# Macromolecular Reactions and Sensory Perception at the Air-Water-Human Interface

by

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## Abstract

During 20th century main concern was to have sanitary water flowing through the tap. In 21st century constant supply of safe drinking water is common at any home in USA. Hence consumers pay attention to aesthetic quality of tap water. Odorous algal metabolites in source water and metals introduced to drinking water due to corrosion of pipes in the distribution system cause taste, odor and color problems, and result in complaints and perception of tap water as unhealthy. Millions of dollars are spent each year by water industry to address and prevent these issues.

This research focused on some of the taste-and-odor issues associated with drinking water. First aim was to understand when geosmin, 2-MIB, and nonadienal become detectable, employing two-resistance mass transfer theory to determine the concentration of odorants in bathroom air. Results showed that water temperature and odorant concentration in water play an important role. Next focus was to develop an international odor standard to be used for training of sensory analysis panelists. There are many sensory methods to monitor drinking water to detect the off-flavors however an odor standard has been missing. Hexanal was studied with trained flavor profile analysis panels and was proposed as an ideal odor reference standard to be used for training and sensory assessment of water samples. Main focus was to understand metallic flavor of drinking water caused by iron and copper. It was shown that metallic sensation has taste and retronasal components creating the flavor and humans are very sensitive to it. Occurrence of lipid oxidation in the oral cavity was shown when metals were ingested, that produces carbonyls which are responsible for the metallic flavor. Antioxidants and chelators were investigated to study prevention of lipid oxidation and, chelators were determined to be more effective. Oral epithelial cell cultures were developed as a model for oral cavity to further investigate lipid oxidation and effectiveness of the antioxidants and chelators.

This dissertation is a result of inter-disciplinary work and possibly a good example for how problems may be solved by incorporating different methods and point of views from several disciplines.

## Extended Abstract

During the 20th century the main concern related to tap water was to have enough and sanitary water flowing through the tap. As the water cleaning practices and the distribution system improved consumers started to focus on water quality and developing more strict criteria to have safe water. Now in 21st century constant supply of safe drinking water is common at any home in USA. Hence the consumers started to pay attention to the aesthetic quality of tap water. Odorous algal metabolites in source water and metals introduced to drinking water due to corrosion of metal pipes in the distribution system cause taste, odor and color problems and result in consumer complaints and perception of the tap water as unhealthy. Millions of dollars are spent each year by the drinking water industry to address and prevent these issues.

This research has focused on some of the taste-and-odor issues associated with drinking water. First aim was to understand when the common odorants (geosmin, 2-MIB, and nonadienal) become detectable in the bathroom air when contaminated water is used. These odorants occur in source water during warm summer and fall months as a result of algal blooms and cannot be removed by conventional treatment methods. An earlier model developed by using the two-resistance mass transfer theory was utilized to determine the concentration of these odorants in shower stall and bathroom air. Effect of odorant concentration in water and water temperature on the indoor air concentration was investigated. The results showed that water temperature plays an important role. Treatment options may be evaluated based on the detectability of the odorants, depending on chemical properties of the odorants, water use patterns by the customers, and the concentration of the odorous compounds in the raw or treated water. In the second part of this portion of the research the volatilization of geosmin, 2-MIB, and nonadienal was investigated. A more thorough analysis was conducted by varying the odorant concentration, water temperature, water flowrate, air flowrate in the shower and bathroom, shower stall volume, and duration of showering. The results indicated that the main factors that affect the concentration of the odorants in the air are odorant concentration and water temperature. Then a simpler model was developed incorporating the most important factors to determine the air concentration

of the odorants at steady-state. An important finding from this work was an incorrect assumption made by most of the indoor air modeling in showers. Usually a complete mix system is selected for the modeling purposes however this is clearly not a legitimate approach considering the formation of a concentration gradient near the shower head. This should be corrected by modifying the model using a plug flow approach.

The next focus of this research was to develop an international odor standard to be used for training of sensory analysis panelists. There are many analytical and sensory methods available for the monitoring of drinking water to detect the odorous compounds and each has its own advantages and disadvantages. When the methods are compared, the sensory methods can be superior because the human nose is more sensitive than most available instrumentation and instant results enable the utilities to take rapid action. Among the sensory methods, the flavor profile analysis (FPA) is one of the most comprehensive and reliable methods. It was originally designed for the food industry and later adapted to the water industry to be applied for the sensory characterization of drinking water. Although being a popular and strong method, FPA training lacks an odor standard. For the basic taste training there are established standards to introduce the panelists to basic tastes at various intensities from very weak to strong. However when odors are rated the panelists are asked to define the intensity of an odor by attributing the perceived intensities of taste standards and translate them to odor intensities. Most panelists find this difficult and subjective so the precision of the evaluation is reduced. Hence hexanal having a pleasant smell was studied with four very well trained FPA panels. The results obtained from the panels paralleled indicating that different groups of people give similar odor ratings to hexanal at defined concentrations with linearly increased odor intensity ratings with increasing concentration. The stability of hexanal during the training session was determined by analyzing the headspace concentration of hexanal after couple sniffs and over several hours. The results indicated that hexanal concentrations in the headspace of the flasks remain fairly constant. Also odor threshold of hexanal was determined by this work indicating that humans are very sensitive to hexanal. Because hexanal possesses all the necessary properties, it was proposed as an ideal odor reference standard to be used for FPA training and sensory panel assessment of water samples.

The main focus of this research was to understand the metallic flavor of drinking water caused by iron and copper. Although taste thresholds of iron and copper and metallic taste intensities have been investigated the mechanism that causes it haven't been researched yet. The hypothesis of this research states that there are two components to metallic flavor: 1) the taste of metal ions on the tongue; 2) an odor component due to metal-catalyzed lipid oxidation of the oral tissues that produces odorous aldehydes and ketones. To fully understand the metallic sensation, its prevention, and application to human health, three specific objectives were developed: 1) to determine the mechanism that causes metallic flavor in mouth when drinking water containing iron and copper; 2) to determine ways to prevent metallic flavor generation in mouth; 3) to compare the sensory thresholds, recommended nutritional levels, and adverse health effect levels of iron and copper in water and relate to health-based problems such as persistent metallic tastes of patients receiving chemotherapy. To address these objectives first sensory studies were conducted with human panelists to determine the taste thresholds of ferrous and cuprous in drinking water. Panelists were tested with one-of-five test with ascending or descending concentrations of the respective ion solutions. The determined taste thresholds indicated that humans are very sensitive to iron and copper at low levels, and EPA SMCL's may be reviewed based on these findings as they are fairly higher than the determined population thresholds. The second step was to determine the components of the metallic sensation. Panelists tasted ferrous, ferric, cuprous, and cupric solutions with and without nose-clips and described their taste and flavor perceptions for each ion. The results indicated that none of the panelists could detect a metallic flavor when their noses were occluded. The only reported bitter, sour or astringents tastes and mouthfeel. However when they tasted the same samples with their nose open there was a strong metallic sensation for ferrous, then followed with lower sensations for cupric, and then cuprous. Ferric did not have any taste in this case as well. This finding supports the hypothesis of this research concluding that there is a significant odor component of metallic flavor perceived retronasally. This finding also let the way to investigate the lipid oxidation.

The lipid oxidation in mouth was investigated by analyzing the saliva samples collected from the healthy subjects after they rinsed their mouth with a control (reagent

water) and then with a metal (iron or copper ion) solution. It was hypothesized that the unsaturated fatty acids (that form the phospholipids) in the cell membrane may be oxidized by the metals. Thiobarbituric acid reactive substance (TBARS) method (which is a commonly used method by the food and medical industry to study lipid oxidation in food, blood, saliva, and tissue samples) was employed for this work. Malondialdehyde (MDA) is a common lipid oxidation by-product and reacts with thiobarbituric acid to form a pink colored complex. The extent of oxidation is determined by measuring the absorbance of the samples. Results from this portion of this research paralleled the findings from the taste perceptions for all of the ions. Ferrous caused the highest lipid oxidation in mouth; followed by cupric, cuprous, and ferric caused a little oxidation. Total protein concentrations of the saliva samples collected from the subjects were measured to report MDA values and make the results more specific to the panelists. The total protein in subjects' saliva varied and did not show a trend for age or gender. For both of the MDA results reported as per L saliva or per gram of protein it was noted that younger panelists produce more MDA than older, however gender did not affect MDA production. This may show that younger people are more prone to lipid oxidation. Another interesting finding was that MDA production varied among panelists, hence this was further investigated by measuring the salivary antioxidants as they may interfere with lipid oxidation. Total antioxidant capacities in the subjects' saliva varied as well and no correlation could be established between the MDA production and antioxidant concentration. Also a trend was not observed for the antioxidant content of saliva depending on the age or gender.

Although the major cause of off-flavors in food is production of carbonyls by lipid oxidation, the effect of ferrous on salivary proteins was investigated by Western Blotting as well. The protein-carbonyls, which may be an indicator of protein oxidation, in the control and metal saliva samples collected were derivatized by 2,4-dinitrophenyl hydrazine (DNPH, a common derivatization reagent used to detect carbonyls in air or water) and then the proteins were separated by electrophoresis. Then the proteins were transferred to a membrane and chemiluminscence was used to detect the protein-carbonyls. Results showed that proteins were carbonylated and were observed more in the metal samples. The carbonylation of proteins may be a result of reaction of the

proteins with carbonyls produced by lipid oxidation, or proteins may be oxidized with ferrous and hence have carbonyl adducts. Although proteins may be oxidized, it is not expected to have them contribute to the flavor development. As a complement to this work the carbonyls in the saliva were analyzed by derivatization with DNPH. Only compounds that were detected were formaldehyde, acetaldehyde, and propionaldehyde. The concentrations of these compounds were significantly higher in metal saliva samples than the controls. Although more aldehydes and ketones were expected to be detected, they may be lost through breathing, and some may be absorbed onto the oral tissues or precipitate with the proteins they are attached to. Another important point is remembering that the humans are very sensitive to metallic flavor, and hexanal as mentioned above. Hence even if the flavor compounds are formed at a much lower concentration, humans could detect them.

As the medical and food literature suggest, antioxidants and chelating agents may be a solution to preventing lipid oxidation. As another aim of this research was to investigate ways to prevent metallic flavor production by administration of antioxidants such as vitamin E and vitamin C and chelating agents such as ethylenediamine tetraacetic acid (EDTA) and lactoferrin. Panelists were asked to rinse their mouths either before or after they rinsed their mouth with ferrous solution. Then they were asked to rate the intensity of the metallic flavor. When antioxidants and chelators were used as an initial rinse the intensity of metallic flavor did not change. In this case the amount of antioxidants or chelating agents remaining in the mouth may not be enough to prevent the lipid oxidation. However when the antioxidants and chelators were admistered after the panelists rinsed their mouth with ferrous solution there was a notable decrease in the metallic flavor intensity. Although vitamin E and vitamin C were not very effective, EDTA reduced the metallic flavor intensity to a very weak rating whereas lactoferrin immediately and completely removed the sensation. These findings suggest that use of a chelating agent may be a better solution in preventing metallic flavor production. Also literature has conflicting information on vitamin E and vitamin C indicating that they may enhance the oxidation by recycling ferric to ferrous. However, it should be noted that the antioxidants may be effective for cancer patients as the ferrous concentration in their mouth may be at much lower concentration.

The final part of this research was to develop a model system that would mimic the oral cavity of humans. Primary oral epithelial tissues were purchased and KB cells (which are cancerous oral epithelial cells) were purchased and cultured in lab. TBARS method was used to detect the lipid oxidation in the samples collected from the cell cultures. The cells were exposed to ferrous, ferric, cuprous, and cupric solutions. Also effect of saliva on lipid oxidation was investigated by using saliva or reagent water in controls and in metal samples. Antioxidants such as vitamin E, vitamin C, vitamin E+C, α-lipoic acid, and chelating agents EDTA and lactoferrin were evaluated to prevent lipid oxidation. Similar results were obtained for primary oral tissues and KB cells for saliva versus reagent water samples and ferrous. For both cultures ferrous caused a significant increase in MDA, however no effect of saliva was observed. The rest of the experiments were conducted with the KB cells. The results paralleled the findings from humans. All of the metals caused a significant increase in MDA concentration, ferrous causing the highest increase followed by cupric, then cuprous, and ferric. The results for the antioxidants and chelating agents indicated that vitamin E and vitamin C did not prevent oxidation, and a mixture of vitamin E and C slightly enhanced the oxidation. However, α-lipoic acid significantly reduced MDA production when compared to results of ferrous alone. Again EDTA and lactoferrin were effective in reducing the lipid oxidation; in this case EDTA was more effective than lactoferrin. These findings also suggest that chelating agents could be a solution for prevention of lipid oxidation. Also preliminary experiments were conducted with ferric and selected antioxidants to determine whether it was reduced to ferrous form. Individual solutions of ferric and vitamins E, C, and α-lipoic acid were prepared and incubated, then the ferrous concentrations in the solutions were measured. The results showed that α-lipoic acid does not reduce ferric whereas 8% and 13.3% reduction of ferric were observed for vitamin E and vitamin C respectively. This also suggests that these antioxidants recycle iron and does not improve the conditions for lipid oxidation. Another conclusion from this work is that KB cells may be cultured in lab indefinitely and may be used as a model for the oral cavity as the cell membranes of KB cells are very similar to healthy oral epithelial cells.

The ultimate goal of this work as it evolved during the course of research and literature review became to be the initial steps of understanding the metallic flavor perception by the cancer patients undergoing chemotherapy and radiotherapy, and investigating ways to prevent it. Collaboration was formed with Hematology and Oncology Department in Wake Forest University Baptist Medical Center and the findings from this research will lead the way to conduct research with cancer patients and evaluate intervention methods. This will greatly benefit the patients suffering from malnutrition and have a decreased quality of life due to metallic taste dysfunction.

As a conclusion, this research investigated some of the off-flavors associated with drinking water and tried to contribute to the body of knowledge as being the first steps with some components of the work conducted. This dissertation is a result of inter-disciplinary work and possibly a good example for how problems may be solved by incorporating different methods and point of views from several disciplines.

## Dedication

This dissertation is dedicated to memory my dear grandmother ***Halime Aydın***.

I remember you always wanted me to be a doctor. Hopefully I will be a good one although not in the way you meant ☺

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Christine and Andrew I will always keep the special card you made for me. Andrew I might be able to use the “antioxidant” you developed for me. When its time to pursue your graduate studies you know where I will be.

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# Chapter I

## Introduction

During the 20th century the main concern related to tap water was to have enough and sanitary water flowing through the tap. As the water cleaning practices and the distribution system improved consumers started to focus on water quality and developing more strict criteria to have safe water. Now in 21st century constant supply of safe drinking water is common at any home in USA. Hence the consumers started to pay attention to the aesthetic quality of tap water. Odorous algal metabolites in source water and metals introduced to drinking water due to corrosion of metal pipes in the distribution system cause taste, odor and color problems and result in consumer complaints and perception of the tap water as unhealthy. Millions of dollars are spent each year by the drinking water industry to address and prevent these issues. This research focused on some of the taste-and-odor issues associated with drinking water. As the title of the dissertation indicates, chemical reactions and sensory perceptions related to drinking water were studeied.

The chapters of this dissertation were written in manuscript format. Chapters II and III present the **literature review** conducted on the major topics of this research.

Chapter IV - **Ability of humans to smell geosmin, 2-MIB and nonadienal in indoor air when using contaminated drinking water** focuses on understanding when selected odorants become detectable in bathroom air. This paper was published in Water Science and Technology.

Chapter V - **Using air-water partitioning model to determine when odorous compounds in drinking water become detectable during showering** studied the factors that affect the concentration of the odorants in bathroom air and developed a simpler model to predict the air concentrations of the selected odorants.

Chapter VI - **Developing hexanal as an odor reference standard for sensory analysis of drinking water** studied the conformity of hexanal, with trained flavor profile analysis panels, to be used as an international odor standard for sensory training of panelists to analyze drinking water. An odor standard to determine odor intensities is lacking and although this issue was highlighted in the literature, no work has addressed it. This paper was published in Water Research.

Chapter VII – **Retronasal perception and taste thresholds of metallic flavored drinking water with iron and copper** studied the components of the metallic flavor by occluding nose using nose-clips. Also taste thresholds of ferrous and cuprous were determined with a 1-of-5 test.

Chapter VIII - **Understanding the metallic flavor development in mouth when drinking water with iron and copper is consumed and investigating the methods to prevent off-flavor formation** focused on determining the mechanism that produces metallic flavor in mouth. Occurrence of lipid oxidation was proven to be the reason causing the sensation. Effectiveness of selected antioxidants and chelating agents were tested in preventing lipid oxidation.

Chapter IX - **Using KB cell cultures as a model to study and prevent the metallic flavor production in mouth as a result of oxidation of the oral epithelial cell membrane phospholipids by iron and copper** studied primary oral epithelial cell tissues and KB cell cultures to develop a model for studying the metallic flavor development in the oral cavity. Effect of saliva and selected antioxidants and chelating agents were also investigated.

Appendix A - **Taste and odor abnormalities in cancer patients** present the abstract of the literature review conducted on taste dysfunctions of cancer patients. This paper is in press by Journal of Supportive Oncology.

Appendix B – **Data** that was not presented in detail is given in this section.

Appendix C – **Institutional Review Board** approvals for the work involved human subjects are included in this section.

# Chapter II

## Literature Review

### 1. Taste and Odor of Drinking Water

Taste and odor perception is a very complex phenomenon and is highly unexplored. Scientists Linda Buck and Richard Axel significantly advanced the understanding of the sense of smell and won the 2004 Nobel Prize in medicine for their work on odorant receptors and the organization of the olfactory system. Taste and odor perception varies depending on age, gender, race, health status, prior exposure and experience. Human senses can recognize and accept essential nutrients but reject toxins that may harm them. Water quality and chemistry affects taste and odor perception. For example, ingestion of distilled water causes oral epithelial cells to release ions and then water is transducted into the cells affecting a "salty" taste perception (Gilbertson et al., 2006). Off tastes and odors are a major concern of water utilities worldwide that aim to serve high-quality drinking water to their customers. Unacceptable flavor of drinking water is perceived as unhealthy by consumers and results in loss of confidence in the utilities and increased number of complaints (McGuire, 1995).

If odorous compounds are present in drinking water the odor concentration in the air may reach a detectable level that concerns and annoys the residents when water is used for showering and washing dishes. Off taste and odor of drinking water may be caused by the water supply reservoir, the treatment plant, the distribution system, or the home plumbing (Stinson and Carns, 1983; Mallevialle and Suffet, 1987; Dietrich *et al.,* 2004). A typical water supply reservoir contribution to off flavors is the production of odorous compounds by actinomycetes, cyanobacteria, and other algae in the surface waters, particularly during warm summer and fall months. Three of the most common odorous compounds produced are geosmin, 2-methylisoborneol (2-MIB), and nonadienal (Zaitlin *et al.,* 2003). Earthy/musty odors are caused by geosmin and 2-MIB, and cucumber/fishy odors are caused by nonadienal. These compounds cannot readily be removed by conventional treatment operations and they require advanced

treatment methods such as ozonation or activated carbon. The aqueous odor threshold concentration for geosmin is 6 to 10 ng/L at 45 $^{o}$C (Rashash *et al.,* 1997), the intensity of the odor is temperature dependent (Whelton and Dietrich, 2004), and customer complaints start at 7 ng/L (Simpson and MacLeod, 1991). Similar to geosmin, 2-MIB has an odor threshold of 2 to 20 ng/L at 45 $^{o}$C (Rashash *et al.,* 1997), and customer complaints start at 12 ng/L (Simpson and MacLeod, 1991). Nonadienal is detectable at 2 ng/L, and its odor characteristic changes from cucumber to fishy as its concentration rises to 13 ng/L (Rashash *et al.,* 1997).

There are many analytical and sensory methods available for the monitoring of drinking water to detect the off-flavor compounds and each has its own advantages and disadvantages (Mallevialle and Suffet, 1987; Bae et al., 2002; Devesa et al., 2004; Bruchet, 2006). When the methods are compared, the sensory methods can be superior because the human mouth and nose is more sensitive than most available instrumentation and instant results enable the utilities to take rapid action. Among the sensory methods, the flavor profile analysis (FPA, Standard Methods 2170: APHA, AWWA, WEF 2005) is one of the most comprehensive and reliable methods. It was originally designed for the food industry (Caircross and Sjostrom, 1950) and later adapted to the water industry (Krasner et al., 1985; Suffet et al., 1988) to be applied for the sensory characterization of drinking water. FPA panelists are highly trained to basic tastes at various intensities, mouthfeel and various odors. This way a water sample may be analyzed for off tastes and odors as well as abnormal mouthfeel.

Iron and copper may be present in drinking water due to corrosion of metal pipes and/or because of the minerals in the source water, and hence tap water can be an important source of these micronutrients. Recommended daily intakes for iron and copper were established as 15 and 2 mg respectively, although these values vary depending on age and gender, 5 % of the required values are supplied by most of the tap water in the US (WHO, 1996). Iron and copper participate in functioning of enzyme systems, required for oxygen transport, and healthy growth. Anemia, impaired immunity and development are common problems related to copper and iron deficiency. Iron and copper participate in complicated oxidation-reduction reactions in

many systems. Iron is more anodic and thus more readily corrodible than copper. Iron and copper in drinking water can cause an annoying metallic sensation which results in consumer dissatisfaction, loss of trust in water safety, reduced consumption of this vital beverage, and possibly a health threat due to too little or too much ingestion of a metal (Dietrich, 2006; Dietrich et al., 2004; Suffet et al., 1996). Individual taste thresholds vary from 0.1 to > 10 mg/L for cupric ($Cu^{2+}$) and from 0.04 to 3 mg/L for ferrous ($Fe^{+2}$) (Cuppett et al., 2006; Tucker et al., 2007; Cohen et al., 1960). Soluble copper species are more readily tasted and chlorine does not interfere with their taste. Metallic flavor is not only a problem for drinking water consumers, but also has been a serious concern for cancer patients that undergo chemotherapy, affecting their nutrition and health. If ingested at higher concentrations (>3 mg/L) iron and copper may cause nausea, vomiting, diarrhea, kidney and liver damage. EPA secondary maximum contaminant levels for ingestion are <1 mg/L for copper, and <0.3 mg/L for iron.

So far only a few researchers have studied the oral sensory perceptions caused by metallic compounds. First it was reported that the metallic salts cause a "metallic" taste as they create a surface electrical potential on the tongue (Plattig, 1988). Later, Lawless et al. (2003) described high concentrations of divalent cations (such as calcium and magnesium) as having bitter, salty, metallic, astringent, sour, and sweet tastes, but Keast (2003) reported zinc had little taste. The first study that connected metallic taste with smell (Hettinger et al., 1990) found that the metallic taste of ferrous sulfate ($FeSO_4$) was decreased when the nose was occluded. Lawless et al. (2004) confirmed that a retronasal smell is a component of oral metallic sensation of ferrous iron but not for copper or zinc sulfate. Cuppett et al. (2006) reported that copper speciation did not significantly affect taste but soluble species were more readily tasted than particulate species. Also a retronasal effect of copper was observed in the unpublished studies. In oral studies with copper, the time to intensity of copper's metallic flavor varied from 10-30 seconds and was pH dependent. Copper binds to salivary proteins; and hence the presence of copper reduces the volatility and thus flavor of n-hexanal in a model mouth system (Hong et al., 2006). One study reported that at very high concentrations of ferrous and cupric TRPV1 taste receptors are activated which may be related to metallic taste perception (Riera et al., 2007).

Although researchers have focused mainly on taste thresholds of metals, recently a few studies started evaluating the effect of olfaction on metallic sensation. These studies have conducted taste tests by occluding the panelists' noses with nose-clips and reported a significant decrease in perceived perception for ferrous and cupric even when they were tested at very high concentrations (Hettinger et al. 1990; Lawless et al. 2004). Epke and Lawless (2007) determined taste thresholds with and without occluding noses of the panelists by nose-clips and reported that taste thresholds determined with closed nose were 10 to 30 times higher than the open nose values. This suggests that metallic sensation has an important odor component. A recent work that investigated the metallic odor of skin and analyzed the carbonyls in the headspace of the skin after ferrous or cupric solutions were rubbed on it. A series of aldehydes and ketones were reported including n-hexanal and 1-octen-3-one (Glindemann et al. 2006). This suggests that the metallic flavor and odor may be caused as a result of oxidation of phospholipids in the cell membranes of the skin.

## 2. Oral Mucosa and Composition of Cell Membranes

The oral cavity as well as having a digestive role is also involved in speech, sensory reception, facial expression and breathing. Lips, tongue, teeth, salivary glands and oral mucosa are the main structures of the oral cavity. Oral mucosa, a mucous membrane, lines the entire oral cavity and contains the sensory receptors. Stratified squamous cells form the epithelium of the oral mucosa and the cells covering the palate are keratinized due to friction as a result of mastication. Stratified epithelia have two or more layers of cells starting with a cubodial basal layer going up to a flattened surface layer. The loss of surface layer cells does not affect the underlying tissue (Burkitt et al., 1993; Terashi et al., 2000). The surface of oral epithelium consists of several layers with distinct characteristics. The basal cell layer is made up by the cornified layers and as the cells form the upper layers they undergo biochemical and morphologic changes (Oda and Watson, 1990).

The plasma (cell) membranes of the epithelial cells are composed of a phospholipid bilayer, proteins and carbohydrates. Phospholipid molecules have a polar head and a

non-polar tail. The polar heads are derived from glycerol conjugated to choline, serine, or ethanolamine. The tails are made up of two fatty acids (usually one saturated and one unsaturated in mammalian cell membranes) covalently bonded to glycerol. Cholesterol molecules are also abundant in the membranes and regulate the fluidity. Proteins may be found as incorporated to the membrane or maybe held to the outer and inner surfaces of the membrane by electrostatic forces. The proteins function as pores and allow the passage of hydrophilic molecules across the membrane. Some of the membrane lipids and most of the membrane proteins are conjugated with polysaccharides forming glycoproteins and glycolipids and these components function in the formation of intercellular adhesions (Burkit et al., 1993).

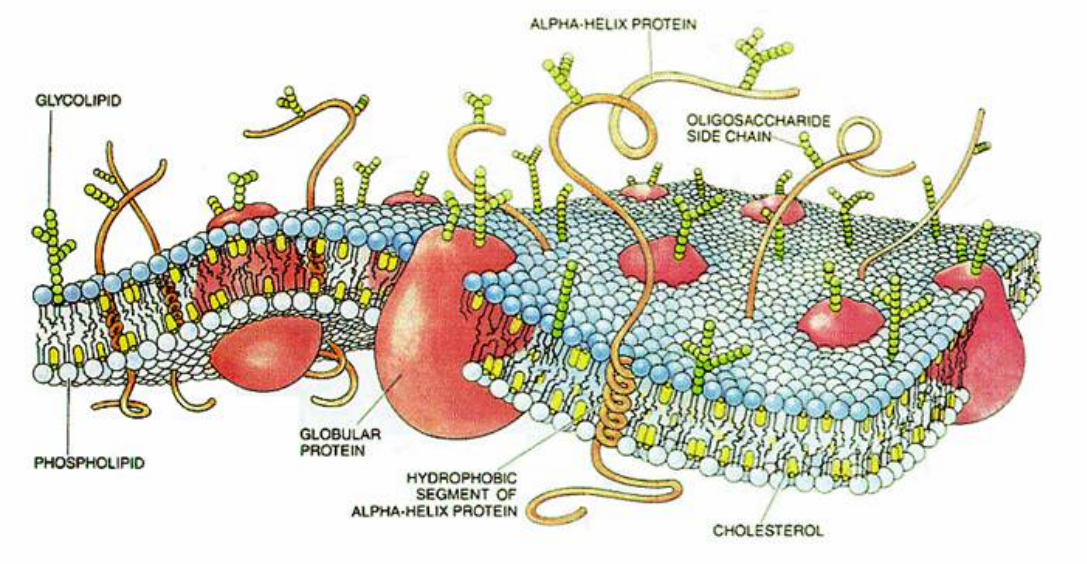



**Figure-2.1.** An illustration of a cell membrane (wikimedia.org)

The phospholipids of the cell membrane have structural and functional significance as well as a role in keratinization of the epithelium (Singer and Nicholson, 1972; Terashi et al, 2000). Fatty acids are an important part of the phospholipids. Humans can synthesize saturated fatty acids such as palmitic (16:0) and stearic (18:0) acids (Marcelo and Dunham, 1993). Palmitoleic (16:1) and oleic (18:1) acids which are the main monounsaturated fatty acids in humans may be synthesized from the saturated fatty acids by the desaturation enzymes (Ziboh and Chapkin, 1988). However, linoleic (18:2) and arachidonic (20:4) acids which are the major essential fatty acids cannot be

synthesized and should be supplied by the diet (Chapkin and Ziboh, 1984). The oxidation of linoleic acid leads to a series of aldehydes and ketones. Acetaldehyde, propionaldehyde are among the aldehydes reported as well as hexanal being the major by-product. Pentanal, hexanal, 1-octen-3-one, and trans-2,cis-4-decadienal were reported as the major oxidation by-products from arachidonic acid (Chan, 1987).

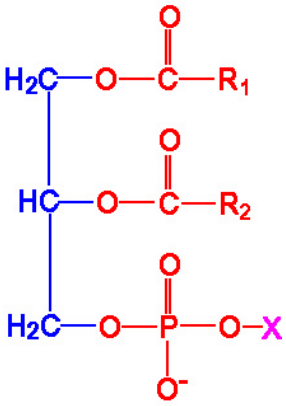



**Figure-2.2.** Basic structure of a phosholipid molecule in a cell membrane (where X may be a number of different substituents such as choline, ethanolamine, glycerol and Rs are fatty acids) (http://dwb.unl.edu/basic-phospholipid.jpg)

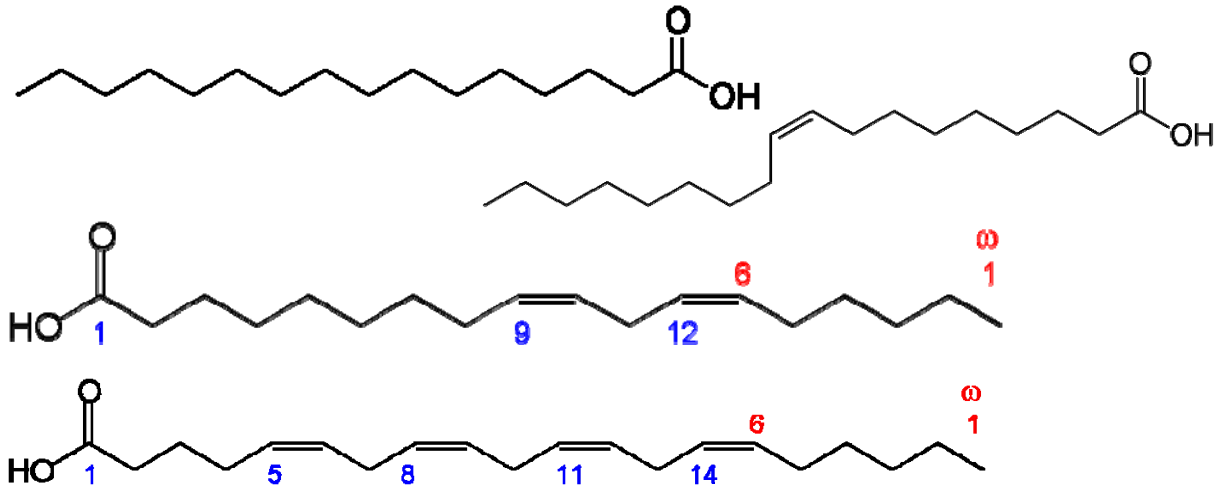



**Figure-2.3.** Structure of palmitic, oleic, linoleic and arachidonic acids (wikipedia.org)

Lekholm and Svennerholm (1977) investigated the lipid pattern of the oral epithelium of humans and reported oleic, linoleic and arachidonic acids as the major

fatty acids detected in the largest fraction of the phospholipids. It was also noted that the composition of the fatty acids were not affected by the age and gender of the subjects. Terashi et al. (2000) suggested that the type of the fatty acids forming the phospholipids affect the cell growth, differentiation and function. The study of the fatty acid composition of cell membranes of epidermal, oral mucosal, and hair follicle cell samples from healthy adult humans revealed that epidermal cell membranes contain a significantly higher amount of linoleic acid whereas the hair follicle cell membranes have higher amount of palmitic acid and lower amount of linoleic and arachidonic acids than the oral mucosal cell membranes. It was also noted that the cell differentiation may be achieved by the linoleic acid accumulation in the cellular membrane.

Effects of metallic dental materials used for orthodontic appliances such as brackets, molar bands and implants on epithelial cells and tissues were studied recently. These materials are in close contact with the cells and tissues and have been reported to cause allergic, inflammatory, toxic and mutagenic reactions. The findings indicated that ions released from there materials especially copper and zinc affect the growth and vitality of oral cells and hence the tissues. However it was also noted that the extent of effects of the metals depends on the corrosion of the alloys, proteins in the saliva and ion release from the appliances (Cortizo et al., 2004).

## 3. Lipid Oxidation

Polyunsaturated fatty acids (PUFA) in biological systems are inert to lipid peroxidation to be initiated above under normal conditions since reactions require a high activation energy and occur very slowly. However in the presence of a metal ion the reactions become kinetically feasible and the initiation of lipid peroxidation occurs (Aust and Swingen, 1982). Heavy metals such as iron and copper that can be present in two or more valency states possess oxidation and reduction potentials and hence are among the powerful catalysts of lipid oxidation. When the metal in its lower oxidation state reacts with the lipid and the oxygen a chain reaction is started and radicals are formed. The metal in its higher oxidation state may also undergo this reaction however the reaction kinetics are much slower than the previous one (Chan, 1987; Frankel, 1998). Iron has

been shown to be the most active catalyst in vivo and in vitro lipid peroxidation reactions. It is found at a much higher level in humans and animals, and some chelators enhance the ability of iron to promote lipid peroxidation (Aust and Swingen, 1982). In the human body, unsaturated fatty acid oxidation is may be catalyzed by the iron-containing heme compounds. In the cellular environment the lipid peroxides are quickly decomposed and metabolized by the tissue homogenates (O'Brien, 1969).
The steps of lipid oxidation are given below.

***Initiation:***

$RH \rightarrow R\cdot + H\cdot$ (catalyzed with heat, light, and/or metals)

$ROOH + M^{3+} \rightarrow ROO\cdot + H\cdot + M^{2+}$ (slow)

$ROOH + M^{2+} \rightarrow RO\cdot + H\cdot + M^{3+}$ (very fast)

$2\ ROOH \rightarrow RO\cdot + ROO\cdot + H_2O$

***Propagation:***

$R\cdot + O_2 \rightarrow ROO\cdot$

$ROO\cdot + RH \rightarrow ROOH + R\cdot$ (slow)

$RO\cdot + RH \rightarrow ROH + R\cdot$

***Termination:***

$R\cdot + R\cdot \rightarrow 2R$

$R\cdot + ROO\cdot \rightarrow ROOR$

$ROO\cdot + ROO\cdot \rightarrow ROOR + O_2$

where RH, R·, RO·, ROO·, ROOH, and M represent unsaturated fatty acid, alkyl radical, alkoxy radical, peroxyl radical, hyroperoxide, and transition metal (such as iron, copper, and zinc) respectively.

During the initiation, free radicals (RO· and ROO·) are formed. Because the initiation reaction of fatty acids with oxygen or radicals is thermodynamically not favored, the reaction should be activated either by light or heat exposure, or catalysis by metals. In the propagation step, unsaturated fatty acids are converted to hydroperoxides

by reacting with free radicals and oxygen. Hydroperoxides are tasteless and odorless however when they decompose off-flavors are generated such as fishy, metallic, grassy, and etc. During propagation, metal ions again play a role and react with the hydroperoxides produced. Then hydroperoxides decompose into aldehydes, ketones, alcohols, hydrocarbons, and acids. During termination non-radical species are formed and the oxidation is stopped (Kochhar, 1996).

An earlier work studied the catalysis of arachidonic acid oxidation by ferrous iron. In the presence of ferrous and oxygen, superoxide is formed, then the superoxide reacts with hydrogen ions to produce hydrogen peroxide. After this step ferrous and hydrogen peroxide react to produce hyroxide and hydroxyl radical. This way iron promoted the reaction between hydrogen peroxide and superoxide and caused the generation of a strong oxidant by reduction of hydrogen peroxide. This reaction is known as the Haber-Weiss or Fenton rection (Fridovich and Porter, 1981). This work also reported that presence of xanthine oxidase also promoted the oxidation of the arachidonic acid. Another study by Braughler et al. (1987) also demonstrated that ferrous catalyzes the oxidation of linoleic and arachidonic acids.

## 4. Measuring the Extent of Lipid Oxidation by Thiobarbituric Acid Reactive Substances (TBARS) Method

Oxidative stress in the cellular environment, which is caused by metal ions in this case, results in the generation of highly reactive and unstable lipid hydroperoxides. The peroxides are derived from the polyunsaturated fatty acids (Aust and Svingen, 1982). Metals promote decomposition of these hydroperoxides as shown below for iron:

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO\cdot + OH^{-}$$
$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO\cdot + H^{+}$$

The decomposition of the hydroperoxides results in the formation of carbonyls including malondialdehyde (MDA). Determination of MDA is a widely used method to monitor lipid oxidation in diseases (Maboudou et al., 2002). Formation of MDA from α-

linolenic acid is given below (Figure-2.4). MDA can be quantified colorimetrically after its controlled reaction with thiobarbituric acid (Figure-2.5). The measurement of TBARS has been a widely used method for screening and monitoring lipid peroxidation since the early 80's. TBARS method has been used to evaluate samples that include human and animal tissues and fluids, drugs and foods to study the effect of nutrition on health (Yagi, 1998; Armstrong and Browne, 1994).

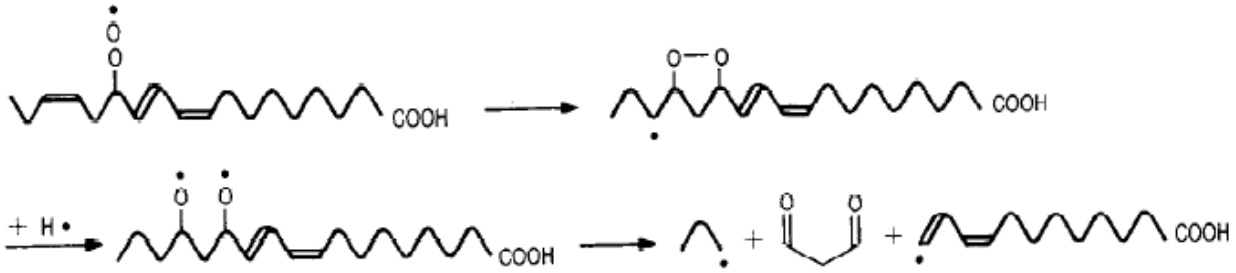
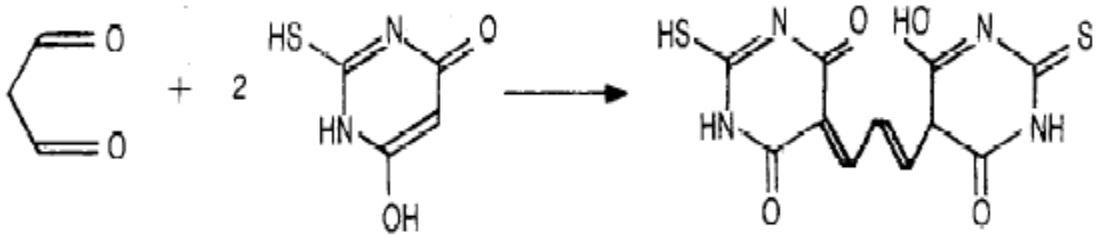



**Figure-2.4.** Abstraction of H from C11 of α-linolenic acid yields malondiadehyde via a cyclic peroxide formed with an addition of OO· at C13 (first compound) to the C15-16 double bond (second compound)




MDA TBA Pink Product

**Figure-2.5.** Reaction of thiobarbituric acid with malondialdehyde

Several studies focused on the MDA concentration in saliva of periodontitis patients, cancer patients, and smoking people. Hodosy and Celec (2005) studied the effect of tooth-brushing on salivary MDA values and reported that the values are reduced after brushing the teeth. It was also noted that the values vary during the day from 0.055 to 0.08 μM. Another work by Celec et al. (2005) studied the effect of smoking on oral oxidative stress for patients with inflammatory periodontal diseases. The MDA values increased to 0.3 μM for non-smokers and were significantly lower than smokers' values

(0.55 μM). The TBARS values of the saliva of the peridontitis patients reported were very higher than the values stated above. The values went up to 1.2 to 1.3 μM. In this study the total antioxidant power in saliva was also measured by ferric reducing antioxidant power (FRAP) test. The determined values were significantly lower in patients compared to healthy controls (Mashayekhi et al., 2005). MDA is also measured by other methods (explained in section 6) to monitor lipid oxidation in biological samples.

## 5. Measuring the Extent of Lipid Oxidation by Other End Products

Lipid peroxidation causes injury to brain and other tissues in vivo. There are several metabolically and chemically stable oxidation by-products. These biomarkers include the isoprostanes and isofurans derived from oxidation of arachidonic acid, and neuroprostanes derived from oxidation of docosahexaenoic acid (Montine et al., 2004). Nonenzymatic free radical induced oxidation of arachidonic acid produces F-2 isoprotanes (Morrow et al., 1990). Formation of isoprostanes from lipid oxidation of arachidonic acid and lipid hydroperoxides are shown below.

