Sensory Aspects of Astringency and Changes of the Oral Environment in the Mechanism of Astringency

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ABSTRACT

Astringency, a tactile sensation felt in the mouth after exposure to various foods, is not well understood. The underlying mechanism is not fully known, and it remains a challenging attribute to assess in sensory tests. Additionally, while most food astringency is caused by polyphenolic compounds, the cause of astringency in some other foods is not known. For the first part of my research, my objective was to improve the understanding of the mechanism of astringency by determining if it was related to a loss of saliva's ability to lubricate, the precipitation of specific classes of salivary proteins, or to the removal of oral lubricating films that coat the inside of the mouth. My results show that while astringency may be related to the latter, astringency is not related to a loss of salivary lubricity or the precipitation of any one class of salivary proteins. The second part of my research aimed to improve palate cleansing strategies for astringent foods. Palate cleansers are often used in an attempt to reduce build-up of astringency intensity that occurs over repeated exposures to astringent foods, but it is unknown how, or if, commonly used palate cleansers affect a person's sensitivity to astringency. Although I did not find that any of the cleansers were superior in their ability to limit the build-up of astringency intensity, it was clear that panelists were better able to discriminate among the astringency of various strength solutions when they used nothing or water to cleanse their palates. The aim of part III of my research was to determine the cause of astringency in acidic whey protein beverages. Although some believe that the whey proteins directly cause the astringency, there was reason to suspect that their high acid concentration was instead responsible. The results of my study found this to be true.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1
INTRODUCTION

The research presented in this dissertation has several distinct components that touch upon many areas of food science; one even delves into matters that are more typical of research performed in dental schools. The common thread that ties it all together is the sensory attribute of astringency.

Some of the work presented here was funded by a grant to explore the cause of astringency in acidic whey protein beverages. Although this work is presented in the last chapter of this dissertation, it was the first study completed. It was during this study that I recognized the lack of knowledge about the mechanism of astringency and the best sensory approaches to measure its perceived intensity in foods. My activities in the past four years have sought to answer these unknowns.

The goal of my first study was to understand the cause of astringency in acidic whey protein beverages. At the time I started this study, there were several 'protein water' beverages on store shelves that were relatively new to the market. They contained low levels of whey proteins of around 1% and were acidified to a pH of 3.4 using phosphoric acid. They were all markedly astringent. Although low levels of astringency are accepted in some products, like red wine, astringency in the acidified beverages was undesirable and may be the reason why they've since been discontinued. If the cause of astringency in these beverages could be determined, food scientists would be given direction towards reducing or eliminating it.

Because my panelists in the acidified whey protein beverage study had to sample many highly astringent beverages in one session, I questioned what cleansing and sampling protocol they should use. The second study I ran, on palate cleansing protocols for improved discrimination among astringent samples, was born from this question. Because I was most interested in knowing if there were differences in the astringency

among the solutions I was serving, I wanted my panelists to be able to have the best discrimination ability possible. Current literature on palate cleansing strategies and timing of samples was lacking and did not provide the answers I was looking for. Palate cleansing protocols in astringency research were focused on determining which cleansers reduced the astringency carryover, but they generally did not consider the flip side: did they still allow for adequate discrimination among samples? Perhaps they were effective at reducing astringent simply because they were masking it.

My last study, and certainly the most complex and challenging part of my research, was fundamental research aimed at understanding the cause of astringency. For years, based on the study of tannins, the key to understanding astringency focused on the reaction between tannins with a class of salivary proteins called the PRPs. While that reaction most certainly affects the intensity of the astringency experienced when consuming tannin-containing foods, I believed that the answer to understanding astringency needed to look beyond that and into other areas including the effects of astringency on saliva's characteristics, the lubricating coatings in the mouth, and on oral tissues directly. Although ultimately I did not get a chance to study everything that I wanted to (graduation eventually became more appealing to me), progress was made towards a better understanding of astringency.

LITERATURE REVIEW

Astringency

Astringency is an enigmatic sensory attribute present in many foods and is described as a drying-out, roughening, and puckery sensation felt in the mouth (Lee & Lawless, 1991). At low levels, such as experienced with many wines and teas, astringency can add a desirable complexity to foods; at high levels, however, people find the sensation unpleasant and avoid consuming the food (Lesschaeve & Noble, 2005). Unfortunately, many of the foods that are highly astringent are also nutritious. These include foods such as fruits, fruit products, teas, and soy products, all of which contain polyphenolic compounds (Haslam & Lilley, 1988) that are reported to have antioxidant properties and may provide protection against cardiovascular disease in humans (Fraga, 2007). Other nutritious foods, including beverages made with whey proteins, can also be highly astringent (Beecher, 2006; Sano et al., 2005). In foods such as wines, teas, and fruits, astringency is attributed to the polyphenolic compounds they contain. The cause of astringency in other foods, like whey protein beverages, remains unclear.

The overwhelming majority of studies on astringency support the notion that astringency is primarily a tactile sensation. In a simple and clever experiment to prove tactility of the sensation, Breslin et al. (1993) tasked subjects with distinguishing between astringent and non-astringent mixtures in two situations: when solutions were applied to the inside upper lip – a non-gustatory surface – and when their tongues were dipped into the solutions. Subjects could correctly identify astringency when solutions were applied to the inner upper lip and the surfaces were moved against each other, but not when their tongues were dipped in the solutions.

The term astringency is derived from the Latin words ad (to) and stringere (bind) and its definition has many variations. In pharmacology, astringent compounds are defined as those that bind to and precipitate proteins. The medical field defines astringents as compounds, such as styptics, that cause a constriction of organic tissues. The American Society for Testing and Materials (ASTM) has stated that astringency is

"the complex of sensations due to shrinking, drawing, or puckering of the epithelium as a result of exposure to substances such as alums of tannins". Although the formation of protein-astringent complexes is believed to play a role in the development of astringency, an understanding of the fundamental physiological mechanism underlying the astringent sensation caused by foods remains unknown. If astringency's mechanism can be elucidated, food producers will gain knowledge that will enable them to manufacture, alter, or treat the food in a way to retain its healthful attributes while minimizing the astringency. This knowledge will also benefit sensory scientists interested in the intensity perceptions of the sensations and will allow them to devise improved methods of palate cleansing for the assessment of the attribute.

The Mechanism of Astringency

The binding of salivary proline-rich proteins may instigate astringency

Astringency is said to be engendered by four general classes of compounds including polyphenols, metal salts, acids, and dehydrating agents such as alcohols (Green, 1993), though much of what is known about the mechanism of astringency is based on studies of tannins, which are bitter, astringent plant-based polyphenols. Though polyphenols are known to reversibly complex with proteins, polysaccharides, nucleic acids, and certain alkaloids (Haslam et al., 1986), astringency research has concentrated on the interaction of polyphenols with proline-rich proteins (PRPs), a specific class of salivary proteins that are high in the amino acid proline. This focus can be traced back to the discovery that the production of a class of proteins rich in proline was greatly increased when rats and mice were fed diets high in astringent tannins, and that the deleterious effects of the tannins including lost body weight abated once PRP concentration of the rodents' saliva was increased (Mehansho et al., 1987). Additionally, mice treated with beta-adrenergic agonist in order to increase their concentration of salivary PRPs have been shown to lose their aversion to 0.5M tannic acid solutions (Glendinning, 1992). Mehansho and Glendinning together provided evidence of a relationship between tannins and PRPs that seemed to suggest that high levels of salivary PRPs prevent or minimize astringency; however, since tannins are also strongly bitter

(Drobna et al., 2004), it is possible that the mice were simply avoiding the bitter taste of the tannins.

Astringent tannins have been shown to be capable of binding human salivary PRPs (Baxter et al., 1997; Kallithraka et al., 1998; Gambuti et al., 2006), but few studies have explored whether PRPs are precipitated by other astringent classes including acids and metal salts. de Wijk & Prinz (2005) have reported that pilot trials in their lab indicated that PRPs are not precipitated by aluminum potassium sulfate (alum), a common astringent metal salt. Recent publications have reported that manufactured foods containing whey and soy are astringent, particularly acidic beverages containing whey protein isolates (Beecher, 2006; Sano et al., 2005). Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) techniques, Beecher found no evidence that PRPs were precipitated by acidic whey protein beverages. To date, no studies on astringency have explored the interaction between acids and salivary PRPs. In general, it is not clear whether all astringent compounds, or only polyphenols, bind to and precipitate salivary PRPs.

Determining if all classes of astringents are capable of precipitating the PRPs will provide an important clue towards understanding the mechanism of astringency, since it is possible that the mechanism is common among all astringent compounds. A common modality of the sensation is likely because sub-quality attributes of astringency, including dryness and roughing, have been found to describe equally well the sensations caused by alum, tannic acid and tartaric acid (Lee & Lawless, 1991). Additionally, the time-course of the astringent sensations caused by these compounds show only very subtle differences, suggesting a similarity in the underlying physiology of the mechanism (Lee & Lawless, 1991). If PRPs are not precipitated by other astringents, there must exist another explanation for the underlying or principle cause of astringency that leads to the astringent sensation.

Establishing whether or not other astringents bind and precipitate the PRPs will also help to clarify the role that the binding reaction plays in the mechanism of astringency. Although it has long been known that astringent polyphenols bind and precipitate PRPs, it is unclear if this binding reaction directly results in astringency or if it

is protective and prevents its development. Analyzing saliva collected from subjects before and after they tasted wine, Kallithraka et al. (2001) found several significant and positive correlations between astringency ratings and the decrease in a specific salivary PRP protein fraction, suggesting that the binding reaction was causing astringency. However, they also observed the opposite trend with a second PRP fraction and found that astringency ratings were lower as the precipitation of this PRP increased. Recent work by Schwarz & Hofmann (2008) suggested that the PRP-binding reaction is protective. They assayed 13 polyphenolic solutions with saliva and found that some of the most astringent polyphenols had very low binding activities with salivary proteins. The relative astringency of the polyphenols seemed to be related to the amount of unbound polyphenol stimuli in the mixtures, although the astringency rankings of the polyphenols they used were obtained by a questionable 'half-tongue test' method (Scharbert et al., 2004), which likely ranked the compounds in order of their bitterness rather than astringency. Regardless, it is not at all clear if the precipitation of PRPs contributes to astringency development. If other astringents, including acids and alum, do not precipitate the PRPs, it can be established that their precipitation is not required for astringency to develop.

There are two popular hypotheses proposing explanations for the mechanism of astringency. One is that the precipitation of PRPs from saliva reduces its ability to lubricate, and this loss of lubricity is perceived as an increase in oral friction (Clifford, 1997). A second hypothesis suggests that the sensation is caused by a direct effect of astringents on the oral epithelium and that PRPs play a protective role and prevent astringency by binding the astringent compounds before they can interact with the oral mucosa (Horne et al., 2002). Both hypotheses predict that people with high salivary flow rates and protein/PRP concentrations will report lower ratings of astringency. Several studies support this prediction (Fischer et al., 1994; Horne et al., 2002; Imm & Lawless 1996; Kallithraka et al., 2001).

Diminished salivary lubricity and astringency

Three studies have measured the effect that astringents have on saliva's lubricity in an effort to determine if astringency is related to diminished salivary lubricity. Prinz & Lucas (2000) found that adding tannic acid to pooled saliva in vitro decreased its viscosity and increased friction, supporting the hypothesis. Conflicting evidence, though, was later obtained from the same lab when using a different friction tester; de Wijk & Prinz (2005) observed a decrease in friction when pooled saliva was mixed with tannic acid, suggesting that saliva's lubricating ability was actually improved when mixed with the polyphenols. They also observed that mixing alum into saliva increased the coefficient of friction, the opposite effect of tannic acid. More recently, Rossetti et al. (2009) found that epigallocatechin-gallate, an astringent tea polyphenol, diminished salivary lubricity while another, epicatechin, did not alter the lubricating properties of saliva from an individual. The inconsistent findings of these studies seem to suggest that a loss of lubricity is not required for astringency to develop, but they may instead indicate that instrumental methods used to assess salivary lubricity are inaccurate or have been too variable to be comparable.

Measuring Salivary Lubricity

Lubrication and Friction Defined

The Modern tribology handbook defines tribology as the study of the interaction between surfaces that are in contact with each other, and it encompasses the concepts of friction and lubrication (Bhushan, 2001). Friction, a phenomenon that is still not completely understood, refers to the force that resists the motion of the two surfaces that are in contact (Bhushan, 2001). High friction is sometimes desirable, e.g., when walking down a very steep hill, but is often undesirable too, such as when it causes premature wear in mechanical parts or when it experienced orally in mouth as may be the case with astringency. The friction, f, generated when two surfaces rub against each other can be drastically decreased by using a lubricant (Figure 1). In mechanical applications, such as

between parts of a motor or ball bearings, lubricants are often oil-based or synthetic polymers. In the mouth, saliva serves this purpose and lowers the friction between opposing hard and soft tissue contacts (Tabak, 1995).

The ability of a lubricant to reduce friction can be mathematically quantified by its coefficient of friction (COF, or μ) - a unitless parameter that can be described by the equation:

$$\mu = f/N \qquad \text{(Eq. 1)}$$

where:

f = frictional force required to move an object resting on a flat surface

N = applied normal force between the surfaces

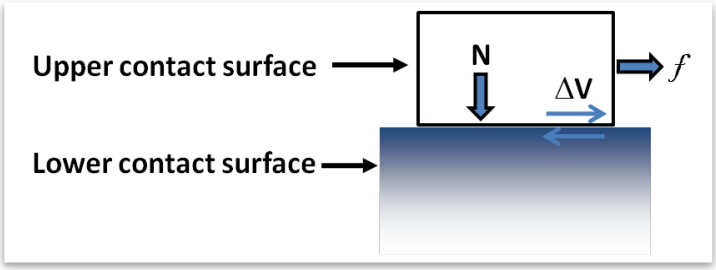

Figure 1. Tribological system
The friction force, *f*, required to move an object resting on a flat surface is a function of the materials of the contact surfaces, the normal force (N) applied, and the speed that the two surfaces travel relative to one another (ΔV). Lubricants can be used between contact surfaces to lower the friction force.

A lubricant's COF is a measurement that is specific to a particular tribological system and generally should only be compared to other lubricants tested on the same system (Barwell & Milne, 1951). When measured on the same tribological system (i.e., friction tester), a greater COF indicates that the lubricant is not as effective and that there is higher friction, *f*, between the contact surfaces. Changing any of the tribological parameters – relative speed between the contact surfaces (ΔV), normal force applied (N), contact materials, etc. – can have different effects on lubricants. The effectiveness of a

lubricant is a function of all the parameters, and the most effective lubricant under one set of conditions may not be the best lubricant under another.

Instrumental Measurements: Friction Tester Design Considerations

Accurate characterization of changes in salivary lubricity in response to astringents poses a number of challenges, especially because the tribological system that needs to be modeled is biological. Careful consideration of and control over the many variables that could potentially impact the friction measurements is critical and extends beyond the selection of the system's contact materials and running parameters like speed and force. When trying to model a biological system such as the mouth, the source of the lubricant – the saliva – is another important factor that can impact the resulting friction measurements.

The source of saliva used in any experiment is an important consideration since the concentrations and relative proportions of proteins in saliva can vary greatly across individuals and collection procedures (Tenovuo, 1989). Most studies have collected stimulated saliva for their experiments, and this seems like a natural choice since astringency is experienced during eating, which is a stimulated condition. Since the protein content and profile of individuals' saliva can vary greatly among people, it is likely wise to use pooled saliva samples. Rossetti (2009) used saliva from a single male subject to conduct all experiments, but the single sample may not be representative of the overall impact astringents have since it is known that individuals vary in their perceptions of astringency (Fischer et al., 1994; Horne et al., 2002; Imm & Lawless 1996).

Secondly, the friction system that is used to collect the measurements needs to be biologically relevant in order to obtain meaningful data. Biological relevance in a tribological system includes the application of forces and speeds that mimic *in vitro* conditions. Forces and speeds experienced in the mouth have been measured and friction testers can be easily designed to apply similar parameters. Ranges of forces and speeds experienced between soft tissues under typical eating, swallowing, and sample evaluation conditions are between 0.3-8N and 1-10mm/s, respectively (Prinz et al., 2007; Ono et al., 2004; Reeh, 1993). Unlike the ranges in the mouth, however, friction testers generally

operate at finite forces and speeds, and comparing saliva mixtures at discrete settings can lead to inaccurate conclusions regarding their ability to lubricate under physiological conditions. For example, Malone et al. (2003) were able to correlate perceived slipperiness of several guar-gum solutions to mechanical friction measurements only when the speed of the friction tester was between 10 to 100mm/sec while applying a 3N load. When speeds were above and below this range, correlation between sensorial and instrumental measurements was poor. Additionally, it is known that a specific lubricant's coefficient of friction has a unique relationship with the stress conditions (speed and load) applied during testing (Barwell & Milne, 1951); this is to say that while one lubricant may have a lower coefficient of friction compare to others at a particular speed and load, the same ranking may not hold true when the load or speed is changed. Therefore, careful consideration of the most appropriate load and speed to use when trying to mimic the motions subjects may use when assessing astringency is imperative to obtaining meaningful data. This knowledge sheds additional light on the inconsistent results among the three experiments that tested the hypothesis that astringency was related to a loss of salivary lubricity. In both the Prinz & Lucas (2000) and De Wijk & Prinz (2005) studies, the data were collected using different friction testers, both of which were run at a single speed and load. In both studies, the actual speed and load applied were unreported and likely unknown to the experimenters. In Rossetti et al. (2009), a normal load of 1N was applied and the relative speed between the contact surfaces was 5mm/sec; these values are comparable to physiological levels reported in literature.

Biological relevance in a tribological system also includes the proper choice of contact surfaces, although there seems to be no consensus on what that should be when mimicking the mouth. There has been little consistency or even justification in what researchers have used for the contact materials even though it is known that the materials influence the friction measurements (Ranc et al., 2006b). In studies on astringency, contact materials have included steel against latex (Prinz and Lucas, 2000), metal (unspecified type) against rubber (deWijk and Prinz, 2005), and polydimethylsiloxane (PDMS), which is a type of silicon-based organic polymer, against PDMS (Rosetti et al., 2009). Glass, gel agarose, and mica surfaces, among others, have been used in studies on

food lubrication and slipperiness. Often times, the materials are chosen because they have a hardness or roughness similar to oral tissues. In a recent study that investigated the effect of surface structure on friction measurements, hard steel and Teflon were both tested against a soft silicone surface. The hard materials was chosen to represent the palate; the soft silicone was chosen because of its similar pliability and roughness compared to the human tongue (Ranc et al., 2006b). In a few studies, biological surfaces, including pig tissues and tooth enamel, have also been used.

It is very likely that the design of the friction tester also impacts the quality of the data collected. Although all friction testers are based on the same principal—produce motion and measure the resulting frictional force generated—they can vary widely on how this is accomplished. The style of motion can vary among friction tester designs - reciprocating vs. continuous and rotating vs. linear motion – and the shape of the contact surfaces can also differ. In the three studies that have measured salivary lubricity as it related to astringency, two different tester designs were used. Both Prinz & Lucas (2000) and de Wijk & Prinz (2005) used boothroyd-style friction devices, which consist of a rubber band looped around the shaft of an electric motor. Friction between the band and the shaft was measured. Rossetti (2009) used a commercially available ball-on-disk friction tester to assess saliva's lubricity when exposed to astringents. In their tester, both the sphere and disc rotated at different speeds relative to each other to achieve a relative speed difference. It is unknown how the different styles of motions and contact surface shapes affect the quality of the data, but it is highly probable that differences in these tester designs contributes to inconsistent findings among the studies.

Measuring Salivary Lubricity using Human Subjects

Often times, instrumental measurements of mechanical or rheological food properties are obtained in an attempt to predict the sensory attributes of that food, but as Brennan (1980) states:

> *"Nonsensory techniques can never be more accurate than sensory methods. The accuracy of the former can only be judged by their ability to predict the sensory quality being studied."*

Thus far, friction measurements obtained using saliva-astringent assays as lubricants have been unable to explain the sensory attribute of astringency. This could be because:

1. Instrumental methods used to assess salivary lubricity are accurate but perceptions of astringency are unrelated to a loss of salivary lubricity
2. Instrumental methods used to assess salivary lubricity are inaccurate

In regards to the latter, instrumental measurements of friction have been successfully correlated to another percept of oral friction – 'slipperiness' – by other researchers. Malone et al. (2003) were able to find a direct, inverse correlation between the slipperiness of four model guar-gum solutions to instrumental measurements of their friction. Earlier work by Kokini et al. (1977) found that slipperiness perception was inversely related to a combination of both viscosity and friction (i.e., total force) in 11 model gum-based solutions and 5 syrupy foods, Figure 2. The success of these studies in correlating instrumental friction measurements to slipperiness suggests that friction measurements could accurately assess salivary lubricity; however, recent work out of our lab has not been able to relate slipperiness to friction when testing a wide array of commercially available food products, Appendix 5C. Thus, from our perspective, it is unclear if oral friction – whether it is from changes in salivary lubricity or from a food's slippery inducing qualities – can be measured instrumentally.

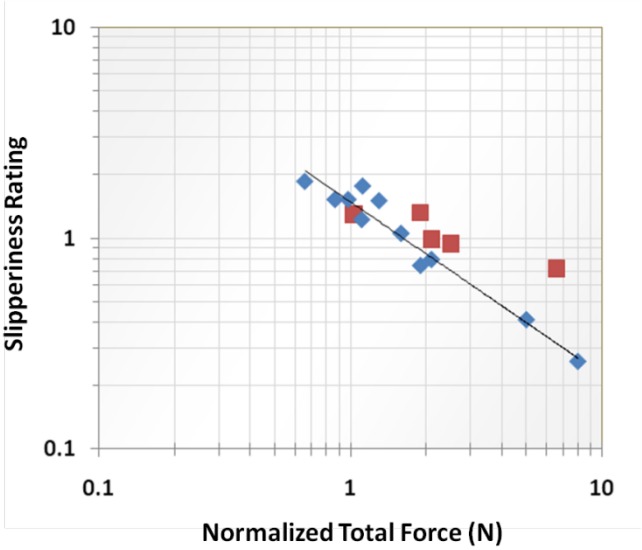

Figure 2. Relationship between slipperiness and total force of various foods
Normalized total force includes viscous plus frictional force. Foods tested were 11 gum-based solutions (◆) and 5 foods including honey and four food syrups (butterscotch, pancake, vanilla and chocolate) (■). Graph recreated from data in Kokini et al. (1977).

Another approach to determining if salivary lubricity is affected by astringents is to have panelists assess the lubricity of saliva using their fingers. This avoids many of the limitations of the instrumental approach and still allows salivary lubricity to be assessed independent of other oral effects that may occur with astringency like the loss of the mucus coating or directly binding of the astringent to the oral mucosa. It also avoids confusion panelists may have between astringency and the tastes that may accompany it, like bitterness and sourness. Before this approach is taken, however, it would be important to establish that panelists are equally sensitive to differences in friction or slipperiness when they use their fingers to assess lubricants or foods compared to when they used their mouths. Additionally, even if a friction tester can detect a difference in salivary lubricity, it is still unknown whether the effect is of any practical significance. In this situation, determining if panelists can also feel the difference may indicate whether or not the change in salivary lubricity would be an important and contributing factor to the sensation of astringency in the mouth.

Astringents may bind salivary glycoproteins

Since all astringents are known protein precipitators and because of the similarity in the quality of the astringent sensation they induce, it is also possible that there exists a class of salivary constituents other than PRPs that are precipitated and result in altered oral tactile sensations (Bate-Smith, 1954). Another class that warrants consideration is the mucins, which are large salivary glycoproteins. Two mucins have been identified in whole mouth saliva: mucous glycoprotein 2 (MG2) - the low molecular weight mucins of 200-250 kDa, and MG1 - the high molecular weight mucins greater than 1000 kDa comprised of disfulfide-linked subunits (Tabak, 1990). It has also been suggested that additional glycoproteins are present in saliva collected from the buccal mucosa and other oral surfaces that are not found in whole mouth saliva (Proctor et al., 2006). The relative lubricating ability of mucins is greater than PRPs, and they are imperative for maintaining the viscoelastic properties of saliva (Slomiany et al., 1989). Precipitation of the mucins could affect saliva's rheological properties, possibly leading to reduced lubricity. Many astringent compounds, including acids and tannins, have been shown to precipitate mucins (Dawes, 1964; Pizzolato & Lillie, 1973), though this relationship has not been directly explored in the context of astringency.

Studies on astringency have focused solely on the interaction of astringents with PRPs though it is possible that mucins are involved. Gambuti et al. (2006) found evidence that tannins do precipitate mucins when analyzing tannin-saliva assays using SDS-PAGE, but because the focus on the study was on other salivary proteins including PRPs, they largely ignored this information. Exploring the effects that different classes of astringent compounds have on the precipitation of both PRPs and mucins, in combination with studying the effects on lubricity, will clarify the role of salivary proteins and lubricity in the mechanism of astringency.

Astringents may remove lubricating oral coatings and cause desquamations of the oral mucosa

Mucins are a primary structural component of the acquired salivary pellicle and the oral mucosa mucus coat that cover the surfaces of the hard and soft tissues, respectively, within the oral cavity (Tabak, 1990; Hannig & Joiner, 2006). Besides offering protection from exogenous chemicals, microbes or mechanical damage (Tabak, 1990), the other major function of these coatings is to provide lubrication between the oral surfaces (Bertram, 1967; Hannig & Joiner, 2006). When it is altered or lost, such as in patients with salivary dysfunction, sensations of oral dryness are a common complaint (Wolff et al., 1990).

The acquired salivary pellicle, which coats the hard tissue surfaces of the mouth including the teeth, is comprised mostly of proteins, glycoproteins and lipids that are selectively absorbed from saliva (Hannig & Joiner, 2006). The exact composition of the pellicle on teeth differs slightly throughout the mouth and is related to the composition of the saliva prevalent in the immediate vicinity (Carlen et al., 1998). The pellicle provides lubrication between the teeth and the mucosa and has been shown to reduce the coefficient of friction between opposing teeth by a factor of 20 (Berg et al., 2003). This has led others to conclude that it is the pellicle layer that is mostly responsible for lubrication of the mouth rather than the bulk salivary fluid (Hannig & Joiner, 2006). The ability of the pellicle to effectively lubricate has been attributed to MG1 and MG2, the two glycoproteins of saliva, along with statherin, an 8 kDa salivary protein also found within the pellicle (Hannig & Joiner, 2006). Some have suggested that other proteins, including PRP-1, also have pronounced lubricating effects (Hahn Berg et al., 2004).

The oral mucosal mucus coat is a visco-elastic hydrogel that covers the soft surfaces in the oral cavity. Approximately 95-99% of the mucus coat is water by weight, but it is the mucins that give the coat its gel-like structure and characteristic properties (Slomiany et al., 1986). Mucins are the second most abundant constituent of the gel layer, comprising 1-5% of its total weight (Slomiany et al., 1986), with other constituents including salivary proteins, lipids, and electrolytes. It is unknown if the mucins interact

specifically and directly with the soft tissues of the mouth or whether they passively coat the surfaces (Tabak, 1995). The coat's thickness is estimated to be 0.07 to 0.1mm (Collins & Dawes, 1987).

The mucus coat that lines the mouth covers the oral epithelium, which serves to protect underlying organs and tissues from environmental insults. The oral epithelium is a stratified squamous epithelium consisting of flattened (squamous) cells in many layers upon a basement membrane (Nanci & Ten Cate, 2003). Structural integrity is maintained by a process consisting of continual cell renewal within the deepest layers, upward migration of maturing cells, and the eventual shedding, or desquamation, of the cells at the surface (Nanci & Ten Cate, 2003). Cell turnover time, from cellular replication to desquamation, is estimated to be 4-14 days (Schubert, 2008).

Although the role of the pellicle, mucus coat, or the desquamation of the oral mucosa in the context of astringency has never been explored, the qualities of the sensation and its temporal profile fit with the idea that astringents remove part or all of these coatings and layers. Perceptually, astringency has been described as including a drawing and tightening sensation sensed inside the mouth plus a feeling of dryness or roughness experienced during movement (Lawless et al., 1994). An increased loss of surface cells and the mucus coating and also the pellicle could conceivably result in these sensations since there would be a loss of oral lubrication throughout the mouth and hydration layer on the soft tissue surfaces. The manner in which astringency intensity builds over repeated exposures also fits with this theory. Guinard et al. (1986) showed that repeated sampling of wine caused an increase in the intensity and duration of the astringent sensation. This is most likely the case if astringents remove more of the pellicle or mucus coat and surface cells upon repeated exposures, leading to heightened intensities and extending the time required to return to a baseline state. A third reason to suspect that these coatings may be stripped away by astringents is the long-lasting nature of astringency, which suggests that a physiological process must occur within the mouth to reestablish baseline conditions. Although little is known about how the mucus coat forms on soft tissues, the pellicle layer has been shown to reform in various stages, with the initial stage taking place over a period of 30 seconds to 3 minutes (Hannig & Joiner,

2006). In the second stage, which lasts a few more minutes, biopolymers from saliva adsorb onto the surface to form a thicker layer (Hannig & Joiner, 2006). Lastly, many of the palate cleansers that have been found to alleviate astringency, like pectin and carboxy-methyl cellulose, have also been identified as bioadhesive polymers (Salamat-Miller et al., 2005). These compounds are able to coat and stick to the oral mucosal lining, in essence replacing the coatings that are perhaps stripped away by astringents.

Palate Cleansers for Astringent Foods

Astringency is a difficult sensory attribute to evaluate, owing to particular characteristics of the sensation. Described as a drying-out, roughening, and puckery sensation felt in the mouth (Lee & Lawless, 1991), astringency is engendered by four general classes of compounds including polyphenols, metal salts, acids, and dehydrating agents such as alcohols (Green, 1993). The feeling can take over 15 seconds to fully develop and is known to build in intensity and become increasingly difficult to clear from the mouth over repeated exposures (Guinard et al., 1986; Lyman & Green, 1990), rendering popular tests like the 2- or 3- alternative forced choice (AFC) or triangle test unsuitable.

Various sensory approaches have been suggested as means to overcome or avoid artifacts introduced into the data from these issues. One suggested approach is the use of statistics to unearth "true" ratings without carryover artifact, but this does nothing to reduce the cumulative effects of the sensation that can lead to taster fatigue (Arnold, 1983). A second option is to delay subsequent tastings of astringent samples until the oral environment has returned to a baseline state. Guinard et al. (1986) has shown that increasing the intersample period from 20 to 40 seconds during repeated exposures reduces but does not eliminate the carryover effect. Lyman & Green (1990) saw large carryover effects in samples spaced 1 minute apart. Extension of the interstimulus period to 5 minutes while rinsing with water has been shown to mostly eliminate carryover (Lee & Vickers, 2008), though a slight, but not significant, trend of increasing ratings was still observed. Astringency has been reported to linger for over 6 minutes in some instances

(Lee & Lawless, 1991). Forcing panelists to wait such long times is not practical as it limits the number of samples that can be assessed in a single session and likely leads to a loss of stimuli memory, particularly with panelists unfamiliar with astringency.

Another approach that can be employed to avoid the introduction of artifacts into astringency ratings is to use a palate cleanser between samples in an attempt to reestablish baseline oral conditions; several palate cleansers have been identified for their ability to alleviate astringency. Breslin et al., (1993) hypothesized that because astringency was a tactile sensation, lubricating rinses should markedly decrease astringency. They found that six rinses including sucrose, corn oil + xanthan gum, Salivart® artificial saliva, subjects' own saliva, water, and alum immediately reduced the sensation of astringency induced by 10 g/L alum solutions. A corn oil + xanthan gum rinse had the most impact; water had the least. All of the rinses, however, were ineffective at preventing build-up over repeated samplings. Brannan et al. (2001) also found a corn oil-xanthan gum solution to be most effective at reducing astringency immediately after panelists sampled a 1 g/L solution of alum, but they later observed that discrimination in a 2-AFC test was improved when panelists rinsed using a 0.55% CMC solution instead. The data from Brannan et al. (2001) suggested that some of the rinses may have been masking astringency and decreasing panelist sensitivity. Upon a replicate sampling of a 1 g/L alum solution, astringency ratings of the second presentation decreased when any of 5 viscous gum and gum-oil rinses were used between the two samplings. Only when water was used as the rinse did astringency ratings increase for the second presentation. Others have found CMC, along with pectin and crackers, to be effective palate cleansers based on their ability to reduce the lingering intensity of astringency (Colonna et al., 2004; Ross et al., 2007).

An ideal palate cleanser for astringency would eliminate or minimize the buildup of the sensation over repeated exposures, and it would enhance discrimination of the astringency among samples. Most studies that have explored the use of palate cleansers for astringency have focused solely on astringency reduction, but the data of Brannan et al. (2001) show that cleansers capable of reducing astringency can mask the sensation during subsequent tastings. Although astringency reduction is a desirable feature of a

palate cleanser, impairment of perception in subsequent samples is extremely undesirable.

Astringency of Whey Protein Beverages

The use of whey proteins in beverages is becoming increasingly common because of their high nutritional value and wide functional versatility. Though whey proteins are soluble over a wide pH range, whey-protein beverages are often formulated at low pH for improved beverage clarity and stability (Miller, 2007). These acidic whey protein beverages, however, have been shown to be astringent (Sano et al., 2005; Beecher et al., 2008; Kelly et al., 2010; Vardhanabhuti et al., 2010), which may limit consumer acceptance of these products.

Some studies that have investigated the astringency of whey protein beverages have concluded that the whey proteins are directly responsible for their astringency. Sano et al. (2005) hypothesized that the whey proteins, which are highly soluble at the low pH levels found in the beverages, precipitate when sips of beverage are taken and the pH rises to around 5. He suggested that astringency is the perception of the whey protein precipitate. Others have suggested that at low pH, positively charged whey proteins are capable of binding with and aggregating salivary proteins, causing astringency (Beecher et al., 2008; Kelly et al., 2010). The studies used primarily sensory-based methods to investigate the cause of astringency in the beverages. Though the approaches used in these studies were generally valid, conclusions were based on comparisons between acidified whey protein beverages to control solutions matched for either pH and buffering capacity (Beecher, 2006), pH and phosphate concentration (Kelly et al., 2010) or total protein content (Sano et al., 2005). They neglected to use controls matched for total acidity, which would have shown in the acid content of the beverages was causing their astringency.

The astringency of acidic whey protein beverages could also be caused by the acid they contain. The previous studies that have implicated the whey proteins as being responsible for beverage astringency used control beverages that did not account for the total acidity of the beverages, nor did any of the studies account for or measure the

differences in the buffering capacity or titratable acidity of any of the solutions. Acids are known astringents (Corrigan Thomas & Lawless, 1995; Lawless et al., 1996; Sowalsky & Noble, 1998), and the effectiveness of the whey proteins to buffer necessitates a high acid concentration to reduce the beverage's pH to targeted levels. Researchers have postulated that the mechanism of acid astringency is directly related to their acidic properties (Lawless et al., 1996), and it has been shown that even when buffered, acids are capable of precipitating salivary proteins (Dawes, 1964). If the acids do cause the astringency of the whey protein beverages, it seems plausible that buffering of acids by whey proteins would not necessarily decrease their astringency.

In a very recent study out of the same lab as the Beecher et al. (2008) and Kelley et al. (2010), there is indication that the high acid content of the beverages is, at least partially, causing their astringency. Results from Vardhanabhuti et al. (2010) suggest that the high astringency in acidified protein solutions is related to their low pH and high buffering capacity. In their study, the astringencies of 4% beta-lactoglobulin (β-Lg) solutions acidified with phosphoric acid to pH levels of 3, 4, and 6 were compared to carefully formulated phosphate buffer controls that were matched for pH and phosphate content. Titratable acidities were measured for all solutions. Titratable acidities and of the β-Lg-containing solutions were much higher than those of the controls, and the β-Lg solutions were also more astringent.

In addition to their sensory-based experiments, Beecher (2006) and Vardhanabhuti et al. (2010) used electrophoretic methods in an attempt to understand the interactions between salivary proteins and whey proteins beverages. While Beecher (2006) was unable to find any differences in the salivary protein profiles of saliva samples mixed with various acidified whey protein beverages, Vardhanabhuti et al. (2010) observed significant mucin precipitation when saliva was mixed with 4% β-Lg solutions at pH levels of 2.6 and 3.5. After mixing, the pH values of the saliva-β-Lg solutions increased to 3.7 and 5.3, respectively. When the pH of pure saliva was reduced to a pH of 3.7 and 5.3 using phosphoric acid, the mucins did not precipitate. Thus, Vardhanabhuti et al. (2010) concluded that charged β-Lg molecules precipitated the mucins; however, the comparison between the saliva-β-Lg mixtures to the acidified pure

saliva may not have been the right one to make. In the mixtures, the saliva was added into a protein-buffered system that started at a low pH (2.6 or 3.5). The salivary proteins buffered the acid in the solution, causing the pH to rise. For the acidified pure saliva, saliva (likely at a near-neutral pH) was lowered to a pH of either 3.7 or 5.3 and was never subjected to high acid concentrations or a low pH. Furthermore, if β-Lg was responsible for precipitating the salivary mucins, there should have been an increased concentration of β-Lg observed in the precipitant samples that were electrophoresed, and this did not appear to be the case.

Research Objectives and Hypotheses

Part I: The role of salivary proteins, diminished lubricity, and changes of the oral mucosa in the mechanism of astringency

Objective: To gain a better understanding of the mechanism of astringency by determining if the astringency of three distinct classes of compounds (polyphenols, acids, and metal salts) is related to:

1. The precipitation of common salivary protein fractions
2. A change in salivary lubricity
3. Desquamation of the oral mucosa and removal of the oral coatings

Hypothesis I.1:

The three classes of astringent compounds tested (polyphenols, acids, and metal salts) will bind with and precipitate salivary mucins, and higher concentrations of astringent solutions will cause greater losses of mucins from saliva. Salivary PRPs will not be precipitated by all classes of astringent compounds.

Hypothesis I.2:

Salivary lubricity will diminish at low levels of astringency, but the reduction will be limited and will not continue with higher concentrations of astringent solutions.

Hypothesis I.3:

Astringent compounds will cause desquamation of the oral mucosa and a loss of the mucus coating. Higher astringency will be related to a greater loss of these coatings.

Part II: Palate Cleansers for Astringent Foods

Objective: To improve sensory methodologies for the assessment of astringent foods by characterizing the usefulness of various palate cleansers. They will be evaluated on their ability to limit astringency carryover during repeated exposures and to improve discrimination among samples during sensory tests.

Hypothesis 2:

Common palate cleansers used to alleviate astringency will reduce carry-over but diminish the ability to discriminate between astringent samples. Stimulation of saliva flow between samples will result in the greatest degree of discrimination among samples.

Part III: Astringency of Acidic Whey Protein Beverages

Objective: To determine if the acid in whey protein beverages, and not the whey proteins directly, is responsible for their astringency.

Hypothesis 3:

Acidic solutions made with and without whey protein will be equally astringent when formulated at equal acid concentrations, and whey protein solutions will precipitate the same salivary proteins as their acid-only matched solutions.

CHAPTER 2

Part I: The Role of Salivary Proteins, Diminished Lubricity, and Changes of the Oral Mucosa in the Mechanism of Astringency

Introduction

The purpose of this multi-phase study was to gain a better understanding of the mechanism of astringency by determining if it was related to: (1) the precipitation of common salivary proteins; (2) diminished salivary lubricity; or (3) removal of lubricating films from the oral cavity. The hypotheses and the procedures used to test them are described in the following sections. Data were gathered through a variety of methods.

To determine if astringency was related to (1) the precipitation of common salivary proteins, precipitates and supernatants from saliva-astringent mixtures were analyzed for PRP and mucin content through electrophoretic methods; to determine if astringency was related to (2) diminished salivary lubricity, instrumental frictional measurements and subjective assessments of friction were collected on saliva-astringent mixtures; to determine if astringency was related to (3) desquamation of the oral mucosa and loss of the mucus coat, panelists were recruited to swish and expectorate astringent samples, which were analyzed for mucins using electrophoretic methods and for DNA content using fluorescence spectroscopy.

I.1 The precipitation of common salivary protein fractions

Introduction

The objective and hypotheses of this phase of the study were as follows:

Obj. I.1: To determine if the astringency of three distinct classes of compounds (polyphenols, acids, and metal salts) is related to the precipitation of common salivary proteins.

