Formation of Copper-Salivary Component Complexes and Its Effect on Sensory Perception

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ABSTRACT

Copper in drinking water elicits a persisting bitter, metallic, or astringent taste. Characteristics and perception mechanisms of copper sensation have not been fully understood. Saliva is assumed to influence copper sensations via binding of salivary electrolytes or proteins with copper. The interaction between salivary components and copper is thought to influence sensory perception by affecting volatility of aroma compounds, de-lubricating salivary proteins, and by controlling solubility of copper. A recent study suggested that intensity of copper taste may be dependent on the amount of solubilized copper, which increases at lower pH. This research was performed to identify 1) the temporal sensory characteristics of copper; 2) the effect of pH on perception of copper sensation; 3) the nature of copper-protein interaction and its impact on sensory perception.

The effect of copper on the volatility of aroma compounds and the role of copper-protein interaction in volatile chemistry were investigated using a model mouth system containing artificial saliva at different pH levels. Headspace concentration of each volatile was measured using SPME-GC analysis. Copper (2.5 mg/L) in the model system increased headspace concentration of volatiles (hexanal, butyl acetate, 2-heptanone, and ethyl hexanoate, 0.5 μL/L each) at pH 6.5, but no change in volatility was observed at pH 7.0. At pH 7.5, presence of copper in the artificial saliva decreased headspace volatile concentration. Effect of copper on volatiles at pH 6.5 may be due to increased solubility of copper at lower pH. Copper seems to facilitate hydrophobic binding between mucin and aroma compounds at pH 7.5, possibly by exposing hydrophobic sites of mucin.

A time-intensity (TI) test was performed to identify the effect of pH on temporal characteristics of copper sensation. Metallic taste, bitterness, and astringency were major attributes of drinking water containing 2.5 mg/L and 5 mg/L Cu. All three attributes were responsible for the lingering aftertaste of copper. TI test results of copper solutions did not show a common TI

pattern of astringency that is characterized with slow onset and longer duration time. Increase in pH of water from 5.5 to 7.5 inhibited metallic taste of copper, but did not reduce bitterness and astringency. The level of soluble copper at pH 7.5 decreased by 50 % compared to that at pH 5.5. Soluble copper concentration and temporal profile of sensory attributes of copper solutions at different pH levels suggest that soluble copper species decide the perception of copper sensation by controlling metallic taste.

The nature of copper-protein interaction and its implication on mechanisms of sensory perception were studied by investigating binding of copper to high molecular weight fractions of human saliva. At the copper concentration < 10 mg/L, most copper exists as unbound copper form while about 60 % of copper was found in protein fractions or with precipitated salivary debris. This result suggests that copper is in a soluble unbound form in saliva at low concentration (<10 mg/L) and assumed to be available for taste receptors. At higher concentration, copper either becomes insoluble or binds with proteins. Insoluble copper species are thought to cause astringency. When copper was added at the concentration ≥ 10 mg/L, two salivary proteins of molecular weight 29 kDa and 33 kDa formed insoluble complexes with copper. Low molecular weight mucin (MG2), α-amylase, basic proline-rich proteins (PRPs), and a protein of MW 45 kDa also bound with copper.

In summary, sensations elicited or influenced by copper are thought to be determined by what copper species are dominant in the mouth. Soluble copper species and insoluble copper species are assumed to interact with different sensory receptors, resulting in metallic taste or astringency. This speciation process is influenced by pH conditions, composition of other electrolytes, and organic chelators such as proteins.

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Chapter I . Introduction and Research Rationale

IMPLICATIONS OF COPPER IN DRINKING WATER ON AESTHETIC QUALITY

Biochemistry and Physiology of Copper

Copper consumed through foods or water is absorbed in the stomach or the small intestine. In the stomach, copper becomes solubilized because of the acidic conditions. Antacids are known to interfere with absorption of copper by raising gastric pH. The small intestine is where the primary absorption of copper occurs. Copper is absorbed by an active transport system that uses binding ligands including histidine, citrate, and gluconate (Deshpande, 2002).

Absorbed copper is bound with albumin or amino acids and then transported to the liver where 4 ~ 6 Cu^{2+} ions bind with apo-protein to form ceruloplasmin. Ceruloplasmin is the main carrier of plasma copper within the body. In the human body, copper exists predominantly as Cu^{2+}, but it is reduced to Cu^{+} when extrahepatic tissues uptake copper. Once within the cells, copper is carried by small polypeptides called as metallochaperones. These chaperones have cysteine-rich ligands where Cu^{+} loosely binds. Copper is transported to intracellular enzymes such as superoxide dismutaste, cytochrome oxidase, and amine oxidases by rapid exchange between chaperones and enzymes (NRC, 2000; Deshpande, 2002).

Copper is an essential dietary nutrient which is necessary for the function of certain enzymes. Copper-containing enzymes generate ATP, protect cells and cell membranes against oxidative damage, produce neurotransmitters and hormones, and help connective tissues keep their integrity. Also, copper-binding protein, ceruloplasmin, is related to the metabolism of iron

(O'Dell, 1976; Deshpande, 2002). The World Health Organization (WHO, 1998) recommends a daily intake of Cu of 30 μg/kg body weight for an average adult (NRC, 2000).

However, excessive copper consumption or consumption from an inappropriate source can have toxic effects on the human body. Vomiting, abdominal pain, diarrhea, gastrointestinal ulceration, dizziness, and comas are common symptoms in acute copper toxicity. Levels of copper cause acute symptoms, based on case reports, varied between 2.8 mg/L to 30 mg/L (NRC, 2000). Pizzaro et al. (1990) suggested that greater than 3 mg/L of copper can be related to acute toxicity based on the experimental study. Major targets of chronic copper toxicity are the liver and the central neuvous system. Hemolytic anemia and renal failure are other symptoms resulting from excessive copper intake over long term (Scheinberg and Sternlieb, 1976; NRC, 2000).

Copper in Drinking Water

Copper uptake from drinking water is one of the common ways to consume copper, but hardly recognized as a major source of copper by the general public. Tap water can be a source of dietary copper, especially if the water contacts copper pipes. Copper is present in fresh water, usually in low amounts (typically less than 0.075 mg/L), but the concentration can increase substantially when water travels through copper pipes to residential homes (Dietrich et al., 2004; Edwards et al., 2004). An estimated 70-80% of drinking water pipes currently in or being installed in new homes in the USA is made of copper. A variety of water quality parameters including pH, mineral content, pipe age, type of scale, oxygen content, natural organic matter, and stagnation time as well as many other variables can affect the corrosion rate of copper plumbing system (Edwards et al., 1996; Dietrich et al., 2005).

Recent problems with pinhole leaks in Maryland, Washington, D.C., and elsewhere have raised awareness and concerns as to the specific causes of copper pipe corrosion (Edwards et al., 2004). According to USEPA databases, in 2003 there were 471 individual drinking water systems that violated the copper action level of 1.3 mg/L Cu and potentially affected 622,000

people (USEPA, 2003). In the survey of 4,500 water systems in 1999, the EPA reported that the copper concentration for water systems to serve recreational areas and schools ranged from 1.3 mg/L to 30 mg/L, which exceeded the safety level. This result suggests that there is possibility of chronic exposure (NRC, 2000).

The International Programme on Chemical Safety (IPCS, 1998) recommended the upper level of copper concentration in water to be 2 ~ 3 mg/day. In the U.S., copper is regulated under the Lead and Copper Rule of 1991. This rule requires water utilities to sample up to 100 locations in the distribution system, even if the population served is >1,000,000. A utility is in compliance with the Lead and Copper Rule if not more 10 % of the samples exceed 1.3 mg/L Cu. Thus, some locations can have more than 1.3 mg/L in the drinking water; there is no upper limit to the amount of copper in the water as long as $\leq$ 10 % of the samples does not exceed 1.3 mg/L Cu. An aesthetic based standard is 1 mg/L Cu because copper above this level can stain plumbing fixtures and laundry as well as contribute to metallic or bitter tasting water (USEPA, 1997).

In the research of Cohen et al. (1960), a copper concentration of more than 3 mg/L changed the sensory qualities of water. Pizarro et al. (1999) reported that this concentration could be related to nausea, vomiting, and abdominal pain. Copper in drinking water may cause a metallic, acidic, astringent, salty, or bitter taste in individuals (Zacarias et al., 2001). Because odor and taste have been important indicators of potential contamination (Zoeteman, 1980), these unpleasant sensations are assumed to be an initial biological protection mechanism from this chemical hazard.

Chemistry of Copper in Drinking Water

General Aspects. Copper is a transition metal that has molecular weight of 63.5. There are two stable isotopes of copper, ^{63}Cu and ^{65}Cu. Because of its malleability, ductile-ability, conductivity of heat and electricity, copper has applications in many products, such as electronics, plumbing, and cookware. Copper has two major oxidation states, the less stable Cu^{+}

and the more stable Cu^{2+}. The oxidation states of Cu^{3+} and Cu^{4+} are found very rarely (NRC, 2000).

Redox reaction of copper and thermodynamic data (Jensen, 2003) is:

$Cu^{2+} + e^- = Cu^+$ $\quad E^0 = 0.158$

$Cu^{2+} + e^- = Cu(s)$ $\quad E^0 = 0.340$

(E^0 (cell potential) is calculated from $\Delta G^0_{rxn} = -nFE^0$,

where ΔG^0_{rxn} = Gibbs Free Energy

n = number of moles of electron transferred

F= faraday = 96,845 coulombs/mol)

This shows that copper is a reductant, but not as strong a reductant as zinc (E^0 = -0.763) or iron (E^0 = -0.409). Copper favors the reduced form in the standard state (25°C, 1 atm) (Jensen, 2003).

Copper Chemistry in Drinking Water. In water, copper loses electron pairs and oxidizes into cupric ion (Cu^{2+}). In theoretical pure water system, uncomplexed free cupric ion (Cu^{2+}) forms hydroxo complexes ($Cu(OH)^+$, $Cu(OH)_2$, $Cu(OH)_3^-$, and $Cu(OH)_4^{2-}$) with OH^- ion. This complexation is dependent on pH as pH controls redox reactions of copper and the amount of hydroxo ion in the system. At acidic pH (typically < 6), most copper exists as cupric ion and complexation with hydroxo ion is not likely to occur due to low concentration of hydroxo ion at low pH. On the other hand, $Cu(OH)_2$ is a major species in water at pH 6.5 ~ 12 (Cuppett et al., 2006). The complex of $Cu(OH)_2$ becomes insoluble ($K_{sp} = 10^{-19.32}$) when it is saturated in water (Jensen, 2003). Insoluble copper either exists as dispersed particulates whose size is greater than 0.45 μm or begins to precipitate as particle size grows. The speciation of copper in water is summarized in Figure 1. It should be noted that copper speciation summarized here is an equilibrium process, which is different from what would happen in kinetic conditions.

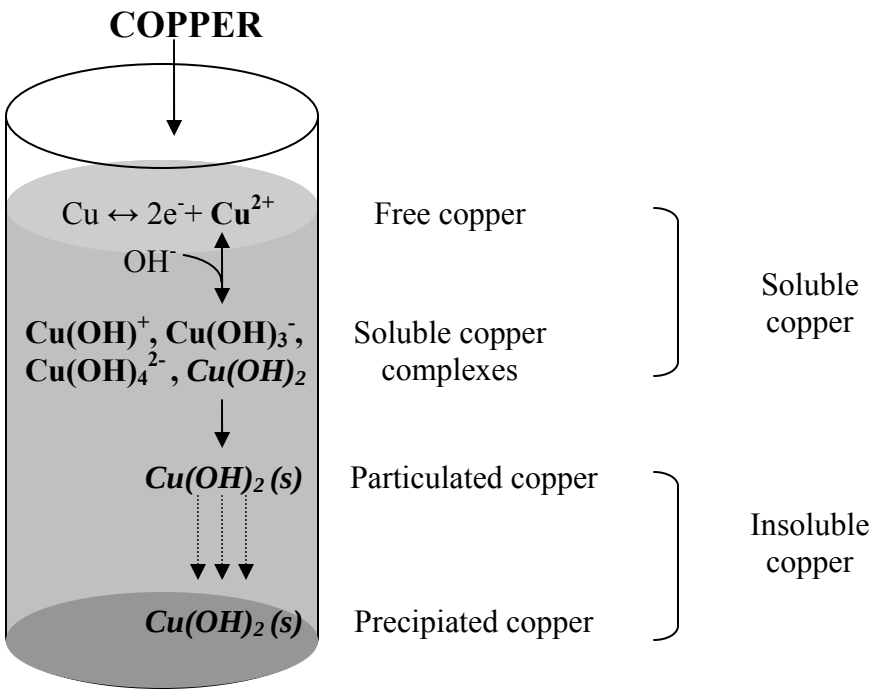

Figure 1. Copper speciation model in hypothetical pure water system.

The reaction becomes more complicated in drinking water that usually contains other electrolytes and natural organic matter (NOM). Anions commonly found in drinking water, such as O^{2-}, OH^-, HCO_3^-, SO_4^{2-}, PO_4^{3-}, and CO_3^{2-}, form insoluble copper complexes that include tenorite (CuO), malachite ($Cu_2(OH)_2CO_3$, K_{sp} = 5.48), and brochantite ($Cu_4SO_4(OH)_6$, K_{sp} = -15.38) (Dietrich et al., 2005). Soluble organic matter in drinking water (i.e. humic acid) binds with copper to form soluble copper-organic complexes, while insoluble colloidal NOM adsorbs copper and becomes dispersed in drinking water (Edwards and Sprague, 2001). pH is also an important factor in copper speciation in drinking water. When copper was added up to 8 mg/L level, copper existed 100% as free cupric ion at pH 5.5. However, treatments such as addition of other minerals and raising pH above 6 decreased cupric ion concentration by forming soluble copper complex and fine copper precipitates (Cuppett et al., 2006).

RATIONALE AND IMPORTANCE OF THE PROPOSED RESEARCH

Copper is an essential micronutrient but can be toxic when an excessive amount is consumed. There is a high potential of increased copper intake in daily life through consumption of drinking water. High copper concentration in water causes metallic, bitter, acidic, and astringent sensations. These sensory qualities are related to innate defense mechanisms for protection against potential hazards. Thus, understanding sensory attributes associated with copper intake is important not only as a guideline for judging user acceptance of drinking water, but also as an alarm system to toxicity.

Only a few studies about copper sensation have been published. The sensory perception mechanism and characteristics of metals such as copper are still not clear. Current research suggests that the perception mechanism of astringency is found in relation to salivary proteins. Also, there has been evidence that salivary proteins form complex compounds with metals. Consequently, interaction between salivary proteins and copper is one of the possible explanations of metal sensation.

Copper in drinking water changes its form depending on pH and electrolytes of water. Recent studies of sensory threshold of copper in drinking water suggested that copper speciation is an important factor deciding the sensory perception of copper.

This research was the first trial to study the interrelationship between copper sensation and the salivary components. The relationship identified is expected to shed light on further studies for understanding sensory systems as defense mechanism of the body.

Objectives of Proposed Research

The objective of this research was to determine if the copper in water influences the perception of taste and aroma under the influence of pH and the presence of salivary components. This research is expected to suggest an explanation about how the human perception mechanism

can help the human prevent the potential danger of toxic exposure from environmental copper contamination.

Objective 1: **Identification of the effect of copper on the release of aroma compounds in the mouth.**

The behavior of volatiles in the mouth is closely related to flavor perception. For aroma to be perceived, compounds must be released from the food matrix and volatilized so they can reach the olfactory cells. During this process, the volatility of aroma compounds is influenced by several factors. Factors include the chemical composition of saliva, salivary flow, mastication, and other food components such as proteins, fats, carbohydrates, fibers and salts. Changes in volatility cause different flavor impressions.

Copper from drinking water may affect the behavior of aroma compounds and, further, play a role in the change of flavor perception. Copper may affect flavor perception by associating with salivary components, especially salivary proteins.

This hypothesis was examined by investigating:

- Changes in the partitioning behavior of aroma compounds between saliva and air when copper is incorporated;
- Effect of salivary pH on the volatility of aroma compounds and copper binding.

Objective 2: Characterization of temporal sensation of copper and the effect of copper speciation at different pH on perception of metallic sensation.

Metallic sensation generated by copper causes aftertaste that lingers in the mouth. Unlike other taste sensations, the temporal profile of copper sensation has not been fully investigated. The recent threshold study of copper in drinking water implies the possible effect of copper speciation on perceived intensity of copper sensation.

The temporal characteristics of copper sensation were investigated by determining:

- Duration and magnitude of the sensory attributes of copper using time-intensity sensory technique;
- Copper speciation in drinking water at different pH and its effect on sensory perception.

Objective 3: Investigation of copper speciation in human saliva.

Human saliva is a complicated biological system containing various proteins, electrolytes, sugar, and other compounds. Since interaction with saliva is the first stage in flavor perception process, copper consumed through drinking water is expected to interact with salivary compounds. Binding of copper to a specific compound in the saliva has been investigated, but to the best of our knowledge, there have been no study on interaction between copper and the components in the saliva as a whole.

The speciation of copper in the human saliva was explored by investigating:

- Changes in salivary proteins due to addition of copper using HPLC;
- Molecular weight of salivary proteins that were changed by addition of copper;
- Quantification of copper mass distributed in different salivary fractions obtained by centrifugation and ultrafiltration before and after drinking copper solutions.

The literature reviewed in Chapter II further describes the current scientific knowledge related to perception of copper sensations, role of human saliva in perception of copper sensations, and methodologies used for investigating copper perception. This information will facilitate the understanding of the methodologies and interpretations of the research investigations. References from Chapter I are included at the end of Chapter II.

Chapter II . Literature Review

PERCEPTION OF COPPER SENSATION

Human Perception Mechanisms of Odor and Taste

The human perception mechanism of odor and taste has been studied for a long time. The taste and smell sensations are called chemical senses, as these sensations are perceived when stimulants bind chemically to the receptor cell membranes in sensory organs (Plattig, 1988).

The process to perceive taste and odor during eating has several stages. First, an object is located in the mouth. At this stage, diffusion of volatiles into the nasal cavity occurs and only smell becomes the major perception mechanism. Second, the mastication releases volatiles and taste compounds from the food matrix. Volatiles are partitioned into the saliva and air in the mouth, while taste compounds are dissolved in the saliva. Both taste and smell are equally important at this stage. Third, expiration during chewing or after swallowing carries volatiles onto a respiratory stream through the back of the mouth to the nasopharynx, thus creating the retronasal sensation (Maruniak, 1988). Finally, a substantial amount of foods remains in the mouth after swallowing, contributing to aftertaste (Prinz and de Wijk, 2004).

Olfaction. Human olfaction can occur only when the odorant is volatile. Only chemicals that have volatility can reach the olfactory epithelium in the nose. The olfactory epithelium is located at the ceiling of the inner nose. The epithelium contains three million receptor cells whose terminals form the ciliated protrusion called the olfactory knob at the surface of the epithelium (Figure 2 (A)) (Maruniak, 1988).

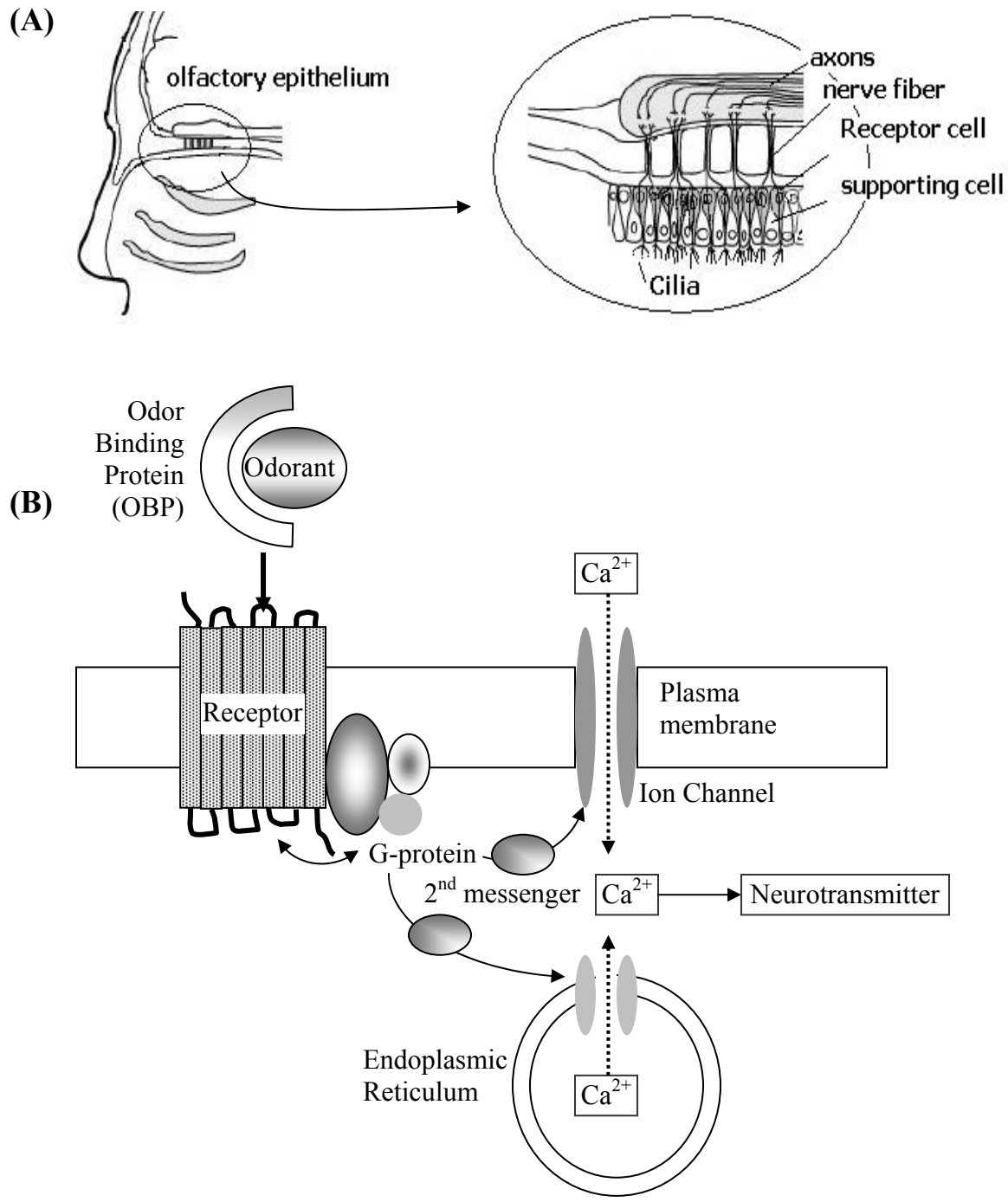

Figure 2. Diagram of human olfactory epithelium and signal transduction. (A) The structure of human olfactory epithelium (modified from Maruniak, 1988). (B) Olfactory signal transduction in receptor cells (modified from Pernollet and Briand, 2004). Odorant molecules interact with receptors through binding with olfactory binding proteins. Binding of odorants on the receptor activates G-protein to start a series of enzyme reactions that ultimately permit influx of calcium cations from extracellular or intracellular reservoir. Rise in intracellular Ca^{2+} level induces release of neurotransmitter.

Once a volatile compound reaches the olfactory epithelium, it is bound to a hydrophobic pocket of odor binding proteins (OBP). OBPs are water-soluble proteins found in mucosa layer that covers olfactory receptors (Figure 2 (B)). The hydrophilic nature of OBPs helps hydrophobic odorants dissolve in the mucosa so that they can be transported to receptor cells. Receptor cells are bipolar neurons of olfactory, trigeminal, and terminal nerves. Within the neurons, chemical signals of an odorant are transduced via G protein-coupled receptors that trigger cascade enzyme reactions. This series of reactions induce a Ca^{2+} influx into a neuron, which causes electrical signals. Then the signal is transferred to the nerves and causes the excitation of the olfactory cortex of the brain (Maruniak, 1988; Lawless and Heymann, 1998; Pernollet and Briand, 2004).

Gustation. Human gustation occurs in taste buds located on the papillae that are nipple-like elevations on the surface of the tongue (Figure 3 (A)). Taste receptors are placed in the taste buds, extended below from the tongue surface to make small channel-like structures called taste pores. Taste receptor cells have microvilli structures in their upper part to increase the surface area, allowing for greater interaction with stimuli. The primary process of taste is initiated by the binding of stimulant molecules to microvilli of the taste receptor cells after molecules pass through saliva by diffusion. Taste receptor cells form synaptic contact with the axons from taste nerves. The stimulation of receptor cells is transported to the cortical taste area in the brain via the seventh, the ninth, and the tenth cranial nerve (Plattig, 1988; Lawless and Heymann, 1998).

More than one type of receptor is found in a taste cell. Each receptor uses a variety of signal transduction mechanisms for different tastes. For example, sour taste and salty taste are mediated through ion channel, while bitterness, sweetness, and umami taste are transduced via G-protein coupled receptors (Figure 3 (B)). In the transduction process of salty and sour taste, Na^+ or H^+ enters directly into a taste cell through amiloid-sensitive epithelial ion channels. It has been reported that H^+ ion uses two additional transduction mechanisms in addition to direct entrance through ion channel. H^+ ion binds with the ion channel to change channel structure so that it can open to a specific ion or block the K^+ channel. These two mechanisms result in an increase of protons inside a cell. Accumulation of ions inside a receptor cell triggers influx of Ca^{2+} that releases neurotransmitters.

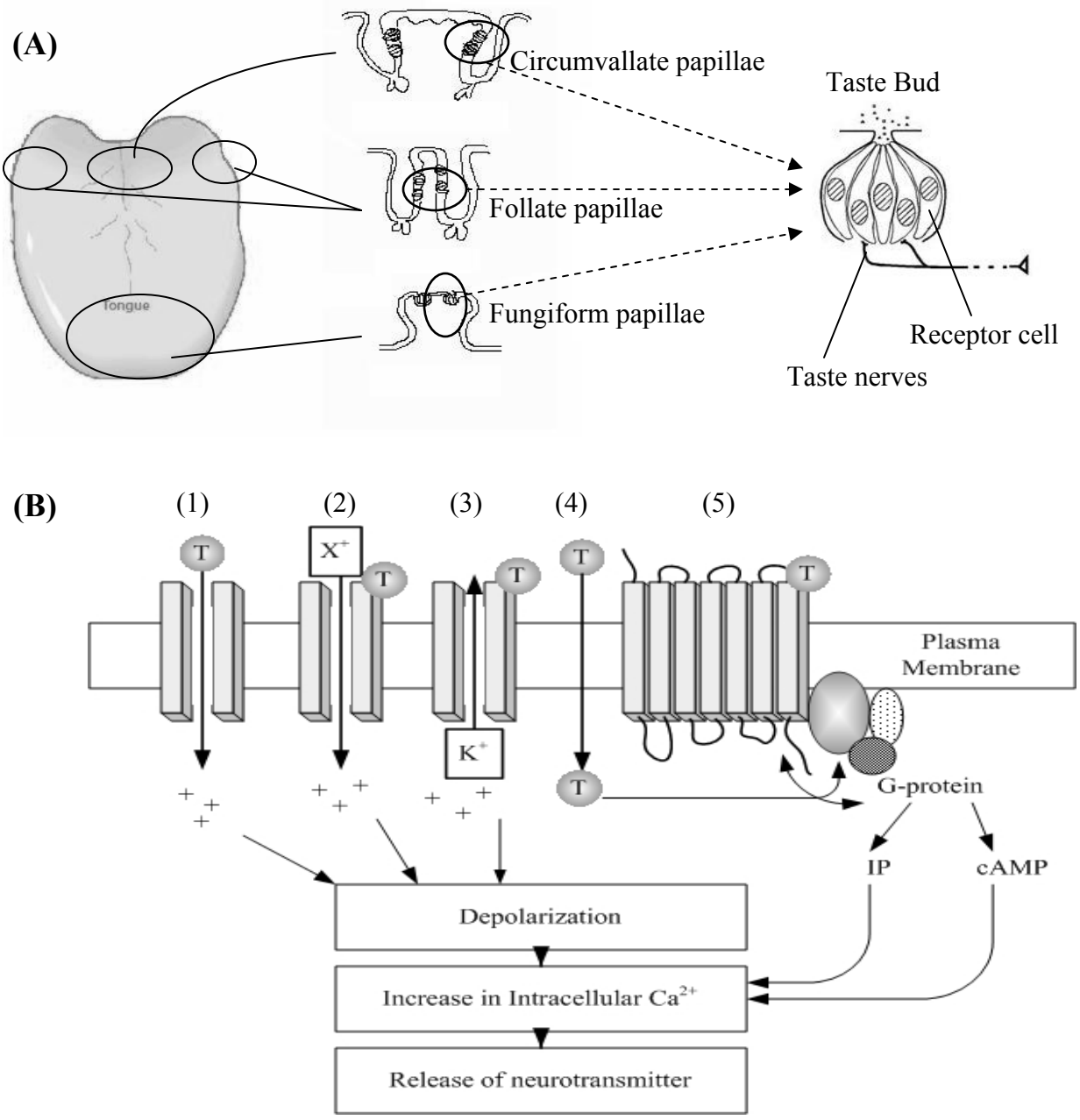

Figure 3. Diagram of taste buds and gustatory signal transduction. (A) The structure of human taste buds (Plattig, 1988). (B) Gustatory signal transduction in receptor cells. (1) Tastant (T) molecules pass through ion channel. Na^+ and H^+ ions are mediated in this way. (2) Tastants such as sugars and H^+ bind to ion channel to open it to channel-specific cations (X^+). (3) Tastants cause depolarization by blocking K^+ channel. Quinine divalent cations and H^+ are main chemicals using this mechanism. (4) Caffeine and quinine permeate the cell membrane and activate G-protein without binding to the receptor. (5) Sugar, sweeteners, amino acids, and nicotine activate G-protein coupled receptor. Sugars are transduced via cyclic-adenosine monophosphate (c-AMP) pathway while artificial sweeteners are mediated through inositol triphosphate (IP). Interaction between tastant and receptors leads to increase in intracellular Ca^{++} concentration, which causes neurotransmitter secretion (Boughter and Gilbertson, 1999; Lindemann, 2001; Rawson and Li, 2004).

In G-protein mediated receptors, tastants directly interact with receptors to start cascade reactions, unlike the olfactory receptor that requires soluble OBPs as odorant carriers. Once activating the G-protein, sweet tastants trigger two different second messengers. Sugars activate cyclic adenosine monophosphate (c-AMP), while artificial sweeteners use inositol trisphosphate. Some bitter or sweet tastants are mediated through ion channel or directly diffuse through membrane. Sugars and quinine cations are mediated by structural change of ion channels that leads to enterance of a channel-specific ion or block of the K^+ channel. Caffeine and quinine permeate membrane without binding to G-protein coupled receptor to directly activate G-protein (Boughter and Gilbertson, 1999; Lindemann, 2001; Rawson and Li, 2004).

Chemesthesis. There are other sensations that also are described as "taste", such as astringency from tannins or alum, burning or spiciness from capsaicin, cooling sensations from menthol, and tingling sensations from carbonated beverages. These sensations are generally referred as "mouthfeel", and called trigeminal sensitivity or more specifically, chemesthesis. Chemesthesis sensations are not classified as "taste" because they are elicited via stimulating pain or temperature nerve fibers on the skins and mucous membranes instead of being mediated through taste receptors (Lawless, 1996).

Chemesthesis is transduced by the trigeminal (cranial V) nerves, the Glossopharyngeal (cranial IX) nerves, and the vagus (craninal X) nerves that have somatosensory neurons. The receptors related with chemesthesis are found to mediate several different somatosensory qualities of other compounds (Lawless, 1996; Green, 2004). This suggests that one sensation can induce other sensations, for example, pain from capsaicin is related with hotness. Also these receptors are located near the taste receptors on the tongue, which means chemsthesis is integrated into flavor (Green, 2004). It has been recently recognized that there may be specific taste receptors for fatty acids, starch, and tannins based on animal studies (Prinz and de Wijk, 2004). If it is proven that there are real "taste" receptors for these compounds that have been regarded as major compounds of chemesthesis, the definition of chemesthesis may be changed.

Metallic Sensation in the Mouth

Metallic sensations have been described as a complex of different sensations (Lawless et al., 2005). Common descriptors for the sensation of metals are metallic, bitter, astringent, sour, salty, and tingling/sting (Zacarias et al., 2001; Keast, 2003). Subjective impressions can lead to different descriptions. Understanding the perception of "metallic sensations" is complicated by a combination of basic tastes (salty, sour, and bitter), trigeminal sensations (astringent and tingling), and "metallic" associated with metals. For example, iron, zinc and copper produced bitter, astringent and metallic tastes as sulfate compounds, but zinc sulfate and copper sulfate were more bitter and astringent and less metallic than ferrous sulfate (Lawless et al., 2004; Lawless et al., 2005). The true nature of metallic sensation has not been clearly identified yet.

Metallic taste is caused by direct contacting with metals, metal oxide fumes, and dental restoration. Response to other compounds or materials, such as blood, artificial sweeteners, and lipid oxidation products may have each been described as "metallic sensation" (Lawless et al., 2005). Taste abnormalities called as "dsygeusia" may also cause a metallic taste. These abnormalities occur when taste cells or the nervous system are impaired by drugs, pesticides, radiation exposure, or physiological disorders such as Sjögren's syndrome (dry mouth syndrome) (Mott and Mann, 2004).

Elucidating the nature of metallic sensation is important because quantitative and qualitative changes in sensory perception serve as important indicators of potential hazard from not only deterioration and contamination of dietary substances but also physiological disorder. Lawless et al. (2004) suggested that retronasal smell is an important component of metallic taste for ferrous compounds. Nasal occlusion drastically decreased the metallic taste of 0.3 and 1 mM ferrous sulfate solution compared to an open nasal passage, even impairing the panelists' ability to discriminate ferrous sulfate solution from water. On the other hand, metallic taste of copper sulfate and zinc sulfate was not influenced by nasal occlusion. The SPME-GC analysis of headspace over 0.3 M ferrous sulfate solution identified 1-octen-3-one and 1-nonen-3-one as potent chemical compounds that cause retronasal metallic smell (Lubran et al., 2005). Since these compounds are byproducts of lipid oxidation, it was suggested that ferrous sulfate might

develop a metallic smell during mastication through oxidation of lipid compounds in the oral environment (Lawless et al., 2004).

Recent research on the smell of iron (Glindemann et al., 2006) suggested two mechanisms of iron smell occurrence. Iron can cause two different odors, musty and garlic that are assumed to be responsible for metallic flavor perception. Musty metallic odor is the odor we can smell on our hands after touching a coin or a metal key. The major compounds of musty metallic odors are carbonyls such as aldehydes and ketones. They are produced from lipid peroxides on the human skin via oxidative degradation caused by ferrous ion. Garlic metallic odor was noted on a cast iron plate by human subjects when metal was contacted by weak acid. The key odorant of garlic metallic odor is metal-phosphorus-carbon compounds. It is assumed that phosphoric or carbonic impurities in metals were released by acidic decomposition of metal, causing metallic odor (Glindemann et al., 2006).

Another important characteristic of metallic sensation was identified as electrical stimulus (Lawless et al., 2005). Plattig (1988) suggested that metals or metallic compounds created surface electrical potential, which is related to electrical sensation. Low levels of electric current generated by small batteries produced a sensation when the anterior part of the human tongue was stimulated. The electrical stimulus was described as sour, salty and metallic. However, the descriptive term "metallic" was selected more frequently when a list of attributes containing the word "metallic" was provided to panelists compared to when panelists generated their own descriptor and no list of descriptors was offered. The intensity of electrical stimulus was the greatest at the anterior part of tongue, and weakest inside the upper lip and cheek. Fungiform papillae populates densely on the anterior part of the tongue; the authors suggested that electrical stimulus is mediated by gustatory pathways rather than trigeminal pathways (Lawless et al., 2005).

Unlike the retronasal smell which characterizes metallic taste of ferrous sulfate, astringency is a common sensation for metallic compounds. Astringency is a tactile sensation rather than gustatory sensation (Plattig, 1988). The astringency from metal compounds was proven to be an oral tactile sensation (Lim and Lawless, 2005). When copper sulfate solution and ferrous sulfate

solution were applied on the non-gustatory region that is between the upper lip and gum, panelists perceived astringency from both compounds. However, astringency from ferrous sulfate solution was decreased by nasal occlusion, suggesting that ferrous sulfate may have a different tactile sensation from astringency or ferrous sulfate may involve a gustatory quality as well as the tactile quality. Astringency results from the loss of oral lubrication (Sarni-Manchado et al., 1999). It is widely known that astringency-causing compounds change the structure of salivary proteins or cause precipitation by binding with proteins, resulting in de-lubrication of the oral environment (Lu and Bennick, 1998; Sarni-Manchado et al., 1999; Bacon and Rhodes, 2000; Charlton et al., 2002).

It has been reported that metal causes a lingering aftertaste. In the protocols for sensory evaluation of metallic compounds, it was encouraged to rinse the mouth with deionized water or 5 mM sucrose solution, or consume non-salted soda crackers to get rid of lingering sensations (Keast, 2003; Lim and Lawless, 2005; Cuppett et al., 2006). In the research of Keast (2003), the lingering effect of various zinc salts was tested by introducing a series of three solutions consisting of one 50 mM zinc salt solution and subsequent two deionized water rinses over one min. Bitterness, sourness, savoriness, sweetness, and saltiness were greatly decreased or removed by water rinses, but intensity of astringency lingered significantly after rinse with 10 mL deionized water twice.

Factors Influencing Metallic Sensation

Effect of Anions that Bind to Metals in Metallic Compounds. Metals are usually incorporated into dietary substances as a form of metallic compounds. Various anions can bind to metallic cations to form metallic compounds. These anions are known to modify the taste of metallic cations by "anionic inhibition" (Lawless et al., 2003). In the research of Lawless et al. (2003), calcium lactate was less intense in bitterness and sourness, while slightly sweeter than the equimolar concentration of calcium chloride. Calcium salts of the organic anions, glycerophate and gluconate, were also evaluated as less bitter, irritative, salty, and sour than calcium chloride. Those anions which have larger molecular weight than chloride were

assumed to interrupt the contact of calcium to taste receptor sites or to barely diffuse into the receptors.

Yang and Lawless (2006) reported the simliar tendency of anionic inhibition in the study of time-intensity characteristics of three ferrous salts having anions with different molecular weights: chloride (molecular weight (MW):35.45), sulfate (MW: 96.1), and gluconate (MW: 195.18). Chloride salt exhibited higher intensity of bitterness than that of gluconate and sulfate salt. A decrease in bitterness in ferrous gluconate was attributed to anionic inhibition.

Keast (2003) showed that taste qualities of metallic compounds were affected by the anions of various zinc salts such as zinc chloride ($ZnCl_2$), zinc acetate ($Zn(CH_3COO)_2$), zinc iodide (ZnI_2), zinc sulfate ($ZnSO_4$), and zinc bromide ($ZnBr_2$). Saltiness, sourness, tingling/sting sensation, and astringency of the salts were significantly changed by the anions. Zinc iodide and zinc chloride were more intense in all four tastes described above, while zinc acetate was significantly weaker in those tastes compared to other salts. Zinc sulfate and zinc bromide had stronger tingling sensation than other salts, but showed less intensity in saltiness, sourness, and astringency. There was no significant difference among zinc salts for bitterness. Unlike the studies reported by Lawless and colleagues (2003; 2005), anionic inhibition on zinc taste was not evident in the zinc salts used in this research. The molecular weight of anions are increasing in the order of $2Cl^-$ (70.9) < SO_4^{2-} (96.1) < $2CH_3COO^-$ (114) < $2Br^-$(160) < $2I^-$ (253.8), but anionic inhibition was only evident between zinc chloride and zinc acetate. Iodide, the largest anion tested, and sulfate, the second smallest anion, did not follow that tendency. Yang and Lawless (2006) reported that a shell of water molecules is formed around sulfate anion because of its double negative charge. They postulated that a hydration sphere around the anion may increase its apparent molecular size, thus contribute to its anionic inhibition effect (Yang and Lawless, 2006).

Effect of pH and Other Electrolytes. Zinc, iron, and copper, which are common metals consumed through dietary substances, are good reductants in aerobic medium. These metals can be consumed either as solid metal forms or as a salt of a metal ion, depending on how metallic compounds are incorporated into dietary substances. They are oxidized into various cation

states, and ready to form the complexes with several ligands including OH^-, HCO_3^-, SO_4^{2-}, and so on. The solid metal or metallic salts are assumed to go through the redox process and ionized in an oral environment which is an aqueous buffer system, becoming ionic taste stimuli like NaCl and acids. This redox reaction is greatly influenced by pH condition (Jensen, 2003).

In the signal transduction system of NaCl and acids, the channels specific to Na^+ ions and H^+ ions mediate the current of Na^+ or H^+ into taste cells, causing action potentials that trigger synaptic events (Boughter and Gilbertson, 1999; Lindemann, 2001). Thus ionization of metallic compounds is thought to be important in perception of metallic taste.

pH of the food media or oral environment can influence perception of metallic compounds through controlling redox reactions of metallic compounds. pH of drinking water controls ionization, solubility of copper, and chemical complexation of copper in drinking water. In the study by Cuppett et al. (2006), the copper solubility diagram of pure water showed that copper has minimal solubility by forming a complex with hydroxy group (OH^-) at pH 8 ~ 11. In tap water, various electrolytes participate in the redox process and produce complexes with copper. Some anions such as phosphate, sulfate, and carbonate form highly insoluble complexes with copper, resulting in precipitation of copper (Dietrich et al., 2005).

It was suggested that the changes in copper chemistry by pH is related to change in perceived metallic taste (Cuppett et al., 2006). In the series of sensory threshold tests of copper, threshold values of copper in synthetic tap water at different pH were investigated. Panelists had lower thresholds for copper in water at lower pH, where more copper is solubilized. The comparison of pH 7.4 water and pH 8.5 water showed that particulated copper also played a role in perception of copper. Both waters had the same amount of soluble copper but pH 8.5 water had much higher particulated copper. It was hypothesized first that panelists would be able to detect copper in pH 7.4 water better, but panelists tasted copper better in pH 8.5 water. It was assumed that particulated copper reacted with saliva over time, and became solubilized to contribute to copper sensation.

ROLE OF HUMAN SALIVA IN PERCEPTION OF COPPER SENSATIONS

Effect of Saliva on Sensory Perception

Composition and Role of Saliva. Saliva is a hypotonic fluid excreted from parotid (inside the cheek near the ears), submandibular (beneath the floor of the mouth), sublingual (under the tongue), and other minor salivary glands. Saliva consists of water, electrolytes, glucose, ammonia, urea, and proteins such as enzymes, glycoproteins, and immunoglobulins (Table 1, Table 2) (Tenovuo, 1989; Lawless and Heymann, 1998). The submandibular saliva consistutes 70% of total volume of resting saliva and is composed of seromucous secretions (Becerra et al., 2003). The parotid gland secretes serous, watery fluid containing α-amylase, proline-rich proteins, and histatins. The sublingual gland produces mainly mucous secretion (Prinz and de Wijk, 2004). The composition of saliva varies depending on several factors including genetic differences among individuals, time within a day, time over long periods, and physiological and nutritional conditions (Tenovuo, 1989).

Saliva has several physiological functions. Saliva coats tissues to protect them from drying and from abusive conditions, and further plays an important role in the enamel pellicle formation. Also, saliva lubricates oral tissues so that tissues can be protected from the mechanical damage of mastication. Since electrolytes endow saliva buffer capacity, saliva can maintain a range of pH from 6.7 to 7.5 (for whole saliva). Saliva is related to control of the microflora composition in the oral cavity, with some components contributing antimicrobial activity (Tenovuo, 1989). Saliva plays a role in perception of flavor in the mouth (Plattig, 1988; Weiffenbach et al, 1995; Delwiche and O'Mahony, 1996; Hutteau and Mathlouthi, 1998; Lawless and Heymann, 1998).

