# Role of Lipid Oxidation, Chelating Agents, and Antioxidants in Metallic Flavor Development in the Oral Cavity

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**ABSTRACT:** This study investigated the production of metallic flavor, which is a combination of taste and retronasal odor. Chemical reactions in the oral cavity and saliva of healthy subjects were investigated after ingesting iron and copper solutions above and near threshold levels. Significant increase in lipid oxidation ($p < 0.001$) occurred after metal ingestion, detected as TBARS values. Ferrous ion caused the greatest flavor sensation and lipid oxidation, followed by cupric and cuprous ions. Ferric ion did not cause metallic sensation. Occurrence of oxidation was supported by damage to salivary proteins, detected as protein-carbonyls, and by a significant increase of odorous lipid oxidation related aldehydes. Sensory evaluation demonstrated that antioxidants (vitamins E and C) minimally reduced metallic flavor but that chelating agents (EDTA and lactoferrin) removed the metallic flavor. The role of lipid oxidation is essential for the production of a metallic flavor from ingestion of ferrous, cupric, and cuprous ions.

**KEYWORDS:** *metallic flavor, iron, copper, lipid oxidation, TBARS, antioxidants, chelating agents*

## ■ INTRODUCTION

A displeasing metallic sensation can be caused by the essential micronutrients, e.g., iron and copper, in foods, beverages, and drinking water which may result in consumer complaints or loss of trust in the product.[1] Metallic flavor problems are also an issue for about 2 million cancer patients receiving chemotherapy and radiotherapy who encounter metallic flavor perception after consuming foods and beverages.[2] Reported individual thresholds vary from 0.1 to >10 mg/L (from 1.57 to >157 $\mu M$) for cupric ($Cu^{2+}$) ion and from 0.003 to 3 mg/L (from 0.054 to 53.7 $\mu M$) for ferrous ($Fe^{2+}$) ion; sensory characteristics are described as metallic, bitter, and astringent.[2−5] Recent studies showed that soluble metals are readily perceived, but particulate or precipitated metals cause low or no sensory perception.[3,5]

Olfactory (retronasal) perception related to metallic flavor was documented first by Hettinger et al.[6] When occluding noses of the panelists, taste thresholds of iron were found to be 10 to 30 times higher than the flavor threshold determined with the nose open.[7] Concentrations, in the range of 0.001 to 1 mg/L (from 0.018 to 18 $\mu M$), of ferrous, cupric, and cuprous ions were recently demonstrated to provide a strong retronasal metallic odor with weaker bitter, salty, and astringent taste perceptions.[4] Studies of metallic odor on human skin demonstrated the formation of odorous carbonyls including $C_6−C_{10}$ aldehydes and 1-octen-3-one.[8] The food industry has studied the metallic-smelling compounds generated as a result of oxidation of unsaturated fatty acids in foods. *trans*-4,5-Epoxydecenal, 1-octen-3-one, *cis*-1,5-octadien-3-one, and 1-penten-3-one were detected in foods and beverages.[9,10] Hexanal is a common lipid oxidation byproduct in meat products.[11]

There is little information on oxidation of human oral tissues by ingested metals, even though metallic flavor occurs during ingestion of metals. Metallic sensation in the mouth has two components: (1) taste of metal ions on the tongue and (2) the retronasal odor perception of carbonyls produced as a result of lipid oxidation catalyzed by metals in the mouth. It was shown that the phospholipids in cell membranes may be oxidized by the iron redox cycle.[12] Unsaturated fatty acids (that form the phospholipids) are very prone to oxidation leading to the formation of carbonyls including malondialdehyde (MDA),[13] which is an indicator of oxidative stress that can be measured colorimetrically through a reaction with thiobarbituric acid reactive substances (TBARS).[14,15] Specific carbonyls can be identified by derivatization with 2,4-dinitrophenylhydrazine (DNPH).

In the food literature, oxidation of lipids is indicated as a main reason for off-flavor production; however, oxidation of proteins is very important as well, especially for monitoring disease progress for patients and texture and taste quality in food.[16] The byproduct of lipid oxidation, including carbonyls and hydroperoxides, can bind to proteins and inactivate them.[17] One of the methods to detect protein oxidation is measuring protein carbonyls through derivatization with DNPH followed by Western blotting.[18,19] Lipid oxidation and damage to proteins may be prevented or reduced by the administration of antioxidants and chelating agents to the oral cavity. Vitamins C and E, and $\alpha$-lipoic acid are important cofactors of enzymatic systems in the body and are good radical scavengers.[20] Vitamins C and E, as well as EDTA, BHT, and BHA are among the antioxidant food additives.[21−23] Vitamin C (L-ascorbic acid) is water-soluble, whereas vitamin E ($\alpha$-tocopherol) is fat soluble.[23] While antioxidants donate hydrogen to scavenge

radicals, chelating agents inactivate the redox active metals before they create free radicals.[21] EDTA chelates with iron and copper.[22] Lactoferrin, a protein mainly found in milk and other secretions by humans such as tears and saliva, binds to iron.[24]