Hypothesis I.1: The three classes of astringent compounds tested (polyphenols, acids, and metal salts) will bind with and precipitate salivary mucins, and higher concentrations of astringent solutions will cause greater losses of mucins from saliva.

Hypothesis I.2: Salivary PRPs will not be precipitated by all classes of astringent compounds.

Overview:

Electrophoretic methods were used to characterize the salivary proteins present in the supernatants and pellets from mixtures of pooled saliva that were assayed with astringent solutions. Qualitative analysis of the protein patterns on polyacrylamide gels was used to determine which saliva proteins were precipitated by each of the astringent compounds.

Although all astringents precipitated some of the salivary proteins, there were distinct differences in the patterns of salivary proteins present in the supernatants and pellets from the various saliva-astringent mixtures. As expected, the tannins precipitated many of the PRPs. Alum was also shown to precipitate the PRPs, but there was no evidence that the PRPs were precipitated by HCl acid. Mucins were precipitated by the acid and by alum, but the tannins had little effect on mucin solubility. It is possible that the precipitation of salivary proteins is involved in the mechanism of astringency, but evidence collected here shows that the precipitation of PRPs is not requisite to the development of astringency.

Materials & Methods

Astringent solutions

"High" and "low" concentrations of alum (food processing grade, Barry Farm, Wapakoneta, OH, USA), tannins (Tanin VR Supra, Scott Laboratories, Petaluma, CA), and hydrochloric acid (Sigma Aldrich, St. Louis, MO) were prepared the day before experiments were run. Alum-high was dissolved into distilled water at 1.0 g/L. Tannin-high was made by adding tannins into distilled water at a concentration of 3.0 g/L and mixing the solution for 1 hour to dissolve the tannins. Acid-high was prepared by titrating 0.5M HCl into distilled water until the pH reached 1.9. The concentrations of the "high" solutions were chosen because they are known to induce astringency at high intensities (Lee & Vickers, 2010; Lee & Lawless, 1991). The lower concentration tannin and alum solutions were 1.5 and 0.5 g/L, respectively, and the lower concentration acid solution was at a pH of 2.2. All three of the "low" astringent solutions were made by preparing a two-fold dilution of the "high" concentration astringent solution. These lower concentrations are known to cause astringency at low to medium levels (Lee & Vickers, 2010; Lee & Lawless, 1991).

Saliva

Approximately 7mL of stimulated saliva was collected from 5 panelists simultaneously. Panelists were asked to refrain from eating, drinking, or brushing their teeth during the hour prior to the collection session. Upon arrival, they rinsed their mouths with distilled water and started chewing a 2 x 4 cm piece of Parafilm® (Pechiney Plastic Packaging, Chicago, IL). After an initial swallow, panelists continued to chew the wax while periodically expectorating their saliva into a 15-mL tube held on ice. The tubes were centrifuged (10,000g, 30 min, 4°C), and the supernatants were pooled and thoroughly mixed in a pre-chilled beaker held on ice. Pooled saliva samples were prepared for SDS-PAGE by mixing and vigorously vortexing 200uL aliquots with 190uL laemmli buffer + 10uL β-mercaptoethanol (βME) in 1.5mL tubes. The tubes were placed in a boiling water bath for 5 minutes and then frozen overnight.

Saliva-Astringent Mixtures

Mixtures were assayed at both a 2:1 and a 1:1 ratio of astringent solution to pooled saliva. Preliminary benchtop experiments suggested that when panelists swished and expectorated 10mL of an astringent solution, the ratio of astringent solution to saliva in the expectorant was greater than a 2:1 ratio; however, it is known that high concentrations of many compounds, including some astringents, can interfere with SDS-PAGE. High tannin concentrations have been shown to cause severe blue streaking in Coomassie Brilliant Blue (CBB)-stained gels (Proctor et al., 2005), and high alum concentrations, like other salts, cause protein band blurring and distortion. This limited the proportion of astringent that could be used in the mixture. The mixtures were made by adding 400μL aliquots of saliva into 1.5mL tubes containing either 800μL (2:1 mixture) or 400μL (1:1 mixture) of one of the six astringent solutions or distilled water.

The tubes were vortexed and held for 5 minutes at room temperature before they were centrifuged (13,000g, 10 minutes) and placed on ice. From each of the tubes, 400μL of the supernatant was transferred into new 1.5mL tubes and frozen at -20°C before being lyophilized. The pellets remaining in the original tubes were then isolated. The remaining supernatant was decanted and 0.5mL of ice-cold acetone was added. The tube was inverted 5 times and centrifuged at 13,000g for 2 minutes. After decanting the acetone, a second acetone wash and centrifugation was performed. The tubes were drained and allowed to air dry. The pellets were resolubilized with 95μL laemmli buffer + 5μL βME, vortexed vigorously, and placed in a boiling water bath for 5 minutes. They were then frozen until electrophoresis was carried out the following day. Immediately before electrophoresis was run, the lyophilized supernatants were resolubilized with 190μL laemmli buffer + 10μL βME, placed in a boiling water bath for 5 minutes, and allowed to cool to room temperature.

SDS-PAGE

Salivary PRPs

Samples were briefly vortexed and 20 µL aliquots were loaded on pre-cast 10.5-14% polyacrylamide Tris-HCl gels (Criterion Tris-HCl gel, Bio-Rad, Hercules, CA). A pre-mixed Tris-glycine-SDS running buffer (Bio-Rad, Hercules, CA) was used. Electrophoresis was run at 70V for approximately 10 minutes until the protein bands had migrated into the resolving gel, at which time the voltage was increased to 150V for approximately 2 hours. To prevent the temperature from rising, the electrophoresis tank was placed on ice and electrophoresis was carried out inside a 40°F (4.4°C) cooler. Gels were stained with Coomassie Brilliant Blue (CBB) R-250 and destained following the methods of Beeley et al. (1991). Using CBB R-250 and omitting organic solvents from the destaining solution causes PRP bands to appear pink-violet instead of blue due to a high ratio of proline (Beeley et al., 1991). Molecular weights of the proteins were estimated based on comparisons to prestained broad-range protein standards (Bio-Rad, Hercules, CA). Gel images were captured in color using a Canon Rebel T1i digital SLR camera. Initially, only the samples from the 2:1 assays were run, but high levels of alum in one of the assays caused streaking of the protein bands, Figure 4. The 1:1 assays were run the following day.

Salivary Mucins: MG1 and MG1

To analyze the samples for the two salivary mucins, 20µL aliquots were loaded onto pre-cast 7.5% polyacrylamide Tris-HCl gels (Criterion Tris-HCl gel, Bio-Rad, Hercules, CA). Electrophoresis was run at 200V for approximately 50 minutes using a pre-mixed running buffer of Tris-glycine-SDS buffer. Gels were stained with periodic acid-Schiff reagent (PAS) (Sigma Aldrich, St. Louis, MO) and destained following the methods of Becerra et al. (2003). Gel images were captured in color using a Canon Rebel T1i digital SLR camera. Because of the known interference of high alum in the 2:1 assays, only the 1:1 assays were run.

Results

PRPs: CBB-stained gels

Several blue and many pink/violet stained protein bands were observed on the CBB-stained gels (Figure 3, Figure 4 and Figure 5). Bands that stained pink/violet were PRPs. A summary of the bands observed in the CBB-stained gels is given in Table 1.

Not all of the astringents precipitated the PRPs, Figure 6. As expected, the tannins precipitated a greater amount of PRPs than did the saliva assayed with water (Figure 6, lanes 5 and 6 vs. lane 4). Alum also precipitated more PRPs than water (Figure 6, lanes 9 and 10 vs. lane 4). The pink/violet-stained bands of the glycosylated (bands 5 and 8), acidic (bands 9), and basic (bands 10) PRPs were diminished or absent from the supernatants of the alum and tannin mixtures (Lanes 5, 6, 9, and 10), particularly in the higher strength tannin and alum mixtures (see boxes a (glycosylated) and b (acidic and basic) in Figure 6). The presence of many of the PRPs in the pellets was confirmed (boxes c and d), but as mentioned previously, the tannin-high and the alum-high assays resulted in pellets that could not be fully resolubilized. This is likely why the PRPs in these two pellets (lanes 14 and 18) appear fainter than in the pellets from the tannin-low and acid-low mixtures (lanes 13 and 17, respectively). Regardless, it is clear that the pellets from both the low and high alum and tannin mixtures contained more PRPs than did the pellet from the water assay. Unlike tannin and alum, acid did not appear to precipitate the PRPs, but rather precipitated amylase and the proteins with a molecular weight greater than amylase (box e, Figure 7).

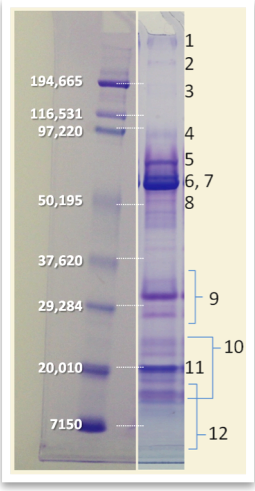

Figure 3. Salivary proteins observed on CBB-stained gels
Molecular weight standards and salivary proteins observed on the CBB-stained gels. Molecular weight standards appear in the left column with their approximate molecular weight in daltons (white text). Salivary protein bands appear in the right column and are labeled with numbers 1-12 (see Table 1). Pink/violet bands are the PRPs (e.g., band 9), and the blue bands (e.g., band 11) are non-PRP protein**s.**

Table 1. Salivary proteins identified on CBB-stained gels
The band numbers refer to those labeled in Figure 3 and observed in Figure 4 and Figure 5. Bands 1 and 2 are also observed on the PAS stained gels in Figure 9. Identification of bands is based solely on their color and apparent molecular weight compared to published work that positively identified the proteins.

Band Number	Approximate observed MW (kDa)	Description
1	>>195	MG1[1]
2	200	MG2[1]
3	190	Unknown
4	86	Lactoferrin[1,2]
5	65	Two bands: Pink diffuse band is glycosylated basic PRP and blue band is possibly one or both of α-heavy chain and secretory component[2]
6, 7	55-60	Glycosylated and non-glycolsylated forms of α-amylase[2]
8	51	Glycosylated PRP[1]
9	26-29	Acidic PRPs – 2 distinct bands; one or more very diffuse, faint bands [2]
10	11-22	Basic PRPs – many bands[2]
11	17	Blue distinct band of unknown protein
12	7-10	Numerous small, blue bands. Likely includes histatin family of proteins [1]

1 Becerra et al., 2003
2 Beeley, 1991

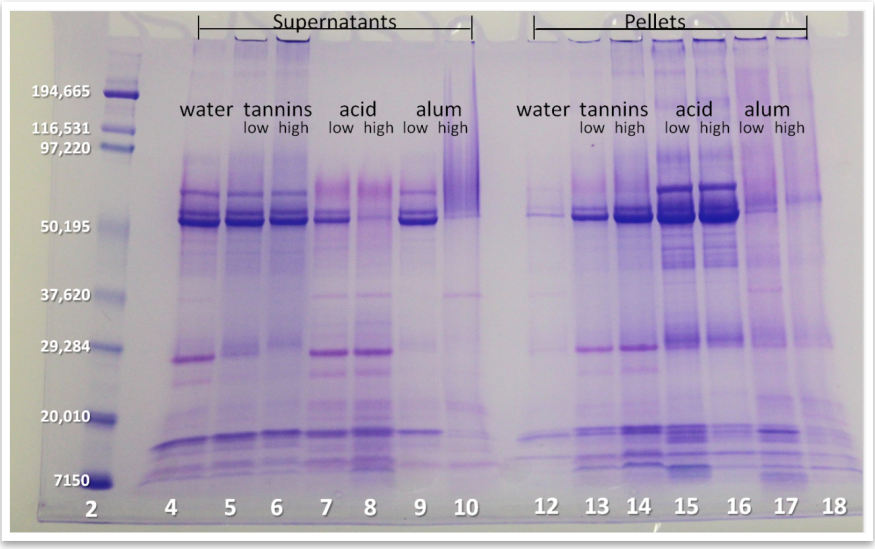

Figure 4. CBB-stained gel of supernatants and pellets from astringent-saliva assays mixed in 2:1 ratio

CBB-R250 stained 10.5-15% polyacrylamide gel showing multiple salivary proteins and the effect of astringents or water on their solubility. Bands that appear pink/violet are PRPs. Assays were mixed in a 2:1 astringent-to-saliva ratio. The high alum concentration in lane 10 caused smearing of protein bands. Lanes are labeled by number and by the sample in the lane except for lane 2, which contains pre-stained broad-range molecular weight standards (Bio-Rad, Hercules, CA). The standards are labeled with weight in units of Daltons.

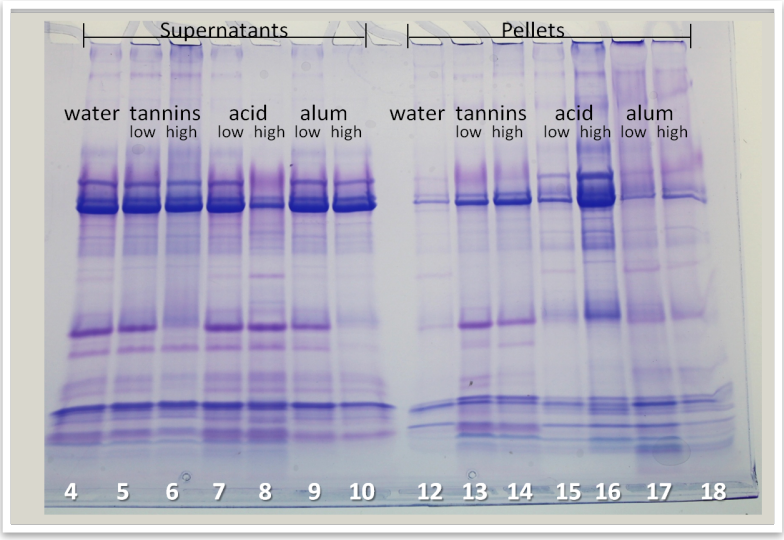

Figure 5. CBB-stained gel of supernatants and pellets from astringent-saliva assays mixed in 1:1 ratio

CBB-R250 stained 10.5-15% polyacrylamide gel showing multiple salivary proteins and the effect of astringents or water on their solubility. Assays were mixed in a 1:1 astringent-to-saliva ratio. Bands that appear pink/violet are PRPs. Lanes are labeled by number and according to the sample in the lane.

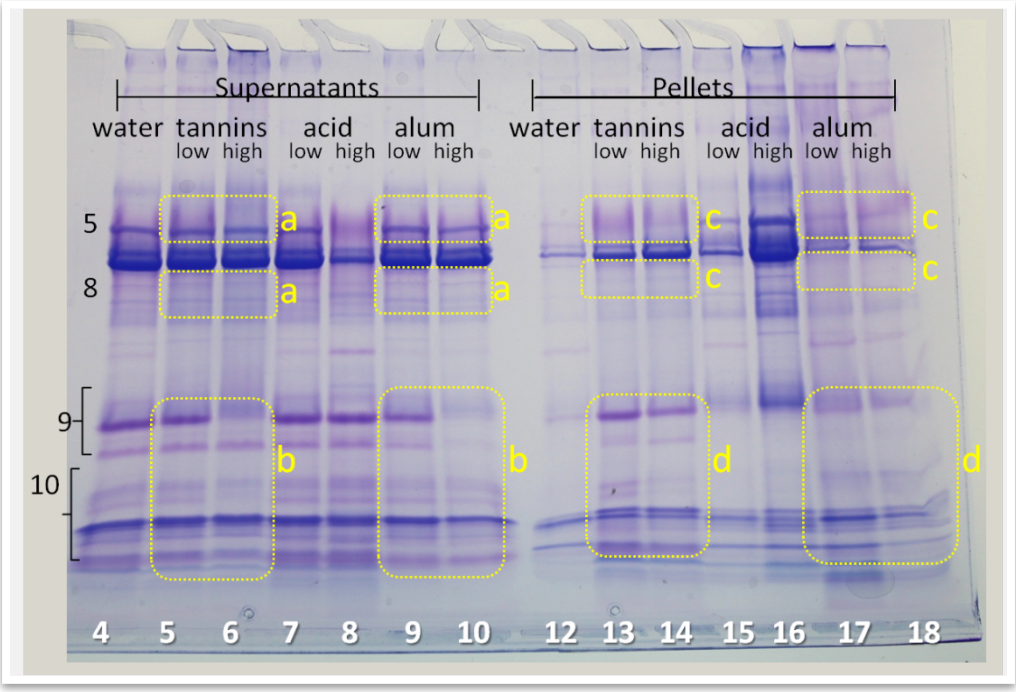

Figure 6. Figure 5 with differences in PRP concentrations highlighted

Cropped view of Figure 5 with key areas circled to highlight the diminished and increased presence of PRP bands in the supernatants and pellets from the tannin and alum assays, respectively. Boxes labeled 'a' highlight glycosylated PRP bands that are less intense in areas of the tannin and alum supernatant lanes (5, 6, 9, and 10), sometimes more noticeable in their reappearance in the pellets (pink within the 'c' boxes in lanes 13, 14, 17, and 18). Boxes 'b' highlight the acidic and basic PRP bands in the tannin and alum lanes that diminish in intensity. Many of the acidic and basic PRP bands appear in the corresponding pellets outlined by boxes 'd'. The same trends are not observed in the acid assays.

Both acid and alum precipitated many of the higher molecular weight proteins, box f in Figure 7. Included among the high-molecular weight proteins precipitated by acid and alum were MG1 and MG2 (bands 1 and 2, respectively). The bands for MG1 (band 1) and MG2 (band 2) are very faint in the supernatant from the high-alum assay (lane 10) and are essentially absent in the high-acid lane (lane 8). Correspondingly, many high molecular weight proteins are observed in the pellets from the acid and alum mixtures, boxes h and i, respectively, in Figure 7.

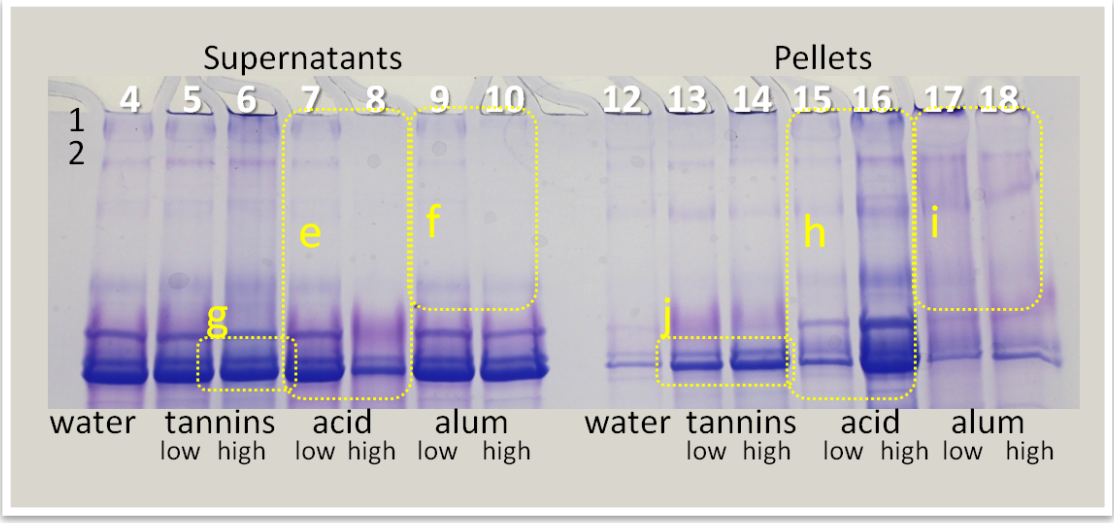

Figure 7. Cropped view of Figure 5 highlighting differences in high molecular weight protein concentrations

Cropped view of Figure 5 with key areas circled to highlight the diminished and increased presence of high molecular weight proteins in the supernatants (e and f) and pellets (h and i) from the acid and alum assays, respectively. Acid precipitated all proteins with a molecular weight equal and greater than amylase, with the notable exception of glycosylated PRP (pink/violet bands within box e). Tannins did not appear to precipitate the majority of these same proteins except for a portion of salivary amylase (g (supernatant) and j (pellet)).

One commonality shared by all astringents was their ability to precipitate many of the low-molecular weight proteins when compared to the water-saliva assay, Figure 8, although the band patterns among the astringents were slightly different. In lanes 13 and 14 of Figure 8, it is clear that tannins precipitate some additional low-molecular weight PRPs, since there are pink bands in this region of the gel. The bands also appear in the alum lanes (17 and 18), albeit faintly.

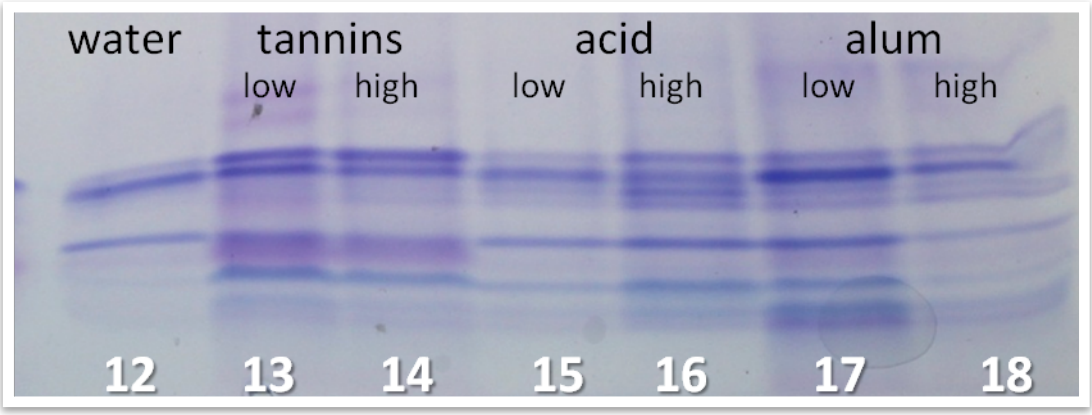

Figure 8. Cropped view of Figure 5 highlighting differences in low molecular weight protein concentrations in the pellets.
Cropped view of Figure 5 showing the low molecular weight proteins (~ 6kDa to 15kDa) observed in the pellets of the samples. It is clear that all astringents precipitate more of these low molecular weight proteins compared to water as demonstrated by the increased intensity and quantity of the protein band that are present. Pink bands present in lanes 13 and 14 and also faintly in lanes 17 and 18 are low-molecular weight PRPs precipitated by tannins and alum, respectively.

Mucins – PAS stained gels

The observations from the CBB-stained gel are mirrored in the PAS-stained gel, which shows that both alum and acid, but not tannins, effectively precipitate MG1 (band 1) and MG2 (band 2), Figure 9. In the acid-high supernatant lane (8), the two mucins bands are barely visible, indicating that very little MG1 and MG2 remain in solution. This increasing precipitation of MG1 and MG2 by acid is reflected in lanes 15 and 16, which show the resolubilized pellet from the acid-low and acid-high assays, respectively. Likewise, increasing alum (lanes 9 and 10) is shown to diminish the amount of both MG1 and MG2 in the supernatant. This is not readily observed in the pellet lanes, but this is likely due to the difficulty encountered in resolubilizing the pellets of these samples.

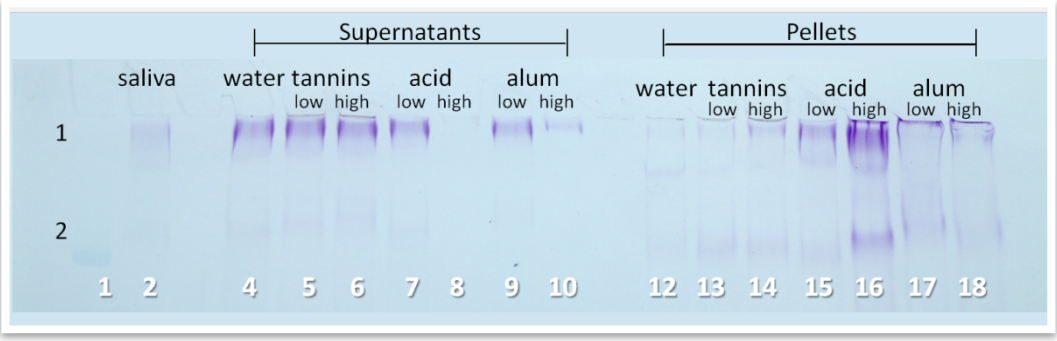

Figure 9. PAS-stained gel of supernatants and pellets from astringent-saliva assays
PAS stained 7.5% polyacrylamide gel showing two high molecular weight salivary glycoproteins and the effect of astringents on their solubility when mixed in a 1:1 astringent to saliva ratio. The smaller of the two glycoproteins (row 2 at the bottom of the gel image) is assumed to be MG2 since it migrated approximately the same distance as a 194kDa molecular weight standard, which is close to MG2's reported weight of between 180 and 200kDa (Becerra et al., 2003; Groenink et. al 1996; Mehrotra et al., 1998). The larger of the two bands, located at the top of the gel, is likely MG1 based on its location compared to MG2 and as observed in other studies (Becerra et al., 2003). Lanes are labeled by number (in white at the bottom) and according to the sample in the lane (in black at the top).

Tannins had a smaller effect on the solubility of mucins, which is observed in the supernatant (5 and 6) and pellet lanes (13 and 14), Figure 9. If the mucins were precipitated by the tannins, the bands in lanes 5 and 6 would appear less intense as compared to the water-assay (lane 4). Similarly, an increase in the band intensities of the tannin pellet lanes (13, 14) compared to water (lane 12) would be expected. While the MG1 band intensity does slightly increase in lane 14 (tannin high assay) compared to lane 12 (water assay), the change is minimal compared to the differences observed between lanes 15-18 (alum and acid) vs. lane 12 (water).

Discussion

The key to understanding astringency has long been focused on the precipitation of the PRPs, but evidence collected here shows that their precipitation is not requisite to the development of astringency. Acids are known to be astringent at the pH levels tested in this study (Lee & Vickers (2008), Lee & Vickers (2010)), yet there was no evidence that they precipitated the PRPs. The dependence of astringency on the ability of polyphenols

to precipitate PRPs has come into question recently with the evidence that certain astringent polyphenols show little to no binding affinity for the PRPs (Schwarz & Hofmann, 2008). The commonality among all astringents in this study was that they were all capable of precipitating some of the salivary proteins.

Contrary to my study's hypothesis, there was no evidence that the mucins were precipitated by the tannins. It seems that the precipitation of these proteins is also not requisite to astringency development. It is somewhat surprising that the tannins did not precipitate mucins since other studies have found that they do (Dawes, 1964; Pizzolato & Lillie, 1973; Gambuti et al., 2006); however, in light of recent evidence that not all astringent polyphenols precipitate PRPs (Schwarz & Hofmann, 2008), it should not be expected that all astringent polyphenols precipitate the mucins.

Subsequent phases of this study may help to answer the new questions brought up here: does the precipitation of salivary proteins (regardless of type) cause a loss of lubricity great enough to be experienced by people and identified as astringency? Are the higher astringent:saliva ratios that are experienced when panelists swish astringent solutions enough to remove the mucin coating lining the oral cavity? Certainly, this is likely the case with acid and alum, shown here to have a very high affinity for MG1 and MG2, though the question remains for tannins.

I.2 A change in salivary lubricity

Introduction

The purpose of this study was to determine if astringency was related to the loss of salivary lubricity. Two complementary approaches were used:

Obj. I.2a: To instrumentally determine if astringents result in decreased salivary lubricity

Obj. I.2b: To determine if human subjects can detect changes in salivary lubricity

The hypotheses to these objectives were as follows:

Hypothesis I.2a: Salivary lubricity will diminish at low levels of astringency, but the reduction will be limited and will not continue with higher concentrations of astringent solutions.

Hypothesis I.2b: Panelists will be unable to detect a difference in salivary lubricity for all astringent types and concentrations

Overview:

For part I.2a, saliva collected from a single subject was assayed with a variety of astringent compounds (alum, acid, and tannins, each at two concentrations), and the saliva-astringent mixtures were analyzed for their friction on a custom-built device. The procedure was repeated a total of five times using saliva from different subjects. To support I.2b, eighteen panelists were recruited to participate in a single sensory session in which they used their fingers to assess the friction of their saliva when it was mixed with the same astringent solutions as used in part I.2a. Panelists used their fingers instead of their mouths to assess the lubricity of saliva-astringent mixtures in order to isolate the

effects that astringents may have had on salivary lubricity from the effects that they may have on the oral tissues or coatings.

Because panelists used their fingers to assess salivary lubricity, it was imperative to establish that their fingers were as sensitive as their mouths in detecting changes in friction. A preliminary study was performed in order to determine if panelists' fingers were as sensitive as their mouth was to perceptions of friction experienced with a variety of foods. The preliminary study provided confirmation that finger assessment of friction could be used in lieu of the mouth, and the details of this study are first presented.

Results from the main study suggest that a loss of salivary lubricity is not a central component to astringency. While instrumentally measured friction of saliva-tannin mixtures was found to be greater than the friction of saliva-water mixtures, the differences were not detectable by human subjects. Saliva-alum and saliva-acid mixtures did not result in increased friction either instrumentally or by human subjects.

Preliminary Study

Objective: To determine if panelists were equally sensitive to differences in friction when assessing several foods using their saliva-moistened fingers versus their mouths

Subjects

Twenty subjects (5 males, 15 females) were recruited from the University of Minnesota to participate in a single test session that was held at the Sensory Center Testing Facility located on the St. Paul campus of the University. All panelists were paid a cash incentive for their participation. The University of Minnesota Institutional Review Board approved all procedures of the study.

Test Samples

Ten food samples were assessed for both mouth and finger friction. The foods that were served included 100% vegetable oil (Cub Food Brand, SuperValu Inc., Eden Prairie, MN), vanilla pudding (Hunts, ConAgra Foods, Omaha, NE), distilled water, creamy peanut butter (Cub Food Brand, SuperValue, Eden Prairie, MN), Hershey's Chocolate Syrup (The Hershey Company, Hershey, PA), honey (The Honey House Grade A pure honey, St. Croix Falls, WI), Miracle Whip (Kraft, Northfield, IL), heavy whipping cream (Land O'Lakes, Arden Hills, MN), and two pectin-containing solutions. The more concentrated pectin solution contained 1.5% pectin (Pectin 800AM, Tic Gums, White Marsh, MD) and 10% sucrose (C&H Sugar Company, Inc., Crockett, CA); the less concentrated pectin solution was made by diluting the concentrated pectin solution in half. Mouth samples were served in 2-oz Solo® cups (Solo Cup Company, Lake Forest, Illinois), and finger samples were served in 7mL general-purpose polyethylene pipettes (Samco Scientific, San Fernando, CA), Figure 10. All samples were served at approximately 37°C. Samples were warmed in a dry proofing box held at 37°C (approximately body temperature) for 2 hours prior to serving. To keep samples warm during evaluation, samples were served on top of bagged sand that was also warmed to

37°C, and dry towels were used to cover the trays in between sample evaluations, Figure 10. All samples were labeled with random 3-digit codes.

Figure 10. Sample presentation for salivary lubricity preliminary study
Presentation of finger samples (left) in pipettes (left) and mouth samples in 2-oz cups (right). All ten finger samples were served on one tray. The mouth samples were served on two trays. Trays were covered with a clean cloth to retain heat during sample evaluation.

Test Session

Upon arriving for the session, each subject was trained to rate food samples for the friction they produced. Friction was defined as "the amount of force resisting the motion of your fingers/mouth" and they were told that "foods that are 'slippery' result in low levels of friction". Friction was rated on a 15-point line scale ranging from low to high friction, and the scales used for the finger and mouth assessments were identical (Figure 11). At the center of both scales, corresponding to a friction rating of 7.5 was a reference point of 'saliva only'.

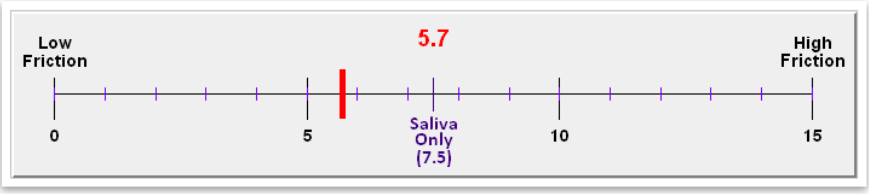

Figure 11. Scale used in salivary lubricity preliminary study
15-pt scale that was used to collect panelist ratings of mouth and finger friction. All ratings were made relative to the 'Saliva Only' reference point, which was anchored at the center of the scale (7.5).

In the mouth, the 'saliva only' reference point was defined as the level of friction experienced when rubbing the tongue along the roof of the mouth from front to back and then from back to front while applying a light force between the two surfaces. The instructions directed panelists to take 2 seconds to complete the back-and-forth motion and to repeat the motion several times. To assess a sample, panelists removed the solo cup from the covered tray and then placed a level spoonful of the sample in their mouth. They were then instructed to evaluate the resulting friction by following the same instruction and were told that they should have a thin layer of the sample between their tongue and palate. Water was provided to panelists so they could rinse between samples.

For the fingers, the saliva-only reference point was defined as the level of friction experienced when rubbing the index finger and thumb together in a circular motion 3 times after they had both been thoroughly moistened with saliva. To evaluate a food sample, panelists were instructed to pipette 2-3 drops of food onto their saliva-moistened thumb and finger and to rub them together in a circular motion several times to first spread the food and then to evaluate friction. They were asked to try to maintain a consistent speed of motion and force between the fingers for all samples. A water bath and napkins were provided for cleansing the fingers between samples.

Half of the panelists were randomly chosen to receive the ten finger samples first, while the other half received the ten mouth samples first. The orders of the mouth and finger samples served to the panelists were based on 10x10 Latin square designs balanced for order and carryover effects (Macfie et al., 1989). All ratings of friction were collected using SIMS 2000. Panelists were paid for their participation. Study documents, including panelists' instructions and definitions of friction, are provided in Appendix 2.

Data Analysis

To determine the extent to which finger friction ratings could predict friction perception in the mouth, linear regression was performed using the mean finger and mouth friction values.

Results

A positive correlation of r = 0.84 between mouth and finger friction ratings was observed after analyzing the data from 19 panelists for each food item, Figure 12 and Table 2. One data set from among the 20 panelists was excluded from the analysis after a panelist reported to have provided incorrect ratings for some of the samples.

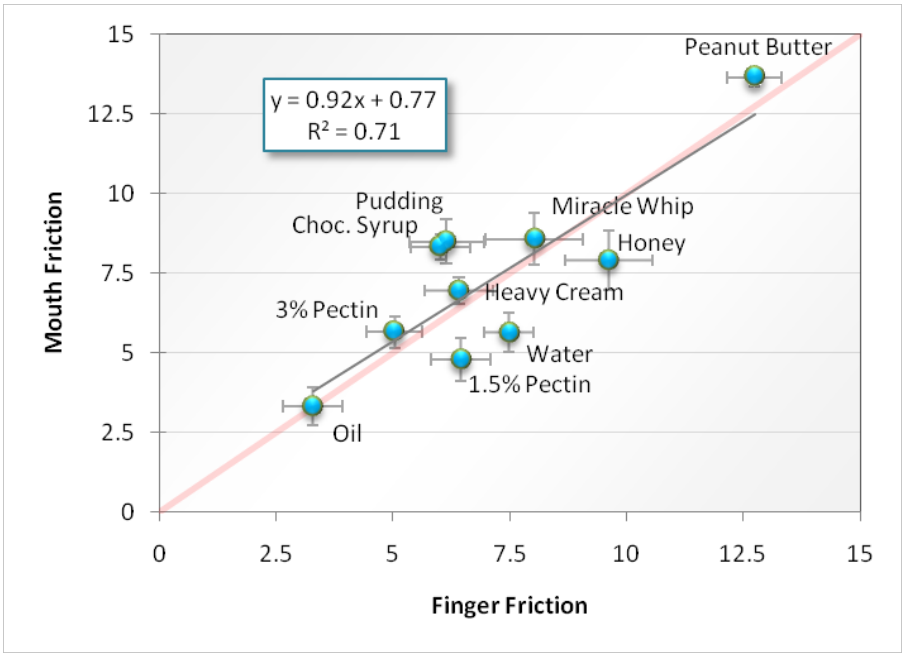

Figure 12. Plot of the correlation between panelists' ratings of mouth vs. finger friction

Correlation between finger friction and mouth friction ratings from 10 different foods. Error bars represent standard errors. The faint line running between the points (0,0) and (15,15) represents the situation where the sensitivity of the mouth would exactly predict the reported friction when panelists used their fingers (i.e., having a slope of 1). The trendline equation had the slope 0.92, indicating that the mouth and finger sensitivity was similar.

Table 2. Mean mouth and finger friction data

Mean mouth and finger friction data and their associated standard errors. Ratings collected on a 15-point line scale labeled with the adjectives 'low friction' and 'high friction' at the scale end-points of 0 and 15, respectively. Low ratings represent foods that are slippery and produced low levels of friction. N = 19 for all data points. SE = Standard error.

	Fingers		Mouth	
Sample	Friction	SE	Friction	SE
Oil	3.29	0.63	3.31	0.59
1.5% Pectin + Sugar	6.46	0.64	4.78	0.67
Water	7.49	0.52	5.64	0.62
3% Pectin + Sugar	5.04	0.60	5.65	0.50
Heavy Whipping Cream	6.42	0.74	6.94	0.42
Honey	9.62	0.94	7.9	0.95
Chocolate Syrup	6.02	0.63	8.31	0.40
Vanilla Pudding	6.15	0.80	8.48	0.69
Miracle Whip	8.03	1.1	8.56	0.81
Peanut Butter	12.7	0.59	13.6	0.28

Discussion

Although some foods received slightly different finger vs. mouth friction ratings (e.g., chocolate syrup in Table 2), the major concern of this preliminary study was establishing equal friction sensitivity (i.e., close to a slope of 1 and high correlation, Figure 12) between fingers moistened with saliva and the mouth. The results from the study do support that panelists' finger assessment of friction is a reasonable substitute, in terms of their sensitivity, to oral perceptions of friction. These findings agree with other studies that have established similarity between the finger tips and various locations within the oral cavity in their ability to discriminate among texture differences (Ringel & Fletcher, 1967) and their sensitivity to touch (Ringel & Ewanowski, 1965).

A change in salivary lubricity

With confirmation that panelists were similarly sensitive to differences in friction when using their fingers as compared to their mouths, the main study to determine if astringency was related to the loss of salivary lubricity was executed.

Materials & Methods

Subjects

Eighteen subjects were recruited from the University of Minnesota to participate in a single test session held at the Sensory Center Testing Facility located on the St. Paul campus of the University. At the session, panelists performed a sensory test that was designed to determine if they could detect a change in salivary lubricity in response to astringents when using their fingers. Five subjects (1 male, 4 females) were asked to attend a separate, individual session during which 12 mL of whole mouth, stimulated saliva was collected. The saliva was used for instrumental measurements of salivary lubricity. For the sensory test and for the saliva collection session, subjects were asked to refrain from eating, brushing their teeth, using a mouthwash, or drinking anything other than water in the hour preceding the session. All panelists were paid a cash incentive for their participation. The University of Minnesota Institutional Review Board approved all procedures of the study.

Test Solutions

A total of eight pairs of solutions were evaluated by each panelist during the sensory sessions (Table 3). The astringent solutions (Solutions B in pairs 1-6) were also used in the instrumental experiments for the assessment of salivary lubricity.

The high concentration alum solution (Alum High) was prepared by dissolving alum at 1.0 g/L into distilled water. The high concentration tannin solution (Tannin High) was made by first adding tannins into distilled water at a concentration 3.0 g/L, mixing the

solution for 6 hours to dissolve the tannins, and then filtering the solution to remove any remaining visible particulates. The strong acid solution (Acid High) was prepared by titrating 0.5M HCl into distilled water until the pH reached 1.9. All three of the Low astringent solutions were made by preparing a two-fold dilution of the "high" concentration astringent solution. To make the colored water solutions (3A and 4A) that paired with the tannin-containing solutions, caramel coloring (D.D. Williamson, Louisville, KY) was added into distilled water until it matched its corresponding high or low strength tannin solution.

