Biomarkers of Lipid Oxidation in the Oral Cavity

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Part I: Biomarkers of Lipid Oxidation in the Oral Cavity

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ABSTRACT

Measuring lipid oxidation is useful as a means of monitoring oxidative stress, such as that induced by clinical conditions or environmental exposure. Characteristic volatile compounds, often with low threshold odors, are secondary products of lipid oxidation reactions. Metallic flavor in food and beverages has been linked with oxidation of lipids in the oral cavity. Breath, an emerging medium for analysis of internal condition, is one means of measuring the metal-induced lipid oxidation responsible for this flavor. This project analyzes the breath of human subjects, as well as lipid oxidation of in vitro samples to identify compounds responsible for producing metallic flavor, which result from the oxidation of lipids in the oral cavity. Because these analytes are found at extremely low (picomolar to nanomolar) concentrations, preconcentration of samples prior to gas chromatography-mass spectrometry analysis is crucial. This study utilizes both solid phase microextraction (SPME) and micromachined silicon micropreconcentrators to concentrate compounds in breath to optimize analysis.

Dedication

This thesis is dedicated to Mom and Becky:
thank you for helping me get to where I am today by offering unyielding love and support during every leg of this convoluted journey.

I love you guys.

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Table of Contents

List of Figures

Part I

List of Tables

Introduction

I. Gas Chromatography Analysis and Preconcentration

Gas chromatography (GC) was developed in the 1950's as a means of analyzing temperature-stable volatile substances. This method achieves separation of components in complex mixtures, using a gaseous phase to move analytes through a liquid or solid stationary phase in packed or capillary columns. A carrier gas (high purity hydrogen, helium, or nitrogen) is used to transport the sample through the column. Columns vary in internal diameter, length, and composition of the stationary phase. The latter is chosen based on the polarity and elution temperatures of analytes of interest. Nonpolar analytes are separated based on their boiling points, while polar compounds are distinguished based on polarity. These characteristics affect compounds' affinities for the stationary phase and, consequently, the time at which they arrive at the detector (at the end of the column). These times, generally characteristic of the compound and analysis parameters (column length, carrier gas flow, etc.), are called retention times. Each detector exhibits a different level of sensitivity and linear range and responds best to varying categories of compounds. For example, organic molecules are commonly analyzed using flame ionization detectors (FID), while halide analysis incorporates an electron capture detector (ECD). Identification of analytes using such detectors requires peak comparisons with standards. Alternatively, a mass spectrometer (MS) provides the most flexibility, especially when identifying unknowns without the need for standards. Using a library search feature, an MS tentatively identifies the chemical structure of the compounds based on the mass/charge ratios of characteristic fragments produced when compounds are ionized.

With the advent of GC, detection limits were lowered from parts per million (ppm) to parts per billion (ppb), allowing for the measurement of analytes previously undetectable with any degree of certainty. Currently, detection limits of parts per trillion or lower are achievable (Miekisch et al., 2006), owing at least partly to the development of methods for preconcentrating samples prior to GC analysis. Preconcentration allows for the extraction of diffuse samples from a large volume, allowing for the detection of much lower concentrations of analytes.

The two primary forms of preconcentration are cryogenic and sorption trapping. Cryogenic concentration involves the cooling of a sample as it passes through a glass tube cooled by cryogenic fluids (e.g., liquid nitrogen) at temperatures typically between -150 and -170˚C

(Kolb, 1999). Sorption trapping utilizes an adsorbent to collect analytes as a sample is actively pumped across the adsorbent for several minutes or passively collected by the adsorbent over a period of days (Ras et al., 2009). This method relies on the phenomena of adsorption, absorption, chemical adsorption, capillary condensation, or dissolution (Alfassi and Wai, 1992).

