Temporary Modification of Salivary Protein Profile and Individual Responses to Repeated Phenolic Astringent Stimuli

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Abstract

The extent of the change in salivary protein characteristics after repeated stimulations was shown to be correlated to differences in perceived astringency. Salivary characteristics of 77 subjects were compared after masticatory (S1) and taste/masticatory (S2) stimulations. The variations (S2 minus S1) of protein concentration and saliva haze-forming capacity (HFC) were used to define 3 subject groups: low responding (LR, $n = 20$), medium responding (MR, $n = 37$), and high responding (HR, $n = 20$). Salivary protein concentration did not change in LR subjects; decreased a little, but significantly, in MR subjects; and strongly decreased in HR subjects. After S2, HFC increased in LR subjects, slightly decreased in MR subjects, and strongly decreased in HR subjects. Salivary protein electrophoresis patterns for HR and LR subjects were analyzed. No significant modifications of glycosylated proline-rich proteins (PRPs), PRPs, and amylases and a slight decrease in cystatins and histatins were found when S2 and S1 samples were compared in LR subjects, whereas HR subjects showed a strong decrease in all the above proteins after S2. Significant modifications of mucins were not found. Tannic acid (TA, 3 g/L) astringency ratings after S1 from HR subjects were significantly higher than those from the other 2 groups, whereas no differences were found comparing LR and MR ratings. The "carryover" effect due to 4 sequential exposures to TA samples (1.4 g/L) was observed in both HR and MR groups, whereas no significant astringency rating variation was found in the LR group. The results support the inhibiting role of proteins with strong phenol-binding activity on astringency elicitation. Individual physiological variations of parotid gland functionality might account for differences in sensitivity to astringent phenolic stimuli.

Key words: carryover, electrophoresis, parotid glands, protein/phenol interactions, sensory evaluations, tannic acid

Introduction

Astringency is a tactile sensation described as drying and puckering of the oral surface experienced when ingesting phenol-rich plant foods and beverages. Various antinutritional effects have been reported for phenols (Mueller-Harvey 2006), and it has been proposed that the sensation of astringency represents a sensory warning cue that would discourage the ingestion of foods that contain high concentrations of these compounds. In fact, astringency is perceived as a negative attribute responsible for the lowering of acceptability for some plant food products (Lesschaeve and Noble 2005).

It is generally accepted that phenol/salivary protein interactions represent the key step in the physiological mechanism of astringency elicitation (Breslin et al. 1993;

Kallithraka et al. 1998). A long-held belief is that astringency is due to the stimulation of mechanoreceptors by precipitated salivary proteins. Based on this theory, it has been assumed that astringency perception parallels the salivary concentration of proteins with strong phenol-precipitating capacity (de Freitas and Mateus 2001; Kallithraka et al. 2001). On the other hand, a number of studies support the hypothesis that astringency arises from rupture of the lubricating saliva film that lines the oral cavity (Prinz and Lucas 2000; de Wijk and Prinz 2006). A molecular mechanism based on modifications of viscous elastic properties of glycosylated proteins, having a weak phenol-precipitating capacity, underlies this latter hypothesis (Rossetti et al. 2008; Sarni-Manchado et al. 2008; Schwarz and Hofmann 2008).

Moreover, a phenol-sequestering role and an inhibiting effect on astringency perception for proteins with strong phenol-precipitating capacity have been suggested (Horne et al. 2002; Condelli et al. 2006; Nayak and Carpenter 2008; Dinnella et al. 2009). A 2-step salivary protein/dietary phenol interaction has been hypothesized in which saliva is considered to be composed of 2 different phases, a thin dynamic film coating the internal oral surfaces and an adsorbed layer of proteins on the hard and soft tissues (Nayak and Carpenter 2008). The first step of protein/phenol interaction might involve the dynamic film consisting of proteins with the highest phenol-binding affinity (proline-rich proteins [PRPs], amylases, cystatins, and histatins) that exert a sequestering and protecting role. The second step might be based on phenol interactions with the adsorbed glycoprotein layer with the consequent oral cavity delubrication and astringency elicitation.

Salivary proteins are secreted as a complex mixture from 3 pairs of major exocrine glands (parotid, submandibular, and sublingual) plus numerous minor salivary glands. Parotid glands consist of serous acinar cells and produce a wide protein class showing high phenol-binding capacity (PRPs, amylases, histatins, cystatins) (Bennick 2002; Dodds et al. 2005). Submandibular glands consist of both serous and mucous acinar cells, whereas only mucous cells are present in sublingual glands. Mucous cells produce a viscous mucin-rich secretion mainly responsible for mouth lubrication (Becerra et al. 2003). The release of salivary components by acinar cells is a continuous process amplified by neuronal stimuli from both sympathetic and parasympathetic nerve fibers (Castle D and Castle A 1998; Mese and Matsuo 2007). In both resting and stimulated cells, nearly all newly synthesized proteins are accumulated in secretory granules for storage (more than 85%), the remainder being released without storage. Exocytosis is the process by which cells release the content of their secretory granules. A "constitutive" exocytosis continuously takes place, but it can be greatly accelerated following an appropriate neural stimulus thus providing "regulatory" exocytosis. Saliva secreted in the absence of apparent sensory stimuli related to eating is referred to as unstimulated, and it consists of a small spontaneous secretion and a small reflex secretion evoked by dryness of the oral mucosa and by low-grade mechanical stimulation caused by movements of the tongue and jaw. Low flow rate, high viscosity, and high mucin concentration characterize unstimulated whole saliva. Stimulated saliva is reflexly secreted following certain visual, olfactory, oropharyngeal, and esophageal stimuli (gustatory, mechanical, and thermal). The type of taste stimuli and the intensity of chewing, for example, strongly affect parotid gland functionality and induce modifications of saliva composition (Neyraud et al. 2006; Mese and Matsuo 2007). An increasing flow rate, mainly due to parotid activity, characterizes stimulated saliva.