The goal of this study was to investigate the chemical reactions that cause and mitigate metallic flavor production in the oral cavity. Three specific objectives were pursued: (1) effects of ingested ferrous iron were studied through sensory analysis and measurement of lipid oxidation in the oral cavity; (2) oral lipid oxidation from iron and copper salts was compared; and (3) antioxidants and chelating agents were evaluated for their ability to prevent and/or reduce oxidation and metallic off-flavor production.

## ■ MATERIALS AND METHODS

**Subjects.** This study (IRB#06-395) was approved by the Institutional Review Board at Virginia Tech. Subjects were selected from the students, faculty, and staff of Virginia Tech that had no chronic oral or general health problems, no known taste or smell dysfunctions, and that were nonsmokers. All subjects read and signed the consent form before the sample collection began. Twenty-two subjects with an age range of 19 to 53 participated in various portions of the study over the period of two and a half years.

**Saliva Collection and Sensory Testing.** Subjects were instructed to refrain from eating and drinking at least 30 min prior to saliva collection, which occurred twice a week at midmorning to prevent bias from diurnal variations. For each collection, subjects rinsed their mouths with reagent water (from the Barnstead Nanopure filter (NJ)) and rested for 2 min to have standard conditions in the mouth. Whole saliva was collected as explained below. Subjects received two 3 oz Solo cups: one containing 2 mL of control (reagent water) and the other containing 2 mL of metal solution (solution of either iron or copper ions in reduced or oxidized states) at pH values around 5.5. High purity ferrous sulfate, ferric sulfate, cuprous chloride, and cupric chloride were purchased from Fisher Scientific (Pittsburgh, PA), and metal solutions were prepared in reagent water fresh daily prior to testing. Subjects sipped the entire (2 mL) control solution, swished it around their mouths for 15 s, and expectorated until 4 mL of saliva was collected in a 15 mL polypropylene centrifuge tube. After a short rest, subjects completed the same procedure for the metal solution. Saliva samples were either immediately analyzed or frozen to −18 °C or to −80 °C for storage until the subsequent analysis could be performed. For the antioxidant or chelating agent studies, the subjects rinsed their mouths with 3 mL of the selected antioxidant or chelating agent solution for 10 s. This rinse step was completed: once before or once after sampling the metal solution during two different experiments conducted on different days.

**Detecting Lipid Oxidation: TBARS Method.** After exposure to 180 $\mu$M ferrous iron, saliva samples were collected from 19 subjects (age range: 24 to 53, 8 females—11 males) three to five different times over 8 months as described above. Later, 180 $\mu$M ferric, cuprous, and cupric ion solutions were tested with 13 subjects (age range: 24 to 53, 6 females—7 males) over 12 months. The thiobarbituric acid reactive substances (TBARS) method as adapted from Spanier and Traylor[25] was used to measure lipid oxidation in the saliva samples (control and metal). 2-Thiobarbituric acid, sodium dodecyl sulfate, glacial acetic acid (99% pure), 1-butanol, pyridine, and 1,1,3,3-tetramehoxypropane were purchased from Fisher Scientific (Pittsburgh, PA). Saliva samples (1 mL) were reacted with 4 mL of the 0.375% thiobarbituric acid (TBA), 0.506% sodium dodecyl sulfate (SDS), and 9.370% glacial acetic acid solution in 15 mL of polypropylene tubes in a water bath (10-L, Fisher Scientific, Pittsburgh, PA) at 95 °C for 60 min. Samples were immediately cooled in an ice bath, and the pink colored complex was extracted with 5 mL of n-butanol/pyridine solution (at 15:1 ratio). Extracts were separated by centrifuging the samples at 3000 rpm for 15 min at room temperature. The absorbance of the supernatant was measured with a spectrophotometer at 532 nm. The standard curve was obtained with 1,1,3,3-tetramethoxypropane standards at 0.025 to

0.5 $\mu$M. Each sample was duplicated. The 50% dilution factor due to 2 mL rinse (control or metal) and 2 mL saliva collection was factored in the calculations to obtain the actual concentrations of malondialdehyde (MDA) in the samples.