In sorption trapping methods (the focus of this paper), the amount of sorbent surface area available correlates directly with the amount of analyte that will be adsorbed. The adsorbent is selected based on the analytes of interest. Volatile organic compounds (VOCs) are best captured based on their volatility: light VOCs (C2-C5) require cryogenic trapping, mid-range VOCs (C5-C12) would typically be captured well on carbon-based and polymer adsorbents (Tenax TA, Carbopack X, Carboxen 1021), while semi-volatiles (> C12) are adsorbed by silica gels or polyurethane foam plugs (Helmig, 1999). The adsorbent of choice may be packed into glass or stainless steel tubes, which are roughly 1-10 cm in length, (Russell 1975; Brown and Purnell, 1979; Pellizzari et al., 1976) or used to coat a 1 cm polymer fiber. The latter method, developed in 1989 (Belardi) is termed solid phase microextraction (SPME). The fiber is contained in a syringe-like holder from where it can be released and retracted, using a plunger. Sorbent tubes require a specialized apparatus for desorption, while SPME fibers can be desorbed directly in the injector port of a GC. The concentration technique choice depends on the analytes of interest, at what concentrations they are found in the sample, complexity of analysis, time, and whether the analysis is intended to be quantitative and/or qualitative.

While SPME has been popular as a preconcentrator in a variety of applications, other preconcentrators have been borne of a field aiming to decrease the size and increase the portability of the entire GC analysis process. Current bench top GC apparati have been streamlined and their size reduced significantly over the years; however, the method still has limited portability for direct use. As applications diversify, this generally requires that samples from the field be collected there and transported to the lab for analysis, which increases the risk for sample loss and contamination. Though GC miniaturization was introduced in the 1970s (Terry et al., 1979), new technologies in microfabrication have allowed the field to expand. Micromachining technology of silicon wafers has allowed for the development of micro gas chromatography (μGC) systems, including preconcentrators (Alfeeli et al., 2008; Alfeeli et al., 2009; Alfeeli and Agah, 2011; Kim and Mitra, 2003; Tian et al., 2005), separation columns (Agah et al., 2006, Ali et al., 2009), and detectors (Cruz et al., 2007, Narayanan et al., 2010) that

require little power, small sample volumes, and few consumables. Such advances will allow the technology to move toward a portable, real-time analysis method, more easily used in clinical and field settings (Miekisch et al., 2006).

Micropreconcentrators (μPCs) may be used in series with other micromachined components or implemented independently as a preconcentrating mechanism for use with bench top GCs. The devices are designed on a silicon wafer, sealed with a with Pyrex wafer. A variety of designs exist, including those with a hollowed-out microcavity packed with granular adsorbent (Tian et al., 2005) and others with etched structures coated by a thin layer of liquid adsorbent (Alfeeli and Agah, 2011; Alfeeli et al., 2008; Alfeeli et al., 2009). As previously mentioned, adsorption efficiency depends on the ability of the gaseous sample to interact with the solid adsorbent; therefore, adsorbent surface area is key to optimal performance. Device design considers type of adsorbent, adsorptive surface area, and maximization of flow to ensure optimal contact time between sample and adsorptive surfaces and maximum adsorption of desired analytes (Alfeeli et al., 2008). Preconcentrators accumulate sample over a period of time and then release them at once, in the form of a concentrated plug. Rapid desorption occurs on a heater with an extremely fast ramp rate as carrier gas flows through the μPC and onto the column for analysis (Alfeeli and Agah, 2011; Alfeeli et al., 2008). This provides a detectable amount of even diffuse, low concentration analytes and introduces the sample into the GC in a way that enhances separation. The use of paired concentrators has further allowed for the removal of unwanted analytes, while also significantly reducing water vapor from samples (Cho et al., 2005; Alfeeli et al., 2009).

II. Breath Analysis

One application for the use of GC that often requires preconcentration of samples is that of breath analysis (Vereb et al. 2011). Pauling et al. (1971) used GC to identify 250 compounds in human breath. In the last few decades, uses of breath analysis by GC have diversified to include monitoring clinical conditions (Song et al., 2010; Deng et al., 2004; Van den Velde et al., 2008; Aghdassi et al., 2000), measuring occupational exposures (Chen et al., 2002; Engstrom et al., 1978; Ghittori et al., 2004; Perbellini et al., 2003), and evaluating exposure to compounds in the course of daily life (Egeghy et al., 2000; Kim, 2008; Park and Jo, 2004; Schreiber et al., 2002).

