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# RADIAL DIFFUSION METHOD FOR DETERMINING TANNIN IN PLANT EXTRACTS

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**Abstract**—Tannin in plant extracts can be determined by reacting the tannin with a protein and quantitating the precipitated complex. In the new assay described here, a tannin-containing solution is placed in a well in a protein-containing agar slab. As the tannin diffuses into the gel and complexes with protein, a visible ring of precipitation develops. The area of the ring is proportional to the amount of tannin in the extract. The detection limit of the method is 0.025 mg tannic acid or condensed tannin and the precision is 6% (relative standard deviation). Tests with extracts of a variety of plants show that the new method gives results comparable to other precipitation methods and that the new method is superior for samples of unusual composition, such as aspen buds. The method has several advantages over other methods for determining tannin: The new method is very simple and requires neither complex reagents nor instruments. Components of the plant extract such as non-tannin phenolics or water-insoluble compounds do not interfere with the method. The assay is not subject to interference from the organic and aqueous solutions which are commonly used to extract tannin from plants.

**Key Words**—Tannin, proanthocyanidin, protein precipitation, digestibility-reducing substances, phenolic analysis.

## INTRODUCTION

Tannins, like many other secondary compounds, are thought to defend plants from herbivores (Swain, 1979). To establish the role of tannin as a defensive compound, the tannin content of various plants must be correlated with patterns of herbivory. Sensitive, specific tannin assays which can easily be run on large numbers of samples are required for such studies of tannin as a defensive compound.



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The analytical methods currently available for determining tannin have several disadvantages. The functional group methods do not have satisfactory specificity. For example, the redox methods such as the Folin-Denis assay (Folin and Denis, 1915) are not specific for tannin, but detect any phenolic compound. On the other hand, the proanthocyanidin and vanillin assays (Bate-Smith, 1975; Price et al., 1978) are too selective. The hydrolyzable tannins, which are gallic acid derivatives (Haslam, 1979), do not react with acidic butanol or vanillin. Only the flavonoid-based condensed tannins (Haslam, 1979) can be detected with these reagents.

Precipitation assays also have disadvantages. Several methods for determining protein precipitated by the tannin have been described (Bate-Smith, 1973; Hagerman and Butler, 1980a; Martin and Martin, 1983). Although selective, these methods are inconvenient; they may include multiple steps for forming and isolating the precipitate, or they may require special materials such as radiolabeled compounds. Two simple precipitation methods have been described. In one method, a dye-labeled protein is used, and the amount of protein precipitated by the tannin is determined spectrophotometrically (Asquith and Butler, 1985). In another method, the tannin precipitated by excess protein is measured spectrophotometrically after reaction with ferric chloride (Hagerman and Butler, 1978). Although these methods are straightforward, sample preparation for these assays is complicated. Some solvents, such as acetone, interfere with the precipitation and must be removed from the extract before analysis. In addition, water-insoluble compounds frequently found in the tannin extract interfere with precipitation assays (Hagerman and Butler, 1978; Asquith and Butler, 1985).

A new protein precipitation assay that overcomes these problems is described here. In the assay, tannin diffuses through a protein-containing gel, and a visible disk-shaped precipitate develops as the tannin interacts with the protein. The method is simple, sensitive, and specific, and should be especially applicable to studies in which large numbers of samples are to be analyzed.

## METHODS AND MATERIALS

*Reagents.* All reagents were analytical grade or the best grade available. Agarose (type I), bovine serum albumin (BSA) (fatty acid-free fraction V), and catechin were obtained from Sigma Chemical Co. (St. Louis, Missouri). Condensed tannin was prepared from *Sorghum vulgare* IS 4225 by the method of Hagerman and Butler (1980b). Hydrolyzable tannin was purified from commercial tannic acid as described by Hagerman and Klucher (1986). Buffer A consisted of 50 mM acetic acid and 60 $\mu$M ascorbic acid adjusted to pH 5.0.

*Assay Method.* A 1% (w/v) solution of agarose was prepared in buffer A

by heating the suspension of agarose to boiling while stirring. The solution was cooled to 45°C in a water bath, and the protein [0.1% (w/v) BSA] was added while the solution was gently stirred. The solution was dispensed in 9.5-ml aliquots into standard plastic Petri dishes (8.5 cm diameter) and allowed to cool. The agarose solidified on cooling; the plates were always cooled on a level surface to obtain slabs of uniform thickness. Once prepared, the plates were stored at 4°C to prevent bacterial growth.