**Total Salivary Protein Concentration.** The Bradford assay[26] was used to determine the total protein concentration in the saliva samples collected for the control (reagent water) and metal (180 $\mu$M ferrous iron) solutions. The Bradford reagent and bovine serum albumin were purchased from Sigma Aldrich (St. Louis, MO). The standard curve was prepared with bovine serum albumin at 1 mg/mL concentration. The samples were transferred to the 96-well plate, and absorbance was read by a plate reader at 599 nm. Three different samples were analyzed for each subject, and each sample was duplicated.

**Total Salivary Antioxidant Capacity.** Total salivary antioxidant capacity was measured in the saliva of subjects with the Antioxidant Assay Kit (709001) (Cayman Chemical Company, Ann Arbor, MI). Whole saliva, collected without any stimulation, was either immediately analyzed or stored frozen at −80 °C. The frozen samples were thawed in a cold water bath. Saliva samples were diluted by 1:3 with the 10× assay buffer just before the analysis. Trolox standards were used for the standard curve. The samples were prepared in a 96-well plate and absorbance read by a plate reader at 750 nm. Each sample was duplicated.

**Detecting Protein-Carbonyls: Western Blotting.** Saliva was collected from 8 subjects (age range: 24 to 53, 4 females—4 males) after rinsing with control and then metal (18 $\mu$M ferrous iron) solutions. OxyBlot protein oxidation detection kit (S7150) was purchased from Chemicon International Company (Billerica, MA), and all the supplies for SDS—PAGE and Western blotting were obtained from Invitrogen (Carlsbad, CA). Protein carbonyls were determined in the control and metal saliva samples with a slightly modified procedure based on Nagler et al.[19] Briefly, 6 $\mu$L of saliva samples (containing 24 $\mu$g of protein) were denatured and then derivatized with 2,4-dinitrophenyl hydrazine (DNPH) solution. For the electrophoresis, the method developed by Laemli[27] was followed with some modifications. Briefly, the prepared control and metal saliva samples were loaded to the wells of the NuPAGE Novex 10% Bis-Tris gel. The inner and outer chambers were filled with 1× NuPAGE SDS running buffer. After the run was complete, the membrane was blocked, and it was incubated with the specific anti-DNPH antibodies. The chemiluminescent reagent (Amersham ECL Plus Western Blotting Detection System) was prepared according to the instructions. The membrane was fully covered with the chemiluminescent reagent and was incubated for 5 min at room temperature. The membrane was exposed for 30 s and imaged by Fujifilm LAS-3000 Imaging System.

**Detecting Carbonyls in Saliva: Derivatization with DNPH.** Saliva was collected from 10 subjects (age range: 24 to 53, 4 females—6 males) for control and metal (180 $\mu$M ferrous iron) samples. Separately, the 10 control saliva samples and 10 metal saliva samples were combined to form pooled samples from which subsamples were allocated into polypropylene tubes to a volume of 10 mL for each sample. Solid high purity DNPH and its hydrazone derivatives of formaldehyde, acetaldehyde, propionaldehyde, high purity acetonitrile, pentane, and 1 M hydrochloric acid (HCl) were purchased from Sigma Aldrich (St. Louis, MO). Solid DNPH was purified by recrystallizing from acetonitrile two times under nitrogen. Saturated DNPH solution was prepared in 1 M HCl. The pooled saliva for control and metal samples were immediately reacted with 2 mL of the DNPH solution for 1 h at room temperature with intermittent shaking. Mixtures were extracted with 10 mL of pentane for 30 min, mixing by vortex intermittently. Pentane was separated by centrifuging the samples for 45 min at 3,000 rpm. The extracts were fully evaporated in the fume hood under a nitrogen stream on a hot plate at 60 °C. The residues were dissolved in 300 $\mu$L of acetonitrile, and 2 $\mu$L of each solution was injected to GC/MS as control and metal samples. The standard curve was obtained with hydrazones of formaldehyde, acetaldehyde, and propionaldehyde as well. GC/MS was performed with a DB-17 MS, 15 m × 0.25 mm i.d. capillary column with 0.25 $\mu$m film thickness. Carrier gas was 1 mL/min He. The injector

temperature was set to 240 °C under splitless mode. The oven was programmed to start at 60 °C (1 min) and then ramped at 10 °C/min to 280 °C. The transfer line of MS was set to 280 °C, source temperature was set to 250 °C, and the quadrupole instrument was set to 120 °C. The MS was run under electron ionization mode with an ion energy of 70 eV.

