
Kerri K. Martin

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Food Science and Technology

Susan E. Duncan, Chair
Andrea M. Dietrich
Sean F. O’Keefe

June 19, 2012
Blacksburg, VA

Keywords: metallic flavor, human salivary proteins, two-dimensional gel electrophoresis, lactoferrin, time-intensity
Metallic flavors are of concern for many industries including food, health, and water. Metallic off-flavor, induced by ferrous sulfate solution (10mg/L), and its remediation using pre- and post-rinse treatments of water (control) or metal chelators, were studied. Metal chelators included lactoferrin (1 µM), a natural metal-binding protein in milk and saliva, and EDTA (36 µM), a synthetic chelator. Time-intensity (TI) evaluation (n=6, 4 female; age 40-70) of lingering metallic flavor indicated that metallic flavor decreased with a post-rinse adjuvant treatment of lactoferrin as indicated by a reduced maximum intensity and area under the curve compared to a pre-rinse treatment; EDTA and water post-rinses were equally effective for three of the TI parameters.

Alterations in salivary components were studied in saliva collected (n=8; 5 female, age 40-70) after sipping a lactoferrin solution (1µM) followed with a ferrous sulfate sample (10 mg/ml) to stimulate metallic flavor, as compared to unstimulated whole saliva. Protein concentration, oral lipid oxidation as indicated by thiobarbituric acid reactive substances assay, and iron concentration were determined on individual saliva samples, with no significant differences found between treatments (p>0.05). Protein patterns were qualitatively characterized for each pre-rinse and metallic stimuli from four panelists by two-dimensional gel electrophoresis. A consistent pattern of regions containing major salivary components was observed. This research has shown that lactoferrin protein is a potential natural alternative to synthetic EDTA for reducing iron-induced metallic off-flavors. This study provides a foundation of method development to better understand
salivary protein interaction with metals and flavor perception.
Acknowledgements

First of all, I would especially like to thank Dr. Duncan for her guidance through graduate school and life! She was able to provide numerous suggestions for completing my projects and encouraged me to go above and beyond. She really knows how to work with people and juggle numerous projects at once. A special thanks to my committee members, Dr. Andrea Dietrich and Dr. Sean O’Keefe, who were willing to understand the heavy influence of biochemistry for my project and were fun and encouraging people to work with. Thank you to all of the organizations that funded myself and research projects: College of Agriculture and Life Sciences at Virginia Tech, the Virginia Tech Water for Health ICTAS Center of Excellence, and Fralin Life Sciences Institute.

I would like to thank everyone in the metallic flavor panel at Virginia Tech for continually providing me with samples and participating in several sensory data collection studies. They showed great patience and willingness to participate. I would also like to thank those at Wake Forest who were involved in collection of samples for the first protein gels that we ran. Without them, this project could not be completed!

I want to give credit and much thanks to Will Slade, a PhD student at Virginia Tech, and Kris Lee, an employee in the Core Lab at VBI at Virginia Tech. They were able to provide me with tons of assistance and advice. Especially Will, who was willing to help me get the most out of my data and being there as someone who understood the struggles I was experiencing.

A big thanks to the food science department faculty and staff: everyone has helped me in some way with finding materials, equipment, understanding statistics, or just having a friendly conversation. It was great to know that someone was always there to help or be more than willing to participate in a sensory study! A huge thanks to Tina Plotka who helped me with saliva collection, running SIMS, ordering materials, and more; she was definitely responsible for a lot of the success of completing my projects! Also, thanks to Dr. Henjian Wang for helping me with my statistical data.

Thanks to everyone in the Dean Lab, especially Dennis Dean and Valerie Cash. I wouldn’t be here now if Dennis had not invited me to work in his lab and encouraged me to return to graduate school. Valerie taught me all the basics of working a laboratory setting and always had great tips and advice. I really enjoyed all the friendly conversation with all of my lab mates from Brazil to China and more!

Special thanks to everyone across campus who let me use their laboratory space or helping me with my project: the Dean Lab for 2D gel equipment, Dr. Jianyong Li in Litton Reaves for imaging equipment, Civil & Environmental Engineering for MS-ICP prep along with Jeff Parks for performing MS-ICP, and the many employees in the core lab at VBI for letting me into the building all the time to run analysis on the software there.

Thanks to my awesome friends and family for pushing me to fulfill my goals in life and being patient with me! A special shout out to the girls at Elite Style Salon, that was where it all started and I also don’t know what I’ll do about my hair and nails! Thanks to all of my fellow graduate students for helping me with sensory studies, encouraging me, motivating me, and becoming some of the best friends for the rest of my life! You all were literally right there by my side! We made it!
# Table of Contents

ABSTRACT  .................................................................................................................. ii
Acknowledgements .................................................................................................... iv
Table of Contents ....................................................................................................... v
List of Figures .............................................................................................................. vi
List of Tables .............................................................................................................. vii

CHAPTER I. INTRODUCTION AND RESEARCH RATIONALE ............. 1

CHAPTER II. LITERATURE REVIEW ................................................. 5

CHAPTER III. ROLE OF HUMAN SALIVARY PROTEINS IN METALLIC FLAVOR PERCEPTION ................................................................................................. 34

CHAPTER IV. COMPARISON OF METAL CHELATORS, LACTOFERRIN AND EDTA, AS ADJUVANTS FOR REDUCING METALLIC FLAVOR ........ 68

CHAPTER V. IN SEARCH OF SALIVARY BIOMARKERS FOR METALLIC FLAVOR PERCEPTION IN CANCER PATIENTS AND HEALTHY HUMANS ......................................................................................................................... 95

APPENDIX A. SUPPLEMENTAL INFORMATION FOR CHAPTER III .... 115
APPENDIX B. SUPPLEMENTAL INFORMATION FOR CHAPTER IV .... 127
LIST OF FIGURES

CHAPTER III

Figure 1. 2DGE Pattern of Unstimulated Whole Human Saliva Proteins…… 66
Figure 2. Saliva Collection Sequence……………………………………………… 67

CHAPTER IV

Figure 1. Time-Intensity Panelist Procedure……………………………………… 93
Figure 2. Time-Intensity Curves of Mean Intensity Values of Iron-Induced
Metallic Flavor for All Panelists per Second for Each Treatment……………… 94

CHAPTER V

Figure 1. 2DGE of Pooled Unstimulated Whole Saliva Sample from Healthy
Human Subject…………………………………………………………………… 108
Figure 2. Visual Comparisons of Protein Spots from Gels Produced from All
Groups……………………………………………………………………………… 111
Figure 3. Close Comparison of Visually Noticeable Differences in Spots from
Gels Produced from High-Responding Cancer Patients (CCHR) and Healthy
Subjects Saliva After Metallic Flavor Induced……………………………….. 113
LIST OF TABLES

CHAPTER III

Table 1. Salivary protein concentration (ug/ul) (and change in protein) in whole unstimulated saliva (baseline), and in saliva collected after a lactoferrin (1 µM) protein rinse and compared to protein concentration after a metallic stimulus (ferrous sulfate, 10 mg/L) ................................................................. 63

Table 2. Oral lipid oxidation measured using the TBARS (Thiobarbituric Acid Reactive Substances Assay) method (and change in TBARS) of whole unstimulated saliva (baseline), and in saliva collected after a lactoferrin (1 µM) protein rinse and compared to protein concentration after a metallic stimulus (ferrous sulfate, 10 mg/ml) ............................................................................. 64

Table 3. Concentration of iron (mg Fe/L) of Saliva 1 (and change in iron) in whole unstimulated saliva (baseline), and in saliva collected after a lactoferrin (1 µM) protein rinse and compared to protein concentration after a metallic stimulus (ferrous sulfate 10 mg/ml) ............................................................................. 65

CHAPTER IV

Table 1. Time-Intensity Parameters for Iron-Induced (Ferrous Sulfate 99.2 µM, 10 mg/L) Metallic Flavor Perception as Affected by Pre-Rinse and Post-Rinse with 1.0 µM Lactoferrin, 36 µM EDTA, and Distilled Water ................................................................. 91

Table 2. Comparison of Metallic Flavor Perception Using Paired Comparison for Water (Control), Lactoferrin (1 µM), and EDTA (36
μM)........................................................................................................... 92

CHAPTER V

Table 1. Number of Differences in Protein Spots with 5-fold Differences

Found in Comparisons of Groups.............................................................. 109

Table 2. Normalized Quantities of Spot Intensities Generated in Software for

Protein Spots Shown in Figure 2................................................................. 110

Table 3. Normalized Quantities of Spot Intensities Generated in Software for

Protein Spots Shown in Figure 3................................................................. 112

Table 4. Demographic, Treatment, and TBARS Data for Each Panelist

Represented in Pooled Saliva Samples for Protein Gels Produced.............. 114
CHAPTER I. INTRODUCTION AND RESEARCH RATIONALE

Metallic off-flavors are caused by oxidation of metals in the oral cavity and are influenced by retronasal effects (Epke 2007; Lawless 2004; Lawless 2005, Omur-Ozbek et al. 2012). Undesirable metallic off-flavors are associated with products produced across several industries including foods, nutritionals, pharmaceuticals, personal care, and healthcare.

There are many challenges with foods and beverages due to metallic flavor defects caused by ingredients, processing, and more; consumers are not likely to purchase products that are not aesthetically pleasing due to unpleasant metallic flavors (Lawless 2004). Metallic flavors in water are caused by groundwater or infrastructure that contains dissolved copper or iron, providing unsatisfactory water, which is displeasing to consumers (Dietrich 2006). Numerous medications cause metallic flavor side effects that possibly lead to patients’ unwillingness to undergo a treatment or take a medicine (Bigeleisen 1999; Doty 2008). In a study by (Shinkai et al. 2006) described in a review by Doty et al. (2008), it was reported that 33% of individuals on prescriptions experienced altered taste perception and 57% experienced astringency. Plus, medical treatments, such as lidocaine injections, as described in a case report by Bigeleisen (1999) have been known to cause taste dysfunction. About two-thirds of cancer patients experience taste defects due to treatment as reviewed by Hong, Omur-Ozbek et al. (2009). Alterations in flavor perception were also shown to result in malnutrition or anxiety. In another review, Porter et al. (2010) reported that taste dysfunction was commonly experienced by head and neck cancer patients who received radiotherapy.
Typically, as people age, their sensitivity to metals decreases, but these associations may be affected during treatments or under certain circumstances as discussed above (Mirlohi et al. 2011). Further investigation is needed in this area to provide a better understanding of how metallic flavor is perceived in specific age groups, in patients undergoing cancer therapy, and other groups that experience metallic off-flavors.

The role of salivary proteins in metallic flavor perception is not described. There are many metalloproteins in human saliva, suggesting that the interaction of minerals from endogenous and exogenous sources with salivary proteins may be important in advancing the understanding of metallic flavor perception. Understanding the role of these proteins may lead to discovery of a biomarker protein involved in metallic flavor perception and provide knowledge of how to treat this side effect. Salivary lactoferrin is an iron-binding protein found at low concentrations throughout the human body, including saliva, and in the milk of many mammals (Rodrigues et al. 2009). This protein has potential to alter metallic flavor by binding iron found in the diet. Studies have also shown that lactoferrin has many other benefits such as antimicrobial activity, iron uptake, and cancer prevention (Levay and Viljoen 1995; Lonnerdal 2009; Rodrigues et al. 2009).

To understand how lactoferrin can be used to reduce metallic flavor perception, comparisons to ethylene diamine tetraacetic acid (EDTA), a metal chelator typically used to reduce iron overload in foods can be useful in determining if lactoferrin has similar or enhanced ability to reduce metallic flavor perception (Miret et al. 2008). Time-intensity sensory studies are useful in observing the lingering effects of metallic flavor and are useful for studies concerning metallic flavor reduction (Hong et al. 2010). Previous studies have indicated that rinses with lactoferrin or EDTA can be used to reduce metallic
flavor (Omur-Ozbek et al. 2012). Further studies can confirm whether a pre- or post-rinse is best for reducing metallic flavor.

Further insight into the saliva proteome and metallic flavor reduction will provide more information for treating, diagnosing, and monitoring disease and expanding the scope of knowledge provided by past and current research. Combining protein research with sensory evaluation studies will provide benefits towards development of a treatment to reduce or prevent metallic flavor, thus improving nutrition and quality of life for cancer patients and others who experience this off-flavor. The many benefits of lactoferrin may also support the overall health of patients and other humans. By gaining additional knowledge of metallic flavor through an interdisciplinary effort, the food, water, and pharmaceutical industries will be able to provide more desirable products.

**Goal**

Understanding the interactions of human salivary proteins with metals will provide knowledge for potential discovery of a protein biomarker involved in metallic flavor perception and for future development of a treatment for metallic off-flavors. This research will also aid in understanding of protein-metal interactions in food formulations, water filtration, and treatment. Research is also applicable in the dental industry.

Data for lactoferrin concentrations in saliva and in rinses used in a therapy for reducing or preventing metallic flavor is particularly useful for individuals undergoing cancer treatment and experience this unpleasant side-effect. Comparisons of proteins present in healthy saliva versus head and neck cancer patient saliva, using 2DGE, will assist in identifying unique protein patterns with differences in protein expression and contribute to knowledge of possible biomarker proteins for susceptibility to metallic
flavor. Changes in salivary protein patterns and protein concentration when exposed to iron-binding proteins (e.g., lactoferrin) and metals in water will provide indications of the role of metal-binding proteins in controlling metallic flavor and oxidation in the oral cavity.

Finally, sensory studies to determine if lactoferrin, a natural metalloprotein chelator, is comparable to EDTA for reducing metallic flavor, providing insight for potential replacement of this chemical chelator. This will expand current understanding of how flavors interact within the oral cavity. The medical, pharmaceutical, and food industries, as well as consumers can utilize this information to consider a natural ingredient as an antimicrobial agent with functionality for reducing metallic flavor, or possibly to aid in iron absorption.
CHAPTER II. LITERATURE REVIEW

Impacts of Metallic Flavor

The food industry faces many challenges from metallic off-flavors. Foods containing polyunsaturated fatty acids are particularly affected by lipid oxidation influenced by oxidized metals, which contributes to off-flavors (Medina et al. 2002; Nielsen et al. 2004). Fortifying foods with iron (ferrous sulfate) has excellent health benefits, but can also cause an undesirable metallic flavor (Hurrell 2002). Artificial sweeteners such as acesulfame-K, saccharin, and aspartame can cause metallic flavor or bitterness at certain concentrations (Riera et al. 2007). Studies to understand metallic flavor will help develop solutions to this food quality issue, thus providing better products to consumers; this includes understanding how metallic flavor is perceived and what compounds may be useful for reduction of metallic flavor.

Water chemistry also influences metallic flavor, affecting spring water more than tap water as observed in a sensory study of ferrous sulfate in multiple water sources, and may affect specific groups of consumers have greater sensitivity to metallic off-flavors (Hoehl et al. 2010). Water quality can therefore affect public perception of water safety; reduced water consumption may arise from perception that water is unsafe due to unpleasant taste or appearance (Dietrich 2006; Doria 2010), and can lead to other health issues such as dehydration.

Metallic off-flavor is a negative side effect resulting from altered taste perception of patients undergoing medical treatment or taking certain medications. This can lead to other complications such as decrease in quality of life, appetite, and emotional state plus
non-compliance of medication use as mentioned in a review by Doty et al. (2008). Numerous drugs, varying from antimicrobials to cardiovascular medications to psychoactive drugs and others, can cause taste alteration. This has a major impact on the many people who are on prescription drugs; Doty et al. (2008) describes that doctors prescribe medications for about 90% of office visits. Metallic off-flavors are also experienced by patients with burning mouth syndrome (BMS) (Grushka et al. 1987); additionally, saliva from those experiencing BMS also have a lower quantity of protein in their saliva (de Moura et al. 2007).

Head or neck cancer patients often experience metallic flavor side-effects resulting in complications similar to those mentioned above from certain drugs. Radioactive therapy and chemotherapy can affect the salivary glands causing taste or odor dysfunction or dry mouth, as described in reviews by Epstein (2010a) and Hong et al. (2009b). Alterations in taste may be due to failure of molecules to react with receptors in the mouth (Epstein 2010a). Taste or odor dysfunction can last in the first two weeks post-treatment, but has also been known to persist up to six months or even years after treatment (Epstein 2010a; Logan 2008). There is little information in research literature concerning methods of prevention or reduction of this side-effect. Recommendations for treating taste defects such as avoiding foods associated with metallic flavor or zinc supplement have proved inconvenient and not as successful as preferred (Epstein 2010a; Hong et al. 2009b; Porter et al. 2010). Therefore, a solution to this serious side-effect is needed.

Perception of Metallic Flavor and Sensory Attributes
Metallic flavor is induced by lipid oxidation caused by metallic compounds in the mouth (Lawless 2004; Lawless 2005). In the case of iron, both ferric and ferrous forms can oxidize to form hydroxyl radicals or superoxide that cause off-flavors, but ferrous iron is primarily responsible (Nilsson et al. 2002). This unpleasant off-flavor can also result from lipid oxidation within foods or beverages that are consumed. Metallic flavor is influenced by many factors discussed later in this review such as metal concentration, pH, and individual perception affected by differences in saliva composition and taste buds.

Metallic flavor is generally described as bitter, astringent, or metallic and is experienced at low concentrations for iron and copper compounds (Cuppett et al. 2006; Epke 2007; Hong et al. 2010; Lim and Lawless 2005; Omur-Ozbek and Dietrich 2011; Yang and Lawless 2006). Perception of metallic flavor persists at a high intensity longer than other taste perceptions such as bitter, sour, salty, or sweet; the intensity of astringency is also persistent and may even increase over time (Yang and Lawless 2006). Iron salts are known to have the highest intensities of metallic perception (Lawless 2004; Omur-Ozbek and Dietrich 2011). Metallic flavor threshold for ferrous sulfate, an iron salt, is estimated at 99.2 µM, and more recently the range was confirmed at 0.007 mg/L Fe^{2+} to 14.14 mg/L Fe^{2+} (Lim and Lawless 2005; Mirlohi et al. 2011).

Metallic flavor is not only experienced in the oral cavity, but also retronasally (Epke 2007; Lim and Lawless 2005; Omur-Ozbek and Dietrich 2011). Retronasal effects are experienced as volatiles release from food or beverage in the oral cavity and enter the nasal cavity via the pharynx (Bojanowski 2012). Interestingly, salivary response has
shown to decrease over time when exposed to one odor is continually experienced unlike when exposed to different odors that result in continuous stimulation.

Research has indicated that metallic flavor is not purely sensed by retronasal effects, as evident by the remaining ability to distinguish between water and iron sulfate solutions even with the nose closed (occluded) (Lim and Lawless 2005). Interestingly, researchers found that with the nose occluded, threshold levels were much higher (Omur-Ozbek and Dietrich 2011). Because of the contributions of retronasal effects on detecting metallic flavor, threshold values of ferrous sulfate samples were shown to increase with nasal occlusion; threshold values were 29.9 µM for open nose and 161 µM for closed nose (Epke 2007). However, nasal occlusion is not a reliable method for reducing metallic flavor since there is still perception and is an inconvenient solution. Although the combined retronasal and oral perception of metallic flavor is recognized in scientific literature, there is minimal information on oral contact and metallic flavor (Epke 2007).

Metallic flavor induced via electrical sensation also indicates that oral components are involved in flavor perception. There is likely an oral mechanism that causes rapid lipid oxidation in the mouth leading to retronasal odor compounds (Epke 2007). Oral astringency is often linked with metallic flavor in the oral cavity and may be the result of a decrease in secretion of mucoproteins in saliva, that create a lubrication in the mouth and are shown to play a role in flavor perception (Green 1993).

A modified TBARS method (thiobarbituric acid reactive substances assay) for human saliva (Mirlohi 2012; Omur-Ozbek et al. 2012) has been used as measure of oral lipid oxidation, reported as µM TBARS/g protein. A three-fold TBARS increase was observed from saliva samples collected (n=19) after sipping nanopure water and sipping
ferrous sulfate solutions (Omur-Ozbek et al. 2012). An increase in lipid oxidation was also observed by (Mirlohi 2011) after induction of metallic flavor confirming that metallic compounds induce oral oxidation (n=46). From the same study, researchers found TBARS values of saliva samples collected after sipping nanopure water ranged from 0.013 to 1.73 µM/g total protein, while samples collected after sipping ferrous solution ranged from 0.149 to 5.54 µM/g total protein. The average of the TBARS values for water and ferrous samples were significantly different, indicating that metallic salts influence oral lipid oxidation.

Studies have shown that taste and flavor perception is an intricate process of interactions with proteins, receptors, and other molecules. Under normal circumstances, most iron is bound to proteins such as transferrin or albumin and can also bind to DNA or citrate which likely leads to prevention of metallic flavor perception (Nilsson et al. 2002; Weijl et al. 2004). TPRV1 receptors are thought have a role in metallic flavor sensation since metallic salts and artificial sweeteners were found to activate these receptors (Riera et al. 2007).