Table 1. Electrolyte components and their concentrations[a] in unstimulated whole saliva.

Electrolyte	Mean ± s.d.[b]	Range
Sodium (mmol/L)	12.03 ± 8.90	0.93 ~ 31.15
Potassium (mmol/L)	21± 4	0.02 ~ 40
Calcium (mmol/L)	2.16 ± 1.11	0.001 ~ 2.8
Magnessium (mmol/L)	0.83 ± 0.33	0.13 ~ 1.78
Copper (μg/L)	19.5	6.3 ~ 460
Lead (μg/L)		0.8 ~ 18.8
Cobalt (μg/L)	0.03 ± 0.03	
Zinc (μg/L)	49.2	46.9 ~ 1627
Barium (μg/L)		5.7 ~ 23.2
Cadmium (μg/L)		0.47 ~ 3.5
Chloride (mmol/L)	29.0 ± 11.9	0.01 ~ 43.2
Chromium (μg /L)	230 ± 30	
Iodide (μmol/L)	5.5 ± 4.2	2 ~ 22
Nitrate(μmol/L)	10	2 ~ 84
Phosphate (mmol/L)	2.2 ± 1.5	
Sulfate (μmol/L)	1.45 ± 0.6	

[a] Data pooled from literature (Tenovuo, 1989; Vaughan et al., 1991; Chicharro et al., 1991; Menegário et al., 2001; Hershkovich and Nagler, 2004; Agha-Hosseini et al., 2006).
[b] Standard deviation.

Table 2. Classification of salivary proteins according to molecular weight and their characteristics.[a]

Range (kDa)	Proteins	Concentration	Molecular weight (kDa)	Function
>1000	High molecular weight salivary mucin	13.3 ± 11.6 mg% (in submandibular saliva)	>1000	Lubrication Antimicrobial Inhibition of demineralization
100 ~ 1000	Fibrinolectin	0.2 ~ 2 μg/mL (in whole saliva)	450	
	Fucose-rich glycoprotein		440	Antimicrobial
	Immunoglobulin A	96 ~ 102 μg/mL (in parotid saliva)	385	Antimicrobial
	Agglutinin		340	Antimicrobial
	Low molecular weight salivary mucin	14 ~ 203 μg/mL (in submandibular saliva)	200 ~ 250	Lubrication Antimicrobial Inhibition of demineralization
50 ~ 100	Lactoperoxidase		80	Antimicrobial
	Lactoferrin	1 ~ 2 μg/mL (in parotid and submandibular saliva)	76.5	Antimicrobial
	Peroxidase	5 ~ 6 μg/ml (in parotid saliva)	72 ~ 78	Antimicrobial
	Serum albumin		60 ~ 66.5	
	α-amylase	650 ~ 800 μg/mL (in parotid saliva)	55 ~ 60	Digestion Antimicrobial

Table 2 (continued). Classification of salivary proteins according to molecular weight and their characteristics.

Range (kDa)	Proteins	Concentration	Molecular weight (kDa)	Function
30 ~ 50	Carbonic anhydrase		42 ~ 71	Maintenance of HCO_3^- buffering
	Proline-rich glycoproteins (PRGs)		38.9 ~ 67	Lubrication Antimicrobial Protection against dietary tannin
	Basic PRPs (isomer Ps)		37 ~ 43	Protection against dietary tannin
	Gustin	1.40 ± 0.18 mg/dL (in parotid saliva)	37	Taste sensation
10 ~ 30	Acidic proline-rich proteins (PRPs)	7.7 mg% (in whole saliva)	15 ~ 24	Remineralization
	Lysozyme		14.7	Antimicrobial
	Cystatin	0.73 μg/L (in whole saliva)	11 ~ 20	Remineralization Protease inhibition
5 ~ 10	Basic PRPs (isomer IB)		6 ~ 22	Protection against dietary tannin
	Statherin	12.8μM	5.38	Remineralization
3 ~ 5	Histatins	44.4μM (in whole saliva)	3.1 ~ 4.8	Antifungal
1~3	Kallikrein		2.7 ~ 9.6	Protease

[a] Data pooled from literature (Minaguchi and Bennik, 1989; Tenovuo, 1989; Beeley, 1993; Castagnola et al. 2001; Becerra et al., 2003; van Nieuw Amerongen et al. 2004).

Effect of Saliva on Aroma. Saliva volume and components are important factors in aroma sensation. A group of people who had high parotid salivary flow rate (> 0.36 g/min) took longer time to reach the maximum intensity of cherry flavor than the low-flow group (< 0.36 g/min) in the time-intensity (TI) sensory test of chewing gum (Guinard et al., 1997). Release of volatile compounds from rehydrated bell peppers in a model mouth system was reduced when saliva volume increased (van Ruth and Roozen, 2000). Salivary enzymes are responsible for the release and changes of aroma compounds in the mouth. Alpha-amylase facilitates the release of methylpropanal and methylbutanal by breaking the starch that forms helical structures to surround volatiles (van Ruth and Roozen, 2000). Human salivary enzymes are assumed to cause the enzymatic degradation of carboxyl esters (ethyl butanoate, ethyl hexanoate, and ethyl ocatanoate) and thiols (2-phenyl-ethanethiol, 2-furfurylthiol, and 3-mercapto-3-methyl-1-butanol) as well as the reduction of aldehydes (hexanal, octanal, decanal, methional, and phenylacetaldehyde) to corresponding alcohols. These investigations explain aftertastes as an induced enzymatic change (Buettner, 2002a; Buettner, 2002b).

As a protein molecule, salivary α-amylase diminishes the release of 1-octen-3-ol. Salivary proteins change the volatility of aroma compounds in the mouth by binding hydrophobic molecules or by salting out hydrophilic molecules (van Ruth and Roozen, 2000; van Ruth et al., 2001). Friel and Taylor (2001) showed that there were three volatile groups that were assumed to use different binding mechanisms. Group 1 compounds (2-methyl butanol, dimethyl cyclohexanone, dimethyl pyrazine, linalool, menthone, and methyl acetate) showed no decrease in headspace concentration in the presence of mucin. Headspace concentration of group 2 compounds (cymene, decanal, decanol, and heptanal) was decreased by mucin. Partition of group 3 compounds (benzaldehyde, ethyl hexanoate, diacetyl, and heptyl acetate) into headspace also was affected by both mucin and salts. The association of mucin and aroma compounds was decreased when sugar was added to artificial saliva prior to adding the volatiles. This suggests that sugar hinders the binding of volatiles by either blocking binding sites or changing the conformation of mucin.

Effect of Saliva on Taste. Saliva is also very important in taste sensations. The primary sensory process in the mouth starts from the dissolution of taste molecules into saliva, allowing dissolved molecules to approach receptor sites (Plattig, 1988). Weiffenbach et al. (1995) observed that the threshold sensitivity of sweetness, saltiness, sourness, and bitterness decreased in patients of Sjorgen's syndrome, which causes reduction in salivary excretion, compared to normal individuals. In Sjorgen's syndrome, decreased salivary gland functions may be related to impaired taste sensation.

Salivary flow is important in gustation. There were significant differences between high-parotid salivary flow rate (HF) group and low flow rate (LF) group in temporal characteristics of the sweetness of chewing gum (Guinard et al., 1997) and temporal characteristics of bitterness and astringency of wines (Fischer et al., 1992). For bitterness and astringency of wines, LF subjects took longer time to perceive and higher maximum intensity than HF subjects. However, for sweetness of chewing gum, HF group demonstrated a longer time to maximum intensity than LF group. Fischer et al. (1992) suggested that astringency and bitterness decreased as tastants were desorbed from receptors. Thus it is assumed that desorption rate of different tastant molecules may cause different trends in TI study with LF group and HF group.

As a medium between taste molecules and receptors, saliva affects the taste sensitivity of sodium chloride (NaCl). Taste sensitivity to sodium chloride was reduced when the sodium cation (Na^+) level in saliva increased. Taste sensitivity was affected by even a very minor elevation of Na^+ content induced by chewing. When salivary Na^+ contents of subjects were increased by 2.8 ~ 32.2 mM, which was induced by chewing flavorless gum, the ability of subjects to discriminate NaCl signal from water "background" noise was decreased by up to 80% (Delwiche and O'Mahony, 1996).

Just as the sodium ion in saliva, components of saliva such as proteins and salts have an effect on the sensory process. The salivary calcium cation (Ca^{2+}) hinders hydration of sugars and sugar alcohols by attracting water to the Ca^{2+} with electrostatic force. The addition of salivary proteins such as mucin and α-amylase reduced the surface tension of a sweetener solution, which increased spreadibility of the solution. Thus it was suggested that salivary

components control the accessibility of sweetener molecules to receptor sites by modifying hydrating activity of the sweetener molecules, further influencing the sweet taste perception (Hutteau and Mathlouthi, 1998).

Astringency arises when salivary proteins lose the lubrication effect as phenolic compounds bind to salivary proteins to form a complex compound. Kallithraka et al. (1998) studied the protein-phenolic compounds interaction in human saliva using HPLC. After subjects drank tannin solution, new soluble salivary protein-phenolic compound complexes appeared in saliva while the amount of salivary protein decreased. This new complex was suggested to be the intermediate of tannin-induced protein precipitation process. The salivary protein-phenolic compound complex was not degraded in the condition mimicking the human digestive system and is assumed not to be absorbed in a gastrointestinal tract. Hence complex formation is regarded as the mechanism to protect humans from toxicity of phenolic compounds (Lu and Bennick, 1998).

Among salivary proteins, proline-rich proteins (PRPs) are known to be related to astringency. PRPs, especially basic PRPs, bind to tannins through hydrophobic interactions or hydrogen bonds, or the combination of both. More specifically, the hydrophobic faces of the aromatic rings of polyphenols stack onto the pyrrol ring of prolines in PRPs and result in the soluble protein-tannin complexes. As more phenolics are bound to proteins, phenolics on the surface of proteins provide intermolecular bridges between proteins, and then proteins become polymerized and precipitate (Lu and Bennick, 1998; Sarni-Manchado et al., 1999; Bacon and Rhodes, 2000; Charlton et al., 2002).

Histatins, a family of histidine-rich polypeptides (HRP), also have been reported to bind tannins. Tannins bind to the N and C terminal of histatins via hydrophobic interaction. This interaction occurs between the aromatic rings of tannins and aromatic (Phe and Tyr) and basic (His) residues of histatins. The histatin-tannin complex is insoluble at the condition similar to that of a human intestine, thus it is suggested that histatins participate in the defense system against tannin compounds. However, epigallocatechin gallate exhibits less precipitation than non-antioxidant tannins do at pH 7.4 and 37^{o}C. This suggests that the antioxidant phenolic

compound may be absorbed in a human gastrointestinal tract more easily compared to toxic tannin compounds (Naurato et al., 1999; Wroblewski et al., 2001).

Interaction between Salivary Proteins and Metals

Salivary proteins are expected to have an important role in the sensation of metals. Nickel dissolved in saliva from dentures causes a metallic sensation and dryness in the mouth (Pfeiffer and Schwickerath, 1991). Thus it can be postulated that metal uptake by saliva is related to sensory attributes caused by metals. Chapman and Lawless (2004) proved that copper sulfate formed a haze in human saliva at the concentration of 0.3 mM and 1.0 mM. Increase in turbidity of human saliva had a positive correlation with astringency caused by copper sulfate. It was suggested that copper ions caused astringency in the mouth by precipitating and de-lubricating PRP proteins.

Metal-salivary protein interactions have been studied mostly in dentistry research in relation with corrosion products of metal dentures. Mueller (1985) reported that corroded copper, nickel, and cobalt ions, from a dental alloy bound to a protein fraction of saliva as well as a non-protein fraction of saliva. Glycoprotein and mucin showed a high binding affinity to copper and nickel, and α-amylase had moderate binding affinity to these metals (Mueller, 1983). Copper from the dental alloy becomes soluble as salivary compounds bind to copper (Mueller, 1987).

Many salivary proteins exhibit metal-binding capacity for their biological functions. Salivary proteins carry calcium, iron, and zinc. Metals play an important role as co-factors of salivary enzymes, and binding of metals cause conformational changes in salivary proteins that eventually leads to important functionality such as antimicrobial activity or mineralization of dental enamel (Shatzman and Henkin, 1980; Argarwal and Henkin, 1987; Tenovuo, 1989; Brewer and Lajoie, 2000). Salivary proteins that show metal-binding capacity are mucin, α-amylase, PRPs, histatins, gustin, carbonic anhydrase, lactoferrin, statherin, and lysozyme.

Acidic PRPs and Statherin. Statherin is secreted from parotid and submandibular salivary glands. Statherin contains high proportion of acidic amino acids and two phosphate groups per

mole. Acidic PRPs are one of the sub-groups of the PRP family (acidic PRPs, basic PRPs, and glycosylated PRPs) that are present in parotid saliva. Since PRPs are polymorphic proteins, acidic PRPs also consists of several isoforms including PRP1~4, PIFs, PIFf, Dbs, Dbf, and Pa. Acidic PRPs are mainly composed of acidic amino acid residues and proline residues (Beeley, 1993; Madapallimattam and Bennik, 1990).

Metal-binding capacity of acidic PRPs and statherin is related to enamel pellicle formation for remineralization of the teeth. They are known to adsorb to calcium phosphate generically formed in the oral environment to prevent calcium phosphate crystals from growing and precipitating instead of attaching to dental enamel. The common structural feature of these proteins are acidic (pI of statherin = 4.2; pI of acidic PRPs = 3.5 ~ 4.5) and asymmetric negative charge distribution. These structural features may enable binding of copper cations (Tenovuo, 1989; Minaguchi and Bennik, 1989).

Acidic PRPs are especially of interest in regards to interaction with copper. Acidic PRPs and their post-secretion cleavage derivatives exhibit the ability to bind Ca^{2+} ions while statherin is reported to bind calcium phosphate. This may imply the possible interaction between cupric ion and acidic PRPs. As previously mentioned, PRPs are related to astringency of polyphenolic compounds, which is regarded as a protection mechanism against antidigestion effect of tannins (Minaguchi and Bennik, 1989; Madapallimattam and Bennik, 1990; Beeley, 1993; Lu and Bennik, 1998). Therefore, acidic PRPs may play an important role in perception of copper sensation in the mouth, especially astringency caused by copper as a warning system for potential hazard of copper overdose.

α-Amylase and Carbonic Anhydrase. Two salivary enzymes, α-amylase and carbonic anhydrase, each contain Ca^{2+} and Zn^{2+} for enzyme activity. α-Amylase is the most abundant enzyme in human saliva (Tenovuo, 1989) that requires calcium as a cofactor. Human α-amylase has two metal binding sites, one of which is exclusive binding site for Ca^{2+} and the other site is for loose binding with Zn or Cu cations. Ca^{2+} does not bind to the second binding site. Metal cations, such as Cu^{2+} or Zn^{2+}, can not replace strongly bound Ca^{2+} ion. Copper was

assumed to bind with α-amylase through glycine ligand of the enzyme with the binding constance (log K) of 13.97 (Argawal and Henkin, 1987).

Carbonic anhydrase is zinc-metalloenzyme. There are six isoenzymes in the carbonic anhydrase family. The major function of carbonic anhydrase is maintaining buffer capacity of saliva (Tenovuo, 1989). In the case of carbonic anhydrase II, Zn ion has tetrahedral coordination with three histidine residues located in the hydrophobic cluster of the protein and one hydroxo anion at physiological pH. Copper is coordinated with carbonic anhydrase II in bi-pyramidal configuration instead of tetrahedral mode. Although copper binds more tightly than zinc, binding of copper does not stabilize carbonic anhydrase due to its configuration of coordination, which explains the Zn-specificity of carbonic anhydrase (Hunt et al., 1999). This implies that copper may be less favored by carbonic anhydrase in biological systems.

Mucins and Gustin. Mucins and gustin are anionic glycoproteins at salivary pH (pI of mucin = 3 ~ 5; pI of gustin = 4.73). Mucins are mainly secreted from the sublingual gland, but present less in submandibular saliva and not found in parotid saliva. Mucins are classified into MG1 (molecular weight (MW) > 1000 kDa) and MG2 (MW 200 ~ 250 kDa). Mucins have a brush-like structure that has a linear tandem repeat backbone with outward projection of O-glycosidically linked sialic acid chains (Loomis et al., 1987; Wu et al., 1994). These sialic acid chains, negatively charged in salivary pH, are responsible for the anionic charge of mucins. Viscosity of mucin results from the flexible linear structure of mucins and repulsive forces between negatively charged side chains. It is known that viscosity of mucin can be reduced in the presence of electrolytes because cationic electrolytes neutralize negatively charged sialic acid moieties (Wu et al., 1994). Divalent ions, especially such as Ca^{2+}, induce more compact configuration by cross-linking between sugar chains. Divalent calcium cation is also known as a binding site for mucins on crystalline calcium phosphate generic in the saliva (Mueller et al., 1983; Wu et al., 1994). Mueller (1983) observed that 5 moles of copper bound to 1 mole of the bovine mucin, fetulin, which has 9 % sialic acid content. Based on previous studies, it is assumed that cupric ions (Cu^{2+}) can bind to mucin, by electrostatically binding to sugar chains.

Gustin is present in parotid saliva, constituting 3 % of total parotid salivary proteins. Gustin is identified as carbonic anhydrase VI, which explains its zinc-binding ability (K_d = 4.5 X 10^{-11} M at pH 7.2). One mole of zinc is bound to one mole of the protein, but a second zinc can loosely bind to gustin in the presence of excess zinc. Zinc is related with taste acuity, and the Zn-binding capacity of gustin is important because it controls homeostasis of salivary zinc. It was reported that the patients of hypoguesia (loss of taste acuity) has significantly lower level of gustin as well as salivary zinc than normal control subjects. Treatment of hypoguesia patients with exogenous zinc improved taste acuity significantly by increasing the level of salivary zinc and gustin (Shatzman and Henkin, 1981; Thatcher et al., 1998; Watanabe et al., 2005).

The second Zn binding site was reported to coordinate loosely with Cu, Ni, Fe, or Mn (Shatzman and Henkin, 1980) Watatanabe et al. (2005) suggested that high serum copper level may have an antagonizing effect on zinc metabolism, thus adversely influence taste acuity. Shatzman and Henkin (1980) reported that it was unknown whether copper on the second binding site had physiological functions.

Lactoferrin, Lysozyme and Histatins. These proteins have common characteristics in that they are cationic proteins and have antimicrobial activity. Lactoferrin consists of two domains that each bind one Fe^{3+} reversibly but tightly (binding constant K = 10^{20}). Antimicrobial activity of lactoferrin is due to its affinity to iron that eventually deprives microorganisms of essential nutrient, Fe^{3+}. One ferric ion (Fe^{3+}) coordinates six ligands on lactoferrin. Four ligands are constituted of two oxygen molecules in phenol rings of two tyrosine residues, one oxygen molecule in carboxyl group of one aspartate residue, and one imidazole nitrogen molecule of one histidine residue. Two ligands are provided by carbonate divalent anion that is located in the pocket of arginine and the amino terminal of a helix. It was found that lactoferrin can accommodate other transition metal ions such as Cr^{3+}, Mn^{3+}, Al^{3+}, Co^{3+}, Cu^{2+}, and Zn^{2+} with less binding affinity than that to Fe^{3+}. Binding of other metals other than Fe^{3+} modifies protein configuration, decreasing the binding affinity of the metals to lactoferrin (Baker et al., 1990; van Nieuw Amerongen et al., 2004). Even though this suggests the possibility of copper-lactoferrin interaction, there has been no observation of actual lactoferrin-copper binding in human saliva reported.

Lysozyme is known to hydrolyze polysaccharides of the cell wall of microorganism. Lysozyme is a highly cationic protein (pI = 11.2) at salivary pH range (van Nieuw Amerogen et al., 2004). The interaction between copper and salivary lysozyme has not been reported, but there is some evidence of copper-lysozyme binding. Copper appears to inhibit hydrolysis activity of egg white lysozyme by coordinating with a carboxyl group on aspartate and glutamate, and tryptophane with association constant (K_a) of 1.8 X 10^2 M^{-1} (Teichberg et al., 1974). In the study to investigate affinity of immobilized metal ion to lysozyme (Lin et al., 1999), Cu^{2+} formed more a stable association with lysozyme than Fe^{3+} did. Mueller (1983) reported that a very low binding of Ni^{+} to lysozyme due to high pI of proteins. These observations suggest that copper consumed through drinking water is not likely to interact with lysozyme at salivary pH.

Histatin is a family of histidine-rich polypeptides (HRP) secreted from the parotid and submandibular glands (Tenovuo, 1989). Twelve histatins, histatin 1-12 have been found, and the most common histatins found in human saliva are histatin 5, histatin 3, and histatin 7. These polypeptides are basic low molecular weight proteins of 3 ~ 4.5 kDa, which are positively charged in neutral and acidic pH. For example, histatin 5 consists of 24 residues of amino acids among which 14 residues are basic (7 histidine, 4 lysine, and 3 arginine) (Oppenheim et al. 1988; Melino et al. 1999). Histatins show a high binding affinity to copper at pH 7.4, with a binding constant of 2.6×10^7 M^{-1}. At pH 7.5, the histidine residues are presumably in deprotonated form, which is better for metal coordination. A high-affinity binding site for copper and nickel, an ATCUN (Amino Terminal CU (II) - and Ni (II) - binding) motif, is located near the N-terminal of histatin 3 and 5. The unique feature of ATCUN motif is an amino acid sequence of Asp-Ser-His. Another binding site of lesser affinity to copper is the C-terminal of histatin with which copper ions is assumed to associate nonspecifically (Brewer and Lajoie, 2000; Gusman et al., 2001; Grogan et al., 2001). Binding of metal ions to histatins are known to induce structural change which is responsible for their antifungal activity. The zinc-histatin 5 complex has the ability to fuse the membrane of microorganisms, which is a key feature of its antimicrobial ability (Oppenheim et al., 1988; Tenovuo, 1989; Melino et al., 1999; Gusman et al., 2001).

METHODOLOGIES FOR INVESTIGATION OF COPPER PERCEPTION MECHANISMS

Measurement of Orthonasal and Retronasal Aroma Perception

The study of aroma perception initially focused on qualifying/quantifying aroma compounds in dietary substances. Because flavor perception is dependent on how much aroma compounds can become available to olfactory receptors, interests in flavor study was shifted to partitioning behavior of aroma compounds to the headspace of foods. Introduction of gas chromatography-olfactometry (GC-O) reveals that not all aroma compounds, but only major key compounds contribute to flavor impression. Since it has been widely recognized that flavor compounds detected from foods cannot entirely explain perceived flavor, the focus of study is now in transition to investigating concentration and behavior of aroma compounds in-nose or in-mouth situations. This approach is expected to give better understanding in aroma perception by the human than headspace analysis (van Ruth et al., 2003; Roberts et al., 2003).

Aroma is perceived not only through the nostrils but also from the mouth via the pharynx. Orthonasal aroma, perceived through the nostrils, is related to inhalation of the air phase containing volatile aroma compounds ("sniffing"). On the other hand, retronasal aroma is perceived during food consumption. It is widely accepted that static headspace analysis is useful to study orthonasal aroma perception because it can provide useful thermodynamic data that can explain flavor release from the food matrix to the headspace. However, aroma release in the human mouth is influenced by several factors such as mastication, swallowing, existence of saliva, nasal airflow, and body temperature. Thus these factors should be considered in investigation of retronasal aroma (Roberts and Acree, 1995).

Orthonasal Aroma. Static headspace methods have been used to investigate the aroma release from different phases including water, oil, emulsion, and gels that are found in real food systems as well as the effect of several food components on release of aroma compounds at equilibrium (Andriot et al., 2000; Guichard and Langourieux, 2000; Fabre et al., 2002; Gianelli

et al., 2003; Jung and Ebeler, 2003). Physicochemical parameters, such as partitioning coefficient between two different phases, have been determined to establish the model for flavor release in the studies of static headspace analysis (Taylor, 2002).

Recently, solid-phase microextraction (SPME) methods have been used for sampling of volatile compounds for static headspace-GC analysis. SPME methods use a fused silica fiber coated with a very thin layer of stationary phase to adsorb volatiles from the matrix. SPME method is simple, rapid, solvent-free, economical, and sensitive compared to traditional extraction methods (Yang and Peppard, 1994; Steffen and Pawliszyn, 1996). On the other hand, SPME fibers have some disadvantages in sampling representative volatile profiles at equilibrium. First, SPME is more selective than a gas-tight syringe injection. In the SPME-headspace analysis, aroma compounds partitioned first between food matrix to headspace, and then between headspace and SPME fiber. The compounds of high partition coefficient between air and the fiber ($K_{\text{air-fiber}}$) will be extracted better than others. Also affinity of a volatile compound to a SPME fiber is dependent on a characteristic of solid phase. For example, a polydimethylsiloxane (PDMS) solid phase has high affinity to nonpolar compounds, while a divinylbenzen (DVB) solid phase adsorbs polar compounds. This makes some volatiles overloaded in short extraction time while other volatiles do not reach equilibrium. Moreover, overloaded volatiles show nonlinear behavior. Second, it has been observed that volatile compounds compete with each other for the polymer coating during the extraction. Over time, a volatile of higher affinity to SPME fiber can "kick out" other volatiles that had been already adsorbed when the high-affinity volatile is adsorbed above the upper limit of linear range. Third, the exhaustive SPME extraction, when extraction continues until the maximum amount of volatiles is bound to a fiber, can disrupt equilibrium status as adsorption of volatiles actually decrease volatile concentration in the headspace, which in turn, induces additional volatilization from the food matrix (Roberts et al., 2000).

"True" headspace sampling methods (Roberts et al., 2000) are suggested to overcome these disadvantages. In true headspace sampling, a SPME fiber is exposed to headspace only for a very short time (1 min). Even though the short sampling time decreases sensitivity, it can prevent additional volatilization of compounds that have high $K_{\text{air-fiber}}$ and allows volatiles to be

extracted in linear mode. Therefore, the true headspace sampling represents equilibrium headspace concentration better than exhaustive SPME sampling.

Despite the static headspace method is not capable of offering kinetic information, it can provide a good starting point for retronasal perception by serving as a tool to determine partitioning behavior of flavor compounds between food matrix and different phases in oral cavity such as saliva and headspace in the mouth (Leland, 1997; Friel and Taylor, 2001).

Retronasal Aroma. Measurement of retronasal aroma is expected to bridge the gap between flavor chemistry and sensory evaluation (Roberts et al., 2003). Nosespace analysis has been developed to study the retronasal aroma compounds actually delivered to olfactory receptors. In nosespace analysis, the air that flows through the nose passage is sampled by inserting a tube into the panelists' nostril. Exhaled and inhaled air is transferred on-line to a gas chromatograph-mass spectrometer (GC-MS) through the fused silica capillary tube inserted to the gas sampling tube. Nosespace analysis makes *in vivo*, on-line, and breath-by-breath analysis of retronasal aroma possible (van Ruth et al., 2003; Taylor and Hort, 2004).

Nosespace analysis requires instrumentation that is compatible with real-time, *in vivo* analysis. In nosespace analysis, the aroma compounds are present in the exhaled air phase that contains high humidity. A high level of water in the sample is an obstacle for GC-MS analysis. Also, the very low concentration of volatiles and interference of other compounds in the breath requires very sensitive detection equipments. Real-time analysis requires speedy sampling (every 0.01-0.1 sec) and simultaneous, fast detection (Taylor et al., 2000).

Recently developed direct inlet MS enables the instrumentation for the real-time retronasal analysis. Two techniques, atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) and proton transfer reaction-mass spectrometry (PTR-MS) have been used for nosespace analysis. These techniques are based on "soft" chemical ionization in which an initial reactant ion (RH^+) transfers protons to other molecules (X) to produce molecular ions (XH^+) of compounds. Analytes are sprayed into an atmospheric interface between an inlet and a standard quadropole MS by a heated nebulizer. Ionization occurs by the plasma created by a corona

discharge at atmospheric pressure, and ions formed are sampled into a quadropole MS. APCI uses protonated water ion (H_3O^+) as a reactant ion, so high humidity in exhaled breath plays an important role in analysis rather than becomes an obstacle (Taylor et al., 2000). PTR-MS shares basic operation theory with APCI, but has an advantage that does not require external standards for quantification because ionization of volatiles is individually controlled (van Ruth et al., 2003).

Another trend in investigation of retronasal aroma is development of model mouth systems. Model mouth systems have been suggested as an alternative for nosespace analysis to overcome poor reproducibility of *in vivo* sampling. There are huge variances found within a subject as well as between the subjects in nosespace analysis due to several uncontrollable factors including physiological and psychological condition of human subjects, and complicated nature of food matrix (Rabe et al., 2002). Model mouth systems simulate dynamic conditions of the human mouth by incorporating artificial saliva, shear forces, air flow, and the human body temperature (Roberts and Acree, 1995; van Ruth and Roozen, 2000). Although model mouth systems generate reproducible data, they should be examined for whether the data can be a good approximation for *in vivo* analysis. A study to validate a model mouth system (Deibler et al., 2001) showed that there are meaningful correlations between data generated by nosespace analysis and data from the model mouth system. However, unlike nosespace analysis which generates breath-by-breath aroma profile, model mouth systems are not capable of providing temporal information. Recently, a computerized model mouth system was developed by Rabe et al. (2002) to improve time-intensity aspects of the traditional model mouth systems.

A current trend for measurement of retronasal aroma is combining several techniques to investigate complex physiological process during retronasal aroma perception. Simultaneous measurement of exhaled breath, nasal flow and muscle movement (Hodgson et al., 2003) showed that release and delivery of aroma are influenced by air flow inside the mouth. Oral movements such as mastication and swallowing modify air flow from the mouth to the nose by pumping or creating fluctuation of the air flow.

Time-Intensity Sensory Test

Time-Intensity Test. Time-intensity (TI) test is defined as "the measurement of the intensity of a single sensory sensation over time in response to a single exposure to a product or other sensory stimulus" (ASTM, 2003). Sensory perception is a dynamic process as flavor compounds are elicited from the food matrix and influenced by various physicochemical factors, such as salivary composition and flow, mastication, and interaction between several food components. However, most sensory evaluation methods use single-point rating techniques that require panelists to process huge amounts of temporal sensory information into a generalized single value (Lee and Pangborn, 1986). A problem with the single-rating techniques is that important information related to the changes over time such as duration of taste, time to maximum intensity, and so on, is lost (Lawless and Clark, 1992). The TI test method is of interest especially when the traditional single-rating methods are not sufficient to reveal the difference between products that have different temporal characteristics (ASTM, 2003).

Information about temporal characteristics can be extracted from a TI-curve. A TI-curve is drawn by plotting the rated intensities versus time. Data can be obtained by either Cued technique or Real-Time technique. With Cued technique, panelists record the intensity on separate scales whenever they hear audible cues provided at the interval of several seconds. Conversely, Real-Time technique requires panelists to record their response continuously over time using devices such as a chart recorder or a computer device (ASTM, 2003). A TI curve can provide many parameters that can explain dynamic changes in sensory attributes: when stimulus starts to appear, how long it lasts, how fast it can reach its maximum intensity, how long maximum intensity lasts, and so on. A representative TI curve and the parameters from the TI test are summarized in Figure 4 and Table 3.

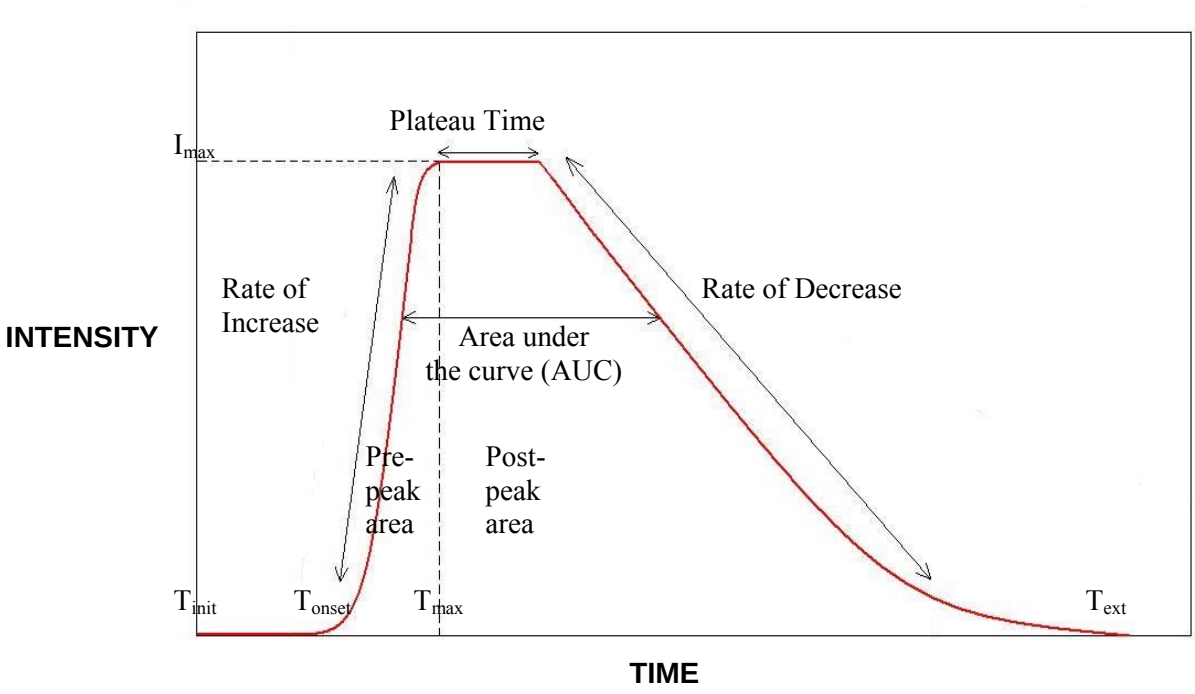

Figure 4. Representative TI curve (Reprinted, with permission, from E1909-97(2003) Standard Guide for Time-Intensity Evaluation of Sensory Attributes, copyright ASTM International, 100 Barr Harbor Drive, West Conshohocken, PA 19428).

Table 3. Definition of TI parameters (Extracted, with permission, from E1909-97(2003) Standard Guide for Time-Intensity Evaluation of Sensory Attributes, copyright ASTM International, 100 Barr Harbor Drive,West Conshohocken, PA 19428.)

Term	Abbreviation	Definition
Peak Intensity	I_{max}	Maximum observed intensity during the time of measurement.
Area Under the Curve	AUC	Area under the TI curve.
Pre-Peak Area		Area before $I_{max.}$
Post-Peak Area		Area after $I_{max.}$
Perimeter		Measured distance of perimeter of area delineated by TI curve.
Plateau Time		Duration of peak intensity.
Rate of Increase		Rate of intensity increase before peak intensity (slope).
Rate of Decrease		Rate of intensity decrease after peak intensity (slope).
Duration Time	T_{dur}	Time from onset of sensation until it can no longer be perceived ($T_{ext} - T_{onset}$).
Extinction Time	T_{ext}	Time from initial exposure to the stimulus (T_{init}) until it can no longer be perceived.
Initial Exposure Time	T_{init}	Time of initial exposure to the stimulus, typically when the clock starts.
Time to peak intensity	T_{max}	Time to reach maximum intensity of the sensation after exposure to the stimulus.
Onset Time	T_{onset}	Time point when the stimulus is first perceived after initial exposure to the stimulus.

Time-Intensity Curve Summarization. TI curves from each individual are usually averaged into a mean curve to enable inspection of perception patterns. A mean curve is usually obtained by either connecting main TI parameters with straight lines or averaging the intensity responses at the given time points (ASTM, 2003). The problem with these methods is that the resultant mean curve does not represent the majority because individual curves have different patterns (MacFie and Liu, 1992). Methods to overcome this problem have been suggested by Overbosch et al. (1986), Liu and MacFie (1990), and van Buuren (1992) (MacFie and Liu, 1992; ASTM, 2003).

Overbosch et al. (1986) suggested normalization of intensity of each curve according to the geometric mean of maximum intensities. Time dimensions of each curve were then split in equal steps before and after T_{max}. The geometric mean of normalized intensities for each time segment was calculated. The final mean curve was produced by plotting the calculated intensity values against the time segements (MacFie and Liu, 1992; ASTM, 2003).

Liu and MacFie (1990) suggested the modified Overbosch method. The traditional Overbosch method did not allow the plateau and multiple peaks, and was not applicable when there is missing intensity value or zero-end at the end of curves. In Liu and MacFie's method (1990), both the intensity axis and the time axis of each curve are normalized in order to keep original traits in individual curves in the averaged curve (MacFie and Liu, 1992; ASTM, 2003). The steps of Liu and MacFie's method to average TI curves are illustrated in Figure 5.

Van Buuren (1992) used Principal Component Analysis (PCA) to analyze TI curves. All TI data was converted into three different PCA curves, each explaining major variances of TI data matrix. The plots themselves were not the average curve, but could be used to understand the difference between individual curves (MacFie and Liu, 1992; ASTM, 2003). MacFie and Liu (1992) suggested that the van Buuren method can be used as an initial tool to examine the variance of data for deciding whether conventional univariate analysis or more sophisticated method should be used for averaging TI curves.

Investigation of Protein-Copper Binding

Metals are tightly bound to proteins as cofactors of enzymes or coordinate less tightly with protein ligands to be transported to target tissues. Proteins, amino acids, and peptides are good ligands for metals because of functional groups in protein. The most common functional groups that coordinate with metals are sulfhydryl groups (SH-) in cysteine, disulfide group (S—S) in cystine, and deprotonated nitrogen group in histidine. Metal-carbon bonds are found in biosynthesized selenoproteins (Szpunar, 2000).

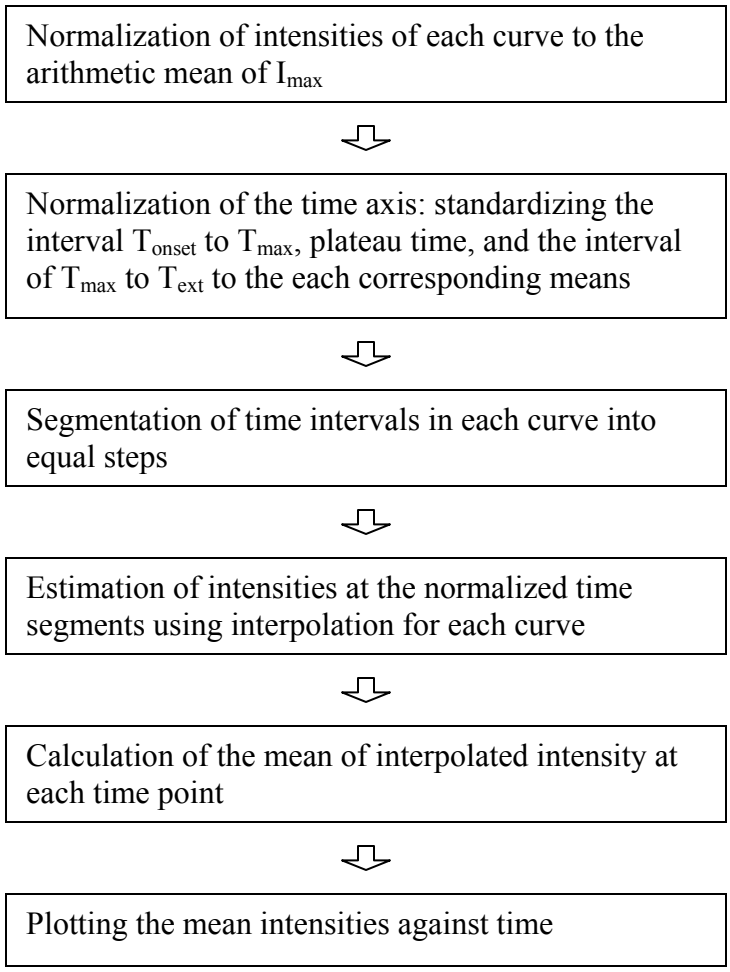

Figure 5. The steps to average time-intensity curves in Liu and MacFie's method (ASTM, 2003)

Binding behavior of copper to a certain protein has been determined by calculating binding constant or plotting binding curves from the molar concentration of protein ligand, free copper, and copper bound to ligands. Equilibrium dialysis or ultrafiltration has been widely used to separate free copper from bound copper based on physical molecular weight cut-off fractionation. Copper concentration of free copper fraction and bound copper fraction is determined using atomic absorption spectroscopy (AAS), inductively coupled plasma (ICP), calorimetry, or spectrophotometry (Arnaud and Favier, 1992; Hunt et al., 1999; Babaeva et al., 2006). While equilibrium dialysis takes a long time until equilibrium is reached, ultrafiltration can be performed in a comparatively short time due to the recent development of centrifugal

ultrafiltration devices that use centrifugal force to induce tangential flow filtration (Kanovian and Chernokalskaya, 2006). Bohrer et al. (2004) developed solid phase extraction (SPE) based on the phenomena that proteins adsorb strongly on the surface of plastic polymers. SPE was performed with a microcolumn packed with high molecular weight polyethylene to separate free copper from copper bound to serum ceruloplasmin, and showed a good agreement with ultrafiltration data.

Hyphenated techniques are used to identify speciation of copper in complex biological systems. Hyphenated techniques are on-line/off-line coupling of protein separation techniques, metal detection techniques, and identification techniques. Various proteins in biological systems are separated with high-performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE), and gel electrophoresis. Separated protein fractions are transferred on-line or off-line to AAS or ICP for determining metal concentration in each fraction. In case of gel electrophoresis, copper is determined on the gel before or after blotting with radioactive methods (Szpunar, 2000).

Several HPLC techniques including size exclusion chromatography (SEC), ion exchange chromatography (IEC), and reversed phase chromatography (RPC) are being used for protein separation. SEC has been used for studying speciation of metallothionein in fish tissue and speciation of transitional metals in blood and saliva (Pomazal et al., 1999; Muniz et al., 2001; Infante et al., 2003). Compared to IEC and RPC, SEC is more advantageous to study biological systems because it does not denature proteins during elution. However, SEC does not provide good separation because of small numbers of theoretical plates, especially for low molecular weight proteins (Pomazal et al., 1999). It should be considered that finding metals in protein fractions does not necessarily mean binding of metals to proteins. Binding of metals to proteins can be verified only when the presence of metal attached to the protein that is bound to the antigen is confirmed by affinity chromatography (Szpunar, 2000).

ICP-MS has been gaining popularity in detection/identification of metallic species for its high sensitivity and capability of multi-element analysis (Infante et al., 2002; Hagege et al., 2004). In most cases of analyzing unknown speciation in biological samples, the lacks of

commercially available standards hinder quantification of possible biometal complexes. Isotope dilution methodology, which spikes samples with isotopes after chromatographic separation, provides reliable quantification results (Muniz et al., 2001). Electrospray ionization MS (ES-MS) is gaining interest recently because it enables accurate determination of molecular weight of metal-protein species by generating molecular ions from biometal complexes by soft ionization technique (Szupnar, 2000; Benson et al., 2003; Hagege et al., 2004).

Unlike investigating binding behavior of a single protein or a protein whose binding behavior has been well identified, exploring unknown metal complexation requires a great deal of consideration and precaution for setting up the right methodology. As previously mentioned, quantification can be problematic since many standards for metalloproteins are not commercially available (Muniz et al., 2001). There are always possibilities that metals are dissociated from the complex and dissociation intermediates are mistaken as the target complex during the analysis procedure. Since dissociation of complexes is greatly influenced by pH and ionic strength of solvents, these factors should be controlled carefully throughout the analysis (De Cremer et al., 1999). However, a complex biological system has various proteins that differ in their optimal pH and ionic strength. This complexity can make it difficult to find optimal conditions for all possible metal-protein complexes.