**Sensory Testing with Antioxidants and Chelating Agents.** α-Tocopherol (vitamin E) and L-ascorbic acid (vitamin C) were purchased from Sigma Aldrich (St. Louis, MO). Ethylenediaminetetra-acetic acid (EDTA) was purchased from EMD (Gibbstown, NJ), and lactoferrin was purchased from Netnutri (West New York, NJ). Ferrous sulfate solution was prepared at 18 μM, vitamin E, vitamin C and EDTA solutions were prepared at 36 μM, and lactoferrin solution was prepared at 0.13 μM in reagent water fresh daily prior to testing. Sensory tests were conducted in two ways: (1) Subjects sipped all of the 3 mL of the antioxidant or the chelating agent solution, swished it around their mouths for 10 s, then immediately sipped 2 mL of the ferrous solution, swished it around their mouths for 10 s, swallowed it, and recorded their taste and flavor perceptions with intensity ratings on the scorecards provided with a guide for basic tastes and the intensity scale; (2) Subjects sipped all of the 2 mL of ferrous solution, swished it around their mouths for 10 s, then swallowed it, and immediately sipped all of the 3 mL of the antioxidant or chelating agent solution, swished it around their mouths for 10 s, swallowed it, and recorded their perceptions. Reagent water was also tested as a control to understand the removal of the metallic flavor from the mouth as a result of a rinse.

The samples were presented at room temperature (~22 °C) in 3 oz Solo cups randomly coded by 3-digit numbers. The subjects were trained to use an intensity scale from 0 to 12; "0" corresponding to "no perception" and "12" corresponding to "strong perception". The subjects were not informed about the specific aim of the study; however, they became familiar with the metallic flavor sensation through participation in the previous portions of this study. An odor free room was selected for testing, and the subjects tasted the samples sitting around a table. No discussions or interactions were allowed among subjects. The subjects' profiles for each trial were as follows: water, 19 to 37 years old, 5 males−6 females; vitamin E, 19 to 53 years old, 5 males−5 females; vitamin C, 19 to 37 years old, 5 males−6 females; EDTA, 19 to 37 years old, 5 males−6 females; lactoferrin, 19 to 53 years old, 9 males−10 females.

**Statistical Analysis.** Depending on the experimental design, either a $t$ test or one-way ANOVA was selected and performed using SAS software (v8.1, SAS Institute, Cary, NC) or a two-way ANOVA was conducted using NCSS 2007 (NCSS Ltd., Kayserville, UT). The α value was set to 0.05. The normality and homogeneity of the results were checked, and log transformation was performed when data was not normally distributed or homogeneous. The type of statistical analysis performed and the results are discussed for each portion of the study.

## ■ RESULTS AND DISCUSSION

**Metallic Flavor Sensation from Ferrous Iron.** All subjects who participated in this research used the word "metallic" to describe the sensation from ingesting 2 mL of either 18 or 180 μM ferrous solution in reagent water. Thus, the ferrous concentrations selected were suprathreshold and caused metallic flavor perception in the subjects of this study.

**Ferrous Induced Lipid Oxidation in the Oral Cavity.** Lipid oxidation was indicated by a nearly 3-fold increase in TBARS (measured as MDA concentration) in saliva collected after ingestion of metal (180 μM ferrous iron) compared to saliva samples from ingestion of reagent water (Figure 1). An increase in oral lipid oxidation was observed for each of the 19 subjects. The TBARS values were consistent over time as coefficients of variation were typically between 2 and 20% for both control and metal samples for individual subjects. A paired $t$ test analysis on log transformed TBARS[28] indicated a
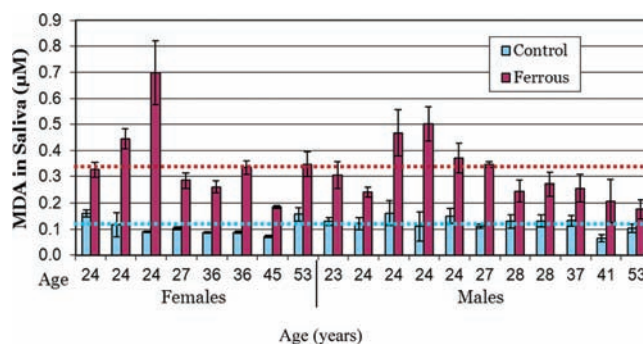


**Figure 1.** Measurement of lipid oxidation reported as MDA (μM) in control (reagent water) and metal (ferrous iron at 180 μM) saliva samples (mean ± SD) collected from healthy subjects (mean MDA concentrations for control (0.12 ± 0.03 μM) and metal (0.33 ± 0.12 μM) are shown as dotted lines).

significant increase in oral lipid oxidation in saliva obtained after rinsing the mouth with the ferrous iron solution ($p < 0.001$). A power analysis indicated that results from 19 subjects had sufficient power (>95%) to project the results to the general population when the standard deviation of the differences was 0.0942 μM MDA.