Furthermore, research has revealed that non-protein bound iron (NPBI) was found in the plasma of cancer patients undergoing therapy (Weijl et al. 2004), which may be a likely cause of the increase of metallic flavor perception in cancer patients undergoing therapy. Transferrin, an iron-binding protein found in the blood serum is also found in saliva, indicating that compounds from the serum are likely to diffuse in and out of saliva (Gonzalez-Chavez et al. 2009; Jou et al. 2010; Levay and Viljoen 1995; Lonnerdal 2009; Nuijens et al. 1996; Rodrigues et al. 2009). Transferrin or other iron-binding proteins, are potentially not able to bind the iron or not expressed at normal levels during treatment.
NPBI was not found at the beginning of treatment or after two months post-treatment (Weijl et al. 2004).

**Analysis of Saliva Proteins**

Studying the human salivary proteome will allow researchers to further investigate protein and metal interaction in the mouth and to aid in understanding how metallic flavor is perceived in the oral cavity. Humphrey and Williamson (2001) claim that research with saliva can help us understand changes in saliva flow and composition changes due to radiation, disease, or other conditions. To do this, two dimensional-polyacrylamide gel electrophoresis (2DGE) coupled with mass spectrometry (MS) can be used and was performed in many previous studies of human saliva with many of the major components identified (Dowling et al. 2008; Ghafouri et al. 2003; Hardt 2005; Hu 2005; Hu et al. 2008; Huang 2004; Jou et al. 2010; Vitorino 2004). By using 2D-PAGE versus 1D-PAGE, proteins are separated by pI in the first dimension and by weight in the second dimension, thus providing improved separation of the proteins. This provides an overall protein profile of the sample and mass spectrometry can aid in identification of proteins and their expression levels. Some proteins, described later, have been studied further, with some already recognized as biomarkers for diseases such as cancer.

Many researchers have studied proteins using Coomassie Brilliant Blue (CBB) or silver staining techniques, but Sypro Ruby (BIO-RAD, Hercules, CA), a fluorescent stain, has provided the best results (Dowling et al. 2008; Hardt 2005; Hu 2005; Huang 2004; Jou et al. 2010; Yao 2003). Huang (2004) suggests using a fluorescent stain, which would allow for detection of proteins at lower concentrations. Flamingo stain, a fluorescent stain (BIO-RAD, Hercules, CA), has detection levels of protein levels as low
as 0.5 ng versus Coomassie staining at 5 ng (Nishihara 2002). Silver staining has lower
detection levels than Coomassie staining and similar detection levels to that of
fluorescent staining, but requires more difficult methods to stain the gels and special
procedures must be followed in order to identify proteins via MS due to interfering
compounds.

The 2DGE technique does have some drawbacks; thus, care should be taken in
creating a standardized protocol to provide the best quality resolution of proteins. There
may be issues with detecting small molecular weight proteins, dealing with proteins with
extreme pH values, hydrophobic proteins, glycosylated proteins, and proteins in low
abundance (Hu et al. 2008; Huang 2004; Schipper et al. 2007). Human saliva is also
highly variable among individuals regarding protein expression, protein concentration,
viscosity, and flow rate. Bacterial metabolism in the sample may also affect protein
concentration over time; when identifying proteins from 2D gels via MS, bacterial genes
can be excluded from the search in order to identify only human saliva proteins (Hu
2005). Centrifugation for removal of highly abundant proteins that are typically heavily
glycosylated including amylases, albumins, mucins, and immunoglobulins is necessary
for improved analysis of low abundance proteins and a more sharply focused gel image.

Several research projects have shown that protein gel comparisons of saliva
before and after treatments are useful for analyzing differences in composition and can
lead to biomarker discovery (Hu et al. 2008; Huang 2004; Jou et al. 2010). Analysis using
2DGE has been used to compare protein profiles with and without bleeding in the oral
cavity and found that cystatin C is a useful a biomarker for predicting healing responses
of tissues in the oral cavity (Huang 2004). It was also found that other cystatins function
in regulation and protection in tumor formation and pathogen infection. Comparisons of protein profiles in 2D gels of saliva from cancer patients and healthy humans revealed 52 unique proteins in only oral cancer patients and 29 unique proteins in only the healthy patient samples (Hu et al. 2008). Subsequent identification via mass spectroscopy, immunoassays, and other analyses of some of the differing proteins was performed to identify potential protein biomarkers of oral cancer. M2BP, profilin, CD59, MRP14, and catalase were confirmed proteins that were different when comparing healthy and cancer patient saliva samples. Another study also found an increase in abundance of beta fibrin, S100 calcium binding protein, transferrin, immunoglobulin heavy chain constant region gamma and coflin-1 in head and neck cancer patients compared to healthy humans (Dowling et al. 2008). Additionally, transferrin was confirmed as a possible biomarker of oral cancer (Jou et al. 2010).

Saliva protein analysis has shown that saliva composition alters based on emotional states (Grigoriev 2003), which would apply directly to the major emotional changes that cancer patients may experience. Currently, saliva is useful in other applications as well, particularly in the medical sector. Analysis of saliva is more simple than other complicated tests that also impose on the patient; examining changes in saliva composition can lead to early diagnosis of diseases, monitoring for diseases, or treating diseases (Huang 2004; Messana et al. 2008a; Messana et al. 2008b; one 2005; Rai et al. 2008).

Gender and age has also been found to affect saliva composition using 2D-PAGE analysis (Fleissig et al. 2010). Six differences were found when comparing gels prepared from healthy human saliva based on gender, which consisted of greater than two-fold
increase of expression in the female group (n=27, males=14, females= 13, age range= 22-88). To compare gels, they used imaging software to detect spot intensities, normalized the data, and ran statistical analysis on differences between gels. When comparing gels based on age, the researchers found decreased expression of eight proteins in the male group as age increased. In the female group, they found that two proteins decreased in expression with aging, five proteins increased with aging, and one protein increased during middle-age, but decreased with age. The data from this study can be used to correlate disease, age, and gender.

More recent studies to identify potential biomarkers within human saliva are utilizing technology to investigate salivary gene expression by exploring the transcriptome or mRNA by using qRT-PCR (Hu et al. 2006; Troxler et al. 1997; Zhang 2010). The latest technology, 2D-DIGE (two dimensional-differential imaging gel electrophoresis), can allow researchers to more easily observe differences between samples by using several fluorescent dyes for each treatment. In a study conducted by Zhang et al. (2010), the researchers revealed that there were 35 up-regulated genes and 32 down-regulated genes in the saliva of breast cancer patients from 2D-DIGE analysis. Several studies have shown that saliva from cancer patients contains differences compared to healthy human saliva, which may cause alterations in metallic taste perception (Dowling et al. 2008; Zhang 2010).

**Salivary Roles, Protein Interactions, and Responses**

Saliva plays a role in human health through constant secretion of saliva provides defense against pathogens, breakdown of food, swallowing, and cleansing of the oral cavity (Hardt 2005; Huang 2004; Kaplan and Baum 1993). If this flow is disrupted, it
may lead to problems such as dryness and changes in taste that can be very uncomfortable. Head and neck cancer patients typically experience these issues because treatment may affect the salivary glands and surrounding tissues thus, saliva production is affected. There are also specific proteins that are secreted at particular levels to maintain these roles of saliva, as well as electrolytes and other compounds (Humphrey and Williamson 2001). Saliva flow rate in healthy individuals was observed to be 1.15 ml/5 min (Huang 2004) and average protein concentration has been measured at 0.35 mg/ml (Hu 2005). The normal pH of saliva has been reported as between 6 and 7 (Humphrey and Williamson 2001), likely due to majority (about 99%) of saliva being composed of water.

There are many proteins in saliva with varying functions, some of the key proteins found in saliva that are of interest in this study are described in Appendix: A3. Salivary proteins important roles include the first defense against harmful materials or pathogens and may also send important signals to other parts of the body as reviewed by Messana, Inzitari et al. 2008. Proteins are known to function in DNA replication and repair, many different protein interactions, transport, metabolism, immune defense, and more (Hu 2005). Hundreds of proteins have been identified in saliva and make up 99% of the total weight of salivary peptides and proteins (Messana et al. 2008b). The majority of proteins in saliva are proline rich proteins (PRPs), which account for 60% of the protein content (Messana et al. 2008b). The second greatest majority of identified proteins in saliva consist of amylases (20%), which are involved in digestion (Hu 2005; Messana et al. 2008b). However, a large percentage of the proteins in saliva (28.7%) are hypothetical proteins, meaning currently not having identification. Current literature indicates that
continued research is necessary to improve gel electrophoretic methods and possibly combine with other methods, such as mass spectrometry, to aid in clear identification of the saliva proteome (Hardt 2005; Hu 2005; Messana et al. 2008b). In a review by Helmerhorst and Oppenheim, 2007, they explain that saliva is made up of many similar proteins and that many differences in those proteins arise from genetics and post-translational modifications (Helmerhorst and Oppenheim 2007). These modifications include glycosylation, phosphorylation, and proteolytic processing or cleaving of proteins; numerous modified proteins can be found via 2D-MS mapping of the saliva proteome (Hardt 2005).

Post-translational modifications and polymorphism is an issue in identification of proteins due to altered protein structure (Hardt 2005; Messana et al. 2008b). Gene polymorphisms are thought to be involved in changes of salivary secretions, causing the varying taste and flavor perceptions among individuals (Padiglia 2010). Padiglia et al. (2010), describes gustin as a protein that is known to have alterations caused by gene polymorphism, resulting in an altered protein structure. This altered structure affects binding of molecules, and therefore, changes the interaction with taste bud receptors and causes altered taste or flavor perception. It is suggested that other proteins may undergo similar changes and may be of interest in future studies involving taste perception.

Gustin is a zinc metalloprotein that is thought to be involved in flavor and taste development in the oral cavity as described by Padiglia et al. (2010). Since gustin activity depends on zinc presence in the oral cavity, zinc has been used as a therapy to treat taste disorders as described in reviews (Epstein 2010a; b; Hong et al. 2009b; Padiglia 2010). Salivary zinc concentrations were shown to be significantly higher in subjects known as
less sensitive to taste than those identified as sensitive to taste. Therefore, this research suggests that for less sensitive tasters, translation of the protein blocks binding of zinc to the gustin protein. Furthermore, research has shown that those with gustin inhibited by zinc blockage or lower levels of gustin expression may not be responsive to zinc treatment for taste disorders.

Many proteins aid in protection of the oral cavity, either as antimicrobials or other immune defense roles (Grigoriev 2003); these proteins bind metals making them unavailable for microbial growth. Antimicrobial proteins include lysozyme, mucins, lactoferrin, immunoglobulins, cystatin, statherin, and histatin. PRPs (proline-rich proteins), histatin, statherin, and cystatin are known to bind calcium (Messana et al. 2008a).

Mucin creates a protective layer or barrier in the oral cavity by concentrating these proteins in a matrix along with PRPs (Hu 2005; Messana et al. 2008b; Schipper et al. 2007). Many other proteins such as lactoferrin, immunoglobulin A (IgA), and lysozyme have been found to interact with the gel matrix. Schipper et al. (2007) also describes the discovery of salivary micelles made up of mucins, lactoferrin, sIgA, lysozyme, and PRPs.

Transferrin and lactoferrin are two related proteins that have metal binding capabilities, with transferrin transporting iron throughout the body via blood serum (Gonzalez-Chavez et al. 2009; Jou et al. 2010; Levay and Viljoen 1995; Lonnerdal 2009; Nuijens et al. 1996; Rodrigues et al. 2009). Analysis of healthy human whole-saliva has shown that metal-protein binding occurs with copper and is likely to occur with other metals such as iron (Hong et al. 2009a). Copper-protein interaction was affected by pH
and temperature, but further research is needed to explore these factors for other metal-protein interactions. Lactoferrin typically binds ferric iron (Fe$^{3+}$) molecules and binds them more strongly at a lower pH, but can also bind other metals as well and is described later in this literature review (Anderson et al. 1989).

Salivary flow rate, concentration, and composition are affected by time of day, diet, age, gender, pharmacologic drugs as well as different stimuli (Dawes 1984; Mandel 1974; Neyraud et al. 2009). Different molecules were shown to have a different effect on saliva in a study using citric acid, sucrose, mono-sodium glutamate, magnesium sulfate, and sodium chloride (Neyraud et al. 2009). After each stimulus was introduced, the researchers found that salivary flow rate rapidly increased from 10 ul/min to about 1,200 ul/min for approximately 30 seconds following. Studies show that protein concentration decreases for approximately two to five minutes, but slowly increases over time (Dawes 1984; Neyraud et al. 2009). The buffering capacity of saliva due to the presence of sodium bicarbonate balances the pH (Mandel 1974), which may in turn affect protein binding and interactions.

**Lactoferrin**

Lactoferrin is a metal binding glycoprotein that has two globular lobes, each having a metal binding site (Anderson et al. 1989; Medina et al. 2002). This protein is part of the transferrin protein family, which transports iron in the blood (Gonzalez-Chavez et al. 2009). When bound to iron, lactoferrin undergoes a large conformational change (Anderson et al. 1989); this is largely due to the spacing between the residues with which iron binds and involves an opening and closing of the binding cleft. Metal binding neutralizes the positive charges of ions, possibly reducing metallic flavor.
perception. It typically binds the ferric form of iron \((Fe^{3+})\) but can also bind many other metal ions, especially those with strong positive charges \((Cr^{3+}, Mn^{3+}, Co^{3+}, Ga^{3+}, Al^{3+}, Cu^{2+}\) and more) (Anderson et al. 1989). A review by Anderson et al. (2008) describes that iron is released from the protein at low pH values.

Lactoferrin is also found in milk, with the highest level being in human milk at 1-6 mg/ml (Nuijens et al. 1996). In saliva, the normal concentration for adults has been reported as 0.11 μM (Weinberg 2007). Lactoferrin has many functions and is secreted throughout the body (Nuijens et al. 1996). Several review articles describe lactoferrin functions; it plays a role in the immune system, is important for iron absorption, has anticarcinogenic effects, and functions as an antimicrobial (Gonzalez-Chavez et al. 2009; Lonnerdal 2009; Nuijens et al. 1996; Rodrigues et al. 2009).

Lactoferrin functions as an antimicrobial by reducing the availability of iron to microbes or disrupting cell membranes (Gonzalez-Chavez et al. 2009; Nuijens et al. 1996). This protein also plays a role in gene regulation during immune response when transferred to areas of inflammation and bound to DNA where it alters gene expression (Nuijens et al. 1996). Nuijens, van Berkel et al. (1996) reported that lactoferrin uptake in humans is still not completely understood, but more recent studies describe that lactoferrin has the ability to regulate iron absorption in the bowel (Gonzalez-Chavez et al. 2009; Lonnerdal 2009). Rodrigues, Teixeira et al. (2009) stated in a review that lactoferrin influences the bioavailability of iron in the body; therefore, lactoferrin is an important protein for regulation of iron in human bodies. Lactoferrin is a naturally occurring protein that has potential benefit to the immune system, mainly acting in the small intestines where it has shown potential in prevention of tumor development and
stimulating other immune responses. The exact action that lactoferrin has on the immune system is currently not well understood.

In a review by Rodrigues et al. (2009), the authors discuss the use of bovine lactoferrin as a chemopreventative agent. Research has indicated that use of metal chelators during treatment can reduce the serious side-effects of chemotherapy such as toxicity due free iron in the body (Weijl, 2004). Although metal chelators or antioxidants are known to aid in reducing side-effects of chemotherapy, a communication by Moss (2006) indicates that caution should be taken when using antioxidants for therapy due to the protective effects these compounds may have on cancer cells (Moss 2006). However, Moss also discusses that antioxidant use may benefit the nutritional status, allowing patients to have a higher intake of vitamins.

Bovine lactoferrin is currently utilized in oral applications, but recombinant DNA technologies could be utilized to increase the amount of human lactoferrin available for oral applications and research purposes (Nuijens et al. 1996). Several lactoferrin purification methods have been assessed and have found that cation-exchange chromatography yields the highest amounts of purified lactoferrin protein (Gonzalez-Chavez et al. 2009). Gonzalez-Chavez et al. (2009) states that there is an interest in purifying lactoferrin protein for use as a food additive or therapeutic agent. The many functions of lactoferrin make it a versatile protein with many benefits.

**Metal Chelators: Reduction of Metallic Flavor and Potential Uses in Industry**

The majority of this literature review has focused on reduction of metallic flavor induced by iron with the metal chelator lactoferrin, but there are also other advantages to use of this protein. As discussed previously, reduction of metallic flavor is a concern in
many industries. Iron is an essential nutrient but creates an undesirable taste, which can be challenging in creating nutritious products or for providing safe, enjoyable water supply. Metal-chelators that are more affordable, non-toxic, and stable over time can increase iron uptake, iron storage in the body, and consumer acceptance (Hunt 2005; Miret et al. 2008; Tripathi and Platel 2011). Current research has developed methods for determining iron absorption in the body and would aid in research pertaining to food fortification with iron (Hoppe et al. 2007). This section discusses other issues surrounding metallic compounds, off flavors, and how to solve these problems.

Several research articles state that iron deficiency is the most common nutritional issue in the world (Hunt 2005; MacPhail et al. 1994; Miret et al. 2008; Pizarro et al. 2002; Tripathi and Platel 2011), as iron is an essential nutrient for many bodily processes, this poses a great issue. Most of the iron in the diet has low bioavailability, mainly due to the fact that most diets in countries suffering iron deficiency have a diet consisting of plants that have substances such as phytic acid and polyphenols that inhibit iron uptake (Hunt 2005). Iron uptake is clearly a complex process and creates a challenge in fortification of foods with iron to increase availability (Hoppe et al. 2007). In a review by Alderova and others (2008), the authors explain that iron absorption of iron from lactoferrin depends on the organism’s need for iron. One pathway of iron absorption involves lactoferrin binding to an enterocyte which becomes degraded when bound to a cell and the iron is released into the cell (Alderova et al. 2008).

Metal chelators such as ethylene diamine tetraacetic acid (EDTA), used in conjunction with iron salts, such as FeSO₄, have been used for fortification of foods; thereby, increasing the bioavailability of iron even with the presence of inhibitors of iron
uptake (Hunt 2005; MacPhail et al. 1994; Miret et al. 2008; Tripathi and Platel 2011). However, one study evaluated the use of disodium EDTA for fortification and observed that there was no significant increase in iron absorption (Fairweather-Tait et al. 2001). The researchers were also unsure whether any increase in iron absorption was from reduced free iron or from iron bound to EDTA. The search for iron chelators is a current area of research, seeking compounds with lower affinities for iron that create equilibrium with the environment, which would help to release the iron for bioavailability (Miret et al. 2008). The formation constant for ferric-EDTA is $1.3 \times 10^{25}$ whereas the formation constant for ferric-lactoferrin is $1.0 \times 10^{20}$, which researchers suggest is the reason why EDTA has a stronger antioxidative effect (Huang et al. 1999; Skoog and West 1976; Masson and Heremans 1968). Iron chelators are currently used in food products for sequestering iron for prevention of microbial growth have a relatively strong affinity for iron, which may decrease bioavailability of the metal; increasing bioavailability of iron to humans would be an added benefit, but could be a challenge.

Lactoferrin is a useful protein for iron chelation and is a natural ingredient that can be used to increase bioavailability of iron, act as an antioxidant, and has potential to decrease lingering metallic off-flavors. Nielson et al. (2004) discovered that lactoferrin addition to foods or beverages has shown antioxidant activity at levels lower than 12 µM, but has prooxidant effects at levels higher than 12 µM (Nielsen et al. 2004). No changes in sensory attributes were found, but researchers postulate that this is likely due to low volatiles and addition of more appealing flavor disguising the off-flavors that may have been present. EDTA at concentrations as low as 16 µM has antioxidant effects. Lactoferrin’s iron-binding capacity is claimed to be 0.025uM of metal per mg. Research
has also confirmed that lactoferrin’s chelating ability is decreased when iron concentration increased due to saturation of the binding sites (Gutteridge 1981; Medina et al. 2002). Phenolic compounds such as α-tocopherol can aid in antioxidant activity of lactoferrin, as well as bicarbonate ions that support the metal-protein complex (Anderson et al. 1989; Masson and Heremans 1968; Medina et al. 2002). In a study where panelists sipped lactoferrin (0.13uM) or EDTA (36 µM) after sipping ferrous sulfate solution (18 µM), metallic off-flavor decreased significantly; there was no significant effect when chelators were sipped as a rinse before the ferrous sulfate solution and duration of flavor was not evaluated (Omur-Ozbek et al. 2012).

