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Interactions of enological tannins with the protein fraction of saliva and astringency perception are affected by pH

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ABSTRACT

The effects of pH on both tannin-induced astringency and tannin–salivary protein interactions were investigated. A trained sensory panel evaluated astringency perception. Tannin–salivary protein interactions were assessed *in vitro* by examining the effects of either a condensed enological tannin or an hydrolyzable enological tannin on two physicochemical properties of the protein fraction of saliva, namely, its mode of diffusion on cellulose membranes and its precipitation. Comparative assays mimicking the degree of dilution experienced by saliva during a tasting assay were performed at pH 3.5 and pH 7.0. Results indicated that both enological tannins were perceived as clearly more astringent at pH 3.5 compared with pH 7.0. In addition, the effects of tannins on protein diffusion and protein precipitation were markedly exacerbated at pH 3.5.

1. Introduction

Phenolic compounds constitute one of the most important quality parameters of wines and other foods since they contribute to a variety of organoleptic characteristics, such as color, aroma, bitterness and astringency (Monagas, Bartolomé, & Gómez-Cordovés, 2005). Astringency, a sensation that is described as a puckering, rough, or drying mouth-feel, has been associated with interactions between some phenolic compounds (tannins) and salivary proteins (Bacon & Rhodes, 2000). Tannins responsible for wine astringency consist mostly of flavan-3-ol polymers that are commonly referred to as proanthocyanidins or condensed tannins (De Freitas & Mateus, 2001). These compounds are extracted from grape skins and seeds during various phases of wine-making (Obreque-Slier, Peña-Neira, López-Solís, Zamora-Marín, et al., 2010; Ribéreau-Gayon, 1972). Although astringency is considered to be a tactile sensation rather than a taste (Breslin, Gilmore, Beauchamp, & Green, 1993), its perception is markedly affected when overlapped with taste stimuli (Kallithraka, Bakker, & Clifford, 1997). A number of physical and chemical properties have been involved in the complex mechanisms of astringency perception. In addition to the concentration and composition of proanthocyanidins (Vidal et al., 2004), both organic and inorganic acids (Hartwig & McDaniel, 1995), ethanol (DeMiglio, Pickering, & Reynolds, 2002; Fischer & Noble, 1994; Obreque-Slier, Peña-Neira, & López-Solís, 2010), sweetness (Smith, June, & Noble, 1996), viscosity (Peleg & Noble, 1999), some minerals (Lawless et al., 2004) and pH (Fischer & Noble, 1994; Kallithraka et al., 1997; Lawless, Horne, & Giasi, 1996; Sowalsky & Noble, 1998) have also been shown to contribute to astringency perception.

A number of authors have studied the effect of pH on astringency perception. Fischer and Noble (1994), observed that dealcoholized white wine at pH 3.0 was perceived more astringent than the same wine at pH 3.6. Similar results were obtained for red wines across a series of pH between 2.2 and 2.8 (DeMiglio et al., 2002) and by using different acids (Kallithraka et al., 1997). Guinard, Pangborn, and Lewis (1986) did not observe such a relationship between pH and astringency in high-phenol red wines subjected to progressive acidification. However, these latter authors suggested that high levels of astringency in red wines may have resulted in precipitation of salivary proteins at the start of the assay thus preventing a change in astringency perception by the addition of acids. Also, astringency perception produced by aqueous solutions of phenolic compounds (i.e. grape seed tannins, tannic acid, catechin, gallic acid) and by a number of model solutions have been shown to be affected by a decrease in pH (Guinard et al., 1986; Kallithraka et al., 1997; Peleg, Bodine, & Noble, 1998). From a molecular perspective, the increased intensity of perceived astringency at lower pH has been tentatively related to an increase in undissociated phenol groups, which may form hydrogen bonds

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with salivary proteins (Guinard et al., 1986; Sowalsky & Noble, 1998). On the other hand, Peleg and Noble (1999) reported that astringency of alums is decreased by the addition of acids and invoked that because of chelation by organic acids the interaction of aluminum ions with salivary proteins is reduced. Anyhow, most of these studies oriented to assess the impact of pH on astringency have been restricted to or primarily based on sensory evaluation by a group of wine taster experts (Valentova, Skrovánková, Panovska, & Pokorny, 2002). Significant efforts have been made to find more objective parameters to describe or to measure astringency (Llaudy et al., 2004; Obreque-Slier, Mateluna, Peña-Neira, & López-Solís, 2010).