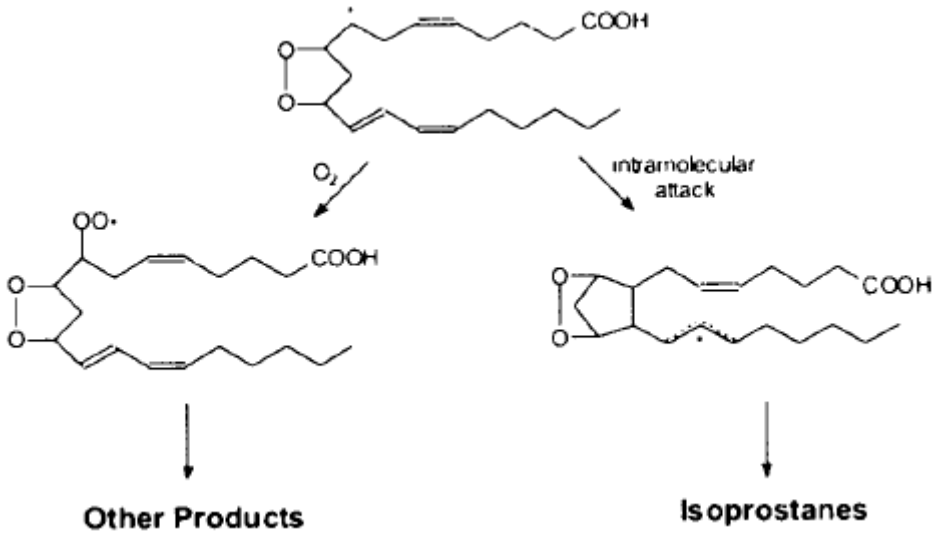



**Figure-2.6.** Formation of isoprostanes from arachidonic acid oxidation

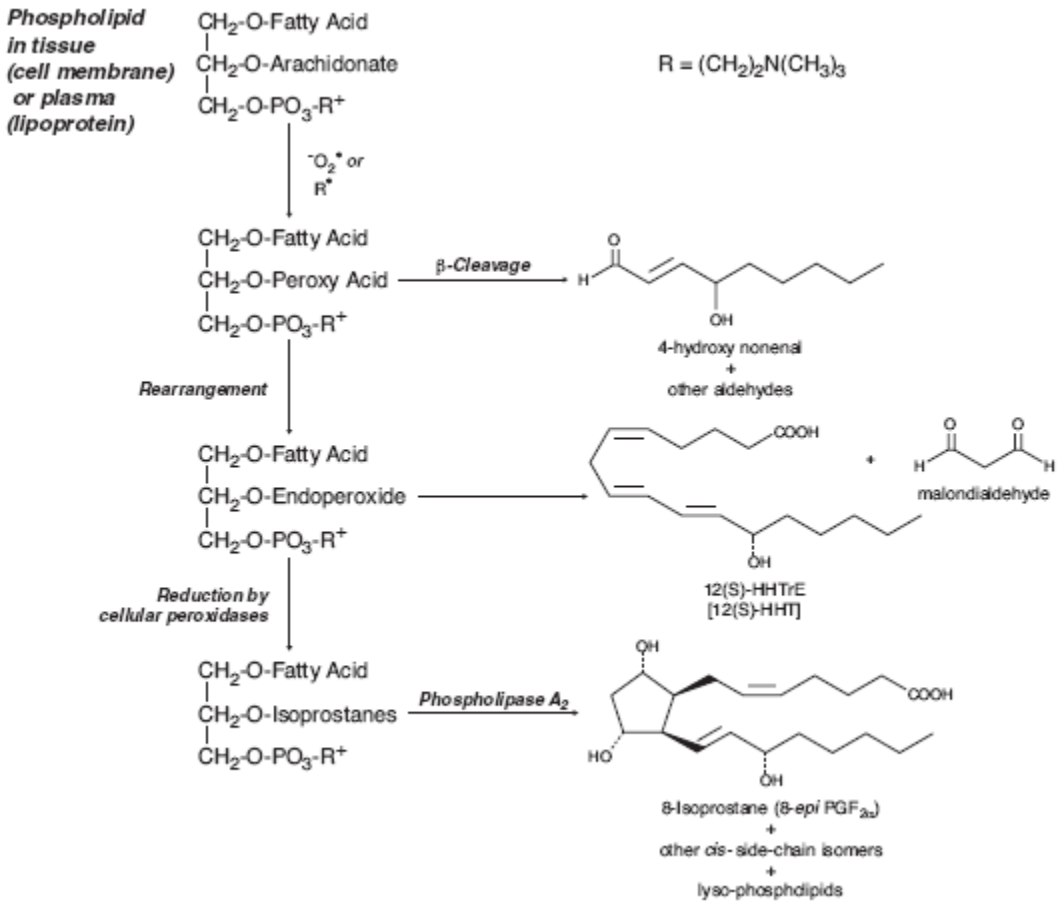



**Figure-2.7.** Oxidation of arachidonic acid in phospholipids and formation of malondialdehyde and 8-isoprostanes

As seen from Figure-2.6 above, isoprostanes are formed when a cyclopentane ring is obtained with an intracellular attack of a double bond to the carbon centered radical. However, at this step molecular oxygen competes and attacks the carbon centered radical to form other end products such as isofurans. Hence in matrices with high oxygen isoprostane may not be a good measure for sensitive determination of lipid oxidation (Fessel et al., 2002).

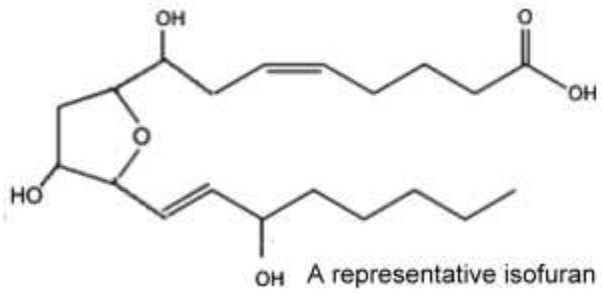



**Figure-2.8.** Isofuran structure

Oxidation of docosahexaenoic acid (DHA), which is found in high quantities in brain, leads to the formation of F2-isoprostane-like compounds which are called F4-neuroprostanes. Initially DHA radicals are generated and with addition of a molecular oxygen peroxyl radicals are formed. More addition of molecular oxygen to the peroxides result in endocyclization and formation of bicyclic endoperoxides. Finally the endorperoxides are reduced to neuroprosanes (Roberts et al., 1998).

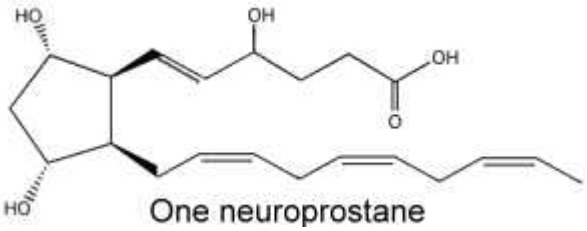



**Figure-2.9.** Neuroprostane structure

Some of the studies that investigated lipid oxidation in vivo measured isoprostanes, isofurans, and neuroprostanes to identify the extent of oxidation. A recent study measured F2-isoprostanes and F4-neuroprostanes in brain tissue samples by GC/MS and concluded that monitoring these compounds may be useful in finding the causes of Alzheimer's and Parkinson's diseases (Bernoud-Hubac and Roberts, 2003). When the tissues of brains from patients died from advanced Alzheimer's or Parkinson's disease were analyzed, increased levls of isoprostanes, neuroprostanes and isofurans were detected (Montine et al., 2004). Wolfram et al. (2006) measured the isoprostanes (8-epi-PGF and 6-oxo-PGF) in the saliva of periodontitis patients who are smokers and

nonsmokers. The results showed that isoprostane concentrations were elevated as the periodontitis state worsened. The patients who were smokers also had much higher levels of isoprostanes. McKinney et al. (2000) measured the isoprostane (8-epi-PGL) levels in plasma, urine and saliva of normotsensive and preeclamtic pregnant women. Non pregnant women were also tested as controls. The samples were deproteinated, centrifuged, diluted with deionized water and acidified before they were passed through a C-18 Supelclean. After the cartridge was rinsed with deionized water and hexane, the isoprostane was eluted with ethyl acetate. The eluate was dried under nitrogen and then the isoprostane precipitate was reconstituted with enzyme immunoassay buffer. The prepared samples were analyzed by a commercial kit. It was reported that isoprastane levels were significantly increased for the pregnant women with the condition for all the samples tested. A recent study determined the isoprostane concentrations in exhaled breath condensate (EBC) and saliva samples from asthma patients to monitor and manage the disease. The values detected were comparable for both specimens and the researchers noted that even though EBC was a simple method the results were variable and hence analysis of saliva samples may be a better measure (Simpson et al., 2005).

## 6. Carbonyls Produced as a Result of Lipid Oxidation and their Measurement

In a recent study, it was demonstrated that ferrous iron reacts with skin lipids to produce the “metallic” odor and a reproducible series of n-aldehydes, including n-hexanal, and ketones, including 1-octene-3-one. The procedure included rubbing palm skin with ferrous iron solution at 50 mM and sampling the headspace with SPME-GC/MS and GC-O (Glindemann et al., 2006). Volatile compounds from oxidized pork phospholipids were studied by several researchers. Meynier et al. (1998) employed purge and trap method while Im et al. (2004) determined the carbonyls and alcohols by SPME-GC/MS that cause metallic off-flavor. An array of n-aldehydes, dienals, and alcohols were reported by either study.

There is little literature available on metal-catalyzed oxidation of human tissue, however the food literature describes the generation of metallic smelling compounds

(aldehydes and ketones) as a result of the oxidation of unsaturated fatty acids. Many compounds having a metallic note, including trans- 4,5-epoxydecenal, (Z)-1,5-octadien-3-one and 1-octen-3-one, were detected in foods and beverages by analyzing the samples with gas chromatographic sniff-port technique (Guth and Grosch, 1990; Hinterholzer and Schieberle, 1998; Hinterholzer et al., 1998; Buettner and Schieberle, 1999). 1-Penten-3-one and (E,Z)-2,6-nonadienal were also detected as by-products of lipid oxidation and are reported as off-flavor compounds having a metallic note (Venkateshwarlu et al., 2004). Hexanal is a major lipid oxidation by-product of meats as well (Mielnik et al., 2006).

Kumasawa and Masuda (1999) analyzed Japanese green tea (Sen-cha) by using aroma extract dilution technique and GC/MS to detect the main odorants. One of the highest flavor dilution value determined was for (Z)-1,5-octadien-3-one. In a previous study made by Guth and Grosh (1993) it was suggested that the compound was an important odorant in the Japanese green tea. Both of the studies documented the compound as having a metallic odor.

In a study conducted by Heiler and Schieberle (1997), it was concluded that the shelf-life of sour cream buttermilk is shorter than the fermented buttermilk due to generation of off-flavor in the former one. The off-flavor that develops during the storage of sour cream buttermilk was described as metallic by Frank, 1984. Heiler and Schieberle (1997) identified the compounds responsible for the metallic note as (E,Z)-2,6-nonadienal, 4,5-epoxy-(E)-2-decenal, and 3-methylindole by using comparative aroma extraction dilution analysis. Hatanaka et al. (1975) suggested that (E,Z)-2,6-nonadienal may be formed in cucumbers by an enzymic reaction with alpha-linolenic acid as a precursor.

Buettner and Schieberle (2001) reported trans-4,5-epoxy-(E)-dec-2-enal as the main compound having a metallic odor identified in fresh, hand squeezed White Marsh seedless grapefruit juice and reported the odor threshold values for epoxydecenal as 0.12 ppb in water orthonasally and 0.015 ppb retronasally. Trans-4,5-epoxydecenal was suggested as an oxidation by-product of linoleic acid by Guth and Grosch (1990).

The carbonyls produced as a result of lipid oxidation may be analyzed by several methods. A common method employed to capture the carbonyls in complex matrices (including air and water) is derivatization of carbonyls with 2,4-dinitrophenylhydrazine (DNPH). This method has been widely used due to its high reactivity and selectivity. During derivatization, the carbonyl loses its oxygen and forms a stable complex with the DNPH as shown in Figure-12.

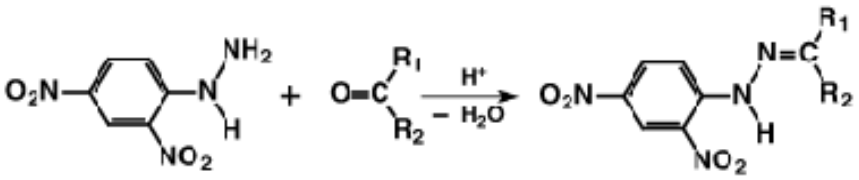



**Figure-2.10.** Derivatization of carbonyls with DNPH (van Leeuwen et al., 2004)

The DNPH-carbonyl derivatives can be analyzed by high pressure liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS) after extraction with a suitable solvent. The HPLC method has been preferred over the GC-MS due to its good replication and robustness. However, in complex samples such as saliva, the HPLC separation is poor due to interferences, and also it is difficult to determine the carbonyl compounds at low levels. In addition, the GC coupled with MS has better separation of the derivatives when compared to HPLC, and each DNPH-carbonyl compound can easily be identified (Dong and Moldoveanu, 2004; Maboudou et al., 2002). The oxidative stress as a result of ischemia-reperfusion syndrome causes production of formaldehyde and malondialdehyde in tissues and plasmas. These two compounds can be derivatized with DNPH and analyzed by GC/MS. Maboudou et al. (2002) employed this method to study the oxidation in rat brains and concluded this method could be used to monitor oxidative stress.

Another method is derivatizing the carbonyls with 0-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine (PFBOA). The resulting derivatives are fairly volatile and a headspace GC/MS method is ideal to detect them, although HPLC maybe employed as well.

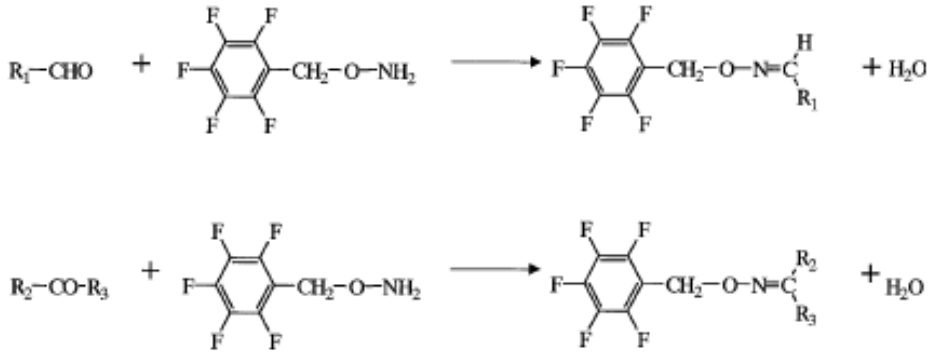


**Figure-2.11.** Derivatization of aldehydes (above) and ketones (below) with PFBHA (Sugaya et al., 2004)

The oxime, the end product of the reaction of PFBHA and the carbonyl, is insensitive to light and thermally stable. PFBHA derivatization has been used for more than a decade to analyze the carbonyls in drinking water produced as disinfection by-products due to ozonation; airborne carbonyls in the atmosphere; and carbonyls in biological matrices produced as a result of lipid peroxidation. The *in situ* PFBHA derivatization enables the researchers to stabilize the carbonyls present in the matrix which may not be stored until analysis as well as improving the sensitivity of the gas chromatography methods (Sugaya et al., 2004).

In practice it is very hard to determine the unknown carbonyls in environmental samples. Le Lacheur et al. (2004), have identified and studied the mass spectra of the selected aldehydes which are important environmental pollutants or disinfection/oxidation by-products. The main ions were presented with corresponding structures to identify the unknown compounds in the samples rather than running standards to pinpoint the compounds detected by GC/MS. Several methods used to detect carbonyls include direct headspace-GC/MS analysis (Sugaya et al., 2001), purge-and-trap GC/MS analysis, HPLC based on the concentration and the matrix of the samples. Annovazzi et al. (2004) developed HPLC and capillary electrophoresis methods to detect the aldehydes inhuman saliva after smoking. For the HPLC portion of the study saliva samples collected from subjects were derivatized with DNPH and were separated by HPLC and monitored by UV absorption. Electrophoresis separation was conducted with whole saliva collected and the aldehydes were measured with a UV-Vis

scanning detector. Formaldehyde, acetaldehyde and acrolein were detected in the samples. Another study analyzed the aldehyde-DNPH derivatives in the saliva samples from smoker with a polymer monolith microextraction couple to HPLC. Same aldehydes as the work above, as well as butyraldehyde were detected in the samples (Zhang et al., 2006). Recently a new method was developed to analyze the aldehydes by on SPME fibre derivatization with PFBHA. In this method SPME fiber was exposed to PFBHA in headspace, then the samples were incubated with the SPME fiber. The derivatized samples were desorbed at the GC/MS port. Asthma and chronic obstructive pulmonary disease patients have elevated levels of n-aldehydes (C6 to C10) in their breaths. It has been suggested that presence of hexanal and heptanal in blood may be used to monitor lung cancer. With the method explained the aldehydes mentioned above were successfully detected in the samples by two separate studies (Svenson et al., 2007; Deng et al., 2004).

## 7. Antioxidants and Chelating Agents to Prevent Lipid Oxidation

Cells and biological fluids have their own antioxidative mechanisms to prevent free radical formation and oxidative damage. The compounds include glutathione, vitamins E and C, β-carotene, and enzymatic systems such as superoxide dismutase, catalase, and glutathione peroxidase (Dille and Joenje, 1991; Koracevic et al., 2001).

Antioxidants slow down or delay the initiation of lipid oxidation *in vivo* and in food systems by donating their hydrogen atom and forming non radical species. The reactions of antioxidants (AH) and free radicals are shown below:

$$AH + RO\cdot \rightarrow ROH + A\cdot$$
$$AH + ROO\cdot \rightarrow ROOH + A\cdot$$
$$RH + A\cdot \rightarrow AH + R\cdot$$
$$A\cdot + R\cdot \rightarrow RA$$
$$A\cdot + A\cdot \rightarrow 2A$$
$$A\cdot + ROO\cdot \rightarrow ROOA$$

There are several natural and synthetic antioxidants used by the food industry as food additives to preserve the color, flavor and texture of the products. Vitamins C and E, as well as EDTA, BHT, and BHA are among the food additives. Vitamins E and C, and α-lipoic acid are important cofactors of enzymatic systems in the body and good radical scavengers. They donate their hydrogen atom and hence stop or slow down the oxidation reactions caused by the free radicals (Rajalakshml et al., 1995). These compounds are essential for human health because they participate in metabolic and enzymatic systems, regenerate oxidized enzymes (i.e. superoxide dismutase) and should be supplied by the diet. Vitamin C (L-ascorbic acid) is water soluble and may be destroyed by heat and light. Vitamin E (α-tocopherol), however, is fat soluble, and is susceptible to oxidation by oxygen in the air (Denisov et al., 2005). Vitamin E reduces lipid peroxide radicals by a nonenzymatic reaction (Gille and Joenje, 1991). α-lipoic acid is both fat and water soluble and hence has the ability to scavenge radicals in fatty and watery matrices (Challem, 1996).

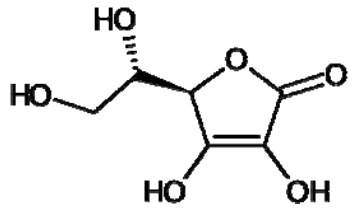



**Figure-2.12.** Structure of Vitamin C

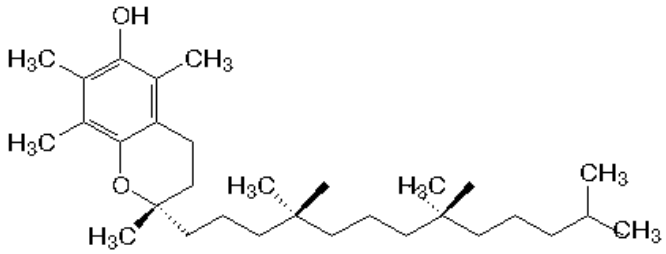



**Figure-2.13.** Structure of Vitamin E

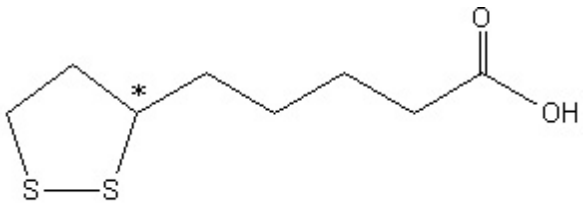



**Figure-2.14.** Structure of α-lipoic acid

While antioxidants scavenge radicals by donating hydrogen, chelating agents inactivate the redox active metals before they create free radicals (Nortemann, 2005). EDTA chelates with iron and copper. Another chelating agent that binds to iron is lactoferrin. Lactoferrin is a protein and mainly found in milk and other secretions by humans such as tears ad saliva (Uchida et al., 2006). In the body metals are bound to specific chelating proteins that reduce their redox potential.

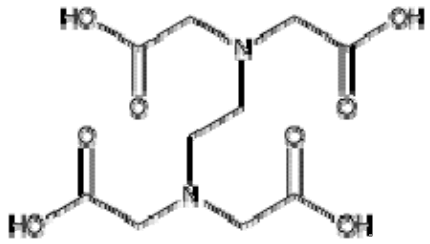



**Figure-2.15.** Structure of EDTA

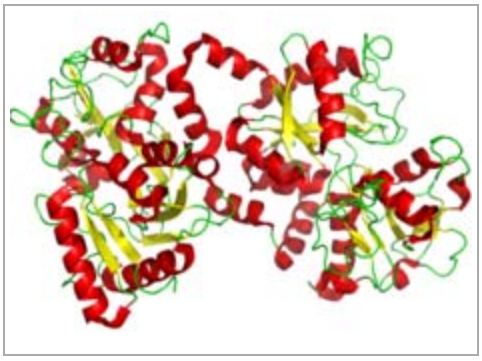


**Figure-2.16.** A lactoferrin molecule

Kawatsu et al. (1984) showed that ferrous causes phospholipid oxidation, and when ferrous and ascorbic acid (vitamin C) were present in the matrix the oxidation was

enhanced. However it was noted that this was not due to reduction of ferric back to ferrous (more reactive species) but due to formation of more free radicals. Another recent study determined the impact of iron and vitamin C in milk. Some supplements are added to human milk to improve the growth of premature infants. These supplements include iron and vitamin C. However it was not considered that the presence of these compounds may cause lipid oxidation in milk. This study showed that lipid oxidation occurs immediately after the supplements were added to milk, and the oxidation (measured by TBARS) was higher when vitamin C and iron were added simultaneously compared to addition of iron alone (Friel et al., 2007).

In a medical note the contribution of vitamin C to inflammation was discussed. The authors suggested that vitamin C is involved in the the redox cycling of metal ions and convert them back to more reactive species. The first suggested pathway was Fenton reaction which forms strong radicals (hydroxyl radical) by the reaction of a metal ion in reduced form and hydrogen peroxide. During this reaction, vitamin C may play a role in oxidation promoter by recycling oxidized metal ion to reduced form. Another mechanism produces hydrogen peroxide when a metal ion in oxidized form oxidizes vitamin C, ths leads the way to Fenton reaction. The last suggested mechanism, Undenfriend's system speculates the formation of hydroxyl radical suggesting a reaction between the reduced state metal ion, vitamin C and molecular oxygen. In this case vitamin C is accepted as a two electron donor (Fisher and Naughton, 2003).

Osborn and Akoh (2003) determined the effect of vitamin E, quercetin, and gallic acid on iron catalyzed lipid oxidation of lipids. As opposed to expected, all of these natural antioxidants enhanced lipid oxidation. They noted that for metal catalyzed lipid oxidation, antioxidants may have a prooxidant effect and may not be added to the food to prevent oxidation. In a recent study antioxidant effects of α-lipoic acid on lead catalyzed lipid oxidation was investigated. TBARS values in serum were compared for the rats which received water with lead acetate. During the feeding of the rats, control group was supplemented with α-lipoic acid as well. The results indicated that the TBARS values for the rats which were exposed to lead only were higher than the other group (Caylak et al., 2008). α-lipoic acid was also studied as a preoperative treatment to

prevent post operative oxidative stress. Patients were administered α-lipoic acid in conjunction with polyunsaturated fatty acids, selenium and magnesium orotate. The patients had reductions in oxidative stress and hence myocardial damage (Pepe et al., 2008). As shown by the previous studies α-lipoic acid has good antioxidant properties. However, as discussed for vitamin C and vitamin E, it recycles the oxidized metal ions back to reactive reduced form and hence has prooxidant effect for lipid oxidation catalyzed by metal ions (Packer et al., 1995).

EDTA is shown to prevent lipid oxidation when it exceeds metal the concentration. However if the concentration is not greater than EDTA cannot prevent oxidation (Gutteridge, 1984). Let et al. (2007) reported that lipid oxidation was greatly prevented in the fish oil samples were fortified with EDTA. Another recent study showed that TBARS values of beef samples were significantly lower when iron catalyzed lipid oxidation was prevented by EDTA, catalyse and superoxide dismutase (Decker et al., 2006). Lactoferrin is able to bind to ferric and cupric, and lactoferrin has a high affinity to iron at pH 6.4 to 6.7 (Teuwissen et al., 1972). A study investigated the effect of lactoferrin on MDA and total antioxidant power levels in serum when piglets were fed with a diet fortified with 2.5 g/kg for a month. The results indicated that the MDA levels were significantly lower than controls whereas total antioxidant power was significantly higher (Xu et al., 2006). Infant formulas lack the important antibacterial protein, lactoferrin. Satue-Gracia et al. (2000) studied the effect of lactoferrin on oxidation wen the formulas were supplemented with iron as well. The results indicated that even lactoerrin was at lower concentrations than iron, it still acted as a antioxidant and prevented oxidation.

The information presented above indicate that although vitamin C and E and α-lipoic acid works well in vivo scavenging free radicals by supplementing the antioxidant enzyme systems, it cannot work well for the lipid oxidation induced by metals. For the prevention of the lipid oxidation catalyzed by metals preventive antioxidants may be a better solution. Metal inactivators such as ethylenediamine tetraacetic acid (EDTA), phosphorc acid, and 8-hydroxy-quinoline suppress redox reactions and prevent oxidation. (Frankel, 1998). It should be noted however, the concentrations of these

compounds in the matrix is very critical for the prevention of lipid oxidation. When the metals are at higher concentrations the chelating agents may promote oxidation (Braughler et al., 1987).

## 8. Constituents of Human Saliva

Saliva plays a very important role in taste sensation. The compounds in the food dissolve in the saliva and undergo enzymatic degradation/reaction. Then the dissolved compounds reach to the receptors where a signal is generated and transmitted to the brain (Plattig, 1988). Enzymes in the saliva react with the compounds in foods and release the aroma compounds. Major enzymes found in saliva are α-amylase, lysozyme, and lingual lipase. Amylase starts the digestion of starches in the mouth by breaking them down to maltose and dextrin. The enzymes are responsible for the reduction of aldehydes to alcohols, and degradation of carboxyl esters and thiols as well.

Saliva is consisted of water, electrolytes, glucose, ammonia, urea, enzymes, ammonia, lipids and proteins. The electrolytes found in saliva include sodium (2-21 mmol/L), potassium (10-36 mmol/L), calcium (1.2-2.8 mmol/L), chloride (5-40 mmol/L), bicarbonate (25 mmol/L), and phosphate (1.4-39 mmol/L). The major proteins found in saliva are histatin (44.4 μM), lactoferrin (2 μg/mL), statherin (12.8 μM), and mucin (14- 203 μg/mL) (Lawless and Heymann, 1998; Tenovuo, 1989; Castagnola et al., 2001). Due to sloughing and microbial conditions oral epithelial cells and bacteria are present in saliva as well (Celec et al., 2005). Average daily saliva production varies between 1 to 1.5 L. Bicarbonates, phosphates and urea define the pH and buffer capacity of saliva. Proteins and mucins attach to microorganisms and contribute to the dental plaque metabolism. Proteins, enzymes and immunoglobins serve as antibacterials. Phosphate, calcium, and proteins also regulate de- and re-mineralization in saliva (Humphrey and Williamson, 2001).

One of the physiological functions of saliva is to coat the tissues to prevent them from drying. Saliva is also important for enamel pellicle formation. Oral tissues are lubricated by saliva and hence are protected against any damage caused by mastication.

Because the saliva contains many electrolytes that enhance the buffer capacity, the pH can be preserved between 6.7 to 7.5 (Tenovou, 1989). The constituents of saliva such as salts and proteins, effect the perception of flavors. They may alter the hydration and/or spreadability of the compounds on the tongue yielding influenced flavor perception (Hutteau and Mathlouthi, 1998). Salivary proteins may alter the volatility of aroma compounds as well either by binding to the hydrophobic molecules or by salting out the hydrophilic molecules (van Ruth et al., 2001; Hong et al., 2006).

Salivary proteins also affect the metallic taste perceived. In a study conducted by Pfeiffer and Schwickerath (1991) it is reported that the dissolved nickel (coming from the dentures) created a metallic taste and dryness in the mouth. Salivary proteins have binding affinity to the metals present in dentures (Mueller 1983; Argarwal and Henkin, 1987). Glycoprotein binds to copper and nickel, whereas gustin binds to zinc (Shatzman et al., 1980). One of the salivary constituents that enhance the metal sensation is histatin. The histatin peptides are positively charged in solutions having a pH equal to and below 7 (Melino et al., 1999). Histatins can bind to various divalent metal ions such as $Zn^{2+}$, $Ni^{2+}$, and $Cu^{2+}$ and cause a structural change supporting biological activity (Melino et al., 1999; Gusman et al., 2001).

A recent study measured the total lipids and proteins in saliva of 44 female subjects in their early twenties. Bradford assay was used to measure total proteins and reported the values to be between 1.7 to 2.7 g/L. Bonilla (1972) reported that the salivary total protein concentration varies over time and sated that average protein concentration of healthy humans to be around 1.65 g/L and varied from 0.8 to 8.5 g/L. The total lipid concentration of saliva was measured by the Folch method. About 40 mg/L of total lipids was detected in the samples. Then the lipids were characterized by thin layer chromatography. Free fatty acids, cholesterol, glycolipids, and phospholipids were detected in saliva at about 15 mg/L, 5 mg/L, 12 mg/L, and 8 mg/L respectively (Tomita et al., 2008). The salivary lipids determined by Larsson et al. (1996) include cholesteryl esters, triglycerides, free fatty acids, cholesterol, and monoglycerides at 1 to 4.3 mg/L. The total lipids in the saliva for healhy subjects was determined to be 13.1 mg/L.

Oral squamous cell carcinoma is one of the most common types of cancer with a high morbidity rate. Recently it was demonstrated that oxidative stress plays a role in oral carcinoma development (Ma et al., 2006). Free radicals such as reactive oxygen species cause oxidative stress and result in DNA damage, tumor suppressor gene damage and strand breaks that lead to cancer. Through evolution, humans developed mechanisms to minimize the effects of oxidative stress. Salivary antioxidants such as peroxidase, superoxide dismutase, and uric acid inhibit the reactive oxygen species. Other antioxidants include vitamins E and C, and glutathione, albumin and trasferrin. Bahar et al. (2007) studied the salivary antioxidant components, and oxidatively damaged salivary DNA and proteins of 25 oral squamous cell carcinoma patients and 25 healthy subjects. It was shown that the antioxidant capacity of saliva obtained from the patients were significantly lower (~49%) than the healthy subjects' saliva whereas the oxidized DNA and protein levels were much higher (~65%) for the patients. It was also noted that these salivary components have anti- and pro-oxidant properties and regulate the conditions in the oral cavity.

## 9. Protein Oxidation

The main pathway of oxidative damage to proteins is the attack of metal catalyzed radicals on amino acids. Metal ions selectively bind to proteins and as they may still remain redox active, they can generate hydroxyl radical at the site of binding. Then the formed radical reacts in the vicinity of the bound metal and induce site-selective damage. Hydoxyl radical abstracts a hydrogen and reaction with the molecular oxygen produces a peroxyl radical. Breakage of a subsequent strand, formation of an aldehyde and generation of a new N-terminal amine are the end results of the site specific oxidation. Lipid oxidation may also cause site-specific damage. The by-products of lipid oxidation can bind to the proteins and inactivate them (Davies and Dean, 1997). One of the methods to detect protein oxidation is measuring protein carbonlys. Protein carbonyls may be formed by oxidative cleavage of the protein backbone, oxidation of amino acids (histidine, lysine, arginine, praline, threonine, and glutamic acid), or binding of carbonyls produced by lipid oxidation by covalent bonds (Kikugawa et al., 1991; Blakeman et al., 1998). Derivatization of the protein carbonyls with DNPH makes

it possible to easily detect and quantify the oxidative modification of proteins. The hydrazone derivatives may be detected by spectroscopy or Western Blotting (Castegna et al., 2003). Another method to detect protein carbonyl employs the alcohol dehydrogenase enzyme and reduces the carbonyls. Then the carbonyls are quantified by spectroscopy (Kingu and Wei, 1997). Proteins may also be oxidized by the reaction with hydroperoxides formed as a result of lipid oxidation (Reyftmann et al., 1990).

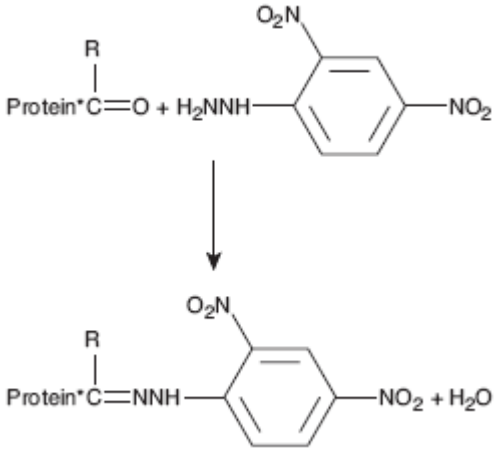



**Figure-2.17.** Derivatization of protein carbonyls with DNPH

Blakeman et al. (1998) investigated ferrous and ferric induced protein carbonylation. In the presence of ferric and ascorbate protein carbonylation was not enhanced, however in the presence of ferrous these was a significant increase in protein carbonyls. During this study TBARS values in the samples were also determined. When lipids were absent in bovine serum albumin (model protein) samples with ferrous, there was no significant TBARS formation. However, there was a small but significant increase in protein carbonyls. This indicates that Fenton reactions caused by ferrous may also carbonylate the proteins.

Several studies focused on effects of cigarette smoke on salivary proteins. Nagler et al. (2000) measured the protein carbonyls in saliva exposed to cigarette smoke. The reported that carbonyls were increased with increased exposure time and the aldehydes in the smoke may increase the protein carbonylation. The protein carbonyls were

quantified by spectroscopy and separated by Western Blotting. Another study later investigated the effect of cigarette smoke on oral peroxidase activity which is a mjor antioxidant enzyme in saliva. A significant decrease in peroxidase was detected, with a significant increase in protein carbonyls in saliva (Reznick et al., 2003). A recent study focused on the salivary profile of smokers. Salivary electrolytes in saliva samples of control subjects and smokers were not different. Unlike other salivary proteins and enzymes, superoxide dismutase and salivary antioxidant measures were very high compared to controls. However, protein carbonyls were much higher than controls as well. Although the antioxidant defense system of saliva was increased it was not enough to protect the proteins (Nagler, 2007). Another reason for changes in salivary antioxidant profile may be age related. Hershkovich et al. (2007) studied saliva samples from 44 subjects (in two groups: 20 to 25 and 70 to 80 years old). Flow of saliva was significantly lower for the older subjects than younger. Also overall total antioxidant level was significantly lower for older subjects, however individual concentrations of antioxidants were significantly higher than the younger. Salivary nitrogen species and protein carbonyls were also significantly higher for the older group. It was concluded that the antioxidant capacity in saliva decreases and oxidative stress increases. This may lead to DNA modification in oral epithelial cells and ultimately may cause oral cancer.

The abovementioned studies employed Western Blotting (SDS-PAGE) to determine protein carbonyls. After the salivary protein carbonyls were derivatized with DNPH the proteins were separated by electrophoresis. SDS-PAGE is a widely used method in biochemistry, genetics, molecular biology, and forensics. Sodium dodecyl sulfate (a detergent) denatures the proteins and gives them a negative charge. Polyacrilamide gel separates the proteins based on molecular weight and charge. Then the proteins are transferred to a PVDF membrane and blocked. The specific proteins are then marked by primary and secondary antibodies. Then the marked proteins on the membrane may be determined by colorimetric, chemiluminescent, radioactive, or fluorescent detection. Originally these procedures were developed by Laemli (1970) and Towbin et al (1978) and have been modified and extensively used for many biological samples since.

## 10. Cell Cultures as a Model for the Oral Cavity

Oral epithelial cells of animals have been cultured *in vitro* since late 60's to aid studies on oral cavity and mucosa. Studies mainly were conducted with oral tissue samples from hamsters and rats. White and Tankersley (1969) cultured the transplants from hamster cheek pouches on plasma clots, while Schuster et al. (1985) developed a simpler method to culture the epithelial cells in plastic tissue flasks. It has been already shown that culture of buccal oral epithelial cells form an organized structure even in the absence of a connective tissue underlying the cells (Flaxman, 1967). Numerous studies later have tested the use of fibroblastic feeder layer cells, epidermal growth factor, collagen gels, varied growth temperatures and pH, and modified media to culture disaggregated oral epithelial cells (Schuster et al., 1985). Many researchers also focused on culture of oral epithelial cells of humans. Taichman et al. (1979), Reinwald and Green (1977), and Peehl and Ham (1980) focused on optimizing the conditions for culturing cell close to *in vivo* properties. A commonly used method for human oral epithelial cell cultures was developed by Oda and Watson (1990) using gingival tissues from healthy adults. The cell cultures were maintained up to 7 passages (100 days). In this study positive effect of physiologic calcium was noted on the growth and development of the cells.

MatTek® (Ashland, MA) has recently developed multilayered and highly differentiated tissue models that consist of primary human epithelial cells. The tissue models closely parallel the normal human oral tissues morphologically with an organized basal layer and multiple non-cornified layers. The tissue cultures overlap the normal lipid profiles of the *in vivo* tissues with the *in vitro* tissues having slightly higher phospholipids (17%) than the *in vivo* tissues. The cells obtained from normal human tissues and are grown on collagen coated standard Millipore Millicell® culture plate inserts at the air liquid interface. After a certain time the proprietary culture medium is removed from the apical (top) surface and the cultures are fed through the basolatheral (bottom) surface. The growth medium is Dubelco's Modified Eagle's Medium (DMEM) with epidermal growth factor (EGF) and other proprietary factors. The tissues may be used for many purposes including toxicology and drug testing.

In order to ensure the survival of cells to study the same cell line, researchers started to transform the primary cells with viruses to make them immortal which would make it possible to passage them indefinitely and also to study carcinogenesis. In an earlier study primary human oral epithelial cells were infected with human papillomavirus, which is found in 90% of the head and neck cancers, and the cells could be maintained in culture for over 4 years whereas the primary cells survived up to 5 to 9 passages (Oda et al., 1996a). It has been reported that transformed cells lines have chromosomal changes and may not pass malignancy tests (Oda et al., 1996b). Another study compared the primary human oral epithelial cells and squamous cell carcinoma cell lines and concluded that the primary cells have higher saturated fatty acids and lower unsaturated fatty acids than the carcinoma cells (Gasparoni et al., 2004).

An earlier work by Macleod et al (1990) has compared the fatty acid composition of healthy and malignant oral epithelial cells. A reduction in relative proportions of palmitoleic and oleic acids were observed whereas there was an increase in palmitic, stearic and arachidonic acids. There was no significant change in linoleic acid composition.

**11. Metallic Taste Dysfunction of Cancer Patients**

About 2 million cancer patients receiving chemotherapy and radiotherapy encounter metallic flavor perception after consuming foods and beverages (Comeau et al., 2001). In some patients, the taste dysfunction starts even before the treatments begin (Johnson, 2001). However, abnormalities in taste acuity occur most commonly after chemotherapy or radiotherapy (Redda and Allis, 2006). Chemotherapy and radiotherapy may cause taste and odor disturbance by destroying taste and olfactory receptor cells. Cell damage may occur in three ways: 1) decrease in numbers of normal receptor cells; 2) alteration of cell structure or receptor surface changes; and 3) interruption of neural coding. Turnover rate of regular human taste bud cells is 10 days whereas the life span of olfactory receptor cells is about 1 week (Bernhardson et al., 2007; Nahikian-Nelms, 1990). Radiation may alter taste bud structure, such as taste pores, resulting in disrupted delivery of flavor molecules to receptor cells (McEntire and

Pixley, 2000). Cancer treatments may affect neuronal activities as well as receptor cells. Abnormal sensitization of the chorda tympani nerve can result in specific taste sensation without stimulating the taste receptors or the presence of the corresponding flavor molecules (Chang et al., 2002).

It was reported that metallic taste was experienced in 16% of 352 lung cancer patients (Sarhill et al., 2003) and 32.2% of 196 cancer patients with cancer at various places such as breast, head-and-neck, stomach, and lung (Newell et al., 1998). Metallic sensation may be connected to low levels of irradiation and chemotherapy with administration of cyclophosphamid, doxorubicin, 5-fluorouracil, methotrexate, and cisplatin (Capra et al., 2001; McDaniel and Rhodes, 1998). Red meats are often related to metallic taste, suggesting that the iron in the meat may be a reason for the metallic sensation (Johnson, 2001). Another reason may be the imbalance between oxidants and antioxidants that may enhance lipid peroxidation especially in the presence of iron and copper, and this can contribute to the metallic flavor production. Vitamins C and E are commonly found in human blood and act as antioxidants by reacting with reactive oxygen species and free radicals (Packer, 1991). However, decreases in antioxidant levels from cancer or its therapy may result in increased irreversible lipid peroxidation in blood and body tissues. In a recent study, the plasma levels of vitamins E and C, TBARS and cholesterol of male cancer patients were determined and compared to healthy subjects. The results showed that the cancer patients have significantly lower plasma levels of vitamin C and higher plasma levels of TBARS indicating that they have higher oxidative stress (Saygili et al., 2003). Another study also determined the oxidative stress of advanced cancer patients and analyzed the blood and bone marrow plasma samples and reported that the TBARS levels were significantly higher in lung and breast cancer patients whereas α-tocopherol levels were significantly lower in breast cancer patients when compared to healthy controls (de Cavanagh, 2002).

Bitter and metallic taste may also come from the taste of chemotherapeutic drugs. Many of these drugs have bitter tasting compounds that can enter the mouth through cervicular fluid from plasma or may diffuse from capillaries to the posterior of taste or odor receptor cells (Bernhardson et al., 2007). Some anticancer drugs work by a

free-radical-mediated mechanism which may lead to increased oxidative stress in patients (Samir and Kholy, 1999) For example, the anthracycline Adriamycin is known to increase lipid peroxidation in the heart (Look and Munsch, 1994). As mentioned above, patients prone to lipid peroxidation potentially face formation of unpleasant taste perceptions (Nowak and Janczak, 2006). A recent work reported interesting findings. Iron levels in plasma samples were measured for the patients before and they started chemotherapy with cisplatin. The results showed that plasma iron significantly increased for 65% of the patients within one day following the treatment. In the mean time vitamin C and E concentrations in the plasma decreased significantly suggesting that they were consumed by the iron in the blood (Weijl et al., 2004). These findings indicate that cancer patients suffering from metallic taste dysfunction may perceive that sensation due to increased levels of iron in their blood and may be consequently in their saliva.

Another reason for altered taste perception may be oral mucositis which is a common complication of head and neck radiotherapy and certain chemotherapeutic agents. Mucositis is the painful inflammation and ulceration of the mucous membranes lining the digestive track. In the bone marrow transplant setting, high dose chemotherapy regimens cause oral and GI mucositis in almost 100% of treated patients, and about 40% have ulcerative oral mucositis. Not surprisingly, oral complications such as mucositis, dry mouth, and blisters are significantly correlated with taste and smell abnormalities in patients with cancer. Mucositis damages the oral epithelial cell membranes and may cause cells to be more prone to oxidation as the oxidative stress is increased at the mucositis site. As explained in the section above, lipid peroxidation of oral epithelial cells result in formation of carbonyls which may cause off-flavor perception by patients (Rubensein et al., 2004; Rosen et al., 2006).

More than 40 % of the hospitalized cancer patients suffer from malnutrition due to taste and smell dysfunction. Unfortunately, these problems are underestimated and understudied by oncologists. Suggested treatments are naïve and only provide temporary solutions (Comeau et al., 2001).

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# Chapter III

# Literature Review for the Taste Threshold, Drinking Water Regulations, Recommended Daily Intake Values, and Adverse Health Effects of Selected Metals


## Abstract

When water gets in contact with soil and rocks, inorganics are naturally incorporated (NAS, 1977). However, water treatment methods, distribution system pipe materials, contamination from industry and agricultural practices may also contribute to the chemical composition of the source water (WHO, 1993, 1996, 1998; Letterman, 1999). Corrosion of copper, cast iron, steel, and zinc galvanized pipes introduce copper, iron and zinc to the drinking water (WHO, 1996; Alam and Sadiq, 1989). Water hardness is caused by the presence of calcium and magnesium. Presence of these metals in drinking water may alter its aesthetic quality (taste and color) as well as making it a source of micronutients. Hence a literature review was performed to understand the sensitivity of humans to iron, copper, zinc, manganese, calcium, magnesium, and aluminium, how they are regulated in drinking water, the recommended daily intake values and their adverse health effects when consumed at higher concentrations.


