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ORIGINAL PAPER

Operationally defined solubilization of copper and iron in human saliva and implications for metallic flavor perception

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Abstract Metals such as copper and iron cause unpleasant taste perceptions. Metallic compounds come in contact with saliva before delivery to taste receptors. Therefore, it is assumed that interactions between saliva and metallic compounds affect the perceptions of metals. The aim of this study was to determine the solubilization of metals in saliva and to examine whether or not perceptions of metallic flavor are influenced by metal solubility in saliva. Ten trained panelists evaluated the sourness, bitterness, astringency, electric sensation, and rusty nail-like retronasal aroma of copper sulfate ($CuSO_4 \cdot 5H_2O$, Cu) and ferrous sulfate ($FeSO_4 \cdot 7H_2O$, Fe) dissolved in ultrapure water at different concentrations. Total and soluble metals were measured in the subjects' saliva collected after tasting the samples using an inductively coupled plasma spectrometer. Approximately, 4.5–6.4% of Fe and 4.0–6.6% of Cu were retained in the saliva after expectoration. The proportion of soluble metal to total metal retained in saliva decreased from 0.68 to 0.29 for Cu and 0.019 to 0.0016 for Fe, as the metal concentration increased. In particular, Fe was solubilized in saliva at a maximum level of 4.5–4.6 μM, regardless of the metal concentration of the solution. The perceived intensities of sensory attributes showed positive linear relationships with log concentrations of total Cu, soluble Cu, and total Fe, but they did not have any relationship with soluble Fe. These results indicate that sensory perceptions of Fe were influenced mainly by the total Fe retained in saliva, whereas the perception of Cu was affected by soluble Cu as well as total Cu.

Keywords Copper · Iron · Metallic sensation · Sensory evaluation · ICP · Saliva

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Introduction

Metallic compounds such as salts of iron (Fe) and copper (Cu) are incorporated into food and beverage systems purposefully for fortification or accidentally by coming in contact with metal packaging. These metallic compounds cause sensations, often described as metallic, bitter, salty, sour, savory, astringent, tingling, or stinging [1, 2]. An extensive line of work on metallic sensations has reported that sensations of metal are transduced through the olfactory, gustatory, and trigeminal pathways [3–17]. More specifically, retronasal perception of metal-catalyzed oxidation by-products of oral tissue and electric tongue stimulation are suggested as two distinct perception mechanisms [4, 7–9, 15].

Recent studies suggest that metallic sensations may be influenced by not only the metal concentration but also the speciation of metallic compounds. In drinking water systems, there is a strong positive association between intensity and duration of metallic sensations and soluble copper concentration [18, 19], indicating the importance of soluble copper in the perception of metallic sensations. Soluble copper concentration in water is influenced by pH and other electrolytes present in the water [20].

Upon contact with saliva, approximately 2.6–24% of consumed copper becomes insoluble by interacting with salivary proteins or salivary electrolytes [21, 22]. Approximately, 76–98% of copper remains soluble, either as free copper or soluble copper complexes [23]. Soluble metal

species, such as free metal ions or unstable metal complexes, may contribute to metallic flavor by facilitating oxidative activity in the oral cavity. On the other hand, insoluble metallic species may be associated with astringency. Studies on polyphenolic compounds [24–26] suggest that insoluble salivary protein–polyphenolic compound complexes may be responsible for astringency by de-lubricating the oral cavity. For metallic compounds such as Fe, Cu, and Zn, a positive correlation between the turbidity of saliva and astringency perception has been observed [27]. However, these assumptions on the role of different metal species in the perception of metallic sensations have not been fully elucidated.

This study was conducted to investigate the influence of the solubilization of metals in saliva on the perception of metallic sensations using iron and copper, the two metals most commonly used in food systems. The outcomes of this study will contribute to a deeper understanding of the perception mechanisms of metallic sensations.

Materials and methods

Sensory evaluation

Stimuli

Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, Showa Chemical Co. Ltd., Tokyo, Japan) and copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, Showa Chemical Co. Ltd., Tokyo, Japan) were selected as sources of iron and copper, respectively. Each metallic compound was dissolved in ultrapure water purified with AquaMAX^{TM}-350 (Younglin Anyang-si, Kyeonggi-do, Korea) at various levels. The concentrations of copper were 0.05, 0.25, 0.5, and 1 mM. Iron was dissolved at levels of 0.5, 1, 5, and 10 mM. These concentrations of metallic compounds were decided by selecting within ranges from subjects' detection thresholds of metal sensations to the concentration at which the subjects began to show a strong rejection response, based on the results of preliminary tests. The samples were prepared within 1 h before the sensory evaluations to prevent precipitation due to aging. They were stored in 1-L amber Pyrex screw-cap storage bottles at room temperature (22 ± 2 °C) until used.

Sample presentation

The sample solutions were presented in 50-mL amber glass bottles to eliminate any visual differences, since solutions of ferrous and cupric salts each can have reddish-brown and blue hues. An aliquot of 30 mL of each sample was presented at room temperature (22 ± 2 °C), and the sample bottles were coded with random 3-digit numbers. Spitting cups and rinsing agents including ultrapure water, 1 mM ethylenediamine tetraacetic acid (EDTA) disodium salt (Kanto Chemical Co., Inc. Tokyo, Japan) solution, and saltine crackers (Charm Crackers, unsalted top, Crown Confectionery Co. Ltd., Seoul, Korea) were provided.

