JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 8375–8379 8375
DOI:10.1021/jf100631k

Quantitative Determination of Interactions Between Tannic Acid and a Model Protein Using Diffusion and Precipitation Assays on Cellulose Membranes

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Astringency perception has been associated with interactions between tannins present in some foods and salivary proteins. A variety of laboratory methods to measure tannin−protein interactions have been designed. Most of them, however, do not differentiate clearly between tannins and the protein fraction. The aim of this work was to characterize a method to measure tannin−protein interactions by following the behavior of the protein fraction. Experiments were performed with a representative hydrophilic globular protein, bovine serum albumin (BSA), and a gallotannin-rich commercial product, tannic acid (TA). Using cellulose membranes, concentration dependency of the inhibitory effect of TA upon the diffusion of BSA on the membrane and the ability of TA to precipitate BSA were assayed. Selective staining of the protein fraction was obtained using Coomassie Blue. TA inhibited BSA diffusion in a concentration-dependent manner in the range from a 0.1−0.2 up to a 1 μg of TA/μg of BSA ratio. Likewise, maximal precipitation of BSA (equivalence point) occurred at a 0.55 μg of TA/μg of BSA ratio, that is, when about 36 representative molecules of TA (average molecular weight = 1000) interact with every molecule of BSA (molecular weight = 66 000). The procedure may be readily adapted to measure interactions between different types of tannins and proteins that may be of relevance for taking decisions during food manufacturing.

KEYWORDS: Astringency; tannin protein; tannin−protein interaction; tannin−protein precipitation; bovine serum albumin

INTRODUCTION

Perceived astringency during food tasting is an important determinant of consumer behavior. Astringency is a drying, roughing, and sometimes puckering sensation that is experienced on the various oral surfaces during or immediately after tasting foods (*1*, *2*). For some foods, such as red wine, both the absence and excess of astringency may represent negative features of the product. Biochemical mechanisms underlying the physiological basis of astringency perception by humans have usually involved physicochemical interactions between phenolic compounds present in the foods and some particular families of salivary proteins, such as the salivary proline-rich proteins and histatins (*1*−*3*). Also frequently, those interactions have been associated with the formation of insoluble protein/polyphenol complexes (*4*−*7*). Thus, a number of widely used *in vitro* laboratory assays have been designed to estimate objectively the strength of the astringency sensation, whereas direct assessment of astringency is performed in parallel by panels of trained tasters (*2*, *5*, *6*, *8*−*12*). Currently, the gelatin index is the most common laboratory test in the winemaking industry to assess protein/polyphenol interactions and, therefore, to optimize production conditions (*13*). Such an assay measures the fraction of procyanidins (measured as optical density at 550 nm following acid hydrolysis) of a sample that remains unprecipitated upon adding an excess of gelatin and incubating for 3 days (*13*). Besides its lengthy time, this procedure has been the focus of controversies associated with the lack of both reproducibility and correlation with sensory tests, mostly as a consequence of the use of gelatin, a highly diverse protein product derived from collagen. In a recent study, Llaudy et al. (*12*) have proposed to assess protein/polyphenol interactions and, therefore, to estimate astringency, by means of the replacement of gelatin by a globular soluble pure and abundant protein, ovalbumin. The assay is based on measuring the degree of ovalbumin precipitation by spectrophotometry at 280 nm and has been reported to be more reproducible and better correlated with sensory analysis than the gelatin index. However, with respect to the specific purpose of estimating protein−polyphenol interactions, this procedure fails to distinguish between the protein and polyphenol component of the complexes because both types of molecules do exhibit light absorption at 280 nm. In addition, that procedure takes into consideration only the formation of protein/tannin precipitates with no regard of the eventual formation of soluble protein/tannin complexes.

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Published on Web 06/29/2010

Highly selective protein-binding dyes have been widely used in analytical biochemistry. The most conspicuous example is protein detection by Coomassie Blue R-250 on matrices of polyacrylamide gels used for fractionating proteins by electrophoresis (*11*, *14*). Such a colorant has been successfully used to stain protein against a background of pure cellulose (*15*−*17*). Furthermore, protein staining on cellulose membranes has also been productive in the description of differential modes of diffusion displayed on that surface by the protein fraction of a variety of biological fluids (*17*, *18*). The present study was aimed at quantitating interactions between a model globular pure protein, bovine serum albumin (BSA), with an extract of a model tannin, tannic acid (TA). The assay was based on observing the effect of the tannin on the diffusion of the protein on a cellulose membrane (diffusion assay) and studying the tannin-induced disappearance of the protein from a supernatant (precipitation assay). Protein detection was performed using a selective protein-binding dye with unsignificant ability to bind the tannin reactant.