Table 3. Solution pairs for salivary lubricity sensory session
Solution pairs evaluated by subjects in the salivary lubricity discrimination test. The astringent solutions (Solution B of pairs 1-6) were also used in instrumental testing of salivary lubricity.

Pair	Solution A	Solution B	Name of Solution B
1	Water	Alum at 0.50 g/L	Alum Low
2	Water	Alum at 1.0 g/L	Alum High
3	Colored Water	Tannin at 1.5 g/L	Tannin Low
4	Colored Water	Tannin at 3.0 g/L	Tannin High
5	Water	HCl at pH = 2.2	Acid Low
6	Water	HCl at pH = 1.9	Acid High
7	Water	2.5 % Sodium lauryl sulfate	SLS
8	Colored Water	Colored 2.5 % Sodium lauryl sulfate	cSLS

Two of the pairs used in the sensory session (pairs 7 and 8, Table 3) contained solutions that were easily distinguishable from each other and were included as a means of boosting panelists' confidence since it was expected that they would have a difficult time discriminating between the solutions in pairs 1-6. Solution B in pair 7 contained 2.5% Sodium Lauryl Sulfate (SLS) (Fischer Scientific, Pittsburgh, PA) dissolved in distilled water. Solution B of pair 8 was 2.5% SLS with caramel coloring added so that it was similar in color to the high concentration tannin solution. The colored water solution that paired with the colored SLS solution was made by adding caramel coloring (D.D. Williamson, Louisville, KY) into distilled water until its color matched. Benchtop testing confirmed that people were easily able to determine that the SLS solution resulted in less friction than the water solutions.

Test Session

Upon arrival, panelists received instructions for assessing the 8 pairs of solutions. The instructions given were as follows:

1. Thoroughly moisten your fingers with your saliva
2. Rub your index finger and thumb together with very light pressure between them. You should not be able to feel your fingerprints.
3. Pour or pipette a few drops of solution onto your fingers
4. Rub your index finger and thumb together in a circular motion
5. Pay close attention to the level of friction
6. Rinse fingers in bath & wipe off
7. Repeat the procedure with the second sample (do not reuse pipettes)
8. Select the sample that results in the higher level of friction (i.e., the one that is the least slippery) by circling its code on the paper ballot.

The booth setup for the sensory portion of the study is shown in Figure 13. All samples were served at room temperature in 1-oz Solo® cups labeled with 3 digit-codes. A water bath and napkins were provided for panelists to rinse and dry their fingers in between samples. Panelist responses were collected on paper ballots.

Figure 13. Booth setup for salivary lubricity sensory test
Booth setup showing the eight sample pairs. Pipettes used for sample delivery onto fingers are shown on the left (in cup). In front, a water bath is shown that was used to cleanse fingers in between samples. The ballot appears on the lower right.

Panelists arriving for their session were assigned a number from 1-18 according to the order in which they arrived. All panelists received the colored SLS solution pair (pair 8, Table 3) first and the clear SLS solution pair (pair 7, Table 3) in the fifth position. The order of the remaining pairs was determined according to multiple 6 x 6 latin squares that accounted for order effects (MacFie et al., 1989). The order of the samples within each of the eight pairs (i.e., Solution A and B) were assigned randomly for odd numbered panelists. For even numbered panelists, the order of the samples within each pair was opposite of the order assigned to the odd numbered panelist who arrived just before them. Blinding codes, the complete sample rotation plan, and the ballot presented to panelists can be found in **Appendix 4**.

Saliva Collection Sessions

Stimulated saliva was collected from a total of five subjects during individual sessions held over a two-week time period. At each of the five sessions, approximately 12mL of saliva was collected from a single subject. The subject first rinsed their mouth with water and then began to chew a 2 x 4" strip of wax (Parafilm® M, Pechiney Plastic Packaging, Chicago, IL). After an initial swallow after 15 seconds of chewing, saliva collection began. The panelist continued to chew the wax while expectorating into two 15-mL pre-weighed tubes (BD Falcon, San Jose, CA) that were held in an ice-filled beaker. They were instructed not to swallow during the collection period. The time it took to collect the saliva was recorded, and the tubes were weighed after the collection was complete.

Saliva-Astringent Mixtures

After each saliva collection, the two tubes of saliva from the single panelist were centrifuged (10,000g, 20 min, 4°C) and the supernatants were pooled. The saliva was held on ice throughout the duration of the instrumental friction measurement session, which lasted approximately 2 hours.

Initial friction readings were obtained using 1mL of water and then using 1mL of pure saliva, both of which were heated in a 37°C water bath for 2 minutes prior to

measurement. These measurements were duplicated at the end of the session. Friction measurements of eight saliva-astringent mixtures and of the pure astringent solutions were collected next. The eight astringent solutions that were used in the experiment included the same six solutions used in the sensory portion of this study (Solutions "B" in pairs 1-6, Table 3, page 47) plus two replicates that used water as the astringent control. The mixtures were prepared by assaying 0.8mL of saliva with 0.2mL of the astringent in 1.5mL tubes. The ratio was chosen based on results from preliminary tests, Figure 14. The tubes were vortexed and heated for 2 minutes in a 37°C water bath immediately prior to loading the mixture onto the friction tester. After the friction from each saliva-astringent mixture was measured, the friction of the corresponding astringent solution (i.e., not assayed with saliva) was measured. For this, 1 mL of the astringent solution was warmed for 2 minutes in at 37°C water bath prior to being pipetted onto the friction tester. The order of the assays was randomized during each of the five sessions, Table 4.

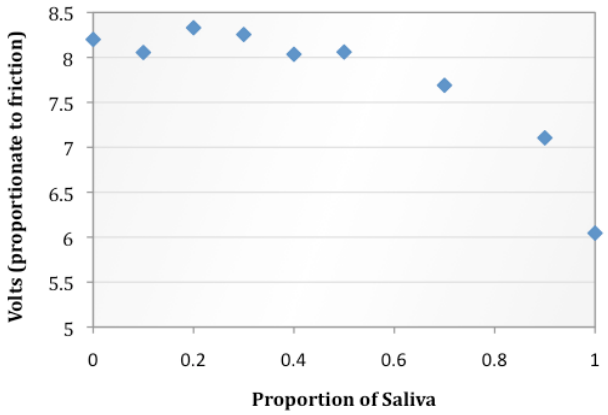

Figure 14. Friction vs. the proportion of saliva in saliva-tannin mixtures
Volts measured by the friction tester on saliva-tannin mixtures as a function of the proportion of saliva. Tannin solutions of 3g/L were used, and stimulated saliva was collected from an individual panelist, centrifuged, and held on ice for the duration of the experiment (~1.5 hours). Volts are proportional to the frictional force. As the proportion of saliva increased to 1 (pure saliva), the measured voltage (friction) decreased. At saliva proportions of < 0.6 (i.e., high proportion of tannin solution), there was little difference among the friction values as compared to the tannin-only solution (proportion of saliva = 0). Thus, at these lower proportions, differences in friction could not be observed between tannin-only solutions and saliva-tannin mixtures. Based on these findings, a saliva:astringent proportion of 0.8 was chosen.

Table 4. Mixing order of astringent solutions in friction study
Order of the astringent mixtures (assigned randomly). For each mixture, 0.8mL of the panelist's saliva was assayed with 0.2mL of the astringent solution. The solutions are as defined in Table 3. Following the friction measurement of the saliva-astringent mixture, friction was measured using the astringent solution only.

Order Number	Panelist Number				
	1	**2**	**3**	**4**	**5**
1	Tannin High	Water	Water	Tannin Low	Water
2	Tannin Low	Tannin Low	Alum High	Alum High	Tannin Low
3	Acid Low	Alum High	Acid Low	Water	Alum High
4	Water	Alum Low	Water	Acid High	Alum Low
5	Acid High	Tannin High	Alum Low	Alum Low	Tannin High
6	Alum High	Water	Tannin Low	Acid Low	Water
7	Alum Low	Acid Low	Tannin High	Water	Acid Low
8	Water	Acid High	Acid High	Tannin High	Acid High

Friction Measurements

Friction measurements were obtained using a custom friction tester that was designed and fabricated at the University of Minnesota Physics and Astronomy Machine Shop, Figure 15. This device was designed to assess salivary lubricity under tribological conditions that mimic those in the oral cavity. The system outputs voltage readings, which can be translated into units of force by application of an equation obtained through calibration of the system. Complete details of the machine, its calibration procedure, and runtime protocol can be found in Appendix 5.

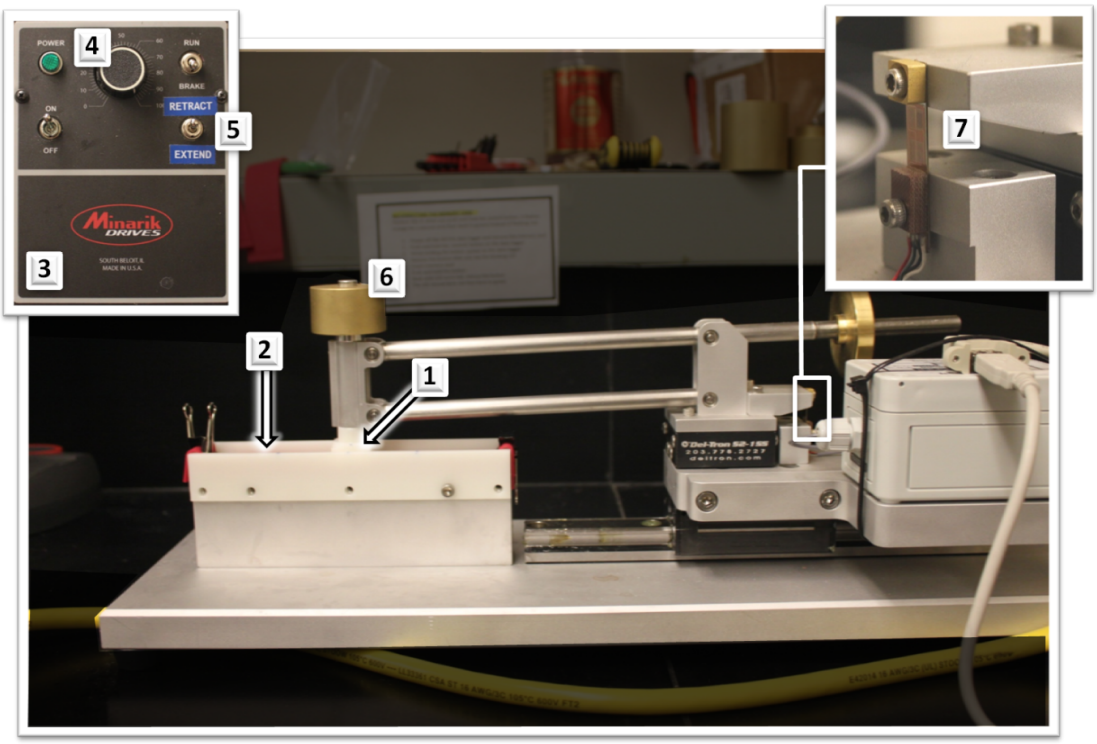

Figure 15. Friction tester used to collect lubricity data
During its operation, the upper nylon contact pad [1] of the friction tester is in contact with and travels back-and-forth along a length of the nylon sample tray [2], in which a known volume of sample resides (usually 1mL). Via the device's controller [3], the travel speed of the upper contact pad can be adjusted from 1 to 10mm/sec [4], and the direction of its motion can be controlled manually using a flip-switch [5]. Weights placed on a platform [6] centered above the upper contact pad apply a known normal force between the two contact surfaces. The resulting friction of the system is detected by a single strain gage [7] that flexes proportionate to the amount of friction between the two contact surfaces. Raw output from the strain gauge is fed into a signal conditioner (not shown), where the signal is amplified and normalized to ±10V.

The friction tester's settings were identical for all five sessions, and it was calibrated at the start and end of the experiment, Appendix 5B. During data collection, a travel speed of 10mm/sec was used with an applied normal force of 2N. The motion of the friction tester was limited to a distance of ±35 mm relative to a starting center-point location, Figure 16. For all runs, the friction tester was allowed to travel back-and-forth within the 70 mm range of motion for 1.5 minutes. A single piece of silicone (Silicone Solutions™ 100% Silicone Red Baking Sheet, Minnesota Rubber and Plastics, Minneapolis, MN) was used to line the sample tray of the friction tester, Figure 16. Silicone was chosen because

it possesses a young's modulus (a measure of stiffness) similar to the tongue, which was the rationale behind its selection in a previous study that examined oral mouth texture perceptions (Ranc, 2006). Additionally, silicone is chemically inert and stable in the presence astringent compounds including acid. Before each mixture's friction was measured, the silicone liner was removed from the system, washed using a detergent, soaked in a 10% ethanol solution, rinsed thoroughly with distilled water, and allowed to air dry.

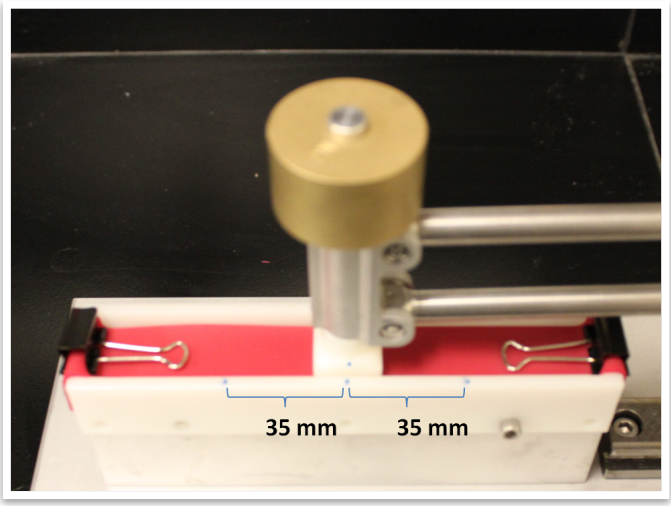

Figure 16. Close-up picture of friction tester
Close-up view of the sample tray and upper contact pad showing markings for travel distance of ±35mm. Also visible in the photo is the piece of red silicone pad used to line the sample tray.

Data Analysis

Sensory

The number of panelists who correctly identified the astringent sample in pairs 1-6 and the water samples of pairs 7 & 8 was recorded and tabulated. To determine if the number of panelists who identified the astringent-containing solution as having more friction was statistically greater than chance, a one-sided z-test using the normal approximation to a binomial was used, with the probability of a correct guess set at 0.5.

Instrumental

To determine if astringents resulted in a higher level of friction (i.e., greater loss of saliva lubricity) when measured instrumentally, an analysis of variance (ANOVA) procedure in SAS v9.1 (Proc MIXED) was used. The friction values ($Friction_{assay}$, in force units of Newtons (N)) calculated for each saliva-astringent mixture were used as the dependent variable in the model. $Friction_{assay}$ values were calculated using the average voltage outputs from the last two complete back-and-forth motions of the friction tester, Appendix 5D. The model predictors included the categorical fixed effect of astringent type (of which there were seven levels total, including the water control) and the random effect of subject. Two covariates were included in the model: one was the friction value of the astringent-only solution, and the other was the run number of the sample on the friction tester, which ranged from 1 (the first friction measurement made collected with saliva from the first subject) to 48 (the final friction measurement collected with saliva from the last panelist). The run number factor was included based on preliminary experiments that showed friction to increase linearly over repeated runs when using the same piece of silicone. The increased friction is believed to be caused by subtle changes to the silicone surface, and this phenomenon has been observed in other studies (Ranc, 2006). The predictable increase of this change in friction over time was favored over the highly unpredictable variability observed when using different pieces of silicon for repeated measurements (Figure 42, page 143).

Results

Sensory

As a group, panelists did not find the astringent solutions to produce more friction compared to the water controls ($z = -0.18$, $p = 0.57$). This was true for the alum ($z = -0.33$, $p = 0.63$), tannin ($z = 0.67$, $p = 0.25$) and acid solutions ($z = 1.33$, $p = 0.09$). Out of the 120 astringent pairs served during the session, 59 astringent samples were identified as producing higher friction than the water controls – a proportion of 0.49 and almost equal to the chance proportion. Panelists were, however, able to differentiate between the

water controls and the samples containing SLS ($z = 3.0$, $p < 0.001$): the SLS containing solutions were found to result in less friction than water 27 out of 36 times.

Instrumental

Friction values of the mixtures were not found to be different among the individual astringent types ($F = 1.83$, $p = 0.13$), but contrasts revealed that, as a group, the assays of the two tannin solutions resulted in higher friction values than did the saliva-water assay ($F = 6.84$, $p = 0.01$), Figure 17. Additionally, although the effect was modest, the higher concentration astringent assays showed a tendency to result in friction values 0.21N higher than the lower concentration assays ($F = 2.8$, $p = 0.11$). The friction of the astringent only (i.e., not mixed with saliva) did not predict the friction of the assay ($F = 0.07$, $p = 0.79$). Lastly, there was a near-significant effect of run number, with friction values increasing with the run number of the measurement ($F = 3.69$, $p = 0.06$), Figure 18. The complete SAS code and order of sample measurements is provided in Appendix 3.

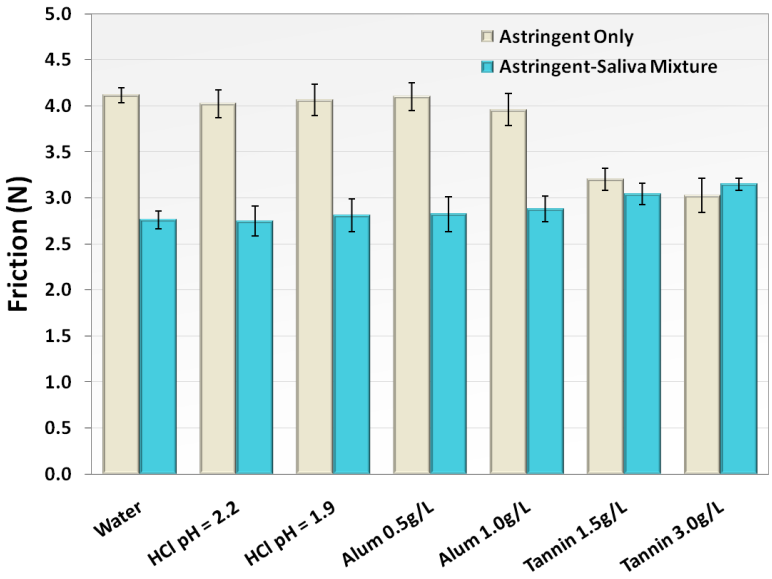

Figure 17. Friction measured on astringent solutions and saliva-astringent mixtures Graph showing the mean friction values of saliva, the seven different saliva-astringent mixtures, and of the astringents by themselves as given in **Table 5**. Error bars represent standard errors. For all water solutions, n = 10 (performed in duplicate for each panelist); for the remaining astringent solutions, n = 5.

Table 5. Mean instrumental friction values of astringent solutions and saliva-astringent mixtures

Mean friction values of the seven different saliva-astringent mixtures and the astringent solutions by themselves. The mean friction values among all saliva-astringent mixtures were not found to differ ($F = 1.83$, $p = 0.13$), but contrasts revealed that when tannins were mixed with saliva, the resulting friction was greater when compared to water ($F = 6.84$, $p = 0.01$). Water-saliva mixtures were measured in duplicate for each of the five panelists (number of measurements total (n) = 10); the astringent-saliva mixtures were measured once for each panelist (number of measurements total (n) = 5).

		Mean friction (N)			
Astringent:	n	saliva-astringent mixture	SE	astringent only	SE
Water	10	2.73	0.12	4.08	0.13
HCl at pH = 2.2	5	2.72	0.18	3.99	0.19
HCl at pH = 1.9	5	2.78	0.20	4.03	0.21
Alum at 0.5 g/L	5	2.80	0.19	4.06	0.22
Alum at 1.0 g/L	5	2.85	0.21	3.92	0.17
Tannin at 1.5 g/L	5	3.01	0.15	3.17	0.15
Tannin at 3.0 g/L	5	3.12	0.22	3.00	0.10

SE = standard error

n = total number of measurements obtained on both saliva-astringent mixture and astringent solution only

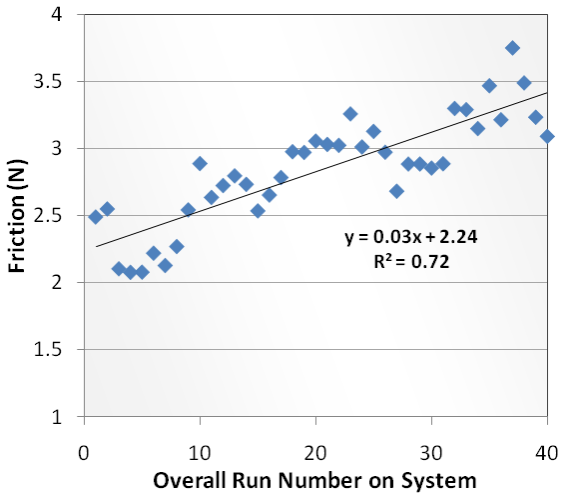

Figure 18. Friction vs. overall run number on the friction tester

Friction values increased as a function of the order of the measurement. Overall run number 1 refers to the first friction measurement on day 1 of data collection, and overall run number 40 refers to the last friction measurement on the last day of data collection.

An interesting and clear relationship between salivary friction and the subject's salivary flow rate was observed: saliva acted as a better lubricant (i.e., resulted in lower friction levels) when the subject's salivary flow rate was low, Figure 19.

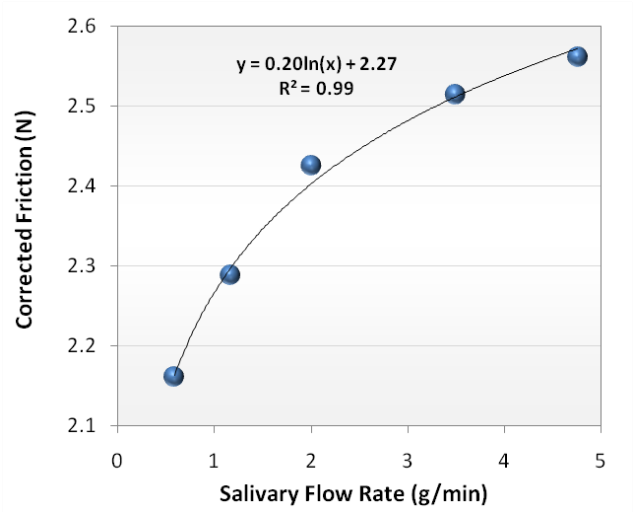

Figure 19. Corrected friction of panelists' saliva vs. their salivary flow rate
Salivary friction vs. salivary flow rate of the 5 subjects. Salivary flow rates were 0.59, 1.17, 2.00, 3.50, and 3.75 g/min. Friction values were corrected based on average friction value of the astringent-only solutions during that session. For example, in session 1, the average friction values obtained for all astringent-only solutions (including water) was 9.53. During session 2, the average was 9.98. The salivary friction measurement from session 2 was then divided by a correction factor of 1.047 (=9.98/9.53) to account for the general increase in friction during that session. The same approach was used to correct for the salivary friction measurements from sessions 3, 4, and 5.

Discussion

Like previous studies on the effects of astringents on salivary lubricity, this study found that astringents had different effects on saliva's lubricity. Of the astringent types tested, only tannins were found to result in increased friction; no change in lubricity was detected among the other astringents. As mentioned previously, this could be because the methods used to detect changes in lubricity are inaccurate, or because astringency is not caused by a loss of lubricity. Because the sensory data suggests that lubricity is not so important, however, the latter is most likely the case.

One factor that may impact the accuracy of studies such as this is the relevance of the ratios used in the saliva-astringent mixture. Based on preliminary experiments, mixing saliva and the astringent in a 4:1 ratio appeared to be the best ratio for detecting the impact that tannin solutions had on salivary lubricity, Figure 14; however, the proportion of astringent to saliva in the mouth is likely much higher when panelists swish 10mL of an astringent solution. This may explain why only a trend was observed in increased friction when saliva was mixed with the higher vs. lower concentration astringent solutions; however, it should be noted that even if this observation were found to be significant, the increase in friction is still very modest, and it is questionable if this difference would be perceptible by subjects.

The inability of the panelists to detect differences among the lubricity of their saliva when mixed with astringents versus water provides additional support that there is an oral phenomenon other than a decrease in salivary lubricity that causes astringency. Panelists assessed salivary lubricity using saliva-moistened fingers in order to isolate the effects that astringents have on salivary lubricity from the other effects that may be occurring within the mouth. Such effects may include a direct binding of certain astringents on the oral tissues or a loss of the lubricating coating of the oral cavity.

I.3 Loss of the mucus coat and desquamation of the oral mucosa

Introduction

The purpose of this study was to determine if astringency was related to the loss of oral coatings and desquamations of the oral mucosa. I hypothesized that astringent compounds would cause a loss of the oral mucus coating accompanied by increased desquamation of the oral mucosa and that this effect would be more pronounced at higher astringency intensities. Six astringent solutions were tested: tannins, alum, and HCl acid at high and low concentrations. Water was used as a control. Although sensory ratings of astringency were not collected for this study, the high and low concentrations of astringent compounds used during this study are known to have distinguishable intensities (Lee & Vickers, 2010; Lee & Lawless, 1991).

For the study, two different groups of panelists each attended a series of sessions during which they swished and expectorated a sample of water and a single astringent solution. The expectorants were collected and later analyzed for cellular and mucin content.

Although neither hypothesis was supported by the data collected, some interesting trends were observed. None of the astringent rinses were found to cause increased desquamation compared to water, but more DNA was removed from the mouth by the high-strength astringent solutions compared to the low-strength solutions. Evidence that astringents remove the mucus coating of the mouth was also not found. The amount of mucins in the expectorated astringent solutions was not found to be higher than in expectorated water samples. Instead, visual observation of the data collected suggested that in some cases, particularly with acids, there were fewer mucins in the expectorated astringent solution. The reason for this remains unknown.

Materials & Methods

Subjects

Five subjects were recruited from the University of Minnesota to participate in a series of eight 5-minute sessions in which they swished and expectorated astringent solutions that were later analyzed for their mucin content. To determine if astringents also caused increased desquamation of the oral mucosa, six subjects (only one of whom participated in the first study) were recruited to participate in a similar series of seven 5-minute sessions. The samples collected during this study were quantified for their DNA content. Sessions for both studies were held at the Sensory Center Testing Facility located on the St. Paul campus of the University. Subjects were asked to refrain from eating, brushing their teeth, using a mouthwash, or drinking anything other than water in the two hours preceding each session. Exclusion criteria included having food allergies or wearing dentures. All panelists were paid a cash incentive for their participation. The University of Minnesota Institutional Review Board approved all procedures of the study.

Samples

I.3.1 Loss of the mucus coating

A total of four 10mL expectorated samples were collected from each panelist at every session: one water pre-rinse, one astringent solution (Table 6), and two water post-rinses. The astringent solutions were the same solutions as used in the tests of salivary lubricity and made as described on page 28 (Test Solutions). The astringent solutions were labeled with 3-digit codes and the water rinses were labeled with the same 3-digit codes and 'WPre', 'W1Post', or 'W2Post' for the pre- and post-rinses, respectively. All samples were presented inside of 15mL centrifuge tubes (BD Biosciences, San Jose, CA) and served at room temperature.

I.3.2 Desquamation of the oral mucosa

A total of two 10mL expectorated samples were collected from each panelist at every session: one water pre-rinse and one astringent solution (Table 6). At each session,

panelists swished one 10mL portion of distilled water and one 10mL aliquot of an astringent solution (Table 6). The samples were presented at room temperature in 1-oz Solo® cups. The water sample was labeled '000', and the astringent solution was labeled with a single digit (1-7), Table 6. The expectorated samples were collected in 15mL centrifugal tubes (BD Biosciences, San Jose, CA). All astringent solutions were the same solutions as used in the tests of salivary lubricity and made as described on page 28 (Test Solutions).

Table 6. Astringent solutions used during the experiments. For all three astringent types, the high astringency sample contained twice the concentration of the low astringency sample.

3-digit code (I.3.1)	Solution # (I.3.2)	Astringent Solution	Solution Name
387	1	Water	Water
872	n/a	Water	Water
826	2	Alum at 0.50 g/L	Alum low
392	3	Alum at 1.0 g/L	Alum high
580	4	Tannin at 1.5 g/L	Tannin low
162	5	Tannin at 3.0 g/L	Tannin high
176	6	HCl at pH = 2.2	Acid low
951	7	HCl at pH = 1.9	Acid high

Session Procedure

I.3.1 Loss of the mucus coating

Panelists were required to attend eight sessions during the course of one week and could complete up to two sessions per day as long as the sessions were over 3 hours apart. Panelists began each session by swishing distilled water for 10 seconds. This rinse was not collected, and panelists were instructed to swallow it. Next, the 10mL water pre-rinse expectorant was collected. Panelists placed the 10ml of water pre-rinse from the 15mL tube into their mouth, swished for 10 seconds, and then expectorated back into the same tube with the aid of a small funnel. Immediately afterwards, they then performed the same procedure with the astringent sample (one of the six astringents or one of the two water controls, Table 6) and then with the two water post-rinses. Clean funnels were used for each tube. The only deviation from this procedure occurred with the acid low, acid high, and one of the water control samples. Instead of expectorating back into the

same tube, the expectorant was collected in a tube containing 75uL of 0.5M or 0.25M NaOH (for acid high (sample 951) and low (sample 176), respectively) or water (sample 872). Neutralizing the acid samples at this stage aided the recovery of the mucins for subsequent analyses, Appendix 8. Collecting one of the expectorated water samples in a similar manner was done to control for the change in procedure. After all samples were expectorated, 2 x 1 mL of distilled water was pipetted onto each of the four funnels to dislodge any salivary proteins off of the funnels and into the tubes. The four tubes were capped and placed into a freezer.

I.3.2 Desquamation of the oral mucosa

Panelists were required to attend seven out of nine possible sessions offered throughout a 2-week period. Each session was open for one hour, and panelists could attend at any time within the hour. Seven of the sessions were held starting at 9:30AM on weekday mornings, and two of the sessions were held starting at 1:30PM on weekday afternoons. Panelists began each session by swishing 10mL of distilled water for 10 seconds, which they then swallowed. They repeated this twice. They then swished the water labeled '000' for 10 seconds and expectorated it into a 15mL tube. Immediately afterwards, they swished the astringent solution assigned to them for that session for 10 seconds and then expectorated it into a separate 15mL tube. The astringent solutions served to the panelists at each session were assigned a random order. All panelists were served each astringent solution once throughout the experiment. Both expectorated solutions were collected using small funnels inserted into 15mL tubes, which were held on ice. Funnels were rinsed with 2mL of distilled water to wash any debris clinging to the funnels into the 15mL tubes. The tubes were capped and placed on ice.

Sample Analysis

I.3.1 Loss of the mucus coating

Sample Preparation

Expectorated samples were allowed to freeze overnight and were then lyophilized for 48 hours. Proteins were precipitated by adding 3mL of 15% trichloroacetic acid (TCA) into

each tube, which was then vortexed and incubated on ice for 30 minutes. This step isolated the mucins from astringents that otherwise interfered with SDS-PAGE. Preliminary work showed mucins to be effectively precipitated by this method, Appendix 8. Tubes were centrifuged (4500 RPM, 10 min, 4°C) to pelletize the proteins. The supernatant was decanted and the tubes were allowed to drain before washing the pellets with 2mL of ice-cold acetone. The tubes were vortexed, centrifuged (4500 RPM, 3 min, 4°C), drained, and allowed to air dry. To each tube, 0.285mL of laemmli sample buffer was added along with 0.02mL of 1.0M tris-HCL buffer at pH = 8.0 and 0.015mL of β-mercaptoethanol (βME). To resolubilize the proteins, the tubes were alternately vortexed and boiled. On the first day, tubes were vortexed, boiled for 3 minutes, vortexed again, boiled for another 3 minutes, and then vortexed once again. Tubes were allowed to sit overnight, and the procedure was repeated.

The tubes were then centrifuged (5000RPM, 5 minutes, 4°C) to remove cellular and other debris that otherwise would cause severe streaking in protein bands during SDS-PAGE. From the water pre-rinse tubes, 150μL of supernatant was transferred to a 1.5mL centrifuge tube. Supernatants from the expectorated astringent samples were combined with the two corresponding water post-rinses. From each of the three tubes, 50μL of the supernatant was transferred into a single 1.5mL tube. All tubes were stored at -20°C until SDS-PAGE was performed.

SDS-PAGE

To analyze the samples for the two salivary mucins, 20μL aliquots were loaded on pre-cast 4-15% polyacrylamide Tris-HCl gels (Criterion Tris-HCl gel, Bio-Rad, Hercules, CA) . All samples collected from a subject were run on a single gel, Table 7. Electrophoresis was run at 150V for approximately 75 minutes using a pre-mixed Tris-glycine-SDS running buffer. Gels were stained with periodic acid-Schiff reagent (PAS) (Sigma Aldrich, St. Louis, MO) and destained following the methods of Becerra et al. (2003). Gel images were captured in color using a Canon Rebel T1i digital SLR camera and in grayscale using a Bio-Rad Universal Hood II.

Table 7. Gel lane assignments for removal of mucus coating experiment
Gel lane assignments for the samples collected to determine if astringents remove the mucus coating. All samples collected from a single subject were run on a single gel.

Lane	Sample
1	Bio-Rad (Hercules, CA) broad range molecular weight standards
2	Laemmli sample buffer (Bio-Rad, Hercules, CA)
3	Water pre-rinse before water rep 1 (lane 4)
4	Water rep 1 sample combined with water post rinses
5	Water pre-rinse before tannin low (lane 6)
6	Tannin low sample combined with water post rinses
7	Water pre-rinse before tannin high (lane 8)
8	Tannin high sample combined with water post rinses
9	Water pre-rinse before acid low (lane 10)
10	Acid low sample combined with water post rinses
11	Water pre-rinse before acid high (lane 12)
12	Acid high sample combined with water post rinses
13	Water pre-rinse before alum low (lane 10)
14	Alum low sample combined with water post rinses
15	Water pre-rinse before alum high (lane 12)
16	Alum high sample combined with water post rinses
17	Water pre-rinse before water rep 2 (lane 18)
18	Water rep 2 sample combined with water post rinses

I.3.2 Desquamation of the oral mucosa

DNA Extraction

DNA was extracted immediately following each session according to the GENTRA PureGene DNA Purification Protocol for Buccal Cells in Mouthwash (Qiagen, 2007) with minor modification: 15mL centrifuge tubes were used for all centrifuge steps, and all centrifuge operations were run at 5500 RPM using a JA 7.5 swinging bucket rotor. Additionally, because the tannin astringent solutions were difficult to dissolve, all solutions were incubated for 45 minutes following the addition of Puregene Proteinase K instead of the 10 minutes recommended in step number 11 of the official procedure. Additionally, at each 15 minute increment within the 45 minute incubation, samples were vortexed vigorously for 20 seconds. Following the final overnight incubation, tubes were transferred to a 40°F cooler until DNA quantification. Extracted DNA samples prepared

according to these procedures are expected to be stable for over 1 year when held at refrigeration temperatures.

DNA Quantification

To quantify the extracted DNA, an ultrasensitive fluorescent nucleic acid staining kit (Quant-iT^{TM} PicoGreen® dsDNA Kit) was used according to the published protocol (Invitrogen, 2008). All samples were quantified in duplicate within a 2 hour time period at 3 weeks and then at 6 weeks after all DNA extractions were completed. During the first DNA quantification procedure, two DNA standard curves (one high-range and one low-range) were prepared according to instructions using the Lambda DNA standard included in the kit. During the second DNA quantification procedure, only the high-range standard curve was prepared.

Fluorescence of the samples was measured using an LS 50B Perkin Elmer (Waltham, Massachusetts) fluorescence spectrometer, which was configured using FL WinLab Software. Excitation was fixed at a wavelength of 480nm, and emission was measured between 500-550 nm. A typical emission curve, showing the high-range DNA standards, is shown in Figure 20.

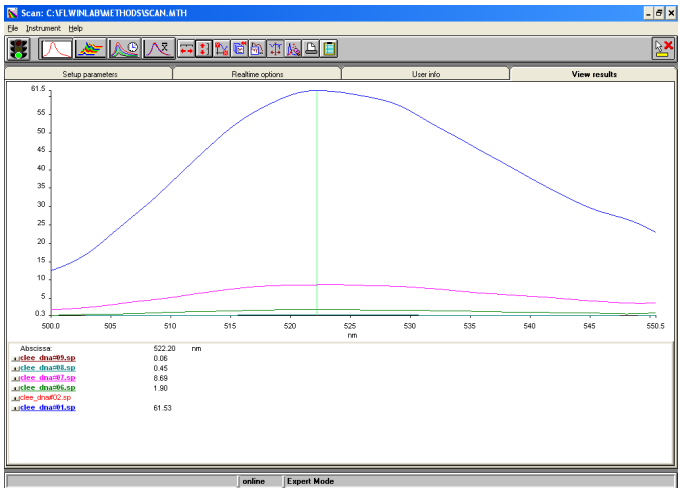

Figure 20. Typical emission spectra of DNA samples
Screen capture showing a typical emission spectra of samples containing DNA at various concentrations. Samples shown are the DNA standards prepared for the high-range standard curve.

Data Analysis

I.3.1 Loss of the mucus coating

Two bands were identified in each of the lanes within the gels from all five panelists, Figure 21. These two bands were presumed to be MG1 and MG2 based on their location in the gel relative to molecular weight standards. The trace intensity of each mucin band was determined using Quantity One v.4.6.7 software, and it is proportional to the amount of protein present. It is determined by measuring the total area underneath a band's intensity profile, Figure 22. The SAS code, in addition to other supporting documents (panelist instructions, blinding codes, sample rotation plan, band intensity data) can be found in Appendix 7.

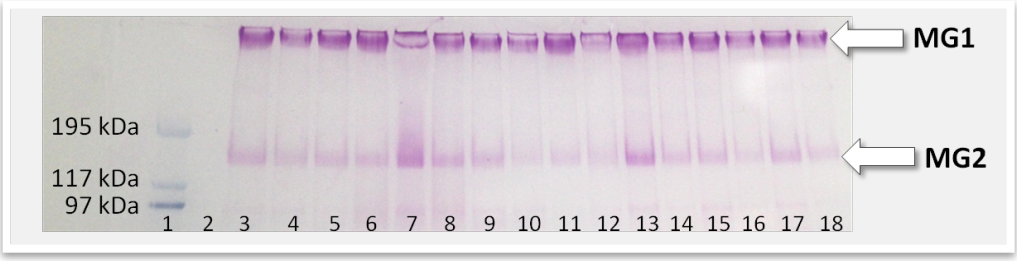

Figure 21. Typical appearance of PAS-stained gel in loss of mucus coating study
Example gel showing typical band pattern observed. Lane numbers are labeled at the bottom of the image and correspond to the samples in Table 7. Two bands were quantified in lanes 3 through 18 and are most likely MG1 and MG2 based on their location relative to molecular weight standards. The molecular weight standards shown in the lane 1 are myosin, β-galactosidase, and bovine serum albumin and their apparent molecular weights (in kDa) in this type of gel are as shown in the image.

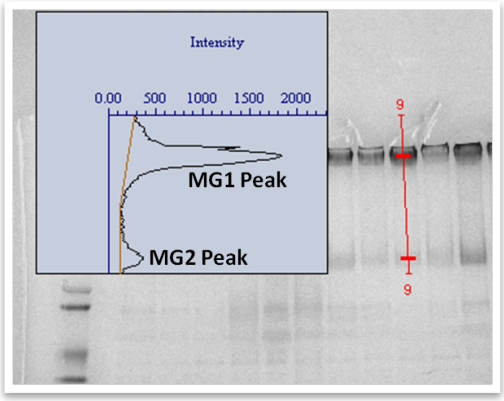

Figure 22. Typical band intensity profile in loss of mucus coating study
Typical intensity profile of the lanes in the gels. Two peaks were easily identified in each gel and most likely corresponded to MG1 and MG2 protein bands. The trace intensity of a band was proportionate to the total area underneath the band's intensity curve and the quantity of protein that is present.