There are large individual variations in saliva characteristics in healthy subjects (Huang 2004; Dodds et al. 2005).

A number of studies indicate the importance of individual variation of saliva characteristics in modulating the sensitivity to phenolic astringent stimuli. The relevance of flow rate in modulating astringency perception is well documented, even if somewhat with conflicting results (Fisher et al. 1994; Ishikawa and Noble 1995; Guinard et al. 1998; Peleg et al. 1999; Horne et al. 2002; Condelli et al. 2006). Salivary volume does not seem to account by itself for differences in astringency perception. Different rates of oral cavity relubrication (Bajec and Pickering 2008) as well as modification of protein salivary profile induced by the adopted experimental conditions (whole vs. parotid flow, mechanical vs. gustatory stimulation, type and concentration of gustatory stimuli) could account for the differences in sensitivity found in subject groups differing for salivary flow rate. Significant negative correlations have also been demonstrated between whole salivary protein capacity to form insoluble aggregates with phenols and the intensity of perceived astringency (Horne et al. 2002; Condelli et al. 2006).

The ability to maintain constant salivary characteristics after repeated stimulation was found to be an effective criterion on which to base differences in sensitivity to astringent phenolic stimuli (Dinnella et al. 2009). A nearly constant protein concentration and an unchanged capacity to bind and precipitate phenols, after both masticatory and taste stimulations, characterize a subject group with lower sensitivity to astringency, whereas a strong reduction in the value of both these salivary characteristics was found in the more sensitive group.

The aim of the present work was to gain further insight on the physiological base modulating the individual response to phenolic astringent stimuli. The effect of repeated saliva stimulation on electrophoresis salivary protein profile was investigated with the aim of clarifying the role of the different classes of proteins in the interaction with dietary phenols. Moreover, the consequences of different salivary protein profiles on the individual responses to phenolic stimuli were investigated to better understand the molecular mechanism of astringency elicitation. The buildup of astringent sensation upon repeated ingestions of phenolic stimuli (carryover effect) has been hypothesized to be related to the subsequent binding of different layers of mouth proteins (Guinard et al. 1986). Thus, the development of the carryover effect was evaluated in subject groups with different salivary protein profile.

Materials and methods

The experimental plan for saliva characterization and sensory data collection was designed according to Dinnella et al. 2009 with a few modifications.

Subjects

Seventy-seven subjects, 33 males and 44 females, aged from 21 to 33 years were recruited from the University

of Firenze students. The subjects had no history of disorders in oral perception. They were paid for their participation in the study. The Ethic Committee of the Dipartimento di Biotecnologie Agrarie, Università di Firenze, approved the protocol. Written informed consent was obtained from each subject after a full explanation of the experiment.

Participants were instructed to avoid food and beverage with high phenolic content for at least 8 h before the session started. A list of these foods was provided. They were also instructed to refrain from smoking or having food or beverage for 2 h before the session started.

Stimuli

Two tannic acid (TA, Sigma-Aldrich) sample sets were used to induce astringency and to stimulate the reflex parotid gland salivation. The first set was composed of one TA sample only at 3.0 g/L in aqueous solution of 1% ethanol, and the second set was 4 TA samples at the same concentration (1.4 g/L in aqueous solution of 1% ethanol). Samples were presented at room temperature.

Sensory procedure

Training

Prior to their participation in the experiment, subjects were trained to recognize and rate the perceived intensity of the following different sensations: sourness, bitterness, and astringency using the following standard (Sigma-Aldrich) aqueous solutions: citric acid: 0.25, 0.38, 0.50 g/L; quinine monohydrochloride dihydrate 0.025, 0.037, 0.050 g/L; aluminum potassium sulfate: 0.3, 0.6, 0.9 g/L. During training sessions, the subjects were asked to rate the perceived intensity on a Labeled Magnitude Scale (LMS, 100-mm line) (Green et al. 1996) with the bottom of the scale labeled as "barely detectable" and the top as "strongest imaginable" oral sensations, including pain. Subjects participated in a total of 4 training sessions.

Evaluation

Subjects received tap water to rinse their mouth. Subsequently, they were instructed to mechanically evoke saliva by chewing parafilm (first saliva collection). These saliva samples were indicated as stimulated samples S1. After a 30-min break, subjects were stimulated with 3 g/L TA sample. Subjects held each sample in their mouths for 10 s, spit it out, waited for a further 20 s, and rated the intensity of astringency, bitterness, and sourness on LMS. Subjects were again asked to rinse their mouths with water and to collect saliva (second saliva collection) as previously described. These saliva samples were indicated as stimulated samples S2. Immediately after saliva collection, subjects received four 1.4 g/L TA samples and were asked to taste and rate the perceived astringency, bitterness, and sourness of each sample as described above. The second sample set evaluation was performed without a resting or rinsing procedure between the 4 TA samples.

Across subjects, the order of attribute evaluation was balanced in order to minimize a possible "proximity" effect. The evaluations were performed in individual booths under red lights to eliminate visual clues. The session started at 9.00 AM with an average duration of 60 min.

