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Enhancement of Both Salivary Protein−Enological Tannin Interactions and Astringency Perception by Ethanol

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Red wine astringency has been associated with interactions of tannins with salivary proteins. Tannins are active protein precipitants. Not much evidence exists demonstrating contribution of other wine components to astringency. We aimed to investigate an eventual role of ethanol both in astringency and salivary protein−enological tannin interactions. A trained sensory panel scored perceived astringency. Salivary protein−tannin interactions were assessed by observing both tannin-dependent changes in salivary protein diffusion on cellulose membranes and tannin-induced salivary protein precipitation. Proanthocyanidins and gallotannins in aqueous and hydroalcoholic solutions were assayed. A biphasic mode of diffusion on cellulose membranes displayed by salivary proteins was unaffected after dilution with water or enological concentrations of ethanol. At those concentrations ethanol was not astringent. In aqueous solution, tannins provoked both restriction of salivary protein diffusion, protein precipitation, and astringency. Those effects were exacerbated by 13% ethanol. In summary, enological concentrations of ethanol exacerbate astringency and salivary protein−tannin interactions.

KEYWORDS: Astringency; alcohol; salivary proteins; enological tannin

INTRODUCTION

Salivary protein−tannin interactions have been assumed to underlie astringency perception (*1*). Salivary proteins are a complex mixture of macromolecules that are differentially produced by diverse secretory glands, tissues, and cells (*2*). Given such a complexity, salivary protein−tannin interactions have been modeled using single proteins (*3*−*9*). Gelatin is a protein material that has been widely in wine-making practice to estimate the potential astringency of tannin-containing natural beverages with particular reference to wine (*10*). However, gelatin itself is a highly complex and diverse protein material whose use in the assessment of tannin−gelatin interactions has been a subject of controversy (*3*). Thus, other simpler proteins, even short synthetic peptides, have been recently used as reference standards for tannin−protein interactions (*3*−*9*, *11*). However, no single protein can replace structurally or functionally the wide diversity of macromolecular components of human saliva (*12*). Precipitation of tannin−protein complexes, such as the well-known Glories gelatin index, has been routinely used to estimate protein−tannin interactions (*10*). However, this approach does not take into consideration the formation of soluble tannin−protein complexes. In addition, these functional methods to assess tannin−protein interactions are usually performed under experimental conditions excluding ethanol (*6*, *9*). Certainly, that seems to be an oversimplification considering that ethanol produces protein denaturation and protein precipitation and that some tannin-containing beverages, such as wine, may contain as much as 15% ethanol (*13*). Some authors have reported that some particular salivary proteins, such as members of the proline-rich proteins, display a reduced affinity for tannins in the presence of ethanol (*4*). Also, several studies have shown that ethanol reduces astringency perception through an increase in saliva viscosity (*14*), an increase in the lubricating power of saliva (*15*), or through its own bitter or sweet taste masking astringency perception (*16*). Contrarily, by assessing astringent subqualities of drying, roughing, and puckering, other authors have shown that after dealcoholization wines are perceived as more grippy/adhesive, aggressive, and drying (*17*). Altogether, the effect of ethanol on both salivary protein−tannin interactions and astringency perception remains highly controversial.

The present study was aimed at conducting a parallel assessment of the effect of ethanol on tannin-induced astringency and tannin−salivary protein interactions. A trained sensory panel evaluated astringency perception. Tannin−salivary protein interactions were assessed under *in vitro* conditions reflecting both the degree of dilution experienced by saliva during wine tasting and the expected ethanol concentration in the wine−saliva mixture in mouth (*18*). We examined the effect of two different enological tannins, a proanthocyanidin tannin and a hydrolyzable tannin, on two physicochemical properties of the salivary protein, namely, the mode of diffusion on cellulose membranes and precipitation (*19*).

MATERIALS AND METHODS

Materials. A hydrolyzable tannin (Tanin Gallique a l′alcool) and a proanthocyanidinic tannin (Protanin R) were purchased from Vinicas,

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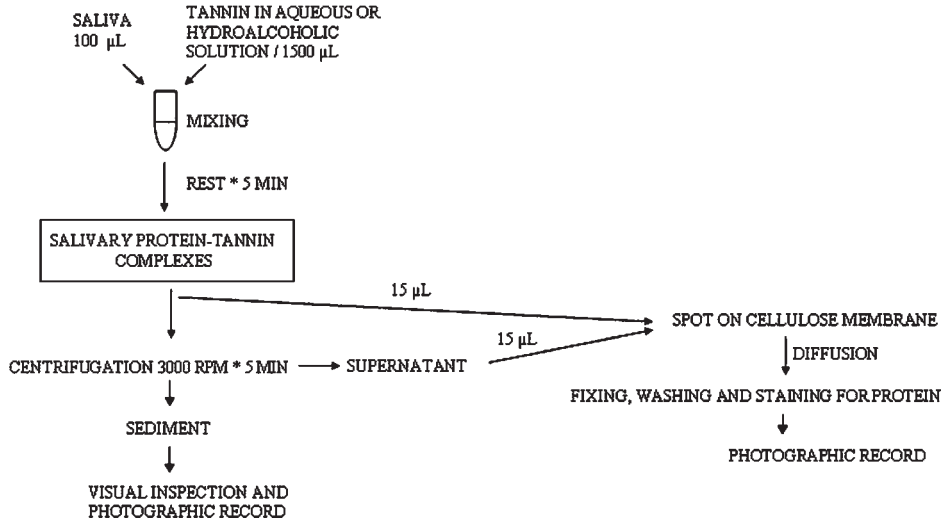