Uniform wells were punched in the plates with a punch 4.0 mm in diameter (Biorad Co., Richmond, California). The wells were spaced 1.5 cm apart on the plates. The tannin-containing solutions were added to the wells with a Hamilton microsyringe; the capacity of 4.0-mm wells was slightly greater than 8 $\mu$l, so the samples were applied in 8-$\mu$l aliquots. For dilute samples, several successive 8-$\mu$l aliquots were added to a single well as the liquid was absorbed by the gel. Although any organic or aqueous solution could be applied to the wells, solutions containing at least 10% (v/v) water were easier to dispense than neat organic solvents.

After placing the samples in the wells, the Petri dishes were covered and sealed with Parafilm. The dishes were incubated at 30°C for 96–120 h. The diameters of the rings were then measured; for each ring, two diameters at right angles to one another were measured to minimize errors due to nonuniform ring development. Tannin concentration was calculated from the square of the average of the two diameters using an appropriate calibration curve.

*Plant Extraction.* Plant tissue was extracted for an hour at room temperature with 50% (v/v) aqueous methanol, using a solvent-to-tissue ratio of 0.5 ml solvent per 100 mg tissue. For diffusion assays, 100 mg tissue was an adequate sample size; if other assays were run, the sample size was increased to 300–500 mg. The extracts were applied directly to the diffusion gels without any sample cleanup. Two or three successive aliquots of 8 $\mu$l were sufficient to form rings for the plants tested.

If the extracts were to be analyzed with other precipitation tests, the samples were centrifuged (5000*g*, 15 min) and the extract removed from the pelleted tissue. For the protein precipitation assays (Hagerman and Butler, 1978, 1980a), 0.20 ml of the methanol extract was added to 7.0 mg iodine-125-labeled BSA in 2.00 ml acetate buffer. After 15 min, the mixtures were centrifuged. An aliquot of each supernatant was removed for radioactive counting, and the remainder of the supernatants discarded. The pellets were dissolved in triethanolamine reagent (Hagerman and Butler, 1978), ferric chloride was added, and the $A_{510}$ was determined. The amount of protein precipitated was calculated from the radioactive counting data, using a calibration curve prepared with the labeled BSA. The amount of tannin precipitated was determined from a standard curve run with purified tannic acid (Hagerman and Klucher, 1986).

## RESULTS

When a solution containing condensed or hydrolyzable tannin was placed in a protein-containing gel, the tannin diffused into the gel and formed a disk-shaped tannin–protein precipitate (Figure 1). No ring formed if the protein was omitted from the gel. Under the conditions described here, the ring was always clearly visible without any staining. The rings appeared similar for condensed tannin obtained from *Sorghum* seeds, for hydrolyzable tannin purified from commercial tannic acid, and for tannin-containing extracts from a variety of plants (Figures 1 and 2).