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Chapter III . Effect of Copper on the Volatility of Aroma Compounds in a Model Mouth System

ABSTRACT

Copper is thought to influence aroma perception by affecting volatility of aroma compounds in the mouth through interaction with salivary components, especially proteins. Our objective was to identify the effect of copper on the volatility of aroma compounds and the role of copper-protein interaction in volatile chemistry in the mouth. Copper (2.5 mg/L) and four aroma compounds (hexanal, butyl acetate, 2-heptanone, and ethyl hexanoate, 0.5 μL/L each) were added to model systems containing water, electrolytes, and artificial saliva at different pH levels. Headspace concentration of each volatile was measured using SPME-GC analysis. Copper in the model systems increased headspace concentration of volatiles at pH 6.5 by 5 ~ 10 %, but no change in volatility was observed at pH 7.0. At pH 7.5, presence of copper in the artificial saliva system containing mucin and α-amylase decreased headspace volatile concentration by 5 ~ 10 %, whereas histatin did not cause any changes in volatility. Effect of copper on volatiles at pH 6.5 may be due to increased solubility of copper at lower pH. Copper seems to facilitate hydrophobic binding between mucin and aroma compounds at pH 7.5, possibly by exposing hydrophobic sites of mucin.

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INTRODUCTION

In the U.S. there is a high potential for excessive copper intake from drinking water through corroded copper plumbing systems. An estimated 70-80% of drinking water pipes currently in or being installed in new homes in the USA is made of copper. Copper is usually present in fresh water in low amounts (typically less than 0.075 mg/L), but the concentration can increase substantially when water travels though copper pipes within residential homes (Dietrich et al., 2004; Edwards et al., 2004). According to USEPA databases, in 2003 there were 471 individual drinking water systems that violated the safety based standard of 1.3 mg/L Cu, potentially affecting 622,000 people (USEPA, 1991; USEPA, 2003). An aesthetic based standard is 1 mg/L Cu because copper above this level can contribute to metallic or bitter tasting water (USEPA, 1997).

Copper has bitter, astringent, sour, salty, metallic tastes, or an electric sensation (Zacarias et al., 2001; Lawless et al., 2005). Pizarro et al. (1999) reported that more than 3 mg/L of copper in drinking water could cause nausea, vomiting, and abdominal pain. Because odor and taste have been important indicators of potential contamination (Zoeteman, 1980), the unpleasant sensations of copper are assumed to be an initial biological protection mechanism from acute copper toxicity. The threshold levels of copper sensation range from 1 to 13 mg/L (Cohen et al., 1960; Bequin-Bruhin, 1983; Zacarias et al., 2001; Cuppett et al., 2006), depending on the testing method and demographic composition.

Studies on metal taste suggest that saliva plays an important role in perception of metal. Nickel, from dentures, dissolved in saliva causes a metallic sensation and dryness in the mouth (Pfeiffer and Schwickerath, 1991). Chapman and Lawless (2004) determined that 0.3 mM copper sulfate formed a haze in human saliva, suggesting that copper ions caused astringency in the mouth by precipitating and de-lubricating proline-rich proteins (PRPs).

Saliva is a hypotonic fluid of pH 6.7 ~ 7.5. Saliva consists of water, electrolytes, glucose, ammonia, urea, and proteins such as enzymes, glycoproteins, and immunoglobulins (Tenovuo, 1989). Each salivary component serves several physiological functions, such as tissue coating,

lubrication, buffer capacity, antimicrobial activity, and perception of flavor (Tenovuo, 1989; Van Nieuw Amerogen et al., 2004).

Human salivary proteins have been reported to change partitioning behavior of aroma compounds in the mouth, resulting in potential changes in aroma perception (van Ruth and Roozen, 2000; van Ruth et al., 2001). Salivary proteins change the volatility of aroma compounds by binding hydrophobic molecules or by salting out hydrophilic molecules (van Ruth et al., 2001; Friel and Taylor, 2001). Salivary glycoproteins such as mucin and α-amylase showed a moderate or high binding affinity to copper (Mueller, 1983; Mueller, 1985; Argarwal and Henkin, 1987). Histatins, a class of salivary polypeptides, also bind copper. Histatin is a family of histidine-rich polypeptides (HRP) (Tenovuo, 1989). Twelve histatins, identified as histatin 1 through 12 have been found, and the most common histatin found in human saliva are histatin-5. Histatin-5 showed a high binding affinity to copper at pH 7.4, with a binding constant of 2.6×10^{7} M^{-1} (Tenovuo, 1989; Oppenheim et al., 1988; Brewer and Lajoie, 2000; Grogan et al., 2001; Gusman et al., 2001). This suggests that copper might influence flavor perception through an interaction with salivary components, especially copper-binding proteins.

OBJECTIVES

Different flavor impression can be caused when partitioning behavior of aroma compounds between the oral headspace and saliva is modified. Several factors, such as the chemical composition of saliva, salivary flow, mastication, and food components, influence portioning behavior of aroma compounds in the mouth. When copper is consumed from drinking water, it may affect the portioning behavior of aroma compounds through interaction with salivary electrolytes or salivary proteins. This research was performed to determine the influence of copper on release of aroma compounds from saliva through interaction with salivary components using a model system.

MATERIALS AND METHODS

Model Mouth System

Artificial Saliva. Artificial saliva was formulated as described by van Ruth and Roozen (van Ruth and Roozen, 2000). The list of ingredients and their final concentration in the artificial saliva model system is shown in Table 4. $NaHCO_3$ (5.208g), $K_2HPO_4 \cdot 3H_2O$ (1.369g), NaCl (0.877g), KCl (0.477g), and $CaCl_2 \cdot 2H_2O$ (0.411g) were separately dissolved in 100 mL ultrapure water (Barnstead nanopure water purification system, Barnstead International, Dubuque, IA) to make ten-fold concentrated stock solutions. Both mucin (from porcine pancreas, Sigma, St.Louis, MO) 0.216g and 20,000 units of α-amylase (from *Aspergillus oryzae*, Aldrich, Milwaukee, WI) in combination were dispersed in 40 mL of ultrapure water. Ten milliliters of each salt stock solution were added to the protein dispersion with stirring and the total volume of artificial saliva was brought to 100 mL with ultrapure water. The pH of the artificial saliva was adjusted with dropwise addition of 6 N HCl solution to pH 6.5, pH 7.0, or pH 7.5. Artificial saliva was used within one hour of formulation.

Sample Preparation. Four aroma compounds, hexanal, butyl acetate, 2-heptanone, and ethyl hexanoate (Sigma, St. Louis, MO), were selected from different chemical species such as aldehydes, ketones, and esters to study the effect of saliva on the various classes of aroma compounds. These volatiles showed the most changes in their headspace concentrations in the presence of proteins compared to in the presence of water (van Ruth and Roozen, 2000; Friel and Taylor, 2001; Jung and Ebeler, 2003). The four aroma compounds were prepared into one stock solution by adding 1 μL of each aroma compound to ultrapure water in a 100mL volumetric flask. After adding aroma compounds, the flask was instantly sealed and sonicated (Ultrasonic cleaner FS20, Fisher Scientific, Pittsburgh, PA) for 30 min at room temperature (22^{o}C) to dissolve aroma compounds. The final concentration of each aroma compound in the model systems was 0.5 μL/L (v/v). $CuSO_4 \cdot 5H_2O$ (Sigma, St. Louis, MO) was prepared as 200 mg/L concentration of stock solution. The stock solution was diluted to give a final copper concentration of 2.5 mg/L

(3.9×10^{-5} M) as Cu in a model mouth system. The copper concentration represents an estimate of sensory threshold level of copper in drinking water (Zacarias et al., 2001).

Table 4. Final concentration of each ingredient in the artificial saliva model system.

	Ingredients	Concentration (w/v)	Molar concentration (M)
Artificial saliva	Sodium bicarbonate ($NaHCO_3$)	5.208 g/L	0.062
	Potassium phosphate dibasic trihydrate ($K_2HPO_4 \cdot 3H_2O$)	1.369 g/L	0.006
	Sodium chloride (NaCl)	0.877 g/L	0.015
	Potassium chloride (KCl)	0.477 g/L	0.006
	Calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$)	0.411 g/L	0.003
	Mucin[a] (from porcine pancreas)	2.160 g/L	NA[b]
	Alpha-amylase[c] (from Aspergillus oryzae)	200,000 unit/L	NA
Aroma compounds[d]	Hexanal ($C_6H_{12}O$)	0.42 mg/L	4.16×10^{-6}
	Butyl acetate ($C_6H_{12}O_2$)	0.44 mg/L	3.79×10^{-6}
	2-heptanone ($C_7H_{14}O$)	0.41 mg/L	3.59×10^{-6}
	Ethyl hexanoate ($C_8H_{16}O_2$)	0.43 mg/L	3.01×10^{-6}
Copper	Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$)	10 mg/L (2.5 mg/L as Cu)	3.94×10^{-5}

[a] The molecular weight of porcine pancreatic mucin is not provided by the manufacturer.
[b] Not applicable.
[c] Concentration is based on unit, not weight.
[d] The final concentration (v/v) of aroma compounds in the model systems is 0.5 μL/L each.

Artificial saliva (36 mL), copper stock solution (2 mL), and aroma compounds stock solution (2 mL) were pipetted into a pre-chilled 40-mL clear glass bottle. Bottles were immediately

sealed with Teflon septa (Supleco, Bellefonte, PA). Bottles were sonicated at 37°C for 5 min to ensure complete mixing and then chilled in the ice bath for another 20 min. Ultrapure water, and a salt solution that has the same electrolyte composition as artificial saliva but no proteins, were used as control samples. Because ultrapure water does not have sufficient ionic strength to generate electric conductivity for pH measurement and results in drift of pH value (ASTM, 2004), sodium bicarbonate was dissolved in ultrapure water at the level of 1 mM to generate stable pH value. The pH of control samples was adjusted to the same level as pH of artificial saliva.

Model Mouth System. The model mouth system was set up as a modification of the method described by van Aardt et al. (2001). Each sample (4 mL) was pipetted into 7-mL clear glass bottles containing small magnetic stir bars. Bottles were immediately sealed with Teflon septa (Supelco, Bellefonte, PA). All operations were performed using pre-chilled glassware in ice bath to prevent evaporation of volatile compounds.

Headspace Solid Phase Microextraction Gas Chromatography

Solid-Phase Microextraction (SPME). Volatiles were trapped using a 75-µm carboxen-polydimethylsiloxane fiber (Supelco, Bellfonte, PA). Adsorption/desorption of the volatiles was performed using the true-headspace sampling method (Roberts et al., 2000; Jung and Ebeler, 2003). After the equilibrium between aqueous phase and gas phase was achieved by stirring samples at 37°C for 15 min in the SPME heating unit (Supelco, Bellfonte, PA), volatiles in the headspace were adsorbed to the fiber for 1 min. The SPME fiber was instantly moved to a gas chromatography (GC) injector port and thermally desorbed at 280°C with the splitless mode. Fiber was left in the injector port for 9 min with purging for cleaning. To improve resolution of the chromatogram, desorbed volatiles were cryofocused at -30°C for 1 min, and then temperature of the cryofocusing unit (Micro Cryo-Trap 981LN_2, Scientific Instrument Services Inc., Ringoes, NJ) was instantly raised to 270°C and held for 10 sec to facilitate rapid transfer of volatiles from the unit to the column. The linearity between FID response and headspace concentration was validated by checking the calibration curve for each aroma compound and combined aroma

compound solution. The calibration curve was determined by adding 0.01, 0.1, 0.5, 1, and 2 mg/L of each aroma compound into the model mouth system that contained water and artificial saliva, respectively. The R^2 values of the linear plots for each aroma compound were 0.90~0.96, indicating that SPME-GC analysis can detect changes in headspace volatile concentration quantitatively. There was no interference effect on SPME adsorption of individual volatile compounds when combined with other volatile compounds.

Gas Chromatography (GC). Volatiles were analyzed using a Hewlett Packard gas chromatograph (model 5890A, Hewlett Packard, Avondale, PA) equipped with a Hewlett Packard 5895A Chemstation. Analysis was performed on an HP-5 column (25m X 0.32mm, 1.05μm film thickness, Supelco, Bellfonte, PA) with a 1.0 mL/min (linear flow velocity: 18.6 cm/sec) helium carrier gas flow. The initial oven temperature was 40°C, and then increased to 90°C at the rate of 30°C/min, followed by a rate of 8°C/min to 150°C. Compounds were detected using a flame ionization detector (FID) at 300°C.

All samples were analyzed in triplicate. Since a known amount of volatile compounds was added, and partitioning of volatiles is expected to be influenced by the treatment, quantitation using the external standard curve is not useful for identifying the effect of the salivary components. Thus peak area was used as raw data for statistical analysis and then the mean peak area for each compound was expressed as a ratio of the peak area to that in the water model system. Data obtained at different pH levels were subjected to two-way analysis of variance (ANOVA) (α=0.05) using JMP IN® statistical software (*ver.* 4.0, SAS, Cary, NC). Mean values of different treatments were compared using Tukey's HSD test.

Effect of Histatin-5 on the Headspace Concentration of Aroma Compounds

Histatin-5 (American Peptide Co. Sunnyvale, CA) was added to the artificial saliva model system of pH 7.5 at the level of 49.5 μM, which represents the median value of histatin concentration in human saliva (Castagnola et al., 2001). An artificial saliva model system of pH 7.5 that contained no histatin was used as a control. Headspace concentration of aroma

compounds was measured with SPME-GC analysis. All samples were analyzed in duplicate. The result of each treatment was expressed as the FID response value. Data obtained were subjected to t-test to find significant differences between the control and the histatin treatment (α=0.05).

Copper Speciation in the Model System

Recent research of copper taste thresholds (Cuppett et al., 2006) reported that solubility of copper may be related to perception of copper. Solubilization of copper depends on pH, so all added copper is not soluble within salivary pH range (Jensen, 2003; Cuppett et al., 2006). In order to identify the effect of copper speciation on volatilization of aroma compounds in the model systems, the concentrations of soluble and precipitated copper in the water and the salt solution model systems were estimated by MINEQL+ chemical equilibrium modeling software (ver. 4, Environmental Research Software, Hallowell, ME) The soluble copper concentration in the artificial saliva model system could not be calculated by the MINEQL+ software because thermodynamic data of salivary proteins-electrolytes interaction required for calculation are not available.

RESULTS AND DISCUSSION

Effect of the Model Mouth System Composition, Copper Concentration and Their Interaction at Different pH Levels

Table 5 shows F-values calculated by ANOVA of headspace concentrations of four aroma compounds (hexanal, butyl acetate, 2-heptanone, and ethyl hexanoate) in three different model systems (ultrapure water, salt solution, and artificial saliva) measured at pH 6.5, 7.0, and 7.5. Effect of the model system composition was significant for all four volatiles at different pH. Effect of copper on volatility of selected compounds was significant only at pH 6.5, while

interaction effects between composition and copper was only significant for selected compounds at pH 7.5.

Table 5. Probability levels[a] associated with F values[b] of three variables (composition of the model system, copper treatment, and interaction between composition and copper treatment) on headspace volatile concentration of four aroma compounds.

Effect / Aroma Compounds	Probability levels associated with F values								
	Composition of the model system (Water, Salt solution, Artificial Saliva) [c]			Copper concentration (control, 2.5 mg/L)			Interaction (Composition × Copper)		
	pH 6.5	pH 7.0	pH 7.5	pH 6.5	pH 7.0	pH 7.5	pH 6.5	pH 7.0	pH 7.5
Hexanal	**< 0.001**	**< 0.001**	**< 0.001**	**0.045**	0.549	0.158	0.231	0..975	**0.009**
Butyl Acetate	**< 0.001**	**< 0.001**	**< 0.001**	**0.071**	0.415	0.961	0.509	0.851	0.180
2-Heptanone	**< 0.001**	**< 0.001**	**< 0.001**	**0.042**	0.107	0.811	0.542	0.837	**0.057**
Ethyl Hexanoate	**< 0.001**	**< 0.001**	**< 0.001**	0.135	0.120	0.577	0.145	0.811	**0.021**

[a] In bold : significant or nearly significant at α=0.05 within a column (within same pH).
[b] ANOVA of experiment data in triplicate.
[c] Water : ultrapure water buffered with 1mM $NaHCO_3$.
Salt solution : $NaHCO_3$ (0.5208 g), $K_2HPO_4 \cdot 3H_2O$ (0.1369 g), NaCl (0.0877 g), KCl (0.0477 g), and $CaCl_2 \cdot 2H_2O$ (0.0411 g) in 100 mL of ultrapure water.
Artificial saliva : mucin (from porcine pancreas) 0.216 g and 20,000 unit of α-amylase (from Aspergillus oryzae) in 100 mL of salt solution.

Effect of the Model System Composition. Composition of the model system had significant effects on volatility of all aroma compounds at pH 6.5, 7.0, and 7.5. Significant differences were mainly observed between the water and the salt solutions, but not between the salt solutions and the artificial saliva. Salivary electrolytes significantly increased headspace concentration of volatiles by 20 ~ 30 %. Salivary proteins lowered volatility by 5 ~ 10%, but only for hexanal at pH 7.5 (Figure 6).

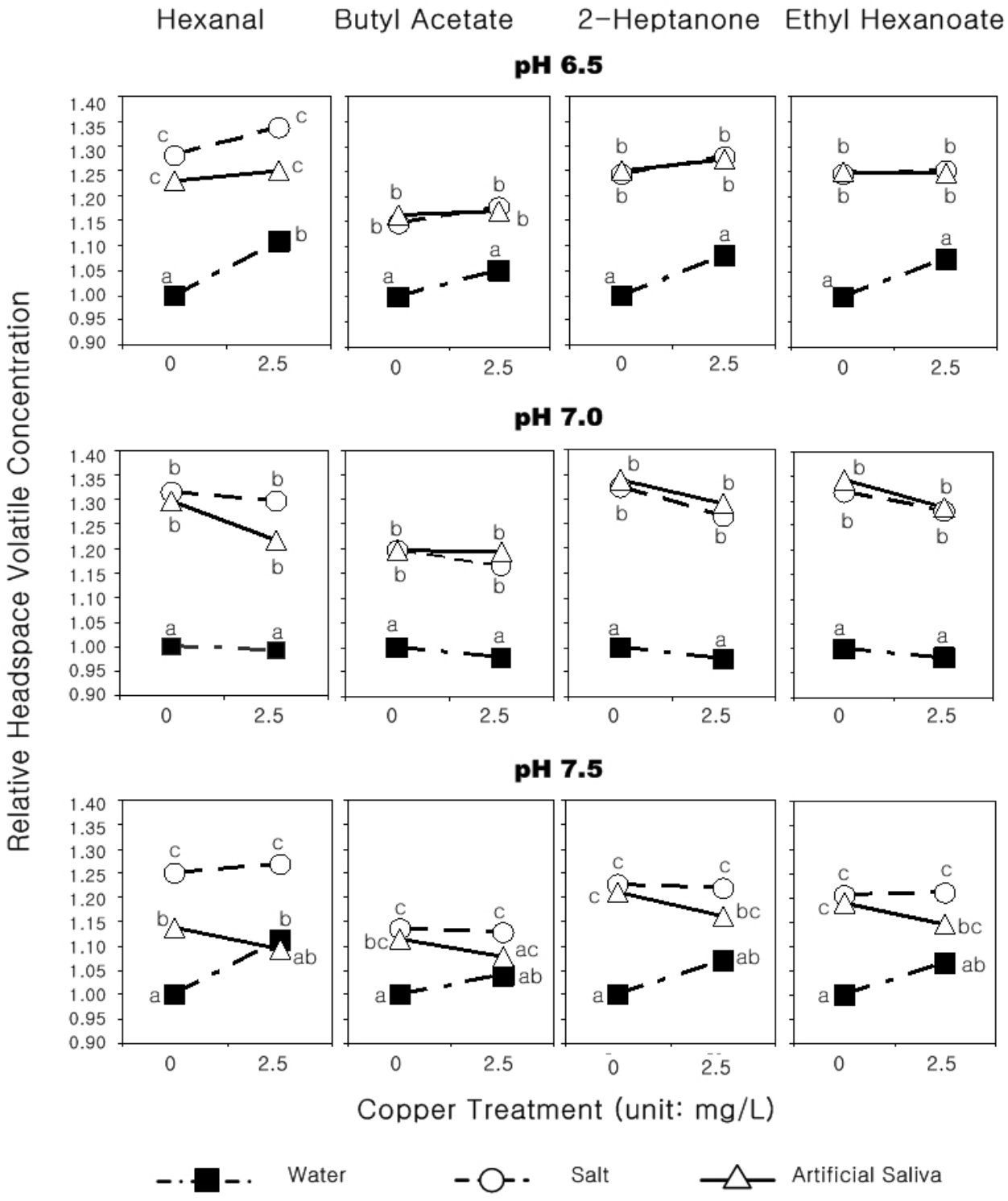

Figure 6. Relative headspace concentration of four aroma compounds at pH 6.5, 7.0, and 7.5 in ultrapure water (control), salt solution (salt), and artificial saliva with and without 2.5 mg/L copper. For each compound, results are normalized to the response of water (control). Different superscript letters indicates a significant effect ($p = 0.05$) between six treatments for an aroma compound at a given pH.

The increase in volatility of aroma compounds by salivary electrolytes is consistent with the previous studies where electrolytes competed for water with volatiles, causing a salting-out effect (van Ruth et al., 2001; Friel and Taylor, 2001). However, the effect of proteins on the partitioning behavior is different from the result that mucin in artificial saliva reportedly decreased volatilization of aroma compounds (van Ruth et al., 2001; Friel and Taylor, 2001). Proteins are known to bind with aldehydes, ketones, esters, and terpenes reversibly by hydrophobic interaction or irreversibly by covalent bonding (O'Keefe et al., 1991; Leland, 1997; Taylor, 2002). The main mechanism of aldehyde-protein association is known to be covalent bond formation via Schiff's base (Taylor, 2002). However, in research on the binding of hexanal to soy proteins (O'Keefe et al., 1991), there was evidence of reversible binding via hydrophobic interactions as well as covalent bonding. Interaction between esters and proteins was suggested to be hydrophobic in nature, especially binding into the hydrophobic pockets of the protein (Jung and Ebeler, 2003; Guichard and Langourieux, 2000). Ketones are well known organic ligands for proteins, especially β-lactoglobulin, and affinity of methylketones to β-lactoglobulin increased as the length of hydrophobic chains of ketones increased. (Jung and Ebeler, 2003).

The results of our work are not consistent with the previous studies, except hexanal which is known to form covalent Schiff-bases with lysine (O'Keefe et al., 1991). In the research of van Ruth et al. (2001), where the same composition of the artificial saliva as that in our research was used, the headspace concentration of hexanal, butyl acetate, and 2-heptanone in the artificial saliva system was significantly decreased compared to those in water. Friel and Taylor (2001) also reported that ethyl hexanoate was retained more in the aqueous phase in the presence of mucin, and retention of ethyl hexanoate was increased by adding electrolytes to the mucin solution.

Our results suggest that hydrophobic interaction of salivary proteins with the esters (butyl acetate and ethyl hexanoate) and the ketone (2-heptanone) were not formed while hexanal bound to the salivary proteins, possibly through covalent interactions. This inconsistency may originate from differences in methodology from those suggested in previous studies. Previous studies used longer times (up to 24 hr) or stronger mechanical forces (750 rpm) for incubation compared to our research. It is possible that differences in shear forces may cause increased protein

unfolding and different binding kinetics in some model systems or that cooperative binding may occur over longer time periods than those used in our study. Interaction times used in our study, which were more realistic with regards to effects in the mouth than those in previous studies, may not be long enough to see flavor binding.

Effect of Copper. Copper showed significant effect on hexanal and 2-heptanone ($p < 0.05$) volatility, and a moderate effect on volatility of butyl acetate ($0.05 < p < 0.1$) at pH 6.5 (Table 5). Headspace concentrations of those compounds were increased by ~10% by addition of copper. Copper did not change volatility of aroma compounds at pH 7.0 and it increased headspace concentration of four volatile compounds by ~10% only in the water model system at pH 7.5 (Figure 6). The significant effect of copper at at pH 6.5 may be related to the amount of soluble copper. The simulation result of copper speciation using MINEQL+ showed that the water and the salt solution model system has higher concentration of solubilized copper at pH 6.5, which includes cupric ion (Cu^{2+}) and soluble copper complexes, compared with soluble copper concentration at higher pH (Figure 7). Recent research of copper taste thresholds (Cuppett et al., 2006) reported that soluble copper complexes in drinking water may be the major copper species that are perceived by humans. The possible mechanisms explaining the effect of soluble copper on aroma compounds are: 1) catalyzing oxidation, 2) interacting with aroma compounds, 3) a salting-out effect. The common role of copper in flavor chemistry is as a catalyst for oxidation, facilitating the production of off-flavors (Bruhn et al., 1976; Leland et al., 1987; Jeng et al., 1988). However, in this research, reaction time and temperature appear to minimize measurable oxidation. Likewise, copper would not likely form complexes with the aroma compounds because copper forms stable soluble complexes with carbonates that were present in the water model system to stabilize the pH; its stability constants are $10^{6.77}$ for $CuCO_3$ and $10^{10.2}$ for $Cu(CO_3)_2^{2-}$ (Jensen, 2003). Thus it is assumed that solubilized copper may participate in a salting-out effect of electrolytes at pH 6.5 in the water system.

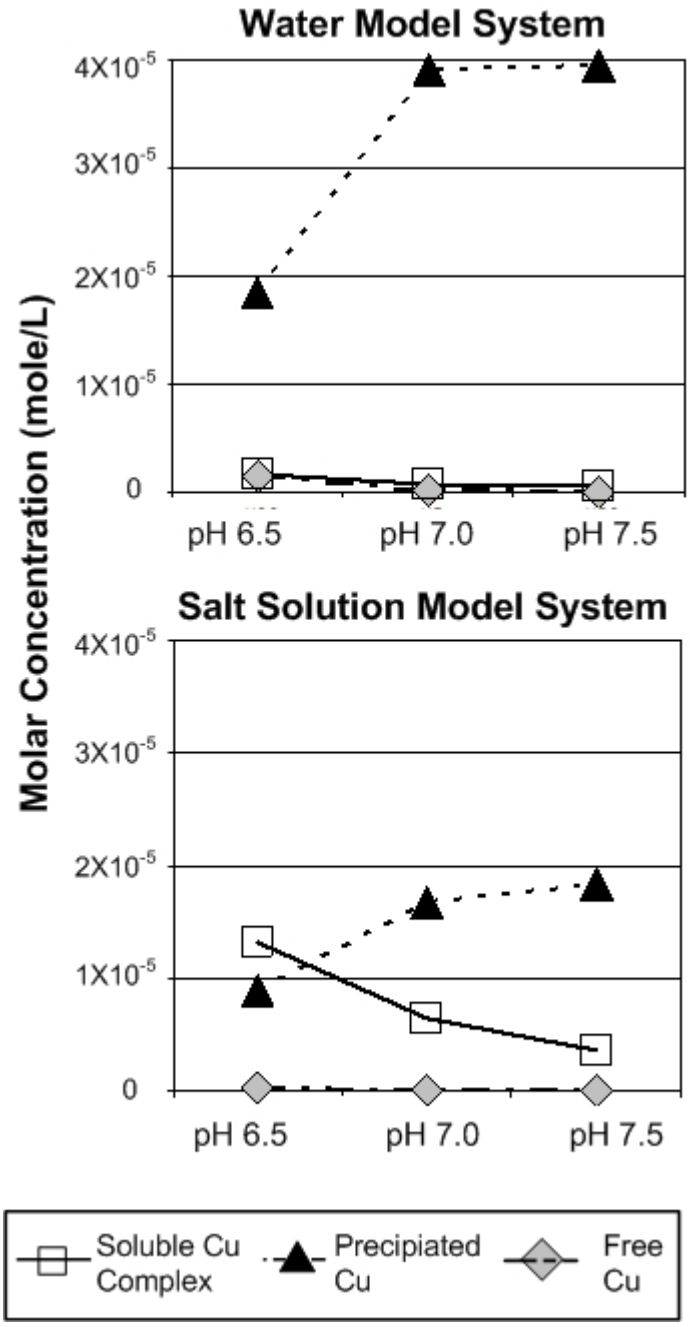

Figure 7. Concentration of soluble and precipitated copper in the water and the salt solution model system calculated by MINEQL+ chemical equilibrium modeling software.

This assumption is supported by the fact that the increase in headspace volatile concentration was most evident in the water model system. Aroma compounds used in this study are hydrophobic in nature (log P > 1) and have relatively low solubility in water (Table 6). Most

carbonyl aroma compounds are well known for behavior of becoming less volatile in hydrophobic food systems such as oil and milk fat, because the more the hydrophobic the matrix is, the more these compounds are retained (van Ruth and Roozen, 2000; Leland,1997; Philippe et al., 2003; Meynier et al., 2003). In preparation of aroma stock solution, aroma compounds were solubilized in water by an aid of mechanical force (i.e. sonication). The forced solubilization of aroma compounds in water was disrupted when water molecules favored the interaction with added copper sulfate, resulting in the salting-out effect. Volatility of aroma compounds in the salt and the artificial saliva model systems are not influenced by copper as much as the water system is, possibly because the salting-out of the copper salt had less contribution than the effect of other electrolytes.

Table 6. Physicochemical, thermodynamical[a] (Physprop database demo, 2006) and sensory threshold characteristics (Stahl, 1973) of the four aroma compounds.

Aroma Compounds	BP [b] (^oC)	P_v [c] (mmHg)	S[d] (mg/L)	Log P[e]	P[f] ($atm \cdot m^3/mole$)	Sensory Threshold (mg/L)
Hexanal	131	11.3	5640	1.78	2.13×10^{-4}	$5 \times 10^{-3} \sim 0.4$[g]
Butyl acetate	126.1	11.5	8400	1.78	2.81×10^{-4}	6.6×10^{-4}[g]
2-heptanone	151	3.86	4300	1.98	1.69×10^{-4}	$8.97 \times 10^{-4} \sim 3$[h]
Ethyl hexanoate	167	1.56	629	2.83	7.23×10^{-4}	$0.021 \sim 0.85$[i]
Acetic acid[j]	117.9	15.7	10^6	-0.17	1.00×10^{-7}	
Hexane[j]	68.7	151	9.5	3.9	1.8	

[a] All data were collected at 25^{o}C.
[b] Boiling point.
[c] Vapor pressure.
[d] Solubility in water.
[e] Hydrophobicity (octanol-water).
[f] Henry's law constant.
[g] Threshold value in water.
[h] Threshold value in air.
[i] Threshold value in milk.
[j] Used as references to provide the general concept of magnitude for solubility and hydrophobicity of aroma compounds. Acetic acid represents water soluble - hydrophilic compounds and hexane represents water insoluble - hydrophobic compounds.

The increase in headspace volatile concentration at lower pH seems to contradict previous studies. In the research of Cao et al. (1999), dynamic light scattering (DLS) showed that mucin was transfigured from isotropic random coil to more relaxed, anisotropic structure below pH 4. These results imply that a decrease in pH drives the relaxation of the mucin structure resulting in exposure of hydrophobic sites. Since volatile compounds usually bind to hydrophobic sites on proteins, lower pH is expected to cause lower volatilization of aroma compounds into headspace. The pH used in this study, however, did not approach this pH, which may be one reason for different observations. Also, there may be additional factors involved in volatile chemistry. First, copper and other electrolytes may help volatilization by influencing the salivary protein structure. They may act to suppress relaxation of mucin structure at lower pH by forming ionic bonding with salivary proteins. The other assumption is that mucin may not have a distinctly relaxed structure at pH 6.5 as it does at pH 4.0. Even though DLS study (Cao et al., 1999) showed steady changes in configuration over pH range of 2 to 7, an apparent transition occurred at pH 4.0. This suggests that the level of exposure of hydrophobic sites on mucin at pH 6.5 may not be enough to overcome the salting-out effect of electrolytes.

Interaction Effect. At pH 7.5, for hexanal, 2-heptanone, and ethyl hexanoate, copper increased headspace concentration of aroma compounds in the water while it decreased volatility in the artificial saliva. The result for the water model system is very intriguing, because headspace concentration of aroma compounds was increased despite similar amount of precipitated copper species at pH 7.5 as at pH 7.0 (Figure 6, Figure 7). It suggests that volatility of aroma compounds are affected by speciation of other ions at different pH, such as sodium bicarbonate that was added to create ionic strength.

ANOVA results showed a significant interaction between copper and model system composition effects (Table 5). This implies that there is association of copper with the salivary proteins in the artificial saliva model system, which leads to facilitation of volatile compounds - salivary protein binding. One of the salivary proteins in the artificial saliva model system, mucin, has high density of sialic acid chains that charge the protein negatively. The isoelectric point (pI) of mucin is pH 3 ~ 5 (Budavari et al., 1989), so mucin was always negatively charged at the pH levels used in this research. Electrolytes can change the polarity at the protein surface

by binding to negatively charged groups. The ionic binding results in decrease of charge repulsion between protein molecules, in turn, reducing hydrodynamic radius of mucin and changing protein structure (Friel and Taylor, 2001; Wu et al., 1994). Friel and Taylor (2001) suggested that the modification of mucin structure influences interactions between volatiles and the protein by changing the number and configuration of binding sites. Copper, as a divalent cation (Cu^{2+}), is expected to interact with mucin by forming ionic bonds at pH 7.5. The interaction between copper and mucin is assumed to affect the binding sites for volatiles on mucin. It can be deduced that hydrophobic binding sites on mucin may be exposed upon binding of copper, because more hydrophobic volatiles such as ethyl hexanoate and 2-heptanone (Table 6) were engaged in the interaction effect.

It is interesting that the artificial saliva treatment at pH 7.5 showed copper-protein interaction effects while there was no copper-protein interaction effect observed at pH 6.5, even though it is postulated that soluble copper species are much higher at pH 6.5 than at pH 7.5 (Figure 6). A possible explanation for this phenomenon is that higher pH increases the chance of copper-protein binding that can restructure binding sites for aroma compounds in the protein. The anionic charge of mucin is expected to be increased due to dissociation of hydrogen ions (H^+) from the carboxyl acid groups (COOH) at the sialic acid chains resulting in the conjugated bases (COO^-) as pH is raised from pH 6.5 to 7.5. This can increase the chance of ionic bonding between Cu^{2+} and negatively charged binding site of mucin. Also the simulation result of MINEQL+ program for electrolyte speciation showed the increasing complexation of other cations such as Ca^{2+}, Na^+, and K^+ at pH 7.5 (data not shown), which may bring about the shielding effect for these cations from anionic binding sites. Thus, there may be much less competition for ionic binding sites, increasing the probability of electrostatic binding despite low concentration of copper cations.

The other possibility is opposite from the first one. Less soluble copper species at increased pH reduces the chance of copper binding to the protein. It was observed that binding of copper to glycoproteins occurred much more at a lower pH in the research of binding of metallic ions to salivary type proteins employing equilibrium dialysis (Mueller, 1983). Less binding of copper to salivary mucin may help aroma compounds interact with the protein better than when there is

more copper available for binding. It will require more intensive research on the mechanism of copper-protein-aroma compound interaction to determine if either of these hypotheses is accurate.

It is not clear what role α-amylase plays in terms of protein-salt-volatile compound interactions. Agarwal and Henkin (1987) found that copper can bind to α-amylase. Human and porcine pancreatic amylase each have two metal binding sites. One binding site is exclusively for calcium, which is strongly bound to the enzyme as a cofactor. The other binding site is the glycine ligand where copper or zinc can bind. This observation may imply a possible interaction between copper and α-amylase in the artificial saliva at pH 7.5. However, little is known about implication of the protein binding of copper or zinc to flavor chemistry or physiology in the mouth.

Effect of Histatin on the Volatile Chemistry in the Model Mouth System

The aritificial saliva model system at pH 7.5 was selected to investigate effect of histatin, based on the observation that protein-copper interaction was significant only at pH 7.5. Histatin was added to the model system at two levels, 0 (control) and 49.5 μM. The latter concentration is within the normal range of histatin level (15.9 μM ~ 102.1 μM) found in human saliva (Castagnola et al., 2001).

Histatin is regarded as the most potent protein associated with copper sensation in the mouth because of its copper binding capacity (Oppenheim et al., 1988). Binding of copper to histatin has been suggested to induce conformational change, which may be responsible for biological activity (Brewer and Lajoie, 2000; Grogan et al., 2001). Thus it was expected that changes in conformation of histatin would contribute to volatilization of aroma in the model systems. However, histatin at 49.5 μM did not significantly change volatility of aroma compounds in the artificial saliva model system at pH 7.5. Several explanations can be offered to explain this observation. First, histatin concentration used in this research may be too low to see any effect of histatin or to generate detectable differences that can be measured using SPME. Second, structural change may require a certain ratio of copper to histatin. In the research of Brewer and

Lajoie (2000), circular dichroism (CD) measurement showed that Cu^{2+} induced subtle changes in histatin structure when the molar ratio of copper to histatin was 5:1. No structural change was observed in the sample at a ratio of 2:1 metal to peptide. The ratio of copper to histatin used in this research was about 1:1.25 (copper : histatin = 39.4 μM : 49.5 μM), which means there was much less copper per histatin molecule compared to Brewer and Lajoie's research. Finally, histatin-copper binding may not be the crucial feature for changes in volatile chemistry in the mouth, for its physiologically low concentration and/or its physiological role is related to antimicrobial activity rather than sensory perception.

Implications of the Change in Headspace Concentrations of Aroma Compounds on Flavor Perception

Aroma released from a food matrix in the mouth has been investigated using different methods, such as static headspace (van Ruth et al., 2001; Friel and Taylor, 2001), and dynamic systems mimicking the human mouth (van Ruth and Roozen, 2000; Roberts and Acree, 1995). Retronasal aroma perception is a dynamic process which is influenced by many factors (mastication, salivation, and body temperature). Thus dynamic systems containing these factors are evaluated to fit better to investigate retronasal aroma perception while static systems are useful for studying orthonasal aroma perception (Roberts and Acree, 1995). Even though studies using static headspace systems cannot explain retronasal aroma perception fully, these studies can still provide useful information and serve as a good starting point because quantities and types of aroma molecules are important in aroma perception (Leland, 1997). The static artificial model saliva system used in this study can help to understand retronasal aroma perception by providing information of interaction between aroma compounds and saliva.

Psychophysics functions, which explain relationships between sensory stimuli and human response, are useful tools to find the implications of changes in headspace concentrations of aroma compounds to flavor perception of the human. In our study, the concentration of aroma compounds used was 0.5 μL/L and changes in headspace concentration by copper were 5 ~ 15% compared to that of the control. When our headspace GC analysis data for hexanal were

interpolated into the Weber-Fechner plot for hexanal (Khiari et al., 2002), increase of headspace concentration by 5 ~ 15% did not cause considerable changes in the predicted perceived intensity. However, a sigmoidal psychophysics function (Keast et al., 2004) shows different implication of changes in headspace concentration on aroma perception. Keast et al. (2004) suggested that a psychophysics curve of a flavor compound has sigmoidal shape which has three distinctive sections: exponential growth at threshold ~ very low concentration; linear increase at low ~ medium concentration; plateau region at high concentration. Each section follows Steven's power function but has different *n* values (Keast et al., 2004):

$I = kC^n$

I = perceived intensity
k = constant
C = concentration of a compound,
n = exponent

Threshold ~ very low concentration: $n > 1$
Low ~ medium concentration: $n = 1$
High concentration: $n < 1$

It's not clear where the level of aroma compounds in our study is located on the psychophysics curve, but based on the threshold values of compounds (Table 6), it is assumed that the concentration used in our study is placed within threshold ~ low concentration range. Even though changes are relatively small, such changes may be able to alter aroma impression if the concentration is positioned in the exponential increase zone in the curve.

In most cases, perceived flavors from food or beverage systems results from a combination of different aroma compounds. It was reported that a psychophysics curve of an original compound shifted in the direction of synergy or suppression when another aroma compound was added, implying interaction between two aroma compounds (Keast et al., 2004). Changes in

headspace concentration of each aroma compound may cause more complicated interaction pattern and possibly lead to changes in aroma impression.

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Chapter IV . Identification of Temporal Copper Sensation and Effect of pH on Sensory Perception of Copper

ABSTRACT

Copper in drinking water causes a persisting bitter, metallic, or astringent taste. A recent study suggested that intensity of copper taste may be dependent on the amount of solubilized copper, which increases at lower pH. This research was performed to identify the effect of copper solubility on temporal characteristics of copper sensation. Samples were prepared by adding $CuSO_4 \cdot 5H_2O$ to water at the level of 2.5 mg/L and 5 mg/L as Cu. pH levels of samples were pH 5.5 (ultrapure water) and pH 7.5 (1 mM $NaHCO_3$ in ultrapure water). This level of $NaHCO_3$ created detectable, but not recognizable stimulus. Bitterness, astringency and metallic taste were evaluated for 2 min using time-intensity (TI) test by ten trained panelists. Twelve TI parameters for each attribute were extracted from TI curves and analyzed using two-way ANOVA ($\alpha = 0.05$). Concentration of soluble copper in samples was measured with atomic absorption spectroscopy (AAS) after filtering samples through 0.2 μm filter to remove insoluble copper particles. Metallic taste of 5 mg/L Cu was significantly inhibited by increase of pH. Copper concentration and pH condition had lesser effect on bitterness compared to metallic taste. Astringency was not significantly affected by copper concentration, but changed as a function of pH. TI test results of copper solutions did not show a common TI pattern of astringency that is characterized with slow onset and longer duration time. The level of soluble copper at pH 7.5 decreased by 50 % compared to that at pH 5.5. Soluble copper concentration and temporal profile of sensory attributes suggested that soluble copper species decide the perception of copper sensation by controlling metallic taste.

INTRODUCTION

Occurrence and Nature of Metallic Sensation

Metallic sensation is caused by exposure to metallic compounds. Medications and organic compounds such as artificial sweeteners, lipid oxidation compounds, and blood have been reported to result in metallic sensation. Metallic sensations were also perceived by patients who have taste abnormalities such as Sjögren's syndrome (dry mouth syndrome) or burning mouth syndrome (Weiffenbach et al., 1995; Mott and Mann, 2004).

Metallic sensation is not a traditional oral stimulus. The sensation caused by metallic compounds has been reported to be multimodal, encompassing olfactory sensation, trigeminal sensation, and gustatory sensation (Keast, 2003; Lawless et al., 2004; Lawless et al., 2005; Lim and Lawless, 2005a; Lim and Lawless, 2005b; Lim and Lawless, 2006; Yang and Lawless, 2006a; Yang and Lawless, 2006b).

Trigeminal sensation, or chemesthesis, has been considered as a single important modality of metallic sensation because astringency and tingling/sting mouthfeel were common descriptors for metals (Keast, 2003; Lim and Lawless, 2005a). Astringency of metals is related with de-lubrication of salivary proteins (Kallithraka et al., 1997a; Kallithraka et al., 1997b; Sarni-Manchado et al., 1999; Chapman and Lawless, 2004). However, Schiffman (2000) suggested that metallic sensations are not only trigeminal sensations but also include a number of sensory qualities based on several clinical and experimental observations: 1) sensory information of metal salts was mediated through taste nerves in frogs, rats, carps, mice, and gerbils; 2) two patients whose trigeminal nerves were impaired could identify ferrous salts as "metallic".

Recent studies found that perception of metallic sensation caused by ferrous sulfate was greatly decreased by nasal occlusion, which suggests olfactory stimuli of ferrous compounds served as an important cue (Hettinger et al., 1990; Lawless et al., 2004). Metallic retronasal aroma is assumed to result from oxidation of lipids in the epithelial tissues (Lubran et al., 2005;

Glindemann et al., 2006). The presence of copper in saliva was shown to alter the volatility of aroma compounds by altering structure of mucin at salivary pH 7.5 (Hong et al., 2006). Thus, the presence of metals in the oral cavity may not only generate the oxidative retronasal metallic smell but also affect sensory impression by changing partitioning behavior of aroma compounds incorporated into the mouth.

