ABSTRACT

BEECHER, JASON W. Factors Regulating Astringency of Whey Protein-Fortified Beverages. (Under the direction of Dr. E. Allen Foegeding.)

Whey proteins are added to a variety of foods and beverages for functionality and added nutrition. A rapidly growing area of whey protein use in foods and beverages is the sports drink category. There are two categories of whey protein-fortified drinks: those at neutral pH and those at low pH. The drinks at low pH have a clear and refreshing appearance, compared to the shake-style drinks at neutral pH. Astringency is very pronounced at low pH. Thought to be caused by compounds in foods that bind with precipitate salivary proteins, astringency at high levels is an undesirable characteristic in foods and beverages. The mechanism of astringency of whey proteins is not understood and has not been investigated. Salivary flow rate, viscosity, and pH are a few variables that have been reported to alter perceived astringency of red wine, tannic acid, alum, chitosan, and cranberry juice.

In order to investigate factors regulating astringency of whey proteins, a market survey was conducted and a model beverage was formulated. Trained sensory panelists evaluated the viscosity and pH effects on astringency of whey protein-fortified model drinks (n=8). Changes in optical density of saliva and drink mixtures before and after centrifugation were also investigated to see if a relationship existed between aggregation, precipitation, and astringency. Increasing viscosity (1.6 mPa s – 7.7 mPa s) did not alter maximum intensity, time to maximum, duration, or area under the curve of astringency time-intensity profile. Significant changes were observed over the pH range investigated (pH 2.6 – 6.8). Acidic drinks were higher in astringency and sourness compared to the drink at neutral pH. Astringency decreased from pH 3.4 to pH 2.6. Saliva and drink mixtures showed that
aggregation and precipitation were taking place, and the degree of precipitation correlated with perceived astringency. Electrostatic interactions between positively charged whey proteins at low pH and saliva proteins with low isoelectric points are thought to be responsible for aggregation and precipitation, resulting in the perception of astringency.
FACTORS REGULATING ASTRINGENCY OF WHEY PROTEIN-FORTIFIED BEVERAGES

by

JASON W. BEECHER

A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Master of Science

FOOD SCIENCE

Raleigh, North Carolina

2006

APPROVED BY:

Dr. MaryAnne Drake

Dr. Clyde Sorenson

Dr. E. Allen Foegeding
Chair of Advisory Committee
Dedication

This work is dedicated to my beautiful wife, Christine, and our daughters, Corrina and Stella. You are my life. May this be an example of what can be accomplished through hard work, determination, and perseverance. Chris, this would not have been possible without your enduring love, confidence, and support. Thank you.
Biography

Jason Weld Beecher was born May 30, 1971, in Cambridge, MA, to Jacqueline and Russell Beecher. Jason has three younger siblings, one sister and two brothers. Jason earned an AOS degree in Culinary Arts from Johnson & Wales University in 1993. He then earned a BS degree in Hospitality Administration from Boston University in 1996. After working in the restaurant industry for some time, Jason decided to return to school to pursue a Masters degree in Food Science at NC State University. Jason and his wife Christine are the proud parents of two beautiful girls, Corrina and Stella.
Acknowledgements

• Mom and Dad, thank you for your love and support throughout the years. I would not be where I am today without it.

• Dr. Allen Foegeding, my experience in your lab was invaluable. Thank you so much for all of your guidance and support, both as a leader and a friend.

• Dr. MaryAnne Drake, you are an inspiration and a good friend. Thank you for all of your help and kindness.

• Dr. Clyde Sorenson, thank you for serving on my committee and for sharing your Food Safety expertise with me. Your knowledge and passion for the subject make your lectures second to none.

• Thank you to the sensory panelist who dedicated so much of their valuable time to help make this project a success: Allen Foegeding, Jeab, Andrea Krause, Michelle Lloyd, Paige Luck, Paula Schneider, Laurie Steed, and Xin Yang.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>List of Tables</th>
<th>vi</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Literature Review</td>
<td>2</td>
</tr>
<tr>
<td>Astringency Defined</td>
<td>2</td>
</tr>
<tr>
<td>Mechanisms</td>
<td>3</td>
</tr>
<tr>
<td>Salivary Flow Rate</td>
<td>6</td>
</tr>
<tr>
<td>Instrumental Evaluation and Prediction of Astringency</td>
<td>8</td>
</tr>
<tr>
<td>Sensory Evaluation of Astringency</td>
<td>10</td>
</tr>
<tr>
<td>Whey and Whey Proteins</td>
<td>14</td>
</tr>
<tr>
<td>Whey Protein-Fortified Beverages</td>
<td>16</td>
</tr>
<tr>
<td>Controlling Astringency</td>
<td>18</td>
</tr>
<tr>
<td>Beverage Formulation</td>
<td>20</td>
</tr>
<tr>
<td>Market Survey</td>
<td>23</td>
</tr>
<tr>
<td>Model Beverage</td>
<td>23</td>
</tr>
<tr>
<td>Research Hypothesis</td>
<td>24</td>
</tr>
<tr>
<td>Research Objectives</td>
<td>24</td>
</tr>
<tr>
<td>References</td>
<td>26</td>
</tr>
<tr>
<td>CHAPTER 2: Manuscript</td>
<td>37</td>
</tr>
<tr>
<td>Factors Regulating Astringency of Whey Protein-Fortified Beverages</td>
<td>38</td>
</tr>
<tr>
<td>Abstract</td>
<td>39</td>
</tr>
<tr>
<td>Introduction</td>
<td>40</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>44</td>
</tr>
<tr>
<td>Results</td>
<td>52</td>
</tr>
<tr>
<td>Discussion</td>
<td>54</td>
</tr>
<tr>
<td>Conclusions</td>
<td>57</td>
</tr>
<tr>
<td>References</td>
<td>59</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>85</td>
</tr>
<tr>
<td>Appendix 1. Sweet, sour, and bitter panel ballot.</td>
<td>85</td>
</tr>
<tr>
<td>Appendix 2. Astringency panel ballot.</td>
<td>86</td>
</tr>
<tr>
<td>Appendix 3. Reference solutions used in sensory panels</td>
<td>87</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1. Market survey of whey protein-fortified beverages . . . . . . . 67
Table 2. Salivary flow rates of panelists . . . . . . . . . . . . 68
Table 3. Sweet, sour, and bitter intensity results for experiment 1 . . . . 68
Table 4. Astringency parameter results for experiment 1 . . . . . . . 68
Table 5. Sweet, sour, and bitter intensity results for experiment 2 . . . . 69
Table 6. Astringency parameter results for experiment 2 . . . . . . . 69
Table 7. Pellet results of saliva – drink mixtures after centrifugation . . . . 69
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>Viscosities of selected acidic whey protein isolate fortified beverages</td>
<td>70</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Saliva flow rates</td>
<td>71</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Viscosities of treatments used in experiment 1</td>
<td>72</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>Sweet, sour, and bitter intensity results for experiment 1</td>
<td>73</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>Astringency time-intensity results for experiment 1</td>
<td>74</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>Maximum astringency versus viscosities for experiment 1</td>
<td>75</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>Sweet, sour, and bitter intensity results for experiment 2</td>
<td>76</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>Astringency time-intensity results for experiment 2</td>
<td>77</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>Maximum astringency of treatments used in experiment 2.</td>
<td>78</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>Sweet, sour, bitter, and astringency intensity results for experiment 2</td>
<td>79</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>Optical density of saliva and drink mixtures</td>
<td>80</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>Optical density of saliva – drink mixtures versus max astringency</td>
<td>81</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>SDS-PAGE of saliva – drink mixtures before and after centrifugation</td>
<td>82</td>
</tr>
<tr>
<td>Figure 14.</td>
<td>Comparison of alum and whey protein TI astringency curves</td>
<td>83</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

Whey proteins and amino acid supplements have a strong position in the sports nutrition market due to the high quality protein they provide (Ha and Zemel 2003). Increasing consumer awareness of the potential health benefits associated with whey protein will continue to expand whey protein use in functional foods. High-protein sports drinks make up a considerable portion of the growing functional foods market. AC Nielsen (2004) reported that the sports/energy drink category grew 10% in 2003-2004 and was one of the top ten fastest growing food and beverage categories worldwide. They identified three key growth drivers, two of which were a continued focus on health and the need for convenience. An increase in worldwide demand for whey protein resulted in a 31% increase in total U.S. exports from 2004 to 2005, bringing the total to 275,540 metric tons (USDEC 2005).

There is a flavor concern, however, due to the astringency in whey protein-fortified drinks, which is most significant at higher protein concentrations and at low pH (Sano et al. 2005). Since flavor is an important factor affecting consumer acceptance of foods and beverages, astringency is likely to limit widespread consumer acceptance.

Astringency has been most extensively studied in red wine and is fairly well understood in this regard. Very little work has investigated protein astringency. With the demand for protein-fortified foods and beverages increasing steadily, controlling astringency in order to improve overall flavor is important. The goal of this research was to investigate factors regulating astringency in whey protein-fortified model beverages.
LITERATURE REVIEW

Astringency Defined

The physiological origin of astringency dates back to Aristotle (384-322 B.C.) and is still being investigated today (Green 1993). In the medical context, astringency is defined as “an agent producing contraction of organic tissues or the arrest of a discharge” (Bate-Smith 1954). Astringency has been investigated in wine (Cliff et al. 2002; Cliff and Dever 1996; Fukui et al. 2002; Gawel et al. 2001; Guinard et al. 1986; Ishikawa and Noble 1995; Kallithraka et al. 1996; Llaudy et al. 2004; Mateus et al. 2004; Pickering et al. 2004; Simon et al. 2003; Vidal et al. 2004a; Vidal et al. 2004b; Vidal et al. 2004c), tea (Scharbert et al. 2004; Yau and Huang 2000), cranberry juice (Peleg and Noble 1999), soy beans (Courregelongue et al. 1999; Kim and Lee 2003), persimmons (Harima et al. 2003; Oshida et al. 1996; Taira et al. 1997; Taira et al. 1998; Yamada and Sato 2002; Yamada et al. 2002), cocoa (Bonvehi and Coll 1997; Misnawi et al. 2004), and whey proteins (Sano et al. 2005).

Astringency in foods is generally attributed to compounds that have the ability to bind with and precipitate proteins. Lee and Lawless (1991) describe astringency as a complex group of sensations involving dryness, roughness of oral surfaces and tightening, drawing or puckering of the mucosa and muscles around the mouth. Smith and Noble (1998) define astringency as the friction developed as the tongue is moved laterally across the inside of the lower lip when exposed to astringent stimuli. Bate-Smith (1954) described “puckeriness” as that which separates astringency from the classical sense of taste. Although tactile sensations (Green 1993), such as astringency, differ from true tastes and smells, astringency is an important component of overall flavor (Lawless and Heymann 1998). Astringency is an accepted characteristic in certain foods, while it is undesirable in others or when perceived at
high levels (Courregelongue et al. 1999; Harima et al. 2003; Owuor and McDowell 1994; Yamada et al. 2002).

**Mechanisms**

Astringent sensations are brought about by the trigeminal nerves in the nose and the mouth via interactions between saliva and compounds in foods (Lawless and Heymann 1998). These interactions can be partially attributed to the unusual chemical composition of human saliva with an unusually high concentration of proline (Bennick 1982).

