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

JAE HEE HONG,*,† SUSAN E. DUNCAN,† ANDREA M. DIETRICH,‡ AND SEAN F. O'KEEFE†

Department of Food Science and Technology, Food Science and Technology Bldg. (0418), and Department of Civil and Environmental Engineering, 413 Durham Hall, Virginia Tech, Blacksburg, Virginia 24061

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, but no change in volatility was observed at pH 7.0. At pH 7.5, the presence of copper in the artificial saliva system containing mucin and α-amylase decreased headspace volatile concentration, 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. Salivary proteins seem to interact with copper at pH 7.5. The interaction may change configuration of binding sites for aroma compounds in mucin.

KEYWORDS: Copper; flavor; salivary proteins; saliva; SPME

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 U.S. 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 (*1*, *2*). 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 (*3*, *4*). An aesthetic based standard is 1 mg/L Cu because copper above this level can contribute to metallic- or bitter-tasting water (*5*).

Copper has bitter, astringent, sour, salty, and metallic tastes or an electric sensation (*6*, *7*). Pizarro et al. (*8*) 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 (*9*), the unpleasant sensations of copper are assumed to be an initial biological protection mechanism from acute copper toxicity. The threshold level of copper sensation ranges from 1 to 13 mg/L (*6*, *10*−*12*), 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 (*13*). Chapman and Lawless (*14*) 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 delubricating 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 (*15*). Each salivary component serves several physiological functions, such as tissue coating, lubrication, buffer capacity, antimicrobial activity, and perception of flavor (*15*, *16*).

Human salivary proteins have been reported to change partitioning behavior of aroma compounds in the mouth, resulting in potential changes in aroma perception (*17*−*19*). Salivary proteins change the volatility of aroma compounds by binding hydrophobic molecules or by salting out hydrophilic molecules (*18*, *19*). Salivary glycoproteins such as mucin and α-amylase showed a moderate or high binding affinity to copper (*20*−*22*). Histatins, a class of salivary polypeptides, also bind copper. Histatin is a family of histidine-rich polypeptides (HRP)

* Corresponding author. Tel: (540) 231-0950. Fax: (540) 231-9293. E-mail: jhhong@vt.edu.
† Department of Food Science and Technology.
‡ Department of Civil and Environmental Engineering.

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Table 1. Final Concentration of Each Ingredient in the Artificial Saliva Model System

ingredient	concentration w/v	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]
α-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.

(*15*). Twelve histatins, identified as histatin-1−12, have been found, and the most common histatin found in human saliva is 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} (*15*, *23*−*26*). This suggests that copper might influence flavor perception through an interaction with salivary components, especially copper-binding proteins.

The aim of this research is 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

Artificial Saliva. Artificial saliva was formulated as described by van Ruth and Roozen (*17*). The list of ingredients and their final concentration in the artificial saliva model system are shown in **Table 1**. $NaHCO_3$ (5.208 g), $K_2HPO_4 \cdot 3H_2O$ (1.369 g), NaCl (0.877 g), KCl (0.477 g), and $CaCl_2 \cdot 2H_2O$ (0.411 g) were separately dissolved in 100 mL of ultrapure water (Barnstead nanopure water purification system, Barnstead International, Dubuque, IA) to make 10-fold concentrated stock solutions. Both 0.216 g of mucin (from porcine pancreas, Sigma, St. Louis, MO) 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 was 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, 7.0, or 7.5. Artificial saliva was used within 1 h of formulation.

Aroma Compounds. 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 (*17*, *19*, *27*). The four aroma compounds were prepared in one stock solution by adding 1 μL of each aroma compound to ultrapure water in a 100 mL 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 °C) to dissolve aroma compounds. The final concentration of each aroma compound in the model systems was 0.5 μL/L (v/v).

Copper Source. $CuSO_4 \cdot 5H_2O$ (Sigma, St. Louis, MO) was prepared as a 200 mg/L 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 (*6*).