The present study was aimed at conducting a parallel assessment of the effect of pH 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 pH in the wine–saliva mixture in mouth (Lagerlöf & Dawes, 1984; Müller et al., 2010). We examined the effect of two different enological tannins, a condensed enological tannin and a hydrolyzable enological tannin, on two physicochemical properties of the salivary protein, namely, the mode of diffusion on cellulose membranes and precipitation (López-Cisternas, Castillo-Díaz, Traipe-Castro, & López-Solís, 2007; Obreque-Slier, Peña-Neira, & López-Solís, 2010; Obreque-Slier, Mateluna, et al., 2010).

2. Materials and methods

2.1. Materials

A hydrolyzable enological tannin (Tanin Gallique a l'alcohol) and a condensed enological tannin (Protanin R-instantáneo) were obtained as kind gifts from Vinicas Industry Co., Santiago, Chile. Cellulose membranes (Whatman # 1) were purchased from Whatman Ltd., Maidstone, England. Tartaric acid, gallic acid, (+)-catechin and Coomassie blue were from Sigma Chemical Company, Saint Louis, Missouri, USA. HPLC grade acetic acid and acetonitrile were purchased from Merck, Darmstadt, Germany. Pro-analysis solvents were obtained from Oxiquim-Chile.

2.2. Tannin extract solutions

Polyphenol compositions of both the hydrolyzable enological tannin and the condensed enological tannin were thoroughly characterized as described elsewhere (Obreque-Slier, Peña-Neira, & López-Solís, 2010). Both the gallotannin-rich enological tannin (47.7 mg g^{-1} gallic acid equivalent) and the proanthocyanidin-rich enological tannin (29.3 mg g^{-1} catechin equivalent) were dissolved (6 mg mL^{-1}) under agitation for 20 min at 20 °C in 5 g L^{-1} tartaric acid. Each tannin solution was divided in two halves and the pH of each subfraction was adjusted with sodium hydroxide either to pH 3.5 or pH 7.0. Just prior to the experiments, concentrations of all the four solutions of tannins were normalized by dilution with distilled water to an absorbance at 280 nm of 0.40. Concentrations of the resulting solutions corresponded to 1 mg mL^{-1} (hydrolyzable tannin) or 4.2 mg mL^{-1} (condensed tannin).

2.3. Sensory evaluation

All the four tannin solutions were rated for astringency by a 13-member trained sensory panel (7 men, 6 women; age range 34–56) whose training was performed according to Cross, Moen, and Stanfield (1978). Assessment of the hydrolyzable tannin was independent from that of condensed tannin. 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 5 g L^{-1} of tartaric acid both at pH 3.5 and pH 7.0 served as controls. Pectin dissolved in distilled water (1 g L^{-1}) was used for mouth rinsing between consecutive samples.

2.4. Whole saliva collection

A single 31-year-old healthy male with no evidence of illness in the past 60 days and with values of serum biochemical profile, hemogram and urine analysis within reference ranges, was a permanent voluntary saliva donor throughout the study. A conventional procedure for collection of saliva with no use of sialagogues (unstimulated whole saliva) was carried out under standardized conditions always between 9.00 and 10.00 AM and just before each experiment (Nordbö, Darwish, & Bhatnagar, 1984). Briefly, saliva was accumulated in mouth during 1 min and then expectorated into a sterile glass container. Saliva collected in three successive procedures was pooled and maintained in ice during the experiment.