Amino acid analysis of unfractionated human saliva has shown the proline portion of total amino acids in saliva to be unusually high, ranging between 16 and 33%, compared to 2–3% proline for most proteins (Bennick 1982). For this reason, the salivary proteins are commonly referred to as proline-rich-proteins (PRPs) (Dodds et al. 2005; Jobstl et al. 2004; Simon et al. 2003). The PRPs are further divided into three groups: acidic, basic, and glycosylated (Bennick 1982). Astringency has been attributed primarily to hydrogen bonding, hydrophobic, and ionic interactions between the salivary PRPs and other compounds that have a particularly high affinity for the PRPs (Guinard et al. 1986). These other compounds are described in the literature and include acids (Lawless et al. 1996; Sowalsky and Noble 1998), ionic compounds (Peleg et al. 1998; Yang 2005), charged polysaccharides (Rodriguez et al. 2003), polyphenols (Bennick 2002; Lesschaeve and Noble 2005; Luck et al. 1994), and whey proteins (Sano et al. 2005).

Bananas, persimmons, apples, pears, peaches, grapes, coffee, wine, tea, beer, and soybean paste are only some of the foods and beverages that contain polyphenols (vegetable tannins) (Bate-Smith 1954; Kim and Lee 2003; Murray et al. 1994). Approximately 8000
phenolic structures have been identified, characterized by at least one aromatic ring with one or more hydroxyl groups (Papadopoulou and Frazier 2004). These are divided up into 15 major classes according to their chemical structure (Bennick 2002; Papadopoulou and Frazier 2004). The flavonoid polyphenols have recently been found to have beneficial antioxidant, anticarcinogenic, and antimutagenic properties (Papadopoulou and Frazier 2004), but tannins are still thought to have harmful effects, including sequestration of dietary iron and inhibition of digestive enzymes (Charlton et al. 2002). Salivary PRPs seem to prevent bioavailability of harmful polyphenols by binding to them, and Charlton et al. (2002) suggest that the astringent sensation is a signal of this interaction.

Polyphenol-PRP associations causing astringency have been extensively studied, and a mechanism is generally accepted (Bate-Smith 1954; Jobstl et al. 2004). Polyphenols are thought to reduce saliva lubrication by binding with and precipitating PRPs, which results in friction in the oral cavity, perceived as astringency (Jobstl et al. 2004; Scharbert et al. 2004). Hydrogen bonding of polyphenol hydroxyl groups to PRPs are assumed to be responsible for aggregation and precipitation, thereby delubricating the oral cavity (Guinard et al. 1986; Jobstl et al. 2004; Lawless et al. 1996; Murray et al. 1994).

Due to the cyclic nature and high concentration of proline in the salivary proteins, the PRPs have a relatively flat, open structure (Guinard et al. 1986; Luck et al. 1994), allowing for a combination of hydrogen bonding and hydrophobic interactions with other flat hydrophobic structures, such as tannic acid, to take place (Lawless et al. 1996).

The aggregation and precipitation attributed to hydrogen bonding and hydrophobic interactions between PRPs and polyphenols have been reported to cause turbidity in beer (Asano et al. 1982; Siebert et al. 1996) and wine (Yokotsuka et al. 1983). Siebert and Chassy
(2003) showed saliva and tannic acid mixtures to increase in turbidity as the concentration of tannic acid was increased, indicating aggregation.

Charlton et al. (2002) described the three steps of polyphenol-peptide binding: “Initially, added polyphenol binds to the peptide, and in general several polyphenol molecules can bind to the same peptide. This continues as more polyphenol is added, until the second stage is reached, by which point enough polyphenol is bound to the peptide to act as a linker between two peptide molecules. The peptide then forms a polyphenol-coated dimer, which starts to precipitate. As it precipitates, more molecules are added, and in the third and final stage, the complex aggregates into either a small particle or a large particle.”

Lawless et al. (1996) suggested that acidic conditions may be responsible for astringency due to PRP denaturation and conformational changes which are possible even in the presence of a buffer (Dawes 1964), leading to a decrease in the natural lubricating ability of saliva.

In an effort to explain ionic interactions causing astringency, Smith and Noble (1998) looked to the leather industry. Collagen is a major leather-making protein with a high proportion of proline amino acids (Li et al. 2003). In leather tanning, aluminum ions are believed to form complexes with the collagen carboxyl groups (Bieànkiewicz 1983). Smith and Noble (1998) suggested that this may result in aggregation and precipitation of polyvalent cations with salivary PRPs. More recently, De Wijk and Prinz (2005) noted that flocculation of the salivary PRPs or other dead cells in the oral cavity by alum could cause the perception of astringency.

Rodriguez et al. (2003) investigated the relationship between astringency and chitosan-saliva solutions at different pH values. Chitosan [(1-4)-2-amino-deoxy-β-d-glucan]
is the deacetylated form of chitin, an abundant natural biopolymer, and is soluble in acidic solutions (Laplante et al. 2005). Chitosan exhibits astringency when dissolved in an acidic solution (Rodriguez et al. 2003). Rodriguez et al. (2003) hypothesized that the mechanisms involved in protein-chitosan interactions were similar to those of protein-tannin interactions (hydrophobic, electrostatic, and/or hydrogen bonding). Chitosan is positively charged at low pH due to protonated amine groups, which they suggest could be responsible for the PRP-chitosan binding. Rodriguez found that astringent intensity increased with a decrease in pH (pH 6.7 – 3.9) and that an increase in astringency correlated with an increase in turbidity of saliva – chitosan mixtures. The authors suggest molecular interaction between the positively charged chitosan and the salivary PRPs to be responsible for this increase in turbidity.

Using a model substrate coated with pig gastric mucin, Malone et al. (2003) showed a strong binding affinity of chitosan at pH 3.5 (net positive charge) to mucin (net negative charge). The isoelectric point of salivary mucin is 2.75 – 2.95 (Inouye 1930). Exley (1998) showed aluminum to bind with bovine salivary mucin above pH 3.0. Peleg et al. (1998) showed a decrease in astringency of alum with a drop in pH. If salivary mucin’s negative charge decreases as pH drops, the binding affinity of alum with the salivary mucin will decrease with a drop in pH. If binding of alum to salivary mucin is related to or causes astringency, this would explain why the astringency decreases as the pH is lowered.

**Salivary Flow Rate**

Ingestion of food stimulates the flow of saliva, which in turn lubricates the oral cavity and ingested material (food) (Bonnans and Noble 1995). Saliva acts as a buffering system and affects the way we perceive certain flavors (Fischer et al. 1994; Guinard et al. 1997).
Since saliva is part of the chemoreception process, differences in salivary flow rate have been reported to cause variation in individual differences in taste perception (Bonnans and Noble 1995; Fischer et al. 1994). Acids (responsible for sour taste) and astringent stimuli are most effective in stimulating salivary flow rate (Ericson 1971; Froehlich et al. 1987). Molecules, such as polyphenols, that have a high affinity for PRPs in the saliva will influence viscosity, lubrication, friction, and the tactile perception of astringency (Green 1993).

Since astringency may be related to friction in the mouth caused by precipitation of PRPs (De Wijk and Prinz 2005; Peleg and Noble 1999; Prinz and Lucas 2000; Smith et al. 1996b), it has been hypothesized that an increase in salivary flow rate might increase lubrication or dilute the astringent-causing compounds significantly enough, so that individuals with higher salivary flow rates may show lower levels of astringent response (Lee and Lawless 1991). This has been reported with mixed results. Fischer et al. (1994) showed that “high flow” subjects scored bitterness and astringency lower than “low flow” subjects in the same study. Smith et al. (1996b) investigated bitterness and astringency of grape seed tannins and found no significant influence of salivary flow rate on perception of either one. Bonnans and Noble (1995) found no significant correlation between salivary flow rate and sourness, sweetness, or fruity flavor; however, the authors acknowledge that the study was done with relatively few subjects and could yield different results with a larger population. Differences in saliva flow rates may also result in altered perception of time-intensity parameters including time to maximum intensity and total duration.

Prinz and Lucas (2000) showed that adding tannic acid to human saliva significantly increased friction while reducing viscosity and the lubricating qualities of the saliva. Recently, DeWijk and Prinz (2005) showed that only certain substances perceived as
astringent actually decreased salivary viscosity, while others increase salivary viscosity. They also found salivary friction to be unrelated to salivary viscosity.

The lubricating properties of solutions of different viscosities have been evaluated for their ability to quench the astringent sensation by rinsing after-tasting astringent samples. They have been shown to have an effect on the time-intensity perception of astringency. Green (1993) showed water to be less effective in relieving astringency than more viscous substances. Breslin et al. (1993) used lubricating rinses such as water, saliva, sucrose, oil, and xanthan gum as a rinse for removing astringency after sampling astringent aluminum sulfate (alum) solutions and concluded that the more viscous lubricants had a greater effect on rapidly reducing astringency.

In summary, salivary flow rate is variable and does not appear to be clearly correlated with individual perception of astringency.

Instrumental Evaluation and Prediction of Astringency

Researchers have proposed models for predicting astringency with limited success. The vast majority of the work done has focused on astringency of polyphenols. Differences in salivary flow rate and salivary protein content between individuals along with the wide array of compounds that elicit astringent responses make this particularly complex.

Cliff et al. (2002) investigated the relationship between sensory-determined astringency and compositional analysis of red wines using multiple linear regression. A model was used to predict astringency based upon total phenolic content and did so to a reasonable degree.
Kaneda et al. (2001) used a quartz crystal microbalance (QCM) in two model systems to investigate a) polyphenol-protein interactions between each other and b) polyphenol-protein interactions with the tongue. A lipid-coated QCM was used to mimic the tongue. Not only were there significant interactions between astringent polyphenols and proteins, but also there were significant interactions between the polyphenol-protein complexes and the lipid-coated membrane. These results support the ideas that the aggregation between tannins and peptides result in astringency and that the oral membranes are important to the sensation of astringency.

The gelatin index is a method that approximates astringency in red wines by evaluating the extent of tannin precipitation with gelatin. Llaudy et al. (2004) proposed a method for predicting astringency of red wine using ovalbumin instead of gelatin. Ovalbumin is one of several proteins used for fining wine. Ovalbumin binds and precipitates excess proteins and tannins in wine via electrostatic interactions and hydrogen bonding. These aggregates settle out, resulting in a wine that exhibits desired clarity. Ovalbumin was added to tannic acid solutions (0.2, 0.4, 0.6, and 0.8 g/l). After precipitation and centrifugation, the change in turbidity of the supernatant was greatest for the higher concentrations of tannic acid and least for the most dilute. A greater change in turbidity as equal amounts of ovalbumin were added to the tannic acid solutions correlated with higher levels of astringency as determined by sensory evaluation.

Although these studies are useful to a certain extent, human sensory data provides the best models for how consumers are likely to perceive and react to food products (Lawless and Heymann 1998).
Sensory Evaluation of Astringency

Stone and Sidel (1993) define sensory evaluation as a scientific method used to evoke, measure, analyze, and interpret those responses to products as perceived through the senses of sight, smell, touch, taste, and hearing. Sensory evaluation is the most common method of evaluating astringency in foods. Measuring human response to astringency is difficult for at least two reasons: 1) Many people confuse astringency with bitterness or sourness, since phenolic compounds can have bitter attributes and astringent acids are also sour (Corrigan Thomas and Lawless 1995; Lawless et al. 1994; Lee and Lawless 1991), and 2) astringency is characterized as having a build-up of intensity (Guinard et al. 1986).