Figure 1. Diffusion and precipitation assays for tannin−protein complexation. Aliquots (100 μL) of freshly collected whole saliva were mixed with 1500 μL of aqueous or hydroalcoholic (containing 13% v/v ethanol) solutions of either proanthocyanidin tannin or hydrolyzable tannin extracts. Either water, aqueous solvent, or hydroalcoholic solvent served as controls. After a 15-μL aliquot from each experimental condition was spotted on a cellulose membrane (diffusion assay), the tubes were centrifuged to produce a supernatant and to assess the occurrence of sediment. Aliquots of the supernatants were used for protein detection on a cellulose membrane (precipitation assay).

Santiago, Chile. Tartaric acid as well as standards of gallic acid and (+)-catechin were purchased from Sigma Chemical Company, Saint Louis, MO, USA. Cellulose membranes (Whatman No. 1) were purchased from Whatman Ltd., Maidstone, England. HPLC grade acetic acid and acetonitrile were purchased from Merck, Darmstadt, Germany. Pro-analysis solvents were obtained from Oxiquim-Chile.

Tannin Extract Solutions. Each enological tannin was dissolved at concentrations of either 0.75 mg/mL (hydrolyzable tannin) or 3 mg/mL (proanthocyanidinic tannin) under agitation for 20 min at 20 °C both in 0.5% w/v tartaric acid (aqueous solution) and in 13% v/v ethanol/0.5% w/v tartaric acid (hydroalcoholic solution). The four resulting tannin solutions were filtered and adjusted to pH 3.7 with sodium hydroxide and to final concentrations of 0.4 absorbance units at 280 nm.

Sensory Evaluation. All four tannin extract solutions were rated for astringency by a 13-member trained sensory panel. Tannin solutions (15 mL) at 20 °C (±0.1 °C) in black cups were presented at random to the panel members, who were asked to describe the intensity of the perceived astringency for each sample on a 0−15 score scale. Each sample was evaluated twice. Solutions of both 0.5% w/v tartaric acid and 13% v/v ethanol/0.5% w/v tartaric acid served as controls (level 0 on the score scale). Pectin dissolved in distilled water (1 g/L) was used for mouth rinsing between consecutive samples.

Characterization of Tannin Extracts. Total phenol content was determined by UV absorptiometry at 280 nm (*10*) using gallic acid as standard. The method of Ribéreau-Gayon and Stonestreet was used to measure the relative content of proanthocyanidins using (+)-catechin as standard (*20*). Characterization of phenolic compounds was performed using an Agilent 1200 HPLC system (quaternary pump model Quat G1311A, autosampler model ALS G1329A, and photodiode-array detector model G1315B) fitted with a reversed-phase Nova Pack C_{18} column (4 μm, 3.9 mm i.d. × 300 mm) (Waters Corporation, Milford, MA, USA). Briefly, tannin extract solutions (50 mL) were extracted successively with ethyl ether (3 × 20 mL) and ethyl acetate (3 × 20 mL). Total extracts were evaporated to dryness at 30 °C, redissolved in 2 mL of 50% (v/v) methanol/water and membrane-filtered (0.45 μm pore size). Aliquots (100 μL) of the extracts were reversed-phase fractionated at 20 °C with detection at 280 nm. Two mobile phases were used: A, water/acetic acid (98:2 v/v) and B, water/acetonitrile/acetic acid (78:20:2 v/v/v). A two-step gradient was carried out at a constant flow rate of 1.0 mL per min: 0−55 min, 100−20% A and 55−70 min, 20−10% A. Equilibration times of 15 min were allowed between injections (*21*). For reference, a few major peaks in the HPLC chromatograms of the tannin extracts were characterized both by retention time and absorption spectrum (from 210 to 360 nm). Identification of specific compounds was achieved by comparison against pure standards. Calibration curves were constructed by using gallic acid (gallotannins) and (+)-catechin (proanthocyanidins). All qualitative and quantitative analyses of phenolic composition of tannins were performed in triplicate.