## 1. Overview of the Selected Metals

### 1.1. Iron

Five percent of the earth's crust is made up of iron. In ground water and at the dead ends of the distribution system iron is found in ferrous form, and it is converted to ferric when it is exposed to a disinfectant or oxygen causing colored water. Iron is found as oxides, hydroxides, sulfides and carbonates in natural waters (Elinder, 1986). The main sources of iron in drinking water are the corroding cast iron and galvanized steel distribution pipes and iron in the source water which does not usually exceed 10 mg/L but may be found up to 50 mg/L (Marier et al., 1979). Iron is an essential nutrient and its deficiency causes anemia. Iron is naturally found in

liver, kidney, fish, red meats and egg. Food is the major source of iron intake (NRC, 1989) whereas drinking water may supply up to 5% of the daily intake, and this could be important for the populations that do not consume animal products. Iron in drinking water is managed by secondary standards set by EPA as it does not cause major health problems. Presence of iron in drinking water at approximately 0.3 mg/L causes an unpleasant metallic taste and rusty colored water that result in consumer complaints. Iron may be removed from the drinking water by conventional methods such as coagulation/flocculation, filtration, aeration and activated carbon, as well as non-conventional methods such as softening, reverse osmosis, and electrodialysis (Sommerfeld, 1999). As indicated above source water is not the only cause of iron in drinking water so corrosion control measures should be taken to reduce iron concentration at the tap as well as to protect the distribution system.

### 1.2. Copper

Copper is a common metal found in soil, water, and air. Copper is a micronutrient and foods like nuts, seeds, meat and seafood are good sources of copper. It has been a popular material for the distribution system pipes for its durability and bacteriacidal properties (Feng et al, 1996). It is also used in making of electrical wires, coins, cooking utensils, and building materials. Copper sulfate is also added to lakes and reservoirs as algicides (NSF, 2000). In natural waters copper may be found as oxides and hydroxides and its fate depends on pH, presence of oxidizing agents and chelators (USEPA, 1995). In US, copper may be found up to 1 mg/L in surface waters (ATSDR, 2002). At the tap, copper was detected at concentrations varying from 0.005 to 18 mg/L (USEPA, 1991). Copper is an essential nutrient for proper function of enzymes systems, however exposure to copper in drinking water may cause health problems such as nausea, vomiting, upset gastrointestinal track in short term and liver or kidney damage in long term. Copper is mainly introduced to drinking water by the corroding distribution system pipes and causes metallic taste and stained fixtures. Soft and acidic waters enhance copper corrosion. Several studies in the US, Canada and Europe reported copper concentrations at the tap up to 30 mg/L levels (USEPA, 1991; Health Canada, 1992; IPCS, 1998; NRC, 2000). As

copper may cause health effects it is regulated by the EPA. The Lead and Copper Rule was established by EPA in 1991 to reduce water corrosivity and hence to reduce copper in drinking water. Drinking water, especially when distributed by copper piping may contribute daily copper intake up to 1 mg/day whereas the average copper intake of adults usually vary from 1 to 3 mg/L (IPCS, 1998). Absorption of copper occurs mainly in the upper gastrointestinal tract. In the body copper is bound to proteins to prevent free radical reactions catalyzed by cuprous/cupric redox reactions (Pena et al., 1999). In the body copper is mainly found in the liver, brain, heart and kidneys (Barceloux, 1999). Excretion of copper from the body is mainly through bile, faeces, and urine (Cox, 1999).

### 1.3. Zinc

Zinc is found at very low quantities in nature, however, it may be introduced to drinking water by the commercial industries during mining, smelting, and dissolution of galvanized pipes. Zinc may be present in the soil at high concentrations due to improper disposal of wastes and may seep to the ground water. Zinc is usually below 10 μg/L in surface waters and 40 μg/L in ground water (Elinder, 1986). Iron pipes that are used in the drinking water distribution system are usually galvanized (coated with zinc) to prevent corrosion and zinc may end up in the tap water through the corrosion of pipes (Nriagu, 1980). Humans may be exposed to zinc through consumption of food and water, and breathing contaminated air. The drinking water may supply up to 10% of the recommended daily intake if the water is corrosive (Gillies and Paulin, 1982). Zinc is an essential nutrient for growth, bone development, wound healing and metabolism however deficiency and over dose may cause adverse health effects. Humans may have lowered taste and smell sensitivity, skin sores and lowered ability to fight infections due to zinc deficiency. On the other side, zinc overdose may cause nausea and vomiting in the short term and anemia, pancreas damage, and nervous system disorders in the long term. Zinc imparts an astringent metallic taste to drinking water and causes opalescent color. EPA has secondary standards for zinc to prevent aesthetic problems.

### 1.4. Manganese

Manganese is one of the most abundant metals on earth can be naturally found in air, soil, and water. In fresh waters it may be present up to 200 μg/L (Barceloux, 1999) and the reducing or acidic conditions may increase the concentration of manganese in ground water to about 10 mg/L levels (ATSDR, 2000). Manganese is an essential nutrient (Leach and Harris, 1997) and its deficiency and over dose may cause adverse health effects (Keen et al., 1999 and Keen et al., 2000). Manganese has important role in several enzyme systems that regulate lipid, carbohydrate and amino acid metabolism (IOM, 2001). The main intake of manganese is thorough food consumption. Because oral ingestion of manganese is not considered a serious health threat and consequently it is managed by secondary standards by EPA for aesthetic problems as it may cause black water problems (Bean, 1974). Above 0.1 mg/L manganese causes off-flavors and stains the fixtures (Griffin, 1960). Manganese may be removed from drinking water by conventional treatments such as coagulation, flocculation, sedimentation, and filtering, as well as other methods such as ion exchange, reverse osmosis, lime softening, and chemical precipitation.

### 1.5. Aluminium

Making up eight percent of the crust, aluminium is the most abundant metal on earth. It is naturally found as oxides, hydroxides and complexes with organic matter. Aluminium has been used as a coagulant for water treatment to remove turbidity, organics and microorganism levels; however this may cause increased levels in the finished water. The residual aluminium may settle in the distribution system and changes in flowrate may cause turbid water at the tap (WHO,1996). Aluminium is used in construction, automotive and electric industries, cooking utensils, and food packaging. It is also used to fortify food, and added to antiperspirants and antacids (ATSDR, 1992). In water, aluminium may be found in many different forms such as complexes with organic compounds and inorganic ligands including hydroxy species. The solubility of aluminium depends on many factors including pH (ISO, 1994). Near neutral pH aluminium may be found up to 0.05 mg/L in surface waters, but as the pH drops it may rise up to 1 mg/L. (WHO, 1997). In drinking water, aluminium levels depend on the concentrations in the source water and the treatment methods

and aluminium may be found up to 2.7 mg/L (Miller et al., 1984). Aluminium is found in food naturally, or introduced by food additives, aluminium utensils and cookware (Pennington and Schoen, 1995). Depending on the concentration in the drinking water, aluminium intake may reach up to 4% of daily recommended value. Absorption of aluminium in the body depends on the solubility, pH and other chemical species and is effectively removed by urine (WHO, 1997). Some research studies suggest that there is a relationship between Alhzeimer's disease development and aluminium intake from drinking water however these studies have not reported all sources for total intake (WHO, 1997). To minimize aluminium concentration in drinking water coagulation process applying aluminium based coagulants should be minimized by selecting an optimum pH, dosage, providing good mixing conditions and employing an efficient filtering process. With these considerations the finished water may have aluminium concentrations below 0.1 mg/L (Letterman and Driscoll, 1988).

### 1.6. Calcium

Calcium in drinking water causes hardness and affects the way water reacts with soap. The main source of calcium in water is sedimentary rocks; chalk and lime stone being the most common. Calcium is naturally found in food. Dairy products are very rich in calcium and an average diet supplies about 1000 mg of calcium per day (WHO, 1973). The taste threshold of calcium varies from 100 to 300 mg/L and is aesthetically unacceptable at 500 mg/L levels (Zoeteman, 1980). Natural waters may contain up to 100 mg/L of calcium. The hardness of finished tap water is usually in the range of 10 to 500 mg/L reported as calcium carbonate (Marier et al., 1979). Drinking water may supply up to 20 % of recommended daily intake value of calcium (Neri and Johansen, 1978). A few epidemiological studies reported a significant relation in water hardness and reduced cardiovascular disease rates however this has not been proven sufficiently considering other variables (Dzik, 1989). While hard waters may cause scale deposition in the distribution system, soft waters may cause corrosion of the metals pipes hence presence of calcium in finished water may affect the copper and iron concentrations in tap water. Calcium deficiency has been linked to bone resorption which causes osteoporosis in humans.

### 1.7. Magnesium

Magnesium in drinking water causes hardness and affects the way water reacts with soap. The main source of magnesium in water is sedimentary rocks, seepage and run-off from soils. Meat is a good source of magnesium and a typical diet provides about 400 mg of magnesium per day (WHO, 1973). Typically natural water sources contain 10 m/L magnesium. The hardness of finished tap water is usually in the range of 10 to 500 mg/L reported as calcium carbonate (Marier et al., 1979). Drinking water may supply up to 20 % of recommended daily intake value of magnesium (Neri and Johansen, 1978). A few epidemiological studies reported a significant relation in water hardness and reduced cardiovascular disease rates however this has not been proven sufficiently considering other variables (Dzik, 1989). While hard waters may cause scale deposition in the distribution system, soft waters may cause corrosion of the metals pipes hence presence of magnesium in finished water may affect the copper and iron concentrations in tap water.

## 2. Taste Thresholds of the Selected Metals

As metallic taste of drinking water and food became a concern researchers started to focus on human sensitivity to various metals found in these matrices. Studies mainly focused on iron and copper as they are mainly found in drinking water and may cause consumer complaints. A summary of the taste thresholds of the selected metals are presented below.

Many researchers have studied the metallic taste caused by iron and copper. Various methods employed were modified triangle test, paired difference, and 1-of-5 forced choice tests. Cohen et al. (1960) reported that ferrous sulfate in distilled water may be tasted at 0.04 mg/L by 5% of the population and at 3.4 mg/L by 50% of the population. The threshold concentrations changed to 0.12 and 1.8 mg/L respectively in spring water. In latter studies with ferrous sulfate, the threshold values were reported as 0.6 mg/L in distilled water by 50% of the population (Zacharias, 1979), 1.58 mg/L in mineral water by geometric mean (Gonzales, 1998), 5.54 mg/L in deionized water by geometric mean (Lim, 2006), 1.67 and 0.46 mg/L in deionized water by geometric mean and logistic

regression respectively (Epke and Lawless, 2007), and 0.052 and 0.031 mg/L in deionized water with geometric mean and logistic regression respectively (Tucker et al., 2007). The taste thresholds reported for cupric were 2.46 and 4.73 mg/L for cupric chloride in distilled and spring water respectively by 50 % of the population (Cohen, 1960), 0.2 mg/L for copper sulfate in mineral spring water (Beguin-Bruhin, 1983), 0.61 and 0.89 mg/L distilled and uncarbonated mineral water by 50 % of the population (Zacarias, 2001), 0.48 and 0.41 mg/L by geometric mean and 1.5 and 1.96 mg/L by logistic regression in distilled and mineralized water respectively (Cuppett, 2006), and 0.49 and 1.2 mg/L by geometric mean and logistic regression in deionized water (Epke and Lawless, 2007). As can be seen from the results of the numerous studies, taste thresholds vary and are greatly affected by the water type, sensory method and data analysis. Also sensitive people detect the presence of iron and copper in water at $10^3$ magnitudes less than the less sensitive people and hence may complain about off-tastes at much lower concentrations.

Zinc taste threshold has not been much studied, in an earlier work by Cohen (1960) several zinc salts were tested in distilled and spring water. The minimum concentrations detected by 5 and 50% of the population were reported as 4.3 and 18 mg/L in distilled, and 6.8 and 27 mg/L in spring water for zinc sulfate; 5.2 and 22 mg/L for zinc nitrate in distilled water; and 6.3 and 25 mg/L in distilled, and 8.6 and 33 mg/L in spring water for zinc chloride. WHO has guidelines for zinc in drinking water to be below 4 mg/L considering aesthetical issues. The same study also analyzed manganous sulfate and reported the detection concentrations by 5 and 50 % of the population as 3.6 and 45 mg/L. However WHO (1997) has reported the taste threshold of manganese to be at 0.1 mg/L and sets guidelines based on this value. Young et al. (1996) reported that aluminum sulfate has a taste threshold at 7.4 mg/L in soft water by geometric mean and the lowest detectable concentrations were 4 and 10 mg/L in soft and hard water.

The taste thresholds of calcium and magnesium are relatively high compared to other metals and are 100 to 300 mg/L and 100 mg/L respectively (WHO, 1997; NAS, 1977). An earlier study reported taste threshold of calcium to be 125 mg/L in distilled

water (Lockhart et al., 1955). Even though these two metals have a taste at these concentrations, they may be acceptable up to 500 mg/L (WHO, 1997).

## 3. Guidelines for the Selected Metals in Drinking Water

EPA regulates certain metals in drinking water to prevent health related and aesthetical problems. The table below shows the secondary maximum contaminant levels for the selected metals set by the EPA. These standards are set to prevent aesthetical problems related to taste and color of water and are not based on health effects as aesthetical problems arise at much lower concentrations. Calcium and magnesium are not regulated by the EPA whereas copper has and action level of 1.3 mg/L indicating that it should not exceed this concentration to prevent adverse health effects.

**Table-3.1.** EPA Guidelines for the selected metals in drinking water

| Metal | SMCL (mg/L) |
|---|---|
| Iron | 0.3 |
| Copper | 1 |
| Zinc | 5 |
| Manganese | 0.05 |
| Aluminium | 0.05-0.2 |

The concentrations of iron found in drinking water are not at levels that would cause adverse health effects and the concentrations that cause aesthetical problems are also below the health based levels. WHO (2006) recommends iron concentrations to be below 0.3 mg/L. For similar reasons WHO (2006) recommends zinc levels to be below 3 mg/L in drinking water.

Based on the gastrointestinal effects of copper IPCS (1998) has concluded that the acceptable oral intake range is within 2 to 3 mg/day. However this indicates an acceptable mass of copper that may be ingested within a 24 hour period. Studies showed

that one cup of water containing 3 mg/L of copper may induce adverse health effects immediately whereas no adverse effects were observed if the same amount (mass) of copper was consumed throughout the day. The guidelines for drinking water consider other intake sources and consumption of copper and should allow 2 to 3 liters of water consumption a day and aim a daily consumption of less than 10 mg of copper per day (IOM, 2001). Sidhu et al. (1995) suggested the copper levels to be below 0.3 mg/L to prevent toxicity and based this guideline to required daily intake and water consumption by different age groups.

A provisional guideline has established by the World Health Organization (WHO) as 0.1 mg/L for manganese in water both to prevent a potential health hazard as well as aesthetical problems although studies indicate that health effects start around 0.4 mg/L levels. The Food and Drug Administration (FDA) recommends manganese concentration in bottled water to be below 0.05 mg/L.

Although aesthetical problems are considered guidelines for certain metals in drinking water, recent studies suggest that iron and copper may be detectable by the majority of population at much lower concentrations. Better sensory studies may be conducted to establish more reliable guidelines to prevent consumer dissatisfaction.

It may be noted that the metals found in drinking water may be a good source of micronutrients. Calcium, magnesium, copper and zinc are found in drinking water in moderate amounts and they are moderately bioavailable in the form they are present. Manganese is also moderately bioavailable when consumed through drinking water however it is usually found at lower levels. Iron on the other hand is not very bioavailable although it may be found in moderate levels in drinking water.

A comparison of the guidelines for the selected metals is given below in Table 2 for WHO, European Union and several countries in Europe (Claes et al., 1997).

**Table-3.2.** Comparison of Drinking Water Regulations or Guidelines (mg/L) for the selected metals

| | WHO | EU | UK | Germany | Belgium | Austria | Switzerland | Poland | Russia | USA |
|---|---|---|---|---|---|---|---|---|---|---|
| Fe | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.3 | 0.5 | 0.3 | 0.3 |
| Cu | 1 | 3 | 3 | 3 | 2 | 0.1 | 1.5 | 0.5 | 1 | 1 |
| Zn | 5 | 5 | 5 | 5 | 5 | 3 | 5 | 5 | 3 | 5 |
| Mn | 0.1 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.1 | 0.1 | 0.05 |
| Al | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.3 | 0.2 | <0.2 |
| Ca | - | 100 | - | 400 | - | - | - | - | - | |
| Mg | - | 50 | - | 50 | - | - | 50 | - | - | - |

## 4. Recommended Daily Intake Values of Selected Metals

In this section intake, absorption, and health problems related to deficiencies of the selected metals are briefly discussed and the recommended daily intake values suggested by the WHO/FAO/IAEA (1996) are presented in the tables.

### 4.1. Iron

Iron functions in vital processes in the body and acts as carrier of oxygen from the lungs to the tissues by hemoglobin, medium for electron transfer, part of enzyme systems (Hallberg, 1982; Mascotti et al., 1995). Iron is not excreted from the body in urine or feces; it is lost by the loss of cells on skin and interior surfaces of the body. Females also lose iron during their menstruation (Hallberg and Rossander-Huthen, 1991). Infants are born with a high iron content which is usually sufficient for 4 to 6 months. Pregnant women especially during their third trimester require the highest amount of iron of their life to supply the demand by the fetus. Iron requirement of elderly decreases as they get less active and their blood volume decreases. Iron is mainly obtained from meat products, poultry and fish, as well as iron fortified foods such as cereals. Calcium negatively affects iron absorption in the body (Hallberg et al., 1993). Iron absorption also depends on the iron stores of the body and increases with deficiency. Ascorbic acid enhances iron absorption (Hallberg et al., 1997). Infants, children, adolescents, and young women are at risk of iron deficiency. Iron

deficiency affects brain development and hence the learning capacity and memory functions (Scrimshaw, 1984; Lozoff et al., 1991). It also causes decreased physical working capacity and impaired defense systems against infections. Iron intake depends on the bioavailability of iron and the types of foods consumed. It has been reported that absorption of iron may vary from 5 to 40% (Hallberg and Rosander, 1982). Recommended daily intakes of iron for different groups are given in the table below.

**Table-3.3.** Recommended intake levels for iron based on bioavailabilities

| Group | Age (years) | Avg. body weight (kg) | Recommended intake (mg/day) for a bioavailability of | | | |
|---|---|---|---|---|---|---|
| | | | 15% | 12% | 10% | 5% |
| Infants/children | 0.5-1 | 9 | 6.2 | 7.7 | 9.3 | 18.6 |
| | 1-3 | 13 | 3.9 | 4.8 | 5.8 | 11.6 |
| | 4-6 | 19 | 4.2 | 5.3 | 6.3 | 12.6 |
| | 7-10 | 28 | 5.9 | 7.4 | 8.9 | 17.8 |
| Females | 11-14* | 46 | 9.3 | 11.7 | 14 | 28 |
| | 11-14 | 46 | 21.8 | 27.7 | 32.7 | 65.4 |
| | 15-17 | 56 | 207 | 25.8 | 31 | 62 |
| | 18+ | 62 | 19.6 | 24.5 | 29.4 | 58.8 |
| Males | 11-14 | 45 | 9.7 | 12.2 | 14.6 | 29.2 |
| | 15-17 | 64 | 12.5 | 15.7 | 18.8 | 37.6 |
| | 18+ | 75 | 9.1 | 11.4 | 13.7 | 27.4 |
| Lactating | | 62 | 10 | 12.5 | 15 | 30 |
| Post menopause | | 62 | 7.5 | 9.4 | 11.3 | 22.6 |

* Pre-menarche

### 4.2. Copper

Copper participates in multiple enzyme systems that have structural and catalytic properties to ensure normal functioning of the body (Uauy et al., 1998). Copper is an

essential nutrient for infant growth, bone strength, iron transport, red and white cell maturation and brain development (Olivares et al., 2000). Copper deficiency causes anemia, altered immunity and hypotonia (Cordano, 1998). A few studies suggested that copper deficiency may increase cardiovascular mortality (Ford, 2000; Klevay, 2000). Young infants are the main group that may be susceptible to copper deficiency however, children and adults have also been diagnosed (Olivares et al., 2000). Copper is mainly absorbed in the large intestine, and the amount of copper absorbed depends on the body's requirements (Olivares et al., 2002). Copper metabolism is mainly controlled by the live, it may be stored bound to proteins, excreted in the bile or secreted to the plasma (Bremner, 1987).

**Table-3.4.** Recommended intake levels for copper

| Group | Age (years) | Intake (mg/day) |
|---|---|---|
| Infants/children | 0-0.5 | 0.2 |
| | 0.5-1 | 0.22 |
| | 1-3 | 0.34 |
| | 4-8 | 0.44 |
| Females | 9-13 | 0.7 |
| | 14-18 | 0.89 |
| | 19+ | 0.9 |
| Males | 9-13 | 0.7 |
| | 14-18 | 0.89 |
| | 19+ | 0.9 |
| Pregnant | | 1 |
| Lactating | | 1.3 |

### 4.3. Zinc

Zinc is an important component of the enzymes that are responsible for the synthesis and degradation of proteins, lipids, carbohydrates. It also has role in metabolism of other micronutrients. Zinc enhances the stability of cell structures and has an essential role in the immune system (Shankar and Prasad, 1998).

Humans that are severely zinc deficient have problems with growth, bone development, diarrhea, and infections (Hambridge et al., 1987). Impaired taste sensation and prolonged wound healing are other symptoms related to milder zinc deficiency. Zinc is mainly absorbed in the small intestines and excreted by urine and feces. Zinc is not stored in the body and infections may reduce the zinc in the plasma (Agett and Favier, 1993). Red meat and whole grain cereals are good sources of zinc. Iron and copper may affect zinc absorption if supplements are taken by the individuals. Infants, children and pregnant women may suffer from zinc deficiency.

**Table-3.5.** Recommended intake levels for zinc based on bioavailabilities

| Group | Age (years) | Avg. body weight (kg) | Recommended intake (mg/day) for bioavailability extent | | |
|---|---|---|---|---|---|
| | | | High | Moderate | Low |
| Infants/children | 0-0.5 | 6 | 1.1 | 2.8 | 6.6 |
| | 0.5-1 | 9 | 0.8-2.5 | 4.1 | 8.4 |
| | 1-3 | 12 | 2.4 | 4.1 | 8.3 |
| | 4-6 | 17 | 2.9 | 4.8 | 9.6 |
| | 7-9 | 25 | 3.3 | 5.6 | 11.2 |
| Females | 10-18 | 47 | 4.3 | 7.2 | 14.4 |
| | 19+ | 55 | 3 | 4.9 | 9.8 |
| Males | 10-18 | 49 | 5.1 | 8.6 | 17.1 |
| | 19+ | 55 | 4.2 | 7 | 14 |
| Pregnant | 1st trimester | | 3.4 | 5.5 | 11 |
| | 2nd trimester | | 4.2 | 7 | 14 |
| | 3rd trimester | | 6 | 10 | 20 |
| Lactating | 0-3 months | | 5.8 | 9.5 | 19 |
| | 3-6 months | | 5.3 | 8.8 | 17.5 |
| | 6-12 months | | 4.3 | 7.2 | 14.4 |

### 4.4. Manganese

Main sources of manganese are nuts, fruits, grains, leafy vegetables, tea and some meat and fish rather than drinking water (USEPA, 2002). Supplements can also be accepted as a source for about 12% of the population (Moss et al., 1989). Average manganese intake for adults through diet and or supplements in US is about 2.4 mg/day (IOM, 2002) with a range from 0.7 to 10.9 mg/day (Greger, 1999). Breast milk contains sufficient levels of manganese and its deficiency is unlikely for infants (Davidsson et al., 1989). However for children and adults, exposure to manganese requires consideration of concentration in food and bioavailability (Kies, 1994). Fiber, tannins, and oxalic acids decrease manganese absorption (Gibson, 1994). It was also reported that high levels of iron may also inhibit manganese uptake (Finley, 1999). Adequate intake values for different age groups and gender were determined by the Food and Nutrition Board of the Institute of Medicine and given in the table below (IOM, 2002).

**Table-3.6.** Adequate Manganese Intake

| Group | Age (years) | Intake (mg/day) |
|---|---|---|
| Infants/children | 0-0.5 | 0.003 |
| | 0.5-1 | 0.6 |
| | 1-3 | 1.2 |
| | 4-8 | 1.5 |
| Females | 9-18 | 1.6 |
| | 19+ | 1.8 |
| Males | 9-13 | 1.9 |
| | 14-18 | 2.2 |
| | 19+ | 2.3 |
| Lactating/pregnant | | 2.6 |

### 4.5. Aluminium

Aluminuim has not been proven as an essential nutrient so there are no guidelines for daily recommended intake values.

### 4.6. Calcium

Calcium is an essential nutrient that makes up about 2% of the body weight of humans and almost all of it is located in the skeleton. A minor amount of calcium is present in the teeth and soft tissues. Calcium salts make the skeleton rigid while calcium ions are a part of metabolic and neuromuscular functions, blood clotting and enzyme mediated processes (NRC, 1989; IOM, 1997). The amount of the recommended daily intake values of calcium mainly depend on the absorption by the body as well as gender and age. Infants up to 2 years of age, pregnant and lactating women, menopausal women, and elderly men constitute the groups that may suffer from calcium deficiency as it may cause problems with bone and teeth development for the infants and osteoporosis in the elderly. Recommended calcium allowances are given in the table below (WHO, 1997). Numerous studies indicate reduced cardiovascular disease mortality when calcium is present in drinking water (Calderon and Craun, 1978; Monarca et al., 2004). No major health problems have been associated with calcium and a recommended upper intake limit is set to 3 g per day.

**Table-3.7.** Recommended Calcium Intake

| Group | Age (years) | Intake (mg/day) |
|---|---|---|
| Infants/children | 0-0.5 | 300 |
| | 0.5-1 | 400 |
| | 1-3 | 500 |
| | 4-6 | 600 |
| | 7-9 | 700 |
| Females | 10-18 | 1300 |
| | 19-65 | 1000 |
| | 65+ | 1300 |
| Males | 10-18 | 1300 |
| | 19-65 | 1000 |
| | 65+ | 1300 |
| Pregnant | | 1200 |
| Lactating | | 1000 |

### 4.7. Magnesium

In the body most of the magnesium is found in the skeleton then in the muscles and soft tissues (Webster, 1987). In the soft tissues, magnesium is involved in the enzyme systems that regulate energy metabolism, protein synthesis, and maintenance of cell membranes (Al-Gamdi, 1994). Magnesium depletion onsets decrease in potassium and calcium levels in the body, however except for the elderly, magnesium may be restored by drawing it from the bones (Breibart et al., 1960). Magnesium deficiency is unlikely for humans except for general malnutrition and loss of magnesium by prolonged diarrhea (Shils, 1988; Gibson, 1990). Nausea, muscular weakness, anorexia, and lethargy may be observed for humans that have a decline in magnesium status. If the duration of deficiency is prolonged symptoms such as tetany, hyperirritability, muscular spasms, and convulsions are also seen (Shils, 1988). Magnesium absorption by the body depends on intake amount and increases with increased consumption. However higher fiber consumption as well as higher zinc and calcium intake may negatively affect magnesium absorption (Seelig,

1982; Andon et al., 1996; Abrams et al., 1997; Sojka et al., 1997; Spencer et al, 1994). Although there are studies that researched the biochemical criteria to determine the magnesium status and requirement, the data is insufficient and hence a better approach is necessary (Gibson, 1990). The recommended daily intake values by WHO are given in the table below.

**Table-3.8.** Recommended intake for magnesium based on relative intake ratios

| Group | Age (years) | Avg. body weight (kg) | Intake (mg/day) | Relative intake ratios | | |
|---|---|---|---|---|---|---|
| | | | | mg/kg | mg/g protein | mg/kcal.day |
| Infants/children | 0-0.5 | 6 | 26 | 4.3 | 2.5 | 0.05 |
| | 0.5-1 | 9 | 54 | 6 | 3.9 | 0.06 |
| | 1-3 | 12 | 60 | 5.5 | 4 | 0.05 |
| | 4-6 | 19 | 76 | 4 | 3.9 | 0.04 |
| | 7-9 | 25 | 100 | 4 | 3.7 | 0.05 |
| Females | 10-18 | 49 | 220 | 4.5 | 5.2 | 0.1 |
| | 19-65 | 55 | 220 | 4 | 4.8 | 0.1 |
| | 65+ | 54 | 190 | 3.5 | 4.1 | 0.1 |
| Males | 10-18 | 51 | 230 | 3.5 | 5.2 | 0.09 |
| | 19-65 | 65 | 260 | 4 | 4.6 | 0.1 |
| | 65+ | 64 | 224 | 3.5 | 4.1 | 0.09 |

*Lactating women may increase intake by 50 mg/day.

## 5. Adverse Health Effects of Selected Metals

### 5.1. Iron

Animal studies conducted with different animal species indicated that iron is lethal above 800 mg/kg body-weight for rats and caused depression, coma, respiratory failure and cardiac arrest. For humans, the average lethal dose is around 200 mg/kg body-weight however incidence of death was reported even at 40 mg/kg

levels with damaged mucosa in the stomach (NRC, 1989). A genetic disorder (hemochromatosis) may cause chronic iron overload (Bothwell et al., 1979). Healthy adults may take supplements up to 1 mg/kg body-weight.day without any adverse health effects (Finch and Monsen, 1972). Provisional maximum tolerable daily intake value for iron was established as 0.8 g/kg body-weight.day to prevent excessive iron storage in the body. Even if 10 % of this value is allocated to drinking water which allows it to be at 2 mg/L levels, aesthetical problems arise at much lower concentrations and toxicity from drinking water becomes unlikely.

### 5.2. Copper

Acute lethal doses of cupric for adults have been reported as 4 to 400 mg per kg body weight. Symptoms such as gastrointestinal bleeding, acute renal failure, oliguria, and hematuria has been observed for the individuals who ingested high doses of copper (Agarwal et al., 1993). At lower doses copper causes symptoms like food poisoning such as nausea, vomiting and diarrhea that generally appear within 15 to 60 minutes (Stenhammar, 1999). Long term ingestion of copper at higher doses results in irreversible liver and kidney damage (O'Donohue et al., 1993). However, long term dietary intake of 1 to 10 mg/L copper has no proven adverse health effects for individuals without any genetic disorders of copper homeostasis and Wilson disease gene. When copper intake is below recommended values it may lead to anemia, bone demineralization in poorly fed children and neutropenia (IOM, 2001).

### 5.3. Zinc

Consumption of excessive amounts of zinc salts causes acute toxicity and vomiting occurs (usually for amounts above 500 mg). Symptoms such as fever, nausea, vomiting, abdominal cramps and diarrhea occurs within 3 to 12 hours after consumption of acidic beverages that are kept in galvanized containers. Also use of galvanized containers for food preparation may cause similar symptoms (Elinder, 1986). Chronic ingestion of zinc for therapy causes copper deficiency. Zinc may be administered at 150 to 400 mg/day to patients with celiac disease and sickle cell anemia (Prasad et al., 1987). Long term exposure to zinc at very high levels also impairs the immune responses of healthy adults (Chandra, 1984). Upper intake level

for zinc is suggested to be 45 mg/L for adults and 28 mg/L for children (WHO, 1996).

### 5.4. Manganese

Manganese intake of humans varies from 0.7 to 10.9 mg/day (Greger, 1999) and the IOM (2002) has set 11 mg/L as tolerable upper intake level for adults. Health effects such as lethargy, mental and tremor disturbances and increased muscle tonus were reported as a result of exposure to high levels of manganese (around 28 mg/L) by 25 individuals who consumed well water that was contaminated due to improper disposal of batteries near the well (Kawamura et al., 1941). Elderly people were affected more than the younger adults. Autopsy of the brains of the dead individuals revealed extreme changes in the brain tissue. Although the symptoms above were attributed to manganese toxicity, the rapid onset of health problems was considered inconsistent with toxicity symptoms from inhaled manganese. It was suggested that there may be other contributors to the reported symptoms above (ATSDR, 2000). Exposure to airborne manganese for a long term has neurological effects such as weakness, muscle pain, anorexia, apathy and slow speech (Roels et al., 1999). Oral uptake of manganese has not been considered as a toxic threat. There are various studies that suggest that long term exposure to higher levels of manganese (about 2 mg/L) in drinking water may cause neurological impairment and an early onset of Parkinson's disease in the elderly people (Kondakis et al., 1989; Goldsmith et al., 1990). On the contrary there are several studies that concluded that manganese in drinking water does not cause adverse health effects (Vieregge et al., 1995; Suzuki, 1970). Sensitivity of humans to manganese uptake may depend on the liver condition as manganese is mainly eliminated through it as well as genetics, age, nutritional status (Devenyi et al., 1994).

### 5.5. Aluminium

Aluminium is commonly found in food and drinking water and there is little evidence that it is acutely toxic by oral ingestion (WHO, 1997). It was reported that elevated levels of aluminium in drinking water consumed by a population of 20,000 for 5 days caused nausea, vomiting, oral and skin ulcers, diarrhea, abdominal pain,

and skin rashes however all of the symptoms disappeared in a short time (Clayton, 1989). It has been also suggested that exposure to aluminium in drinking water accelerates onset of Alzheimer's disease. WHO (1997) evaluated numerous high quality epidemiological studies to test this argument. Although a positive relationship was found, the studies failed to consider other factors such as exposure from all sources, controls for genetic factors, etc. It was concluded that the evidence is not enough to confirm a relationship between aluminium in drinking water and Alzheimer's disease.

### 5.6. Calcium

Toxicity is only an issue when calcium is consumed in carbonate form at high doses. In this case, calcium salts precipitate in the renal tissue, cause constipation and may onset formation of kidney stones. Hence an upper intake level of 3 g/day is recommended (Burnett et al., 1949). Excess amounts of calcium in the body may affect absorption of other minerals such as iron and copper and may result in anemia. Consumption of very high levels of calcium may cause nausea, vomiting, abdominal cramps, and even seizure and coma.

### 5.7. Magnesium

Contaminated drinking water consumption may cause hypermagnesaemia, nausea, diarrhea, and hypotension. Recommended upper magnesium intake limits from water and food are 65 mg/day for children aged 1–3 years, 110 mg for children aged 4–10 years, and 350 mg for adolescents and adults (FNB, 1997).

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# Chapter IV

## Ability of Humans to Smell Geosmin, 2-MIB and Nonadienal in Indoor Air When Using Contaminated Drinking Water


### Abstract

The most common compounds responsible for off flavors are geosmin, 2-MIB, and nonadienal which are poorly removed by conventional water treatment operations and hence result in customer complaints. Because these odorants are moderately volatile and have very low odor threshold values, it is necessary to determine their concentrations in air when water is used indoors. If the detectable aqueous concentrations for these odorants are known, the utilities may take action to treat their water at times when the concentration of the raw water exceeds the threshold concentration. To predict the concentration in the shower stall and bathroom air after showering, recently published Henry's law constants for the selected odorants and a model developed to determine the volatilization of the odorous compounds by applying two-resistance theory were used. Then the results were compared with the threshold concentration data from another study to determine under which conditions the odorants become detectable. For parameters representing a typical bathroom and shower stall setting, the results showed that the odorants become detectable when the aqueous concentration of geosmin and nonadienal exceed 10 ng/L at 42 $^{o}$C. As the aqueous concentration increases, geosmin and nonadienal become detectable at lower temperatures, however 2-MIB is only detectable above 20 ng/L and at 42 $^{o}$C.



***Keywords:*** *2-MIB; drinking water; geosmin; Henry's law; indoor air concentration; nonadienal; odor threshold*


### 1. Introduction

Off tastes and odors are a major concern of water utilities worldwide that aim to serve high-quality drinking water to their customers. Unacceptable flavor of drinking water is

perceived as unhealthy by consumers and results in loss of confidence in the utilities and increased number of complaints (McGuire, 1995). When drinking water is used for showering and washing dishes the concentration in the air may reach a detectable level that concerns and annoys the residents.

Off taste and odor of drinking water may be caused by the water supply reservoir, the treatment plant, the distribution system, or the home plumbing (Stinson and Carns, 1983; Mallevialle and Suffet, 1987; Dietrich *et al.,* 2004). A typical water supply reservoir contribution to off flavors is the production of odorous compounds by actinomycetes, cyanobacteria, and other algae in the surface waters, particularly during warm summer and fall months. Three of the most common odorous compounds produced are geosmin, 2-methylisoborneol (2-MIB), and nonadienal (Zaitlin *et al.,* 2003). Earthy/musty odors are caused by geosmin and 2-MIB, and cucumber/fishy odors are caused by nonadienal. These compounds cannot readily be removed by conventional treatment operations and they require advanced treatment methods such as ozonation or activated carbon. The aqueous odor threshold concentration for geosmin is 6 to 10 ng/L at 45 $^{o}$C (Rashash *et al.,* 1997), the intensity of the odor is temperature dependent (Whelton and Dietrich, 2004), and customer complaints start at 7 ng/L (Simpson and MacLeod, 1991). Similar to geosmin, 2-MIB has an odor threshold of 2 to 20 ng/L at 45 $^{o}$C (Rashash *et al.,* 1997), and customer complaints start at 12 ng/L (Simpson and MacLeod, 1991). Nonadienal is detectable at 2 ng/L, and its odor characteristic changes from cucumber to fishy as its concentration rises to 13 ng/L (Rashash *et al.,* 1997).

Odorous compounds in drinking water should be volatile and exceed the human odor threshold concentration in order to be detected by the human nose. The amount of the odorant volatilizing is determined by Henry's law. The law states that every volatile compound has its unique Henry's law constant, which gives the ratio of headspace concentration of an odorant to its aqueous concentration at equilibrium. The equations corresponding to the two Henry's law constants; m (dimensionless) and $K_H$ (L-atm/mol) and the relationship between the constants are given below;

$$m = C_{gas} / C_{liq} \quad (1)$$

$$K_H = P_v / C_{liq} \quad (2)$$

$$K_H / m = RT \quad (3)$$

where $C_{gas}$ is the concentration in the headspace (mass/volume or mol/L), $C_{liq}$ is the concentration in aqueous phase (mass/volume or mol/L), $P_v$ is the partial pressure in the vapor phase (atm), R is the universal gas constant (8.314 J/mol-K), and T is temperature (K). The dimensionless Henry's law constants of geosmin, 2-MIB and nonadienal at 20 $^oC$ are presented in Table-4.1.

**Table-4.1.** The Henry's law constants (m) and enthalpy of vaporization values ($\Delta H^o$) for geosmin, 2-MIB, and nonadienal (Ömür-Özbek and Dietrich, 2005).

| **Compound** | **m at 20 $^oC$** | **$\Delta H^o$ (kJ/mol)** |
|---|---|---|
| Geosmin | 0.0023 | 80 |
| 2-MIB | 0.0027 | 89 |
| Nonadienal | 0.0035 | 57.5 |

Factors affecting Henry's law constant values include temperature, pH, compound hydration, compound concentration, complex mixtures, dissolved salts, suspended solids, dissolved organic materials, and surfactants (Staudinger and Roberts, 1996; Lee *et al.,* 2004). The temperature dependence of Henry's law constants is given by the van't Hoff equation:

$$\ln (m_1 / m_2) = - (\Delta H^o / R) \times [(1/ T_2) – (1/ T_1)] \quad (4)$$

where $m_1$ is the dimensionless Henry's law constant at $T_1$ ($^oK$), $m_2$ is the dimensionless Henry's law constant at $T_2$ ($^oK$), $\Delta H^o$ is the enthalpy of aqueous vaporization reaction (J/mol), and R is the universal gas constant (8.314 J/mol-K). By using the van't Hoff equation, Henry's law constants at a desired temperature can be calculated if the

constant at another temperature and $\Delta H^o$ are known. Henry's constants generally increase with increasing temperature.

When contaminated drinking water is used in the home, the concentration of the odorants in the air increases and can become noticeable. Especially when hot water is used, the amount of odorant volatilizing to the air inside the house increases as determined by Henry's law. There are several models developed to predict the exposure to the volatile contaminants due to household use of drinking water. Mass transfer coefficients for many water-using devices in homes such as baths, washing machines, dishwashers, faucets, and showers were measured by recent studies over a range of operating conditions, enhancing the prediction of exposure to the volatile contaminants (Kim *et al.,* 2004). The concentration of the odorants in the house depends on the volatility of the compound, mass transfer coefficients, water usage frequency, water temperature and flowrate, air flowrate in the house, and volume of the house (Little, 1992). The model for volatilization of aqueous compounds in a shower is explained below.

The volatilization rate of the compounds is influenced by the two resistance theory which gives the overall resistance based on the two resistances in gas and liquid phase. The corresponding equation is:

$$1/K_{OL}A = 1/K_LA + (1/m)(1/K_GA) \qquad (5)$$

where $K_{OL}$ is the overall, $K_L$ is the liquid phase, and $K_G$ is the gas phase mass transfer coefficients (L/min), A is the interfacial area, and m is the dimensionless Henry's law constant. For those compounds with a high m, the mass transfer is controlled by the liquid phase resistance, whereas the gas phase resistance becomes significant for the compounds that are less volatile. The overall mass transfer coefficient ($K_{OL}A$) is affected by the shower system and the chemical properties of the odorant. The mass transfer coefficients are mainly a function of fluid turbulence, but water temperature and the liquid- and gas-phase diffusion coefficients of the compound are also important.

An ideal bathroom/shower system, as shown in Figure-4.1, assumes the water and air flow rates to be constant, and uses a plug flow model to represent the water flowing through the shower stall and a completely mixed flow model to represent the air in the shower stall and bathroom.

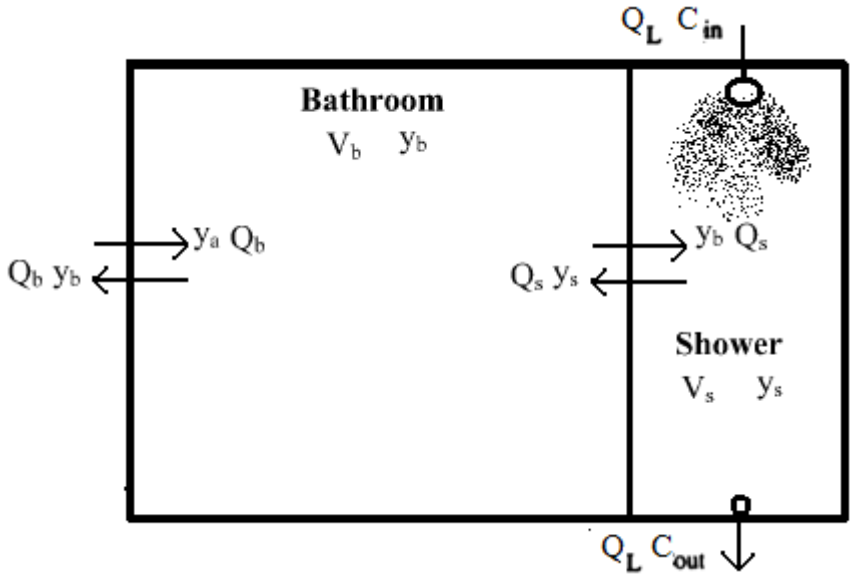


**Figure-4.1.** Schematic of the bathroom system

For the system shown above, the change in the water concentration as it passes through the shower stall is given by:

$$dc / dt = - K_{OL}(A/V_L)(c - y_s/m) \qquad (6)$$

where $V_L$ is the volume of water falling through the shower stall, c is the concentration of the odorant in water, and $y_s$ is the concentration of the odorant in the shower air. Assuming that $y_s$ is constant during the relatively short period of time it takes the water to pass through the stall, the above equation may be integrated to give:

$$c_{out} = c_{in}\, e^{-(K_{OL}A / Q_L)} + y_s/m\,(1 - e^{-(K_{OL}A / Q_L)}) \qquad (7)$$

where $Q_L$ is the water flowrate. A transient mass balance on shower stall air gives:

$$(dy_s / dt)V_s = Q_L (c_{in} - c_{out}) - Q_s (y_s - y_b) \quad (8)$$

where $V_s$ is the volume of the shower stall, $Q_s$ is the air flowrate through the shower stall, and $y_b$ is the concentration of the air coming into the shower stall from the bathroom. If a mass balance for the bathroom air is made, then:

$$(dy_b / dt)V_b = Q_b (y_a - y_b) - Q_s (y_b - y_s) \quad (9)$$

where $V_b$ is the bathroom volume, $Q_b$ is the air flowrate in the bathroom, and $y_a$ is the concentration of the air coming into the bathroom from the house.