In order to evaluate astringency successfully, panelists should be trained and provided with definitions and references for astringency, sour, and bitter flavor profiles (Lee and Lawless 1991). In order to address the time-intensity behavior, different scaling and sampling techniques and statistical methods have been developed to minimize these effects (Lee and Lawless 1991). Time-intensity training and evaluation of astringency has been done in a variety of ways.

Lee and Lawless (1991) used alum (0.5 g/l and 1.0 g/l) for low and high astringent sensations respectively. Scoring was done on a 9-point hedonic scale used to scale astringent, puckering, drying, and roughing. A warm-up session was conducted to familiarize participants with the scales and procedures, to provide verbal definitions of attributes, and to answer questions. Participants swirled samples in the mouth for 15 seconds, expectorated, and rated every 30 seconds for 6 minutes. They tasted lower concentrations first, followed by higher concentrations, taking a 15-minute break between low and high concentrations. They consumed distilled water and crackers between samples.
Lawless et al. (1994) evaluated two-component mixtures of astringent compounds over time. A 15-point scale was used and marked at specific time intervals. Alum (0.5 g/l) was used as a reference in the study. Panelists swirled 20 ml of the sample for 15 seconds, then expectorated, and immediately began rating 7 attributes simultaneously. Ratings were made every 15 seconds for 1 minute and every 30 seconds for 4 additional minutes. Two or three solutions were tested each session and a 15-minute break was taken between samples. Panelists were instructed to eat a cracker and rinse at least twice with spring water between samples.

Corrigan Thomas and Lawless (1995) used alum (1.0 g/l) as an astringent reference in evaluating acid astringency. Three samples were tested on a given day. Panelists took the entire 20 ml sample in their mouth and swished. They expectorated at 15 seconds and immediately began to rate astringency. A 15-point category scale was used to rate astringency at expectoration, at 30 seconds, and then every 30 seconds for 3½ minutes. A 5-minute break was required between samples, and panelists ate a plain cracker and rinsed with spring water.

Lee and Lawless (1996) used alum (1 g/l) as an astringent reference. Fifteen-point category scales anchored with “none” and “strong” in one experiment, and “not astringent” and “extremely astringent” in a second experiment, were used. Subjects took samples into their mouths for 15 seconds, expectorated, and rated nine stimuli,—including astringency, drying, roughing, and puckering—at 30, 60, and 120 seconds following expectoration. Four to five stimuli were rated in each session with a total of four sessions.

Smith et al. (1996b) used alum (0.1 g/l and 1.0 g/l) for low and high reference standards for astringency of grape seed tannins. Judges were instructed to take the entire
sample in their mouth, expectorate at 10 seconds, and rate astringency until the sensation dissipated completely. Panelists rinsed 3 times with deionized water and waited 30 seconds between samples. Five solutions were presented each on 2 separate days.

Smith and Noble (1998) used alum at 0.5 g/l and 1.0 g/l for low and high astringent anchors presented to judges prior to evaluating the additional solutions. Training consisted of a discussion of astringency and 3 training sessions on the computerized time-intensity system. Eight alum solutions were rated in 1 session. One replication was performed in a second session. A 1000-point scale using a time-intensity computer program was used. Judges sipped 15 ml of sample, expectorated at 10 seconds, and astringency was rated constantly until the sensation dissipated completely. Judges rinsed 3 times with water and waited 30 seconds between samples.

Sowalsky and Noble (1998) evaluated astringency and sourness of organic acids. Alum (2.8 g/l) was used as high anchor, and deionized water was used as low anchor. Panelists rated maximum intensity of astringency after expectoration. Panelists rinsed 3 times with deionized water between samples. Samples were presented using Williams Latin Squares, balanced for carry-over effects (Schlich 1993). Two sessions were performed for formal evaluations.

Horne et al. (2002) evaluated astringency of acids. Three samples were evaluated per session. A forced 5-minute rest was required between samples, during which time panelists rinsed with water and ate a cracker. Tasting involved taking the entire sample in the mouth, swishing for 15 seconds, and expectorating. Compusense was used to evaluate time intensity on a vertical 10-point line scale.
Valentova et al. (2002) used unstructured line scales 100 mm long with descriptors “imperceptible astringency” and “very strong astringency” at 0 and 100 mm respectively. Panelists who had experience with time-intensity of bitterness were trained on time-intensity of astringency in 4 introductory sessions. Four samples were served at a time, with 2 minutes between samples after astringency had dissipated completely.

Rodriquez et al. (2003) evaluated astringency of chitosan at varying pH, using an ordinal scale (0 to 5). Six samples were evaluated by untrained panelists. Panelists sipped 10 ml of sample, swirled for 10 seconds, and then rated astringency immediately after expectoration. Four minute breaks were taken between samples, at which time panelists ate a cracker and sipped deionized water.

Drobna et al. (2004) used alum as a reference for evaluating astringency in tea. Time-intensity evaluations were performed using Compusense. A 20 ml sample was tasted, swished for 5 seconds, and then swallowed. Time-intensity evaluation began immediately after swallowing. Data was collected every 5 seconds from 5 to 160 seconds after swallowing. Panelists waited 90 seconds between samples, at which time they sipped soda water and ate a cracker. Three replications were performed.

Yang (2005) rated maximum intensity of astringency on a 15-point scale, anchored “none” to “very strong.” Testing was conducted with Compusense. Five samples were evaluated per session, and 3 replications were performed. Panelists rinsed with deionized water and 0.05 M sucrose during a 2-minute forced break between samples. Crackers were also provided.

Since build-up of astringency can be problematic in these studies, researchers have looked for ways to reduce carry-over effects of astringency. Colonna et al. (2004) evaluated
different rinses for their effect on reducing or preventing build-up of astringency of red wine. Carboxymethylcellulose (CMC) (1.0 g/l) was shown to be effective at minimizing carry-over of astringency. Brannan et al. (2001) showed 5.5 g/l CMC to be very effective at minimizing carry-over effects. In an informal evaluation with astringent whey protein-fortified acidic beverages, CMC (5.5 g/l) was also shown to be extremely effective at minimizing carry-over effects of astringency.

**Whey and Whey Proteins**

Whey is the liquid fraction of milk that remains following removal of fat and casein during the manufacture of cheese or acid and rennet casein (Sienkiewicz and Riedel 1990). The liquid whey contains approximately 20% of the original milk protein (McIntosh et al. 1998). The term “whey proteins” applies to the milk proteins that remain soluble in milk at pH 4.6 and 20ºC (Farrell et al. 2004). Historically, whey was considered a waste-product and disposed of or processed into relatively low-value commodities such as whey powder (McIntosh et al. 1998). Today whey powder is further processed, dried or condensed, and made into a highly nutritious powder composed of protein, lactose, lipid, ash, and moisture (Morr and Foegeding 1990; Sienkiewicz and Riedel 1990). Whey protein concentrate (25-80% protein) and whey protein isolate (≥90% protein) are two forms of whey protein powders commonly used in the food industry today (Sienkiewicz and Riedel 1990).

Whey proteins contribute many functional properties to food formulations and are used as ingredients in several foods. Whey protein functionality contributes to the stability of dispersed systems, such as gels or foams (Sienkiewicz and Riedel 1990), and to the ability to bind and emulsify fat, to bind and entrap water, and to entrap and stabilize air (Phillips and
Williams 2000). These functional properties also contribute to the textural, rheological and sensory properties of the foods. Whey proteins are commonly used as the main component in infant formulae, weight-gain and weight reduction diet foods, protein-fortified fruits juices, and other nutritious foods and drinks (Phillips and Williams 2000).

The primary proteins in whey, in order of abundance, are β-Lactoglobulin (β-LG), α-Lactalbumin (α-LA), bovine serum albumin (BSA), immunoglobins (Ig), proteose-peptones, and lactoferrin (LF) (Farrell et al. 2004; Sienkiewicz and Riedel 1990). The functional properties of whey protein concentrates can, for the most part, be attributed to the properties of β-LG (Sienkiewicz and Riedel 1990). β-LG is well characterized with respect to molecular and physiochemical properties (Phillips et al. 1994). Its three-dimensional structure has been determined using X-ray crystallography and high resolution nuclear magnetic resonance (NMR) spectroscopy (Creamer et al. 2002). β-LG, the most abundant globular protein in milk (~50-60% of total whey protein), consisting of 162 amino acid residues (Farrell et al. 2004; Phillips et al. 1994). At least eleven genetic variants have been identified: A, B, C, D, E, F G (Phillips et al. 1994), H, I, J, and W (Papiz et al. 1986), with A and B being the most prevalent (Farrell et al. 2004). The variants possess the same number of amino acids, but they differ in amino acid residue positions. The calculated molecular weight for the β-LG B monomer is 18,277 Da. Papiz et al. (1986) determined the two disulfide bonds in the native protein to be Cys$_{66}$ - Cys$_{160}$ and Cys$_{106}$ - Cys$_{119}$, with one free thiol group, Cys$_{121}$. β-LG normally exists as a dimer at 25°C between pH 4.0 and 6.5, while dissociation of the dimer occurs below pH 3.5 and above pH 7.5 (Phillips et al. 1994). Although β-LG is one of the few globular proteins that remains soluble at its isoelectric point (pI~5.1), it is more likely to
become insoluble when it dissociates into monomers since non-polar groups are increasingly exposed as the protein unfolds (Phillips et al. 1994).

Alpha-Lactalbumin is a globular protein consisting of 123 amino acid residues (Demarest et al. 1999). Three variants have been reported: A, B, and C, with A and B being most prevalent, each having four disulfide bonds (Farrell et al. 2004). The B variant has a molecular weight of 14,178 Da (Farrell et al. 2004).

Hirayama et al. (1990) identified BSA (~8% of total whey protein) to have 583 amino acid residues with 17 disulfide bonds and Mw=66,399 Da. The immunoglobins represent ~6% of total whey protein. Lactoferrin has a calculated Mw~76,110 Da with 17 disulfide bonds and represents a very small fraction of total whey protein.

**Whey Protein-Fortified Beverages**

Products, such as sports drinks, that use whey protein as a protein source contain between 20 and 50 grams of protein per 20 ounce serving. In high protein beverages, this equates to 3 - 7%+ protein. Protein drinks can be further classified by pH. The neutral pH beverages are shake-style and are available in chocolate, orange cream, vanilla, and other “creamy” flavors. The low pH (acidic) drinks are relatively clear and are available in grape, lemon-lime, fruit punch, and other fruity flavors. In order for the drink to retain its clarity, the whey proteins must remain soluble. Proteins are least soluble at their isoelectric point (pI), the point at which the protein-protein interactions increase, because the electrostatic forces are at a minimum (Pelegrine and Gasparetto 2005). Generally, whey proteins are more soluble at pH values below or above their pI due to electrostatic repulsion (Pelegrine and Gasparetto 2005). Holsinger et al (1973) showed whey proteins to be completely soluble
between pH 2.0 and 3.5, resulting in a clear whey protein-fortified beverage. Low pH not only allows for clarity in the beverages, but also has important processing implications (pH < 4.6).

There are four categories of shelf-stable ready-to-drink beverages grouped by thermal process: 1) Aseptically processed commercially sterile beverages, 2) retort processed commercially sterile beverages, 3) tunnel pasteurized, and 4) hot-filled or pasteurized cold-filled beverages (Rittmanic 2006). Aseptic processing involves sterilizing the container prior to filling, then filling with a commercially sterile beverage, and sealing the container. Retorting involves filling a container with the beverage, sealing the container, and heating the entire container to achieve commercial sterility. Tunnel pasteurization involves passing the filled bottled beverages on a conveyor through a hot water spray. Drinks that are heated and bottled while hot are referred to as “hot fill.” Drinks that are heated and cooled immediately before filling are “cold-filled.” The thermal processing method one utilizes depends on the particular objectives of the processor, along with process and packaging costs associated with each.