**Sensory Evaluation and Time-Intensity to Compare Metal Chelators**

Sensory perception of a final product is very important to creating a desirable product in industry, whether fortification of iron or removal of iron for improving taste of water supply is the goal. Rating a sample using a time-intensity scale has proved to be a method for evaluating lingering tastes from metallic flavor induction (Hong et al. 2010). Time-intensity (TI) studies can both describe the qualitative attributes and quantify those attributes (Meilgaard et al. 2007). Curves, typically generated in software, can tell a researcher the initial response to stimuli, when it reaches its peak, how long it lasts, and the rate and shape of the response (Lawless et al. 2010). Time-intensity has previously been used to evaluate the flavor release, lingering flavors, and how flavors interact within a formulation (Lawless et al. 2010). Additionally, TI studies can aid in understanding texture attributes of foods. Although extremely helpful in understanding intensity and duration of a flavor, the process of how a panelist generates a response is not well understood and is thought to not be continuous. Therefore, different curves may be
produced with the same stimuli. Planning and understanding what types of responses are expected beforehand can help explain TI curves.

Our research involves evaluating reduction of flavor induced by ferrous sulfate using metal chelators. Iron detection limits are described earlier in this literature review and included 0.007 mg/L Fe$^{2+}$ to 14.14 mg/L Fe$^{2+}$ (Mirlohi et al. 2011). Additionally, research has shown that about 90% of panelists can detect iron at or below the secondary maximum contaminant levels set at 0.3 mg/L by the USEPA. As discussed throughout this literature review, the total composition of salivary constituents and oral pH can affect taste and flavor perception. The concentration of the stimulus, viscosity of saliva, rate, duration, and presence of other tastants can affect overall perception and should be considered when studying sensory responses and lingering perception with time-intensity curves (Meilgaard et al. 2007). The overall difference in reduction of metallic flavor using lactoferrin as an alternative to EDTA can be determined using time-intensity graphs to determine how long the metallic aftertaste lingers using continuous data collection. Sensory evaluation using time-intensity curves can confirm if lactoferrin provides similar or greater benefits as EDTA.
REFERENCES


Cuppelt JD, Duncan SE, Dietrich AM. 2006. Evaluation of copper speciation and water quality factors that affect aqueous copper tasting response. Chem Senses. 31: 689.


24


Chapter III. Role of Human Salivary Proteins in Metallic Flavor Perception

ABSTRACT

Flavor perception is a complex mechanism involving salivary components, taste receptors in the oral cavity, retronasal effects, and may change as we age due to individual variability. Metallic flavor is a concern for many industries including food, health, and water. Metallic off-flavors are a serious side-effect for those with alterations in taste and flavor perception caused by drugs or therapies. Metal-binding salivary proteins, such as lactoferrin, are suspected to control perception of metallic flavor. To study the alterations in salivary components and potential adjuvant therapy, eight panelists were chosen (5 female, age 40-70 years) contributed 4 ml of unstimulated whole saliva or after sipping a lactoferrin solution (1µM), then collected a total of 4 ml of saliva after sipping 2 ml a ferrous sulfate sample (10 mg/ml) to stimulate metallic flavor. The sequence was presented in two occasions and sample collection was replicated. Protein concentration, total oral lipid oxidation as indicated by thiobarbituric acid reactive substances assay, and iron concentration were determined on individual saliva samples. Protein patterns were qualitatively characterized in saliva for each pre-rinse and metallic stimuli from four panelists by two-dimensional gel electrophoresis. A consistent pattern of regions containing major salivary components including mucin, alpha-amylase, prolactin inducible proteins, immunoglobulins, secretory component precursors, and cystatin precursors was observed. Variability in protein profiles of individuals was a major factor in differences found between gel sets. This study provides a foundation of
method development to better understand salivary protein interaction with metals and other flavor perception.

**INTRODUCTION**

Metallic off-flavors are produced by oxidation in the oral cavity and are influenced by retronasal effects, receptors in the oral cavity, and human salivary proteins (Epke 2007; Lawless 2004; Riera et al. 2007, Mirlohi et al. 2011, Omur-Ozbek and Dietrich 2011). Knowledge of the complete process of metallic perception is not completely understood at this time (Riera et al. 2007). More research is needed in order to gain a better understanding of how perception occurs and how to resolve the problem. Metallic off-flavors are an issue that affects many industries including food, health, and water. Off-flavors in foods may be affected by certain ingredients that are susceptible to oxidation or foods in foods fortified with iron (Lawless 2004). Additionally, water resources contain dissolved copper and iron leading to unpleasant metallic flavors (Dietrich 2006). Altered taste is a side-effect to numerous medications, leading to patients’ unwillingness to undergo treatment or properly take medication needed (Bigeleisen 1999; Doty 2008). Cancer patients that experience metallic flavors as a side-effect of treatments are of particular interest for this research. Close to two-thirds of cancer patients undergoing therapy experience altered taste perception (Hong et al. 2009c). Additionally, cancer patients experience a loss in quality of life and may suffer malnutrition due to this side-effect (Epstein 2010b; Hong et al. 2009c; Hutton 2007).

Several studies have indicated that oral components, such as receptors in the oral cavity and salivary proteins, are likely involved in metallic flavor perception as is retronasal effects (Epke 2007; Green 1993; Lawless 2005). There are few studies that
have explored metal interaction with human salivary proteins; copper is known to interact with salivary proteins, resulting in complexes that may play a role in perception of metallic flavor (Hong et al. 2009b). Salivary composition is highly variable among individuals as a result of genetic variations (Bartoshuk 2000). This may explain why some individuals are sensitive to certain tastes or flavors, especially bitter, while others are not (Duffy 2000). Different taste stimuli have shown to affect overall protein concentration and salivary flow rate (Neyraud et al. 2009). Protein concentration decreases for approximately two to five minutes after stimuli, but slowly increases over time (Dawes 1984; Neyraud et al. 2009). Studying the role of human salivary proteins in perception of metallic flavor may lead to a better understanding of flavor perception in the oral cavity.

Metal-binding proteins found in saliva are suspected to be involved in metallic flavor perception including gustin, amylase, mucins and histatin (Agarwal and Henkin 1987, Oppenheim 1988, Padiglia 2010, Wu et al. 1994). Iron-binding proteins lactoferrin and transferrin are of interest in this particular study. Lactoferrin is an iron-binding protein that can bind two positive metal ions, possibly reducing perception of flavor from oxidized metals in the oral cavity. Transferrin is found in the blood serum, and therefore likely able to diffuse into the blood stream to transport iron throughout the body (Gonzalez-Chavez et al. 2009; Jou et al. 2010; Levay and Viljoen 1995; Lonnerdal 2009; Nuijens et al. 1996; Rodrigues et al. 2009). Researchers have observed that non-protein bound iron (NPBI) was found in the plasma of cancer patients undergoing therapy (Weijl et al. 2004), which may be a likely cause of the increase of metallic flavor perception in cancer patients undergoing therapy. Transferrin and other iron-binding proteins were
potentially not able to bind the iron or not expressed at normal levels during treatment. NPBI was not found at the beginning of treatment or after two months post-treatment, suggesting that this is an effect of treatment (Weijl et al. 2004). Astringency, typically experienced along with metallic flavor, are likely a result of decreased production of mucoproteins that lubricate the oral cavity and are involved in perception of other flavors (Green 1993). Further proteomics research may reveal other proteins that interact with metals in the oral cavity and help us to further understand abnormal metallic flavor as a side-effect.

Two-dimensional gel electrophoresis (2DGE) is useful for mapping and comparing the salivary proteome. Many major components of saliva have been identified using 2DGE (Dowling et al. 2008; Ghafouri et al. 2003; Hardt 2005; Hu 2005; Hu et al. 2008; Huang 2004; Jou et al. 2010; Vitorino 2004). Many of these studies revealed several proteins that have altered presence when comparing before and after bleeding in the oral cavity, as well as comparing healthy human and cancer patient saliva. Research has also concluded that protein concentration and composition are affected by gender, age, and emotional state (Fleissig et al. 2010; Grigoriev 2003, Mirlohi et al. 2011).

Advancing the knowledge of the interaction of salivary proteins with pro-oxidant metals can lead to further understanding perception of metallic flavor as well as providing direction for resolving this problem for foods, pharmaceuticals, and cancer patients. The goal of this research was to expand on knowledge of protein interaction with iron when a metallic flavor is induced. Qualitative characterization of salivary proteins was completed by comparing 2D gels produced from human saliva from healthy, middle-aged panelists before and after introduction of ferrous sulfate into the oral cavity.
The specific objectives of this study were to analyze protein gels produced from healthy, middle-aged adult saliva before and after induction of metallic flavor by ferrous sulfate, by comparing the whole human saliva proteome without a protein chelator to reduce metallic flavor and the use of lactoferrin solution to potentially reduce metallic flavor; and 2) to evaluate changes in metal concentration as affected by ferrous sulfate and lactoferrin supplementation in each of these samples using mass spectrometry-inductively coupled plasma (MS-ICP), and oxidation, using a modified thiobarbituric acid reactive substances assay (TBARS).

**MATERIALS AND METHODS**

**Chemicals**

All chemicals not specified in this section were purchased from Sigma-Aldrich (St. Louis, MO), BIO-RAD (Hercules, CA), or GE Healthcare Life Sciences (Pittsburgh, PA). Iodacetamide (IAA) was purchased from Invitrogen (Grand Island, NY).

**Preparation of Ferrous Sulfate and Lactoferrin Solutions**

Samples were prepared fresh before saliva collection to prevent degradation or precipitation of compounds in clean glassware, which was rinsed thoroughly beforehand to remove any residual minerals. The concentration of lactoferrin and ferrous sulfate was determined based on preliminary trials and previous studies, respectively (Mirlohi 2011); each compound was dissolved in distilled water (The Kroger Co., Cincinnati, OH, 45202) with agitation. Lactoferrin was prepared at 1µM and ferrous sulfate at 99.2 μM (10 mg/L).

**Saliva Collection**
The Virginia Tech Institutional Review Board approved collection of saliva samples from human subjects (IRB #10-156). Panelists were first asked to collect unstimulated whole saliva (4 ml) then subsequently sip 2 ml of ferrous sulfate to induce metallic flavor. On a second occasion, the panelist sipped 2 ml of lactoferrin for reducing metallic flavor, then subsequently sipped a ferrous sulfate sample. The panelist took the entire rinse sample provided into the mouth and swished it around the oral cavity for 10 seconds without swallowing, then expectorated whole saliva plus the rinse treatment sample into a pre-chilled graduated, conical centrifuge tube (10 ml, Greiner Bio-One GmbH, Bridgeport, NJ). They continued expectorating until a total volume reached the 4 ml mark on the tube, which took anywhere from (10-15 minutes per sample). Panelists rested for approximately 2 minutes to allow salivary function to recover, then sipped and swished the metallic stimuli and followed the same saliva collection procedures. Unstimulated whole saliva served as the baseline sample for analyses. The two treatment sequences (unstimulated whole saliva collection, metallic stimuli; lactoferrin pre-rinse, metallic stimuli) were in separate sessions on separate days. Samples were collected on ice with pre-chilled tubes and then frozen at -80°C for subsequent analysis.

**Analysis of Salivary Proteins and Detection of Reactive Substances**

Frozen samples were thawed on ice to prevent degradation of proteins. First, the saliva samples were mixed well using a vortex, then 1 ml of sample was removed and placed into a pre-chilled microcentrifuge tube. Samples were placed in a chilled (4°C) centrifuge for 15 minutes at 14,500 rpm to reduce viscosity and remove debris. The concentration of proteins were quantified in each sample using the 2D-Quant Kit (GE Healthcare #80-6483-56), following the protocol given in the brochure. The protein
sample was then precipitated and cleaned using 90% acetone/10% TCA/20mM DTT (Hu 2005), at twice the volume of saliva sample, and chilled overnight at -20°C. The next day, the samples were placed in a chilled centrifuge (14,500 rpm, 4°C) to pellet the protein. The pellet was washed a second time using a 20mM DTT/acetone wash and placed in the chilled centrifuge to pellet the protein once again.

The protein samples were separated in the first dimension (IEF, isoelectric focusing) using 11cm IPG strips (GE Healthcare, Pittsburgh, PA) with GE Healthcare Ettan IPGphor 3 after first including a 15 hour rehydration step at 20°C to absorb the protein sample into the strip for proper movement of proteins through the gel matrix. Following IEF, strips were equilibrated with a three step process, 15 minutes per step, first with the equilibration buffer alone, then with addition of 130mM DTT (dithiothreitol), and then the addition of 130 mM IAA. Proteins were separated in the second dimension (molecular weight) using 11cm Criterion Precast 12.5% Polyacrylamide Gels in Tris-HCl buffer (BIO-RAD, Hercules, CA) for timely movement of large proteins through the gels in a charged cell holding the gel with a tris-glycine SDS (sodium dodecyl sulfate) running buffer to charge and linearize the proteins. Gels were stained using Flamingo fluorescent stain (BIO-RAD, Hercules, CA) for low detection limit by following the manufacturer’s instructions, and imaged for viewing using Molecular FX Imager (BIO-RAD, Hercules, CA). Protein spots were detected and gels were compared using PDQuest 2-D Analysis Software (BIO-RAD Version 8.0, Hercules, CA) changing the parameters as needed for optimal resolution and removal of speckles. Notable spots and regions of related proteins were confirmed by comparison to mapped saliva proteome gels found in current literature.
Samples were analyzed for lipid oxidation using a modified TBARS procedure for saliva samples as described by Mirlohi (2012). Protein content varies among individuals and to therefore report oxidation in the oral cavity more accurately, we reported these values as µM TBARS per gram of protein. Salivary samples were also prepared for metals analysis using mass spectrometry- inductively coupled plasma (MS-ICP, Thermo Electronic Corporation, X-Series ICP-MS, Waltham, MA) to quantify iron concentration in saliva samples using using the method described by Mirlohi (2012). We also reported these measurements as mg Fe per g protein to relate concentration and further understand iron availability for oxidation and for binding to proteins.

Data Analysis of Salivary Protein Concentration, Oral Lipid Oxidation, and Iron Concentration

Statistical analysis for protein concentration, TBARS, and iron concentration was performed using SAS software (SAS vs 9, Cary, NC). The overall model included main effects of panelist (n=8), and treatment (baseline, metallic sample 1, lactoferrin, or metallic sample 2) and was analyzed by the GLM procedure with mean separations by least significant difference. An alpha level of 0.05 was pre-established for statistical significance. Analyses were also completed on the change (difference) in protein, change in TBARS, and change in iron, with change defined as the concentration of baseline treatment subtracted from concentration for each treatment for each individual for each treatment, following the same statistical model. By applying a dilution factor of two to lactoferrin, metallic 1, and metallic 2 samples, we could compare the treatments to the baseline sample. Treatments were paired (baseline and metallic sample 1; metallic sample
1 and metallic sample 2; lactoferrin and metallic sample 2) and analyzed by t-tests. Outliers were excluded in statistical analysis, but are shown in data tables.

RESULTS

Protein Concentration

Protein concentration was not statistically different among panelists or among treatments (p>0.05). Average protein (± SE) in baseline, metallic sample 1, LF rinse, and metallic sample 2 saliva samples, averaged across all panelists, was 1.24 (± 0.36), 1.88 (± 1.03), 1.75 (± 0.85), and 1.59 (± 0.63), respectively (Table 1). We must consider that the dilution factor applied to our protein concentration, which was based on the 2 ml sample (pre-; post-; or metallic stimuli) plus the saliva excreted (2 ml) during the collection (total 4 ml), panelists may have swallowed some of the sample that would not have been expectorated into the collection tube. The baseline sample, which was whole unstimulated saliva, was not diluted.

Oral Lipid Oxidation

Statistical analysis of measurements for TBARS revealed no statistical differences among each treatment (p>0.05, Table 2). Average µM TBARS/g protein (± SE) in baseline, metallic sample 1, LF rinse, and metallic sample 2 saliva samples, averaged across all panelists, was 0.78 (± 1.27), 0.85 (± 1.06), 3.18 (± 6.07), and 1.05 (± 1.08), respectively (Table 1). For samples reported as 0 µM TBARS/g protein, the level of oxidation was well below our limit of detection (0.125 µM TBARS). There were statistically significant differences between each panelists’ measurement due to individual variability in salivary constituents, as discussed later.
Iron Concentration

No statistical differences found when comparing the iron content or change in iron content across for all treatments (p>0.05, Table 3). Average mg Fe/g protein (± SE) in baseline, metallic sample 1, LF rinse, and metallic sample 2 saliva samples, averaged across all panelists, was 7.38 (± 19.81), 0.64 (± 0.96), 0.96 (± 1.89), 1.70 (± 3.64), respectively (Table 3). For samples reported as mg Fe/g protein, the amount of iron was well below the detection limit. Although there was not a significant increase, when considering the iron concentration by individuals, most saliva samples that were collected after sipping a ferrous sulfate sample showed in increase in iron content.

Salivary Protein Analysis using 2DGE

A consistent protein spot pattern was observed when analyzing whole saliva proteomic gels. When comparing to other proteome maps, we were able to pinpoint major protein regions and potential proteins of interest. We were able to detect an average of about 300-400 protein spots on each gel using software, which has been reported in other saliva proteome research (Fleissig et al. 2010). When comparing to current maps of the salivary proteome, overall gel analysis revealed a recurring pattern of regions containing albumin, α-amylase, secretory component precursors, zinc-α-2-glycoprotein, immunoglobulins, glyceraldehyde-3-phosphatase, and cystatins including their precursors (Ghafori 2003, Hu 2005, Huang 2004). Comparisons of gels produced in our study to other studies are a clear indication that these protein spots and regions are abundant and identified using mass spectrometry.

Gel images that had the best resolution were chosen from four panelists (panelist 1, 3, 4, and 7); a representative gel produced from a baseline sample from panelist 1 is
shown in Figure 1. Within individual variation does exist, but when comparing all gels produced from a single individual, there appears to be a protein pattern for that individual. Some individuals have a strong pattern of mucins, alpha-amylase, and cystatin precursors and proteins, whereas others have a more faint spot pattern, suggesting there may be differences in concentration. This may also be due to individual genetic variation, discussed later. When comparing to gels found in literature, we were able to obtain more separation within the molecular weight region from 25-50 kD. We were also able to identify our target protein for this study, lactoferrin, based on identified spots in literature and the molecular weight of approximately 97 kD and pI of approximately 8.7 (Ghafari 2003, Hong et al. 2009, Levay 1995).

In further comparisons within individuals, we were able to find some notable differences in gels produced from different saliva samples. In the gel produced from Metallic 2 sample for panelist 1, we observed deeper spots, suggesting a higher concentration, for prolactin inducible proteins and cystatin precursors. Presence of lactoferrin varied by individual also; we found the spot in only some gels when comparing gels produced from all samples from one individual. In gels produced from metallic samples or lactoferrin sample for all panelists, in the region containing stratifin, immunoglobulin J-chain, and proline rich proteins, the spots appeared to deepen, indicating a higher concentration.

**DISCUSSION**

**Protein Concentration**
We found that the average protein concentration of baseline saliva samples was 1.24 ug/ul. The mean protein concentration for whole unstimulated saliva has been observed at 1.27 ug/ul, which is very similar to our results (Hong et al. 2009). Other studies have observed lower values: 0.35 ug/ul for unstimulated saliva used for 2DGE, and 0.62 ug/ul for baseline saliva and 0.58 ug/ul for saliva stimulated with metal (Hu 2005, Omur-Ozbek et al. 2012). These variations in protein concentration may be due to differences in saliva collection and protocol, such as time sample was collected after eating, and centrifugation that could lead to loss of proteins. The mean concentration did not vary by much from sample to sample resulting in no statistical differences; the mean protein concentration of all samples ranged from 1.24 ug/ul to 1.88 ug/ul.

Previous research has shown a significant decrease in the flow rate of saliva and protein concentration observed after different stimuli, however we did not see a significant decrease in our protein concentration (Neyraud 2009, Dawes 1984). Another study found that there was no significant difference in protein concentration levels in samples treated with copper (Hong 2009). The researchers concluded that this is because the copper levels were too low to have any effect on protein concentration. This may be true for the iron concentrations used in this study as well.

