

Turbidity as a Measure of Salivary Protein Reactions with Astringent Substances

John Horne, John Hayes and Harry T. Lawless

Department of Food Science, Cornell University, Ithaca, NY 14853, USA

Correspondence to be sent to: Harry Lawless, Department of Food Science, Stocking Hall, Cornell University, Ithaca, NY 14853, USA.
e-mail: htl1@cornell.edu

Abstract

Binding of tannins to proline-rich proteins has been proposed as an initial step in the development of astringent sensations. In beer and fruit juices, formation of tannin–protein complexes leads to the well-known effect of haze development or turbidity. Two experiments examined the development of turbidity in human saliva when mixed with tannins as a potential *in vitro* correlate of astringent sensations. In the first study, haze was measured in filtered human saliva mixed with a range of tannic acid concentrations known to produce supra-threshold psychophysical responses. The second study examined relationships among individual differences in haze development and the magnitude of astringency ratings. Mostly negative correlations were found, consistent with the notion that high levels of salivary proteins protect oral tissues from the drying effects of tannic acid.

Introduction

Oral astringency is a complex group of sensations involving dryness, roughness of oral surfaces and tightening, drawing or puckering sensations of the mucosa and muscles around the mouth (Lee and Lawless, 1991; Corrigan Thomas and Lawless, 1996; Gawel *et al.*, 2000). Astringency may also involve the classical taste pathways, as demonstrated by recordings from taste nerves (Schiffman *et al.*, 1992; Critchley and Rolls, 1996). However, much of what human observers report is tactile in nature and is perceived in areas of the mouth innervated only by trigeminal nerve endings, a ‘diffuse sensation’ in the words of Bate Smith (Bate Smith, 1954; Breslin *et al.*, 1993). Astringency is produced by a variety of oral chemical stimuli, including tannins and other polyphenols (Gawel, 1998), tannin substituents such as catechins (Thorngate and Noble, 1995), acids (Rubico and McDaniel, 1992; Hartwig and McDaniel, 1995; Lawless *et al.*, 1996) and aluminum salts (Lee and Lawless, 1991).

Polyphenolic compounds such as tannins form complexes with salivary proteins and mucopolysaccharides. Protein–tannin complexes result in the precipitation and/or aggregation of salivary proteins causing them to lose their lubricating properties. These interactions have led to a theory that astringency is at least initially due to delubrication via removal of the slippery coating on oral surfaces. Thus the mouth feels rough, tightened and dry. Support for the delubrication theory comes from recent work showing increases in coefficients of friction when tannins are added to saliva (Prinz and Lucas, 2000). Thorngate and Noble (Thorngate and Noble, 1995) pointed out that mechano-

receptors are likely involved in some of the astringent sensations, which seems reasonable given the movement involved in appreciating sensations of roughness or dryness.

A large family of proline-rich proteins (PRPs) is found in human saliva (Hay and Oppenheim, 1974; Kaufman and Keller, 1979) that are thought to serve functions of wetting, lubrication and protection of the oral epithelium. Due to the disruption of α -helical structures by proline and exposed sites such as the carbonyl groups in the keto-imide linkages, the PRPs provide a good substrate for the formation of hydrogen bonds with tannins and for hydrophobic interactions (McManus *et al.*, 1981; Murray *et al.*, 1994). Animals on high tannin diets may produce increased levels of salivary proteins, which may in turn modulate their sensory responses to astringency (Glendinning, 1992). This increase in secretions might represent a protective mechanism since high concentrations of tannins have deleterious effects on nutrient uptake (Mehansho *et al.*, 1983; Kim, 1994). However, Prinz and Lucas (Prinz and Lucas, 2000) proposed that the increased friction and its related sensations of dryness and roughness may serve as a sensory warning cue to foraging animals to avoid plant substances that are high in tannins.