Whole Saliva Collection. Throughout the study, samples of saliva were provided by a single young adult healthy volunteer displaying normal salivary polypeptide profiles (*22*). A conventional collection procedure with no use of sialagogues (unstimulated saliva) was carried out under standardized conditions, always between 9.00 and 11.00 a.m. and just before the experiments (*23*). Whole saliva accumulated in mouth for two successive 1-min intervals was expectorated into a single sterile glass container. Samples were maintained in ice during the experiments.

Salivary Protein−Tannin Complexation. A. Diffusion assay. One hundred microliters of a fresh sample of whole saliva were mixed with 1500-μL aliquots of aqueous or hydroalcoholic solutions of either proanthocyanidin (PaT) or hydrolyzable tannin (HT). Saliva mixed with water at a volume ratio of 1:15 served as control. After incubation for 5 min at room temperature, 15-μL aliquots of the mixtures were placed punctually on a cellulose membrane and allowed to diffuse. The dry membrane was fixed in 5% trichloroacetic acid and stained for protein with Coomassie blue as described elsewhere (*19*). Both diffusion area and stain intensity of the protein spots were semiqualitative estimates for protein−tannin interaction. **B. Precipitation assay.** The rest of the whole saliva−tannin extract mixtures of the diffusion assay were centrifuged at 3000 rpm for 5 min in a Sorvall microcentrifuge. Fifteen-microliter aliquots of each supernatant were placed punctually on a cellulose membrane, allowed to diffuse, and processed for protein staining, as indicated above. In this assay, reduced protein staining was taken as indicative of protein precipitation. This observation was complemented by a direct visual inspection of the centrifuge tubes (**Figure 1**).

RESULTS

Characterization of Enological Tannins. Table 1 shows a chemical characterization of both enological tannins used in this study. On a weight basis, the content of total phenols in the hydrolyzable tannin was 4 times that in the proanthocyanidinic tannin, while the concentration of tannins in the proanthocyanidinic tannin, as measured by the Ribereau-Gayon and Stonestreet method, was 11.4 times that in the hydrolyzable tannin. HPLC-DAD analysis and UV absorptiometry showed marked composition differences between both enological tannins (**Figure 2**). Thus, gallotannins were identified only in the hydrolyzable tannin, whereas

proanthocyanidins were present only in the proanthocyanidinic tannin. Gallic acid was far more abundant in the hydrolyzable tannin extract (**Table 1**).

Sensory Analysis. Proanthocyanidinic tannin and hydrolyzable tannin extracts were assayed for astringency both in the presence and absence of 13% (v/v) ethanol. As shown in **Table 2**, both aqueous extracts of tannins were recognized by the trained sensory panelists as being astringent. However, both the proanthocyanidin tannin extract and the hydrolyzable tannin extract were perceived as clearly more astringent in the presence of 13% ethanol (ANDEVA, $p < 0.05$).

Diffusion of Salivary Protein on Cellulose Membranes. When an aliquot of whole saliva is spotted onto a cellulose membrane, radial diffusion of the salivary fluid participates in a chromatographic fractionation of the protein component of saliva, thus producing a biphasic mode of diffusion. In effect, once diffusion has ended, a protein-binding dye shows an intense blue-stained roughly circular area close to the spotting site (nondiffusible fraction of salivary protein), which becomes surrounded by a weaker blue-stained outer band (diffusible fraction of salivary protein) (**Figure 3**).

Effect of Dilution and Ethanol upon Salivary Protein Diffusion on Cellulose Membranes. To analyze whether tannins affected diffusion of salivary proteins on the cellulose membranes, we first examined any eventual effect of the corresponding tannin solvents on that parameter. To that end, saliva was diluted gradually with either water or ethanol up to 1:11 v/v (up to 9% in saliva). Dilution of saliva with water resulted in no major effect upon salivary protein diffusion on cellulose membranes, excepting the expected decrease in the intensity of protein staining (**Figure 4**, **A**−**I**). Over all the assayed range of dilutions (up to 9% in saliva) the biphasic mode of diffusion was fully preserved. By contrast, parallel dilutions of saliva with ethanol produced no effect at ethanol concentrations lower than 9%, that is, over the range of

Table 1. Chemical Composition of Commercial Enological Tannins Used in the Study[a]

	hydrolyzable tannin	proanthocyanidin tannin
total phenols[b]	308.2 ± 0.3	77.4 ± 0.2 *
tannins[c]	2.2 ± 0.6	25.1 ± 3.9 *
gallic acid[d]	1.1 ± 0.3	0.1 ± 0.0 *
gallotannin[d]	47.7 ± 3.5	0.0 ± 0.0 *
proanthocyanidins[d]	0.0 ± 0.0	29.3 ± 1.3 *

[a] Figures represent mean ± standard deviation (triplicates). Asterisks indicate significant difference between both enological tannins (Tukey test, $p < 0.05$). [b] Milligram equivalent gallic acid/g tannin. [c] Milligram equivalent procyanidin/g tannin (Ribereau-Gayon and Stonestreet method). [d] Milligram per gram enological tannin (HPLC-DAD data).