Using the trace intensity data of the mucin bands, proportions were calculated:

$$MUCIN_{prop} = MUCIN_{astr}/MUCIN_{water}$$

where:

$MUCIN_{water}$ = Trace intensity of the bands in the water pre-rinse expectorant

$MUCIN_{astr}$ = Trace intensity of the bands in the sample from the combined astringent and post-water rinse expectorants

$MUCIN_{prop}$ was calculated for the MG1 and MG2 bands separately and for the two bands combined. A $MUCIN_{prop}$ equal to one would indicate that the amount of mucin(s) in the astringent expectorant was equal to that found in the water pre-rinse expectorant. A $MUCIN_{prop}$ greater than one would indicate that there was more mucin in the astringent expectorant than there was in the water expectorant. A $MUCIN_{prop}$ for an astringent that was greater than the $MUCIN_{prop}$ for the water control (i.e., water pre-rinse followed by water "astringent" rinse) would indicate that that particular astringent removed more mucins than the control.

To determine if there were differences in the trace intensity proportions ($MUCIN_{prop}$) among the astringent rinses, an ANOVA procedure (Proc MIXED) was performed in SAS v9.1. $MUCIN_{prop}$ was the dependent variable, and solution type and panelist were used as fixed and random effects, respectively. Contrasts were performed to reveal differences among the rinses or groups of rinses.

I.3.2 Desquamation of the oral mucosa

Emission at 522nm was recorded for each water expectorant and its corresponding astringent solution expectorant. From these data points, proportions were calculated:

$$DNA_{prop} = DNA_{astr}/DNA_{water}$$

where:

DNA_{water} = *DNA quantity in the water expectorant*

DNA_{astr} = *DNA quantity in the astringent expectorant*

A DNA_{prop} equal to one would indicate that the same amount of DNA was found in the astringent solution as was found in the water rinse expectorated just prior to it. A DNA_{prop} greater than one would indicate that there was a higher level of DNA in the astringent expectorant than there was in the water expectorant. DNA_{prop} values obtained in the two days of DNA quantification were averaged ($DNA_{prop(mean)}$)for use in statistical analysis.

To determine if there were differences in the DNA proportions among the astringent rinses, an ANOVA procedure (Proc MIXED) was setup in SAS v9.1. $DNA_{prop(mean)}$ was the dependent variable, and solution type and panelist were used as fixed and random effects, respectively. Contrasts were performed to reveal differences among the rinses or groups of rinses (e.g., high strength vs. low strength astringent solutions).

Results

I.3.1 Loss of the mucus coating

If the hypothesis that astringents removed the mucus coating of the mouth were true, $MUCIN_{prop}$ for the astringents would be expected to be higher than the $MUCIN_{prop}$ of water, but differences between the $MUCIN_{prop}$ for water and the $MUCIN_{prop}$ for any astringent were not detected for either MG1 ($F = 0.68$, $p = 0.67$) or MG2 ($F = 2.07$, $p = 0.08$). Instead, For MG1, although the trend was far from significant for most astringents, $MUCIN_{prop}$ may actually be lower for astringents as compared to water, Figure 23a. Contrasts between acid (as a group) and water showed this to be near significance ($F = 3.48$, $p = 0.07$). For MG2, no clear pattern emerged in the band analyses, Figure 23b. Visual observations of the gels match the results from the band analyses, Figure 24.

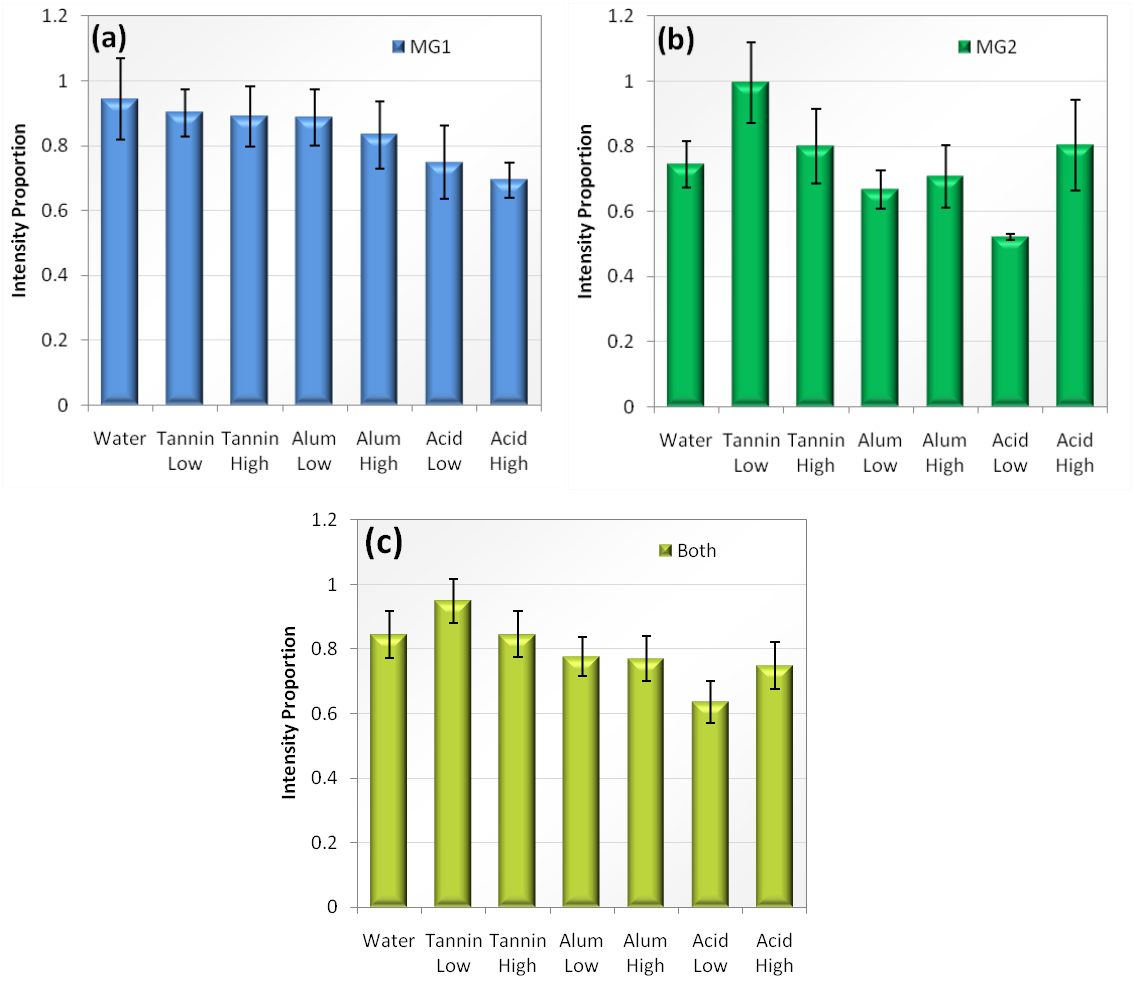

Figure 23. Relative proportion of MG1, MG2, and MG1+MG2 band intensities in the astringent rinses

$MUCIN_{prop}$ vs. astringent solution type for MG1 (a), MG2 (b), and both MG1 & MG2 combined (c). If the astringents removed the mucus coating as hypothesized, $MUCIN_{prop}$ for the astringents would be expected to be higher than $MUCIN_{prop}$ for water. For MG1, the opposite trend was observed, although the trend was far from significant. No differences in $MUCIN_{prop}$ among the water and astringent expectorants were found for either MG1 ($p = 0.67$) or MG2 ($p = 0.08$).

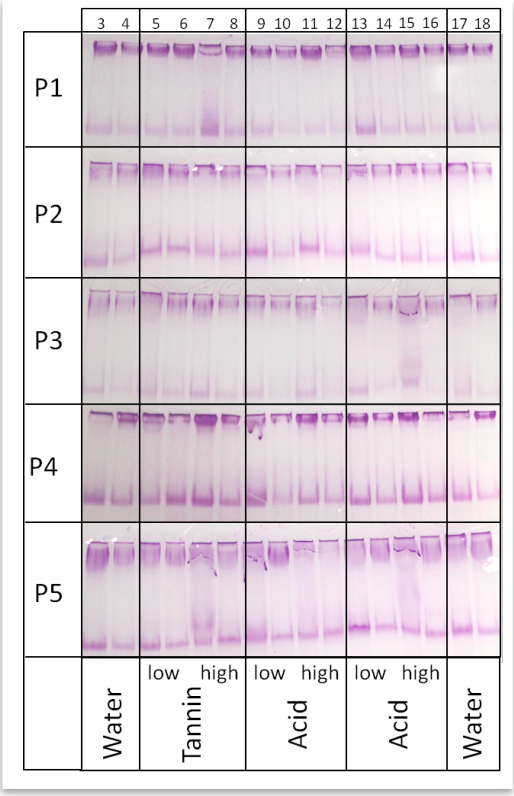

Figure 24. PAS-stained gels from all five panelists in loss of mucus coating study
Color images of the PAS-stained 4-15% polyacrylamide gels from each of the five panelists showing MG1 and MG2 bands. Lane numbers are labeled at the top. Lanes 1 and 2 are not shown and contained molecular weight standards or Laemmli buffer only, respectively. Lane contents are as detailed in Table 7 and are labeled at the bottom of the image. Odd numbered lanes contain expectorated water pre-rinses, and the even lanes contain expectorated astringent solutions (mixed together with water post-rinses as previously described). For example, lane 3 contains the expectorated water pre-rinse solution collected before the expectorated water solution in lane 4. Lane 5 contains the expectorated water pre-rinse solution collected before the expectorated tannin-low solution in lane 6.

I.3.2 Desquamation of the oral mucosa

While none of the astringent rinses' DNA_{prop} values were found to be different when contrasted with DNA_{prop} of water (all $p > 0.09$), DNA_{prop} was higher (i.e., more DNA was removed from the mouth) following rinsing with the high-strength astringent solutions when compared to the low-strength solutions (contrast, $F = 8.25$, $p = 0.008$), Figure 25. The DNA_{prop} of water (DNA in a water expectorant relative to the DNA in a preceding water expectorant) was 0.93 (with a 95% confidence interval that included 1.00),

indicating that the levels of DNA removed in both water rinses were not found to be different. The complete data set can be found in Appendix 6.

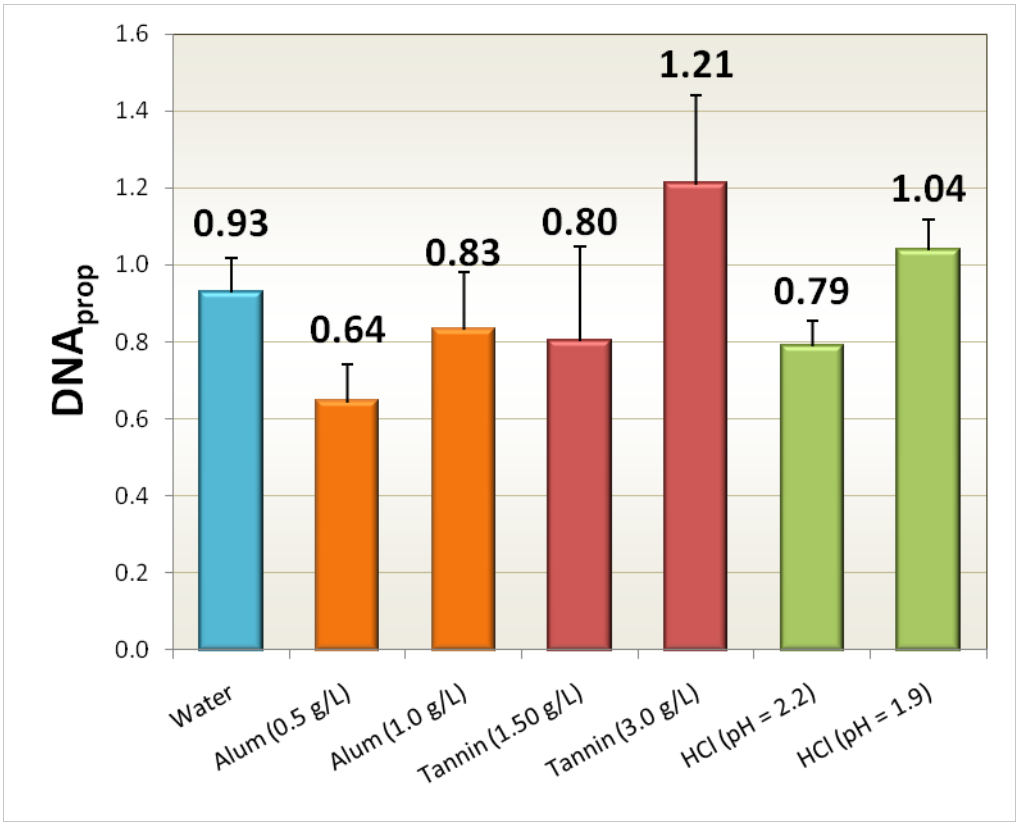

Figure 25. **Relative proportion of DNA in astringent rinses**
DNA_{prop} for all seven astringent rinses. A $DNA_{prop} = 1$ indicates that the quantity of DNA in the expectorated astringent rinse was the same as in the water prerinse expectorant. $DNA_{prop} < 1$ indicates that less DNA was found in the astringent expectorant compared to the water prerinse expectorant.

Discussion

Results from these two studies do not support the hypothesis that astringents caused increased desquamation of the oral mucosa and loss of the mucus coating, but the data pattern observed suggests that the astringents do have some type of predictable oral effect. Compared to water, DNA_{prop} and $MUCIN_{prop}$ were expected to increase when astringent solutions were expectorated (more DNA and more mucins being removed compared to water), but in some cases, the opposite trend was observed.

It was surprising that there were fewer mucins and less DNA in many of the expectorated astringent samples compared to the water controls, and it is unknown why this occurred. The decreased DNA and mucins in many of the alum and acid expectorated samples could be due to an unknown oral phenomenon or it could be that the astringent compounds interfered with the quantification methods. In the electrophoretic-based methods used to analyze for mucins, preliminary work established that astringents did not interfere in mucin quantification (Appendix 8), so this cannot explain why there were less mucins in these samples. It is possible, however, that astringents interfered with DNA quantification, and this is discussed in greater detail below.

It is very possible that something was occurring orally that would explain the decreased presence of mucins and DNA in the expectorated acid and alum samples. It was obvious, from visual observations of the expectorated samples, that alum and acid rinses were having profound effects on the salivary proteins. In these expectorated samples, large flocculated chunks were present and likely contained proteins and other oral debris. This was especially true in the acid samples. Perhaps this phenomenon was occurring inside the mouth, causing proteins and debris to flocculate and stick onto oral surfaces. This could explain why, in general, higher amounts of mucins were observed in the second study in which panelists followed the astringent rinse with 2 water post-rinses (compared to the first study where no water post-rinses were used, Appendix 8).

It is unclear why the high-strength astringent solutions removed more DNA than the low-strength solutions, yet none were found to have removed more DNA that water alone. It is possible that low levels of astringents bind to the oral surfaces and cells in the mouth and prevent them from being removed with the rinse, but as the concentration of the astringent is increased, some of the cellular debris starts to slough off; however, support for this theory is not provided by the mucin experiment. As previously mentioned, it is also possible that the astringents and other sample contaminants influenced the signal strength in assay used to quantify the DNA. For example, 200mM of sodium chloride is

known to decrease the signal by 30%. The signal strength is affected by other common salts, so it seems likely that alum could also interfere. Proteins can also affect the signal strength. Immunoglobulin G concentrations of 0.1% have been shown to increase the signal by 19%, while bovine serum albumin at 2% decreases the signal by 16% (Invitrogen, 2008). Unfortunately, the extent to which this occurred was not easily tested, since there was no way of knowing how much protein or astringent was left in the solution that was used for DNA quantification. This is because, prior to being quantified, the DNA was purified following a procedure that included numerous rinse and pelletization procedures. To test if and how astringents affected the entire procedure (both DNA purification and quantification), a known quantity of oral cells would need to be added into astringent solutions, and these solutions would have to be treated as the expectorated solutions were.

The variability in the data from the tannin expectorants was noticeably higher than with the acid, alum, or water expectorated samples. In the DNA purification procedure, after cells were initially pelletized, cells were lysed, incubated for 15 minutes, and then a proteinase was added and incubated again. More so than any of the other samples, the expectorants from the tannin solutions resulted in large pellets that were very difficult to break up. The incubation step following the addition of the proteinase was extended (for all samples) because of the excess time required to process the tannin samples. It is also known that tannins can bind cells (Payne et al., 2009), and this could have interfered with the analysis and increased variability. It is also possible that the natural variation among people's salivary PRP concentrations affected the actions of the tannins when inside the mouth.

Part I Final Thoughts

The overall goal of Part I of my research was to better understand the mechanism of astringency. Although much remains to be understood, some long-standing questions have been answered. Most notably, I provide evidence that the precipitation of PRPs or diminished salivary lubricity does not explain astringency.

If diminished salivary lubricity is not the cause of astringency, then what is? Based on the results from my studies that analyzed the DNA and mucin content in expectorated astringent samples, it does seem that astringents are affecting the oral coatings in certain ways that may contribute to the sensation. The coincidence is too strong that higher DNA levels were found in the high vs. low concentration of astringent solutions and that mucin concentrations were often lower in expectorated acid and alum solutions compared to water. Further investigation into this phenomenon is warranted. I also tend to believe, based on my personal experience in feeling astringency hundreds of times during the past 4 years and after viewing imagery of porcine tissue exposed to alum, that a direct tissue effect is a large component of astringency with certain (but probably not all) astringents.

When I initially started my research into the mechanism of astringency, I believed there to be one mechanism among all astringent compound types. I no longer think this is the case. Although the sensations are very similar across acids, alum, and tannins, based on what I've observed through my studies – both in data, my personal perceptions, panelist feedback, and viewing expectorated solutions of astringents – I believe that there must be differences. Rubbing the peel of an unripe banana on the inner part of the lips, for example, causes a constricting sensation that is not experienced with acid. This, along with results showing acid to precipitate the mucins quite effectively, suggests that a loss of oral coatings is most important to acid astringency. On the other hand, the constricting sensation that comes with tannin astringency and their inability to precipitate the mucins as effectively as acids and alum suggests that their astringency is perhaps more closely

related to direct tissue effects. There are likely multiple contributing factors leading to the astringent sensation, with some being more relevant than others depending on the astringent.

CHAPTER 3

Part II. Palate Cleansers for Astringent foods

My objective was to assess six palate cleansers and two cleansing protocols for their ability to limit buildup and enhance discrimination of tannin-containing and acidic astringent solutions. Palate cleansers included water, carboxymethylcellulose, crackers, milk, chewing wax, or nothing. Twenty-nine panelists, randomly divided into two groups, participated in a 12-session series in which they rated the astringency of either six tannin or hydrochloric acid solutions. Panelists used a single palate cleanser at each session according to one of two cleansing protocols. The palate cleansers did not differ in their ability to prevent astringency buildup from occurring. Panelists were best able to discriminate among the astringency of the tannin solutions when water or nothing was used as a palate cleanser. Water or nothing also improved discrimination among the acid samples, but only at low levels of astringency. Astringency discrimination and buildup were not affected by the cleansing protocol. My results indicate that using water or nothing as a palate cleanser will facilitate detection of sensory differences of astringency.

Foreword: The bulk of the narrative presented in this chapter has been previously published (Lee & Vickers, 2010); some minor revisions and additions are included.

Introduction

The purpose of this study was to determine if palate cleansers improved panelists' ability to discriminate among the astringency in tannin and acid-containing samples, and if they prevented astringency build-up from occurring. I hypothesized that palate cleansers commonly used to alleviate astringency would result in less astringency buildup, but that they would also diminish panelists' discrimination ability. Six cleansers were tested and included water, nothing at all, skim milk, crackers, chewing wax, and carboxymethylcellulose. Panelists used these cleansers according to either an ad-lib or standardized procedure between astringent solutions that either contained tannins or HCl acid. Taste (either bitterness or sourness) and astringency ratings were collected.

Although the ability of a palate cleanser to enhance discrimination among samples is perhaps best evaluated using a difference test (e.g., alternative forced choice or triangle tests), the assessment of astringency in foods is typically done using scaling methods. As such, they represent a realistic scenario used to find differences among samples and were used in this study.

The study had three main objectives:

- The first was to evaluate six palate cleansers for their ability to improve discrimination among astringent samples containing tannins or acids; we hypothesized that common palate cleansers used to alleviate astringency would diminish the ability to discriminate between astringent samples when compared to no cleanser or cleansing with water.

- Our second objective was to evaluate the same six cleansers for their ability to limit astringency buildup from occurring; we hypothesized that common

palate cleansers used to alleviate astringency would reduce buildup.

- Our third objective was to compare two cleansing protocols for their effect on astringency buildup and discrimination among samples; we hypothesized that when panelists were allowed to cleanse until they could no longer perceive astringency (as compared to a shorter, timed cleansing period), there would be less astringency buildup.

Materials and Methods

Subjects

Twenty-nine subjects (8 males, 21 females) were recruited from the University of Minnesota to participate in a series of twelve test sessions that were held at the Sensory Center Testing Facility located on the St. Paul campus of the University. Subjects were selected from a pool of thirty-three trained subjects based on their ability to correctly distinguish astringency from sourness and bitterness, and all were paid a cash incentive for their participation. The University of Minnesota Institutional Review Board approved all procedures of the study.

Training

Panelists attended two training sessions during the first week of the study. During the first training session, panelists were familiarized with the definitions of astringency, bitterness, and sourness and were taught how to differentiate these attributes. Astringency was defined as 'a tactile sensation felt in the mouth that is commonly described as a drying-out or roughing sensation' (Lee & Vickers, 2008). Bitterness was defined as an unpleasant, disagreeable taste sometimes found in coffee, beer, citrus peels, and certain vegetables that tends to be slow to develop and is often most noticeable near the back of the tongue. Sourness was definite as a tart and tangy taste. Subjects were presented with examples of astringent (0.2% alum, McCormick, Hunt Valley, MD), sour (0.075% citric acid, Fisher Scientific, Fair Lawn, NJ), and bitter (0.057% caffeine, Eastman Kodak, Rochester, NY).

During the first session, subjects learned a specific sampling technique that was designed to improve their ability to separate bitterness and sourness from astringency (Lee & Vickers, 2008). The procedure required that they first rate sourness or bitterness after placing a sample in their mouth while holding still for 5 or 15 seconds, respectively. Ratings were collected on horizontally-oriented 15-point line scales labeled with the adjectives 'not' or 'extremely' at the scale end-points. They then swished the sample gently for 10 seconds, expectorated, and performed a standardized mouth movement consisting of three exaggerated mouthings of the word 'why'. Because astringency is a tactile sensation, standardization of movements served to minimize differences in the perception of the sensation. Astringency ratings were then collected on the same style scale as used for the taste attributes, but instead of a single, discrete rating, astringency ratings were collected continuously for 20 seconds. Panelists marked the line scale at the location corresponding to their initial perceived astringency intensity and were instructed to adjust their rating during the 20 seconds if they perceived a change in the sensation's intensity by changing the location of the mark on the line scale. Panelists practiced the sampling and rating technique using solutions containing tannins and dilute hydrochloric acid (HCl) that were prepared the same way as those used in the test sessions. Panelists were also introduced to and practiced using all palate cleansers.

At the second training session, panelists practiced the sampling procedures while interfacing with the SIMS 2000 computerized data collection system, and data collected during this session was used for screening panelists. Four sets of astringent samples were served, and panelists used different cleansers with each set of samples. All panelists received the same samples and cleansers in the same order. The first set of astringent samples included two tannin solutions (0.6 and 3.0 g/L) and panelists cleansed with crackers. The second set of astringent samples was two solutions that contained a mix of alum and caffeine, and panelists cleansed with milk. One alum-caffeine solution was made with high bitterness (0.1% caffeine) and low astringency (0.025% alum), and the other solution was made with high astringency (0.25% alum) and low bitterness (0.025%

caffeine). The third set of astringent samples included two acid solutions (pH = 1.9 and 2.4), and panelists cleansed by chewing on wax. The fourth set of samples was identical to the first, although panelists cleansed with 1% carboxymethylcellulose. Panelists passed screening if they successfully rated the astringency of strongest tannin and acid samples greater than corresponding weaker samples and if they ranked the intensity of the bitterness and astringency of the alum-caffeine solutions in the correct order.

Test Samples

Astringent Samples

A series of 6 bitter-astringent and a series of 6 sour-astringent samples were used throughout the 12 test sessions. The bitter samples contained 0, 0.6, 1.2, 1.8, 2.4 and 3.0 g/L of tannins (Tanin VR Supra, Scott Laboratories, Petaluma, CA) and were flavored using root beer concentrate (McCormick, Sparks, MD) and sweetened with 0.015% sucralose (Splenda® sucralose granular, Tate & Lyle, Decatur, IL). Caramel coloring (D.D. Williamson, Louisville, KY) was added to solutions to mask color differences caused by varying levels of tannins. To make five of the sour samples, HCl (Sigma-Aldrich, St. Louis, MO) was added to distilled water to reach pH's of 3.0, 2.7, 2.4, 2.15, and 1.9. The sixth sour sample was distilled water with no added acid. Sour samples were flavored with raspberry flavoring (Natural raspberry flavor, Givaudan, Cincinnati, OH) and sweetened with 0.015% sucralose. Individual 10-ml samples were served at room temperature in 1-oz (29.6 mL) cups labeled with random, 3-dgit codes. New codes were generated for each test session.

Palate Cleansers

Six palate cleansers were used in the study and included distilled water, crackers (Premium® Crackers with unsalted tops, Nabisco, Northfield, IL), skim milk (Minnesota Creamery™, Edina, MN), 1% carboxymethylcellulose (Carboxymethylcellulose sodium salt, medium viscosity, Sigma-Aldrich, St. Louis, MO), wax (Parafilm® M, Pechiney Plastic Packaging, Chicago, IL) plus lemons, and nothing. Palate cleansers were used before and in between all astringent samples. Liquid palate cleansers were swished in the

mouth and expectorated. Crackers were chewed and then either swallowed or expectorated and followed with an optional brief rinse with water. To use the wax palate cleanser, panelists chewed on a 2"x 4" (50.8 x 101.6 mm) piece of wax while sniffing a freshly cut lemon wedge to induce salivation (Pangborn et al., 1979). All palate cleansers were served at room temperature.

Palate cleansers were served according to one of two protocols. In half of the test sessions, panelists used the cleansers following a strictly timed (fixed) protocol, and in the other sessions, they used them ad libitum. The amount of palate cleanser served for the fixed cleansing protocol was controlled. The liquid palate cleansers (water, milk, CMC) were served in 10-ml portions in 1-oz (29.6 mL) cups, and 6 pieces each of wax and crackers were served. When the cleansing protocol was ad-lib, palate cleansers were served in greater amounts, and panelists could also request more palate cleanser if needed. Liquid palate cleansers were served in full, 4-oz (118.3 mL) cups and multiple cups were given. Twelve pieces each of crackers or wax were served. For the ad-lib protocol, panelists were instructed to continue to use the palate cleanser until astringency could no longer be perceived. The timeline for the sampling procedures using the different cleansing protocols is show in Figure 26.

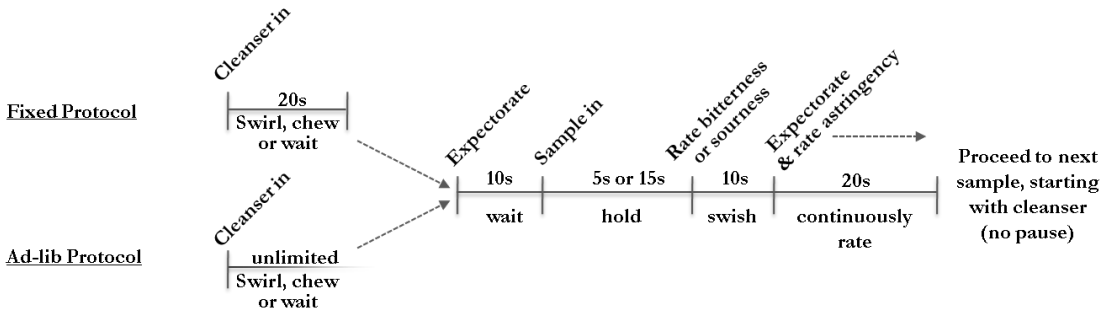

Figure 26. Timeline for sampling procedure in palate cleanser study
Timelines of sampling procedures for set protocol and self-determined cleansing strategies. Procedures were identical except for the time spent and quantity of palate cleanser used. Panelists received timed prompts automatically from the SIMS system.

Test Sessions

Panelists attended 12 test sessions, two per week, beginning the week after training. For these sessions, the 29 panelists were randomly divided into two groups. Fifteen were assigned to the 'bitter' group and received the six tannin-containing solutions at every test session. The other fourteen subjects were assigned to the 'sour' group and received the acid solutions. The bitter group rated the bitterness and astringency of the tannin solutions, and likewise, the sour group rated the sourness and astringency of their samples.

At each session, panelists were served the six astringent test samples and one of the six palate cleansers, which they used according to either the 'ad-lib' or 'fixed' protocol. Unbeknownst to the panelists, the time they spent using the cleansers was recorded. Every panelist used each of the twelve possible cleanser-protocol combinations once, and the session in which they were assigned a specific cleanser-protocol combination was determined according to a 12 x 12 Latin square design balanced for order (MacFie et al., 1989). The order in which the astringent test samples were served was balanced across the twelve times each cleanser-protocol combination was used according a 6 x 6 Latin square design balanced for order and carry-over effects.

All test sessions were run in an identical fashion except for the last session. At the end of the last session, a liking questionnaire was presented to panelists asking them to rate how much they liked or disliked using each of the palate cleansers. Ratings were collected using a horizontally-oriented labeled affective magnitude scale (Schutz & Cardello, 2001) ranging from 'greatest imaginable disliking' to 'greatest imaginable liking' on the left and right scale ends, respectively.

Data Analysis

Mixed model analysis of variance procedures in SAS® v9.1 (Proc Mixed) were used to determine if the samples differed in their astringency and in their bitterness or sourness. For astringency, which was rated continuously for 20 seconds, the maximum rating was

used as the dependent variable in the model. Apart from the palate cleanser liking data, data from the sour and bitter groups were analyzed separately. Fixed effects included the sample's tannin or acid concentration, serving position, palate cleanser type, and cleansing protocol (fixed or ad-lib). Random effects included panelist and a panelist-session-cleanser interaction factor. Overall mean ratings for astringency, bitterness and sourness by palate cleanser were compared using a Bonferroni-adjusted least-significant means procedure within the Proc Mixed procedure.

To determine if palate cleansers affected panelists' ability to discriminate among the varying levels of astringency in the samples and to test our first hypothesis, we included a cleanser-by-concentration interaction term. A significant effect would indicate that the slope of the lines (taking into account the variability associated with the data) describing the relationship between astringency ratings and tannin concentrations changed depending on the cleanser type. To determine if there were differences among the palate cleansers in their ability to prevent build-up of astringency from occurring and to test our second hypothesis, the cleanser-by-position interaction term was added to the model. If significant, this would indicate that the relationship between astringency ratings and serving position had changed depending on the cleanser that was used. To see if the palate cleansing protocol used (fixed or adlib) affected the level of buildup that occurred and to test the third hypothesis, a protocol-by-position term and its three-way interaction with cleanser type were added to the model. Additionally, protocol-by-concentration and its three-way interaction with cleanser type were included to determine if the cleansing protocol affected discrimination ability. Contrasts were performed within all significant interactions to reveal specific differences among the cleansers' or protocols' abilities to prevent buildup or enhance overall discrimination among samples.

To determine if certain cleansers were liked more than others, we setup Proc Mixed using labeled affective magnitude (LAM) scale values as the dependent variable. Scale values ranged from 0 to 120, which corresponded to 'greatest imaginable disliking' and 'greatest imaginable liking', respectively. Fixed effects in the model included group type (bitter or

sour), cleanser, and a group by cleanser interaction term. We included the group by cleanser interaction term to determine if liking scores from panelists in the 'bitter' group differed from those in the 'sour' group. A random effect of panelist, nested within group type (bitter or sour) was also included. The mean liking scores of the six palate cleansers were compared using a Bonferroni-adjusted least-significant means procedure within the Proc Mixed procedure.

To determine if the cleansing protocol (fixed or adlib) and cleanser type influenced the time panelists spent using the cleansers, we performed t-tests (Proc ttest in SAS®) to compare the total time elapsed between the first and sixth samples. Data from the sour and bitter groups were compared separately since there were slight differences in their tasting procedures.

The SAS code, in addition to other supporting study documents (training handout, blinding codes, and sample serving orders) can be found in Appendix 9.

Results

The Effect of Palate Cleanser: Discrimination among Astringent Samples

Panelists' ability to discriminate among the astringency of the six tannin samples was best when water was used for cleansing as compared to all other cleansers (all p-values from contrasts within cleanser-by-concentration interaction < 0.05) except for nothing ($p = 0.19$) (Figure 27). Using nothing for cleansing allowed for better discrimination than when using milk or crackers (both $p < 0.001$) but not wax ($p = 0.40$) or CMC ($p = 0.10$). The palate cleansers did not appear to affect panelists' ability to discriminate among the astringency of all acid samples ($p = 0.52$); however, when mean astringency ratings were plotted vs. acid concentration, discrimination differences among the cleansers appeared at low acid concentrations (Figure 27). To determine whether the palate cleansers effected discrimination at the lower concentrations, the same mixed model analysis of variance procedure was performed on the data from only the three weakest samples. Panelists'

ability to discriminate among the 3 weakest solutions differed and was best when they cleansed with water or nothing compared to each of the other cleansers (all p-values from contrasts within cleanser-by-concentration interaction < 0.01). Cleansing with water or crackers resulted in the highest and lowest average astringency ratings, respectively, across all sessions and samples ($p < 0.001$) (Table 8).

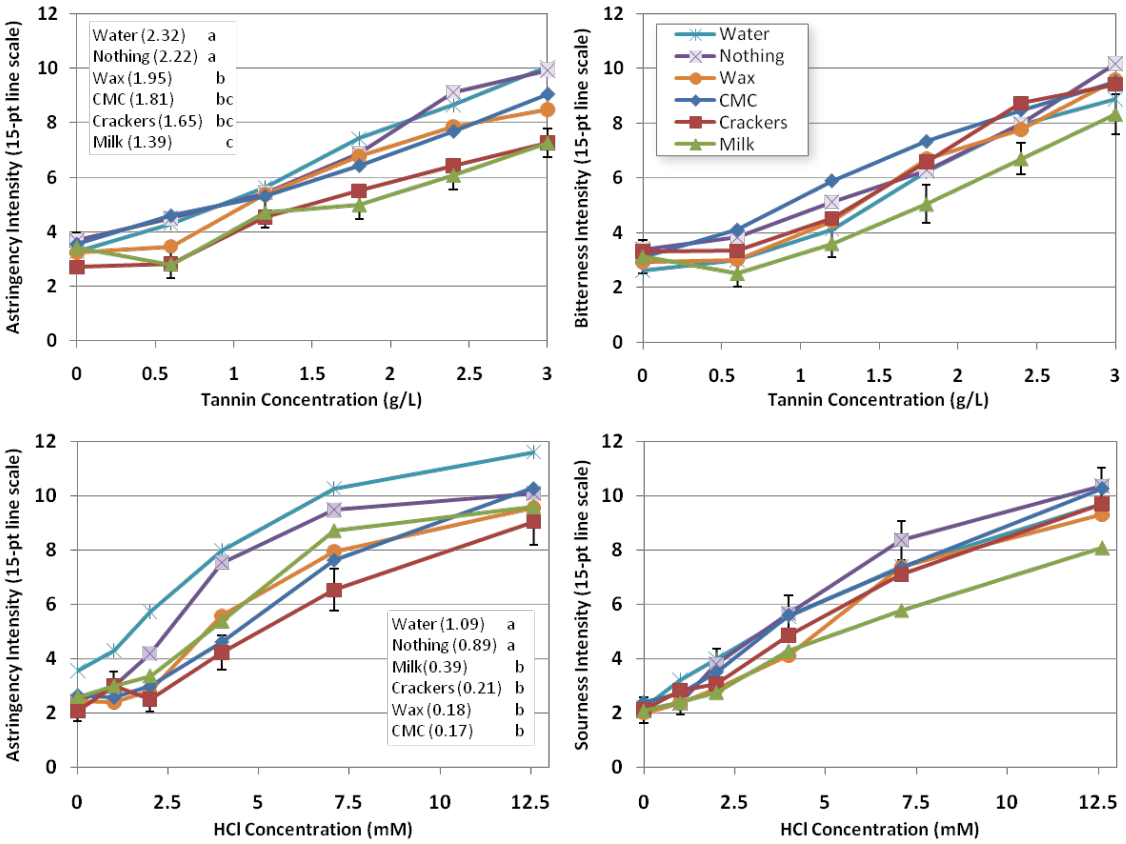

Figure 27. Astringency and taste ratings vs. astringent concentrations in palate cleanser study

Mean astringency (a) and bitterness ratings (b) vs. tannin concentration and mean astringency (c) and sourness ratings (d) vs. HCl concentration as a function of palate cleanser. Cleansers with the same letter did not differ in enabling discrimination among all samples in (a) and among the 3 weakest solutions in (c) ($p < 0.05$); slopes of the rating vs. concentration function for each cleanser type are shown in parenthesis. All ratings were obtained on a 15-pt line scale labeled with the adjectives 'not' and 'extremely' at the scale end-points. Standard errors of ratings were similar for all palate cleansers and are shown for one palate cleansers on each graph.

Table 8. Mean astringency and taste ratings by palate cleanser type
Mean astringency, bitterness, and sourness ratings (and associated standard errors) collapsed over all samples and sessions by palate cleanser for acid and tannin solutions. All ratings collected on a 15-point line scale labeled with the adjectives 'not' and 'extremely' at the scale end-points.

	Tannin Solutions				Acid Solutions			
	Astringency		Bitterness		Astringency		Sourness	
Palate cleansers:	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Water	6.7	0.27	5.6	0.28	7.2	0.36	5.3	0.31
Nothing	6.6	0.25	6.1	0.29	6.1	0.35	5.4	0.34
Skim Milk	4.9	0.23	4.9	0.28	5.4	0.35	4.2	0.28
Carboxymethylcellulose (CMC)	6.0	0.24	6.5	0.28	5.1	0.32	5.3	0.31
Wax + lemons	5.7	0.26	5.6	0.29	5.1	0.31	4.7	0.31
Crackers	5.0	0.23	5.7	0.29	4.6	0.32	5.0	0.31

The Effect of Palate Cleanser: Astringency Buildup

For the tannin-containing solutions, astringency increased as a function of the serving position of the sample ($p < 0.001$) (**Figure 28**), indicating that astringency buildup was occurring, but the extent to which this occurred did not differ among the six palate cleansers tested (cleanser-by-position interaction, $F(25, 829) = 1.40$, $p = 0.09$). The astringency of the acid samples also increased as a function of serving position ($p < 0.001$) (**Figure 28**), but again, differences were not found among the palate cleansers ability to prevent astringency buildup from occurring (cleanser-by-position interaction, $F(25,758) = 0.81$, $p = 0.73$)

The Effect of Cleansing Protocol

The particular cleansing protocol employed – either fixed or ad-lib – did not impact astringency buildup in either the bitter (protocol-by-position interaction, $F(5, 829) = 1.69$, $p = 0.14$) or sour (protocol-by-position interaction, $F(5, 758) = 1.36$, $p = 0.24$) groups. This was true for all cleanser types (protocol-by-position-by-cleanser interaction, $p > 0.64$ for bitter and sour groups). Likewise, cleansing protocol did not appear to impact astringency discrimination ability in either the bitter (protocol-by-concentration interaction, $F(1, 830) = 2.36$, $p = 0.13$) or sour (protocol-by-concentration interaction, F

(1, 758) = 0.33, p = 0.57) groups, and this was again true for all cleanser types (protocol-by-concentration-by-cleanser interaction, p > 0.36 for bitter and sour groups). The cleansing protocol was also not found to directly influence astringency intensity in either the tannin (p = 0.24) or acid (p = 0.49) group. In both groups, for every cleanser type, panelists spent less time cleansing when the protocol was adlib vs. fixed (all p < 0.01) (Table 9).