Breath is a complex matrix dominated by common gases (e.g., CO_2, N_2, H_2O, etc.) interspersed among hundreds to thousands of other compounds, including those produced within the body (Risby and Solga, 2006) and as those internalized from the environment (Egeghy et al., 2003; Kim, 2008). Breath from various portions of the respiratory system has differing value for researchers. Studies of flavor (Denker et al., 2006) or conditions of the mouth (Phillips et al., 2005; Van den Velde et al., 2007b) may focus on the first 150 – 200 mL of an exhaled breath, which represents the volume of air occupying the oral cavity and, potentially, the nasal passages. Procedures focusing on breath as an indication of the internal environment, however, are much more likely to discard such "dead space" air in favor of the end-tidal volumes. This air comes from deep within the lungs where it has contacted the blood at the diffusive barrier of the alveoli. Thus, it is more representative of internal conditions (Mendis et al., 1994; Van den Velde et al., 2007).

Breath analysis does present its own challenges. The low concentration of analytes, hundreds of unique compounds present (Phillips et al., 1997; Van den Velde et al., 2007), and high humidity (10,000s of ppm) (Cho et al., 2006) of breath can make it difficult to extract specific analytes of interest. Unwanted air from other sections of the respiratory system has the potential to dilute the sample of interest and complicate results. Further, many of the compounds are found in ambient air at similar concentrations (Larsted et al., 2006; Svensson et al., 2007). Consequently, such background levels must be accounted for in a consistent, reliable manner (Kim, 2008; Phillips, 1997; Qin et al., 2006; Van den Velde et al. 2007).

Though many challenges still exist with widespread application of breath analysis (Vereb et al., 2011), breath is increasingly used as a matrix of measuring subjects' dosage of various agents (Chen et al., 2002; Egeghy et al., 2003; Engstrom et al., 1978; Fantuzzi et al., 2000; Ghittori et al., 2004; Ong et al., 1991). Studies have shown that breath and blood concentrations correlate (Chen et al., 2002; Engstrom et al., 1978). Breath offers a less invasive alternative to traditional blood sampling methods for analyzing internal conditions.

III. Lipid Oxidation

Once refined, breath analysis will be valuable in a variety of fields. Common to several of these, including monitoring disease state and environmental exposure, is an interest in measuring products of oxidative stress. Oxidative stress is a condition that results when there is an imbalance between radical oxygen species (responsible for initiating lipid oxidation) and

antioxidant processes in the body (Gille and Joenje, 1991). Oxidative stress is measurable by the level of lipid oxidation occurring in an individual. Lipid oxidation is a chain reaction, which involves three phases: initiation, propagation, and termination.

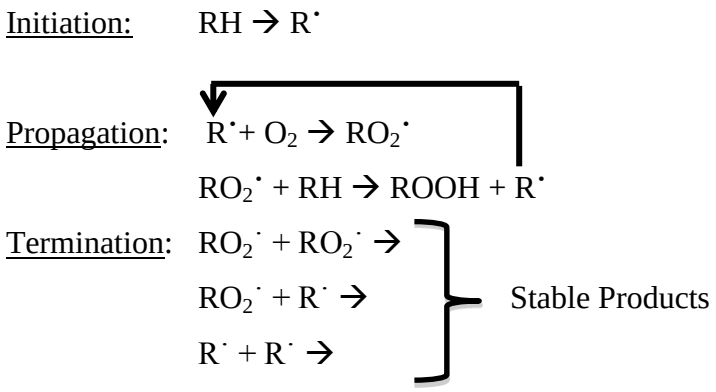
Radicals are chemical species with unpaired electrons. Though highly unstable, such species are able to pull hydrogen atoms from other molecules, initiating a chain reaction that can lead to cell damage. Radicals may be generated by a variety of mechanisms, including light, heat, redox reactions, and transition metals. Though the nature of this initial radical formation is not completely understood, it is thought that transition metals reduce oxygen, forming the superoxide radical ($O_2^{\cdot -}$), as follows:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -}$$