Table 2. Intensity of Perceived Astringency of Enological Tannins in Aqueous and Hydroalcoholic Solutions[a]

	aqueous	13% ethanol
proanthocyanidinic tannin	4.9 ± 1.1	9.5 ± 1.8 *
hydrolyzable tannin	4.0 ± 1.2	9.4 ± 1.9 *

[a] Figures represent mean ± standard deviation of duplicate scores by a 13-member sensory panel. Asterisks indicate significant difference ($p < 0.05$) between scores for each tannin at different ethanol concentrations.

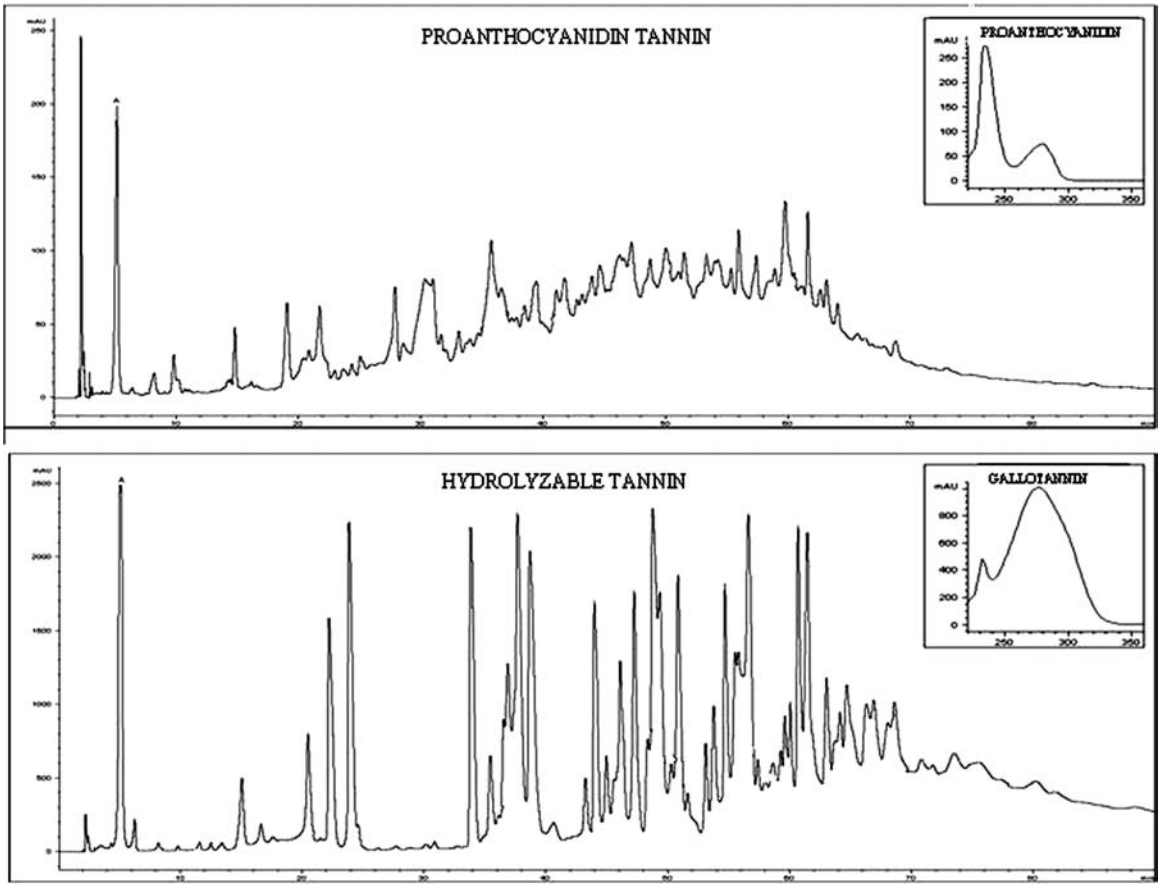

Figure 2. HPLC-DAD chromatograms of the enological tannins used in the study. A proanthocyanidin tannin extract (PaT) and a hydrolyzable tannin extract (HT) were prepared from commercially available enological products and characterized by HPLC-DAD fractionation and UV absorptiometry. Representative UV spectra of all components of each extract are shown in the corresponding insets.

90−99% in saliva (**Figure 4**, **K**−**N**). When the ethanol concentration was increased to 20%, a minor antidiffusive effect on the diffusible salivary protein fraction together with a minor condensing effect of the non-diffusible salivary protein fraction became apparent (**Figure 4**, **O**). At ethanol concentrations above 50% (**Figure 4**, **P**−**S**), the diffusible salivary protein fraction fully disappeared and all of it seemed to become part of the non-diffusible salivary protein material. Both solvents showed no reactivity with the protein dye (**Figure 4**, **J** and **T**).