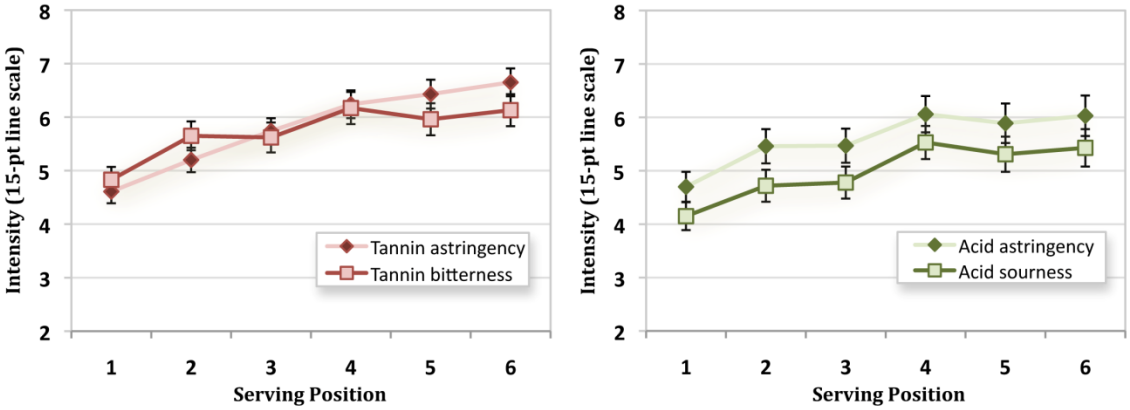

Figure 28. Ratings vs. sample serving position in palate cleanser study
Mean ratings as a function of serving position for all cleansers and sessions for tannin solutions (left) and acid solutions (right). Ratings for astringency, bitterness, and sourness all increased as a function of serving position (all p < 0.001). All ratings were obtained on a 15-pt line scale labeled with the adjectives 'not' and 'extremely' at the scale end-points. Error bars represent standard errors.

Table 9. Mean time panelists spent using palate cleansers for ad-lib and fixed protocols in palate cleanser study
Average total minutes elapsed between 1st and 6th sample for each palate cleanser (CMC = carboxymethylcellulose). Longer times indicate that panelists spent more time using the cleanser. Times between 'bitter' and 'sour' groups cannot be compared directly due to slight differences in tasting procedures. Standard errors in the table are for the time spent cleansing. Standard errors of the astringency ratings obtained through the two protocols types were not found to differ (p = 0.85).

		CMC		Cracker		Milk		Nothing		Water		Wax	
	Protocol:	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Bitter	**Fixed**	10.4	0.06	10.5	0.10	10.4	0.09	10.4	0.06	10.5	0.04	10.5	0.05
	Ad-lib	8.4	0.26	9.3	0.27	8.2	0.37	8.4	0.6	8.6	0.37	9.1	0.39
Sour	**Fixed**	9.6	0.05	9.8	0.17	9.7	0.07	9.7	0.04	9.7	0.10	9.7	0.05
	Ad-lib	7.4	0.37	8.6	0.39	7.2	0.27	6.8	0.41	7.7	0.45	8.1	0.38

Bitterness and Sourness

Bitterness, like astringency, increased as a function of the serving position of the tannin samples ($p < 0.001$) (Figure 28), and we observed no differences in the palate cleansers' abilities to prevent bitterness from building up (cleanser-by-position interaction, $F(25, 827) = 0.53$, $p = 0.97$). Unlike with astringency, however, we observed no differences among the palate cleansers in their ability to facilitate bitterness discrimination (cleanser-by-concentration interaction, $F(5, 828) = 1.70$, $p = 0.13$).

The sourness of the acid samples increased by serving position ($p < 0.001$) (Figure 28), and buildup was not affected by the cleanser type (cleanser-by-position interaction, $F(25, 758) = 0.70$, $p = 0.86$). At high sourness levels, cleansing with milk diminished sensitivity compared to all other cleansers (all p-values from contrasts within cleanser-by-concentration interaction < 0.04) (Figure 27).

Palate Cleanser Liking Data

Water and crackers were the most-liked cleansers, and both received mean liking scores corresponding to just under 'like moderately' on the LAM scale (Table 10). The only other cleanser to receive an above-neutral liking score was wax. The cleanser 'nothing' was rated at near neutral on the scale. Milk and CMC were disliked, with CMC receiving the lowest mean liking score. Panelists in the sour and bitter groups preferred the same cleansers ($p = 0.98$).

Table 10. Mean palate cleanser liking scores

Mean liking scores of palate cleansers (CMC = carboxymethylcellulose) with associated standard errors. Liking scores range from 0 (greatest imaginable dislike) to 120 (greatest imaginable liking). Intermediate scale anchors and their ratings (in parenthesis) included: dislike extremely (13), dislike very much (25), dislike moderately (39.5), dislike slightly (53), neutral (60), like slightly (67), like moderately (81), like very much (93), and like extremely (104). Means with the same letter are not significantly different ($p > 0.05$) as determined by a Bonferroni-adjusted least-significant means procedure.

	CMC	Milk	Nothing	Wax	Crackers	Water
Liking Score	41_a	56_{ab}	61_b	77_{bc}	81_c	81_c
Standard Error	5.1	5.1	3.2	4.0	3.6	3.3

Discussion

We set two criteria to assess palate cleanser efficacy: a palate cleanser was defined as effective if it was able to reduce buildup and if it enhanced discrimination among samples. Based on these measures, we identified water and nothing as the most effective palate cleansers for astringency. Others have not come to the same conclusions, likely due to differences in their definition of efficacy. Colonna et al. (2004) tested a variety of cleansers including water, crackers, and a 1% solution of CMC. Opposite to our findings, they concluded that CMC and crackers were both more effective than water. They, however, defined a cleanser's effectiveness as its ability to reduce or prevent the buildup of astringency from occurring. Likewise, Brannan et al. (2001), found CMC to be more effective than water but defined efficacy as being related to a cleansers ability to prevent buildup. Palate cleanser effectiveness has multiple aspects, and palate cleansers that are effective at reducing astringency and buildup are not necessarily effective for improving discrimination.

Palate cleansers that are most effective at reducing astringency or buildup may not the best at enhancing discrimination for several reasons. Some cleansers that are capable of reducing astringency may act by masking the sensation. Astringency is believed to be a tactile sensation caused by increased oral friction, and viscous cleansers, like CMC, may simply coat and lubricate oral surfaces, diminishing the friction. This would explain why panelists in the study by Brannan et al. (2001) consistently gave lower astringency ratings to the 2nd presentation of an identical, astringent solution when they used viscous rinses, including CMC, in between presentations. Other cleansers may interfere with the development of astringency. In our study, astringency ratings were suppressed at higher stimulus levels when panelists used milk, wax or crackers as compared to when they cleansed with nothing or water (Figure 27). Milk, which is often added to tea or coffee to reduce its bitterness and astringency via tannin binding by the casein proteins, may have had the same effect in the mouth. Likewise, chewing wax or eating crackers may have caused a similar phenomenon. Salivary proteins are also capable of binding tannins (Baxter, Lilley, Haslam & Williamson, 1997; Kallithraka, Bakker & Clifford, 1998), and

the increased salivary flow rate from chewing wax would have increased the amount of proteins present (Dawes & Jenkins, 1964; Froehlich et al., 1987). Fischer et al. (1994) and Horne et al. (2002) have correlated higher salivary flow rates to lower astringency levels. Milk proteins and increased salivary flow rates may have also interfered with the development of astringency caused by HCl, albeit though increased buffering of the acid within the mouth.

Although panelists' ability to discriminate among the astringency of all six acid samples was the same regardless of the palate cleanser used, at lower astringency levels, this was not true (Figure 27). The acid concentrations used in this study were distributed according to log-steps (with some minor adjustments based on preliminary data), and at the higher acid concentrations, this resulted in stimuli that were spread too far apart. Panelists had no problems discriminating among the strong acid solutions, regardless of the palate cleanser used. The choice of palate cleanser for the weaker solutions, which were more difficult for panelists to discern, was important. We believe that this is relevant for sensory evaluation of food products since the lower stimulus levels are the most representative of the levels found in foods.

Astringency ratings of the acid solutions were relatively high when panelists used water as a palate cleanser. Though ratings were largely determined by the acid concentration of the solutions, the significant effect of palate cleanser suggests that the cleanser can affect the acids' ability to elicit astringency. Residuals from some of the palate cleansers may have partially buffered the acid. Buffering capacity could have been increased by the proteins in skim milk, the carboxyl groups of CMC or by the increased quantity of saliva from chewing wax. Other cleansers, like crackers, may have raised the oral pH between samples (Vickers et al., 2008) and limited the subsequent pH drop from the following acid solution. Using water as a cleanser, however, likely diluted the saliva that was present and diminished its buffering capacity. With less ability to buffer acids, intraoral pH could have dropped to lower levels and greater astringency would have resulted.

The buildup of sourness ratings that we observed was unexpected. Though bitterness is known to linger and build with repeated exposures (Lea & Arnold, 1978; Brannan et al., 2001; Johnson & Vickers, 2004), we had not observed a similar trend with sourness ratings in previous studies of a similar nature (Lee & Vickers, 2008; Vickers et al., 2008). However, in both of those studies, we used citric and phosphoric acids, which are both weak acids. While self- and cross-adaptation occurs with the perception of sourness in weak acids, the phenomena does not happen with strong acids like HCl (Ganzevles & Kroeze, 1987). Instead, they found that the perceived sourness of an HCl solution actually increased following adaptation to HCl. Based on our data and those of Ganzevles & Kroeze (1987), it appears that sourness can build over repeated exposures when the acid that elicits the sour taste is strong.

We expected, but did not observe, an effect of palate cleansing protocol on astringency buildup in the study, likely because panelists did not adhere to cleansing instructions for the ad-lib protocol. The fixed cleansing protocol was designed to ensure that panelists sampled the astringent solutions in a relatively rapid fashion in order to induce astringency buildup. Thus, if a palate cleanser was superior to others in its ability to reduce buildup, the effect could be observed. For the ad-lib cleansing protocol, panelists were instructed to wait in between samples until their perceived astringency level was zero, and we expected that little to no buildup would be observed in this situation. Panelists did not appear to wait long enough, as significant buildup was still observed, and panelists completed sessions faster when they were assigned the ad-lib vs. the fixed cleansing protocol. This was true for all of the palate cleansers (Table 9). Time spent cleansing appeared to be heavily influenced by the format of the cleanser rather than a direct reflection of the lingering astringency intensity or the overall liking score given to the cleanser. When assigned to the ad-lib protocol, panelists spent the least amount of time cleansing when their assigned cleanser was nothing. They spent roughly equal amounts of time using each of the three liquid cleansers, and they spent the most time cleansing when having to chew either wax or crackers.

Standard errors associated with astringency ratings increased by position within sessions in both the sour and bitter groups (Table 11). The increasing variability in panelists' ratings as the test sessions progressed may indicate that panelists became less capable of judging astringency intensity levels due to taster fatigue. It is also possible that panelists experienced different levels of astringency due to their inherent physiological differences, and that these effects became more exaggerated with repeated tastings throughout each session. For example, salivary flow rates are known to affect astringency perception, and people with high flow rates have been shown to report lower astringency ratings (Fischer et al., 1994; Horne et al., 2002). Perception differences between people with high and low flow rates may have become amplified with increasing sample repetitions, causing the higher variation in ratings.

Table 11. Standard errors in astringency ratings by position of sample in palate cleanser study

Standard errors of astringency ratings by serving position averaged across all 12 sessions for solutions containing tannins and acid. Means with the same letter within a row are not significantly different as determined using a Bonferroni-adjusted least-significant means procedure ($p > 0.05$).

	Serving Position					
	1	2	3	4	5	6
Tannin Solutions	0.76_{a}	0.81_{ab}	0.81_{ab}	0.91_{b}	0.94_{b}	0.92_{b}
Acid Solutions	1.00_{a}	1.14_{ab}	1.13_{ab}	1.19_{bc}	1.32_{c}	1.35_{c}

Conclusions

Of the six palate cleansers tested in this study, discrimination among astringent samples was enhanced when water or no palate cleanser was used for all tannin samples and for the weaker acid solutions. None of the cleansers were able to prevent astringency buildup from occurring. Because the objective of sensory testing is often to find differences among samples, an essential function of a palate cleanser is its ability to allow for discrimination. The findings from this study indicate that forgoing any palate cleanser, or using only water, will facilitate detection of astringency differences.

CHAPTER 4

Part III. The Astringency of Whey Protein Beverages

Panelists rated acidic whey protein and acid-only solutions for astringency and sourness. Acidic whey protein solutions contained 6% or 1% whey protein isolate and phosphoric acid at a pH of 3.4. Acid-only solutions were formulated to match the whey-protein solutions for either total acidity or for pH. The acid-only solutions matched for total acidity were more astringent than the whey-containing solutions, while those matched for pH were significantly less astringent. Sourness was reduced by the whey proteins, most likely because of the decreased concentration of free hydrogen ions. The astringency of acidic whey protein solutions appears to be caused by their high acidity and not directly by the whey proteins.

Foreword: The bulk of the narrative presented in this chapter has been previously published (Lee & Vickers, 2008). The narrative has been updated to include experiments performed using electrophoretic methods to study the interactions between salivary proteins and astringent whey proteins beverages.

Introduction

The purpose of this study was to determine, through sensory and electrophoretic methods, the cause of the astringency in acidic whey protein beverages. There were two objectives:

Obj. III.a: To determine if the acidity of whey protein solutions was responsible for their astringency

Obj. III.b: To determine if the salivary proteins precipitated by acids were also precipitated by the whey protein solutions

The hypotheses were as follows:

Hypothesis III.a: Solutions containing equal acid concentrations will be equally astringent, regardless of their whey protein content and pH.

Hypothesis III.b: Astringent whey protein solutions will precipitate the same high-molecular weight proteins as acids

Materials and methods

III.a: Sensory Test

Subjects

Twenty subjects (5 males, 15 females) were recruited from the University of Minnesota and were paid a cash incentive for their participation during this two-day sensory study. Sessions were held at the Sensory Center located in the Food Science and Nutrition building on the St. Paul campus of the University.

Training

Panelists attended a training session on the first day of the study. The training session served to familiarize them with the concept of astringency and to teach them to differentiate between sour taste and the tactile sensation of astringency. Astringency was defined as 'a tactile sensation felt in the mouth that is commonly described as a drying-out or roughing sensation'. This definition was modified from the definition of Lee & Lawless (1991) by omission of the term 'puckery' to avoid confusion with sourness. Subjects then tasted astringent (alum, food processing grade, Barry Farm, Wapakoneta, OH, USA) and sour (citric acid, Sigma-Aldrich Co., St. Louis, MO, USA) samples and were introduced to the 15-pt category scales that would be used during the test session for intensity ratings. Alum at 0.5 g L-1 was used as an astringency reference standard, and was defined to have a rating of 5 on the15-pt scale.

During training, subjects were introduced to and practiced a specific sampling technique that was designed to improve their ability to distinguish between sourness and astringency. This technique required that subjects rated sourness immediately before astringency as a strategy to avoid their dumping of sour sensations into ratings of astringency. The entire sample was first placed in the mouth, and panelists were instructed to hold the sample in their mouth without moving while making a sourness rating. Subjects were then instructed to swish gently for 10 seconds, expectorate the

sample, and immediately perform a standardized mouth movement that consisted of three exaggerated mouthings of the word 'why'. The manner in which this movement was performed forced the gums and the buccal surfaces of the teeth to rub against the inner anterior lips and cheeks. Because astringency is a tactile sensation, standardization of movements after sampling served to minimize differences in the perception of the sensation. Subjects then rated the maximum astringency perceived during sampling of the solution. They practiced this procedure using the SIMS 2000 (Sensory Computer Systems, Morristown, NJ, USA) computerized data collection interface used during the test session.

Test samples

Four test solutions and a control were prepared for the test session (Table 12). Two test solutions, W6 and W1, contained whey protein isolate (WPI, Instantized BiPro®, Davisco, Eden Prairie, MN, USA). To prepare these solutions, WPI was dissolved into distilled water at 6% and 1% (w/v), and 0.5M phosphoric acid (Fisher Scientific, Fair Lawn, NJ, USA) was titrated into each solution until its pH reached 3.40. Measurements of pH were made using a Corning model 340 digital pH meter (Lowell, MA, USA) and a calomel electrode. The volume of 0.5M acid required to achieve a pH of 3.40 in the W1 and W6 solutions was recorded. Two other test solutions, A1 and A6, were made by adding these same volumes of acid into distilled water. Thus, A1 and A6 contained the same acid concentration as W1 and W6, respectively, but the A samples did not contain WPI. They were, however, at different pH levels. In order to control for the effects of pH, a control solution, ALo, was prepared by titrating 0.5M phosphoric acid into distilled water until the pH reached 3.40. Control solutions containing whey protein isolate without any acid were not included in the test design since these have been shown not to be astringent and because the majority of whey protein beverages are formulated to be acidic (Beecher, 2006).

Table 12. Formula, pH, and titratable acidity of test samples

Sample	Formula	pH	Titratable acidity[a]
W6	6% WPI with phosphoric acid	3.40	0.26
A6	Diluted phosphoric acid at concentration matched to W6	1.84	0.25
W1	1% WPI with phosphoric acid	3.40	0.046
A1	Diluted phosphoric acid at concentration matched to W1	2.37	0.041
ALo	Diluted phosphoric acid to match pH 3.4 (control)	3.40	0.002

[a]Measured titratable acidity is expressed as % phosphoric acid and was measured by titrating 0.5 M NaOH into 30 mL of sample (100 mL of ALo) to a pH endpoint of 7.0. NaOH was standardized using potassium hydrogen phthalate.

All solutions were prepared the day before the test session and held at 4°C until the morning of the test. We coded 2 oz. plastic cups (Solo® brand) with random 3-digit numbers and filled them with 10mL aliquots of the sample solutions. Presentation order of the solutions during the test sessions was based on a Latin Squares design balanced for order and carryover effects. Solutions were allowed to equilibrate for 2 hours at room temperature prior to serving.

Test session

On the second day of the study, subjects attended an hour-long testing session. They first sampled the alum reference standard and were reminded of its assigned intensity rating of 5 on the 15-pt scale. After a 5 minute wait that included rinsing with distilled water, panelists were served the four test solutions, the pH-matched control, and a distilled water control. Sourness and astringency ratings were collected for all samples. After rating each sample using the standardized procedure, a mandatory 5 minute waiting period was enforced in which panelists were instructed to rinse a minimum of 5 times using distilled water. Panelists were encouraged to extend the waiting period and continue rinsing until they no longer perceived an astringent sensation before proceeding to the next solution.

Data analysis

A mixed model analysis of variance procedure (Proc Mixed) in SAS® v9.1 (SAS Institute Inc., Cary, NC, USA) was used to determine if the solutions differed in their astringency and sourness. The solution type and serving position were set as fixed effects, and judges were treated as random effects. Contrasts were performed to compare the astringency and sourness of individual solutions and among groups of solutions. To determine the relationship between average sourness ratings and titratable acidity and pH, linear regression (Proc REG) in SAS® was performed using sourness as the dependent variable and titratable acidity and pH as the predictors. The SAS code, in addition to other supporting documents (training handout, blinding codes, sample serving order), is provided in Appendix 10.

III.b: SDS-PAGE analysis of saliva-astringent assays

Astringent solutions

W1 and ALo were used for this study, Table 12. Additionally, for this portion of the experiment, a neutral whey protein solution (W1n) was prepared by dissolving 1% (w/v) WPI into distilled water and stirring for 30 minutes.

Saliva

The pooled saliva that was collected for part I (page 28) was used for these experiments.

Saliva-Astringent Assays

Assays were at 1:1 ratio of astringent solution to pooled saliva. 400uL aliquots of saliva were added into 1.5mL tubes containing 400uL of W1, W1n, ALo, or distilled water. The tubes were vortexed and held for 5 minutes at room temperature before they were centrifuged (13,000g, 10 minutes) and then placed on ice. From each of the tubes, 100uL of the supernatant was transferred into new 1.5mL tubes and frozen at -20°C before being lyophilized. The pellets from each of the tubes were then isolated. The remaining

supernatant was decanted and 0.5mL of ice-cold acetone was added. The tube was inverted 5 times and centrifuged at 13,000g for 5 minutes. After decanting the acetone, a second acetone wash and centrifugation was performed. The tubes were drained and allowed to air dry. The pellets were resolubilized with 190uL laemmli buffer + 10uL βME, vortexed vigorously, and placed in a boiling water bath for 5 minutes. They were frozen until electrophoresis was carried out the following day. Immediately before electrophoresis was run, the lyophilized supernatants were resolubilized with 190uL laemmli buffer + 10uL βME, placed in a boiling water bath for 5 minutes, and allowed to cool to room temperature.

SDS-PAGE

Two gels were run to understand the interactions between the salivary mucins and the PRPs with the acidic whey protein solutions, and both the pellets and supernatants from the assays were run each of the gels. The PRP gel was stained using Coomassie brilliant blue R-250 (CBB), and a periodic acid-Schiff (PAS) stain was used for the mucin gel. The same methods used to analyze saliva-astringent assays in Part I (page 30) were used in this portion of the study.

Results

III.a: Sensory Test

Panelists rated the acid-only solutions A1 and A6, as a group, more astringent than the solutions containing whey protein, W1 and W6 ($t = 1.96$, $p = 0.05$) (Figure 29). The pH alone was not the determinant of astringency because subjects rated the ALo sample (water adjusted to pH = 3.4) much less astringent than either of the whey protein solutions at the same pH ($p =<0.0001$). Subjects assigned low astringency ratings to both ALo and water, and they did not distinguish between the astringency of the two ($t = 1.36$, $p = 0.19$). Although the presentation order of the solution did not have a statistically significant impact on the astringency ratings ($F = 0.41$, $p = 0.84$), we observed a slight tendency for the mean astringency rating to increase vs. position (Figure 30).

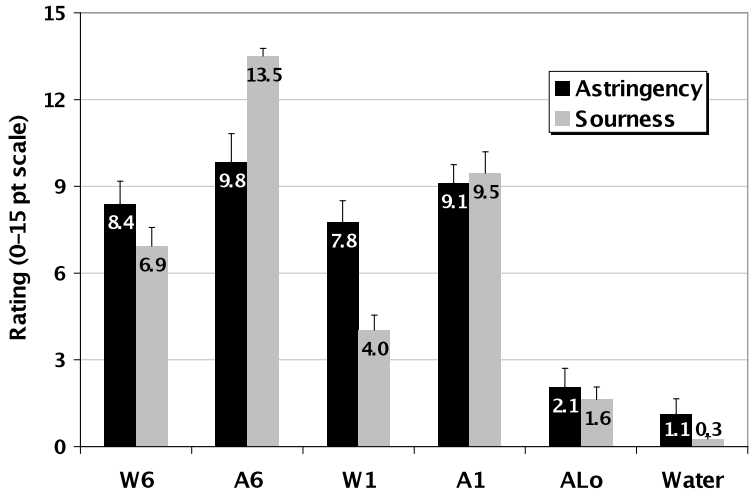

Figure 29. Astringent and sour ratings vs. sample type in whey protein beverage study
Mean astringency and sourness ratings (n = 20) on a 15-pt scale. See Table 12 for abbreviation explanations. Error bars represent standard errors.

Subjects rated both of the acid-only solutions as much more sour relative to their corresponding WPI-containing samples: A6 was more sour than W6 ($t = 9.36$, $p < 0.0001$) and A1 was found to be more sour than W1 ($t = 5.99$, $p < 0.0001$) (Figure 29). Low sourness ratings were given to both ALo and water, and subjects found water to be less sour than ALo ($t = 3.10$, $p = 0.007$). Sourness ratings were unaffected by the serving position ($F = 0.36$, $p = 0.88$).

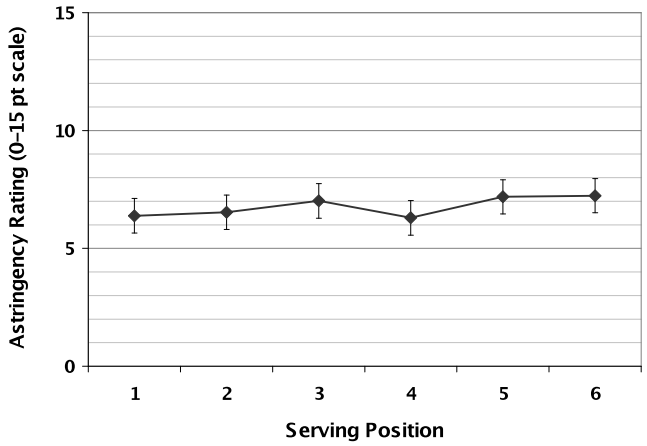

Figure 30. Astringency ratings vs. serving position of sample in whey protein beverage study
Mean astringency rating by serving position averaged over all solutions. Error bars represent standard errors.

III.b: SDS-PAGE analysis of saliva-astringent assays

No obvious differences were observed among the supernatant lanes corresponding to the acid, whey protein solution, or water-assays on the CBB-stained gel, Figure 31. Subtle differences were present among the density and types of proteins appearing in the pellet lanes, but this difference is likely attributable to the degree of which the pellet could be resolubilized. Pellets containing whey proteins tended to be more difficult to resolubilized. Similarly, in the mucin gel, the supernatant lanes showed MG1 to be of similar intensity across all assays, Figure 32. There was a noticeable difference in the pellet lanes of this gel, however, and acid appeared to precipitate a high proportion of the two mucins compared to all other assays. Acid was also observed to effectively precipitate the mucins in part I (Figure 5).

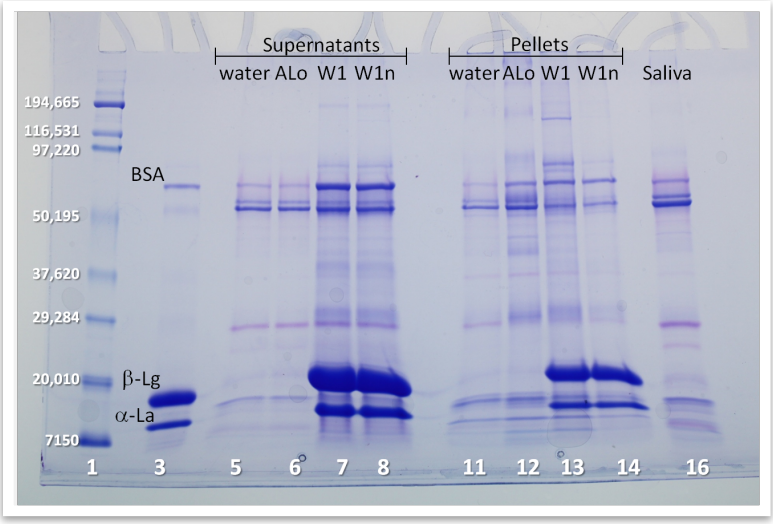

Figure 31. CBB-stained gel from whey protein beverage study
CBB-R250 stained 10.5-15% polyacrylamide gels showing multiple salivary proteins and the effect of astringents on their solubility. Bands that appear pink are PRPs. Assays were mixed in a 1:1 astringent-to-saliva ratio. Lanes are labeled by number and according to the sample in the lane except for lane 1 and lane 3. Lane 1contains pre-stained broad-range molecular weight standards (Bio-Rad, Hercules, CA). The standards are labeled with weight in units of Daltons. Lane 3 is whey protein isolate, which was prepared for electrophoresis in the same manner as the other samples. Whey proteins are labeled with abbreviations: BSA (bovine serum albumin), β-Lg (beta-lactoglobulin), α-La (alpha-lactalbumin).

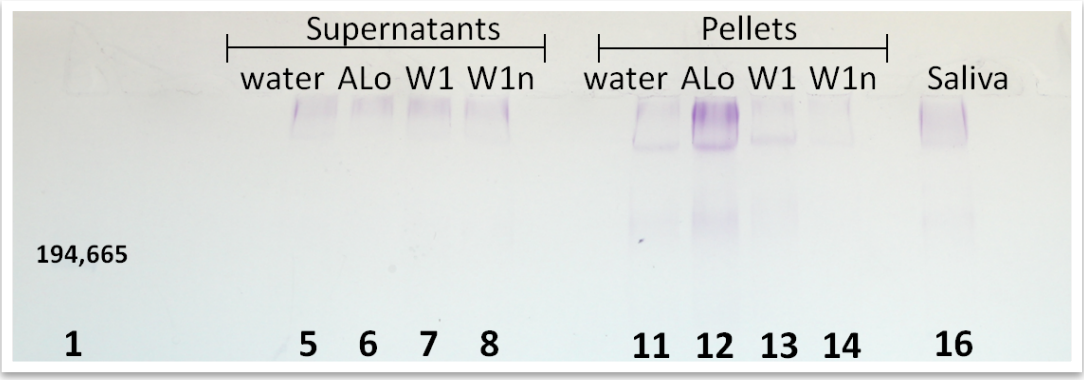

Figure 32. PAS-stained gel from whey protein beverage study
PAS stained 7.5% polyacrylamide gel showing two high molecular weight salivary glycoproteins and the effect of astringents on their solubility. The smaller of the two is assumed to be MG2 since it migrated approximately the same distance as a 194kDa molecular weight standard, which is close to MG2's reported weight of between 180 and 200kDa (Becerra et al., 2003; Groenink et al., 1996; Mehrotra et al., 1998). The larger of the two (at the top of the gel) is likely MG1 based on its location compared to MG2 and as observed in other studies (Becerra et al., 2003). Lanes are labeled by number and according to the sample in the lane except for lane 1, which contains pre-stained broad-range molecular weight standards (Bio-Rad, Hercules, CA). Only one standard is visible and it is labeled with weight in units of Daltons.

Discussion

Whereas others have suggested that the whey proteins are directly responsible for the astringency in acidic whey protein beverages, our sensory evidence strongly suggests that astringency is caused by high acidity. The two previous studies that have implicated the whey proteins as being responsible for beverage astringency used control solutions matched for pH and either protein level (Sano, 2005) or buffering capacity (Beecher, 2006). Neither study controlled for the total acidity of the solutions. Though it is evident from our results and the results of Sano and Beecher that pH alone does not predict astringency, the role of total acidity had not been considered. Our use of acidity-matched controls enabled us to see that the addition of the whey proteins (W1 or W6) to the acid did not increase the astringency of the acid-only solutions. The addition of the whey proteins did cause a rise in pH, which may explain why W6 and W1 were found slightly less astringent than A6 and A1. Our observation that the acidity matched control

solutions were slightly more astringent than the samples containing whey proteins further supports the hypothesis that the acid is primarily responsible for astringency in whey protein beverages.

Since having completed our study, additional literature from the same lab as Beecher (2005) has been published that agrees with our findings. In the most recent study by Vardhanabhuti et al. (2010), the astringencies of 4% β-Lg solutions acidified with phosphoric acid to pH levels of 3, 4, and 6 were compared to carefully formulated phosphate buffer controls that were matched for pH and phosphate content. Titratable acidities were measured for all solutions, and as expected, the β-Lg-containing solutions had much higher titratable acidities due to increased buffering. Not surprisingly, they also were more astringent. In our study, titratable acidities were matched between protein-containing solution and control, and ours were found to be equally astringency. Although the semantics between our paper and theirs are slightly different in use of the terms 'buffering capacity' and 'titratable acidity', our results are in agreement: the high levels of whey proteins in solution are effective buffers that require high acid concentrations for pH reduction, and this is the main contributor to the astringency in the beverages.

The astringency of most acids cannot be explained by the proposed mechanism for polyphenol astringency, which is said to initiate with hydrophobic interactions between polyphenols and proline-rich salivary proteins (Charlton et al., 2002). This has led other researchers to postulate that the mechanism of acid astringency is directly related to the acid's acidic properties (Lawless et al., 1996). Dawes (1964) has shown that salivary proteins can be precipitated by acids in buffered solutions, even near a physiological pH. Thus, if the acids do cause the astringency of the beverages by precipitation of salivary proteins, perhaps leading to decreased salivary lubricity, it seems plausible that buffering of acids by whey proteins would not necessarily decrease their astringency, which is what we observed in our study.

Sourness ratings of the solutions covered almost the entire range of scale intensities, and unlike astringency, sourness was strongly affected by whey protein buffering. Sourness ratings increased with higher titratable acidities (e.g. W6 vs. W1) and with lowered pH (eg. A6 vs. W6). The concentration of free hydrogen ions (i.e. the pH) and titratable acidity together explained over 98% of the variance observed in the ratings, with pH alone explaining 76% of the variance. These observations suggest that the sourness of the solutions depended on the immediately available free hydrogen ions and on the hydrogen ions that dissociated in the higher pH environment of the mouth. This is in agreement with other studies that have shown that both the amount of free and potential hydrogen ions affect sourness (Hartwig & McDaniel, 1995; Sowalsky & Noble, 1998).

Carry-over of astringency during sensory testing is difficult to avoid and has been the focus of a number of studies. The build-up phenomenon has been well-established and has been observed using different astringents including red wine and tannic acid solutions (Guinard et al., 1986; Lyman & Green, 1990; Colonna et al., 2004). In these studies, repeated ingestions of astringent samples were spaced a minute or less apart, and increased astringency ratings were observed. There have been few studies which have explored the minimal wait needed to eliminate the carry-over effect. The inter-stimulus protocol performed in this study included an enforced 5 minute wait, which was partly effective in limiting carry-over effects; however, a trend of increasing ratings was still observed over serving position (Fig. 2). Designing a protocol that can eliminate build-up with minimal inter-stimulus waiting would be of great value. Oral rinses such as carboxymethyl cellulose have been shown to reduce carry-over (Brannan et al., 2001; Colonna et al., 2004), but the reduced carry-over may come at the price of diminished sensitivity.

While the results from our sensory test provide evidence that acids cause the astringency in whey protein beverages, the gels from SDS-PAGE do not provide additional proof to support this finding. There are likely several contributing factors as to why differences were not observed. First, it is entirely possible that astringency is not at all related to

protein precipitation, in which case SDS-PAGE would not be expected to be useful. Second, there are practical limits to the quantity of proteins that can be electrophoresed, and this put constraints on the experimental parameters that could be used. The percent WPI solution, the assay ratio, and the dilution factor (i.e., how much supernatant was freeze-dried and the subsequent amount of sample buffer used for resolubilization) all had to be limited because too much whey protein in the samples to be electrophoresed resulted in smearing and gross band distortion,

Figure 33. Preliminary experiments were run in order to determine the maximal amount of protein that could be used. It was determined that, at most, a 1% whey protein solution could be used at a 1:1 assay ratio; additionally, the freeze-dried supernatant sample needed to be 4 times more dilute than used in part I (page 28). As a result, many of the salivary proteins were too dilute for visual observations.

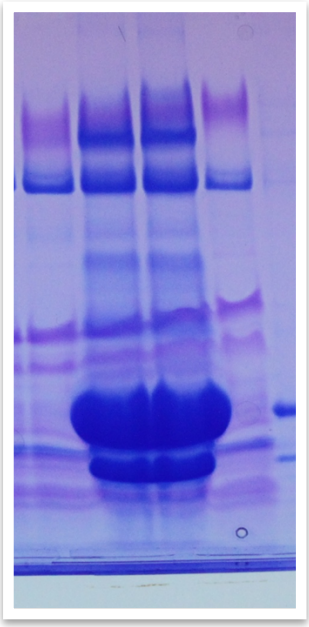

Figure 33. Effects of high whey protein concentration in CBB-stained gel.
Band distortion as a result of protein quantities that are too high. In this case, too much whey protein was in the sample.

Perhaps the biggest issue was that the assay ratios were not adequately representing true oral conditions. Benchtop experiments revealed that when subjects swished a 10mL solution of a 1% whey protein solution at a pH of 3.4, the magnitude of pH rise was the same as when astringents were mixed with saliva in 4:1 to 5:1 ratio. Ideally, a higher astringent:saliva ratio should have been used for the experiment, but because of the protein overload problem, could not be used. Because our experiment was looking for qualitative differences instead of quantitative, and because all other researchers performing similar experiment have used 1:1 ratios (Beecher, 2006; Beecher et al., 2008; Gambutti et al., 2006), this ratio was used. In retrospect, however, it seems clear why this lower 1:1 ratio would not reveal many differences: if astringency does cause a precipitation of salivary proteins, and if acid is the mechanism by which the effect occurs, the pH of the resulting assay mixture will likely be the most important determinant in controlling which proteins precipitate. In other words, while a 5:1 assay will result in a much lower pH than a 1:1 assay, and it would not be expected that the same proteins would precipitate at a much higher pH. If the positively charged whey proteins are truly the cause of astringency, the same issue would hold true: at a 1:1 assay ratio, the pH would be relatively high, and it is likely that the whey proteins would be close to their isoelectric point. With the realization of the importance of a higher astringent-to-assay ratio, it is recommended that an approach other than SDS-page be employed to explore this topic.

SDS-PAGE electrophoresis to understand interactions between acidified whey protein solutions and saliva was also used in the recent study by Vardhanabhuti et al. (2010). When saliva was mixed in a 1:1 ratio with β-Lg solutions at pH levels of 2.6 and 3.5, salivary mucins precipitated and were evident in the lanes where the pellets from the mixtures were electrophoresed. The pH values of the 1:1 mixtures were 3.7 and 5.3, respectively. When the pH of saliva was reduced to these same pH levels, the mucins were not precipitated. From this, it was concluded that β-Lg had precipitated the mucins, which may be the case, but should not be concluded due to lack of appropriate control. In the 1:1 mixtures, the saliva was mixed into a protein-buffered system that started at a

low pH (2.6 or 3.5). The salivary proteins buffered the acid in the solution, causing the pH to rise. The control used for comparison purposes was saliva at a biological pH level (near neutral) that was lowered to a pH of either 3.7 or 5.3. It was not subjected to high acid concentrations and low pH. Additionally, if β-Lg is responsible for precipitating the salivary mucins, one would expect there to be an increase in the amount of β-Lg in the pellet, and this did not appear to be the case. Given that acids are known to be quite effective at precipitating the mucins, this would explain why the mucins were precipitated.

Conclusions

The astringency of acidic whey protein beverages appears to be caused by their high acidity rather than by the whey proteins directly.

CHAPTER 5
Final Conclusions

Four years have passed since I first started studying astringency. While there is still much to be understood about the sensation, many discoveries have been made during this time. It is now apparent that the loss of saliva's ability to lubricate is not the fundamental key to astringency, nor is the precipitation of the PRPs. Although their precipitation is involved in tannin astringency, evidence obtained here (and through other labs in the past few years) shows that this precipitation most likely prevents astringency from developing rather than being a direct cause of it. Since the precipitation of PRPs does not in itself lead to astringency, and since other astringents do not precipitate the PRPs, there must be another oral phenomenon causing astringency. Based on this and on the tissue imaging work detailed in Appendix 1, it seems highly probable that a direct effect of the astringents on oral tissues is likely occurring with some of the astringents, including alum and tannins. It is unknown if other astringents, like acid, also have this effect. Future research should continue to explore how astringents interact with the lubricating films that cover the oral surfaces and on their direct tissue interactions.

My research has also shed light on the value of palate cleansers in sensory tests. Palate cleansers are very popular with panelists, especially in descriptive analysis work. There is huge debate in the sensory world as to what, if any, palate cleansers should be used, and everyone has their theory on what works best. Some of the more interesting suggestions I've come across have included alcohol and beer, chocolate, sour cream, pineapple/papaya sherbet, and even Hershey's Kisses. Though I'm sure panelists enjoy palate cleansers – especially if they come in the form of a chocolate kiss or beer - based on the observations from my studies and through discussions with sensory professionals, most palate cleansers seem to be more of a mental break from the repetitiveness of the stimuli being assessed more so than a true palate cleanser. The danger in this comes when the palate cleanser interferes with the sensory attribute being measured. In my study, it was clear that certain palate cleansers, like milk, prevented astringency from

fully developing. Because of this, in my opinion, water or nothing at all is the safest palate cleanser to use.

Lastly, it now seems clear that the acid in whey protein beverages is what causes their astringency. While this information comes too late for the discontinued commercial 'protein waters', this information suggests that approaches to reducing beverage astringency should be targeted at either replacing the phosphoric acid with a stronger and less astringent acid, or by reducing the acid content of the beverages by lowering the buffering capacity of the whey proteins. Unfortunately, additional work performed in our laboratory by another graduate student has shown that replacing phosphoric acid with HCl acid (which tends to be the least astringent of the acids at most pH levels) does not lower the astringency of the beverages. If whey protein is to be used in acidified protein beverages, it seems that the whey proteins will need to be modified in a way that allows the pH to be reduced more readily.