Panel selection

Ten panelists (ages 24–30, all women) were selected among a panelist pool of graduate students in the Dept. of Food Science and Engineering at Ewha Womans University (Seoul, Korea) through a series of triangle tests to find those who had the ability to discriminate 0.01 mM ferrous sulfate and copper sulfate from ultrapure water. The panelists had previous experience in sensory evaluations of various food products, but were naive to sensations of metallic compounds. They reported no tasting or health problems related to metals.

Panel training

The panelists were trained 3–4 times per week for 4 months, for approximately 1 h each session. During the training period, the panelists developed descriptors, their definitions, reference standard materials (Table 1), and evaluation procedures by consensus using copper sulfate and iron sulfate solutions with varying concentrations, including the samples to be tested in the main session. The ballot training [28] technique, in which panelists develop descriptors based on a given list of possible descriptors, was applied. The possible descriptor list was compiled from previous studies [2, 4–10, 15].

The training continued until all of the panelists reached a consensus agreement and produced consistent results over replications. Panelist performance was checked by performing analysis of variance (ANOVA) on the data set of three practices. The discriminating ability of the panel, reproducibility, and concept alignment were assessed by examining F values for samples of each panelist, panelist by replication interactions, and panelist by sample interactions, respectively. Individual panelists who showed different rating tendencies or poor reproducibility were identified through Duncan's multiple range test and received additional training.

Evaluation procedure

Generic descriptive analysis, a descriptive analysis that uses general guidelines of quantitative descriptive analysis (QDA®) and spectrum analysis™ with slight adaptations [28], was applied. The intensities of the sensory attributes were evaluated using a 15-point category scale labeled with weak and strong at the left and right ends, respectively.

While wearing a nose clip, the panelists sipped approximately 10 mL of a sample. They were asked to

Table 1 Definitions and reference standards for sensory descriptors developed for copper and iron solutions

	Attributes	Definitions	Reference standards
Taste	Sour taste	Fundamental taste sensation of which citric acid is typical	0.1 mM citric acid solution
	Bitter taste	Fundamental taste sensation of which caffeine is typical	1 mM caffeine solution
Trigeminal Sensation	Astringency	The feeling which shrivels the tongue associated with tannins	1 mM alum solution
Gustatory sensation	Electric sensation	A sensation evoked by a weak electrical stimulation	Aluminum-core exposed copper coin, ∅ 18 mm
Retronasal aroma	Rusty nail	Aromatic associated with rusty iron nails	Smelling the palm after rubbing a rusty nail[a] 5–6 times with 2–3 drops of water
Aftertaste	Rusty nail	Aromatic associated with rusty iron nails	Smelling the palm after rubbing a rusty nail[a] 5–6 times with 2–3 drops of water
	Bitter	Fundamental taste sensation of which caffeine is typical	1 mM caffeine solution
	Astringency	The feeling which shrivels the tongue associated with tannins	1 mM alum solution

[a] A 10-cm length iron nail that rusted previously by covering with wet cloth for 6 h

swirl it around in their mouth for 10 s and expectorate. Then, the panelists instantly recorded the taste and mouthfeel perceived at the moment of expectoration on a score sheet. Upon completing the ratings of taste and mouthfeel approximately 1 min after sipping, the panelists removed their nose clips and evaluated retronasal aroma and aftertaste. This procedure was developed to minimize sensory interactions between gustatory and tactile sensations and ortho/retronasal sensations [2, 5, 13, 15].

The panelists rinsed their mouths out with a 1 mM EDTA solution, which was shown to be the most effective rinsing agent for metallic solutions in previous studies [29], and then ultrapure water. The panelists chewed and expectorated saltine crackers as a supplementary rinsing agent to reduce carry-over effects. The panelists were asked to take a 2-min mandatory break between the samples.

The sensory tests were conducted in individual booths in a sensory testing room. All samples were evaluated in triplicate, and each session took approximately 20 min. Each metallic compound was tested in separate sessions, and samples of different concentrations were presented in a random order during each session. The panelists were not allowed to consume any food or drink other than water, or use any oral care products or strong perfumes 2 h before the test.

Operational determination of soluble and total copper and iron in saliva

Saliva collection

Unstimulated whole saliva was collected from the 10 subjects who had participated in the sensory test for 1 month following the test. The subjects were asked not to consume any food, drink, or oral care products other than drinking water for 1 h before collecting the saliva. If necessary, the subjects were asked to brush their teeth without using toothpaste and rinse their mouths thoroughly with tap water 30 min before the collection, in order to remove any foreign material in the mouth.

Right before the collection, the subjects rinsed their mouths with ultrapure water three times and waited for 3–5 min until salivary secretion was brought back to its usual level. Saliva was collected twice, before and after swirling a sample around in the mouth. The subjects collected saliva behind closed lips and expectorated once every 20–30 s for 1 min. After waiting for 3–5 min again, the subjects sipped and swirled around 10 mL of a sample or ultrapure water (control) for 10 s and expectorated. Saliva was collected for 1 min immediately after expectoration.

