Vanillin Assay for Proanthocyanidins (Condensed Tannins): Modification of the Solvent for Estimation of the Degree of Polymerization

Larry G. Butler,* Martin L. Price,[1] and Jeffery E. Brotherton[2]

When the reaction of flavanols with vanillin is carried out in glacial acetic acid, the absorbance produced is much greater than in methanol, the conventional solvent. In glacial acetic acid, but not in methanol, the time course of the vanillin reaction with catechin is similar to that of tannin. The absorbance produced in glacial acetic acid, but not in methanol, is approximately proportional to the concentration of flavan-3-ol end groups and thus measures the concentration of oligomeric molecules rather than the total concentration of flavan-3-ol units. By use of the reaction in glacial acetic acid, the degree of polymerization of purified tannin samples can be determined. The results agree well with literature values obtained independently by more laborious techniques. However, methanol is the solvent of choice for determination of tannin content because in methanol the reaction is much less sensitive to monomer units such as catechin than it is to the polymeric tannins.

The vanillin assay is widely employed as a method for quantitative determination of condensed tannin (proanthocyanidins) in plant materials such as fruits (Goldstein and Swain, 1963b), sorghum grain (Burns, 1971), and forage legumes (Broadhurst and Jones, 1978). It is a sensitive, relatively simple assay specific for flavan-3-ols, dihydrochalcones, and proanthocyanidins (Sarkar and Howarth, 1976; Gupta and Haslam, 1980). For convenience, catechin, a monomeric flavan-3-ol unit of condensed tannins, is often used to standardize the assay rather than purified condensed tannin, although this leads to a considerable overestimation of tannin content (Price et al., 1978; Gupta and Haslam, 1980). In methanol, the usual solvent for the assay of extracts of sorghum, catechin and tannin react with vanillin with quite different kinetic patterns (Price et al., 1978; Gupta and Haslam, 1980).

We now report that in glacial acetic acid, the reactions of catechin and tannin with vanillin are kinetically similar. In addition, the reaction produces more chromophore than in methanol, and the concentrations of chromophore produced is proportional to the concentration of flavan-3-ol end groups present. Thus, the assay can be used to estimate the degree of polymerization of a proanthocyanidin.

EXPERIMENTAL PROCEDURES

Materials. Catechin, phloroglucinol, and vanillin were obtained from Sigma, and epicatechin was from Aldrich. All were used without further purification. Catechin and epicatechin oligomers were generously provided by Dr. E. Haslam, Department of Chemistry, Sheffield University, U.K. Purified tannin from sorghum grain (NK 300) was provided by Dr. Haslam. Tannin from a high-tannin sorghum grain (BR 54) was purified by our standard technique (Hagerman and Butler, 1980) by A. Hagerman. Samples to be used for determining extinction coefficients were dried overnight over P_2O_5 at room temperature.

Assays. Vanillin assays in methanol were carried out as recommended by Price et al. (1978), in a 30 °C water bath with a reaction time of 20 min. The vanillin reagent contained 4% concentrated HCl and 0.5% vanillin in methanol. Absorbance was read in a Zeiss PMQ-II spectrophotometer, at 500 nm, the wavelength of maximum absorbance, by using a 1-cm cell. Samples were dissolved in methanol.

For assays in glacial acetic acid, the vanillin reagent contained 4% concentrated HCl and 0.5% vanillin in glacial acetic acid. The absorbance was read at 510 nm as described above. Samples were dissolved in glacial acetic acid, except for tannins, which were dissolved in a minimum volume of methanol and diluted in glacial acetic acid. The reaction time was shortened to 5 min, and the methanol content was kept as low as possible to minimize the effect of methanol on the reaction (see below).

RESULTS

We examined several parameters of the vanillin assay for tannin in addition to those we previously investigated (Price et al., 1978). A brief survey of vanillin (4-hydroxy-3-methoxybenzaldehyde) analogues demonstrated that 2,4-dimethoxybenzaldehyde gave absorbance values 2–5 times greater than those obtained with vanillin and may therefore warrant further investigation if greater sensitivity is needed. All data presented in this paper were obtained by using vanillin.

The rate and extent of color development in the reaction between vanillin and catechin or tannin were found to be strongly solvent dependent. Conventional solvents for this reaction are sulfuric acid (Swain and Hillis, 1959) and methanol (Burns, 1971). In both glacial acetic acid and acetonitrile the reaction produced much more intense absorption near 500 nm than was produced in methanol. Glacial acetic acid was chosen for further investigation because the kinetics of the reaction are less complex in this solvent and the colored product is more stable.