Besides trigeminal and olfactory qualities, gustatory sensation is also involved in metallic sensation. Lim and Lawless (2005a) suggested that gustatory sensation is at least a part of metallic sensation based on the finding that ferrous sulfate could be perceived by panelists without a retronasal cue. Multidimensional scaling (MDS) of ferrous and zinc compounds (Lim and Lawless, 2005b; Stevens et al., 2006) showed that the non-traditional gustatory qualities other than four basic tastes were required to explain metallic taste. Lawless et al. (2005) suggested that electrical stimuli, which are widely accepted to be transduced through gustatory pathways, could play an important role in gustatory perception of metallic taste. However, the possibility that electrical stimuli are trigeminal sensations cannot be entirely ruled out because somatosensory receptors are located near the taste receptor on the tongue (Green, 2004; Lawless et al., 2005).

The term "metallic" is used to generally describe many metals and their salts. However, sensations of various metallic compounds are distinguishable from one another. Ferrous compounds were reported to possess distinctive "metallic" flavor compared to other metallic compounds such as calcium, magnesium, and zinc. Strong metallic flavor from ferrous compounds may be because $Fe^{2+}/Fe^{3+}/Fe^{0}$ is more easily oxidized and reduced so it can contribute to lipid oxidation than copper and zinc (Glindemann et al., 2006). Zinc compounds were related to strong astringency and a hint of umami taste. Alkaline metals, magnesium and calcium, had pronounced bitterness (Keast, 2003; Lawless et al., 2003; Yang and Lawless, 2006a).

Metallic Sensation of Copper

Copper can be detected by humans at very low concentration. Reported detection threshold concentrations of copper range from 0.5 ~ 13 mg/L, depending on the water composition, variation among subjects, and protocols (Cohen et al., 1960; Bequin-Bruhn et al., 1983; Zacarias et al., 2001; Cuppett et al., 2006). Descriptive terms including metallic, bitter, salty, sour, and astringent were used to explain sensory qualities of copper (Zacarias et al., 2001; Lawless et al., 2004). Compared to ferrous compounds, copper was more bitter and astringent (Lawless et al., 2004). Retronasal aroma was not perceived well from copper compounds, but copper had a strong tactile sensation (Lawless et al., 2004; Lim and Lawless, 2005a).

Another important characteristic of copper sensation is a lingering aftertaste. Lingering aftertaste is commonly found for several metallic compounds. Zacarias et al. (2001) reported lingering "bitter and other off-flavor" from copper. It seemed that the strongest sensations of metallic compounds may become a major aftertaste. For example, metallic aftertaste was found from ferrous compounds, bitter aftertaste was detected from alkaline metals, and persisting astringency was experienced with zinc compounds (Keast, 2003; Yang and Lawless, 2006a). Time-intensity test of ferrous compounds showed that metallic taste and astringency were lingering long after consumption (Yang and Lawless, 2006b). Because copper was observed to have more bitterness and astringency than iron, astringency would be a major characteristic of a lingering aftertaste (Yang and Lawless, 2006a; Cuppett et al., 2006).

Factors Influencing Metallic Sensations

Perception of metallic sensation is influenced by several factors. Since "metallic taste" comprises of several different sensory qualities, concentration of metallic compounds affects the impression of sensation by changing the proportion of each sensory quality as well as intensity of total sensation (Lim and Lawless, 2006). Anions in metallic compounds suppress bitterness of metal cations. Large organic anions influence taste of metals by: 1) hindering metallic compounds from diffusing into the taste receptor cells (Lawless et al., 2003); or 2) rendering

additional characteristics such as sourness or sweetness (Yang and Lawless, 2006a; Yang and Lawless, 2006b).

Recently, Cuppett et al. (2006) reported that metal speciation is important in perception of copper in drinking water. Because copper favors the oxidized form under standard conditions (25^{o}C, 1 atm) (Jensen, 2003), common conditions in drinking water allow copper to go through several chemical reactions. Depending on pH and availability of other electrolytes, copper becomes free cupric ion, and forms soluble complexes, and insoluble complexes. Free cupric ion and soluble complexes are dominant species under acidic (< pH 6) conditions, while insoluble form is prevalent at basic pH range (pH 8 – 12) (Jensen, 2003). A series of experiments performed by Cuppett et al. (2006) provided evidence supporting that soluble copper species are readily perceived by panelists.

OBJECTIVES

Copper concentration in drinking water is regulated under Lead and Copper rule (1991) of the USEPA. An action level of 1.3 mg/L is recommended to prevent adverse effects on human health; the copper concentration of 1.0 mg/L is used for an aesthetic-based standard (USEPA, 1991). Cuppett et al. (2006) suggested that 75 % of panelists could detect the health-based standard level of copper. Since unpleasant metallic and bitter tastes of copper is regarded as a detection mechanism against potential hazards, perception of copper in drinking water at the threshold level has important implications on public health. This leads to the needs for better understanding of the relationship between copper speciation and perception of copper sensations. Descriptive sensory methodologies can provide more valid and reliable analysis through consensual descriptors and extensive training. Especially, the time-intensity (TI) descriptive analysis can be used to measure changes of attributes over time (Meilgaard et al., 1999), which is very useful in identifying the lingering aftertaste of copper.

This research was performed to investigate the effect of copper speciation in drinking water on temporal sensory characteristics of copper. This research is expected to provide more

comprehensive perspectives on the role of copper chemistry in the perception mechanism of copper sensations.

METHODS AND MATERIALS

Panelist Selection

Seventeen subjects among students and staff at Virginia Tech were recruited for screening for copper sensitivity based on their interest, health condition, and availability. Panelists for time-intensity (TI) sensory tests were selected based on their sensitivity to copper using 1-of-5 test, a modification of 3-alternative forced choice (AFC) method (Lawless and Heymann, 1998), as described by Cuppett et al. (2006). In this method, panelists were asked to choose one different sample among five samples, in order to reduce the chance of selecting the right answer by guessing.

Five samples consisting of four control samples (deionized water) and one copper containing sample were given to panelists. The concentration of copper used in the screening test was 1.3 mg/L, which is the EPA regulatory level for copper in drinking water. This level was expected to ensure the selection of panelists who were sensitive enough to discriminate the level of copper used in this research (2.5 mg/L, copper threshold in the research of Zacarias et al. (2001)). Samples were presented in 20 mL plastic cups each, with randomly generated 3-digit codes, at room temperature (approximately 21 ± 2^{o}C). The order of samples was randomly assigned. Panelists were asked to rinse their mouth with deionized water between samples. The screening test was performed in triplicates, and panelists who selected the correct sample more than twice were chosen for TI test. This detection acuity level was based on the recommendation of Meilgaard et al. (1999) that panelists should achieve 50 ~ 60 % of correct replies in triangle tests to pass the screening test for descriptive analysis. The sensory ballot used is attached in Appendix 1.

Ten subjects (four male and six female) between the ages of 22 and 55 were selected. All panelists were reported to have no physical conditions that might interfere with tasting. They were familiar with the theory and practice of sensory evaluation, but had no previous experience with TI testing protocols before. The sensory protocol was approved by the Institutional Review Board at Virginia Tech (IRB # 05-368) and informed consent forms were obtained from all panelists (Appendix 2).

Panelist Training

Ten panelists who were selected through the screening procedure were trained in TI testing protocols and sensory attributes of copper solution in nine sessions (one hour per each session) for three weeks. First, orientation was given to panelists to introduce the concept and protocols of TI testing. In the orientation, panelists also were familiarized with metallic sensations using copper pennies and 7.5 mg/L $CuSO_4 \cdot 5H_2O$ solution. Copper pennies were soaked in 5 % acetic acid solution to remove rust on the surface and rinsed with deionized water. All pennies were sanitized by being immersed in 70 % ethanol solution (FCC grade, not denatured) and then both sides of pennies were air-dried for 30 seconds each. Two types of copper pennies were prepared; one type was an ordinary copper coin, the other was a copper coin on which the surface was filed to expose zinc core for generating an electrical sensation (Lawless et al., 2005). Over the next two sessions, panelists developed the terms and definition for sensory attributes and reference standards.

A series of practice sessions were held for two weeks over six sessions. For the first three sessions, panelists tested temporal characteristics of sweetness using sugar, xylitol, and aspartame. A chart recorder (Fisher recordall series 5000, Fisher Scientific, Pittsburgh, PA) was used as a recording device, in order to help panelists understand the TI test procedure. Sugar (Great Value Pure Cane Sugar, Wal-Mart Stores, Inc., Bentonville, AR) was dissolved at the level of 5 % in filtered water (Brita Mägnum, Brita, Oakland, CA). Equivalent sweetener concentration for xylitol and aspartame were calculated based on manufacturer's information to generate similar sweetness intensity as that of 5 % sugar solution (Portmann and Kilcast, 1996).

Xylitol (KAL dietary supplement, Park City, UT) was made into 5 % solution and aspartame (NutraSweet, Chicago, IL) was dissolved at the level of 0.625 %. Solutions were provided in 20 mL plastic cups coded with 3-digit random numbers at room temperature (approximately 21 ± 2^{o}C). Panelists were asked to track the intensity for sweetness of each sweetener solution over time using a chart recorder (Fisher recordall series 5000, Fisher Scientific, Pittsburgh, PA). Panelists marked a continuous line depicting intensity of perceived sweetness for 1 min as chart recorder paper scrolled continuously at 5 cm/min. The scales were 150 mm long and each end designated the intensity of the sensory characteristic (0 mm = no sensation, 150 mm = extremely strong sensation). A white cardboard barrier with a 2 cm X 16 cm slit was placed on the chart recorder so panelists could see only the small portion of the TI curve they were drawing through the slit. The cardboard barrier was used to avoid bias that panelists tend to develop what they have drawn into a certain shape (Lee and Pangborn, 1986).

The next three sessions were held for one week in order to: 1) help panelists to understand the concept of astringency and differentiate it from bitterness; 2) train panelists for a computerized TI test procedure. The ability to grade astringency is important in our sensory evaluation because one of the important characteristics reported in threshold tests of copper was astringency (Zacarias et al., 2001; Chapman and Lawless, 2004; Lawless et al., 2004; Yang and Lawless, 2006a). TI practice for astringency was carried out on a computerized TI system operated with SIMS 2000 software *ver.* 3.3 (Sensory Computer System LLC, Morristown, NJ) using a green tea infusion. Prior to the TI tests of green tea infusion at the first session for the TI tests of astringency, panelists tasted alum solution (0.0175 %) and caffeine solution (0.025 %) to distinguish astringency from bitterness.

Green tea infusion was prepared by the method suggested by Drobna (2004). Green tea leaves (Republic of Tea, Novato, CA) (4 g) was soaked in 500 mL of filtered tap water at 80 ^{o}C for 5 min and then strained. After being cooled down to room temperature (approximately 21 ± 2 ^{o}C), the infusion was diluted with filtered water to 12.5 %. Green tea samples of two different concentrations (12.5 % and 100 % infusion) were provided to panelists at room temperature. Alum solution of 0.0175 % concentration was offered as a reference standard. Panelists tasted samples following the instruction suggested by Valentová et al. (2002). At 0 second, panelists

were asked to place the sample in the mouth and began to rate the initial intensity. While keeping the solution in the mouth, panelists swirled solution around using their tongue for 10 seconds. After 10 seconds, panels expectorated the sample. To remove carry-over effect, saltine crackers and filtered water were consumed during two-minute break between samples.

The TI tests for training were carried out in triplicate to check panelists' performance. TI curve parameters such as time to maximum intensity (T_{max}), maximum intensity (I_{max}), and total duration (T_{tot}) were extracted from each TI curves and variance within panelists and between panelists was calculated using ANOVA. Additional training was given to panelists who showed large variance in their result or showed different trends from others.

Determination of pH Conditions for Time-Intensity Test

Increased pH of water may hinder human from perceiving copper by decreasing solubility of copper (Cuppett et al., 2006). Copper is least soluble at the pH range of 8.5 ~ 11.2, thus it was presumed that copper might be perceived least in water at this pH range (Cuppett et al., 2006). Three pH conditions, pH 5.5, 7.5, and 8.5 that each represents pH conditions that are expected to cause all copper soluble, part of copper soluble, and most of copper insoluble, were selected based on the result of Cuppett et al. (2006)'s study. The pH range was adjusted by adding sodium bicarbonate to ultrapure water (Barnstead nanopure water purification system, Barnstead International, Dubuque, IA) at the levels of 0, 1, or 100 mM. Actual pH of the solutions was checked with a standardized pH meter (Accumet AR15 pH meter, Fisher Scientific, Pittsburgh, PA) following the ASTM standard test method (2004).

To investigate the effect of sodium bicarbonate on water taste, a triangle test was performed for 1 mM and 100 mM sodium bicarbonate solutions. It was tested that 1 mM or 100 mM sodium bicarbonate solution could be distinguished from ultrapure water. Twenty panelists were recruited from the students and staff in the Department of Food Science and Technology. Panelists were comprised of nine panelists trained for TI test and eleven untrained panelists. Untrained panelists, consisting of three males and eight females between the age of 20 ~ 40, had

experience in sensory testing but were not familiar with sensory stimuli of water. Samples were presented in 20 mL plastic cups marked with 3-digit codes at room temperature (approximately 21 ± 2°C). The sensory ballot used in this research is attached in Appendix 3. Significance for a difference between sodium carbonate solutions and ultrapure water was determined at α risk of 5%, β risk of 10%, and P_d of 50% (Meilgaard et al., 1999).

Masking effect by background taste of sodium bicarbonate on copper sensation was investigated using a rating test. Eight panelists trained for TI test participated in the rating test. The level of copper and sodium bicarbonate concentration used for identifying masking effect were 2.5 mg/L and 1 mM, respectively. A total of five samples comprised of sodium bicarbonate 1 mM solution, copper 2.5 mg/L solution, sodium bicarbonate 1 mM – copper 2.5 mg/L solution, and two deionized water samples were tested. The first deionized sample was used as a control, while the second deionized water sample was included to validate panelists' performance. Panelists were instructed to rate metallic taste and taste arisen from sodium bicarbonate. A 10-point intensity scale (0 – no perception, 9 – extremely strong; Meilgaard et al., 1999) was used and the rating test was prepared in the same way as the preparation for the triangle test (Appendix 4). Samples were provided in random order at the room temperature (21 ± 2°C). Panelists took a two-minute break between samples and rinsed their mouth with deionized water. The test was performed in triplicate.

The statistical analysis was performed using JMP IN® statistical software (*ver.* 4.0, SAS, Cary, NC). The effects of treatments (copper and sodium bicarbonate) on each attribute was examined using two-way ANOVA ($\alpha = 0.05$). Mean values of different treatments were compared using Tukey's HSD test. Assuming that the background taste of sodium bicarbonate acts as "noise" that interferes with perception of metallic taste ("signal") (Lawless and Heymann, 1998), paired t-test between metallic taste and electrolyte taste was performed to examine if metallic taste can be significantly discriminated from electrolyte taste ($\alpha = 0.05$).

Sample Preparation

Samples were prepared using $CuSO_4 \cdot 5H_2O$ and ultrapure water. The pH of sample was adjusted to two levels, pH 5.5 and 7.5. For the lower pH, ultrapure water was used without modification because pH of ultrapure water is around 5.5 when dissolved CO_2 in the water is equilibrated with that in the air. To increase pH of water to around 7.5, 0.1 M $NaHCO_3$ stock solution was added to deionized water to make a final concentration of 1 mM prior to addition of copper. Copper (II) sulfate pentahydrate stock solution (2 %, w/v) was added to deionized water and 1 mM $NaHCO_3$ solution to make final concentrations of 2.5 mg/L (3.94×10^{-5} M) and 5 mg/L (7.88×10^{-5} M) as Cu.

All glassware used for sample preparation were filled with distilled water and autoclaved for 15 min at 121°C to get rid of any possible remaining smell after washing using tap water and a detergent. The glassware was rinsed with ultrapure water three times and then air dried. Samples for TI tests were prepared and stored at room temperature until they were served to panelists. To prevent aging effect of electrolytes, samples were made daily two hours before the test.

Sensory Evaluation Protocol for Evaluation of Taste Sensations of Copper

The sensory attributes and reference standards developed for copper solutions are summarized in Table 7. They were developed during the two sessions for total two hours at the early stage of training process. For the bitterness and astringency, the representative chemicals for each stimuli suggested by Meilgaard et al. (1999) were prepared into several concentrations. For metallic taste, panelists chose ferrous sulfate solution as a reference standard material among the materials used for familiarizing panelists with metallic taste such as a copper penny, a zinc-core exposed copper penny, ferrous sulfate solution, and a piece of aluminum foil. Ferrous sulfate solution was also prepared into several concentrations. Panelists selected one or two concentrations of each reference standard that served as calibration points on the intensity scale by consensus.

All tests were performed in triplicate. To prevent fatigue, two samples of different pH but of the same copper concentration were evaluated for single attribute per one session per day. Samples were presented in random order within a session. A total of sixty sessions were identified and panelists were asked to select three available days for performing sensory evaluations because only three computerized sensory stations were available. Each panelist performed 18 sessions total over six weeks.

Table 7. Sensory attributes and reference standards used for evaluation of copper sensation.

Attributes	Reference Standard			
	Materials	Source	Concentration	Scale value [a]
Metallic	$FeSO_4 \cdot 7H_2O$	USP grade, Sigma	R1 – 5.9 µM R2 – 118 µM	30 128
Bitter	Caffeine, anhydrous	USP grade, Sigma	R1 – 0.0125 % R2 – 0.025 %	22 116
Astringency	Alum ($AlK(SO_4)_2 \cdot 12H_2O$)	Kroger	R - 0.0175 %	55

[a] Intensity scale: 0 (none) to 150 (extremely strong)

Sensory evaluation was conducted in isolated booths at approximately 21 ± 2°C. The samples were presented in 20 mL plastic cups coded with randomly selected 3-digit numbers at room temperature (21 ± 2°C). TI tests were completed using a computerized TI system operated with SIMS 2000 software *ver.* 3.3 (Sensory Computer System LLC, Morristown, NJ).

Tasting protocol was developed by panelists during training sessions based on the protocol used for training with green tea infusion. The intensity of each attribute over time was graded on an unstructured 150 mm line scale with anchors on opposing ends (0 mm - none, 150 mm - extremely strong), and in the middle. Short vertical lines representing intensity of reference standards were positioned on the scale and identified as R1 and R2. At the beginning of each

session, panelists tasted reference standards and confirmed their location on the intensity scale. After a two-minute break, panelists placed a pointer on the 0 point of the scale when they were ready to sip the sample. They initiated rating by clicking the 0 point at the same time they sipped the sample. Panelists were instructed to rate the intensity for the given attributes by moving the pointer around on the scale. While evaluation continued on, panelists swirled the sample around for 10 seconds, then expectorated. The intensity for a single attribute was recorded in one second interval for 120 seconds.

Panelists were asked not to drink or consume any beverage, food, and oral care products one hour before the test. During the test, panelists took rest for two minutes between samples to remove carry-over effect of the previous sample. While panelists were taking rest, they cleaned their pallet with saltine crackers and deionized water.

Data Analysis of Time-Intensity Test

Time-intensity data was collected automatically by the computerized sensory test system equipped with SIMS 2000 software. Collected data was exported to MATLAB computing software (ver. 7.0, The Mathworks, Inc., Natick, MA) for averaging and plotting TI curves for each attribute following the mathematical procedure suggested by MacFie and Liu (1992).

Twelve TI parameters were extracted from TI curves by SIMS software. TI parameters investigated in this research were defined in Table 3 in Chapter II. TI parameters extracted was exported to SAS®9 software (SAS, Cary, NC) and analyzed using two-way ANOVA for testing the main effects: panelists, copper concentrations, pH conditions, replication and their cross effect. Panelists were treated as random effects. The significant difference between means for each TI parameter was determined by pairwise comparison of least squares means using Student's t-test (significant level $\alpha = 0.05$).

Measurement of Soluble Copper Concentration in Time-Intensity Samples

Concentration of soluble copper in TI samples was measured using atomic absorption spectroscopy (Perkin Elmer 5100, Wellesley, MA) following the method suggested by Cuppett et al. (2006). Analysis scheme is illustrated in Figure 8. Samples were filtered through 0.2 μm filter (Acrodisc HT tuffyn membrane, diameter 25 mm, Gelman Science, Ann Arbor, MI) to remove precipitated copper particles and then filtrate was subjected to the AAS. Samples for a standard curve were prepared by dissolving $CuSO_4 \cdot 5H_2O$ in deionized water at the level of 0, 1.0, 2.0, 4.0, and 8.0 mg/L as Cu. All tests were performed in triplicate. The results were analyzed with t-test. ($\alpha = 0.05$).

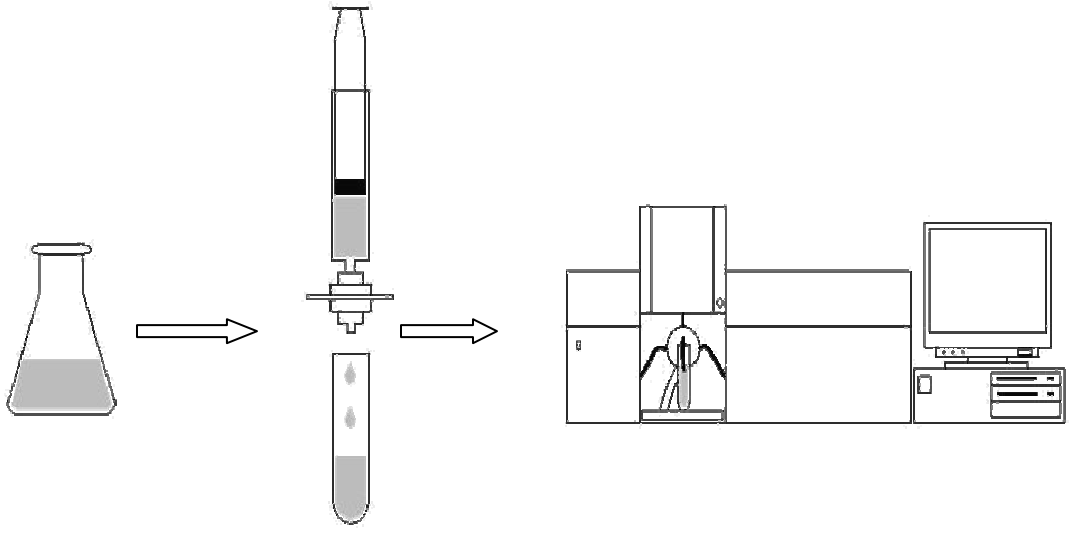

Figure 8. AAS analysis of soluble copper concentration in time-intensity sensory test samples of Cu 2.5 mg/L and 5 mg/L in ultrapure water and 1 mM $NaHCO_3$ solution.

RESULTS

Determination of pH Conditions for Time-Intensity Test

To investigate the effect of pH on the temporal profiles of 2.5 mg/L and 5 mg/L copper solutions, pH conditions that generated different soluble copper amounts but no changes in taste were explored. Three pH conditions, pH 5.5, 7.5, and 8.5 were examined. The pH conditions were adjusted by adding sodium bicarbonate at the level of 0, 1 mM, and 100 mM level.

Measured pH of 0, 1, and 100 mM sodium bicarbonate solution was 5.60 ± 0.01, 7.42 ± 0.02, and 8.31 ± 0.01, respectively. The 1 mM sodium bicarbonate (pH 7.42) was selected for TI test because the sodium bicarbonate 1 mM solution was not distinguished from ultrapure water (9 correct answers/total 20 answers) (Table 8). Among nine trained panelists, six panelists chose the correct answer, showing that the difference was discriminated by trained panelists. However, the panelists who picked the correct sample reported that the difference was detection threshold level, detectable but not identifiable. Therefore, we concluded that sodium bicarbonate did not have a significant interference effect on copper taste at the level of 1 mM. Panelists could discriminate 100 mM bicarbonate solution from the ultrapure water. The strong taste was described as saltiness, "baking soda" taste, metallic taste, bitterness, tingling sensation or sourness. The background taste of 100 mM sodium bicarbonate was so intense that it could cover the copper sensation.

Detecting Copper Sensation above Background Taste of Sodium Bicarbonate

Even though the intensity of taste was threshold level, sodium bicarbonate might influence the taste of copper. It was investigated if 1 mM of sodium bicarbonate increased metallic taste of copper using a 10-point scale rating test. Before the test, one open session was held to gain consensus on the term describing the taste of 100 mM sodium bicarbonate solution. Panelists agreed on expressing the "metallic", "salty" or "tingling" taste associated with sodium

bicarbonate as "electrolyte" taste and defining it as the taste arisen from the electrolytes existing in water.

Table 8. Discrimination between sodium bicarbonate solutions (1 mM and 100 mM) compared to ultrapure water using triangle test.

Panelist group (number of the panelists)	Sample	Number of correct answers	Critical number of correct responses in a triangle test[a] (α=0.05)	Difference from water (α=0.05)
Total (n=20)	1 mM $NaHCO_3$	9	11	NS[b]
	100 mM $NaHCO_3$	20		S[c]
Trained (n=9)	1 mM $NaHCO_3$	6	6	S
	100 mM $NaHCO_3$	9		S

[a] The minimum number of correct answers that are required for significance at α = 0.05 for 20 respondents (Total panelist group) or 9 respondents (Trained panelist group) (Meilgaard et al., 1999).
[b] Not significant.
[c] Significant.

The result of the rating test is illustrated in Figure 9. Metallic taste showed a significant difference (α = 0.05) among samples. Copper solution of 2.5 mg/L had the highest mean intensity of 3.7 for metallic taste. Metallic taste was slightly ($0.05 < p < 0.1$) decreased by addition of 1 mM sodium bicarbonate. Mean value for metallic taste of sodium bicarbonate 1 mM solution was rated comparable to that of deionized water samples. Electrolyte taste did not significantly change among samples with intensity ratings near the low end of the scale.

The primary effect, based on two-way ANOVA for metallic taste (Table 9), was addition of copper. Sodium bicarbonate 1 mM treatment did not influence metallic taste perception. An interaction effect between copper and sodium bicarbonate treatment was observed, because

sodium bicarbonate increased metallic taste in 1 mM sodium bicarbonate solution while it decreased metallic taste in the Cu 2.5 mg/L – sodium bicarbonate sample.

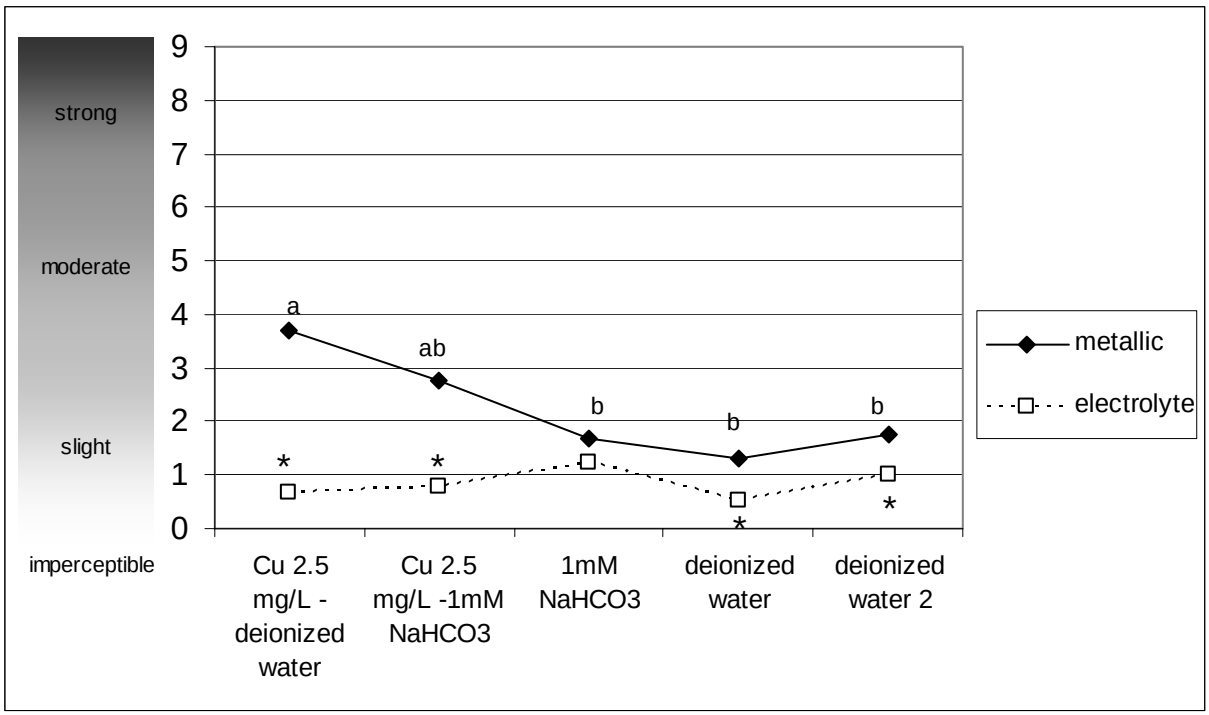

Figure 9. Effect of background taste of 1 mM sodium bicarbonate on metallic taste perception. Different letters (a, b) indicate a significant difference among samples containing 2.5 mg/L copper at different pH (deionized – pH 5.5; 1 mM sodium bicarbonate – pH 7.5) for metallic taste ($\alpha = 0.05$). Asterisk (*) over the open square (□) indicates a significant difference between metallic taste and electrolyte taste tested by paired t-test ($\alpha = 0.05$) within a sample solution.

Table 9. Two-way ANOVA for testing effect of copper treatment and sodium bicarbonate treatment on metallic taste perception ($\alpha = 0.05$).

Source of Variation	DF	Prob > F
Copper (0, 2.5 mg/L)	1	< 0.001
$NaHCO_3$ (0, 1 mM)	1	0.1396
Copper X $NaHCO_3$	1	0.0456

The paired t-test result (Figure 9) showed that the expected signal (metallic taste) was significantly higher than the background noise (electrolyte taste) for all samples, including deionized water samples. This result is in agreement with the observations from the previous triangle test that sodium bicarbonate 1 mM had a detection threshold level of stimuli. Deionized water samples were used to validate the panelists' performance. The mean values of deionized water samples 1 and 2 were 1.29 and 1.75 for metallic taste, 0.54 and 1.00 for salty taste, respectively. The mean value above 0 (imperceptible) resulted because some panelists (five out of eight for metallic taste; two out of eight for electrolyte taste) had "false alarms", which means that panelists respond that stimulus (metallic or electrolyte taste) is present when there is no stimulus (Lawless and Heymann, 1998). The false alarm seemed to be caused by a carry-over effect from the previous samples or an expectation error in which panelists might expect metallic or electrolyte taste from all presented samples (Meilgaard et al., 1999).

The addition of sodium bicarbonate did not increase the metallic taste of copper solution. Based on this evidence and assuming that metallic taste of copper in sodium bicarbonate solution was generally higher than that of sodium bicarbonate solution and of water, we proceeded to study the effect of pH on taste of copper with an assumption of no interference from sodium bicarbonate.

Time-Intensity Test of Copper in Drinking Water

The time-intensity curve of metallic taste, bitterness and astringency in 2.5 and 5 mg/L copper solution at pH 5.5 and 7.5 are illustrated in Figure 10, Figure 11, and Figure 12. All three attributes reached a maximum intensity around 30 sec after consumption after which the intensity decreased over time. The maximum intensity of each of the three attributes ranged 4 ~ 5 on the 15 cm-line scale due to the level of copper close to sensory threshold. All three sensations did not totally disappear over the test duration, implying that copper sensation is likely to linger after 2 min. It is surprising to see that astringency did not exhibit common pattern of a delayed onset and a slow decaying rate, because persisting astringency has been reported as an important sensory characteristics from other metallic compounds (Keast, 2003;

Yang and Lawless, 2006b). One possible explanation is that the level of copper used in this study may be too low to elucidate astringency that can show a common pattern found in astringency TI curves (Drobna, 2003; Yang and Lawless, 2006b). The samples of different pH and copper concentrations seemed to be differentiated by area under curve (AUC) that represents the overall response to the stimulus (Naish et al., 1993), rather than by a set of distinctive TI parameters that render a unique curve shape.

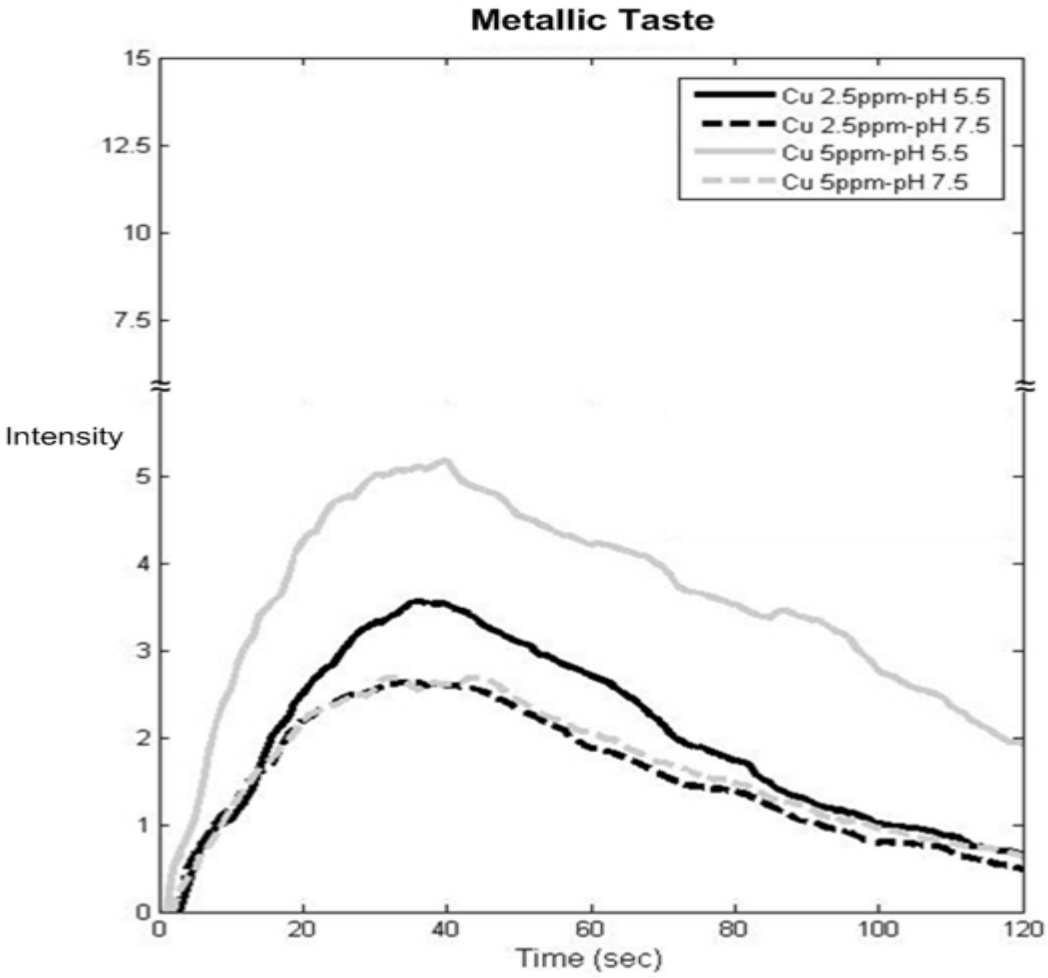

Figure 10. Time-intensity curves for metallic taste of copper solutions (2.5 mg/L and 5 mg/L) at pH 5.5 and 7.5 (n = 30; 10 subjects X 3 replications).

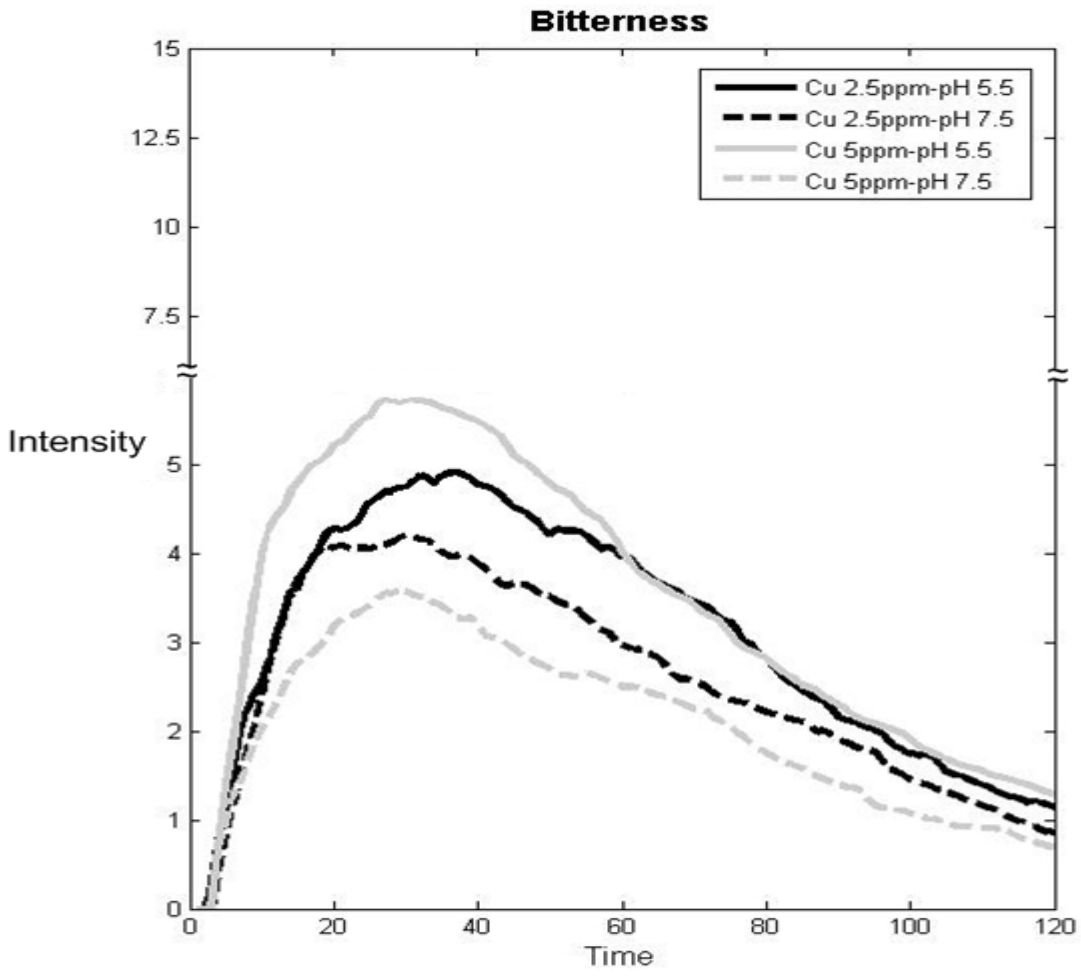

Figure 11. Time-intensity curves for bitterness of copper solutions (2.5 mg/L and 5 mg/L) at pH 5.5 and 7.5 (n = 27; 9 subjects X 3 replications).

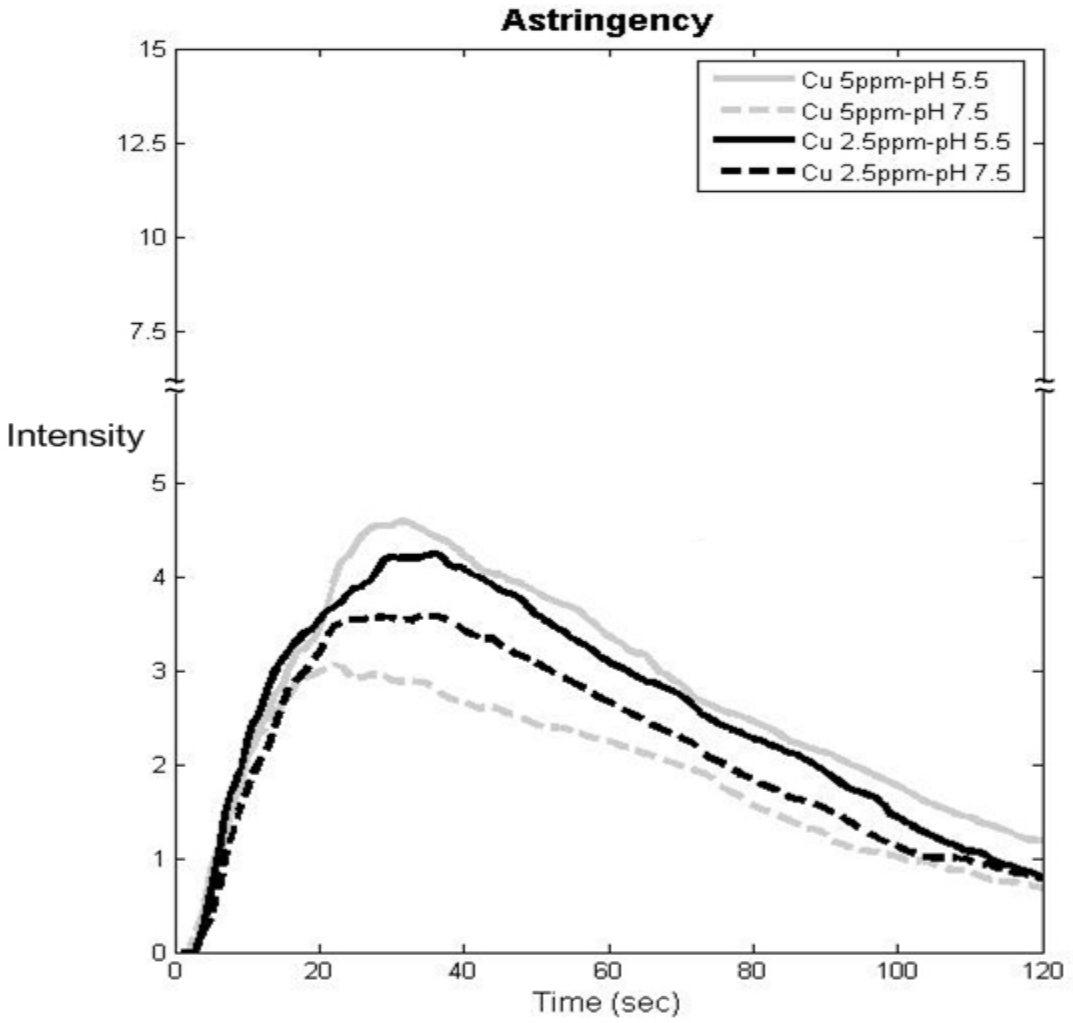

Figure 12. Time-intensity curves for astringency of copper solutions (2.5 mg/L and 5 mg/L) at pH 5.5 and 7.5 (n = 27; 9 subjects X 3 replications).

Effect of pH on Temporal Sensory Profile of Copper in Drinking Water

The main effect and interaction effect of panelists, replication, copper, and pH were tested with 2-way ANOVA with the significance level of 5%. Mean values for TI parameters are summarized in Table 10. There were no significant effects of replication and interaction between replication and other factors for all TI parameters for metallic, bitter, and astringent taste, validating consistency in the performance of panelists in TI test. Panelists were a significant source of variance for all TI parameters in all three attributes. TI data is usually reported to show large panelist-to-panelist variance which cannot be eliminated by training, because individual panelists differ in their sensitivity to stimuli (Guinard et al., 1995). This uniqueness of individual panelists in TI curve shape often results in "curve signature" (ASTM, 2003).

Metallic Taste. Metallic taste was influenced by copper concentration and pH condition (Table 10). Seven parameters (maximum intensity (I_{max}), area under curve (AUC), pre-peak area, post-peak area, perimeter, rate of decrease, and time to maximum intensity (T_{max})), were significantly affected by both copper concentration and pH conditions ($p < 0.05$). Rate of increase, and onset time (T_{onset}) were influenced by only the interaction of copper and pH. Metallic taste was perceived more strongly when copper concentration was increased from 2.5 mg/L to 5 mg/L, but adjustment of pH to 7.5 suppressed the perception of metallic taste. T_{onset} was significantly decreased by increasing the level of copper concentration, pH, or both, which suggests that these treatments made metallic taste perceived quicker.