In this paper, the shower volatilization model (Little, 1992) was combined with the previously measured Henry's law constants (Ömür-Özbek and Dietrich, 2005) to determine the indoor air concentration of odorous compounds during showering. The influent aqueous concentration of the odorant in the tap water and the water temperature were varied to determine under which conditions the odor threshold concentration would be exceeded.

## 2. Methods

### 2.1. Calculation of Henry's Law constants of the odorants at selected temperatures

The model by Little (1992) requires the Henry's law constants for the odorants at the selected temperatures. In a previous study by Ömür-Özbek and Dietrich (2005), the constants and enthalpy values were measured for the three odorants at four different temperatures (20, 25, 32, and 39 $^{o}$C). The data obtained are presented in Table-4.1. As explained, when the enthalpy of vaporization and the Henry's law constant at a certain temperature are known, constants at any temperature can be calculated using the van't Hoff equation.

## 2.2. Predicting the indoor air concentration of the odorants at selected environments and water use patterns

To calculate the gas-phase concentration of the selected odorants under different conditions when using contaminated tap water, the model developed by Little (1992) is used. The model applied the two-resistance theory to determine the volatilization of the odorants from water. A transient mass balance model was used, and the mass transfer coefficients were estimated from data provided in other studies. Five different bathroom systems were investigated with varying bathroom volumes, air and water flowrates, water temperatures, and duration of showering. The mass transfer coefficients were obtained for environmentally important drinking water contaminants.

The equations used to calculate gas-phase concentrations in both the shower stall and bathroom are (Little, 1992):

$$y_s = (A_2B_1 - A_1B_3) / (A_3B_3 - A_2B_2) \quad (10)$$

$$y_b = (A_1B_2 - A_3B_1) / (A_3B_3 - A_2B_2) \quad (11)$$

where $A_1 = (Q_L c_{in})(1 - e^{-(K_{OL}A / Q_L)})/V_s$ , $A_2 = Q_s/V_s$, $A_3 = ((-Q_L/m)(1-e^{-(K_{OL}A / Q_L)})-Q_s)/V_s$, $B_1 = Q_b y_a/V_b$, $B_2 = Q_s/V_b$, and $B_3 = (-Q_b-Q_s)/V_b$. The model parameters, either selected by the authors or obtained from the data in the referenced article, are shown in Table-4.2.

**Table-4.2.** Selected parameters for calculation of indoor air concentration of volatile odorants

| **Parameter** | **Selected Values** |
|---|---|
| Shower volume, $V_s$ (L) | 2000 |
| Bathroom volume, $V_b$ (L) | 8000 |
| Water flowrate, $Q_L$ (L/min) | 10 |
| Concentration in water, $c_{in}$ (ng/L) | 10, 20, 30 |
| Air flowrate in shower, $Q_s$ (L/min) | 50 |
| Air flowrate in bathroom, $Q_b$ (L/min) | 50 |
| Overall mass transfer coefficient, $K_{OL}A$ (L/min) | compound dependent |
| Initial concentration in air (house/bathroom), $y_a$ and $y_b$ (ng/L) | 0 |
| Water temperature, T ($^oC$) | 22, 33, 42 |
| Showering duration (min) | 10 |
| Henry's law constant, m | compound dependent |

For our typical bathroom, the $K_LA$ is taken as 28 L/min with a $K_GA$ of 480 L/min based on an assumed $K_G/K_L$ ratio of 17 (Little, 1992). Then the overall mass transfer coefficient is calculated using equation (5) for each compound and the values are corrected for each temperature by using the relationship between $K_LA$, $K_GA$, and m and presented in Table-4.3.

**Table-4.3.** Calculated overall mass transfer coefficients ($K_{OL}A$) for the three odorants at selected temperatures

| | $K_{OL}A$ (L/min) | | |
|---|---|---|---|
| **Compound** | **22 °C** | **33 °C** | **42 °C** |
| Geosmin | 1.3 | 3.8 | 7.9 |
| 2-MIB | 1.6 | 4.4 | 9.4 |
| Nonadienal | 1.8 | 2.7 | 4.8 |

### 2.3. Threshold concentrations of the odorants

To determine the threshold concentration that is detectable by the consumers, flavor profile analysis data from previous studies by Rashash *et al.* (1997) and Simpson and MacLeod (1991) were used. The detectable and displeasing aqueous concentrations of the odorants were found to be 7, 10, and 5 ng/L for geosmin, 2-MIB, and nonadienal respectively at 45 °C. These represent typical concentrations that humans could detect, although some individuals may be sensitive to lower levels. By using these values and the enthalpies and Henry's law constants from Ömür-Özbek and Dietrich (2005), the detectable gas phase concentrations for the odorants were calculated.

## 3. Results and discussion

### 3.1. Henry's Law constants calculated for selected water temperatures

Using the data in Table-1 and the van't Hoff equation, the constants for geosmin, 2-MIB, and nonadienal were calculated at 22, 33, and 42 °C. The results are presented in Table-4. These temperatures represent a cold, warm, and hot shower. As expected, the m values increased with increasing temperature, although the constants for geosmin and 2-MIB increased more with temperature than for nonadienal.

**Table-4.4.** Calculated Henry's law constants of the three odorants

| | **Dimensionless Henry's law constant, m** | | | |
|---|---|---|---|---|
| **Compound** | **22 °C** | **33 °C** | **42 °C** | **45 °C** |
| Geosmin | 0.0029 | 0.0093 | 0.0228 | 0.0304 |
| 2-MIB | 0.0035 | 0.0109 | 0.0295 | 0.0477 |
| Nonadienal | 0.0041 | 0.0063 | 0.0120 | 0.0224 |

### 3.2. Indoor air concentration of the selected odorants after showering

The parameters presented in Tables-4.3 and 4.4 were entered into the model to predict the indoor air concentrations of the odorants. The selected parameters represent a typical size bathroom and shower stall, with typical air and water flowrates, and water temperatures. The results are shown in Table-4.5 for varying concentrations of odorants and temperature in drinking water where $y_s$ represents the shower stall air concentration and $y_b$ represents the bathroom air concentration.

**Table-4.5.** Calculated indoor air concentrations of the odorants for selected aqueous concentrations and temperatures (concentrations exceeding odor threshold values are shown in bold italics)

| Environmental Media | Indoor Air Concentration, ng/L | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Geosmin | | | 2-MIB | | | Nonadienal | | |
| | 22 °C | 33 °C | 42 °C | 22 °C | 33 °C | 42 °C | 22 °C | 33 °C | 42 °C |
| **Water (10 ng/L)** | | | | | | | | | |
| Shower Air ($y_s$) | 0.03 | 0.09 | ***0.21*** | 0.03 | 0.10 | 0.26 | 0.04 | 0.06 | ***0.11*** |
| Bathroom Air ($y_b$) | 0.01 | 0.04 | 0.10 | 0.02 | 0.05 | 0.13 | 0.02 | 0.03 | 0.06 |
| **Water (20 ng/L)** | | | | | | | | | |
| Shower Air ($y_s$) | 0.05 | 0.17 | ***0.41*** | 0.07 | 0.20 | ***0.53*** | 0.08 | ***0.12*** | ***0.22*** |
| Bathroom Air ($y_b$) | 0.03 | 0.09 | ***0.21*** | 0.03 | 0.10 | 0.26 | 0.04 | 0.06 | ***0.11*** |
| **Water (30 ng/L)** | | | | | | | | | |
| Shower Air ($y_s$) | 0.08 | ***0.26*** | ***0.62*** | 0.10 | 0.30 | ***0.79*** | ***0.12*** | ***0.18*** | ***0.33*** |
| Bathroom Air ($y_b$) | 0.04 | 0.13 | ***0.31*** | 0.05 | 0.15 | ***0.39*** | 0.06 | 0.09 | ***0.17*** |
| **Threshold (ng/L)** | 0.21 | | | 0.48 | | | 0.11 | | |

### 3.3. Threshold concentration of odorants in air

As mentioned, the odor of the drinking water becomes displeasing when the aqueous concentrations of the odorants are at or above 7, 10, and 5 ng/L for geosmin, 2-MIB, and nonadienal respectively at 45 °C. Using these data and the m values at 45 °C from Table-4, the detectable gas phase threshold concentrations for geosmin, 2-MIB, and nonadienal are calculated as 0.21, 0.48, and 0.11 ng/L, respectively. In Table-5, calculated air threshold concentrations are compared and the conditions when odorants become detectable are shown in bold italics. A graphical representation of the data for geosmin is given in Figure-4.2.

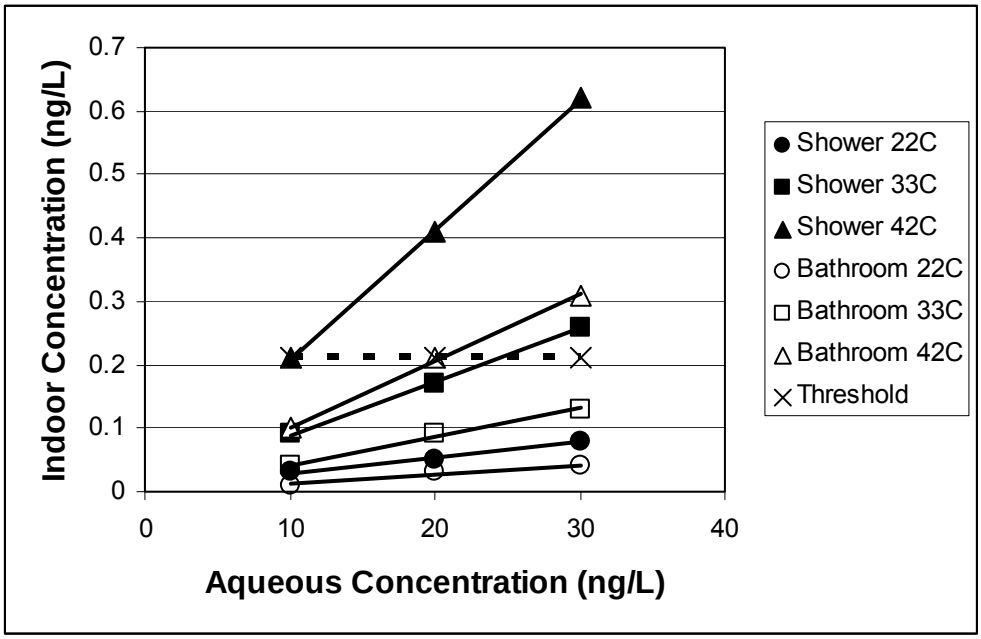


**Figure-4.2.** Relationship between aqueous concentration, water temperature and indoor air concentration for geosmin

As shown in Table-4.5, the odorants become detectable in the shower at different temperatures and at different concentrations for the three odorants. Odorants are detectable by smell in the shower before they are detectable in the bathroom. None of the odorants is detectable in the bathroom air when the aqueous concentrations of the odorants are at or below 10 ng/L. However, geosmin and nonadienal can be detected in the shower stall if the water temperature is 42 $^{o}$C. All three of the compounds become detectable in the shower at 42 $^{o}$C at or above 20 ng/L aqueous concentration, and in the bathroom at 30 ng/L.

The selected aqueous concentrations are common concentrations encountered during taste and odor episodes. The showering temperatures selected are in the normal range. This means that the odorants become detectable if the aqueous concentration exceeds 20 ng/L and assuming that the bathroom is similar to the model system. The concentration in the gas phase depends on the shower and bathroom volume as well as the air and water flowrate. The water temperature, aqueous concentration of the odorant and the Henry's constant play a very important role in determining the indoor

air concentration as well. Although it is hard to generalize to all possible bathroom configurations, the results shown here represent a reasonably typical situation.

## 4. Conclusions

For water utilities, satisfied customers are very important. To serve the most acceptable drinking water to the public, utilities need to understand how and when the taste and odor of the water reaching the customers will be displeasing. This research greatly expands the understanding of this issue by simultaneously considering both aqueous and gas-phase concentrations of odorants. Treatment options may be evaluated based on the detectability of the odorants, depending on chemical properties of the odorants, water use patterns by the customers, and the concentration of the odorous compounds in the raw or treated water.

One drawback of this research is that the Henry's law constants were measured for distilled water. It is reported in the literature and by Ömür-Özbek and Dietrich (2005) that the constants are affected by the presence of other substances such as humic or fulvic acid in the water matrix. This phenomenon may affect the results and hence the accuracy of the data presented.

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# Chapter V

## Using Air-Water Partitioning Model to Determine when Odorous Compounds in Drinking Water Become Detectable During Showering


### Abstract

This study employed a mass-transfer model to determine the indoor air concentrations of geosmin, 2-MIB, and nonadienal when water is used for showering under varying conditions (shower stall volume, water flow rate and temperature, odorant concentration, duration of shower) as this is the main place that the water utilities receive complaints for off-smelling drinking water. By using the existing data on detection of the selected odorants in literature and predicted air concentrations by the developed model, the reliability of the model predictions was determined. To prevent the odorous water from reaching their customers, the water utilities need to know when to treat the water with advanced methods as these odorants cannot be removed by conventional treatment methods. For better understanding of the occurrence of detectable odors, parameters that affect the indoor concentration are varied and the results are modeled by using statistical R software.



***Keywords:*** *Odor; aqueous volatilization; geosmin; 2-MIB; nonadienal, drinking water use by household*


### Practical Implications

Naturally occurring water-borne odorants, which have no-known adverse health effects but are nuisances to drinking water customers and utilities, offer efficient means to calibrate indoor air/water shower models and also can act as biomarkers for human exposure. Through modeling human exposure to the microbially-related aqueous odorants: geosmin (earthy), 2-Methylisoborneol (2-MIB) (musty), and (trans, cis)-2,6-nonadienal (cucumber, fishy) this study improves understanding of water-air odorant transfer by accounting for most scenarios for water use and other parameters.

Concentrations at which the odorants become detectable to the human senses is controlled by aqueous concentration and temperature for the steady state model, but shower volume, air flow, and water flow are also important for the dynamic model and initial exposure to the odorant when the shower is started.

## 1. Introduction

There is significant interest in predicting human exposure due to inhalation of contaminants that partition from drinking water into the indoor air. Inhalation exposure to aqueous contaminants is a major exposure route when water is used for showering (McKone, 1987; Ott and Roberts, 1998; Weisel et al., 1999). A recent study demonstrated that humans' exposure to trichloromethane during showering had significant increases in blood and exhaled breath concentrations of trichloromethane (Gordon et al., 2006). Inhalation was also demonstrated to be a means of exposure and increased risk for other drinking water disinfection by-products, including haloketones and haloacetic acids (Xu and Weisel, 2003). Dynamic and steady-state models at the interface of air and water modeling have been developed to assist in calculating human exposure to water-borne contaminants such as regulated compounds like disinfection by-products and components of gasoline (Andelman, 1985; Giardino et al., 1992; Little, 1992; Tancrede et al., 1992; Moya et al., 1999; Chen et al., 2003).

While much of the air-water modeling has focused on compounds with adverse human health effects, consumers are also plagued by annoying odorous compounds that disrupt their lives but do not necessarily have health risks. Worldwide, geosmin (trans-1,10- dimethyl-trans-9-decalol), 2-methylisoborneol (2-MIB), and trans,cis-2,6-nonadienal (nonadienal) are major drinking water odorants that cause complaints, loss of trust in utilities, and concern for water safety (McGuire, 1995; Zaitlin *et al.,* 2003, Dietrich, 2006). Chemical data for these compounds are presented in Table-5.1.

Geosmin, 2-MIB and nonadienal are primarily introduced to the source water by microbes, but may also be generated in the distribution system at the dead ends. Geosmin and 2-MIB are bicyclic alcohols that have earthy and musty odors,

respectively, and are produced by cyanobacteria or actinomycetes usually during the warm summer and fall months. Their aqueous odor threshold values at 45 $^{o}$C are 1-10 ng/L for geosmin and 2-20 ng/L for 2-MIB (Rashash *et al.,* 1997, Piriou et al, 2007). Their concentrations in drinking water can range from a few ng/L to over 800 ng/L (Yagi, 1988; McGuire et al. 1991). A survey of water utilities found that during earthy smelling geosmin episodes which caused customer complaints, the median aqueous concentration was 11.3 ng/L and the mean aqueous concentration that elicited noticeable customer complaints in homes was 12.8 ng/L (temperature was not recorded) (Graham et al, 2000). Nonadienal is produced by chrysophyte algae that proliferate in water supplies in late winter and spring. Its odor threshold concentration is 2 ng/L at 45 $^{o}$C, and the odor descriptor changes from cucumber to fishy as the concentration exceeds 13 ng/L (Burlingame et al, 1992; Rashash *et al.,* 1997). Nonadienal is the source of cucumber and fishy odor events in drinking water at concentrations of 2-10 ng/L (Hayes and Burch, 1989; Burlingame et al, 1992; Khiari et al., 1992). The odor intensities of these odorants generally increase with increasing water temperatures (Whelton and Dietrich, 2004).

Drinking water utilities depend on their customers to report problems in the drinking water quality, including off-tastes and odors (Whelton et al, 2007). For odorants to be detectable by the human nose, the amount of odorant in air has to reach or exceed an individual's odor threshold concentration. The concentration at which an odorant becomes detectable to a person varies with the compound's structure and the person's sensitivity. Variability in the normal human population and variability in the methods to determine typical odor threshold concentration values lead to a "range" of concentrations being a more acceptable way to represent thresholds in the human population (Lawless and Heymann, 1998; Gallagher and Cuppett, 2007).

The selected odorants are persistent in natural waters and chlorinated drinking waters which is why they are problematic. Conventional water treatment operations are not adequate for removing these odorants and hence advanced treatment processes such as oxidation by ozone, hydrogen peroxide-UV or activated carbon filters are necessary (Meunier et al., 2006; Kruithof et al., 2007; Peter and vonGunten, 2007;

Liang et al., 2008). Because off-flavor of drinking water is a concern for water utilities throughout the world, understanding when the odorants become detectable to the consumers in air and water will be beneficial to explain or prevent the problems before they arise. The non-toxic odorants (such as geosmin, 2-MIB and nonadienal) can also serve as "biomarkers" for air exposure to compounds with more serious adverse-health effects such, as trihalomethanes, when knowledge of aqueous concentration and air-water partitioning are available.

Henry's Law defines the amount of odorant that volatilizes to the air stating that every volatile compound has a unique constant that gives the ratio of concentration of odorant in air to concentration of odorant in water under equilibrium in a closed system at a certain temperature. The equation for dimensionless Henry's Law constant, m is:

$$m = \frac{C_G}{C_L} \tag{1}$$

where $C_G$ is the concentration of the odorant in the air (mass/volume or mol/L), and $C_L$ is the concentration of the odorant in liquid phase (mass/volume or mol/L). Henry's law constants are most affected by temperature, but also can be affected by pH, compound concentration, dissolved salts, dissolved organic materials, and surfactants (Staudinger and Roberts, 1996; Lee *et al.,* 2004; Omur-Ozbek and Dietrich, 2005). To calculate the constants over a range of temperatures the relationship given by the van't Hoff equation may be used:

$$\ln\left(\frac{m_1}{m_2}\right) = -\left(\frac{\Delta H^o}{R}\right)\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \tag{2}$$

where $m_1$ is the dimensionless Henry's law constant at first temperature, $T_1$ ($^oK$), $m_2$ is the dimensionless Henry's law constant at second temperature, $T_2$ ($^oK$), $\Delta H^o$ is the enthalpy of aqueous vaporization reaction (J/mol), and R is the universal gas constant (8.314 J/mol-K). With the van't Hoff equation, Henry's law constants can be calculated at a desired temperature when one constant (at a defined temperature) and the $\Delta H^o$ are known. The dimensionless Henry's law constants and $\Delta H^o$ for geosmin, 2-MIB and nonadienal were determined by a previous study by Omur-Ozbek and Dietrich (2005) and are given in Table-5.1.

**Table-5.1.** Physical/Chemical data and Dimensionless Henry's law constants at 20 °C and $\Delta H^o$ of the selected odorants

| Odorant | Chemical Formula | Molecular Weight (g/mol) | Vapor Pressure (atm) | Water Solubility (mg/L) | m @ 20°C | $\Delta H^o$ (J/mol) |
|---|---|---|---|---|---|---|
| **Geosmin** | $C_{12}H_{22}O$ | 182.3 | $5.49*10^{-5}$ | 150.2 | 0.0023 | 80,000 |
| **2-MIB** | $C_{11}H_{20}O$ | 154.2 | $7.26*10^{-5}$ | 194.5 | 0.0027 | 89,000 |
| **Nonadienal** | $C_9H_{14}O$ | 138.2 | $13.16*10^{-5}$ | Insoluble | 0.0035 | 57,500 |

Although drinking water is used for showering, bathing, laundry, dishes, and washing in sinks in the house, the main location where most consumers would detect off-odors is the shower considering higher water temperature and the higher amount of water flowing through the shower head in a confined area. Mathematical models that predict human exposure to volatile contaminants indoors due to use of tap water incorporate mass transfer coefficients for showers, baths, faucets, and other water-using devices (Kim *et al.,* 2004), Henry's Law constant, mass transfer coefficients, water temperature and flow rate, air flow rate in the house, water usage frequency, and volume of the house (Little, 1992). The model developed by Little (1992) is adopted in this study to determine the concentration of the selected odorants during showering as explained below.

Two-resistance mass-transfer theory defines the volatilization rate of odorants with the overall resistance that depends on the gas and liquid phase resistances (Little, 1992). Parameters that are correlated with gas and liquid phase mass transfer coefficients and hence the overall mass transfer coefficient are the water flow rate ($Q_L$, volume/time), air flow rate ($Q_G$, volume/time), and water temperature (T, °C). The relationship is given by the equation below:

$$\frac{1}{K_{OL}A} = \frac{1}{K_L A} + \frac{1}{m}\frac{1}{K_G A} \tag{3}$$

where $K_{OL}$ is the overall, $K_L$ is the liquid phase, and $K_G$ is the gas phase mass transfer coefficients (length/time), A is the interfacial area (length$^2$), and m is the dimensionless

Henry's law constant. As can be interpreted from the equation, for highly volatile compounds with a high m the mass transfer is controlled by the liquid phase resistance, and the gas phase resistance controls the volatilization of the semi-volatile compounds with a low m. The overall mass transfer coefficient may also be affected by the chemical properties of the odorant.

The mass transfer coefficients depend on diffusivity by a power relationship such as $K_L \propto D_L^p$ and $K_G \propto D_G^q$. In order to determine the mass transfer coefficients and hence the overall mass transfer coefficient of a volatile chemical either analytical experiments may be conducted or modified version of equation 3 with a reference chemical may be used:

$$\frac{1}{K_{OL}A_i}\left(\frac{D_{Li}}{D_{Lr}}\right)^p = \frac{1}{K_L A_r} + \frac{1}{K_G A_r}\frac{1}{m_i}\left(\frac{D_{Gr}}{D_{Gi}}\right)^q\left(\frac{D_{Li}}{D_{Lr}}\right)^p \quad (4)$$

where i represents the odorant of interest and r represents the selected reference compound. The relationship between the gas and liquid phase mass transfer coefficients and the diffusivity of the volatile chemicals were given in the literature as $K_G \propto D_G^{2/3}$ and $K_L \propto D_L^{2/3}$ hence p = 2/3 and q = 2/3 in the above equation (Cho and Wakao, 1988; Munz and Roberts, 1989; Corsi and Howard, 1998).

Kottegoda and Rosso (1997) conducted studies to determine mass transfer coefficients of trichloroethylene (TCE) under varying conditions such as temperature, air and water flow rates. For the shower system, $K_LA$ is expected to increase with water flow rate and temperature:

$$K_L A \propto Q_L^{\beta_1}\beta_2^{(T-20)} \quad (5)$$

After taking the logarithm of both sides and regression analysis the equation becomes:

$$\log K_L A = 0.84\log Q_L + 0.0057(T-20) + 0.20 \quad (6)$$

$K_G$ mainly depends on the air flow rate, and the interfacial surface area (A) depends on the water flow rate. So $K_GA$ depends on air and water flow rates and given by the equation below:

$$K_G A \propto Q_L^{\beta_1} Q_G^{\beta_2} \tag{7}$$

After taking the logarithm of both sides and regression analysis the equation becomes:

$$\log K_G A = 0.52 \log Q_L + 0.74 \log Q_G + 0.83 \tag{8}$$

The bathroom system shown in Figure-5.1 below assumes that the water flow rate through the shower head and the air flow rates in the shower stall and bathroom are constant, the air coming from the house into the bathroom and shower stall has no odorant, and the water flowing through the shower stall follows a plug flow model system whereas the air in the shower stall and bathroom follows a completely mixed flow model.

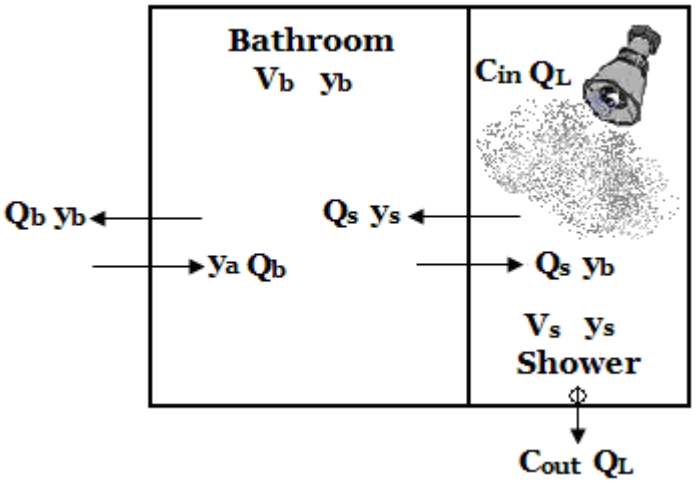


**Figure-5.1.** Schematic of the bathroom

Considering the shower stall alone, the rate of volatilization of the odorant from the water into the air while the water flows through the stall is given by:

$$\frac{dC}{dt} = -K_{OL}\left(\frac{A}{V_L}\right)\left(\frac{C - y_s}{m}\right) \tag{9}$$

where $V_L$ is the volume of water flowing through the shower stall, c is the odorant concentration in water, $y_s$ is the odorant concentration in shower air. Equation above may be rewritten with the assumption that $y_s$ remains constant during the short period of time the water remains in the shower stall until it reaches the drain as:

$$C_{out} = C_{in} e^{-\left(\frac{K_{OL}A}{Q_L}\right)} + \frac{y_s}{m}\left(1 - e^{-\left(\frac{K_{OL}A}{Q_L}\right)}\right) \tag{10}$$

where $Q_L$ is the water flowrate. The transient mass balance on shower stall air yields:

$$\left(\frac{dy_s}{dt}\right)V_s = Q_L\left(C_{in} - C_{out}\right) - Q_s\left(y_s - y_b\right) \tag{11}$$

where $V_s$ is the shower stall volume, $Q_s$ is the air flowrate in the shower stall, and $y_b$ is the concentration of the odorant in air coming into the shower stall from the bathroom. Similarly a transient mass balance for the bathroom air yields:

$$\left(\frac{dy_b}{dt}\right)V_b = Q_b\left(y_a - y_b\right) - Q_s\left(y_b - y_s\right) \tag{12}$$

where $V_b$ is the bathroom volume, $Q_b$ is the air flowrate in the bathroom, and $y_a$ is the concentration of the odorant in air coming into the bathroom from the house. When equation 10 is substituted into equation 11 and the equation 12 is rearranged and the following simultaneous differential equations are obtained:

$$\frac{dy_s}{dt} = \frac{Q_L c_{in}\left(1 - e^{-\left(\frac{K_{OL}A}{Q_L}\right)}\right)}{V_s} + \frac{Q_s}{V_s} y_b + \frac{\left(\frac{-Q_L}{m}\left(1 - e^{-\left(\frac{K_{OL}A}{Q_L}\right)}\right)\right) - Q_s}{V_s} y_s \tag{13}$$

$$\frac{dy_b}{dt} = \frac{Q_b y_a}{V_b} + \frac{Q_s}{V_b} y_s + \frac{-Q_b - Q_s}{V_b} y_b \tag{14}$$

The above differential equations are solved and using $y_{si}$ and $y_{bi}$ at t = 0 as initial conditions, the dynamic-state values for the odorant concentration in the shower stall and the bathroom air are determined by:

$$\frac{y_s}{y_{si}} = \left(\frac{a_2}{r_1 r_2}\right) - \frac{\left(r_1^{\,2} + a_1 r_1 + a_2\right)}{r_1\left(r_2 - r_1\right)} e^{(r_1 t)} + \frac{\left(r_2^{\,2} + a_1 r_2 + a_2\right)}{r_2\left(r_2 - r_1\right)} e^{(r_2 t)} \tag{15}$$

$$\frac{y_b}{y_{bi}} = \left(\frac{b_2}{r_1 r_2}\right) - \frac{\left(r_1^{\,2} + b_1 r_1 + b_2\right)}{r_1\left(r_2 - r_1\right)} e^{(r_1 t)} + \frac{\left(r_2^{\,2} + b_1 r_2 + b_2\right)}{r_2\left(r_2 - r_1\right)} e^{(r_2 t)} \tag{16}$$

The steady-state values for the odorant concentration in the shower stall and the bathroom air are determined by:

$$y_s = \frac{\left(A_2 B_1 - A_1 B_3\right)}{\left(A_3 B_3 - A_2 B_2\right)} \tag{17}$$

$$y_b = \frac{\left(A_1 B_2 - A_3 B_1\right)}{\left(A_3 B_3 - A_2 B_2\right)} \tag{18}$$

where $A_1 = \dfrac{Q_L C_{in}\left(1 - e^{-\left(\frac{K_{OL}A}{Q_L}\right)}\right)}{V_s}$, $A_2 = \dfrac{Q_s}{V_s}$, $A_3 = \dfrac{\left(-\dfrac{Q_L}{m}\left(1 - e^{-\left(\frac{K_{OL}A}{Q_L}\right)}\right)\right) - Q_s}{V_s}$, $B_1 = \dfrac{Q_b y_a}{V_b}$,

$B_2 = \dfrac{Q_s}{V_b}$, $B_3 = \dfrac{-Q_b - Q_s}{V_b}$ and, $a_1 = \dfrac{\left(A_1 + A_2 y_{bi} - B_3 y_{si}\right)}{y_{si}}$, $a_2 = \dfrac{\left(A_2 B_1 - A_1 B_3\right)}{y_{si}}$,

$b_1 = \dfrac{\left(B_1 + B_2 y_{si} - A_3 y_{bi}\right)}{y_{bi}}$, $b_2 = \dfrac{\left(A_1 B_2 - A_3 B_1\right)}{y_{bi}}$,

$r_{1,2} = \dfrac{1}{2}\left(A_3 + B_3\right) \pm \dfrac{1}{2}\left(\left(A_3 + B_3\right)^2 - 4\left(A_3 B_3 - A_2 B_2\right)\right)^{0.5}$

The $y_{si}$ and $y_{bi}$ represent the initial concentrations of the odorant in bathroom and shower stall air and should be set to a very small number although assumed to be equal to zero.

In this study the model of Little (1992) is applied under varying conditions (such as water flow rate, temperature, air flow rate, and volume of the system) to predict indoor air odorant concentrations and to obtain a better understanding of when the odorants become detectable indoors when water is used for showering. This was accomplished by analyzing the existing model using R-software with many possible combinations of the

selected parameter values. From data generated by the full model, a new simpler model using the tree regression was developed. The simpler regression model accounts for all major parameters that contribute to the indoor air concentration of the odorants and allows efficient prediction of the concentration for various settings (shower volume, water use patterns, etc).

## 2. Methods

### 2.1. Values for model parameters

To study a broad range of configurations, the selected parameters and the values representing the lower and upper limits with the values in between defined by the selected increments are shown in Table-5.2. The concentration of the compounds in indoor air initially is assumed to be zero and for the modeling purposes set to a very small number.

**Table- 5.2.** Parameters and selected values used for modeling

| **Parameters** | **Symbol** | **Minimum to Maximum* (Increment)** |
|---|---|---|
| Shower volume (L) | $V_s$ | 1000 to 2000 (every 200) |
| Bathroom volume (L) | $V_b$ | 8000 |
| Water flow rate of shower (L/min) | $Q_L$ | 5 to 15 (every 2) |
| Air flow rate in shower (L/min) | $Q_s$ | 20 to 100 (every 20) |
| Air flow rate in bathroom (L/min) | $Q_b$ | 20 to 100 (every 20) |
| Concentration in water (ng/L) | $C_{in}$ | 10 to 30 (every 5) |
| Concentration in bathroom/house air(ng/L) | $y_a$, $y_b$ | 0 |
| Water temperature ($^oC$) | T | 30 to 45 ( every 3) |
| Showering time (min) | t | 1 min, until detection or Steady-state |

* The values selected are based on the values reported by Khanal (1999) and Mayer (1995).

Henry's law constants of geosmin, 2-MIB, and nonadienal at selected temperatures were calculated using equation 2 and the values reported in Table-5.1. The equations and enthalpy and Henry's law constant values were entered to R statistical software and the constants for the temperatures between 30 to 45 $^{o}$C with 3 $^{o}$C increments were determined; this range incorporates typical temperatures used in showering (Keating et al., 1997).

In order to calculate the overall mass transfer coefficient for the selected compounds trichloroethylene (TCE) was selected as the reference compound since there is limited information on the liquid and gas phase mass transfer coefficients of geosmin, 2-MIB, and nonadienal. The liquid and gas phase mass transfer coefficients of TCE were calculated using equations 6 and 7 respectively for each temperature, water and air flow rate combinations by using R. Then equation 4 is used to calculate the overall mass transfer coefficients of the selected odorants at selected temperature and water and air flow rate settings. The diffusion constants for TCE were given by Little (1992) and the constants for geosmin, 2-MIB, and nonadienal were obtained from the EPA's online tool that estimates the coefficients by considering the entered chemical properties of the compounds. The diffusion constants are given in Table-5.3 below.

**Table-5.3.** Liquid and gas diffusion constants of the selected odorants

| Compound | $D_L$ (m²/s) | $D_G$ (m²/s) |
|---|---|---|
| **TCE** | $9.1x10^{-10}$ | $7.9x10^{-6}$ |
| **Geosmin** | $8x10^{-5}$ | $7x10^{-2}$ |
| **2-MIB** | $8x10^{-5}$ | $7x10^{-2}$ |
| **Nonadienal** | $6.16x10^{-6}$ | $6.16x10^{-2}$ |

## 2.2. Determining the concentrations of the odorants in shower air with time and at steady-state

To determine the odorant concentration in air and understand when it becomes detectable equations 15 and 16 were used for the dynamic model and equations 17 and

18 were used for the steady-state model. All possible combinations of the parameters; time in shower, concentration of compound in water, air flow rate in the shower and bathroom, and volume of the shower stall were considered for the calculation of the concentrations of the compounds in air. More than 75,000 unique calculations were made and the results were analyzed and modeled by using the statistical R software.

### 2.3. Development of a simplified predictive model

After all of the indoor air concentrations in shower were calculated for each combination of the parameters, the results were plotted and analyzed by R software using a full factorial linear regression to obtain simplified model equations for the shower for geosmin, 2-MIB, and nonadienal. Also the plots were analyzed to understand which parameters had the most significant effect on the concentration of the odorants in air.

To understand when the odorants become noticeable in air, the detectable air concentrations of the odorants were calculated from the data for the aqueous odor threshold concentrations of geosmin, 2-MIB, and nonadienal assuming an average showering temperature of 40 $^{o}$C, and applying Henry's Law to calculate the corresponding air concentrations. The aqueous odor threshold detection ranges selected for geosmin, 2-MIB, and nonadienal are 1-10 ng/L; 2-10 ng/L and 2-13 ng/L in water respectively. There may be individuals more or less sensitive to the odorants of interest hence considering a detection range rather than one selected value is more realistic. The detectable air concentration ranges are calculated from Henry's law as 0.02-0.19 ng/L; 0.06-0.28 ng/L and 0.03-0.19 ng/L for geosmin, 2-MIB, and nonadienal respectively.

## 3. Results and discussion

Model parameters were varied over a range of values as shown in Table-5.2, and the steady state concentration, the concentration after 1 minute, and dynamic air concentrations over time were determined for each odorant. Also to understand the fate of the odorant in the shower air better, parameters were kept constant for some of the analyses.

### 3.1. Steady-state model

A full factorial linear regression including all interactions was performed to fit the steady state concentration as a function of shower volume, water flow in shower, air flow in shower, air flow in bathroom, concentration in water, and water temperature. Sixty-three total parameters were estimated and the intercept was set to 0. The overall regression was statistically significant (for geosmin: $F=7.17\times10^{4}$, $p<3\times10^{-16}$, $R^2=0.9941$; for 2-MIB: $F=5.42\times10^{4}$, $p<3\times10^{-16}$, $R^2=0.9922$; and for nonadienal: $F=2.34\times10^{5}$, $p<3\times10^{-16}$, $R^2=0.9982$). A reduced model was obtained for each odorant and it was observed that the water temperature and the concentration of the odorant captured most of the explanatory power. Thus the steady-state air concentrations can be very accurately predicted simply based on water concentration and temperature. It should be noted that even with considering only two parameters the determined air concentrations match with the concentrations predicted with the full model ($R^2\sim0.99$).

Geosmin steady-state = $-3.691\times10^{-2} \times C_{in} + 1.398\times10^{-3} \times C_{in} \times T$
($F=1.84\times10^{6}$, $p<3\times10^{-16}$, $R^2=0.9927$)

2-MIB steady-state = $-5.928\times10^{-2} \times C_{in} + 2.178\times10^{-3} \times C_{in} \times T$
($F=1.24\times10^{6}$, $p<3\times10^{-16}$, $R^2=0.9892$)

Nonadienal steady-state = $-2.018\times10^{-2} \times C_{in} + 8.957\times10^{-4} \times C_{in} \times T$
($F=5.10\times10^{6}$, $p<3\times10^{-16}$, $R^2=0.9974$)
where $C_{in}$ is the concentration in the water (ng/L) and T is water temperature ($^{o}C$).

The importance of water concentration and temperature can be observed in the tree regression shown in Figure-5.2 for the steady-state model for geosmin. Same pattern for the parameters were obtained for 2-MIB, and nonadienal as well. Tree regressions used for this analysis use binary recursive partitioning to sequentially split the data into two branches so that the difference in the response variable (steady-state concentration) between the two branches is greatest. This way the important parameters to calculate a

realistic indoor air concentration could be determined by evaluating the effect of parameters on the air concentration and pursuing the higher impact one.

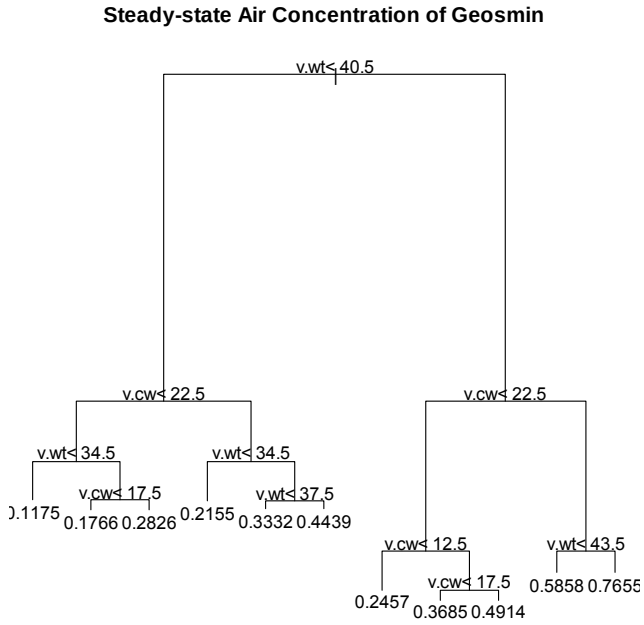


**Figure-5.2.** Tree regression for geosmin for the steady state model

As it was determined that the water temperature and odorant concentration in water are the two main parameters to determine air concentrations of the odorants at steady-state, the quartic surface contour plots given in Figure-5.3 were obtained for geosmin, 2-MIB, and nonadienal.

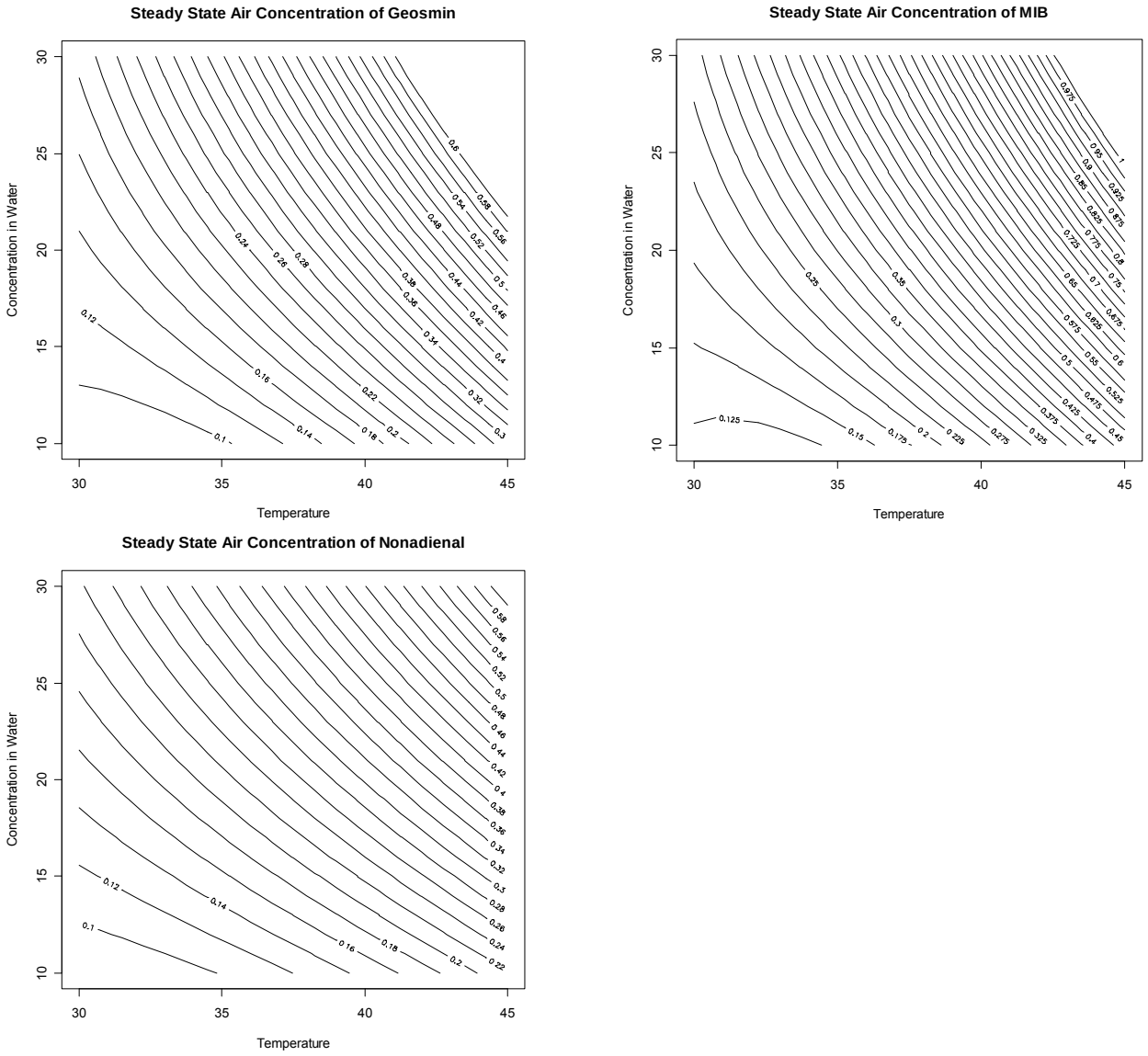


**Figure-5.3.** Contour plots of geosmin, 2-MIB, and nonadienal to determine the odorant concentration in air at steady-state based on water temperature and odorant concentration in water

As can be deducted from the plots, the contours are flat at lower temperatures and lower odorant concentrations in water, as these parameters increase the steady-state air concentration varies significantly with a slight change in water temperature or odorant concentration. This is an important point to consider when determining the extent and type of treatment during algal bloom episodes and high odorant concentrations in

drinking water as the odorants may be removed below detection limits with a minor treatment.