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Appendices

Appendix 1: Preliminary data suggest that astringents have a direct effect on the oral mucosa

This appendix has been included to guide efforts of other researchers interested in exploring the direct effects of astringents on the oral mucosa. The following excerpts (with minor modifications) were taken from a 2007 USDA grant proposal in which I was a co-principal investigator and the primary author. A brief literature review is provided first and is followed by a methods section, which was written with the help of Mark A. Sanders, head of the University of Minnesota's College of Biological Sciences Imaging Center. Much of the work to obtain the microscopy images presented below was performed by Mark Sanders, and the methods detailed below for the proposed work were based on the methods that he used. The images we obtained suggested that alum had a noticeable effect on the appearance of tissue samples when compared to a water control. Use of this methodology to further investigate astringent's effects on oral tissues appears promising.

Literature review: the theory of a direct oral mucosa effect

Many researchers have suggested that astringent compounds affect the oral mucosa directly once the salivary PRPs have all been bound, leaving the tissues susceptible to attack. The characteristics of the sensation appear to support this viewpoint. Perceptually, astringency has been described as including a drawing and tightening sensation sensed on the cheeks inside the mouth plus a feeling of dryness or roughness experienced during movement (Lawless et al., 1994), which could be attributed to altered tissue surface morphology. The manner in which astringency intensity builds over time suggests a mucosal effect. Guinard et al. (1986) showed that repeated exposure to wine caused an increase in the intensity and duration of the astringent sensation. This was most likely the case, they argued, if the tannins were first reacting with the PRPs mainly, but then reacted with an increasing amount of membrane proteins during subsequent exposures, making the return to a baseline state lengthier.

Indirect evidence supporting an oral mucosal effect comes from a study that observed the willingness of mice to drink water mixed with tannic acid (Glendinning,

1992). Four strains of mice were treated with beta-adrenergic agonist to increase levels of PRPs in their saliva. Prior to treatment, all mice avoided tannic acid solutions in favor of plain water. After treatment, only one strain showed an increased willingness to drink the tannic acid solutions. The PRPs of this strain had a high binding affinity to tannins, suggesting that the PRPs were protective and prevented astringency from developing. The three mice strains that continued avoiding tannic acid solutions were shown to have PRPs with a low binding affinity to tannic acid, and so the tannins remained free to interact with the epithelium directly.

Oral epithelial tissues may be altered by astringents in a number of ways. First, astringents may result in a constriction or tightening of the tissues caused by direct binding and cross-linking of the proteins in the mucosal epithelium (McManus et al., 1981). Constriction or other morphological changes in tissues can cause a reorganization of the actin cytoskeleton, a major structural component of cells, and it may alter the internuclear distance between mucosal cells, both of which are quantifiable using confocal microscopy (Marshall, 2003). Secondly, astringents may alter the mucosa by causing a dissolution or reduction in thickness of the oral mucosal mucus coat from the surface of the epithelium. The coat is a thin layer of protective coating that contains a relatively high concentration of mucins (Sarosiek et al., 1988), and whose functional properties are largely attributed to the high-molecular weight mucins (Slomiany et al., 1996). SDS-page analysis of unstimulated whole-mouth saliva and salivary fluid collected directly from oral mucosal surfaces has shown that mucosal surface saliva contains a higher total protein concentration and additional high-molecular weight mucins which are not present in the whole-mouth saliva (Proctor et al., 2006). Loss of these mucins or other proteins in the coat matrix would change the characteristics of this layer or strip it completely, resulting in increased friction or roughness in the mouth. Cryogenic scanning electron microscopy (cryo-SEM) has been used to assess changes in the thickness of mucus films coating tissue surfaces (Wu et al., 1996) and also to view and analyze surface topographies of tissues (Read & Jeffree, 1991). If astringents strip away the mucus coat or cause an increased surface roughness or other morphological change in the tissue, these events can be observed with microscopy.

Only one study has attempted to find direct evidence that the oral mucosa is altered

by astringents. Prinz (unpublished) was able to show an increased coefficient of friction between two porcine epithelial surfaces after application of astringent solutions. Because a similar increase in friction was not observed when rubber surfaces were used in place of the tissues, he attributed the increased friction to a roughening of the epithelial tissues. Pilot trials in our lab also provide evidence of the direct action of astringents on the oral mucosa. Images obtained using confocal microscopy and cryo-SEM of fresh porcine buccal mucosa tissues treated with alum (4.0 g/L), distilled water, or human saliva show distinct differences. From the cyro-SEM images, Figure 34, one can see that alum caused the removal of mucus from the tissue. In the confocal microscopy images, Figure 35, tissues treated with alum have fewer nuclei present at the mucosal surface and have cell edges that appear to have contracted upwards. Fewer nuclei may be an indication that the oral mucous coat was removed by the alum (assuming that the mucus coat contained sloughed-off cells), or that the alum removed a superficial layer of cells. The rough appearance of cell edges on the alum treated samples suggests that alum caused a contraction of the cells, resulting in the cell edges folding upward. This preliminary data provides strong evidence for a direct mucosal effect. It is important to know if this effect can be observed with other astringents including tannins and acids, and if it correlates to ratings of astringency. To effectively control astringency in foods and alleviate the sensation once it develops, it is imperative to know if the perception of astringency is mostly attributable to direct effects on tissue surfaces.

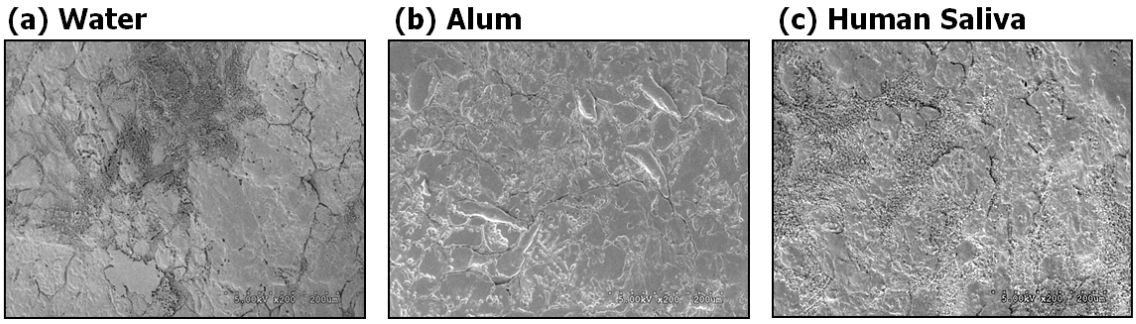


Figure 34. Cryo-SEM images of porcine buccal mucosa.
Roughly 30% of the area of the tissue treated with water was coated with a lattice-like structure (a), while almost none of this feature was observed on the alum-treated sample (b). We suspect that this lattice network was a remnant of the mucus coat, so we imaged a tissue after bathing in human saliva. This image showed extensive lattice networks (c).

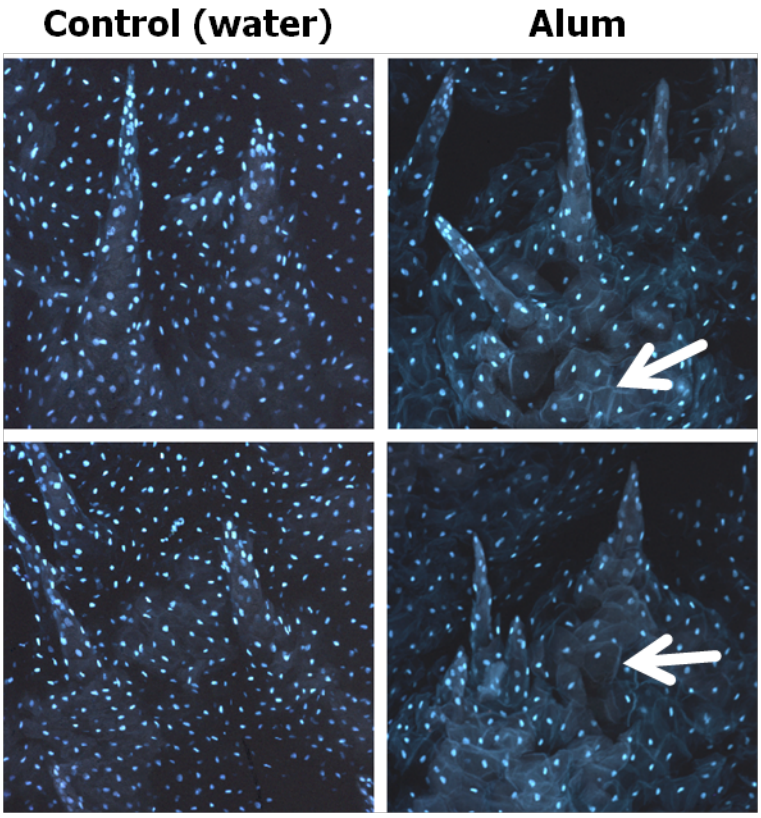


Figure 35. Confocal microscopy images of porcine tissue treated with an astringent Confocal microscopy images of porcine buccal mucosal tissue treated with a control (left two images) or with alum (right two images). Images of the control tissue show a denser population of cell nuclei and no cell boundaries. There are significantly fewer cell nuclei visible on the alum-treated tissues, and cell edges are visible (some of which are indicated by the arrows).

Proposed Methods

Tissue Harvesting and preparation

Porcine mucosal tissue and bovine enamel will be harvested and used in this portion of the study. Porcine buccal mucosal tissue will substitute for human tissue because of its availability and similarity in structure and permeability (Shojaei, 1998). Porcine buccal mucosa tissues will be obtained through the University of Minnesota's Tissue Sharing Program supported by the Research Animal Resources group which follows the policies and guidelines established by the Institutional Animal Care and Use Committee (IACUC). Tissues will be obtained fresh and transported in sterile saline (*Baxter*), and all experiments will be run within four hours to maintain tissue integrity. A 2" x 2" section

of buccal tissue will be used for each experiment. Bovine enamel was chosen because of its comparable properties to human enamel (Esser et al., 1998) and its ability to provide a large, relatively flat surface. Caries-free bovine permanent maxillary central incisors will be obtained through the Meat Lab located on the St. Paul Campus of the University of Minnesota.

Microscopy of porcine buccal mucosa tissues

During each imaging session, four tissue samples from the same specimen will be treated with an astringent (or control) and imaged using either cryo-SEM or confocal microscopy. A total of 8 sessions will be run to collect SEM images, and two sessions will be run to collect confocal microscopy images. Eight sessions are planned for the SEM imaging because two different sample preparation methods will be used and also to allow for balanced order of tissue treatment since tissues will be held in saline for different amount of time prior to imaging. Since tissues will be fixed at the same time during confocal microscopy methods, and since only one preparation method is planned, only 2 sessions are required to complete all treatments.

For all imaging experiments, saliva (previously collected from multiple subjects, centrifuged, and pooled), will be thawed the morning of the experiment and held at 4°C until its use. Just prior to imaging, the tissue sample will be bathed in 10ml of the pooled human saliva in a sterile container for a minimum of 10 minutes. The tissue will be removed from saliva and rinsed with distilled water. The tissue will then be treated with one of three astringent solutions or with a control. The solution will be allowed to sit on the tissue for two minutes before it is rinsed with 10ml of distilled water and imaged using cryo-SEM or Confocal Microscopy.

Cryo-SEM

Fresh tissue samples approximately 2 x 4 x 2 mm (w x l x h) will be dissected from a single tissue piece and analyzed using cryo-SEM. After rinsing with distilled water, tissues will be bathed for 2 minutes in an astringent treatment (or control). Specimens

will be mounted on stubs and inserted into a Hitachi S-3500N scanning electron microscope equipped with a K1150 cyro-SEM preparation unit by Emitech. The tissue will be frozen in sub-cooled liquid nitrogen. Inside the SEM, the sample will be briefly freeze dried to remove extraneous surface water, gold-coated using a Fullam model 840 Sputter-coater, and imaged at 5-15 kv. This method is the same as that used to obtain preliminary data.

In addition to the above method, treated tissue samples will also be prepared by dehydration in a graded series of alcohols followed by critical point-drying in a Tousimis Autosamdri-814 Critical Point Dryer. After drying, tissues will be mounted on stubs, gold coated, and viewed. Tissues prepared by critical-point drying will be viewed using conventional and stereo-pair images (2 separate images taken at 4-6^{o} from each other) which will permit accurate measurements of structural heights and surface changes due to various treatments. ImagePro Plus v.6.2 and Stereo Investigator v. 3.2 analysis softwares will be used to obtain histometric data on the tissues. Images will be collected at various magnifications.

Confocal Microscopy

To obtain preliminary data, tissue were fixed in 2% glutaraldehyde in PBS and then incubated with 4 μg/ml DAPI for 15 minutes to label the DNA in the tissue. For the proposed research, however, we will use a triple stain to allow imaging of actin and tubilin in addition to the DNA in the cells. This will allow us to observe cytoskeletal components and help us to understand the underlying cellular mechanisms as they relate to changes in morphology.

Four fresh tissue sections approximately 2 x 4 x 2 mm (w x l x h) will be dissected from a single fresh tissue section placed into a single treatment. The tissues will be fixed in 4% formaldehyde and extracted with detergent to permeabilize the cells. The detergent treatment allows the antibodies to penetrate the cell membranes. The tissues will be blocked and then incubated with a mouse (1°) antibody directed against tubulin followed with a fluorescent secondary (2°) antibody (Texas Red conjugated anti-mouse antibody).

This staining solution also contains Alexa Fluor 488 phalloidin, which will bind to and stain the F-actin green. Finally, the tissues will be mounted in an anti-fade reagent containing DAPI to stain the DNA in the cell blue. Preparations will be viewed using a Nikon C1si spectral confocal microscope attached to a Nikon TE3000 motorized inverted microscope (Nikon USA, Melville, NY) using the 408nm laser line. An emission filter allowing 415-445 nm range of the fluorescence signal was collected using a 10X, 0.45 n.a. plan apo objective with zoom setting of 1. Optical sections will be collected at 1.25 micron increments. 2 and 3D images will be collected on a Nikon C1si confocal, and cytoskeletal markers will be examined for distribution and quantitative differences using ImagePro Plus Image Analysis software.

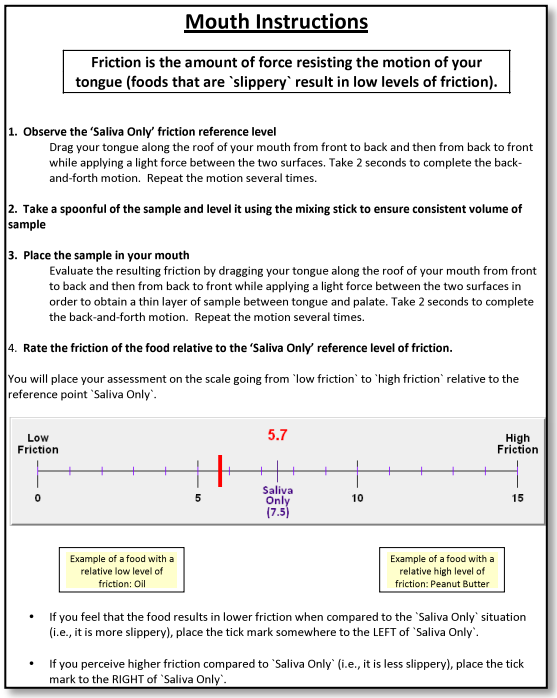
Data Analyses

Various characteristics of the tissue structure will be quantified from SEM and confocal images to determine the effects of the astringents. Some of the parameters which we will measure include: cell area, size and shape of pits and microvilli, microplication length (micropilation is the folds or wrinkles in the cellular structure that cause a shortening of the cell), surface roughness, actin contraction, location and density of nuclei, and presence of mucous residue. An analysis of variance will be used to determine if astringents caused an increase or change in one of the parameters that characterize the tissue's surface or state.

Appendix 2: Supporting Documents for 'Sensitivity of Fingers versus Mouth in Friction Assessment of Foods' preliminary study

A. Ballot Instructions
B. Blinding Codes
C. Sample Rotation Plan
D. SAS Code

(A) Ballot instructions



Mouth Instructions

Friction is the amount of force resisting the motion of your tongue (foods that are `slippery` result in low levels of friction).

1. Observe the 'Saliva Only' friction reference level

Drag your tongue along the roof of your mouth from front to back and then from back to front while applying a light force between the two surfaces. Take 2 seconds to complete the back-and-forth motion. Repeat the motion several times.

2. Take a spoonful of the sample and level it using the mixing stick to ensure consistent volume of sample

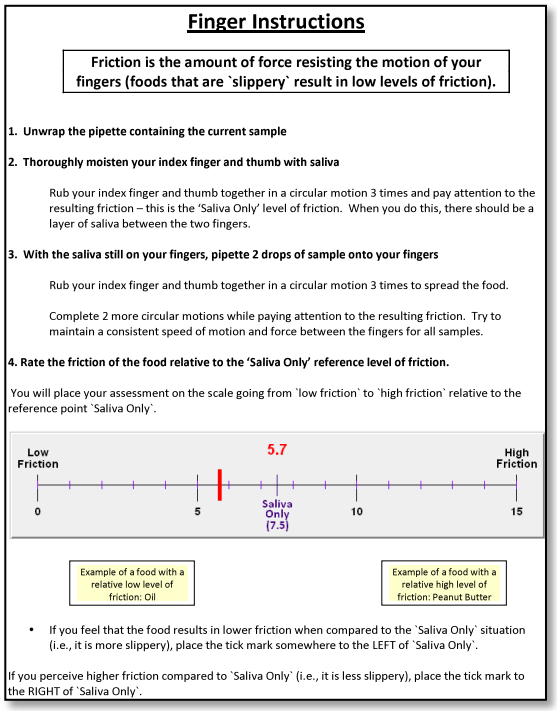
3. Place the sample in your mouth

Evaluate the resulting friction by dragging your tongue along the roof of your mouth from front to back and then from back to front while applying a light force between the two surfaces in order to obtain a thin layer of sample between tongue and palate. Take 2 seconds to complete the back-and-forth motion. Repeat the motion several times.

4. **Rate the friction of the food relative to the 'Saliva Only' reference level of friction.**

You will place your assessment on the scale going from `low friction` to `high friction` relative to the reference point `Saliva Only`.

- If you feel that the food results in lower friction when compared to the `Saliva Only` situation (i.e., it is more slippery), place the tick mark somewhere to the LEFT of `Saliva Only`.
- If you perceive higher friction compared to `Saliva Only` (i.e., it is less slippery), place the tick mark to the RIGHT of `Saliva Only`.



Finger Instructions

Friction is the amount of force resisting the motion of your fingers (foods that are `slippery` result in low levels of friction).

1. Unwrap the pipette containing the current sample

2. Thoroughly moisten your index finger and thumb with saliva

Rub your index finger and thumb together in a circular motion 3 times and pay attention to the resulting friction – this is the 'Saliva Only' level of friction. When you do this, there should be a layer of saliva between the two fingers.

3. With the saliva still on your fingers, pipette 2 drops of sample onto your fingers

Rub your index finger and thumb together in a circular motion 3 times to spread the food.

Complete 2 more circular motions while paying attention to the resulting friction. Try to maintain a consistent speed of motion and force between the fingers for all samples.

4. Rate the friction of the food relative to the 'Saliva Only' reference level of friction.

You will place your assessment on the scale going from `low friction` to `high friction` relative to the reference point `Saliva Only`.

- If you feel that the food results in lower friction when compared to the `Saliva Only` situation (i.e., it is more slippery), place the tick mark somewhere to the LEFT of `Saliva Only`.

If you perceive higher friction compared to `Saliva Only` (i.e., it is less slippery), place the tick mark to the RIGHT of `Saliva Only`.

(B) Blinding codes

	Blinding Code	
Food	**Finger**	**Mouth**
Vegetable Oil	147	512
Vanilla Pudding	964	134
Water	732	684
1.5% Pectin + Sugar	839	541
3% Pectin + Sugar	817	853
Peanut Butter	398	224
Chocolate Syrup	418	915
Honey	215	495
Miracle Whip	279	285
Heavy Whipping Cream	674	578

(C) Sample Rotation Plan

FINGER SAMPLE ORDER

Panelist #	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
90001	398	147	279	839	732	418	964	674	817	215
90002	964	732	817	279	215	398	674	147	418	839
90003	215	817	674	964	418	732	839	279	147	398
90004	279	398	732	147	964	839	817	418	215	674
90005	817	964	215	732	674	279	418	398	839	147
90006	418	674	839	215	147	817	398	964	279	732
90007	147	839	398	418	279	674	732	215	964	817
90008	732	279	964	398	817	147	215	839	674	418
90009	839	418	147	674	398	215	279	817	732	964
90010	674	215	418	817	839	964	147	732	398	279
90011	674	215	418	839	147	964	398	817	732	279
90012	398	147	732	418	279	674	817	215	964	839
90013	964	817	839	279	215	732	674	398	418	147
90014	817	279	964	732	839	398	215	147	674	418
90015	732	398	279	147	817	418	964	674	839	215
90016	279	732	817	398	964	147	839	418	215	674
90017	839	964	215	817	674	279	418	732	147	398
90018	418	674	147	215	398	839	732	964	279	817
90019	215	839	674	964	418	817	147	279	398	732
90020	147	418	398	674	732	215	279	839	817	964

(D) SAS Code

```
libname clib 'G:\FSCN\Vickers_Lab\Lee\SAS_Programs\Sept2009
Study\Saliva Friction Redo (Correct)';

data clib.Salivafrictionredo;
set work.Salivafrictionredo;
run;

Proc Mixed data=clib.Salivafrictionredo Method = REML CovTest;
title 'Mixed ANOVA: Friction';
      class Panelist Astringent;
      model Friction_Assay = Astringent Friction_Astringent
Overall_Position  / ddfm=satterth;
      random Panelist;
      contrast 'High vs Low' astringent 1 -1 1 -1 1 -1 0;
      contrast 'tannin solutions with water' astringent 0 0 0 0 1 1 -2;
      estimate 'tannin solutions with water' astringent 0 0 0 0 1 1 -2;
      estimate 'High vs Low' astringent 1 -1 1 -1 1 -1 0;
run;
```

Appendix 3: Supporting Documents for 'Instrumentally determine if astringents result in decreased salivary lubricity' study

A. Sample Analysis Rotation
B. SAS Code

(A) Sample Analysis Rotation (randomized)

		Order of Analysis							
		1	2	3	4	5	6	7	8
Panelist	1	Tan_Hi	Tan_Lo	Acid_Lo	Water	Acid_Hi	Alum_Hi	Alum_Lo	Water
	2	Water	Tan_Lo	Alum_Hi	Alum_Lo	Tan_Hi	Water	Acid_Lo	Acid_Hi
	3	Water	Alum_Hi	Acid_Lo	Water	Alum_Lo	Tan_Lo	Tan_Hi	Acid_Hi
	4	Tan_Lo	Alum_Hi	Water	Acid_Hi	Alum_Lo	Acid_Lo	Water	Tan_Hi

(B) SAS Code

```
Proc Mixed data=clib.Salivafrictionredo Method = REML CovTest;

title 'Mixed ANOVA: Friction';
class Panelist Astringent;
model Friction_Assay = Astringent Friction_Astringent Overall_Position
/      ddfm=satterth;
random Panelist;
contrast 'High vs Low' astringent 1 -1 1 -1 1 -1 0;
contrast 'tannin solutions with water' astringent 0 0 0 0 1 1 -2;
run;
```

Appendix 4: Supporting Documents for 'Determining if human subjects can detect a change in salivary lubricity' study.

A. Blinding Codes
B. Sample Rotation Plan
C. Ballot

(A) Blinding Codes

Pair	Sample A	A Code	Sample B	B Code
1	Water	475	Alum at 0.50 g/L ("Alum Low")	337
2	Water	837	Alum at 1.0 g/L ("Alum High")	488
3	Colored Water	125	Tannin at 1.5 g/L ("Tannin Low")	634
4	Colored Water	726	Tannin at 3.0 g/L ("Tannin High")	224
5	Water	286	HCl at pH = 2.2 ("Acid Low")	428
6	Water	518	HCl at pH = 1.9 ("Acid High")	441
7	Water	705	2.5 % SLS	189
8	Colored Water	916	Colored 2.5 % SLS	546

(B) Sample Rotation Plan

Panelist	Pair 1	Pair 2	Pair 3	Pair 4	Pair 5	Pair 6	Pair 7	Pair 8
1	7	6	4	2	8	5	1	3
2	7	4	5	6	8	3	2	1
3	7	2	6	1	8	4	3	5
4	7	1	2	3	8	6	5	4
5	7	5	3	4	8	1	6	2
6	7	3	1	5	8	2	4	6
7	7	3	6	5	8	2	1	4
8	7	1	5	4	8	3	2	6
9	7	4	1	2	8	5	6	3
10	7	5	3	1	8	6	4	2
11	7	6	2	3	8	4	5	1
12	7	2	4	6	8	1	3	5
13	7	6	1	2	8	3	4	5
14	7	1	3	6	8	5	2	4
15	7	2	6	4	8	1	5	3
16	7	3	5	1	8	4	6	2
17	7	4	2	5	8	6	3	1
18	7	5	4	3	8	2	1	6
19	7	5	1	3	8	6	4	2
20	7	3	5	4	8	1	2	6
21	7	6	2	1	8	4	5	3
22	7	4	3	2	8	5	6	1
23	7	1	6	5	8	2	3	4
24	7	2	4	6	8	3	1	5

= Sample 'A' presented 1st
= Sample 'B' presented 1st

(C) Ballot:

Panelist #_____

Instructions:
You will be assessing 8 pairs of samples for the friction they produce. You will be asked to select the sample that results in the higher level of friction (i.e., the one that is the least slippery).

To assess each pair of samples:

9. Thoroughly moisten your fingers with saliva
10. Rub your index finger and thumb together with very light pressure between them. You should not be able to feel your fingerprints.
11. Pour or pipette a few drops of solution onto your fingers
12. Rub your index finger and thumb together in a circular motion
13. Pay close attention to the level of friction
14. Rinse fingers in bath & wipe off
15. Repeat the procedure with the second sample (DO NOT REUSE PIPETTES)

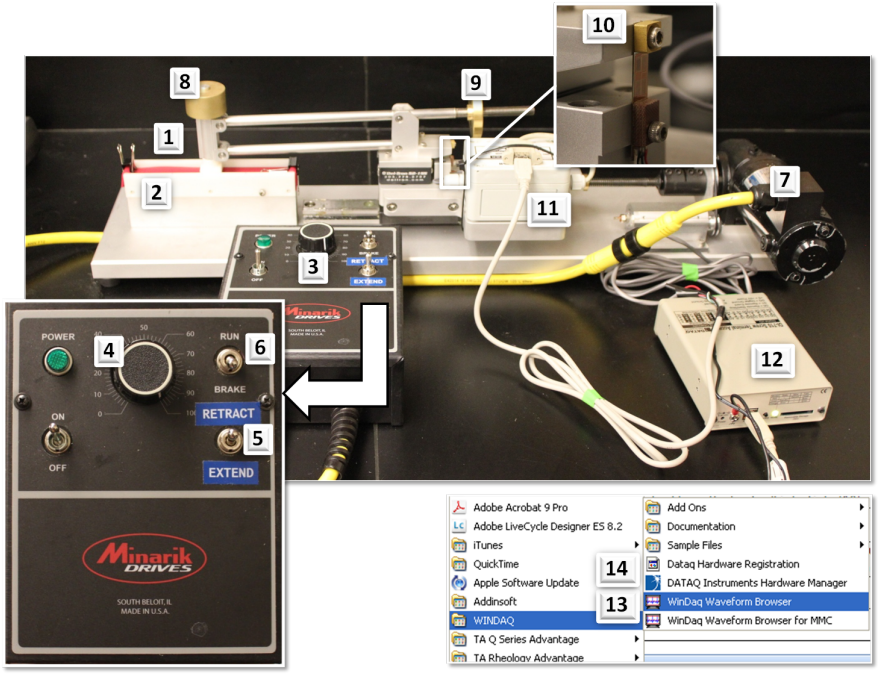
Circle the code corresponding to the sample that results in a higher level of friction (i.e., circle the sample that is less slippery):

Pair 1:	1st: _____	2nd: _____
Pair 2:	1st: _____	2nd: _____
Pair 3:	1st: _____	2nd: _____
Pair 4:	1st: _____	2nd: _____
Pair 5:	1st: _____	2nd: _____
Pair 6:	1st: _____	2nd: _____
Pair 7:	1st: _____	2nd: _____
Pair 8:	1st: _____	2nd: _____

Appendix 5: Friction Tester. University of Minnesota Property ID #200901904.

A. Machine Specifications
B. Calibration Procedure and Calibration Data
C. Qualification of the machine
D. Measurement protocol

A. Machine Specifications



1	Upper contact pad	Constructed of nylon. Square area of 2 x 2 cm in contact with the lubricant that is loaded onto [2]. Leading and trailing edges of upper contact pad slightly rounded to avoid removal of lubricant during motion. The upper contact pad can be moved up or down (for sample loading) by manually raising or lowering the double-beam arm to that it is attached.
2	Sample loading tray	Constructed of nylon. 15.5 cm long by 2.5 cm wide; 0.8cm deep. Removable for easy cleaning.
3	Controller	Minarik (South Beloit, IL) model C1RGD03-D230AC drive. Controls the motion of the upper contact pad via control over the drive motor. Speed of the upper contact pad (0-10mm/sec) is controlled via the analog knob [4], and the direction of travel (either 'extend' or 'retract') is controlled via a flipswitch [5]. Motion can be stopped or started using a second flipswitch [4].
4	Speed dial	Analog dial ranging from 0 to 100 that is used to control the speed of travel from 0 to ~10mm/sec.
5	Direction of travel flipswitch	Controls direction of travel of the upper contact pad. The contact pad travels in the retract or extend directions when the flipswitch is in the up or down position, respectively. Extend refers to motion of the contact pad away from the

		main body of the device (i.e., traveling to the left in the image).
6	Brake flipswitch	Allows motion to be started or stopped.
7	Drive motor	Leeson (Grafton, WI) 985-509E Permanent Magnet DC Gearmotor. Moves the main platform, which is supported on a low-friction ball slide and rail.
8	Weight / weight platform	Weights are placed on the weight platform located directly above the upper contact pad; This applies a known normal force to the system. Weights manufactured for the system include weights for application of normal forces of 0.3N, 2N, 4N, and 8N.
9	Zero adjust counter weight	Adjustable counter weight. Can be rotated to ensure a starting normal force of ~0N.
10	Load Cell	Omega (Stamford, CT) LCL-816G Full Bridge Thin Beam Load Cell that includes an integrated strain gage for measuring frictional force. The gauge flexes proportionate to the amount of friction between the upper contact pad and sample tray. Output from the load cell is fed into [10].
11	Signal Conditioner	Honeywell (Columbus, OH) in-line amplifier, p/n 060-6827-04. Receives input signal from [9]. Amplifies and normalizes signal to ±10V. Output is fed directly into [11].
12	Data logger	DATAQ (Akron, OH) DI-710 data logger. Can be run in stand-alone mode (data is recorded onto SD memory card) or can be connected directly to a PC. Data is recorded as volts, ranging from -10V to +10V.
13	WinDaq Waveform Browser	Can create (record) data files from direct input of [11], or can read data files created and saved on SD memory card when [11] is used in stand-alone mode. Files are of extension .WDC. Software can be used to retrieve average voltage readings while system is in motion. Average voltages are used to compute frictional forces.
14	DATAQ Instruments Hardware Manager	Software for configuration of [11].

B. Calibration Procedure and Calibration Data

The friction tester is calibrated using a set of 3 weights. The weights are 0.1475kg, 0.2950kg and 0.4425kg and correspond to forces of 1.45, 2.89, 4.34 N, respectively. During calibration, there should not be any weights on top of the weight platform, and the system should be balanced properly by adjusting the zero adjust counter weight before proceeding.

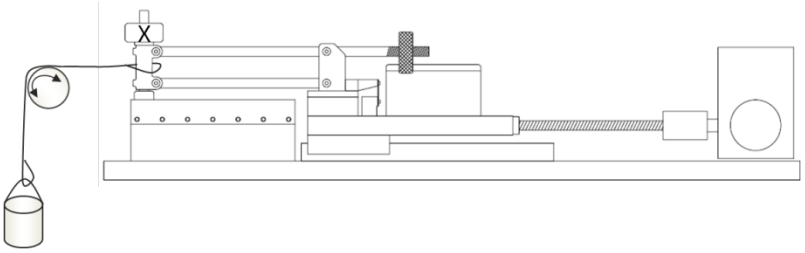


Figure 36. Calibration setup of the friction tester

Using each of the weights successively, the calibration procedure is as follows:

Step 1. Attach weight to machine

One end of cording is looped around the machine's arm, Figure 36. The other end is tied to the weight.

Step 2. Ready machine to record data

The WinDaq Waveform browser is opened and data recording is started.

Step 3. Apply force

The cording, with the weight attached, is draped over a plastic cylinder, which is held using both hand and level to where the cording is looped around the friction tester's arm. The weight is allowed to hang freely.

Step 4. Rotate cylinder

To account for the friction between the cording and the cylinder, the cylinder is rotated clockwise and then counter clockwise. This is repeated 3 times.

Step 5. Extract data

Data recording is stopped. Average voltages are read from the data file, Figure 37. For example, from this file the following data points were read:

Zero ("Z") 1, 2: -0.63, -0.62
Counterclockwise ("CC") 1, 2, 3: -2.99V, -2.96V, -3.00V
Clockwise ("C") 1, 2, 3: -2.39V, -2.42V, -2.43V

Step 6. Calculate average voltage

Using the data in from Step 5, a single average voltage reading is calculated:

Voltage (average) = $[(CC_1+C_1)/2+(CC_2+C_2)/2+(CC_3+C_3)/2]/3 - [(Z_1+Z_2)/2]$

For example, using the data extracted above:

V_{AVE} = [(-2.99+ -2.39)/2 + (-2.96+ -2.42)/2 + (-3.00+ -2.43)/2]/3- [(-0.63+ -0.62)/2]

= -2.07V

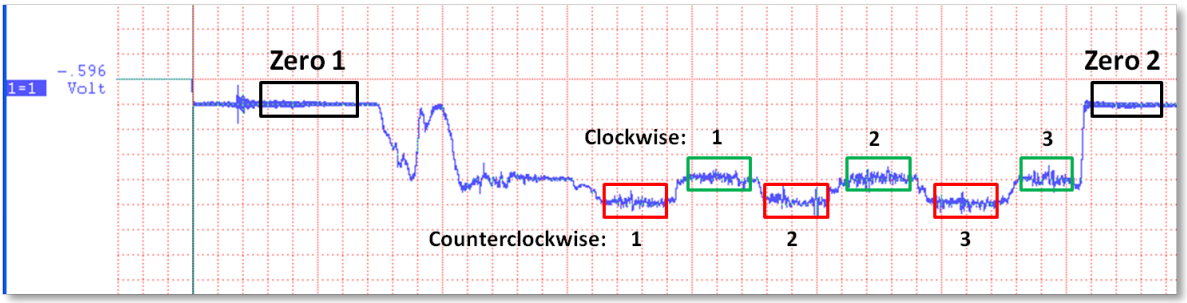


Figure 37. Data file from friction tester for calibration

Data file obtained during calibration using the 0.1475kg weight. Two average voltages are read when no weights are applied (zero 1, zero 2); 3 average voltages are read from both the counterclockwise and the clockwise rotations of the plastic cylinder.

Step 7. Construct calibration curve

The 3 data points (force vs. the average voltages) are plotted for the 3 weights, Figure 38.

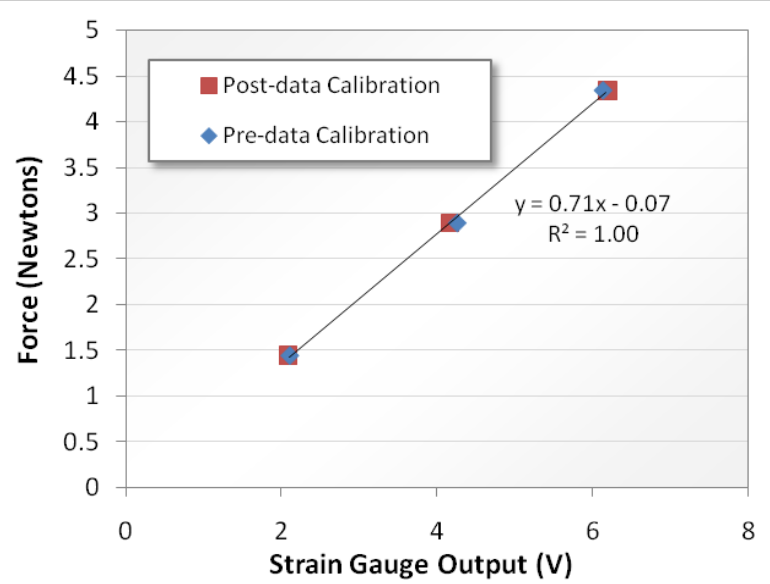


Figure 38. Force vs. strain gauge output curve for calibration of friction tester
Data and the calibration curves constructed before (pre-data calibration) and after (post-data calibration) the collection of the friction data of part I.2b (instrumental detection of a change in salivary lubricity in response to astringents, page 39). The calibration curve can be used to convert the voltage output of the strain gauge (x) to the friction force experienced by the machine (y).

Table 13. Calibration data for the friction tester
Calibrations were performed before any data were collected (pre-data calibration) and after all data collection was complete (post-data calibration). The average of the data points collected during these two calibrations was used to construct the calibration curve (y (force, N) = 0.71*voltage – 0.07)).

	Calibration (V_{AVE})	
Newtons	Pre-data	Post-Data
1.45	2.11	2.09
2.89	4.26	4.17
4.34	6.13	6.19

C. Qualification of the machine

A number of preliminary experiments were run in order to (1) determine if the friction tester measurements were logical and reflected human perceptions of friction and (2) assess its repeatability.

(1) Determining if the friction tester measurements were logical

The first approach we took was to determine if friction measurements (with and without adjusting for viscosity) of various food products could be correlated to slipperiness ratings made orally and using fingers. Food slipperiness has been found to be related to a food's viscosity and its friction (Kokini et al., 1977) or directly to its friction alone (Malone et al., 2003). The foods that were used included apple sauce, chocolate syrup, creamy peanut butter, diluted strawberry Jell-O, baby food (peas), honey, low-fat vanilla yogurt, Miracle Whip, vegetable oil, water and a 0.75g/L solution of alum. These experiments were run by a visiting undergraduate, Bénédicte Pichard, over the summer of 2009.

In brief, we were unable to establish a relationship between the instrumental friction values and panelists' ratings of friction (opposite of slippery) using their mouths, Figure 39, or their fingers (data not shown; finger data was nearly identical to mouth data). When the foods' viscosity was taken into account according to the formula devised by Kokini et al (1977), the correlation improved but it was still poor, Figure 39.

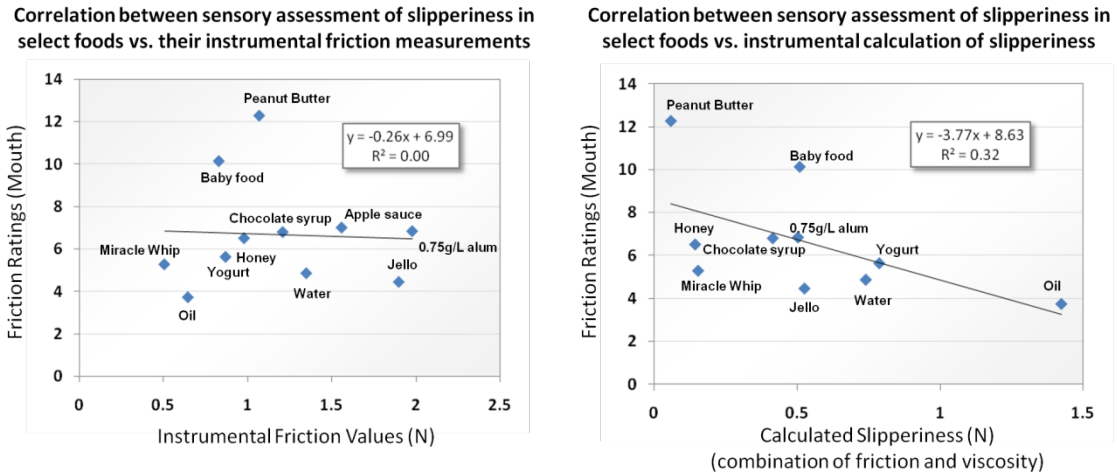


Figure 39. Friction ratings vs. instrumental friction values of various foods
Correlation between sensorial and instrumental measures of friction/slipperiness. Instrumental measurements of friction of foods did not predict measurements obtained in the mouth (left); inclusion of viscosity data to calculate a slipperiness value (Kokini et al., 1977) resulted in a slightly better but still poor correlation.