Superoxide reacts with hydrogen ions and quickly forms hydrogen peroxide (H_2O_2), which reacts with ferrous iron, via the Fenton reaction, to form the hydroxyl radical ($^{\bullet}OH$). The Fenton reaction ($M + A\text{-}B \rightarrow M^+ + A^- + B^{\bullet}$) describes the mechanism by which this occurs. Though $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$ are all capable of oxidizing other molecules, the hydroxyl radical is the most reactive of the three and thought to be primarily responsible for lipid oxidation. Likely, the main role of superoxide and hydrogen peroxide in oxidation is their involvement in the formation of hydroxyl radicals. (Symons and Gutteridge, 1998)

Lipid oxidation results when these radicals react with the fatty acid side chains of cell membrane lipids (Gille and Joenje, 1991). Cell membranes consist of lipids, protein, and oligosaccharides (Singer and Nicholson, 1972). Membrane fluidity, critical for adequate cell function, is maintained by PUFA side chains of membrane lipids (Catala, 2008). Of the fatty acids, long hydrocarbon chains with a carboxyl group at one end, unsaturated fatty acids are more prone to such reactions than saturated fatty acids. Susceptibility to oxidation increases with the number of double bonds in a molecule because the attraction for allylic hydrogen atoms on the former is weakened by the adjacent double bond, making abstraction easier. Radicals, such as $^{\bullet}OH$ remove allylic hydrogen atoms from lipids to form a carbon-centered radical on the fatty acid. This fatty acid radical reacts with molecular oxygen, forming lipid hydroperoxides. These products can then further react with iron, generating more radicals and perpetuating a chain of oxidation reactions. (Symons and Gutteridge, 1998)

The three phases of this reaction, initiation, propagation, and termination, are summarized below (Gunstone, 1996):



Hydroperoxides may decompose quickly to form odorous secondary products, including a variety of alkanes, aldehydes, ketones, alcohols, acids, and esters (Mallia et al., 2009; Meynier et al., 1998; Selke et al., 1971; Withycombe et al., 1971). Though the amounts of products created are minute, picograms per liter (pg/L) to nanograms per liter (ng/L) (Fuchs et al., 2009; Kanoh et al., 2005; Larsted et al., 2002; Van den Velde et al., 2007, Van den Velde et al., 2007b), their sensory threshold in humans is low enough that they are easily sensed (Gunstone, 1996).

Different types of epithelia and, consequently, different parts of the body, have characteristic fatty acid composition. Table 1 shows that the lining of the oral cavity, including the insides of the cheek, tongue, and gums, predominantly consists of oleic acid, palmitic acid, and linoleic acid. Of these, oleic and linoleic acids are of particular interest in relation to lipid oxidation studies because their double bond(s) make them more susceptible to the hydrogen abstraction that initiates the chain reaction. Arachadonic acid is also known to produce odorous lipid oxidation products when oxidized.

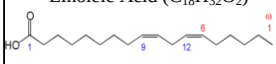
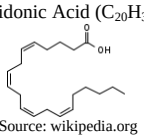
Table 1 - Common Fatty Acids in the Oral Cavity and their Relative Proportions

#of C atoms: # double bonds	Fatty Acid	Relative Proportion +/- std error of mean
C18:1	Oleic Acid	43.80% +/- 1.08
C18:2	Linoleic Acid	10.01% +/- 0.32
C20:4	Arachadonic Acid	1.6% +/- 0.26
C14:0	Myristic Acid	2.04% +/- 0.36
C16:0	Palmitic Acid	21.98 +/- 1.04
C16:1	Palmitoleic Acid	8.81% +/- 0.93
C18:0	Stearic Acid	3.70% +/- 0.11
C22:6	Docosahexanoic Acid	1.77% +/- 0.27

Source: MacLeod et al., 1990

Depending on the fatty acid being oxidized, different characteristic lipid oxidation compounds are produced. Table 2 highlights the categories of products from the oxidation of oleic acid, linoleic acid, and arachadonic acid, respectively, as well as odors associated with compounds in each category and the thresholds of such odors.