### 3.2. Dynamic model

Under a selected constant set of conditions ($Q_L$ = 10 L/min, $C_{in}$ = 20 ng/L, $Q_s$ = 50 L/min, $Q_b$ = 200 L/min, odorant concentration in influent air (shower and bath) = 0, $y_b$ and $y_s$ = 1x10$^{-5}$ ng/L, $V_s$ = 1000 L, $V_b$ = 8000 L and T = 20 and 40 $^{o}$C), assuming an instantaneous complete mixing in the shower volume, the air concentrations over time for the selected odorants determined are shown in Figure-5.4. After about three minutes at 20 $^{o}$C and about ten minutes at 40 $^{o}$C the odorants reach their steady-state concentrations. An interesting finding from this analysis was the relationship between steady-state air concentrations of the odorants at two different temperatures: at lower temperatures nonadienal reached a higher concentration than geosmin and 2-MIB whereas at higher temperatures 2-MIB had the higher steady-state concentration. This is due to higher Henry's law constant of nonadienal at lower temperatures and since it has a much smaller $\Delta H^{o}$ than geosmin and 2-MIB the constant at a higher temperature is smaller compared to the other two. Air-water partitioning of a compound is a thermodynamic trade-off between water solubility and vaporization and temperature affects these parameters differently for odorants with different chemical structures. The impact of temperature on Henry's law constants can readily be seen in Figure-5.5. The constant for nonadienal falls below that for 2-MIB at about 25 $^{o}$C, and below that for geosmin at about 34 $^{o}$C. These non-linear changes in Henry's law constants for different odorants result in different concentration profiles as temperature changes as can be observed in Figure-5.4.

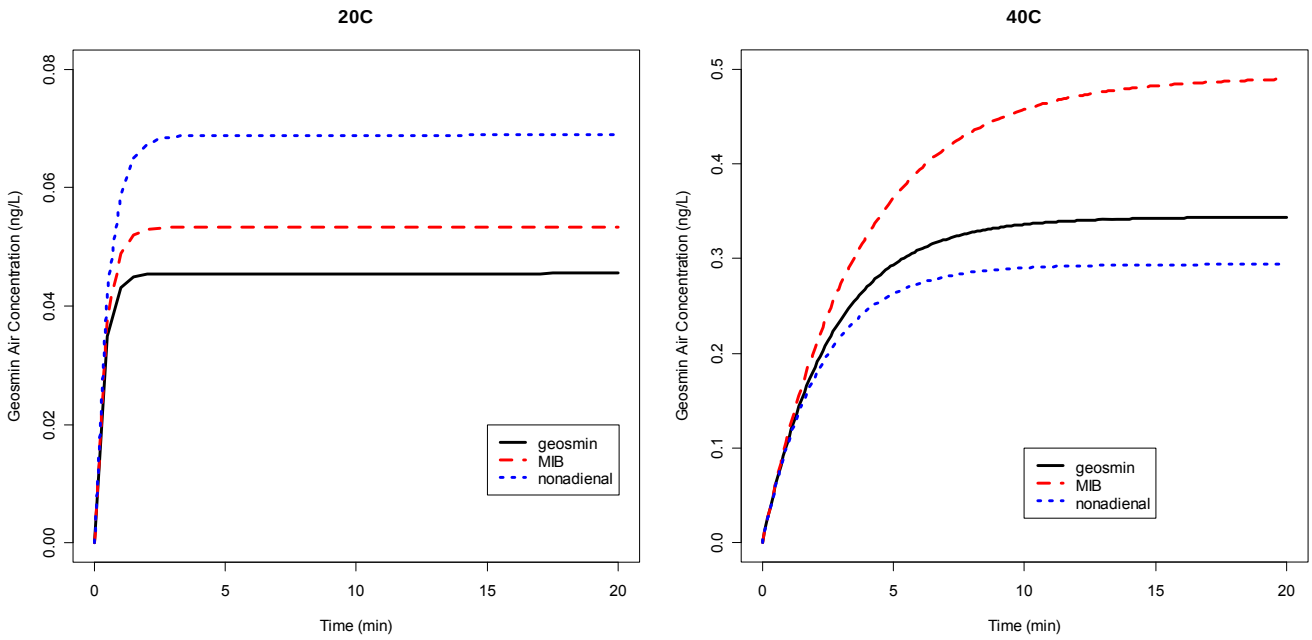


**Figure-5.4.** Air concentrations of the odorants in time at selected parameter values

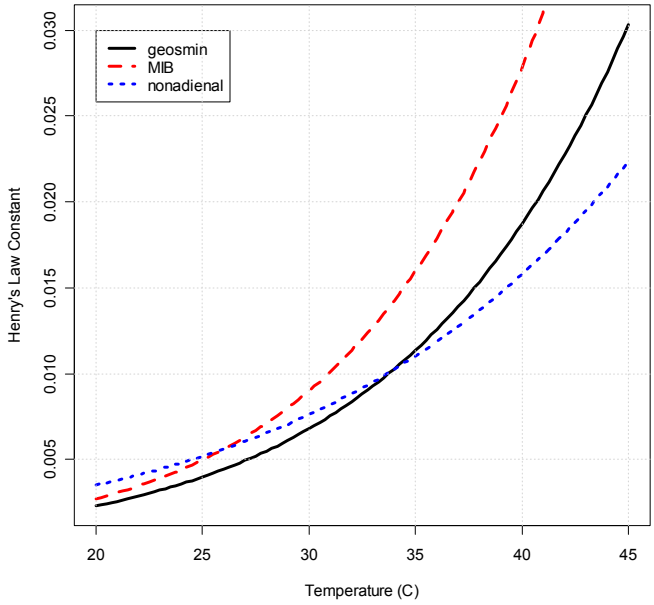


**Figure-5.5.** Effect of temperature on Henry's law constants of geosmin, 2-MIB and nonadienal

At the same conditions given above, the times to reach the detectable air concentrations for the odorants are given in Table-5.4. Although a complete mix model

is assumed for our calculations it may not be valid for the sensory detection. As soon as the water starts to flow through the shower head there will be a concentration gradient in the air with the highest concentration near the water stream. Then the odorants will diffuse to the lower concentrated air in the shower stall. Hence the air concentration may reach detectable limits for a person near the water stream earlier than predicted in Table-5.4.

**Table-5.4.** Duration in shower for the odorants to reach the detection concentration

| Chemical | Air Detection Concentration | Time to reach detection concentration (min) |
|---|---|---|
| **Geosmin** | 0.182 ng/L | 1.9 |
| **2-MIB** | 0.085 ng/L | 0.7 |
| **Nonadienal** | 0.112 ng/L | 1.1 |

To model for the realistic diffusion of the odorants from the shower head to throughout the stall, the shower volume was varied and the results are shown in Figure-5.6 with the concentration of the odorants in air after 1 minute. The upper line represents the steady state concentration and the lower line/bar shows the estimated detectable concentration range for the odorants of interest.

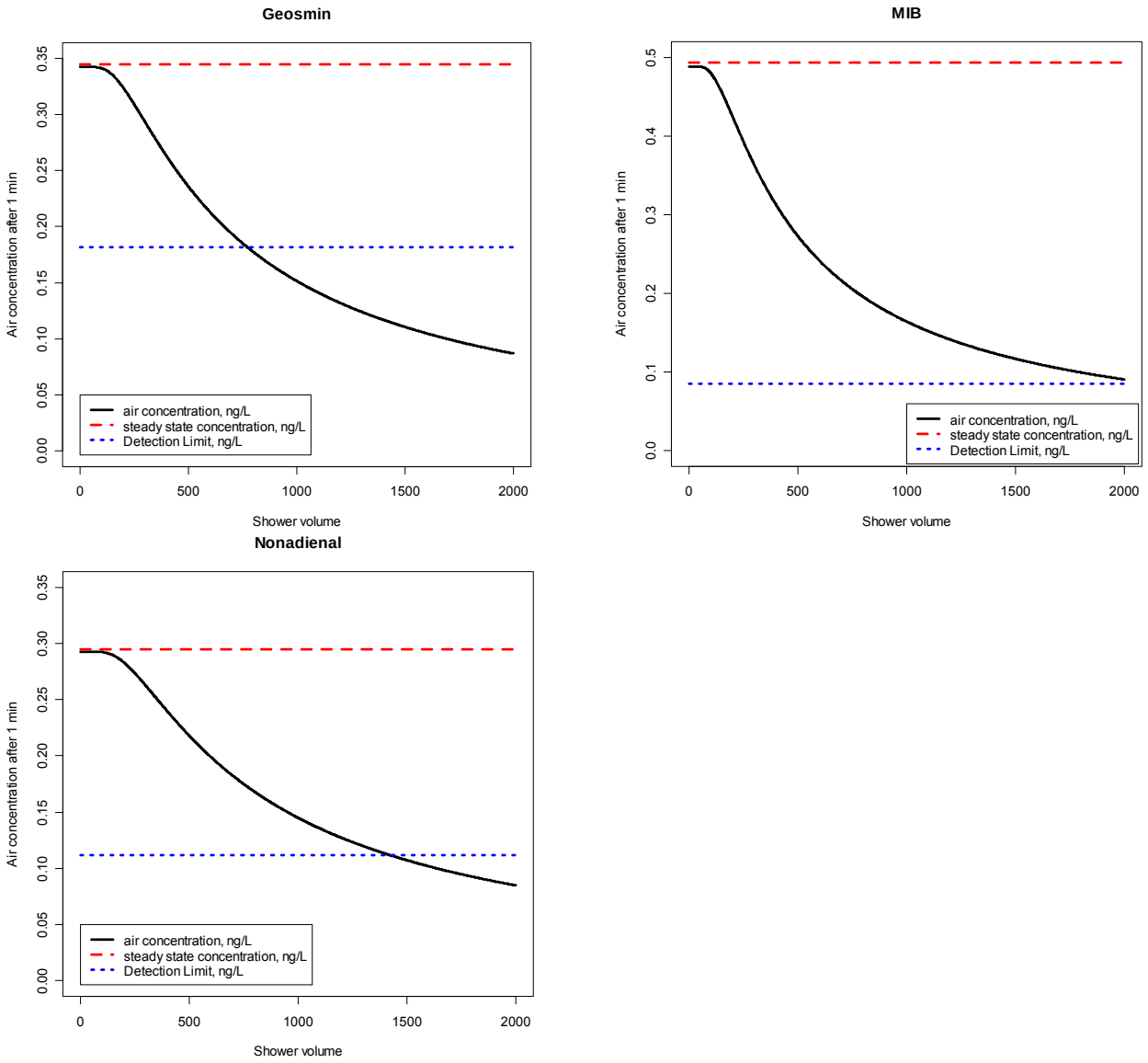


**Figure-5.6.** Effect of "shower" volume on the odorant concentrations in air after 1 minute

As can be seen from the plots, for all of the odorants the concentration in air assuming a minimal shower volume was almost equal to the steady-state concentration and well above the detection limit. This implies that the concentrations predicted by the model are realistic and can be used to predict real-life situations. Also the model needs to be adjusted to account for the concentration gradient created as soon as the faucet is opened. Rather than a complete mix model, a modified plug flow model should be assumed and studied for the diffusion of the odorants in the shower stall. The air around

the shower head will reach a higher concentration in a very short time and then the odorants will start to diffuse to the further sides of the shower. It is important to understand the parameters that determine the air concentration of the odorants in a dynamic model. So the tree regressions for geosmin, 2-MIB, and nonadienal were obtained for the concentrations after 1 minute. These are more complicated than that for steady-state model, as shown in Figure-5.7 for geosmin. Again same pattern for the parameters were obtained for 2-MIB, and nonadienal. Compared to steady state conditions, where only temperature and water concentrations determined the air concentration, the 1 minute concentration was also a function of the shower volume and the water flow rate.

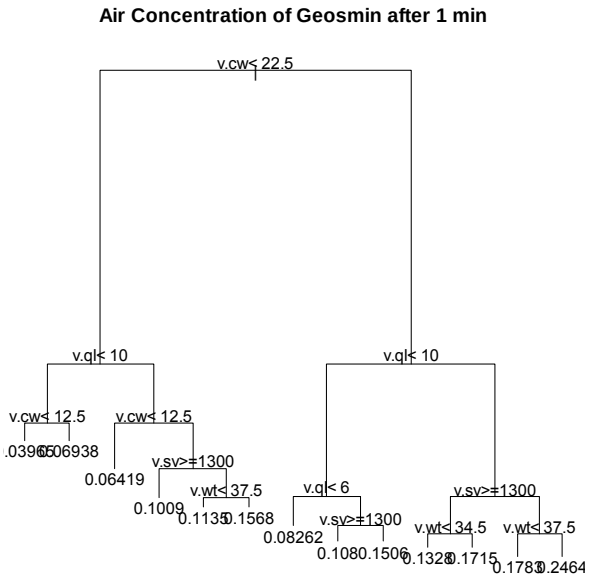


**Figure-5.7.** Tree regression for geosmin at 1 minute for varying shower volume (dynamic model)

A full factorial linear regression including all interactions was performed to fit the 1 minute air concentration as a function of shower volume, water flow in shower, air flow in shower, air flow in bathroom, concentration in water, and water temperature. The linear regression results confirmed the tree regression results shown above. The overall regression was statistically significant (for geosmin:$F=2.98x10^5$, $p<3x10^{-16}$, $R^2=0.9986$; for 2-MIB:$F=2.65x10^5$, $p<3x10^{-16}$, $R^2=0.9984$; and for nonadienal:$F=3.76x10^5$, $p<3x10^{-16}$, $R^2=0.9989$). A reduced model was obtained for each odorant and it was observed that

the shower volume, water flow rate, odorant concentration in water, and the water temperature captured most of the explanatory power.

$$\text{Geosmin 1 min} = 1.816\text{x}10^{-4} \times C_{in} - 2.642\text{x}10^{-6}\text{x}\ V_s \times C_{in} + 4.079\text{x}10^{-4} \times Q_L \times C_{in} + 1.300\text{x}10^{-4} \times C_{in} \times T$$

$(F=7.68\text{x}10^{5}, p<3\text{x}10^{-16}, R^2=0.9913)$.

$$\text{2-MIB 1 min} = 1.169\text{x}10^{-3} \times C_{in} - 3.089\text{x}10^{-6}\text{x}\ V_s \times C_{in} + 4.725\text{x}10^{-4} \times Q_L \times C_{in} + 1.157\text{x}10^{-4} \times C_{in} \times T$$

$(F=8.25\text{x}10^{5}, p<3\text{x}10^{-16}, R^2=0.9919)$.

$$\text{Nonadienal 1 min} = 1.256\text{x}10^{-3} \times C_{in} - 2.535\text{x}10^{-6}\text{x}\ V_s \times C_{in} + 3.952\text{x}10^{-4} \times Q_L \times C_{in} + 9.873\text{x}10^{-5} \times C_{in} \times T$$

$(F=1.18\text{x}10^{6}, p<3\text{x}10^{-16}, R^2=0.9943)$.

Thus for typical ranges of bathroom and shower conditions, only water temperature and concentration determine the maximum air concentrations and whether or not the concentration is detectable. The time to reach this concentration (and hence when the concentration might become detectable) is also determined by these parameters. It may be argued that although the shower volume affects the air concentration and time to detection, the volume that really should be considered is the volume around the shower head near the human's head (and nose). Because the odorant concentration increases immediately around this area and the odorant is detected the time to detection is shorter as the concentration in the smaller volume gets higher faster (when disregarding the complete mix situation).

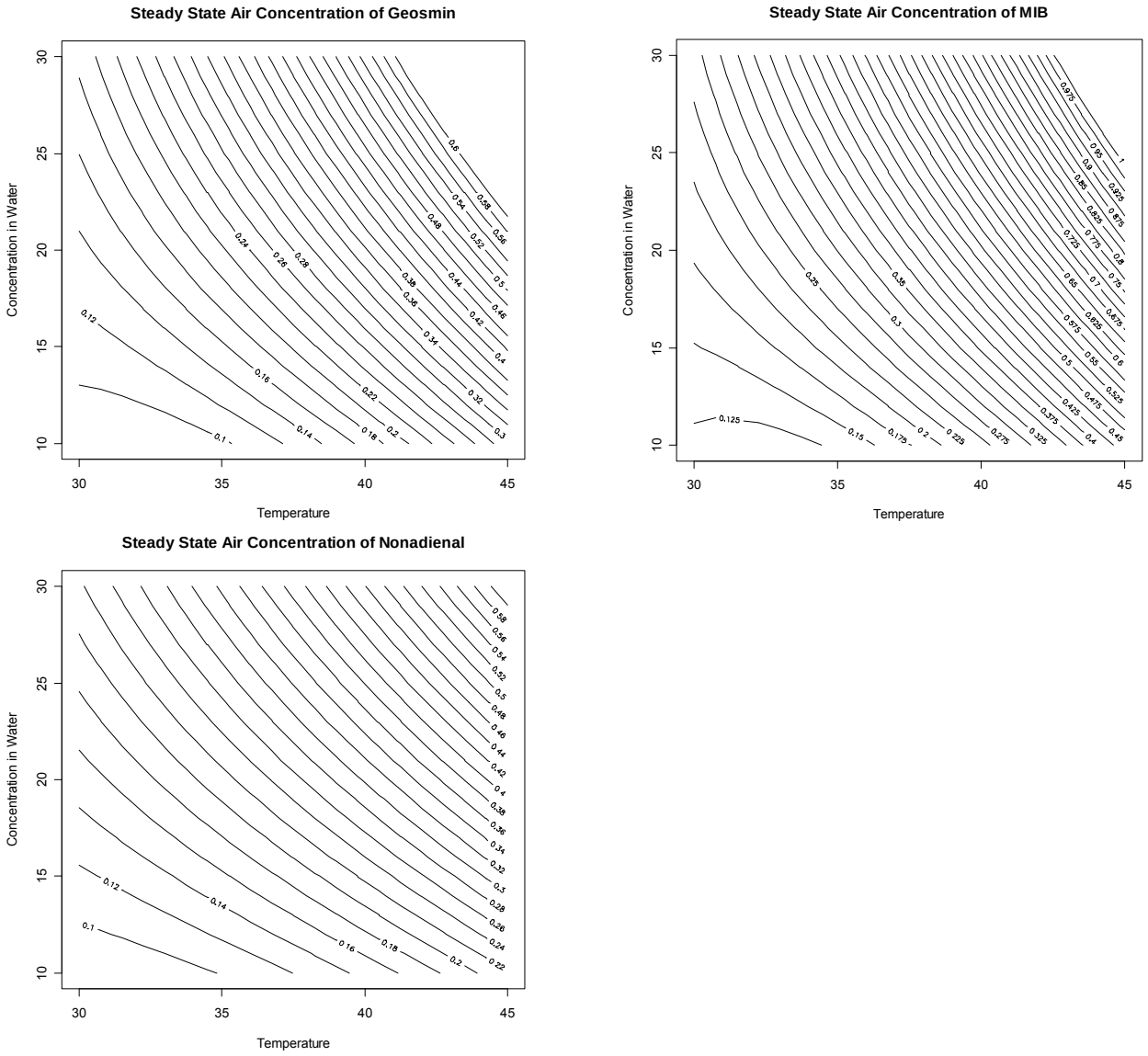


**Figure-5.8.** Contour plots of geosmin, 2-MIB, and nonadienal to determine the odorant concentration in air after 1 minute on water temperature and odorant concentration in water

## 3.3. Strengths and limitations of the model

The values for air concentrations of the odorants obtained from the model and their detectability parallels the data reported in the literature. The regression analyses indicate that ($R^2$>0.99) reduced models that only consider only a few parameters can be used to predict the air concentrations of the odorants without decreasing the accuracy of

the results. This means that the model may easily be used by the drinking water utilities to determine whether the contaminated water during a taste-and-odor episode should be treated or not. Also based on potential of the reduced model approach, other indoor air models may be calibrated and simplified enabling the researchers to predict the air concentrations easily.

One of the weaknesses of this indoor air model is assuming a complete mix system which is clearly not a legitimate approach considering the formation of a concentration gradient near the shower head. This should be corrected by modifying the model using a plug flow approach. Another consideration with the odor detection would be the type of the shower head that could produce aerosols which may affect the odor detection. Also water flow rate may be affected by the shower head design. As indicated by Chen et al. (2003), the shower head design affects the volatilization of the odorants from the water; hence they adopted a hybrid-showering model incorporating a jet-flow stream and spray droplets. The odorant concentration in shower was estimated to be higher for a spray type showerhead than a jet-flow type shower head.

This research could be well connected to the consumer complaints. Although utilities pay attention to consumer complaints, a systematic approach is lacking that would ask necessary questions to understand the place of detection, and other parameters that would clarify the situation. If the place of complaint, water temperature, volume of shower, etc were recorded during a taste-and-odor episode for the community, this model could be fine tuned. The odor threshold values reported in the literature vary significantly with missing information such as water temperature that would determine the air odor threshold concentration of the odorants. Volunteers may be obtained for an area with high occurrence of odor episodes, that would report the detection of odors and other parameters which could be used for fine tuning of this model as well. Laboratory studies conducted so far use synthetic chemicals that may not have the same properties as the odorants found in water supplies. For example geosmin has two isomers and although only the odorous one is present in the environment, the commercially sold synthesized geosmin is a mixture of both which affects the odor threshold studies

conducted with it, falsifying the results by doubling the odor threshold concentration determined.

## 4. Conclusions

A mechanistic model (Little, 1992) was used to develop simple steady-state and simple dynamic regression models that can be readily used by researchers and practitioners to calculate indoor air concentrations of drinking water odorants to determine which concentrations will be detectable to consumers and likely to cause complaints. These simplified models have about 99 % agreement with the mechanistic model. The steady-state model is dependent on only the aqueous concentration and the water temperature, while the dynamic model is influenced by shower volume, water flow rate, air flow rate and water temperature. Water utilities can use these simplified models which will enable them to predict and possibly prevent a problem before the contaminated water reaches the customers and hence will result in better explanations and, possibly more satisfied customers.

Application of indoor air models to odorants represents new and worthwhile use of mechanistic and simplified models. While many odorants that enter indoor air above the threshold odor concentration are only a nuisance, annoying odors are important to consumers and should be avoided whether emitted from aqueous, solids, or gaseous sources. Odorants also can serve as biomarkers for assessing human exposure to contaminants because if the odor threshold is known and humans are detecting the odor, then exposure is certain. This is the case for geosmin, 2-MIB, and nonadienal for which water utilities often receive customer complaints. Use of odor threshold concentration for odorants, which by their very nature elicit a distinct human response, can be used to verify and calibrate indoor air models to asses how well they predict actual human exposure when the human response is known.

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# Chapter VI

## Developing Hexanal as an Odor Reference Standard for Sensory Analysis of Drinking Water


### Abstract

There are many analytical and sensory methods to analyze drinking water for flavor and off-flavors before it reaches consumers. Flavor profile analysis (FPA) is one of the most comprehensive methods. A well-trained panel is essential for FPA and although taste standards are well established, FPA training lacks an odor reference standard. In search of an odor reference standard, four different panel groups were trained and tested for n-hexanal at various concentrations (1-1000 μg/L) over 14 months. The Weber-Fechner plots for n-hexanal showed a linear and overlapping relationship for all panels. Analytical measurements demonstrated that the headspace concentration of n-hexanal was constant after 5 sniffs at 45°C and it remained constant during FPA sessions for up to 4 hours. The panelists liked the grassy odor of n-hexanal, which did not result in fatigue, and testing demonstrated that approximately 95% of the population can detect n-hexanal's odor. n-Hexanal is proposed as an odor reference standard for FPA training to define odor intensities because it is chemically stable, follows Weber-Fechner law, mimics grassy odors found in drinking water, and was acceptable to the human panelists.



***Keywords:*** *Flavor profile analysis, FPA, odor reference standard, n-hexanal, drinking water, Henry's Law constant*


### 1. Introduction

Taste-and-odor of drinking water is a worldwide concern. People desire a palatable drinking water and off-flavors may be caused by the contaminants in source water, new distribution system and plumbing materials, unforeseen chemical reactions and/or corrosion of metal pipes. Off-flavor of drinking water is an annoying aesthetical problem

and is not usually a health concern. If not controlled properly, off-flavors may cause consumer complaints, loss of confidence in the water utilities, and reduced intake of water. Hence millions of dollars are spent each year by the drinking water industry world-wide to remove taste-and-odor compounds (Hosaka et al., 1995; Jones and Korth, 1995; Juttner, 1995; McGuire, 1995; Ventura et al., 1995; Davies at al., 2004; Dietrich, 2006; Bae et al., 2007; Joe et al., 2007). To monitor taste-and-odor compounds, reliable and robust analytical and sensory methods are essential. These methods require defined protocols and reference standards that can be used for calibration (Rigal, 1995; Kenefik et al., 1995; Hochereau and Bruchet, 2004; Dietrich et al., 2004a,b).

There are many analytical and sensory methods available for the monitoring of drinking water to detect the odorous compounds and each has its own advantages and disadvantages (Mallevialle and Suffet, 1987; Bae et al., 2002; Devesa et al., 2004; Bruchet, 2006). When the methods are compared, the sensory methods can be superior because the human nose is more sensitive than most available instrumentation and instant results enable the utilities to take rapid action. Among the sensory methods, the flavor profile analysis (FPA, Standard Methods 2170: APHA, AWWA, WEF 2005) is one of the most comprehensive and reliable methods. It was originally designed for the food industry (Caircross and Sjostrom, 1950) and later adapted to the water industry (Krasner et al., 1985; Suffet et al., 1988) to be applied for the sensory characterization of drinking water.

FPA panelists are selected from healthy human subjects with basic taste and smell ability. Initial training takes 8 to 10 hours including introduction to basic tastes at selected intensities and to common drinking water odorants. The training continues with bi-weekly or weekly meetings that may last for a few weeks to months to obtain a very well trained panel as it is essential for the success of the analyses. The goal of this elaborative training is to be able to assess any taste and odor (and their combinations) at any intensity. At the initial training, panelists receive sweet, salty, sour, and bitter taste standards in plastic cups at room temperature at four intensities; very weak (2), weak (4), moderate (8) and strong (12). The panelists are asked to calibrate themselves according to these taste intensities although their perceptions might be slightly

different, for example, for sensitive panelists a weak intensity may be perceived as moderate, yet they need to learn to report it as weak. After calibration the panelists go through various tests with blind samples to check their accuracy in determining the correct taste and intensity. The next step is the introduction of the mouthfeel samples that trigger physical sensations in mouth and are neither tastes nor odors. These sensations include astringent, drying, and tingling mouthfeels. The last step is the introduction of the common drinking water odorants. Standard 500 mL Erlenmeyer flasks are used for this training and samples are presented at 45 $^{o}$C. Panelists become familiar with odor standards such as geosmin, 2-MIB, benzaldehyde, and chlorine. At this step to define the intensity of an odor the panelists are asked to attribute the perceived intensities of taste standards and translate them to odor intensities. Most panelists find this difficult and subjective so the precision of the evaluation is reduced. If an odor reference standard could be obtained, the panel could be trained accurately and provided an odor reference standard while performing routine odor analysis of the samples. Unfortunately FPA lacks odor reference standards to be used for the odor training and calibration of the panelists.

During a regular FPA session, the samples are presented to the panel in cups or standard Erlenmeyer flasks at 45 $^{o}$C. After tasting and/or smelling, each panelist rates the sample in terms of its descriptor(s) and intensity(ies). The results are collected by the panel leader and the panel discusses the results. The descriptor of the sample is any identifiable characteristic recognized by the panel. To rate the intensities a seven-point scale is used: it starts with odor free (0), then comes the threshold (1), and the even numbers between 2 to 12 are used for the intensity ratings (Krasner et al., 1985; Suffet et al., 1999).

Odor reference standards for qualitative description and quantitative intensity rating are common in the food industry (Young et al., 1999; Karagul-Yuceer et al., 2003). Odor standards that are either the compounds causing the off-flavors in drinking water or the ones that have similar odors have been identified and used in the drinking water industry. However, odor reference standards are still missing that would enable the utilities to precisely report the sensory characteristics and intensities of the odorants.

The need for an odor reference standard in the water industry was previously articulated (Burlingame et al., 1991). Although the water industry has some descriptive odor standards to identify selected notes and characteristics, e.g. geosmin for earthy, toluene for glue, 2-methylisoborneol for soil, nonanal for hay (Suffet et al., 1999), these are not sufficiently developed to be odor reference standards for the purpose of qualitative and quantitative calibration (Khiari et al., 1999). The closest the water industry comes to a qualitative and quantitative protocol for assessing odors is the attribute rating test (Dietrich et al., 2004a). In this protocol, a utility selects a specific odorant, e.g. geosmin, and a specific concentration the utility wants to monitor, e.g. 15 ng/L. The sensory panelists then assess the odor of the sample against the odor standard and rate it less than, equal to, or greater than the odor standard. Utility personnel really like this method because having an odor standard during the sensory test gives them confidence that they are properly calibrated and can produce accurate data.

There are many challenges to finding an odor reference standard for the water industry. The ideal odor reference standard should be readily available and not be a hazard to human health; should have a pleasant smell; should be detected by most of the population; should be stable during sampling maintaining the same intensity; should give reproducible results for every panel; should not cause fatigue after a few evaluations; and should have a linear Weber-Fechner plot (Lawless and Heyman, 1998). The Weber-Fechner plots show the relationship between the log concentration of the odorant and the odor intensity, and are commonly used in the water industry (Rashash et al., 1997; Cancho et al., 2001; Davies et al., 2004; Martin-Alonso et al., 2007). When a straight line with a positive slope is obtained, it means that the odor intensity of the compound increases with increasing concentration. This is an important property for an odor reference standard as the odorant could be introduced to the panelists at various intensities by increasing the concentration at logarithmic intervals.

n-Hexanal, having a pleasant grassy odor (Fabrellas et al., 2004), being an approved food additive by the United States Food and Drug Administration (Burdock, 1995), being available commercially in pure and food grade form, and grassy odor being a common odorant in drinking water (Khiari et al., 1999; Watson, 2004), is a good

candidate to be an odor reference standard. The reported odor thresholds of n-hexanal are variable; from 0.3 to 14 μg/L (Cotsaris et al., 1995; Young and Suffet, 1999; Watson, 2004).

This study investigated the conformity of n-hexanal to being an odor reference standard. Specific objectives of the research were to: determine the odor threshold concentration; obtain the Weber-Fechner plot of n-hexanal; provide concentrations to be used for an odor reference standard for FPA; estimate the percent of the human population that can detect this odorant at the reference standard concentration; and evaluate the compound's chemical stability.

## 2. Materials and methods

### 2.1. Reagents

High purity n-hexanal (CAS 66-25-1) was purchased from Sigma Aldrich (St. Louis, MO) and Nanopure® ultrapure water (Barnstead/Thermolyne, model #D4744, Dubuque, IA) was used as odor-free reagent water for the experiments. The solubility of n-hexanal is 5.64 g/L at 30 $^{o}$C and its vapor pressure is 10 atm. n-Hexanal stock solutions were prepared in methanol at 50 mg/L and 2 g/L concentrations in amber volatile organic analysis (VOA) vials (Fisher Scientific, Pittsburgh, PA) with Teflon® lined caps, sealed with parafilm and stored at 4 $^{o}$C. The experimental n-hexanal solutions were prepared in reagent water at 1, 5, 10, 25, 30, 50, 100, 250, 500, and 1000 μg/L concentrations by adding corresponding μL of stock solutions to the flasks.

### 2.2. Glassware

Kimax® 500 mL wide mouth Erlenmeyer flasks (Fisher Scientific, Pittsburgh, PA) with ground glass stoppers were used for the sensory analyses. All of the flasks were washed with Sparkleen® odor-free detergent (Fisher Scientific, Pittsburgh, PA) after each use, rinsed 3 times with tap water and 2 times with reagent water. The washing steps were repeated until the flasks were odor free. The glassware was stored containing 200 mL of

reagent water to prevent chalky odors. Immediately before use, the glassware was rinsed with reagent water again. For chemical analyses similar flasks with a septum port attachment on the side were used.

### 2.3. Analytical testing equipment

Solid phase micro-extraction (SPME) and gas chromatography with mass spectrometer detector was used to measure the headspace concentration of n-hexanal in the flasks over time and after a few sniffs. 65 μm PDMS/DVB SPME fibers were purchased and conditioned as specified by the supplier (Supelco, Bellefonte, PA). Agilent 6890 Series GC System was operated at splitless mode with a 0.75 mm i.d. injection sleeve (Supelco, Bellefonte, PA); total helium flow of 24 mL/min; inlet temperature of 220°C; 80 kPa pressure; J&W DB-5 capillary (30.0 m x 250 μm i.d. x 0.3 μm film thickness) column with 325°C maximum temperature. The oven was programmed to start at 60 °C with a 1 min hold, then to ramp at 20 °C/min to 140 °C. Elution time of hexanal was at 3.31 min. Agilent 5973 Network Mass Selective Detector was operated under selected ion monitoring mode, and m/z 44, 56, 100 were the selected ions.

### 2.4. Human panelists

#### 2.4.1. General information

The study protocol was approved by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech. The subjects were selected from the students and staff at Virginia Tech who have the basic ability to taste and smell. The panelists were requested not to wear any perfumes, colognes or lotions and to refrain from smoking, eating and drinking at least 30 minutes prior to sessions. Subjects were given incentives such as snacks after each session and gift certificates after completion of the experiments. All of the information obtained was kept anonymous.

#### 2.4.2. FPA session panelists

20 panelists were selected from the students and faculty at Virginia Tech and were grouped into four panels. All of the participants underwent a one day long training

session to learn the Flavor Profile Analysis method. The protocol for FPA was obtained from the *American Society for Testing and Materials (STP 435), Standard Methods for the Examination of Water and Wastewater*, Section 2170 B, and the American Water Works Association (AWWA Manual, 1993). This session helped the panelists to recognize the four basic tastes at various intensities and to familiarize with several odors found in drinking water. Because this method lacks odor reference standards, panelists were asked to interpret the taste intensities as odor intensities when rating odors. They were also screened for their ability to smell. The panelists participating in FPA sessions had an age range of 18 to 51 and consisted of 10 males and 10 females. The mean age for the FPA panelists was 29.6 years.

### 2.4.3. Triangle test panelists

Panelists that participated in triangle testing were also selected from the students and faculty of Virginia Tech and they were not trained as the aim was to determine the ability of the general population to detect n-hexanal at a low concentration. To determine the minimum number of panelists required for statistically significant triangle testing, the α and β values were selected as 0.05 and the $p_d$ (proportion of distinguishers, which defines the percentage of the population that may detect the difference in the samples) was set to a medium level of 30%. The number of panelists required was found to be 66. Based on the selected α value and number of panelists, the number of correct responses to conclude that the general population can detect hexanal was 29 (Meilgaard et al., 1999). 34 males and 32 females participated in the triangle testing with an age range of 18 to 71, and the mean age of the panelists was 30 years.

## 2.5. Sensory test sessions: FPA

Samples presented to panelists were coded with randomly selected 3-digit numbers to prevent bias. Experiments were performed in odor free environments. At least 3 or 4 panelists were present in each session. The 500 mL wide-mouth Erlenmeyer flasks containing 200 mL of test water at selected concentrations from 1 to 1000 μg/L were heated up to 45 °C in a water bath and the temperature kept constant. To prevent the bias of the panelists and to prevent them from expecting ascending or descending

concentrations, in some sessions all of the flasks presented were at the same concentration. The lower concentrations were used to assess the threshold concentration for the panel. No more than six samples were presented at one session to prevent saturation and fatigue, and panelists waited for 2 minutes between samples. Odor free flasks were supplied to refresh the panelists' perceptions. In each session panelists smelled each flask, described and rated the intensities and recorded the results on provided scorecards. Then the results were discussed to come to a panel consensus. The panels were tested over 14 months until each panel tested every concentration at least four times and a total of 650 data points were obtained.

### 2.6. Sensory test sessions: triangle test

Samples presented to panelists in groups of three were coded with randomly selected 3-digit numbers to prevent bias. Experiments were performed in odor free environments. The 500 mL wide-mouth Erlenmeyer flasks containing 200 mL of odor free reagent water or 30 μg/L hexanal solutions were heated up to 45 °C in a water bath and the temperature kept constant. The panelists were asked to smell the three samples in order from left to right, waiting for 2 minutes between samples, and to pick the odd one. The odd sample could be the one with or without the n-hexanal solution. The odd sample (odor-free or n-hexanal) was varied equally, and the order of the samples was also randomly picked to statistically strengthen the experiment.

### 2.7. Chemical stability tests

The stability of n-hexanal was determined by the SPME-GC/MS method. Modified Erlenmeyer flasks with septum ports on the sides were used to measure the headspace concentration of the odorant after 0 to 5 sniffs at 45°C. A series of six flasks were prepared with n-hexanal at 30 and 100 μg/L concentrations in reagent water, the first flask was used as a control to determine the initial concentration in the headspace and the rest of the flasks were analyzed after they were sniffed as usual by a panelist. The first flask was sniffed and then the concentration in the headspace was measured after a 2 minute equilibration time following the sniff. The second flask was sniffed twice with 2

minute equilibration time between sniffs and before sampling. The rest of the flasks were sniffed accordingly up to 5 sniffs to mimic regular FPA panel sessions with 5 panelists. The stability of n-hexanal in water over time was also measured. A series of six flasks were prepared with n-hexanal at 30 and 100 μg/L concentrations in reagent water at 45°C and were tested at 0, 1, 2, 3, 4, and 5 hours. For each trial the SPME fiber was exposed to the headspace for 10 minutes and n-hexanal was desorbed from the fiber at the GC inlet. Each experiment was repeated three times. To make sure the SPME fiber was not saturated with n-hexanal and hence could detect the change in the headspace concentration (if there was), a series of flasks were prepared at 10, 30, 100, and 250 μg/L concentrations in reagent water.

### 2.8. Relating odor threshold to gaseous odorant concentration

In order to better understand the odor threshold concentration of n-hexanal and to relate the odor intensity to the gaseous concentration rather than the aqueous concentration, dimensionless Henry's Law constant of n-hexanal was measured at 23.5 and 37 °C applying the method described by Ömür-Özbek and Dietrich, 2005. The initial aqueous concentration of n-hexanal was 100 μg/L and the SPME fiber was exposed to the headspace for 10 minutes before it was analyzed by GC/MS as described above. The enthalpy of solution was determined using the van't Hoff equation with the two constants measured at 23.5 and 37 °C. The van't Hoff equation was then used again to estimate the dimensionless Henry's Law constant at any temperature and to determine the partitioning between gaseous and aqueous phases.

### 2.9. Statistical analyses

For statistical analyses of the data obtained from the sensory and analytical tests, Microsoft Excel™ was used. Single factor ANOVA was run for each experiment with an α value of 0.05. In each analysis the null hypothesis was that there is no significant difference (among the panels or replicates or results over time).

## 3. Results and discussion

### 3.1. Sensory tests: FPA

The FPA results for four different panels tested with n-hexanal at concentrations from 1 to 1000 μg/L with the mean intensity rating corresponding to each concentration are shown in Table-6.1. The findings indicate that n-hexanal yields similar intensity ratings regardless of the panel tested. Statistical analysis indicated that the results from panels are not different (p=0.99). Hence it is determined that n-hexanal gives reproducible results.

**Table-6.1.** Intensity ratings of solutions of n-hexanal by four human panels

| Conc. (μg/L) | Panel 1 | Panel 2 | Panel 3 | Panel 4 | Overall $\pm$ std dev |
|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 1.0 $\pm$ 0 |
| 5 | 2 | 2 | 1.8 | 1.8 | 1.9 $\pm$ 0.1 |
| 10 | 2.5 | 3.3 | 2.4 | 2.4 | 2.6 $\pm$0.4 |
| 25 | 4 | 3.4 | 3.9 | 4 | 3.8 $\pm$ 0.3 |
| 50 | 4.5 | 4.9 | 4.7 | 4.9 | 4.7 $\pm$ 0.2 |
| 100 | 5.5 | 5 | 5.4 | 5.7 | 5.4 $\pm$ 0.3 |
| 500 | 6.5 | 7.2 | 7 | 6.7 | 6.8 $\pm$ 0.3 |
| 1000 | 7 | 8.2 | 7.5 | 7.3 | 7.5 $\pm$ 0.5 |

To determine the relationship between the odor intensity and odorant concentration, a Weber-Fechner plot was used. As shown in Figure-6.1, the plot for overall results indicates that there is a linear relationship between the log intensity and the concentration. Hence the odor intensity did not level off with increasing concentration which is a required property of an odor reference standard. This finding also agrees with a previous study (Khiari et al., 2002). The equation obtained from the plot may be used to prepare the samples at desired concentrations to yield desired odor intensities to train the panelists.

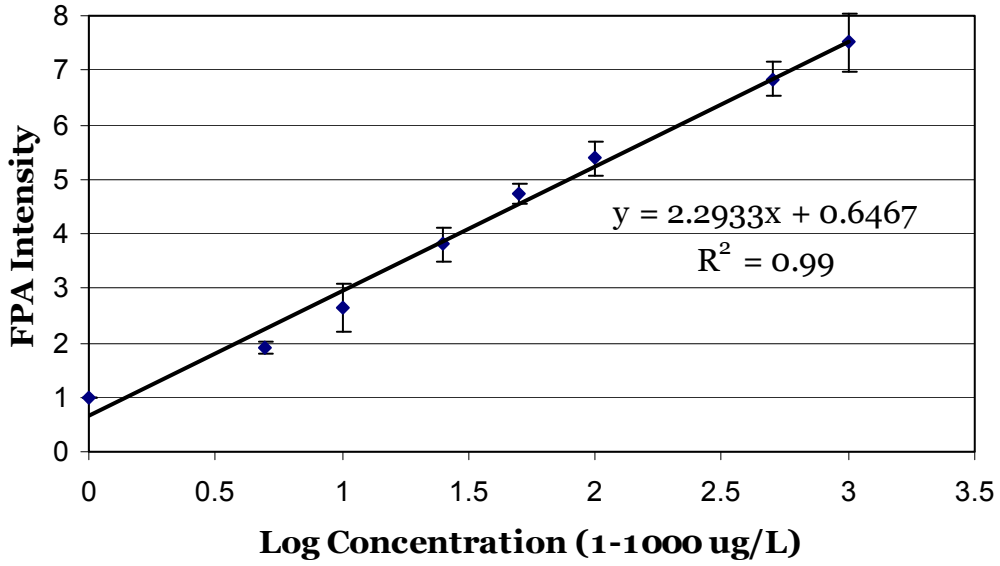


**Figure-6.1.** Weber-Fechner of overall results from four panels (with error bars for the standard deviation between panels)

From the Weber-Fechner plot, the odor threshold of n-hexanal was also determined. The concentration that corresponds to the odor intensity of 2 is accepted as the odor threshold concentration (OTC) (Krasner, 1988). From the equation obtained from the plot, the OTC for hexanal was determined to be 3.9 µg/L.

The panelists liked smelling the n-hexanal samples. One of the panelists even indicated that he would drink water that smelled like that. The general descriptors used by the four panels include grassy, pumpkin, green, sweet, and green apple. All of the panelists however could agree on "grassy" descriptor. These descriptors comply with the ones reported in the literature (Davies et al., 2004; Fabrellas, 2004; Aldrich Fragrance Catalog). From the responses of the panelists and as reported in the literature (Burlingame et al., 1991), n-hexanal does not cause fatigue especially when the panelists rest for 2 minutes between samples.

