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Ecological tannin assays

Evaluation of proanthocyanidins, protein binding assays and protein precipitating potential*

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Summary. Estimations of condensed tannin content are generally based on calibration standard curves from Quebracho condensed tannins. We generated calibration standard curves from eight Sonoran Desert species for comparison with estimates of tannin concentrations derived from the Quebracho standard curve. Estimates of leaf tannin concentrations of each of the eight species using each species standard curve differed significantly with the estimates given by the Quebracho standard curve. Standard curves constructed from tannins from different individuals of three of the species varied significantly between, but not within, species. The efficiency of precipitation of protein bovine serum albumin (BSA) by each different tannin varied up to a factor of fifty for tannins of different species. Ordering species from highest to lowest based on tannin concentrations or binding efficiencies gave two different ranks. We argue that concentration or efficiency alone do not describe adequately tannin ecological activity. Instead, we suggest combining tannin concentrations and binding efficiencies to measure the protein precipitating potential of a leaf. Leaf protein precipitating potential is a more ecologically realistic parameter, we feel, for between-species comparisons than tannin content or binding efficiencies alone.

Key words: Condensed tannins – Quantification – Protein precipitation potential

Tannins are a heterogeneous class of phenolic polymers in the 500 to 30000 molecular weight which are water soluble and are capable of precipitating soluble proteins from solution (Martin and Martin 1982; Swain 1979). These compounds have been proposed as an important component of plant chemical defenses against herbivorous animals, fungi and microbes (Feeny 1976; Rhoades and Cates 1976; Swain 1979; Zucker 1983). Studies of the ecological function of tannins have centered on the concentration of these compounds and their generalized ability to complex and precipitate proteins (Feeny 1968, 1970; Price et al. 1980; Fox and Macauley 1977; Cooper and Owen-Smith 1985; Lindroth and Batzli 1984; Roehrig and Capinera 1983).

The determination of condensed tannin concentrations has relied on two main techniques, colorimetric analysis (with proanthocyanidin measurements) and protein binding assays (Swain 1979; Tempel 1982). The colorimetric, or proanthocyanidin assay, is used to determine the amount of condensed tannin present in a plant extract. The binding assays are used to determine the ability of tannin extracted from plant material to bind and precipitate protein from solution.

In the determination of proanthocyanidin content, it is necessary to use a calibration curve. The result of the colorimetric analysis is a spectrophotometer value, which must then be converted to grams of tannin by the use of the calibration curve (called a standard curve). The value generated from such an analysis is completely dependent on the standard curve used. In general, tannins from two species have been settled on as representative standard curves for condensed tannin analysis: Quebracho (*Schinopsis quebracho-colorado*) (Martin and Martin 1982; Waterman and Janzen 1985) or Sorghum (*Sorghum bicolor*) (Price et al. 1980). Such single standard curve assays have been used for comparing tannin concentrations within and between plant communities (McKey et al. 1978) and tannin concentrations with herbivory rates (Coley 1983). Similarly, protein binding assays have used a single protein type for measuring the ability of a plant extract to precipitate protein from solution, either bovine serum albumin (BSA) (Martin and Martin 1982) or ribulose-1,5-bisphosphate carboxylase (RuBPC) (Martin et al. 1985). Finally, many studies that have assessed tannin antifeedant activity against different herbivores have also used quebracho tannin as a representative of the class of condensed tannin (Bernays et al. 1981; Lindroth and Batzli 1984). A common thread running through these studies is the assumption that condensed tannins exhibit generalized activity from plant to plant (but see Martin and Martin 1982; Zucker 1983).

However, when we constructed a standard curve from condensed tannins extracted from *Prosopis glandulosa* (Mesquite), it yielded different estimations of tannin concentrations in Mesquite leaves than those from a quebracho curve. This led us to question how close quebracho-based tannin estimations are to estimations generated from standard curves from the condensed tannin produced by the species under study. Additionally, how similar are the bind-

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ing efficiencies of tannins of these different species for the same protein? In this study, we have isolated condensed tannins from species of nine different genera for comparison of standard curve parameters between and within species and the efficiency of their binding to the same protein.