2.5. Salivary protein–tannin complexation

Salivary protein–tannin interactions were assessed using a recently reported protein diffusion assay and a precipitation assay (López-Cisternas et al., 2007; Obreque-Slier, Mateluna, et al., 2010; Obreque-Slier, Peña-Neira, et al., 2010), with the following modifications.

2.5.1. Diffusion assay

One-hundred-microliters of a fresh sample of whole saliva were mixed with 1500-μL aliquots of pH 3.5 and pH 7.0 solutions of either condensed (PaT) or hydrolyzable tannins (HT). Saliva mixed with either pH 3.5 water or pH 7.0 water in a ratio of 1:15 served as controls. After incubation for 5 min at room temperature, 15-μL aliquots of the mixtures were dotted on a cellulose membrane and allowed to diffuse. The dry membrane was fixed in 50 g L^{-1} trichloroacetic acid, rinsed in 800 mL L^{-1} ethanol and stained for protein with Coomassie blue for 20 min, destained with several rinses of 73 g L^{-1} acetic acid and dried under a heat lamp. Both diffusion area and stain intensity of the protein spots were semi-qualitative estimates for protein–tannin interaction.

2.5.2. Precipitation assay

The rest of the whole saliva–tannin extract mixtures of the diffusion assay were centrifuged at 750× gravitational force (G) for 5 min in a Sorvall microcentrifuge. Fifteen-μL aliquots of each supernatant were dotted on a cellulose membrane, allowed to diffuse and processed for protein staining, as indicated above. In this latter assay, reduced protein staining was taken as indicative of protein precipitation. This observation was complemented by a direct visual inspection of the centrifuge tubes.

3. Results

3.1. Sensory analysis

Both the condensed tannin extract and the hydrolyzable tannin extract, each of them at pH 3.5 and 7.0, were assayed for astringency by using a descriptive test in two independent successive sessions. As shown in Table 1, both pH 3.5 tannin extracts were recognized by the trained sensory panelists as being significantly

Table 1
Intensity of perceived astringency of enological tannin solutions as a function of pH.

	pH 3.5	pH 7.0
Condensed tannin	10.4 ± 1.2	6.9 ± 0.8*
Hydrolyzable tannin	8.0 ± 1.1	5.1 ± 1.0*

Sensory evaluation as described under Materials and methods. Figures represent mean ± standard deviation of duplicate scores (0 = no astringency; 15 = maximum astringency) by a 13-member sensory panel. Asterisks indicate significant difference (Tukey test, $p < 0.05$) between scores for each tannin at different pH.

more astringent compared to the corresponding pH 7.0 tannin extracts (ANOVA, $p < 0.05$).

3.2. Diffusion of salivary protein on cellulose membranes

When an aliquot of whole saliva is dotted 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 (non-diffusible fraction of salivary protein) which becomes surrounded by a weaker blue-stained outer band (diffusible fraction of salivary protein) (Fig. 1). Aiming to analyze whether tannin solutions at pH 3.5 and 7.0 affected diffusion of salivary proteins on the cellulose membranes we firstly examined any eventual effect of the corresponding solvents on that parameter. To that end, saliva was diluted 1:15 volume ratio with either pH 3.5 water or pH 7.0 water. Dilution of saliva with pH 7.0 water did result in no major effect upon the biphasic mode of salivary protein diffusion on cellulose membranes, excepting the expected decrease in the intensity of protein staining (Fig. 2A). By contrast, dilution of saliva with pH 3.5 water produced a significant anti-diffusive effect on the diffusible salivary protein fraction together with an also significant condensing effect of the non-diffusible salivary protein fraction (Fig. 2B).