The rings expanded slowly, and reached their equilibrium sizes only after

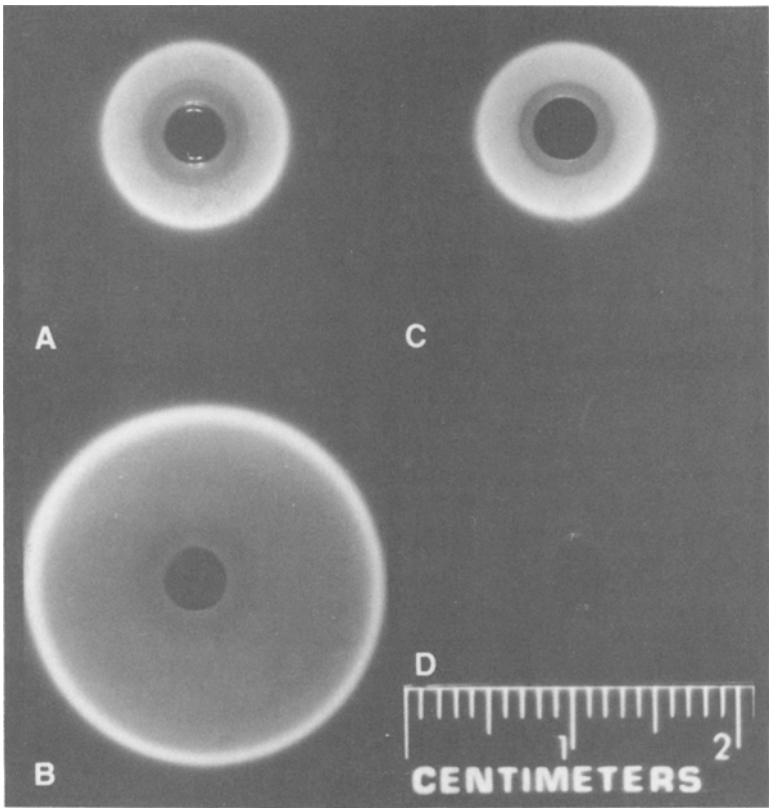


FIG. 1. Radial diffusion assay for tannin. An 8-$\mu$l aliquot of the sample dissolved in 70% acetone was placed in each well and photographed after the rings reached equilibrium. (A) 0.60 mg sorghum tannin; (B) 0.60 mg tannic acid; (C) 0.50 mg catechin plus 0.60 mg sorghum tannin; and (D) 0.50 mg catechin.

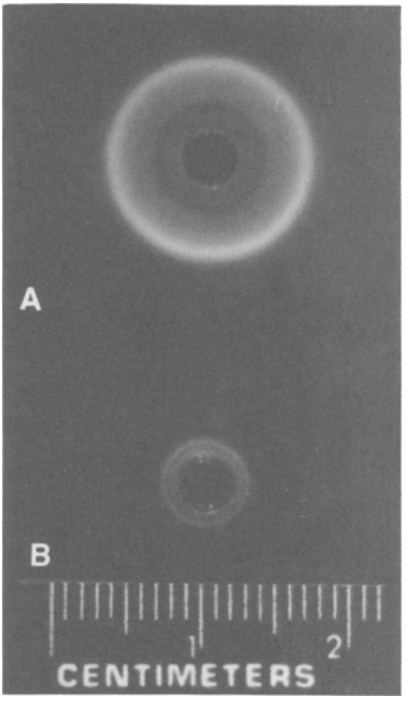


FIG. 2. Plant extracts in radial diffusion assay. Maple leaves (*Acer* sp.) or birch twigs (*Betula* sp.) were extracted with 50% methanol (300 mg tissue/1.5 ml solvent). Two 8-$\mu$l aliquots of the maple extract were placed in well A. Three 8-$\mu$l aliquots of the birch extract were placed in well B.

long incubation (Figure 3). The rings started to form immediately after the sample was placed in the well, but quantitative analysis was possible only after equilibrium was reached. The amount of time required for the rings to reach equilibrium increased with increasing tannin concentration (Figure 3). An incubation time of 96 hr after sample application was adequate for all samples tested here.

The area of the ring was linearly related to the amount of tannin placed in the well (Figure 4). To simplify calculations, the diameter squared was used instead of the area. The calibration lines had nonzero $y$ intercepts because the wells into which the samples were dispensed had finite areas (0.16 $cm^2$). The detection limit of the assay was 0.025 mg tannin, and the precision was $\pm 6\%$ (relative standard deviation). The detection limit of the assay depended on the concentration of protein in the plates. The amount of protein used here was chosen to optimize the limit of detection.