Materials and methods

Plant collection and preparation. Mature foliage of all species (Table 1), except quebracho and oak, were collected in 1985 at the Philip L. Boyd Deep Canyon Desert Research Center, Palm Desert, California. See Table 1 for species that included stems in the analysis. Quebracho and oak condensed tannins were provided by J. Smiley, University of California, Irvine. Plant material was dried at 20° C and ground to a fine powder in a Wiley mill.

Table 1. Names and general distributions of species used in this study. *Krameria, Phorandendron, Cercidium* and *Euphorbia* included for analysis stems and leaves where present

Species	Common name	Location
Schinopis quebracho-colorado	Quebracho	Europe
Acacia greggii	Cat Claw Acacia	Sonoran Desert
Krameria parvifolia	Krameria	Sonoran Desert
Phorandendron californicum	Mistletoe	Sonoron Desert
Prosopis glandulosa	Mesquite	Sonoran Desert
Cercidium floridum	Palo Verde	Sonoran Desert
Quercus agrifolia	Oak	Coastal Oak Grassland
Chilopsis linearis	Desert Willow	Sonoran Desert
Fouquieria splendens	Ocotilla	Sonoran Desert
Euphorbia micromera	Sandmat	Sonoran Desert
Hyptis emoryi	Desert Lavender	Sonoran Desert

Tannin extraction. Dried and ground plant material was extracted according to Dement and Mooney (1974). Condensed tannin powder was prepared from plant extracts in 70% acetone (containing 0.1 g ascorbic acid and 0.02 g potassium cyanide per 100 ml). After filtering, separation of the acetone/water layers was formed by the addition of solid sodium chloride. After removal, an equal volume of ethyl alcohol was added to the acetonic layer. Four volumes of ethel ether were added to the acetone/ethanol mixture, which was then washed three times with 100 ml of water. The water washes were combined and dialyzed against pure water for two days. The dialysate was then freeze-dried and stored as a powder at 4° C.

Standard curve preparation. Standard curves were constructed by adding weighed amounts of each species' condensed tannin powder to 2 ml of 50% methanol. Twenty ml of 95:5 n-butanol:concentrated hydrochloric acid were added to each 2 ml sample and shaken. Half of each sample was then refluxed in marble capped test tubes in a boiling water bath for 40 min. After cooling for ten minutes, each heated sample was measured against its control, the unheated portion of each sample, at 550 nm in a Beckman DU-7 spectrophotometer.

Protein binding efficiencies. The binding efficiency of BSA by each species condensed tannin was measured by the technique of Martin and Martin (1982) with the following modifications. Starting concentration of the protein, bovine serum albumin (Sigma Chemical Co.), was 0.3 mg/ml acetate buffer. After the 15 min complexing period, the solution was centrifuged (12000 xg for 15 min, 5° C) and then the