Repeated stimulation with astringent tannins shows a pattern of partial recovery and build-up of intensity in a stepwise manner (Guinard *et al.*, 1986). From this perspective, astringency may be viewed as a kind of ‘temporarily induced xerostomia’ caused by delubrication of oral tissues. Salivary flow rate has recently been reported to modulate

astringent reactions (Fischer *et al.*, 1994; Ishikawa and Noble, 1995). These studies showed slower onset and slower decay of astringent sensations among groups with low salivary flow rates. Support for the involvement of salivary proteins in astringency comes from recent work by Kallikathraka *et al.* (Kallikathraka *et al.*, 1998), showing changes in the chromatographic profile of salivary constituents following stimulation with tannins, possibly due to formation of protein–tannin complexes. Further work by this group showed correlations between the time parameters of astringent sensations and specific peaks in a salivary chromatogram (Kallikathraka *et al.*, 2001). Direct action of astringents on the oral epithelium and/or taste receptors is also possible.

Recent publications in food chemistry bring to light a surprising parallel between the salivary binding/delubrication hypothesis for astringency and the association of polyphenols and proteins to form ‘chill haze’ in cider, beer and other beverages (McMurrrough *et al.*, 1992). PRPs, particularly hordein, a barley protein, bind with polyphenols to produce turbidity (Asano *et al.*, 1982). This reaction is largely specific to PRPs. Both hydrogen bonding and hydrophobic interactions are likely mechanisms for these associations (Asano *et al.*, 1982; Siebert *et al.*, 1996). A model of polyphenol–protein crosslinking, similar to that proposed for tannin binding to oral proteins (McManus *et al.*, 1981; Haslam and Lilley, 1988), was recently proposed for chill haze (Siebert *et al.*, 1996).

This literature substantiates the possibility that binding of tannins to PRPs is a likely mechanism contributing to astringent sensations. Protein-binding assays such as simple turbidity measurements may be useful as a measure of tannin–protein association in the saliva of experimental subjects who are simultaneously generating psychophysical data. Imm and Lawless (1996) found lowered astringency responses amongst individuals with higher overall protein content. However, establishing relationships between salivary protein content and perceived astringency has not always met with success. Kallikathraka *et al.* (Kallikathraka *et al.*, 2001) found no relationship between total protein and astringency response and Guinard *et al.* (Guinard *et al.*, 1998) found no relationship between parotid protein content and astringency. However, Kallikathraka *et al.* did find significant correlations between the magnitude of specific protein fractions and astringency. An assay that mimics the type of chemical interactions that occur between salivary proteins and tannins might provide a better correlate with sensory responses. Based on the literature from beverage research on chill haze, the development of turbidity in saliva–tannin mixtures could be such a correlate. A preliminary report from our laboratory showed development of haze in saliva–tannic acid mixtures over a range of tannic acid concentrations known to elicit astringent sensations (Lawless *et al.*, 1999). The studies reported here follow up with a larger group.

The objective of the first study was to determine whether human saliva would show measurable haze development over a range of concentrations known to be astringent. A second experiment examined whether individuals with varying haze development would co-vary in their astringency responses. Such a result would implicate salivary proteins as a contributor or modulator of astringent sensations. Flow rate was also examined as a potential correlate of astringency. Subjects with a higher resting rate of salivary flow would be expected to have increased levels of salivary protein available. Increased levels of protein might have a protective coating effect, reducing astringency, and thus a negative correlation between resting flow and astringency as well as a negative relationship between haze and astringency.

Experiment 1. Instrumental measurement of salivary turbidity

Methods

Subjects were 25 volunteers from the Cornell University campus in Ithaca, NY (13 females, 12 males, age range 18–56 years, age median 24 years). Subjects were paid a token cash incentive for their participation.

Whole-mouth saliva was collected by having each panelist expectorate into a 60 ml cup. Panelists were permitted to chew on a square of Parafilm in order to evoke saliva. Panelists reported not having consumed any food or drink other than water for at least 1 h before saliva collection. Panelists were instructed to provide 30–35 g of saliva and had access to a balance to monitor their progress. Saliva from each panelist was individually filtered in a vacuum (2 mm Hg) through Whatman no. 1 filter paper. Aliquots ranging from 20 to 31 ml (median 26 ml) were obtained and stored in individual sterile screw-top vials. All saliva was used and discarded within 48 h of collection. Saliva held overnight refrigerated at 4°C was allowed to equilibrate to room temperature (~20°C) before analysis. Four milliliters of saliva from each individual was combined with 4 ml of tannic acid solution [lot 93H0269, formula weight (FW) 1701.18; Sigma, St Louis, MO] at the following concentrations: 0, 0.5, 0.89 and 1.58 g/ml. All solutions were placed in capped 10 ml clear borosilicate glass test tubes and mixed once by inversion.