Effect of Enological Tannins upon Salivary Protein Diffusion on Cellulose Membranes. Considering the lack of effect of high dilutions of saliva with water on the biphasic mode of diffusion of whole saliva as well as the lack of any significant effect on that mode of diffusion displayed by ethanol concentrations below 20%, we next analyzed the effect of mixing saliva in a 1:15 v/v ratio with either aqueous or 13% ethanol-containing tannin solutions. As expected, dilution of whole saliva with water in a 1:15 v/v ratio followed by spotting of an aliquot of the mixture onto a cellulose membrane resulted in a biphasic pattern of diffusion consisting of a slightly stained diffusible protein fraction and a sort of swollen and structured nondiffusible protein fraction (**Figure 5**, **A** and **D**). By contrast, mixing of an aliquot of whole saliva with an aqueous solution of proanthocyanidin tannins in a 1:15 ratio produced a dramatic aggregation of the nondiffusible salivary component and a partial decrease in the diffusible protein component (**Figure 5**, **B**). Likewise, mixing of an aqueous solution of hydrolyzable tannins with saliva in the same 1:15 v/v ratio resulted only in some increase in the intensity of the nondiffusible salivary component. Under those conditions, the diffusible salivary component was only marginally affected (**Figure 5**, **E**).

Exacerbating Effect of Ethanol on the Enological-Tannin-Induced Inhibition of Salivary Protein Diffusion on Cellulose Membranes. Ethanol concentration in wine ranges from 11% to 14% v/v. On that basis, we investigated whether those concentrations

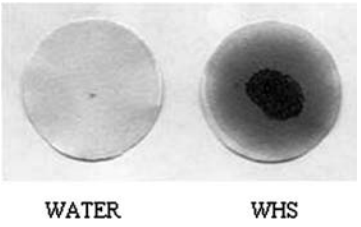

Figure 3. Mode of diffusion of the salivary protein fraction on cellulose membranes. When an aliquot of saliva is placed onto the center of an absorbing cellulose disk, radial diffusion occurs. Upon staining with Coomassie blue, a biphasic distribution of the salivary protein is revealed, that is, a nondiffusible protein fraction is surrounded by a diffusible protein fraction (right). The protein-binding dye does not react with a cellulose disk seeded with an equivalent aliquot of water (left).

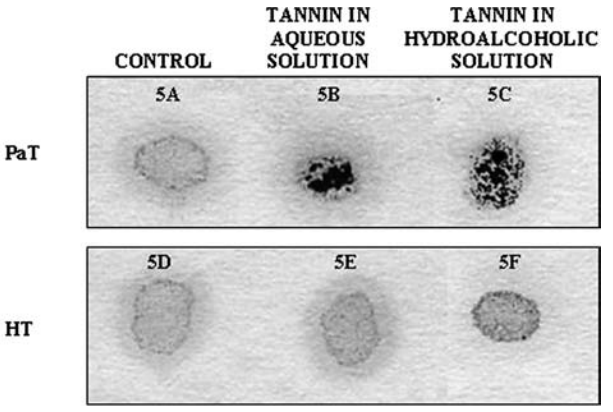

Figure 5. Enhancing effect of ethanol on the tannin-induced inhibition of salivary protein diffusion on cellulose membranes. Aqueous and 13% ethanol-containing extracts of either proanthocyanidin (PaT) or hydrolyzable tannin (HT) were mixed with aliquots of whole saliva at a 15:1 v/v ratio and incubated for 5 min at room temperature. Fifteen-microliter aliquots of the mixtures were placed punctually on a cellulose membrane and allowed to diffuse. The dry membrane was fixed in 5% trichloroacetic acid and stained for protein with Coomassie blue. In the control condition (aqueous solution instead of tannin solution), the non-diffusible fraction of salivary protein exhibiting a relaxed appearance is surrounded by a band of diffusible protein (**5A** and **5D**). PaT in aqueous solution is highly effective in provoking both a marked condensation of the non-diffusible protein fraction and a significant decrease in the diffusible salivary protein fraction (**5B**). In the presence of PaT and 13% ethanol, all of the salivary protein becomes non-diffusible (**5C**). Likewise, HT in aqueous solution induces a perceptible increase in the condensation of the non-diffusible salivary protein, but this remains surrounded by a significant amount of diffusible protein (**5E**). By contrast, in the presence of 13% ethanol, HT provokes a much higher condensation of the non-diffusible protein fraction and a full disappearance of the diffusible salivary protein fraction (**5F**).