The average MDA concentration for the control saliva samples was 0.12 μM, which is comparable to the values reported as 0.055 to 0.2 μM in the saliva of healthy controls who participated in oral oxidative stress studies.[29,30] The reason for the presence of TBARS/MDA in control saliva is not known,[31] but it may be explained by local intraoral production[32] or oral microbial flora causing oxidative stress.[33] Several researchers reported that systemic oxidative stress does not alter salivary TBARS values.[29,34] MDA concentration in control and metal saliva samples (Figure 1) varied among subjects and was investigated further by measuring the proteins and antioxidants in saliva.

**Total Salivary Protein Concentration.** The total protein concentrations of saliva samples collected from 19 subjects were measured by the Bradford assay. A paired $t$ test indicated that there was no significant difference ($p = 0.053$) between the control and the metal saliva samples. The total protein values ranges from approximately 0.2 to 1.2 g/L with average protein concentrations for the group of 0.62 g/L (±0.25) and 0.58 g/L (±0.22) for the control and metal saliva samples, respectively. The protein concentrations were used to incorporate more discriminative factors to the ferrous induced lipid oxidation results as proteins may bind or react with metals. The increase in MDA production was reported per liter of saliva and per gram of protein.

One-way ANOVA with Tukey and Scheffe analyses with the log transformed data suggested that there was no significant difference between genders when normalized data were compared for increase in MDA per liter of saliva ($p = 0.10$) and per gram of protein ($p = 0.054$). This finding suggests that gender did not affect the extent of oxidation caused by ferrous in mouth. Since the subject pool was smaller and the age range was narrow for this study, the effect of age was not investigated. Previous medical research indicated that lipid oxidation in the mouth increases with age as Hershkovich et al.[18] collected saliva samples from 80 healthy subjects (20 to 80 years old) and reported that the elderly group had higher levels of oxidation in their mouths.

**Total Salivary Antioxidant Capacity.** Saliva is known to have antioxidant properties, so its relationship to metal induced oral lipid oxidation was investigated. The salivary antioxidant capacity varied among subjects without following a trend for gender ($p = 0.78$) determined by a two sample $t$ test (Figure 2).
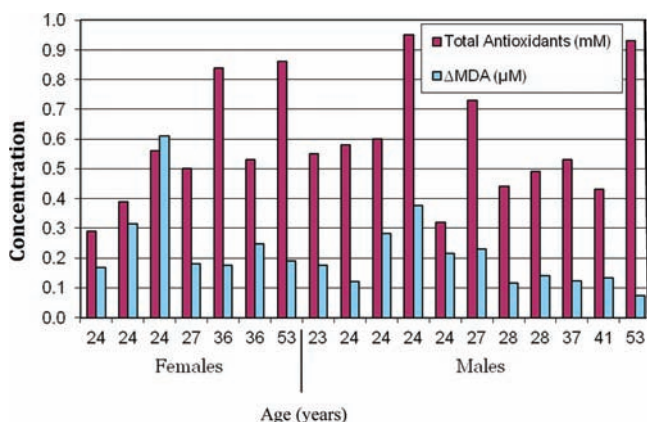


**Figure 2.** Total concentration of antioxidants in saliva (mM) and increase in MDA production [metal (ferrous iron) − control (reagent water)] (μM) for healthy adult subjects.

Some subjects with high antioxidant capacity produced less MDA compared to the others; however, some subjects who had lower antioxidant capacity also produced less MDA. Hence, no correlation ($R^2 = 0.0031$) could be obtained for antioxidant capacity and MDA production. Ingestion of antioxidant supplements were unlikely to have affected the salivary antioxidant capacity as a recent study determined that, although serum antioxidant capacity increased with antioxidant supplementation, salivary antioxidant capacity was not affected.[35]

**Protein Carbonyls in the Oral Cavity.** The protein-carbonyls in the control and metal (18 μM ferrous iron) saliva samples (Figure 3) indicated that, although the size and the concentration of the carbonylated proteins varied among the subjects, an increase in protein-carbonyls was observed after ingesting ferrous iron. The proteins that were most affected were 21, 29, and 68 kDa proteins.