Table 2 - Odorous Lipid Oxidation Products Created from the Oxidation of Different Fatty Acids

Fatty Acid (Chemical Formula)	Autoxidation Products	Representative Compounds With Known Odor Properties		
		Compound	Odor Description	Threshold in Water (ppm)
Oleic Acid ($C_{18}H_{34}O_2$)  Source: wikipedia.org	C6-8 Hydrocarbons C2-11 Alkanals C6-11 Alkenals C1, 6-9 Acids C5-8 C2-8 Alkylformates	• Octanal • Nonanal • Decanal	• fatty, soapy, fruity • soapy, fruity • orange peels	• 0.0007 • 0.001 • 0.001
Linoleic Acid ($C_{18}H_{32}O_2$)  Source: wikipedia.org	C3-5 Hydrocarbons C3-8 Alkanals C7-10 Alkenals C9-10 Alkadienals C6-9 Acids C5-8 Alkanols C2-8 Alkylformates	• Hexanal • Heptanal • Trans-2-heptenal	• green, fruity, bitter, almond • oil, putty • putty, fatty, bitter, almond	• 0.008 • 0.003 • 0.051
Arachidonic Acid ($C_{20}H_{32}O_2$)  Source: wikipedia.org	C5-6 Alkanals C7-10Alkenals C10, 12 Alkadienals C13 Alkatrienal C8 Alkenone C11 Alkadienone	• Pentanal • Trans-2-Nonenal • 1-octen-3-one	• sharp, bitter, almond • tallowy, cucumber, startch, glue • mouldy, mushroom, metallic	• 0.012 • 0.0008 • $8.9X10^{-5}$

Source: Grosch, 1987

Lipid oxidation products are known to be produced during periods of oxidative stress and any time lipids are oxidized. As Table 3 shows (italicized compounds are lipid oxidation

products), the average person has a certain level of background oxidation, measurable in breath, even when healthy.

Table 3 - Compounds Found in Breath of Healthy Individuals (Lipid Oxidation Products in *Italicized* Text)

% of 40 subjects, mean age 41yr	*Compounds Present*
100	2-methyl-butane ethanol acetone isoprene ***2-propanol*** ethyl acetate cyclohexane benzene heptane methyl cyclohexane toluene octane ethylbenzene p-xylene styrene nonane ***heptanal***
97.5	chloroform 2-pentanone 1-methylthio-propane
95	butane ***1-propanol*** 2-methyl-furan
92.5	dimethyl ester carbonic acid 3-methyl-thiophene cyclohexanone
90	2-methyl hexane
85	acetaldehyde dimethoxy methane
80	***2-methyl pentane*** 2-butanone
62.5	***hexane***
40	a-pinene benzaldehyde B-pinene D-limonene ***2-ethyl-1-hexanol*** methyl ester benzoic acid undecane ***nonanal*** 4-trimethyl-3cyclohexene-1-methanol dodecane ***decanal*** methenamine tridecane tetradecane butylated hydroxytoluene pentadecane diethyl phthalate phenol ***octanal*** ***hexanal*** acetophenone decane naphthalene 3-methyl hexane menthol indole