A lower response was obtained with condensed tannin than with hydrolyz-

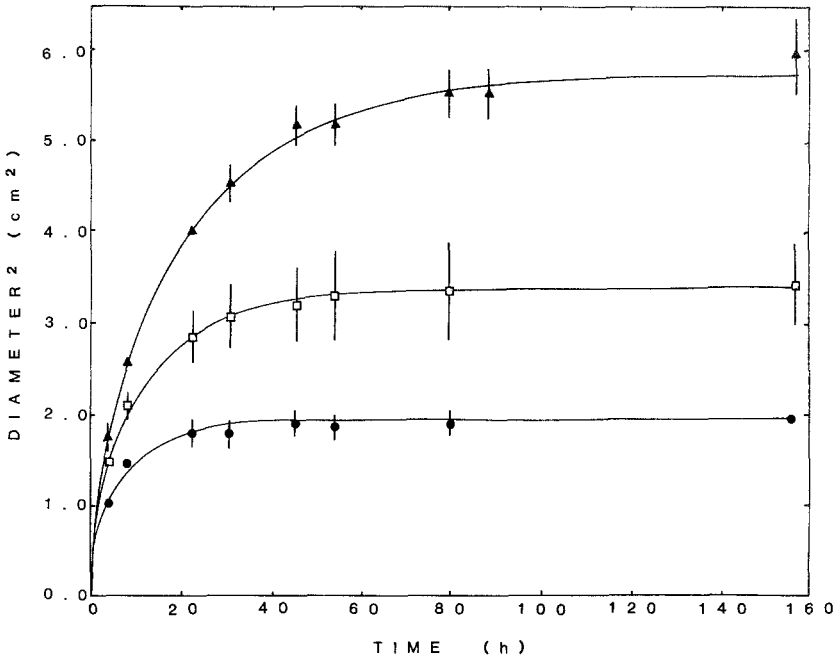


FIG. 3. Kinetics of ring formation. An 8-$\mu$l aliquot containing 1.0 mg tannic acid (▲), 0.50 mg tannic acid (□), or 0.25 mg tannic acid (●) was placed in a well and the diameters of the rings were measured at various times. Each point represents the mean ±SD for at least three replicates.

able tannin (Figure 4). Because the two types of tannin are not chemically identical, it is typical to obtain different sensitivities with the same test. For example, the response to condensed tannin in the protein precipitable phenolics assay is only half as large as the response to hydrolyzable tannin because the yield of colored complex differs for the two types of tannin (Hagerman and Butler, 1978). The amount of protein precipitated by the two types of tannin is different (Hagerman and Klucher, 1986). Therefore, it was not surprising that the two types of tannin responded differently in the diffusion assay.

The response of the two types of tannin to the assay was additive, even though the responses were not equivalent. The size of a ring formed by a mixture of tannins was equal to the sum of the sizes of rings formed by the individual samples (Figure 5). Analysis of an extract containing both types of tannin accurately reflected the total tannin content since the response was additive even when one component was present in excess. The method cannot be used to determine the relative amounts of the two types of tannin. Functional group assays are available for chemical characterization of mixtures of tannins.

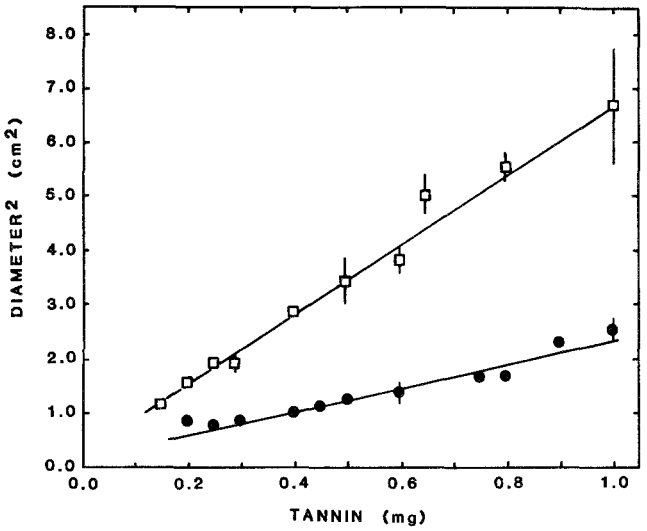


FIG. 4. Calibration curve for condensed and hydrolyzable tannin. An 8-$\mu$l aliquot of 70% acetone containing the purified tannin was placed in the well. The diameters of the rings were measured after equilibrium was reached, and the diameter squared was calculated. Each point represents the mean of at least three replicates $\pm$SD. The data was fit to a line using the method of least squares. The diameter$^2$ is in cm$^2$; the amount of tannin is in mg. For tannic acid (□): diameter$^2$ = 6.5 (amount of tannin) + 0.24 ($r^2$ = 0.94); for sorghum tannin (●): diameter$^2$ = 2.21 (amount of tannin) + 0.17 ($r^2$ = 0.92)

Nontannin phenolics such as flavonoids, benzoic acids, or hydroxycinnamic acids did not interfere with the assay. The addition of catechin or gallic acid to purified tannic acid or sorghum tannin had no effect on the ring size (Table 1). Similar data were obtained for hydroxycinnamic acids. If an aliquot of a saturated solution of catechin or gallic acid was applied to the well, a small ring formed rapidly. However, these rings disappeared within an hour and did not contribute to the final size of the ring. Plant extracts which do not contain tannin but do contain nontannin phenolics, such as extracts of leaves of the spider plant, did not form rings. Addition of such tannin-free leaf extracts to solutions of purified or condensed tannin did not affect the ring formation, indicating that nontannin components extracted from the leaf did not interfere with the tannin assay.