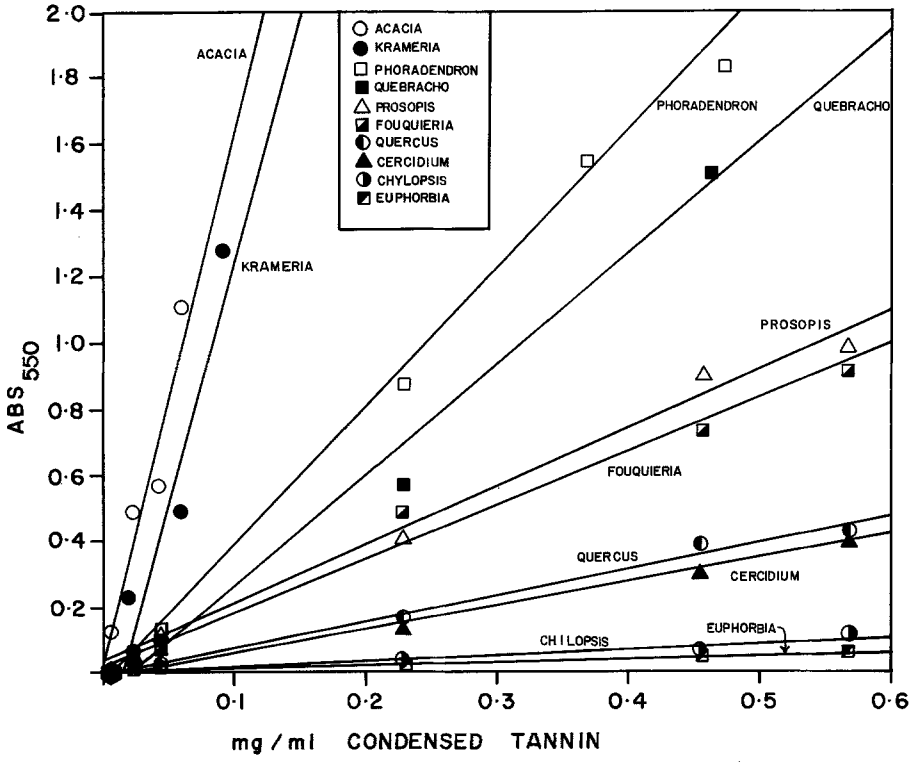

Fig. 1. Standard calibration curves for condensed tannin estimation generated from tannins from the different study species. Weighed amounts of extracted tannins from different species were used to generate each curve by the butanolic-HCl method. See Table 2 for curve parameters

supernatant transferred to an empty test tube. One ml of 10% trichloroacetic acid (TCA) was added to the supernatant and allowed to sit at room temperature for 15 min. This solution was centrifuged under the same conditions, the supernatant discarded and the pellet redissolved in 1 ml of 0.36 N sodium hydroxide. The suspension was then measured by the modified Lowry method (Hartree 1972). Control tubes were 1 ml of buffer instead of the protein solution. Values reported are the tannin precipitated by TCA in the controls subtracted from the experimental solutions of non-complexed protein plus TCA precipitated tannin.

Results

The standard curves generated for all ten species varied over a wide range (Fig. 1), none of them being identical to the curve for quebracho. Eight of the ten estimated curves had an r^2 of 0.98 or better (Table 2). Slopes of the standard curves varied from 0.12 for *E. miromera* to 26.00 for *A. greggii*.

As a measure of the error in estimations between the quebracho standard curve and each species standard curve, we used a typical spectrophotometric value of 0.2 absorbance units to compare the results generated by each species' standard curve to the values generated by the quebracho curve, calculating this comparison as a ratio (species estimate/quebracho estimate). Such ratios showed an under-/overestimation of tannin concentrations from a factor of one fifth underestimate (*A. greggii*) to twenty one times overestimate (*E. micromera*).

Tannins isolated from three individuals (two for *P. glandulosa*) of three different species produced standard curves that could be distinguished statistically between, but not within, species (Fig. 2). *P. glandulosa* individuals produced a wide range of curves, but the slopes are indistinguishable statistically. These results support the proposal that comparisons of plant species to quebracho for tannin estimations, can result in an over-/underestimation of concentrations.

Titration curves of a constant protein concentration with increasing tannin concentrations revealed distinct characteristics for the different species. Of the nine tannins titrated, *Q. agrifolia* and *P. californicum* showed some threshold prior to any significant protein binding and they had the highest saturation points (Fig. 3, Table 3).

Those titration curves with showed no delay in protein binding can be divided into categories based on the linear region slope and the saturation points. Tannic acid and *K. parvifolia* both exhibit a high slope (efficient binding) and a low saturation point (low binding capacity) (Fig. 3). *S. quebracho-colorado* and *F. splendens* show a midrange saturation point and a low slope (Fig. 3). *A. greggii* tannins saturate at midrange values and show high linear range slopes (Fig. 3). Both *H. emoryi* and *E. micromera* saturate at high concentrations (high binding capacity), but *H. emoryi* has a low slope while the slope of *E. micromera* is high (Fig. 3).

Since the titration curves took the form of a sigmoidal relationship, we transformed each curve by inverting each variable with a double logarithmic transformation (Table 3). We have included the double logarithmic plots of high efficiency *P. californicum* and low efficiency *E. greggii* protein binding tannins as examples (Fig. 4). The slope of each plot is related to the efficiency of binding and ranges from 0.40 for *P. californicum* (high binding efficiency) to 0.28 *E. micromera* (low binding efficiency).