Turbidity was measured using a Hach Laboratory Turbidimeter (model 2100N; Hach Co., Loveland, CO). The optical system in this instrument measures light scattering in nephelometric turbidity units (NTUs) and is comprised of a tungsten filament lamp, lenses and apertures to focus the light, a 90° detector, forward scatter detector and a transmitted light detector. The instrument was used for this experiment in ratio measurement mode in which it gathers data from all three detectors and determines a ratio from the signals. The instrument was calibrated prior to the experiment with Formazin primary standards prepared from a 4000 NTU stock (Hach) and HPLC grade deionized water.

The calibration of the instrument was verified periodically during the experiment using Gelex secondary turbidimetry standards.

Turbidity (NTUs) was measured immediately after mixing (0 min) for each test tube and then at 15 min intervals for 105 min. Baseline data were collected by measuring the turbidities (NTUs) of 4 ml of deionized distilled water mixed with 4 ml of the following concentrations of tannic acid: 0, 0.5, 0.89 and 1.58 g/l. Concentrations were chosen to correspond to levels of tannic acid used in previous psychophysical studies (Lee and Lawless, 1991).

A four-way repeated measures ANOVA with tannic acid concentration and time as within subject factors, gender as a between subjects factor and panelists as a random effect was used to detect differences in the data. Statistical analyses were performed using Statistica v.5.1 (Statsoft Inc., Tulsa, OK). Results reported below are significant at $P < 0.01$.

Results and discussion

Figure 1 shows the turbidity development with increasing concentrations of tannic acid. Turbidity increased with increasing concentrations of tannic acid [$F(3,69) = 71.2$] and increased slightly over incubation time, as shown in Figure 2, except for the no tannin control [interaction $F(21, 483) = 18.7$]. Turbidity of solutions with tannin only and no saliva was .5 NTUs (not plotted). No gender effects were observed. The haze development confirmed earlier observations on the potential use of turbidity measurements as an indicator of the interactions of salivary proteins and tannins (Lawless *et al.*, 1999). Measurable haze development is seen over a range of concentrations that are physiologically active in the dynamic range of sensory intensity ratings (Guinard *et al.*, 1986; Lee and Lawless, 1991; Lawless *et al.*, 1994). The method is rapid and simple to execute. This further supports the possibility of using *in vitro* haze development as a predictor of astringency responses in psychophysical tests.

One question of interest is whether haze development should be positively or negatively correlated with sensory responses. Both haze development and astringency responses increase with concentration (Lawless *et al.*, 1994) across the same ranges. Comparing these concentrations to previous psychophysical data (Lee and Lawless, 1991), responses would span the middle third of an intensity scale (roughly from 5 to 10 on a 15 point category scale). This correspondence implies a positive correlation when concentration is the source of variation. However, a negative correlation might be expected when variation across individuals in salivary protein content is the source of variation. If the function of salivary proteins is to lubricate and protect the epithelium from reactions with astringents, then a person with higher levels of salivary protein might be expected to have lower astringency ratings (better protection), but higher levels of haze development. A negative correlation would be consistent with the observation that

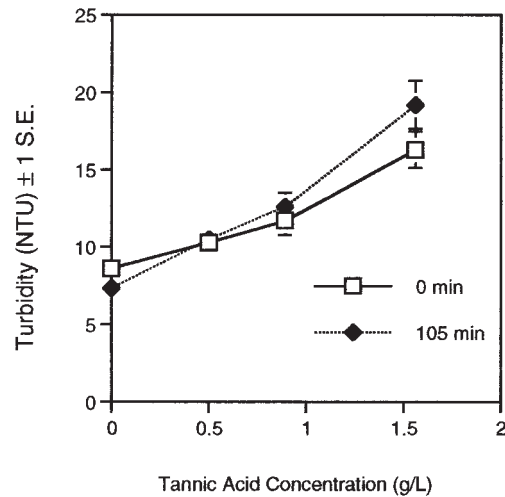


Figure 1 Turbidity of salivary samples mixed with tannic acid as a function of concentration and time. NTUs are nephelometric turbidity units, a measure of light scattering. The x-axis shows concentration of tannic acid in the 4 ml solutions before mixing with 4 ml of saliva. Values are means $\pm$ SE. Standard errors do not appear for the first two points in the series as they are smaller than the plot symbols.