The mechanism of astringency in protein-fortified beverages is not known. Proteins are polyelectrolytes (polymers containing charged groups: negative, positive, or both) (Walstra 2003). Whey proteins are positively charged at pH values below their pl due to positively charged nitrogen-containing groups and protonation of the carboxylic groups. A reasonable explanation for the astringency mechanism is that electrostatic interactions between whey proteins at low pH (net positive charge) and negatively charged salivary proteins (with low pl) aggregate and precipitate, causing astringency similar to that of polyphenol-PRP interactions.
Controlling Astringency

Several factors that appear to influence astringency in model and complex systems are consistently reported in the literature. The predominant variables include pH (Lawless et al. 1996; Peleg and Noble 1999; Rodriguez et al. 2003; Shahidi et al. 1999), viscosity (Cook et al. 2003; Courregelongue et al. 1999; Herceg and Lelas 2005; Peleg and Noble 1999; Smith et al. 1996a; Smith et al. 1996b; Smith and Noble 1998), and degree of polymerization (Lesschaeve and Noble 2005; Vivas and Glories 1996).

Bourne (2002) argues that texture pertains to rheological properties of solid foods, while viscosity, broadly defined as the tendency of a fluid to resist flow, pertains to the texture of fluid foods. Quality and flavor perception of solid or semisolid foods clearly depends to a high degree on the texture of the food. Several molecular phenomena influence viscosity, including particle shape, colloidal interactive forces between particles, swelling of particles, and particle size (Walstra 2003). Viscosity has been shown to alter flavor perception in both model and complex systems (Cook et al. 2003; Courregelongue et al. 1999; Herceg and Lelas 2005; Peleg and Noble 1999; Smith et al. 1996a; Smith et al. 1996b; Smith and Noble 1998).

Ishikawa and Noble (1995) showed that an increase in sucrose concentration in red wine decreased perceived astringency. Lyman and Green (1990) showed that astringency was suppressed more effectively by sucrose than with aspartame. In both of these cases, a reduction in astringency could be partially attributed to the increased sweetness; however, since the viscosity was also increased, it is possible that viscosity was at least partially responsible for suppressing the astringency. Peleg and Noble (1999) demonstrated that as cranberry juice became more viscous due to lower temperature, astringency decreased. This
is probably due to suppressed trigeminal sensations associated with colder temperatures rather than viscosity alone, but viscosity must be considered. Yanes et al. (2002) used κ-carrageenan and sodium alginate to increase the viscosity of a model dairy drink and reported a decrease in flavor as viscosity was increased. They also showed differences for different hydrocolloids, which might mean that molecular interactions could have caused these differences.

Baines and Morris (1987) concluded that sweetness and strawberry flavor in guar gum-thickened solutions were most significantly suppressed at concentrations above coil-overlap concentration (c*). Also referred to as the specific critical concentration (Morris et al. 1981), this is the point at which an abrupt increase in solution viscosity is seen as thickener concentration is increased.

Smith and Noble (1998) used trained judges to evaluate time to maximum intensity and maximum intensity of astringency at constant pH using 0.5 and 1.0 g/l alum thickened with methylcellulose (1 - 29.4 mPa s). Although time to maximum intensity was not significantly affected by viscosity changes, they observed a significant decrease in maximum intensity of astringency as viscosity was increased, particularly in the low viscosity range between 1 and 10 mPa s. The higher concentration of alum (1.0 g/l) showed greater astringency than the low concentration (0.5 g/l) intensity for all viscosities.

Vivas and Glories (1996) showed a greater degree of polymerization of wine tannins to increase the sensation of astringency. Lesschaeve and Noble (2005) showed astringency to increase and bitterness to decrease with degree of polymerization. Polymerization would normally result in an increase in viscosity, but conformational changes due to polymerization will also introduce changes in molecular interactions and affect astringency perception.
Viscosities, particle size, degree of polymerization, temperature, and changes in pH have all been reported to influence astringency. These variables are not independent of one another. A change in temperature or particle size will affect viscosity, and so on. Solvent quality, ionic strength, and protein modification are also important considerations. Physical, chemical, enzymatic, and genetic modification have all been shown to change protein functionality (Phillips et al. 1994).

**Beverage Formulation**

Dickinson (2003) defines food hydrocolloids as high-molecular-weight hydrophilic biopolymers used as functional ingredients in the food industry for the control of microstructure, texture (viscosity), flavor, and shelf-life. These include polysaccharides that are extracted from plants, seaweeds and microbial sources, and gums derived from plant exudates, and modified biopolymers made by chemical or enzymatic treatment of starch or cellulose (Dickinson 2003). Hydrocolloids typically found in dairy products are carrageenan, locust bean gum, carboxymethylcellulose (CMC), pectin, alginate, and modified food starch (Syrbe et al. 1998). These are not all appropriate for beverage use. Hydrocolloids most suitable for use in beverages are those highly soluble in water, with low viscosity in solution (Buffo et al. 2001). Due to whey proteins’ net positive charge at low pH (<5), anionic hydrocolloids such as CMC or xanthan gum would be problematic due to electrostatic attraction. For this reason, a neutral polysaccharide, such as dextran, (Akhtar and Dickinson 2003; Zhang and Foegeding 2003), or a complex charged polysaccharide, such as gum acacia (gum arabica), may minimize protein-hydrocolloid interactions. Dextran is a neutral polysaccharide (~500 kDa) produced by bacteria, consisting primarily of α-1,6-glucosidic
linkages (Akhtar and Dickinson 2003; Zhang and Foegeding 2003). Gum acacia is a high molecular weight (~580 kDa) polysaccharide extracted from *Acacia* trees and shrubs that grow primarily in sub-Saharan Africa (Buffo *et al.* 2002). Hydrophobic affinity chromatography has been used to identify three principal fractions of gum acacia: a low-molecular weight arabinogalactan, a very high-molecular weight arabinogalactan-protein complex, and a low-molecular-weight glycoprotein which represent 88%, 10%, and 1% respectively (Nussinovitch 1997).

Lawless and Heymann (1998) define flavor as a complex group of sensations compromising olfactory, taste, and other chemical sensations such as irritation or chemical heat. McGorrin *et al.* (1996) provide four common characteristics of food flavors: 1) they consist of many components, some present in high proportions; 2) they exert their influence at extremely low levels; 3) they are highly specific with respect to molecular configuration; and 4) they tend to be volatile. Natural and artificial flavors contain compounds that contribute to the overall flavor of a food. Natural flavor systems can be especially complex and difficult to control. For example, there are over 250 compounds responsible for the characteristic flavor associated with “chocolate” (McGorrin *et al.* 1996). More than 350 chemical compounds are responsible for strawberry flavor (Bessiere and Thomas 1990). However, a relatively small number of compounds that dominate the sensory profile can be isolated and/or synthesized and used to create a simple and more reliable flavor system (McGorrin *et al.* 1996). Proteins and other molecules can interact with flavor compounds in foods, influencing flavor perception (Heng *et al.* 2004). Sweeteners can alter beverage flavor by physical interaction with the flavor compounds or by enhancing volatile flavor (Nahon *et al.* 1996). In order to minimize interactions and variability in a model system, it is important
to use a flavor with relatively few compounds with less potential for molecular interaction. (Morr and Foegeding 1990). Smith et al. (1996b) used aspartame (N-L-D-aspartyl-L-phenylalanine-1-methyl ester) instead of sucrose to increase sweetness intensity without affecting viscosity. In the same study, carboxymethylcellulose (CMC) was used to increase viscosity without increasing sweetness. Another benefit of using an artificial sweetener is the lower calorie content of the final product. A drawback of using an artificial sweetener in the final product is the negative perception artificial sweeteners have among many health-conscious consumers. Phosphoric acid has been shown to have less effect on denaturation of whey protein at lower pH than citric acid in soft drink fortification (Holsinger et al. 1973) and is likely a good choice to lower pH in this study. Furthermore, citric acid is an astringent organic acid (Lawless et al. 1994).
MARKET SURVEY

A market survey was conducted in order to develop a model beverage to be used for the study. Viscosity, pH, and protein type and concentration, were documented for 18 randomly selected whey protein-fortified beverages. Of the 18 drinks, 11 were acidic (median pH=3.4) and 7 were neutral (pH>6.4). Of the acidic drinks, protein content ranged between 4.2 and 7%, with an average of 6%. Viscosities at 22°C ranged between 2 and 7 mPa s in the shear rate range of 10 s\(^{-1}\) to 100 s\(^{-1}\), exhibiting mostly Newtonian behavior. The majority of the drinks were acidified with phosphoric acid, and sweetened with various artificial sweeteners.

MODEL BEVERAGE

A model drink was formulated with 6% w/v protein (WPI), pH 3.4, adjusted with phosphoric acid, and the sweetness was adjusted with sucralose. Phosphoric acid was chosen because it is less astringent than organic acids, and sucralose was chosen because it does not interfere with viscosity.

After experimenting with several possible thickening agents, maltodextrin (M100) and prehydrated methylcellulose were chosen for increasing viscosity due to their pH and temperature stability and bland flavor. In addition, the rheological behaviors of the model drinks thickened with maltodextrin and methylcellulose are Newtonian, which is more practical for experimental comparison.
RESEARCH HYPOTHESIS

We hypothesize that electrostatic interactions are responsible for the perceived astringency in whey protein fortified acidic beverages. Interactions between the whey proteins and salivary proteins and the oral cavity may be affected by changes in viscosity, enough to reduce the perception significantly. This could be due to the effect of restoring lubrication in the oral cavity, or by slowing aggregation between the whey proteins and salivary PRPs due to changes in the diffusion rate. Small increases in viscosity may result in reduced levels of astringency, as shown with polyphenols and alum (Smith et al. 1996a; Smith et al. 1996b; Smith and Noble 1998). Changes in pH will also have an effect on astringency due to changes in net charge and the electrostatic interactions between whey proteins and salivary PRPs.

RESEARCH OBJECTIVES

The mechanism responsible for astringency of whey proteins is not known. Whey protein fortified sports drinks are relatively new and gaining in popularity, but acceptance will be limited by taste. Very little research has addressed the astringency problem associated with these drinks. Our objective is to investigate some of the factors regulating astringency with a long term goal of improving overall flavor and consumer acceptance. A market survey will help us design a model beverage to be used in the study. The effects of viscosity and pH on sweet, sour, and bitter tastes and the time-intensity behavior of astringency of whey protein-fortified acidic beverages will be evaluated by trained sensory panelists. Additionally, saliva-drink interactions will be investigated.
This study will lay the groundwork for further mechanistic studies on astringency of whey proteins within our lab. Having the ability to control astringency will allow manufacturers to develop better tasting drinks fortified with whey proteins for them to compete more effectively in the global beverage market.
REFERENCES


CHAPTER 2: MANUSCRIPT
FACTORS REGULATING ASTRINGENCY
OF WHEY PROTEIN-FORTIFIED BEVERAGES

Jason W. Beecher, MaryAnne Drake and E. Allen Foegeding*
Department of Food Science, North Carolina State University
Raleigh, NC 27695, USA

*Correspondence to be sent to:
E. Allen Foegeding, Ph.D.
Department of Food Science
North Carolina State University
Box 7624
236 Schaub
Raleigh, NC 27695-7624
USA

e-mail: allen_foegeding@ncsu.edu
Phone: +1 (919) 513-2244
FAX: +1 (919) 515-7124
ABSTRACT

A rapidly growing area of whey protein use in foods and beverages is the sports drink category. There are two types of whey protein-fortified drinks; those at neutral pH and those at low pH. Astringency is very pronounced at low pH. Astringency is an undesirable characteristic thought to be caused by compounds in foods that bind with, and precipitate salivary proteins. The mechanism of astringency of whey proteins is not understood and has not been investigated. Salivary flow rate, viscosity, and pH have been reported to influence astringency of red wine, tannic acid, alum, and chitosan solutions, and cranberry juice.