Table 2. Parameters of the estimated standard curves generated by acid degradation of weighed condensed tannin material extracted from the different species. As quebracho tannins are frequently used as a general standard, we calculated the ratio of predicted tannin amount from each species to that predicted by quebracho, at a hypothetical absorbance of 0.200 absorbance units. This represents the degree of over- or underestimation of actual tannin content when only quebracho standards are used

Species	Slope	Intercept	r^2	ABS (0.200) Species/ Querbracho
A. greggii	26.00	−0.21	0.86	0.20
K. parvifolia	14.21	−0.19	0.80	0.35
P. californicum	4.16	−0.02	0.99	0.67
Quebracho	3.54	−0.08	0.99	1.00
P. glandulosa	2.67	−0.02	0.99	1.05
C. floridum	0.69	−0.004	0.99	3.78
Q. agrifolia	0.79	−0.001	0.99	3.24
C. linearis	0.19	−0.0003	0.98	13.56
F. splendens	1.60	0.03	0.98	0.33
E. micromera	0.12	−0.001	0.99	21.58

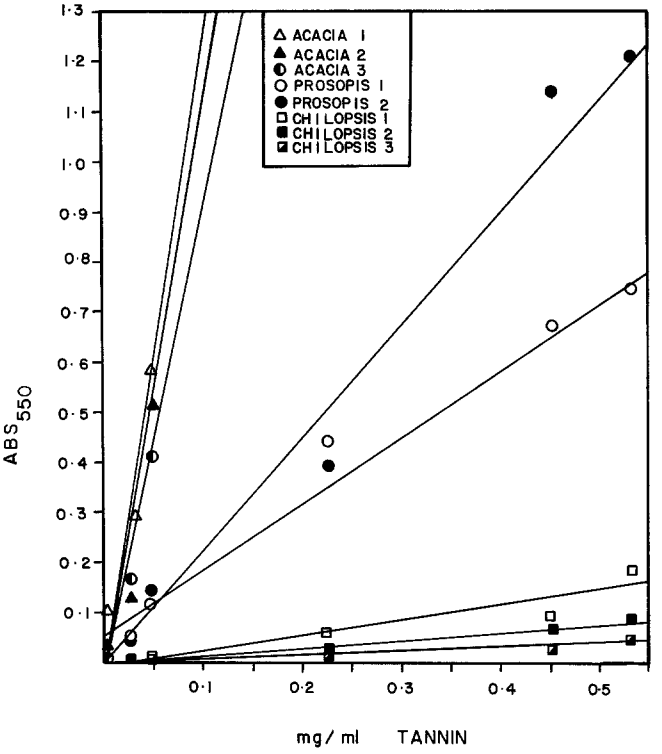

Fig. 2. Standard calibration curves for condensed tannins extracted from different individual trees from three different species. The curves cannot be distinguished statistically between species, but do differ significantly between species

Discussion

Estimations of unknown concentrations of leaf tannins have until now been conducted using a standard calibration curve derived from a single tannin type, typically that of quebracho. However, tannin estimates generated from curves from the extracted tannins of different species yield