Saliva collection was performed approximately for 10–15 min, twice each day at 10:30 am and 4:00 pm. Only one sample was presented during a collecting session. Samples of various copper and iron concentrations were presented randomly within each replicate. However, in order to minimize any potential influence of residual metals on the subsequent saliva collection, the presentation order was modified, so that a solution of higher concentration was not presented before the solution of lower concentration. Saliva was collected in triplicate from each subject four times per week over 1 month.

The saliva collected from each subject was transferred to 1.5-mL Eppendorf tubes and stored at −20 °C until analysis within 2 months. All collection procedures were performed on ice to prevent any enzymatic degradation of

salivary compounds that might influence the solubility of metals in saliva. All glassware and plasticware used for saliva collection had been soaked in 3% nitric acid overnight and thoroughly rinsed with ultrapure water to remove any copper or iron residue.

Analysis of soluble and total metal concentrations in saliva

The saliva samples were defrosted at room temperature for 2–3 min right before analysis and then mixed with a vortex mixer (Genius3, IKA Works, Inc., Wilminton, NC, USA) for 30 s to re-disperse any insoluble matter [30]. Equal volumes (0.5 mL) of saliva collected from each subject were pooled. Another 0.5 mL of saliva, which was collected from each subject before sipping a sample, was commingled, and the pH of this pooled saliva was measured using a pH meter (ph-200L, iSTEK, Inc., Seoul, Korea).

An aliquot of 1 mL was taken from the pooled saliva for total metal analysis. To measure soluble metal concentration, 1.5 mL of the pooled saliva was centrifuged for 30 s at 16,000×*g* (Micro 17TR, Hanil Science Industrial Co., Ltd., Inchun, Korea) to remove large insoluble particles, after which the supernatant was filtered through a 0.2-μm syringe filter (DISMIC-25, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). One milliliter of the filtered sample was analyzed to determine the soluble metal concentration.

The samples were wet-washed for inductively coupled plasma (ICP) spectrometry by the method of Hong et al. [23]. A volume of 1.0 mL of nitric acid (Duksan Pure Chemical Co., Ltd., Ansan, Kyeonggi-do, Korea) was added to saliva samples placed in Pyrex tubes. The tubes were closed with Teflon-lined caps and heated at 90 °C for 45 min in a heating block (HB-1, Wealtec Corp, Sparks, NV, USA). After cooling the tubes to room temperature, 0.5 mL of hydrogen peroxide (35%, Duksan Pure Chemical Co., Ltd., Ansan, Kyeonggi-do, Korea) was added. The tubes were gradually heated to and held at 130 °C for 2 h without caps. The samples were then brought to a volume of 10 mL with ultrapure water. The metal concentrations of the samples were determined using an ICP-optical emission spectrometer (OES; ICPS-7510, Shimadzu, Kyoto, Japan). The conditions for analysis are shown in Table 2. The analyses were performed in triplicate.

Statistical analysis

Analysis of variance (ANOVA) was performed to examine the effects of different metal concentrations on the intensities of sensory attributes in each metallic compound solution ($p < 0.05$). Duncan's multiple range test was carried out as a post hoc comparison ($p < 0.05$). Regression analysis was conducted to identify the relationships between the intensities of sensory attributes and the concentrations of soluble or total metal in saliva ($p < 0.05$). All statistical analyses were performed using SPSS for Windows (ver. 15.0, SPSS Inc., Chicago, IL, USA).

Table 2 The condition for ICP-OES analysis

Description	Conditions
R.F. Generator	27.12 MHz 0.05% (ISM band)
R.F. Power	1.2 kW
Plasma torch	3 concentric, fassel type
Nebulizer	Conical type
Gas flow rate	Carrier gas 0.7L/min
	Coolent gas 14L/min
	Purge gas 3.5 mL/min
Observation height	15 mm above load coil
Wavelength	Cu 324.754 nm, Fe 259.940 nm
	Entrance 20 μm
Slit width	Exit 30 μm
Torch unit	Cyclonic chamber
Number of grooves	3,600/nm for 160–458 nm
	1,800/nm for 458–850 nm

Results and discussion

Sensory evaluations

The sensory attributes of copper and iron and their perceived intensities are shown in Fig. 1. The panelists developed bitter taste, sour taste, electric sensation, rusty-nail retronasal aroma, astringency, and residual sensations of bitterness, astringency, and rusty-nail flavor as sensory descriptors for copper and iron sulfate by consensus. During the process of concept formation and alignment [31], panelists agreed that the metallic nature of copper sulfate and iron sulfate was explained well by the terms rusty nail flavor and electric sensation. Other attributes reported in previous studies, such as sweet taste, umami, salty taste, and spiciness, were not chosen in this study. These attributes were derived from other divalent metal ions (Zn, Ca, or Mg) or various ligands [2, 9]. Since this study used only two metals (Fe and Cu) with the same anionic ligand (SO_4^{2-}), those descriptors only suitable to describe the attributes of copper and iron were selected during the attribute development session.