Saliva measurements

Salivary flow

Whole salivary flow was measured according to the procedure described by Gaviao et al. 2004. Subjects mechanically evoked saliva by chewing on a square of parafilm (3 × 3 cm) while spitting saliva into a weighed container for 5 min. Then, a rest of 5 min was given before a further saliva collection with a new piece of parafilm. The entire procedure was repeated twice for a total saliva collection time of 15 min. The collected saliva was weighed on an analytical balance and the flow expressed as grams of saliva per minute.

Saliva samples were put in an ultrasonic water bath at the maximum output for 5 min at 37 °C. The pellet eventually still present in the salivary sample was discarded, whereas the clear upper phase was recovered, diluted 1:2 (v/v) with water and analyzed.

Haze-forming capacity

Saliva phenol-precipitating capacity was measured as protein reactivity with TA solution and expressed in terms of haze-forming capacity (HFC) as described by Horne et al. (2002). Aliquots of diluted saliva (0.6 mL) were mixed with 2.4 mL of TA solution (0.23 g/L) in 1% ethanol. A reference sample was prepared by mixing 0.6 mL of diluted saliva with 2.4 mL of 1% ethanol solution. The mixtures were allowed to stand for 1 min at 37 °C. The turbidity was determined in a HACH 2100N laboratory turbidimeter (Hach Co) and expressed in nephelometric turbidity unit (NTU). Saliva HFC value was calculated as the difference between NTU measured in saliva/TA mixture and NTU measured in the relevant saliva reference sample. Each sample was analyzed in triplicate.

Protein content

Total salivary protein concentration (SPs) was determined by the biuret method (Kallithraka et al. 2001). Bovine serum albumin (BSA) was used as the protein reference. Each sample was analyzed in triplicate.

Phenol content

Salivary phenolic concentration (Phs) was determined by using a modified Folin–Ciocalteau assay (Siebert and Chassy 2003). Diluted saliva samples (0.25 mL) were mixed

with 1.25 mL of Folin–Ciocalteau reagent (1:10 v/v with water) and left to react for 15 min at 45 °C. Sample absorbance at 760 nm was determined, phenolic content calculated, and expressed as gallic acid concentration (mg/mL saliva).

Electrophoresis

A saliva volume corresponding to 250 or 150 μg of proteins (for protein or glycoprotein detection, respectively) was freeze-dried, dissolved in a nonreducing loading sample buffer (0.5 M Tris–HCl, pH 6.8, 15% v/v glycerol, and 1.5% w/v sodium dodecyl sulfate [SDS]), boiled for 5 min, and loaded on tricine–SDS–polyacrylamide gel electrophoresis (PAGE) using a 16% acrylamide separating gel and 4% acrylamide staking gel. Electrophoresis runs were performed on a BioRad Protean III apparatus at 25 mA/gel. Apparent molecular weights were estimated by comparison with the migration rates of standard proteins (broad range, BioRad).

After electrophoresis, gels were stained for 18 h with Coomassie brilliant blue R250 (Sigma-Aldrich) and then destained with 7% acetic acid for 24 h to detect the whole salivary protein profile. The periodic acid Schiff (PAS) method was used to stain glycoproteins as described by Segrest and Jackson (1972).

Protein concentration in the electrophoresis patterns was calculated by a semiquantitative technique. The optical density of protein bands was measured by densitometry with Quantity One (BioRad) program using BSA as internal standard for Coomassie-stained gels and mucin (from bovine submaxillary glands, Type I–S, Sigma-Aldrich) for the PAS-stained ones. The optical density of the band corresponding to BSA (3 μg) and mucin (5 μg) was used to calculate protein concentration of saliva sample electrophoresis patterns. Results are expressed in terms of micrograms per milliliter of BSA equivalent and bovine mucin equivalent, respectively. Each saliva sample was analyzed in triplicate.

Results

Effect of repeated stimulation on saliva characteristics

Mean salivary characteristics and D values (arithmetic difference between S2 and S1 samples for each considered salivary variable) measured in 77 subjects are reported in Table 1. Salivary protein concentration and composition were significantly modified by repeated stimulation. SPs was significantly lower in S2 than in S1 samples ($t_{76,1.66}$ = –2.70, P < 0.01). Also, the second stimulation induced a significant decrease in saliva reactivity with TA solution measured in terms of HFC ($t_{76,1.66}$ = 2.26, P = 0.03). As expected, Phs significantly increased after tasting astringent phenolic stimuli ($t_{76,1.66}$ = –5.56, P < 0.01). No significant change in flow rate was found ($t_{76,1.66}$ = –0.20, P = 0.84).

Subject grouping

HFC and SPs D values have recently been demonstrated to be factors that relate to individual sensitivity to phenolic astringent stimuli (Dinnella et al. 2009). Subjects were grouped according to 3 levels of variation (low, medium, and high) of saliva HFC or SPs D values. Characteristic values of a percentile distribution (first and third quartiles) were used in order to define 3 groups: low-responding (LR, n = 20), medium-responding (MR, n = 37), and high-responding (HR, n = 20) subjects. Subject groups did not differ in their composition when either HFC or SPs D values were used.

The salivary characteristics of the 3 groups are reported in Table 2. SPs did not significantly change in the LR group ($t_{19,2.09}$ = –1.55, P = 0.14), whereas an increase in HFC after the second stimulation was found ($t_{19,2.09}$ = –4.20, P < 0.001). In the MR group, the second stimulation induced a small but significant decrease in both SPs ($t_{36,2.02}$ = –5.69, P < 0.001) and HFC ($t_{36,2.02}$ = 2.28, P = 0.02) values. Finally, in HR subjects, both SPs and HFC values were strongly lowered after the second stimulation ($t_{19,2.09}$ = –6.06, P < 0.001; $t_{19,2.09}$ = –6.89, P < 0.001).