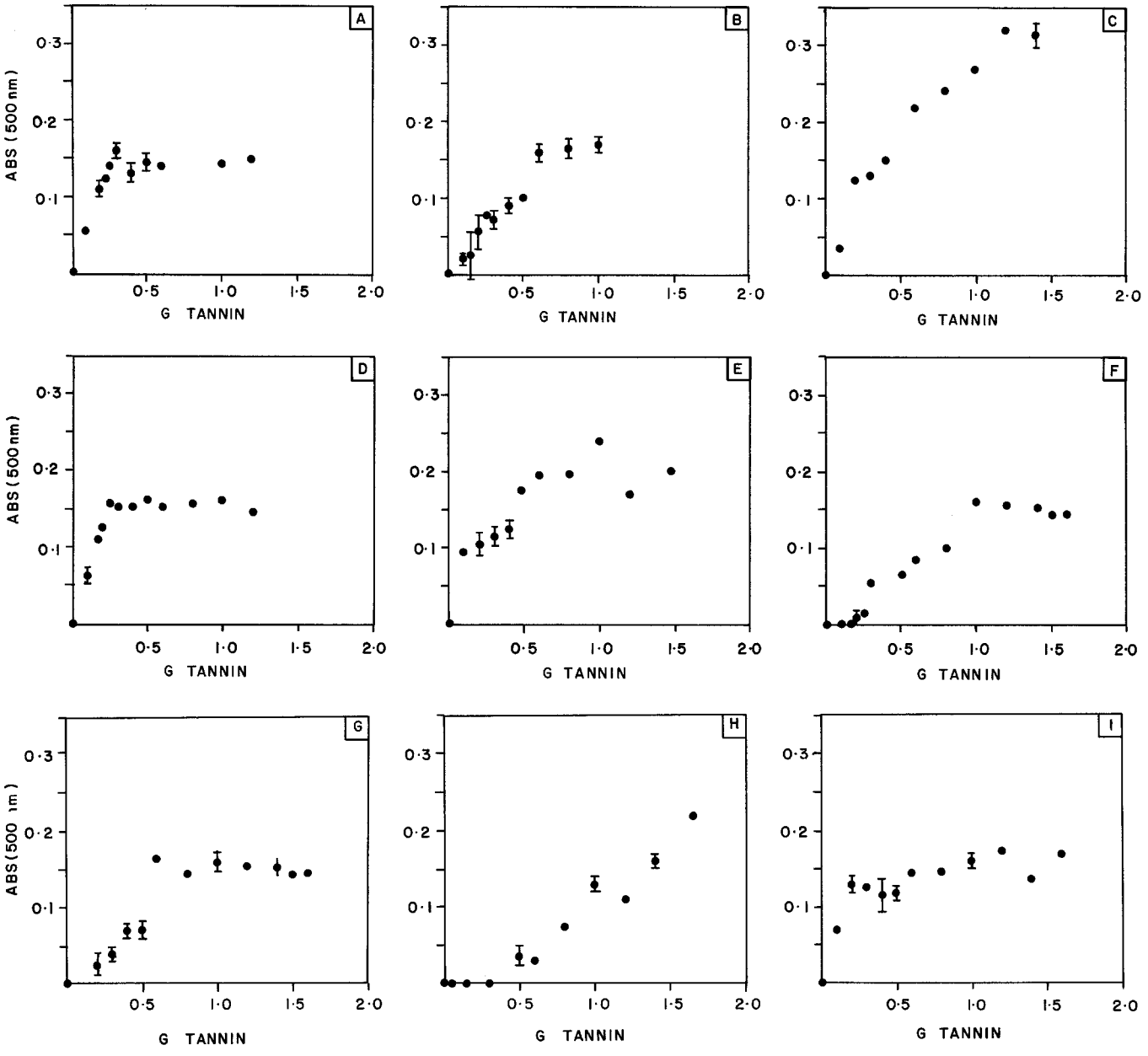

Fig. 3A–I. Titration curves for constant protein concentrations against increasing tannin concentrations. See Table 3 for curve parameters. **A** Tannic acid, **B** Quebracho, **C** *H. emoryi*, **D** *A. greggii*, **E** *K. parvifolia*, **F** *Q. agrifolia*, **G** *F. splendens*, **H** *P. californicum*, **I** *E. micromera*

very different numbers. Asquith and Butler (1985) found large differences in standard curves from tannins isolated from sorghum and quebracho. A hypothetical absorbance of 0.2 absorbance units gives tannin estimates from the different species' curves a range of five times lower to up to twenty one times higher than those of quebracho. Analyzing dried and ground leaf and stem material of six species gives both different concentrations and different rank orders, comparing quebracho estimates with those of each species' curve (Table 4). *E. micromera* tannin concentrations are estimated at 23.3% dry weight of the plant (ranking second in overall concentration) for the *E. micromera* curve versus 1.1% dry weight (ranking sixth in overall concentration for the quebracho curve). *Q. agrifolia* moves from the highest tannin containing leaves for its own curve to fourth with the quebracho curve. *K. parvifolia* goes from 9.9% dry weight tannins (*K. parvifolia* curve) to over 37% dry weight with a quebracho curve.

Such radical over- and underestimates of tannin concentrations and species ranking by use of a single standard curve appears to invalidate its use in both previous and future studies. Use of a quebracho curve when measuring tannin concentrations within leaves of one species will retain a proper ordering of highest to lowest while not reporting an accurate estimate of tannin amounts. However, relationships between the condensed tannin concentrations of different species can never be correctly estimated by the use of any one standard curve.