For copper, metal concentration had a significant effect on the intensities of all sensory attributes ($p < 0.001$ for all attributes). Duncan's multiple range test showed that there were significant differences among all of the samples for all attributes, except sour taste. For iron, ANOVA indicated that there were significant differences among the samples

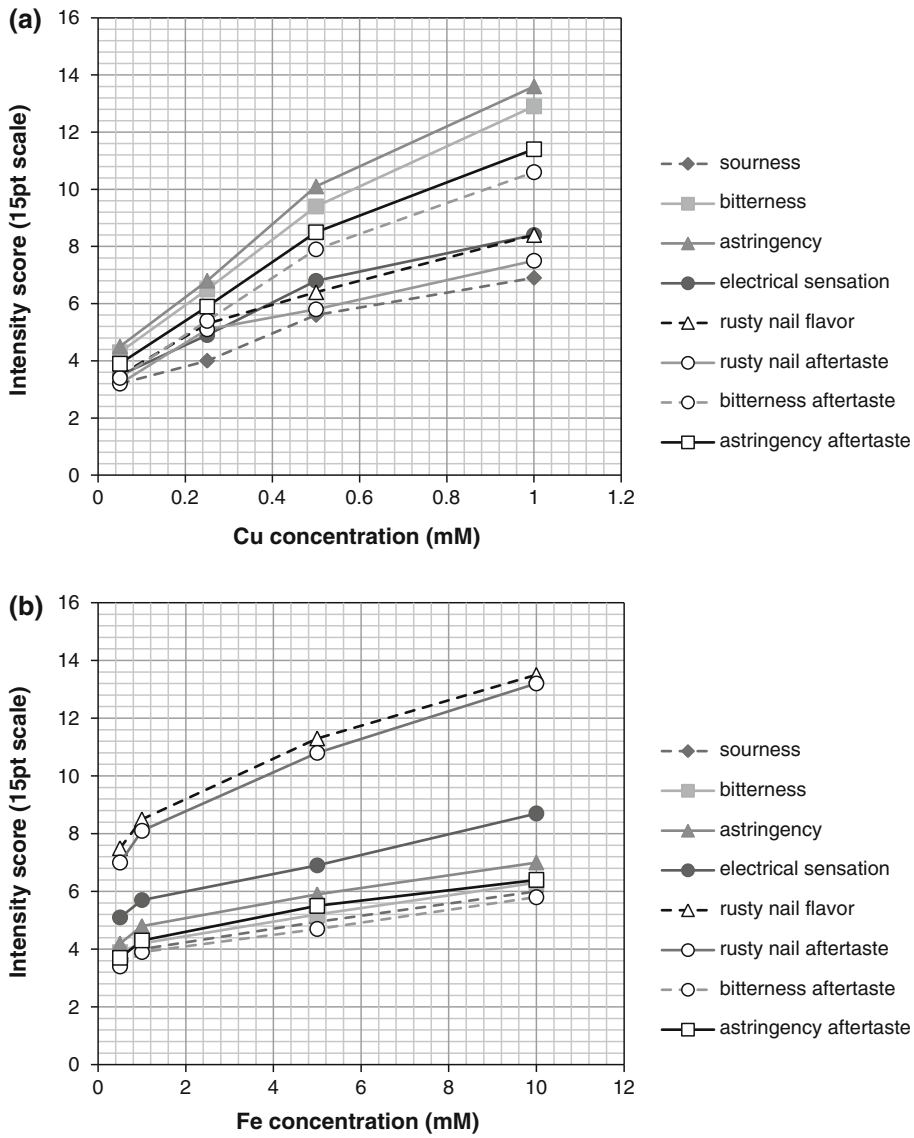

Fig. 1 Mean intensities of sensory attributes for **a** cupric sulfate, **b** ferrous sulfate

in all sensory attributes ($p < 0.001$ for all attributes), except bitter taste and bitter aftertaste. Intensities of bitter taste and aftertaste were not significantly different between the 0.5 and 1 mM Fe solutions.

Figure 1 indicates that the subjects perceived strong bitter taste, astringency, and aftertaste from copper, whereas iron was characterized with a pronounced rusty nail-like retronasal aroma. These results were consistent with previous studies that investigated the sensory qualities of metallic compounds [4, 6, 8, 9, 15]. The elimination of retronasal aroma had a greater negative impact on perceived intensities of metallic in ferrous sulfate solution than cupric sulfate solution [13]. In particular, oral contact is necessary for the development of a metallic flavor [15]. Glindemann et al. [16] detected several volatile compounds, including hexanal and 1-octen-3-one, from the headspace of skin rubbed with solutions of copper and iron compounds. These compounds are generally described as metallic and are also known to be lipid oxidation products [32–36]. Omur-Ozbek [29] investigated the roles of copper and iron ions with different valence states in metallic flavor perception by determining taste thresholds and malondialdehyde (MDA) values in saliva after drinking iron and copper solutions. Ferrous (Fe^{2+}) ion produced the most MDA in saliva, followed by cupric (Cu^{2+}) and cuprous (Cu^{+}) ions. On the other hand, ferrous was detected at concentrations as low as 0.9 μM in a nose-open condition, whereas cupric and cuprous were detected at 7.6 and 9.6 μM, respectively. These data support the hypothesis that the retronasal perception of carbonyls, by-products of oral lipid oxidation, plays an important role in metallic flavor perception. In addition, the results provide an

Table 3 Mean and standard deviation values of total and soluble copper concentrations (μM) and recovery rates (%) remaining in saliva after holding cupric sulfate solution for 10 s in the mouth and expectorating (10 subjects, repeated in triplicates)