**Oral Lipid Oxidation**

We expected that there would be an increase in TBARS from baseline saliva samples compared to the saliva collected after metallic flavor induction. The iron (ferrous sulfate) in our study induces lipid oxidation in the oral cavity and should yield higher TBARS values (Mirlohi et al. 2011; Omur-Ozbek et al. 2012). However, we did not find any statistical differences among TBARS values or change in TBARS.
The range for TBARS for the baseline saliva samples is larger than previous data in a study that found the average was 0.12 µM, however our study used a higher concentration of ferrous sulfate (180uM, our study used 99.2 µM) (Omur-Ozbek et al 2012). Another study that used the same concentration of iron that we used found the range to be 0.011 to 1.24 µM (Mirlohi et al. 2011, Omur-Ozbek et al. 2012). Both of these studies found that there were significant differences in TBARS from baseline saliva to saliva collected after metallic stimuli were used. Five out of the seven saliva samples from our study showed an increase in TBARS from baseline to the subsequent ferrous sulfate sample, and five out of the eight samples had an increase in TBARS from the lactoferrin sample to the subsequent ferrous sulfate sample. Replicates would improve our range and variability of observed in our study. It may be possible to provide a better relative evaluation by normalizing the protein concentration across all panelists first, then calculating TBARS based on the normalized protein levels. This would account for variability in sample collection protocols.

**Analysis of Salivary Proteins Using 2DGE**

We were successful in using a reliable protocol for ongoing research concerning changes in overall salivary proteome. In comparing individual gels and based on our literature review, it is possible that metallic flavor perception may vary among individuals based on variability in one’s salivary proteome. Additionally, based on literature searches, metal-binding proteins are likely involved in these changes (Hong et al. 2009a). We were able to identify cystatin and their precursors and lactoferrin in our gels, which are metal-binding proteins and may be involved in metallic flavor perception; these proteins are discussed further in the next section. Due to limitations of this study,
quantitative results were challenging to obtain without replicates. Further research is needed to confirm that these proteins are responsible for perception of metallic flavor.

The human whole saliva proteome is complex and typically only the most abundant protein spots are detected and identified using mass spectrometry (Hirtz 2005). In our study, it is evident that replication would improve the ability to gain more quantitative results. Our study has indicated that a clean, clear gel image is difficult to obtain and has been noted in other studies as well (Fleissig et al. 2010). In our study, we used 11 cm gels, whereas larger format would provide improved resolution of protein spots (Hirtz 2005). Numerous factors can affect the quality of a protein gel produced starting from the beginning with handling as described in a review by Schipper et al. (2007). In this study, the effects of handling were observed in the resulting protein gels in preliminary experiments. Thus, care was taken in producing a sound protocol to prevent degradation by pre-chilling collection tubes, collecting on ice, immediate storage, and minimal freeze-thaw cycles. There was continued care in handling the sample properly for producing gels such as reducing the viscosity, which removes many mucin proteins that are a large portion of saliva. We confirmed that this increased clarity in preliminary runs including standards that indicated that highly glycosylated proteins, such as mucins, lactoferrin, amylase, and albumin, do reduce the clarity of gel images by creating smeared regions seen on gel images.

The many variations in overall protein profiles of saliva as observed in this study can lead to a challenging analysis to determine potential biomarkers for metallic flavor perception. We found that when comparing the protein gels from baseline samples provided by panelists, that each panelist had a some unique protein patterns such as a
more heavy spot of mucin compared to others. Many salivary proteins undergo post-translational modifications, sometimes due to gene polymorphisms, which results in altered protein structure (Hardt 2005; Messana et al. 2008). These altered proteins may also travel differently in a protein gel, leading to variation in gels due to different movement through the gel that could cause streaking or different pattern observed (Hirtz 2005); the observed molecular weight and pI have altered from the theoretical value by 10%. However, as in our study, the researchers report that there is a consistent pattern, but the complexity of saliva is also apparent in gel analysis. Replicates would reduce this variability and aid in confirmation of identity of protein spots. Current literature suggests that age, gender, genetic variation, and possibly different cancers or other diseases can cause alterations in protein expression or taste perception (Bartoshuk 2000; Fleissig et al. 2010; Zhang 2010). The variation that we observed in our gels may be a result of these factors.

Stress has been shown to cause changes in overall salivary protein concentration (Grigoriev 2003). In stress induced subjects, α-amylase, cystatin S and light chain IgA were increased and there were significant increases in glutathione S-transferase and prolactin inducible protein (Trueba 2012). In our study, everyday stress levels might lead to changes in the protein profile of individual saliva.

From current literature, we have found that taste and flavor perception is an intricate process that is not completely understood. Numerous receptors have been discovered, including receptors that specifically detect bitterness which is associated with metallic flavor (Chandrashekar et al. 2000; Hong et al. 2010; Riera et al. 2007). Those with altered taste perception who are negatively affected by metallic flavor, such as
cancer patients undergoing therapy, likely have altered taste receptors. It is known that cancer therapies can affect salivary glands leading to hyposalivation and taste dysfunction (Epstein 2010a). Alterations of taste receptors and salivary components can lead to failure of molecules to interact with receptors in the mouth. Our study shows that using 2DGE, we can observe some of these differences in saliva, but some of these differences may be due to variation in gels due to procedure or variation in individual saliva as discussed in this paper.

A biochemical approach to studying the transcriptome and how it is controlled would lead to further understanding of how and why proteins are expressed and secreted (Zhang 2010); this will allow us to understand if protein expression is changed by a particular molecule, genetic variation in individuals, or alterations due to medication or other treatment. Regulation of calcium ions in saliva was found to be controlled by ATP (adenosine triphosphate) levels (Park 2012). Activated channels that regulate fluid and ion secretion into the oral cavity have been found to be responsible for calcium levels found in saliva (Romanenko 2010). This may be a potential mechanism that releases metals that induce off-flavors in the oral cavity. Studying in-cell mechanisms could provide insight to how metals are regulated in saliva. This would further supplement our studies involving oxidation in the oral cavity, measurement of metals in saliva, and use of 2DGE to examine overall protein profiles.

Performing 2DGE analysis for saliva from cancer patients undergoing therapy would be particularly useful for determining which proteins have changed in abundance and may be responsible for patients’ altered taste perception. Current research has already discovered differences in healthy humans when compared to cancer patient saliva which
could potentially lead to a better understanding of increased metallic flavor perception during cancer therapies; these include several mRNA and proteins that are increased or decreased in abundance (Dowling et al. 2008; Zhang 2010).

Advanced options are available for protein analysis that can lead to improved results. The latest approach to biomarker discovery is the use of SELDI ProteinChip Array to observe differences in before and after treatments (Shintani 2010). Analysis of saliva of cancer patients before and after therapy using SELDI ProteinChip revealed that nine proteins had decreased expression and 17 proteins had increased in expression levels. Also more recently, the use of two dimensional- difference gel electrophoresis (Ettan™ DIGE) has improved reproducibility and quantitative analysis of protein spots in gels by using samples labeled using fluorescent dyes for mass and charge (Alban 2003). Quantification is improved with this technique because of the increase in sensitivity and reduced variability caused by experimental error; replicates are still necessary with this technique for statistical significance (Van den Bergh 2004). This method is advantageous for observing two to three samples for differences by using different dyes that are visible at different wavelengths. The dyes are also useful for observing differences caused by post-translational modifications. Proper training, equipment available, and funding can are an issue when choosing this method.

**Lactoferrin and Other Metal-Binding Proteins**

Lactoferrin protein, used as an adjuvant to reduce metallic flavor, was provided as a pre-rinse to ferrous sulfate to induce metallic flavor. As an iron-binding protein, lactoferrin is capable of binding ferric ions (Fe$^{3+}$). This binding potentially prevents the ferric ions from being reduced to the ferrous form (Fe$^{2+}$) that can induce lipid oxidation
Lactoferrin is present in human saliva at low-levels: 0.007 mg/ml to 0.01 mg/ml (Rodrigues et al. 2009). We were able to suggest that there is a lactoferrin protein spot observed on our gels using a fluorescent staining technique; however, with lactoferrin present at such low levels to start, it may be at a more dilute concentration in samples other than the baseline sample. It is also interesting to note that a lactoferrin protein spot could be found only in some gels, and sometimes not detected at all in protein gels produced from saliva samples obtained after sipping the lactoferrin rinse.

We are unsure if lactoferrin was still present at levels that would reduce metallic flavor since saliva was collected between the lactoferrin and ferrous sulfate rinse. If the lactoferrin we obtained for this experiment was fully saturated with iron, it would have been unable to bind more iron to reduce oral lipid oxidation in our study. Lactoferrin may have also became oversaturated in our study, as research has shown that the rate of oxidation increases as lactoferrin becomes unable to bind more iron molecules that participate in oxidative processes (Gutteridge 1981, Medina et al. 2002). Lactoferrin’s antioxidant capability and metal-binding is enhanced by phenolic compounds such as α-tocopherol and bicarbonate ions support the metal-protein complex (Anderson et al. 1989; Masson and Heremans 1968; Medina et al. 2002). We did not include these compounds in our lactoferrin rinse solution, but this may improve its ability to decrease oxidation by enhancing iron-binding. Previous research has suggested that copper-protein complexes may not be stable under heat or SDS, which may have caused precipitation and decreased detection of protein spots in our study (Hong et al. 2009b). Reduction of metallic flavor using metal chelators is explained further in Chapter IV.
Metallic taste perception has been shown to be affected by the protein gustin binding to zinc (Padiglia 2010). Conformational changes in the gustin protein structure were shown to alter zinc binding. This suggests that individuals who have diminished taste sensitivity likely express proteins that are unable to bind zinc. We have been unable to identify gustin in our protein gels due to lack of information in the literature; it would be interesting to find differences in saliva gustin concentration in individuals.

Research has found that there are four forms of transferrin that exist (Makey and Seal 1976). By using 1D-PAGE, researchers were able to separate a buffer mixture containing transferrin and were able to observe four forms present with some bound to iron. This may explain why we observe several spots or streaks that contain one protein. Transferrin is also difficult to detect in protein gels due to streaking caused by glycosylated mucins and alpha-amylase proteins.

There is also value to measuring OLO, as many proteins are involved in oxidation and binding to metals in saliva and may affect metallic flavor perception (Elias 2008, Mirlohi et al. 2011). Metal-binding proteins within saliva include lactoferrin, transferrin, ferritin, haptoglobin, hemopexin, albumin, and ceruloplasmin; these may bind metals and reduce perception of metallic flavor. These proteins may act as antioxidants that scavenge free radicals that would otherwise induce lipid oxidation. Mirlohi et al. (2011) also tells that byproducts of lipid oxidation may contribute to a metallic off-flavor sensation indicating lipid oxidation has occurred, regardless of TBARS measurement of lipid oxidation.

**CONCLUSIONS**
Using 2DGE and literature available, we have identified several metal-binding proteins present in saliva that may participate in prevention of oxidative reactions such as lactoferrin, transferrin, or cystatins. We also observed individual variation in human whole saliva proteome that may be responsible for changes in flavor perception. Metallic flavor stimuli did not show a significant effect on protein concentration. Measures of oral lipid oxidation before and after metallic flavor induction were not statistically significant, but indicated that there is an increase in oxidation levels after ferrous sulfate to stimulate metallic flavor. Increases in iron concentration were likely responsible for the slight increase in oral lipid oxidation, as measured by TBARS. Changing the concentration of lactoferrin to ferrous sulfate would allow us to further investigate the use of lactoferrin’s ability to bind iron and reduce metallic flavor.

By perfecting a protocol for detection of specific proteins, we can improve analysis to examine differences before and after treatments and provide input into the increasing use of 2DGE to detect protein biomarkers for metallic flavor perception or more. By exploring the use of 2DGE to examine overall protein profile or changes in protein profiles, we have found that there is potential for more research to further analyze salivary proteins and their interactions with metals in the oral cavity.

ACKNOWLEDGEMENTS

This work was supported by the Institute for Critical Technology and Applied Science at Virginia Tech, an integrated, internal competitive grant from the College of Agriculture and Life Sciences at Virginia Tech, and Fralin Life Sciences Institute. Special acknowledgement is given to all panelists for their participation. Thanks to Tina Plotka,
Food Science & Technology, for her assistance in sample collection and more. Thanks to Dr. Hengjian Wang, Food Science & Technology, for assistance with statistics. I am also very thankful to have had the support and technical knowledge of Susan Mirlohi, Civil and Environmental Engineering; Will Slade, Biological Sciences; and Kris Lee, Virginia Bioinformatics Institute.
REFERENCES


Hong JH, Duncan SE, O'Keefe SF, Dietrich AM. 2009b. Ultrafiltration as a tool to study binding of copper to salivary proteins. Food Chemistry. 113: 180-184.


Park HS. 2012. Regulation of Ca²⁺ release through inositol 1,4,5-trisphosphate receptors by adenine nucleotides in parotid acinar cells. Amer J Physiol, Gastrointestinal Liver Physiol 302: G97-G104.


Table 1. Salivary protein concentration (ug/ul)\(^1\) (and change in protein) in whole unstimulated saliva (baseline), and in saliva collected after a lactoferrin (1 µM) protein rinse and compared to protein concentration after a metallic stimulus (ferrous sulfate, 10 mg/L)

<table>
<thead>
<tr>
<th>Panelist</th>
<th>Saliva Collection 1</th>
<th></th>
<th></th>
<th>Saliva Collection 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Metallic 1</td>
<td>(\Delta^2)</td>
<td>Lactoferrin</td>
<td>(\Delta) Protein</td>
<td>Metallic 2</td>
</tr>
<tr>
<td>1</td>
<td>1.07</td>
<td>1.18</td>
<td>0.11</td>
<td>1.57</td>
<td>0.50</td>
<td>1.26</td>
</tr>
<tr>
<td>2</td>
<td>1.26</td>
<td>2.96</td>
<td>1.70</td>
<td>2.27</td>
<td>1.02</td>
<td>1.28</td>
</tr>
<tr>
<td>3</td>
<td>1.20</td>
<td>1.77</td>
<td>0.57</td>
<td>1.25</td>
<td>0.05</td>
<td>0.89</td>
</tr>
<tr>
<td>4</td>
<td>1.10</td>
<td>2.33</td>
<td>1.23</td>
<td>1.50</td>
<td>0.40</td>
<td>1.72</td>
</tr>
<tr>
<td>5</td>
<td>1.62</td>
<td>1.09</td>
<td>-0.53</td>
<td>2.60</td>
<td>0.98</td>
<td>2.10</td>
</tr>
<tr>
<td>6</td>
<td>0.55</td>
<td>0.58</td>
<td>0.03</td>
<td>2.25</td>
<td>1.70</td>
<td>2.85</td>
</tr>
<tr>
<td>7</td>
<td>1.46</td>
<td>1.50</td>
<td>0.05</td>
<td>0.04</td>
<td>-1.41</td>
<td>1.09</td>
</tr>
<tr>
<td>8</td>
<td>1.68</td>
<td>3.64</td>
<td>1.96</td>
<td>2.51</td>
<td>0.83</td>
<td>1.54</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>1.24 ± 0.36</td>
<td>1.88 ± 1.03</td>
<td>1.75 ± 0.85</td>
<td>1.59 ± 0.63</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1,2\) Whole saliva protein concentration was measured for each participant for each sample that was collected on two separate events: a baseline sample (4ml), followed by sipping ferrous sulfate (10mg/ml) to induce metallic flavor on day one, then after sipping a lactoferrin rinse (1µM), followed by ferrous sulfate. Participants sipped 2ml samples of ferrous sulfate and lactoferrin and collected a total of 4 ml, giving a dilution of one-half which is applied to each diluted sample. \(\Delta\) Protein is the change in protein concentration by subtracting the baseline measurement from the measurement for each treatment. Mean values for each sample set were evaluated using LSD; no statistical differences were found. SE: standard error.
Table 2. Oral lipid oxidation measured using the TBARS (Thiobarbituric Acid Reactive Substances Assay) method (and change in TBARS) of whole unstimulated saliva (baseline), and in saliva collected after a lactoferrin (1 µM) protein rinse and compared to protein concentration after a metallic stimulus (ferrous sulfate, 10 mg/ml)

<table>
<thead>
<tr>
<th>Panelist</th>
<th>Saliva Collection 1</th>
<th>Saliva Collection 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Metallic 1</td>
</tr>
<tr>
<td>1</td>
<td>0.48</td>
<td>2.89</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>0.15</td>
</tr>
<tr>
<td>3</td>
<td>0.12</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>0.20</td>
<td>1.55</td>
</tr>
<tr>
<td>5</td>
<td>0.07</td>
<td>0.26</td>
</tr>
<tr>
<td>6</td>
<td>3.48</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>1.91</td>
<td>0.99</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0.11</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>0.78 ± 1.27</td>
<td>0.85 ± 1.06</td>
</tr>
</tbody>
</table>

¹Oxidation levels in the oral cavity were measured using a modified TBARS method for saliva and shown in µM TBARS/g protein. TBARS was used to measure oxidation of each saliva sample for each participant that was collected on two separate events: a baseline sample (4ml), followed by sipping ferrous sulfate (10mg/ml) to induce metallic flavor on day one, followed by sipping lactoferrin (1uM), and after sipping ferrous sulfate. Participants sipped 2ml samples of ferrous sulfate and lactoferrin and collected a total of 4ml, giving a dilution of one-half for TBARS; measurements that are 0 were well below the detection limit of 0.125 µM TBARS. Mean values for each sample were evaluated using LSD, outliers were removed; there were no statistical differences found. Δ is the change in µM TBARS/g protein by subtracting the baseline measurement from each measurement. – missing data. SE: standard error.
Table 3. Concentration of iron (mg Fe/L) of Saliva \(^1\) (and change in iron) in whole unstimulated saliva (baseline), and in saliva collected after a lactoferrin (1 \(\mu\)M) protein rinse and compared to protein concentration after a metallic stimulus (ferrous sulfate 10 mg/ml)

<table>
<thead>
<tr>
<th>Panelist</th>
<th>Saliva Collection 1</th>
<th>Saliva Collection 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Metallic 1</td>
</tr>
<tr>
<td>1</td>
<td>1.100791</td>
<td>0.596481</td>
</tr>
<tr>
<td>2</td>
<td>0.308304</td>
<td>0.036183</td>
</tr>
<tr>
<td>3</td>
<td>0.147595</td>
<td>0.264923</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0.185494</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0.574014</td>
</tr>
<tr>
<td>6</td>
<td>56.37955</td>
<td>2.765053</td>
</tr>
<tr>
<td>7</td>
<td>0.02815</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>1.05157</td>
<td>0.087088</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>7.38 ± 19.81</td>
<td>0.64 ± 0.96</td>
</tr>
</tbody>
</table>

\(^1\)Salivary iron content was measured using MS-ICP (mass spectrometry-inductively coupled plasma). Iron was measured for each sample that each participant provided on two separate events: a baseline sample (4ml), after sipping ferrous sulfate (10mg/ml) to induce metallic flavor on day one, then after sipping lactoferrin, and after sipping ferrous sulfate. Participants sipped 2ml samples of ferrous sulfate and lactoferrin and collected a total of 4 ml, giving a dilution of one-half for iron content shown in table. Mean values for each sample were evaluated using LSD, outliers were removed; no significant differences were found between samples. Δ is the change in iron content determined by subtracting the baseline iron content from each measurement. – missing data. SE: standard error.
1. Secretory Component Precursors, Albumin
2. Transferrin or Transferrin Precursor
3. Lactoferrin
4. Mucin, alpha-amylase
5. Zinc proteins, actin β
6. Carbonate dehydrogenase v1 precursors
7. Glyceraldehyde-3-phosphatase
8. Immunoglobulins
9. Stratifin, Immunoglobulin J, proline rich proteins
10. Prolactin inducible proteins
11. Cystatin precursors
12. Cystatin precursors

**Figure 1. 2DGE Pattern of Unstimulated Whole Human Saliva Proteins.** Protein profile of a baseline saliva sample from panelist 1 with labeled regions of areas identified using mapped proteins identified in literature (Ghafouri 2003, Hu 2005, Huang 2004). Proteins were separated in the first dimension using 11 cm strips (non-linear (NL), pI 3-11), and then were separated using 12.5% SDS–PAGE and stained using Flamingo fluorescent stain.
**Figure 2. Saliva Collection Sequence.** Boxes represent saliva samples collected and analyzed: Baseline, Metallic 1, Lactoferrin, and Metallic 2. Arrows represent rinses or rest periods. Lactoferrin was prepared at 1μM and ferrous sulfate at 10 mg/ml (Sigma-Aldrich, St. Louis, MO). Panelist rested approximately 2 minutes between samples to recover salivary proteins and flow rate; each sample took approximately 15-20 minutes to collect.
Chapter IV. Comparison of Metal Chelators, Lactoferrin and EDTA, as Adjuvants for Reducing Metallic Flavor

ABSTRACT

Although an essential nutrient, iron in foods and beverages can cause an unpleasant and lingering metallic flavor. Metal chelators may be useful in reducing metallic flavor by binding iron in the mouth, and may assist in increasing iron bioavailability within the body. Ethylene diamine tetraacetic acid (EDTA) is a synthetic chelator used to bind metals in foods, thus providing antimicrobial and antioxidant functions. Lactoferrin, a naturally occurring protein found in human saliva and in milk, is a metal-binding protein that has several health benefits. Lactoferrin (1.0 µM) and EDTA (36 µM) samples were prepared in distilled water; a two-sided directional difference test (n=37; α = 0.10; β=0.20; p_{max}=0.70) was implemented to determine if either had a perceptible metallic flavor, compared to water and to each other. Lactoferrin had a perceptible metallic flavor (p=0.10) compared to water but not compared to EDTA; EDTA was not different than water. Lactoferrin and EDTA, when used as an oral rinse after tasting ferrous sulfate solution (99.2 µM), were both effective (p<0.05; n=6) at reducing metallic flavor intensity and duration, based on time intensity analysis. Time-intensity evaluation indicates that lactoferrin, EDTA, and water are similar (p<0.05) when comparing the mean for maximum intensity, area under the curve, the area post-maximum intensity, and total time duration as pre- and post-rinse. This study indicates that lactoferrin protein is a potential natural alternative to synthetic EDTA for reducing iron-induced metallic off-flavors.
INTRODUCTION

Undesirable metallic off-flavors are associated with products produced across several industries including foods, nutritionals, pharmaceuticals, personal care, and healthcare. Iron is an essential nutrient but creates undesirable metallic flavors and retronasal effects, due to oxidation of metals in the oral cavity (Epke 2007; Lawless 2004; Lawless 2005), which can be challenging in creating nutritious and stable products and for providing a safe, enjoyable water supply. Metal-binding compounds or chelators can aid in reduction of off-flavors produced by oxidation in foods (Nielsen, Petersen et al. 2004). Phytates and polyphenols can bind iron and are inhibitors of iron-uptake, whereas other iron-binding compounds like EDTA, ascorbic acid, and some other organic acids and amino acids have shown to increase bioavailability of iron; currently, deferoxamine, deferiprone, and deferasirox are commonly used metal-chelators that have a high affinity for iron (Miret, Van Buuren et al. 2008). Additionally, most metal-chelators have an optimum pH that allows the compound to bind and release iron to reduce oxidative effect, but also increase bioavailability throughout the body (Cho, Alamed et al. 2003; Nielsen, Petersen et al. 2004; Miret, Van Buuren et al. 2008).

