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Determination of Both Tannin and Protein in a Tannin–Protein Complex

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A method for determination of both protein precipitation capacity of tannins and protein-precipitable phenolics and the ratio of protein to tannin in a tannin–protein complex is presented. The relationships of both bovine serum albumin (BSA) precipitated and the protein-precipitable phenolics with tannic acid and the ratio of BSA and tannins in the protein–tannic acid complex agreed well with those obtained from the methods already available. The above parameters were also determined for *Quercus incana* leaves at different stages of maturation. The protein precipitation capacity and protein-precipitable tannins decreased substantially with maturation, whereas the ratio decreased slightly. As the present method allows determination of these parameters from a tannin–protein complex, it is convenient, takes less time, can handle a large number of samples at a time, and requires less tannin-containing extracts and chemicals compared to other available methods.

Protein-precipitation methods for quantitation of tannins involve the formation of a tannin–protein complex. The method of Hagerman and Butler (1978) measures tannins, and those of Makkar et al. (1987) and Marks et al. (1987) measure proteins in the complex. Not much attention has been given to another parameter, i.e., the ratio of protein to tannin in a tannin–protein complex, probably because of nonavailability of simple methods to quantitate protein in a tannin–protein complex. This ratio represents the amount of protein bound by an unit of phenolics/tannins in the complex. This could provide valuable information regarding changes in the nature of tannins, vis-à-vis their protein-binding capacity associated with, for example, development, maturity (Makkar et al., 1988), and postharvest storage of foods and feeds. In addition, insight can be had into the differences in the nature of tannins that bind proteins in bird- and mold-resistant and susceptible varieties. One way of obtaining the protein to tannin ratio in the complex is to measure protein in a complex (Makkar et al., 1987) and tannins (Hagerman and Butler, 1978) in another complex formed under the identical conditions. This takes more time and labor and requires more chemicals and extracts of the sample containing tannins. The present paper reports simple modifications of the methods of Hagerman and Butler (1978) and Makkar et al. (1987), which enable measurements of both tannin and protein in a complex. Besides alleviating the above-mentioned problems associated with separate measurements of tannins and proteins, a large number of samples can be handled and the variation in the protein to tannin ratio is expected to be smaller by the present method. The utilization of *Quercus incana* leaves as feed for ruminants is a common practice in hilly regions of India and various other countries (Makkar et al., 1988). The protein precipitation capacity, protein-precipitable phenolics, and ratio of protein to tannin in protein–tannin complex were measured for *Q. incana* leaves at different stages of maturation by the method reported here.

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MATERIALS AND METHODS

Materials. Chemicals and equipment were the same as described earlier (Makkar et al., 1987).

The leaves of oak (*Q. incana*) were collected from a tree in the vicinity of the Research Station. On leaf sprouting, the first collection of leaves (∠4 days old) was made in April 1987. The collected leaves were divided into two groups of sizes 3–5 and 6–9 cm. Next collections were made 3 and 10 days after the first collection. Two more collections were made at an interval of 14 days. The old leaves (aged leaves), which were about to fall after the sprouting of new leaves, were also collected. The leaves were dried at 50 °C.

Methods. The extracts were prepared as described by Martin and Martin (1982).

Preparation of the Complex and Solubilization. The protein–tannin complexes were formed as described earlier (Makkar et al., 1987). The complex was dissolved in 1.5 mL of sodium dodecyl sulfate (SDS) (1% (w/v) in distilled water).

Assay of Tannins in the Complex. The method of Hagerman and Butler (1978) was used with slight modi-

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Table I. Effect of Increasing Concentration of Sodium Dodecyl Sulfate (SDS) and Triethanolamine (TEA) on the Color Development by Ninhydrin Assay[a]

	% inc A_{570nm}
glycine	0
glycine + 0.1 mL of SDS–TEA	3.5
glycine + 0.2 mL of SDS–TEA	19.9
glycine + 0.3 mL of SDS–TEA	41.5
glycine + 0.5 mL of SDS–TEA	77.5

[a] Aliquots (0.1 mL) of glycine (2 mM) and (0–0.5 mL) of SDS–TEA solution (1% SDS, 5% (v/v) TEA in distilled water) were dried in test tubes. To these were added 0.3 mL of NaOH (13.5 N), 0.5 mL of glacial acetic acid, and 1 mL of ninhydrin reagent in that order. Color development and its measurement were as described in Materials and Methods. The value obtained with glycine alone was taken as 100%.

fication. An aliquot (1 mL) of the above dissolved complex was taken, and to it was added 3 mL of SDS–triethanolamine solution (1% SDS (w/v) and 7% (v/v) triethanolamine in distilled water). A 1-mL portion of ferric chloride reagent (0.01 M ferric chloride in 0.1 N HCl) was added, and the solution was vortexed thoroughly. The absorbance at 510 nm was taken 15–30 min after the ferric chloride reagent was added. The absorbance due to the tannin in the complex was calculated by $(A_0 - A_b) \times 1.5$, where A_0 and A_b are the absorbance of the sample and the blank, respectively.