The solvent did not affect ring size. Common solvents used to prepare extracts include 50% methanol (methanol–water, 1:1 v/v), 70% acetone (acetone–water, 7:3 v/v), and 1% HCl in methanol (HCl–methanol, 1:99 v/v). None of these solvents affected the response to tannic acid obtained with the assay (Table 2). Similar data were obtained for condensed tannin in the same

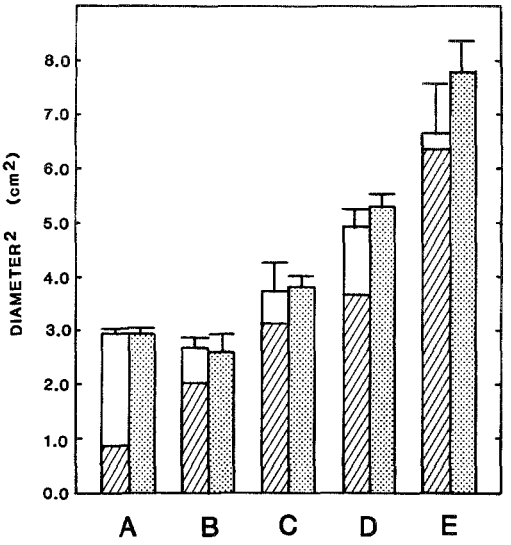


FIG. 5. Analysis of mixtures of condensed and hydrolyzable tannin. An 8-$\mu$l aliquot of 70% acetone containing either tannic acid (▨), sorghum tannin (□), or a mixture of the two (⬚) was placed in the well. The diameters of the rings were measured after equilibrium was reached. For each pair of bars, the bar to the left represents the sum of the diameters squared for the two individual solutions, and the bar on the right represents the diameter squared for the mixture. Each value is based on three replicate determinations, and error bars represent 1 SD. (A) 0.091 mg tannic acid, 0.91 mg sorghum tannin; (B) 0.33 mg tannic acid, 0.33 mg sorghum tannin; (C) 0.50 mg tannic acid, 0.25 mg sorghum tannin; (D) 0.50 mg tannic acid, 0.50 mg sorghum tannin; and (E) 0.91 mg tannic acid, 0.091 mg sorghum tannin.

solvents. The addition of antioxidants such as ascorbic acid did not interfere with the assay.

The results obtained with the radial diffusion assay were highly correlated ($r^2 = 0.95$) with the results obtained with precipitation assays (Figure 6). The radial diffusion assay, the protein precipitable phenolics assay, and the labeled BSA protein precipitation assay were used to analyze extracts of a variety of plants and two *Vaccinium*-containing diets (C. T. Robbins, personal communication). All the assays gave qualitatively similar results. One group of samples was classified as low tannin based on all of the assays (*Ceanothus*, *Sorghum*, 50% *Vaccinium*); another group was classified as intermediate tannin (oak, 75% *Vaccinium*, green tea, dogwood); and a third group was classified as high tannin (fireweed flowers). Quantitatively, the assays gave different values since, with each assay, a different chemical characteristic of tannin is measured. There was a twofold difference between the tannin content determined with the radial diffusion method and the content determined with the protein

TABLE 1. EFFECT OF NONTANNIN PHENOLICS ON SIZE OF RING IN RADIAL DIFFUSION ASSAY[a]

| Tannin | Phenolic | Diameter[2] ($cm^2$)[b] |
|---|---|---|
| Tannic acid | | |
| 0.30 mg | none | 1.98 + 0.04 |
| 0.30 mg | catechin | 2.10 + 0.10 |
| 0.30 mg | gallic acid | 2.10 + 0.15 |
| Tannic acid | | |
| 0.60 mg | none | 4.88 + 0.06 |
| 0.60 mg | catechin | 4.66 + 0.22 |
| 0.60 mg | gallic acid | 5.18 + 0.41 |
| Sorghum tannin | | |
| 0.30 mg | none | 0.83 + 0.03 |
| 0.30 mg | catechin | 0.87 + 0.05 |
| 0.30 mg | gallic acid | 0.83 + 0.03 |
| Sorghum tannin | | |
| 0.06 mg | none | 1.25 + 0.07 |
| 0.60 mg | catechin | 1.27 + 0.10 |
| 0.60 mg | gallic acid | 1.28 + 0.10 |

