Hydrolysis for as much as 120 h did not release additional ellagic acid. The release of gallic acid from gallotannins under the same conditions requires 26 h (Inoue and Hagerman, 1988).

To determine the stability of ellagic acid under the hydrolysis conditions, ellagic acid was suspended in 2 N H_2SO_4 and hydrolyzed under vacuum for 120 h at 100 °C. The average recovery obtained by method 1 was 99 ± 7% (n = 3) and by method 2 was 94 ± 4% (n = 3). Ellagic acid was added to a sample of chestnut tannin before hydrolysis to determine whether components in the mixture of hydrolysis products influenced ellagic acid recovery. The average recovery of ellagic acid was 100 ± 2% (method 2, n = 4).

The limited solubility of ellagic acid (Press and Hardcastle, 1969) made it simple to prepare hydrolysates for analysis. The solids recovered from the hydrolysates were washed with cold acetone/H_2O containing HCl to eliminate colored compounds which interfered with the determination. Ellagic acid was insoluble in the cold solvent.

Both the recovered ellagic acid and the filter used in method 1 were dissolved in pyridine for analysis. The absorbance obtained by assaying a solution of ellagic acid containing dissolved filter was not statistically different from that obtained with a solution not containing the filter ($P > 0.05$, n = 3), indicating that the dissolved filter did not interfere in the assay.

Method 2 is convenient for large numbers of samples, but care must be taken to avoid loss of ellagic acid in the siphoning step. Centrifuging steps should be performed at 4 °C to minimize loss of ellagic acid in the wash solvent.

Analysis of a commercial preparation of chestnut tannin revealed no condensed tannins, 4.6% free gallic acid, and 3.5% esterified gallic acid (gallotannin). According to the results of the Bate-Smith assay, the chestnut tannin is 37% ellagitannin. The nitrosylation reaction described here was used to analyze the chestnut tannin for ellagic acid released by hydrolysis. Crude and purified preparations of chestnut tannin contained 7.5% and 11.3% ellagic acid respectively (n = 3).

Several attempts were made to extract ellagitannins from leaves. Leaf samples (*Quercus macrocarpa*) were extracted with water; acetone/H_2O (7/3 or 1/1 v/v); absolute acetone; ethyl acetate/acetone (2/1 v/v); methanol/H_2O (7/3, 4/1, or 1/1 v/v); or absolute methanol (Hagerman, 1988; Teel and Martin, 1988; Klocke et al., 1986; Scalbert et al., 1988; Swain, 1979). Organic solvents were removed by evaporation under reduced pressure, and the samples were acidified to 2 N H_2SO_4 with concentrated H_2SO_4, hydrolyzed at 100 °C for 15 h, and assayed for ellagic acid. Ellagic acid was detected in only five of the extracts, and only about 15% of the ellagic acid that could be released by direct hydrolysis of the leaves was detected in those extracts.

Ellagic acid was liberated by hydrolysis of leaves of sugar maple (*Acer saccharum*) and oak (two *Quercus* sp.) (Table II). Hydrolysis of shellbark hickory (*Carya laciniosa*), tulip poplar (*Leriodendron tulipifera*), and sumac (*Rhus typhina*) did not release any ellagic acid. All of the species tested contained condensed tannin (Table II). Sumac and maple, the only species to contain gallotannins (Table II), also contained trace amounts of unesterified gallic acid.

Table II. Tannin in Leaves[a]

species (common name)	% by weight: ellagic acid	esterified gallic acid	condensed tannin
R. typhina (sumac)	0.0	7.28	0.32
C. laciniosa (shellbark hickory)	0.0	0.0	0.86
L. tulipifera (tulip poplar)	0.0	0.0	1.35
A. saccharum (sugar maple)	4.62 ± 0.20	3.35	1.12
Q. macrocarpa (burr oak)	1.47 ± 0.20	0.0	1.32
Q. alba (white oak)	1.02 ± 0.25	0.0	0.80

[a] Leaf samples were hydrolyzed in sulfuric acid, and the hydrolysates was analyzed in triplicate for ellagic acid. For other analyses, tannins were extracted from the leaves with acetone/water (7/3 v/v). Gallotannins were determined by measuring esterified gallic acid (Inoue and Hagerman, 1988), and condensed tannins were measured as anthocyanidins (Porter et al., 1986) by using purified sorghum tannin (Hagerman and Butler, 1980) to standardize the assay.

DISCUSSION

When ellagitannins are reacted with $NaNO_2$ in acidic solution in the absence of oxygen, a blue compound (λ_{max} = 600 nm) slowly forms. A quantitative method for determining ellagitannins based on this reaction (Bate-Smith, 1972) is inadequate, since it gave negative results for several plants known to contain ellagitannin (Mole and Waterman, 1987). Gallic acid interferes with the Bate-Smith determination (Scalbert et al., 1988). Furthermore, the procedure is rather insensitive, and the need to exclude oxygen from the reaction makes it inconvenient.

Hydrolysis of ellagitannins produces hexahydroxydiphenic acid, which spontaneously forms the lactone ellagic acid (Figure 1). A method for determining the hydrolysis product would provide an indirect method for estimating ellagitannins. Ellagic acid does not react with $NaNO_2$ under the conditions described by Bate-Smith (1972). However, modification of the conditions for nitrosylation provided a sensitive, selective method for ellagic acid determination.