MATERIALS AND METHODS

Materials. BSA (molecular weight = 66 000), TA (T0125, batch 66F-0751, average molecular weight = 1000), and gallic acid were purchased from Sigma Chemical Company, St. Louis, MO. Cellulose membranes (Whatman number 1) were purchased from Whatman Ltd., Maidstone, U.K. Solvents used for cellulose membrane processing were purchased from Merck, Santiago, Chile. High-performance liquid chromatography (HPLC)-grade acetic acid and acetonitrile were purchased from Merck, Darmstadt, Germany. Other pro-analysis-grade solvents and reagents were obtained from Oxiquim, Santiago, Chile. The HPLC system consisted of a photodiode-array detector, model G1315B, a quaternary pump, model Quat G1311A, and an autosampler, model ALS G1329A (Agilent Technologies 1200) fitted with a reversed-phase Nova Pack C_{18} column (4 μm, 3.9 mm inner diameter × 300 mm) (Waters Corporation, Milford, MA).

BSA and TA Solutions. BSA was dissolved in water at 40 °C using mechanical agitation for 20 min. The resulting solution was centrifuged at 3000 rpm for 5 min, and the 280 nm absorbance of the supernatant was adjusted to 0.7, which corresponds to 1 mg/mL. TA was dissolved in water with mechanical agitation at a final concentration of 10 mg/mL.

Characterization of TA. Total phenol content was determined by UV absorptiometry at 280 nm (*13*) using gallic acid as a standard. For characterization of phenolic compounds, a 50 mL aliquot of TA was extracted successively with ethyl ether (3 × 20 mL) and ethyl acetate (3 × 20 mL). The total extract was evaporated to dryness at 30 °C, dissolved in 2 mL of 1:1 (v/v) methanol/water, and filtered through 0.45 μm pore-size membranes. Aliquots of 20 μL were subjected to HPLC fractionation. Two solutions were used to produce mobile phases at a constant flow rate of 1 mL/min: A, 98:2 water/acetic acid (v/v); and B, 78:20:2 water/acetonitrile/acetic acid (v/v/v). The gradient profile was 100−20% A for 0−55 min, 20−10% A for 55−57 min, and 10−0% A for 57−90 min. Detection was performed by UV absorptiometry at various wavelengths in a range from 210 to 360 nm, with an acquisition speed of 1 s^{-1} (*19*). Characterization of compounds corresponding to individual HPLC peaks was performed by comparing their UV absorption spectra to those of pure standards (*19*−*21*). Both fractionation and composition analysis were performed in three independent experiments.

Protein Diffusion Assay on Cellulose Membranes. Aliquots of BSA and aliquots of TA solutions, representing each experimental condition, were thoroughly mixed in an Eppendorf tube and allowed to rest for 5 min. BSA alone and TA alone were used as controls. A total of 20 μL aliquots from the BSA plus TA mixtures were placed punctually on a cellulose membrane (Whatman number 1), allowed to diffuse freely, air-dried at room temperature for 10 min, fixed for 5 min in 5% trichloroacetic acid, rinsed for 5 min in 80% ethanol, and stained for 20 min in 0.25% Coomassie Blue R-250 dissolved in 45% isopropanol/10% acetic acid. The cellulose membrane was washed thoroughly in 7% acetic acid until it displayed a clear background. After a final rinse with water, the membrane was dried under a heat lamp. Blue spots, which represent the protein distribution area corresponding to particular experimental or control conditions, were visually compared (*15*−*17*). A decreased distribution area of BSA in the presence of TA with respect to the one displayed by BSA alone was considered indicative of the occurrence of TA−BSA complexes. A diagram of the procedure is shown in **Figure 1**.