[a]The indicated amount of tannin or tannin plus 0.50 mg catechin or gallic acid was dissolved in 70% acetone. An 8-$\mu$l aliquot was placed in each well and the diameter was measured after equilibrium was reached.
[b]The values shown are the means of three replicates ± 1 SD.

precipitable phenolics method (Figure 6), although tannic acid was used as the standard for both assays. The radial diffusion assay depends only upon the ability of the tannin to interact with protein to form a visible precipitate. The protein precipitable phenolics assay depends both on interaction with protein to form a precipitate and on reaction with ferric chloride to form a colored complex. Be-

TABLE 2. EFFECT OF SOLVENT ON SIZE OF RING IN RADIAL DIFFUSION ASSAY

| Solvent[a] | Diameter[2] ($cm^2$)[b] |
|---|---|
| 50% acetone | 3.49 + 0.21 |
| 50% methanol | 3.74 + 0.23 |
| Buffer A | 3.13 + 0.51 |
| 1% HCl in methanol | 3.49 + 0.21 |

[a]The indicated solvent, 8 $\mu$l, containing 0.50 mg purified tannic acid was placed in the well. The diameter of the ring was measured after the system was at equilibrium.
[b]Values are the mean of three replicates ± 1 SD.

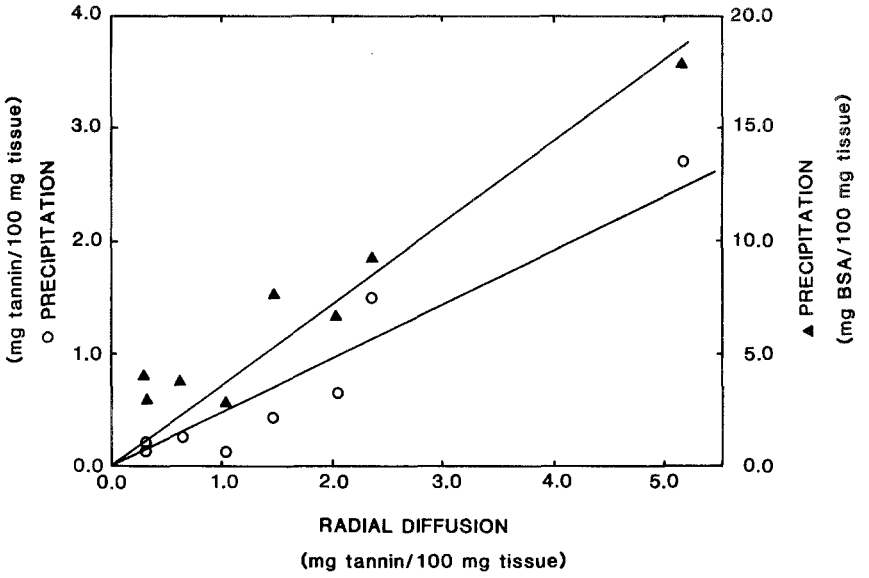


FIG. 6. Comparison of the radial diffusion assay to precipitation assays. A variety of plant tissues were extracted with 50% methanol (300 mg tissue/1.5 ml solvent). The extracts were analyzed by the radial diffusion assay, the protein precipitable phenolics assay, and the labeled protein precipitation assay as described in the text. The data was fit to a line using the method of least squares. For protein precipitable phenolics (○): $y = 0.49x$, $r^2 = 0.95$; for labeled protein precipitation (▲): $y = 4.56x$, $r^2 = 0.95$. The samples analyzed and their tannin content by the radial diffusion method are: *Ceanothus* leaves (0.308 mg tannin/100 mg tissue); *Sorghum vulgare* grain (0.313 mg tannin/100 mg tissue); 50% *Vaccinium* diet (0.642 mg tannin/100 mg tissue); oak leaves (*Quercus* sp.) (1.03 mg tannin/100 mg tissue); 75% *Vaccinium* diet (1.46 mg tannin/100 mg tissue); green tea leaves (2.03 mg tannin/100 mg tissue); red-osier dogwood leaves (*Cornus stolonifera*) (2.36 mg tannin/100 mg tissue); and fireweed (*Epilobium angustifolium*) (5.15 mg tannin/100 mg tissue).

cause the two assays are based on different chemistry, it was not surprising that the assays gave different responses with the plant extracts.