Table 1 Mean salivary protein concentration (SPs), phenol concentration (Phs), HFC, flow rate, and relevant D values of 77 subjects determined after first (S1) and after second stimulation (S2)

	SPs (mg/mL)			Phs (mg/mL)			HFC (NTU)			Flow rate (g/min)		
	S1	S2	D	S1	S2	D	S1	S2	D	S1	S2	D
Mean	4.42	3.31	–1.12	0.14	0.17	0.03	1.74	1.47	–0.27	1.55	1.56	0.01
SE	0.27	0.17	0.17	0.01	0.01	0.01	0.18	0.15	0.12	0.07	0.07	0.04
Min	1.55	1.40	–7.24	0.07	0.09	–0.07	0.31	0.18	–4.53	0.57	0.50	–1.39
Max	15.74	8.50	1.01	0.32	0.53	0.26	8.10	8.21	3.54	4.27	3.65	0.80
P value			0.01			0.01			0.03			0.84

SE, standard error; min, minimum value; max: maximum value.

Table 2 Mean salivary protein concentration (SPs), phenol concentration (Phs), HFC, flow rate, and relevant D values determined in LR ($n = 20$), MR ($n = 37$), and HR ($n = 20$) subjects after first (S1) and after second stimulation (S2)

	SPs (mg/mL)			Phs (mg/mL)			HFC (NTU)			Flow rate (g/min)		
	S1	S2	D	S1	S2	D	S1	S2	D	S1	S2	D
LR subjects												
Mean	3.67	3.40	−0.26	0.13	0.17	0.04	1.59	2.30	0.71	1.38	1.38	−0.01
SE	0.23	0.28	0.17	0.01	0.01	0.01	0.37	0.43	0.17	0.11	0.10	0.08
Min	2.39	1.67	−1.76	0.10	0.11	0.00	0.31	0.65	0.20	0.72	0.67	−0.89
Max	6.57	6.62	1.01	0.19	0.25	0.11	7.95	8.21	3.54	2.52	2.08	0.80
P-value			0.14			<0.001			<0.001			0.96
MR subjects												
Mean	3.59	2.79	−0.79	0.12	0.15	0.03	1.08	0.99	−0.08	1.64	1.64	0.00
SE	0.24	0.18	0.14	0.01	0.01	0.01	0.10	0.10	0.04	0.10	0.10	0.05
Min	1.55	1.40	−2.90	0.07	0.09	−0.05	0.32	0.23	−0.52	0.59	0.50	−0.74
Max	7.77	5.46	0.54	0.20	0.39	0.26	2.53	2.40	0.52	2.70	3.04	0.72
P value			<0.001			<0.001			0.02			0.93
HR subjects												
Mean	6.76	4.22	−2.54	0.18	0.21	0.03	3.13	1.59	−1.54	1.55	1.59	0.04
SE	0.69	0.38	0.42	0.01	0.02	0.01	0.41	0.26	0.22	0.19	0.17	0.10
Min	3.01	2.18	−7.24	0.11	0.12	−0.07	0.99	0.18	−4.53	0.57	0.62	−1.39
Max	15.74	8.50	−0.06	0.32	0.53	0.20	8.10	4.79	−0.68	4.27	3.65	0.66
P value			<0.001			0.03			<0.001			0.67

SE, standard error; min, minimum value; max: maximum values.

As expected, all 3 groups showed a significant increase in Phs values ($P < 0.05$), whereas no significant modifications of flow rate were found ($P > 0.05$).
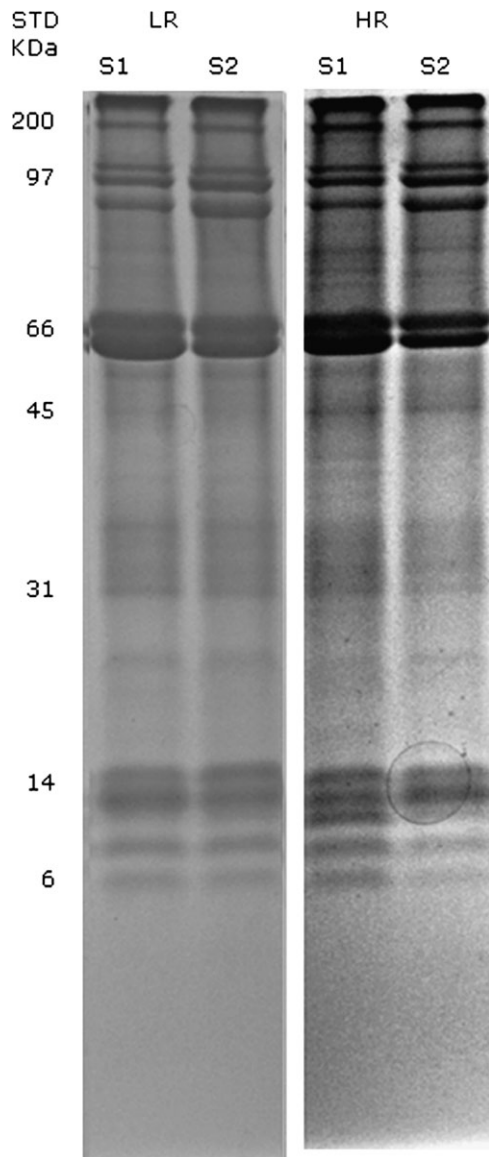
Effect of repeated stimulations on electrophoretic salivary protein profile

The effect of repeated stimulation on the major salivary protein class profile was investigated by analyzing the electrophoresis patterns of the 2 extreme groups (HR and LR). Three salivary samples gave poorly resolved electrophoresis runs; therefore, results relevant to 19 subjects from LR group and 18 subjects from HR group are reported.