3.3. pH-dependence of the effect of both enological tannins upon salivary protein diffusion on cellulose membranes

Considering the different effect of diluting saliva with pH 3.5 water and 7.0 water on the biphasic mode of diffusion of the salivary protein we then analyzed the effect of mixing saliva with tannin solutions at either pH 3.5 or pH 7.0. Mixing of whole saliva with an aqueous solution of the condensed tannin at pH 7.0 in a 1:15 volume ratio followed by spotting of an aliquot of the mixture onto a cellulose membrane resulted in a typical biphasic mode of diffusion, that is, a diffusible protein fraction and a more

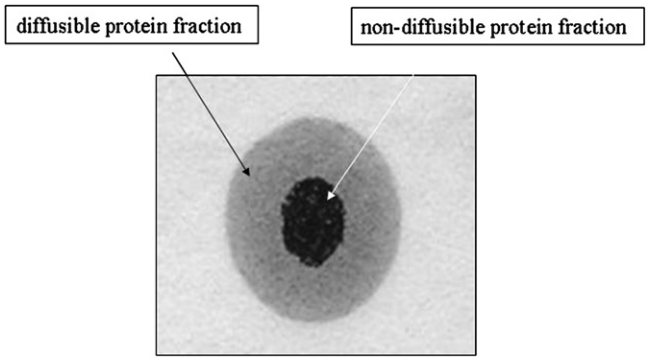

Fig. 1. Mode of diffusion of the salivary protein fraction on cellulose membranes.

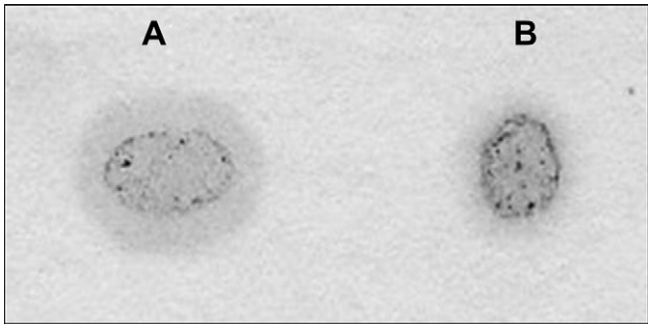

Fig. 2. Effect of pH on the mode of diffusion of the protein fraction of diluted whole saliva on cellulose membranes. (A) saliva diluted with pH 7.0 water, and (B) saliva diluted with pH 3.5 water.

intensely stained non-diffusible protein fraction (Fig. 3A). By contrast, mixing of an aliquot of whole saliva with an aqueous solution of the condensed tannin at pH 3.5 in a 1:15 volume ratio produced a dramatic aggregation of the non-diffusible salivary component and a marked decrease in the diffusible protein component (Fig. 3B). Likewise, mixing saliva with an aqueous solution of hydrolyzable tannin at pH 7.0 in the same 1:15 volume ratio did not affect the biphasic mode of salivary protein diffusion whereas mixing saliva with an aqueous solution of the same hydrolyzable tannin at pH 3.5 resulted in a marked aggregation of the non-diffusible salivary protein fraction together with a complete disappearance of the diffusible salivary protein fraction (Fig. 3C and D).

3.4. pH-dependence of the effect of both enological tannins upon salivary protein precipitation

Interactions between tannins and salivary proteins result in the formation of soluble and insoluble complexes that may underlie the above-described tannin-induced alterations in the mode of diffusion of the salivary protein on cellulose membranes. In order to