### 3.1.1. Proposed concentrations for an odor reference standard

Based on the Weber Fechner plot shown in Figure-1, a concentration of 30 µg/L n-hexanal in odor free water corresponds to an FPA 4 rating which is a "weak" intensity on the FPA scale (Standard Methods, 2710). This would be a good concentration for an odor reference standard as many of the odors commonly found in drinking water are on the low end of the FPA scale, or at "weak" intensities. A second odor reference standard of FPA 6 rating which is "weak-moderate" could be prepared at 102.5 µg/L concentration. Using a slight extrapolation of the Weber-Fechner plot, a concentration of 1608.5 µg/L would equal an FPA 8 (moderate) rating.

## 3.2. Sensory tests: triangle test

The concentration of hexanal (30 µg/L) selected for this study was at an FPA intensity of 4 (see Figure -1) which corresponds to a weak intensity and is at a level typically found in drinking water. Thus, this is an appropriate concentration for an odor reference standard. As described before, 66 panelists have participated in the triangle testing and only 3 people could not correctly identify the odd sample. To be statistically significant only 29 correct answers were required. This indicates that the general public is very sensitive to n-hexanal and most of the population can detect the grassy odor.

## 3.3. Chemical stability tests

To be an effective odor reference standard, n-hexanal must be stable in water and the headspace. In order to make sure that the concentration and hence the odor intensity remains constant during sensory testing the headspace concentration of hexanal in flasks after 5 sniffs in the FPA setting were determined (with three replicates) and plotted in Figure-6.2. Statistical analysis of the data indicated that the results from each sniff are not different ($p=0.20$). So it can be concluded that the concentration remains constant and hence all the panelists will be exposed to the same concentration of the odorant during an FPA panel session.

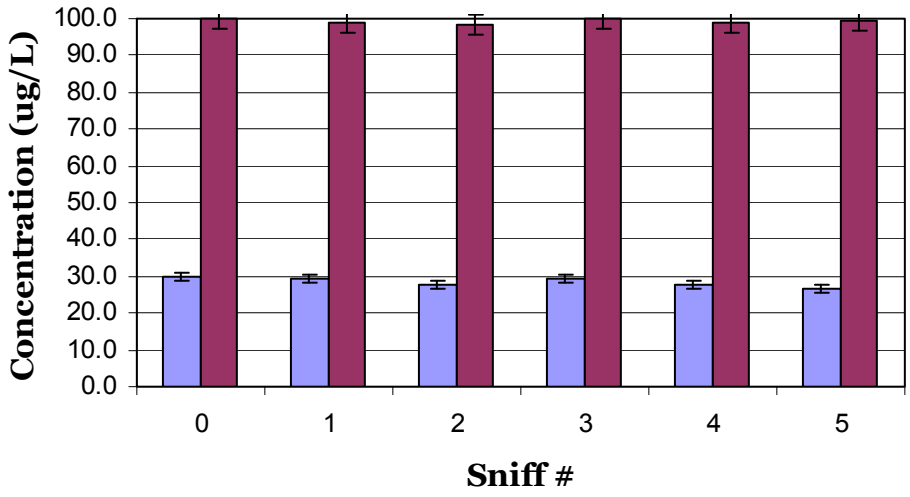


**Figure-6.2.** Concentration of n-hexanal at 30 and 100 µg/L (error bars represent standard deviations for three replicates) in the FPA Erlenmeyer flasks after each of 5 sniffs by the human nose at 45 $^{o}$C

The stability of aqueous n-hexanal over time was also determined and plotted in Figure-6.3. Again the statistical analysis of the data indicated that the results from each tested hour are not different ($p=0.67$). The results demonstrated that n-hexanal is stable over several hours at 45 $^{o}$C. This allows the researchers to prepare the samples ahead of time or test different panels over a couple of hours without diminishing the quality of the samples.

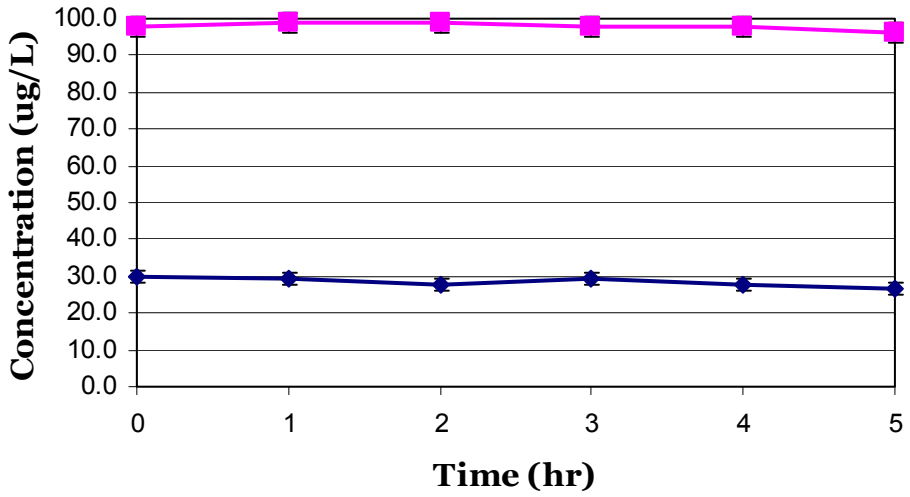


**Figure-6.3.** Stability of n-hexanal in headspace over time for 30 and 100 µg/L n-hexanal solutions in water at 45 °C over 5 hours (error bars represent standard deviations for three replicates)

The experiment conducted with various concentrations (10 to 250 µg/L) to determine whether the SPME fiber was saturated indicated that the concentration of n-hexanal in the headspace increases linearly with increasing concentration in reagent water. Hence the SPME fiber was not saturated and yielded reliable results.

### 3.4. Henry's Law constant of n-hexanal and the gaseous odor threshold

Dimensionless Henry's Law constants of n-hexanal were measured as 0.0109 and 0.0233 at 23.5 and 37 °C respectively. The constant at 23.5 °C is in agreement with the constant reported as 0.011 at 25 °C (Sander, 1999). The enthalpy of solution was determined by using the measured constants and the van't Hoff equation as 43 kJ/mol. The calculated enthalpy and one of the measured constants were used to determine the constant at 45 °C as 0.0355. From Henry's Law the gaseous odor threshold concentration of n-hexanal may be determined. Since the aqueous odor threshold of n-hexanal was 3.9 µg/L-water, the gaseous odor threshold is determined as 0.14 µg/L-air which is a better means to report odor threshold concentrations.

## 4. Conclusions

n- Hexanal, a food additive of no known adverse health effects, has a pleasant grassy smell; can be detected by a large percent of the population (~95.5%) ; is readily soluble in water and stable during sampling yielding similar intensities among the panelists; gives reproducible results for different human panels; does not cause fatigue at higher concentrations or after a few sample evaluations; and has a linear Weber-Fechner plot indicating that the odor intensity increases with increasing odorant concentration. Because n-hexanal possesses all the necessary properties, it is proposed as an ideal odor reference standard to be used for FPA training and sensory panel assessment of water samples. A concentration of 30 μg/L n-hexanal in odor free water is equivalent to an FPA 4 intensity or a "weak" rating.

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# Chapter VII

## Retronasal Perception and Taste Thresholds of Metallic Flavored Drinking Water with Iron and Copper


### Abstract

Metallic flavor has been an issue for drinking water consumers and is caused by the dissolved iron and copper in drinking water. Iron and copper are commonly found in groundwater and may be introduced to the drinking water by the corroding infrastructure. US Environmental Pollution Agency has secondary maximum contaminant levels for iron and copper set as 0.3 and 1.0 mg/L in drinking water to prevent aesthetical problems such as metallic taste and stains on clothes and fixtures. An action level of 1.3 mg/L has been established for copper to prevent health effects. Taste thresholds of iron and copper have been investigated by several sensory studies however reported results and test methods were varied considerably. This study determined the taste thresholds of ferrous and cuprous in reagent water by using a one-of-five test. For ferrous and cuprous individual thresholds ranged from 0.003 to >5 mg/L and 0.035 to >5 mg/L, respectively. The population thresholds were determined by logistic regression and geometric mean methods as 0.031 and 0.05 mg/L respectively for ferrous ion and as 0.61 mg/L for cuprous ion by both methods. The components of metallic sensation were investigated by use of nose-clips while panelists tasted iron and copper solutions. The results showed that metallic taste has a significant odor component and should be treated as a flavor instead.



***Keywords:*** *Iron, copper, taste threshold, drinking water, metallic flavor, retronasal*

# 1. Introduction

## 1.1. Iron and copper in drinking water

Dissolved iron and copper in drinking water cause a metallic sensation. Iron and copper are essential micronutrients. Recommended daily intakes for iron and copper were established as 15 and 2 mg respectively, although these values vary depending on age and gender, 5 % of the required values are supplied by most of the tap water in the US (WHO, 1996). Iron and copper participate in functioning of enzyme systems, required for oxygen transport, and healthy growth. Anemia, impaired immunity and development are common problems related to copper and iron deficiency. Due to anaerobic conditions iron and copper are commonly found in groundwater. Although some of the treatment methods, such as coagulation and precipitation, remove iron and copper, corroding infrastructure (as a result of soft and heavily acidic or alkaline water) may introduce them back to the drinking water. SMCLs have been established by the USEPA at 0.3 and 1 mg/L for iron and copper respectively to prevent aesthetical problems such as metallic taste, colored water, stains on clothes and fixtures, and nausea. Because copper causes health problems when consumed at higher levels, an AL was set to 1.3 mg/L. When consumed at higher doses for longer periods iron and copper may cause irreversible damage to kidney and liver, and may be lethal (NRC, 1989; O'Donohue et al., 1993).

## 1.2. Taste thresholds of iron and copper

Metallic sensation has been an issue for the drinking water industry due to aesthetical issues. Recently it also started to draw attention by the medical industry as the cancer patients receiving chemotherapy suffer from malnutrition because of the metallic taste dysfunction. Until recently researchers have only focused on taste thresholds of iron and copper. First work was conducted in 1960 by Cohen et al. Numerous sensory methods have been used including 1-of-5 forced choice test, modified triangle test, and paired difference test. Cohen et al. (1960) reported that ferrous sulfate may be tasted by 5 and 50 % of the population at concentrations as low as 0.04 and 3.4 mg/L in distilled water.

The values change for spring water as 0.12 and 1.8 mg/L. Another study reported a taste threshold value of 0.6 mg/L for ferrous in distilled water that can be detected by 50 % of the population (Zacharias, 1979). More recent studies reported higher values for ferrous as 1.58 mg/L in mineral water (Gonzales, 1998); and as 5.54 mg/L in deionized water by using the geometric mean method (Lim, 2006). The reported taste threshold values are higher for cupric. Cohen et al. (1960) also studied cupric and reported the taste thresholds that can be detected by 50 % of the population as 2.46 and 4.73 mg/L in distilled and spring water respectively. Recent studies however, reported lower values for cupric taste threshold. It was reported that cupric may be detected by 50 % of the population at 0.61 mg/L(Zacarias, 2001). Another work reported taste thresholds for cupric as 0.48 and 0.77 mg/L in distilled water by using the geometric mean method and and as 1.5 and 1.96 mg/L in mineralized water by using the logistic regression method (Cuppett, 2006). Similar values were obtained by a recent study for cupric as 0.49 and 1.2 mg/L in deionized water by using the geometric mean method and logistic regression method (Epke and Lawless, 2007). As can be concluded by comparing the values reported in literature, taste thresholds vary significantly by the sensory methods employed, the experimental water that the samples were prepared with, concentrations of metals tested and data analysis method used. It should also be noted that sensitive people (who represent 5% of the population) may be able to detect metals in water at 1000 times lower concentrations compared to the less sensitive people. Hence even though the iron and copper concentrations are very low in drinking water this group may still complain.

### 1.3. Retronasal perception of iron and copper in drinking water

Although researchers have focused mainly on taste thresholds of metals, recently a few studies started evauating the effect of olfaction on metallic sensation. These studies have conducted taste tests by occluding the panelists' noses with nose-clips and reported a significant decrease in perceived perception for ferrous and cupric even when they were tested at very high concentrations (Hettinger et al., 1990; Lawless et al., 2004). Epke and Lawless (2007) determined taste thresholds with and without occluding noses of the panelists by nose-clips and reported that taste thresholds determined with closed

nose were 10 to 30 times higher than the open nose values. This suggests that metallic sensation has an important odor component. A recent work that investigated the metallic odor of skin and analyzed the carbonyls in the headspace of the skin after ferrous or cupric solutions were rubbed on it. A series of aldehydes and ketones were reported including n-hexanal and 1-octen-3-one (Glindemann et al., 2006).

### 1.4. Iron and copper chemistry

Iron may be present in ferric or ferrous forms in drinking water. Complexation and speciation of iron depends on the pH, dissolved oxygen, pE and minerals in the water. Due to anaerobic conditions ferrous, reduced form of iron is the dominant species in ground water, and has no color. Ferric, the oxidized form of iron is more commonly found in surface waters and has a rusty orange color (Nealson and Saffarini, 1994). Copper may also be found in cupric and cuprous forms in drinking water. As for iron, pH, dissolved oxygen, pE and minerals in water play an important role in speciation and complexation of copper. Cuprous is colorless whereas cupric, which is the dominant species in surface waters, has a blue green color.

The aim of this research was to determine taste thresholds for ferrous and cuprous in deionized water and compare the results to standards set by the drinking water industry. So far no work has studied taste thresholds for cuprous, hence this work will be the first to compare threshold value of cupric and cuprous. As the data from literature suggests, the threshold values are much lower than the set standards, hence drinking water industry may revise them based and may consider other control measures to prevent corrosion. The taste and odor components of the metallic sensation were investigated for ferrous, ferric, cuprous and cupric by occluding nose with nose-clips.

## 2. Materials and methods

### 2.1. Reagents

High purity ferrous sulfate, ferric sulfate, cuprous chloride, and cupric chloride were purchased from Fisher Scientific (Pittsburgh, PA). Deionized reagent water was obtained from the Nanopure® filter (NJ). Nose clips were purchased from Speedo™. The metal salt solutions were prepared fresh daily prior to testing. The concentrations of the metal salt solution were verified by either a flame atomic adsorption spectrometry (Perkin Elmer 5100) or an inductively coupled plasma/mass spectroscopy (Thermo X-Series). For the threshold studies the concentrations tested for ferrous sulfate were 0.002, 0.005, 0.01, 0.02, 0.05, 0.1 0.2 0.5 1, 2, 3, 4, and 5 mg/L; and the concentrations tested for cupric chloride were 0.025, 0.05, 0.075, 0.125, 0.2, 0.325, 0.5, 0.75, 1, 1.3, 2, 3, 4, and 5 mg/L. For the nose-clips studies ferrous sulfate, ferric sulfate, cuprous chloride, and cupric chloride were prepared at 20 mg/L concentrations. For both studies 30 mL of samples were presented at room temperature (~22 $^{o}$C) in 3 oz Solo cups coded with randomly selected 3-digit numbers. Water for rinse and cups for expectoration were also provided. For the taste threshold tests panelists were asked to sip and swish the samples around their mouth for 15 seconds and then to expectorate the sample. Then they waited for two minutes before tasting the next sample. For nose-clips testing the panelists were asked to sip and swish the samples around their mouth for 15 seconds and then to expectorate the samples. This procedure was repeated twice: with and without nose-clips and right after each tasting panelists were asked to report their taste and flavor perceptions.

### 2.2. Subjects

The research protocol for this work was approved by the Institutional Review Board at Virginia Tech. Healthy subjects were selected from the faculty, staff and students at Virginia Tech. All of the subjects read and signed the consent form prior to testing. The panelists were untrained and were not informed about the aims of the sudy. For ferrous threshold testing 27 panelists (14 females and 13 males) were tested with an age range of

18 to 60 years old. Another group of 27 panelists (12 females and 15 males) participated to cuprous threshold testing with an age range of 19 to 72 years old. For the nose-clips studies 25 panelists (12 males and 13 females) with an age range of 22 to 53 years old participated to ferric and ferrous studies. 17 (7 males and 10 females) and 21 (9 males and 12 females) panelists with and age range of 18 to 53 years old participated the cupric and cuprous tests. None of the subjects reported problems in taste or smell pereptions.

### 2.3. Taste thresholds for ferrous and cuprous

One-of-five forced choice test was selected for this research which is a modified version of triangle test. It is a stronger discriminative test as it has only 20% of chance of guessing correct. For each session panelists received 5 cups: four containing reagent water and one containing the metal salt solution (at around pH 6). The panelists were asked to taste the samples from left to right without going back, to take a 2 minute break in between samples in case of a delayed taste development, and to pick the odd (metallic tasting) sample and mark it on their scorecard. Order of the samples was randomly selected. Testing was complete for a subject when there correct answers were obtained following an incorrect answer.

Individual taste thresholds for the panelists were calculated as the geometric mean of the highest incorrectly picked concentration and the lowest correctly identified concentration. The population thresholds were determined by using the geometric mean method and logistic regression (as the threshold value detectable by 60% of the population determined by Abbot's formula).

### 2.4. Components of metallic sensation from iron and copper

The taste and odor perceptions were isolated by occluding the noses of the panelists by nose-clips. For each iron and copper ion solution panelists first placed the nose-clips on their noses and made sure no airflow through their noses were permitted. Then they tasted the solutions as described above, when they completed reporting their perceptions they removed the nose-clips and rated their perceptions again. Discussions

were not allowed between panelists during the testing. A scale from 0 to 12 was used to rate the taste, flavor and mouthfeel perceptions, "0" corresponding to "no perception" and "12" corresponding to "strong perception". A guideline for basic tastes (sweet, salty, sour, bitter, umami) and mouthfeels (astringent, tingling, drying) were supplied at the bottom of the scorecards.

## 3. Results and discussion

### 3.1. Taste thresholds for ferrous and cuprous

During the sensory sessions panelists described the taste of iron mostly as metallic and bloody, and copper as metallic and penny-like. However, these descriptors were also interchangeably used for both iron and copper.

Individual taste thresholds determined by the geometric mean method for ferrous ranged from 0.003 to >5 mg/L. The population thresholds determined by geometric mean method and logistic regression for ferrous were 0.052 mg/L and 0.031 respectively. Figure-7.1 below shows the taste threshold values for ferrous.

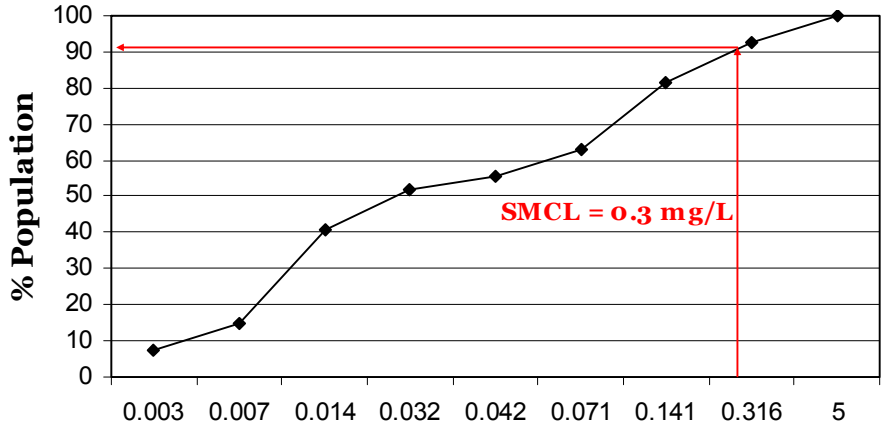


**Figure-7.1.** Ferrous Threshold Concentrations for 27 panelists (mg/L)

Individual taste thresholds determined by the geometric mean method for cuprous ranged from 0.035 to >5 mg/L. The population threshold determined by geometric mean method and logistic regression for cuprous was 0.61 mg/L for both methods. Figure-7.2 below shows the taste threshold values for cuprous.

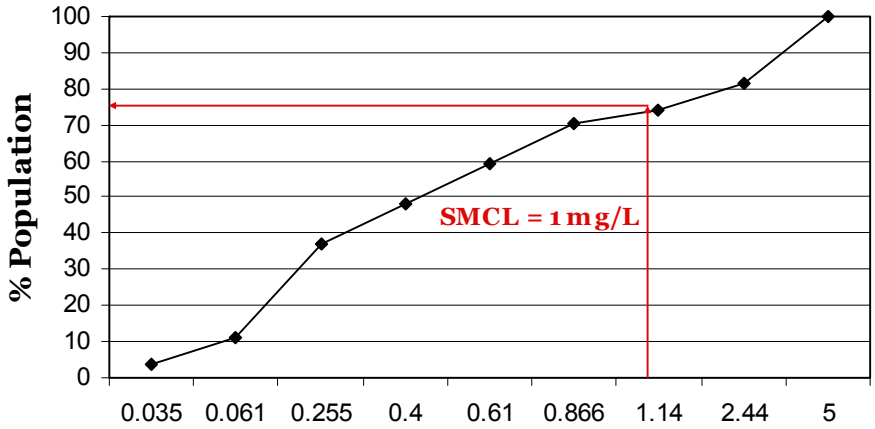


**Figure-7.2.** Cuprous Threshold Concentrations for 27 panelists (mg/L)

A recent study with cupric sulfate have also reported similar values threshold. The determined threshold values varied from 0.04 to >8 mg/L for thirty six panelists (15 males and 21 females) with an age range of 22 to 54 years old. The population threshold concentrations were determined as 0.48 and 1.50 mg/L by geometric mean method and logistic regression (Cuppett et al., 2006). Data from Cuppett et al. (2006), not shown, indicated that cupric is more detectable at lower concentrations up to 0.61 mg/L, then detection of cuprous and cupric parallel each other very closely. When population thresholds compared for geometric mean method cupric has slightly lower threshold concentration.

When the above figures are observed it was noted that about 90 % and more than 75 % of the panelists can detect ferrous and cuprous in water at or below the SMCLs set by the USEPA as 0.3 mg/L and 1.0 mg/L or iron and copper. This means that the standards may be revisited to prevent complaints and off-flavor problems. Although sensory studies conducted over a decade ago reported higher threshold values for iron and copper, recent studies using more powerful sensory tests and testing lower

concentrations of metal salts indicate that iron and copper may actually be detected at much lower concentrations.

### 3.2. Components of metallic sensation from iron and copper

The panelists did not report a metallic sensation for any of the iron or copper ion solutions when they tasted the samples with closed noses. Taste perceptions such as bitter, sour and salty were reported at very weak intensities as well as mouthfeel such as astringent and drying for ferrous, cuprous, and cupric. Ferric did not cause much taste or mouthfeel perception. Iron and copper are known to have different gustatory and tactile components such as astringent, drying, bitter, salty, and sour and the intensities and descriptors depend on the concentrations and anions (Hettinger et al., 1990; Lim and Lawless, 2005; Lawless et al., 2005; Lim and Lawless, 2006).

Upon removal of the nose-clips panelists reported a moderate-strong bloody/metallic flavor for ferrous, followed by a moderate penny-like/metallic flavor for cupric, and weak-moderate metallic flavor for cuprous. Panelists expected a bad taste when they noticed the yellow colored solution of ferric and were surprised to perceive no flavor. The flavor development took about 10 to 15 seconds for cuprous and cupric and got stronger in time however final ratings were always lower than intensity ratings ferrous. The results for ferrous, cuprous, and cupric are presented in Figures-7.3, 7.4, and 7.5.

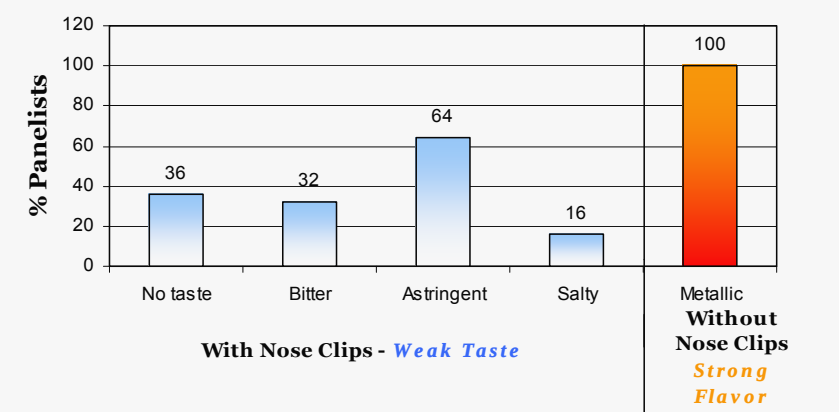


**Figure-7.3.** Taste and flavor perceptions reported for $FeSO_4$ at 20 mg/L (n=25)

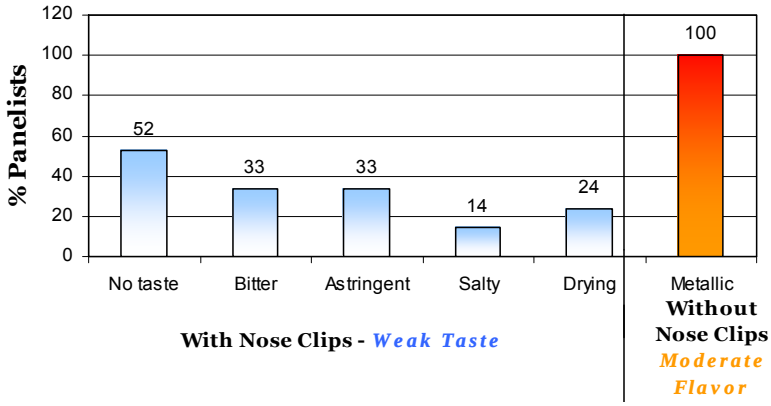


**Figure-7.4.** Taste and flavor perceptions reported for CuCl at 20 mg/L (n=21)

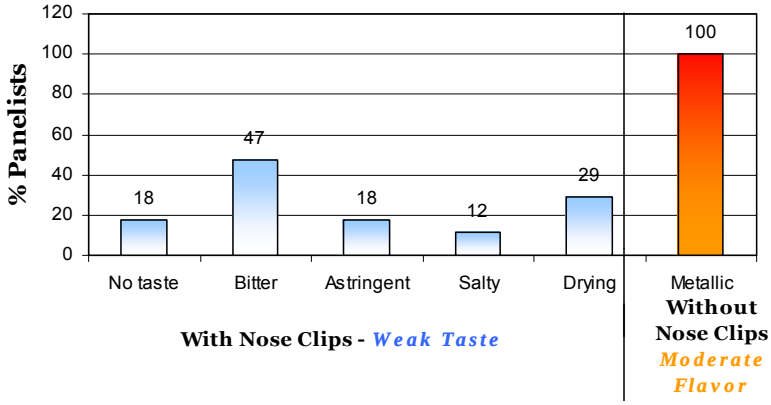


**Figure-7.5.** Taste and flavor perceptions reported for $CuCl_2$ at 20 mg/L (n=17)

These findings agree with the retronasal component of metallic flavor reported by Hettinger et al. (1990), Lawless et al. (2004), and Epke and Lawless (2007). It should be noted that in these studies panelists still reported metallic perception even when their noses were closed however the concentrations of the iron and copper were about 10 fold higher than the concentrations we tested. A recent article reported that at very high concentrations (30 fold higher concentrations than tested here) activate TRPV1 taste receptors and suggest that these receptors may be related to metallic taste perception (Riera et al, 2007). Nevertheless, it should again be noted that the concentrations tested by this study were at unrealistically high concentrations and the sulfates may have activated these receptors as well.

## 4. Conclusions

Recently determined threshold values in literature and our findings suggest that ferrous, cupric and cuprous may be detected at concentrations that are much below the USEPA guidelines. Sensitive people can taste these metals at low μg levels and hence may complain. Water utilities should consider this and develop a system to address this issue. Also the drinking water guidelines may be revised. Although treated water may comply with the standards corrosion of infrastructure may increase the iron and copper concentrations at tap. Attention should be given to this issue and corrosion control measures may be taken. It may also be noted that even though reagent water may not contain the cdonstituents of a typical tap water the conditions in the mouth (buffers and salts in saliva) override the complexation conditions hence control the speciation and result in similar results for taste and flavor perception.

As proven with the nose-clips studies metallic flavor perception has a significant odor component that is perceived retronasally. As mentioned above, carbonyls were detected over the skin that had a metallic odor. It was suggested that oxidation of the phospholipids in the cell membranes produces these carbonyls that cause the metallic odor and hence the metallic flavor. Hence it should be noted that rather than taste receptors, carbonyls produced as a result of lipid oxidation may be the cause of the metallic flavor.

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# Chapter VIII

## Metallic Flavor Development in the Oral Cavity with Iron and Copper Ions and the Effects of Antioxidants and Chelating Agents


### Abstract

Metallic flavor is caused by iron and copper ions in water or food and results in consumer complaints and dissatisfaction. Metallic flavor has been an issue for cancer patients undergoing chemotherapy and radiotherapy as well, affecting their nutrition and quality of life. Although metallic flavor has been a concern, biochemical mechanisms that produce metallic flavor have not been studied until now. This work focused on determining the mechanisms that cause metallic flavor. Saliva samples were collected from healthy subjects before and after ingestion of iron and copper ion solutions. There was a significant increase in lipid oxidation in the mouth ($p<0.001$), detected as TBARS values, when metal ions were ingested. Ferrous ion caused the strongest flavor sensation and caused the greatest increase in lipid oxidation (0.22 μM) followed by cupric (0.12 μM), and cuprous (0.06 μM) ions. Although ferric ion did not cause any metallic sensation it caused a slight increase in lipid oxidation (0.02 μM). No correlation could be determined between lipid oxidation caused by ferrous ion and the total salivary antioxidant capacity, as there was no increasing or decreasing trend in TBARS values as the antioxidant capacity increased or decreased. Occurrence of oxidative stress was supported by damage to proteins as detected by protein-carbonyls by Western Blotting. There was a significant (two to four fold) increase in C1 to C3 aldehydes in saliva as well. Effect of antioxidants (Vitamin E and C) and chelating agents (EDTA and lactoferrin) on reducing or preventing the oxidative stress and hence the lipid oxidation in mouth was evaluated by sensory methods. Chelating agents removed the metallic flavor when ingested after metals, whereas antioxidants only slightly reduced the perception.



***Keywords:*** *metallic flavor, lipid oxidation, TBARS, SDS-PAGE, antioxidants, chelating agents*

## 1. Introduction

Iron and copper may be present in source water as it filters through soil and rocks also corrosion of metal pipes may introduce these metals into drinking water. Iron and copper are essential micronutrients and required for proper enzyme functioning, healthy growth, and metabolism. Deficiencies may cause anemia, impaired mental development, and impaired immunity. Recommended daily intake values for iron and copper were established as 15 and 2 mg respectively as a general value, however actual requirements are specific to individuals and vary by age, gender and absorption efficiency (WHO, 1996). It has been reported that most tap waters in the US may supply about 5% of daily requirements of iron and copper (WHO, 1996). If consumed at higher concentrations (i.e. 2 L of water at 3 mg/L) iron and copper may cause adverse health effect such as nausea, vomiting, and diarrhea. In extreme cases kidney and liver damage may occur (WHO, 1996). To prevent health and aesthetical problems EPA set secondary maximum contaminant levels as <1 mg/L for copper, and <0.3 mg/L for iron.

Ingested iron and copper can cause an annoying metallic sensation in drinking water. This results in consumer complaints, loss of trust in cleanliness and safety of water and in return may caused reduced consumption of tap water (Dietrich, 2006; Suffet et al., 1996). About 2 million cancer patients receiving chemotherapy and radiotherapy encounter metallic flavor perception after consuming foods and beverages. More than 40 % of hospitalized cancer patients suffer from malnutrition due to taste and smell dysfunction (Comeau et al., 2001; Nitenberg and Raynard, 2000). Sjogren's disease patients are known also to complain about metallic off-flavors (Soto-Rojas et al., 1998). Unfortunately, these problems are underestimated and understudied by doctors and oncologists. Suggested treatments are naïve and only provide temporary solutions.

Unlike the medical industry, food and drinking water industries have been studying metallic taste of food and drinking water and taste thresholds of iron, copper, and zinc. Initially, production of an electrical potential on the tongue by metals was identified as a metallic taste (Plattig, 1988). In a later study, Lawless et al. (2003) reported that calcium and magnesium have bitter, metallic, salty, and astringent tastes. Reported

individual taste thresholds vary from 0.1 to above 10 mg/L for cupric ($Cu^{2+}$) ion and from 0.04 to 3 mg/L for ferrous ($Fe^{+2}$) ion (Cuppett et al., 2006; Tucker et al., 2007; Cohen et al., 1960).

One recent study reported that TRPV1 taste receptors are activated at very high concentrations (~550 mg/L) of ferrous and cupric ions (Riera et al., 2007). This may indicate that rather than just bitter and salty tastes, there may actually be a metallic taste perception. However, the concentrations of the metals tested in this study were unrealistically high and the activation of the receptors may be caused by the anions. Olfactory (retronasal) perception related to metallic taste was documented first by Hettinger et al. (1990) indicating a decrease in taste of ferrous sulfate when the nose was occluded. Another study later confirmed this finding by testing copper and iron with and without nose-clips (Lawless et al., 2004). Recently Epke and Lawless (2007) determined taste thresholds of iron and copper by occluding noses of the panelists. The results indicated that the taste thresholds determined with noses occluded were 10 to 30 times higher than the normal taste thresholds. These findings mean that metallic sensation has a significant odor component contributing to the full metallic perception. Metallic odor on skin was investigated by analyzing the headspace of skin with SPME-GC/MS after rubbing it with iron and copper salt solutions. Several carbonyl peaks were identified including hexanal and 1-octen-3-one (Glindemann et al., 2006). These carbonyls are lipid oxidation by-products and are very common in foods that are high in unsaturated fats.

Although there is little information on oxidation of human tissues with metals, the food literature has been studying 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, 1-penten-3-one and trans,cis-2,6-nonadienal were detected in foods and beverages (Guth and Grosch, 1990; Hinterholzer and Schieberle, 1998; Hinterholzer et al., 1998; Buettner and Schieberle, 1999). Hexanal is a common lipid oxidation by-product (Mielnik et al., 2006).

The hypothesis of our study states that metallic sensation in the mouth has two components: taste of metal salts on the tongue, and the retronasal perception of the carbonyls produced as a result of lipid oxidation catalyzed by metals in the mouth. The phospholipids in the cell membranes may be oxidized by metals in the oral cavity. Unsaturated fatty acids (that form the phospholipids) are very prone to oxidation as free radicals attack the double bonds and abstract a hydrogen atom. Formation of lipid hydroperoxides leads the way to formation of carbonyls including malondialdehyde (MDA) (Kochhar, 1996). One of the most commonly used methods to detect lipid oxidation is measurement of malondialdehyde (MDA) with thiobarbituric acid reactive substances (TBARS) method. MDA, which is a by-product of lipid oxidation, complexes with thiobarbituric acid and forms a compound that can be quantified colorimetrically (Armstrong and Browne, 1994; Yagi, 1998). A common method employed to capture the carbonyls in complex matrices (including air and water) is derivatization of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) (Dong and Moldoveanu, 2004). This method has been widely used due to its high reactivity and selectivity. During derivatization, the carbonyl loses its oxygen and forms a stable complex with DNPH (van Leeuven et al., 2004). Then the derivatized compounds may be separated and detected by GC/MS or HPLC/MS. Maboudou et al. (2002) employed DNPH derivatization coupled to GC/MS to study the oxidation in rat brains and measure MDA and formaldehyde and concluded this method could be used to monitor oxidative stress (and hence lipid oxidation).

In the literature oxidation of lipids is indicated as the main reason for off-flavor generation and hence studied and monitored in depth; however oxidation of proteins are very important as well for monitoring disease progress for patients, and texture and taste quality in food. The main pathway of oxidative damage to proteins is the attack of metal catalyzed radicals on amino acids. Metal ions selectively bind to proteins and as they still may remain redox active, they can generate hydroxyl radicals at the site of binding. Then the formed radical reacts in the vicinity of the bound metal and induce site-selective damage. Lipid oxidation also may lead to site-specific damage in proteins. The by-products of lipid oxidation can bind to proteins and inactivate them (Davies and Dean, 1997). One of the methods to detect protein oxidation is measuring protein carbonyls. Derivatization of the protein carbonyls with DNPH makes it possible to easily

detect and quantify the oxidative modification of proteins. The hydrazone derivatives may be detected by spectroscopy or Western Blotting (Castegna et al., 2003). Another method to detect protein carbonyls employs the alcohol dehydrogenase enzyme that reduces the carbonyls. The carbonyls then are quantified by spectroscopy (Kingu and Wei, 1997). Western Blotting is a widely used method in biochemistry, genetics, molecular biology, and forensics. Sodium dodecyl sulfate (a detergent) denatures the proteins and gives them a negative charge. Polyacrylamide gel separates the proteins based on molecular weight and charge. The proteins then are transferred to a polyvinylidene fluoride membrane and blocked. Specific proteins are marked by primary and secondary antibodies and the marked proteins on the membrane may be determined by colorimetric, chemiluminescent, or fluorescent detection. Originally these procedures were developed by Laemli (1970) and Towbin et al. (1978) and have been modified and extensively used since for many biological samples.

Human saliva has many constituents including water, electrolytes, glucose, ammonia, urea, enzymes, ammonia, lipids and proteins. Hence saliva may also contribute to the metallic flavor formation due to oxidation or affect the reactions by its antioxidant enzyme system. Total proteins in saliva, measured by the Bradford assay, are in the range of 0.8 to 2.7 g/L (Bonilla, 1972). Free fatty acids, cholesterol, glycolipids, and phospholipids were detected in saliva at about 15 mg/L, 5 mg/L, 12 mg/L, and 8 mg/L respectively (Tomita et al., 2008; Larsson et al., 1996). Total antioxidant capacity of saliva may be measured by several methods that employ spectrophotometric, chemiluminescence, or cyclic voltammetry assays. (Ferreiro et al., 2002). Human saliva typically has an antioxidant capacity of 0.3 to 1 mM (Miller et al., 1997).

Lipid oxidation and damage to proteins may be prevented or reduced by the administration of antioxidants and chelating agents to the oral cavity. Food and medical industries extensively use such compounds to prevent diseases caused by oxidative stress, and preserve flavor of food. Vitamins E and C, and α-lipoic acid are important cofactors of enzymatic systems in the body and are good radical scavengers. Vitamins C and E, as well as EDTA, BHT, and BHA are among the food additives. Vitamin C (L-

ascorbic acid) is water soluble and may be destroyed by heat and light. Vitamin E (α-tocopherol), is fat soluble, and is susceptible to oxidation by oxygen in the air (Denisov et al., 2005). Vitamin E reduces lipid peroxide radicals by a nonenzymatic reaction (Gille and Joenje, 1991). α-Lipoic acid is both fat and water soluble and hence has the ability to scavenge radicals in fatty and watery matrices (Challem, 1996). While antioxidants scavenge radicals by donating hydrogen, chelating agents inactivate the redox active metals before they create free radicals (Nortemann, 2005). EDTA chelates with iron and copper. Another chelating agent that binds to iron is lactoferrin. Lactoferrin is a protein and mainly found in milk and other secretions by humans such as tears ad saliva (Uchida et al., 2006). In the body, metals are bound to specific chelating proteins that reduce their redox potential. Oral administration of these antioxidants and chelating agents may stop or reduce metallic off-flavor formation.

The goals of this study were to: 1) investigate the biochemical reactions that cause the metallic flavor sensation and 2) determine a way to prevent it. To achieve these objectives lipid oxidation catalyzed by iron and copper ions in the oral cavity were investigated as this is the main cause of off-flavor production. The occurrence of oxidative stress in the oral cavity was supported by the analysis of protein-carbonyls as well. For the prevention of oxidation, antioxidants and chelating agents were evaluated.

## 2. Materials and methods

### 2.1. Subjects

This study 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 and who were non-smokers. All subjects read and signed the consent form before the sample collection began. Twenty-two panelists with an age range of 19 to 53 participated in various portions of the study.

## 2.2. Saliva collection and sensory testing

Panelists were instructed to refrain from eating and drinking at least 30 minutes prior to testing. The saliva collection sessions were scheduled twice a week in the morning between 10 and 11:30 am to prevent bias from diurnal variations. Before every saliva collection, panelists rinsed their mouth with reagent (deionized) water and waited for 2 minutes to have standrd conditions in the mouth. Whole saliva was collected as explained below. Subjects were presented with 3 oz Solo® cups containing 2 mL of control (reeagent water) and metal solutions (solutions of iron and copper ions in reduced or oxidized states) each. High purity ferrous sulfate, ferric sulfate, cuprous chloride, and cupric chloride were purchased from Fisher Scientific (Pittsburgh, PA) and metal salt solutions were prepared in reagent water (from the Nanopure® filter) fresh daily prior to testing. subjects were asked to sip the entire (2 mL) control solution, swish it around their mouth for 15 seconds and start expectorating until 4 mL of saliva was collected in a 15 mL polypropylene centrifuge tube. After resting for a few minutes, subjects were asked to sip the metal solution, and again to swish it around their mouth for 15 seconds and start expectorating until 4 mL of saliva was collected. Saliva samples were either analyzed or frozen immediately to -18 $^{o}$C or to -80 $^{o}$C for storage until the subsequent analysis could be performed. For the antioxidant or chelating agent studies the subjects were asked to rinse their mouth with 3 mL of the selected antioxidant or chelating agent solutions for 10 seconds and to either expectorate or swallow them. This rinse step was completed either before or after sampling the metal sample.

## 2.3. Detecting lipid oxidation – Thiobarbituric acid reactive substances

For the first portion of this study ferrous iron solution was used at 180 μM concentration. Saliva samples were collected from 19 panelists (with an age range of 24 to 53, 8 females and 11 males) three to five different times as described above and either analyzed immediately or frozen to -18 $^{o}$C. Later ferric, cuprous, and cupric ion solutions were tested at 180 μM with 13 panelists (with an age range of 24 to 53, 6 females and 7 males). Lipid oxidation was measured by analyzing saliva samples (control and metal) with the thiobarbituric acid reactive substances (TBARS) method adapted from Spanier

and Traylor (1991). 2-Thiobarbituric acid, sodium dodecyl sulfate, glacial acetic acid (99% pure), 1-butanol (CAS# 71-36-3), pyridine (CAS# 110-86-1), 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 polypropylene tubes in a water bath (10-L, Fisher Scientific, Pittsburgh, PA) at 95 $^{o}$C for 60 minutes. Samples were immediately cooled down in an ice bath and pink colored complex formed from the reaction was extracted with 5 mL of n-butanol/pyridine solution at a 15:1 ratio. Extracts were separated by centrifuging (Model PR-2, International Portable Refrigerated Centrifuge, International Equipment Company, Boston, MA) the samples at 3000 rpm for 15 minutes at room temperature. The absorbance of the supernatant was measured with a spectrophotometer (Model 21D Spectronic, Milton Roy Co., Rochester, NY) at 532 nm. The standard curve was obtained by running 1,1,3,3-tetramethoxypropane standards at 0.025 to 0.5 μM concentrations. 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 real concentrations of MDA in the samples.

### 2.4. Total protein concentration in saliva

Bradford assay (Bradford, 1976) was used to determine the total protein content of the saliva samples collected as described above for the control (reagent water) and metal (180 μM ferrous iron) solutions. Bradford reagent and bovine serum albumin were purchased from Sigma Aldrich (St. Louis, MO). Glass tubes were prepared and filled with 1 mL of the Bradford reagent. The standard curve was prepared with bovine serum albumin at 1 mg/mL concentration and the samples were prepared by adding 10 μL of the collected control or metal saliva. Three different samples were analyzed for each panelist and each sample was duplicated. The tubes were mixed by vortxing for a few seconds and 200 μL of the mixtures were transferred to the 96-well plate and the absorbance was read by a plate reader at 599 nm.