The change in absorbance at 510 nm vs. time for the reaction between vanillin and catechin in a solution of 4% concentrated HCl in glacial acetic acid is shown in curve A of Figure 1. When the same reaction was carried out in methanol (Price et al., 1978), the absorbance after 5 min was approximately 3-fold lower.

Addition of methanol to the reaction in glacial acetic acid caused a rapid decrease in absorbance (curves B and C in Figure 1). The change in A_{510} is not due to a single second-order reaction for either the initial color formation or the decrease following the addition of methanol, as shown by the nonlinearity of the appropriate semilog plots [log $(A_{max} - A_t)$ vs. t or log $(A_t - A_{min})$ vs. t, not shown].

In methanol, vanillin reacts more slowly and in a more complex fashion with tannin than with catechin (Price et al., 1978; Gupta and Haslam, 1980). However, in glacial acetic acid purified tannin behaved kinetically in a manner

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907.

[1]Present address: ECHO, N. Fort Meyers, FL 33903.

[2]Present address: Department of Agronomy, University of Illinois, Urbana, IL 61801.

0021-8561/82/1430-1087$01.25/0

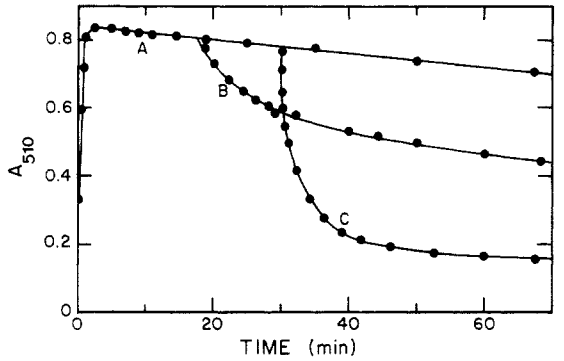

Figure 1. Reaction of catechin with vanillin in glacial acetic acid. Conditions were as described under Experimental Procedures. At the times indicated, methanol was added to aliquots of the reaction mixture (sample A) to the final concentration of 3.8% (B) and 12.7% (C) (v/v).

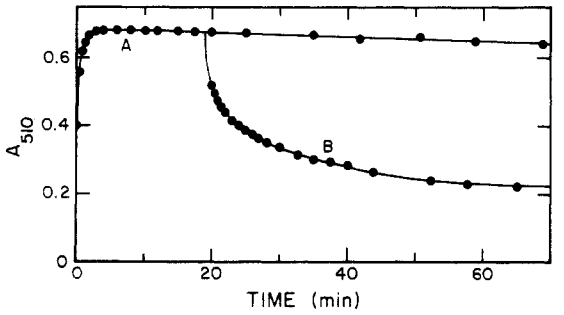

Figure 2. Reaction of sorghum tannin with vanillin in glacial acetic acid. Conditions were as described under Experimental Procedures; the methanol concentration was 3.8% (v/v) in the original solution (A). After 19-min reaction, methanol was added to an aliquot from sample A to a final concentration of 16% (v/v) (B).

similar to that of catechin [curve A of Figures 1 and 2; compare to Figure 4 in Price et al. (1978)]. Rate constants cannot be compared because of nonlinear semilog plots as described above. A small amount of methanol was present during the initial reaction of tannin with vanillin because tannin could not be dissolved in glacial acetic acid. As with catechin, methanol diminishes the absorbance of the reaction product (curve B of Figure 2).

We reported earlier (Price et al., 1978) that use of catechin for the standard curve in the vanillin assay (methanol solvent) results in considerable overestimation of tannin content because more chromophore is produced per milligram of tannin than is produced per milligram of catechin (Figure 3, curves C and D). However, when glacial acetic acid is the solvent, the absorption produced by the polymeric tannin is considerably less than that from the monomeric catechin (Figure 3, curves A and B). Thus, in glacial acetic acid some of the flavan-3-ol units of the tannin polymer apparently do not react with vanillin.