Nagler et al.[19] and Reznick et al.[36] investigated the protein carbonyls in saliva exposed to oxidative stress from cigarette smoking and reported that carbonyls increased with increased exposure time and that aldehydes in the smoke may increase the protein carbonylation. Blakeman et al.[37] studied ferrous and ferric iron-induced TBARS production and protein carbon-

ylation of bovine serum albumin. Ferrous iron caused a significant increase in protein carbonyls, whereas no change was observed for ferric iron. TBARS formation was not significant in the lipid free bovine serum albumin samples that were exposed to ferrous iron. However, there was a significant increase in protein carbonyls indicating that Fenton reactions caused by ferrous iron may carbonylate the proteins. For this work, it was hard to conclude whether the proteins were oxidized by the metals or were modified as a result of lipid oxidation carbonyls as it was impossible to exclude lipid oxidation and study only protein oxidation.

**Low Molecular Weight Carbonyls in Saliva.** It is very challenging to measure trace amounts of carbonyls in mL volumes of saliva or the approximate 150 mL volume of the oral cavity, which is moisture and enzyme rich. Additionally, the measurements must be rapid and occur within 30 s of carbonyl formation, which is the approximate time a person can hold his/her breath before exhaling the lipid oxidation products. We expended much effort and had some success in capturing, characterizing, and quantitating the specific carbonyls that resulted from exposure of the oral cavity to ferrous iron. The amount of formaldehyde, acetaldehyde, and propionaldehyde in control saliva samples was determined as 6, 7, and 3.6 μM, respectively. The amount of formaldehyde, acetaldehyde, and propionaldehyde in metal (180 μM ferrous iron) saliva samples was determined as 24, 15, and 7.9 μM, respectively. A paired $t$ test demonstrated that formaldehyde and propionaldehyde were statistically different between control and metal treatments but that there was no difference for acetaldehyde. These three aldehydes are among those frequently reported for lipid oxidation. Cordis et al.[38] studied the oxidation of rat heart tissue with iron and suggested that monitoring MDA, formaldehyde, acetaldehyde, propionaldehyde, and acetone is a good approach to monitor oxidative stress.

It should be noted that some of the carbonyls may be lost through breathing, absorption onto the oral tissues and proteins in saliva, and reactions in the oral cavity or may be produced below the detection limit of GC/MS. Buettner[39] reported that salivary enzymes reduced 30 to 50% of aldehydes to alcohols. Hong et al.[40] determined that the headspace concentrations of the aldehydes and other aroma volatiles were decreased when salivary proteins and copper were present. Possible carbonyls that may be present in the oral cavity after exposure to metals are those found in the work of Glindemann et al.[8] who detected $C_6-C_{10}$ aldehydes and 1-octen-3-one in the headspace above skin that had a metallic odor after exposure to ferrous and cupric/cuprous ions. An increase of hexanal, octanal, and
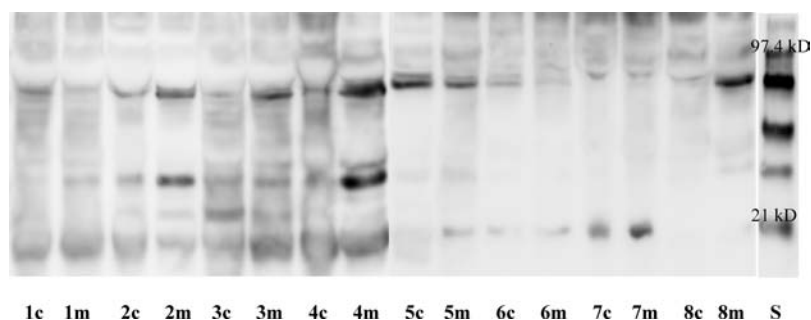


**Figure 3.** Protein-carbonyl detection by SDS−PAGE and chemiluminescence in saliva of 8 healthy subjects before and after rinsing mouth with ferrous iron solution (at 18 μM) (where "c" labeled lanes represent controls, "m" labeled lanes represent metal samples, and the last lane (S) represents the protein standard indicating the protein sizes of 97.4, 68, 43, 29, and 21 kDa from top to bottom).

nonanal in the oral cavity was observed when human subjects swished 180 $\mu$M solution of ferrous iron in their mouths.[41]