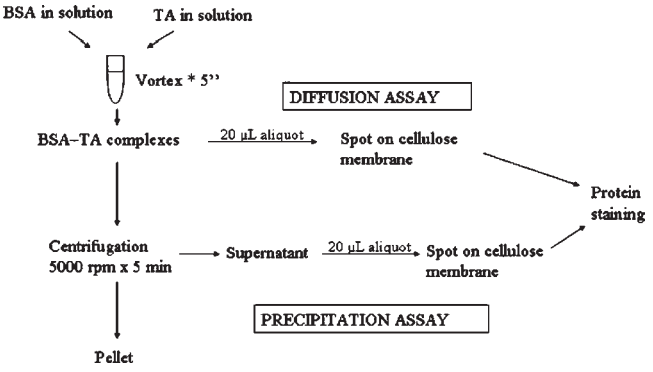

Figure 1. Diffusion and precipitation assays for detection of interactions between TA and BSA. A series of aliquots of TA were thoroughly mixed with a constant amount of BSA. After a 5 min incubation period, 20 μL aliquots of each mixture were placed punctually on a cellulose membrane and allowed to diffuse freely. Once dried, the membrane was fixed and stained with Coomassie Blue to assess protein distribution (diffusion assay). The rest of the TA plus BSA mixture of each tube was centrifuged at 5000 rpm for 5 min at room temperature to produce a supernatant and a sediment. Aliquots were taken from each supernatant and spotted on a cellulose membrane for protein detection as indicated above. In this latter assay, the absence of Coomassie Blue staining represents full precipitation of TA−BSA complexes (precipitation assay).

Table 1. Chemical Characterization of TA

	concentration[a]
total phenols[b]	1099.3 ± 13.6
gallic acid[c]	6.6 ± 0.3
gallotannin[c]	94.8 ± 15.3

[a] Values represent mean ± standard deviation (triplicates). [b] Milligrams of equivalent gallic acid per gram of TA. [c] Milligrams per gram of TA [data from HPLC−diode array detector (DAD) analysis of a low-molecular-weight extract of TA].

Protein Precipitation Assay Using Cellulose Membranes. After mixing BSA with TA, as above, the Eppendorf tubes were centrifuged at 5000 rpm for 5 min at room temperature. Aliquots (20 μL) were taken from the supernatants and spotted on a cellulose membrane. The membrane was processed as described in the previous section (**Figure 1**). In this assay, a decreased staining intensity was considered indicative of precipitation of insoluble TA−BSA complexes. Also, the equivalence point represents the lowest amount of TA needed to produce the full disappearance of the protein from the supernatant. Assays were performed in triplicate.

RESULTS

Characterization of TA. Table 1 shows a chemical characterization of TA used in this study. The HPLC profile of low-molecular-weight phenolic compounds extracted from TA is shown in **Figure 2**. Absorption spectra of the most prominent peaks in the profile showed with no exception that they correspond to gallotannins; that is, the absorption peak occurs around 276−278 nm and absorption is almost completely attenuated at wavelengths longer than 320 nm. Also, a prominent peak of gallic acid displaying the shortest retention time and a maximum absorption at 270 nm was observed.

Differential Detection of BSA and TA on Cellulose Membranes. Figure 3 shows the homogeneous distribution of BSA when placed punctually as 10−30 μL aliquots of a 1 mg/mL solution on a cellulose membrane and allowed to diffuse. When the protein is stained with the protein binding dye Coomassie Blue,

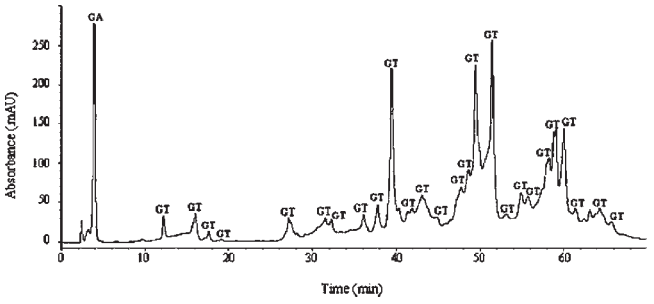

Figure 2. HPLC fractionation of TA. Representative chromatogram of an extract of low-molecular-weight phenolic compounds extracted from TA. Detection was performed at 275 nm. The absorption spectrum of each individual peak was compared against those of gallic acid (λ_{max} = 270 nm) and gallotannin (λ_{max} = 276−278 nm). With no exception, only gallotannin (GT) and gallic acid (GA) were identified.