Sample Preparation. Artificial saliva (36 mL), copper stock solution (2 mL), and aroma compounds stock solution (2 mL) were pipetted into a prechilled 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 (*28*), sodium bicarbonate was dissolved in ultrapure water at the level of 1 mM to generate stable pH value. The pH of the 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. (*29*). 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 prechilled glassware in an ice bath to prevent evaporation of volatile compounds.

Solid-Phase Microextraction. Volatiles were trapped using a 75-μm carboxen-polydimethylsiloxane fiber (Supelco, Bellfonte, PA). Measurement of the adsorption/desorption of the volatiles was performed using the true-headspace sampling method (*27*, *30*). 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 s 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 (25 m × 0.32 mm, 1.05 μm 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) ($\alpha = 0.05$) using JMP IN statistical software (version 4.0, SAS, Cary, NC). Mean values of different treatments were compared using Tukey's HSD test.

Table 2. 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

	probability levels associated with *F* values								
	composition of the model system[c]			Cu concn (control, 2.5 mg/L)			interaction (composition × Cu)		
aroma compd	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 are values that are significant or nearly significant at $\alpha = 0.05$ within a column (within the same pH). [b] ANOVA of experiment data in triplicate. [c] Water, salt solution, or artificial saliva. Water is ultrapure water buffered with 1 mM $NaHCO_3$. Salt solution is $NaHCO_3$ (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 is 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 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 (*31*). 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 ($\alpha = 0.05$).

Copper Speciation in the Model System. Recent research of copper taste thresholds (*12*) 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 the salivary pH range (*12*). 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 (version 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

Table 2 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. The effect of the model system composition was significant for all four volatiles at different pH values. The effect of copper on volatility of selected compounds was significant only at pH 6.5, while interaction effects between composition and copper were only significant for selected compounds at pH 7.5.

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 1**).

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 (*18*, *19*). 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 (*18*, *19*). Proteins are known to bind with aldehydes, ketones, esters, and terpenes reversibly by hydrophobic interaction or irreversibly by covalent bonding (*32*−*34*). The main mechanism of aldehyde−protein association is known to be covalent bond formation via Schiff's base (*34*). However, in research on the binding of hexanal to soy proteins (*32*), 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 (*27*, *35*). Ketones are well-known organic ligands for proteins, especially β-lactoglobulin, and affinity of methyl ketones for β-lactoglobulin increased as the length of the hydrophobic chains of ketones increased (*27*).

The results of our work are not consistent with the previous studies, except for hexanal, which is known to form covalent Schiff bases with lysine (*32*). In the research of van Ruth et al. (*18*), 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 (*19*) 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) was 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 h) 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 regard to effects in the mouth than those in previous studies, may not be long enough to see flavor binding.

Effect of Copper. Copper showed a significant effect on the volatility of hexanal and 2-heptanone ($p < 0.05$) and a moderate effect on the volatility of butyl acetate ($0.05 < p < 0.1$) at pH 6.5 (**Table 2**). Headspace concentrations of those compounds were increased by ∼10% by addition of copper. Copper did not change the volatility of aroma compounds at pH 7.0 and it increased the headspace concentration of four volatile com-