Bitterness. Only two TI parameters of bitterness, I_{max} and T_{max} were significantly influenced by copper concentration. As seen in the case of metallic taste TI parameters, increase in copper concentration resulted in higher I_{max}, but neutralization of pH interfered with increase of I_{max}. T_{max} was decreased by addition of copper, addition of sodium bicarbonate, or combination of both. Acidic pH (5.5) increased AUC, pre-peak area, and post-peak area significantly, which implies that overall bitterness response was greater at acidic pH than neutral pH.

Table 10. Mean values* for time-intensity parameters for metallic taste, bitterness, and astringency of copper at two levels (Cu 2.5 mg/L and Cu 5 mg/L) in ultrapure water (pH 5.5) and sodium bicarbonate 1 mM solution (pH 7.5).

Cu (mg/L)	pH	I_{max}	AUC	Pre-peak area	Post-peak area	Peri-meter	Plateau time	Rate of increase	Rate of decrease	T_{dur}	T_{ext}	T_{max}	T_{onset}
						METALLIC TASTE							
2.5	5.5	**3.97**^{a†}	**249.05**^{a}	**83.22**^{a}	**165.83**^{a}	**120.48**^{a}	6.60	**0.15**^{ab}	**-0.04**^{ab}	84.60	93.97	**32.90**^{ab}	**9.37**^{b}
	7.5	**3.03**^{a}	**192.42**^{a}	**43.47**^{a}	**148.96**^{a}	**120.50**^{a}	4.83	**0.2**^{ab}	**-0.03**^{a}	87.73	92.63	**27.10**^{a}	**4.90**^{a}
5	5.5	**7.05**^{b}	**463.51**^{b}	**148.26**^{b}	**315.25**^{b}	**122.82**^{b}	6.40	**0.28**^{b}	**-0.052**^{b}	100.00	104.87	**37.87**^{b}	**4.87**^{a}
	7.5	**3.24**^{a}	**199.21**^{a}	**68.91**^{a}	**130.30**^{a}	**120.39**^{a}	7.66	**0.12**^{a}	**-0.034**^{a}	89.35	95.52	**31.41**^{ab}	**6.17**^{a}
						BITTERNESS							
2.5	5.5	**5.82**^{ab}	**373.33**^{bc}	**111.18**^{c}	**262.14**^{ab}	122.24	5.00	0.21	-0.05	98.22	103.93	**32.07**^{b}	5.70
	7.5	**5.66**^{ab}	**294.89**^{ab}	**65.36**^{ab}	**229.53**^{a}	122.37	4.78	0.38	-0.05	95.74	100.48	**24.30**^{a}	4.74
5	5.5	**6.65**^{b}	**419.84**^{c}	**89.62**^{bc}	**330.22**^{b}	122.54	10.52	0.35	-0.05	104.44	108.56	**22.67**^{a}	4.11
	7.5	**4.56**^{a}	**232.23**^{a}	**46.45**^{a}	**185.78**^{a}	121.10	7.48	0.27	-0.04	97.00	100.93	**22.63**^{a}	3.93
						ASTRINGENCY							
2.5	5.5	**4.80**^{bc}	**289.69**^{b}	63.75	**225.94**^{b}	**121.36**^{b}	**8.19**^{ab}	0.24	**-0.05**^{bc}	**105.41**^{b}	**111.19**^{b}	26.89	5.78
	7.5	**4.17**^{ab}	**249.56**^{ab}	65.74	**183.82**^{ab}	**120.84**^{ab}	**6.89**^{ab}	0.18	**-0.04**^{ab}	**98.26**^{a}	**102.82**^{a}	27.96	4.56
5	5.5	**5.28**^{c}	**326.73**^{b}	71.44	**255.29**^{b}	**121.49**^{b}	**12.70**^{b}	0.24	**-0.05**^{c}	**107.04**^{b}	**111.83**^{b}	27.65	4.78
	7.5	**3.67**^{a}	**218.47**^{a}	52.22	**166.25**^{a}	**120.62**^{a}	**4.78**^{a}	0.21	**-0.03**^{a}	**95.39**^{a}	**99.70**^{a}	24.83	4.30

* Standard deviation values for metallic taste, bitterness, and astringency in samples are summarized in Appendix 5.

† Different subscript letters (a, b, and c) for each attribute indicate a significant difference among combinations of two pH conditions and two Cu concentrations calculated using 2-way ANOVA followed by pairwise Student's t-test ($\alpha = 0.05$).

Astringency. TI parameters of astringency were not influenced by copper concentration at all. Changes in I_{max}, AUC, post-peak area, perimeter, plateau, rate of decrease, duration time (T_{dur}) and extinction time (T_{ext}) were a function of pH. Means for I_{max}, AUC, post peak area, and perimeter at pH 5.5 were greater than those at pH 7.5, which means that total astringency over time was perceived better at pH 5.5. Mean values of TI parameters that are related with aftertaste qualities, such as rate of decrease, T_{dur}, and $T_{ext,}$ showed that astringency decayed more slowly at acidic pH than at neutral pH.

Measurement of Soluble Copper Concentration

Soluble copper concentration at different pH conditions is shown in Figure 13. Copper became less soluble (by ~ 50%) by raising pH. When copper was added at the level of 2.5 mg/L, soluble copper in the solution was decreased from 2.42 mg/L to 1.03 mg/L (Δ 1.39) as pH was increased from 5.5 to 7.5. For 5 mg/L copper solution, solubilized copper was 2.29 mg/L at pH 7.5 compared to 4.87 mg/L at pH 5.5 (Δ 2.58). The majority of soluble copper species are assumed to be in free cupric ion form at pH 5.5, but at pH 7.5 turned into precipitated copper by complexing with bicarbonate or carbonate ions (Cuppett et al., 2006). Soluble copper concentration of a Cu 5.0 mg/L – pH 7.5 sample was almost two fold higher than that at the same pH (1.3 mg/L) reported by Cuppett et al. (2006). Copper solubility is determined by not only pH but also electrolyte composition and time to thermodynamic equilibrium (Jensen, 2003). Higher levels of soluble copper of a Cu 5.0 mg/L – pH 7.5 sample in our study is thought to be due to differences in these factors between our study and the study of Cuppett et al. (2006).

pH significantly affected temporal parameters of all three attributes, while copper concentration significantly influenced TI parameters for metallic taste and bitter taste. Soluble copper concentration was compared to three TI parameters of metallic taste, I_{max}, T_{max}, and AUC, which are known to best summarize a TI curve (MacFie and Liu, 1992): the magnitude of change for mean values were significantly greater in the Cu 5 mg/L – pH 5.5 sample compared to other samples; mean values of the Cu 2.5 mg/L – pH 5.5 sample, the Cu 2.5 mg/L – pH 7.5 sample, and the Cu 5 mg/L – pH 7.5 sample were not much different from one another (Figure 14). This

observation confirms the previous result showing that soluble copper species determine the sensory perception of copper (Cuppett et al., 2006). Because 2-way ANOVA showed that bitterness and astringency were affected by pH rather than copper concentration, it is postulated that copper speciation influences the perception of copper sensation through changing intensity of metallic taste.

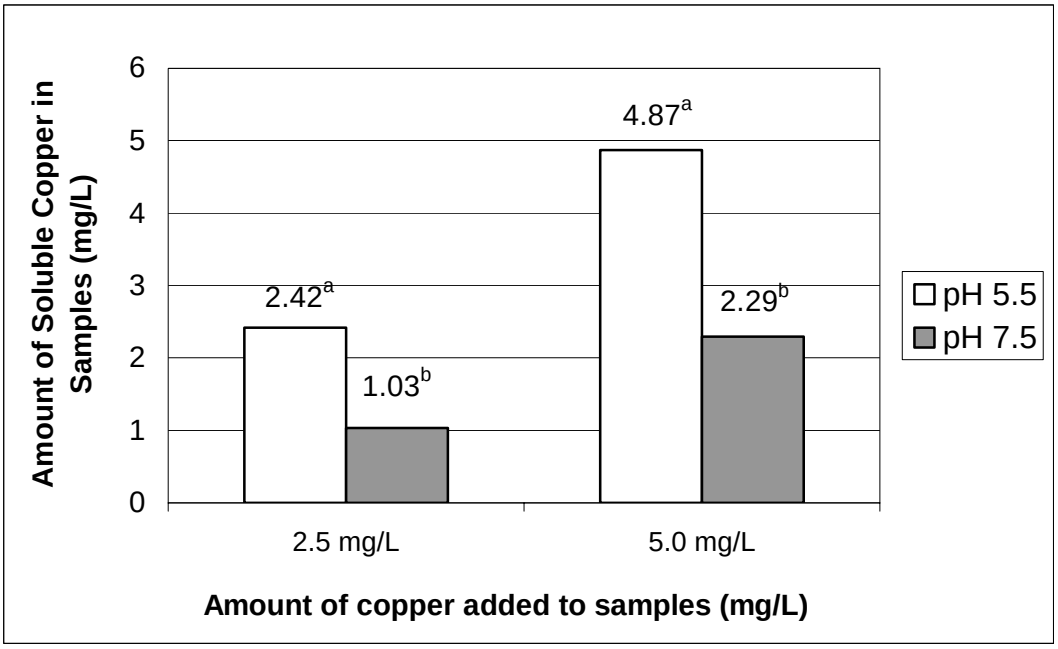

Figure 13. Concentration of apparent solubilized copper in time-intensity test samples at pH 5.5 or 7.5. Different superscript letters (a, b) within copper concentration indicates a significant effect ($p < 0.05$) of pH.

DISCUSSION

Temporal Characteristics of Copper Sensation

A time-intensity sensory test was performed to investigate influence of copper speciation on temporal profile of copper sensation. Also, the TI test was expected to identify characteristics of

lingering aftertaste of copper. Summarized TI curves for metallic, bitter, and astringent taste showed similar curve shapes, which reach the maximum intensity of 2.5 ~ 5 at 20 ~ 30 sec after consumption and last for at least 2 min with the final intensity of 0.5 ~ 2 (Figure 10, Figure 11, and Figure 12). Common TI parameters that showed significant differences between samples are related with intensity or overall response, such as I_{max}, AUC, and post-peak area (Table 10). Therefore, increase or decrease in curve area or maximum height is thought to explain the majority of variation in temporal characteristics of copper and rest of variation can be understood from changes in time-course related terms such as T_{onset} or T_{dur}.

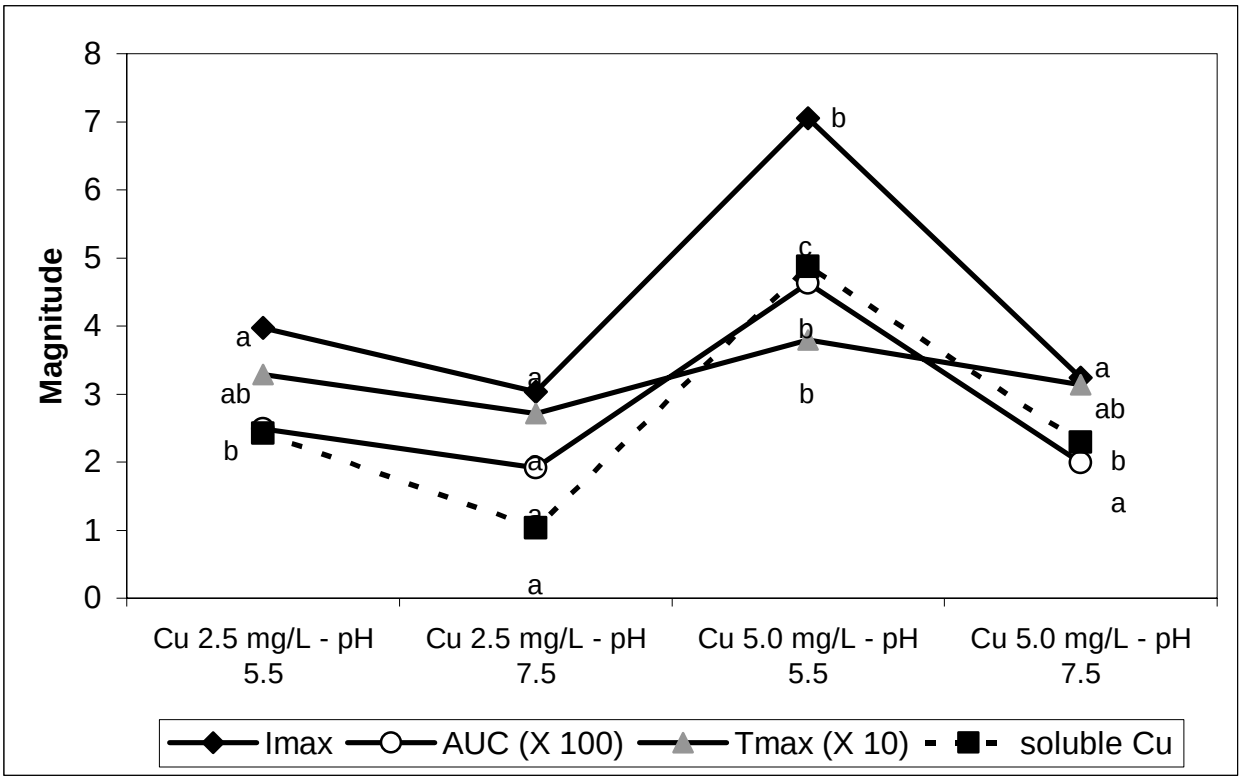

Figure 14. Pattern of changes in measurements of three representative time-intensity sensory test parameters and soluble copper concentration from samples of Cu 2.5 mg/L and 5.0 mg/L at pH 5.5 and pH 7.5. Units for area under curve (AUC) and time to maximum intensity (T_{max}) are displayed in hundreds and tens, respectively. A unit for soluble copper concentration is mg/L. Different superscript letters (a, b) within a measurement indicate a significant effect ($p < 0.05$) of copper and pH treatments.

We hypothesized that a TI curve of astringency would show distinctively slow onset, long duration time, and very slow decaying rate that are typical for lingering aftertaste (Peleg et al., 1999; Valentova et al., 2002; Drobna, 2003), because this typical time-intensity course of astringency has been observed from several other metallic compounds (Keast, 2003; Yang and Lawless, 2006b). However, our results showed that the decaying pattern of astringency was not different from those of metallic taste and bitterness. This result means that the aftertaste of copper includes not only astringency, but also metallic taste and bitterness.

Effect of Copper Speciation in Drinking Water on Copper Sensation

Copper speciation controlled by pH significantly influences metallic taste most, and bitterness to some degree. Increase in pH had an inhibitory effect on perception of metallic taste and bitterness by reducing soluble copper species in drinking water. Inhibitory effect was not statistically significant at lower copper concentration (2.5 mg/L), but metallic taste and bitterness of Cu 2.5 mg/L samples tended to be perceived less at pH 7.5 than pH 5.5. The relationship between copper speciation and metallic taste perception suggests that metallic taste may be an important attribute that can explain copper perception. However, we should consider that this suggestion is only valid in the range of the copper concentration close to the level used in this study, because the taste qualities of metallic compounds can be changed depending on concentration of metals (Lawless et al., 2003; Lim and Lawless, 2006b).

Another effect of copper speciation is reducing T_{onset} for metallic taste and T_{max} for bitterness, resulting in a more rapid perception of metallic taste and quicker perception of the bitterness. This finding is contradictory to the concept that soluble copper is more readily tasted. Although ANOVA showed that copper concentration influenced significantly these TI factors, decrease in T_{onset} or T_{max} may not have an actual connection with copper speciation. Changes in these factors may be related with sodium bicarbonate that was used to increase pH, because some panelists reported that they detected a sensation associable with minerals in 1 mM sodium bicarbonate solution right after drinking the solution. It is possible, in some cases, that this mineral-like sensation was mistaken as metallic taste.

Compared to metallic taste that was under the influence of copper speciation and astringency that was controlled by pH, the effect of treatments was more ambiguous for bitterness. Besides I_{max} and T_{max} that were influenced by copper speciation, three TI parameters that are related with total gustatory response, AUC, pre-peak area, and post-peak area, were affected by only pH. They were perceived better at acidic pH. The effect of pH on bitterness perception is thought to be induced by sodium bicarbonate. Sodium ion is known to suppress the bitterness of various bitter compounds including urea, quinine, caffeine, amino acids, and Mg_2SO_4 (Breslin and Beauchamp, 1997; Delwiche et al., 2001; Keast and Breslin, 2002). Sodium bicarbonate is dissociated into Na^+ and HCO_3^- at pH 7.5 ($pK_{a,1}$ of H_2CO_3 = 6.3) (Jensen, 2003), and then Na^+ possibly suppresses the bitterness of copper. However, Keast and Breslin (2002) observed that bitterness of Mg_2SO_4 was not effectively suppressed by sodium. Because copper is an inorganic stimulus like magnesium sulfate and the concentration of both copper and sodium is very low in our study, the suppression effect of sodium may not be the main reason of low copper bitterness at pH 7.5.

Astringency was perceived greater and lasted longer at acidic pH, regardless of copper concentration. Again it should be emphasized that this is only valid in the range of the copper concentration close to the level used in this study. Increase in astringency under acidic pH (pH $\leq$ 5) has been reported in several studies (Lawless et al., 1996; Kallithraka et al., 1997a; Kallithraka et al., 1997b). Astringency is a tactile sensation caused by loss of lubrication due to binding of polyphenolic compounds to salivary proteins or direct attack of epithelial tissues by polyphenols. Lawless et al. (1996) suggested that conformational changes or partial precipitation of salivary proteins caused by low pH could be another perception mechanism of astringency. Metals can contribute to lower pH in saliva by forming complexes with hydroxo ion (OH^-) and thus releasing H^+ from H_2O (Jensen, 2003)

Future Studies

Soluble copper species, including soluble copper complex and free copper, have been identified as the major species that are perceived by the humans. However, the role of precipitated/particulate copper has not been understood yet. Cuppett et al. (2006) suggested that particulated copper would play a small role in perception of copper by becoming solubilized upon incoming salivary flow. Saliva is known to be capable of changing sensory perception over time through its buffer capacity, protein and electrolyte composition, and dilution effect. The effect of salivary flow on temporal sensory characteristics has been investigated using several food systems (Fisher et al., 1992; Guinard et al., 1995; Guinard et al., 1998; Peleg et al., 1999). However, the effect of salivary flow is not consistent due to variations in food system tested, sensory test setting, and variability in human subjects. Also, copper speciation would be affected by removal of insoluble copper residue by swallowing as well as salivary flow. To understand how particulated copper contributes to copper sensation, dynamics of copper speciation under constantly changing oral environment, including re-dissolution, removal, complexation, and attachment to the oral tissues, should be investigated.

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Chapter V . Interaction between Copper and Salivary Proteins and Role of Human Saliva in Perception of Copper Sensation

ABSTRACT

Interaction with saliva is the first step in flavor perception process. Saliva is assumed to influence perception of copper taste by controlling solubility of copper or causing astringency via binding of proteins with copper. This study was performed to identify the nature of copper-protein interaction and its impact on sensory perception. Unstimulated whole saliva samples were collected from five healthy subjects. pH and total protein content of the collected samples, and salivary flow rate were determined. Collected saliva samples were pooled and treated with copper sulfate at levels of 0, 2.5, 10, 20, or 40 mg/L. Saliva samples were analyzed for changes in salivary protein peaks using high performance liquid chromatography (HPLC). Protein peaks that showed changes were characterized with sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). Copper bound to salivary protein fractions and unbound copper were determined using ultrafiltration coupled with inductively coupled plasma – mass spectrometry (ICP-MS). HPLC analysis revealed that copper treatment up to 40 mg/L decreased the largest protein peak at retention time 6.8 min by ~ 70%. A new small peak at retention time 3.0 min appeared when copper was added at the level of ≥ 2.5 mg/L. SDS-PAGE result showed that proteins of molecular weight 29 kDa and 33 kDa precipitated when copper was added at concentrations ≥ 10 mg/L. At copper concentrations ≤ 10 mg/L, most of copper exists as unbound copper form while about 60 % of copper was found in protein fractions or with precipitated salivary debris at copper level above 10 mg/L. The results suggest that copper is in soluble unbound form in saliva at low concentration and assumed to be available for taste receptors. At higher concentration, copper either becomes insoluble or binds with proteins. These copper species are thought to cause astringency. This study provides biochemical information for understanding perception mechanisms of copper taste.

INTRODUCTION

Perception Mechanism of Copper

The perception mechanism of copper at the cellular level has not been clearly identified. Plattig (1988) suggested that metallic compounds created electrical taste by generating a surface electrical potential. In the study of Lawless et al. (2005), small batteries could yield metallic taste, especially on the anterior part of tongue. Since fungiform papillae are densely located on this part of the tongue, electrical taste was suggested as a gustatory sensation, not a trigeminal sensation.

At the cellular level, taste stimuli are transduced using two major mechanisms: ion channels and G-protein coupled receptors. Sweet taste, bitter taste, and umami taste activated G-protein coupled receptors. Binding of tastants on the receptors triggers a series of cellular events including generation of second messengers. Second messengers such as cyclic nucleotides or inositol triphospate raise the level of intracellular Ca^{2+} and eventually lead to release of neurotransmitters. Salty taste and sour taste are mediated through the channels specific to Na^{+} ions and H^{+} ions, respectively. Influx of Na^{+} or H^{+} into taste cells causes action potential that trigger increased level of intracellular Ca^{2+} (Boughter and Gilbertson, 1999; Lindemann, 2001; Rawson and Li, 2004). Many ionic species can elicit a salty taste, even though Na^{+} is the most important stimuli of salty taste (Figure 3).

These studies provide a starting point for investigation of copper taste perception. Copper is assumed to be ingested directly or go through the redox process in the mouth, which generates of Cu^{2+} ion. Thus copper is expected to act as ionic stimuli such as Na^{+} or H^{+}. Redox process within the mouth can generate a weak electric current, which produces electric taste as reported by Lawless et al. (2005). The redox process of copper in the mouth starts with solubilization of copper in saliva. A complicated aqueous buffer system, saliva can greatly influence the electrochemical reactions of copper in the mouth by controlling pH and having salivary components interact with copper.

Salivary pH is thought to control solubility of copper in the mouth. Generally copper is dissolved well in acidic pH (pH <6) (Jensen, 2003). In the study of Cuppett et al. (2006), no more than 1.3 mg of copper could be dissolved in 1 L of synthetic tap water at pH 7.4., while 8 mg of copper was soluble in 1 L of water at pH 5.5. Therefore copper is assumed to have limited solubility in the salivary pH range of pH 6.5 ~ 7.4. Salivary electrolytes may play a role in solubilization of copper by complexing with copper ions. Phosphate, sulfate, and carbonate anions were reported to form insoluble complexes with copper, thus precipitate copper in drinking water (Dietrich et al., 2005). It is hypothesized that a decrease in solubility reduces the ionic form of copper available to taste receptors, eliciting less perception of copper. Low intensity of copper taste at salivary pH range has been observed in our previous TI sensory studies (Hong et al., 2006) and the study of Cuppett et al. (2006).

Salivary proteins also are expected to have an important role in the sensation of copper. It was observed that copper formed a haze in human saliva at the concentration of 0.3 mM and 1.0 mM. Increase in turbidity of human saliva had a positive correlation with astringency caused by copper sulfate (Chapman and Lawless, 2004). Chapman and Lawless (2004) suggested that copper ions caused astringency in the mouth by precipitating and de-lubricating proline-rich proteins (PRPs). This suggestion is based on the perception mechanism of phenolic compounds (Kallithraka et al., 1998; Lu and Bennick, 1998; Sarni-Manchado et al., 1999; Charlton et al., 2002). Astringency arises when salivary proteins lose the lubrication effect as phenolic compounds bind to salivary proteins to form a complex compound. Kallithraka et al. (1998) studied interaction of protein-phenolic compounds in human saliva using HPLC. After subjects drank wine model solution containing polyphenolic compounds, a new soluble salivary protein-phenolic compound complex appeared in saliva while the amount of salivary protein decreased. This new complex was suggested to be an intermediate in tannin-induced protein precipitation process. Sarni-Manchado et al. (1999) reported that condensed tannins (grape seed proanthocyanidins) precipitated salivary PRPs. Polyphenolic compounds bind to PRPs by hydrophobic interaction or hydrogen bonding. More specifically, hydrophobic faces of the aromatic rings of polyphenols stack onto the pyrrol ring of prolines in PRPs and result in the soluble protein-tannin complex. As more phenolics are bound to proteins, phenolics on the surface of proteins provide intermolecular bridges between proteins, and then proteins become

polymerized and precipitate (Lu and Bennick, 1998; Sarni-Manchado et al., 1999; Bacon and Rhodes, 2000; Charlton et al., 2002). However, unlike the perception mechanism of astringency of polyphenolics, interaction between copper and proteins in regards to astringency has not been clearly understood yet.

Salivary Proteins that Interact with Copper

Mueller (1985) reported that corroded copper, nickel, and cobalt ions from a dental alloy bound to a protein fraction of saliva as well as a non-protein fraction of saliva. Salivary proteins that have been reported to have metal binding capacities are mucin, α-amylase, PRPs, histatins, gustin, lactoferrin, and lysozyme.

Mucin. Mucins are glycoproteins secreted from submandibular-sublingual gland. Two major species of mucin are known. MG1 has very high molecular weight (> 1,000 kDa) and MG2 has molecular weight of 200 ~ 250 kDa (Loomis et al., 1987; Wu et al., 1994). Mucins are negatively charged in salivary pH range due to negatively charged sialic acid residues. It was reported that divalent ions such as Ca^{2+} formed cross-linking between the negatively charged molecules (Mueller et al., 1983; Wu et al., 1994). It is possible that cupric ions (Cu^{2+}) form ion bridges between mucin molecules through ionic bonding.

α-Amylase. Divalent calcium ion is an important cofactor of α-amylase. α-amylase has two metal binding sites. One is an exclusive binding site for Ca^{2+}, and the other binding site containing glycine. It was reported that Cu^{2+} bound to the second binding site in the molar ratio of 1:1, without influencing the binding between Ca^{2+} and α-amylase (Argawal and Henkin, 1987).

Proline Rich Proteins (PRPs). A major amino acid residue of PRPs is proline, which consistutes 28 ~ 40 % of total amino acid residues. PRPs are classified into acidic (pI 3.5 ~ 4.5), basic (pI > 8.0), and glycosylated (pI > 8.0). These three PRPs constitute approximately 70% of total proteins found in parotid saliva (Beeley, 1993). Acidic PRPs are known to bind Ca^{2+} due to negatively charged amino residues such as aspartate and glutamate. The calcium-binding ability of acidic PRPs is associated with dental pellicle formation, which is important in

protection of dental enamel (Tenovuo, 1989). As seen in cases of mucin and α-amylase, negative charge and Ca^{2+} binding ability may increase PRPs capacity to interact with Cu^{2+} (Minaguchi and Bennik, 1989).

Histatins. Histatin is a family of histidine-rich proteins (HRP) secreted from the parotid and submandibular glands (Tenovuo, 1989). Twelve histatins, histatin 1-12 have been found, and the most common histatins found in human saliva are histatin 5, histatin 3, and histatin 7. These peptides are basic low molecular weight peptides, which are positively charged in neutral and acidic pH (Oppenheim et al., 1988; Melino et al., 1999). Histatins have been found to possess antifungal activity to *Candida albicans*. This ability has been suggested to be relevant to its metallopeptide characteristics. Histatins exhibit binding ability with several divalent metal ions, such as Zn^{2+}, Ni^{2+}, and Cu^{2+} (Oppenheim et al., 1988; Tenovuo, 1989; Melino et al., 1999; Gusman et al., 2001). Especially, histatins have a high binding affinity to copper at pH 7.4, with a binding constant of $2.6\times10^{7}\ M^{-1}$. Copper binds to an ATCUN (Amino Terminal CU (II) - and Ni (II) - binding) motif that has a unique amino acid sequence of Asp-Ser-His. Another binding site for copper is C-terminal of histatin 3 and histatin 5 with which copper ions are assumed to associate nonspecifically (Brewer and Lajoie, 2000; Gusman et al., 2001).

Gustin. Gustin constitutes 3% of total parotid salivary proteins. Gustin is an aicidic glycoprotein (pI = 4.73) which strongly binds with Zn with the dissociation constant of 4.5×10^{-11} M at pH 7.2. The Zn-binding capacity is known to control homeostasis of salivary zinc. Low salivary zinc concentration causes loss of taste acuity (hypoguesia) and pathological changes in taste buds (Shatzman and Henkin, 1981; Thatcher et al., 1998). Like α-amylase, gustin has two Zn-binding sites. Zinc binds very tightly with one binding site, but binds loosely to the second site. Copper and other metal ions could bind with the second site loosely (Shatzman and Henkin, 1980).

Other Proteins. Lactoferrin and carbonic anhydrase are also known to bind with metals. Lactoferrin is famous for its iron-binding ability. Lactoferrin is composed of two subunits that each binds with one molecule of Fe^{3+} reversibly (Tenovuo, 1989). Lactoferrin bound with Cu^{2+} with much less binding affinity than that for iron. Decrease in binding affinity was assumed to

be related with structural change of lactoferrin caused by changes in metal coordination (Baker et al., 1990; van Nieuw Amerongen, 2004). Carbonic anhydrase is a zinc-binding metalloenzyme controlling hydration of carbon dioxide. There are six isoenzymes in the carbonic anhydrase family, and it was recently found that gustin is carbonic anhydrase VI (Tenovuo, 1989; Thatcher et al., 1998). It was reported that copper coordinated with carbonic anhydrase II *in vitro* and this binding destabilized the enzyme (Hunt et al., 1999). Even though this suggests the possibility of interaction between copper and lactoferrin or carbonic anhydrase, there has been no observation of actual binding of copper to these proteins in the human saliva reported.

Most of the studies focused on binding of calcium or corroded metals from dentures (Mueller, 1983; Mueller, 1985; Argarwal and Henkin, 1987; Mueller, 1987; Wu et al., 1994), not on protein-copper binding in regard with sensory perception. In addition, a binding study was performed with a single compound and a purified protein ligand at ideal conditions (Shatzman and Henkin, 1980; Baker et al., 1990; Melino et al. 1999; Hunt et al., 1999; Brewer and Lajoie 2000; Gusman et al. 2001). Hence interaction between copper and proteins in real saliva should be investigated to understand the nature of binding such as competition between molecules and ligands within the complex system like the oral environment.

OBJECTIVES

Study of the perception mechanism of metallic taste is a relatively new area in sensory science. There have been only a few studies to investigate how metallic taste is perceived. Studies reported so far have focused on mostly perception itself, such as modality and sensory characteristics. Biochemical interactions behind the perception have not been studied intensively.

Since sensory stimulants should be dissolved in the saliva phase at the first stage of the perception process for further interaction with taste receptor cells, it is important to understand what impact saliva has on delivery of sensory stimulants to taste receptors. Saliva influences

copper perception by controlling pH and interaction with salivary components, especially salivary proteins. It is postulated that copper precipitates salivary proteins and this will lead to changes in perception of copper by creating astringency and limiting solubilized copper available for interaction with taste receptors.

This research was performed to investigate the interaction of copper and salivary proteins and the role of copper-salivary protein interaction in copper perception mechanism. This research suggests an explanation about what roles copper speciation in the mouth play in generating different sensory attributes.

A series of hypotheses was set up to scrutinize copper-salivary protein interaction and its role in copper taste perception:

- Copper, when consumed, binds to salivary proteins and salivary proteins precipitate upon binding with copper;
- Salivary proteins that participate in binding with copper are proteins that have shown metal-binding ability, such as mucin, α-amylase, PRPs, histatins, gustin, and lactoferrin;
- Precipitation of proteins by copper creates an astringent sensation in the mouth.

These hypotheses were examined by: 1) assessing the amount of copper bound to proteins and free (unbound) copper with ultrafiltration coupled with ICP-MS; 2) determining quantitative and qualitative changes in salivary proteins with HPLC caused by copper treatment; 3) characterizing salivary proteins changed by copper using gel electrophoresis.

METHODS AND MATERIALS

Human Saliva Collection

Human saliva was collected from five healthy subjects (two males and three females) between the ages of 30 and 45 following the protocol suggested by Tenovuo (1989). All subjects demonstrated an ability to detect copper in water based on pervious sensory test. Three subjects were the panelists who had participated in the time-intensity (TI) test of copper solution, and two had participated in the sensory study for background taste of copper solution of pH 7.5 (Chapter IV). All subjects were reported to be non-smoking and to have no physical condition that may influence the results. The saliva collection protocol was approved by the Institutional Review Board at Virginia Tech (IRB #05-368) and informed consent forms were obtained from all subjects (Appendix 6).

Total 60 mL of saliva was collected from each subject for five consecutive days. Saliva was collected for 10 min each day. Human subjects were requested not to consume any food, beverage, and oral care products for two hour before saliva collection. Saliva was collected between 10 ~ 11 am or 2 ~ 4 pm. Subjects were asked to rinse their mouth with ultrapure water (Barnstead nanopure water purification system, Barnstead International, Dubuque, IA) three times, and then spit three times to get rid of any dilution effect of rinsing. Unstimulated whole saliva was collected behind closed lips, and then expectorated once per one or two min in a 50 mL glass beaker chilled on ice. Saliva samples were aliquoted by 2.5 mL in 5 mL pre-sterilized polypropylene cryogenic vials (Corning Inc., Acton, MA) after weighing total collection weight. After labeling with the name of the subject, date, and weight, saliva samples were stored at -75^{o}C until analyzed. Stored samples were analyzed within two months of collection. All procedures were performed on ice to prevent further degradation by generic hydrolytic enzymes in saliva. All glassware and plasticware used for saliva collection had been washed first with detergent and tap water, and then soaked in 3 % nitric acid overnight to get rid of copper residue, followed by rinsing with ultrapure water.

General Characteristics of Collected Saliva: pH, Total Protein Concentration, and Flow Rate

Immediately after collecting saliva, pH of individual saliva samples were measured using a pH meter (Accumet AR15 pH meter, Fisher Scientific, Pittsburgh, PA) equipped with an automatic temperature compensation probe. During measurement of pH, saliva samples were kept on ice. Flow rate of saliva of each individual was calculated by dividing yield of collected saliva (mL) by collecting time (min).

Total protein content of saliva was determined using Biuret assay (Copeland, 1994). Saliva samples (1 mL) were mixed with 4 mL of Biuret reagent. Mixture was mixed well using a vortexer and incubated for 20 min at room temperature. Bovine serum albumin (Sigma, St.Louis, MO) was diluted in 0.15 M NaCl solution at the concentration of 0, 0.1, 0.5, 1.0, and 2.0 mg/mL for preparing a standard curve. Sodium chloride solution of 0.15 M was used as a blank solution. The absorbance of standards and samples were measured at 540 nm using a UV spectrophotometer (Milton Roy Co., Rochester, NY).

All samples were analyzed in quintuplicate. Mean and standard deviation for individual subject samples as well as pooled samples from all five subjects were calculated.

Assessment of Binding of Copper to Salivary Proteins with Ultrafiltration

Sample Preparation. The scheme for saliva sample preparation is illustrated in Figure 15. Total 15 tubes of saliva aliquot (2.5 mL each) from five subjects were defrosted as described previously. Equal volumes of saliva from each subject were combined together and pH of pooled saliva was measured instantly. Pooled saliva was divided into six portions of 5 mL volume in 15 mL polypropylene centrifuge tubes.

Copper sulfate pentahydrate stock solution (2 %, w/v) was added to saliva samples to give the concentration of 0, 2.5, 5, 10, 20, and 40 mg/L Cu, followed by vortexing for 10 sec for

complete mixing. When copper stock solution was added to saliva at the level of 40 mg/L Cu, total volume was increased by only 0.8 % of initial saliva volume. Dilution effect caused by this amount of volume increase was considered to be negligible.

Aliquots of 0.5 mL saliva containing different copper concentrations were transferred to Pyrex tubes for copper analysis. This uncentrifuged saliva was referred as crude saliva. Remaining crude saliva samples were transferred into several microcentrifuge tubes for centrifugation at 13,200 rpm (16,100g) for 5 min to separate any insoluble matter. After pipetting supernatant into clean tubes, precipitates were transferred using a micropipetter to Pyrex tubes for wet ashing. Because precipitate was highly dense and viscous, any residue remaining on tubes was rinsed with 0.5 mL of 2 % nitric acid solution twice and transferred completely to Pyrex tubes.

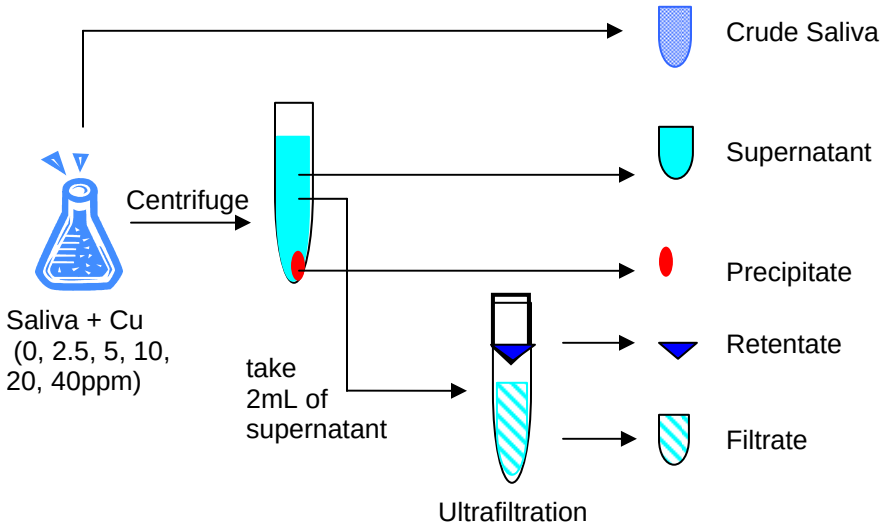

Figure 15. Saliva sample separation scheme for evaluating copper-protein interaction.

Samples were kept on an ice bath to prevent hydrolytic degradation of major salivary components during all operations. All tubes used in this study had been washed first with detergent and tap water, and then soaked in 3 % nitric acid overnight to remove any copper

residue, followed by rinsing three times with ultrapure water. After drying at 80°C for 3 hrs in an oven (Precision Scientific Thelco Glassware Dryer, Thermo Electron Corporation, Waltham, MA), tubes were cooled down to room temperature to measure the initial weight of empty tubes. Yields of crude saliva, supernatants, and precipitates were obtained by subtracting the weight of empty tubes from the weight of tubes containing samples.

Ultrafiltration. Supernatants of saliva that had different copper concentrations were subjected to ultrafiltration to separate free copper from copper that binds with high molecular weight components of saliva. Two milliliters of supernatant were transferred to Amicon Ultra 4 centrifugal filter devices (5 kDa molecular weight cut-off (MWCO), Millipore, Billerica, MA) and centrifuged at 3,500 rpm (2180 g) at 4°C using a refrigerated high-speed centrifuge (IEC Refrigerated Centrifuge PR-2, International Equipment Company, Boston, MA) for ~ 2 hrs until volume of retentate decreased to ~ 0.1 mL. Initial weight of supernatant, and yields of retentates and filtrates were calculated by subtracting the initial weight of empty centrifuge tubes from the tubes containing samples. Retentates and filtrates were recovered from devices using a micropipetter to Pyrex tubes after weighing. Retentates remaining on the filter parts were washed with 0.5 mL of 2 % nitric acid twice and combined with previously transferred retentates.

Recovery of proteins was calculated by the formula suggested by the manufacturer (Millipore, 2004) based on the observation that density of saliva is close to 1 (density of saliva measured from five subjects in triplicate = 0.97).

Recovery of retentate (%)

$$= 100 \times \frac{(\text{weight of retentate}) \times (\text{protein concentration in retentate})}{(\text{weight of supernatant}) \times (\text{protein concentration in supernatant})}$$

Recovery of filtrate (%)

$$= 100 \times \frac{(\text{weight of filtrate}) \times (\text{protein concentration in filtrate})}{(\text{weight of supernatant}) \times (\text{protein concentration in supernatant})}$$

Total recovery (%) = recovery of retentate + recovery of filtrate

Validation of Ultrafiltration. Normal pH of saliva is 6.7 ~ 7.5 (Tenuvou, 1988), and solubility of copper can be limited at this range of pH (Cuppett et al., 2006). Thus copper in retentates may be particulated copper which cannot pass through membrane. An experiment was implemented to determine whether copper in retentates is particulated or in a bound form.

Copper was added to combined saliva supernatant at the level of 10 and 50 ppm. Ultrapure water containing the same amount of copper was prepared as control. Before copper was added to water or saliva samples, pH of samples was adjusted to either 5.5 (pH of ultrapure water) or pH of saliva (6.9 ~ 7.1). pH of ultrapure water was brought to salivary pH by adding sodium bicarbonate at the level of 1 mM and then fine-tuning with 0.1 N HCl. To decrease pH of saliva to 5.5, a few drops of 0.1 N HCl was added to saliva. Aliquotes (1.5 mL) were placed into two microcentrifuge tubes for centrifugation at 13,200 rpm (16,100g) for 5 min to separate any insoluble matter. Top 1 mL of each tube (supernatant) was carefully transferred into an ultrafiltration filter device to make the final volume of 2 mL. Remaining 0.5 mL at the bottom was discarded to prevent any large particle from clogging filter pores.

Two milliliters of each sample were filtered using Amicon Ultra 4 centrifugal filter devices (5 kDa MWCO) at 3,500 rpm (2180 g) at 4^{o}C until volume of retentate decreased to ~ 0.1 mL. Retentate was rinsed by adding 2 mL of identical water or non-protein saliva but containing no copper and centrifuging again, based on the assumption that particulated copper was washed out by newly added water or buffer while copper bound to salivary proteins would remain unaffected. Non-protein saliva was prepared by removing proteins from 15 mL combined saliva with 5kDa MWCO Amicon Ultra 15 centrifugal filter device followed by adjusting pH to 5.5 or salivary pH with 0.1 N HCl.

Copper concentration of retentates, filtrates before rinsing, and filtrates after rinsing was determined using an atomic absorption spectroscope (Perkin Elmer 5100, Wellesley, MA). Filtrates (~1.9 mL) were filled up to 10 mL with ultrapure water. Total copper in filtrates was stabilized by adding 0.2 mL nitric acid (trace metal grade, Fisher scientific, Pittsburgh, PA). Retentates were transferred to Pyrex tubes as described previously. Organic matter in retentates was degraded by heating at 90^{o}C for 1 hr with 1 mL nitric acid. AAS analysis was performed

following the method suggested by Cuppett et al. (2006). Analysis was performed in triplicate. Mass (μg) and recovery (%) of copper in each fraction were calculated.

Total Protein Assay. Total protein content in supernatants, filtrates, and retentates was determined using Bradford assay (Copeland, 1994). Supernatants and retentates were diluted in 0.15 M NaCl solution in the ratio of 1:1 and 1:20, respectively. Saliva samples (100 μL) were mixed with 5 mL of Bradford reagent. Mixture was mixed well using a vortexer and incubated for 5 min at room temperature. Bovine serum albumin (Sigma, St.Louis, MO) was diluted in 0.15 M NaCl solution at the concentration of 0, 0.1, 0.25, 0.5, 1.0, and 1.4 mg/mL for preparing a standard curve. Sodium chloride 0.15 M solution was used as blank solution. The absorbance of standards and samples was measured at 595 nm using a UV spectrophotometer (Milton Roy Co., Rochester, NY). All samples were analyzed in triplicate. The result was analyzed with the analysis of variance (ANOVA) (α=0.05) using JMP IN® statistical software (*ver.* 4.0, SAS, Cary, NC) to test if there was significant difference in protein concentration between different copper treatments within a fraction.