This point was further investigated by using a series of defined flavan-3-ol oligomers provided by Dr. E. Haslam. Table I reports extinction coefficients for the vanillin adduct of monomeric, oligomeric, and polymeric flavan-3-ols (proanthocyanidins) obtained in both methanol and glacial acetic acid. For all the compounds tested, more absorption is produced by the reaction in acetic acid than by the reaction in methanol. Chromophore production in methanol seems to be dependent upon several factors, including the degree of polymerization, the type of linkage between monomer units, and possibly the nature of the monomer units. No regularities occur in the extinction coefficient, either on a molar or on a weight basis. In contrast, for all

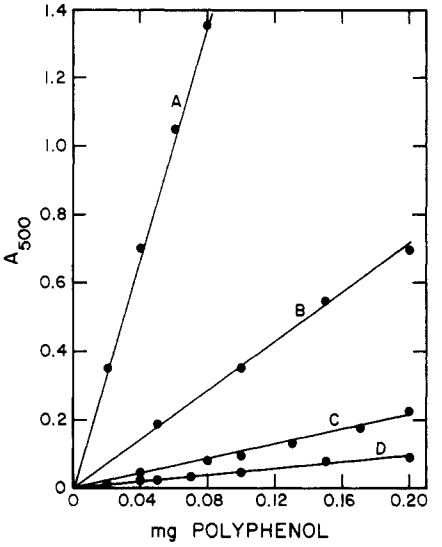

Figure 3. Standard curves for catechin and sorghum tannin in methanol and in glacial acetic acid. (A) Catechin in acetic acid; (B) tannin in acetic acid; (C) tannin in methanol; (D) catechin in methanol.

Table I. Extinction Coefficients of Reaction with Vanillin

compound[a]	$E_{500}{}^{1\%}$ (methanol)	$E_{510}{}^{1\%}$ (HAc)	$\epsilon_{500}{}^{M} \times 10^{-3}$ (methanol)	$\epsilon_{510}{}^{M} \times 10^{-3}$ (HAc)
monomers				
epicatechin	11.8	271	0.43	9.8
catechin	8.2	243	0.30	8.5
dimers				
B-2 (epicatechin)$_2$	34.9	155	2.52	11.2
B-3 (catechin)$_2$	25.2	170	1.82	12.3
A-2 (epicatechin)$_2$	2.8	134	0.20	9.7
trimers				
B-9 (epicatechin)$_3$	38.4	113	4.16	12.2
polymers				
NK 300 tannin		48		
BR 54 tannin	22.8	56		

[a] Structural details of dimers can be found in Thompson et al. (1972) and in Jacques and Haslam (1974). The trimeric nature of B-9 was a personal communication from Dr. E. Haslam. The structure of NK 300 tannin is presented in Gupta and Haslam (1978).

the flavan-3-ols and their oligomers the molar extinction coefficient in glacial acetic acid is similar. This suggests that reaction with vanillin in glacial acetic acid occurs only at end groups. The relatively low extinction of the polymeric tannins, on a weight basis, is in agreement with this conclusion.

DISCUSSION

The vanillin assay would be most useful for determining proanthocyanidins if it could be carried out under conditions where reaction occurs either at all of the flavan-3-ol units of the polymer or only at the terminal units. The first condition, which is usually assumed in using the assay, would result in equivalent values for all proanthocyanidins on a weight basis. The second condition would result in equivalence on a molar basis. The data in Table I show that in methanol, the conventional solvent for this reaction, neither condition holds. Methanol, in fact, profoundly inhibits chromophore production in this reaction, and addition of small amounts of methanol result in rapid color loss (Figures 1 and 2). Furthermore, methanol affects the

reaction with flavan-3-ol monomers and their oligomers and polymers quite differently, producing complex kinetic patterns that make standardization of tannin analysis with monomers such as catechin tenuous at best.

Replacing methanol with glacial acetic acid as the solvent results in similar kinetics for monomers and polymers, produces severalfold more intense absorption, and most importantly gives extinction coefficients that are approximately equivalent on a molar basis.

The reaction in both solvents involves condensation of vanillin with the proanthocyanidin, without depolymerization of the proanthocyanidin (Watterson and Butler, 1983), so different chromophoric products are formed from different proanthocyanidins. Our data obtained with monomers, dimers, and trimers suggest that in glacial acetic acid the absorbance of the product of the reaction with catechin and epicatechin is essentially equivalent to that of oligomers and the polymeric proanthocyanidin. We conclude that only terminal units of the polymer react with vanillin in acetic acid. Goldstein and Swain (1963a) have previously shown that a series of compounds containing only one phloroglucinol ring all have about the same molar extinction coefficient in the vanillin assay using H_2SO_4 as the solvent. These workers also demonstrated that polymerization of catechin reduces its ability to react with vanillin more than its reaction with a nonspecific reagent for phenolic groups, an observation consistent with preferential reaction at terminal units.