Metallic flavor is generally described as bitter, astringent, or metallic and is experienced at low concentrations for iron and copper compounds (Lim and Lawless 2005; Yang and Lawless 2006; Epke 2007). Perception of metallic flavor persists at a high intensity longer and a longer duration than basic taste perceptions such as bitter, sour, salty, or sweet; bitter and astringent intensity, which are associated with metallic flavor from copper are also persistent over time (Yang and Lawless 2006; Hong, Duncan et al. 2010). Iron salts, such as ferrous sulfate, are known to have the highest intensities of
metallic perception (Lawless 2004; Omur-Ozbek and Dietrich 2011). Metallic flavor threshold for ferrous sulfate has been estimated at 99.2 µM or about 10 mg/L (Lim and Lawless 2005); more recently, the range of detection was observed at 0.007 mg/L Fe$^{2+}$ to 14.14 mg/L Fe$^{2+}$ (Mirlohi, Dietrich et al. 2011).

Foods containing polyunsaturated fatty acids are particularly affected by lipid oxidation and influenced by oxidizing metals that contribute to off-flavors (Medina, Tombo et al. 2002; Nielsen, Petersen et al. 2004). Iron fortification with ferrous sulfate and artificial sweeteners such as acesulfame-K, saccharin, and aspartame can cause metallic flavor or bitterness at certain concentrations (Hurrell 2002; Riera, Vogel et al. 2007). Additionally, potable water resources contain metals such as iron or copper that can cause metallic off-flavors (Dietrich 2006).

Metallic flavor is a negative side effect often experienced by patients undergoing medical treatment or those taking certain medications that cause changes in taste perception; this effect can lead to other complications such as decreased quality of life, appetite, and emotional state plus non-compliance of medication use as mentioned in a review by Doty (2008). A study found that 33% of individuals on prescriptions experienced altered taste perception and 57% experienced astringency (Shinkai, Hatch et al. 2006). About two-thirds of cancer patients experience taste defects due to treatment as reviewed by Hong, Omur-Ozbek et al. (2009). Another review by Porter, Fedele et al. (2010) reports that taste dysfunction is commonly experienced by head and neck cancer patients who received radiotherapy.

Ethylene diamine tetraacetic acid (EDTA) is a synthetic metal chelator that is commonly used in the food industry to preserve products; it binds and removes metals
that microbes must utilize for survival. It is commonly added to reduce metallic off-flavors, especially with foods that are fortified with iron (Miret, Van Buuren et al. 2008). Omur-Ozbek et al. (2012) confirmed that EDTA was able to reduce off-flavors induced by oxidation.

Lactoferrin is an iron-binding protein present at low levels in human saliva at 0.007 to 0.01 mg/ml and in other mammalian fluids such as cow’s milk at about 0.02 to 0.2 mg/ml (Rodrigues, Teixeira et al. 2009). This protein has potential to aid in reducing or preventing metallic off-flavors induced by oxidized iron compounds in the oral cavity (Omur-Ozbek 2012). Studies also have shown that lactoferrin has many other benefits such as antimicrobial activity, enhancing iron uptake, and protecting against cancer (Levay and Viljoen 1995; Lonnerdal 2009; Rodrigues, Teixeira et al. 2009).

Consumers desire products with natural ingredients that are functional for health (Miret, Van Buuren et al. 2008; Gruenwald 2009). Use of naturally occurring lactoferrin protein in products, as an alternative to synthetic EDTA, can fulfill these needs. However, sensory studies are necessary to provide information regarding replacement of ingredients to determine if lactoferrin provides similar or greater benefits as EDTA.

The main objective of this study was to compare lactoferrin and EDTA in reducing metallic off-flavor induced by ferrous sulfate as well as the potential of these iron-binding molecules to induce a metallic flavor. Additionally, we determined if a pre-rinse or post-rinse of either compound is optimal for reducing metallic flavor. And last, based on preliminary observations of metallic flavor induced by lactoferrin in some panelists, we determined if either of these compounds was capable of inducing metallic flavor.
MATERIALS AND METHODS

Perception of Metallic Flavor Induce From Lactoferrin or EDTA

Sample Preparation

Based on preliminary observations of metallic flavor induced by lactoferrin in some panelists, we evaluated the potential of lactoferrin and EDTA as sources of metallic flavor (Duncan et al. 2011). All samples were prepared daily to prevent degradation or precipitation of compounds in clean glassware, which was rinsed thoroughly beforehand to remove any residual minerals. The concentration of each compound was determined based on preliminary trials and previous studies (Omur-Ozbek et al. 2012); each compound was dissolved in distilled water (The Kroger Co., Cincinnati, OH, 45202) with agitation. Lactoferrin, from a nutritional supplement (Naturade Operating Corporation Symbiotics Lactoferrin Supplement, 250 mg per serving, purity unknown), was prepared to 1µM based on molecular weight of lactoferrin by breaking the gelatin capsules and weighing. EDTA was prepared at 36 µM and ferrous sulfate at 99.2 µM (10 mg/L) (Sigma-Aldrich F8048).

Sensory Testing

This sensory study was approved through Virginia Tech IRB #12-241. Panelists (n=37) were recruited from a general population from the Virginia Tech campus, with a high proportion of the panel being young to middle-aged adults. Participants completed directional difference tests in individual sensory booths equipped with touchscreen computer monitors under white light. Samples were presented at room temperature, based on previous studies (Omur-Ozbek et al. 2012).
Two sessions were held and panelists expectorated all samples to reduce biasing effects from carryover of lingering metallic flavor from sampling of ferrous sulfate; Samples (approximately 25 mL) were presented at room temperature in portion cups (2 oz.) identified with 3-digit codes. Prior to sample evaluation, panelists were familiarized with metallic sensation through identified reference samples. Each panelist initially rinsed their mouths with distilled water, which was identified as “not metallic,” and then with a ferrous sulfate, identified as “metallic.” After a rest period, panelists were presented with sample pairs, from which they identified the “more metallic” sample. The sequence of sessions was held constant across all panelists. In the first day, one pair was evaluated: water and lactoferrin. In the second day, two pairs were evaluated: water and EDTA, and lactoferrin and EDTA in a balanced order of presentation. Water was always presented first, as to not induce potential carryover of lingering aftertaste from lactoferrin or EDTA.

In each session, the panelists sipped and swished each sample in their mouth for 10 seconds before expectorating. A sensory computer system (Sensory Information Management System, SIMS, Morristown, NJ, vs 6, 2000) instructed the panelists throughout the testing sequence displayed on the computer monitor. Panelists chose the “more metallic” by marking the corresponding 3-digit code on a paper ballot.

**Data Analysis**

Data was analyzed by counting the number of responses for each sample. Statistical test parameters for the overall difference, which were chosen based on the number of panelists that were available for testing were set at $p_{\text{max}}=0.70$, $\alpha=0.10$, $\beta=0.20$ (Meilgaard, Civille et al. 2007); the $p_{\text{max}}$ was set based on a previous study that reported
75% of panelists were able to detect metallic flavor (Omur-Ozbek et al. 2012). In order to limit the Type II risk and have a relatively high power in the study, we decided to allow the alpha level to increase to 0.10, with 25 out of 37 responses needed for a significant result and for one sample as “more metallic.”

**Time Intensity of Ferrous Sulfate-Induced Metallic Flavor as Influenced by Pre- and Post-Oral Rinses with Lactoferrin and EDTA**

**Sample Preparation**

Sample preparation was as described previously except that lactoferrin was obtained from a chemical supplier (Sigma-Aldrich L476, ≥85% purity).

**Panelist Selection and Training**

This research study was approved by the Virginia Tech Institutional Review Board (IRB # 12-041). Panelists (n=6) that were able to detect metallic flavor in 10mg/L ferrous sulfate solution were recruited (aged between 40 and 70 years). Panelists over the age of 40 were recruited based on studies that suggests possible changes in metallic flavor perception as we age (Mirlohi et. al 2011). Panelists (4 female, 2 male) were all faculty and staff in the Virginia Tech Department of Food Science. For all sessions, panelists were asked to refrain from consuming food or beverage at least one hour prior to testing to reduce variability associated with other stimuli and improve detection of metallic flavor.

Panelists were trained for time-intensity data collection in several sessions. Initially, they were asked to identify and rate intensity of basic taste solutions to establish panelist sensitivity to basic tastes and introduce scaling concepts. Subsequently, panelists were introduced to ferrous sulfate solutions using various levels of stimuli (2.5 mg/L, 5
mg/L, and 10 mg/L; approximately 2 ml per sample, room temperature). During these sessions, panelists became familiar with the changes in perception associated with metallic flavor intensity and the effects of lingering aftertaste. Panelists were familiarized with time-intensity recording by following standard procedures for training (Meilgaard, Civille et al. 2007) by chewing cinnamon flavored gum and hand-recording intensity over a 120 second time period, followed by discussing results as a group with the panel leader.

The final training sessions, which were conducted in individual sensory booths using the touchscreen monitors and SIMS software, were used to validate panelist performance. Panelists recorded the intensity of metallic flavor induced by ferrous sulfate (99.2 µM, 10 mg/L) in three sessions. Individual panelist performance was evaluated using analysis of variance (ANOVA, JMP 9, SAS, Cary, NC) to compare performance against the entire panel for time-intensity results generated by SIMS (maximum intensity, area under the curve, and total duration of metallic flavor). All panelists completed additional training due to variance among all individuals for each parameter chosen; the panelists were asked to record the intensity of three different ferrous sulfate concentrations (as described above) in order to demonstrate their ability to differentiate and rate intensities. Discussions with panelists aided in design of the final protocol to ensure panelist understanding of the time-intensity protocols.

**Sensory Testing**

Panelists attended separate sessions to prevent fatigue and carryover of metallic flavor. Several sessions were held to evaluate and compare the use of the metal chelators, lactoferrin and EDTA, as both a pre- and post-rinse to metallic flavor induction with ferrous sulfate in comparison to a water rinse. Each combination of lactoferrin or EDTA
in sequence as pre- or post-rinse with ferrous sulfate solution, as metallic flavor stimulus, were performed in duplicate (n=6, Figure 1).

The panelists sipped the first sample provided (metal chelator pre-rinse or metallic flavor stimuli), swished for 10 seconds, expectorated, then immediately repeated the procedure with the second sample. Panelists began rating the perceived metallic flavor intensity after the second sample was in the mouth (expectorating after 10 seconds of evaluation) on a 15-point scale (15= highest intensity) by using their finger on the touch screen monitor. Data was recorded with each change in perception or at least every 10 seconds, whichever was shorter, over a 120 second duration. Time-intensity data were recorded using SIMS software.

**Data Analysis**

Time-intensity data was collected automatically and extracted from SIMS software. TI curves were generated for each individual panelist for each second over the 120-second time interval for each replication. Individual curves were analyzed for Imax, AUC, area post max (APostMax), plateau time, rate of decrease, Tdur, and time of maximum intensity (Tmax). Data for each parameter (6 panelists, 2 replications) were then used for statistical analyses as a way to compare the reduction in metallic flavor as a function of pre- or post-rinse sequence with the metallic stimuli and for chelator treatment (water (control), lactoferrin, EDTA). Statistical analysis for each parameter was completed using SAS software (SAS vs 9, Cary, NC) using the GLM procedure and analyzing means using least significant differences for each of the treatments (pre- and post-rinse for water, lactoferrin, and EDTA). An alpha of 0.05 was used for determining significant differences. A TI curve for each panelist for each stimuli sequence was
generated by averaging the individual responses and graphically illustrating the average response over time (Appendix B3). An average TI curve was generated by averaging the individual TI curves (Figure 2), to graphically illustrate the differences by treatment.

RESULTS

Results from the two-sided directional difference tests indicate that lactoferrin (from supplemental source) induced a detectable metallic sensation compared to water (p<0.10), but not in comparison to EDTA (p>0.10). EDTA was not found to induce a metallic sensation (p>0.10).

The time-intensity curves for all treatments are shown in Figure 2. Individual time-intensity results for each session have variability in maximum intensity and time and are shown in Appendix B3.

The most pertinent data was extracted from SIMS software and is included in Table 1. The averages for these parameters for all panelists for each session were used to create the data table; the average values for each post-rinse parameter were less than the average for each value for pre-rinse value. There were no significant differences observed for rate decrease, Tmax, or the plateau time. There were statistical differences found for the Imax, AUC, APostMax, and the Tdur. The first four parameters indicated significant differences between the pre-rinse and post-rinse groups, with water post-rinse similar to the both the pre- and post-rinse results for all other rinses. The Tdur showed significance between the same grouping, except for EDTA and water as a post-rinse, which was similar to both the pre-rinse and post-rinse groups.
The averages of the parameters for the post-treatments are less than the average for the pre-treatments when comparing treatment groups. The average and standard error for the pre-treatment parameters were 9.22 ± 4.06, 417.70 ± 244.72, 328.35 ± 182.56, 8.72 ± 7.92, -0.076 ± 0.04, 86.03 ± 30.13, and 12.56 ± 10.93 for Imax, AUC, APostMax, Plateau Time, Rate Decrease, Tdur, and Tmax, respectively. The average and standard error for the post-treatment parameters were 5.47 ± 4.47, 238.76 ± 296.82, 168.67 ± 205.41, 4.97 ± 4.71, -0.06 ± 0.10, 68.28 ± 40.14, 12.25 ± 18.82, for Imax, AUC, APostMax, Plateau Time, Rate Decrease, Tdur, and Tmax, respectively.

The post-rinse treatments appear to reduce metallic flavor intensity sooner than the pre-rinse (Figure 2). Pre-rinse treatments (lactoferrin and EDTA) had significantly higher mean values for Imax and AUC when compared to post-rinse treatments, with lactoferrin having the lowest intensity and area under the curve observed. Water as a pre-rinse was statistically similar to that of EDTA and lactoferrin as a pre-rinse, as were the post-rinses.

**DISCUSSION**

In preliminary studies performed to determine the concentration of lactoferrin use for metallic flavor studies, it was anecdotally suggested that lactoferrin might induce a metallic flavor. Preliminary studies with time-intensity testing revealed that some panelists detected a metallic flavor after a lactoferrin rinse, with one panelist experiencing an intense metallic sensation at about 10 (intensity scale of 0 to 15, 0=none, 15=high). This observation appeared to be unusual but, since there was no literature evidence that described if lactoferrin had any metallic flavor, we investigated this effect.
further. Based on the results of the directional difference test, lactoferrin may contribute to a metallic or metallic-like flavor in comparison to distilled water. Some authors suggest that there are no changes in sensory characteristics of foods containing lactoferrin because the flavor of lactoferrin is disguised by that of other ingredients (Nielsen, Petersen et al. 2004). It is also important to note that EDTA did not have a metallic flavor in comparison to water, therefore, making it a practical compound for use without contributing other off-flavors. There was no difference in metallic flavor when comparing lactoferrin and EDTA ($\alpha=0.10$, $\beta=0.20$); this is an interesting finding and indicates that lactoferrin has only a low level of flavor, and is comparable to EDTA. We must also recognize that lactoferrin for this study is from a supplement source, which may have been partially or fully saturated with iron, contributing to the potential or excess of iron. The supplement may have had peptides or other contaminants that may have contributed astringent or bitter sensations identified as metallic when compared to distilled water. The panelists were not trained for distinguishing metallic flavor from bitter or astringent sensations.

Variability in individual responses is influenced by genetics and is likely a factor in this study (Bartoshuk 2000). Some individuals are more sensitive to bitterness and intensity associated with metallic flavor, and others may not experience these sensations at all (Duffy 2000). This study had a fairly high power (80%; $1-\beta=0.20$) although the Type I error risk, of saying there was no difference between the chelating solution (EDTA) and the water, or between the two-chelating solutions (EDTA and lactoferrin), when there actually was a difference was higher ($\alpha=0.10$) than traditionally accepted ($\alpha=0.05$).
Lactoferrin as an oral rinse agent for reducing metallic flavor has exhibited similar, with potential for better, results compared to EDTA, when used after a metallic (iron) flavor stimuli. As expected, iron-induced metallic sensation was not affected when water was used as a pre-rinse agent, having a high intensity and lingering aftertaste, but water has some effective function when used after a metallic stimuli, in reducing metallic flavor, based on this study. A reduction in metallic flavor intensity also was observed when water was used as a post-rinse although not as effective as lactoferrin or EDTA (Omur-Ozbek et al. 2012). Time-intensity curves for the post-rinse samples show a lower initial intensity; this is due to the delay in data recording since metallic samples were presented first (Figure 1). However, the short delay after sipping did not appear to affect the results as there was a significant change in the intensity experienced even with the delay and is consistent in graphs of the pre-rinse treatments which were statistically similar. We did observe a slight delay within the first few seconds for all treatments, except lactoferrin post-rinse, which was likely due to panelists’ decision-making process to evaluate the intensity. When asked if any changes were observed in metallic taste after the second rinse, panelists noted that they observed an immediate decrease in metallic flavor after sipping a metal chelating post-rinse.

Although the statistical evidence for some parameters does not indicate a difference among the various post-rinse treatments, the collective comparison suggests that lactoferrin, when used as a post-rinse adjuvant, provides relief for metallic flavor. The lactoferrin post-rinse treatment had lower numeric values for AUC and Imax compared to the EDTA and water post-rinse treatments, although it was not statistically different. The area under the curve captures the initial intensity of the metallic flavor and
the rate of decrease. Although there was no statistical evidence that the time at maximum intensity was different in any of the treatments, the lactoferrin had the lowest Tmax (9.917 sec) compared to the highest for water post-rinse (17.083 sec). The total duration is more difficult to consider as the metallic flavor reaches its threshold and is more difficult for panelists to perceive metallic flavor. Numerous factors have been found to affect this detection limit such as nasal occlusion and genetic variability (Epke 2007).

Our study supports that metal-chelators are successful at diminishing metallic flavor that is likely the result of oxidation of metals and lipids in the oral cavity (Omur-Ozbek et al. 2012); additionally, our study indicates that a post-rinse containing a metal chelator significantly decreases metallic flavor compared to a pre-rinse, but is also similar to use of water as a post-rinse when comparing the mean values for AUC (Table 1). The average of all pre-treatments compared to the average of post-treatments were similar to that of each individual treatment; this indicates that there is not a notable difference between each use of EDTA, lactoferrin, or water as a pre-treatment or post-treatment.

Previous studies pertaining to metal-chelators as a post-rinse have been found to significantly reduce metallic flavor, but were not evaluated over a duration of time (Omur-Ozbek et al. 2012). Panelists (n=12 for EDTA and water, n=19 for lactoferrin, age= 19-37) EDTA (36 µM), lactoferrin (0.13 µM), and water were evaluated for metallic flavor on a scale from 0 (none) to 12 (strongest flavor) as pre- and post- rinses to 18 µM ferrous sulfate; the researchers found that the use of a chelating post-rinse was able to decrease metallic flavor significantly. Although the panelists are in a younger age bracket and they were not asked to evaluate over time, our study supports that using a
chelator as a post-rinse treatment is more effective than as a pre-rinse treatment. We also used a much higher concentration of ferrous sulfate (99.2 µM).