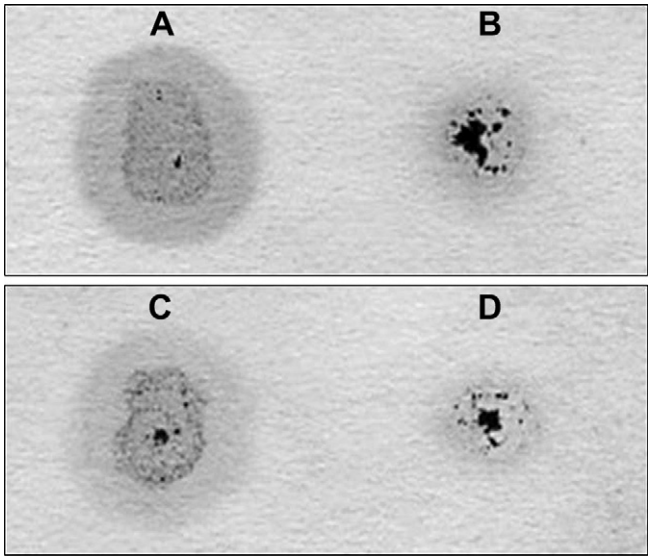

Fig. 3. Enhancing effect of low pH on the tannin-induced inhibition of salivary protein diffusion on cellulose membranes. Solutions of either condensed tannin (A, B) or hydrolyzable tannin (C, D) both at pH 7.0 (A, C) and pH 3.5 (B, D) were mixed with whole saliva and analyzed for protein diffusion on cellulose membranes and for protein–tannin precipitation.

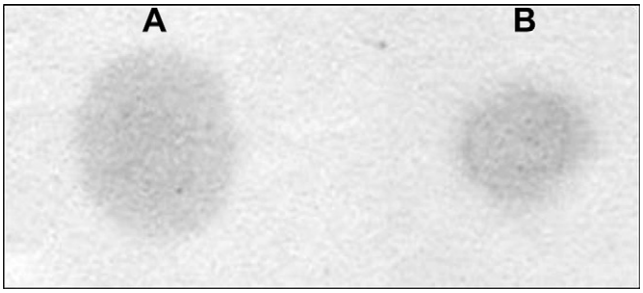

Fig. 4. Soluble protein in diluted whole saliva at pH 7 and 3.5. The experiment is similar to the one described in Fig. 3, except that after dilution with either pH 7.0 or pH 3.5 water, saliva was centrifuged and aliquots of the supernatants were analyzed for protein on cellulose membranes.

further substantiate and extend this observation, reaction tubes containing saliva–tannin mixtures in a 1:15 volume 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). Simple dilution of 100 μL of whole saliva with 1500 μL of water at pH 7 followed by centrifugation at 750×G for 5 min produced a supernatant whose assessment on a cellulose membrane showed a readily visible and mostly monophasically distributed blue-stained protein material (Fig. 4A). Under these conditions a minor, whitish, cell-rich and mucinous sediment could be also observed (unshown). When the procedure was performed by diluting saliva with water at pH 3.5, the supernatant was also markedly positive for protein but diffusion of the salivary protein component on the cellulose membrane was significantly reduced (Fig. 4B). No differences could be appreciated in the corresponding sediment (unshown). By contrast, mixing whole saliva with an aqueous solution of the condensed tannin at pH 7.0 in a 1:15 volume ratio followed by centrifugation produced a supernatant displaying an intense positive reaction for protein on the cellulose membrane (Fig. 5A) together with a clearly visible dark precipitate comprising insoluble proanthocyanidin–salivary protein complexes (Fig. 5C). When whole saliva was mixed with an aqueous solution of the condensed tannin at pH 3.5 and processed as before, the supernatant showed a weak reaction when probed for protein on the cellulose membrane (Fig. 5B). Under these conditions, a big dark sediment of insoluble proanthocyanidin–salivary protein complexes was observed (Fig. 5D).

In parallel assays, whole saliva was mixed with aqueous solutions of the hydrolyzable tannin both at pH 7.0 and pH 3.5. At pH 7.0 the supernatant was clearly positive for protein (Fig. 5E) whereas at pH 3.5 the absence of protein reactivity was indicative of its complete precipitation as hydrolyzable tannin–salivary protein complexes (Fig. 5F). In effect, a somewhat bigger and darker gray precipitate was observed at pH 3.5 compared with the one observed at pH 7.0 (Fig. 5G and H). Altogether, these observations suggest that precipitation occurring after interaction of both hydrolyzable and condensed tannins with the protein fraction of saliva is also greatly enhanced at pH 3.5 compared to 7.0.