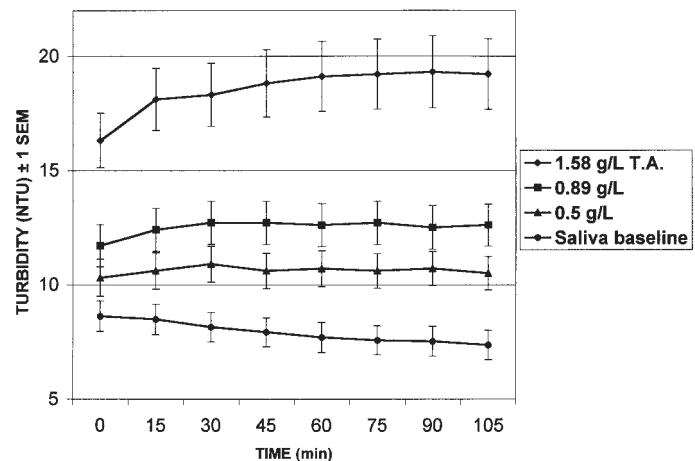


Figure 2 Changes in turbidity as a function of incubation time of saliva with tannic acid, plotted at different concentrations of added tannic acid (T.A.)

less responsive individuals have higher overall salivary protein content and more responsive individuals have lower protein content (Imm and Lawless, 1996). If, on the other hand, the binding of tannins to proteins produces a precipitate that itself causes astringency, then a positive correlation would be expected. Experiment 2 was conducted to examine the relationship of haze and astringency ratings across individuals.

Experiment 2. Correlation of instrumental turbidity and perceived astringency

The objective of Experiment 2 was to examine the relation-

ship between instrumental turbidity and perceived astringency. To the extent that salivary proteins are bound by tannins in the initial steps of the development of astringent sensations, and assuming that the salivary haze development seen in Experiment 1 is an indicator of the extent of the saliva–protein interactions, a correlation across individuals is predicted, i.e. individuals with higher levels of protein would show greater haze development. If tannin–protein complexes or precipitates are themselves responsible for the astringent sensations a positive correlation would be expected. However, to the extent that salivary proteins provide a protective mechanism, an inverse relationship would be expected. The relationship between an individual's salivary protein content and the magnitude of their astringency responses is not yet clear (Guinard *et al.*, 1998; Kallikathraka *et al.*, 2001).

Methods

Subjects were 18 volunteers from the Cornell University campus community in Ithaca, NY (10 females). Informed consent was obtained. Subjects were instructed not to eat or drink anything for at least 1 h prior to each experimental session. Subjects were paid a token cash incentive for their participation.

Samples used for evoking saliva were 1.58 g/l tannic acid (lot 93H0269; Sigma Chemical Co.) and 3.16 g/l citric acid (lot 11K0238, FW 210.1; Sigma Chemical Co.). Samples used to measure perceived astringency were 0.5, 0.89 and 1.58 g/l tannic acid and 1.0, 1.78 and 3.16 g/l citric acid. All samples were 15 ml and were presented at room temperature ($20 \pm 1^\circ\text{C}$).

All subjects participated in three experimental sessions. In the first session unstimulated saliva was collected by the whole mouth method for 20 min. Subjects chewed a 2 cm² piece of Parafilm to evoke the saliva. Following mechanical stimulation of saliva subjects rested for 10 min and saliva was collected again for 150 s, evoked by either 1.58 g/l tannic acid or 3.16 g/l citric acid. Lastly, after another 10 min rest period, saliva was again collected for 150 s, evoked by either the citric acid or the tannic acid stimulus, whichever was not used previously.