In our study, we have not included the intensity rating of metallic flavor without any rinse agent. In a previous time-intensity study, panelists (n=12, age= 21-58) rated the intensity of metallic flavor induced by ferrous sulfate (0.05M) on a 15 cm scale (Yang and Lawless 2006). The panelists initially rated the intensity as approximately 8; the researchers observed that the metallic flavor continued to persist at above a 4 on the scale until the 2 minute study was over. This research involves a much higher ferrous sulfate concentration and panelists were likely trained differently to rate this concentration of iron on a 15-point scale; however, there are similar results that there is a high initial intensity which decreases, but continues to persist over time.

The lactoferrin level (1.0 µM) in this study was chosen based on preliminary studies. For lower levels (at 0.25 µM), there was not a notable reduction in intensity of metallic flavor when used as a pre-rinse. Lactoferrin at 0.13 µM was determined to reduce metallic flavor as a post-rinse (Omur-Ozbek et al. 2012). When increased to 1.0 µM, the best results were observed with using minimal product, which is important when incorporating this as an ingredient in foods. Higher levels, around 12 µM, of lactoferrin have shown to increase the oxidation in foods (Nielsen, Petersen et al. 2004). With lactoferrin present at 0.13 µM, subjects have reported complete removal of metallic flavor within seconds of rinsing with lactoferrin (Omur-Ozbek et al. 2012). Based on Omur-Ozbek et al. (2012), we chose to evaluate EDTA at 36µM; however, literature reports that EDTA levels as low as 16µM have had notable effects in reducing metallic flavor (Nielsen, Petersen et al. 2004). Other antioxidants such as vitamins E and C have
been evaluated for use as reducing metallic flavor, but have been unsuccessful. The lactoferrin obtained for this experiment may have also been fully saturated with iron, preventing it from binding additional iron molecules to aid in reduction of metallic flavor perception.

There are numerous factors that may affect the detection of metallic flavor including age, genetic variability, and interactions within the oral cavity (Bartoshuk 2000; Hong, Duncan et al. 2010). These factors may affect the variability in individual time-intensity curves and variability from session to session. All panelists in our study claimed to have dental fillings or other metal devices in the mouth, which also may affect their sensitivity to metallic flavor. Metallic flavor is known to be sensed retronasally, but there are indications that saliva and receptors in the oral cavity also play a role in taste and flavor perception (Lim and Lawless 2005; Epke 2007).

Previous studies concerning metallic flavor induced by copper have indicated that pH also affects intensity over time due to solubility at different pH levels (Hong, Duncan et al. 2010); the oxidized form of iron, ferric, is much less soluble in water than its counterpart, ferrous (Nealson 1994). Lactoferrin can bind both ferrous (Fe$^{2+}$) and ferric (Fe$^{3+}$) ions, but reversibly binds ferric ions in synergy with carbonate (CO$_3^{2-}$) (Gonzalez-Chavez, Arevalo-Gallegos et al. 2009). We did not include carbonate ion in our study, but this may enhance lactoferrin’s capability of binding of ferric ions so that these will not participate in oxidation. Oxidized ferrous ions are responsible for inducing metallic flavor, unlike ferric ions that have been reported as having inducing no flavor or taste (Omur-Ozbek and Dietrich 2011). Further studies to investigate the roles of saliva and its
components, such as lactoferrin or other metal-binding proteins, in sensing metallic flavor can aid in forming a complete understanding of how to reduce metallic flavor.

Another study involving the use of chelators to reduce oxidation in emulsions report that the ratio of chelator: iron for EDTA 2:1 with EDTA at 62.8 µM and iron at 31.4 µM, meaning that in our study the ratio is approximately 1/3:1 (Nielson et al. 2004). Additionally, they report that the ratio of chelator: metal for lactoferrin is 2:1 for a lactoferrin concentration of 5.6 µM and iron content of 2.9 µM, meaning that the ratio in our study is much lower. With lactoferrin binding two molecules of iron and EDTA binding only one molecule of iron, at the ratios we used, these chelators may have become oversaturated with iron. This may explain why we did not see significant differences when comparing rinses with chelators to rinses with water. Increasing this ration in future studies could yield more accurate results.

Lactoferrin has been shown to be an excellent antioxidant within the body and incorporated into foods (Nielsen, Petersen et al. 2004). Our study indicates potential to inhibit oxidation in the oral cavity that leads to metallic off-flavors. Lactoferrin has many other benefits including use as an antimicrobial and to increase bioavailability of iron (Nuijens, van Berkel et al. 1996; Gonzalez-Chavez, Arevalo-Gallegos et al. 2009; Lonnerdal 2009; Rodrigues, Teixeira et al. 2009).

CONCLUSIONS

This research supports that lactoferrin has similar effects to that of EDTA in reducing metallic flavor, and may be an excellent natural replacement in some applications. However, lactoferrin itself may induce a low-level of metallic or metallic-
like sensation, and industry applications also must consider the effects of pH, solubility, and heat sensitivity of this protein. This study does not definitely conclude that lactoferrin contributes a metallic flavor, but using supplements as an adjuvant for reducing metallic flavor may not be as effective in reducing metallic flavor. Our study concludes that a post-rinse was most useful in reducing metallic flavor, but water as a post-rinse was statistically similar when comparing the mean values for AUC. However, industry will likely continue to seek natural options, such as lactoferrin, for incorporation into their product. Lactoferrin has additional benefits such as antimicrobial activity and increasing bioavailability of iron. Future studies pertaining to metallic flavor perception could lead to better understanding of the mechanisms in the oral cavity and how lactoferrin could be used in other systems besides water.

ACKNOWLEDGEMENTS

This work was supported by the Institute for Critical Technology and Applied Science at Virginia Tech, an integrated, internal competitive grant from the College of Agriculture and Life Sciences at Virginia Tech, and Fralin Life Sciences Institute. Special acknowledgement is given to all panelists for their participation. Thanks to Tina Plotka, Food Science & Technology, for her assistance in setting this project up. Thanks to Dr. Hengjian Wang, Food Science & Technology, for assistance with statistics. Also, thanks to all who helped me to prepare for each sensory session.
REFERENCES


Hong JH, Duncan SE and Dietrich AM. 2010. Effect of copper speciation at different pH on temporal sensory attributes of copper. Food Qual Pref. 21:132-139.


Table 1. Time-Intensity Parameters for Iron-Induced (Ferrous Sulfate 99.2 µM, 10 mg/L) Metallic Flavor Perception as Affected by Pre-Rinse and Post-Rinse with 1.0 µM Lactoferrin, 36 µM EDTA, and Distilled Water

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Imax (mean ± SE)</th>
<th>AUC (mean ± SE)</th>
<th>APostMax (mean ± SE)</th>
<th>Plateau Time (mean ± SE; seconds)</th>
<th>Rate Decrease (mean ± SE)</th>
<th>Tdur (mean ± SE; seconds)</th>
<th>Tmax (mean ± SE; seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactoferrin Pre-Rinse</td>
<td>9.13^b ± 3.96</td>
<td>428.70^a±286.39</td>
<td>358.43^a±216.39</td>
<td>7.50 ±4.19</td>
<td>-0.07 ± 0.03</td>
<td>87.58^a ± 32.31</td>
<td>9.91 ± 8.92</td>
</tr>
<tr>
<td>Lactoferrin Post-Rinse</td>
<td>5.10^b ± 3.50</td>
<td>175.01^b ± 206.80</td>
<td>151.23^b ± 184.77</td>
<td>5.50 ± 4.50</td>
<td>-0.03 ± 0.03</td>
<td>67.67^bc ± 42.07</td>
<td>9.50 ± 10.13</td>
</tr>
<tr>
<td>EDTA Pre-Rinse</td>
<td>9.35^c ± 3.86</td>
<td>391.07^a±218.28</td>
<td>301.94^a±149.79</td>
<td>9.25 ±9.13</td>
<td>-0.07 ± 0.03</td>
<td>84.17^abc ± 32.69</td>
<td>11.92 ± 9.69</td>
</tr>
<tr>
<td>EDTA Post-Rinse</td>
<td>5.23^a ± 4.59</td>
<td>235.70^a±300.62</td>
<td>180.07^a±221.53</td>
<td>4.58 ±3.73</td>
<td>-0.04 ± 0.05</td>
<td>75.00^abc ± 40.31</td>
<td>10.17 ± 11.21</td>
</tr>
<tr>
<td>Water Pre-Rinse (Control)</td>
<td>9.18^a±4.69</td>
<td>433.33^a±244.10</td>
<td>324.66^a±187.13</td>
<td>9.42 ±9.81</td>
<td>-0.08 ± 0.16</td>
<td>86.33^abc ± 26.76</td>
<td>15.92 ± 13.34</td>
</tr>
<tr>
<td>Water Post-Rinse (Control)</td>
<td>6.08^a±5.43</td>
<td>305.56^a±371.33</td>
<td>174.73^a±224.78</td>
<td>4.83 ±6.00</td>
<td>-0.09167 ± 0.17</td>
<td>62.17^a±40.51</td>
<td>17.08 ± 28.64</td>
</tr>
</tbody>
</table>

a,b,c Different subscripts indicate significant differences between treatments. The mean values for each measure listed in the table were compared using LSD (n=6, p<0.05): maximum intensity (Imax), area under the curve (AUC), area post max (APostMax), plateau time, rate of decrease, total time duration (Tdur), and time of maximum intensity (Tmax). Imax is based on a scale of 0 to 15 (0=none, 15=high). Time is in seconds.
Table 2. Comparison of Metallic Flavor Perception Using Paired Comparison for Water (Control), Lactoferrin (1 µM), and EDTA (36 µM)

<table>
<thead>
<tr>
<th></th>
<th>Number of Responses</th>
<th>Number of Responses Needed For Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Lactoferrin*</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>EDTA</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>EDTA</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

*Significant; $\beta = 0.20$, $p_{\text{max}} = 0.70$, $p<0.10$, $n=37$. 
Figure 1. Time-Intensity Panelist Procedure. Sequence of time-intensity sample collection. Panelists were asked to refrain from eating or drinking one-hour before data collection and did not rinse with water during sampling. Data collection began immediately after expectorating the second sample. Panelists were asked to rate intensity as it changed or every 10 seconds, whichever came first for a total of 120 seconds. Intensity was rated on a 15-point scale (0=none, 15=high).
Figure 2. Time-Intensity Curves of Mean Intensity Values of Iron-Induced Metallic Flavor for All Panelists per Second for Each Treatment. Comparison of time-intensity curves generated from mean values of intensity per second for all panelists (n=6, 2 repetitions averaged together). For pre-rinse treatments, panelists sipped and swished either lactoferrin (1.0 µM), EDTA (36 µM), or distilled water for 10 seconds before expectorating and sipping and swishing a ferrous sulfate sample (99.2 µM, 10 mg/ml) for 10 seconds and expectorating. Panelists began rating metallic flavor intensity on a 15-point scale (0=none, 15=high) immediately after expectorating the ferrous sulfate sample and continued to rate as intensity changed or every 10 seconds, whichever was first, for a total of 120 seconds. For post-rinse treatments, the panelists tasted the ferrous sulfate sample first and then the treatment, giving a delay of about 20 seconds after the iron-inducing sample.
Chapter V. In Search of Salivary Biomarkers for Metallic Flavor Perception in Cancer Patients and Healthy Humans

ABSTRACT

Metallic flavor is produced by a chemical reaction in the mouth and its occurrence may be influenced by salivary constituents. Cancer patients experiencing this unpleasant side effect may suffer malnutrition due to loss of appetite and loss in quality of life. Saliva was collected from cancer patients with primary malignant gliomas during treatment cycle and grouped according to taste and smell abnormalities (TSA) throughout treatment. Healthy subject saliva was collected before and after induction of metallic flavor with ferrous sulfate. Protein concentration and presence in saliva was compared between sample sets using two dimensional gel electrophoretic methods. Five-fold differences were found between comparisons of “high responders”, “low responders”, healthy patients, and healthy patients exposed to metallic flavor ranging from 69 to greater than 100 differences; only 18 differences were found when comparing the healthy patient pooled (water vs. ferrous) saliva. Several unique proteins were found only in healthy saliva compared to cancer patient saliva and vice versa. These differences indicate that saliva protein composition is possibly affected by cancer treatment as well as by exposure to iron and may play a role in metallic flavor perception. This will lead to development of solutions to reduce or prevent metallic taste defects for cancer patients and others who experience the negative effects of changes in metallic flavor perception.
INTRODUCTION

Taste and flavor perception is a complex mechanism involving salivary components, taste receptors in the oral cavity, retronasal effects; alterations in taste or flavor perception can be an undesirable and serious side-effect of medications or treatments. Research has indicated that many factors affect taste and smell such as age, environmental exposure, medications, head trauma, neurological diseases, and cancer treatment (Bigeleisen 1999; Bromley 2000; Doty 2008; Epstein 2010; Hong et al. 2009; Mirlohi et al. 2011).

Taste and odor disorders are experienced by about two-thirds of cancer patients undergoing treatment (Hong et al. 2009). Patients with head or neck cancers most commonly suffer from taste and smell disorders (Porter et al. 2010). These undesirable conditions may lead to malnutrition, anxiety, or reduced quality of life (Hong et al. 2009; Hutton 2007). There has been minimal research to aid in preventing or reducing taste and odor issues.

Metallic off-flavors are of particular concern for food, water, pharmaceutical, and health industries. Cancer patients undergoing therapy typically describe a metallic or chemical taste (Epstein 2010). Most patients are advised to attend counseling or use therapies such as zinc supplement to help reduce the side-effect, however, most therapies have not been sufficiently tested and are often unsuccessful or inconvenient such as nasal occlusion or chewing gum (Epstein 2010; Hong et al. 2009; Logan 2008).

Metals can induce lipid oxidation in foods or the oral cavity resulting in metallic flavor sensation (Epke 2007; Lawless 2005). Ferrous sulfate has shown to cause an increase in oral lipid oxidation in the oral cavity and can be measured using a modified
TBARS (thiobarbituric acid reactive substances assay) method (Mirlohi 2012). Metallic sensations are not only experienced in the oral cavity, but also are perceived retronasally; metallic flavor is difficult to remove, leaving an unpleasant, lingering aftertaste (Epke and Lawless 2007; Yang and Lawless 2006). The relationship of production of oxidized metals and perception of metallic flavor is not completely understood at this point.

Studying the human salivary proteome will aid in further understanding the process of metallic perception in the oral cavity. Two-dimensional gel electrophoretic methods can provide a closer examination of the overall salivary proteome and allow for detailed observation in the changes that may occur after metallic flavor is induced. Salivary protein research has led to the discovery of several biomarkers that indicate presence of cancer in the body and is also being used to monitor the overall health and well-being of the body (Schipper et al. 2007). Iron-binding proteins such as lactoferrin or transferrin are likely to be involved in metallic flavor perception.

Studies have shown that in cancer patients reporting chemosensory changes, more than half of those patients reported changes in taste and odor perception, with more sensitivity to bitter and sour tastes (Hutton 2007). Chemosensory changes are directly related to quality of life and enjoyment of food. Malnutrition is a severe concern for patients experiencing chemosensory changes (Hong et al. 2009; Hutton 2007). More research is needed in this area to fully understand perception of off-flavors and odors to provide the best solutions. The primary goals of this project were to quantify taste and smell disorders in a small group of newly diagnosed primary malignant glioma patients primary undergoing treatment, to assess the role of oxidative stress and salivary components, and to compare salivary components in cancer patients and healthy patients.
MATERIALS AND METHODS

Human Subjects

This study was approved by the Institutional Review Board at Virginia Tech and the Wake-forest School of Medicine; as described by Mirlohi (2012). In the brain tumor clinic at the Comprehensive Care Center of Wake Forest University, 22 patients (10 female) were recruited, with ages ranging from 20 to 79 years (median 60 years). The patients had to be 18 years of age or older, recently diagnosed with malignant brain tumor, anticipated combined modality therapy, and an expected survival of at least 6 months. Patients with extreme dry mouth syndrome that prevented them from producing at least 2 mL of saliva in 15 to 20 minutes, were HIV positive, or had any of the following diseases: untreated gastroesophageal reflux disease; uncontrolled diabetes; active oral infections; or evidence of active mucositis were excluded. All subjects read and agreed to participate by signing an informed consent form approved under the IRB protocols.

Cancer Treatment Plan and Saliva Collection Points

Cancer patients were undergoing a combined modality treatment (CMT), consisting of a standard radiation therapy (RT) and temozolomide (TEM) over 6 weeks and followed by adjuvant TEM for 5 days each month over a period of 6 months. Saliva was collected from the patients prior to CMT and 3, 6, 10, 18, and 30 weeks after treatment. Saliva was collected from one healthy subject at 0, 3, 6, 10, 18, and 30 weeks. At the beginning of treatment (baseline) and for every week saliva was collected, cancer patients were asked to complete a self-questionnaire assessing their taste and smell.
function as described by Mirlohi (2012). Patients were grouped based on existence of a taste or smell abnormality (TSA): 1) no TSA at baseline or during treatment, 2) no TSA at baseline but developed with treatment, 3) TSA at baseline and persisted throughout treatment. Saliva samples were pooled based on saliva that was available from patients with “high TSA” and “low TSA” based on the three TSA groups (Table 4).

Saliva Collection and Analysis

For a baseline sample, saliva was collected from subjects that had not consumed any food or beverage or smoked within 1 hour prior to testing. Subjects first rinsed their mouths with purified water (Aquafina®) and rested for 1 minute. The subjects then sipped 2 mL of water, swished for 20 seconds, then expectorated the sample and saliva into a clean test tube for a total of 4 mL. The subjects were given a short period to rest, then sipped 2 mL of 10mg/ L ferrous sulfate, swished in the mouth, then expectorated the sample and saliva for a total of 4 mL. Subjects wore nose clips during the ferrous sulfate sample to evaluate the retronasal component of metallic flavor perception. Saliva samples were frozen and stored immediately at -50°C.

Salivary Protein Analysis

Saliva collected at 0, 3, 6, 10, 18 weeks from one healthy subject (Virginia Tech, saliva collection procedure similar to that for cancer patients) was pooled, based on available sample remaining after other analyses were completed to yield two samples: “before” and “after” metal rinse (Table 4). TSA grouping were used to pool saliva from cancer patients at 6, 10, and 30 weeks without metal rinse into “high TSA” (n=10 0.5 mL samples from 6 patients) and “low TSA” (n=9 0.5 mL samples from 7 patients); “low” meaning having reported TSA less often throughout treatment or “high” considered as
having reported TSA more often throughout treatment. Table 4 includes details on age, gender, and cancer treatment of this pooled group.

The four pooled samples were each mixed in a 1:1 ratio with Laemmli buffer, boiled for 5 minutes, and placed in a centrifuge for 10 minutes at 3,000 g before using the final protein pellet for two dimensional gel electrophoresis (2DGE). Protein concentration was measured using 2D-Quant Kit (GE Healthcare 80-6483-56), then 50-80 ug were precipitated using the ReadyPrep 2D Cleanup Kit (BioRad 163-2130) following the protocol provided for each kit. Isoelectric focusing (IEF) was performed using Ready Strip 3-10NL IPG strips, 17 cm (BioRad 163-2009). Sodium dodecyl sulfate (SDS)-PAGE was performed using precast 20cm 12% SDS-PAGE gels (Jule, Inc. 12D1BXLC1G). The gels were stained with Flamingo fluorescent stain (BIO-RAD 1610491) and imaged using Typhoon Trio Imager (GE Healthcare). BIO-RAD PDQuest 8.0 was used to detect protein spots and observe notable differences between pooled saliva protein gels.

RESULTS

Saliva Proteome

Analyses of the whole saliva proteome via two-dimensional gel electrophoretic methods indicate some changes in protein presence and expression level. Based on comparisons to gels produced in published studies, areas of prolactin inducible protein, α-amylase, mucin, albumin, glyceraldehyde-3-phosphate dehydrogenase, immunoglobulins, and cystatin precursors were distinguishable on all gels of pooled saliva samples (Hu 2005; Huang 2004) (Figure 1).
When comparing all four protein gels, five-fold differences for presence and absence of proteins indicated numerous differences (Table 1). Boxed regions in Figure 1 point out areas where notable differences exist and these spots have been identified in previous studies (Hu 2005; Huang 2004). Closer analysis revealed more notable differences as highlighted in Figures 2 and 3, with quantities for spot intensities generated by software in Tables 2 and 3. When examining notable differences, quality scores for protein spots that were normalized were utilized in determining protein spots or regions that were potential changes in saliva or variation in gel quality.