Concentration of metallic compounds incorporated in saliva (mM)	Total metal		Soluble metal		% Soluble metal[a]
	Concentration (μM)	Recovery rate[b] (%)	Concentration (μM)	Recovery rate (%)	
Before[c]	0.58 (0.18)[d]	–	0.23 (0.11)	–	40.0 (0.12)[e]
0[f]	0.31 (0.01)	–	0.17 (0.03)	–	55.4 (6.98)[e]
0.05	3.61 (0.45)	7.27 (0.91)	2.40 (0.32)	4.82 (0.64)	67.8 (8.08)
0.25	14.68 (2.74)	5.78 (1.10)	6.30 (0.47)	2.52 (0.19)	43.8 (9.93)
0.5	20.35 (3.43)	4.07 (0.69)	9.43 (1.69)	1.89 (0.34)	46.5 (6.21)
1	55.91 (1.60)	5.59 (0.16)	16.29 (1.70)	1.63 (0.17)	29.1 (3.79)

[a] [(Mass of soluble metal (mole) in saliva − mass of soluble metal in saliva after drinking ultrapure water)/(total mass of metal (mole) in saliva − total mass of metal in saliva after drinking ultrapure water)] × 100

[b] [Mass of metal (mole) found in a fraction/(total mass of metal taken orally − mass of metal in saliva after drinking ultrapure water)] × 100

[c] Saliva obtained before taking metal solutions

[d] Mean (standard deviation)

[e] (Mass of soluble metal (mole)/mass of total metal) × 100

[f] Ultrapure water containing no metal

Table 4 Mean and standard deviation values of total and soluble iron concentrations (μM) and recovery rates (%) remaining in saliva after holding ferrous sulfate solution for 10 s in the mouth and expectorating (10 subjects, repeated in triplicates)

Concentration of metallic compounds incorporated in saliva (μM)	Total metal		Soluble metal		% Soluble metal[a]
	Concentration (μM)	Recovery rate[b] (%)	Concentration (μM)	Recovery rate (%)	
Before[c]	17.04 (5.37)[d]	–	4.64 (0.94)	–	29.97 (14.81)[e]
0[f]	4.12 (0.40)	–	3.80 (0.58)	–	93.48 (21.07)[e]
500	31.62 (0.32)	6.38 (0.07)	4.20 (0.34)	0.85 (0.07)	1.90 (1.59)
1,000	58.93 (5.24)	5.92 (0.53)	4.64 (0.14)	0.46 (0.005)	1.48 (1.14)
5,000	231.91 (6.99)	4.64 (0.14)	4.48 (1.28)	0.09 (0.03)	0.68 (0.16)
10,000	445.52 (26.36)	4.46 (0.26)	4.51 (0.42)	0.05 (0.004)	0.16 (0.14)

[a] [(Mass of soluble metal (mole) in saliva − mass of soluble metal in saliva after drinking ultrapure water)/(total mass of metal (mole) in saliva − total mass of metal in saliva after drinking ultrapure water)] × 100

[b] [Mass of metal (mole) found in a fraction/(total mass of metal taken orally − mass of metal in saliva after drinking ultrapure water)] × 100

[c] Saliva obtained before taking metal solutions

[d] Mean (standard deviation)

[e] (Mass of soluble metal (mole)/mass of total metal) × 100

[f] Ultrapure water containing no metal

explanation of the different sensory profiles between copper and iron, particularly in terms of the differences in rusty nail-like retronasal aroma.

Operational determinations of soluble and total copper and iron in saliva

Mean pH of saliva pooled from 10 human subjects was 7.7 ± 0.22, showing a slightly higher pH value than previously reported values (pH 6.7–7.5) [22, 37]. It was often observed that the pH of unstimulated whole saliva is usually 6.5–6.9 [38], whereas the pH of stimulated whole saliva ranges from 6.30 to 8.08 [39]. The pH of the saliva used in this study was close to that of stimulated saliva, suggesting that the rinsing procedure with ultrapure water might have disturbed resting status.

Tables 3 and 4 show concentrations of residual copper and iron in saliva, respectively. When 10 mL of ultrapure water was taken into the mouth, approximately half (0.31 μM) of the copper in the saliva (0.5 μM) remained, indicating significant dilution. For saliva in contact with copper solution, approximately 4–7% of the copper incorporated in the mouth remained after expectoration (Table 3). Among the total residual copper in saliva, approximately 30–70% was in soluble form. The recovery rate of soluble copper in saliva decreased from 4.8% in