Subjects were given a practice trial with water to familiarize themselves with the time–intensity module of Compusense and how to respond with the computer mouse. Astringency was not defined for them, although subjects had participated in previous studies of astringency in our laboratory and none enquired about further definition of the term. In those previous studies, astringency was generally defined as a complex of drying, roughing and puckery sensations experienced in the mouth after rinsing with alum or tannins, with 1 g/l alum generally given as an example. In the second and third sessions subjects rated three concentrations of tannic acid and three concentrations of citric acid for perceived astringency by continuous time intensity for 2 min each. Citric acid was included for several reasons.

Sour acids are also astringent (Rubico and McDaniel, 1992; Hartwig and McDaniel, 1995; Lawless *et al.*, 1996), although it is unclear whether they share the same mechanisms as polyphenols, and so a different pattern of correlations could occur. Also, sour acids are potent sialogens (Dawes *et al.*, 2000), so a different relationship with stimulated flow rate might be observed. All three concentrations of each acid were presented in single sessions and the order of presentation within and between sessions was randomized. There was a 5 min rest interval between each sample during which time subjects were instructed to rinse their mouths with deionized water and chew on table water biscuits. For each sample, subjects were instructed to place the entire sample into their mouths and swish it about for 15 s, being sure to cover all oral surfaces. At 15 s subjects were verbally signaled by the experimenter to expectorate the sample and to begin rating. Data were collected using the Compusense 5 v.4.0 time–intensity module. Ratings were made by moving the mouse, which marked off height on a vertical line scale labeled only 'astringency'. The time–intensity module scores the data from 0 to 10 with resolution set at 0.01.

In assessing mechanically stimulated salivary flow, whole mouth saliva was collected in pre-weighed 60 ml plastic cups for 20 min. Immediately following collection individual saliva samples were weighed and flow rates (g/min) were calculated. Individual saliva samples were then filtered through Whatman no. 1 paper under vacuum. The filtrate was dispensed into individual 60 ml sterile screw-top plastic vials and the mass was again measured. Finally, haze was measured in individual saliva samples by combining 4 ml of filtered saliva with 4 ml of 1.58 g/l tannic acid. Haze was measured using the same apparatus and protocols as used in Experiment 1, except measurements were made at 0, 5, 10 and 15 min. Following analysis the saliva was discarded.

In measuring chemically evoked salivary flow, subjects were instructed to place the entire sample into their mouths and swish it about for 15 s, being sure to cover all oral surfaces. At 15 s subjects were verbally signaled by the experimenter to expectorate the sample and to begin collecting saliva. Whole mouth saliva was collected for 150 s using the same protocols as those described above. Immediately following collection individual saliva samples were weighed and flow rates (g/min) were calculated. Haze was not measured in these samples due to the presence of residual tastant chemicals.

Subjects were separated into either a high flow or low flow group and into a high haze or low haze group around the medians of mechanically stimulated salivary flow rates (median 1.5 g/min, range 0.53–2.16 g/min) and of measured haze at 15 min (median 15.2 NTU, range 7.7–28.1 NTU). Individual maximum intensities (I_{max}) were obtained from the time–intensity data for each sample. Correlation matrices were calculated that included I_{max} of perceived astringency for all samples, measured haze levels at each time interval, mechanically stimulated salivary flow rate,

chemically evoked salivary flow rates, flow group and haze group.

Two-way split plot repeated measures ANOVAs were performed that compared $I_{\max}$ for each acid across the concentrations of each acid and among the two different flow groups and the two different haze groups. A two-way split plot repeated measures ANOVA was performed that compared haze levels (NTU) across all time intervals and among the different flow groups. Statistics reported below are significant at $P < 0.05$.

Results and discussion

Correlations among salivary flow rates, haze measures and intensity scores from tannic acid and citric acid are shown in Table 1. Haze development was positively correlated with mechanically stimulated salivary flow rate. This 'resting' (i.e. chemically unstimulated) rate was negatively correlated with astringency ratings of tannic acid but not with astringency ratings of citric acid. Astringency was negatively correlated with haze development for tannic acid but not for citric acid. These results are consistent with a pattern of low responding subjects having more salivary protein and therefore better protection of the oral surfaces against the stimulation by tannins. Conversely, high flow subjects may have higher amounts of salivary protein, more haze development and are less susceptible to the influences of tannins on the oral epithelium. Responses to citric acid did not correlate significantly with haze development, although astringency was negatively correlated with the stimulated flow rate to citric acid itself. This suggests a simple dilution effect for citric acid and a different mechanism than the haze-developing protein interactions seen with tannic acid.