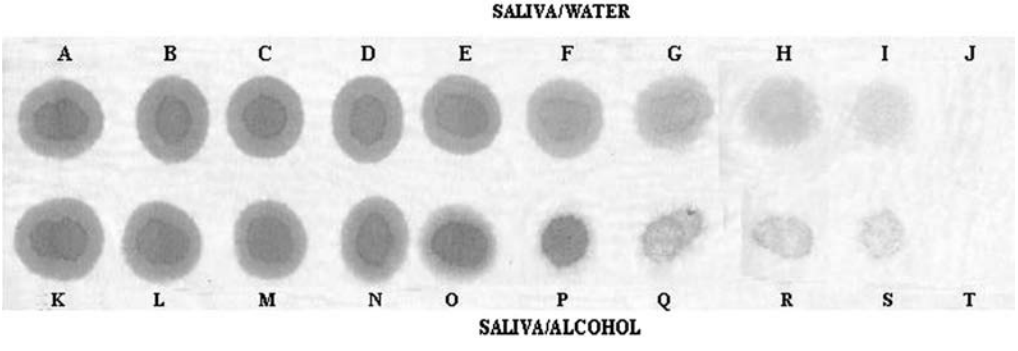

Figure 4. Effect of dilution of saliva by water or ethanol on salivary protein diffusion on cellulose membranes. Five hundred microliters of saliva were mixed with 0, 5, 25, 50, 125, 500, 1000, 2000, or 5000 μL of water (**A**−**I**) or ethanol (**K**−**S**) and incubated at room temperature for 5 min. Fifteen microliters of each mix were placed punctually onto a cellulose membrane and allowed to diffuse. Once dry, the cellulose membrane was dipped into 5% trichloroacetic acid for 5 min and into 0.25% Coomassie blue for 20 min. Finally, the membrane was rinsed with successive changes of 7% acetic acid until clear background. Note that the biphasic mode of diffusion of the salivary protein (**A** and **K**) remains unaltered throughout the whole range of dilutions with water (**B**−**I**) and after dilutions with low concentrations of ethanol (20% v/v or less) (**L**−**O**). At ethanol concentrations of 50% v/v or more, the diffusible fraction of salivary protein fully disappears (**P**−**S**). **J** and **T** correspond to 15-μL aliquots of water and ethanol, respectively.

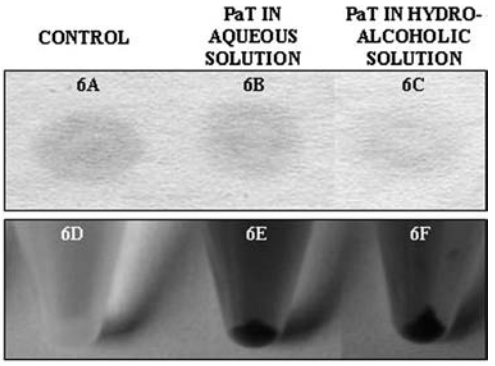

Figure 6. Enhancing effect of ethanol on the proanthocyanidin-induced precipitation of salivary protein. Aqueous and 13% ethanol-containing extracts of proanthocyanidin (PaT) (aqueous solution served as a control) were mixed with aliquots of whole saliva at a 15:1 v/v ratio and incubated for 5 min at room temperature. The mixtures were centrifuged at 3000 g for 5 min to produce sediments (bottom panel) and supernatants. Fifteen-microliter aliquots of the supernatants were placed punctually on a cellulose membrane and allowed to diffuse. The dry membrane was fixed and stained for protein as indicated previously (top panel). In the control condition a minor whitish sediment of salivary material containing exfoliated mouth cells (**6D**) and a significant protein-positive supernatant (**6A**) were observed. In contrast, a major dark sediment (**6E**) together with a still protein-positive supernatant (**6B**) were observed after centrifuging the aqueous PaT/saliva mix. When hydroalcoholic PaT was mixed with saliva, an even major dark sediment (**6F**) together with an almost protein-free supernatant (**6C**) were obtained.

of ethanol affect the interaction between tannins and salivary proteins. To that end, we analyzed the effect of including 13% v/v ethanol in the reaction medium for observing tannin-induced changes in protein diffusion on cellulose membranes. Mixing of whole saliva with proanthocyanidin tannins in a 1:15 v/v ratio in the presence of 13% ethanol resulted in full disappearance of the diffusible protein component of saliva and in keeping maximum aggregation of the non-diffusible salivary component (**Figure 5**, **C**). Likewise, in the presence of 13% ethanol the interaction between hydrolyzable tannins and whole saliva was evidenced by an increased aggregation of the nondiffusible protein component and in a corresponding full disappearance of the diffusible salivary protein component (**Figure 5**, **F**). Altogether, 13% ethanol was found to provoke a significant exacerbation of the antidiffusion effects of both types of enological tannins.