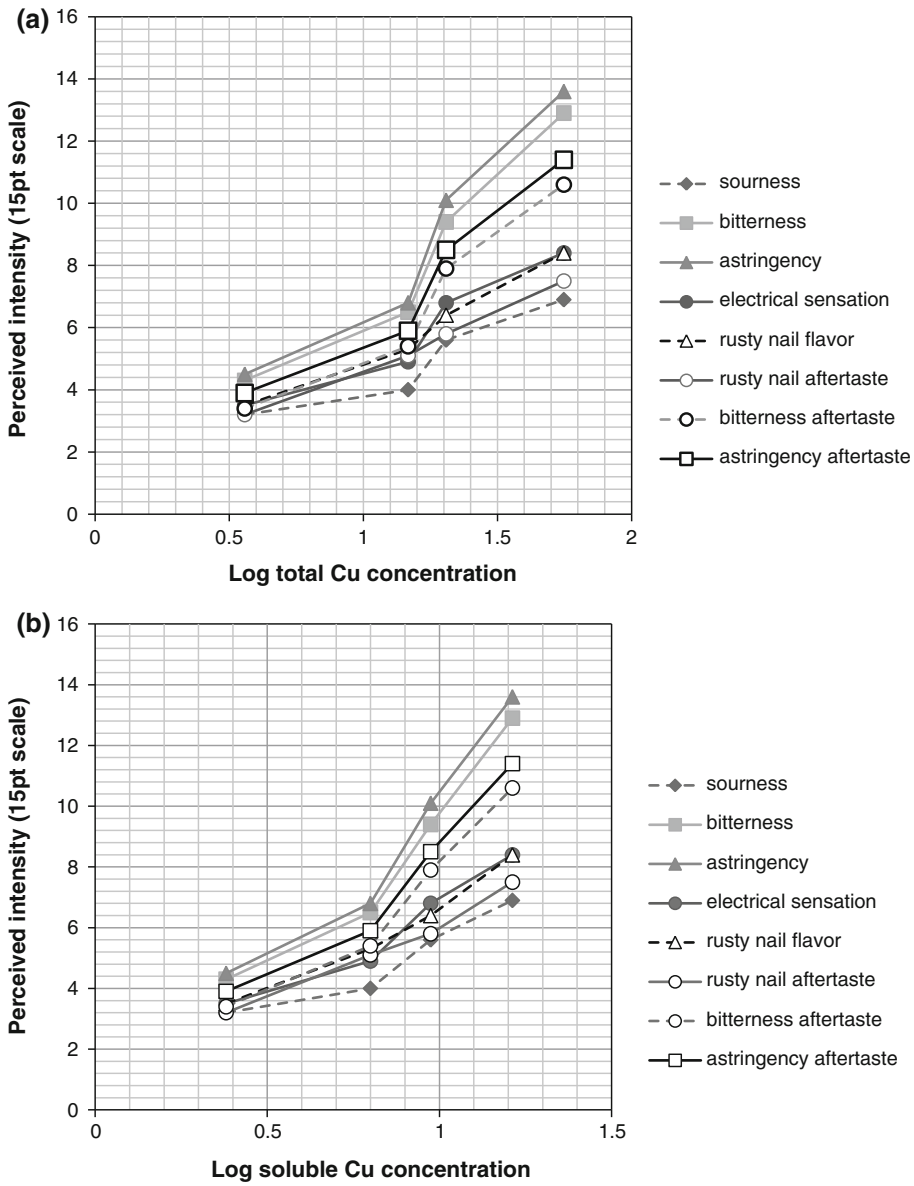

Fig. 2 A semi-log plot of relationship between perceived intensities of sensory attributes and copper concentration in saliva: **a** total copper, **b** soluble copper

saliva in contact with 0.05 mM Cu to 1.6% in saliva collected after holding 1 mM Cu. This result indicates that copper was dissolved in saliva only to a certain degree. Hong et al. [21] reported that copper forms insoluble malachite ($Cu_2CO_3(OH)_2$) by interacting with salivary anions, bicarbonate (HCO_3^-), and hydroxide (OH^-). In addition, copper has low solubility in salivary pH ranges [20]. Cuppett et al. [18] observed that copper has a maximum solubility of 1.3 mg/L in water at pH 7.4, but copper becomes more soluble at pH 5.5. When copper was added to saliva at a level of 10 mg/L at pH 7.04, only 58.7% of added copper was solubilized [23].

The iron concentration of saliva collected before sipping the samples, which represents the iron naturally existing in saliva, was 17.0 μM (Table 4), and approximately 30% of total iron (4.6) was soluble. This result is consistent with the salivary iron concentration of 18.6 μM (103.9 μg/dL) reported by Mishra et al. [40]. After sipping ultrapure water, the total iron content of saliva decreased by approximately 76%, from 17.0 to 4.1 μM. The recovery rate of total iron in saliva was 4.5–6.4%. Soluble iron was recovered at concentrations of 4.2–4.6 μM, regardless of the concentration of iron in the samples. Iron seemed to have a maximum solubility of approximately 4 μM in saliva. In an aqueous medium, iron exists in the ferrous or ferric (Fe^{3+}) form, depending on the pH and redox potential of the medium. Ferrous (Fe^{2+}) ion is generally present with a colorless dissolved status, whereas ferric ion has low solubility and forms orange-brown precipitates [41]. At pH levels below 8, Fe^{2+} can be dissolved at more than 100 mg/L in water [20]. However, in the presence of air, Fe^{2+} can be easily oxidized to Fe^{3+} [42]. Since air constantly flows in and out of the oral