**Sensory Testing of Ferrous Iron with Antioxidants and Chelating Agents.** To further investigate ferrous induced oral lipid oxidation and metallic sensation, the effect of chemical additives likely to mitigate lipid oxidation was studied. Subjects reported metallic flavor sensation at a moderate to high intensity after the ferrous iron solution (at 18 $\mu$M) was ingested, even though they prerinsed their mouths with an antioxidant or chelating agent solution beforehand (data not shown). However, when antioxidants and chelating agents were ingested after the ferrous iron solution (at 18 $\mu$M), the perceived metallic flavor intensity decreased significantly for all treatments ($p < 0.001$ for reagent water, EDTA, and lactoferrin, $p = 0.006$ for vitamin C, and $p = 0.017$ for vitamin E) (Figure 4). The mean differences (before−after) indicated the
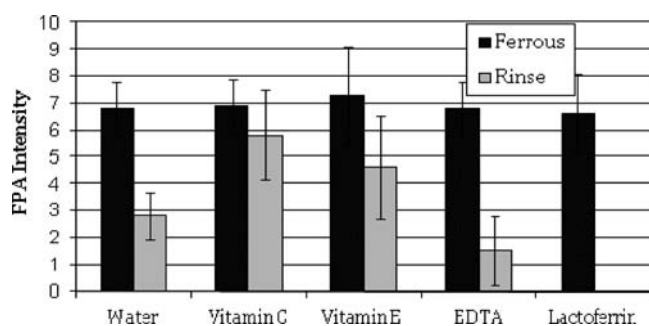


**Figure 4.** Intensity ratings (mean ± SD) on a scale of 0 to 12 for metallic flavor after ingesting 18 $\mu$M ferrous iron solution then rinsing the mouth with selected antioxidant or chelating agent. Metallic flavor was completely removed after lactoferrin rinse for all subjects.

following order of difference: lactoferrin > EDTA > rinsewater > vitamin E > vitamin C. The corresponding mean differences were 6.6, 5.3, 4.0, 2.7, and 1.1. A Tukey HSD multiple comparison indicated that any two adjacent mean differences were not statistically different but that other differences were. For example, lactoferrin was not statistically different from EDTA but was statistically different from all other chelators.

For reagent water and vitamin E rinses, all subjects reported a decrease in sensation, whereas 2 subjects reported increased sensation, and 1 subject reported the same intensity after the vitamin C rinse. While only 4 subjects reported that the metallic sensation was completely removed by an EDTA rinse, all of the subjects reported that the metallic flavor was completely removed in seconds after the lactoferrin rinse.

Although vitamins E and C seemed to reduce the metallic sensation, it should be noted that the intensity ratings were still higher when compared to results from the reagent water rinse. This indicates that they were not very effective in removing the metallic flavor. On the contrary, it may be argued that they even enhanced the flavor, even though the metals in the mouth were rinsed out with the antioxidant solution that did not contain metals. Although vitamins E and C are accepted as good free radical scavengers, there are occasions where they promote lipid oxidation, especially in the presence of metals.[23] Kawatsu et al.[42] showed that ferrous causes phospholipid oxidation, and when ferrous iron and ascorbic acid (vitamin C) were present in the matrix, the oxidation was enhanced. Osborn and Akoh[43] determined the effect of vitamin E, quercetin, and gallic acid on iron-catalyzed oxidation of lipids. As opposed to expected, all of these natural antioxidants enhanced lipid oxidation. However,

EDTA has been successfully incorporated into food to preserve the flavor and color of the products.[22] Let et al.[44] reported that lipid oxidation was greatly prevented in fish oil fortified milk samples that were supplemented with EDTA. Satue-Gracia et al.[45] studied the effect of lactoferrin on oxidation when baby formulas were supplemented with iron as well. The results indicated that even though lactoferrin was at lower concentrations than iron, it still acted as an antioxidant and prevented oxidation.

**Correlation of Metallic Flavor and Lipid Oxidation for Reduced and Oxidized Iron and Copper.** The previous sections presented ferrous iron as a model metal to study metallic flavor production. Ferrous iron causes oral lipid oxidation plus increased levels of protein-carbonyl and low molecular weight aldehydes in saliva samples. Lipid oxidation as MDA formation was also investigated for ferric, cuprous, and cupric ions to understand the reactivity of these ions and to compare their sensory characteristics.