Protein precipitating ability is an alternative characteristic of condensed tannins, which has been proposed as a measure of tannin activity (Goldstein and Swain 1965; Bate-Smith 1973; Hagerman and Butler 1978). Most re-

Table 3. Parameters of the protein binding titration curves and the double logarithmic transformation of each curve. The saturation point is the tannin concentration beyond which no further precipitation occurs. The slope of the double logarithmic line is a direct measure of the binding efficiency of a tannin for Bovine Serum Albumin. The greater the slope the less tannin required to bind an equivalent amount of tannin, thus a more efficient tannin

	Titration plot saturation point	Double logarithmic plot		
		Slope	Intercept	r
E. micromera	1.2	0.28	0.29	0.87
Tannic acid	0.3	0.30	0.31	0.81
A. greggii	1.0	0.31	0.33	0.81
K. parvifolia	0.6	0.37	0.24	0.91
H. emoryi	1.2	0.54	0.05	0.99
Quebracho	0.8	0.47	−0.09	0.97
F. splendens	0.6	0.38	−0.05	0.93
P. californicum	1.6	0.40	−0.026	0.89
Q. agrifolia	1.5	0.33	−0.15	0.93

cently, Martin and Martin (1982) showed that estimates of proanthocyanidin concentrations by a quebracho standard curve showed no correlation with BSA precipitating ability of extracts of six different oak species. They propose also that measuring tannin protein precipitating ability is a more appropriate criterion of tannin presence.

An additional component of tannins as measured by the Martin and Martin method is the efficiency of protein precipitation by that tannin. Differential binding affinity of sorghum tannin has been demonstrated for three different proteins (Hagerman and Butler 1981). Additionally, different tannins can have different binding affinities for the same protein (Asquith and Butler 1985). Using extracted tannin powder, we determined that the nine different tannins of this study have very different efficiencies for binding a single protein type, BSA (Table 4). Ranking species from high to low based on their BSA binding efficiency yields a very different rank than either proanthocyanidin assay. Additionally, no correlation exists between the slopes of the species standard curves and the BSA binding efficiencies.

In a tannin-containing plant, we consider that the key

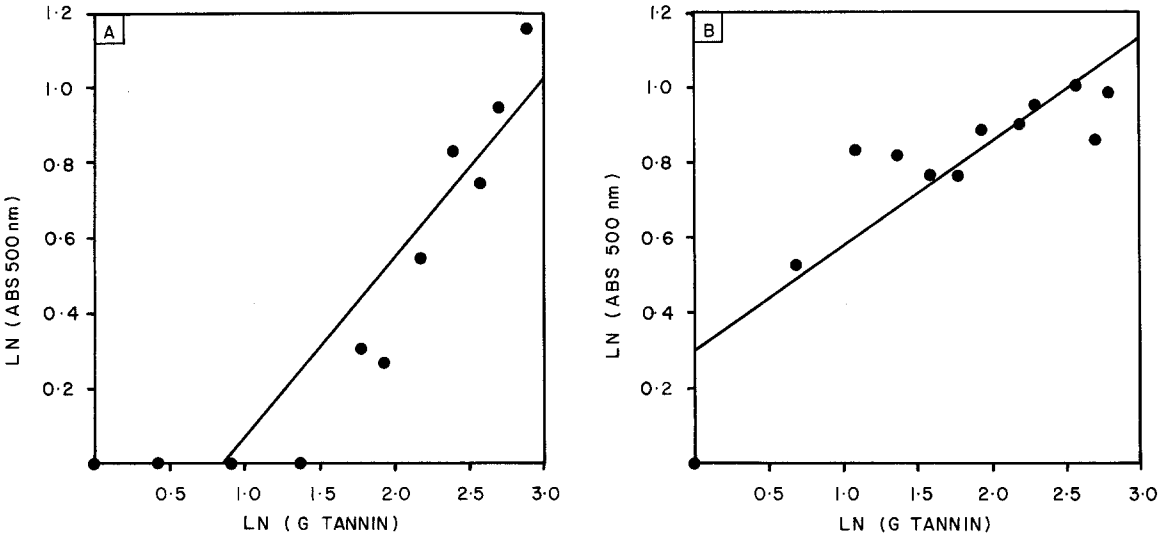

Fig. 4A, B. Double logarithmic plots of the titration curves shown in Fig. 3. Examples of high and low protein efficiencies have been chosen for plotting. Transformations are $\ln(x+1)$ to adjust zero values. See text for details. **A** *P. californicum*, **B** *E. micromera*