**DISCUSSION**

Analysis with two-dimensional protein gel electrophoretic methods provided insight to how we can better understand oxidation within the oral cavity by observing presence and absence of proteins, changes in expression levels of proteins, and interaction of proteins with metals in the oral cavity. We were able to identify several regions of major protein groups in human saliva and several notable changes in spots shown in Figure 1, 2, and 3. Glyceraldehyde-3-phosphate dehydrogenase could change in quantity based on normal changes in saliva composition throughout the day as we consume food and beverage. Also, notable differences were found in regions with immunoglobulin chains, which could also vary by individual and could change with disease. The overall salivary proteome is known to vary based on gender, age, diet, time of day, or genetic variations and would affect our study that collected saliva over time and pooled samples from several subjects undergoing different treatments (Dawes 1984; Mandel 1974; Neyraud et al. 2009).
It is interesting to note differences among cancer patients and healthy patients; further examination of proteins in regions with notable changes in spot intensities may lead to discovery of protein biomarkers for metallic flavor perception or other issues cancer patients may experience (Tables 2 and 3, Figures 2 and 3). Statistical analysis could not be performed as this was a preliminary research project without replicates. Further research should include replicates of protein gels produced from several subjects and identification of proteins that are potentially involved in altered taste and flavor perception in both cancer patients and healthy subjects.

There is a clear indication that there are differences that exist in saliva composition of healthy subjects and cancer patients. Several protein spots were observed at differing quantities of spot intensities or were present or absent in either cancer patients or healthy subjects. Comparisons of protein profiles in 2D gels of saliva from cancer patients and healthy humans has revealed 52 unique proteins in only oral cancer patients and 29 unique proteins in only the healthy patient samples (Hu et al. 2008). Further analysis is needed to confirm that these are true differences or if these are differences based on individual variability. We have to consider that the samples used to produce protein gels were from pooled samples that were collected over a period of time, which can have an effect on salivary protein composition mentioned earlier.

In Figures 2 and 3, we observed several differences when comparing protein gels produced from healthy human saliva and cancer patients. This suggests that there are differences that may exist between healthy human saliva and cancer patient saliva that may play a role in metallic flavor perception. There is also an indication (Figure 3), that some protein presence or perhaps an interaction exists after metallic flavor induction.
**CONCLUSION**

We have found that differences in the overall human saliva proteome may exist between healthy human subjects and cancer patients by using 2DGE and measure of OLO. Further studies pertaining to human metallic flavor will lead to better understanding of the role of proteins in taste and smell perception as we narrow our understanding of protein and metal interaction in the mouth. This will be especially applicable to developing a therapy for reducing metallic flavor for cancer patients, who suffer the most from taste and smell disorders. Investigation of naturally occurring proteins that bind metal and act as antioxidants to reduce lipid oxidation such as lactoferrin could lead to potential therapy for this side-effect.

**ACKNOWLEDGEMENTS**

This work was supported by the Institute for Critical Technology and Applied Science at Virginia Tech and the Comprehensive Cancer Center of the Wake-Forest School of Medicine for funding support. Special acknowledgement is given to all the human subjects for their participation. Also, thanks to the Oncology staff at the Wake-Forest University School of Medicine for collection of saliva samples and sensory data collection and Virginia Tech research assistant, Tim Smiley and REU fellow, Mr. Shannon Flynn for laboratory support. Also, thanks to Susan Mirlohi at Virginia Tech, whose dissertation project is supplemented by this additional information, and Kris Lee at Virginia Bioinformatics Institute at Virginia Tech for support in completing the two-dimensional gel analysis of this project.
REFERENCES


Figure 1. 2DGE of Pooled Unstimulated Whole Saliva Sample from Healthy Human Subject. Protein profile of a pooled healthy human subject saliva sample from weeks 0, 3, 6, 10, and 18 collection points. Labeled regions were identified using mapped proteins from literature sources (Hu 2005, Huang 2004). Proteins were separated in the first dimension using 17 cm strips (NL pI 3-10) and in the second dimension using 12% SDS-PAGE.

1. Secretory component precursors
2. Alpha-amylase, mucins, albumins
3. Glyceraldehyde-3-phosphate dehydrogenase
4. Ig Kappa Chains
5. Prolactin inducible proteins
6. Cystatins and their precursors
Table 1. Number of Differences in Protein Spots with 5-fold Differences Found in Comparisons of Groups

<table>
<thead>
<tr>
<th>Matchsets Compared</th>
<th>Number of Differences in Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCLR-CCHR</td>
<td>68</td>
</tr>
<tr>
<td>CCLR-HC</td>
<td>81</td>
</tr>
<tr>
<td>CCHR-HC</td>
<td>108</td>
</tr>
<tr>
<td>CCLR-HM</td>
<td>100</td>
</tr>
<tr>
<td>HC-HM</td>
<td>18</td>
</tr>
</tbody>
</table>

The number of differences of each pair were found using BIO-RAD PDQuest 8.0 Software (Hercules, CA). CCHR: Cancer Control High Responder, CCLR: Cancer Control Low Responder, HC: Healthy Control, HM: Healthy after metallic flavor induced with ferrous sulfate (10 mg/ml). High Responders were defined as having taste and smell abnormalities (TSA) more often, low responders were defined as having TSA less often.
Table 2. Normalized Quantities of Spot Intensities Generated in Software for Protein Spots Shown in Figure 2.

<table>
<thead>
<tr>
<th>Spot Number</th>
<th>CCHR</th>
<th>CCLR</th>
<th>HC</th>
<th>HM</th>
</tr>
</thead>
<tbody>
<tr>
<td>4503</td>
<td>0</td>
<td>0</td>
<td>561</td>
<td>902</td>
</tr>
<tr>
<td>4504</td>
<td>845</td>
<td>0</td>
<td>1107</td>
<td>1188</td>
</tr>
<tr>
<td>4506</td>
<td>0</td>
<td>0</td>
<td>1134</td>
<td>1258</td>
</tr>
<tr>
<td>5402</td>
<td>0</td>
<td>0</td>
<td>672</td>
<td>719</td>
</tr>
<tr>
<td>5501</td>
<td>0</td>
<td>0</td>
<td>1200</td>
<td>1342</td>
</tr>
<tr>
<td>5507</td>
<td>0</td>
<td>0</td>
<td>800</td>
<td>643</td>
</tr>
</tbody>
</table>

Quantity output was generated using BIO-RAD PDQuest 8.0 (Hercules, CA). Quantities that are 0 are undetectable in those gels. Findings indicate that protein gels produced from cancer patients have several undetected spots when compared to those produced from healthy patient saliva. Protein spots are shown in Figure 2. CCHR: Cancer Control High Responder, CCLR: Cancer Control Low Responder, HC: Healthy Control, HM: Healthy after metallic flavor induced with ferrous sulfate (10 mg/ml). High Responders were defined as having taste and smell abnormalities (TSA) more often, low responders were defined as having TSA less often.
Figure 2. Visual Comparisons of Protein Spots from Gels Produced from All Groups. Gel images were analyzed using BIO-RAD PDQuest 8.0 software (Hercules, CA). Spots in the area shown is between pI ~ 8-10 (horizontal) and MW~60 kD (vertical). Quantities for each spot are shown in Table 2. CCHR: Cancer Control High Responder, CCLR: Cancer Control Low Responder, HC: Healthy Control, HM: Healthy after metallic flavor induced with ferrous sulfate (10 mg/ml). High Responders were defined as having taste and smell abnormalities (TSA) more often, low responders were defined as having TSA less often.
Table 3. Normalized Quantities of Spot Intensities Generated in Software for Protein Spots Shown in Figure 3.

<table>
<thead>
<tr>
<th>Spot Number</th>
<th>Normalized Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCHR</td>
</tr>
<tr>
<td>3507</td>
<td>4387</td>
</tr>
<tr>
<td>3606</td>
<td>5513</td>
</tr>
<tr>
<td>4608</td>
<td>6668</td>
</tr>
</tbody>
</table>

Quantity output was generated using BIO-RAD PDQuest 8.0 (Hercules, CA). Quantities that are 0 are undetectable in those gels. Findings indicate that protein gels produced from saliva of healthy subjects after metallic flavor induction have several undetected spots when compared to those produced from cancer patient saliva. Protein spots are shown in Figure 3. CCHR: Cancer Control High Responder, HM: Healthy after metallic flavor induced with ferrous sulfate (10 mg/ml). High Responders were defined as having taste and smell abnormalities (TSA) more often, low responders were defined as having TSA less often.
Figure 3. Close Comparison of Visually Noticeable Differences in Spots from Gels Produced from High-Responding Cancer Patients (CCHR) and Healthy Subjects Saliva After Metallic Flavor Induced. Gel images were analyzed using BIO-RAD PDQuest 8.0 software (Hercules, CA). The approximate pI is 9-10 (horizontal dimension) and MW ~75-80 (vertical dimension). Quantities for each spot are shown in Table 3. CCHR: Cancer Control High Responder, HM: Healthy after metallic flavor induced with ferrous sulfate (10 mg/ml). High Responders were defined as having taste and smell abnormalities (TSA) more often, low responders were defined as having TSA less often.
<table>
<thead>
<tr>
<th>Protein Gel Group</th>
<th>Subject ID</th>
<th>Week Saliva Collected</th>
<th>Age</th>
<th>Gender</th>
<th>TSA Group</th>
<th>Cancer Treatment</th>
<th>Amount of Saliva Pooled</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>H001</td>
<td>0</td>
<td>42</td>
<td>F</td>
<td>1</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1ml</td>
</tr>
<tr>
<td>HM</td>
<td>H001</td>
<td>0</td>
<td>42</td>
<td>F</td>
<td>1</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1ml</td>
</tr>
<tr>
<td>CCLR</td>
<td>C003</td>
<td>6</td>
<td>55</td>
<td>F</td>
<td>2</td>
<td>CMT 0.5ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td>chemo 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C005</td>
<td>30</td>
<td>62</td>
<td>M</td>
<td>1</td>
<td>chemo 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C006</td>
<td>6</td>
<td>20</td>
<td>F</td>
<td>1</td>
<td>CMT 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td>chemo 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C012</td>
<td>30</td>
<td>42</td>
<td>M</td>
<td>3</td>
<td>chemo 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C014</td>
<td>30</td>
<td>58</td>
<td>M</td>
<td>1</td>
<td>chemo 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C020</td>
<td>10</td>
<td>50</td>
<td>F</td>
<td>1</td>
<td>post-CMT 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C021</td>
<td>6</td>
<td>72</td>
<td>M</td>
<td>2</td>
<td>CMT 0.5ml</td>
<td></td>
</tr>
<tr>
<td>CCHR</td>
<td>C007</td>
<td>30</td>
<td>65</td>
<td>F</td>
<td>2</td>
<td>chemo 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C008</td>
<td>6</td>
<td>79</td>
<td>M</td>
<td>3</td>
<td>CMT 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>post-CMT 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C011</td>
<td>6</td>
<td>75</td>
<td>M</td>
<td>3</td>
<td>CMT 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>post-CMT 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C013</td>
<td>6</td>
<td>54</td>
<td>F</td>
<td>2</td>
<td>CMT 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>post-CMT 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C023</td>
<td>6</td>
<td>46</td>
<td>F</td>
<td>2</td>
<td>CMT 0.5ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>post-CMT 0.5ml</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Demographic, Treatment, and TBARS Data for Each Panelist Represented in Pooled Saliva Samples for Protein Gels Produced. Saliva samples were chosen based on amount of sample available and grouped according to taste and smell abnormality. High Responders were defined as having taste and smell abnormalities (TSA) more often, low responders were defined as having TSA less often. HC= Healthy Control, HM= Healthy after induction of metallic flavor with ferrous sulfate (10 mg/L), CCHR: Cancer Control High Responder, CCLR= Cancer Control Low Responder. The week number defines how far in treatment cycle the patients were, and are also defined as chemo (chemotherapy), CMT (combined modality treatment), and post-CMT. The amount of each saliva sample that was combined in the final sample used for gel electrophoresis is also defined in the last column.
APPENDICES

Appendix A. Supplemental Information for Chapter III

A1. Approval Form and Informed Consent Form for Saliva Collection

Office of Research Compliance
Institutional Review Board
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, VA 24060
540/231-4606 Fax 540/231-0959
e-mail irb@vt.edu
website http://www.irb.vt.edu

MEMORANDUM
DATE: May 22, 2012
TO: Susan E Duncan, Sarah Lynn Fong, Kim M Waterman, Tiffany A Drape, Virginia C Fernandez-Plotka, Ellen Ewell, Kerri Katherine Martin
FROM: Virginia Tech Institutional Review Board (FWA00000572, expires May 31, 2014)

PROTOCOL TITLE: Use of Facial Recognition Software for Sensory Evaluation of Foods

IRB NUMBER: 10-156
Effective May 21, 2012, the Virginia Tech Institution Review Board (IRB) Administrator, Carmen T Green, approved the Amendment request for the above-mentioned research protocol. This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:
http://www.irb.vt.edu/pages/responsibilities.htm
(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:
Approved As: Expedited, under 45 CFR 46.110 category(ies) 6,7
Protocol Approval Date: February 23, 2012
Protocol Expiration Date: February 22, 2013
Continuing Review Due Date*: February 8, 2013
Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

**FEDERALLY FUNDED RESEARCH REQUIREMENTS:**
Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee. The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

IRB Number 10-156 page 2 of 2 Virginia Tech Institutional Review Board

Date* OSP Number Sponsor Grant Comparison Conducted?
* Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this IRB protocol is to cover any other grant proposals, please contact the IRB office (irbadmin@vt.edu) immediately.

Virginia Polytechnic Institute and State University

**Informed Consent for Participants in Research Projects Involving Human Subjects (Sensory Evaluation)**

**Title Project:** Time Duration of Metallic Flavor with Use of Facial Recognition Software for Application in Sensory Evaluation – **Phase 2**

**Investigators:** Susan E. Duncan, PhD, RD, Virginia Fernandez-Plotka, Ellen Ewell, Tiffany Drape, Sarah Fong

**I. Purpose of this Research/Project**
You are invited to participate in a study to evaluate the potential use of the facial recognition software, FaceReader and Observer, for applications pertaining to the sensory evaluation of foods.

Facial recognition software (FaceReader and Observer), designed to collect real time emotional response by videotaping facial features in response to
information or stimuli, has not been used previously for evaluating sensory response to foods. The intensity and duration of metallic flavor (ferrous sulfate) in water will be correlated with facial recognition software data. This activity will also demonstrate how the milk protein lactoferrin affects the perception and intensity of metallic flavor in water.

II. Procedures
There will be a total of four sessions, which will take place in Room 125 Food Science and Technology on four separate days to prevent sensory fatigue. Each session will last no longer than 15 minutes. On sessions 1 and 3, panelists will provide saliva samples before and after a pre-rinse and a ferrous sulfate sample are administered. On sessions 2 and 4, participants will be given the same pre-rinse/sample and their response will be video recorded during a period of four minutes. During the video recorded sessions, no saliva will be collected. Saliva samples collected during sessions 1 and 3 will be frozen and saved for TBARS and protein analysis.

III. Risks
There are only minimal risks associated with this study. Individuals with allergies to certain food components or sensitivity to iron may be at risk. Consumption of metals, specifically iron (in the form of ferrous sulfate), may have minimal risks, although the concentration used in this study (10 mg/L) is far below toxic levels. The Videotaping may cause concern to some individuals. Participants are able to withdraw from this study for any reason, including emotional stress resulting from being videotaped.

IV. Benefits
The potential to reduce flavor perception of metallic flavor and metallic flavor aftertaste with the use of lactoferrin, a milk protein, could be beneficial to those who are more sensitive to metallic flavors, including persons over the age of 40, cancer patients, and persons who are on prescription medications that are known to cause sensitivity to metals.

Your participation in this study will provide valuable information about the application of facial recognition software as a tool in sensory evaluation, which will be useful to the food and related consumer industries. If you would like a summary of the research results, please contact the researcher at a later time.

V. Extent of Anonymity and Confidentiality
The results of your performance as a panelist will be kept strictly confidential except to the investigator. Individual panelists will be referred to by a code number for data analyses and for any publication of the results.

If you are willing to permit the video data associated with your responses to foods to be used for demonstration purposes and/or production of a marketing video about this sensory application, please indicate so by checking the box below:
“By marking this box, I am giving permission for the researchers on this project to use video data associated with my responses during this project to be used for demonstration purposes and/or production of a marketing video about this sensory application.”

VI. Compensation
No direct compensation is provided for this activity.

VII. Freedom to Withdraw
If you agree to participate in this study, you are free to withdraw from the study at any time without penalty. There may be reasons under which the investigator may determine you should not participate in this study. If you have allergies to dairy products, or are under the age of 40, you are asked to refrain from participating.

VII. Subject’s Responsibilities
I voluntarily agree to participate in this study. I have the following responsibilities:

- Assist in adjustment of the video camera used to capture video footage for facial recognition evaluation.
- Taste the same prerinse/ferrous sulfate powder described in the Procedures section above and use the scale provided on the touch-screen monitors (SIMS software) to record metallic taste perception over a period of four minutes.
- Provide saliva samples and measure oral cavity pH before and after tasting the pre-rinse/ferrous sulfate sample described above. Saliva samples will be frozen for further analysis (protein and TBARS).

IX. Subject's Permission
I have read the Consent Form and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent:

Date: ________________________

Subject Signature: __________________________________________

Subject Printed Name: _________________________________________

- - - - - - - - - - For Human Subject to Keep - - - - - - - - - -
Should I have any pertinent questions about this research or its conduct, and research subjects’ rights, and whom to contact in the event of a research-related injury to the subject. I may contact:

Susan Duncan, Faculty/ Investigator (540) 231-8675; duncans@vt.edu

Virginia Fernandez-Plotka, Research Associate, Investigator (540) 231-9843; tplotka@vt.edu

Tiffany Drape, PhD Graduate Student, Agriculture and Extension Education Investigator (540)231-6836; tdrape@vt.edu

David Moore Chair, Virginia Tech Institutional Review Board for the Protection of Human Subjects (540) 231-4991; moored@vt.edu

Office of Research Compliance
1880 Pratt Drive, Suite 2006 (0497)
Blacksburg, VA 24061
### A2. F-Tables

#### Protein Concentration (panelist n=8)

**Full Model (treatments n=4)**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>5.129847</td>
<td>0.732835</td>
<td>1.38</td>
<td>0.2629</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>3</td>
<td>1.824759</td>
<td>0.608253</td>
<td>1.15</td>
<td>0.3523</td>
<td></td>
</tr>
</tbody>
</table>

**Change in Protein Full Model (treatments n=4)**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>7.504096</td>
<td>1.072014</td>
<td>1.63</td>
<td>0.2067</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>2</td>
<td>0.337408</td>
<td>0.168704</td>
<td>0.26</td>
<td>0.7773</td>
<td></td>
</tr>
</tbody>
</table>

**Protein Concentration Baseline vs. Metal 1**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>5.539594</td>
<td>0.791371</td>
<td>1.98</td>
<td>0.1941</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>1.632006</td>
<td>1.632006</td>
<td>4.08</td>
<td>0.0832</td>
<td></td>
</tr>
</tbody>
</table>

**Protein Concentration Baseline vs. Lactoferrin**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>3.033444</td>
<td>0.433349</td>
<td>1.02</td>
<td>0.4891</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>1.025156</td>
<td>1.025156</td>
<td>2.42</td>
<td>0.164</td>
<td></td>
</tr>
</tbody>
</table>

**Protein Concentration Lactoferrin vs. Metal 2**
<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>6.047</td>
<td>0.863857</td>
<td>3.26</td>
<td>0.0708</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>0.099225</td>
<td>0.099225</td>
<td>0.37</td>
<td>0.5599</td>
<td></td>
</tr>
</tbody>
</table>

**Change in Protein Concentration Lactoferrin vs. Metal 2**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>9.281544</td>
<td>1.325935</td>
<td>5.04</td>
<td>0.0245</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>0.100806</td>
<td>0.100806</td>
<td>0.38</td>
<td>0.5556</td>
<td></td>
</tr>
</tbody>
</table>

**Protein Concentration Metal 1 vs. Metal 2**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>3.212375</td>
<td>0.458911</td>
<td>0.46</td>
<td>0.8383</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>0.3364</td>
<td>0.3364</td>
<td>0.34</td>
<td>0.5808</td>
<td></td>
</tr>
</tbody>
</table>