A similar pattern of results was provided by between groups analyses of variance. Subjects were divided into high and low flow groups based on resting flow rate and into high and low haze groups based on median splits. This is not

meant to imply that there was a bimodal distribution in flow or haze, but simply to provide another way of looking at statistical relationships among salivary flow rate, haze development and psychophysical responses. High flow subjects had lower responses to tannic acid astringency [$F(1,16) = 10.04$], with greater differences at higher concentrations [interaction $F(2,32) = 5.42$]. A parallel pattern was seen based upon haze group, with higher haze reflecting lower astringency [$F(1,16) = 5.38$], and the effect was also greater at higher concentrations [interaction $F(2,32) = 5.64$]. These relationships are shown in Figure 3. There was also greater haze development in the saliva of the high flow group [$F(1,16) = 7.23$]. No significant group effects were seen for citric acid.

General discussion

The studies reported here show a relationship between the production of turbidity in saliva–tannin mixtures and astringency responses. Our initial thought was that haze development might parallel astringency as haze increased across the range of tannin concentrations that were psychophysically active. However, pilot work from one author (J. Hayes) showed a surprising negative correlation between haze development and the initial height of time–intensity functions. That led to consideration of the delubrication hypothesis and the notion that salivary protein would indeed serve as a protectant against the direct attack of tannins on oral tissues. Therefore, the inverse relationship of haze development and the rated intensity of astringency can be explained by increased protein content among low responding individuals, who are 'better protected'.

The inverse relationship of flow rate to astringency intensity (high flow, lower intensity) as shown in Figure 3 might suggest a simple dilution effect rather than protection by salivary proteins. However, flow rate (from tannic acid stimulation) and haze were positively correlated, implicat-

Table 1 Correlations among salivary measures and perceived intensity

	Flow			Haze	
	Parafilm	Tannic acid	Citric acid	0 min	15 min
Flow (tannic acid)	0.87 ^a				
Flow (citric acid)	0.18	0.26			
Haze (0 min)	0.48 ^a	0.31	−0.16		
Haze (15 min)	0.49 ^a	0.42	−0.12		
0.5 g/l tannic acid	−0.65 ^a	−0.69 ^a	−0.49 ^a	−0.31	−0.47 ^a
0.89 g/l tannic acid	−0.71 ^a	−0.61 ^a	−0.37 ^a	−0.44 ^a	−0.57 ^a
1.58 g/l tannic acid	−0.76 ^a	−0.67 ^a	−0.19	−0.58 ^a	−0.67 ^a
1 g/l citric acid	−0.21	−0.17	−0.53 ^a	−0.11	−0.09
1.78 g/l citric acid	−0.17	−0.19	−0.60 ^a	−0.21	−0.07
3.16 g/l citric acid	−0.21	−0.20	−0.80 ^a	−0.10	−0.06

Salivary measures are flow rates in g/min (see text). Haze measurements are in NTUs (see text) at time 0 and after 15 min incubation.

^aSignificant at $P < 0.05$.