## 2.5. Total salivary antioxidant capacity

Total salivary antioxidant capacity was measured in the saliva of subjects with the Antioxidant Assay Kit (709001) purchased from Cayman Chemical Company (Ann Arbor, MI). All the necessary reagents were supplied by the kit. This assay relies on the ability of the antioxidants in saliva to prevent the oxidation of ABTS® (2,2'-azino-di-[3-ethylbenzhiazoline sulphonate]) to $ABTS^{®\cdot+}$ by metmyoglobin. The amount of oxidized $ABTS^{®\cdot+}$ was measured by reading the absorbance of blue-green end product formed by the reaction.

The whole saliva collected without any stimulation was either analyzed or stored frozen at -80 $^{o}$C. The frozen samples were thawed in cold water bath. The saliva samples were diluted by 1:3 with the 10X Assay Buffer just before the analysis. Trolox standards were prepared as indicated in the kit's manual to be used for the standard curve. A 96 well plate was used for the analysis. For the standard curve: 10 µL of each Trolox standard, 10 µL of metmyoglobin, and 150 µL of chromogen were added to the designated wells. For the samples: 10 µL sample (diluted saliva), 10 µL of metmyoglobin, and 150 µL of chromogen were added to the designated wells in duplicates. Reactions were initiated by addition of 40 µL of hydrogen peroxide working solution to all of the wells within a short time. The plate was covered and incubated on a shaker for 5 minutes at room temperature. Then the cover was removed and the absorbance was read by a plate reader at 750 nm.

## 2.6. Detecting protein-carbonyls – Western Blotting

Saliva was collected from 8 subjects with an age range of 24 to 53 (4 females, 4 males) both after rinsing with control and metal (ferrous ion at 18 µM) solutions as described above. 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. (2000). The OxyBlot™ protein oxidation detection kit (S7150)

was used to derivatize the protein carbonyls. Recipes for all of the buffers were given in the manual of the kit. Briefly; 6 µL of saliva sample (containing 24 µg of protein) were denatured with 6 µL of 12% SDS solution in a 0.5 mL Eppendorf tube. The mixture was derivatized with 12 µL of 1X DNPH solution for 15 minutes at room temperature. The derivatization was completed by adding 9 µL of neutralization solution. For the electrophoresis portion, the method developed by Laemli (1970) was followed with some modifications. Briefly; 4 µL of 10X NuPAGE reducing agent and then 3 µL of 4X NuPAGE LDS sample buffer were added to the derivatized samples to a total of 40 µL. The prepared control and metal samples were loaded to the wells of the NuPAGE Novex 10% Bis-Tris gel. The inner and outer chambers were filled with 1X NuPAGE SDS running buffer. Immediately prior to electrophoresis 500 µL of NuPAGE antioxidant was added to the inner buffer chamber. The gel was run at 200 V for 35 minutes (with <0.2 A) to separate the proteins. Then proteins were electroblotted to a polyvinylidene fluoride (PVDF) membrane. Briefly; blotting pads were soaked until saturated in 1X NuPAGE Tris-Glycine transfer buffer. The transfer membrane and filter papers also were prepared by soaking in methanol, distilled water and the transfer buffer sequentially. The gel was removed from the cassette and placed on the filter paper. The transfer membrane then was placed on the gel. Another filter paper was placed on the membrane and air bubbles were removed. The sandwiched gel and membrane were placed between two layers of blotting pads. The assembly was placed in the blotting chamber. The inner chamber was filled with the transfer buffer and the outer chamber was filled with distilled water. Electroblotting was completed in 16 hours at 25 V (with < 0.13 A). The membrane was then removed onto a suitable plastic container and blocked in 20 mL of 1% BSA/TBS-T blocking/dilution buffer for 1 hour with gentle shaking. The primary antibody incubation was performed with 15 mL of primary antibody stock (rabbit anti-DNP antibody) diluted by 1:150 with 1% BSA/TBS-T for 1 hour at room temperature with gentle shaking. After the incubation was complete the membrane was rinsed with 20 mL of TBS-T dilution buffer for each rinse: two times for 1 minute, once for 15 minutes, and twice for 5 minutes at room temperature with gentle shaking. For the secondary antibody incubation the secondary antibody stock (goat anti-rabbit IgG, HRP conjugated antibody) was diluted by 1:300 with 1% BSA/TBS-T. The membrane was incubated with 15 mL of the antibody solution for 1 hour at room temperature with

gentle shaking. After the incubation was complete the membrane was rinsed with 20 mL of TBS-T dilution buffer for each rinse: two times for 1 minute, once for 15 minutes, and twice for 5 minutes at room temperature with gentle shaking. Then the membrane was drained and placed on a plastic sheet with the protein side up. The chemiluminescent reagent (Amersham ECL Plus Western Blotting Detection System) was prepared according to the instructions. The membrane was fully covered with 2 mL of the chemiluminescent reagent and was covered with an aluminum foil (not touching the surface) and incubated for 5 minutes at room temperature. The membrane was placed on a new plastic sheet after removing the excess reagent, covered with a plastic wrap and the air bubbles were removed. The membrane was exposed for 30 seconds and imaged by Fujiilm LAS-3000 Imaging System.

### 2.7. Detecting carbonyls – Derivatization with 2,4-dinitrophenyl hydrazine

Carbonyls possibly responsible for the metallic flavor were analyzed by derivatization with 2,4-dinitrophenyl hydrazine (DNPH). Saliva was collected from 10 panelists as described above for control and metal (ferrous ion at 180 μM) and pooled to have 10 mL. Solid high purity 2,4-dinitrophenylhydrazine and its hydrazone derivatives of formaldehyde, acetaldehyde, propionanaldehyde, high purity acetonitrile, pentane, and 1 M hydrochloric acid 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 hydrochloric acid (HCl). The pooled saliva for control and metal samples were immediately reacted with 2 mL of the DNPH solution. The mixtures were reacted for 1 hour at room temperature in a polypropylene centrifuge tube with intermittent shaking and mixing by vortex. Mixtures were extracted with 10 mL of pentane for 30 minutes, by shaking and mixing by vortex intermittently. The pentane was separated by centrifuging the samples for 45 minutes at 3,000 rpm. The extracts were fully evaporated in the hood under a nitrogen stream on a hotplate at 60 °C. Then the residues were dissolved in 300 μL acetonitrile and 2 μL of each solution was injected to GC/MS as control and metal samples. To identify the carbonyls, a standard curve was obtained with hydrazones of formaldehyde, acetaldehyde and propionanaldehyde as well. GC/MS was performed with a DB-17 MS, 15m x 0.25mm i.d.

capillary column with 0.25um 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 ramp 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.

### 2.8. Sensory testing with antioxidants and chelating agents

As antioxidants and chelating agents have been used by the food and medical industry to prevent oxidation in the food products and body, several antioxidants and chelating agents were evaluated to prevent metallic flavor formation. α-tocopherol (vitamin E) and L-ascorbic acid (vitamin C) were purchased from Sigma Aldrich (St. Louis, MO). Ethylenediaminetetraacetic 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: first the panelists were presented with 3 mL of the antioxidant or the chelating agent, asked to sip whole solution and swish it around their mouth for 10 seconds, then to swallow or expectorate it, and immediately sip all of the 2 mL of the ferrous solution, swish it around their mouth for 10 seconds, swallow or expectorate it, and record their taste and flavor perceptions with intensity ratings on the scorecards provided with a guide for basic tastes and the intensity scale. The other version was the opposite of this procedure. The panelists were presented with 2 mL of ferrous solution, they were asked to sip the whole solution and swish it around their mouth for 10 seconds, then to swallow or expectorate it, and immediately sip all of 3 mL of the antioxidant or chelating agent solution, swish it around their mouth for 10 seconds and record their perceptions with intensity ratings on the scorecards provided with a guide for basic tastes and the intensity scale. For this second version, 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 $^{o}$C) in 3 oz Solo® cups randomly coded by 3-digit numbers. The

panelists were trained to use an intensity scale from 0 to 12; “0” corresponding to “no perception” and “12” corresponding to “strong perception”. The panelists were naïve to the aim of the study, however, they were familiar with the metallic flavor sensation as they have participated to the previous portions of this sudy. An odor free room was selected for testing and the panelists tasted the samples sitting around a table. No discussions or interactions were allowed between panelists. The panel profiles for each trial were as follows: Water – 11 panelists, 19 to 37 years old, 5 males, 6 females; Vitamin E – 10 panelists, 19 to 53 years old, 5 males, 5 females; Vitamin C – 11 panelists, 19 to 37 years old, 5 males, 6 females; EDTA – 11 panelists, 19 to 37 years old, 5 males, 6 females; Lactoferrin – 19 panelists, 19 to 53 years old, 9 males, 10 females.

### 2.9. Statistical analysis

Statistical analyses were performed for all the data collected. Depending on the experimental design either a t-test or one-way ANOVA was selected and were performed using the SAS software (version 8.1, SAS Institute, Cary, NC). 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 statistical analyses performed and the results are discussed for each portion of the study below.

## 3. Results and discussion

### 3.1. Lipid oxidation – Malondialdehyde (MDA) production by ferrous

Lipid oxidation in the oral cavity was determined by measuring the concentration of MDA (a common lipid oxidation by-product) in control and ferrous iron containing saliva samples (Figure-8.1). A paired t-test analysis was performed to compare the log transformed TBARS results for the control and metal saliva samples to obtain normality and homogeneity. In the literature TBARS data is usually log transformed for data analysis (Oake and van der Kraak, 2003; Yousefzahdeh et al., 2006). There was a significant increase in MDA concentration in saliva after rinsing the mouth with the

ferrous iron solution ($p<0.001$). Also a power analysis indicated that results from 19 subjects have enough power (>95 %) to project the results to te general population. It may be noted that the concentration of MDA in control and metal treated saliva samples varied among panelists and was investigated further as described below by measuring the total antioxidants in saliva. Studies that focused on oral oxidative stress due to smoking have reported MDA concentrations similar to these findings for the control saliva samples. The average MDA concentration for the control samples was 0.12 µM which is comparable to the values reported as 0.055 to 0.08 µM in the saliva of healthy controls by other researchers (Hodosy and Celec, 2005). The reason for presence of MDA in control saliva is not known (Meucci et al., 1998) but it may be explained by local intraoral production (Nagler et al., 2001) or oral microbial flora causing oxidative stress (Marton et al., 1993). It should be noted that systemic oxidative stress does not alter salivary TBARS values as the serum and salivary TBARS values were compared and could not be correlated (Ponte et al., 2001; Celec et al., 2005; Moore et al., 1994; Qurino et al., 1995).

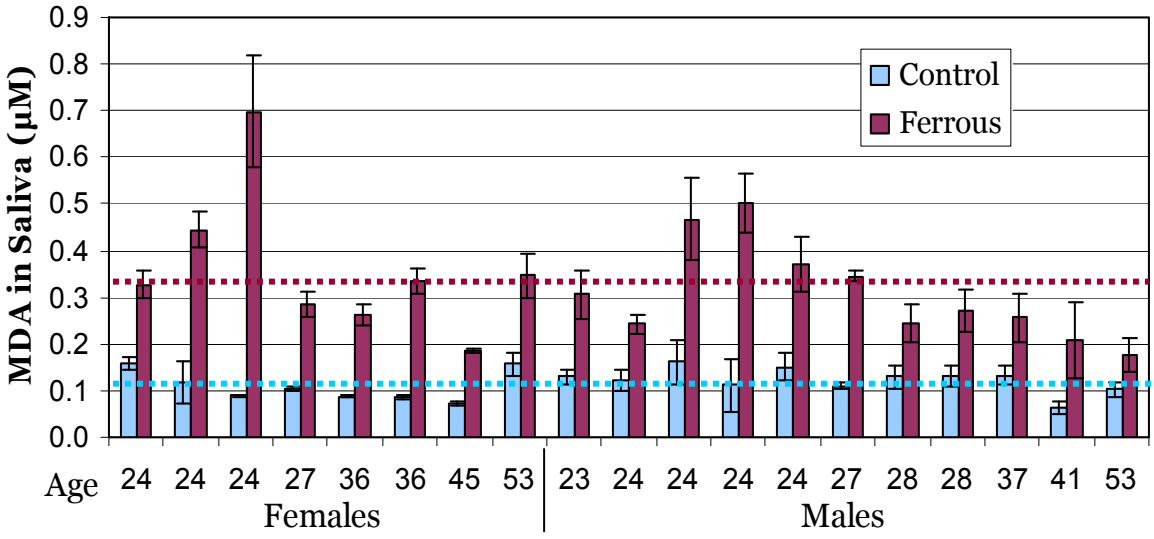



**Figure-8.1.** Measurement of lipid oxidation reported as MDA (µM) in control (reagent water) and metal (ferrous iron) treated saliva samples (mean ± std dev) collected from healthy panelists (average concentrations ± std dev for control (0.12 ± 0.03 µM) and metal (0.33 ± 0.12 µM) are shown in dotted lines)

### 3.2. Total protein concentration of saliva

The total protein concentration of saliva samples collected from 19 panelists were measured by the Bradford assay and are presented in Figure-8.2.

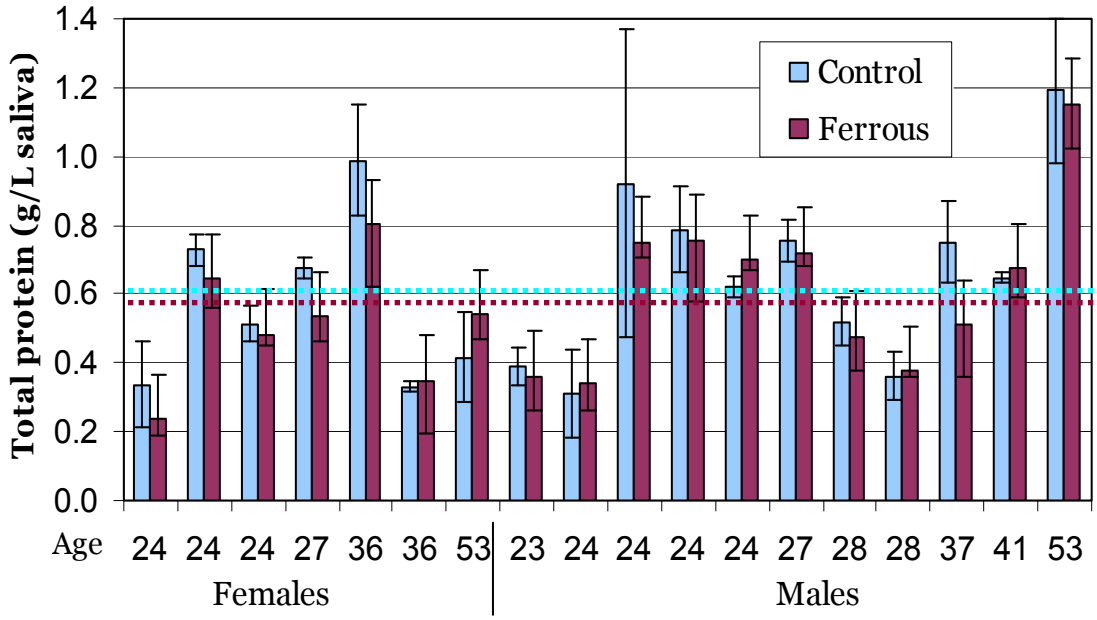



**Figure-8.2.** Total protein concentration (g/L) in control (reagent water) and metal (ferrous iron) treated saliva samples (mean ± std dev) collected from healthy panelists (average concentrations ± std dev for control (0.62 ± 0.25 µM) and metal (0.58 ± 0.22 µM) are shown in dotted lines)

A paired t-test was used to analyze the total protein results. There was no significant difference (p=0.053) between the control and the metal saliva samples. The average protein concentrations for the group were calculated as 0.62 g/L (±0.25) and 0.58 g/L (±0.22) for the control and metal saliva samples respectively. The measured protein concentrations were used to incorporate more discriminative factors to the TBARS results. The increase in MDA production was reported per L of saliva and per gram of protein and the effect of age and gender on MDA production was investigated (figures-8.3 and 8.4).

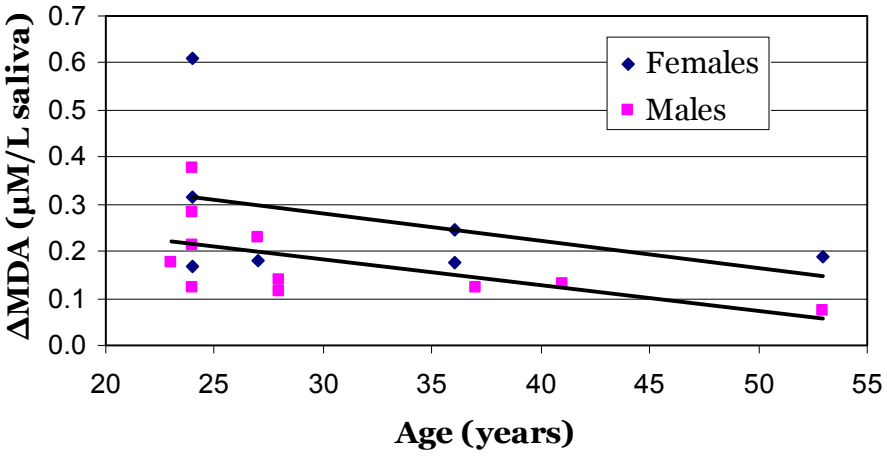



**Figure-8.3.** Increase in MDA concentrations in saliva per L of saliva after ingestion of ferrous solution across gender and age for 19 human subjects

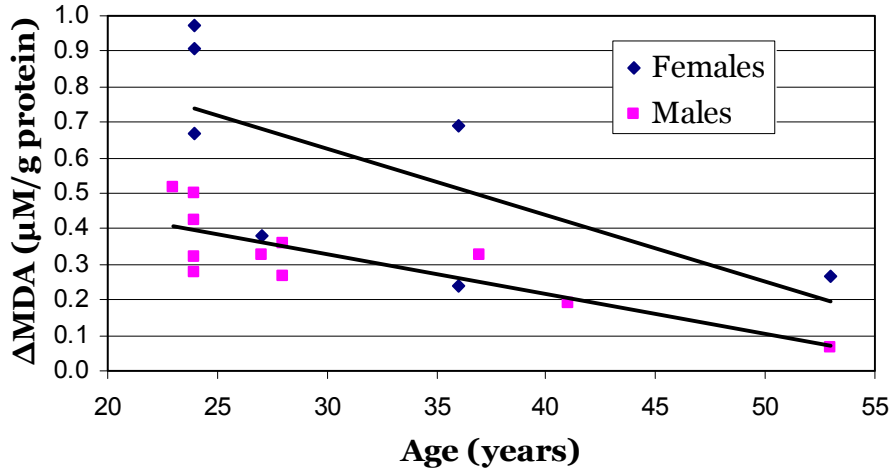



**Figure-8.4.** Increase in MDA concentrations in saliva per g of protein after ingestion of ferrous solution across gender and age for 19 human subjects

One-way ANOVA with Tukey and Scheffe analyses with the log transformed data indicated that there was a significant difference between the increase in MDA concentration for younger (<36) versus older (>36) panelists when the mouth was rinsed with ferrous iron solution for both per L saliva (p= 0.03) and per g protein (p=0.037). However no significant difference was observed between genders when normalized data were compared for increase in MDA per L saliva (p= 0.10) and per g protein (p=0.054). This shows that younger panelists are more prone to lipid oxidation

in the mouth however gender does not affect the extent of oxidation caused by the metals in mouth. In contrast Celec et al. (2005) reported that older panelists produced higher MDA concentrations. In that work a much larger (217 stomatologic patients) subject pool was tested. This may explain the differences in the results. In this study the oldest panelist was 53 years old and only a few "older" subjects were tested. In order to further investigate this finding, total antioxidant capacities of the saliva of the subjects were measured.

### 3.3. Total salivary antioxidant capacity

The differences in MDA production among panelists were further investigated by the measurement of the total antioxidant capacity of the whole unstimulated saliva and correlating it to the increase in MDA in saliva after ingesting ferrous iron solution. The results are shown in Figure-8.5.

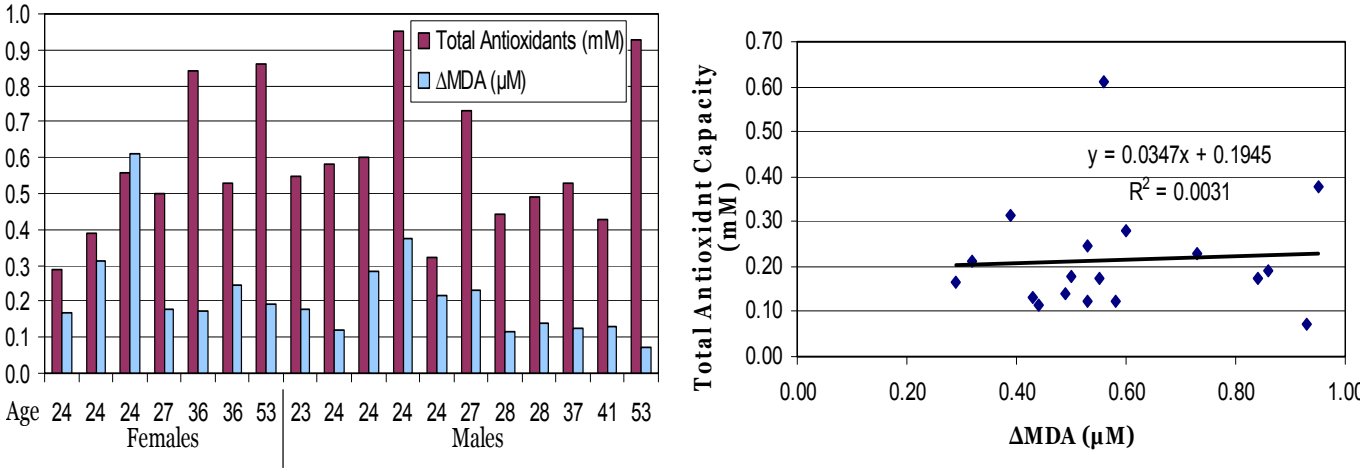



**Figure-8.5.** Correlation of total antioxidant capacity of saliva (mM) and increase in MDA production (metal (ferrous ion) - control) for healthy adult panelists

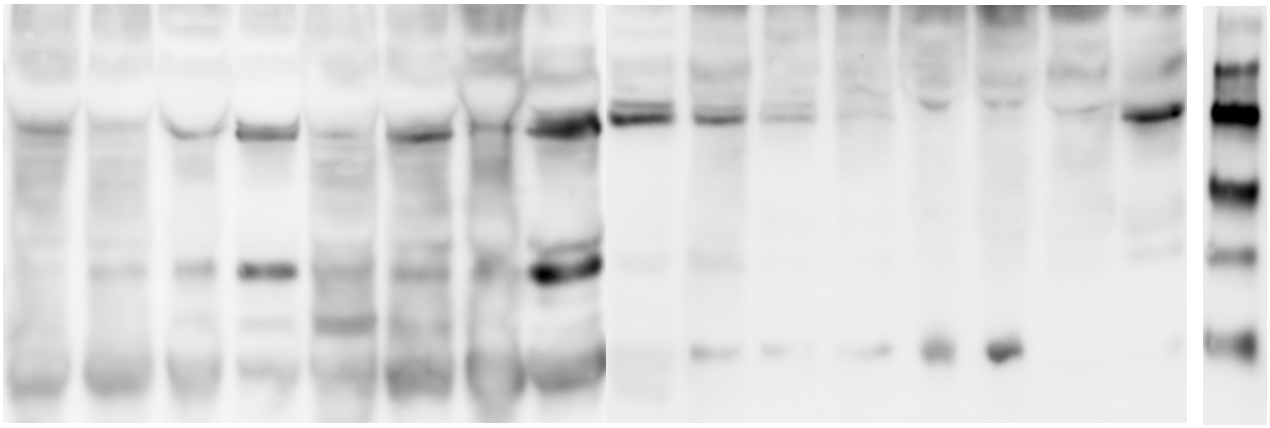
As can be seen from the results shown above, the salivary antioxidant capacity varied among panelists without following a trend for age ($p=0.13$) or gender ($p=0.78$) determined by one-way ANOVA. A comprehensive study by Hershkovich et al. (2007) however reported that the total antioxidant levels in elderly (age 70 to 80) were significantly lower than the younger group (age 20 to 25). Our study examined only a

small group of subjects and hence may require more work to conclude whether there is a gender or age effect on the total salivary antioxidant levels. Sculley and Langley-Evans (2002) investigated the relationship between antioxidant supplements and salivary and serum antioxidant capacity levels and reported that, although serum antioxidant capacity increased with antioxidant supplementation salivary antioxidant capacity was not affected.

Although some panelists with high antioxidant capacity produced less MDA compared to the others, there were some panelists who also had less MDA production even though their antioxidant capacity was determined as low. Hence no correlation could be obtained for antioxidant capacity of saliva and MDA production. However it should be noted that the ferrous iron solution was prepared at a very high concentration and the effectiveness of the antioxidants may be superseded. A recent study by Nagler (2007) reported that even though oxidative stress in the oral cavity was high, hence the oxidation of lipids and proteins was high for smokers, salivary antioxidant measures were also high. This also may indicate that salivary antioxidants are not very effective in preventing oxidation in the mouth.

### 3.4. Oxidation or modification of the proteins –Protein-carbonyls

Oxidation or modification of proteins was measured as the protein-carbonyls in saliva. When proteins undergo oxidation or are exposed to carbonyls or hydroperoxides formed as a result of lipid oxidation, amino groups and the carbonyls react. The proteins then become inactive and may be detected by DNPH derivatization. The protein-carbonyls in the control and metal (ferrous ion) treated saliva samples were measured by derivatization and Western Blotting and are presented in Figure-8.6.



**1c 1 2c 2 3c 3 4c 4 5c 5 6c 6 7c 7 8c 8 S**

**Figure-8.6.** The protein-carbonyl detection by SDS-PAGE and chemiluminescence in saliva of 8 healthy subjects before and after rinsing mouth with ferrous solution (at 18 μM) (where "c" labeled lanes represent controls, just numbered lanes represent metal samples, 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.)

As may be observed from the SDS-PAGE results above although the size and the concentration of the proteins carbonylated varied among the panelists, and increase in protein-carbonyls was observed. The lanes 1 and 2 and to 5 and 6 belong to female subjects, and 3 and 4 and 7 and 8 belong to male subjects. It may be noted that by qualitative observation males have higher concentrations of protein-carbonyls than females. However, more panelists may be tested to conclude the effect of age and gender on carbonyl formation. Several studies focused on effects of cigarette smoke on salivary proteins. Nagler et al. (2000) and Reznick et al. (2003) investigated the protein carbonyls in saliva exposed to cigarette smoke by Western Blotting. They reported that carbonyls were increased with increased exposure time and the aldehydes in the smoke may increase the protein carbonylation. Herskovic et al. (2007) reported age has an effect on protein-carbonyls and that elderly subjects (70 to 80 years old) have higher protein-carbonyls than younger subjects (20 to 25 years old).

Blakeman et al. (1998) investigated ferrous and ferric induced protein carbonylation of bovine serum albumin. In the presence of ferric and ascorbate protein carbonylation was not enhanced, however in the presence of ferrous there was a significant increase in

protein carbonyls. During this study TBARS values in the samples were also determined. When lipids were absent in bovine serum albumin (model protein) samples with ferrous, there was no significant TBARS formation. However, there was a small but significant increase in protein carbonyls. This indicates that Fenton reactions caused by ferrous may also carbonylate the proteins. For our results it is hard to conclude whether the proteins were oxidized by the metals or were modified as a result of lipid oxidation carbonyls. In the mouth it is impossible to exclude lipid oxidation and study only protein oxidation. However it may be concluded that the presence of protein-carbonyls supports the oxidative stress in the oral cavity.

### 3.5. Detecting carbonyls by DNPH

As the protein-carbonyls were detected by derivatization by DNPH, free carbonyls in saliva were also analyzed by DNPH derivatization. Saliva samples collected for control and metal (ferrous iron) were derivatized by DNPH and analyzed by GC/MS. The results indicated an increase in formaldehyde, acetaldehyde and propionaldehyde concentrations in the saliva. The amount of formaldehyde, acetaldehyde, and propionaldehyde in control saliva samples were identified as 6, 7, and 3.6 μM respectively. The amount of formaldehyde, acetaldehyde, and propanaldehyde in metal saliva samples were identified as 24, 15, and 7.9 μM respectively. Hence there was about a four fold increase for formaldehyde, two fold increases for acetaldehyde and propionaldehyde after the mouth was rinsed with ferrous solution. Although a wide array of aldehydes and ketones are reported for lipid oxidation, these three aldehydes were the only ones detected. An earlier study conducted by Cordis et al. (1994) suggested that monitoring MDA, formaldehyde, acetaldehyde, propionaldehyde and acetone is a good method to monitor oxidative stress. They developed a method to oxidize rat heart tissue with iron and derivatized the samples with DNPH. The aldehydes were separated with HPLC and detected by UV absorbance. The above mentioned compounds were detected in the samples.

It should be noted that some of the carbonyls may be lost through breathing and some may be absorbed onto the oral tissues or precipitate with the proteins they are

attached to. Also a recent work by Omur-Ozbek and Dietrich (2008) reported that humans are very sensitive to hexanal which is a common lipid oxidation by-product suggesting that even the carbonyls are produced at low μM concentrations humans may detect them. It was reported that humans can detect hexanal at 014 μg/L in air. Buettner (2002) studied the effect of human saliva on odorant including aldehydes and ketones. It was reported that the aldehydes were reduced by 30 to 50 % by the salivary enzymes to their corresponding alcohols. Hong et al. (2006) evaluated the volatility of aroma compounds in the presence of copper in artificial saliva. It was determined that the headspace concentration of the volatiles were decreased when salivary proteins and copper was present. Another suggested mechanism for reduction in aldehydes was precipitation with proteins (protein-carbonylation). Hence these two mechanisms may be interfering with the measurements of metallic flavor compounds in saliva.

### 3.6. Sensory testing with ferrous, antioxidants and chelating agents

Panelists reported metallic flavor at a moderate to high intensity after the ferrous iron solution rinse even though they rinsed their mouth with an antioxidant or chelating agent solution before it. However, when antioxidants and chelating agents were sampled after rinsing mouth with the ferrous solution, the perceived metallic flavor intensity decreased significantly for all treatments ($p<0.001$ for resgent water, EDTA, and lactoferrin, $p=0.006$ for vitamin C, and $p=0.017$ for vitamin E) (Figure-8.7). For reagent water and vitamin E rinses all panelists reported a decrease in sensation, whereas 2 panelists reported increased sensation and 1 panelist reported same intensity after vitamin C rinse. While only 4 panelists reported that metallic sensation was completely removed by EDTA rinse, after lactoferrin rinse all of the panelists reported that the metallic flavor was completely removed in seconds. Although vitamins E and C seemed to reduce the metallic sensation, it should be noted that the intensity rating are still higher when compared to results from reagent water rinse. This indicates that they are not very effective in removing the metallic flavor. On the contrary it may be argued that they even enhance the flavor even though the metals in the mouth are rinsed out with the antioxidant solution that does not contain metals. In the literature conflicting results have been presented. Although vitamin E and C are accepted as good free radical

scavengers there are occasions where they promote lipid oxidation, especially in the presence of metals (Denisov et al., 2005). Kawatsu et al. (1984) showed that ferrous causes phospholipid oxidation, and when ferrous ion and ascorbic acid (vitamin C) were present in the matrix the oxidation was enhanced. Osborn and Akoh (2003) determined the effect of vitamin E, quercetin, and gallic acid on iron catalyzed lipid oxidation of lipids. As opposed to expected, all of these natural antioxidants enhanced lipid oxidation. On the other hand EDTA has been successfully incorporated into food to preserve the flavor and color of the products (Myers, 2007). Let et al. (2007) reported that lipid oxidation was greatly prevented in fish oil samples that were fortified with EDTA. Satue-Gracia et al. (2000) studied the effect of lactoferrin on oxidation when the baby formulas were supplemented with iron as well. The results indicated that even lactoferrin was at lower concentrations than iron, it still acted as an antioxidant and prevented oxidation.

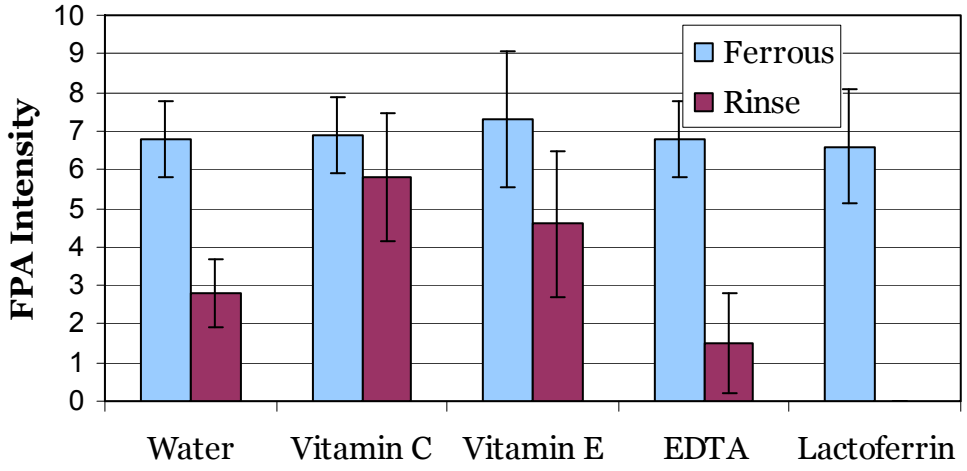



**Figure-8.7.** Intensity ratings (mean ± std dev) on a scale of 0 to 12 for metallic flavor after rinse with ferrous iron solution at 18 μM then another rinse with selected antioxidant or chelating agent

*Metallic flavor was completely removed after lactoferrin rinse for all panelists

The lipid oxidation measured by TBARS (data not shown) for the administration of antioxidants before and after metal (ferrous iron) solution rinse did not give conclusive

results. When the metal solution was tasted before antioxidants or chelating agents the lipid oxidation had already occurred in the mouth. When the metal solution was tasted after, the residual antioxidant or chelating agent concentration was not enough to prevent lipid oxidation and a significant increase in MDA concentration was observed. Hence sensory tests were selected to study the effectiveness of these compounds.

### 3.7. Metallic flavor and lipid oxidation for reduced and oxidized iron and copper

As explained in the sections above ferrous ion was used as a model metal to study metallic flavor production. It was demonstrated that lipid oxidation, measured by TBARS method as MDA production, is the best indicator of ferrous iron reacting in the oral cavity. The occurrence of oxidative stress caused by ferrous ion in the oral cavity was supported by increased protein-carbonyl levels in ferrous saliva samples, and by detection of low molecular aldehydes in saliva. MDA formations for ferric, cuprous, and cupric were also investigated to understand the reactivity of these ions and to compare them to sensory perceptions.

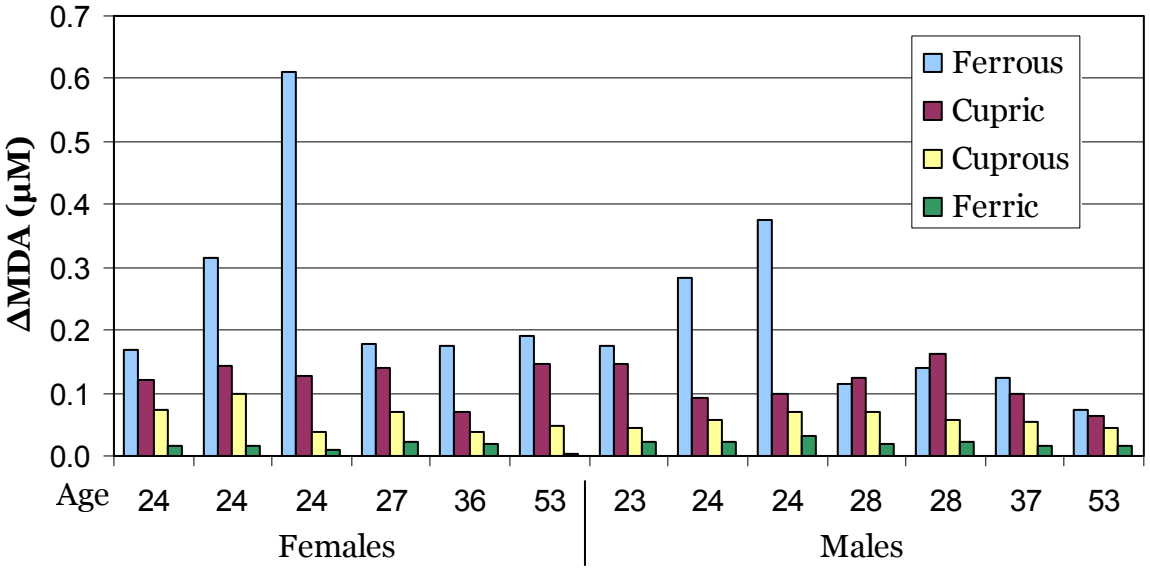



**Figure-8.8.** Comparison of lipid oxidations, as measured by MDA production in the saliva of healthy adult subjects, when the oral cavity is exposed to 180 μM iron or copper ions (n=13)

The data presented in Figure-8.8 indicate the increase in MDA concentrations (metal saliva MDA – control saliva MDA). One-way ANOVA with Tukey and Scheffe analyses with the log transformed data indicated that there was a significant difference between the increases in MDA concentration for all of the metal salt solutions. Ferrous caused the most increase in MDA production followed by cupric, cuprous. Ferric produced very little MDA. This was expected as ferrous iron is more redox active than copper.

To investigate the role of lipid oxidation and its role in metallic flavor production, formation of MDA (for the data shown above) and flavor thresholds were investigated for iron and copper ions (except for ferric as subjects could not detect a metallic flavor even at 360 μM, and only a few subjects rated it sour or salty) and given in Table-8.1. Taste threshold values shown below were determined in reagent water around pH 5.5, using a forced choice 1-of-5 test with metal concentrations of 0.01- >5 mg/L, testing 25 to 36 subjects, and have been previously reported (Cuppett et al., 2006; Tucker et al., 2008; Omur-Ozbek et al., 2008).

**Table-8.1.** Taste thresholds of iron and copper ions in reagent water

| Metal | Mean MDA production (μM) | Threshold (μM) by geometric mean method | Threshold (μM) by logistic regression method | ~75% of the population detected flavor at or below (μM) |
|---|---|---|---|---|
| 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 |

Ferrous being the most “flavorful” also causes the most lipid oxidation, followed by cupric, cuprous, and then ferric. The threshold results indicate that majority of the population can detect ferrous at concentrations as low as 1.79 μM, whereas this value increases to 15.74 μM for cupric and cuprous. This almost 10 fold difference in detection concentration may be due to carbonyl production as a result of lipid oxidation in mouth.

The results shown in Figure-8.8 clearly demonstrate that ferrous causes the highest lipid oxidation, which also means the highest carbonyl formation in the oral cavity. Hence even at lower concentrations than cuprous and cupric, the lipid oxidation (and flavor production) by ferrous is significant.

**Conclusions**

Although there have been numerous works focusing on metallic "taste" perception, the reactions causing the metallic sensation have little been investigated. This work is the first attempt to study biochemical reactions to understand and solve the metallic "flavor" perception of healthy humans and, cancer and Sjogren's disease patients suffering from metallic taste dysfunction. It was demonstrated that iron and copper in the oral cavity cause oxidative stress and result in lipid oxidation (determined by a significant increase in MDA concentrations in metal saliva samples ($p<0.001$)) and formation of carbonyls (such as formaldehyde, acetaldehyde and propionaldehyde) that ultimately contribute to the metallic flavor formation. Occurrence of oxidative stress in the mouth was also supported by increased protein-carbonyls in metal saliva samples. As expected, ferrous iron caused the most lipid oxidation because it is the most redox active ion among those tested. Ferrous ion was followed by cupric and cuprous ions in producing the most 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. As discussed, ferrous has the lowest taste threshold followed by cupric and cuprous that inversely parallel the MDA formation in the oral cavity.

Food literature has documented an array of carbonyls (such as hexanal, 1-octen-3-one, and trans-4,5-epoxydecanal) produced as a result of oxidation of unsaturated fats in food causing metallic off-flavors. Hence, the metallic sensation caused by the iron and copper ions in mouth is not just 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 carbonyls produced as a result of lipid oxidation. Our findings focused on preventing the metallic flavor suggests that chelating agents are more

effective in inactivating the metals than antioxidants, and are able to remove metallic flavor from mouth. A solution to metallic flavor generation may be found in the chelating agents.

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# Chapter IX

## Using KB Cell Cultures as a Model to Study and Prevent the Metallic Flavor Production in Mouth as a Result of Oxidation of the Oral Epithelial Cell Membrane Phospholipids by Iron and Copper


### Abstract

It was reported that the metallic flavor is a combination of taste and retronasal perception (odor) and the compounds responsible for the retronasal perception are produced by the oxidation of the oral epithelial cell membrane phospholipids. To investigate the metallic flavor production and its prevention in human oral cavity, KB cell cultures were used as a model and were compared to primary oral tissues for their response to reduced and oxidized copper and iron ions. The effect of saliva and selected antioxidants and chelating agents on oxidation mediated by iron and copper ions were also investigated. Lipid oxidation was similar for both the primary and KB cells and increased in the presence of the divalent cations (ferrous and cupric). Except α-lipoic acid, antioxidants (vitamin E and C) were not found to be very effective in reducing the oxidation of the phospholipids. Chelating agents (EDTA and lactoferrin) however were more effective in reducing the lipid oxidation.



***Keywords:*** *metallic flavor, KB cells, lipid oxidation, antioxidants, chelating agents*


### 1. Introduction

Metallic taste dysfunction is a quality of life issue for patients undergoing chemotherapy, radiotherapy, and humans suffering from Sjogren's disease (Nitenberg and Raynard, 2000; Soto-Rojas et al., 1998). Metallic taste of drinking water is a concern for the water industry. Corrosion of metal pipes introduces iron and copper into the water and causes aesthetical problems. US Environmental Pollution Agency set secondary maximum contaminant levels for iron and copper as 0.3 and 1.0 mg/L to prevent off-flavors and discoloration of drinking water.

Recent studies (Omur-Ozbek et al., 2008; Lawless et al., 2004) reported that the metallic sensation for copper and iron is a combination of taste of the metal ions on the tongue and the retronasal perception of the odorous compounds produced as a result of oxidation of oral epithelial cell membrane lipids. Researchers have conducted sensory and analytical tests to understand the perception and the role of lipid oxidation in the oral cavity on production of the metallic flavor. Thiobarbituric acid reactive substances (TBARS) method was employed to detect the malondialdehyde (MDA) production in the oral cavity to determine the extent of lipid oxidation occurring in mouth as a result of ingestion of iron and copper salt solutions (Armstrong and Browne, 1994; Yagi, 1998). The results indicated an increase in MDA concentrations after healthy human subjects rinsed their mouth with iron or copper solutions. This suggests that the fatty acids of phospholipids in cell membranes are oxidized in the presence of these metal ions and odorous aldehydes and ketones create the unpleasant metallic sensation.

Oral mucosa is formed by the stratified squamous epithelial cells that are para-keratinized in the hard palate (Terashi et al., 2000). The plasma (cell) membranes of the epithelial cells are composed of a phospholipid bilayer, proteins and carbohydrates. Phospholipid molecules have a polar head and a non-polar tail. The polar heads are derived from glycerol conjugated to choline, serine, or ethanolamine. The tails are made up of two fatty acids (usually one saturated and one unsaturated in mammalian cell membranes) covalently bonded to glycerol (Burkitt et al., 1993). Phospholipids of the cell membrane have structural and functional significance as well as a role in keratinization of the epithelium (Singer and Nicholson, 1972; Terashi et al, 2000). Fatty acids are an important part of the phospholipids in the cell membranes. Humans can synthesize saturated fatty acids such as palmitic (16:0) and stearic (18:0) acids (Marcelo and Dunham, 1993). Palmitoleic (16:1) and oleic (18:1) acids which are the main monounsaturated fatty acids in humans may be synthesized from the saturated fatty acids by the desaturation enzymes (Ziboh and Chapkin, 1988). However, linoleic (18:2) and arachidonic (20:4) acids which are the major essential fatty acids cannot be synthesized and should be supplied by the diet (Chapkin and Ziboh, 1984).