4. Discussion

Saliva is the first physical contact of polyphenols with a mouth structure just before astringency is perceived. Two highly diverse families of salivary proteins, namely, histidine-rich and proline-rich proteins, have been recurrently mentioned in the past few years as part of a frontline for polyphenol neutralization in the upper part of the gastrointestinal tract (Bacon & Rhodes, 2000; Lu & Bennick, 1998). Interaction of other salivary proteins with polyphenols has not been discarded (Gawel, 1998; Nautaro, Wong, Lu, Wroblewski, & Bennick, 1999). In addition, all of those interactions would underlie astringency perception. As a sort of corollary to this statement, local conditions in the mouth that may affect polyphenol–tannin interactions during degustation, such as pH, ethanol, sugars and polysaccharides, would play a major influence on astringency perception (DeMiglio et al., 2002; Fischer & Noble, 1994; Guinard et al., 1986; Hartwig & McDaniel, 1995; Kallithraka et al., 1997; Lawless et al., 1996; Lawless et al., 2004; Peleg & Noble, 1999; Peleg et al., 1998; Sowalsky & Noble, 1998; Vidal et al., 2004).

In the present study we assessed the effect of pH on the ability of two enological tannins to interact *in vitro* with salivary proteins as well as on the ability of a trained sensory panel to score the astringency those tannins provoke. We used two independent phenomena as indicative of interaction between tannins and the protein fraction of saliva, namely, the restrictive effect of tannins on protein diffusion on cellulose membranes and salivary protein

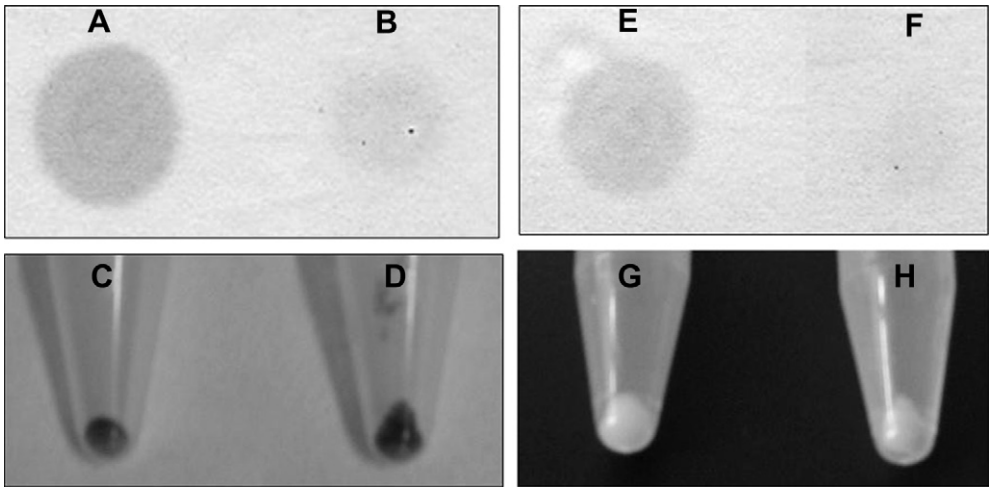

Fig. 5. Enhancing effect of low pH on the tannin-induced precipitation of salivary protein. Solutions of either condensed tannin (A through D) or hydrolyzable tannin (E through H) both at pH 7.0 (A, C, E and G) and pH 3.5 (B, D, F and H) were mixed with whole saliva and centrifuged to produce sediments (bottom panels). In addition, aliquots of the supernatants were spotted onto cellulose membranes for protein detection as indicated in Materials and methods (top panels).