Table 4. Rank order relationships of six species for tannins from dried and ground leaves based on different measures. Estimations of leaf tannin concentrations have been calculated using the calibration curves generated from both the Quebracho standard curve and from each individual species standard curve from Table 2. Ranks based on these concentrations differ greatly between the two calibration curves (highest – 1/lowest – 6). BSA binding efficiencies are the slopes of the transformed titration curves (double logarithmic plots) and are a direct indicator of ability to bind BSA protein (data from Table 3). Ranking the six proteins based on binding efficiency yields a different order from each of the tannin estimations using the standard curves. Protein precipitating potential values are the product of multiplying the tannin concentrations (species curves) and the binding efficiencies

	Protein precipitating potential (BSA)		BSA binding efficiency		Species std. curve		Quebracho std. curve	
	PPP	Rank	$\frac{\ln(\text{g protein})}{\ln(\text{g tannin})}$	Rank	$\frac{\text{g tannin}}{\text{g leaf}}$	Rank	$\frac{\text{g tannin}}{\text{g leaf}}$	Rank
Q. agrifolia	0.081	1	0.33	4	0.244	1	0.057	4
E. micromera	0.065	2	0.28	6	0.233	2	0.011	6
K. parvifolia	0.037	3	0.37	3	0.099	3	0.378	1
P. californicum	0.032	4	0.40	1	0.081	4	0.095	3
A. greggii	0.015	5	0.31	5	0.047	5	0.317	2
F. splendens	0.008	6	0.38	2	0.020	6	0.028	5

ecological factors are both tannin levels and biochemical efficiency. This is because plants can compensate for low protein affinity by high concentrations of tannins. The effective amount of protein bound is a combination of both affinity and concentration (Zucker 1983). We have discussed above the importance of accurate estimations of tannin content based on species-specific standard curves and the differential protein binding abilities of different tannins. We now want to introduce the "protein precipitating potential" of a plant, an ecological parameter that combines both tannin concentrations and binding efficiency. Presuming that binding efficiency for a specific protein does not vary between individuals within a species (Martin and Martin 1982), then the amount of protein precipitable by the extractable contents of a leaf can be generated by multiplying the protein binding efficiency (i.e. the slope of the transformed binding curve) by the leaf proanthocyanidin content. This measure of tannins will require the extraction of leaf tannin powder for the establishment of a protein binding efficiency and a species standard curve for every species of interest. This measure is equivalent to directly measuring protein binding of each leaf extract, but only requires a spectrophotometric analysis of the extract once the potential equation is established.

We propose that combining tannin concentration and protein binding affinity into a single measure will allow a more representative comparison of effective tannin presence between species. Measurements of protein precipitating potential of leaves from different species most closely models the biochemical barriers faced by a herbivore. Herbivores consuming tanniferous leaves must deal with both tannin content and the protein binding ability of that tannin. Consequently, studies of plant communities that compare tannin content between species need to measure both aspects of tannin presence.

This measure of the ecological potential of tannin presence in a leaf comes closest to the role of tannins, we feel. The protein precipitating measure proposed by Martin and Martin (1982) is the slope of the linear portion of the untransformed titration curve. By using the transformed binding efficiency measure we propose here, a more complete description of the titration curve (protein binding) is included, such as thresholds and saturation levels in addition to the linear region slope.

P. californicum exhibits a threshold or binding, but has a high slope and a high saturation level ranking first in the transformed binding efficiencies. *Q. agrifolia* exhibits a similar threshold, but saturates quickly and ranks fourth in binding efficiencies. However, the measured *Q. agrifolia* samples contained high levels of condensed tannins, compensating for low binding affinities and ranking first in protein precipitating potential.

Due to the differential binding efficiencies of different tannins for different proteins (Hagerman and Butler 1981; Asquith and Butler 1985), it is important to use a standard protein for protein precipitating determinations. We agree with Martin and Martin (1983) that use of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCo) is the most ecologically significant standard protein to use for establishing relevant efficiencies since this is the most common plant protein constituent. Use of a plant protein conforms to the action of condensed tannins being at the "host-substrate level" – binding to plant substances to prevent digestion by herbivores and microbes (Zucker 1983).