Effect of Enological Tannins on Salivary Protein Precipitation. Interaction between tannins and salivary proteins may result in the formation of soluble and insoluble complexes that may underlie marked tannin-induced alterations in the mode of diffusion of the salivary protein on cellulose membranes, as shown in **Figure 5**. To further substantiate and extend this observation, reaction tubes containing saliva-tannin mixtures in a 1:15 v/v ratio were subjected to centrifugation experiments aimed at simultaneously detecting both salivary protein in the supernatants (protein-dye assay on cellulose membranes) and the eventual occurrence of tannin−salivary protein precipitates (direct visual inspection of the sediments). Thus, a simple dilution of 100 μL of whole saliva with 1500 μL of distilled water followed by centrifugation at 3000 rpm for 5 min produced both a minor, whitish, and mucinous sediment and a supernatant containing a readily visible blue-stained protein material (**Figure 6**, **A** and **D**; **Figure 7**, **A** and **D**). By contrast, mixing a 100-μL aliquot of whole saliva with an aqueous solution of proanthocyanidin tannins in a 1:15 ratio produced a clearly visible dark precipitate as well as a supernatant displaying a still intense positive reaction for protein

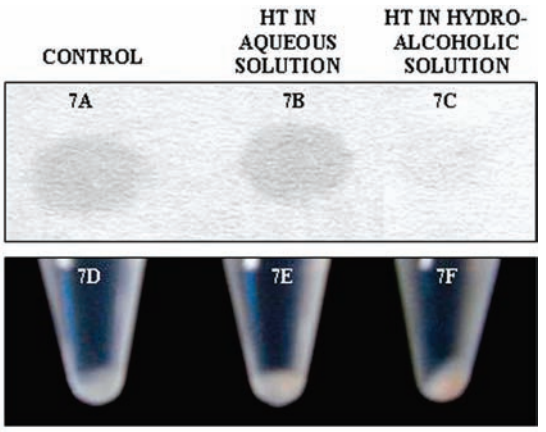

Figure 7. Enhancing effect of ethanol on the hydrolyzable-tannin-induced precipitation of proteins from whole saliva. The experiment is equivalent both in design and in results to the one shown in **Figure 6**, except that the reaction was performed by mixing whole saliva with the hydrolyzable tannin extract. Unprecipitated protein was detected by Coomassie blue staining on cellulose membrane, and the corresponding sediments were examined by direct visual inspection (bottom panel). Note the full disappearance of protein reactivity in the supernatant of the HT−13% ethanol condition (**7C**) compared to the supernatant of the HT−aqueous solution condition (**7B**), which is consistent with the occurrence of a larger precipitate in the hydro-alcoholic reaction medium compared to the one observed in the aqueous medium (**7F** versus **7E**).

on the cellulose membrane (**Figure 6**, **B** and **E**). Likewise, mixing a 100-μL aliquot of whole saliva with an aqueous solution of the extract of hydrolyzable tannins in a 1:15 v/v ratio also produced a somewhat more compact white precipitate as compared to the one observed in the absence of tannins (**Figure 7**, **E**). Under these conditions, a supernatant displaying a readily visible reactivity with the protein-binding dye was also observed (**Figure 7**, **B**).

Exacerbating Effect of Ethanol upon the Tannin-Induced Precipitation of Salivary Proteins. In this study we analyzed the effect of including 13% v/v ethanol in the reaction medium for the assessment of tannin-dependent salivary protein precipitation. Mixing of 100 μL of whole saliva with proanthocyanidin tannins in a 1:15 v/v ratio in the presence of 13% ethanol results in a significantly bigger dark precipitate compared to the precipitation observed in the absence of ethanol (**Figure 6**, **F**). In this assay, precipitation of the salivary protein by proanthocyanidin tannins in the presence of 13% ethanol provoked the almost full depletion of the protein-dye reacting material from the corresponding supernatant (**Figure 6**, **C**). In a parallel assay with the extract of hydrolyzable tannins, we also observed an increased protein−tannin precipitation as well as an almost complete protein loss from the corresponding supernatant (**Figure 7**, **C** and **F**). Altogether, 13% ethanol provoked a significant boost of the precipitating effect of salivary proteins by both types of enological tannins.

DISCUSSION

Astringency, a complex tactile sensation of dryness and roughness of the oral surfaces, has been attributed to interactions of polyphenolic compounds with particular protein components of the salivary fluid (*1*, *24*−*26*). Studies aiming to find quantitative parameters for astringency have associated that sensation with the ability of polyphenols to precipitate a number of nonhuman animal proteins, particularly gelatins and, more recently, serum albumin (*3*−*8*). Since the ability of polyphenols to precipitate different proteins varies considerably, associations between astringency