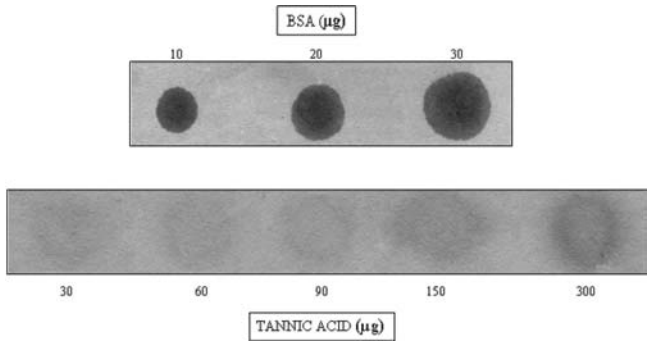

Figure 3. Selective detection of BSA in the presence of TA on cellulose membranes. Aliquots of 10, 20, and 30 μL of 1 mg/mL BSA (left to right, upper figure) and 30 μL aliquots of a series of solutions containing 1, 2, 3, 5, and 10 mg/mL TA (left to right, lower figure) were placed punctually on separate cellulose membranes, fixed, stained with Coomassie Blue, and destained. Note the intense staining of BSA in contrast with the poor reactivity of TA.

the protein is clearly revealed as a roughly blue circle, whose area is a direct function of the aliquot volume. In contrast, when an aliquot of a similar concentration (1 mg/mL) of TA was placed on the cellulose membrane, Coomassie Blue failed to stain the tannin. Poor reactivity of the protein dye with TA was observed even at concentrations as high as 10 mg/mL.

Effect of TA upon BSA Diffusion on Cellulose Membranes. As shown before, BSA in aqueous solution diffuses freely when placed punctually on a cellulose membrane. Because tannin and proteins may interact and eventually form insoluble tannin−protein complexes, protein diffusion on cellulose membranes may be affected. In a preliminary assay, we mixed 100 μL of 1 mg/mL BSA with 10 μL of either distilled water (control condition) (**A** and **B** of **Figure 4**) or 10 mg/mL TA (**C** and **D** of **Figure 4**) and then we placed 10 and 30 μL aliquots on a cellulose membrane. When TA and BSA had been mixed in a 1:1 ratio, TA provoked a dramatic suppression of BSA diffusion (**C** and **D** of **Figure 4**). To quantify the effect of TA on BSA diffusion, aliquots of a 1 mg/mL BSA solution were mixed with a series of aliquots of a 10 mg/mL TA solution to produce TA plus BSA mixtures in a range of TA/BSA mass ratios from 0 to 1. Aliquots of each mixture were then placed on a cellulose membrane and processed for protein detection. As shown in **Figure 5**, in this assay, TA provoked a decrease in BSA diffusion in a concentration-dependent manner. In addition, in this study, a non-diffusible protein material became progressively more apparent.

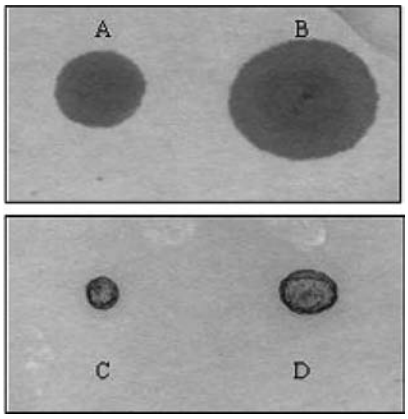

Figure 4. Inhibitory effect of TA on BSA diffusion on cellulose membranes. A 100 μL aliquot of 1 mg/mL BSA was thoroughly mixed with 10 μL of either distilled water (control condition) or 10 mg/mL TA. Aliquots of 10 and 30 μL of both the control mixture (A and B, respectively) and the TA plus BSA mixture (C and D, respectively) were placed punctually on a cellulose membrane and processed for protein staining. Note the homogeneous distribution of BSA all over the roughly circular surface of the membrane that was wetted by the aliquots of the control mixture (A and B). In contrast, in the presence of TA, BSA did not diffuse and remained close to the spotting site (C and D).

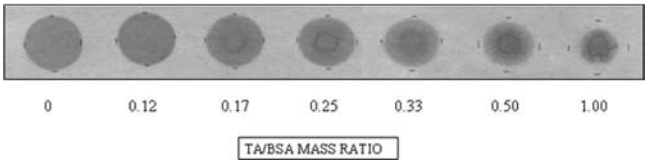

Figure 5. Titration of the inhibitory effect of TA on BSA diffusion. Aliquots (20 μL) of TA plus BSA mixtures in a range of ratios from 0 to 1 were placed punctually on a cellulose membrane, and after the boundaries of the wetted surface were marked with a carbon pencil, the membrane was processed for protein staining. Note that, with a progressive increase in the TA/BSA ratio, BSA diffusion became progressively diminished, a non-diffusible protein fraction became increasingly more evident, and the surface of the originally wetted area remained mostly unaffected.