Analysis of Copper. Copper in five fractions of saliva (Figure 15) was analyzed with an Inductively Coupled Plasma Mass Spectroscopy (ICP-MS). Saliva fractions were wet-ashed for ICP analysis following the method of Kilic et al. (2002) with modification. Concentrated nitric acid (trace metal grade, Fisher scientific, Pittsburgh, PA) was added to each saliva fraction placed in Pyrex round bottom tubes (Table 11). Tubes were closed with Teflon-lined caps and heated at 90^{o}C for 45 min in a heating block (Fisher Scientific, Pittsburgh, PA). After cooling to room temperature, hydroperoxide (35 %, Fisher Scientific, Pittsburgh, PA) was added to crude saliva, supernatants, retentates, and precipitates. Wet-ashing of filtrates was stopped before hydroperoxide treatment because most of organic matters did not exist in filtrates due to ultrafiltration. Tubes containing remaining four fractions were heated slowly to 130^{o}C without caps and maintained at that temperature for about two hours or until no more bubbling was observed. Finally samples were filled up to 10 mL with ultrapure water.

Samples were analyzed for copper using a Thermo Electron X-Series inductively coupled plasma with mass spectrometry (ICP-MS) per Standard Method 3125 (APHA, AWWA, and

WEF, 1998). ICP analysis was performed in duplicate. Concentration (mg/L) and mass (μg) of copper in each fraction were calculated.

Table 11. Amount of nitric acid and hydroperoxide added to each saliva fraction in wet ashing procedure for inductively coupled plasma.

Saliva fraction	Sample volume (mL)		Nitric acid (mL)	Hydroperoxide (mL)
	Fraction only	Fraction + 2% HNO_3		
Crude saliva	0.5	0.5	1.0	0.5
Supernatant	1.0	1.0	1.0	0.5
Precipitate	~ 0.1	~ 1.1	2.0	1.0
Retentate	~ 0.1	~ 1.1	1.0	0.5
Filtrate	~ 1.9	~ 1.9	0.2	-

Assessment of *In Vivo* Copper Binding to Salivary Protein in Real Drinking Situation

Saliva was collected from five subjects for 2 min each for three consecutive time intervals: before drinking copper solutions; after drinking 20 mL copper solutions of 2.5, or 5 ppm or ultrapure water; and 2 min after drinking 20 mL copper solutions or water (Figure 16). These intervals were selected to interpret the result of copper-protein binding in relation with the result of the time-intensity test (Chapter IV). Subjects were asked to expectorate copper solutions after swirling them around for 10 sec in the mouth. Only one solution of a certain copper concentration was provided per a day so that saliva collected after drinking a solution of low copper level would not be influenced by preceding collection with a solution of higher copper level. Saliva was collected in duplicate over six days within two weeks. Samples of varying copper concentration were presented randomly for each subject using a random complete block design in which subjects was treated as a block (Table 12). Day was treated as random effect.

Saliva samples were fractionated, ultrafiltrated, and analyzed for copper as described previously. The result was analyzed with the analysis of variance (ANOVA) (α=0.05) using JMP IN® statistical software (*ver.* 4.0, SAS, Cary, NC) to test if there was significant difference in protein concentration between different copper treatments and collection times within a fraction.

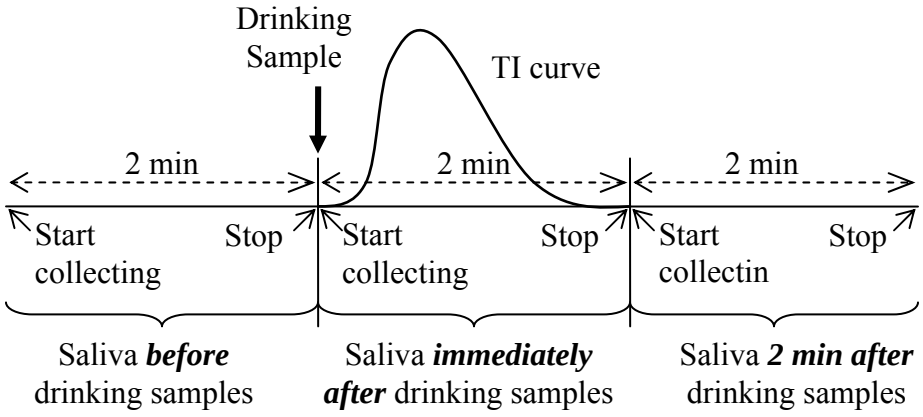

Figure 16. Scheme for saliva collection before, immediately after, and two min after drinking Cu 0, 2.5, and 5 mg/L solution.

Table 12. Randomized complete block design for collecting saliva before, immediately after, and 2 min after drinking Cu 0, 2.5, and 5 mg/L solution. Replication and subjects were treated as blocks. Days were treated as random effect.

Subject	Replication					
	1st			2nd		
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
1	2.5 mg/L	control	5 mg/L	5 mg/L	2.5 mg/L	control
2	5 mg/L	2.5 mg/L	control	control	2.5 mg/L	5 mg/L
3	control	5 mg/L	2.5 mg/L	2.5 mg/L	5 mg/L	control
4	2.5 mg/L	control	5 mg/L	2.5 mg/L	control	5 mg/L
5	2.5 mg/L	5 mg/L	control	5 mg/L	2.5 mg/L	control

Investigation of Effect of Copper on Salivary Proteins with High Performance Liquid Chromatograpy (HPLC)

Sample Preparation. Saliva aliquots (2.5 mL each) from five subjects were defrosted at room temperature (21 ± 2^{o}C) for 20 min. Saliva vials were vortexed (Vortex-2 Genie Vortexer G-560, Scientific Industries, Bohemia, NY) vigorously for 30 sec to re-solubilize precipitates resulting from freezing-thawing (Francis et al., 2000). One milliliter of each sample was immediately transferred to a microcentrifuge tube. A portion (0.2 mL) of saliva from each individual was pipetted into a vial and then vortexed vigorously for 10 sec to make a pooled saliva sample. Copper sulfate pentahydrate stock solution (2 %, w/v) was added to saliva at the level of 0, 2.5, 5, 10, 20, and 40 mg/L as Cu. This range of copper concentration was selected based on preliminary HPLC analysis (Appendix 7). Sample tubes were centrifuged at 13,200 rpm (16,100 g) for 5 min (IEC Centra-M Centrifuge, Thermo Electron Co., Whaltham, MA). Supernatants obtained from centrifugation were filtered with 0.45 μm syringe filters (Fisher Scientific, Pittsburgh, PA) and then placed in HPLC autosampler vials that contained 250 μL polypropylene inserts (National Scientific, Rockwood, TN).

HPLC. Changes in protein pattern caused by copper were analyzed using an Alliance HPLC system consisting of Waters 2695 separation module and Waters 2487 dual Λ absorbance UV detector (Waters, Milford, MA) following the method suggested by Kallithraka et al. (1998). This HPLC method was verified for its efficacy to show changes in proteins (Appendix 8). Analysis was performed on a C-18 reverse phase column (Luna C18(2), 150 × 4.6mm, particle size 5 μm, pore size 100Å, Phenomenex, Torrance, CA) with a 1.0 mL/min mobile phase flow rate. Mobile phase consisted of 0.1 % (v/v) trifluoroacetic acid (TFA; Pierce, Rockford, IL) in water (solvent A) and 0.1 % (v/v) TFA in 65 % (v/v) acetonitrile (HPLC grade, Fisher Scientific, Pittsburgh, PA) aqueous solution (solvent B). Separation was performed using the gradient conditions summarized in Table 13. Injection volume was 25 μL.

Table 13. Mobile phase gradient condition for HPLC analysis of salivary proteins.

Solvent / Retention time (min)	A[a] (%)	B[b] (%)
0	100	0
30	0	100
32	0	100
33	100	0
40	100	0

[a] 0.1 % (v/v) trifluoroacetic acid (TFA) in water.

[b] 0.1 % (v/v) TFA in 65 % (v/v) acetonitrile aqueous solution.

Signals were detected at dual wavelength of 230 nm and 280 nm. Peaks in chromatograms were integrated using Waters Millennium chromatography manager software (*ver* 3.20, Waters, Milford, MA). Proteins are usually detected at 210 ~ 220 nm which is specific for peptide bonds or at 280 nm where aromatic amino acid residues have high absorbance. PRPs in saliva are not detected at 280 nm due to lack of aromatic amino acid residues (Aguilar, 2004). If a protein peak had high absorbance at 230 nm but was not detected at 280 nm, this protein could be considered to have high proportion of non-aromatic amino acid residues. This relationship can be expressed as the ratio of peak area at 230 nm to that at 280 nm (A_{230}/A_{280}) which can be used as an index to identify PRPs. Because of hydrophilic nature of proline residues, PRPs are expected to be eluted early in the given mobile phase gradient condition. Therefore, if A_{230}/A_{280} of a peak is greater than 1 and eluted at early RT, the protein peak is assumed to be a PRP (Kallithraka et al., 1998).

All samples were analyzed in triplicate. Area of each peak was subjected to analysis of variance (ANOVA) (α=0.05) using JMP IN® statistical software (*ver.* 4.0, SAS, Cary, NC) to identify the effect of copper on peak area. Mean values of different treatments were compared using Tukey's HSD test. Data obtained as peak area was transformed to the ratio of the mean

peak area at a certain treatment to that of control for data presentation, since quantification of peaks were extremely limited by a lack of adequate reference standard materials.

Characterization of Salivary Proteins Influenced by Copper with Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sample Preparation. Control and saliva containing 40 mg/L copper were injected to the HPLC system and peaks that showed changes in HPLC analysis were collected. Collected peak fractions were stored at -20^{o}C and used within one week. Fractions were defrosted at room temperature for 20 min and then divided into 200 μL aliquots in microcentrifuge tubes. Fractions were concentrated to dryness using a Speedvac concentrator (SVC-100H, Savant, Waltham, MA). Dried samples were reconstituted by adding 12 μL ultrapure water and 4 μL four-fold concentrated Laemmli sample buffer [127.2 mM Tris-HCl (pH 6.8) containing 40 % (v/v) glycerol, 10 % (w/v) SDS, 20 % (v/v) mercaptoethanol, and 0.0075 % (v/v) bromophenol blue] to the fraction of RT 2 ~ 4 min and 18 μL ultrapure water and 6 μL sample buffer to the fraction of RT 6 ~ 7 min. After adding sample buffer, tubes were vortexed for 5 sec and boiled at 100^{o}C for 5 min. All reconstituted fractions were combined in one tube and centrifuged at 13,200 rpm for 2 min to remove any insoluble precipitate.

Non-fractionated saliva samples were prepared in the same manners as HPLC sample fractions, except three parts of saliva supernatants were mixed with one part of four-fold concentrated Laemmli sample buffer. Mixture was boiled at 100^{o}C for 5 min, and centrifuged.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4 ~ 20 % gradient Tris-HCl gels (Ready-gel, Bio-Rad, Hercules, CA) with Mini-Protean III system (Bio-Rad, Hercules, CA) following the method of Laemmli (1970). The dimension of gels was 7 × 8.6 cm, 1.0 mm thickness. Gel running buffer was 0.1 % (w/v) SDS in 25 mM Tris base and 192 mM glycine. Non-fractionated saliva samples (15 μL) were loaded with 5 μL of molecular weight standard (Mark 12 unstained wide range molecular weight standard, MW range 2.5 ~ 200

kDa, Invitrogen, Carlsbad, CA) in all gels. Proteins in collected peak fractions were detected with silver staining, so molecular weight standard was diluted 20 fold and samples were diluted so that all fractions could express same intensity when stained with silver nitrate. Gels were run at 15 mA constant current per gel for about 2 hr.

Commassie Blue Staining. Non-fractionated saliva samples were stained with 0.1 % (w/v) Coomassie brilliant blue R-350 reagent (PhastGel Blue R, Amersham Bioscience, GE Healthcare, Uppsala, Sweden) in 30 % (v/v) methanol and 10 % (v/v) acetic acid. Destaining was performed by the method suggested by Beeley et al. (1993) using 10 % acetic acid for 2~ 3 days until bands of proline-rich proteins (PRPs) developed violet-pink color to verify those proteins.

Silver Staining. Proteins from collected peak fractions separated by HPLC were stained poorly with Coomassie brilliant blue (CBB) in a preliminary gel electrophoresis due to their concentration under the detection limit (0.2 ~ 0.5 μg) (Hames, 1990). Therefore, proteins from collected peak fractions were stained with PlusOne silver staining kit (Amersham Bioscience, GE Healthcare, Uppsala, Sweden) by the method of Heukeshoven and Dernik (1985). Gels were fixed with 40 % (v/v) ethanol in 10 % (v/v) acetic acid for 30 min followed by sensitizing for 30 min in 6.8 % (w/v) sodium acetate in 30 % (v/v) ethanol containing 0.2 % (w/v) sodium sulfate and 0.125 % (w/v) glutaraldehyde. Gels were washed with deionized water for 5 min three times, and then stained in 0.25 % (w/v) silver nitrate solution containing 14.8 mg% (w/v) formaldehyde for 20 min. After washing with deionized water for 1 min twice, gels were developed in 2.5% (w/v) sodium carbonate solution containing 29.6 mg% (w/v) formaldehyde for ~ 2 min. Staining was stopped by transferring gels to 1.46 % (w/v) EDTA-$Na_2 \cdot 2H_2O$ solution and leaving them for 10 min. Gels were rinsed with deionized water for 5 min three times. Finally gels were preserved by treating in 4 % (v/v) glycerol in 30 % (v/v) ethanol for 30 min twice. The staining process was performed at room temperature under constant gentle shaking in a shaking water bath (Precision, Thermo Electron Corporation, Waltham, MA).

Image Processing and Data Analysis. Images of protein bands were preserved by taking a photograph using a digital camera (DSC-W5, Sony, San Diego, CA), and then drying gels at 80^{o}C for 30 ~ 40 min with a gel dryer (Model 583, Bio-Rad, Hercules, CA). Molecular weights

of proteins were determined by comparing migration distance of bands to that of molecular weigh standards. Protein bands were further identified based on molecular weight and the band pattern reported previously (Beeley et al., 1991; Beeley, 1993; Schwartz et al., 1995; Francis et al., 2000; Banderas-Tarabay et al., 2002; Carlen et al., 2004).

RESULTS

General Characteristics of Collected Saliva: pH, Total Protein Concentration, and Flow Rate

pH, flow rate, and protein concentration of saliva collected from five subjects are summarized in Table 14. Mean salivary pH was 7.08 ± 0.10. pH value reported from several studies varied depending on the collecting method, timing, and population. This result is higher than the range of pH 6.5 ~ 6.9 reported for unstimulated whole saliva (Tenovuo, 1989), but similar to the mean pH 7.01 ± 0.26 reported by Rehak et al. (2000).

Table 14. Mean values (± standard deviation) of pH, flow rate, and total protein concentration obtained from collected human saliva. Analysis was performed in quintuplicate (n = 5).

Subject	Mean ± S.D.		
	pH	Flow rate (mL/min)	Protein (mg/mL)
1	7.04 ± 0.12	1.24 ± 0.36	1.03 ± 0.12
2	6.94 ± 0.19	1.29 ± 0.32	1.38 ± 0.16
3	7.20 ± 0.15	2.15 ± 0.23	1.20 ± 0.20
4	7.14 ± 0.14	1.13 ± 0.26	0.97 ± 0.39
5	7.10 ± 0.05	1.07 ± 0.10	1.80 ± 0.57
Grand Mean	***7.08 ± 0.10***	***1.38 ± 0.44***	***1.31 ± 0.37***

Mean flow rate was 1.38 ± 0.44 mL/min. The range of flow rate reported was 0.25 ~ 0.5 mL/min for unstimulated whole saliva (Tenovuo, 1989; Rehak et al., 2000; Banderas-Tarabay et al., 2002) while flow rate of stimulated saliva was 1 ~ 3 mL/min. Our value is close to the flow rate of stimulated saliva. It seems that rinsing before saliva increased flow rate by diluting saliva or acting as a stimulant, even though diluted saliva portion was supposed to be removed by spitting before collecting.

Mean of total protein concentration in saliva was 1.31 ± 0.37 mg/mL. Total protein values of saliva have been determined using various assays such as Biuret, Lowry, spectrophotometric determination at 280 nm, dye-binding methods, and Kjeldahl (Tenovuo, 1989; Copeland, 1994). Each method has its own advantages and drawbacks. Jenzano et al. (1986) suggested Biuret assay gave the most consistent and accurate value after comparing five methods to determine protein content in saliva. The result in this study is almost half of 2.37 ± 0.51 mg/mL, total protein content obtained using Biuret assay in Jenzano et al. (1986)'s study. Considering the relatively high salivary flow rate, this result may imply that dilution effect of rinsing the mouth cavity with ultrapure water was not totally removed by spitting three times before actual collection of saliva.

Assessment of Binding of Copper to Salivary Proteins with Ultrafiltration

Validation of Ultrafiltration. Binding of copper to ligands is usually investigated by separating bound copper from free (unbound) copper using dialysis or ultrafiltration. In ultrafiltration, copper that is bound with large molecular size of ligands is retained on the membrane of certain molecular weight cut-off size while unbound copper, which is small enough to pass through the membrane pore, is filtered out (Kavonian and Chernokalskaya, 2006)

Copper has limited solubility in solution at certain pH ranges, and forms insoluble particulates (Dietrich et al., 2005). Previous TI test showed that only half of added copper was soluble at pH 7.5 (Hong et al., 2006), which is close to normal salivary pH range (Tenovuo, 1989). Common protocol to measure the concentration of soluble copper in water is to remove

particulated copper with a 0.2 μm pore size filter from solution and then determine soluble copper with AAS or ICP. This response of copper to pH condition means that soluble copper in food or beverage systems can be transformed into particulated form at the pH condition of the human mouth, and the particulated copper can be retained on the ultrafiltration membrane which has smaller pore size than 0.2 μm pore size filter.

Even though previous studies (Mueller, 1983; Mueller, 1985; Mueller, 1987; Argarwal and Henkin, 1987; Brewer and Lajoie, 2000) have observed binding of copper to salivary proteins, it may not be true in the *in vivo* human mouth condition. Most studies were performed under equilibrium conditions that allow long duration conditions to develop binding between salivary proteins and copper to maximum degree. However, binding may not be able to occur when copper is consumed under physiological condition, because copper resides in the mouth for only few second. If copper only changes into particulates without binding with salivary proteins in the mouth, copper retained on ultrafiltration membrane will be particulate, not protein bound copper. A binding study using ultrafiltration protocol will lead to false conclusion in such a case. Therefore, a study was conducted to determine whether the copper retained by ultrafiltration membrane is protein-bound copper or particulate copper.

A validation experiment was designed based on the assumption that particulate copper retained on the membrane will be re-dissolved readily when copper-free buffer is freshly added. On the other hand, bound copper may be still retained after adding fresh buffer. Thus if retentate on ultrafiltration membrane is "rinsed" (re-dispersed in freshly added copper-free buffer and filtered again), copper concentration in retentate will be still substantial or decreased greatly depending on binding status of copper.

The result of validation experiment is summarized in Table 15. Most copper was filtered out in water samples at pH 5.5 while salivary pH water samples still hold substantial amount on the retentate. The recovery rates of pH 7.04 water samples were 69 % and 88 % while those of pH 5.5 water samples were close to 100 %, showing that some of added copper to pH 7.04 water samples had been precipitated out in the centrifugation step performed prior to ultrafiltration.

Table 15. Mass of copper in retentate and filtrate before and after rinsing retentate with fresh copper-free buffer. Filtrate and retentate were obtained from ultrafiltration of 2 mL water and saliva containing 10 mg/L Cu and 50 mg/L Cu at pH of ultrapure water (5.5) and average pH of saliva (7.04) (unit: μg).

Sample \ Treatment		Copper 10 mg/L				Copper 50 mg/L			
		pH 7.04[a]		pH 5.5[b]		pH 7.04		pH 5.5	
		Before[c]	After[c]	Before	After	Before	After	Before	After
Water	Retentate	5.86 (0.42)[d]	4.96 (0.36)	2.79 (0.14)	1.58 (0.08)	20.68 (0.23)	16.44 (0.19)	10.91 (0.10)	1.94 (0.02)
	Filtrate	8.06 (0.58)	0.94 (0.07)	17.70 (0.86)	1.16 (0.06)	67.46 (0.77)	4.38 (0.05)	93.65 (0.90)	7.16 (0.07)
	Total[e]	13.92		20.49		88.14		104.56	
	Recovery[f] (%)	69.60		102.45		88.14		104.56	
Saliva[g]	Retentate	5.58 (0.59)	4.48 (0.47)	9.00 (0.61)	6.67 (0.45)	34.01 (0.77)	22.07 (0.50)	24.15 (0.28)	15.17 (0.18)
	Filtrate	3.88 (0.41)	1.84 (0.19)	5.82 (0.39)	2.34 (0.16)	9.91(0.23)	6.58 (0.15)	61.56 (0.72)	10.88 (0.13)
	Total[e]	9.46		14.82		43.92		85.71	
	Recovery[h] (%)	80.63		80.22		87.39		83.91	

[a] Mean pH of saliva collected three different times.
[b] pH of ultrapure water.
[c] Before and after adding rinsing retentate with fresh copper-free buffer.
[d] Ratio of copper mass in a fraction to total copper mass.
[e] Sum of copper mass in filtrate and copper mass in retentate.
[f] Recovery rate (%) = [(mass of copper in a fraction) / (copper mass initially added to a sample)] × 100.
Mass amount of copper is 20 μg in 2 mL sample for 10 mg/L Cu, 100 μg in 2 mL sample for 50 mg/L Cu.
[g] Saliva supernatant.
[h] Recovery rate (%) = [(mass of copper in a fraction) / (copper mass in supernatant of a sample)] × 100.

The centrifugal ultrafiltration device used in this study was designed to have hold-up volume of 0.1 mL. Thus if no copper particulates are in water samples, 1 μg copper for 10 mg/L samples, and 5 μg copper for 50 mg/L samples will be found in retentates after rinisng. Retentates of water samples at pH 5.5 after rinsing held the amount of copper close to or less than these values, which suggests that only bound copper can be separated from filtrated copper using ultrafiltration at acidic pH range. However, the amount of copper in rinsed retentates of water samples at pH 7.04 was 4 ~ 5 folds higher than these values. This implies that ultrafiltration membrane can hold not only bound copper but also copper particulates if an experimental condition allows formation of copper particulates.

When saliva samples were compared to water samples of the same pH, it was found that more copper was retained on the membrane in saliva samples compared to water samples at both pH 7.04 and pH 5.5. This showed that there were another species of copper besides particulated copper, proving that bound copper is retained on the ultrafiltration membrane along with particulated copper.

This result provides evidence that ultrafiltration can be used to separate bound copper. However, it also demonstrates that particulated copper also can be retained at salivary pH. Thus ultrafiltration may not be able to determine bound copper only, because copper particulates will always be formed at the pH of saliva. On the other hand, copper in filtrate is assumed to be entirely free copper because both bound and precipitated copper are separated by ultrafiltration membrane. Another feature to be considered is the nature of binding. If copper-protein binding is reversible, adding fresh copper-free buffer may cause dissociation of copper from copper-protein complex and free copper concentration would be determined as higher than the actual concentration. Nevertheless, ultrafiltration can determine the amount of free copper in saliva which presents useful information in understanding the role of free copper in perception mechanism.

Binding of Copper to Salivary Protein Fraction. Total protein concentration of saliva was determined with Bradford assay (Copeland, 1994). Biuret assay had been used for analyzing total protein concentration of individual saliva samples in the earlier part of the study,

following the suggestion of Jenzano et al. (1986) that Biuret assay provided the most consistent and accurate value. Bradford assay is known to be more sensitive than Biuret assay, but Bradford assay yielded only one third of the protein concentration determined with Biuret assay in the study of Jenzano et al. (1986). It is known that Bradford assay is not compatible with some proteins. Highly acidic pH of Bradford reagent (pH ~ 1.1) precipitates acidic proteins. Also, Coomassie brilliant blue (CBB) G-250 binds poorly with proteins that can not interact with negatively charged dye molecules (Davies, 1988; Copeland, 1994).

However, protein concentration determined with Biuret assay tended to increase as copper concentration increased in the preliminary study. The mechanism of Biuret assay is not totally identified yet, but it has been found that cupric ion (Cu^{2+}) forms complexes with nitrogen in peptide bonds of proteins under alkaline condition. This complex produces blue color that has absorbance at 550 nm (Davies, 1988). It is assumed that copper treatment of saliva has an interfering effect on Biuret assay by influencing formation of the complex of cupric ions and proteins. Most of total protein assay protocols use the formation of the copper ion – protein complex that absorbs a certain level of wavelength. Bradford reagent which is formulated with dye instead of copper ion was used in this study.

Bradford assay was only applied to the measurement of total proteins in supernatants, retentates, and filtrates because insoluble matter in crude saliva and precipitate made spectrophotometric assay impossible. Protein concentration of crude saliva and precipitate may be able to be determined with Kjeldahl method (Copeland, 1994), but the amount of sample was not large enough for Kjeldahl analysis.

Unlike the study of Jenzano et al. (1986), the total protein concentration in pooled saliva supernatant measured with Bradford assay (1.02 ± 0.55 mg/mL) had almost same value as that determined with Biuret assay (1.31 ± 0.37 mg/mL) (Table 16). Protein concentration of copper was not significantly decreased by copper treatment, but 40 mg/L level of copper seemed to reduce the level of total protein in saliva. Low level of protein in saliva treated with 40 mg/L was consistent with large decrease in peak area in our previous HPLC result.

Table 16. Concentrations of protein and copper in different fractions of combined saliva of five subjects.

Saliva fractions	Concentration	Copper treatment					
		0 mg/L	2.5 mg/L	5 mg/L	10 mg/L	20 mg/L	40 mg/L
Crude	Protein (mg/mL)	unavailable[a]					
	Cu (mg/L)	0.06 ± 0.07[b]	2.33 ± 0.13	5.32 ± 0.32	10.55 ± 0.34	22.46 ± 1.06	49.42 ± 1.21
Supernatant	Protein (mg/mL)	1.02 ± 0.55	1.08 ± 0.64	1.04 ± 0.59	1.02 ± 0.58	1.01 ± 0.57	0.93 ± 0.53
	Cu (mg/L)	0.02 ± 0.01	1.97 ± 0.08	3.90 ± 0.19	7.59 ± 0.34	16.42 ± 0.14	27.12 ± 0.06
Precipitate	Protein (mg/mL)	unavailable					
	Cu (mg/L)	0.07 ± 0.06	5.63 ± 0.78	15.41 ± 0.11	29.41 ± 3.84	52.24 ± 2.57	183.40 ± 2.56
Retentate	Protein (mg/mL)	10.50 ± 2.12	11.22 ± 4.10	10.72 ± 4.80	11.32 ± 5.56	10.67 ± 3.61	11.60 ± 2.80
	Cu (mg/L)	0.19 ± 0.19	8.54 ± 0.53	20.13 ± 1.09	43.52 ± 7.84	125.09 ± 0.89	208.03 ± 5.52
Filtrate	Protein (mg/mL)	0.015 ± 0.021	0.025 ± 0.021	0.025 ± 0.035	0.005 ± 0.007	0.025 ± 0.021	0.004 ± 0.005
	Cu (mg/L)	0.05 ± 0.04	1.42 ± 0.06	2.49 ± 0.04	4.46 ± 0.30	7.48 ± 0.99	9.13 ± 0.07

[a] Spectrophotometric total protein assay is not compatible with measurement of copper concentration in this fraction.
[b] Mean ± Standard Deviation.

Reported concentration of copper naturally existing in unstimulated whole saliva widely varied from 0.0063 mg/L to 3611 mg/L (Table 17). The levels of electrolytes in human saliva show wide variation. Thousands of factors can be attributable to variations in salivary electrolyte concentrations: salivary flow rate, diet, long-term/short-term period, sex and age of subjects, physiological condition, and so on (Tenovuo et al., 1989). Copper in pooled crude saliva containing no copper was 0.06 ± 0.07 mg/L in our study (Table 16), located within the reported range of copper concentrations. The measured concentration had large variance. Because saliva samples from five subjects were pooled, the variation must come from the daily variation in saliva composition due to different day of collecting (variation within subjects), not from variation among subjects.

Table 17. Reprinted concentration ranges and means of copper naturally existing in human whole saliva.

Sources	Reported copper concentration (mg/L)[a]			
	n[b]	Mean (S.D.)	Median	Range
Tenovuo (1989)	– [c]	0.025		0.013 ~ 0.050
Vaughan et al. (1991)	20	0.02		
Chicharro et al. (1999)	40	0.18 (0.28)		
Menegario et al. (2001)	5		0.023[d]	0.006 (0.0007) ~ 0.054 (0.007)
Grahammer et al. (2004)	38		68.5	18.0 ~ 3611.0
Watanabe et al. (2005)	35	0.034 (0.013)		

[a] Other units were converted to mg/L.
[b] Number of human subjects who provided saliva.
[c] Data were pooled from literature sources.
[d] Processed from reported data.

Protein concentration in retentate was approximately ten-fold higher than that in supernatant, while filtrate had very low level of protein. Percent recovery (Table 18) showed that ultrafiltration successfully separated proteins from saliva. Percent recovery greater than 100 % found in saliva fractions treated with copper may be related with particulated copper or copper-

protein complex that was too large to pass through the pores of the membrane. These molecules may build up on the membrane, retaining protein molecules smaller than the membrane pore size (Kavonian and Chernokalskaya, 2006). This may be the reason that protein concentration in retentate was not significantly changed by copper treatment. Percent recovery of saliva treated with 20 mg/L was greater than saliva treated with 40 mg/L, even though the level of particulate copper is greater in the sample of Cu 40 mg/L. It may be because protein concentration in Cu 40 mg/L sample was smaller than that in Cu 20 mg/L sample (Table 16). There is strong positive correlation (average R^2 = 0.993) observed between protein concentration and copper concentration for each copper treatment. Higher copper concentration was found in the retentate, where proteins are concentrated. This can be another strong evidence to support that copper incorporated in saliva mostly interacts with salivary proteins, even though part of copper concentration in retentate possibly resulted from particulated copper.

Table 18. Percent recovery of protein in filtrate and retentate obtained from ultrafiltration of pooled saliva supernatant containing 0, 2.5, 5, 10, 20, and 40 mg/L Cu.

Recovery (%)	Copper treatment (mg/L)					
	0	2.5	5	10	20	40
Filtrate	2.10	2.42	2.39	1.05	4.04	0.21
Retentate	87.11	98.17	104.48	127.02	163.76	124.12
Total	89.21	100.59	106.87	128.07	167.80	124.33

High copper concentration in precipitates (Table 16) was assumed to be caused by three different copper sources: 1) precipitated copper particles due to pH of saliva; 2) precipitated copper-protein complex; 3) physically trapped copper in viscous mucin clot and cell debris. However, it is not clear how much each of these copper sources is in total copper of precipitates. As previously mentioned, copper in filtrate is regarded to consist of soluble copper only, because copper particulates were separated by ultrafiltration membrane.

Since the volume of each fraction was not all the same, the mass of copper distributed in each fraction did not exhibit same tendency as that of copper concentration. Distribution of the mass

of added copper in salivary fractions is illustrated in Figure 17. Absolute amount of copper mass in each fraction increased as saliva was treated with higher level of copper, but the amount of copper distributed in fractions changed according to the level of copper treatments. Most of copper (> 50%) was located in filtrates under 10 mg/L of copper treatment, whereas saliva treated with over 20 mg/L copper had most of copper in retentates. In the saliva treated with more than 20 mg/L copper, about 70 % of copper was found in retentate and precipitate, suggesting most of copper bound with proteins or existed in insoluble form. Because precipitation of salivary proteins is known as the major cause of astringency, large amount of copper found in retentate or precipitate may be the major cause of strong astringency when the high concentration of copper was consumed.

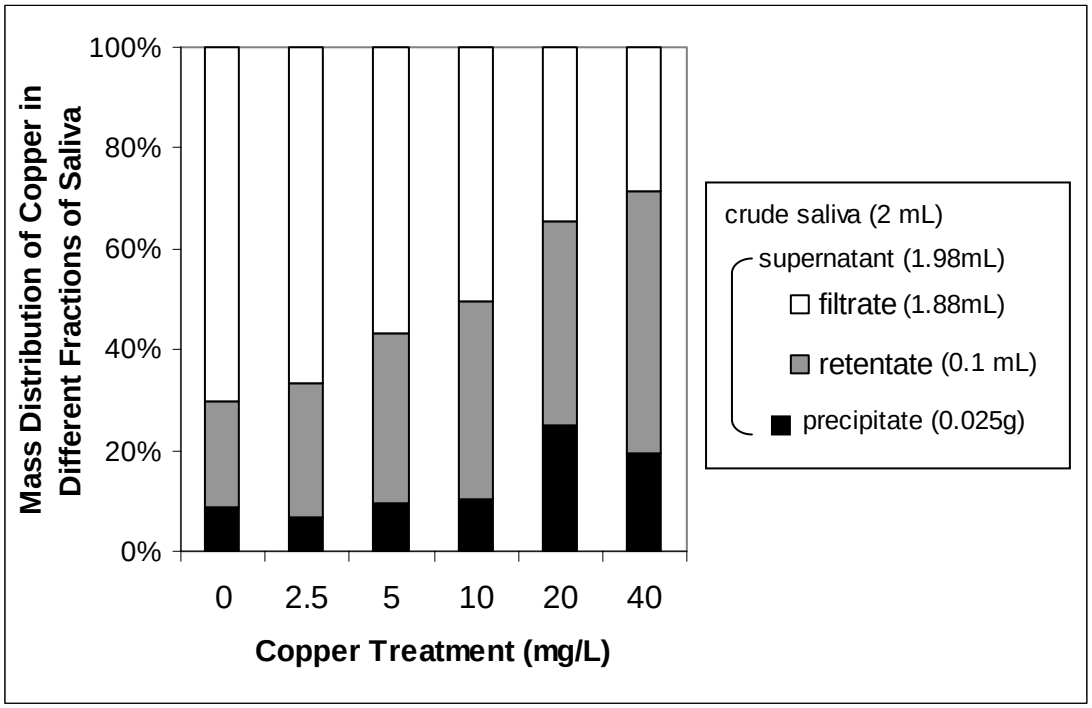

Figure 17. Distribution of copper mass in each fraction of saliva treated with different level of copper.

Assessment of *In Vivo* Copper Binding to Salivary Protein in Real Drinking Situation

The concentrations of copper and protein in fractions of saliva collected before and after drinking copper solutions were determined to provide information about the interaction between copper and proteins in real drinking situation (Table 19). There were no significant differences between protein concentrations collected at different timing. However, saliva collected before drinking solutions showed higher protein concentration than saliva collected after drinking. It may be because drinking solutions diluted saliva. Two minutes after drinking, the level of proteins in saliva returned to the level before drinking solutions. There was no significant effect of copper on protein concentration, but concentration of protein in saliva collected after drinking copper solutions were slightly lower than that in saliva after drinking ultrapure water.

Copper concentration of saliva was raised by drinking water containing copper. Unlike the result of *in vitro* copper treatment, concentration of copper was not high in fractions where most of proteins were found. The distribution of copper mass in fractions showed that most of copper is distributed in filtrate, as unbound soluble form (Figure 18). Because copper in protein fractions are thought to be related with astringency, this result shows that astringency may be not be the main sensory attribute perceived when drinking copper solution of 2.5 mg/L or 5 mg/L concentration.

Table 19. Concentrations of protein and copper in different fractions of combined saliva of five subjects before and after drinking 0, 2.5 mg/L, and 5 mg/L copper solution.

Saliva fractions	Concentration	Copper treatment						
		Before	after			2m after		
			0 mg/L	2.5 mg/L	5 mg/L	0 mg/L	2.5 mg/L	5 mg/L
Crude	Protein (mg/mL)				unavailable[a]			
	Cu (mg/L)	0.036 ± 0.004[b]	0.051 ± 0.011	0.815 ± 0.025	1.299 ± 0.120	0.027 ± 0.005	0.504 ± 0.039	0.956 ± 0.185
Supernatant	Protein (mg/mL)	1.53 ± 0.35	1.19 ± 0.35	1.30 ± 0.32	1.01 ± 0.34	1.55 ± 0.40	1.34 ± 0.58	1.34 ± 0.08
	Cu (mg/L)	0.013 ± 0.008	0.018 ± 0.002	0.718 ± 0.072	1.141 ± 0.106	0.012 ± 0.004	0.413 ± 0.046	0.823 ± 0.145
Precipitate	Protein (mg/mL)				unavailable			
	Cu (mg/L)	0.012 ± 0.002	0.008 ± 0.0005	0.072 ± 0.002	0.113 ± 0.009	0.009 ± 0.002	0.053 ± 0.007	0.084 ± 0.012
Retentate	Protein (mg/mL)	21.00 ± 6.23	20.24 ± 3.64	17.02 ± 1.52	20.36 ± 3.30	25.47 ± 1.50	17.80 ± 0.88	16.31± 1.53
	Cu (mg/L)	0.027 ± 0.003	0.040 ± 0.04	0.330 ± 0.011	0.618 ± 0.115	0.032 ± 0.016	0.175 ± 0.062	0.463 ± 0.184
Filtrate	Protein (mg/mL)				N.D.[c]			
	Cu (mg/L)	0.004 ± 0.002	0.013 ± 0.006	0.545 ± 0.049	0.934 ± 0.140	0.021 ± 0.015	0.330 ± 0.073	0.619 ± 0.109

[a] spectrophotometric total protein assay is not compatible with measurement of copper concentration in this fraction.
[b] Mean ± Standard Deviation.
[c] Not detected.

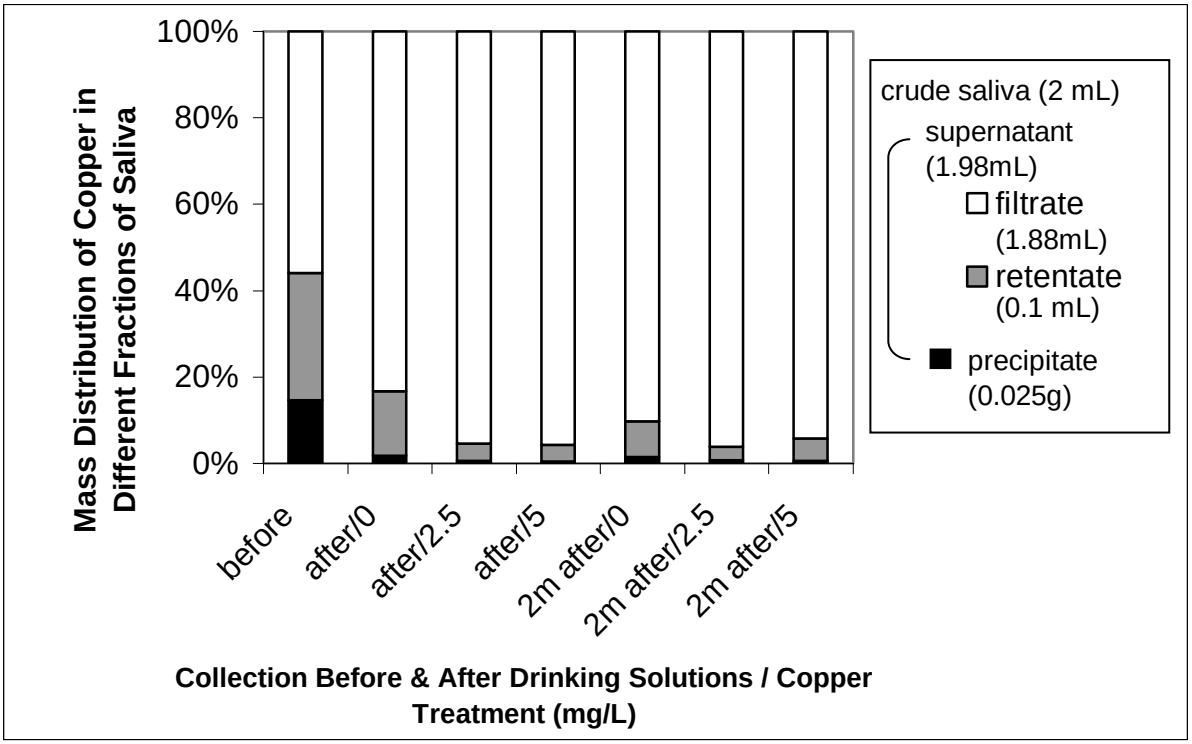

Figure 18. Distribution of copper mass in each fraction of saliva collected before and after drinking 20 mL of ultrapure water, Cu 2.5 mg/L solution or Cu 5 mg/L solution.

Investigation of Effect of Copper on Salivary Proteins with High Performance Liquid Chromatography (HPLC)

General features of the chromatograms are similar to the chromatograms reported by Kallithraka et al. (1998) which could be divided into three parts according to peak eluting pattern. In our chromatogram, the first part was from a retention time (RT) 0 to 9.5 min where three major peaks (peak No. 1, 2, and 4) were observed from all subjects; the second part included minor peaks that varied in their pattern and size between subjects at RT 10 ~ 18 min; the third part spanned from RT 19 min to 25 min and contained three or four major peaks (peak No. 11 ~ 14) (Figure 19 and Figure 20). In the result of Kallithraka et al. (1998), two peaks at RT 2 ~ 2.5 min were the largest among all peaks, but area of these peaks were smaller than another major peak detected at RT 6.8 min (peak No. 5) in our study. This peak with RT 6.8 had the largest peak area among all peaks detected.

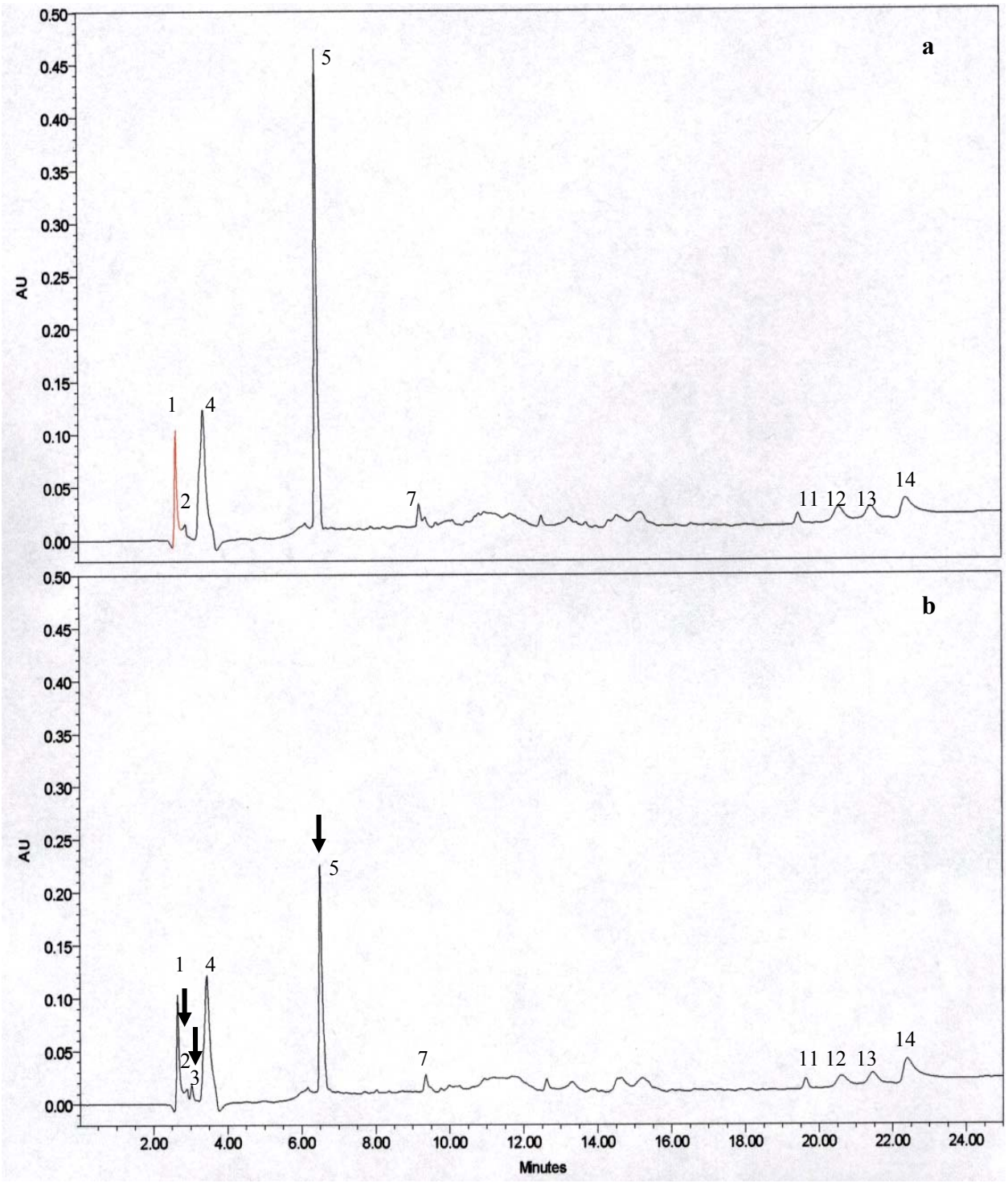

Figure 19. Representative HPLC chromatogram of combined human saliva detected at the wavelength of 230 nm (a – combined human saliva containing no copper; b – combined human saliva containing 40 mg/L of copper). An arrow mark indicates significant change in peak area caused by addition of copper. Peak number 6 and 8 ~ 10 are shown in Figure 20. Peaks are identified with the numbers in Table 20.