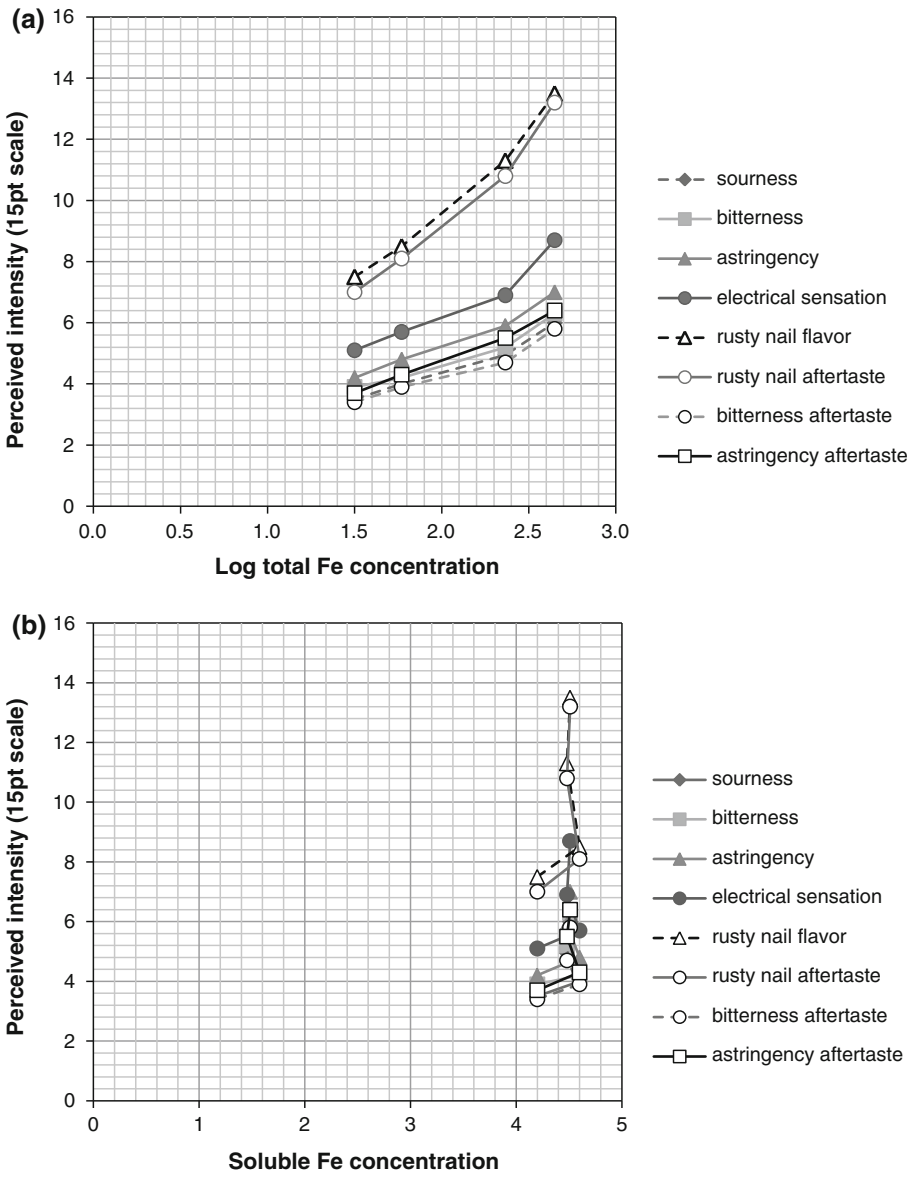

Fig. 3 Relationships between perceived intensities of sensory attributes and **a** total iron concentration of saliva as a semi-log plot, **b** soluble iron concentration of saliva as a linear scale plot

cavity through the upper respiratory tract during breathing and eating, it seems that Fe^{2+} in the saliva is oxidized to Fe^{3+}, leading to decreased solubility. Under higher pH and temperature conditions, oxidation to Fe^{3+} occurs faster. For example, it was shown that 90% Fe^{2+} is oxidized in 30 s at pH 8.0, whereas it takes 100 h at pH 6.0; similarly, it takes 1 h for 90% Fe^{2+} to be oxidized at 21 °C at pH 7.0, whereas it takes 10 h at 5 °C at the same pH [41]. Various ligands in saliva such as lactoferrin, bicarbonate, and phosphate affected the solubility of iron differently. Lactoferrin increases the solubility of iron, while bicarbonate and phosphate form insoluble complexes [42, 43]. This suggests that the oral conditions provide favorable circumstances for the formation of insoluble iron species, since the salivary pH in this study was slightly basic (7.7) and the temperature was warm (36.5 °C). The higher bicarbonate and phosphate concentrations (0.06–3.6 mg/mL, 0.21 mg/mL, respectively) compared to the lactoferrin concentration (1–2 μg/mL) of saliva [37, 44] might also have played a role in the formation of insoluble iron species.

The relationship between the intensities of sensory attributes and the concentrations of total and soluble copper and iron in saliva

The mean intensity ratings for each sensory attribute versus the log of total and soluble metal concentrations in saliva are shown in Figs. 2 and 3. The perceived intensity of each attribute was positively related with total copper, soluble copper, and total iron concentrations (Figs. 2a, b, 3a, respectively). However, the soluble iron concentration did not show any specific relationship with perceived intensity (Fig. 3b).