Figure 1 reports representative SDS–PAGE stained with Coomassie brilliant blue R250. No evident differences were found when comparing patterns from LR subjects (Figure 1). On the other hand, a general lowering of protein band staining was observed in S2 with respect to the S1 pattern for HR subjects (Figure 1). The apparent molecular weight estimated by migration in tricine–SDS–PAGE varied from 94 to 14 kDa showing the expected band pattern for whole saliva in the adopted run conditions (Schwartz et al. 1995; Bacon and Rhodes 2000; Banderas-Tarabay et al. 2002;

Sarni-Manchado et al. 2008). Lactoferrin and glycosylated PRPs show apparent molecular mass between 94 and 67 kDa. The major signal around 66 kDa corresponds to α-amylase glycosylated and nonglycosylated forms, protein bands from 45 to 31 to PRPs fraction, and bands around 14 and 6 kDa to cystatins and histatins, respectively (Yao et al. 2003). Salivary mucins MG1 and MG2 were identified on the basis of their characteristic PAS-stained electrophoresis behavior (Becerra et al. 2003; Nayak and Carpenter 2008). MG1 has an apparent molecular weight higher than 1000 kDa and appears as a tight PAS-reactive band at the top of the staking gel. MG2 has an apparent molecular weight around 200 kDa and appears as the most PAS-reactive band at the boundary between stacking and running gel. MG1 and MG2 distribution appeared to remain constant in both subject groups either in S1 or S2 samples.

The amount of protein in the identified bands was calculated using a semiquantitative approach and expressed in terms of internal standard protein equivalent concentration (μg/mL). Amounts of identified proteins both in S1 and S2 samples were compared within each subject group (Table 3). The LR group showed a slight decrease (less than 10%) in

Figure 1 Salivary proteins molecular patterns of S1 and S2 samples of subjects from LR (*n* = 19) and HR groups (*n* = 18) in a representative SDS–PAGE stained with Coomassie brilliant blue R250.

No significant modifications of mucin concentration were found in either group (HR: $t_{17,1.74} = 0.74$, $P = 0.46$; LR: $t_{18,1.73} = 0.07$, $P = 0.94$).

The difference between LR and HR groups for each tannin-binding protein concentration in S1 and S2 samples was tested by an unpaired *t*-test. Tannin-binding protein concentrations in S1 samples were significantly higher in HR subjects than in LR subjects ($P < 0.001$). On the contrary, phenol-binding protein content did not significantly differ between HR and LR groups in S2 samples ($P > 0.15$).

Sensory ratings

The astringency, bitterness, and sourness intensities of the sample containing 3.0 g/L TA were rated 30 min after the first saliva collection. A one-way analysis of variance (ANOVA) model was computed to estimate the group effect (3 levels: LR, MR, and HR subjects) on ratings. Subjects from both LR and MR groups perceived the astringency induced by TA solution at a significantly lower intensity than subjects from HR group ($F_{2,74} = 5.57$, $P < 0.001$). No significant group effect was found for bitterness ($F_{2,74} = 1.59$, $P = 0.21$) and sourness ($F_{2,74} = 0.32$, $P = 0.32$) ratings.

Sensitivity to phenolic astringent stimuli of the 3 groups was evaluated on the ratings from four 1.4 g/L TA samples tasted immediately after the second saliva collection. Astringency, bitterness, and sourness ratings were independently submitted to a 2-way repeated-measure ANOVA to estimate the group (3 levels: LR, MR, and HR subjects) and the repeated exposures to TA sample (4 levels: TA1, TA2, TA3, and TA4) effects.

The results confirmed that groups differed significantly for the intensity of perceived astringency ($F_{2,74} = 8.48$, $P < 0.001$). Mean astringency ratings from HR subjects (35.74 ± 2.04) were significantly higher than those from the other 2 groups, whereas no differences were found comparing LR (20.98 ± 2.04) and MR (18.48 ± 1.50) ratings. No significant effect was found for sample × group interactions ($F = 0.675$, $P = 0.670$). No significant group effect was found for bitterness ($F_{2,74} = 2.62$, $P = 0.10$) and sourness ($F_{2,74} = 1.03$, $P = 0.36$) ratings.

Figure 2 depicts the development of astringency, bitterness, and sourness induced in 77 subjects by the 4 repeated exposures to 1.4 g/L TA samples. Astringency ratings regularly increased with each successive exposure ($F_{3,222} = 7.23$, $P < 0.001$) thus clearly indicating the carryover effect. As expected, repeated exposures to TA did not result in modification of perceived bitterness or sourness intensities ($P > 0.05$).

The effect of repeated exposure to TA samples was studied in each of 3 subject groups (Figure 3). Perceived astringency progressively increased with repeated TA sample evaluations both in MR and HR groups. The average ratings significantly increased ($t_{36,2.02} = -4.05$, $P < 0.001$) from

tannin-binding proteins induced by the second stimulation. In particular, no significant modifications of lactoferrin and glycosylated PRPs ($t_{18,2.10} = 1.12$, $P = 0.28$), PRPs ($t_{18,2.10} = 2.03$, $P = 0.06$), and amylase ($t_{18,2.10} = 1.62$, $P = 0.12$) concentrations were found. Cystatin and histatin concentrations slightly decreased ($t_{18,2.10} = 2.84$, $P = 0.01$; $t_{18,2.10} = 2.89$, $P = 0.01$, respectively).