**Change in Protein Concentration Metal 1 vs. Metal 2**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>3.7892</td>
<td>0.541314</td>
<td>0.54</td>
<td>0.7824</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>0.3364</td>
<td>0.3364</td>
<td>0.34</td>
<td>0.5805</td>
<td></td>
</tr>
</tbody>
</table>
TBARS Full Model (treatments n=4, panelist n=8)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>24.1775</td>
<td>3.4539</td>
<td>2.78</td>
<td>0.0344</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>3</td>
<td>0.368089</td>
<td>0.122696</td>
<td>0.1</td>
<td>0.9598</td>
<td></td>
</tr>
</tbody>
</table>

Change in TBARS Full Model

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>57.7811</td>
<td>8.2545</td>
<td>10.4</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>2</td>
<td>0.030588</td>
<td>0.015294</td>
<td>0.02</td>
<td>0.9809</td>
<td></td>
</tr>
</tbody>
</table>

TBARS Baseline vs. Metal 1

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>11.0768</td>
<td>1.5824</td>
<td>1.4</td>
<td>0.3478</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>0.16962</td>
<td>0.16962</td>
<td>0.15</td>
<td>0.7115</td>
<td></td>
</tr>
</tbody>
</table>

TBARS Baseline vs. Lactoferrin

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>13.922</td>
<td>1.988857</td>
<td>0.69</td>
<td>0.6809</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>0.161202</td>
<td>0.161202</td>
<td>0.06</td>
<td>0.8197</td>
<td></td>
</tr>
</tbody>
</table>

TBARS Lactoferrin vs. Metal 2

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>27.85449</td>
<td>3.979212</td>
<td>8.33</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td>DF</td>
<td>Type III SS</td>
<td>Mean Square</td>
<td>F</td>
<td>Value</td>
<td>Pr &gt; F</td>
</tr>
<tr>
<td>----------</td>
<td>----</td>
<td>-------------</td>
<td>-------------</td>
<td>-----</td>
<td>-------</td>
<td>--------</td>
</tr>
<tr>
<td>Panel</td>
<td>7</td>
<td>57.65623</td>
<td>8.236604</td>
<td>17.14</td>
<td>0.0007</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>0.01974</td>
<td>0.01974</td>
<td>0.04</td>
<td>0.8451</td>
<td></td>
</tr>
</tbody>
</table>

### TBARS Metal 1 vs. Metal 2

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>14.08213</td>
<td>2.011733</td>
<td>13.52</td>
<td>0.0027</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>0.259488</td>
<td>0.259488</td>
<td>1.74</td>
<td>0.2347</td>
<td></td>
</tr>
</tbody>
</table>

### Change in TBARS Metal 1 vs. Metal 2

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>25.1238</td>
<td>3.589115</td>
<td>8.17</td>
<td>0.0103</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>0.007968</td>
<td>0.007968</td>
<td>0.02</td>
<td>0.8973</td>
<td></td>
</tr>
</tbody>
</table>

| Sample | 1 | 0.015688 | 0.015688 | 0.03 | 0.8613 |
### Iron Concentration Full Model (treatments n=4, panelists n=8)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>21.99527</td>
<td>3.142181</td>
<td>0.56</td>
<td>0.7782</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>3</td>
<td>7.217152</td>
<td>2.405717</td>
<td>0.43</td>
<td>0.7353</td>
<td></td>
</tr>
</tbody>
</table>

### Change in Iron Concentration Full Model

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>6</td>
<td>40.77446</td>
<td>6.795743</td>
<td>0.81</td>
<td>0.5886</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>2</td>
<td>7.422023</td>
<td>3.711011</td>
<td>0.44</td>
<td>0.6564</td>
<td></td>
</tr>
</tbody>
</table>

### Iron Concentration Baseline vs. Metal 1

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>6.226826</td>
<td>0.889547</td>
<td>5.88</td>
<td>0.0343</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>0.062496</td>
<td>0.062496</td>
<td>0.41</td>
<td>0.5487</td>
<td></td>
</tr>
</tbody>
</table>

### Iron Concentration Baseline vs. Lactoferrin

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>9.213664</td>
<td>1.316238</td>
<td>0.52</td>
<td>0.7886</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>1.907942</td>
<td>1.907942</td>
<td>0.75</td>
<td>0.4343</td>
<td></td>
</tr>
</tbody>
</table>

### Iron Concentration Lactoferrin vs. Metal 2

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>49.23754</td>
<td>7.033934</td>
<td>0.57</td>
<td>0.7565</td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td>DF</td>
<td>Type III SS</td>
<td>Mean Square</td>
<td>F</td>
<td>Value</td>
<td>Pr &gt; F</td>
</tr>
<tr>
<td>--------</td>
<td>----</td>
<td>-------------</td>
<td>-------------</td>
<td>-----</td>
<td>-------</td>
<td>--------</td>
</tr>
<tr>
<td>Panel</td>
<td>6</td>
<td>54.67939</td>
<td>9.113231</td>
<td>0.6</td>
<td>0.7231</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>4.929444</td>
<td>4.929444</td>
<td>0.33</td>
<td>0.598</td>
<td></td>
</tr>
</tbody>
</table>

**Change in Iron Concentration Lactoferrin vs. Metal 2**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>7</td>
<td>45.30681</td>
<td>6.472402</td>
<td>0.74</td>
<td>0.6545</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>5.253413</td>
<td>5.253413</td>
<td>0.6</td>
<td>0.4692</td>
<td></td>
</tr>
</tbody>
</table>

**Iron Concentration Metal 1 vs. Metal 2**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>6</td>
<td>52.39332</td>
<td>8.732221</td>
<td>1</td>
<td>0.5123</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>10.62765</td>
<td>10.62765</td>
<td>1.21</td>
<td>0.321</td>
<td></td>
</tr>
</tbody>
</table>
### A3. Proteins of Interest in Human Saliva

<table>
<thead>
<tr>
<th>Protein</th>
<th>$M_r$(kDa)</th>
<th>pI</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>67</td>
<td>6.10</td>
<td>(Yao 2003; Huang 2004; Hu 2005)</td>
</tr>
<tr>
<td>Mucin</td>
<td>*</td>
<td>*</td>
<td>(Hu 2005)</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14</td>
<td>9.28</td>
<td>(Yao 2003; Hong, Duncan et al. 2009)</td>
</tr>
<tr>
<td>cystatin A</td>
<td>11</td>
<td>5.38</td>
<td>(Huang 2004)</td>
</tr>
<tr>
<td>Histatin</td>
<td>2.5-3.5</td>
<td>**</td>
<td>(Hong, Duncan et al. 2009)</td>
</tr>
<tr>
<td>Statherin</td>
<td>5</td>
<td>4.22</td>
<td>(Yao 2003)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>97</td>
<td>8.70</td>
<td>Levay (Hong, Duncan et al. 2009)</td>
</tr>
<tr>
<td>Transferrin</td>
<td>52</td>
<td>6.09</td>
<td>(Huang 2004)</td>
</tr>
<tr>
<td>Amylase</td>
<td>57</td>
<td>5.43</td>
<td>(Huang 2004)</td>
</tr>
<tr>
<td>zinc-$\alpha$-2-glycoprotein precursor</td>
<td>34</td>
<td>5.57</td>
<td>(Huang 2004)</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>varies; avg. 12</td>
<td>avg. 5-6</td>
<td>(Huang 2004)</td>
</tr>
<tr>
<td>proline rich proteins</td>
<td>varies; est. 25</td>
<td></td>
<td>(Huang 2004)</td>
</tr>
</tbody>
</table>

Rounded MW for ease in reading gels.
Highlighted proteins were used for standards.
* Mucin: Has several possibilities at this time due to precursors.
** Histatin: Unavailable. Further searching of literature is necessary at this time.
Appendix B. Supplemental Information for Chapter IV

B1. Informed Consent Form

Virginia Polytechnic Institute and State University
Informed Consent for Participation in Sensory Evaluation

Title of Project: Metallic Flavor Induced by Lactoferrin or EDTA

Principal Investigator: Dr. Susan E. Duncan, Kerri Martin, Virginia Fernandez-Plotka

I. PURPOSE OF PROJECT

The lingering aftertaste of metallic flavor is a common problem for the food, water, pharmaceutical, and health industry. This study will help us compare the use of rinse agents to reduce metallic flavor intensity over time, using common food constituents.

II. PROCEDURES

This sensory study will consist of a training phase and a data collection phase. Training will involve three main parts: basic taste training, metallic intensity recognition, and time-intensity evaluation. Each session should last approximately 45-60 min. For basic taste training, you will be asked to evaluate the intensity of three levels of each taste: sweet, sour, salt, and bitter. The second part will train you to recognize and rate metallic taste intensity at a variety of levels and may take 1-3 sessions. In the final training sessions, you will become familiar with the concept of time-intensity and the use of the touchscreen monitors and sensory software. Depending on your ability to recognize and rate metallic flavor and effectively perform the test protocols, this will require at least one and probably more sessions until you have developed the appropriate understanding and ability to rate the training samples. Data collection sessions (n=8; approximately 15 minute duration, one per day) will be held to rate the time-intensity of the samples being evaluated. Before each session, please do not eat or drink anything at least one hour prior. The time commitment for this project is approximately 6-10 hours, over 3-4 weeks.

Certain individuals are sensitive to some foods; please list any that you are aware of in the space provided. If you are allergic to dairy proteins, reactions to dietary metals, or other food additives, please state specific concerns below.

III. BENEFITS/RISKS OF THE PROJECT

Your participation will help to identify compounds that may help reduce the perception of metallic flavor. You may receive the results or summary of the panel when the project is completed. Excessive intake of iron can cause nausea, vomiting, and abdominal pain. The levels in this study are well below the recommended daily dietary intake of iron. The iron solution can also cause a lingering aftertaste such as metallic, bitter, and astringent. You will be given water and saltine crackers to help diminish lingering taste.
IV. EXTENT OF ANONIMITY AND CONFIDENTIALITY

The results of your performance as a panelist will be kept strictly confidential. Individual panelists will be referred to by code for analyses and in any publication of the results.

V. COMPENSATION

Snacks will be provided at the conclusion of each session as an expression of our gratitude for your participation. At the conclusion of the study, you will be invited to a “thank you” meal provided by the researchers.

VI. FREEDOM TO WITHDRAW

It is essential to sensory evaluation projects that you complete each session in so far as possible. However, there may be conditions preventing your completion of all sessions. If after reading and becoming familiar with the sensory project, you decide not to participate as a panelist, you may withdraw at any time without penalty.

VII. APPROVAL OF RESEARCH

This research project has been approved by the Institutional Review Board for projects involving human subjects at Virginia Polytechnic Institute and State University and by the human subjects review of the Department of Food Science and Technology.

VIII. SUBJECT'S RESPONSIBILITIES

I know of no reason I cannot participate in this study, which will require approximately 6-8 hours of training and eight 15-minute data collection sessions. It is my responsibility to

- Not eat or drink (water is permitted) for at least one hour prior to each training or data collection session
- To be attentive at each session
- To follow the protocols as directed
- To not discuss my observations with other study participants, except during the training sessions as encouraged by the researchers.

Signature/Date

Please provide address and phone number so investigator may reach you in case of emergency or schedule changes.

Address __________________________________________________________

Phone __________________________________________________________

IX. SUBJECT'S PERMISSION (provide tear off for human subject to keep)
I have read the information about the conditions of this sensory evaluation project and give my voluntary consent for participation in this project. I know of no reason I cannot participate in this study which will require: (list sessions to be attended or other requirements.)

___________________________________________________________________

Signature

Should I have any questions about this research or its conduct, I should contact:

Kerri Martin, Master’s Student 540-818-7080 kerrik@vt.edu
Investigator/Phone/E-Mail

Dr. Susan E. Duncan 540-231-8675 duncans@vt.edu
Faculty/Phone/E-Mail

Dr. David Moore (540)231-4991
Chair, IRB/Phone for Research Division
B2. Basic Tastes Training Ballot

Panelist #: 
IRB# 
Date: 

Intensity of Basic Tastes

For the following sets of samples, you are to evaluate three samples, each with the same basic taste, identify the basic taste, and rank order the samples.

Directions:
Take the full volume of each sample into your mouth, swirl/swish for 10 seconds, then expectorate into the appropriately identified (SPIT) cup. Identify the basic taste represented by the set of samples. Rank order the samples, based on intensity of the basic taste, from least (1) to most (3) within each sample set. After you have completed the first set of three, another series (n=3) of samples will be provided to you. There will be four sample sets (total).

Sample Set 1: Basic Taste ________________

Rank order: Sample Code
1 (least) __________
2 __________
3 (most) __________

Sample Set 2: Basic Taste ________________

Rank order: Sample Code
1 (least) __________
2 __________
3 (most) __________

Sample Set 3: Basic Taste ________________

Rank order: Sample Code
1 (least) __________
2 __________
3 (most) __________

Sample Set 4: Basic Taste ________________

Rank order: Sample Code
1 (least) __________
2
3 (most)
B3. Basic Taste Recognition Training

Panelist #: 
IRB# 
Date: 

Basic Taste Training

In this training, you will be asked to name the taste stimuli in six samples. Not all samples contain a basic taste stimuli.

Directions: Take the full volume of a sample into your mouth, swirl/swish for 10 seconds, then expectorate into the appropriately identified (SPIT) cup. Identify the basic taste within each sample. Rinse your mouth with water and wait for a minute. Taste the next sample and repeat the process until you have tasted and identified all the samples.

Sample # ______  Basic Taste_______

Sample # ______  Basic Taste_______

Sample # ______  Basic Taste_______

Sample # ______  Basic Taste_______

Sample # ______  Basic Taste_______

Sample # ______  Basic Taste_______

Sample # ______  Basic Taste_______
B4. Metallic Flavor Intensity Training Ballot

Rating Metallic Flavor Intensity Practice Session

Sip each sample, in no particular order, swish for 10 seconds, and expectorate. Write the sample number and your intensity rating on a scale of 0 – 15, with 0 being least intense and 15 being most intense flavor. Not all samples contain metallic.

Sample #________  Intensity________
Sample #________  Intensity________
Sample #________  Intensity________
### B5. Time-Intensity Concept

#### Training

<table>
<thead>
<tr>
<th>Time (seconds)</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>low</td>
</tr>
<tr>
<td>10</td>
<td>low</td>
</tr>
<tr>
<td>20</td>
<td>low</td>
</tr>
<tr>
<td>30</td>
<td>low</td>
</tr>
<tr>
<td>40</td>
<td>low</td>
</tr>
<tr>
<td>50</td>
<td>low</td>
</tr>
<tr>
<td>60</td>
<td>low</td>
</tr>
<tr>
<td>70</td>
<td>low</td>
</tr>
<tr>
<td>80</td>
<td>low</td>
</tr>
<tr>
<td>90</td>
<td>medium</td>
</tr>
<tr>
<td>100</td>
<td>medium</td>
</tr>
</tbody>
</table>

For this training, you will grade the intensity of the stimulus and rate the intensity for 20 seconds. Record your initial intensity immediately after a mild tone of 20 seconds. If you fail to grade the following grade, take the next grade.
B6. Sample Screen Shot for Time-Intensity Recording

Welcome to the Time Intensity (TI) of Metallic Flavor test!

Please read the instructions carefully BEFORE starting the test.

First you should position yourself adequately for proper video capture. Find a comfortable position and make sure you are facing forward during the entire test. It is critical that your face is not blocked in any way (e.g. by hair or by your arm while reaching the touch screen).

Next, please rinse your mouth with deionized water. Use the cup provided to expectorate.

To start the test, you need to simultaneously taste the first sample provided and touch the zero mark on the intensity scale on the screen. Touching the scale will start a one- or two-minute countdown. To taste the sample, sip the entire content of the cup provided, swirl it around your mouth for 10 seconds, and expectorate. While you do this, use the intensity scale to indicate any changes you perceive in the intensity of metallic sensation over time. You do not need to keep your finger on the scale, just touch it to increase or decrease the rating. After you are done, please rate the second sample following the same procedure.

When you are ready to start, press the pointed finger icon on top of your screen.

Sip sample ‘19B’ and simultaneously touch the scale on the 0 mark for 10 seconds and adjust the intensity rating as you do so. Expectorate around your mouth for 1 minute.
Sip sample "198" and simultaneously touch the scale on the 0 mark. Swirl the sample around your mouth for 10 seconds and adjust the intensity rating as you do so. Expectorate and continue to rate metallic flavor intensity for 1 minute.

---

Now sip sample "402" and rate the intensity of metallic flavor for two minutes.

Do you still perceive a metallic aftertaste?
- Yes  
- No
Now sip sample `402` and rate the intensity of metallic flavor for two minutes.

<table>
<thead>
<tr>
<th>None</th>
<th>Low</th>
<th>Moderate</th>
<th>Moderately High</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.26</td>
<td>7.5</td>
<td>11.35</td>
<td>15</td>
</tr>
</tbody>
</table>

Seconds Remaining: 120

Do you still perceive a metallic aftertaste?
- Yes
- No

Thank you for completing the test!
Please go to the sensory lab to select a treat.
B7. Time-Intensity Curves for All Individuals for Each Treatment Replication (See Title Below)

Lactoferrin Pre-Rinse Replication 1.
Lactoferrin Pre-Rinse Replication 2.
Lactoferrin Post-Rinse Replication 1.
Lactoferrin Post-Rinse Replication 2.
EDTA Pre-Rinse Replication 1.
EDTA Pre-Rinse Replication 2
EDTA Post-Rinse Replication 1
EDTA Post-Rinse Replication 2
Water Post-Rinse Replication 1
Water Post-Rinse Replication 2
Water Pre-Rinse Replication 1
Water Pre-Rinse Replication 2
### B8. F-Tables for TI Parameters (Panelist n=6, treatments n=6, replicates n=2)

#### Imax

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>5</td>
<td>812.6111</td>
<td>162.5222</td>
<td>20.63</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>treat</td>
<td>5</td>
<td>260.3194</td>
<td>52.06389</td>
<td>6.61</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Panel*treat</td>
<td>25</td>
<td>172.7872</td>
<td>6.911489</td>
<td>0.88</td>
<td>0.6288</td>
<td></td>
</tr>
</tbody>
</table>

#### AUC

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>5</td>
<td>3245894</td>
<td>649178.8</td>
<td>19.83</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>treat</td>
<td>5</td>
<td>691678.5</td>
<td>138335.7</td>
<td>4.23</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Panel*treat</td>
<td>25</td>
<td>640122.6</td>
<td>25604.91</td>
<td>0.78</td>
<td>0.7369</td>
<td></td>
</tr>
</tbody>
</table>

#### APostMax

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>5</td>
<td>1378976</td>
<td>275795.1</td>
<td>11.85</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>treat</td>
<td>5</td>
<td>483935.1</td>
<td>96787.02</td>
<td>4.16</td>
<td>0.0044</td>
<td></td>
</tr>
<tr>
<td>Panel*treat</td>
<td>25</td>
<td>401474.9</td>
<td>16059</td>
<td>0.69</td>
<td>0.8323</td>
<td></td>
</tr>
</tbody>
</table>

#### Plateau Time

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>5</td>
<td>824.5694</td>
<td>164.9139</td>
<td>5.45</td>
<td>0.0008</td>
<td></td>
</tr>
<tr>
<td>treat</td>
<td>5</td>
<td>285.5694</td>
<td>57.11389</td>
<td>1.89</td>
<td>0.1209</td>
<td></td>
</tr>
<tr>
<td>Panel*treat</td>
<td>25</td>
<td>1025.681</td>
<td>41.02722</td>
<td>1.36</td>
<td>0.1983</td>
<td></td>
</tr>
</tbody>
</table>

#### Rate Decrease

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>5</td>
<td>0.13135</td>
<td>0.02627</td>
<td>7.32</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>treat</td>
<td>5</td>
<td>0.033467</td>
<td>0.006693</td>
<td>1.87</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>Panel*treat</td>
<td>25</td>
<td>0.124133</td>
<td>0.004965</td>
<td>1.38</td>
<td>0.1832</td>
<td></td>
</tr>
</tbody>
</table>
### Tdur

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>5</td>
<td>51959.24</td>
<td>10391.85</td>
<td>16.01</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>treat</td>
<td>5</td>
<td>6737.736</td>
<td>1347.547</td>
<td>2.08</td>
<td>0.0912</td>
<td></td>
</tr>
<tr>
<td>Panel*treat</td>
<td>25</td>
<td>11762.85</td>
<td>470.5139</td>
<td>0.72</td>
<td>0.798</td>
<td></td>
</tr>
</tbody>
</table>

### Tmax

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>5</td>
<td>5025.167</td>
<td>1005.033</td>
<td>6.39</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>treat</td>
<td>5</td>
<td>649.1667</td>
<td>129.8333</td>
<td>0.83</td>
<td>0.5402</td>
<td></td>
</tr>
<tr>
<td>Panel*treat</td>
<td>25</td>
<td>5236.167</td>
<td>209.4467</td>
<td>1.33</td>
<td>0.2125</td>
<td></td>
</tr>
</tbody>
</table>