Assay of Protein in the Complex. The method used was essentially the same as that of Makkar et al. (1987) with some modification. The method is based on hydrolysis of protein–tannin complex under alkaline conditions and determination of released amino acids by the ninhydrin reaction. An aliquot (0.1 mL) of the dissolved complex was taken in a test tube and dried in an oven at about 80 °C. To it was added 0.3 mL of NaOH (13.5 N), and the protein was hydrolyzed at 120 ± 2 °C for 20 min in an oven. The tubes were cooled, and to these was added 0.5 mL of glacial acetic acid very slowly to neutralize the alkali. One milliliter of ninhydrin reagent (Makkar et al., 1987) was added and the resulting mixture heated in a boiling water bath for 20 min after marbles were placed on the tubes. The tubes were cooled, and 10 mL of distilled water was added. The absorbance was read at 570 nm.

The protein content was determined from a standard curve prepared against BSA (15–150 μg in 0.1 mL of solution in distilled water). The BSA solution was dried and hydrolyzed, and the released amino acids were estimated as described above for the complex.

The protein precipitation capacity and the protein-precipitable phenolics were determined from the regression coefficient (slopes) of linear regressions fitted to measurements performed at different concentrations as suggested by Martin and Martin (1982). The ratio of protein to tannin in the complex was determined from the complexes formed at different concentrations of the tannin-containing extracts by taking the average of the values. The results are expressed as mean ± SD (n).

RESULTS AND DISCUSSION

Initially, the protein–tannin complex was dissolved in 1.5 mL of SDS–triethanolamine solution [1% (w/v) SDS, 5% (v/v) triethanolamine; the reagent used by Hagerman and Butler (1978)]. However, the SDS and triethanolamine present at this concentration interfered in the assay of glycine by the ninhydrin method (Table I). The interference was due to triethanolamine and not SDS (results not shown). Therefore, the protein–tannin complex was dissolved in 1.5 mL of 1% SDS solution.

The tannic acid–BSA complex was formed as described earlier (Hagerman and Butler, 1978; Makkar et al., 1987), and tannins and protein were determined in the complex by the method reported here. The relationship between protein-precipitable phenolics (y, A_{510nm}) and tannic acid (x, mg) was $y = 1.21x - 0.17$ ($r = 0.993$), and that of BSA precipitated (y', mg) and tannic acid (x, mg) was $y' = 2.30x - 0.31$ ($r = 0.992$), which agreed well with the earlier values reported by Hagerman and Butler (1978) and Makkar et al. (1987), respectively. The ratio of protein to tannin (milligrams of protein precipitated/A_{510nm}) in the complex by the present method was 1.92 ± 0.13 (6), and that obtained by using the methods of Hagerman and Butler (1978) and Makkar et al. (1987) on separate complexes formed under identical conditions was 1.97 ± 0.36 (5). By the present method the protein precipitation capacity, protein-precipitable phenolics, and protein to tannin ratio of *Q. incana* leaves at different stages of maturation are presented in Table II. The results agreed well with those obtained by the methods of Hagerman and Butler (1978) and Makkar et al. (1987) (Table II). The significance of these results and those of the changes in total phenolics, condensed tannins, and degree of polymerization in the leaves with maturation has been presented separately (Makkar et al., 1988). The variability in the ratios obtained by the present method was less compared to those obtained by the previous methods, as is evident from the standard deviation (Table II).

The above results suggest that the proposed method can be used for determination of tannin and protein and their ratio in a protein–tannin complex. The method has several advantages (see introduction) and can provide valuable

Table II. Protein-Precipitating Capacity, Protein-Precipitable Phenolics, and Protein to Tannin Ratio of *Q. incana* Leaves at Different Stages of Maturity

	young leaves						aged leaves
	4 days		7 days	14 days	28 days	42 days	
leaf size, cm	3–5	6–9	8–11	12–15	12–15	12–15	12–15
protein-precipitating capacity (mg BSA ppt/g dry wt)							
present method, $n = 3$	235.89 ± 18.71	263.32 ± 15.21	209.82 ± 19.35	105.34 ± 20.32	79.83 ± 10.91	80.39 ± 9.11	72.83 ± 5.56
method of Makkar et al. (1987),[a] $n = 3$	229.50 ± 17.20	266.89 ± 19.13	202.95 ± 20.19	97.35 ± 28.10	86.74 ± 15.82	83.87 ± 18.25	78.35 ± 8.25
protein-precipitable phenolics (A_{510nm}/g dry wt)							
present method, $n = 3$	67.56 ± 6.35	65.21 ± 11.36	52.35 ± 6.92	29.69 ± 3.21	25.39 ± 4.93	23.53 ± 3.19	25.32 ± 4.01
method of Hagerman and Butler (1978), $n = 3$	70.03 ± 8.91	68.50 ± 10.55	56.05 ± 7.98	28.57 ± 4.90	24.36 ± 5.53	24.53 ± 4.20	24.08 ± 4.93
protein to tannin ratio							
present method, $n = 12$	3.91 ± 0.21	3.80 ± 0.19	3.88 ± 0.23	3.71 ± 0.32	3.65 ± 0.18	3.61 ± 0.33	3.50 ± 0.40
separate complexes,[a,b] $n = 6$	4.05 ± 0.45	3.90 ± 0.69	3.80 ± 0.38	3.87 ± 0.57	3.66 ± 0.48	3.70 ± 0.49	3.52 ± 0.52