Effect of TA upon BSA Precipitation. Precipitation of protein−tannin complexes has been frequently used as an objective parameter for tannin−protein interactions. To that end, in this study, we observed on cellulose membranes the fraction of protein that remains unprecipitated after mixing a constant amount of BSA with various amounts of TA. Thus, 90 μL of 1 mg/mL BSA was mixed with 10 μL aliquots of 2-fold serial dilutions containing from 400 to 12.5 μg of TA (**Figure 6**). After 5 min at room temperature, the tubes were centrifuged and aliquots of the corresponding supernatants were placed punctually on a cellulose membrane and allowed to diffuse. After processing the membrane for staining with Coomassie Blue, we could appreciate that the spot corresponding to 25 μg of TA was only partially stained, whereas the spot corresponding to 50 μg of TA was undistinguishable from the ones corresponding to zero protein. Accordingly, this assay indicated that 90 μg of BSA is partially precipitated with 25 μg of TA, whereas the equivalence point, that is, full protein precipitation with the lowest amount of tannin, occurs at a ratio of 50:90 TA/BSA. Levels of TA much higher than the one of the equivalence point (4−8-fold that of the equivalence point) show the well-known mordant effect of tannins that accounts for the observed binding of Coomassie Blue.

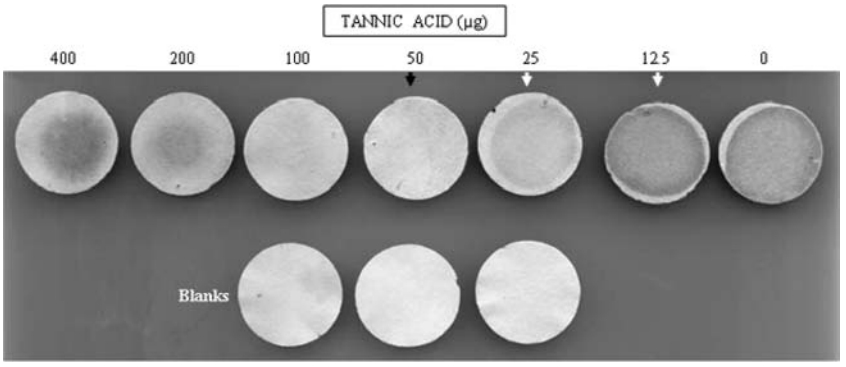

Figure 6. Effect of TA on BSA precipitation. Aliquots (90 μL) of 1 mg/mL BSA were mixed with 10 μL aliquots of 2-fold serial dilutions containing from 400 to 12.5 μg TA. After incubation for 5 min at room temperature, the tubes were centrifuged at 5000 rpm for 5 min and 20 μL aliquots of the corresponding supernatants were placed punctually on cellulose disks (water aliquots were placed on blank disks). The cellulose disks were processed for protein staining. Note the progressive decrease in the BSA present in the supernatant as the amount of TA in the TA plus BSA mixture increases (white arrows). The black arrow indicates the equivalence point. At levels of TA much higher than the one of the equivalence point, the mordant effect of tannins can be appreciated.

DISCUSSION

Tannin−protein interactions have been assayed using cellulose membranes. TA, a commercially available tannin extract from *Quercus infectoria*, and BSA, a widely available protein for research purposes, were used in this study as representative members of both families of molecules. Interactions between TA and BSA were associated primarily to a tannin-dependent attenuation of protein diffusion on cellulose membranes. From a modification of the procedure, we also assayed tannin-dependent protein precipitation on the cellulose membranes. BSA was distinctively detected by Coomassie Blue R-250, a selective protein-binding dye that fails to bind both TA and cellulose. In the study, such a selectivity was used to detect BSA distribution on the cellulose membrane even in the presence of TA.