Trained sensory panelists evaluated the viscosity and pH effects on astringency of whey protein-fortified model drinks (n=8). Changes in optical density of saliva and drink mixtures before and after centrifugation were investigated in an attempt to correlate aggregation and precipitation with astringency. Increasing viscosity (1.6 mPa s – 7.7 mPa s) did not reduce astringency, however there were significant differences across the pH range investigated (pH 2.6 – 6.8). Acidic drinks were higher in astringency and sourness compared to the drink at neutral pH. Saliva and drink mixtures showed that aggregation and precipitation was taking place, and the degree of precipitation correlated with perceived astringency. Electrostatic interactions between positively charged whey proteins at low pH and saliva proteins with low isoelectric points are thought to be responsible for aggregation and precipitation, resulting in the perception of astringency.

Key words: Astringency, Saliva, Time-Intensity Analysis, Whey Protein
INTRODUCTION

An increase in worldwide demand for whey protein resulted in a 31% increase in total U.S. exports from 2004 to 2005, bringing the total to 275,540 metric tons (USDEC 2005). High-protein sports drinks make up a considerable portion of the growing functional foods market. AC Nielsen (2004) reported that the sports/energy drink category grew 10% in 2003 and was one of the top ten fastest growing food and beverage categories worldwide. Whey protein-fortified beverages are designed to be attractive to a variety of markets because of the high quality proteins they provide.

Style of beverages fortified with whey proteins can be controlled by pH. Beverages produced at near neutral (pH ~ 6.8) are milkshake style and are available in such flavors as chocolate, orange cream, and vanilla. Acidic drinks (pH < 3.5) are relatively clear and are available in fruity flavors such as grape, lemon-lime and fruit punch. Whey proteins must remain dispersed and soluble in order for the drinks to retain their clarity. Whey protein ingredients tend to be least soluble at their isoelectric point (pH ~ 5.1) (Phillips et al. 1994), and solubility increases as pH is decreased (Hidalgo and Gamper 1977; Lupano 1994; Pelegrine and Gasparetto 2005). Low pH not only allows for clarity in the beverages, but also has important processing and storage implications.

There is a concern related to flavor attributed to a high level of astringency in drinks containing whey proteins at higher protein concentrations (3 – 7%+) and at low pH (Sano et al. 2005). Since flavor is an important factor affecting consumer acceptance of foods and beverages, high levels of astringency will limit acceptance. Astringency has been extensively studied in red wine (Cliff and Dever 1996; Colonna et al. 2004; Gawel et al. 2001; Guinard et al. 1986; Ishikawa and Noble 1995; Mateus et al. 2004; Pickering et al. 2004; Vidal et al.
2004a; Vidal et al. 2004b; Vivas and Glorres 1996; Yokotsuka et al. 1983). However, very little work has investigated protein astringency. With the demand for protein-fortified foods and beverages increasing steadily, controlling astringency in order to improve overall flavor is important.

Astringency is associated with many foods and beverages and has been investigated in wine (Cliff et al. 2002; Cliff and Dever 1996; Fukui et al. 2002; Gawel et al. 2001; Guinard et al. 1986; Ishikawa and Noble 1995; Kallithraka et al. 1996; Llaudy et al. 2004; Mateus et al. 2004; Pickering et al. 2004; Simon et al. 2003; Vidal et al. 2004a; Vidal et al. 2004b; Vidal et al. 2004c), tea (Scharbert et al. 2004; Yau and Huang 2000), cranberry juice (Peleg and Noble 1999), soy beans (Courregelongue et al. 1999; Kim and Lee 2003), persimmons (Harima et al. 2003; Oshida et al. 1996; Taira et al. 1997; Taira et al. 1998; Yamada and Sato 2002; Yamada et al. 2002), cocoa (Bonvehi and Coll 1997; Misnawi et al. 2004), and whey proteins (Sano et al. 2005). It is generally attributed to specific compounds, such as polyphenols (Bennick 2002; Lesschaeve and Noble 2005; Luck et al. 1994), and organic acids (Corrigan Thomas and Lawless 1995; Lawless et al. 1996; Siebert and Chassy 2003) that have the ability to bind with and precipitate proline rich salivary proteins (PRPs). A popular model for the perception of astringency describes the complexation and precipitation of the astringent compounds with salivary PRPs, which increase friction in the mouth, perceived as astringency (Jobstl et al. 2004; Scharbert et al. 2004). Lee and Lawless (1991) describe astringency as a complex group of sensations involving dryness, roughness of oral surfaces, and tightening, drawing or puckering of the mucosa and muscles around the mouth. Although astringency is considered a tactile sensation (Green 1993) rather than a basic taste, it is clearly a critical component of overall flavor (Lawless and Heymann 1998).
Chitosan exhibits astringency when dissolved in an acidic solution when it is positively charged due to protonated amine groups (Rodriguez et al. 2003). Rodriguez et al. (2003) found that astringency increased with a decrease in pH (pH 6.7 – 3.9) and hypothesized that the mechanisms involved in salivary protein-chitosan interactions were similar to those of saliva-tannin interactions. The researchers also observed an increase in turbidity when chitosan was combined with saliva, suggesting molecular interactions between the positively charged chitosan and salivary PRPs.

There are several studies that have suggested a decrease in astringency to be the result of an increase in viscosity. Lyman and Green (1990) reported that astringency was lowered by adding sucrose—as opposed to aspartame-- to red wine and suggested that the increase in viscosity due to the addition of sucrose may be the reason for this. Smith et al. (1996) thickened grape seed tannin solutions with carboxymethylcellulose (CMC) in order to evaluate the viscosity effects on astringency. They reported significant decreases in maximum astringency in the low viscosity range (~1 – 8 mPa s). Smith and Noble (1998) also showed a significant decrease in astringency in a low viscosity range (~1 – 8 mPa s) of alum thickened with methylcellulose. Peleg and Noble (1999) suggested that astringency of cranberry juice decreased as viscosity increased at lower temperatures. They also pointed out that the trigeminal nerves are suppressed at lower temperatures, which may also result in a reduction in astringency.

Sensory evaluation is the most common method for evaluating astringency in foods. Measuring human response to astringency is difficult. Many people confuse astringency with bitterness or sourness since phenolic compounds can have bitter attributes and astringent acids are also sour (Corrigan Thomas and Lawless 1995; Lawless et al. 1994; Lee and
Lawless 1991). Furthermore, astringency is characterized as having a repeated stimulation pattern of partial recovery and a build-up of intensity (Guinard et al. 1986).

In order to evaluate astringency successfully, panelists must be trained to evaluate sour and bitter tastes, and astringency, and must be provided with definitions and references (Lee and Lawless 1991). Different sampling techniques and statistical methods have been developed to address the time-intensity behavior and to minimize carry-over effects (Lee and Lawless 1991; Schlich 1993).

Like chitosan and alum, whey proteins, carry a net positive charge at low pH. We hypothesize that electrostatic interactions between the whey proteins and the salivary proteins, and the subsequent aggregation and precipitation leads to the astringency sensation. Small increases in viscosity may slow these interactions or increase lubrication sufficiently, resulting in significant decreases in astringency.

The mechanism of whey protein astringency is not known, and very little is understood about what factors regulate the astringency of proteins. Time-intensity evaluations of astringency of whey protein-fortified beverages have not been reported in the literature. The objectives of this study were to investigate the effects of viscosity (experiment 1) and the effects of pH (experiment 2) on the time-intensity development of astringency in whey protein-fortified beverages.
MATERIALS AND METHODS

Procedure

A market survey was conducted in order to develop a model beverage to be used for the study (Table 1). Viscosity, pH, and protein type and concentration, were documented for 18 randomly selected whey protein-fortified beverages. Of the 18 drinks, 11 were acidic (median pH = 3.4) and 7 were neutral (pH > 6.4). Of the acidic drinks, protein content ranged between 4.2 and 7%, with a mean of 6%. Viscosities at 22°C ranged between 2 and 7 mPa s in the shear rate range of 10 s\(^{-1}\) to 100 s\(^{-1}\), exhibiting mostly Newtonian behavior (Figure 1). One drink was shear thinning, due to a higher concentration of protein (7.1%). The most viscous drink contained maltodextrin. The majority of the drinks were acidified with phosphoric acid, and sweetened with various artificial sweeteners.

Trained sensory panelists evaluated the effect of viscosity on the time-intensity (TI) profile of astringency in a whey protein isolate (WPI) fortified model beverage. \((n=8)\) in experiment 1. The panelists evaluated effects of pH, and a modified protein treatment, on sweet, sour, and bitter tastes, and the time-intensity of astringency in experiment 2. Saliva and model drink interactions were also investigated.

Subjects

Eight healthy subjects (6 female and 2 male) were selected for the study based upon their interest and availability. All subjects were either students or faculty in the NC State Department of Food Science. The same eight subjects participated in experiments 1 and 2.
**Experiment 1**

*Sample preparation*

Six treatments were prepared for evaluation. Samples were prepared using whey protein isolate (BiPro, Davisco Foods, Eden Prairie, MN; 91.97% protein) at 6% w/v protein. Maltodextrin at 0%, 10%, 15%, and 20% w/v (National Starch Food Innovation, Bridgewater, NJ), and prehydrated methylcellulose at 0.27% w/v (TIC Gums, Belcamp, MD) were used as thickeners. Powders were hydrated in water in beakers by mixing for approximately 2 hr at approximately 70% of the final volume. Drinks were adjusted to pH 3.4 using 2N H₃PO₄ (Mallinckrodt, Hazelwood, MO), and final volume was adjusted with deionized water. Sweetness was controlled by adjusting with 20% w/w sucralose (McNeil Nutritionals, LLC, Fort Washington, PA) solution to approximate the sweetness equivalent of 10% sucrose. Drinks (300 ml) were poured into 500 ml media jars (Fisher Scientific, Pittsburgh, PA) and immersed in a 90°C water bath (PolyScience Microprocessor Controlled Water Bath, Niles, IL) for 15 min with a dual action shaker set at 30 rpm. Drinks were brought to 4°C in under 30 min by immersing the jars in crushed ice. Samples were then refrigerated prior to pouring. A no-protein control was also prepared. Sucrose (purchased at a local grocery store), citric acid (Tate & Lyle, PLC, London, UK), and caffeine (Sigma-Aldrich, St. Louis, MO) were used for sweet, sour, and bitter reference solutions respectively. Alum (McCormick, Hunt Valley, MD) was used as an astringency reference.
Sensory training

Sensory analysis was conducted in compliance with the NC State University IRB human subject regulations. Panelists were trained to evaluate sweet, sour, bitter, and astringency using the Spectrum® intensity scale (Meilgaard et al. 1999b). Basic tastes (sweet, sour, and bitter) training and evaluation, and astringency training and evaluation were kept separate throughout the study. Panelists were trained 10 hr to evaluate sweet, sour, and bitter tastes, and 20 hr to evaluate astringency. Training lasted for 5 months.