The introduction of the concept of protein precipitating potential yields two advantages. First, population and community surveys will be based on an ecological property of tannins for determining the degree of defense elaborated by plants. Secondly, the establishment of the protein precipitating potential equation for a species will allow the reinterpretation of quebracho based tannin concentrations called into question by our findings.

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References

Asquith TN, Butler LG (1985) Use of dye-labeled protein spectrophotometric assay for protein precipitants such as tannin. J Chem Ecol 11:1535–1544

Bate-Smith EC (1973) Haemanalysis of tannins: the concept of relative astringency. Phytochemistry 12:907–912

Coley PD (1983) Herbivory and defensive characteristics of tree species in a lowland tropical forest. Ecol Monogr 53:209–233

Cooper SM, Owen-Smith N (1985) Condensed tannins deter feeding by browsing ruminants in a South African savanna. Oecologia (Berlin) 67:142–146

Dement WA, Mooney HA (1974) Seasonal variation in the production of tannins and cyanogenic glucosides in the chaparral shrub, *Heteromeles arbutifolia*. Oecologia (Berlin) 15:65–76

Feeny PP (1968) Effect of oak leaf tannins on larval growth of the winter moth *Operophthera brumata*. J Insect Physiol 14:805–817

Feeny PP (1970) Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. Ecology 51:565–581

Feeny P (1976) Plant apparency and chemical defense. In: Wallace J, Mansell L (eds) Recent advances in phytochemistry, vol 10. Plenum Press, New York, pp 1–40

Fox L, Macauley B (1977) Insect grazing on Eucalyptus in response to variation in leaf tannins and nitrogen. Oecologia (Berlin) 29:145–162

Goldstein JL, Swain T (1965) The inhibition of enzymes by tannins. Phytochemistry 4:185–192

Hagerman AE, Butler LG (1978) Potein precipitation method for the quantitative determination of tannins. J Agri Food Chem 26:809–812

Hagerman AE, Butler LG (1981) The specificity of proanthocyanidin-protein interactions. J Biol Chem 256:4494–4497

Hartree EF (1972) Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal Biochem 48:422–427

Lindroth RL, Batzli GO (1984) Plant phenolics as chemical defenses: Effects of natural phenolics on survival and growth of prairie voles (*Microtus ochrogaster*). J Chem Ecol 10:229–244

Martin J, Martin M (1982) Tannin assays in ecological studies: lack of correlation between phenolics, proanthocyanidins and protein-precipitating constituents in mature foliage of six oak species. Oecologia (Berlin) 54:205–211

Martin JS, Martin MM (1983) Tannin assays in ecological studies. Precipitation of ribulose-1,5-biphosphate carboxylase/oxygenase by tannic acid, quebracho and oak foliage extracts. J Chem Ecol 9:285–294

Martin MM, Rockholm DC, Martin JS (1985) Effects of surfactants, pH, and certain cations on precipitation of proteins by tannins. J Chem Ecol 11:485–493

McKey D, Waterman PJ, Mbi CN, Gartlan JS, Struhsaker TT (1978) Phenolic content of vegetation in two African rain forests: ecological implications. Science 202:61–64

Price M, Hagerman A, Butler L (1980) Tannin content of cowpeas, chickpeas, pigeon peas, and mung beans. J Agric Food Chem 28:459–461

Rhoades D, Cates R (1976) A general theory of plant antiherbivore chemistry. In: Wallace J, Mansell L (eds) Recent advances in phytochemistry, vol 10. Plenum Press, New York, pp 168–213

Roehring NE, Capinera JL (1983) Behavioural and developmental responses of the range caterpillar larvae, *Hemileuca oliviae* to condensed tannin. J Insect Physiol 29:901–906

Swain T (1979) Tannins and lignans. In: Rosenthal G, Janzen D (eds) Herbivores – their interactions with secondary plant metabolites. Academic Press, New York, pp 657–682

Tempel AS (1982) Tannin measuring techniques. J Chem Ecol 8:1289–1298

Zucker WV (1983) Tannins: Does structure determine function? An ecological perspective. Am Nat 121:335–365

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