BSA diffuses freely on cellulose membranes, that is, when a microliter drop aliquot of an aqueous solution of BSA is placed punctually on that absorbing membrane, the protein moves radially beyond the area of the original drop together with the aqueous solvent (*17*). From a chromatographic viewpoint, the affinity of BSA for water is much higher than its affinity for the solid cellulose matrix, and this is why it becomes distributed all over the wetted area. That is probably a common behavior of any other highly hydrophilic medium-sized protein. By the same token, interactions of BSA (or any other protein sharing its characteristics) with smaller compounds displaying high affinity to it, such as polyfunctional phenolics, would induce the organization of larger and less diffusible supramolecular structures. It would be expected that, at low tannin/protein ratios, soluble complexes would consist of tannin molecules bound to single protein molecules, whereas at high tannin/protein ratios, tannin molecules would link two or more protein molecules, thus generating a true network of diverse tannin−protein structures (*5*,*22*). In our study, the occurrence of soluble TA−BSA complexes was evidenced by an usual TA-dependent increase in the staining intensity of the protein spots with TA/BSA mass ratios below 0.14. In the study, we also observed that TA provoked a marked attenuation of BSA diffusion on cellulose membranes with TA/BSA mass ratios of 0.25 or over. Accordingly, the organization of a variety of supramolecular TA−BSA structures (complexes) is quite probably underlying the observed changes in protein diffusion. Thus, growing concentrations of TA provoked a progressively higher inhibitory effect on BSA diffusion on cellulose membranes in such a way that, when the mass (mg) of TA/mass (mg) of BSA was around 1, protein diffusion became fully restricted (null diffusion). Under this condition, the surface of the originally wetted area on the membrane remained mostly unaffected, whereas the area between the bulk of the stained protein and the edge of the wetted area was undistinguishable from the membrane background.

Considering that Coomassie Blue is a selective stain for proteins (*11*, *14*) and that tannins are powerful protein precipitants (*2*, *12*, *13*), in this study, we also assayed on cellulose membranes the presence of protein that remains in suspension when a constant amount of BSA is mixed with a growing series of TA-containing aliquots; that is, we assayed the non-precipitated BSA. Thus, to perform this precipitation assay, we just centrifuged the series of TA plus BSA mixtures before placing the corresponding aliquots on the cellulose membrane. In these experiments, we observed that, as the TA concentration in the TA plus BSA mixtures increased (TA/BSA mass ratios of 0.28 or over), the presence of BSA in the corresponding supernatants decreased progressively to a point in which no protein reactivity was detected. In this assay, the "equivalence point" represents the minimal amount of TA that is necessary to fully precipitate a given amount of BSA. This parameter is very important because it represents a stoichiometric relationship between the reactants. In our study, 90 μg of BSA was precipitated by 50 μg of TA (TA/BSA mass ratio = 0.56). Accordingly, considering Avogadro's number and the molecular weight of the reactants, such stoichiometry indicates either that full precipitation of BSA occurs when about 36 representative molecules of TA (average molecular weight = 1000) interact with every molecule of BSA (molecular weight = 66 000) or that the insoluble TA−BSA complexes may have a supramolecular global formula of $TA_{36}BSA$. Certainly, further improvements in accuracy may be achieved through the complementary assay of narrower ranges of TA around the equivalence point. Altogether, for this pair of reactants, there is a range of low relative TA concentrations producing soluble TA−BSA complexes (TA/BSA mass ratios below 0.14), whereas at higher concentrations (TA/BSA mass ratios over 0.28), TA forms insoluble TA−BSA complexes. In our view, both phenomena, that is, the formation of either soluble or insoluble tannin−protein complexes, should be considered separately when looking for eventual associations with results of subjective sensory testing for astringency (*12*, *23*−*25*). Even so, it has not escaped our attention that TA, as it happens with any tannin, is a complex mixture of (gallotannin) polyphenols quite probably differing from each other in their reactivity toward proteins (*1*, *4*, *8*, *26*). Accordingly, our observations do not discard the possibility that, in addition to quantitative considerations, some particular gallotannins may form soluble complexes with BSA, while others may

form insoluble complexes with that protein. That differential reactivity may occur naturally in the mouth during sensory assessment of astringency, where complex tannins are mixed with an array of salivary protein families (*27*, *28*). Anyhow, the procedure that we are describing may be readily adapted to evaluate tannin−protein interactions using either particular polyphenol, polyphenol families, or tannin extracts together with a particular model protein, protein extract (gelatins), or salivary fluid containing proteins of physiological relevance for astringency perception (*29*, *30*). Such an array of possibilities may be of great help for the assay of tannin−protein interactions that are part of technological evaluations in the food industry, such as the assessments of astringency and phenolic maturity of grapes in the wine industry.

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Received for review February 17, 2010. Revised manuscript received June 15, 2010. Accepted June 17, 2010. This study was partially supported by Grants Corfo-Innova Tecnovid-05CTE02-04, DI-Universidad de Chile Mult-05/35-2, and Fondecyt-Chile 1080559.