Sweet, sour, bitter

On the first day of training, subjects were familiarized with sweet, sour, and bitter tastes by tasting a 5% sucrose (sweet 5), 0.08% citric acid (sour 5), 0.05% caffeine (bitter 2). After the first day of training, between 7 and 10 samples of known intensities (0 to 15) were presented to panelists at each training session in 2 oz plastic cups randomly numbered with three-digit codes. Forty ml of the following reference solutions were provided to all panelists at all sessions: Sour 2 (0.05% citric acid), sour 5 (0.08% citric acid), sweet 8 (8% sucrose), sweet 12 (12% sucrose), and bitter 2 (0.05% caffeine) (Meilgaard et al. 1999a). Samples and references were stored at 4°C and tempered to room temperature for approximately 2 hr prior to evaluation.

During the first 12 training sessions, panelists indicated the maximum intensities for each sample using a 15 pt intensity scale labeled “none” at number 0 on the left, and “very strong” at number “15” on the right. Ballots were collected and monitored for panelist accuracy and consistency.
After 12 training sessions, panelists were trained to use Compusense 5 (version 5.0, Compusense, Inc., Guelph, Ontario, Canada). At this point, training continued in sensory booths using the sensory software. All three attributes were scored on a 15 pt category scale exactly as was done during training. When samples of known intensities were evaluated, feedback was provided to the panelists for training and calibration purposes. This feature is part of the Compusense software that indicates the “actual” intensity to the panelist after all attributes for a particular sample have been scored. Whey protein model drinks were added to samples being evaluated during the last 8 training sessions.

*Astringency*

On the first day of training, astringency was defined for the subjects as “the complex of sensations due to the shrinking, drawing, or puckering of the epithelium as a result of exposure to substances such as alums or tannins” (ASTM 1989). Alum solutions of astringent intensities 2 (0.02% alum) and 10 (0.1% alum) were provided to panelists as examples of low and high intensities of astringency. Tea (6 Lipton tea bags steeped in 1 liter deionized water for 30 min) was also provided as an example.

Between 3 and 4 samples of known intensities of alum were presented to panelists at each training session. Panelists were instructed to take the entire sample (20 ml +/- 0.1 ml) into their mouth and swish for 5 seconds before expectorating. After expectorating, maximum astringent intensity was noted on paper ballots using 15 pt category scales as described above. A rinse protocol was established for astringency evaluation in order to minimize carry-over effects. After maximum intensity was reached and recorded, panelists rinsed with carboxymethylcellulose (CMC, 5.5 g/l, Hercules-Aqualon, Wilmington, DE)
(Brannan et al. 2001), sipped deionized water, ate a piece of an unsalted cracker, and sipped water again. Panelists waited two minutes between samples. During training, alum references were provided.

After 22 training sessions, panelists switched to using the Compusense sensory program in booths. Panelists were introduced to the time-intensity module in Compusense, and 8 training sessions were conducted prior to the real evaluation. During this training, 3 unknown samples were evaluated by each panelist at each session. Panelists took the entire sample in their mouth while clicking the “start” button in the time-intensity module. They swished the sample in their mouth and expectorated at 5 seconds, indicated by the clock on the computer screen. Intensity values were recorded automatically at two-second intervals while panelists moved the mouse along a horizontal category scale numbered 0 to 15 for up to 3 minutes.

**Sweet, sour, bitter evaluation**

Formal evaluations took place in booths with samples at room temperature. A sweet and sour warm up (5% sucrose, 0.1% citric acid) was tasted first, followed by all 6 unknown samples. Sweet, sour, and bitter attributes were rated for all samples. Four replications were performed. Panelists were provided with unsalted crackers and deionized water to cleanse the palate between samples.

**Astringency evaluation**

Panelists rinsed with a warm up solution (0.6% alum) at the beginning of each session. The CMC rinse protocol and a forced 3-minute break were required between all samples. Rather
than provide references for panelists to taste during evaluation, an alum solution (0.08%, astringency intensity = 8) was evaluated first as a “dummy” sample. While sipping the entire sample, panelists clicked the “start” button in the time-intensity module. At 5 seconds, indicated by the clock on the screen, samples were expectorated. Intensity values were recorded automatically at two-second intervals, while panelists moved the mouse along a horizontal category scale numbered 0 to 15 for 3 minutes.

Three samples were evaluated by each panelist at each session, so that all 6 samples were evaluated by each panelist every two sessions. Orders of presentation were balanced for first order carry-over effects using Williams Latin Squares (Schlich 1993). Eight sessions (4 replications) were performed.

**Viscosity measurement**

Shear rate sweeps were performed in a StressTech Rheometer (Rheological Instruments AB, Lund, Sweden) with a smooth cup and bob to evaluate sample viscosities. All viscosities were measured at 20°C between shear rates 10 s\(^{-1}\) and 100 s\(^{-1}\). Samples were presheared at 50 s\(^{-1}\) for 60 s and allowed to equilibrate for 20 s prior to measurement. A delay time of 10 s and an integration time of 50 s were set to allow for accurate measurement of low viscosity fluids as recommended by ATS Rheosystems (Bordentown, NJ).

**Experiment 2**

**Sample preparation**

Six treatments were prepared for evaluation. Samples were prepared with 6% w/v protein (WPI), and sweetness was adjusted to an approximate sweetness equivalent of 10% w/v
sucrose with sucralose. Three samples were prepared by adjusting to a final pH of 2.6, 3.0, and 3.4 with 2N H$_3$PO$_4$. A neutral pH drink was prepared without the addition of acid (pH=6.8). A protein-modified drink (3.4R) was prepared using 6% w/v protein and 10% w/v ribose (Bioenergy, Inc., Minneapolis, MN), brought to 70% final volume, and heat treated in a water bath at 60°C for 30 min. (Sucralose was used at 50% compared to the other samples due to the sweetness contribution of ribose.) Acid (2N H$_3$PO$_4$) was added immediately after this initial heat treatment, and the drink was brought to room temperature on ice. Samples were adjusted to final pH (3.4) and volume prior to the final heat treatment. All drinks were heated to 85°C for 10 to 20 s and then brought to 4°C in less than 30 min by immersing in crushed ice and then refrigerated. A control with the same buffering capacity as the pH 3.4 whey protein drink was prepared using 10% v/v 2N H$_3$PO$_4$, adjusted to pH 3.4 with 2N NaOH. Sucralose was used to impart sweetness to the control.

*Sensory evaluation*

Formal evaluations were done exactly as described in experiment 1 with the following two exceptions: 1) Experiment 2 evaluations were performed under red light conditions to eliminate visible differences due to the ribose treatment, and 2) three replications were performed.

*Saliva flow rate*

Saliva flow rate for each panelist was collected. Flow rate was determined as described by Fenoll-Palomares et al. (2004), with slight modifications. Subjects were asked to refrain from eating or drinking anything but water for at least 2 h prior to saliva collection. Subjects were
instructed to swallow all saliva present in the mouth and then to collect all saliva produced over the course of 10 minutes by spitting into a pre-weighed 2 oz portion cup. This procedure was performed in triplicate, and flow rate (g/min) was calculated (Table 2, Figure 2).

**Saliva-drink interactions**

*Saliva Collection and Sample Preparation*

Approximately 25 ml of saliva was collected in less than 3 hr at room temperature. The saliva was centrifuged at 12,000 x g at 23°C for 20 minutes (Sorvall RC-5B Refrigerated Superspeed Centrifuge, DuPont Instruments, Wilmington, DE), and the supernatant was used immediately. Samples of protein drinks and saliva were combined in 1.5 ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) in a 1:1 ratio. The tubes were capped, inverted once, vortexed briefly, and allowed to equilibrate at room temperature for 15 min.

*Spectrophotometric measurement*

Optical density was measured after 15 min at 400 nm using a Shimadzu UV160U spectrophotometer (Shimadzu Co., Kyoto, Japan) prior to and after centrifugation in a Beckman Microfuge 11 (Palo Alto, CA) at 13,500 x g for 15 min.

**SDS-PAGE**

All of the electrophoresis supplies were purchased from Invitrogen Corporation (Carlsbad, CA). Samples of protein drinks and saliva (30 µl) were removed from the microcentrifuge tubes prior to and after centrifugation, diluted, and added to NuPAGE® LDS (4X) sample buffer and NuPAGE® sample reducing agent (10X). Samples were heated to 70°C for 10 min
in a water bath and run on 4-12% acrylamide Bis-Tris gels, with SeeBlue® Plus2 molecular weight marker, and 500 µl NuPAGE® antioxidant in an MES SDS buffer system. Pellets present after centrifugation were resolubilized in 200 µl of NuPAGE® LDS (4X) sample buffer and NuPAGE® sample reducing agent (10X) overnight under refrigeration. Gels were run at 190 V (constant voltage) for 40 min. Gels were agitated in fixing solution (40% deionized water, 50% methanol, and 10% acetic acid) for 10 min at room temperature. The fixing solution was then removed, and the gels were agitated in Colloidal Coomassie Blue reagent for 3 hr. After staining, the gels were rinsed and destained in deionized water overnight. Gels were soaked in gel-dry solution for 5 minutes prior to air drying at room temperature.

All statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC). Maximum sweet, sour, and bitter intensities were analyzed, and four parameters were extracted from the astringency TI curves: maximum intensity (IMAX), time to maximum (TMAX), duration (DUR), and area under the curve (AUC). Analysis of variance (ANOVA) was generated using PROC GLM, and comparisons of means were made using Tukey’s Studentized Range Honestly Significant Difference (HSD) test.

RESULTS

Figure 3 shows the viscosities of the treatments evaluated in experiment 1. The lowest viscosity treatments (< 4 mPa s) exhibited Newtonian behavior, while the others were very slightly shear thinning, but with very little change in viscosity at shear rates ≥ 40 mPa s. Sweet, sour, and bitter intensities were about the same for all treatments except the control (Table 3; Figure 4). Increasing viscosity from 1.6 mPa s to 7.7 mPa s did not result in
significant differences in any astringency parameters (Table 4; Figure 5). Figure 6 shows maximum astringency to be independent of the viscosity changes utilized in this study.

In experiment 2, sourness significantly increased as pH was lowered from 3.4 to 3.0 to 2.6 (Table 5, Figure 7). Maximum intensity of astringency decreased from 8.8 to 7.1 between pH 3.4 and pH 2.6 (Table 5, Figure 8, 9, 10). The neutral drink exhibited very low sourness (0.4) and astringency (1.2) compared to acidic drinks. The maximum astringency of the whey protein drink with ribose (3.4R) was lower than the drink at pH 3.4. The phosphate buffer control exhibited very low astringency, but mean sourness was the same as the drink at pH 3.0.

Saliva-drink mixtures were prepared in a 1:1 ratio as done by Gambuti et al. (2006) and optical density was recorded prior to and after centrifugation. Optical density was observed to be much greater in saliva-drink mixtures when compared to the controls (Figure 11). Furthermore, the optical density of the saliva-drink mixtures after centrifugation was down to an approximate level of the controls. The controls were virtually unchanged. Pellets were observed in the microcentrifuge tubes containing saliva and drink mixtures only (Table 7). The change in optical density was relatively small in the drink at neutral pH. Although the change in optical density after centrifugation correlated well with astringency (Figure 12), the ribose treated whey protein drink (3.4R) did not follow this trend. The change in optical density was greater in this treatment compared to the other drinks, yet the astringency was less.