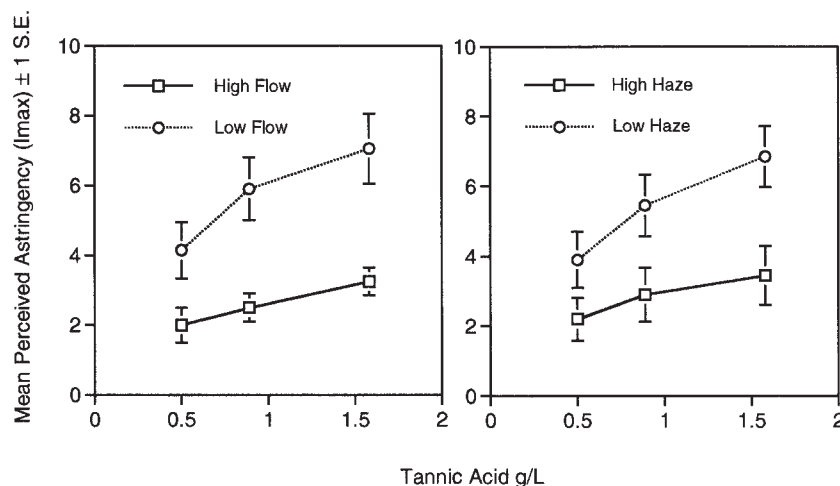


Figure 3 Perceived astringency (means of maximum intensity rating) as a function of tannic acid concentration for high and low flow groups (left) and high and low haze groups (right). Flow groups were determined by a median split of mechanically stimulated salivary flow (median 1.5 g/min). Haze groups were determined by a median split of haze development at 15 min incubation of 4 ml of saliva with 4 ml of 1.58 g/l tannic acid (median 15.2 NTU). High and low groups are significantly different in both plots, as were the interactions of group with tannic acid level (see text).

ing protein interactions as a probable cause of the effect. If there were a negative relationship between haze and flow, simple dilution would be more plausible. However, dilution might still play a role in the relationship to citric acid stimulation due to its ability to evoke copious flow. No significant relationships were observed between haze and citric acid-stimulated flow.

These studies provide no direct evidence of protein–tannin binding. However, that hypothesis seems reasonable in light of the extensive literature showing haze development in solutions of PRPs mixed with polyphenols and in light of the well-known phenomenon of chill haze in beverages, a function of tannin–protein interactions (Siebert *et al.*, 1996; Siebert and Lynn, 1997; Siebert, 1999). Also, recent studies have addressed changes in haze in saliva with a changes in pH, a variable known to affect tannin–protein interactions (K.J. Siebert and A.W. Chassy, submitted for publication). Demonstrating psychophysical parallels to such pH changes may not be straightforward, because lowering pH in itself increases astringency (Lawless *et al.*, 1996).

Kallikathraka *et al.* (Kallikathraka *et al.*, 2001) demonstrated that different salivary protein fractions from HPLC analysis were correlated either positively or negatively with time–intensity measurements of astringency. An early eluting fraction (peak 1 in their study), tentatively characterized as a hydrophilic PRP, was positively correlated with total perceived astringency (measured by area under the curve of the time–intensity response). They also noted a positive correlation with time to maximum, suggesting that individuals with a higher salivary protein content would take longer to achieve their peak intensity of sensation. In a somewhat closer parallel to the current result, subsequent peaks that were tentatively identified as non-PRP and hydrophobic PRP fractions were negatively correlated with

astringent intensity. This would be consistent with a lubricating or protective role that minimized astringent sensations among individuals with greater amounts of protein coating on the oral epithelium. The observation in Experiment 2 of a negative correlation with astringent intensity suggests that haze development may be due to binding these hydrophobic PRPs. Are the individual fractions observed by Kallikathraka's group differentially efficient at producing the salivary haze effect? If such relations are found, they would help substantiate the conclusion that the haze effect bears a mechanistic and predictive relationship to the initial astringency reactions and is not just an epiphenomenon.

In conclusion, the haze development effect adds to the evidence that interactions of salivary proteins and polyphenols are a likely first step in the development of astringency. Whether this interaction is sufficient to explain the entire mechanism of astringency is open to question. As Thorngate and Noble (Thorngate and Noble, 1995) pointed out, mechanoreceptors are likely involved in some of the astringent sensations, which seems reasonable given the movement involved in appreciating sensations of roughness or dryness. Binding with salivary proteins might serve to sequester or inactivate tannins and thus protect the alimentary tract from the deleterious effects of tannins on nutritional uptake. However, it is not known whether such protein–tannin complexes would survive the low pH of the stomach. As an alternative, Prinz and Lucas (Prinz and Lucas, 2000) proposed that the increased friction is simply a sensory warning that would help animals avoid ingesting plants that are high in tannins. The interesting question then arises of why these sensations are preferred by humans in foods and beverages under some circumstances.

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