Our inability to find a good correlation between slipperiness and instrumental measurements of either friction or calculated slipperiness was likely due to the complexity of foods that were chosen for the experiment. For example, some of the foods that we used contained particulates (e.g., baby food and apple sauce), which may have confused panelists, and the foods that we used varied widely in fat content.

The foods used by Kokini et al. (1977) and Malone et al. (2003) were comparably simple. The majority of 'foods' used in these studies were model solutions formulated using gums of various concentrations. Malone et al. (2003) used four guar gum solutions ranging in concentration from 0.05 to 0.6% to assess slipperiness perception. Kokini et al. (1977) used 16 different foods. Eleven were solutions containing varying amounts of maltrin, guar gum, and CMC; five were commercial products and included honey, and butterscotch, pancake, vanilla, and chocolate syrups. In fact, the correlation between instrumental measurements and panelists' ratings of slipperiness does not appear to be convincing when only the commercial foods are considered, Figure 44. Another contributing factor that possibly affected our data during these experiments was discovered in subsequent repeatability tests. During all of these experiments, a piece of silicone was used to line the sample tray, Figure 16. Repeatability testing later revealed

that friction measurements increased steadily with run number when the same piece of silicone was used.

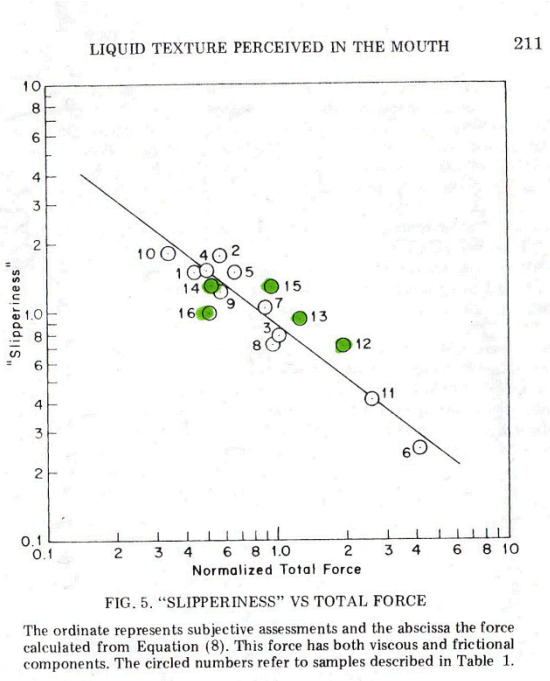


Figure 40. Slipperiness ratings vs. normalized total force from Kokini et al. (1977) Slipperiness ratings vs. normalized total force (i.e., instrumental slipperiness) on a log-log scale from Kokini et al. (1977). Numbers 1-11 represent model solutions, while 12-16 (shaded data points) are commercial foods and include honey, and butterscotch, pancake, vanilla, and chocolate syrups.

Our second approach was to determine if the friction tester could detect differences in lubricity among lubricants with relatively similar viscosities and thicknesses since the friction tester was fabricated for the purpose of measuring slight changes in salivary lubricity in response to astringents. For this experiment, which was run concurrently with the repeatability experiments detailed in the next section, oil, saliva, water, and a saliva-water mixture (8:2 ratio) were tested on the machine, Figure 41. The machine's sample tray was lined using a single piece of silicone for all runs, and it was rinsed and dried between runs. All data were collected using a normal applied force of 2N and a motion speed of 10mm/sec.

The friction tester could reliably detect differences in friction when oil, saliva, or water was on the system. The order of the friction values measured was also logical: oil on the friction tester resulted in the lowest level of friction (i.e., it was the best lubricant), and saliva was found to be a more effective lubricant than water. When saliva and water were mixed, the saliva-water mixture resulted in a slightly higher level of friction than saliva alone.

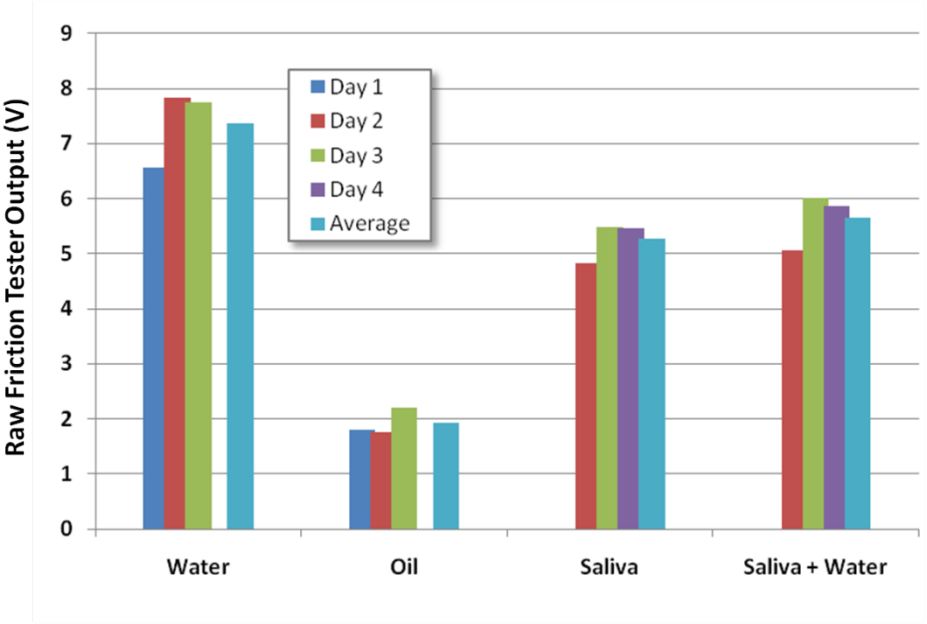


Figure 41. Voltage output of friction tester with various lubricants
Friction tester raw output (V), which is proportional to frictional force, vs. lubricant type. Data were collected over a 4-day period. Oil and water consistently resulted in the lowest and highest friction levels on the tester, respectively. All data were collected with the tester at 100% speed (~10mm/sec), 2N normal force, and with a silicone liner in the sample tray.

(2) Assessment of system repeatability

To assess the friction tester's repeatability, repeated measures of friction were obtained over a 2-week period using water on the system, Figure 42. Friction values steadily rose over run number. Calibrations of the machine during this time were unchanged, implying that one of the surfaces of the friction tester (either the nylon contact pad or the silicon tray liner) was changing over time. Through additional testing (data not shown), I

confirmed that it was the silicone's surface, and not the nylon, that was changing over time.

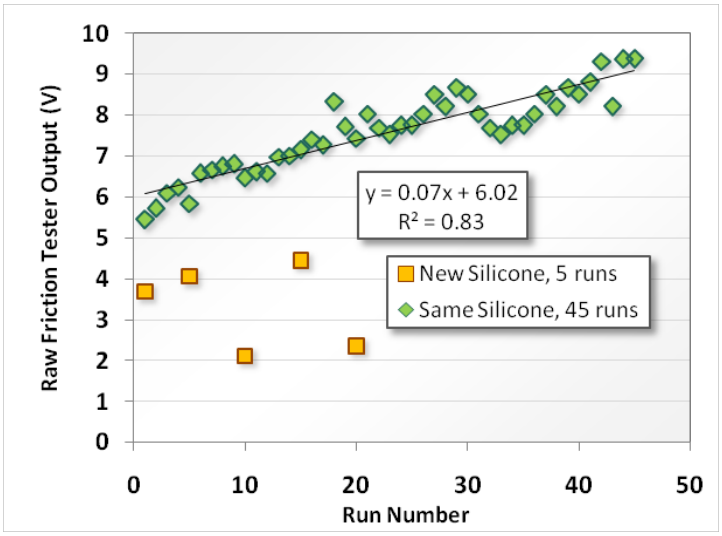


Figure 42. Friction tester raw output (V) of water measurements vs. run number for repeated measures tests
Forty-five runs were made using the same silicone liner, which was always removed and dried in between runs. Before some of the runs (when other lubricants, like oil, were used on the system), the silicone was washed using soapy water and then rinsed with 10% ethanol and distilled water. Five runs (spread out along the x-axis of the graph for easier viewing) were made using a new piece of silicone for each run. For all runs, the friction tester was run using a normal force of2N and a speed of 10mm/sec.

A similar repeated-measures experiment was run using water as the lubricant but with new pieces of silicone for every run, Figure 42. Within a given day, the results were more repeatable when the same piece of silicone was used as compared to using new pieces of silicone for each run; additionally, the increase in friction as a result of the changing surface morphology of the silicone could be, for the most part, adjusted for in the experimental design and ANOVA model used to analyze experimental data. Thus, it was decided that the best setup was to use the same piece of silicone during the experiments and to include runs of only water (and of other 'standard' solutions) so that the expected increase in friction could be accounted for.

D. Measurement protocol

All measurements during the salivary lubricity experiment were collected in the same manner and as follows:

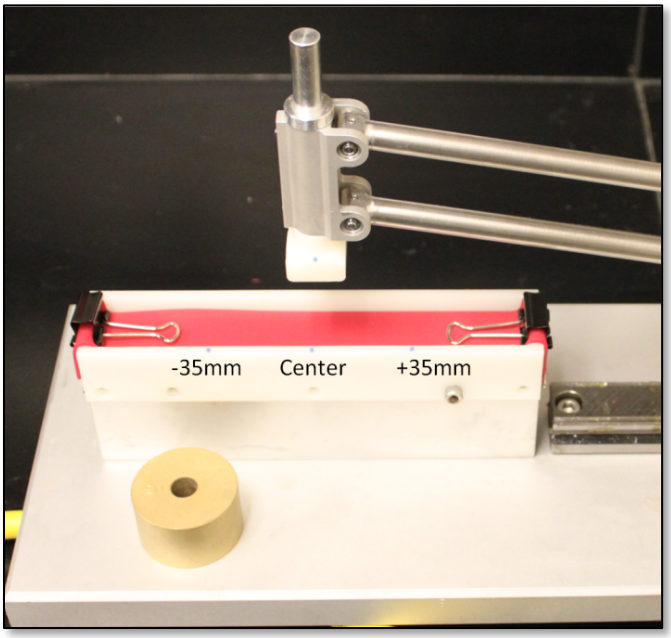


Figure 43. Close-up view of friction tester sample tray
Friction tester in raised position for sample loading, shown with the silicone liner in the sample tray. The blue alignment markings can be seen on the sample tray, just above the labels -35mm, Center, and +35mm. The sample tray and the silicon liner had markings at the center point and at ±35mm. The upper contact pad was also marked. All experimental runs began with the upper contact pad aligned to the center marking of the sample tray.

1. The silicone liner was attached to the sample tray. To ensure precise positioning of the silicone liner for every run, markings on both the silicone liner and the tray were aligned, Figure 43.
2. The speed dial was set to 100 (~10mm/sec).
3. If needed, the upper contact pad was moved so that it was at the center point of the sample tray. Without any weight on the platform, the arm of the tester (attached to the upper contact pad) was raised for sample loading, Figure 43.

4. 3mL of room-temperature water was pipetted onto the silicone liner at the center point location. The upper contact pad was lowered and the 2N weight was placed on the weight platform. The friction tester was then run continuously (retract and then extend; repeat many times over) in between the ±35mm marking for 10 minutes. It was stopped at the center point location. The weight was removed, the arm of the system was raised, and the liner and contact pad were patted dry with a Kimwipe (Kimberly-Clark Corporation, Irving, Texas) and allowed to air dry for 5 minutes.
5. A 1mL water measurement was taken:
    a. 1 mL of distilled water was pipetted into a 2mL tube and placed in a 37°C water bath for 2 minutes
    b. The sample was pipetted onto the silicone liner at the center location
    c. The contact pad was lowered and the 2N weight was placed on the platform
    d. The system was run continuously (retract and then extend; repeat many times over) in between the ±35mm marking for 1.5 minutes. It was stopped at the center point location. The weight was removed, the arm of the system was raised, and the liner and contact pad were patted dry and allowed to air dry for 5 minutes.
6. A saliva-only measurement was taken
    a. Bulk saliva collected from the panelist was first centrifuged (10,000g, 20 min, 4°C) and stored on ice
    b. 1mL of the bulk saliva was pipetted into a 2mL tube and placed in a 37°C water bath for 2 minutes; the remaining protocol for the measurement collection was the same as described for water above.
7. The silicone liner was removed from the system and washed:
    a. The liner was rinsed and then washed in a soapy bath for 1 minute
    b. It was rinsed again and allowed to soak in a 10% ethanol solution for 1 minute
    c. The liner was then thoroughly rinsed with distilled water and patted dry
    d. The upper contact pad was wiped with water multiple times and dried.

8. The saliva-astringent assays were run (in random order)
    a. 0.8mL of saliva was mixed with 0.2mL of water in a 2mL tube
    b. The tube was placed in the 37°C water bath for 2 minutes
    c. The assay was run on the friction tester in the same manner as described above for water
    d. The system was patted dry, rinsed with water, and then patted dry again
    e. 1.0 mL of the astringent solution (i.e., no saliva) was then run on the system in the same manner
    f. The system was completely washed as described in (7); the next assay was run
    g. This process was repeated until all 8 assays (6 astringent solutions, 2 water) were run on the device; the device was again washed
9. A replicate saliva-only and another water-only friction measurement were collected

Data files (*.WDC) were saved from each run, and the voltages that were used for the calculation of friction ($Friction_{assay}$) were extracted, Figure 44. $Friction_{assay}$ was calculated according to the equation below:

$$Friction_{assay} = 0.705*(V_{ave}/2) - 0.0324 \qquad \text{Eq. 2}$$

where:

$$V_{ave} = [(V1 – V2) + (V3 – V4)]/2$$

Equation 2 was derived from the calibration of the system, which was performed on the first day of data collection (Appendix 5B)

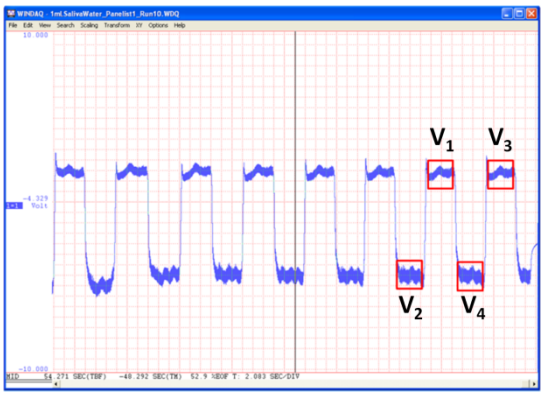
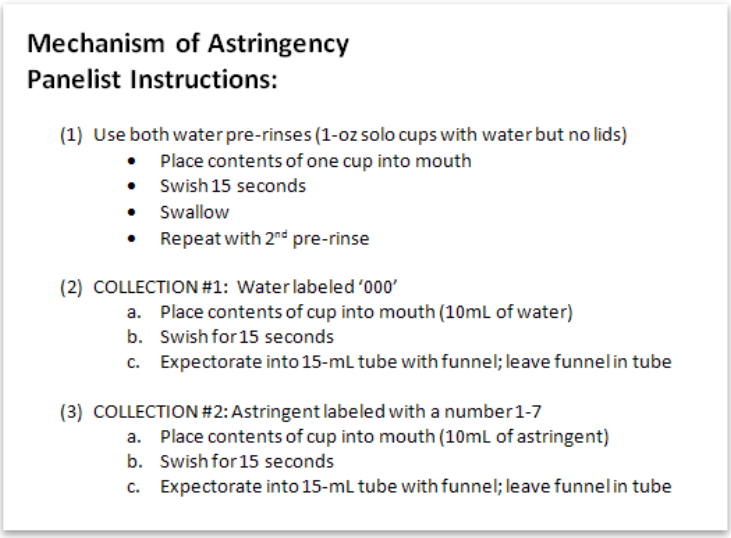


Figure 44. Friction tester output data file from one run on tester
Shown in the image is the output from the friction tester when the saliva + water assay was run on the friction tester. V_{ave} was calculated using the voltage readings from the last two complete back-and-forth motions using the equation: [(V1-V2)+(V3-V4)]/2. V_{ave} is the voltage that represents 2x the friction (i.e., it's the sum of the voltage of the back and forth motion of the machine). V_{ave} was translated into a frictional force by using the calibration information of the system.

Appendix 6: Supporting Documents for 'Do astringents cause a desquamation of the oral mucosa' study

A. Ballot Instructions
B. Sample Codes
C. Sample Rotation Plan
D. Complete data set
E. SAS Code

(A) Ballot Instructions



Mechanism of Astringency
Panelist Instructions:

(1) Use both water pre-rinses (1-oz solo cups with water but no lids)
- Place contents of one cup into mouth
- Swish 15 seconds
- Swallow
- Repeat with 2nd pre-rinse

(2) COLLECTION #1: Water labeled '000'
a. Place contents of cup into mouth (10mL of water)
b. Swish for 15 seconds
c. Expectorate into 15-mL tube with funnel; leave funnel in tube

(3) COLLECTION #2: Astringent labeled with a number 1-7
a. Place contents of cup into mouth (10mL of astringent)
b. Swish for 15 seconds
c. Expectorate into 15-mL tube with funnel; leave funnel in tube

(B) Sample Codes

Number	Rise
1	Water
2	Alum Lo
3	Alum Hi
4	Tan Lo
5	Tan Hi
6	HCl Lo
7	HCl Hi

(C) Sample Rotation Plan

		Session #						
		1	2	3	4	5	6	7
Panelist	1	6	7	2	1	5	4	3
	2	4	1	5	7	3	2	6
	3	4	2	6	1	3	7	5
	4	1	4	6	3	2	5	7
	5	6	5	3	2	1	7	4
	6	7	1	2	3	5	4	6

(D) Complete data set

				Reading			
Day	Session	Subject	Rinse	Pre-rinse	Astringent	DNA_{prop}	$DNA_{prop(mean)}$
1	5	1	1	55.67	56.44	1.01	
2	5	1	1	18.33	19.23	1.05	1.03
1	4	1	2	58.93	36.69	0.62	
2	4	1	2	20.02	17.66	0.88	0.75
1	9	1	3	51.25	18.29	0.36	
2	9	1	3	15.06	14.07	0.93	0.65
1	8	1	4	53.74	31.1	0.58	
2	8	1	4	24.43	16.98	0.70	0.64
1	7	1	5	21.19	31.05	1.47	
2	7	1	5	11.42	23.43	2.05	1.76
1	1	1	6	55.27	42.15	0.76	
2	1	1	6	11.5	18.64	1.62	1.19
1	2	1	7	55.85	40.01	0.72	
2	2	1	7	11.15	27.5	2.47	1.59
1	3	2	1	56.36	40.55	0.72	
2	3	2	1	14.3	13.99	0.98	0.85
1	8	2	2	59.54	49.94	0.84	
2	8	2	2	23.9	23.58	0.99	0.91
1	7	2	3	54.05	45.08	0.83	
2	7	2	3	18.13	9.74	0.54	0.69
1	1	2	4	61.05	2.35	0.04	
2	1	2	4	19.28	3.6	0.19	0.11
1	4	2	5	37.28	16.2	0.43	
2	4	2	5	18.75	9.5	0.51	0.47
1	9	2	6	50.43	24.55	0.49	
2	9	2	6	10.26	10.35	1.01	0.75
1	5	2	7	34.78	28.76	0.83	
2	5	2	7	20.8	11.78	0.57	0.70
1	5	3	1	58.18	59.59	1.02	
2	5	3	1	26.29	15.01	0.57	0.80
1	3	3	2	48.17	5.61	0.12	
2	3	3	2	25.56	5.08	0.20	0.16

1	6	3	3	35.48	50.55	1.42	
2	6	3	3	14.05	13.78	0.98	1.20
1	1	3	4	41.29	54.32	1.32	
2	1	3	4	19.94	11.62	0.58	0.95
1	9	3	5	53.13	53.59	1.01	
2	9	3	5	14.86	30.29	2.04	1.52
1	4	3	6	52.99	34.79	0.66	
2	4	3	6	19.77	10.85	0.55	0.60
1	7	3	7	45.79	54.03	1.18	
2	7	3	7	25.94	19.27	0.74	0.96
1	2	4	1	41.73	56.08	1.34	
2	2	4	1	25.9	14.17	0.55	0.95
1	6	4	2	41.52	25.83	0.62	
2	6	4	2	13.74	18.88	1.37	1.00
1	5	4	3	49.65	40.7	0.82	
2	5	4	3	19.61	17.68	0.90	0.86
1	3	4	4	42.74	53.12	1.24	
2	3	4	4	13.6	18.58	1.37	1.30
1	7	4	5	22.9	40.58	1.77	
2	7	4	5	11.38	9.88	0.87	1.32
1	4	4	6	48.06	40.26	0.84	
2	4	4	6	18.58	22.48	1.21	1.02
1	9	4	7	46.14	36.38	0.79	
2	9	4	7	13.3	21.99	1.65	1.22
1	6	5	1	60.58	47.86	0.79	
2	6	5	1	14.77	13.87	0.94	0.86
1	5	5	2	45.8	28.4	0.62	
2	5	5	2	22.31	10.19	0.46	0.54
1	4	5	3	55.92	28.14	0.50	
2	4	5	3	13.7	13.85	1.01	0.76
1	9	5	4	26.71	43.48	1.63	
2	9	5	4	25.33	13.14	0.52	1.07
1	2	5	5	53.17	47.26	0.89	
2	2	5	5	25.25	26.7	1.06	0.97
1	1	5	6	50.89	28.06	0.55	
2	1	5	6	23.71	16.54	0.70	0.62
1	8	5	7	50.38	55.02	1.09	
2	8	5	7	13.83	10.04	0.73	0.91
1	4	6	1	36.62	39.71	1.08	
2	4	6	1	21.52	23.14	1.08	1.08
1	5	6	2	14.35	7.23	0.50	
2	5	6	2	13.58	6.7	0.49	0.50
1	6	6	3	16.84	12.84	0.76	
2	6	6	3	13.64	12.35	0.91	0.83
1	8	6	4	23.91	11.22	0.47	
2	8	6	4	8.98	8.79	0.98	0.72
1	9	6	6	44.91	17.57	0.39	
2	9	6	6	18.06	12.07	0.67	0.53
1	3	6	7	25.49	19.59	0.77	
2	3	6	7	20.53	19.06	0.93	0.85

(E) SAS Code

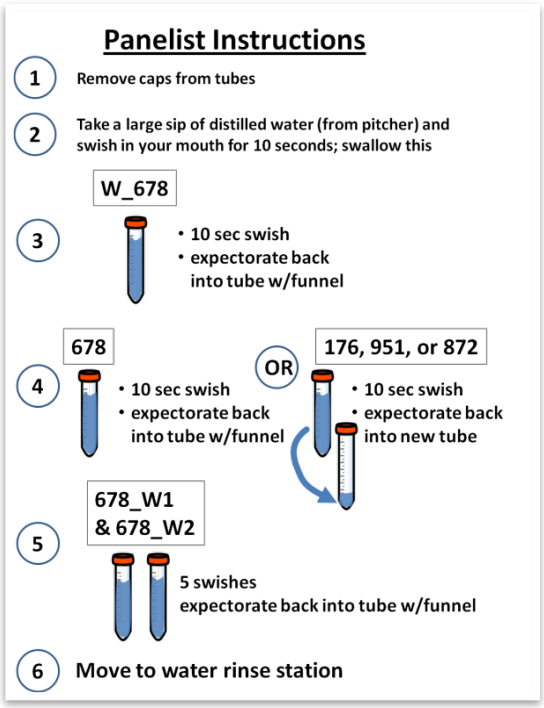
DNA Data Analysis

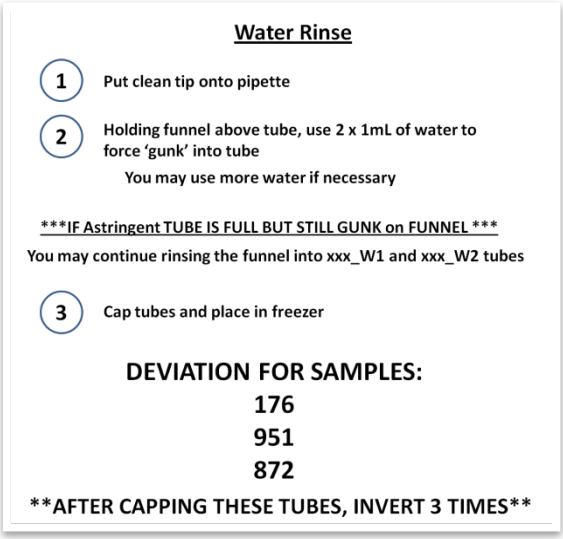
```
libname clib 'G:\FSCN\Vickers_Lab\Lee\SAS_Programs\Sept2009 Study';
run;

data clib.DNA_AvePROP;
set work.DNA_AvePROP;
run;

proc sort data=clib.DNA;
by rinse;
run;

proc means data=clib.DNA maxdec=3 mean stderr;
title 'Ave Astringency by Cleanser';
Class rinse;
var proportion;
run;

/*  Rinse #        Solution
1                  Water
2                  Alum Low (0.5 g/L)
3                  Alum High (1.0 g/L)
4                  Tannin Low (1.5 g/L)
5                  Tannin High (3 g/L)
6                  HCl Low (pH = 2.2)
7                  HCl High (pH = 1.9)*/

proc mixed data=clib.DNA_AveProp
      COVTEST;
title 'ANOVA: 7 Rinses';
class rinse panelists session;
model AverageProp = rinse / ddfm=satterth;
random panelists session;
contrast 'High vs low concentrations of rinses' rinse 0 1 -1 1 -1 1 -1;
contrast 'Water vs Alum Low' rinse 1 -1 0 0 0 0 0;
contrast 'Water vs Alum High' rinse 1 0 -1 0 0 0 0;
contrast 'Water vs Tannin Low' rinse 1 0 0 -1 0 0 0;
contrast 'Water vs Tannin High' rinse 1 0 0 0 -1 0 0;
contrast 'Water vs Acid Low' rinse 1 0 0 0 0 -1 0;
contrast 'Water vs Acid High' rinse 1 0 0 0 0 0 -1;

contrast 'Water vs Low Levels' rinse 3 -1 0 -1 0 -1 0;
contrast 'Water vs High Levels' rinse 3 0 -1 0 -1 0 -1;
run;
```

Appendix 7: Supporting Documents for 'Do astringents cause a loss of the mucus coating' study

A. Panelist Instructions
B. Blinding Codes
C. Sample Rotation Plan
D. Band Intensity Data
E. SAS Code

(A) Panelist Instructions





(B) Blinding Codes

Solution	Code
Water	387
Water	872
Acid Low	176
Acid High	951
Alum Low	826
Alum High	392
Tannin Low	580
Tannin High	162

(C) Sample Rotation Plan

	Session Number							
Panelist:	1	2	3	4	5	6	7	8
1	826	951	162	580	387	872	392	176
2	162	826	387	951	392	580	176	872
3	951	580	826	872	162	176	387	392
4	176	392	872	387	580	162	951	826
5	580	872	951	176	826	392	162	387

(D) Band Intensity Data

The following data are band intensity values ("trace intensity) obtained on each of the 5 gels. $MUCIN_{prop}$ was calculated by dividing the trace intensity of the astringent sample (even lane samples) with the trace intensity from its corresponding water pre-rinse sample (from the odd-numbered lane preceding it).

Panelist	Lane	Sample	Band	Trace Intensity	**$MUCIN_{prop}$**
1	1	Water Pre-Rinse	MG1	4879.9	
1	2	Water	MG1	3226.9	**0.66**
1	3	Water Pre-Rinse	MG1	5380.5	
1	4	Tannin Low	MG1	5581.4	**1.04**
1	5	Water Pre-Rinse	MG1	3880.6	
1	6	Tannin High	MG1	4399.1	**1.13**
1	7	Water Pre-Rinse	MG1	4258.7	
1	8	Acid Low	MG1	3520.5	**0.83**
1	9	Water Pre-Rinse	MG1	5273.2	
1	10	Acid High	MG1	3766.3	**0.71**
1	11	Water Pre-Rinse	MG1	5438.4	
1	12	Alum Low	MG1	4664.6	**0.86**
1	13	Water Pre-Rinse	MG1	4933.2	
1	14	Alum High	MG1	3734.3	**0.76**
1	15	Water Pre-Rinse	MG1	4456.0	
1	16	Water	MG1	3922.2	**0.88**
1	1	Water Pre-Rinse	MG2	1188.5	
1	2	Water	MG2	869.0	**0.73**
1	3	Water Pre-Rinse	MG2	1210.8	
1	4	Tannin Low	MG2	1234.8	**1.02**
1	5	Water Pre-Rinse	MG2	3031.6	
1	6	Tannin High	MG2	1646.9	**0.54**
1	7	Water Pre-Rinse	MG2	1277.5	
1	8	Acid Low	MG2	701.7	**0.55**
1	9	Water Pre-Rinse	MG2	798.5	
1	10	Acid High	MG2	884.0	**1.11**
1	11	Water Pre-Rinse	MG2	2169.8	

1	12	Alum Low	MG2	1370.0	**0.63**
1	13	Water Pre-Rinse	MG2	1138.6	
1	14	Alum High	MG2	792.7	**0.70**
1	15	Water Pre-Rinse	MG2	1549.7	
1	16	Water	MG2	1251.4	**0.81**
2	1	Water Pre-Rinse	MG1	3836.1	
2	2	Water	MG1	4121.7	**1.07**
2	3	Water Pre-Rinse	MG1	5804.8	
2	4	Tannin Low	MG1	4477.9	**0.77**
2	5	Water Pre-Rinse	MG1	3670.7	
2	6	Tannin High	MG1	3621.0	**0.99**
2	7	Water Pre-Rinse	MG1	4001.4	
2	8	Acid Low	MG1	2469.8	**0.62**
2	9	Water Pre-Rinse	MG1	3415.3	
2	10	Acid High	MG1	2193.1	**0.64**
2	11	Water Pre-Rinse	MG1	3698.9	
2	12	Alum Low	MG1	3035.0	**0.82**
2	13	Water Pre-Rinse	MG1	3484.4	
2	14	Alum High	MG1	2966.3	**0.85**
2	15	Water Pre-Rinse	MG1	3685.8	
2	16	Water	MG1	2090.1	**0.57**
2	1	Water Pre-Rinse	MG2	2118.5	
2	2	Water	MG2	1564.3	**0.74**
2	3	Water Pre-Rinse	MG2	2552.0	
2	4	Tannin Low	MG2	2272.2	**0.89**
2	5	Water Pre-Rinse	MG2	2233.9	
2	6	Tannin High	MG2	2053.2	**0.92**
2	7	Water Pre-Rinse	MG2	3609.4	
2	8	Acid Low	MG2	1925.5	**0.53**
2	9	Water Pre-Rinse	MG2	3021.3	
2	10	Acid High	MG2	1821.5	**0.60**
2	11	Water Pre-Rinse	MG2	2563.2	
2	12	Alum Low	MG2	1743.7	**0.68**
2	13	Water Pre-Rinse	MG2	1662.7	
2	14	Alum High	MG2	1199.7	**0.72**
2	15	Water Pre-Rinse	MG2	2208.7	
2	16	Water	MG2	1397.1	**0.63**
3	1	Water Pre-Rinse	MG1	3973.2	
3	2	Water	MG1	3031.2	**0.76**
3	3	Water Pre-Rinse	MG1	4386.8	
3	4	Tannin Low	MG1	4728.4	**1.08**
3	5	Water Pre-Rinse	MG1	4526.4	
3	6	Tannin High	MG1	2961.3	**0.65**
3	7	Water Pre-Rinse	MG1	4065.5	
3	8	Acid Low	MG1	3211.0	**0.79**
3	9	Water Pre-Rinse	MG1	4011.0	
3	10	Acid High	MG1	2116.3	**0.53**
3	11	Water Pre-Rinse	MG1	4727.8	
3	12	Alum Low	MG1	3556.8	**0.75**

3	13	Water Pre-Rinse	MG1	5187.6	
3	14	Alum High	MG1	3902.2	**0.75**
3	15	Water Pre-Rinse	MG1	3263.3	
3	16	Water	MG1	3286.9	**1.01**
3	1	Water Pre-Rinse	MG2	1101.4	
3	2	Water	MG2	769.5	**0.70**
3	3	Water Pre-Rinse	MG2	1214.2	
3	4	Tannin Low	MG2	1212.4	**1.00**
3	5	Water Pre-Rinse	MG2	1575.3	
3	6	Tannin High	MG2	1076.1	**0.68**
3	7	Water Pre-Rinse	MG2	948.8	
3	8	Acid Low	MG2	475.6	**0.50**
3	9	Water Pre-Rinse	MG2	1175.6	
3	10	Acid High	MG2	468.6	**0.40**
3	11	Water Pre-Rinse	MG2	1498.5	
3	12	Alum Low	MG2	812.4	**0.54**
3	13	Water Pre-Rinse	MG2	1404.5	
3	14	Alum High	MG2	509.5	**0.36**
3	15	Water Pre-Rinse	MG2	846.8	
3	16	Water	MG2	1148.4	**1.36**
4	1	Water Pre-Rinse	MG1	2940.0	
4	2	Water	MG1	5677.7	**1.93**
4	3	Water Pre-Rinse	MG1	5359.1	
4	4	Tannin Low	MG1	3760.1	**0.70**
4	5	Water Pre-Rinse	MG1	8413.9	
4	6	Tannin High	MG1	5825.4	**0.69**
4	7	Water Pre-Rinse	MG1	6693.1	
4	8	Acid Low	MG1	2788.5	**0.42**
4	9	Water Pre-Rinse	MG1	5247.1	
4	10	Acid High	MG1	3847.8	**0.73**
4	11	Water Pre-Rinse	MG1	5157.8	
4	12	Alum Low	MG1	4012.5	**0.78**
4	13	Water Pre-Rinse	MG1	6635.1	
4	14	Alum High	MG1	3951.3	**0.60**
4	15	Water Pre-Rinse	MG1	3849.8	
4	16	Water	MG1	4110.8	**1.07**
4	1	Water Pre-Rinse	MG2	3359.2	
4	2	Water	MG2	2112.3	**0.63**
4	3	Water Pre-Rinse	MG2	2375.3	
4	4	Tannin Low	MG2	3359.4	**1.41**
4	5	Water Pre-Rinse	MG2	3731.3	
4	6	Tannin High	MG2	2478.0	**0.66**
4	7	Water Pre-Rinse	MG2	4536.6	
4	8	Acid Low	MG2	2260.2	**0.50**
4	9	Water Pre-Rinse	MG2	2500.0	
4	10	Acid High	MG2	1990.0	**0.80**
4	11	Water Pre-Rinse	MG2	2636.9	
4	12	Alum Low	MG2	2330.2	**0.88**
4	13	Water Pre-Rinse	MG2	3630.7	

4	14	Alum High	MG2	2907.8	**0.80**
4	15	Water Pre-Rinse	MG2	3382.2	
4	16	Water	MG2	2004.5	**0.59**
5	1	Water Pre-Rinse	MG1	7970.4	
5	2	Water	MG1	4621.2	**0.58**
5	3	Water Pre-Rinse	MG1	5127.2	
5	4	Tannin Low	MG1	4729.5	**0.92**
5	5	Water Pre-Rinse	MG1	4809.0	
5	6	Tannin High	MG1	4750.1	**0.99**
5	7	Water Pre-Rinse	MG1	5662.6	
5	8	Acid Low	MG1	6184.7	**1.09**
5	9	Water Pre-Rinse	MG1	3561.7	
5	10	Acid High	MG1	3042.8	**0.85**
5	11	Water Pre-Rinse	MG1	4628.9	
5	12	Alum Low	MG1	5661.9	**1.22**
5	13	Water Pre-Rinse	MG1	4712.3	
5	14	Alum High	MG1	5718.4	**1.21**
5	15	Water Pre-Rinse	MG1	5114.3	
5	16	Water	MG1	4658.2	**0.91**
5	1	Water Pre-Rinse	MG2	2694.7	
5	2	Water	MG2	1665.9	**0.62**
5	3	Water Pre-Rinse	MG2	2484.2	
5	4	Tannin Low	MG2	1619.8	**0.65**
5	5	Water Pre-Rinse	MG2	2020.3	
5	6	Tannin High	MG2	2402.2	**1.19**
5	7	Water Pre-Rinse	MG2	3308.5	
5	8	Acid Low	MG2	1752.8	**0.53**
5	9	Water Pre-Rinse	MG2	2051.1	
5	10	Acid High	MG2	2283.3	**1.11**
5	11	Water Pre-Rinse	MG2	3434.5	
5	12	Alum Low	MG2	2032.0	**0.59**
5	13	Water Pre-Rinse	MG2	1827.6	
5	14	Alum High	MG2	1744.9	**0.95**
5	15	Water Pre-Rinse	MG2	2377.4	
5	16	Water	MG2	1528.3	**0.64**

(E) SAS Code

```
libname clib 'G:\FSCN\Vickers_Lab\Lee\SAS_Programs\Removal of Mucins';
run;

data clib.Mucins;
set work.Mucins;
run;

/* Variables in this data set:
Panelist = 1 - 5
Lane = Lane in gel; don't need this; instead use:
Astringent = Acid Low, Acid High, Alum Low, Alum High, Tannin Low,
Tannin High, Water
MUCINProp = calculated from original data set = intensity of astringent
peak (either MG1 or 2)/ intensity of peak in water pre-rinse
*/

proc sort data=clib.Mucins;
by Band;
/* Band = MG1 or MG2*/
run;

proc means data=clib.Mucins maxdec=3 mean stderr;
title 'Ave MUCINProp by Astringent type';
Class astringent;
var MUCINprop;
by Band;
run;

proc mixed data=clib.Mucins;
title 'ANOVA: MUCINProp';
class astringent panelist;
model MUCINprop = Astringent / ddfm=satterth;
random panelist;
contrast 'Astringents vs water' Astringent 1 1 1 1 1 1 -6;
by Band;
run;
```

Appendix 8: Method development for analyzing mucin content of expectorated samples

A method to prepare and analyze expectorated samples for their mucin content using SDS-PAGE methods was developed in order to investigate if astringency was related to the loss of the mucus coating of the mouth. For adequate resolution of the MG1 and MG2 protein bands in the gels, mucins from the expectorated samples needed to be concentrated and some of the astringents needed to be removed. High concentrations of tannins and alum (a salt) in samples are known to cause smearing, streaking, and band distortion during electrophoresis.

The following sample treatment options were explored:

1. Dialysis to simultaneously concentrate the proteins and decrease astringent concentrations within the samples
2. Centrifugal filtration methods to simultaneously concentrate the proteins and decrease astringent concentrations of the samples
3. Trichloroacetic acid (TCA) protein precipitation method to eliminate astringents in the sample and to concentration proteins
4. Lyophilization of samples to concentration proteins followed by a trichloroacetic acid (TCA) protein precipitation method to eliminate astringents in the sample

Combining lyophilization with a TCA precipitation step (Option 4) was the only successful method. Dialysis was unsuccessful since tannins were not removed even with large (100kDa) pore sizes and because it was a 'messy' method that made complete and repeatable recovery of the mucins difficult. Centrifugal filtration methods were not suitable for these samples because of the oral debris and high molecular weight proteins that clogged the filtration units even when molecular weight cut-offs of 100kDa were used. Option 3 (TCA precipitation of the proteins in the expectorated solutions) seemed to work well with the astringent solutions, but proteins in the expectorated water solution

did not precipitate as easily. Lyophilizing the samples first (Option 4) seemed to resolve this issue. Additionally, by lyophilizing the samples first, much less TCA was required.