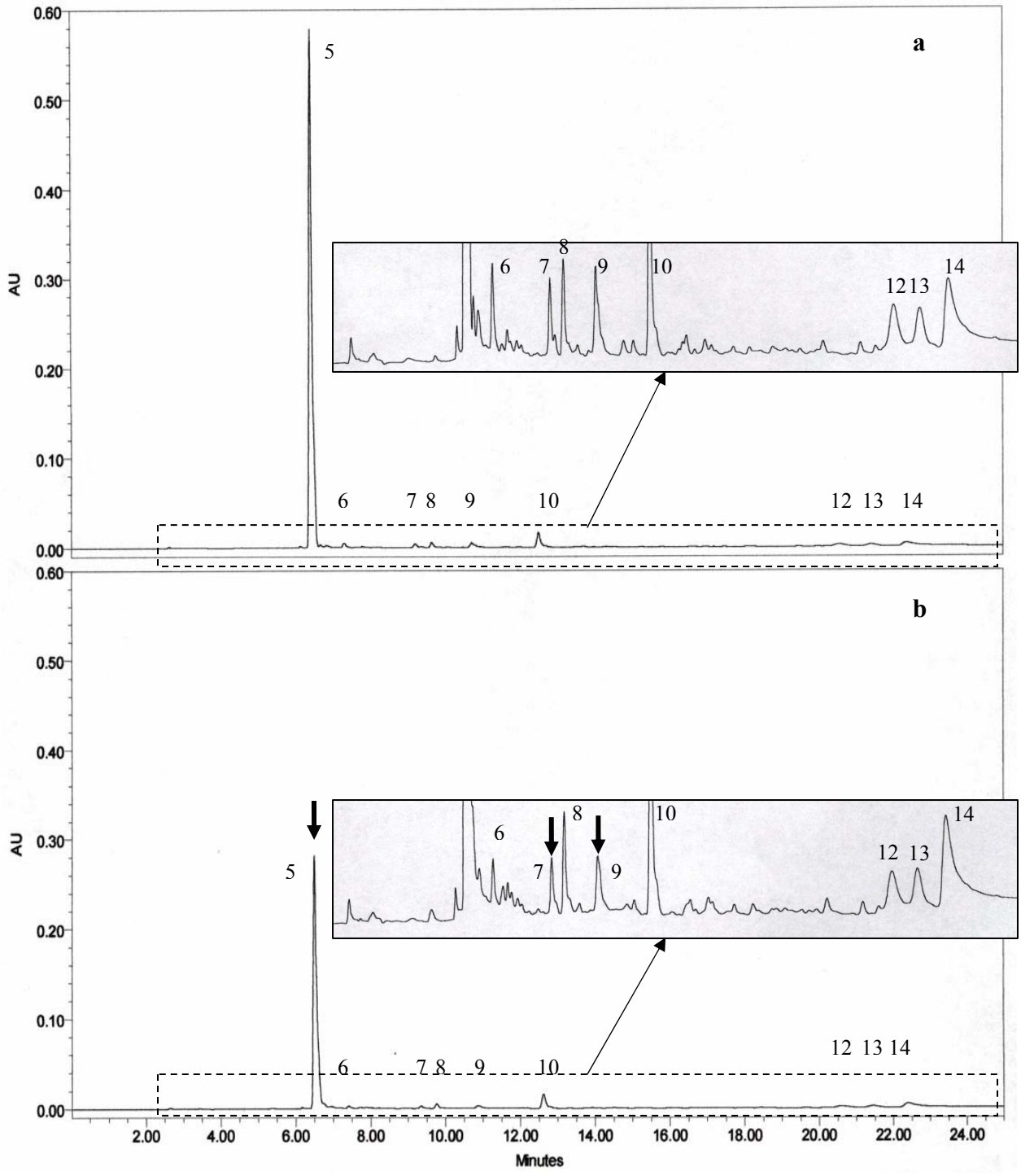

Figure 20. Representative HPLC chromatogram of combined human saliva detected at the wavelength of 280 nm (a – combined human saliva containing no copper; b – combined human saliva containing 40 mg/L of copper). An arrow mark indicates significant change in peak area caused by addition of copper. Peak number 1 ~ 4 are shown in Figure 19. Peaks are identified with the numbers in Table 20.

It was assumed that the peak of RT 6.8 min might be α-amylase based on the largest peak area and the ratio of A_{230}/A_{280} less than 1, because α-amylase usually shows the thickest blue band in SDS-PAGE (Beeley et al., 1991; Schwartz et al., 1995; Banderas-Tarabay et al., 2002; Carlen et al., 2004) suggesting high concentration in whole saliva. The detail discords between two studies were not surprising because it was expected that many factors, such as genetic variations in saliva among human subjects, HPLC instrumentation, and sample preparation methods, would cause the difference in the results.

Even though examination of a chromatogram of each individual revealed that there was large variation in patterns and area of salivary protein peaks among subjects, there were peaks found at the same retention times which can be recognized as identical proteins. The common peaks and their characteristics are listed in Table 20. The peaks with RT earlier than 5 min (peak No. 1 ~ 4) were only detected at the wavelength of 230 nm, while the peaks at RT 6 to 13 (peak No. 6 ~ 10) were found at 280 nm. The peak with RT 6.5 min (peak No. 5) and the peaks eluted at RT 22 ~ 23 min (peak No. 12 ~ 14) were detected at both wavelength, but had different absorbance.

All peaks that showed significant changes in the preliminary study were influenced significantly by addition of copper in the main experiment. The peak with RT 7.4 (peak No. 6), which did not change in the preliminary study, also showed significant decrease caused by copper. It seemed that increased numbers of observation (n=18 per each treatment) brought more power to statistical analysis by reducing variance in data. The peaks that showed significant difference between control and copper treatments were eluted before 15 min. During this elution time, mobile phase was mainly composed of water (Table 13), facilitating partitioning of hydrophilic compounds in a reverse-phased column. It suggests that salivary proteins affected by copper were hydrophilic, maybe more prone to interact with copper than hydrophobic proteins.

Figure 21 and Figure 22 showed the pattern of decrease in area of the peaks at different copper concentrations. The plot of each peak exhibits three distinctive patterns of change: The peak at RT 2.9 (peak No. 2) had plateau at lower concentrations and decreased linearly; the peaks at RT 6.5 min (peak No. 5) had linear slope; the peaks eluted later than 7 min had L-shape

curves. Different patterns of change in peak area suggested that each peak may have different mechanisms of interaction with copper. The new peak appearing at RT 3.0 min increased linearly (Figure 22), but our preliminary result (Appendix 7) suggested the plateau may exist above 30 mg/L copper concentration.

Table 20. Identification of protein peaks on the HPLC chromatogram of pooled saliva samples containing 0, 2.5, 5, 10, 20, and 40 mg/L copper.

Peak No.	Typical retention time (min)	A_{230}/A_{280}[a]	Identification[b]	Significant changes[c]
1	2.6	>>1	Proline-rich proteins (PRPs)	No[d]
2	2.9	>>1	PRPs	Decrease [e]
3	3.0	>>1	New peak for saliva + Cu	Increase[f]
4	3.5	>>1	PRP	No
5	6.5	<1	α-amylase	Decrease
6	7.4	<<1	Non-PRP	Decrease
7	9.5	>1	PRPs	Decrease
8	9.7	<<1	Non-PRPs	No
9	10.9	<<1	Non-PRPs	Decrease
10	12.5	<<1	Non-PRPs	No
11	19.5	>1	PRPs with hydrophobic regions	No
12	20.5	>1	PRPs with hydrophobic regions	No
13	21.5	>1	PRPs with hydrophobic regions	No
14	22.5	>1	PRPs with hydrophobic regions	No

[a] The ratio of peak area detected at 230 nm to peak area detected at 280 nm. The ratio greater than 1 implies that the peak may be one of PRPs while the ratio less than 1 means that the peak may be not PRPs (Kallithraka et al., 1998).

[b] Peaks were identified following the postulation of Kallithraka et al. (1998) based on retention time, peak size, and A_{230}/A_{280}.

[c] Significant decrease or increase in protein peaks by addition of different amount of copper in pooled human saliva ($\alpha = 0.05$).

[d] The peak was significantly decreased by copper treatment.

[e] The peak was significantly increased by copper treatment.

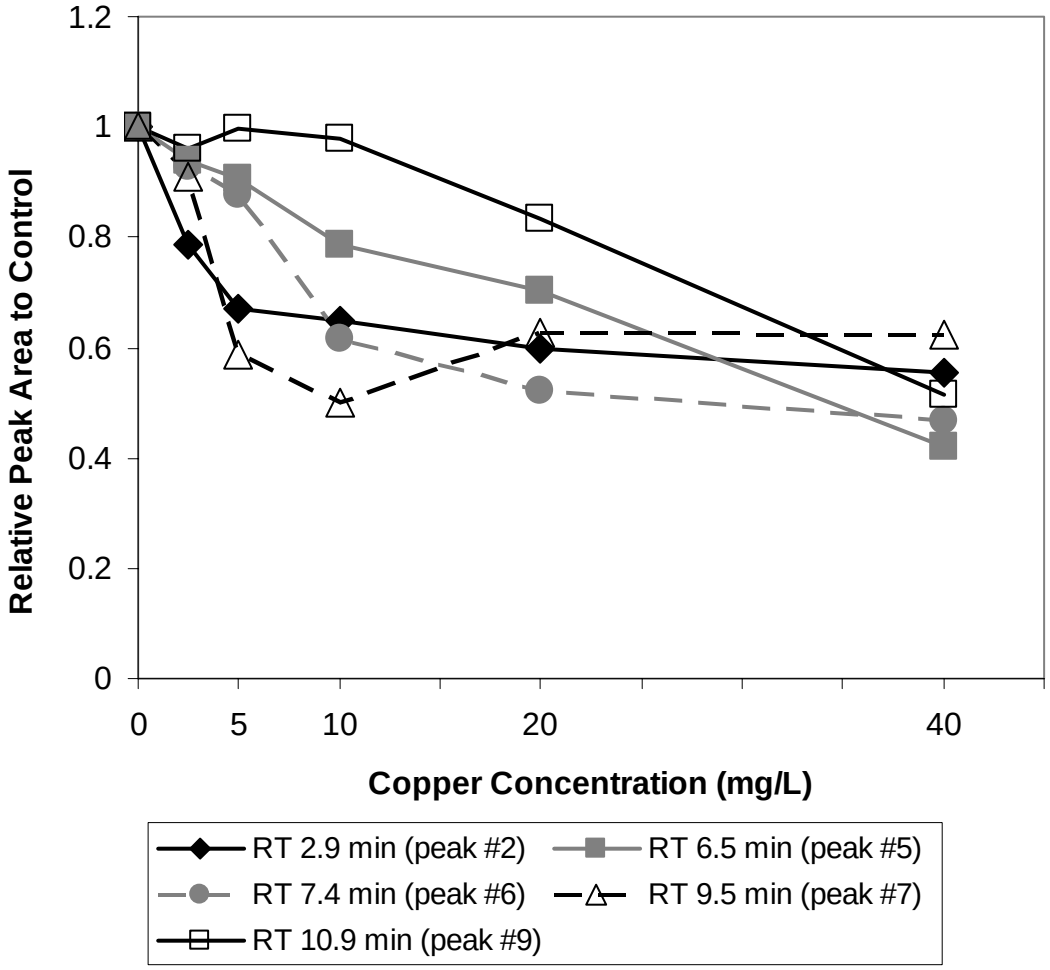

Figure 21. Pattern of decrease in salivary protein peaks from pooled human saliva treated with 0, 2.5, 5, 10, 20, and 40 mg/L *in vitro* separated by reverse-phase HPLC.

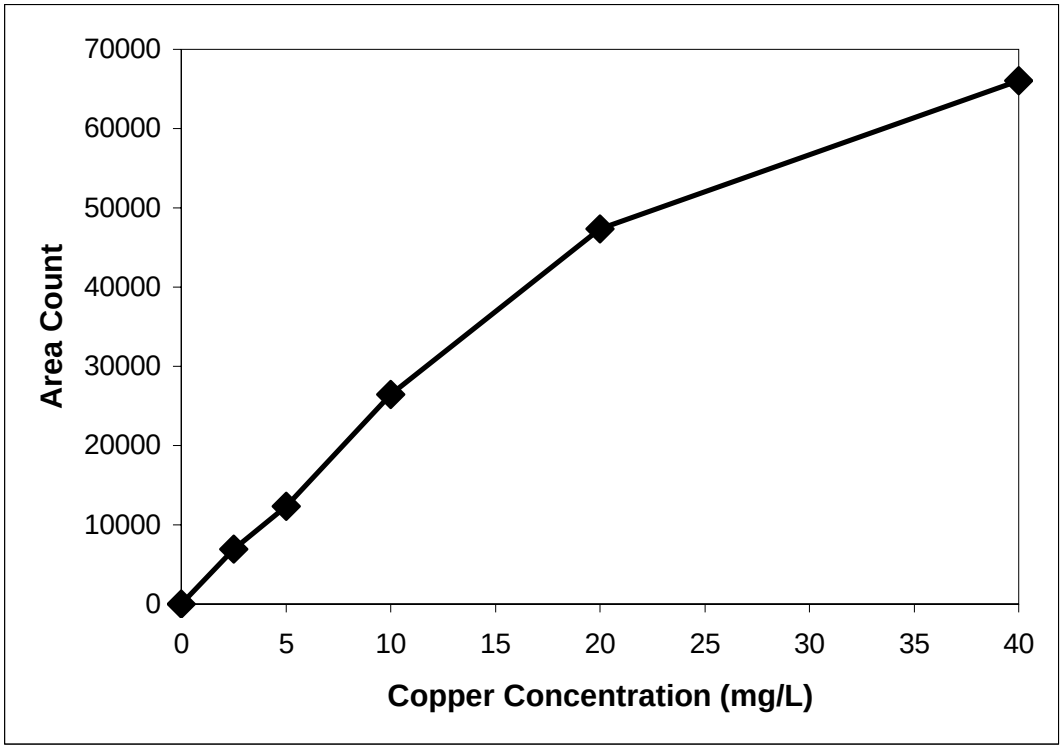

Figure 22. Pattern of increase in the new salivary protein peak at RT 3.0 min (peak No. 3) from pooled human saliva treated with 0, 2.5, 5, 10, 20, and 40 mg/L *in vitro* separated by reverse-phase HPLC.

When 10 ~ 40 mg/L of copper was added to saliva, area of all peaks were decreased by 40 ~ 60 % compared to control (Figure 21). Low levels of copper concentration, 2.5 mg/L and 5 mg/L did not cause changes in peaks significantly, except the appearance of the new peak at 3.0 min. This unique protein peak may be evidence that saliva proteins interact with very low level of copper, even though other peaks did not exhibit noticeable changes. This peak can be identified as a PRP based on the rationale of Kaillithraka et al. (1998) since it was detected only at 230 nm. Moreover, the peak at RT 2.9 min that has A_{230}/A_{280} greater than 1 decreased as the new peak increased. Thus the new peak may be a product of interaction between copper and one of PRPs. However, the pattern of increase in the new peak size was not same as that of the peak with RT 2.9 min, which suggests that other explanations may be possible. First, it is possible that the new peak results from interference of copper with UV absorbance of salivary proteins, rather than formation of complexes between salivary proteins and copper. Copper has

maximum absorbance at 650 ~ 700 nm within visible regions of spectrum, but also absorbs ultraviolet range. Copper chloride ($CuCl_2$) in tetrahydrofuran was reported to have maximum absorbance at 305 nm with molar extinction coefficient (ε) of 3480, and also have absorbance at 220 ~ 250 nm with ε = 830 ~ 1100 (Amuli et al., 1983). Second, a very crude approximation of molar concentration of salivary proteins is ≥ ~ 500 μM, which was calculated based on the information in Table 2 (Chapter II). The estimated molar ratio of copper to salivary proteins, 0.08:1 ~ 1.25:1 (Cu 2.5 ~ 40 mg/L), may imply that copper concentration present in samples is not enough to allow complexation of copper to a salivary protein. This also can lead to the argument that increased absorbance at RT 3.0 is relevant to absorbance of copper rather than appearance of new protein-copper complexes. Nevertheless, hydrophilic nature of copper would make copper eluted earlier than any other proteins in saliva, without partitioning with hydrophobic C-18 stationary phase. Thus retention time of the new peak may support the hyphothesis of soluble copper-protein complex formation. The peaks that showed large changes were collected and analyzed by gel electrophoresis for further identification.

Characterization of Salivary Proteins Influenced by Copper using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed with fractionated saliva from HPLC as well as whole mixed saliva containing different levels of copper to examine effect of copper on salivary proteins. Typical electrophoretic pattern of whole saliva on a 4 ~ 20 % gradient gel is shown in Figure 23. Protein bands were characterized by comparing estimated molecular weight of each protein band and gel eluting pattern with those reported in literature (Table 21). PRPs can be identified easily because PRPs are stained into violet-pinkish color with Coomassie brilliant blue R-250 when destained with 10% acetic acid due to the phenomenon called as “metachromasia”, a shift of spectrum resulting from dye interaction with proline residues (Beeley et al., 1991). Reported molecular weight (MW) values for any salivary protein vary in the literature when gel electrophoresis was performed on different duct saliva under different conditions. Thus band characterization based on literature review may not be accurate and require additional identification methods such as immunoblotting. Nevertheless, identifying the dark blue band

with 60 kDa molecular weight with α-amylase is valid since eluting pattern and molecular weight of α-amylase were consistent with literature values (Tenovuo, 1989; Beeley et al., 1991; Beeley, 1993; Schwartz et al., 1995; Lu and Benick, 1998; Sarni-Manchado, 1999; Beeley and Khoo, 1999; Francis et al., 2000; Bacon and Rhodes, 2000; Milleding et al., 2001; Banderas-Tarabay et al., 2002).

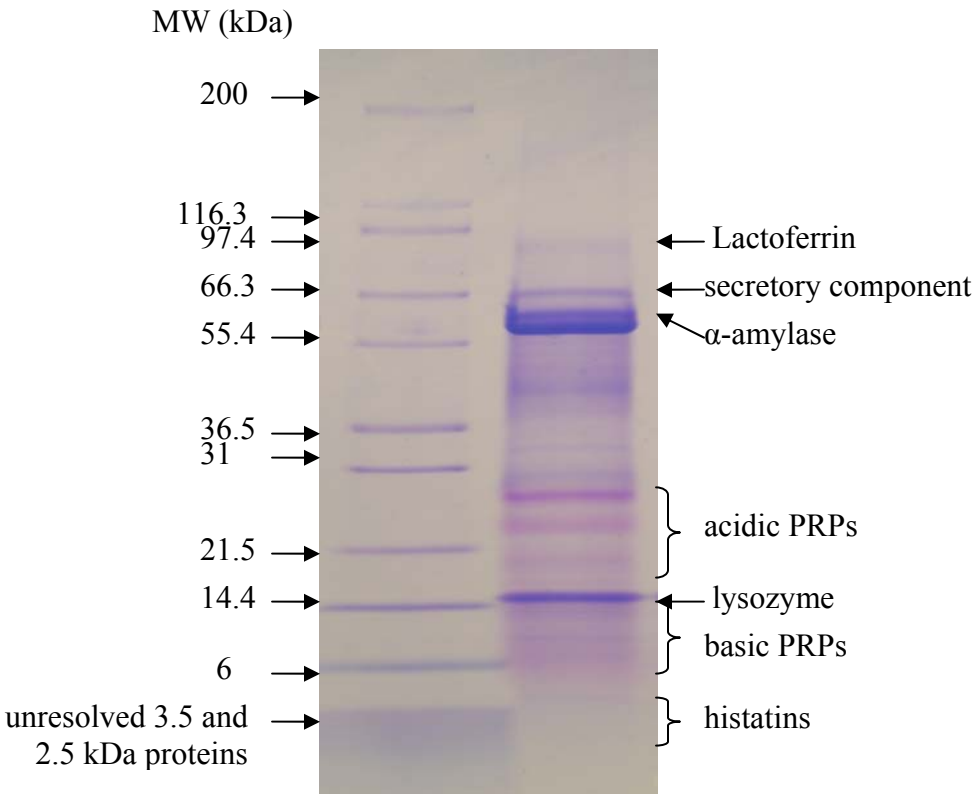

Figure 23. SDS-PAGE of human whole saliva pooled from five subjects containing no copper (protein load = 15 μg). Molecular weight standard was given on the left side of the gel. Protein bands were characterized based on the molecular weight and the previous SDS-PAGE studies (Table 21).

A protein band of 90 kDa MW was identified as lactoferrin (Table 21), based on the result of Beeley et al. (1991). A blue band of 14.3 kDa was determined as lysozyme because MW was identical to the value reported in literature (Beeley et al., 1991; Schwartz et al., 1995; Francis et al., 2000; Banderas-Tarabay et al., 2002). Several violet bands of MW 19 ~ 25 kDa were characterized as acidic PRPs. MW range of acidic PRPs cited in literature has wide variation depending on the method used.

Table 21. Estimated molecular weight ranges of salivary proteins[a]

Proteins	Molecular weight (MW: kDa)			
	Estimated MW in our study	Lowest MW reported	Most frequently reported MW	Highest MW reported
Mucin				
MG1			>1000	
MG2	168		200 ~ 250	
Lactoferrin	90	75	76.5	90
Secretory components	67		67	
Amylase	64	55	59 ~ 60	63
Carbonic anhydrase				
Carbonic anhydrase		38	42, 71	71
Gustin (carbonic anhydrase IV)			37	
Glycosylated PRPs		35	38.9	50 ~ 66
Acidic PRPs	19.2 ~ 25.1	9	24 ~ 30	40
Basic PRPs				
IB 1 ~ IB 6	9.9 ~ 12.5	6 ~ 12	14 ~ 22	
Ps 1, 2			37 ~ 43	
Lysozyme	14.3		14.7	
Cystatin		11	14.4	< 20
Histatin	2.5 ~ 3.5		3.1 ~ 4.8	< 14.4

[a] Values are pooled from literatures (Tenovuo, 1989; Beeley et al., 1991; Beeley, 1993; Schwartz et al., 1995; Lu and Benick, 1998; Sarni-Manchado, 1999; Beeley and Khoo, 1999; Francis et al., 2000; Bacon and Rhodes, 2000; Milleding et al., 2001; Banderas-Tarabay et al., 2002).

Another violet band set of MW 9 ~ 14.3 kDa was characterized as basic PRPs according to reported molecular weight. Two minor blue bands are shown with basic PRPs, suggesting the existence of non-PRPs. Protein bands between α–amylase and acidic PRPs were identified as isoforms of basic PRPs (37 ~ 43 kDa) or glycosylated PRPs (35 kDa) in other studies (Beeley et al., 1991; Schwartz et al., 1995; Becerra et al., 2003), but protein bands in our study did not develop violet-pink color. Therefore they are likely to be non-PRP proteins of 40 ~ 50 kDa, such as gustin. Another possible characterization of the blue bands in PRP zone is related with the staining method. CBB R-350 used in this study has more sensitivity to proteins by 40 % than CBB R-250 that used in other studies (Beeley et al., 1991; Schwartz et al., 1995; Lu and Benick, 1998; Sarni-Manchado et al., 1999; Beeley and Khoo, 1999; Francis et al., 2000; Bacon and Rhodes, 2000; Milleding et al., 2001; Banderas-Tarabay et al., 2002) because it contains more active dye form than CBB R-250 (discussion with technical support of Amersham Biosceince). The different sensitivity may develop different hue in same band from that caused by CBB R-250.

A faint blue unresolved region observed alongside of 2.5 ~ 3.5 kDa molecular weight standard, was assumed to be histatins based on comparison to results of Beeley et al. (1991), which observed binding capacity of this band with ^{65}Zn. It is not surprising that histatins were not detected well because: 1) gel pore size is not small enough to resolve this peptide well; 2) level of acidic PRPs and histatins are considerably reduced in whole saliva compared to parotid saliva (Beeley, 1993).

SDS-PAGE of saliva supernatant samples at different copper levels was performed to characterize HPLC peaks that showed differences. Electrophoretic pattern of salivary proteins changed as copper concentration increased (Figure 24). Two protein bands with estimated molecular weight of 33 kDa and 29 kDa showed changes in higher copper concentrations. These protein bands became blurred upon addition of 10 mg/L copper. The protein band of MW 33 kDa totally disappeared in 40 mg/L copper. Saliva containing 2.5 mg/L copper and 5 mg/L copper did not exhibit differences in electrophoretic pattern from that of control (data not shown). There are not enough data to identify these two bands, but it is assumed that they are non-PRPs as they were stained blue or acidic PRPs that happened to be dyed blue with more sensitive CBB

R-350. It is also possible that these protein bands are composed of several proteins of same molecular weight due to separation mechanism of SDS-PAGE. More advanced techniques, such as 2D gel electrophoresis and LC-MS is expected to give more accurate characterization of these bands.

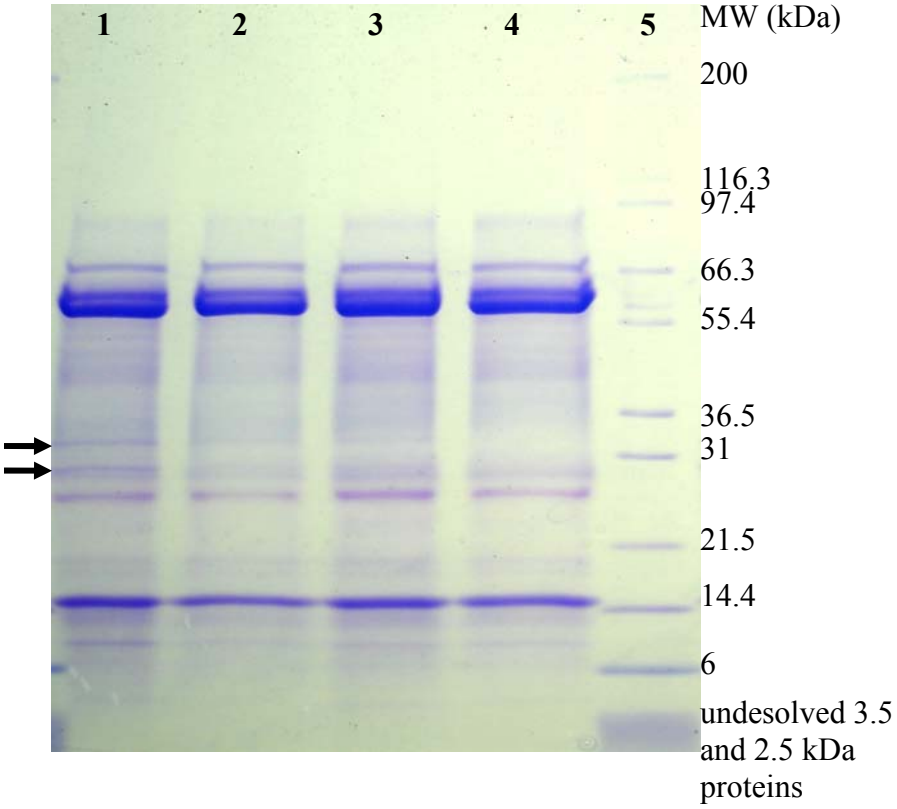

Figure 24. SDS-PAGE of combined human whole saliva treated with 0, 10, 20, and 40 mg/L copper (protein load = 15 μg per a lane). Bold arrow on the left designates the bands changed by addition of copper (lane 1 – control; lane 2 – 10 mg/L Cu; lane 3 – 20 mg/L Cu; lane 4 – 40 mg/L Cu; Lane 5 – molecular weight standard).

One of the hypotheses regarding the mechanism of astringency caused by copper is delubrication resulting from precipitation of PRPs. This hypothesis is based on the perception mechanism of astringency caused by polyphenolic compounds (Sarni-Manchado et al., 1999;

Kallithraka et al., 1998; Lu and Bennik, 1998; Bacon and Rhodes, 2000; Kallithraka et al., 2001; Charlton et al., 2002). If the disappearing bands are acidic PRPs, electrostatic interactions between Cu^{2+} and negatively charged amino acid residues of acidic PRPs would be major binding mechanism. The same mechanism was observed in the binding of Ca^{2+} and acidic PRPs in dental pellicle formation (Tenovuo, 1989). Precipitation may occur by neutralization of protein charge via binding with Cu^{2+} or polymerization of PRPs by forming Cu^{2+} bridges between PRP molecules as seen in Ca^{2+} cross-linking between mucin molecules (Mueller et al., 1983; Wu et al., 1994). However, if these two bands are not acidic PRPs, it suggests that astringency of metal is perceived by a different mechanism from the perception mechanism of polyphenolic compounds.

Since precipitated proteins are only present in minor amounts, their precipitation may not affect the lubrication while the other proteins are remaining in saliva. However, this small extent of precipitation may be able to influence perception of astringency by bringing about a differential threshold of astringency, at which an increase in astringency can be perceived (Lawless and Heymann, 1998). Also, grittiness caused by tannin-protein complexes on oral tissues is assumed to contribute to loss of lubrication (Kallithraka et al. 2001). Thus it can be deduced that other copper species, especially insoluble copper, can generate the impression of de-lubrication by producing grittiness even though de-lubrication was not significant.

The peaks influenced by copper treatment in HPLC analysis were characterized with SDS-PAGE (Figure 25). Two fractions, the early eluting peaks collected at RT 2 ~ 4 min and the largest peak at RT 6.8 min, were loaded. Because the volume of reconstituted samples was very limited, final protein concentration of fractions was not determined. The loading amount of each fraction was adjusted so that all fractions could express same intensity when stained with silver nitrate. The concentration ratio of each fraction was not same since the amount of protein eluted and total elution time were different between fractions. Also evaporation process caused variation in concentration ratio between fractions. Even though solvent in fractions were evaporated to dryness, some tubes still had small amount of liquid that eventually caused dilution in final concentration. Therefore, it is noteworthy that the SDS-PAGE result should not be interpreted quantitatively.

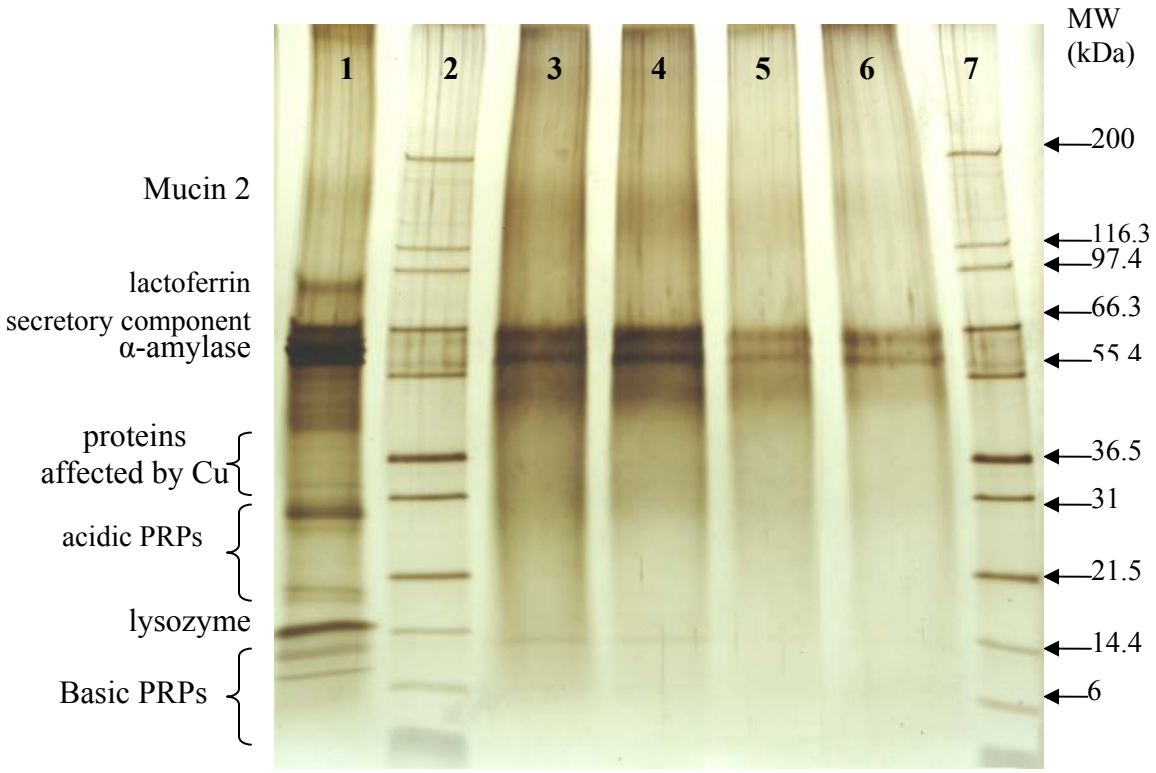

Figure 25. SDS-PAGE of peak fractions collected from HPLC. Proteins were detected using silver staining. Protein load may be different in each lane [lane 1 – unfractionated saliva with no Cu, protein load 0.75 μg; lane 2, 7 – MW standard; lane 3 – fraction of RT 2 ~ 4 min with no Cu (× 1 dilution); lane 4 – fraction of RT 6.8 min with no Cu (× 5 dilution); lane 5 – fraction of RT 2 ~ 4 min with 40 mg/L Cu (× 1 dilution); lane 6 – fraction of RT 6.8 min with 40 mg/L Cu (× 15 dilution)].

Unfractionated saliva in lane 1 showed slight changes in gel electrophoretic pattern compared to that obtained from CBB staining. Acidic PRPs that showed distinctive violet-pink color in the CBB stained gel were not detected well in silver stained gel due to their amino acid composition. Silver staining is more sensitive than CBB staining, but it is reactive less to acidic amino acid residue. Silver staining has more sensitivity to basic residues and sulfur-containing residues, such as cysteine (Hames, 1990). It is postulated that the silver-stained bands found in the region of acidic PRPs are non-PRP proteins that were minor in CBB staining. Bands that had not been shown in the CBB stained gel appeared in the silver-stained gel, especially one wide band of MW 168 kDa. This band is thought to be low-molecular mucin, because it has the

same electrophoretic pattern as the result of immunoblotting with mucin antibody reported by Carlen et al. (2004).

Lane 3 and lane 4 are the fractions with no copper from HPLC, each the peaks eluted in RT 2 ~ 4 min and the peak eluted in RT 6.8 min. It is intriguing that several protein bands were detected in the fraction of RT 6.8 min (lane 4) which had appeared as just one peak in the HPLC chromatogram (Figure 19). Lane 4 contained α-amylase, secretory component, proteins of MW 45 kDa (either basic PRP or gustin), and basic PRPs. Shadowy area was detected in the region where the intense acidic PRPs had appeared in the CBB stained gel and basic PRPs showed up as very faint band under lysozyme.

The fraction of RT 2 ~ 4 min (lane 3) was not distinctively different from the fraction of RT 6.8 min. The comparison between the fraction of control (lane 3) and the fraction with Cu (lane 5) did not show the new band corresponding to the new peak at RT 3. 0. It is attributable to that the minute amount of proteins below the detection limit of silver staining was loaded. Even though characterization of the new peak with one dimensional SDS-PAGE was not successful due to staining method and low concentration, yet it can be assumed that the new peak is an interaction product between copper and the proteins found on the fraction. More specific information on a product of copper-salivary protein interaction will require different approach.

Another interesting fact found in the SDS-PAGE of the HPLC fractions is that the proteins in the fractions did not match to the protein bands that disappeared in the CBB stained gel (Figure 24). It was expected that the fraction of RT 6.8 min would consisted of non-PRP proteins of MW 29 and 33 kDa, because the peak with RT 6.8 min was reduced by the largest amount in the HPLC. Instead, the major compounds of the peak at RT 6.8 min were the proteins of higher molecular weight. The proteins of MW 29 and 33 kDa might be precipitated when copper was added, and centrifugation had removed these precipitated proteins before injection to the HPLC. Thus changes in peak area in the HPLC chromatograms are related with several other proteins such as α-amylase and mucin. However, these proteins are not likely to contribute to astringency because no loss of these proteins was observed in SDS-PAGE of unfractionated saliva.

DISCUSSION

Copper Speciation and Copper-Protein Interaction in Saliva

Saliva is assumed to influence the perception of copper taste by controlling copper speciation as a function of pH or interaction with salivary components. The postulated process of copper speciation in the mouth is illustrated in Figure 26. Copper speciation may be directly related with sensory characteristics perceived.

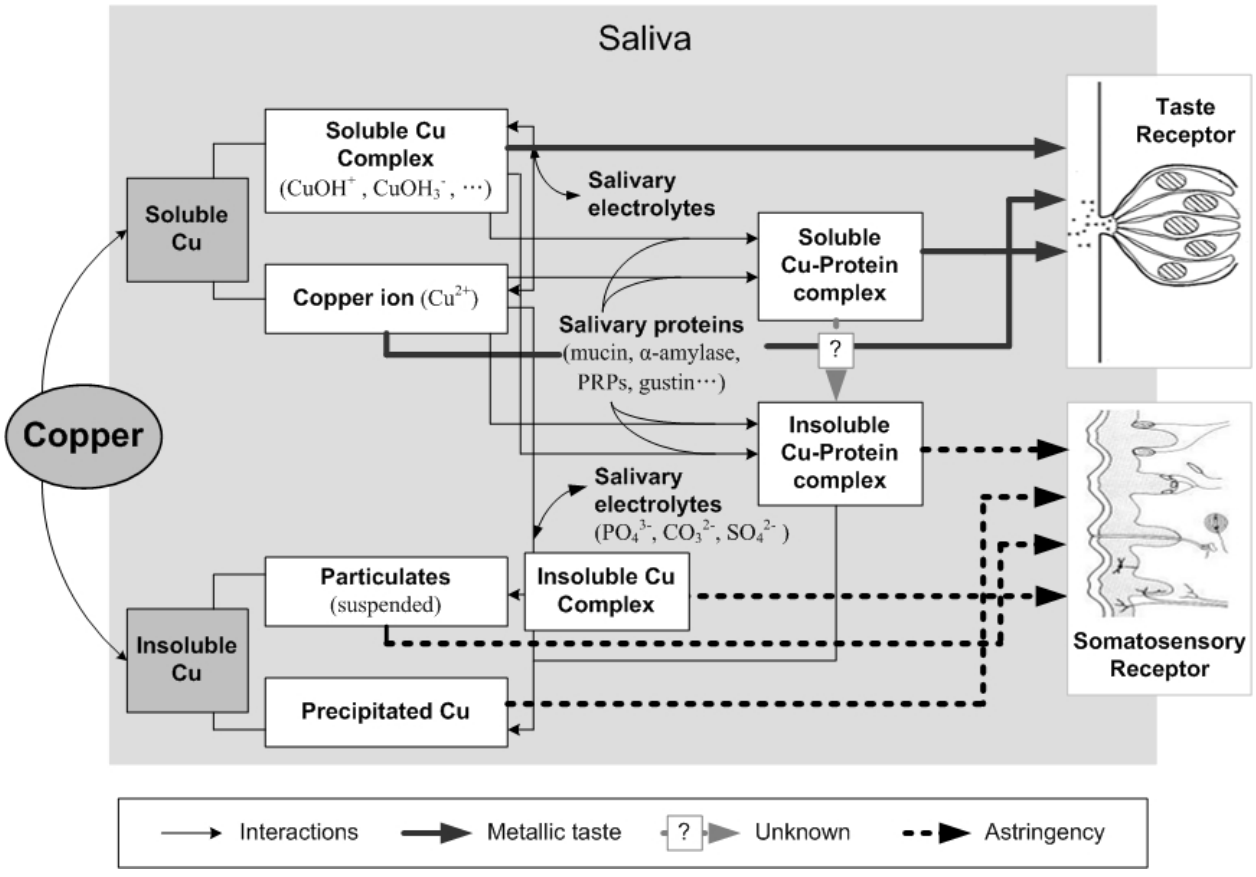

Figure 26. Speciation model of cupric ion in the mouth and its potential effect on perception of copper sensation.

Copper incorporated into the mouth either loses solubility or remains soluble at salivary pH of 6.5 ~ 7.5. Insoluble copper becomes particles and will precipitate with cell debris and mucin clot when saliva is centrifuged at 16,100g for 5 min. In cases when particle size is very fine (< 0.2 μm) (Cuppett et al., 2006), copper particles are suspended in the aqueous phase of saliva even after centrifugation.

Copper that remains soluble is thought to be in ionic form or soluble complex form. Soluble copper interacts with several electrolytes in saliva, and can form insoluble complex with carbonate, phosphate, and sulfate anions (Dietrich et al., 2005). Insoluble copper-electrolyte complexes are thought to cause astringency in the same way that copper particulates or precipitates do.

Soluble copper binds with proteins. Ionic copper or soluble copper complexes, or both, are expected to interact with proteins. The proteins of molecular weight of 29 kDa and 33 kDa form insoluble copper-protein complexes that are removed by centrifugation. These proteins were not clearly identified, but assumed to be either acidic PRPs or other Non-PRPs. Salivary proteins such as MG2, α-amylase, and basic PRPs IB forms also complex with copper. A protein of MW 45 kDa, which is assumed to be basic PRPs Ps forms or gustin, also binds with copper. These protein-copper complexes may be soluble or dispersible because they remain in supernatant after centrifugation, but the size of complexes is large enough to be removed by a 0.45 μm filter. Another protein-copper complex was found as a new peak appearing in the HPLC chromatogram. It is regarded as a real "soluble" protein-copper complex, because it was not removed with a 0.45 μm filter. Interaction between copper and histatins could not be identified because the level of histatins in whole saliva is very low (Beeley, 1993).

The mechanisms of copper binding to salivary proteins have not been clearly identified. The profile of copper-binding proteins estimated from HPLC and SDS-PAGE is that they are hydrophilic and likely to be mostly acidic glycoproteins. Thus, the main binding mechanism would be electrostatic binding between copper cations and negatively charged residues on proteins (Mueller, 1987). Since the binding mechanisms of copper to proteins are not known, it is hard to deduce the process of the copper-protein complex precipitation. One possible

mechanism is that negatively charged glycoproteins at salivary pH may become neutralized upon binding with copper. Other possible explanation is that cupric ions (Cu^{2+}) bridge negatively charged protein molecules (Mueller et al., 1983; Wu et al., 1994). Proteins are polymerized by the copper cross-link and start to precipitate. A similar mechanism was reported for binding of polyphenols to PRPs (Charlton et al., 2002). If the second hypothesis is correct, a "soluble" protein-copper complex may be an intermediate before proteins become polymerized.