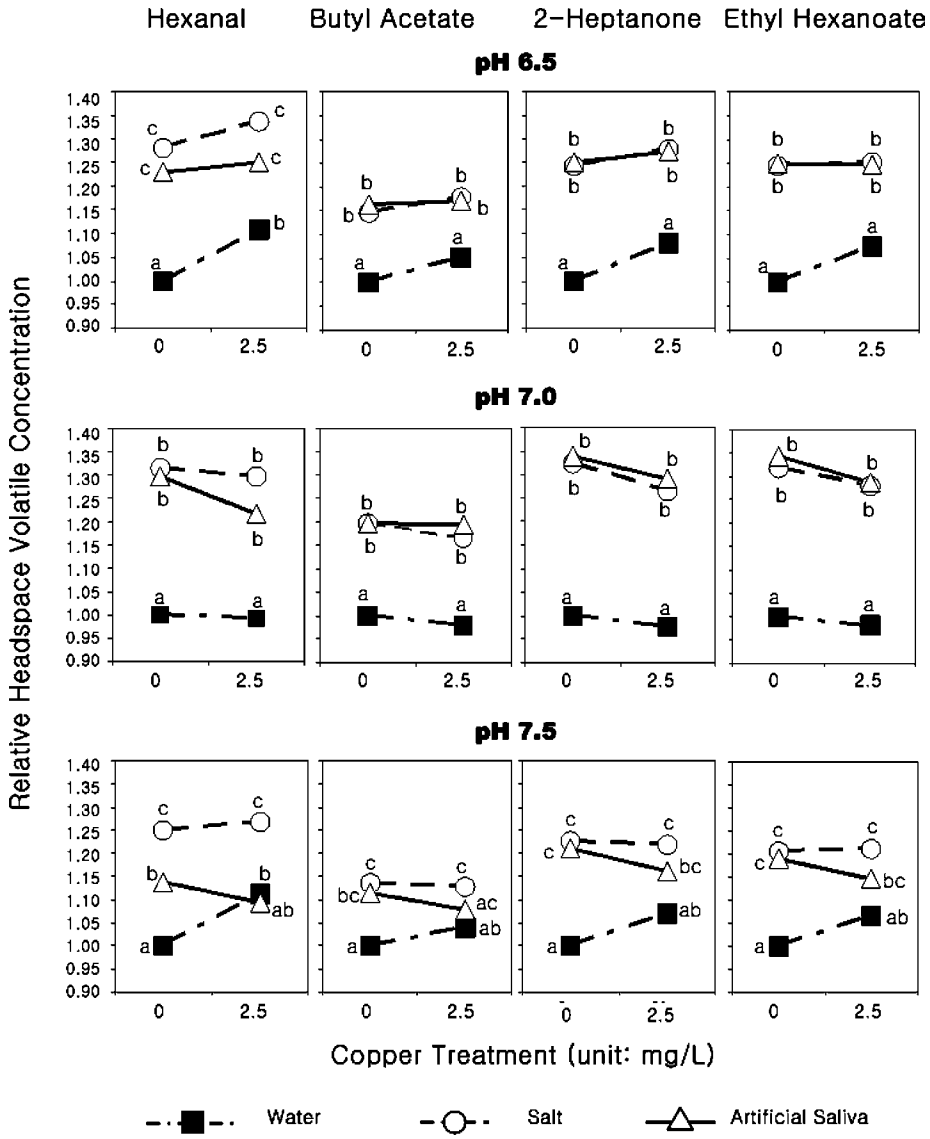

Figure 1. Relative headspace concentration of four aroma compounds at pH 6.5, 7.0, and 7.5 in ultrapure water (control), salt solution (salt), and artificial saliva with and without 2.5 mg/L copper. For each compound, results are normalized to the response of water (control). Different superscript letters indicates a significant effect ($p = 0.05$) between six treatments for an aroma compound at a given pH.

pounds by ~10% only in the water model system at pH 7.5 (**Figure 1**). The significant effect of copper 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 2**). Recent research of copper taste thresholds (*12*) 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, and (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 (*37*−*39*). 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 $Cu(CO_3)_2^{2-}$ (*36*). Thus it is assumed that solubilized copper may participate in a salting-out effect of electrolytes at pH 6.5 in the water system.

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 3**). Most carbonyl aroma compounds are well-known for 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 (*17*, *33*, *42*, *43*). In preparation of aroma stock solution, aroma compounds were solubilized in water by 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. The 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.

The increase in headspace volatile concentration at lower pH seems to contradict previous studies. In the research of Cao et