In HR subjects, protein profile was strongly modified by the second stimulation (lactoferrin and glycosylated PRPs: $t_{17,2.11} = 4.04$, $P < 0.001$; PRPs: $t_{17,2.11} = 4.73$, $P < 0.001$; amylases: $t_{17,2.11} = 4.45$, $P < 0.001$; histatins: $t_{17,2.11} = 5.14$, $P < 0.001$; cystatins: $t_{17,2.11} = 4.9$, $P < 0.001$). An average phenol-binding proteins loss of 40% was determined.

Table 3 Mucins, amylases, glycosylated PRPs, PRPs, histatins, and cystatins mean value concentration and standard error (SE) and relevant *D* values determined in LR (*n* = 19) and HR (*n* = 18) subjects after first (S1) and after second stimulation (S2)

	Mucins (µg/mL bovine–mucin equivalent)			Amylases (µg/mL BSA equivalent)			Glycosylated PRPs (µg/mL BSA equivalent)			PRPs (µg/mL BSA equivalent)			Histatins (µg/mL BSA equivalent)			Cystatins (µg/mL BSA equivalent)		
	S1	S2	*D*	S1	S2	*D*	S1	S2	*D*	S1	S2	*D*	S1	S2	*D*	S1	S2	*D*
LR subjects																		
Mean	312.18	310.59	−1.59	86.42	78.46	−7.96	77.13	72.87	−4.26	73.17	62.57	−10.61	30.73	25.88	−4.85	59.60	53.13	−6.47
SE	37.32	38.48	23.92	7.33	9.19	4.90	7.46	8.42	3.80	5.81	6.26	5.22	2.43	2.45	1.67	3.29	3.74	2.27
P value			0.94			0.12			0.28			0.06			0.01			0.01
HR subjects																		
Mean	387.98	361.17	−26.81	166.91	98.51	−68.41	124.94	88.35	−36.59	123.33	78.83	−44.49	42.55	26.53	−16.02	86.81	58.69	−28.12
SE	51.72	51.37	35.84	14.44	9.69	15.35	12.35	9.36	9.06	12.14	7.32	9.39	4.11	3.28	3.11	7.48	5.26	5.74
P value			0.46			<0.001			<0.001			<0.001			<0.001			<0.001
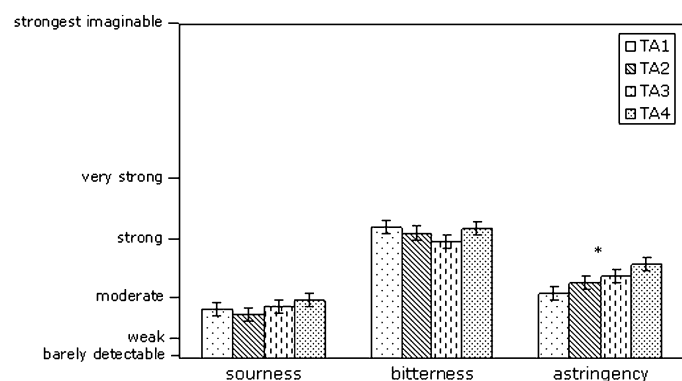
Figure 2 Sourness, bitterness, and astringency ratings of 4 TA samples 1.4 g/L (TA1, TA2, TA3, TA4). Bars represent standard errors. *n* = 77. *$P \leq 0.05$.
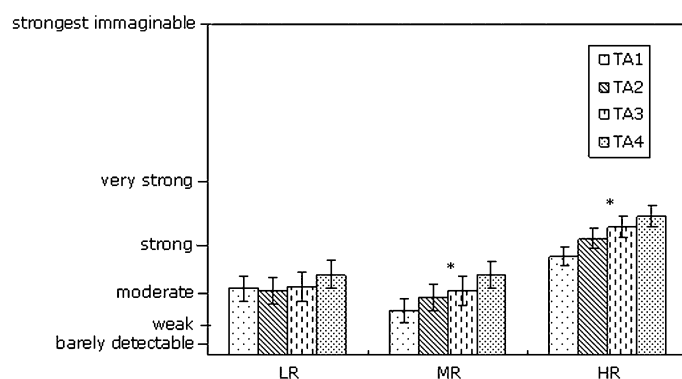
Figure 3 Astringency intensities of 4 TA samples 1.4 g/L (TA1, TA2, TA3, TA4) rated by LR (*n* = 20), MR (*n* = 37), and HR (*n* = 20) subject groups. Bars represent standard errors. *$P \leq 0.05$.

TA1 (13.2 ± 2.7) to TA4 (24.1 ± 3.1) in MR group. An analogous increase was found in HR subjects ($t_{19;2.09} = -2.10$, $P = 0.05$; TA1 rating: 29.7 ± 3.7; TA4 rating: 41.7 ± 4.2). On the other hand, no significant astringency rating variation was found in the LR group ($t_{19,2.09} = -1.1$, $P = 0.29$; TA1 rating: 19.9 ± 3.7; TA4 rating: 24.2.7 ± 4.2).