Reaction Mechanism. The reaction is an electrophilic aromatic substitution in which $NaNO_2$ and acid are added to a solution of pyridine to form the electrophile in situ. The pK_b of pyridine is 8.6, making it a sufficiently weak base that it does not interfere with the formation of the electrophile. In acid, $NaNO_2$ forms the electrophiles HONO, N_2O_3, H_2ONO^+, NOCl, and NO^+ (Ridd, 1978; Williams, 1983). Formation of electrophile(s) probably occurs during the delay in the reaction before the primary product forms (Figures 3 and 4).

Of the possible electrophiles, the principal species in this system are probably N_2O_3 and NOCl. When large amounts of HNO_3 were added to reactions, red-brown gas evolved and the solution became light blue, suggesting that N_2O_3 was present. In an analogous reaction, Cl^- increased the rate of nitrosylation of phenolics, suggesting that NOCl was participating in the reaction (Challis and Higgins, 1973). More nitrosylated ellagic acid accumulates when HCl is the catalyst than when HNO_3 is the catalyst (Table I), consistent with the presence of NOCl in the HCl-catalyzed reaction.

Other electrophiles formed by $NaNO_2$ in solution are probably not important in the nitrosylation of ellagic acid. HONO, detected in the reaction mixtures by its characteristic spectrum between 340 and 390 nm (Figure 1b,c,e; Bayliss and Watts, 1956; Waldorf and Babb, 1964), is a very weak electrophile and probably does not participate in nitrosylation (Ridd, 1978). The basicity of pyridine minimizes the protonation of HONO (Ridd, 1978) and suggests that H_2ONO^+ is not an important reactant. The small amounts of acid catalyst needed for the nitrosylation of ellagic acid suggest that NO^+ is probably not formed in these reactions, since it has been reported that 40% by weight H_2SO_4 or 50% by weight H_3PO_4 is necessary for the formation of NO^+ (Bayliss and Watts, 1956).

There are two equivalent carbons in ellagic acid available for substitution (Figure 1). The two carbons where substitution could occur are chemically identical and are probably both nitrosylated.

The nitrosylation of ellagic acid may involve one of two mechanisms: either a simple substitution of H^+ by NO^+ on the unsubstituted electron-dense carbon to give a nitrosyl compound (A, Figure 1) or electrophilic attack and loss of H^+ from the ortho phenol group to give the neutral nitrosyl dienone (B, Figure 1). The latter mechanism is like the mechanism proposed for the nitrosylation of other phenolics (Challis and Higgins, 1973). Addition of base to reaction mixtures that contained the primary product of nitrosylation of ellagic acid increased the rate of formation of the decay product, consistent with the base catalysis noted by Challis and Higgins (1973). Furthermore, the primary reaction product accumulated only when relatively large amounts of acid were used, consistent with the Challis and Higgins (1973) mechanism.

The decay product is probably the quinone oxime (C, Figure 1). Upon reaction with $NaNO_2$, phenolics other than ellagic acid immediately form yellow nitrosylated products which may be quinone oximes. Presumably the substitution pattern of phenolics other than ellagic acid promotes rapid decay of the nitrosyl dienone to the quinone oxime so that the dienone does not accumulate. With ellagic acid the decay is slower and the dienone accumulates during the initial phase of the reaction.

When NaOH was added to the decay product, a bright red-orange compound (ionized decay product) formed, presumably the ionized salt of the quinone oxime (D, Figure 1). Similar color changes accompanying ionization of the quinone oxime have been observed with other phenolics (the Hoepfner reaction) (Bate-Smith, 1972; Waite and Tanzer, 1981; Stafford et al., 1987).

Sample Analysis. The new method for determination of ellagic acid gives results consistent with the Bate-Smith (1973) method for ellagitannins. Both methods indicated that the commercial preparation of chestnut tannin contained relatively little ellagitannin and large amounts of unidentified impurities. As has been reported for both condensed tannins and gallotannins, some purification of the ellagitannin was achieved with Sephadex LH-20 (Strumeyer and Malin, 1975).

The analyses of leaf tannins reported here are similar to results obtained by other workers. Klocke et al. (1986) found 1.32% (by dry weight) ellagic acid in the aerial parts of *Quercus gambellii*, and Scalbert et al. (1988) reported 0.74% ellagic acid in leaves of *Quercus robur*. Similar levels of ellagic acid were found in two species of *Quercus* by using the new nitrosylation method for ellagic acid (Table II). *Quercus* contains condensed tannin and ellagitannin but not gallotannin (Table II; Haddock et al., 1982; Klocke et al., 1986; Scalbert et al., 1988). Several *Rhus* species contain gallotannin but not ellagitannin (Table II; Haddock et al., 1982). Hydrolyzable tannins are completely absent from *Carya* species (Table II; Wilkin and Cosgrove, 1964). *L. tulipifera*, like other members of Magnoliidae, does not contain ellagitannin (Table II; Klocke et al., 1986). The tannins of *Acer* species have been investigated extensively, and *A. saccharum* contains all three types of tannin (Table II; Haslam, 1965; Bate-Smith, 1977, 1978). These results indicate that the new method for ellagic acid analysis is successful with plant samples containing mixtures of ellagitannin, gallotannin, and condensed tannin. With modification of sample preparation procedures, the method could also be used to determine ellagic acid in animal tissues or secretions.

Some ellagitannins, such as brevilagin 2, contain modified hexahydroxydiphenic acid groups (Haslam, 1981) and yield derivatives of ellagic acid upon hydrolysis. It is not known whether these ellagic acid derivatives would react in the nitrosylation reaction to produce a primary product similar to that obtained with ellagic acid. Similar problems are encountered with methods for determining condensed tannin or gallotannin by quantitating degradation products (Porter et al., 1986; Inoue and Hagerman, 1988).

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