## DISCUSSION

The radial diffusion assay is similar to radial immunodiffusion, in which antibodies are quantitated by forming a ring of precipitation in an antigen-containing agarose gel (Becker, 1969; Vaerman, 1981). Application of principles of immunoassays to tannin analysis was undertaken since tannin–protein interactions are similar in some respects to antigen–antibody interactions. In both reactions, a water-soluble precipitating reagent interacts specifically with pro-

teins to form multivalent, precipitable complexes (Hagerman and Butler, 1981). Radial diffusion assays are particularly suitable for tannin analysis. Tannin is water soluble and readily diffuses through the agarose gel. Tannin apparently does not interact to form precipitable complexes with the carbohydrate medium of the gel, since rings of precipitation are not observed if the protein is omitted from the gel.

BSA was selected as the protein to incorporate in the gel because it is homogeneous, soluble, and inexpensive. Tannin is a selective protein-precipitating agent, binding tightly to some proteins and more weakly to others (Hagerman and Butler, 1981; Hagerman and Klucher, 1986). Precipitation assays conducted with inhomogeneous proteins are ambiguous, since the tannin interacts selectively with the proteins in the mixture. Both condensed and hydrolyzable tannin have moderate affinities for BSA (Hagerman and Butler, 1981; Martin and Martin, 1983; Hagerman and Klucher, 1986).

The assay has several advantages in common with previously described precipitation assays (Hagerman and Butler, 1978, 1980a; Martin and Martin, 1983; Asquith and Butler, 1985). For example, the assay is not subject to interference by low-molecular-weight, nontannin phenolics. In addition, the method can be used to determine either condensed or hydrolyzable tannin or mixtures of the two. However, the assay is an improvement over other precipitation assays because it accommodates a wide variety of solvents including acetone; it can be used to assay extracts containing water-insoluble components; and it is especially appropriate for analyzing large numbers of samples.

Acetone does not affect the new assay, which simplifies analysis of extracts. Acetone is one of the most effective solvents for extracting tannin from plant tissue (Fletcher et al., 1977). Most protein precipitation assays for tannin are very sensitive to solvent. For example, as little as 4% acetone in a reaction mixture containing tannin and protein inhibits precipitation by 50%. Thus, acetone must be removed from extracts by evaporation under reduced pressure before attempting precipitation assays. Because acetone does not interfere with the new assay, plant extracts can be analyzed directly, without preliminary workup.

The new assay is more reliable than other assays for some unusual tissues, such as aspen buds or sorghum, which contain water-insoluble materials that interfere with most precipitation assays (Hagerman and Butler, 1978; Asquith and Butler, 1985; Hagerman, unpublished). The water-insoluble components, which are extracted into the aqueous organic solvents with the tannin, interfere with other assays by forming spurious precipitates in the aqueous buffers used in the assays. However, in the new assay, these water-insoluble materials do not enter the gel and thus do not form precipitation rings.

The assay can easily be used to determine tannin in a large number of samples, since extracts can be analyzed directly without any preliminary cleanup steps. Even centrifugation of the tissue extracts is unnecessary, although very

fine tissue samples may block the syringe used to dispense the extract into the wells. Allowing such samples to settle briefly or centrifuging at low speed clarifies the extract enough for radial diffusion analysis.

The assay could be modified to use proteins which are ecologically more significant than BSA. Although using BSA in the assay is analytically convenient, there may be advantages to using various plant or herbivore gut proteins for determining tannin (Martin and Martin, 1983). Soluble proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase could be incorporated into the agarose gel and used in a radial diffusion assay.

The assay described here provides a convenient, sensitive method for selectively determining tannin in crude plant extracts. The assay can be performed with as little as 100 mg tissue and can be conveniently used with large numbers of samples. Application of this method in studies of herbivory should enable ecologists to establish whether tannin defends plants from insects and other herbivores.

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