This being the case, the degree of polymerization of a sample of purified tannin can be readily obtained as the ratio of absorbance of monomer to polymer, both determinations being made on equal weights of material. In Table I, for example, the average $E_{510}^{1\%}$ of catechin and epicatechin is 257, which when divided by 48, the $E_{510}^{1\%}$ of sorghum tannin (NK 300) purified by E. Haslam, gives a degree of polymerization of 5.4. This is in reasonable agreement with the value of 6–7 flavan-3-ol units/polymer contained by Gupta and Haslam (1978) by an independent method on a sample from the same source. A comparable value of 4.6 units/polymer can be calculated from the data in Table I for sorghum tannin (BR 54) from a different line prepared in our laboratory. This technique is much simpler than the ^{13}C NMR technique recently developed (Czochanska et al., 1980).

For estimation of the tannin content of a sample, the vanillin assay run in methanol as previously described (Price et al., 1978) is the most useful of several methods tested (Earp et al., 1981). The relative insensitivity of the assay in methanol toward monomers (Figure 3; Table I) is an advantage because absorbance due to the polymeric tannins, which are of greatest interest, is maximized and absorbance due to monomers is minimized. Therefore, it is not expected that glacial acetic acid will replace methanol as the solvent for the vanillin assay of tannin content.

Measurement of the degree of polymerization as described above requires purified condensed tannin, which can be weighed to determine the total number of flavan-3-ol units. Estimation of the average degree of polymerization can be carried out in crude extracts without purification of the tannin if the total content of flavan-3-ol units can be determined by an independent method. We estimate total flavan-3-ol units by spectrophotometric assay of the anthocyanidin pigments produced from the proanthocyanidins by heating in HCl/butanol mixtures (Gupta and Haslam, 1980; Lewak, 1968). Because the vanillin assay for terminal flavan-3-ol units detects both polymer units and free monomers such as catechin, but the above assay for total flavan-3-ol units does not detect free monomers, the average degree of polymerization tends to be underestimated in crude extracts that contain catechin and/or epicatechin.

The following paper (Butler, 1982) reports relative values of the average degree of polymerization of proanthocyanidins from sorghum grain as a function of extraction conditions and degree of maturation.

LITERATURE CITED

Broadhurst, R. B.; Jones, W. T. *J. Sci. Food Agric.* **1978**, *29*, 788.
Burns, E. E. *Agron. J.* **1971**, *63*, 511.
Butler, L. G. *J. Agric. Food Chem.* **1982**, following paper in this issue.
Czochanska, Z.; Foo, L. Y.; Newman, R. H.; Porter, L. J. *J. Chem. Soc., Perkin Trans. 1* **1980**, 2278.
Earp, C. F.; Akingbala, J. O.; Ring, S. H.; Rooney, L. W. *Cereal Chem.* **1981**, *58*, 234.
Goldstein, J. L.; Swain, T. *Nature (London)* **1963a**, *198*, 587.
Goldstein, J. L.; Swain, T. *Phytochemistry* **1963b**, *2*, 371.
Gupta, R. K.; Haslam, E. *J. Chem. Soc., Perkin Trans. 1* **1978**, 892.
Gupta, R. K.; Haslam, E. "Polyphenols in Cereals and Legumes"; Hulse, J. H., Ed.; IDRC: Ottawa, Canada, 1980; p 15.
Hagerman, A. E.; Butler, L. G. *J. Agric. Food Chem.* **1980**, *28*, 947.
Jacques, D.; Haslam, E. *J. Chem. Soc., Perkin Trans. 1* **1974**, 2663.
Lewak, S. *Phytochemistry* **1968**, *7*, 665.
Price, M. L.; Van Scoyoc, S.; Butler, L. G. *J. Agric. Food Chem.* **1978**, *26*, 1213.
Sarkar, S. K.; Howarth, R. E. *J. Agric. Food Chem.* **1976**, *24*, 317.
Swain, T.; Hillis, W. E. *J. Sci. Food Agric.* **1959**, *10*, 63.
Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. N. N. *J. Chem. Soc., Perkin Trans. 1* **1972**, 1387.
Watterson, J. J.; Butler, L. G. *J. Agric. Food Chem.*, in press, 1983.

Received for review February 22, 1982. Accepted June 18, 1982. Supported in part by USAID Project No. XII PRF 4. Paper no. 8898 from the Agriculture Experiment Station, Purdue University.