[a] Data from Makkar et al. (1988). [b] Protein and tannins in the complexes were determined by the methods of Makkar et al. (1987) and Hagerman and Butler (1978), respectively, using different complexes formed under identical conditions.

information on the mechanism of tannin–protein interactions and changes associated with various processes.

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Photoactivatable Time–Temperature Indicators for Low-Temperature Applications[1]

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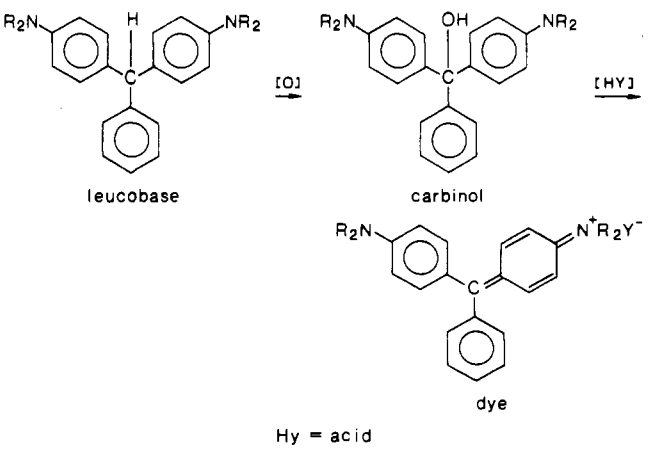
This paper describes a photoactivatable time–temperature indicator based on a leucobase system. A leucobase is mixed in a polymeric matrix with a material that generates acid upon exposure to light. Photoexcitation causes the formation of a thermally sensitive, color-forming product. Following this activation step, a progressive color development occurs at a rate that increases with temperature. The indicator is useful for monitoring the freshness of perishable products, particularly those stored at subambient temperatures.

Many articles of commerce—both food and nonfood—are perishable. Particularly when the perishable is enclosed in packaging, it may not be readily apparent when the article has exceeded its useful lifetime. It is even more difficult to determine precisely where an article is positioned on an imaginary graph that plots its deterioration as a function of time. Since the rate at which a perishable deteriorates is generally a function of its integrated time–temperature exposure—at least within a restricted range of time–temperature—a time–temperature indicator is a useful tool for those who are concerned with the freshness of perishable products. Principal applications seen for these indicators involve monitoring the freshness of perishable products, particularly those stored at subambient temperatures. This paper discusses the development and application of a shelf life monitoring indicator for temperature-sensitive products. The indicator uses color-changing, time–temperature integrating materials that remain inactive until activated by shining actinic radiation.

In the literature, there are several devices available that can respond to temperature in much the same way as perishable commodities in which loss of quality is directly related to the combined effects of the degree and the duration of the storage temperature (Hu, 1972; Blixt and Tiru, 1977; Labuza, 1982; Farquhar, 1982; Fields and Prusik, 1985; Zall and Fields, 1986). The indicator devices described are all visual in nature and feature systems using polymeric materials, enzymes, etc., to measure temperature abuse of the products. However, most are not readily adaptable for use at refrigeration temperatures, primarily because ambient temperatures for storage of the test materials are, in effect, *high* temperatures relative to test conditions. For this reason the "clock" for the indicating materials must be started when low-temperature storage of the monitored sample is begun. The activation mechanism, brief exposure to ultraviolet light of a preselected wavelength, described in this paper is especially convenient to do the same. The photoactivation procedures has potential advantages such as (i) activation of color change at a specified point in time and (ii) totally nonintrusive nature of activation. We have discovered that a suitable photoactivatable time–temperature indicator can be based on color development from a leucobase. Leucobases are the colorless forms that are the precursors to diphenylmethane and triarylmethane dyes (Venkataraman, 1952). Examples of triarylmethane leuco bases include those of Malachite Green dye, Brilliant Green dye, Crystal Violet dye, etc. By selecting from among these and other suitable leucobases, as well as mixtures of two or more of them, a wide variety of desired colors can be obtained. Color selection entirely depends on types of monitoring devices employed to record the growth of color development. Thus, a He–Ne laser, which emits at 632 nm, is a convenient light source for monitoring the reflectivity of the indicators derived from the leucobases.

It is known that colorless leucobase is converted to dye in a two-step process:



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