Implication of Copper Speciation and Copper-Protein Interaction on Perception of Copper Taste.

It is widely accepted that astringency of polyphenolic compounds results from reduced lubrication effect of salivary proteins due to precipitation upon binding with PRPs (Kallithraka et al., 1998; Sarni-Manchado et al., 1999; Kallithraka et al., 2001; Bacon and Rhodes, 2000; Charlton et al., 2002). Other mechanisms, such as direct binding of polyphenolics to epithelial proteins (Kallithraka et al., 1998) or existence of protein-polyphenolic complex on the tongue and soft pallet (Kallithraka et al., 2001), are also assumed to be related with astringency. Therefore, it is postulated that insoluble copper species, including precipitated or particulated copper, insoluble copper complexes, and insoluble protein-copper complex, are related with astringency by de-lubricating the oral cavity and causing grittiness on the palate. The insoluble copper species not only elicits astringency but also may affect metallic taste of copper by controlling amount of copper accessible to taste receptors.

Solubilized copper, especially copper ions, are believed to cause metallic taste. Since copper, as an ionic stimulus, is expected to be transduced through ionic channel receptor cells like Na^+ or H^+, copper ions may be more accessible to taste receptors than complexed forms. Hence it is postulated that copper ions released from these complexes are main contributors to metallic taste. However, Cuppett et al. (2006) reported that both cupric ion and soluble copper complexes influenced perception of copper. In our previous study (Chapter III), the simulation result of copper speciation in a multi-electrolyte system showed that most added copper was changed into soluble copper complexes rather than cupric ions. Soluble copper complexes may be the

dominating copper species in saliva in terms of concentration. These results suggest that not only cupric ion but also soluble copper complexes may play a role in metallic taste perception.

The relationship between bitter taste and copper species in the mouth is not fully understood. However, it is known that anions that complexed with metal ions can influence bitterness of metallic compounds by "anionic inhibition" (Lawless et al., 2003). Anions of large molecular weight such as gluconate and lactate can reduce bitterness of metallic compounds by interrupting the contact of metals to taste receptor sites (Yang and Lawless, 2006). Thus bitterness of copper may be influenced by complexation with anions that are generic in saliva or even complexation with salivary proteins.

A role of soluble protein-copper complexes in copper taste perception would be a precursor of the astringency-causing compounds as it may be an intermediate in formation of precipitated protein-copper complex. However, this assumption needs to be examined since the actual binding mechanism may not support it. A soluble protein-copper complex also can be regarded as an inhibitor of metallic taste, because it is assumed to prevent copper from being diffused into the receptor cells as in the example of the anionic inhibition.

Sensory characteristics of copper could be dose-dependent. At the copper concentration less than 10 mg/L, most of the added copper was soluble copper unbound to proteins. On the other hand, only 25 ~ 30 % of total added copper was soluble unbound copper at > 20 mg/L Cu. This suggests that metallic taste is a main characteristic of copper at the concentration close to threshold while astringency becomes more important as the concentration of copper incorporated in the mouth increases. This supposition is supported by the result of our previous TI test. TI curve of astringency for 2.5 mg/L (0.039 mM) and 5 mg/L (0.079 mM) copper solutions did not show typical pattern of astringency, which has slow onset time and low rate of decrease. Copper concentration less than 2.5 mg/L may only cause a low level of insoluble copper and protein-bound copper that are responsible for development of astringency.

In real drinking condition, copper concentration in saliva is much lower than that observed in our *in vitro* study, probably due to dilution effect of food or beverage matrix and saliva flow.

Saliva collected after drinking 2.5 mg/L and 5 mg/L solutions had only 20 ~ 50 % of copper concentrations determined in the *in vitro* study. About 90% of copper in saliva was soluble unbound copper. This suggests that metallic taste is the most intense characteristics of copper when consuming threshold levels of copper. Our previous TI test showed that metallic taste had the highest maximum intensity score among all three attributes, metallic, bitter, and astringent taste at the level of 2.5 mg/L and 5 mg/L as Cu.

Future Studies

Taste and aroma are perceived under dynamic environments. Mastication, swallowing, and salivary flow keep changing oral conditions where perception occurs. For example, insoluble copper species may become solubilized over time as fresh saliva is continuously secreted from ducts, driving temporal changes in the sensory characteristics of copper. Therefore, effects of these factors should be taken into consideration for the future studies.

Formation of copper-salivary protein complexes may have an important implication on human health. Salivary proteins, such as basic PRPs and histatins are proven to act as a defense against toxic effect of condensed tannin compounds by forming insoluble complexes that remain stable in the digestive tract. Complexation products between these proteins and condensed tannins were stable under *in vitro* conditions mimicking that in the stomach, indicating that these complexes are not likely to be absorbed in the stomach. About 30 ~ 40 % of salivary protein-tannin complexes were degraded under *in vitro* conditions that were similar as that in the small intestine. The degradation at *in vitro* intestinal condition does not necessarily mean that tannin compounds are readily absorbed in the small intestine, because bile acids or digestive enzymes may form insoluble complexes with tannins as soon as tannins are released from the protein-tannin complexes by digestion (Lu and Bennick, 1998; Naurato et al., 1999). These results suggest that interaction between copper and salivary proteins may also serve as a protection mechanism against acute toxicity of copper by forming indigestible complexes. This potential role of salivary proteins should be examined to help understanding of implication of sensory perception on human health.

In addition, the salivary proteins that interact with copper were not clearly identified. It is still not certain whether the major proteins that precipitated at >10 mg/L copper are PRPs or not. Our HPLC and SDS-PAGE results showed that several proteins could be eluted as one peak or detected as a single band. Characterization of salivary proteins requires more advanced separation/identification techniques. Proteomic techniques, such as immunoblotting, two dimensional SDS-PAGE coupled with matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) (Hardt et al., 2005), two dimensional HPLC (Wilmarth et al., 2004), are expected to provide better identification in future studies.

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Appendices

Appendix 1. Sensory ballot used for screening of time-intensity test panelists

Panelist #: _____

1-out of-5 Test for the Sensory Evaluation of a Copper solution

Name: _____ Date: _____

INSTRUCTIONS: Sip the samples in the order they are presented, swirl around for 10 seconds and expectorate in the empty cup. DO NOT SWALLOW the samples. Four samples are identical; one is different. Rinse your mouth with water before tasting the next sample. Select the odd/different sample and indicate by placing an X on the code of the odd sample.

Indicate odd sample

☐	☐	☐	☐	☐
963	274	632	161	442

Remarks:

Thank you for your time!

Appendix 2. Informed consent form

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

Title of Project: Formation of copper-salivary compound complex and its effect on sensory perception.

Principal Investigator: Dr. Susan E. Duncan, Jae Hee Hong

I. THE PURPOSE OF THIS PROJECT

You are invited to participate on a sensory evaluation panel about copper-deionized water solution. The purpose of this project is to identify how copper in water influences on sensory perception via interaction with salivary proteins.

II. PROCEDURES

There will be 18 sessions over a period of 6 weeks involving about 15 minutes at each session. You will be presented with approximately 2 samples at each session. As a panelist, it is critical to the project that you attend each session. Should you find a sample unpalatable or offensive, you may choose to spit it out and continue to other samples.

Certain individuals are sensitive to some foods such as milk, eggs, wheat gluten, strawberries, chocolate, artificial sweeteners, etc. If you are aware of any food or drug allergies, list them in the following space.

III. BENEFITS/RISKS OF THE PROJECT

Your participation in the project will provide the following information that may be helpful to figure out the mechanism of metallic taste perception and its implication of human health. You may receive the results or summary of the panel when the project is completed. Excessive intake of copper can cause nausea, vomiting, and abdominal pain. The levels used in this study are well below the recommended daily dietary intake of copper. Copper solution also can cause an unpleasant lingering sensation such as metallic, bitter, and astringent taste.

IV. EXTENT OF AN0NYMITY 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

Monetary

For participation in the project, you will receive a piece of candy as token of our appreciation for your time and support of this project.

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: (list sessions to be attended or other requirements.)

_____

Signature/Date

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

Address _____

Phone _____

----------(tear off)----------

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:

_____

Investigator/Phone

_____

Faculty/Phone

_____ _____(540)231-6077

Chair, IRB/Phone for Research Division

Appendix 3. Sensory ballot used for the triangle test to investigate the effect of sodium bicarbonate on water taste

Panelist #: _____

Triangle Test for the Sensory Evaluation of a Copper solution

Name: _____ Date: _____

INSTRUCTIONS: Sip the samples in the order they are presented, swirl around for 10 seconds and expectorate in the empty cup. DO NOT SWALLOW the samples. Two samples are identical; one is different. Rinse your mouth with water before tasting the next sample. Select the odd/different sample and indicate by placing an X on the code of the odd sample.

Indicate odd sample

☐	☐	☐
308	953	142

Remarks:

Thank you for your time!

Appendix 4. Sensory ballot used for the rating test to investigate masking effect of sodium bicarbonate on copper sensation

Rating Test for the Sensory Evaluation of a Copper solution

Name: _____ Date: _____

INSTRUCTIONS: Sip the samples in the order they are presented, swirl around for 10 seconds and expectorate in the empty cup. DO NOT SWALLOW the samples. Rinse your mouth with water and saltine cracker, and take two-minute break before tasting the next sample. Taste the first sample and rate the intensity for METALLIC and ELECTROLYTE taste using the following scale, and proceed to the next sample. Please write down the intensity for each taste under the sample code below.

Intensity	Score
Imperceptible	0
Slight	1 2 3
Moderate	4 5 6
Strong	7 8 9

Sample #	953	395	786	180	435
METALLIC					
ELLECTROLYTE					

Remarks:

Thank you for your time!

Appendix 5. Time-intensity sensory data

Table 22. Mean and standard deviation for time-intensity parameters for metallic taste of copper at two levels (Cu 2.5 mg/L and Cu 5 mg/L) in ultrapure water (pH 5.5) and sodium bicarbonate 1 mM solution (pH 7.5).

Cu (mg/L)	pH	I_{max}	AUC	Pre-peak area	Post-peak area	Peri-meter	Plateau time	Rate of increase	Rate of decrease	T_{dur}	T_{ext}	T_{max}	T_{onset}
2.5	5.5	3.97[a]	249.05	83.22	165.83	120.48	6.60	0.15	-0.04	84.60	93.97	32.90	9.37
		(3.73)[b]	(297.11)	(126.66)	(182.64)	(1.76)	(9.40)	(0.16)	(0.04)	(34.86)	(29.81)	(14.31)	(10.87)
	7.5	3.03	192.42	43.47	148.96	120.50	4.83	0.20	-0.03	87.73	92.63	27.10	4.90
		(2.74)	(240.56)	(58.94)	(200.63)	(14.62)	(9.79)	(0.46)	(0.03)	(34.98)	(33.99)	(13.18)	(5.26)
5	5.5	7.05	463.51	148.26	315.25	122.82	6.40	0.28	-0.05	100.00	104.87	37.87	4.87
		(3.97)	(373.58)	(164.15)	(274.87)	(3.34)	(10.78)	(0.34)	(0.04)	(30.25)	(29.02)	(21.38)	(6.78)
	7.5	3.24	199.21	68.91	130.30	120.39	7.66	0.12	-0.03	89.35	95.52	31.41	6.17
		(2.44)	(256.40)	(140.70)	(163.73)	(1.73)	(8.08)	(0.14)	(0.04)	(30.49)	(29.75)	(16.73)	(6.74)

[a] Mean
[b] Standard deviation

Table 23. Mean and standard deviation for time-intensity parameters for bitterness of copper at two levels (Cu 2.5 mg/L and Cu 5 mg/L) in ultrapure water (pH 5.5) and sodium bicarbonate 1 mM solution (pH 7.5).

Cu (mg/L)	pH	I_{max}	AUC	Pre-peak area	Post-peak area	Peri-meter	Plateau time	Rate of increase	Rate of decrease	T_{dur}	T_{ext}	T_{max}	T_{onset}
2.5	5.5	5.82[a]	373.33	111.18	262.14	122.24	5.00	0.21	-0.05	98.22	103.93	32.07	5.70
		(3.99)[b]	(284.43)	(107.26)	(209.43)	(3.09)	(6.60)	(0.15)	(0.05)	(23.80)	(19.95)	(14.97)	(7.45)
	7.5	5.66	294.89	65.36	229.53	122.37	4.78	0.38	-0.05	95.74	100.48	24.30	4.74
		(3.47)	(218.76)	(61.99)	(164.61)	(3.34)	(14.16)	(0.55)	(0.04)	(33.74)	(31.66)	(12.51)	(4.97)
5	5.5	6.65	419.84[c]	89.62	330.22	122.54	10.52	0.35	-0.05	104.44	108.56	22.67	4.11
		(3.94)	(312.96)	(92.39)	(232.99)	(2.97)	(22.80)	(0.28)	(0.04)	(21.50)	(19.71)	(11.85)	(4.42)
	7.5	4.56	232.23	46.45	185.78	121.10	7.48	0.27	-0.04	97.00	100.93	22.63	3.93
		(3.36)	(197.01)	(47.97)	(157.38)	(2.33)	(11.27)	(0.44)	(0.03)	(28.95)	(27.03)	(17.01)	(4.37)

[a] Mean
[b] Standard deviation

Table 24. Mean and standard deviation for time-intensity parameters for astringency of copper at two levels (Cu 2.5 mg/L and Cu 5 mg/L) in ultrapure water (pH 5.5) and sodium bicarbonate 1 mM solution (pH 7.5).

Cu (mg/L)	pH	I_{max}	AUC	Pre-peak area	Post-peak area	Perimeter	Plateau time	Rate of increase	Rate of decrease	T_{dur}	T_{ext}	T_{max}	T_{onset}
2.5	5.5	4.80[a]	289.69	63.75	225.94	121.36	8.19	0.24	-0.05	105.41	111.19	26.89	5.78
		(2.35)[b]	(161.38)	(44.95)	(130.14)	(1.96)	(12.78)	(0.19)	(0.02)	(15.96)	(13.09)	(10.52)	(6.51)
	7.5	4.17	249.56	65.74	183.82	120.84	6.89	0.18	-0.04	98.26	102.82	27.96	4.56
		(2.26)	(159.35)	(50.44)	(131.85)	(1.53)	(8.71)	(0.12)	(0.03)	(24.58)	(23.07)	(13.93)	(4.17)
5	5.5	5.28	326.73	71.44	255.29	121.49	12.70	0.24	-0.05	107.04	111.83	27.65	4.78
		(2.54)	(201.46)	(50.49)	(164.01)	(1.98)	(25.60)	(0.17)	(0.03)	(11.98)	(12.08)	(11.26)	(4.82)
	7.5	3.67	218.47	52.22	166.25	120.62	4.78	0.21	-0.03	95.39	99.70	24.83	4.30
		(2.07)	(188.39)	(83.07)	(150.86)	(1.75)	(7.95)	(0.19)	(0.02)	(29.31)	(27.62)	(16.98)	(4.51)

[a] Mean
[b] Standard deviation

Appendix 6. Informed consent form

Informed Consent for Participants in Research Projects Involving Human Subjects

Title of Project: Formation of Copper-Salivary Component Complex and Its Effect on Sensory Perception

Investigator(s) Dr. Susan E. Duncan, Jae Hee Hong

I. Purpose of this Research/Project

You are invited to participate in the research about copper-salivary protein interaction. The purpose of this project is to identify how copper in water influences sensory perception via interaction with salivary proteins. Five participants (age of 20 ~ 55) selected from students and the staff of Virginia Tech are involved in this project. All participants are non-smokers and report no problem in health and tasting ability.

II. Procedures

You will be asked to collect saliva in the sterile specimen cup before drinking copper solution and after drinking copper solution. Do not consume any food, beverage, and oral care products for 2 hour before saliva collection. Before saliva collection, rinse your mouth with deionized water three times, and spit for three times to get rid of any residual water. Collect saliva for a certain amount of time on cue from the researcher before drinking copper solution. Sip copper solution, swirl it around for 10 seconds, and expectorate. Collect saliva in the same way as describe above. Saliva will be collected six times over six days.

III. Risks

Copper solution may cause an unpleasant lingering sensation such as metallic, bitter, or astringent taste. Deionized water and saltine crackers will be given to help removing unpleasant tastes after saliva collection.

IV. Benefits

Your participation in the project will provide the following information that may be helpful to identify the perception mechanism of metallic sensation and its implication of human health. You may receive the results or summary of the result when the project is completed. Some risk may be involved if you have an unknown sensitivity to copper.

V. Extent of Anonymity and Confidentiality

The results of the research will be kept strictly confidential. Individual participant will be referred to by code for analyses and in any publication of the results.

VI. Compensation

For participation in the project, you will receive a piece of candy as token of our appreciation for your time and support of this project.

II. Freedom to Withdraw

It is essential to the project that you provide saliva in so far as possible. However, there may be conditions preventing your donation of saliva. If after reading and becoming familiar with the saliva collection, you decide not to donate saliva, you may withdraw at any time without penalty.

VIII. Subject's Responsibilities

I voluntarily agree to participate in this study. I have the following responsibilities: providing saliva before and after drinking copper solution, consumption or use of no foods, beverage, and oral care products for 1 hour before saliva collection.

X. Subject's Permission

I have read and understand the Informed Consent 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

_____ Date _____
Witness (Optional except for certain classes of subjects)

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:

_____ Jae Hee Hong _____	_540-231-6806/jhhong@vt.edu_
Investigator(s)	Telephone/e-mail
____Dr. Susan E. Duncan _____	_540-231-8675/duncans@vt.edu_
Faculty Advisor	Telephone/e-mail
____Dr. Susan S. Sumner _____	_540-231-5280/summers@vt.edu_
Departmental Reviewer/Department Head	Telephone/e-mail

David M. Moore 540-231-4991/moored@vt.edu
Chair, Virginia Tech Institutional Review Board for the Protection of Human Subjects Telephone/e-mail
Office of Research Compliance – CVM Phase II (0442)
Research Division

This Informed Consent is valid from _____ to _____.

[NOTE: Subjects must be given a complete copy (or duplicate original) of the signed Informed Consent.]

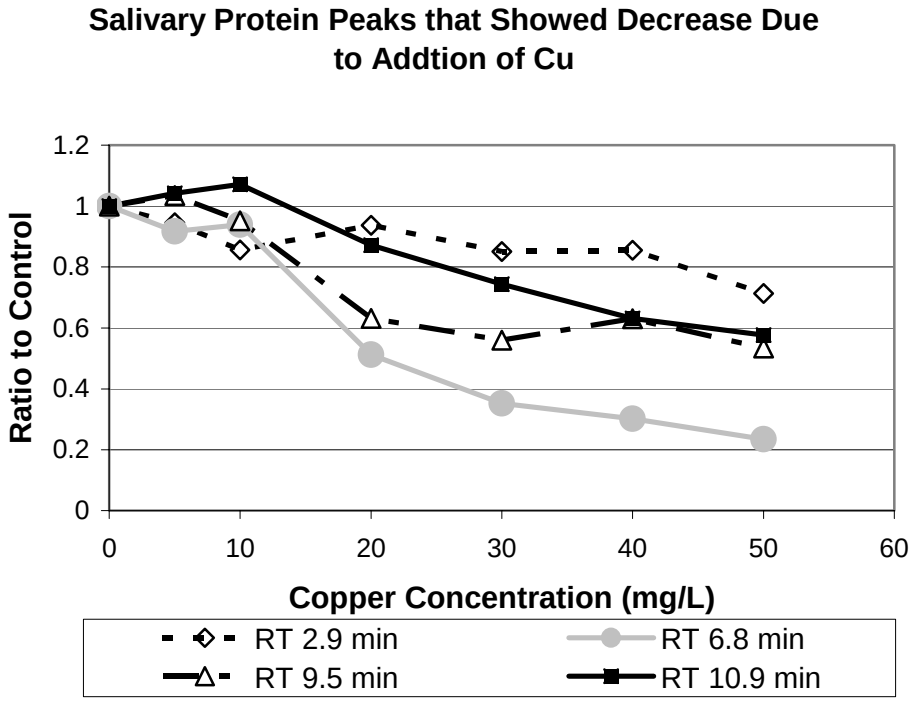
Appendix 7. Preliminary HPLC

Purpose. Preliminary HPLC was performed to determine a range of effective copper concentrations that can cause changes in salivary proteins.

Methods. Copper was added to saliva samples collected from three individuals at the levels of 0, 5, 10, 20, 30, 40, and 50 ppm and changes in salivary proteins were measured with HPLC as described previously (Chapter V, Materials and Methods, HPLC). Saliva samples obtained from three individuals were averaged and subjected to 1-way ANOVA to examine effect of copper ($\alpha = 0.05$).

Results. Major protein peaks detected at 230 nm and 280 nm were integrated and peak area at different copper concentration was analyzed using 1-way ANOVA. The peak with RT 6.8 min (the major peak at both 230 nm and 280 nm), and 10.9 min (detected at 280 nm) were decreased significantly ($p \leq 0.05$). The peaks at RT 2.9 min and 9.5 min, which were major peaks detected at 230 nm, were not significantly changed, but showed significant tendency of decrease ($0.05 < p < 0.1$). The peak at RT 3.0 min (detected at 230 nm) were not detected in control, but appeared when copper was added and increased as more copper was added. The pattern of changes in peak area is plotted in Figure 27. Since the peaks had wide range of area count from 10,000 to 2,500,000, values were normalized to give better representative pattern in a plot. The peak area count was normalized by converting area to the ratio of mean peak area at a copper concentration to the mean peak area of control:

$$\text{Normalized difference in peak area} = \frac{\text{Mean peak area of copper treatement}}{\text{Mean peak area of control}}$$



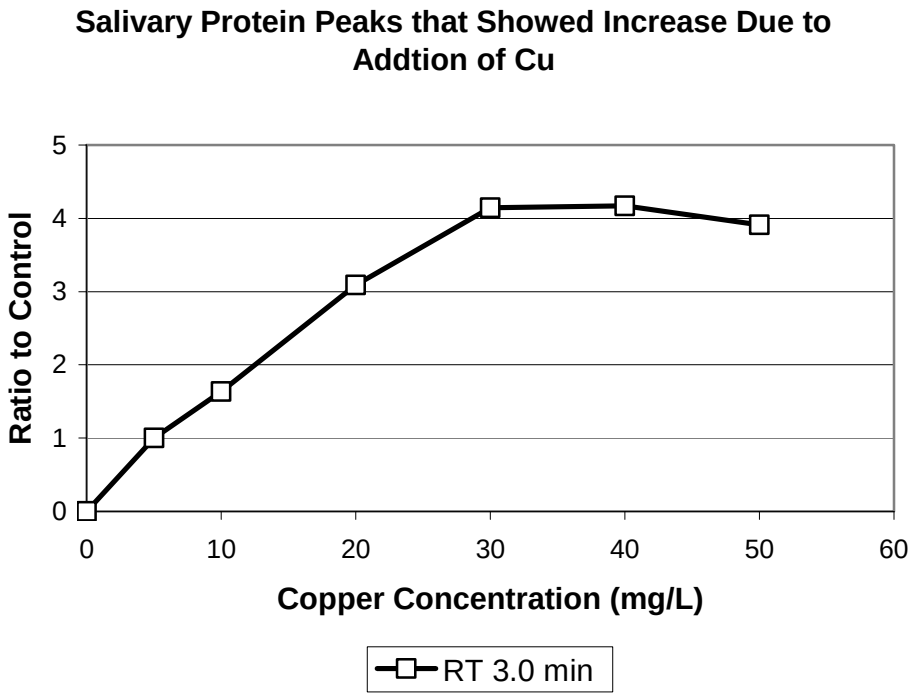


Figure 27. Changes in major protein HPLC peaks of saliva treated with 0, 5, 10, 20, 30, and 40 mg/mL copper. Peak area was normalized by dividing the difference in the mean peak area between a copper treatment and control by the mean peak area of control (n = 3).

The peaks at RT 2.9 min, 9.5 min, and 10.9 min decreased by 30 ~ 40 % compared to control as copper concentration increased. The peak with RT 6.8 min got smaller by 80 %, but a new peak at RT 3.0 min increased as copper was added up to 50 mg/L. The peaks did not exhibit big difference when copper was added at 5 mg/L and 10 mg/L. It is assumed that changes caused by low concentration of copper were so small that it could be compensated by the variance of protein concentration in saliva. The shapes of the plots imply that each salivary protein peak has different kinetics of interaction, but steep slopes were observed between 10 mg/mL and 30 mg/mL in common for the peaks at RT 3.0, 6.8, 9.5, and 10.9 min. Thus copper concentrations of 10, 20, and 40 mg/mL were selected for the main experiment to provide more information about interactions that occur in this range. Copper concentrations that were used in TI test study (2.5 mg/L and 5 mg/L) were also selected for the main experiment to explain changes in the sensory perception in relation to biochemistry in the mouth.

Appendix 8. Validation of HPLC method

Purpose. Reverse-phase chromatography is a commonly used method for quantitative and qualitative analysis of various proteins. In reverse-phase chromatography, proteins are separated with hydrophobic interaction between *n*-alkyl ligands on solid phase of a column and hydrophobic regions of proteins. Separation of protein peaks are improved by using gradient mobile phase condition and ion modifiers such as trifluoroacetic acid (TFA) (Aguilar, 2004).

Because biological systems such as the human saliva are very complex, it is possible that peaks on HPLC chromatograms are not originated from proteins. Therefore, changes in peaks detected by HPLC may not indicate changes in salivary proteins. In order to verify if our HPLC instrumentation also can detect changes in salivary proteins, interaction between grape tannins and salivary proteins, which was already verified in the previous study (Kallitharka et al., 1998) was analyzed using our HPLC instrumentation. If our result showed the similar trend as that was observed in Kallithraka et al. (1998), our instrumentation could be regarded as a suitable setup to detect changes in salivary proteins.

Methods. Grape seed extract (Muscadin plus powder, Nutragon LLC, NC) was added to saliva from two subjects at the level of 10 mg/mL and analyzed with HPLC as described previously (Chapter V, Materials and Methods, HPLC). The result was compared to those from saliva containing no grape skin extract and 10 mg/mL grape skin extract in water. Peaks were identified based on retention time (RT) and the ratio of peak area detected at 230 nm to that at 280 nm (A_{230}/A_{280}) as described previously (Chapter V, Materials and Methods, HPLC).

Results. Chromatograms of saliva containing 10 mg/mL grape seed extract (GSE-saliva) were compared to those from saliva containing no grape seed extract (control) and grape seed extract in water (GSE-water) (Figure 28, Figure 29). Two peaks, which were detected at 3.5 min and 7.7 min from GSE-saliva at 230 nm, were significantly increased compared to those in GSE-water and control ($p \leq 0.05$), indicating that changes occurred in salivary proteins by addition of grape polyphenols. The peak detected at RT 3.5 min was increased by 25% compared to the peaks with the same RT in control and GSE-water. The peak at 7.7 min newly

appeared when GSE was added to saliva. Interestingly, these two peaks were not detected at 280 nm. These peaks were tentatively identified as PRPs following the method of Kallithraka et al.(1998).

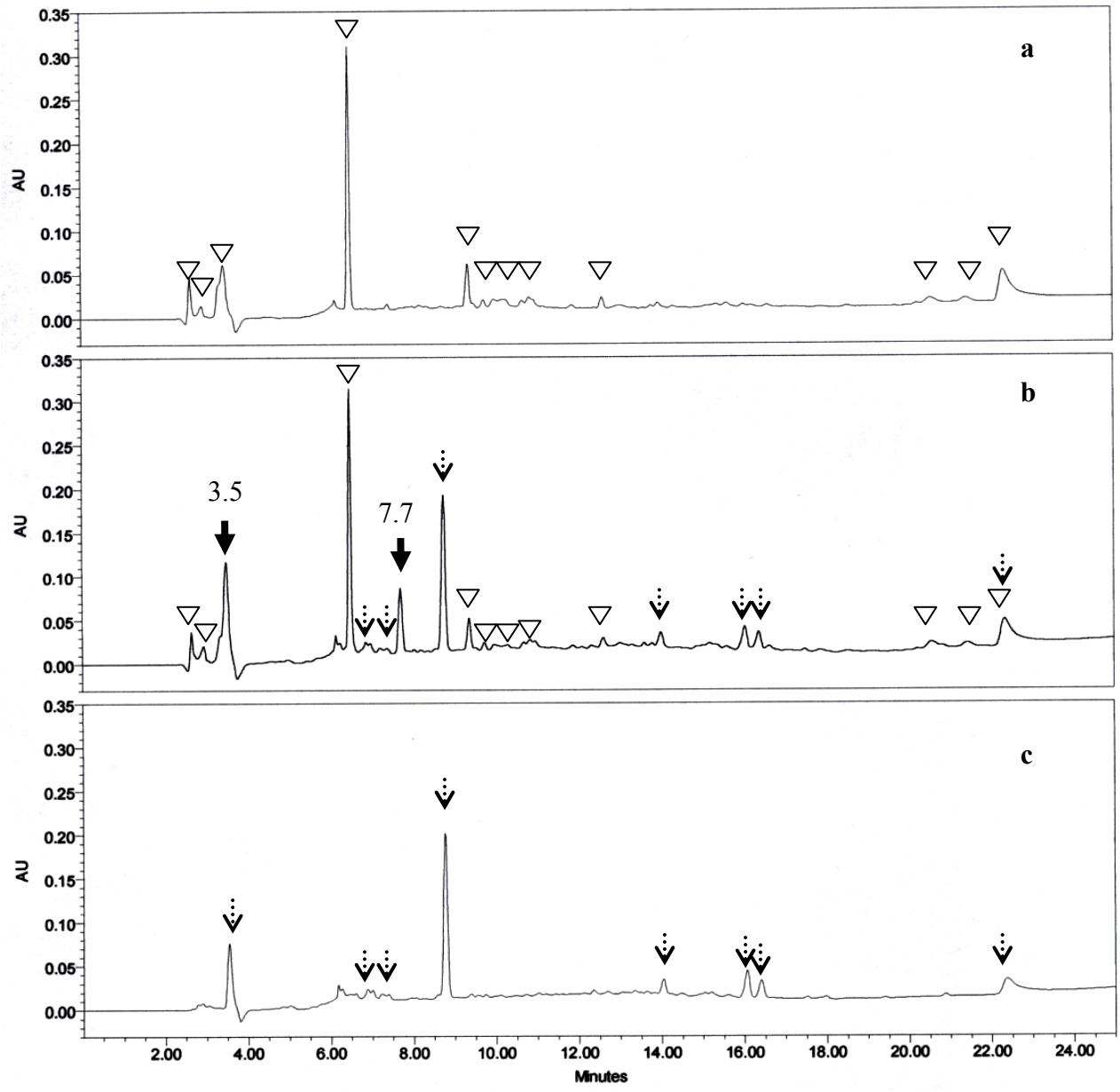


Figure 28. Representative HPLC chromatogram detected at the wavelength of 230 nm (a – saliva containing no GSE; b –10 mg/mL of GSE in saliva; c – 10 mg/mL of GSE in ultrapure water). A bold arrow mark in chromatogram b indicates a peak that shows significant change compared to that in chromatogram a or chromatogram c. A reversed triangle mark in chromatogram a and b indicates protein peaks from saliva. A dashed arrow mark in chromatogram b and c indicates tannin compounds from GSE.

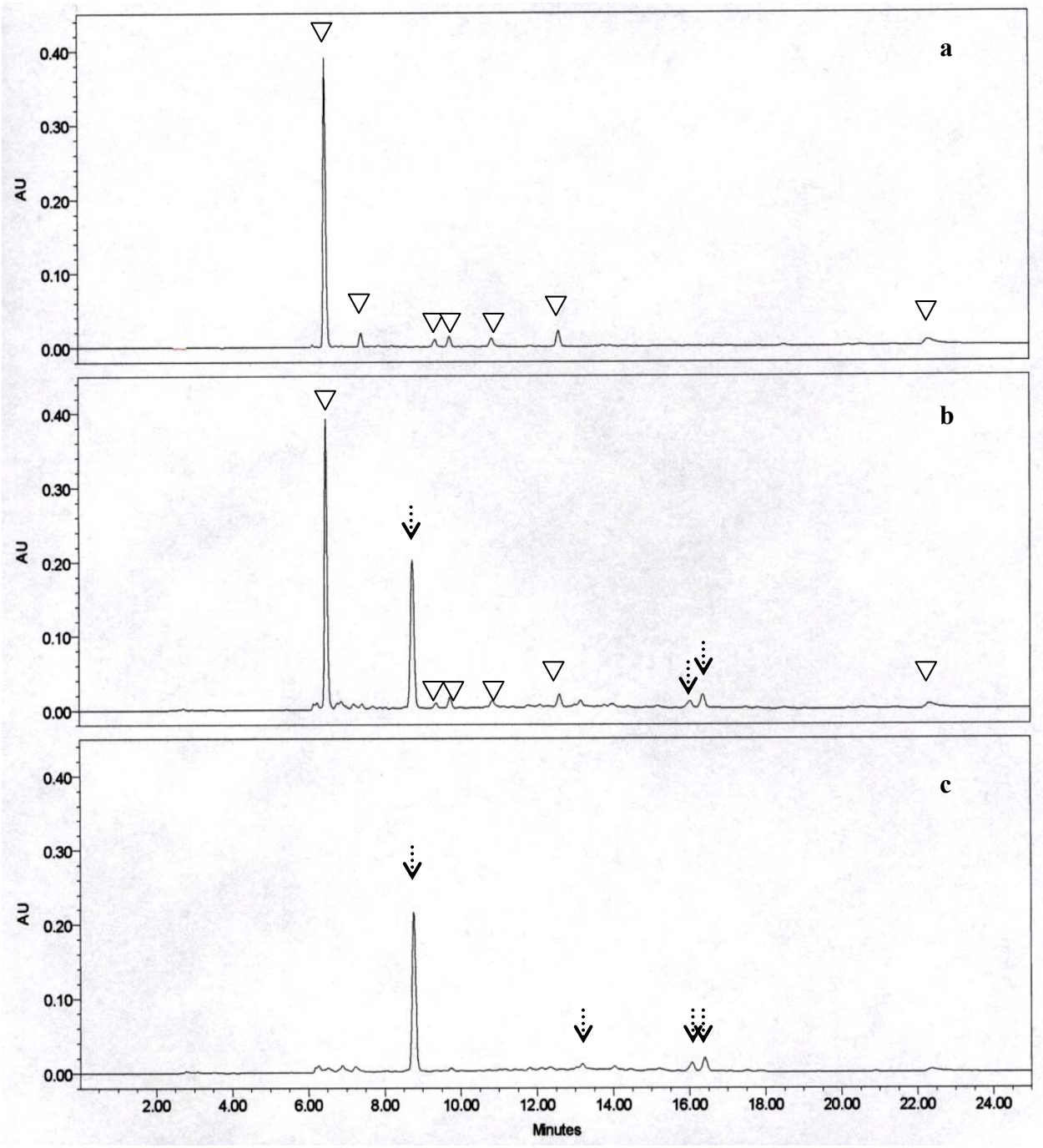


Figure 29. Representative HPLC chromatogram detected at the wavelength of 280 nm (a – saliva containing no GSE; b – GSE 10mg/mL in saliva; c – 10 mg/L of GSE in ultrapure water). A reversed triangle mark in chromatogram a and b indicates protein peaks from saliva. A dashed arrow mark in chromatogram b and c indicates tannin compounds from GSE.

Our result was compared to the study of Kallithraka et al.(1998) for validation. Kallithraka et al.(1998) compared saliva before drinking model wine solution or red wine to saliva after drinking, and observed decreases in peaks with RT 2 ~ 2.5 min corresponding to our peaks at RT 2.5 ~ 4 min and RT 21 min after drinking wine. These peaks were identified as PRPs based on their absorbance ratio (A_{230}/A_{280}). However, the peaks at this retention time in our study did not show any decrease at all. Furthermore, the peak at RT 3.5 in our study was increased by addition of grape polyphenols. There was a new peak at RT 8.0 min which coincides with appearance of a new peak at RT 7.7 min in our study. Kallithraka et al. (1998) explained that salivary PRPs formed soluble complexes with polyphenolic compounds (appearance of new peak at RT 8.0). PRPs-tannin complexes became less soluble and precipitated as polyphenols bound to proteins more and more (decrease in the peaks at RT 2 ~ 2.5 min). Appearance of a new peak at RT 7.7 min in our study was also assumed as formation of salivary protein – tannin complexes, but no formation of insoluble complexes was observed in our study. It may be because complexation of salivary PRPs and tannins in our study were not progressed to the point where insoluble complexes started to be formed. The inconsistency in results is thought to result from different experimental conditions, variety of polyphenolic compounds, and other compounds (acid and alcohol) found in samples in Kallithraka et al. (1998)'s study. It is postulated that the increase in the peaks of 3.5 min were resulting from complexation between non-polyphenolic compounds and proteins.

In spite of some differences in results between our study and Kallithraka et al.(1998), our HPLC study proved that changes in salivary proteins can be detected by the HPLC method suggested by Kallithraka et al.(1998). Thus this method was used for investigating effect of copper on salivary proteins.

Curriculum Vitae

Jae Hee Hong

EDUCATION

Doctorate of Philosophy, Food Science and Technology, December 2006.
Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA.
Dissertation: Formation of Salivary Component-Copper Complexes and its Effect on Sensory Perception.
Advisor: Susan E. Duncan.

Master of Science, Science of Food and Nutrition, February 1996.
Ewha Womans University, Seoul, Korea.
Dissertation: Effect of Hydroxypropylation and Cross-linking of Nonwaxy Rice Grit on the Storage Stability of Julpyun (Korean Plain Rice Cake).
Advisor: Kwang Ok Kim.

Bachelor of Science, Science of Food and Nutrition, February 1994.
Ewha Womans University, Seoul, Korea.

HONORS/AFFILIATIONS

- 2nd place (team captain: Mark Klein), 2006 Danisco Product Development Competition, Spring 2006.
- Graduate Research Development Funding Award, Virginia Tech Graduate Student Association, Spring 2005, Spring 2006.
- Graduate Travel Funding Award, Virginia Tech Graduate Student Association, Fall 2005.
- Virginia Dare Award (3rd in yogurt), 2005 National Collegiate Dairy Products Evaluation Competition, Fall 2005.
- Ewha Welfare Scholarship, Ewha Womans University, 1995.
- Honor Scholarship, Ewha Womans University, 1993.
- Member of International Food Technologiest, 2004 – present.

RESEARCH INTERESTS

- Impact of changes in oral pH due to consumption of acidic foods on metallic taste perception.
- Degradation of metal-salivary protein complexes in the digestive tract and its effect on human health.

- Complexation of salivary components and metallic compounds and its effect on oxidative process in the human mouth.
- Metallic flavor formation as oxidative process caused by drugs, taste disorder, and cancer.

RELATED EXPERIENCE

Research Associate Faculty, Program Manager for MILES (Macromolecular Interfaces with Life Science) – IGERT (Integrative Graduate Education and Research Traineeship) program, September 2006 – present, Department of Food Science and Technology, Virginia Tech, Blacksburg, VA

- Identify the potential granting opportunities and develop research, education, and outreach proposals.
- Interfaces with MILES students, core faculties, university partners, and science/public clientele to achieve the goals of MILES-IGERT program.
- Coordinate assessment activites and reporting.
- Organize events for educational programs, technical meetings, scientific and public clientele related to dissemination of program activities and impacts.

Research Assistant for MUSES (Material Use: Science, Engineering, and Society) Project, January 2004 - May 2005, Department of Food Science and Technology, Virginia Tech, Blacksburg, VA.

- SPME Headspace-GC analysis to identify the effect of copper on changes in aroma in the model mouth system.
- Time-intensity sensory test to determine the influence of pH on taste of copper.
- Biochemical analysis: perform the ultrafiltration of saliva to fractionate proteins, analyze copper in each protein fractions with ICP, and run gel electrophoresis to identify protein composition.
- Experience in working as a member of multidisciplinary project team consisting of researchers from environmental engineering, civil engineering, chemistry, microbiology, public health, and economy.
- Developed the proposal for the USDA-National Research Initiative (NRI) funding (Effect of Copper on Changes in Sensory Quality and Bioactive Compounds in a Protein Based Functional Model Beverage).

Research Assistant for Peanut Hull Extract Research, Summer 2003, Department of Food Science and Technology, Virginia Tech, Blacksburg, VA.

- Decolorization of methanol extract of peanut hull, and skin that have antioxidant activity using preparative liquid chromatography and solid-phase extraction.
- Measurement of changes in antioxidant activity due to decolorization.

Senior Researcher, Functional Food Team, Pulmuone, Co. Ltd. Seoul, Korea, October 2000 – January 2002.

- Searched a new functional compound and examine bioactivity.
- Extracted nonsaponifiable fraction of rice bran and check antioxidant effect.

Researcher/Senior Researcher, Analytical & Sensory Evaluation Team, Pulmuone, Co. Ltd. Seoul, Korea, July 1996 – September 2002.

- Sensory Evaluation: Set up descriptive analysis panel system, Performing sensory tests and market survey for new products, Panel training for R/D and QA.
- HPLC/GC analysis of compounds related to flavor: amino acid, sugars, organic acids, capsaicin, aroma compounds.

Researcher, Diet Center, Pulmuone, Co. Ltd. Seoul, Korea, February 1996 – July 1996.

- Consulted individuals who participated in weight control program.
- Developed diet/exercise therapies for weight control program.

Teaching

Teaching Assistant, Department of Food Science and Technology, Virginia Tech, Blacksburg, VA. Spring 2004; Spring 2005; Fall 2005.

- Prepared undergraduate Food Analysis (FST 4514) lab and mentored lab activity.
- Prepared undergraduate Dairy Product Sensory Evaluation (FST 2104) lab.
- Graded quizzes, test, and lab activity for Dairy Product Sensory Evaluation lab.

Researcher/Senior Researcher, Analytical & Sensory Evaluation Team, Pulmuone, Co. Ltd. Seoul, Korea, July 1996 – September 2002.

- Internal Sensory Evaluation Short Courses for QA personnel.

PUBLICATIONS

Jae Hee Hong, Susan E. Duncan, Andrea M. Dietrich, and Sean F. O'Keefe. Effect of Copper on Volatile Flavor Compounds in a Model Mouth System. In press. J. Agric. Food Chem. (accepted Sep. 17, 2006).

Ewa Kleczyk, Darrell Bosch, Junseok Lee, G. V. Loganathan, Timothy Heim, Andrea Dietrich, Jae Hee Hong, Susan E. Duncan, Abhijeet Dudi, and Mark Edwards. Student Research on Drinking Water Infrastructure. Virginia Water Central, June 2006.

Dong Soon Suh, Shin Hae Kim, Jae Hee Hong and Kwang Ok Kim. 2000. Application of Quantitative Descriptive Analysis to Commercial Soybean Curd. J. Korean Soc. Dietary Culture. 16(1), 58.

Jae Hee Hong, Da Mi Chung, and Kwang Ok Kim. 1996. Effects of Hydroxypropylation and Cross-linking of Nonwaxy Rice Grit on the Storage Stability of Julpyun (Korean Plain Rice Cake). J. Food Sci. Biotech. 5(2), 6.

ABSTRACTS

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[http://ift.confex.com/ift/2005/techprogram/]

Joo Shin Kim, Jae Hee Hong, Kyung Nam Kim, and Hyeon Gyu Lee. 2001. γ-Oryzanol Inhibits the Formation of C-7 Oxidized Cholesterol Derivatives in an Aqueous Model System during Cholesterol Autoxidation. Proceedings of the 67th Korean Society of Food Science and Technology (Kosfost) Annual Meeting. Oct. 18-20, 2001. Cheju National University. Jeju-Si, Jeju-Do, Korea. p77.

Jae Hee Hong. 1998. Application of Sensory Evaluation in the Industry. Proceedings of the 61st Kosfost Annual Meeting. Nov. 6-7, 1998. Ewha Womans University, Seoul, Korea