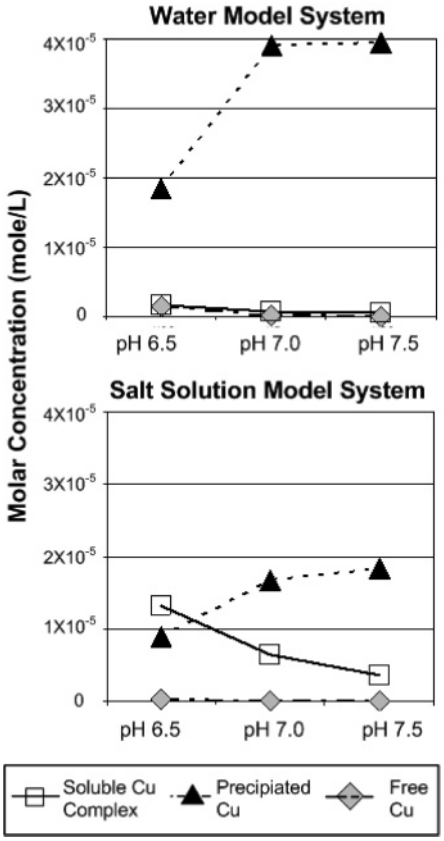

Figure 2. Concentration of soluble and precipitated copper in the water and the salt solution model system calculated by MINEQL+ chemical equilibrium modeling software.

Table 3. Physicochemical, Thermodynamic[a] (*40*), and Sensory Threshold Characteristics (*41*) of the Four Aroma Compounds

aroma compds	bp[b] (°C)	P_V[c] (mmHg)	S[d] (mg/L)	log P[e]	P[f] (atm·m^3/mol)	sensory threshold (mg/L)
hexanal	131	11.3	5640	1.78	2.13×10^{-4}	(5×10^{-3})–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}	(8.97×10^{-4})–3[h]
ethyl hexanoate	167	1.56	629	2.83	7.23×10^{-4}	0.021–0.85[i]
acetic acid[j]	117.9	15.7	10^6	–0.17	1×10^{-7}	
hexane[j]	68.7	151	9.5	3.9	1.8	

[a] All data were collected at 25 °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.

al. (*44*), dynamic light scattering (DLS) showed that mucin was transfigured from isotropic random coil to a 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 (*44*) showed steady changes in configuration over the pH range of 2−7, 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 the 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 a similar amount of precipitated copper species at pH 7.5 as at pH 7.0 (**Figures 1** and **2**). It suggests that volatility of aroma compounds is 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 2**). This implies that there is an 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 (p*I*) of mucin is pH 3−5 (*45*), 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 a decrease of charge repulsion between protein molecules, in turn reducing the hydrodynamic radius of mucin and changing protein structure (*19*, *46*). Friel and Taylor (*19*) 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 3**) 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 2**). 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 the negatively charged binding site of mucin. Also the simulation result of MINEQL+ 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 the 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 (*20*). 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 (*22*) 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 the 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 the effect of histatin, based on the observation that the 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 the histatin level (15.9−102.1 μM) found in human saliva (*31*).

Histatin is regarded as the most potent protein associated with copper sensation in the mouth, because of its copper-binding capacity (*23*). Binding of copper to histatin has been suggested to induce conformational change, which may be responsible for biological activity (*24*, *25*). Thus it was expected that changes in the conformation of histatin would contribute to volatilization of aroma in the model systems. However, histatin at 49.5 μM did not significantly change the 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 (*24*), 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 (*18*, *19*) and dynamic systems mimicking the human mouth (*17*, *47*). Retronasal aroma perception is a dynamic process that 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 (*47*). 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 (*33*). The static artificial model saliva system used in this study can help to understand retronasal aroma perception by providing information on the 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 (*48*), an increase of headspace concentration by 5−15% did not cause considerable changes in the predicted perceived intensity. However, a sigmoidal psychophysics function (*49*) shows different implications of changes in headspace concentration on aroma perception. Keast et al. (*49*) suggested that a psychophysics curve of a flavor compound has sigmoidal shape that has three distinctive sections: exponential growth at threshold to very low concentration, linear increase at low to medium concentration, and a plateau region at high concentration. Each section follows Steven's power function but has different *n* values (*49*)

$$I = kC^n$$

where I is the perceived intensity, k is a constant, C is the concentration of a compound, and n is an exponent that at threshold to very low concentration is >1, at low to medium concentration is $=1$, and at high concentration is <1.

It is 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 3**), it is assumed that the concentration used in our study is placed within threshold to 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 result 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 (*49*). 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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