Table 5 Regression equations and coefficients of determination (R^2) associated with the relationships between total metal concentration in saliva and perceived intensities of sensory attributes, and between soluble metal concentrations in saliva and perceived intensities of sensory attributes

Physical stimulus	Sensory attributes	Cu		Fe	
		Regression equation[a]	R^2	Regression equation	R^2
Total metal	Sourness	$Y = 3.16X + 1.15$	0.89	$Y = 2.06X + 0.36$	0.97
	Bitterness	$Y = 7.26X + 4.03$	0.92	$Y = 2.01X + 0.75$	0.95
	Astringency	$Y = 7.72X + 0.47$	0.91	$Y = 2.31X + 0.68$	0.98
	Electrical sensation	$Y = 4.19X + 0.88$	0.92	$Y = 2.90X + 0.59$	0.93
	Rusty nail flavor	$Y = 4.12X + 0.98$	0.97	$Y = 5.12X - 0.40$	0.98
	Rusty nail flavor aftertaste	$Y = 3.61X + 1.09$	0.99	$Y = 5.22X - 1.04$	0.98
	Bitterness aftertaste	$Y = 6.11X + 0.48$	0.93	$Y = 1.93X + 0.44$	0.95
	Astringency aftertaste	$Y = 6.35X + 0.17$	0.92	$Y = 2.28X + 0.25$	0.99
Soluble metal	Sourness	$Y = 4.48X + 1.15$	0.91	NA[b]	NA
	Bitterness	$Y = 10.20X - 0.31$	0.93	NA	NA
	Astringency	$Y = 10.87X - 0.40$	0.93	NA	NA
	Electrical sensation	$Y = 5.94X + 0.90$	0.94	NA	NA
	Rusty nail flavor	$Y = 5.75X + 1.06$	0.97	NA	NA
	Rusty nail flavor aftertaste	$Y = 5.03X + 1.17$	0.98	NA	NA
	Bitterness aftertaste	$Y = 8.61X - 0.42$	0.94	NA	NA
	Astringency aftertaste	$Y = 8.95X - 0.10$	0.93	NA	NA

[a] Y = perceived intensity measured with a 15-point category scale, X = log concentration of metals

[b] Not applicable

The coefficients of determination (R^2; Table 5), as measures of goodness of fit, showed that Fechnerian semilog linear equations (mean perceived intensity = k_1 log (concentration) + k_2, where k_1 and k_2 are constants) fit well into the relationship between perceived intensities and log concentrations of total copper, soluble copper, and total iron in saliva, within the concentration range used in this study [28]. This suggests that perceived intensity increased at a much slower rate as the metal concentration increased. In addition, the higher slope values observed in the soluble copper data (Table 5) indicate that the changes in perceived intensities were more sensitive to soluble copper concentration compared to total copper concentration.

It was initially hypothesized that olfactory and gustatory sensations of metallic compounds, such as bitter taste, sour taste, metallic flavor, and electric sensation, are influenced by soluble metal concentrations in saliva, whereas astringency exhibits a close relationship with insoluble metallic species such as metal–salivary compound complexes [27]. This hypothesis is based on the assumptions and previous observations that (1) soluble metal species, either free or unstable complexes, are more active in catalyzing oxidation [45]; (2) the concentration of soluble copper species have a positive relationship with metallic flavor [19]; and (3) salivary proteins form insoluble complexes with metallic species [22], which may cause astringent sensations in the mouth by de-lubricating the oral cavity [27].

The results of this study support only part of this hypothesis. For the copper solutions, increases in the total and soluble copper concentrations were positively associated with increases in intensities of sour taste, bitter taste, electric sensation, rusty-nail retronasal aroma, and their aftertaste (Fig. 2; Table 5). These results imply that soluble copper concentration played an important role in the olfactory and gustatory perception of copper. Astringency also increased linearly as total copper concentration increased. It is assumed that, if the increase in astringency was due to an increase in total copper rather than soluble copper, then the relationship between astringency and soluble copper concentration would show a flatter slope, or even a negative slope compared to those of olfactory or gustatory sensations. However, astringency became stronger as the soluble copper concentration increased and exhibited a steeper slope when plotted against log soluble copper concentration. This indicates that there was a stronger linear relationship between log soluble copper concentration and perceived astringency (Fig. 2b; Table 5), implying that astringency was influenced by soluble metallic species, not insoluble species.

Iron showed a different tendency from copper. There was a positive relationship between total iron concentration and perceived intensities (Fig. 3a; Table 5), but an increase in the soluble iron concentration did not have an impact on the perceived intensities (Fig. 3b). It was hypothesized that

the strong rusty-nail flavor of iron is caused by the oxidation of oral tissue lipids induced by soluble iron, a more labile species than insoluble iron. However, the perceived intensities were associated with total metal concentration in saliva. The results of the ICP-OES analysis suggest that the majority of ferrous ions taken in saliva were oxidized to ferric form under aerobic oral conditions. Omur-Ozbek and Dietrich [46] reported that ferric ion was not distinguishable from ultrapure water even at 360 μM in a taste threshold test, and subjects described ferric ion as being tasteless or having a faint sour or salty note. The relationship between iron speciation and metallic sensation observed in this study leads to questions concerning the role of soluble iron species in metallic flavor perception. For a clearer understanding of the relationship between soluble iron species and metallic flavor perception, it may be necessary to develop a real-time, or if possible, in situ analysis method to capture iron speciation in the oral cavity at the moment of contact to taste receptors.

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