Terashi et al. (2000) suggested that the type of fatty acids forming the phospholipids in the cell membrane affect cell growth, differentiation and function. The study of fatty acid composition of cell membranes of epidermal, oral mucosal, and hair follicle cell samples from healthy adult humans revealed that epidermal cell membranes contain a significantly higher amount of linoleic acid whereas hair follicle cell membranes have higher amount of palmitic acid and lower amounts of linoleic and arachidonic acids than oral mucosal cell membranes. It also was noted that cell differentiation may be achieved by linoleic acid accumulation in the cellular membrane.

Oral epithelial cells of animals have been cultured *in vitro* since late 1960's to aid studies of the oral cavity and mucosa. Studies mainly were conducted with oral tissue samples from hamsters and rats. White and Tankersley (1969) cultured transplants from hamster cheek pouches on plasma clots, while Schuster et al. (1985) developed a simpler method to culture epithelial cells in plastic tissue flasks. It has already been shown that culture of buccal oral epithelial cells form an organized structure even in the absence of connective tissue underlying the cells (Flaxman, 1967). Numerous studies later have tested the use of fibroblastic feeder layer cells, epidermal growth factor, collagen gels, varied growth temperatures and pH, and modified media to culture disaggregated oral epithelial cells (Schuster et al., 1985). Many researchers also focused on culture of oral epithelial cells of humans. Taichman et al. (1979), Reinwald and Green (1977), and Peehl and Ham (1980) focused on optimizing the conditions for culturing cells close to *in vivo* properties. A commonly used method for human oral epithelial cell cultures was developed by Oda and Watson (1990) using gingival tissues from healthy adults. The cell cultures were maintained up to 7 passages (100 days).

MatTek® (Ashland, MA) has recently developed multilayered and highly differentiated tissue models that consist of primary human oral epithelial cells. The tissue models closely parallel the primary human oral tissues morphologically with an organized basal layer and multiple non-cornified layers. The tissue cultures overlap the normal lipid profiles of the *in vivo* tissues with the *in vitro* tissues having slightly higher phospholipids (17%) than the *in vivo* tissues. The cells obtained from primary human tissues and are grown on collagen-coated standard Millipore Millicell® culture plate

inserts at the air liquid interface. After a certain time the proprietary culture medium is removed from the apical (top) surface and the cultures are fed through the basolateral (bottom) surface. The growth medium is Dubelco's modified Eagle's medium with epidermal growth factor and other proprietary factors. This oral tissue model may be used for many purposes including toxicology and drug testing.

In order to ensure the survival of cells to study the same cell line, researchers started to transform the primary cells with viruses to make them immortal which would make it possible to passage them indefinitely and also to study carcinogenesis. In an earlier study, primary human oral epithelial cells were infected with human papillomavirus (HPV), which is found in 90% of the head and neck cancers, and the cells could be maintained in culture for over 4 years whereas the primary cells survived up to 5 to 9 passages (Oda et al., 1996a). It has been reported that transformed cells lines have chromosomal changes and may not pass malignancy tests (Oda et al., 1996b). KB cells which were obtained by HeLa contamination of oral epithelial cells have been used since late 60's to study plasma membrane functions and amino acid transport (Charalampous et al., 1973).

Lekholm and Svennerholm (1977) investigated the lipid pattern of the oral epithelium of humans and reported oleic, linoleic and arachidonic acids as the major fatty acids detected in the largest fraction of the phospholipids. It also was noted that the composition of fatty acids was not affected by age and gender of the subjects. A recent study compared primary human oral epithelial and squamous cell carcinoma cell lines and concluded that primary cells have higher saturated fatty acids and lower unsaturated fatty acids than the carcinoma cells (Gasparoni et al., 2004). An earlier work by Macleod et al. (1990) compared the fatty acid composition of healthy and malignant oral epithelial cells. A reduction in relative proportions of palmitoleic and oleic acids were observed in the cell membranes of malignant oral epithelial whereas there was an increase in palmitic, stearic and arachidonic acids. There was no significant change in linoleic acid composition. Transformed cell lines usually have a very similar cell membrane composition to primary cell membranes (Micklem et al., 1976) and employing transformed cells in experiments has been practiced to study primary cells as

transformed cells usually show similar membrane and growth characteristics to the primary cells (Staab et al., 2004).

Effects of metallic dental materials used for orthodontic appliances such as brackets, molar bands and implants on epithelial cells and tissues were studied as these materials are in close contact with the cells and tissues and have been reported to cause allergic, inflammatory, toxic and mutagenic reactions. The findings indicated that ions released from these materials, especially copper and zinc, affect the growth and vitality of oral cells and hence the tissues. However it also was noted that the extent of effects of these metals depends on the corrosion of the alloys, proteins in the saliva and ion release from the appliances (Cortizo et al., 2004).

It has been shown that metals catalyze lipid oxidation reactions and form free radicals. Hence the phospholipids (fatty acids) in the cell membranes may be affected by the presence of metals in the oral cavity. The double bonds in the fatty acids chains are very prone to oxidation and they form unstable products. At the initiation step free radicals are formed then with the attack of free radicals, the fatty acids are converted to hydroperoxides during the propagation step. Metals play another role in oxidation and iron and copper, which can be present in two valence states, react with hydroperoxides and convert them to aldehydes, ketones, alcohols, hydrocarbons, and acids. After these reactions non-radical species are formed at the termination step (Kochhar, 1996).

Lipid oxidation may be reduced or prevented by the administration of antioxidants or chelating agents. In the medical and food industry several compounds that either reduce the availability of the metals or donate a hydrogen atom to form non-radical species have been administered to prevent or reduce lipid oxidation (Rajalakshml and Narasimhan, 1995). Main antioxidants that also are found in food are vitamin C (ascorbic acid), vitamin E (α-tocopherol) and α-lipoic acid. Vitamin C is water soluble and is not very stable. At high concentrations it has been shown to reduce oxidation caused by iron (Denisov and Afanasev, 2005). Vitamin E is fat soluble and has shown to possess good antioxidant properties *in vivo* (Gille and Joenje, 1991). α-Lipoic acid, which is in the family of B-vitamins is both water and fat soluble, which makes it

available in different matrices. It also easily donates a hydrogen atom to stop the free radical chain reactions (Challem, 1996). While antioxidants donate a hydrogen atom to form non-radical species, chelating agents remove the metals from the matrix and prevent oxidation. However efficiency of chelation depends on external factors such as pH and temperature (Nortemann, 2005; Saito et al., 1994). Ethylenediamine tetraacetic acid (EDTA) is a common chelating agent that can bind to iron and copper, and is added to food to prevent the formation of foul tastes (Myers, 2007). Lactoferrin is another chelating agent that binds to iron and is naturally found in milk and tears. It has a very high affinity to iron however its binding efficiency depends on the pH of the matrix (Uchida et al., 2005).

The objective of this work was to study metal-induced lipid oxidation of phospholipids in the cell membranes of primary oral epithelial cells and KB cells. Cell cultures were tested with: 1) reagent water or saliva as controls, and ferrous as the metal samples; 2) reduced and oxidized iron and copper ions with reagent water as control; and 3) selected antioxidants and chelating agents with ferrous.

## 2. Materials and methods

### 2.1. Cell cultures and materials

Primary human oral epithelial cell tissues (ORL-606) and the maintenance medium were obtained from MatTek® (Ashland, MA). KB cells (CCL-17) were obtained from American Type Culture Collection (ATCC). Eagle's minimum essential medium and cell culture flasks were purchased from Cell Applications, Inc. (San Diego, CA). Ferrous chloride, ferric chloride, cuprous chloride, and cupric chloride were purchased from Fisher Scientific (Pittsburg, PA). α-Tocopherol (vitamin E), L-ascorbic acid (vitamin C) and α-lipoic acid were purchased from Sigma Aldrich (St. Louis, MO). EDTA (Ethylenediaminetetraacetic acid) was purchased from EMD (Gibbstown, NJ), and lactoferrin was purchased from Netnutri (West New York, NJ). 2-Thiobarbituric acid, sodium dodecyl sulfate, glacial acetic acid (99% pure), 1-butanol, pyridine, and 1,1,3,3-tetramethoxypopane were purchased from Fisher Scientific (Pittsburgh, PA). Reagent

water was obtained from the Nanopure® filter. Whole saliva was obtained from a single healthy donor and was immediately frozen to -20 $^{o}$C.

Upon receipt, primary oral epithelial cell tissues were transferred into new culture flasks with the maintenance medium and incubated at 37 $^{o}$C for one day. KB cells were cultured in T-75 flasks in Eagle's minimum essential medium with 10% fetal bovine serum, 1% non-essential amino acid, 1% sodium-pyruvate, 1% L-glutamine and 1% Penn-Strep. To subculture the cells, medium was removed and the cells were rinsed with 0.25% trypsin and 0.03% EDTA solution. Then the rinse solution was removed and an additional 2 mL of trypsin-EDTA solution was added to the cells. The flask was incubated at 37 $^{o}$C for 5 to 10 minutes until the cells detached from the flask. Fresh culture medium was added and aspirated and dispensed into one new T-75 culture flask and four six well culture plates (at a subcultivation ratio of 1:5). This procedure was repeated twice per week. Cells were periodically checked under the microscope until 90% confluence was achieved before the treatments were applied to the cells in six well culture flasks (Figure-9.1).

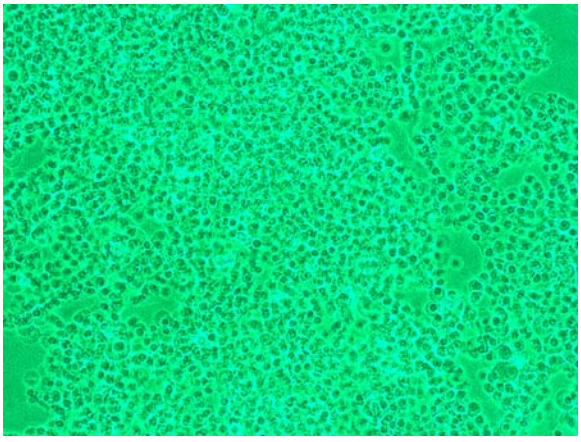

**Figure-9.1.** KB cells under microscope before treatments

## 2.2. Treatments applied to cell cultures

### 2.2.1. Comparing KB versus primary cells and determining the effect of saliva

Primary and KB oral epithelial cell cultures were treated with saliva and reagent water samples with and without ferrous iron. Each culture well first received 1 mL of either saliva (50% diluted with reagent water) or reagent water as a control. The wells were gently swirled for 15 seconds and the samples were collected with an Eppendorf pipettor. Then the same culture received 0.5 mL of saliva or reagent water and 0.5 mL of ferrous iron solution at 36 µM (which is double the concentration shown to cause lipid oxidation *in vivo* considering the dilution) as metal samples. Again the wells were gently swirled for 15 seconds and the samples were collected. The samples were analyzed for lipid oxidation with the TBARS method as described in section 2.3. A schematic is given in Figure-9.2. Experiments were conducted with 8 replicates of primary cells and 12 replicates for KB cells for each treatment. As will be presented and discussed in the next section KB cells gave similar results to primary oral epithelial cell tissues and hence were used as a model culture for the next treatment steps. Also, as no difference was detected for saliva and reagent water samples, reagent water was used for the control samples for the next treatment steps.

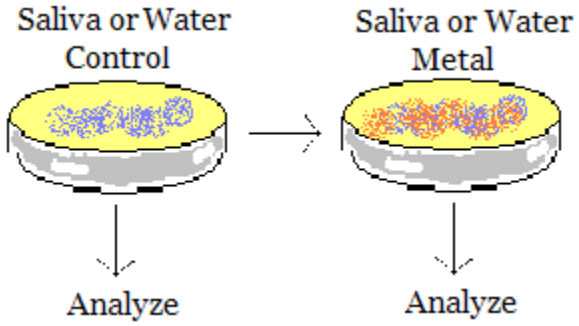


**Figure-9.2.** Schematic of treatments to the cell cultures/tissues

### 2.2.2. Comparing iron and copper ions

Three sets of KB cells with twelve wells each first received 1 mL of reagent water as control and the wells were gently swirled for 15 seconds and the samples were collected.

Then the wells received either ferric, cuprous or cupric salt solutions at 18 μM prepared in reagent water, the wells were gently swirled for 15 seconds and the samples were collected. The samples were analyzed with the TBARS method.

### 2.2.3. Comparing effectiveness of antioxidants and chelating agents

KB cells again first received 1 mL of antioxidant or chelating agent solution at 36 μM (which is the same concentration as the ferrous iron solution) in reagent water as control and the wells were gently swirled for 15 seconds and the samples were collected. Then the wells received 0.5 mL of the selected compound and 0.5 mL of ferrous solution at 36 μM. The wells were gently swirled for 15 seconds and the samples were collected. The concentration of the antioxidants and chelating agents added to the cells were: vitamin C at 72 μM, vitamin E at 72 μM, vitamin C+E at 36 μM (each), α-lipoic acid at 72 μM, EDTA at 72 μM, and lactoferrin at 0.26 μM. The samples were analyzed with the TBARS method. The concentrations of the selected compounds were doubled to account for dilution whereas the lactoferrin concentration was selected lower due to its strong capacity to inactivate ferrous iron.

## 2.3. Measuring lipid oxidation as malondialdehyde production

Lipid oxidation was detected by analyzing the samples obtained with the thiobarbituric acid reactive substances (TBARS) method (Armstrong and Browne, 1994; Yagi, 1998). The samples collected from the cell cultures (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 a water bath (10-L, Fisher Scientific, Pittsburgh, PA) at 95 $^{o}$C for 60 minutes. Then the samples were immediately cooled down in an ice bath and pink complex formed was extracted with 5 mL of n-butanol/pyridine solution at a 15:1 ratio. The extracts were separated by centrifuging (Model PR-2, International Portable Refrigerated Centrifuge, International Equipment Company, Boston, MA) the samples at 3000 rpm for 15 minutes. The absorbance of the supernatant was measured with a spectrophotometer (Model 21D Spectronic, Milton Roy Co., Rochester, NY) at 532 nm. The standard curve was obtained by running 1,1,3,3-tetramethoxypropane standards at 0.01 to 0.075 μM.

### 2.4. Statistical analysis

Data were log transformed to conform to normality and homogeneous distribution. SAS software (version 8.1) was used to run one-way ANOVA and Bonferroni's test to determine the statistical significance of the difference between the treatments. Alpha = 0.05 was the significance level for all tests.

## 3. Results and discussion

### 3.1. Comparing KB versus primary cells and determining the effect of saliva

As shown in Figure-9.3, both primary oral epithelial cell tissues and KB cells yielded similar results for saliva versus reagent water and a significant increase in MDA concentration was detected when metal was added. For primary cells the average increase in MDA concentration was 0.053 μM and 0.059 μM for saliva and reagent water respectively. For KB cells the average increase in MDA concentration was 0.056 μM for both saliva and reagent water. There was no significant difference between saliva and reagent water samples for the primary cells ($p=0.668$) and the KB cells ($p=0.177$). When all data for saliva and reagent water samples were compared for primary and KB cells, again no significant difference was observed ($p=0.908$). Although some effect of saliva on lipid oxidation was expected due to its antioxidant capacity associated with antioxidant enzymes and chelating agents, saliva did not show a difference in our experiments. Free radicals such as reactive oxygen species cause oxidative stress in mouth and through evolution, humans developed mechanisms to minimize the effects of oxidative stress. Salivary antioxidants such as peroxidase, superoxide dismutase, uric acid, vitamin E and vitamin C inhibit the reactive oxygen species (Bahar et al., 2007). However, the effect of the salivary antioxidants may be lessened by the dilution factor to conduct uniform experiments for the controls as well as the saliva added to the metal samples were diluted by the addition of the metal salt solution. A difference between the primary and KB cell cultures was not expected as the literature suggests that they have very similar cell membrane characteristics and fatty acid profiles (Miklem et al., 1976). As the reagent water and KB cells worked similar to saliva and primary cell tissues, they

were used subsequently as a model to study lipid oxidation in the oral cavity as induced by copper and iron ions and selected antioxidants and chelating agents as inhibitors.

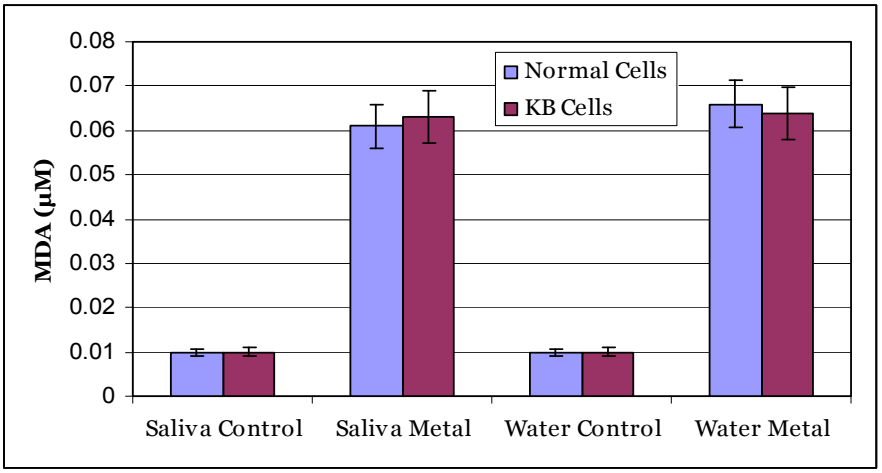


**Figure-9.3.** Lipid oxidation reported as malondialdehyde (µM) when primary oral epithelial and KB cells were treated wtih saliva or reagent water as control and ferrous ion solution (18 µM) as metal sample *n = 8 for primary cells, n=12 for KB cells.

### 3.2. Comparing iron and copper ions

As shown in Figure-9.4, ferric ion caused the least increase in MDA for the metal samples (n=12) whereas ferrous ion yielded the highest. The mean increases in MDA concentration were 0.056 µM, 0.023 µM, 0.044 µM, 0.053 µM for ferrous, ferric, cuprous and cupric ions respectively. A one-way ANOVA indicated that there was a significant difference among the metal ions ($p<0.001$) and Bonferroni's test indicated that ferric ion was significantly different from the others. This indicates that ferric ion is not as reactive as the other ions and hence causes less lipid oxidation, but that ferrous, cupric, and cuprous all induce similar levels of lipid oxidation in cell culture.

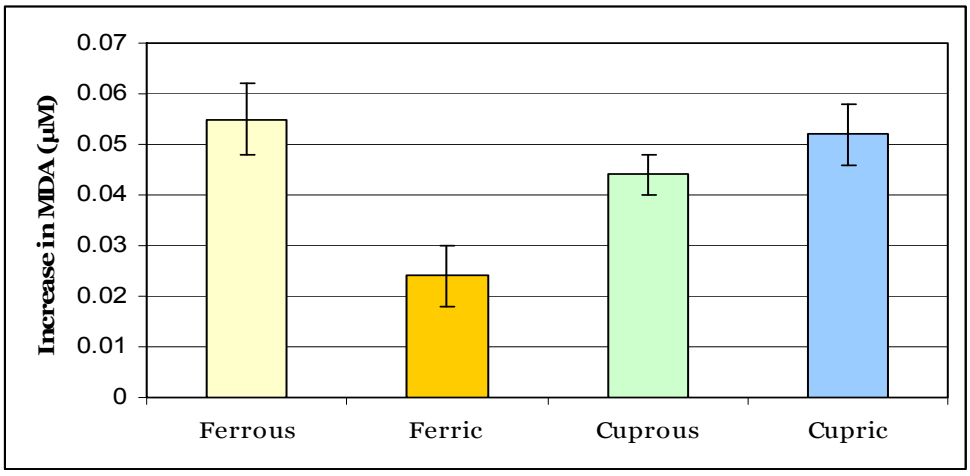


**Figure-9.4.** Increase in lipid oxidation reported as malondialdehyde (µM) for KB cells for selected metal ions (at 18 µM) in reagent water

As reported by Omur-Ozbek et al. (2008) the increase in MDA concentrations in the mouth when human subjects ingested ferrous, ferric, cuprous, cupric ions at 180 µM were 0.22 µM, 0.02 µM, 0.06 µM, and 0.12 µM respectively. When the surface area of the cell cultures (about 4 $cm^2$) and exposed human mouth (about 80 $cm^2$) is considered and the results are normalized for the surface area, the MDA production for KB cells are much higher than *in vivo* results. However there is a trend in MDA formation; higher for ferrous and cupric, followed by cuprous and then ferric. It should be noted that although the effect of saliva could not be determined by these studies, in the real mouth it could be interacting with the metals and affecting their redox activity. It is known that lactoferrin, which is a metal chelator, is present in saliva and can bind and inactivate metals (Satue-Gracia et al., 2000).

### 3.3. Comparing the effectiveness of antioxidants and chelating agents

As depicted in Figure-9.5, the most effective compounds that reduced the MDA production were EDTA and lactoferrin. The average increase in MDA concentrations were 0.053 µM, 0.053 µM, 0.044 µM, 0.056 µM, 0.017 µM, and 0.026 µM for Vitamin C, Vitamin E, α-lipoic acid, Vitamin C+E, EDTA and lactoferrin. Nine replicates were run for each treatment except for vitamin C+E (six replicates). Statistical analyses on the

log transformed data indicated that there was a significant difference between the antioxidants and the chelating agents ($p<0.001$), and Bonferroni's test indicated that results for EDTA and lactoferrin are significantly different than the results for the antioxidants. When α-lipoic acid was compared to ferrous, a statistically significant reduction in the MDA formation was observed ($p<0.001$).

Although antioxidants have been shown to work well in the body to scavenge free radicals and reduce or prevent lipid oxidation (Rajalakshml and Narasimhan, 1995; Gille and Joenje, 1991; Challem, 1996), in the cell culture models (and in human mouth, Omur-Ozbek et al., 2008) a molar amount of antioxidant equal to double the molar amount of metal did not reduce lipid oxidation. Also there has been conflicting data in the literature on the anti- and pro- oxidant capacities of vitamins C and E, supporting our results (Friel et al., 2007; Osborn and Akoh, 2003; Packer et al., 1995). The effectiveness of α-lipoic acid however has been discussed by the medical industry when administered orally to patients undergoing surgery (Pepe et al., 2008). Let et al. (2007) reported that lipid oxidation was greatly prevented in fish oil samples that were fortified with EDTA. Satue-Gracia et al. (2000) studied the effect of lactoferrin on oxidation when the baby formulas were supplemented with iron. The results indicated that even when lactoferrin was at lower concentrations than iron, it still acted as an antioxidant and prevented oxidation.

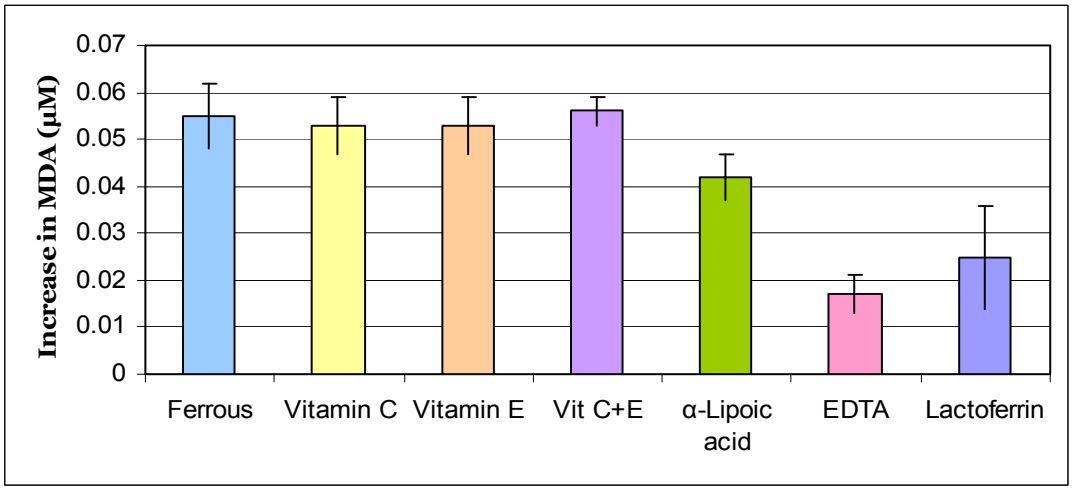


**Figure-9.5.** Increase in lipid oxidation measured as malondialdehyde (µM) for KB cells treated with several antioxidants/chelating agents (36 µM) and ferrous ion in reagent water (18 µM) *EDTA and lactoferrin significantly reduced lipid oxidation

## 4. Conclusions

KB cells and primary human oral epithelial cells were successfully used as a model to study lipid oxidation of cell membrane phospholipids in our laboratory. Statistically no differences were detected for the results obtained for the primary versus KB cells. This enabled us to conduct faster and more controlled tests with the KB cells. We have expected some interference from saliva and its constituents, mainly by the antioxidants, however no statistically significant change in oxidation was observed. It should be noted that for controlled tests whole saliva was collected from one donor and the supernatant was diluted 50% with reagent water and used as the control saliva solution for the treatments. Antioxidants may have an effect on lipid oxidation and hence the metallic flavor production when administered orally and accumulated in the body. The results from the cell cultures paralleled those with human subjects where lipid oxidation was measured in the oral cavity. For both studies, ferrous ion produced the most lipid oxidation, followed by cupric, cuprous, and ferric ions.

The findings indicate that KB cells may be successfully cultured and treated in the laboratory as a model to study the lipid oxidation in the oral cavity. It has been shown

that the results correlate wll with the *in vivo* results. This model will enable the researchers to conduct more controlled experiments to investigate the metallic flavor production in the mouth. Applications may involve intervention methods to prevent production of metallic flavor as well as investigating the effect of certain therapies on the vitality of the oral epithleial cells and hence their vulnerability to lipid oxidation due to the side effects.

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## Appendix A

## Taste and Odor Abnormalities in Cancer Patients

JaeHee Hong, Pinar Omur-Ozbek, Brian Stanek, Andrea M. Dietrich, Susan E. Duncan, YongWoo Lee, Glenn Lesser

### Abstract

Taste and smell abnormalities are major daily concerns for patients with cancer, and more studies are required to understand the causes and mechanisms of these abnormalities. This review summarizes the common taste and odor disorders of cancer patients and provides insight into their possible causes. Cancer and its therapy, including chemotherapy and radiotherapy, may directly alter and damage taste and odor perception. These alterations affect the daily quality of life of these patients and may lead to patient malnutrition and, in severe cases, to significant morbidity. Cancer patients experience decreases in sensitivity to taste and odor, as well as unpleasant metallic and bitter sensations. Complaints relating to taste and odor are not usually addressed as few, if any, effective interventions are available for these problems. Understanding the types and causes of taste and odor dysfunctions will enable researchers and physicians to develop treatments for resolving these conditions so as to improve the quality of life for cancer patients.

## Appendix – B: Data and Scorecards

**Table-B.1.**TBARS Results for humans for all ions (μM)

| ID | $Fe^{2+}$ | $Fe^{3+}$ | $Cu^{+}$ | $Cu^{2+}$ |
|---|---|---|---|---|
| F - 24 | 0.17 | 0.02 | 0.07 | 0.12 |
| F - 24 | 0.31 | 0.02 | 0.10 | 0.14 |
| F - 24 | 0.61 | 0.01 | 0.04 | 0.13 |
| F - 27 | 0.18 | 0.02 | 0.07 | 0.14 |
| F - 36 | 0.17 | 0.02 | 0.04 | 0.07 |
| F - 53 | 0.19 | 0.00 | 0.05 | 0.15 |
| M - 23 | 0.18 | 0.02 | 0.05 | 0.15 |
| M - 24 | 0.28 | 0.02 | 0.06 | 0.09 |
| M - 24 | 0.38 | 0.03 | 0.07 | 0.10 |
| M - 28 | 0.12 | 0.02 | 0.07 | 0.12 |
| M - 28 | 0.14 | 0.02 | 0.06 | 0.16 |
| M - 37 | 0.12 | 0.02 | 0.05 | 0.10 |
| M - 53 | 0.07 | 0.02 | 0.05 | 0.06 |

**Table-B.2.**TBARS Results for humans for ferrous (μM) normalized for protein

| ID | μM MDA/g protein | μM MDA/L saliva |
|---|---|---|
| F - 24 | 0.91 | 0.17 |
| F - 24 | 0.67 | 0.31 |
| F - 24 | 0.97 | 0.61 |
| F - 27 | 0.38 | 0.18 |
| F - 36 | 0.24 | 0.17 |
| F - 36 | 0.69 | 0.25 |
| F - 53 | 0.27 | 0.19 |
| M - 23 | 0.52 | 0.18 |
| M - 24 | 0.32 | 0.12 |
| M - 24 | 0.42 | 0.28 |
| M - 24 | 0.50 | 0.38 |
| M - 24 | 0.28 | 0.21 |
| M - 27 | 0.33 | 0.23 |
| M - 28 | 0.27 | 0.12 |
| M - 28 | 0.36 | 0.14 |
| M - 37 | 0.32 | 0.12 |
| M - 41 | 0.19 | 0.13 |
| M - 53 | 0.07 | 0.07 |

**Table-B.3.** Sensory Results for antioxidants and chelating agents

| Water | | Vitamin C | | Vitamin E | | EDTA | | Lactoferrin | |
|---|---|---|---|---|---|---|---|---|---|
| Before | After | Before | After | Before | After | Before | After | Before | After |
| 6 | 2 | 6 | 4 | 6 | 2 | 6 | 0 | 4 | 0 |
| 6 | 2 | 6 | 4 | 8 | 2 | 6 | 0 | 5 | 0 |
| 6 | 2 | 6 | 4 | 8 | 4 | 6 | 0 | 5 | 0 |
| 6 | 2 | 6 | 4 | 8 | 4 | 6 | 0 | 5 | 0 |
| 6 | 2 | 6 | 6 | 8 | 4 | 6 | 2 | 6 | 0 |
| 6 | 3 | 6 | 6 | 8 | 4 | 6 | 2 | 6 | 0 |
| 7 | 3 | 8 | 6 | 8 | 6 | 7 | 2 | 6 | 0 |
| 8 | 3 | 8 | 6 | 10 | 6 | 8 | 2 | 6 | 0 |
| 8 | 4 | 8 | 8 | 4 | 6 | 8 | 3 | 6 | 0 |
| 8 | 4 | 8 | 8 | 5 | 8 | 8 | 3 | 6 | 0 |
| 8 | 4 | 8 | 8 | | | 8 | 3 | 7 | 0 |
| | | | | | | | | 7 | 0 |
| | | | | | | | | 7 | 0 |
| | | | | | | | | 8 | 0 |
| | | | | | | | | 8 | 0 |
| | | | | | | | | 8 | 0 |
| | | | | | | | | 8 | 0 |
| | | | | | | | | 8 | 0 |
| | | | | | | | | 10 | 0 |

**Table-B.4.** Cell culture results for all ions and $Fe^{2+}$+antioxidant or chelator

| Compound | ΔMDA (μM) |
|---|---|
| $Fe^{+2}$ | 0.055 |
| $Fe^{+3}$ | 0.024 |
| $Cu^{+}$ | 0.044 |
| $Cu^{+2}$ | 0.052 |
| Vitamin C | 0.053 |
| Vitamin E | 0.053 |
| Vititamins C+E | 0.056 |
| Lipoic Acid | 0.042 |
| EDTA | 0.025 |
| Lactoferrin | 0.017 |

**Table-B.5.** Score card for flavor profile analysis with hexanal

Name: _____ Date: __/__/200_

*Please report if you have a cold or allergies that may affect you smelling ability.

1. Please start by smelling the odor free sample.
2. Then proceed with your first sample indicated in the table below.
3. Please hold the flask at the bottom and swirl gently, open the stopper and take couple of brief sniffs.
4. Report your odor perceptions with their corresponding intensities.
5. You may refer to the taste-and-odor wheel provided.
6. Take a two minute break between samples and smell the odor free sample to refresh your nose.
7. Please repeart the steps above until you complete all the samples for today.
8. Please record your perceptions without interacting with the other panelists. Your results will be discussed with the rest of the panel after all of the samples are evaluated.

| Sample Code | Descriptor | Intensity |
|---|---|---|
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |

Thank you very much!

**Table-B.6.** Score card for 1-of-5 analysis with iron and copper

Name: _____ Date: __/__/200_

*Please report if you have a cold or allergies that may affect you tasting and/or smelling ability.

1. Please rinse your mouth with the rinse water provided.
2. Check the order of your samples and make sure they match with the codes provided below.
3. Then proceed with your first sample on the left.
4. Please take a sip from the first sample swish it around your mouth 10 to 15 seconds. You may either swallow or expectorate the samples.
5. You may record your perceptions.
6. Please take a two minute break before you proceed to the next sample.
7. Taste the samples from left to right without going back.
8. Repeat steps 4 to 7 until you taste all the samples.
9. Please pick the **odd** sample that has a different taste/flavor than the others, and check the box corresponding to it.
10. No collaboration between panelists is allowed.

☐ ☐ ☐ ☐ ☐

_____ _____ _____ _____ _____

Thank you very much!

**Table-B.7.** Score card for sensory analysis of metallic flavor with nose-clips

Name: _____ Date: __/__/200_

Please report your own perceptions without interacting with the other panelists.

**Sample 1 – Code _____**

Please place the nose-clip on your nose and make sure you cannot breathe through your nose. Take a sip from the cup with the code for sample 1. Swish it around your mouth 10 to 15 seconds. You may either swallow or expectorate the sample to the provided cup. Please write your **taste** and **mouthfeel** perceptions with their **intensities** below. You may use the guides at the bottom of this scorecard.

Perceptions: Intensity:

**Sample 2 – Code _____**

Please take a sip from the cup with the code for sample 2. Swish it around your mouth 10 to 15 seconds. You may either swallow or expectorate the sample to the provided cup. Please write your **flavor** and **mouthfeel** perceptions with their **intensities** below. You may use the guides at the bottom of this scorecard.

Perceptions: Intensity:

**Basic tastes:** Sweet, salty, sour, bitter, umami
**Mouthfeel:** Astringent, drying, tingling
**Flavors:** Metallic, bloody, penny-like

**Intensity scale:** 0:no perception, T:threshold, 2:very weak, 4:weak, 8:moderate, 12:strong

**Table-B.8.** Score card for sensory analysis of metallic flavor and antioxidants/chelators

Name: _____ Date: __/__/200_

Please report your own perceptions without interacting with the other panelists.

**Sample 1 – Code _____**

Please take a sip from the cup with the code for sample 1. Swish it around your mouth 10 to 15 seconds. You may either swallow or expectorate the sample to the provided cup. Please write your **flavor** perceptions with their **intensities** below. You may use the guides at the bottom of this scorecard. Please proceed with the second sample as soon as possible.

Perceptions: Intensity:

**Sample 2 – Code _____**

Please take a sip from the cup with the code for sample 2. Swish it around your mouth 10 to 15 seconds. You may either swallow or expectorate the sample to the provided cup. Please write your **flavor** perceptions with their **intensities** below. You may use the guides at the bottom of this scorecard.

Perceptions: Intensity:

**Basic tastes:** Sweet, salty, sour, bitter, umami
**Mouthfeel:** Astringent, drying, tingling
**Flavors:** Metallic, bloody, penny-like

**Intensity scale:** 0:no perception, T:threshold, 2:very weak, 4:weak, 8:moderate, 12:strong

## Appendix – C: IRB Approval Letters

Virginia Tech
VIRGINIA POLYTECHNIC INSTITUTE
AND STATE UNIVERSITY

**Institutional Review Board**

Dr. David M. Moore
IRB (Human Subjects)Chair
Assistant Vice President for Research Compliance
CVM Phase II Duckpond Dr., Blacksburg, VA 24061 0442
Office: 540/231-4991; FAX: 540/231-6033
email: moored@vt.edu

DATE: March 9, 2005

MEMORANDUM

TO: Andrea M. Dietrich Civil & Environmental Engineering 0246
Pinar Omur

FROM: David Moore

SUBJECT: **IRB Expedited Approval:** "Determining an Odor Standard for Water Industry" IRB # 05-179

This memo is regarding the above-mentioned protocol. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. As Chair of the Virginia Tech Institutional Review Board, I have granted approval to the study for a period of 12 months, effective March 8, 2005.

Virginia Tech has an approved Federal Wide Assurance (FWA00000572, exp. 7/20/07) on file with OHRP, and its IRB Registration Number is IRB00000667.

cc: File
Department Reviewer: William E. Cox

Virginia Tech
VIRGINIA POLYTECHNIC INSTITUTE
AND STATE UNIVERSITY

**Institutional Review Board**

Dr. David M. Moore
IRB (Human Subjects) Chair
Assistant Vice President for Research Compliance
1880 Pratt Drive, Suite 2006(0497), Blacksburg, VA 24061
Office: 540/231-4991; FAX: 540/231-0959
email: moored@vt.edu

DATE: February 2, 2006

MEMORANDUM

TO: Andrea M. Dietrich Civil & Environmental Engineering
Pinar Omur

FROM: David Moore

SUBJECT: **IRB Expedited Continuation**: "Determining an Odor Standard for Water Industry" IRB # 05-179

This memo is regarding the above referenced protocol which was previously granted expedited approval by the IRB on March 8, 2005. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. Pursuant to your request of last week, as Chair of the Virginia Tech Institutional Review Board, I have granted approval for extension of the study for a period of 12 months, effective as of ?.

Approval of your research by the IRB provides the appropriate review as required by federal and state laws regarding human subject research. It is your responsibility to report to the IRB any adverse reactions that can be attributed to this study.

To continue the project past the 12-month approval period, a continuing review application must be submitted (30) days prior to the anniversary of the original approval date and a summary of the project to date must be provided. Our office will send you a reminder of this (60) days prior to the anniversary date.

Virginia Tech has an approved Federal Wide Assurance (FWA00000572, exp. 7/20/07) on file with OHRP, and its IRB Registration Number is IRB00000667.

cc: File
Department Reviewer: William E. Cox

**Office of Research Compliance**
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FWA00000572( expires 7/20/07)
IRB # is IRB00000667.

DATE: February 9, 2007

MEMORANDUM

TO: Andrea M. Dietrich
Pinar Omur

Approval date: 3/8/2007
Continuing Review Due Date:2/22/2008
Expiration Date: 3/7/2008

FROM: David M. Moore

SUBJECT: **IRB Expedited Continuation 2:** "Determining an Odor Standard for Water Industry" , IRB # 05-179

This memo is regarding the above referenced protocol which was previously granted expedited approval by the IRB. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. Pursuant to your request, as Chair of the Virginia Tech Institutional Review Board, I have granted approval for extension of the study for a period of 12 months, effective as of March 8, 2007.

Approval of your research by the IRB provides the appropriate review as required by federal and state laws regarding human subject research. As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.
3. Report promptly to the IRB of the study's closing (i.e., data collecting and data analysis complete at Virginia Tech). If the study is to continue past the expiration date (listed above), investigators must submit a request for continuing review prior to the continuing review due date (listed above). It is the researcher's responsibility to obtained re-approval from the IRB before the study's expiration date.
4. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

cc: File
Department Reviewer: William E. Cox

**Office of Research Compliance**
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FWA00000572( expires 1/20/2010)
IRB # is IRB00000667

DATE: February 11, 2008

MEMORANDUM

TO: Andrea M. Dietrich
Pinar Omur

Approval date: 3/8/2008
Continuing Review Due Date:2/21/2009
Expiration Date: 3/7/2009

FROM: David M. Moore

SUBJECT: **IRB Expedited Continuation 3:** "Determining an Odor Standard for Water Industry" , IRB # 05-179

This memo is regarding the above referenced protocol which was previously granted expedited approval by the IRB. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. Pursuant to your request, as Chair of the Virginia Tech Institutional Review Board, I have granted approval for extension of the study for a period of 12 months, effective as of March 8, 2008.

Approval of your research by the IRB provides the appropriate review as required by federal and state laws regarding human subject research. As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.
3. Report promptly to the IRB of the study's closing (i.e., data collecting and data analysis complete at Virginia Tech). If the study is to continue past the expiration date (listed above), investigators must submit a request for continuing review prior to the continuing review due date (listed above). It is the researcher's responsibility to obtained re-approval from the IRB before the study's expiration date.
4. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

cc: File
Department Reviewer: William E. Cox

VirginiaTech

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FWA00000572( expires 7/20/07)
IRB # is IRB00000667.

DATE: July 14, 2006

MEMORANDUM

TO: Andrea M. Dietrich
Pinar Omur

Approval date: 7/14/2006
Continuing Review Due Date:6/29/2007
Expiration Date: 7/13/2007

FROM: David M. Moore

SUBJECT: **IRB Expedited Approval:** "Mechanisms of Metallic Flavor From Drinking Water" , IRB # 06-395

This memo is regarding the above-mentioned protocol. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. As Chair of the Virginia Tech Institutional Review Board, I have granted approval to the study for a period of 12 months, effective July 14, 2006.

As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.
3. Report promptly to the IRB of the study's closing (i.e., data collecting and data analysis complete at Virginia Tech). If the study is to continue past the expiration date (listed above), investigators must submit a request for continuing review prior to the continuing review due date (listed above). It is the researcher's responsibility to obtained re-approval from the IRB before the study's expiration date.
4. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

**Important:**
If you are conducting **federally funded non-exempt research**, this approval letter must state that the IRB has compared the OSP grant application and IRB application and found the documents to be consistent. Otherwise, this approval letter is invalid for OSP to release funds. Visit our website at http://www.irb.vt.edu/pages/newstudy.htm#OSP for further information.

cc: File
Department Reviewer: William R. Knocke

**Office of Research Compliance**
Institutional Review Board
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FWA00000572( expires 1/20/2010)
IRB # is IRB00000667

DATE: May 21, 2008

MEMORANDUM

TO: Andrea M. Dietrich
Pinar Omur
Christine Sargent

FROM: David M. Moore

Approval date: 7/14/2007
Continuing Review Due Date:6/29/2008
Expiration Date: 7/13/2008

SUBJECT: **IRB Amendment 1 Approval:** "Mechanisms of Metallic Flavor From Drinking Water", OSP #455475, IRB # 06-395

This memo is regarding the above referenced protocol which was previously granted approval by the IRB on July 14, 2007. You subsequently requested permission to amend your IRB application. Since the requested amendment is nonsubstantive in nature, I, as Chair of the Virginia Tech Institutional Review Board, have granted approval for requested protocol amendment, effective as of May 21, 2008. The anniversary date will remain the same as the original approval date.

As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.
3. Report promptly to the IRB of the study's closing (i.e., data collecting and data analysis complete at Virginia Tech). If the study is to continue past the expiration date (listed above), investigators must submit a request for continuing review prior to the continuing review due date (listed above). It is the researcher's responsibility to obtain re-approval from the IRB before the study's expiration date.
4. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

cc: File

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IRB # is IRB00000667

DATE: June 16, 2008

MEMORANDUM

TO: Andrea M. Dietrich
Pinar Omur
Christine Sargent

FROM: David M. Moore

Approval date: 7/14/2008
Continuing Review Due Date:6/29/2009
Expiration Date: 7/13/2009

SUBJECT: **IRB Expedited Continuation 2:** "Mechanisms of Metallic Flavor From Drinking Water", OSP #455475, IRB # 06-395

This memo is regarding the above referenced protocol which was previously granted expedited approval by the IRB. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. Pursuant to your request, as Chair of the Virginia Tech Institutional Review Board, I have granted approval for extension of the study for a period of 12 months, effective as of July 14, 2008.

Approval of your research by the IRB provides the appropriate review as required by federal and state laws regarding human subject research. As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.
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4. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

cc: File
Department Reviewer: William R. Knocke
OSP