Discussion

Stimuli

TA was used to elicit astringency and the reflex parotid salivation. TA is classified as hydrolyzable tannin, a class of water-soluble phenolic compounds. Nevertheless, dilute alcoholic solutions are often used to fasten and improve tannin dissolution in aqueous media (Kielhorn and Thorngate 1999; Peleg et al. 1999; Ribereau-Gayon et al. 2000; Monteleone et al. 2004). Modifications of stimulated saliva characteristics have been reported after acute alcohol consumption (0.65 g/kg of body weight) (Enberg et al. 2001). In the present study, the TA solutions correspond to an alcohol intake of 0.0025 g/kg of body weight only. Furthermore, astringency elicited by diluted ethanol solution cannot be discriminated from the sensation induced by distilled water (Thorngate and Noble 1995; Monteleone et al. 2004). Thus, it is reasonable to assume that the presence of 1% ethanol does not affect properties of the TA solutions.

Phenolic compounds contribute to different oral sensations. Monomeric flavan-3-ols (catechin and epicatechin) and related oligomers are perceived more bitter than astringent (Thorngate and Noble 1995; Peleg et al. 1999) and also induce a weak sourness in aqueous solutions (Peleg et al. 1998). According to several authors (Lea and Arnold 1978; Guinard et al. 1986; Robichaud and Noble 1990), TA water solutions ranging from 0.5 to 3.0 g/L are described as more astringent than bitter and weakly sour. These evidences justify the generally accepted use of TA as standard for astringency induced by phenolic compounds.

The mouth-feel perception of astringency in phenol-containing foods depends on the presence of individual food

components exerting a synergic (organic acid, acidic pH) (Peleg et al. 1998) or antagonistic effect (polysaccharides, sweeteners, proteins) (Narain et al. 2004; Yan et al. 2009). Flavan-3-ols give a neutral reaction, whereas TA develops acidic pH when dissolved in aqueous solution. However, neutral flavan-3-ols are often experienced as astringent stimuli in acidic food matrices such as wine, fruit, and vegetables.

Based on these considerations, TA in 1% ethanol with its acid pH is assumed as an appropriate model food astringent stimulus.

Effect of stimulation on saliva characteristics

The oral environment can be considered a highly dynamic system susceptible to a variety of physiological and biochemical processes responsible for salivary modifications. Extensive salivary protein modifications can occur, and a number of enzymes are responsible for posttranslational processing involving glycosylation, phosphorylation, and proteolysis (Helmerhorst and Oppenheim 2007). Also, salivary protein secretion is under neural control, with protein output being dependent on the stimulus (Proctor and Carpenter 2007). Based on these considerations, reference conditions for saliva collection were adopted in order to minimize the high variability of whole saliva proteome.

The soluble protein phase present in the oral cavity before stimulation was removed by the 2 min of rinsing with water before saliva collection (Nayak and Carpenter 2008). S1 samples were representative of whole saliva and mainly consisted of proteins synthesized and stored in salivary glands during a prolonged absence of both mechanical and chemical stimulation (Gorr et al. 2005). In fact, the unconditioned response induced by masticatory reflexes activates parasympathetic signals, thus stimulating parotid glands via the glossopharyngeal nerve and submandibular and sublingual glands via the facial nerve inducing a massive exocytotic release of proteins (McManaman et al. 2006).

A selective action of tannins on parotid glands has been reported for animal models (Gho et al. 2007; da Costa et al. 2008). The TA sample tasted before the second saliva collection selectively stimulates parotid glands and induces a massive secretion of protein storage granules consisting of amylase, PRPs, histatins, and cystatins (Castle D and Castle A 1998; Bacon and Rhodes 2000; Kallithraka et al. 2001; Gorr et al. 2005). In the adopted experimental procedure, TA-evoked saliva was discarded during the mouth rinsing procedures immediately preceding the second saliva collection. Thus, the protein profile of S2 samples is affected by de novo protein biosynthesis that occurred in the 30-min break between saliva collections (Palade 1975; Becerra et al. 2003; Neyraud et al. 2006) and by the depletion of parotid protein output due to the prolonged mechanical and chemical gland stimulation (Jensen et al. 1998). Thus, the overall lowering of whole saliva protein concentration found in S2 samples might be due to the decreasing of parotid protein secretion. The observed decreasing of HFC values supports

this hypothesis because parotid secretory proteins are reported to be the most haze-forming salivary proteins (Bennick 2002).

Another consequence of TA exposures is the increased concentration of salivary phenol content in S2 samples due to the ability of ingested phenols to persist in the oral cavity probably because of their capacity to bind to epithelial cells (Siebert and Chassy 2003; Payne et al. 2009).

The experimental data clearly showed a wide variability in subjects' reactions to stimulation. HFC or SPs D values were computed to express an individual's capacity to react to stimulation by restoring the basal saliva composition. The 3 subject groups (LR, MR, and HR) were obtained from a percentile distribution computed on saliva HFC or SPs D values in view of earlier findings (Dinnella et al. 2009) about the strong positive correlation between the 2 considered parameters. Differences in parotid gland characteristics might account for the observed individual variation of concentration and properties of whole saliva proteins in response to stimulation (Ono et al. 2006). The relationship between parotid saliva composition and both taste qualities and chemical properties of oral stimuli are still not clear. In fact, the same modification of parotid saliva protein concentration has been found to be induced either by salty–neutral (NaCl) or sour–acidic (citric acid) stimuli (Neyraud et al. 2009). Analogous results of large variations in salivary protein profile have been reported for other tastants inducing aversive responses (Neyraud et al. 2006).