In an effort to identify which whey proteins may be involved in the aggregation and precipitation, SDS-PAGE was performed on the supernatant of the saliva-drink mixtures before and after centrifugation, and pellets were resolubilized in buffer for comparative
purposes. SDS-PAGE results did not show any visible differences in proteins before and after centrifugation (Figure 13) or in the pellets.

**DISCUSSION**

In experiment 1, sweetness was controlled by adjusting the amount of added sucralose, depending on the amount of maltodextrin used to thicken the drink. Slight variations in sweet intensity were not surprising, particularly the increases observed in the control (no protein) and the most viscous sample (highest level of maltodextrin) (Table 3). In contrast with other studies that have shown viscosity (~1 – 8 mPa s) to decrease astringency of alum and tannic acid (Smith *et al.* 1996; Smith and Noble 1998), this was not observed in the present study (1.6 mPa s – 7.7 mPa s). Alum exhibited a notably different time-intensity profile when compared to the whey protein drinks (Figure 14). The time to maximum astringency for alum was approximately 10 s less than that of the whey protein drinks, and the astringency of alum dissipated much more quickly as well. These differences may be related to why increased viscosity decreases astringency of alum and not whey protein drinks. The proteins may also take longer to rinse off of the tongue. Electrostatic interactions between the positively charged aluminum ions and negatively charged salivary proteins may result in aggregation and precipitation (Exley 1998) perceived as astringency. This may also be the case with whey proteins, which carry a net positive charge at low pH. Furthermore, molecular size and shape of the aggregates influence chemical properties and astringency (De Wijk and Prinz 2006) and are important considerations when comparing alum to whey proteins.

In experiment 2, sourness increased as pH was lowered. Since an increase in sourness can cause a decrease in sweetness perception (Keast and Breslin 2002), the slight decrease in
sweetness observed between pH 3.0 and 2.6 may be attributed to this effect (Table 5, Figure 7).

Although the acidic drinks were much more astringent than the drink at neutral pH, a stepwise decrease in astringency was observed between pH 3.4 and 2.6 (Figure 9). The phosphate buffer control was not astringent, which shows that the whey proteins are involved in causing the astringent sensation.

At pH values above or below their isoelectric points, proteins carry net negative or net positive charges, respectively. As pH is raised or lowered away from the isoelectric points, these net negative or net positive charges increase. Guo et al. (2006) sequenced 5338 distinct peptides, representing 1381 distinct proteins, from human saliva. Approximately 3400 of the 5338 peptides were identified as having mean isoelectric points below 5.0. These proteins are likely to be involved in interactions with whey proteins in acidic beverages due to the general electrostatic charge attraction model. At pH values well above their isoelectric points, the proteins are highly negative, and are less likely to aggregate, and the same is true at very low pH. In the pH range of acidic whey protein fortified drinks (~pH 2 – 3.5), however, the electrostatic attraction between whey proteins and low pI salivary proteins is variable and depends on the charge differences between the proteins. Since the whey proteins carry a net positive charge at these low pH values, an increase in repulsion due to increasing positive charges on the salivary proteins as pH is lowered from 3.4 to 2.6 would result in a decrease in astringency perception, as was observed.

Nonenzymatic browning (the Maillard reaction) plays an important role in food processing, preparation, and storage, and involves a multi-step pathway of reactions between reducing sugars and amino acids (DeMan 1999). Reactive species, moisture, temperature,
and pH influence the rate of this reaction, which can introduce color and flavor changes (DeMan 1999). The Maillard reaction also results in the unavailability of lysine (DeMan 1999; McGorrin et al. 1996) due to its inability to protonate at lower pH. Native β-lactoglobulin has 15 lysines (Farrell et al. 2004), so reacting the whey proteins with ribose (reducing sugar) at neutral pH is likely to reduce the net positive charge, resulting in a decrease in perceived astringency, which was observed.

Sano et al. (2005) hypothesized that when whey proteins are combined with saliva, they approach their isoelectric point and precipitate, resulting in astringency. Our work suggests that electrostatic interactions between whey proteins and salivary proteins may be the cause of the astringent sensation. Aggregation and precipitation were investigated in saliva and drink mixtures. Comparing optical density before and after centrifugation showed that aggregation and precipitation occurred when saliva and the drinks were mixed (Figure 11), which correlated with increased astringency (Figure 12). Pellets were visible in saliva–drink mixtures, but not in the controls, showing that aggregation and precipitation is taking place (Table 7).

Other studies have shown an increase in turbidity to correlate with an increase in astringency of polyphenols (Horne et al. 2002; Yokotsuka et al. 1983), and chitosan (Rodriguez et al. 2003). The whey protein drink treated with ribose, however, showed the greatest change in optical density, with less astringency (Figure 12). This contradicts the model presented, relating aggregation to astringency. At the same time, however, the ribose treatment introduces chemical changes that cannot be accounted for in the current design. Comparing changes in optical density of ribose treatments at varying pH values would be a
better comparison, though it was not done in the current study. This would also be true for varying amounts of alum with saliva compared to astringency.

SDS-PAGE results indicate that the salivary proteins involved in the aggregation and precipitation are not evident as a major loss of one or more protein bands. No differences were observed between the supernatant before or after centrifugation or in the resolubilized pellets. It is possible that the amount of proteins required to cause the astringent sensation is very small, or that it is not detected in this electrophoretic method. Further work is needed to establish what proteins are involved in these interactions.

It seems logical that panelists with different salivary flow rates might perceive astringency differently. Dilution, relubrication, buffering, or rinsing effect of saliva might alter maximum intensity, time to maximum, or duration of astringency. Some researchers have found salivary flow rate to influence astringency (Fischer et al. 1993), while others have not (Smith et al. 1996). In this study, panelists’ salivary flow rates did not correlate with individual means for astringency in any of the following parameters (IMAX, TMAX, DUR, and AUC) for either experiment.

CONCLUSIONS
Changing viscosity is not a viable option for reducing astringency of whey protein-fortified beverages to an acceptable level. Salivary proteins and whey proteins aggregate and precipitate to varying degrees when combined, and the degree of aggregation and precipitation is correlated to the astringency. Astringency decreases as pH is lowered, due to the decrease in attractive charge between the whey proteins and saliva proteins. Controlling the net charge on whey proteins via modification with a reducing sugar such as ribose, or
altering the ionic environment may help reduce both sourness and astringency in acidic whey protein-fortified drinks.
REFERENCES


TABLES AND FIGURES
Table 1. Market survey of 11 acidic, and 7 neutral pH whey protein-fortified drinks.

### ACIDIC

<table>
<thead>
<tr>
<th>Beverage</th>
<th>Beverage Flavor</th>
<th>pH</th>
<th>Protein %</th>
<th>Protein Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tropical Fruit Punch</td>
<td>2.6</td>
<td>6.5%</td>
<td>WPI, WPC</td>
</tr>
<tr>
<td>2</td>
<td>Wild Berry</td>
<td>2.9</td>
<td>6.2%</td>
<td>IX WPI</td>
</tr>
<tr>
<td>3</td>
<td>Grape</td>
<td>3.1</td>
<td>6.6%</td>
<td>WPI</td>
</tr>
<tr>
<td>4</td>
<td>Citrus Splash</td>
<td>3.2</td>
<td>4.5%</td>
<td>WPI</td>
</tr>
<tr>
<td>5</td>
<td>Fruit Punch</td>
<td>3.3</td>
<td>7.1%</td>
<td>WPI</td>
</tr>
<tr>
<td>6</td>
<td>Fruit Punch</td>
<td>3.4</td>
<td>6.4%</td>
<td>IX WPI</td>
</tr>
<tr>
<td>7</td>
<td>Power Punch</td>
<td>3.4</td>
<td>6.4%</td>
<td>WPI and l-glutamine</td>
</tr>
<tr>
<td>8</td>
<td>Original</td>
<td>3.4</td>
<td>4.9%</td>
<td>WPI</td>
</tr>
<tr>
<td>9</td>
<td>Icy Orange</td>
<td>3.4</td>
<td>6.8%</td>
<td>WPI</td>
</tr>
<tr>
<td>10</td>
<td>Arctic grape</td>
<td>3.5</td>
<td>6.8%</td>
<td>WPI, l-glutamine, l-leucine, l-isoleucine, l-valine</td>
</tr>
<tr>
<td>11</td>
<td>Lemon Lime</td>
<td>3.7</td>
<td>4.2%</td>
<td>WPI</td>
</tr>
</tbody>
</table>

Mean 3.2 6.0%
Median 3.4 6.4%

### NEUTRAL

<table>
<thead>
<tr>
<th>Beverage</th>
<th>Beverage Flavor</th>
<th>pH</th>
<th>Protein %</th>
<th>Protein Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vanilla Shake</td>
<td>6.4</td>
<td>6.8%</td>
<td>Nonfat Dry Milk, Calcium Caseinate</td>
</tr>
<tr>
<td>2</td>
<td>Strawberry</td>
<td>6.4</td>
<td>6.8%</td>
<td>Nonfat Dry Milk, Calcium Caseinate</td>
</tr>
<tr>
<td>3</td>
<td>Ice Cream Vanilla</td>
<td>6.6</td>
<td>9.9%</td>
<td>Milk Protein Isolate, Calcium Caseinate</td>
</tr>
<tr>
<td>4</td>
<td>Vanilla Bean</td>
<td>6.7</td>
<td>8.5%</td>
<td>Milk Protein Isolate</td>
</tr>
<tr>
<td>5</td>
<td>Creamy Vanilla</td>
<td>6.8</td>
<td>9.9%</td>
<td>Milk Protein Isolate, Calcium Caseinate, WPC</td>
</tr>
<tr>
<td>6</td>
<td>French Vanilla</td>
<td>6.8</td>
<td>4.5%</td>
<td>Soy Protein Isolate</td>
</tr>
<tr>
<td>7</td>
<td>French Vanilla</td>
<td>6.9</td>
<td>6.1%</td>
<td>Soy Protein Isolate, WPI</td>
</tr>
</tbody>
</table>

Mean 6.7 7.5%
Median 6.7 6.8%

**ABBREVIATIONS:**
- WPI Whey protein isolate
- WPC Whey protein concentrate
- IX WPI Ion-exchange whey protein isolate
Table 2. Salivary flow rates of panelists, 3 replications. Significant differences ($\alpha=0.05$) are denoted by different letters.