To confirm that these methods worked for all astringent expectorated solutions, a series of controlled experiments, written up in the next few pages, were run in which known quantities of saliva were added to 10mL samples of acid, alum, tannins, and water. The procedure as written below was followed, and the mucins observed in the gels for these samples were compared to a pure saliva sample that was frozen, lyophilized, and resolubilized according to the method below. The pure saliva sample was not subjected to TCA precipitation.

Experiment 1

The objectives of this experiment were to determine if:

1. TCA precipitation could be used to eliminate high alum and tannin concentrations from samples prior to electrophoresis
2. TCA precipitation should be carried out before or after sample lyophilization (using alum containing samples)
3. the method also works on acid-containing samples

Experiment 1: Objectives 1 and 2

Stimulated whole saliva was first collected from a single subject and centrifuged to remove oral debris and other particulates. TCA solutions at 35% and 100% (w/v) were prepared. A set of three 15mL centrifuge tubes was prepared by adding 0.5mL of saliva into the tubes that contained a) nothing; b) 2.5mL of 1g/L alum c) 5.0mL of 1g/L alum. This set was replicated three times (set 1, 2, 3). Additionally, a fourth set (set 4) of three 15mL centrifuge tubes was prepared by adding 0.5mL of saliva (collected on a different day from the saliva used in sets 1-3) into 15mL tubes containing a) nothing; b) 2.5mL of 3g/L tannins c) 5.0mL of 3g/L tannins. The alum and tannins in this lab experiment were

the same ones used during the final experiment. After vigorously vortexing the tubes, the samples of the three sets were treated as follows:

Set 1: No removal of astringents.
Samples were frozen, lyophilized, and then resolubilized with 0.285mL laemmli sample buffer, 0.015mL βME, and 0.04mL of 1M Tris-HCl at pH = 8.0. Tubes were then boiled for 5 minutes and vigorously vortexed.

Set 2: Removal of astringents via TCA precipitation preformed after lyophilization.
Samples were frozen and lyophilized. A TCA precipitation step was performed next by adding 2mL of 25% TCA plus an additional 1mL of water. Tubes were incubated on ice for approximately 20 minutes, and then centrifuged using a JS 7.5 swinging bucket rotor at 4500RPM for 10 minutes at 4°C. The supernatant was discarded, the tube was allowed to drain, and the pellet was washed with 2mL of ice cold acetone and vortexed. Proteins were repelletized by centrifuging the tubes for 3 minutes at the previously used settings. The acetone was then decanted, and the tubes were drained and allowed to air dry before being resolubilized with 0.285mL laemmli sample buffer, 0.015mL βME, and 0.04mL of 1M Tris-HCl at pH = 8.0. Tubes were then boiled for 5 minutes and vigorously vortexed

Set 3: Removal of astringents via TCA precipitation.
TCA precipitation was performed first by adding 0.1, 0.6, and 1.1mL of 100% (w/v) TCA into tubes 3A, B, and C, respectively. Tubes were incubated on ice for approximately 20 minutes, and then centrifuged using a JS 7.5 swinging bucket rotor at 4500RPM for 10 minutes at 4°C. Pellets were washed with 2mL of ice-cold acetone and recentrifuged for 3 minutes. Tubes were drained and the pellets were allowed to air dry before being resolubilized with 0.285mL laemmli sample buffer, 0.015mL βME, and 0.04mL of 1M Tris-HCl at pH = 8.0. Tubes were then boiled for 5 minutes and vigorously vortexed

Set 4: Prepared exactly as described for Set 3.

SDS-PAGE was carried out and the gel was stained according to the methods as described on page 30. A 7.5% polyacrylamide precast Tris-HCl gel (Bio-Rad, Hercules, CA) was used. Lane assignments are given in Table 14, and the gel is shown in Figure 45.

Table 14. Lane assignments for experiment 1 (Method development for analyzing mucin content of expectorated samples)

Lane	Sample	Treatment Method
1	1a: pure saliva	Lyophilized and resolubilized in buffer. Samples B and C of this set (C is in lane 18) contain high alum concentrations
2	1b: saliva in 2.5mL 1g/L alum	
4	2a: pure saliva	Lyophilized first, followed by TCA precipitation before resolubilizing in sample buffer.
5	2b: saliva in 2.5mL 1g/L alum	
6	2c: saliva in 5.0mL 1g/L alum	
8	3a: pure saliva	TCA precipitation before resolubilizing in sample buffer. No freeze-dry step.
9	3b: saliva in 2.5mL 1g/L alum	
10	3c: saliva in 5.0mL 1g/L alum	
12	4a: pure saliva	TCA precipitation before resolubilizing in sample buffer. No freeze-dry step.
13	4b: saliva in 2.5mL 3g/L tannins	
14	4c: saliva in 5.0mL 3g/L tannins	
18	1c: saliva in 5.0mL 1g/L alum, lyophilized and resolubilized in buffer (run in this far lane due to very high alum content)	

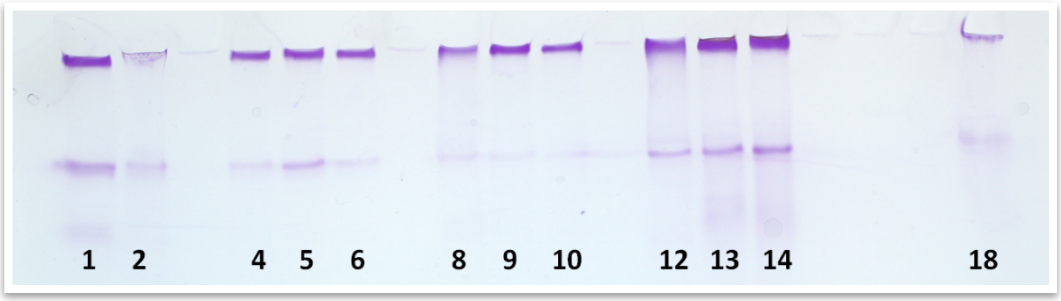


Figure 45. PAS-stained gel for analyzing mucin content of expectorated samples
Gel from experiment 1 to determine if TCA precipitation with or without lyophilization of the sample was adequate to remove astringents for SDS-PAGE analysis. Lane assignments given in Table 14.

Several conclusions can be made from viewing the gel, Figure 45. It is very clear that high alum concentrations cause band interference (lane 2 and 18), and either of the tested protocols sufficiently reduced the concentration of alum to eliminate this problem (see lanes 5 and 6 and 9 and 10). Clear bands are observed in lanes 4-6 (lyophilization plus TCA precipitation) and in lanes 8-10 (TCA precipitation only), which indicated that samples could be treated either way with success. Because of the increased amount of TCA required to prepare the samples when skipping the lyophilization step, it is preferred to start sample preparation with their lyophilization. Lanes 12-14 also show clear bands, indicating that TCA precipitation is also appropriate for tannin-containing samples.

Experiment 1: Objective 3

For this objective, saliva was collected from a single panelist and centrifuged to remove debris. Samples were prepared by adding 0.5mL aliquots of saliva into three 15mL tubes containing 10mL of distilled water and six tubes containing 10mL of HCl acid at pH = 1.9 (acid "high"). Three drops of 1M NaOH were added into 3 of the HCl-saliva tubes. This amount of NaOH raised the pH to around 3.5 - 4 and was determined based on benchtop experiments using saliva from multiple donors. The goal was to raise the pH so that it was closer to neutral, but to avoid overshooting a pH of 7.

The gel image from this experiment is shown below, Figure 46. Raising the pH of the acid samples (HCl plus NaOH) appeared to result in band densities closer to those from the water samples and appears to be an appropriate treatment for the samples. The lack of bands in lanes 1 and 2 show what happens when the pH is highly basic.

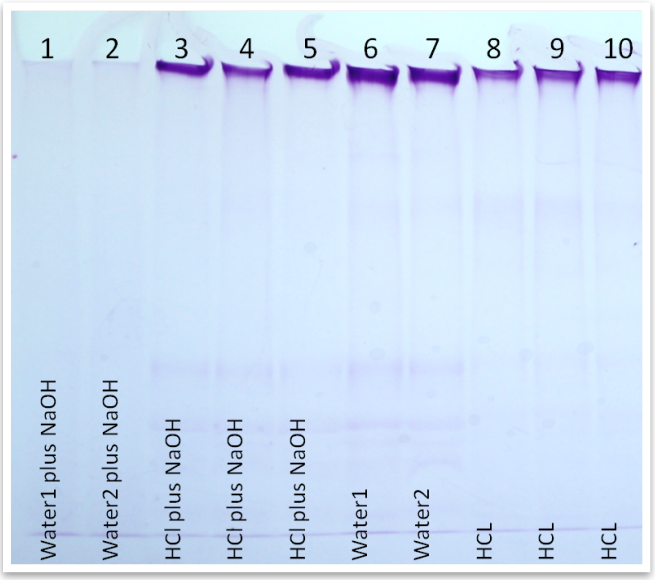


Figure 46. PAS-stained gel showing effect of increasing pH of acid-saliva samples
Effect of adding NaOH into saliva-HCl samples and saliva-water samples. Increasing the pH of the acid samples appears to result in bands similar to those from the water samples. The effect of a basic pH on the water samples can be seen in lanes 1 and 2 where no MG1 band is visible.

Experiment 2

This experiment was the first attempted study for part I.3a: Loss of the mucus coat, page 59. The objective of this experiment was to determine if astringent was related the loss of oral coatings. The results of the study, while consistent, left unanswered questions and therefore, additional work was continued (Experiments 3) before rerunning a modified version of the study.

Subjects

5 panelists were recruited to participate in this study and the sessions were run as described on page 60.

Procedure

Panelists swished and expectorated a water pre-rinse solution and one astringent solution at each session in exactly the same manner as described on page 61; however, water post-rinse expectorants were not collected. Samples were prepared for SDS-PAGE as described on page 62, except that samples were left in the original 15mL tubes. Samples were also not centrifuged (as they were for the final study) prior to being electrophoresed.

Gel Results

Samples were electrophoresed on 7.5% polyacrylamide gels and stained according to the methods of Becerra et al. (2003), Figure 47. If my hypothesis were true – that astringents remove the mucus coating – an increase in MG1 and MG2 would be expected in the lanes for the astringent expectorants when compared to water. Instead, the mucins bands in the acid and alum samples were very faint (lanes 8, 10, 12, 14), Figure 47.

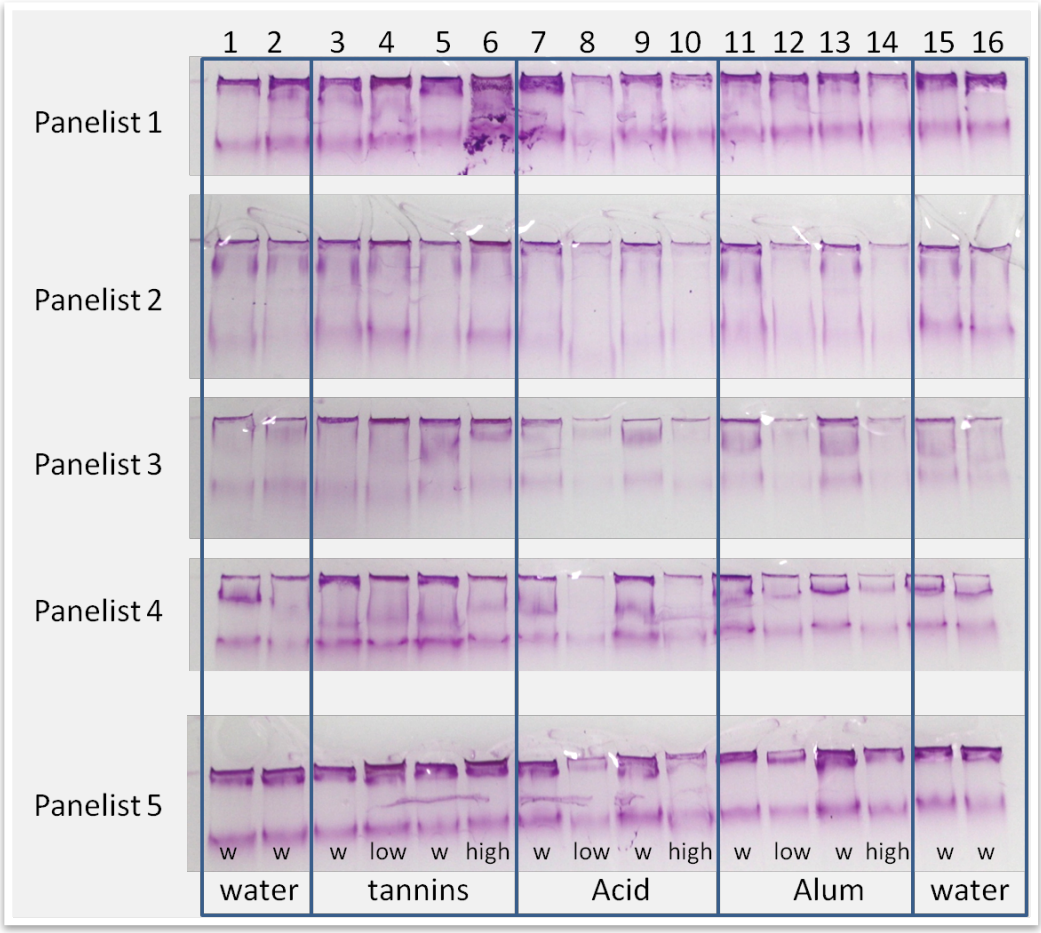


Figure 47. PAS-stained gels of panelists' expectorated solutions
Cropped images of the gels from the 5 panelists' expectorated samples. For each astringent or control solution expectorated (water, tannins low or high, acid low or high, and alum low or high), it was always preceded by a water pre-rinse, labeled 'w'. Astringent expectorated samples are always in even-numbered lanes, and their corresponding water pre-rinse sample is always in the left neighboring lane.

It was unknown why MG1 and MG2 appeared faint in most of the acid and alum expectorated samples, and this was the reason why this study was eventually rerun with additional water post-rinses. Was it possible that the astringent caused the salivary and mucus coating proteins to adhere to surfaces in the mouth and not come out in the initial astringent expectorant? Were there issues with the sample preparation or method of electrophoresis? Perhaps this was why poor band quality and loose debris was also observed in many of the gels.

Experiment 3

Based on the results of Experiment 2, additional work needed to be performed. There were two main objectives to this study:

1. Determine if additional water rinses following the astringent sample are required to capture mucins that may be removed by the astringents but remain in the mouth

2. Determine if cellular or oral debris is distorting the bands of the expectorated samples

To collect expectorated water pre-rinse, astringent (water, alum high, tannin high, or acid high), and water post-rinse samples, a single subject followed the instructions as written up on page 60. The expectorated samples were prepared according to the procedures written up on page 60, but the astringent expectorated solution was not combined with any of the water post-rinses. Instead, each of the samples were electrophoresed in their own lane in order to determine if the bulk of the mucins were being expectorated in any of the post-rinses. The gel from this experiment in shown in Figure 48.

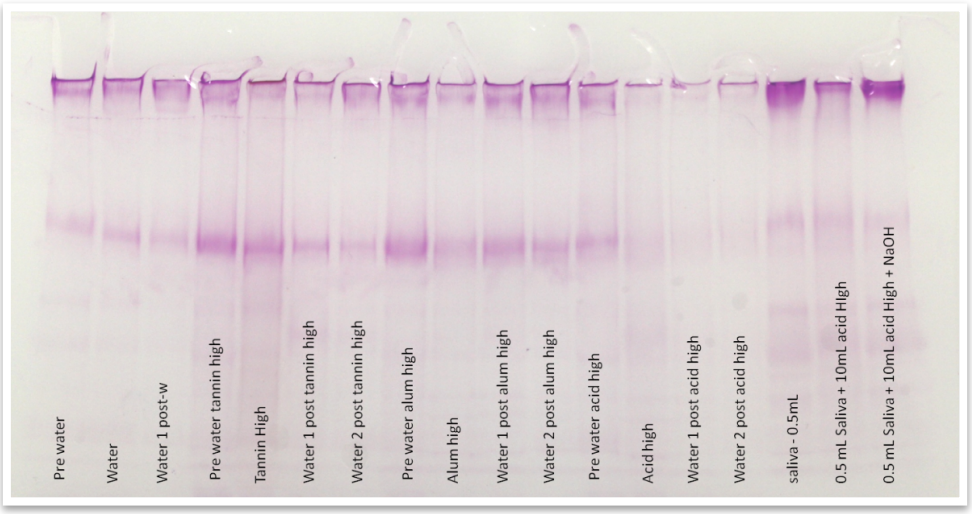


Figure 48. PAS-Stained gel showing mucins in water post-rinses following astringent samples

Gel to determine if the addition of two water post-rinses following the astringent solution would capture the mucins 'missing' from the expectorated acid or alum samples in experiment 2. Lanes are labeled with sample ID. Post-rinses for alum appear to contain higher levels of mucins, but the acid lanes still show low levels of acids. The three right-most lanes repeat the controlled experiment detailed above (Experiment 1: Objective 3), and reconfirm that neutralizing acid samples results in better recovery of the mucins.

Because the acid expectorant and its post water rinses still had little to no mucins, two additional expectorated acid samples were collected, and the pH of one of them was raised by adding NaOH, Figure 49. Although it is not possible to compare the two expectorated samples for mucin content, it is worth noting that the sample with added NaOH showed much stronger MG1 and MG2 bands. This agrees with the observed increase in MG1 and MG2 bands in the controlled samples (10mL of acid + 0.5mL saliva) with added NaOH, Figure 46 and Figure 48.

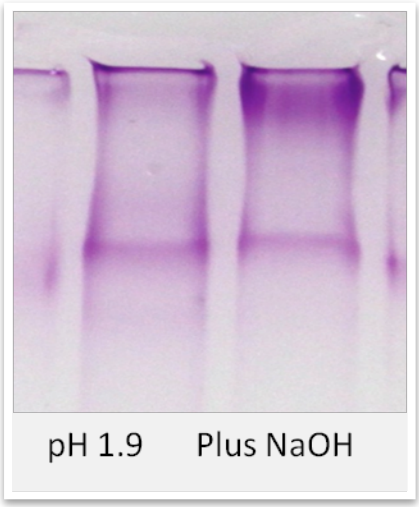


Figure 49. The effect of added NaOH on expectorated acid samples.
When NaOH was added into an expectorated acid sample, MG1 and MG2 bands were clearly visible.

Finally, to determine if cellular or oral debris was distorting the mucin bands of the expectorated samples, some of the samples in Figure 48 were run on a second gel. The samples that were re-run included each of the astringent expectorated samples (water, acid high, tannin high, and alum high) and the first water post-rinses corresponding to each of the astringents. The 15mL tubes were centrifuged at 5000RPM for 5 minutes, and then the samples were loading onto a 7.5% gel. Then, each of the samples was loaded a second time, but the tube was vigorously vortexed first. The gel clearly shows that when the samples were vortexed before loading, debris was present in the samples that resulted in streaking and band distortion, Figure 50.

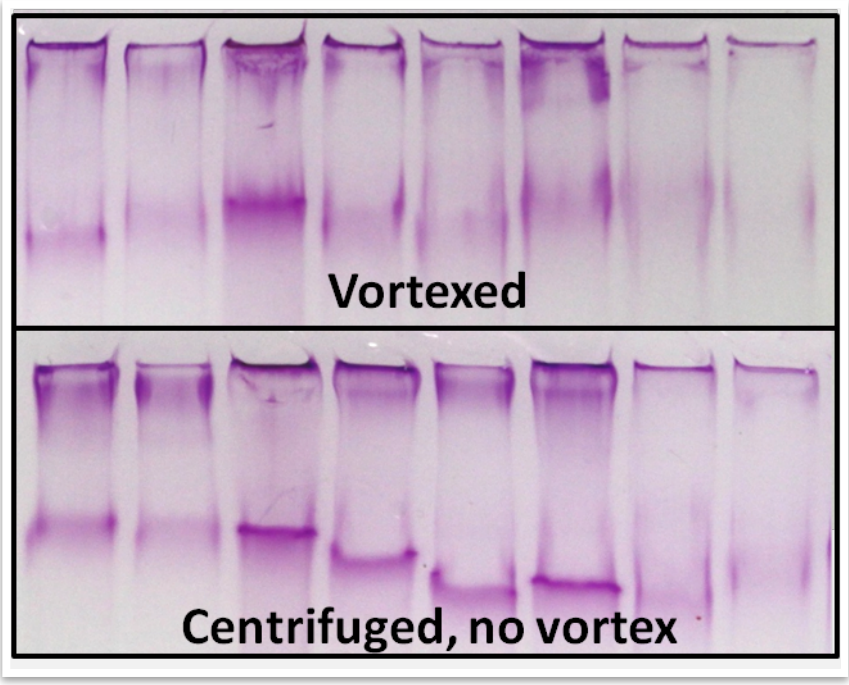


Figure 50. Effects of vortexing vs centrifuging expectorated samples prior to electrophoresis

The effects of vortexing expectorated samples before loading on a gel. The two images are cropped from a single gel and show what happens when samples are vortexed (top) or centrifuged (bottom) before being loaded. The bands were tighter and showed less streaking or smears when the samples were centrifuged.

Appendix 9: Supporting Documents for 'Palate Cleanser for Astringent Foods' study

A. Blinding codes
B. Assignment of rinse option for panelists by session number
C. Assignment of serving order of the six astringent solutions
D. SAS Code

(A) Blinding Codes: New codes were randomly assigned for each session.

Tannin Solutions:

		Tannin Concentration					
		3	2.4	1.8	1.2	0.6	0
Session Number	1	521	846	463	193	645	574
	2	729	657	027	538	158	823
	3	570	271	639	391	791	917
	4	142	681	183	365	254	568
	5	436	384	968	675	781	173
	6	823	158	485	293	512	481
	7	168	726	517	186	148	765
	8	724	389	297	234	042	731
	9	145	493	351	576	784	357
	10	786	618	413	273	845	931
	11	147	276	245	178	715	597
	12	142	495	581	473	287	165

Acid Solutions:

		Acid Sample pH					
		1.6	1.72	1.89	2.16	3.1	Water
Session Number	1	318	495	289	694	432	597
	2	241	935	518	946	348	452
	3	346	562	723	641	914	958
	4	163	617	274	174	674	261
	5	649	738	971	264	763	483
	6	154	832	841	486	692	378
	7	746	715	469	796	825	579
	8	268	285	627	769	891	381
	9	127	417	934	142	314	251
	10	857	231	576	482	763	176
	11	192	243	452	476	584	713
	12	846	631	379	531	425	947

(B) Assignment of Rinse Option for panelists by Session Number

The 12 rinse options (6 cleansers x 2 cleansing protocols) were assigned to panelists according to a 12 x 12 latin square design balanced for order effects (MacFie et al., 1989). The same latin square design was applied for panelists assigned to the bitter group and those in the sour group.

Table 15. Assignment of rinse option by subject and session number in palate cleanser study

The meaning of the colors is explained below and represents the serving order of the astringent solutions within each session.

Subject	Session Number 1	2	3	4	5	6	7	8	9	10	11	12
1	9	5	11	7	3	10	2	8	6	12	4	1
2	12	1	8	4	10	6	7	2	5	3	9	11
3	3	11	2	9	6	5	4	7	1	10	12	8
4	11	9	3	5	2	7	6	10	4	8	1	12
5	8	12	10	1	7	4	5	6	9	2	11	3
6	1	4	12	6	8	2	10	3	7	11	5	9
7	2	3	6	11	4	9	1	5	12	7	8	10
8	6	2	4	3	1	11	12	9	8	5	10	7
9	4	6	1	2	12	3	8	11	10	9	7	5
10	5	7	9	10	11	8	3	12	2	1	6	4
11	7	10	5	8	9	12	11	1	3	4	2	6
12	10	8	7	12	5	1	9	4	11	6	3	2
13	6	1	11	4	7	9	12	8	3	2	10	5
14	1	4	6	9	11	8	7	2	12	5	3	10
15	7	11	12	6	3	1	10	4	5	9	2	8

Rinse Option	Strategy	Cleanser
1	Self-Determined	Nothing
2	Self-Determined	Cracker
3	Protocol	CMC
4	Protocol	Cracker
5	Self-Determined	Skim Milk
6	Self-Determined	CMC
7	Protocol	Wax
8	Protocol	Water
9	Protocol	Skim Milk
10	Self-Determined	Water
11	Self-Determined	Wax
12	Protocol	Nothing

(B) Assignment of serving order of the six astringent solutions

The order of astringent solutions was assigned according to two 6x6 Latin squares balanced for order effects (MacFie et al., 1989) applied across each rinse option. Thus, within each rinse option, the order of astringent samples was fully balanced for their serving order.

For example, each of the 12 panelists (in the complete Latin square above) receives each rinse option 9 once. Rinse option 9 is served only once each day, and always to a different panelist. The two 6 x 6 Latin squares are applied to the rinse option 9 in order of session number:

Table 16. Serving order of astringent solutions for rinse option 9 in palate cleanser study

Session	Panelist	Astringent Solution (1 = weakest, 6 = strongest)						
1	1	6	4	2	5	1	3	1st 6x6 Latin Square
2	4	4	5	6	3	2	1	
3	10	2	6	1	4	3	5	
4	3	1	2	3	6	5	4	
5	11	5	3	4	1	6	2	
6	7	3	1	5	2	4	6	
7	12	3	6	5	2	1	4	2nd 6x6 Latin Square
8	8	1	5	4	3	2	6	
9	5	4	1	2	5	6	3	
10	9	5	3	1	6	4	2	
11	2	6	2	3	4	5	1	
12	6	2	4	6	1	3	5	

The same latin squares were applied in a similar manner to all rinse options, albeit slightly different. This is reflected in the shading applied to Table 15.

(C) SAS Code

The following code is shown for the bitter (tannin) data set, with the dependent variable being astringency. Using the appropriate variable names, the same code was used to analyzed the data from the panelists in the sour (acid group) and to analyze the data with the dependent variable being the taste (either bitterness or sourness).

```
libname clib 'G:\FSCN\Vickers_Lab\Lee\SAS_Programs\Palate Cleanser
Study';
run;

data clib.bitterData;
set work.bitterData;
run;

proc sort data=clib.bitterData;
by sample;
run;

proc means data=clib.bitterData maxdec=2 mean stderr;
title 'Ave Astringency by Cleanser';
Class Cleanser;
var bitterness astringency;
run;

proc means data=clib.bitterData maxdec=2 mean stderr;
title 'Means By Sample/Cleanser';
Class Cleanser Sample;
var bitterness astringency;
run;

proc sort data=clib.bitterData;
by strategy;
run;

proc means data=clib.bitterData maxdec=2 mean stderr;
title 'Time Spent Using Cleanser';
Class Cleanser position;
by strategy;
var minutes_total;
run;


/*  ----> ANOVA FOR ASTRINGENCY DATA <----  */


proc mixed data=clib.bitterData COVTEST;
title 'Astringency Main ANOVA';
class session position panelist strategy cleanser;
```

```
model astringency = cleanser tanninconc strategy position
cleanser*position cleanser*tanninconc cleanser*strategy
strategy*tanninconc strategy*position strategy*tanninconc*cleanser
strategy*position*cleanser/ ddfm=satterth;
random panelist panelist*session*cleanser;
/*lsmeans cleanser / PDIFF ADJUST=BON;
lsmeans position / PDIFF ADJUST=BON;*/

/*Did certain cleansers result in better discrimination among
astringent samples?*/
contrast 'test equal slopes -by tannin concentration'
cleanser*tanninconc 1 -1 0 0 0 0,
cleanser*tanninconc 0 1 -1 0 0 0,
cleanser*tanninconc 0 0 1 -1 0 0,
cleanser*tanninconc 0 0 0 1 -1 0 ,
cleanser*tanninconc 0 0 0 0 1 -1;

/* Yes, so which ones were best?*/
/* Order of cleansers:  CMC Crackers Milk Nothing Water Wax */
/* This proc glm contrasts specific astringency vs. tannin conc slopes
*/

contrast 'Water vs Nothing' cleanser*tanninconc  0 0 0 -1 1 0;
contrast 'Water vs CMC' cleanser*tanninconc -1 0 0 0 1 0;
contrast 'Water vs Wax' cleanser*tanninconc  0 0 0 0 1 -1;
contrast 'Water vs Milk' cleanser*tanninconc  0 0 -1 0 1 0;
contrast 'Water vs Crackers' cleanser*tanninconc  0 1 0 0 -1 0;
contrast 'Nothing vs CMC' cleanser*tanninconc  1 0 0 -1 0 0;
contrast 'Nothing vs Wax' cleanser*tanninconc  0 0 0 -1 0 1;
contrast 'Nothing vs Milk' cleanser*tanninconc  0 0 1 -1 0 0;
contrast 'Nothing vs Crackers' cleanser*tanninconc  0 -1 0 1 0 0;
contrast 'Wax vs Crackers' cleanser*tanninconc  0 -1 0 0 0 1;
contrast 'Wax vs CMC' cleanser*tanninconc  -1 0 0 0 0 1;
contrast 'CMC vs Crackers' cleanser*tanninconc  1 -1 0 0 0 0;
contrast 'Water and nothing vs all else' cleanser*tanninconc -1 -1 -1 2
2 -1;
contrast 'Milk (worst) and wax' cleanser*tanninconc 0 0 -1 0 0 1;
contrast 'Milk (worst) and CMC' cleanser*tanninconc 1 0 -1 0 0 0;
contrast 'Milk (worst) and Crackers' cleanser*tanninconc 0 1 -1 0 0 0;

run;

/* Time spent using cleansers (BOTH SOUR AND BITTER) */

DATA clib.timingdata; /*data includes only position = 6*/
set work.timingdata;
run;

proc sort data=clib.timingdata;
by group strategy;
run;

proc means data=clib.timingdata maxdec=2 mean stderr;
title 'Time spent after samples';
Class cleanser;
var Minutes;
by group strategy;
```

```
run;

proc sort data=clib.timingdata;
by group cleanser;
run;

proc mixed data=clib.timingdata;
title 'Means Comparisons';
class group session panelist strategy cleanser;
model Minutes = strategy cleanser strategy*cleanser/ ddfm=satterth;
random panelist panelist*session*cleanser;
by group;
lsmeans strategy / PDIFF ADJUST=BON;
run;

proc sort data=clib.timingdata;
by group cleanser;
run;

proc ttest data=clib.timingdata;
Class strategy;
var Minutes;
by group cleanser;
run;
```

Liking Data:

```
libname clib 'G:\FSCN\Vickers_Lab\Lee\SAS_Programs\Palate Cleanser
Study';

data clib.cleanserliking;
set work.cleanserliking;
run;

proc means data=clib.cleanserliking maxdec=2 MEAN STDERR;
by cleanser;
var value;
Run;

/* Perform Proc Mixed for LIKING */

Proc Mixed data=clib.cleanserliking;
title 'Liking';
      class Group Cleanser panelist;

      model value = group cleanser group*cleanser / ddfm=satterth; /*--
      random group(panelist);
      lsmeans cleanser / adjust=bon;

run;
```

Appendix 10: Supporting Documents for 'Acidic Whey Protein Beverages' study

A. Training Handout
B. Blinding codes
C. Sample serving order
D. SAS Code

(A) Training handout

~~ Welcome to the Whey Protein Beverage Study! ~~

Whey Protein: The proteins left over in the whey during cheese manufacturing. The proteins are isolated from the whey, purified, and used in a wide variety of products.

Whey Protein Beverages: Many beverages contain whey protein. Acidic whey protein drinks, such as what we're going to taste, have a common problem: they're astringent. These drinks are becoming more common, but no one fully understands the reason why they're astringent.

Session 1: Training

Goals of training:

1. **Understand the difference between sourness and astringency**
2. **Learn how to rate samples for sourness and astringency**
3. **Become familiar with the astringent reference sample and its astringency rating**
4. **Go through a practice session with the computer**

Goal 1: Understand the difference between sourness and astringency

During the testing session tomorrow, you will be rating samples for their astringency and for their sourness. These samples contain whey protein which is a dairy ingredient.

Astringency is a tactile sensation felt in the mouth that is commonly described as a drying-out or roughing sensation. It is experienced on the oral surfaces inside the mouth including the cheeks and inner lip areas, and it is often a delayed sensation taking some time to fully develop.

Sourness is the tart and tangy taste. While astringency is a tactile sensation, sourness is a taste.

The difference between the two will become more apparent when we taste some samples.

The most important distinction between sourness and astringency: sourness is a taste which can be perceived without movement. Astringency is tactile and generally is difficult to perceive prior to moving the mouth.

Goal 2: Learn how to rate samples for sourness and astringency.

This is how samples will be tasted during the study.
Sample procedure:

1. Place contents of entire 10 ml sample in mouth; don't move
2. Rate the sourness
3. Swish gently for 10 seconds
4. Expectorate sample
5. Three "Why's"

Pay attention to maximum perceived astringency throughout

6. Rate the maximum perceived astringency
7. Rinse mouth thoroughly with water for 5 minutes OR LONGER until astringency has returned to a level of zero

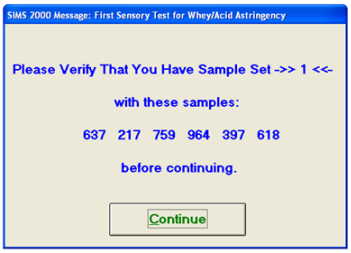
Important!! – Do NOT continue to next sample until perceived astringency is zero!

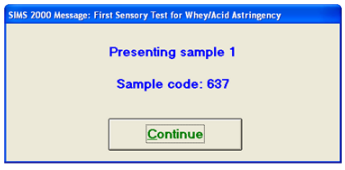
There will be a total of 6 coded test samples tomorrow. You will also receive a reference sample of alum which you will receive first.

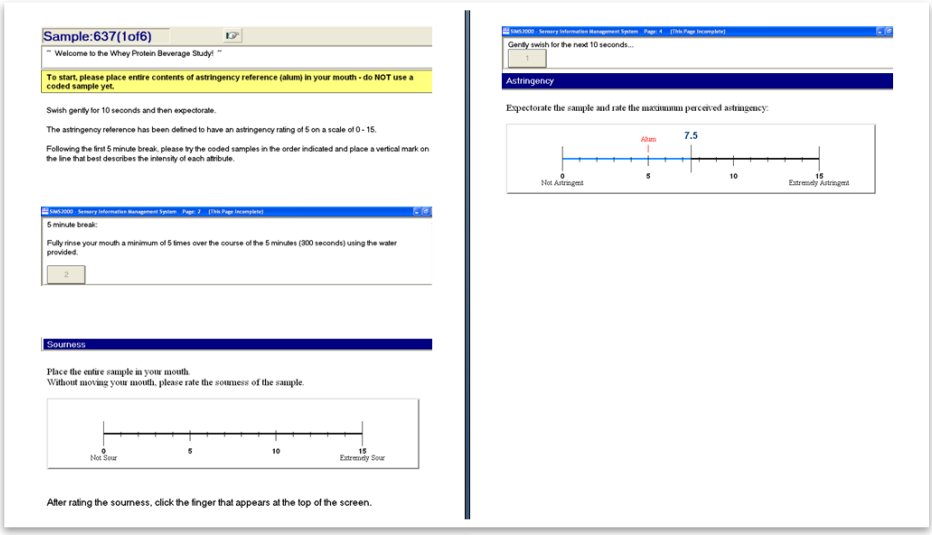
We will now try the technique using a citric acid sample which is both sour and astringent. The sourness is noticed in step 2. The astringency is noticed in step 5.

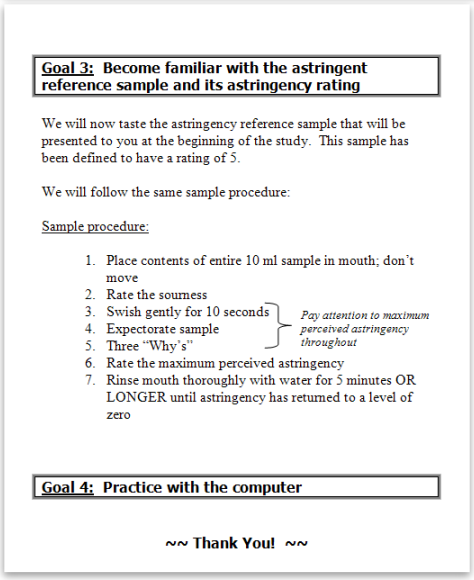
During the test tomorrow, you will rate both the sourness and astringency on a scale of 0-15. We will now go over the procedure that you will follow during testing.

Screen Shots:









Goal 3: Become familiar with the astringent reference sample and its astringency rating

We will now taste the astringency reference sample that will be presented to you at the beginning of the study. This sample has been defined to have a rating of 5.

We will follow the same sample procedure:

Sample procedure:

1. Place contents of entire 10 ml sample in mouth; don't move
2. Rate the sourness
3. Swish gently for 10 seconds
4. Expectorate sample
5. Three "Why's"

Pay attention to maximum perceived astringency throughout

6. Rate the maximum perceived astringency
7. Rinse mouth thoroughly with water for 5 minutes OR LONGER until astringency has returned to a level of zero

Goal 4: Practice with the computer

~~ Thank You! ~~

(B) Blinding codes

Sample	Blinding Code
6% WPI at pH = 3.4	397
Acid at concentration of #1	759
1% WPI at pH = 3.4	618
Acid @ conc. of #3	217
Acid @ pH = 3.4	964
Water	637

(C) Sample serving order

	Sample Order					
Panelist:	1	2	3	4	5	6
1	637	217	759	964	397	618
2	217	964	637	618	759	397
3	759	637	397	217	618	964
4	397	759	618	637	964	217
5	964	618	217	397	637	759
6	618	397	964	759	217	637
7	618	637	964	759	397	217
8	397	964	217	618	759	637
9	217	397	759	964	637	618
10	964	618	397	637	217	759
11	637	759	618	217	964	397
12	759	217	637	397	618	964
13	637	397	759	618	217	964
14	397	618	637	964	759	217
15	759	637	217	397	964	618
16	618	964	397	217	637	759
17	217	759	964	637	618	397
18	964	217	618	759	397	637
19	964	397	618	637	217	759
20	618	964	217	397	759	637
21	637	759	397	217	964	618
22	217	618	759	964	637	397
23	397	637	964	759	618	217
24	759	217	637	618	397	964
25	637	397	964	759	618	217

(D) SAS code

```
libname clib 'G:\FSCN\Vickers_Lab\Lee\SAS_Programs';

data clib.wpitest1;
set work.wpitest;
run;

/* Perform Proc Mixed for ASTRINGENCY */

Proc Mixed data=clib.wpitest1;
title 'Astrigency';
      class prod position;

      model astringency = prod position position*prod / ddfm=satterth;
      random judge;
```

```
      estimate 'Astringency of 6% WPI vs Acid Matched for 6%'
            prod  0 -1 0 0 1 0;
      estimate 'Astringency of 1% WPI vs Acid Matched for 1%'
            prod  -1 0 0 1 0 0;
      estimate 'Astringency of ALo vs W6 & W1'
            prod  0 0 1 -0.5 -0.5 0;
      estimate 'Astringency of last position vs first'
            position  1 0 0 0 0 -1;
      /*estimate '6% WPI vs 1% WPI'
            prod  1 0 -1 0 0 0;*/
      /*estimate '6% group vs 1% group'
            prod  -0.5 0.5 0 -0.5 0.5 0;*/
      estimate 'Whey group vs Acid group'
            prod  -0.5 -0.5 0 0.5 0.5 0;
      estimate 'Acid @ pH 3.4 vs water'
            prod  0 0 1 0 0 -1;
run;

/* Perform Proc Mixed for SOURNESS */

Proc Mixed data=clib.wpitest1;
      title 'Sourness';
      class prod position;
      model sourness = prod position position*prod / ddfm=satterth;
      random judge;

      lsmeans prod / pdiff;  /* adjust=bon; */
            /*eliminated position and position*prod*/

      estimate 'Sourness of 6% WPI vs Acid Matched for 6%'
            prod  0 -1 0 0 1 0;
      estimate 'Sourness of 1% WPI vs Acid Matched for 1%'
            prod  -1 0 0 1 0 0;
      estimate 'Sourness of Acid Matched for 6% vs Acid Matched for 1%'
            prod  -1 1 0 0 0 0;
      estimate 'Acid @ pH 3.4 vs water'
            prod  0 0 1 0 0 -1;

run;
```