and precipitation of particular proteins have proven to be somewhat controversial. However, saliva is the first physical contact of polyphenols with a mouth structure just before astringency is perceived. On that premise, two highly diverse families of salivary proteins, namely, histatins and proline-rich proteins, have been recurrently mentioned in the past few years as single molecular targets for polyphenols causing astringency (*11*, *27*). A role of other individual salivary proteins in polyphenol-activated astringency mechanisms has not been discarded (*11*, *27*−*29*). Recently, it has been shown that human whole saliva is constituted by protein fractions displaying either a diffusible or a nondiffusible character on an absorbing cellulose membrane (*19*). In effect, when an aliquot of saliva is placed as a sharp spot on a cellulose membrane, a nondiffusible salivary protein fraction remains close to the place where the aliquot was spotted, whereas a freely diffusible component moves radially outward in all directions together with the salivary water. This "biphasic" distribution of the protein component of saliva on the cellulose membrane, which can be evidenced by staining with a selective protein-binding dye (*19*), may be altered, for instance, when new molecular interactions give rise to less diffusible supramolecular complexes (*30*). On that basis, we investigated whether enological tannins affected the mode of diffusion of the salivary protein on a cellulose membrane as an indirect way to detect protein−tannin interactions. In a more conventional complementary assay serving the same purpose of detecting tannin−salivary protein complexation, we also assayed the ability of tannin extracts to precipitate salivary protein. Data obtained from these *in vitro* assays on tannin−salivary protein interactions were contrasted against those from in *vivo* sensory assessments of astringency carried out by a trained panel. The experimental design of the study comprised a couple of main distinctive characteristics that in our view are essential for a proper contrast between the *in vitro* and *in vivo* observations. First, we assayed salivary protein−tannin interactions by mixing salivary fluid with tannin solutions in a 1:15 v/v ratio in order to reproduce the degree of saliva dilution occurring during a normal degustation of a liquid fluid (e.g., wine) in which around 15 mL of the fluid become thoroughly mixed with approximately 1 mL of whole saliva in the mouth of the panelist (*31*, *32*). Interestingly, after that dilution in water, saliva preserved its biphasic mode of protein diffusion on cellulose membranes.

A second characteristic of the present study was the comparative use of two different enological tannins considering that they may differ significantly in regard to the astringency they produce (*32*). HPLC-DAD fractionation and UV spectroscopy together with a general chemical characterization showed that one of the enological tannins was composed solely by gallotannins whereas the other consisted only of nonhydrolyzable proanthocyanidins. The proanthocyanidin tannin was rated clearly more astringent than the hydrolyzable tannin by a trained sensory panel. Altogether, both main features of the study design in reference to the assessment of *in vitro* interactions between tannins and salivary proteins represent experimental conditions contributing to a closer correspondence with any independent assessment of astringency by a trained sensory panel. Under these study conditions the aqueous extracts of both the hydrolyzable tannin and the proanthocyanidin tannin displayed a significant reactivity with the salivary protein. That conclusion was supported by both methodological approaches used in this study, namely, by a tannin-dependent increased aggregation of the nondiffusible salivary protein fraction on an absorbing cellulose membrane (and a decrease in the corresponding diffusible salivary protein fraction), as well as by the appearance of tannin−protein sediments (together with a concomitant diminution of the salivary protein present in the corresponding supernatants) obtained after centrifugation of the tannin plus saliva mixtures.

On the basis of these observations, we then studied the effect of ethanol on both tannin-induced astringency and tannin−salivary protein interactions. As another main feature of the study we routinely studied those effects at a constant concentration of 13% ethanol, that is, a condition usually observed in wine composition. Under these conditions, 13% ethanol provoked a significant increase in the perception of astringency produced either by the proanthocyanidinic tannin or by the hydrolyzable tannin. On the other hand, in a dilution study we showed that the mode of diffusion displayed by the salivary protein fraction on cellulose membranes was markedly affected only by ethanol concentrations of over 20% v/v. That observation was consistent with the well-known protein precipitant character of ethanol (*33*). In accordance with the same dilution study, 13% ethanol did not produce any effect on the mode of salivary protein diffusion on cellulose membranes. However, at variance with the aqueous environment, 13% ethanol did produce an unequivocal enhancement of the interactions between given concentrations of tannins and salivary proteins on the basis of all objective indicators used in this study. In effect, upon mixing saliva with tannins in the presence of 13% ethanol, we observed a full disappearance of both the diffusible salivary protein fraction on cellulose membranes and the salivary protein in the supernatants obtained after centrifugation of tannin/saliva mixtures. In addition, the corresponding sediments of tannin/salivary protein complexes obtained in the presence of 13% ethanol appeared to be markedly bigger than those obtained in parallel by mixing tannins with saliva in the absence of ethanol. All of these exacerbating effects of 13% ethanol on tannin/salivary protein interactions, as well as on perceived astringency, were observed regardless the type of tannin used in this study. Altogether, these observations strongly suggest that for a proper sensory assessment of the astringency produced by tannins and for a proper analysis of molecular mechanisms underlying such a complex oral sensation, including salivary protein−tannin interactions, ethanol should be considered as part of the experimental conditions.

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