Salivary protein profiles and astringency sensitivity

Interactions between phenols and specific salivary proteins are responsible for either inhibiting or enhancing effects on the perceived astringency (Kallithraka et al. 1998; Horne et al. 2002; Condelli et al. 2006; Nayak and Carpenter 2008). Overall, the electrophoresis results support the hypothesis that individual differences in parotid gland secretory behavior influence the responsiveness to astringent phenolic stimuli. Analysis of the S1 sample protein profile indicated that HR subjects accumulate a higher concentration of the protein fraction with high phenol-sequestering ability (PRPs, cystatins, histatins) as well as a greater amount of glycosylated protein with lubricating properties (amylases, glycosylated PRPs) with respect to the LR group during resting conditions. In HR subjects, repeated stimulation induced a strong depletion of both these protein fractions thus lowering phenol sequestering capacity and the lubricating properties of saliva with a consequent exposure of the protein mucus layer to phenols. HR subjects showed a higher response than LR subjects to astringent stimuli in both experimental conditions, that is, after the 30-min rest subsequent to the first mechanical stimulation (TA: 3.0 g/L evaluation) and immediately after the second mechanical stimulation (4 TA 1.4 g/L sample evaluation). It seems reasonable to hypothesize that in the absence of oral stimuli the

parotid glands of HR subjects synthesize and accumulate large amounts of proteins that are massively secreted in response to oral chemical/mechanical stimulation. This basal parotid protein level might take time to be restored, thus resulting in a temporary lowering of the overall saliva defense level against phenolic compounds. Thereby, an increased response to astringent stimuli provides a warning cue.

Hypotheses related to physiological differences in salivary protein secretory pathways such as regulation of protein biosynthesis, storage granules formation, as well as sensitivity to exocytosis-inducing stimuli (Castle D and Castle A 1998; Turner and Sugiya 2002) can perhaps explain the different behavior of subject groups in response to astringent phenolic stimuli. Also morphological characteristics of the gland, such as size, might be taken into account for explaining subject group differences in protein parotid secretion after protracted oral stimulation (Ono et al. 2006).

Effect of salivary characteristics on responsiveness to astringency

Results from sensory evaluations support the 2-step model of salivary protein/dietary tannin interaction in astringency elicitation. The almost constant parotid protein output found in MR and LR subjects may prevent the loss of lubrication in the oral cavity and the consequent astringency elicitation that occurs in HR subjects. The increase in the HFC value found in the LR group after the second stimulation supports the sequestering role of proteins to precipitate phenols and their suppressing effect on astringency sensation (Shimada 2006; Nayak and Carpenter 2008).

Astringency is a very long-lasting sensation that exhibits a carryover effect upon repeated ingestion of astringent samples. As expected, the astringency ratings of all subjects regularly increased from the first to the fourth TA sample (Lyman and Green 1990). It is generally well accepted that the different layers of mouth proteins may bind in sequence with phenols upon repeated ingestions of astringent stimuli (Guinard et al. 1986). The gradual lowering of soluble salivary proteins upon subsequent TA exposures implies an increasing involvement of the deeper layer proteins, thus inducing the rupture of the lubricating film and the consequent increase in the perceived sensation. The results of the present study indicate that the ability to react to oral stimulation also affects the development of the carryover effect. Both HR and MR groups showed the same sensation buildup upon repeated TA sample exposures, whereas this effect was not observed in the LR group. The constant amount of soluble proteins after resting and after stimulated conditions in LR subjects could protect the lubricating mucous layer proteins from interacting with TA thus preventing the saliva lubrication loss and the astringency building up.

Several astringent phenols are also able to elicit both bitter and sour sensations (Thorngate and Noble 1995; Peleg et al. 1999; Siebert and Chassy 2003; Lee and Vickers 2008). The individual variability in salivary characteristics considered in the present study does not affect the sensitivity to either of these taste sensations. Experimental data from the present study indicate that individual traits responsible for differences in astringency response are not related to differences in response to bitter and sour stimuli. The lack of carryover effect for bitterness and sourness further underline the independent sensory pathways for tactile and taste sensations.

The results of our work contribute to a better understanding of the different roles of SP in astringency elicitation and support the 2-step mechanism proposed for this sensation. As already found for other animal species (Gho et al. 2007; da Costa et al. 2008), it appears that in humans proteins from parotid glands exert a protective role against dietary phenols and have an inhibitory effect on astringency perception. Furthermore, a new concept arises from our results. The overall analysis of both chemical and sensory data indicate that subject group variations for response to phenolic astringent stimuli are not related to the absolute amount of different salivary protein classes present in the mouth when the stimulus is experienced. The perceived intensity seems to depend on a subjective comparison between basal and stimulated oral conditions. It is possible to hypothesize that HR subjects are used to an extremely well-lubricated oral environment due to the high amount of both glycosylated and phenol-sequestering proteins accumulated in absence of orosensory stimulation. The decreasing of this secretory output due to prolonged stimulation could induce a lowering of the usual level of mouth lubrication further enhanced by the ingestion of the phenolic stimuli. On the other hand, LR subjects only experience the lubrication lowering caused by the phenols because the secretory output of their salivary gland system is only slightly affected by stimulation.

Further works need to be devoted to an accurate characterization of saliva proteome of subject groups with different astringency responsiveness.

Morphological and physiological studies on parotid gland function of subjects with different sensitivity to astringent phenolic stimuli should be performed to support the hypothesis formulated in the present work. The possible phenotypic variation associated to the responsiveness to astringent stimuli let to hypothesize a genetic base regulating the perception of this sensation as well as in the case of other tastants (Bartoshuk et al. 1994; Mennella et al. 2005; Hayes et al. 2008; Mizuta et al. 2008). Finally, because food preferences can be influenced by phenotypic variations (Duffy 2007; Keskitalo et al. 2007; Hayes and Duffy 2008), studies on dietary habits for foods varying in astringency in subject groups with different sensitivities to this sensation can be envisaged.

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