precipitation (López-Cisternas et al., 2007; Obreque-Slier, Mateluna, et al., 2010; Obreque-Slier, Peña-Neira, & López-Solís, 2010). In this study, we used normalized solutions of a gallotannin-rich hydrolyzable tannin and a condensed tannin, both of which had been previously characterized by HPLC chromatography and spectral analysis (Obreque-Slier, Peña-Neira, & López-Solís, 2010; Obreque-Slier, Peña-Neira, López-Solís, Ramírez-Escudero, & Zamora-Marín, 2009). Tannin concentrations in these experiments are part of the range of concentrations that can be assessed for astringency perception by a trained panel. Also, those concentrations are usually in the range of those obtained during the preparation of tannin extracts from grape seeds and skins (Obreque-Slier, Peña-Neira, López-Solís, et al., 2010; Pérez-Magariño & González-San José, 2006). Another distinctive characteristic of our experimental design, which in our view is essential for a proper contrast between the *in vitro* and *in vivo* observations, was the assay of salivary protein–tannin interaction by mixing saliva with tannin solutions in a 1:15 volume ratio (Obreque-Slier, Peña-Neira, & López-Solís, 2010). Such a ratio reproduces the degree of saliva dilution occurring during a wine degustation. In that circumstance, around 15 mL of the beverage become thoroughly mixed with approximately 1 mL of whole saliva in the mouth of the panelist (Lagerlöf & Dawes, 1984; Müller et al., 2010). Under both considerations, in this study a trained sensory panel reported that both the condensed tannin and the hydrolyzable tannin were perceived as clearly more astringent at pH 3.5 compared with pH 7.0. A transition between those pHs may result in significant differences both in the degree of dissociation of carboxyl groups of aspartic acid and glutamic acid side chains and in the imidazole-imidazolium ring of histidine residues of salivary proteins as well as in the degree of dissociation of the carboxyl groups of complex polyphenols, such as those tannins containing gallic acid or gallate residues (Gawel, 1998; Monagas et al., 2005). Thus, significant pH-dependent changes in the electrical charge or in the structure of hydrogen bond forming sites of those potentially interacting molecules may explain marked changes in the ability of tannins and salivary proteins to interact with each other and thence in producing astringent sensations.

In the present study we investigated the ability of two different enological tannins to interact with salivary proteins at the "physiological" pH 7 and at the "enological" pH 3.5. To this aim, we assessed whether those enological tannins affected in a pH-dependent fashion the mode of diffusion of salivary proteins on a cellulose membrane and, in a more conventional complementary assay, whether those tannin extracts affected, also in a pH-dependent fashion, the solubility of the salivary protein fraction. Control conditions of these experiments considered a conventional 1:15 volume ratio of saliva with either pH 3.5 water or pH 7.0 water, as corresponding. Both analytical approaches showed unequivocally a significant reactivity between the aqueous extracts of tannins and the salivary protein fraction, that is, a tannin-dependent increased aggregation of the non-diffusible protein fraction (and a decrease in the diffusible protein fraction) on an absorbing cellulose membrane and a tannin-induced precipitation of salivary protein. All those effects were clearly exacerbated at pH 3.5 on the basis of several objective indicators. Thus, upon mixing saliva with tannins at pH 3.5 diffusion of the diffusible salivary protein was markedly restricted, salivary protein mostly disappeared from the supernatants obtained after centrifugation of tannin/saliva mixtures and the corresponding sediments of tannin/salivary protein complexes were bulkier than those observed by mixing tannins with saliva at pH 7.0. All these exacerbating effects of pH 3.5 were observed regardless the type of tannin used in this study. This observation, which fully agrees with a previous report from other laboratory using the same pH conditions but a different analytical approach and different tannin extracts (Lawless et al., 1996), strongly suggests that salivary proteins may be primary targets of the pH condition affecting their interaction with tannins. Regardless the molecular mechanism by which pH affects salivary protein–tannin interactions, astringency might also be a pH-dependent sensory perception. In this study we observed that the intensity of astringency perceived by a trained sensory panel at pH 3.5 was significantly higher than at pH 7.0. Previous reports from other laboratories have also shown an increased astringency perception at lower pH (DeMiglio et al., 2002; Fischer & Noble, 1994). Altogether, these observations strongly suggest that for a proper sensory assessment of the astringency produced by tannins and for a proper assessment of salivary protein–tannin interactions, pH should be considered a relevant experimental parameter.

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