The data (Figure 5) indicate an increase in lipid oxidation (metal saliva MDA − control saliva MDA) for all four metal
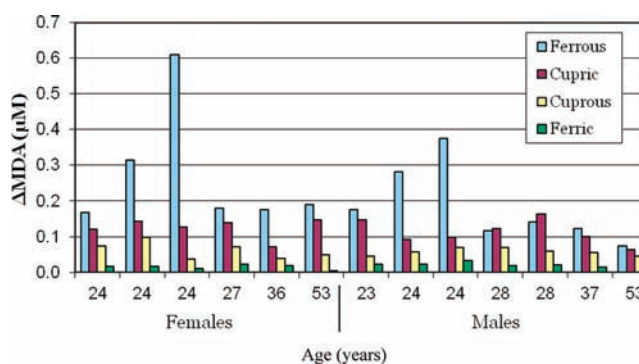


**Figure 5.** Comparison of lipid oxidation, as measured by change in MDA production in the saliva before and after ingesting 180 $\mu$M metal solution ($n = 13$).

ions tested. The change in lipid oxidation before and after metal exposure was evaluated with a 2-way ANOVA, with metals ($n = 4$) as a fixed effect and subjects ($n = 13$, with 2 replications) as a random effect, followed by a Tukey-Kramer multiple comparison of the mean response by metal. The 2-way ANOVA indicated significance for both main effects ($p < 0.0001$) and the interaction ($p < 0.0001$). This interaction was likely driven by a few subjects who had very high responses to ferrous ion ($Fe^{2+}$) as can be seen in Figure 5. Multiple comparisons among the metal ions indicated that ferrous was different from all other metals, cupric was not different from cuprous but was different from ferric, and that ferric was not different from cuprous.

To investigate the role of lipid oxidation on metallic flavor production, the formation of MDA (Figure 5) was compared with flavor thresholds from the literature for ferrous, cupric, and cuprous ions (not ferric iron as subjects could not detect a metallic flavor even at 360 $\mu$M, and only a few subjects rated it sour or salty) as summarized in Table 1. Flavor threshold values ($\mu$M) were determined in reagent water around pH 5.5, using a forced choice 1-of-5 test with metal concentrations of 0.001 to >5 mg/L (approximately 0.015−75 uM), testing 25 to 36 subjects, and have been previously reported.[26,28]

Ferrous ion was found to be the most "flavorful" and also caused the most lipid oxidation, followed by cupric, cuprous,

**Table 1. Literature Values for Flavor Thresholds of Soluble Iron and Copper Ions in Reagent Water[3,4]**

| metal | mean MDA production ($\mu$M) with 180 $\mu$M metal | threshold ($\mu$M) by geometric mean method | threshold ($\mu$M) by logistic regression method | concn ($\mu$M) for ~75% detection[a] |
|---|---|---|---|---|
| ferrous | 0.22 | 0.93 | 0.56 | 1.79 |
| cupric | 0.12 | 7.55 | 12.12 | 15.74 |
| cuprous | 0.06 | 9.59 | 9.59 | 15.74 |

[a]75% of those tested could detect a metallic flavor at or below this concentration.

and then ferric ions. The threshold results indicated that 75% of the population can detect ferrous ion in water at or below concentrations of 1.79 $\mu$M, whereas this value increases to 15.74 $\mu$M for cupric and cuprous ions. This almost 10-fold difference in detection concentration may be due to carbonyl production as a result of lipid oxidation in the mouth. The results shown in Figure 5 clearly demonstrate that ferrous causes the highest lipid oxidation, which also means the highest carbonyl formation in the oral cavity.

Although there have been several works focusing on metallic "taste/flavor" perception, the reactions causing the metallic sensation have been little investigated. This work is the first to simultaneously study biochemical reactions and metallic sensation in the mouth to understand the metallic flavor perception of humans. Ferrous was demonstrated to cause oxidative stress, which results in lipid oxidation, metallic flavor formation, production of at least formaldehyde, acetaldehyde, propionaldehyde, and increased protein-carbonyls in ferrous-treated saliva samples. The ability of chelating agents to reduce or eliminate the metallic sensation through complexation also supports the idea that metals in the oral cavity cause oxidative stress and lipid oxidation. As expected, ferrous iron caused the most lipid oxidation because it is the most anodic ion among those tested. Ferrous ion was followed by cupric and cuprous ions in causing lipid oxidation in the oral cavity. The flavor threshold values also indicated that, as the lipid oxidation in the oral cavity increased, the produced metallic sensation was stronger; hence, the threshold concentration of the metal was lower. Production of the metallic flavor in the oral cavity is mitigated or eliminated by chelating agents which are more effective in inactivating the metals than antioxidants.

The metallic sensation caused by ferrous, cuprous, and cupric ions in the mouth is more than a taste. It is a flavor which is a combination of taste sensation created on the tongue by the salts and retronasal perception created in the olfactory region due to the biochemical reaction of lipid oxidation, which results in odorous compounds including carbonyls. This has implications for understanding metallic flavor issues in healthy and diseased humans.

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