<table>
<thead>
<tr>
<th>Panelist Id</th>
<th>Flow Rate, g/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5747 a,b</td>
</tr>
<tr>
<td>2</td>
<td>0.3050 c</td>
</tr>
<tr>
<td>3</td>
<td>0.6723 a,b</td>
</tr>
<tr>
<td>5</td>
<td>0.6270 a,b</td>
</tr>
<tr>
<td>6</td>
<td>0.7913 a</td>
</tr>
<tr>
<td>7</td>
<td>0.5310 a,b</td>
</tr>
<tr>
<td>8</td>
<td>0.4410 b,c</td>
</tr>
<tr>
<td>9</td>
<td>0.6153 a,b</td>
</tr>
</tbody>
</table>

Table 3. Experiment 1 mean maximum sweet, sour, and bitter intensity. Significant differences ($\alpha=0.05$) are denoted by different letters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Trt Name</th>
<th>Sweet</th>
<th>Sour</th>
<th>Bitter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>8.6 a,b</td>
<td>0.2 b</td>
<td>0.1 a</td>
</tr>
<tr>
<td>2</td>
<td>1.6 mPa s</td>
<td>7.9 b</td>
<td>2.9 a</td>
<td>0 b</td>
</tr>
<tr>
<td>3</td>
<td>3.3 mPa s</td>
<td>8.2 a,b</td>
<td>2.6 a</td>
<td>0 b</td>
</tr>
<tr>
<td>4</td>
<td>5 mPa s</td>
<td>8.5 a,b</td>
<td>2.6 a</td>
<td>0 b</td>
</tr>
<tr>
<td>5</td>
<td>4.9 mPa s</td>
<td>8.0 b</td>
<td>3.0 a</td>
<td>0 b</td>
</tr>
<tr>
<td>6</td>
<td>7.7 mPa s</td>
<td>8.9 a</td>
<td>2.5 a</td>
<td>0 b</td>
</tr>
</tbody>
</table>

Table 4. Experiment 1 mean astringency TI parameter results. IMAX adjusted from 100 pt scale to 15 pt scale. Significant differences ($\alpha=0.05$) are denoted by different letters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Trt Name</th>
<th>IMAX</th>
<th>TMAX, s</th>
<th>DUR, s</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0.5 b</td>
<td>10.4 b</td>
<td>13.4 b</td>
<td>49.2 b</td>
</tr>
<tr>
<td>2</td>
<td>1.6 mPa s</td>
<td>9.1 a</td>
<td>25.5 a</td>
<td>113.0 a</td>
<td>3,325.0 a</td>
</tr>
<tr>
<td>3</td>
<td>3.3 mPa s</td>
<td>8.7 a</td>
<td>27.9 a</td>
<td>119.8 a</td>
<td>3,376.1 a</td>
</tr>
<tr>
<td>4</td>
<td>5 mPa s</td>
<td>9.1 a</td>
<td>27.2 a</td>
<td>119.7 a</td>
<td>3,616.1 a</td>
</tr>
<tr>
<td>5</td>
<td>4.9 mPa s</td>
<td>8.3 a</td>
<td>26.3 a</td>
<td>110.3 a</td>
<td>3,016.1 a</td>
</tr>
<tr>
<td>6</td>
<td>7.7 mPa s</td>
<td>8.8 a</td>
<td>26.3 a</td>
<td>116.0 a</td>
<td>3,431.9 a</td>
</tr>
</tbody>
</table>

IMAX = maximum intensity, TMAX = time to max, DUR = duration, AUC = area under the curve.
Table 5. Experiment 2 mean maximum sweet, sour, and bitter intensity. Significant differences ($\alpha=0.05$) are denoted by different letters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Trt Name</th>
<th>Sweet</th>
<th>Sour</th>
<th>Bitter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pH 2.6</td>
<td>6.9 b,c</td>
<td>6.1 a</td>
<td>0 b</td>
</tr>
<tr>
<td>2</td>
<td>pH 3.0</td>
<td>8.2 a</td>
<td>3.9 b</td>
<td>0 b</td>
</tr>
<tr>
<td>3</td>
<td>pH 3.4</td>
<td>7.9 a,b</td>
<td>2.8 c</td>
<td>0 b</td>
</tr>
<tr>
<td>4</td>
<td>pH 6.8</td>
<td>8.2 a</td>
<td>0.4 d</td>
<td>0 b</td>
</tr>
<tr>
<td>5</td>
<td>3.4R</td>
<td>8.8 a</td>
<td>2.4 c</td>
<td>0.2 a</td>
</tr>
<tr>
<td>6</td>
<td>buffer</td>
<td>6.6 c</td>
<td>4.2 c</td>
<td>0.2 a</td>
</tr>
</tbody>
</table>

Table 6. Experiment 2 mean astringency TI parameter results. IMAX adjusted from 100 pt scale to 15 pt scale. Significant differences ($\alpha=0.05$) are denoted by different letters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Trt Name</th>
<th>IMAX</th>
<th>TMAX, s</th>
<th>DUR, s</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pH 2.6</td>
<td>7.1 b</td>
<td>24.3 a,b</td>
<td>91.2 a</td>
<td>2,348.8 a,b</td>
</tr>
<tr>
<td>2</td>
<td>pH 3.0</td>
<td>7.8 a,b</td>
<td>24.8 a,b</td>
<td>87.2 a</td>
<td>2,270.3 b</td>
</tr>
<tr>
<td>3</td>
<td>pH 3.4</td>
<td>8.8 a,b</td>
<td>26.2 a</td>
<td>97.1 a</td>
<td>2,956.3 a,b</td>
</tr>
<tr>
<td>4</td>
<td>pH 6.8</td>
<td>1.2 c</td>
<td>16.2 b,c</td>
<td>55.6 b,c</td>
<td>367.4 c</td>
</tr>
<tr>
<td>5</td>
<td>3.4R</td>
<td>6.9 b</td>
<td>25.4 a</td>
<td>78.4 a,b</td>
<td>1,947.7 b</td>
</tr>
<tr>
<td>6</td>
<td>buffer</td>
<td>1.0 c</td>
<td>12.0 c</td>
<td>43.3 c</td>
<td>210.7 c</td>
</tr>
</tbody>
</table>

IMAX = maximum intensity, TMAX = time to max, DUR = duration, AUC = area under the curve

Table 7. Presence of pellet (n = no, y = yes) in saliva – drink mixtures after centrifugation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>saliva control</td>
<td>n</td>
</tr>
<tr>
<td>pH 2.6 control</td>
<td>n</td>
</tr>
<tr>
<td>pH 3.0 control</td>
<td>n</td>
</tr>
<tr>
<td>pH 3.4 control</td>
<td>n</td>
</tr>
<tr>
<td>3.4R control</td>
<td>n</td>
</tr>
<tr>
<td>pH 6.8 control</td>
<td>n</td>
</tr>
<tr>
<td>buffer w/saliva</td>
<td>n</td>
</tr>
<tr>
<td>buffer control</td>
<td>n</td>
</tr>
<tr>
<td>alum control</td>
<td>n</td>
</tr>
<tr>
<td>pH 6.8 w/saliva</td>
<td>small</td>
</tr>
<tr>
<td>pH 2.6 w/saliva</td>
<td>y</td>
</tr>
<tr>
<td>pH 3.0 w/saliva</td>
<td>y</td>
</tr>
<tr>
<td>pH 3.4 w/saliva</td>
<td>y</td>
</tr>
<tr>
<td>3.4R w/saliva</td>
<td>y</td>
</tr>
<tr>
<td>alum w/saliva</td>
<td>y</td>
</tr>
</tbody>
</table>
Figure 1. Viscosities of 5 acidic WPI-fortified beverages at 22°C.
Figure 2. Saliva flow rate of panelists.
Figure 3. Experiment 1 treatment viscosities at 20°C.
Figure 4. Sweet, sour and bitter means.
Figure 5. Mean astringency TI profiles.
**Figure 6.** Maximum intensity of astringency of model drinks.
Figure 7. Sweet, sour and bitter means.
Figure 8. Mean astringency TI profiles.
Figure 9. Maximum astringency of treatments. Significant differences ($\alpha=0.05$) are denoted by different letters.
Figure 10. Maximum intensity of sweet, sour, bitter, and astringency for experiment 2.
Figure 11. Differences in optical density of saliva, protein drinks, and alum (0.8 g/l) before and after centrifugation.
Figure 12. Relationship between change in optical density before and after centrifugation, and maximum astringency.
Figure 13. SDS-PAGE of saliva-drink supernatant before and after centrifugation. Molecular weight marker (lanes 1 and 10), WPI (lane 2), saliva (lane 3), pH 3.4 before centrifugation (lane 4), pH 3.4 after centrifugation (lane 5), 3.4R before centrifugation (lane 6), 3.4R after centrifugation (lane 7), pH 6.8 before centrifugation (lane 8), pH 6.8 after centrifugation (lane 9).
Figure 14. Comparison of astringency TI curves for model whey protein beverage (pH 3.4), and 0.8 g/l alum.
APPENDICES
Appendix 1. Sweet, sour and bitter panel ballot.

Basic Tastes Panel Ballot

Note: Always score the maximum perceived intensity.

Sweet Taste Intensity


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

None Very Strong

Sour Taste Intensity


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

None Very Strong

Bitter Taste Intensity


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

None Very Strong
Appendix 2. Astringency panel ballot

Astringency Panel Ballot

Definitions:

The complex of sensations due to the shrinking, drawing, or puckering of the epithelium as a result of exposure to substances as alums or tannins (ASTM 1989).

Astringency is a tactile sensation, not a basic taste. Also described as a “feeling factor”.

Before tasting the first sample:
  1. Sip and swish with CMC, and rinse with water.

Tasting protocol:
  1. Take entire sample in mouth (~20 ml), and swirl.
  2. Expectorate at 5 seconds and immediately begin to note astringent intensity.
  3. Continue to note astringency until the sensation has dissipated completely.

Rinse protocol (between samples):
  1. Swish with CMC, expectorate, and rinse with water.
  2. Eat a cracker and sip some water.
  3. Time between samples should be at least 2 minutes to minimize carry-over effects.

Note: Always score the maximum perceived intensity of astringency even if it is delayed.

Astringency Intensity – “mouth drying” intensity

<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Very Strong</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix 3. Reference solutions used for sensory training and evaluation.

<table>
<thead>
<tr>
<th>Name</th>
<th>Stimuli</th>
<th>Concentration, w/v</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astringent 4</td>
<td>Alum</td>
<td>0.04%</td>
<td>4</td>
</tr>
<tr>
<td>Astringent 6</td>
<td>Alum</td>
<td>0.06%</td>
<td>6</td>
</tr>
<tr>
<td>Astringent 8</td>
<td>Alum</td>
<td>0.08%</td>
<td>8</td>
</tr>
<tr>
<td>Astringent 10</td>
<td>Alum</td>
<td>0.10%</td>
<td>10</td>
</tr>
<tr>
<td>Astringent 12</td>
<td>Alum</td>
<td>0.12%</td>
<td>12</td>
</tr>
<tr>
<td>Bitter 2</td>
<td>Caffeine</td>
<td>0.05%</td>
<td>2</td>
</tr>
<tr>
<td>Bitter 5</td>
<td>Caffeine</td>
<td>0.08%</td>
<td>5</td>
</tr>
<tr>
<td>Sweet 5</td>
<td>Sucrose</td>
<td>5%</td>
<td>5</td>
</tr>
<tr>
<td>Sweet 8</td>
<td>Sucrose</td>
<td>8%</td>
<td>8</td>
</tr>
<tr>
<td>Sweet 10</td>
<td>Sucrose</td>
<td>10%</td>
<td>10</td>
</tr>
<tr>
<td>Sweet 15</td>
<td>Sucrose</td>
<td>16%</td>
<td>15</td>
</tr>
<tr>
<td>Sour 2</td>
<td>Citric acid</td>
<td>0.05%</td>
<td>2</td>
</tr>
<tr>
<td>Sour 5</td>
<td>Citric acid</td>
<td>0.08%</td>
<td>5</td>
</tr>
<tr>
<td>Sweet 6, Sour 4</td>
<td>Sucrose</td>
<td>5.00%</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Citric Acid</td>
<td>0.10%</td>
<td>4</td>
</tr>
</tbody>
</table>


**developed from unpublished astringency scale used in work done by Neta, Edith Ramos Da Conceicao, developed by trained sensory panelists at NC State University

***adapted from Meilgaard et al. (1999)