Source: Van den Velde et al., 2007

IV. Lipid Oxidation and Metallic Flavor

Metal-catalyzed lipid oxidation has been a focus of the food industry for years because the odorous end-products can impart off-flavors to foods and beverages. Another area of interest is within the drinking water industry. Tap water, transported through metal pipes and dispensed from metal faucets, is prone to carrying metal ions with it to the consumer's tap. Changes in the taste and/or odor of tap water are perceived by consumers as different from the norm and often feared to be a threat to public safety, which may be detrimental to the consumer's confidence in their local drinking water authority. Sources of such flavors may be from conditions at the water source, actions taken during treatment, or alterations resulting from the nature of transport and storage. Increases in metallic flavor are likely to stem from the latter, especially corrosion of pipes (Dietrich, 2006). USEPA drinking water standards for iron are set at 0.3 mg/L for aesthetic reasons. However, taste thresholds for solutions of the metal have been identified well below this standard, which can make for an unpleasant drinking experience at levels well below the (voluntary) secondary standard. Further, incidence of a persistent metallic flavor is commonly reported among cancer patients undergoing chemotherapy and radiation treatment (Comeau et al., 2001; Hong et al., 2009). Unfortunately, the perception of the metallic sensation is not yet completely understood.

Senses of taste and smell operate through the detection of chemicals by cells, hence the name "chemical senses." The term flavor is often used interchangeably with the word taste; however, this sensation technically results from the combination of taste perceptions on the tongue and odor perceptions. Odorants may be both inhaled through the nostrils (orthonasally) and detected as they pass through the nasopharynx from the mouth (retronasally) (Dietrich, 2009). "Flavor" of consumed products typically comes from the latter (Shepherd, 2006).

Significant study has been conducted to determine the extent to which metallic sensations are tastes or flavors and at what concentrations metallic salts can be detected. Compounds sensed without the nose occluded but not sensed with the nose occluded likely have a retronasal component. Nasal occlusion inhibits retronasal perception of odors, as exhalation through the nose is what allows gases to enter the retronasal passage from the oral cavity (Shepherd, 2006). Omur-Ozbek and Dietrich (2011) measured detection limits of iron and copper solutions without noses occluded. The threshold, based on the geometric mean, ranged from 0.003 to > 5 mg/L

Fe^{2+} with a population thresholds of 0.052 mg/L Fe^{2+}. No metallic sensation was reported by subjects with occluded noses, however, even at concentrations of 20 mg/L Fe^{2+}. Epke and Lawless (2007) likewise found that nasal occlusion increased the flavor threshold of $FeSO_4$ in subjects by more than a factor of five (30 μM vs. 160 μM), even though it was ranked as the most metallic (compared with copper and zinc salts) when tasted with the nose open (Lawless et al., 2004). The appearance of the metallic flavor upon removal of the nose clips (Omur-Ozbek and Dietrich, 2011) substantiates that the reaction producing the odor has occurred and further supports the notion that the metallic flavor is perceived primarily retronasally. At much higher concentrations (0.05 M), subjects could identify $FeSO_4$ as different from water, even with nose occluded, meaning (at excessively high concentrations) there may be a taste sensation (often bitterness) accompanying what is predominantly recognized as a flavor reliant on retronasal odors (Lim and Lawless, 2005). Tests conducted by placing the metal between the gum and the lip showed no tactile (mouthfeel) perception of the metal (astringency) (Lim and Lawless, 2005), though panelists with noses open reported both bitter tastes and astringent mouthfeel associated with ferrous salts (Omur-Ozbek and Dietrich, 2011). There may be a true chemical reaction-induced taste associated with $FeSO_4$ at such high concentrations. At even higher concentrations (10 mM), $FeSO_4$ activates TRPV1 taste receptors, which detect, among other things, bitterness from capsaicin, the compound that gives hot peppers their "heat" (Riera et al., 2007).

Though it may not be the only mechanism by which metallic flavors are sensed, the retronasal component is strongly supported (Epke and Lawless, 2007; Hettinger et al., 1990; Lawless et al., 2004; Omur-Ozbek and Dietrich, 2011). To ensure that nasal occlusion didn't simply eliminate the possibility of sensing the metallic odor orthonasally, Lawless et al., (2004) showed that there was negligible metallic smell when the headspace over $FeSO_4$ solutions was "sniffed," suggesting that odorous compounds are either produced in the mouth through metal-catalyzed lipid oxidation or that the increased temperature within the mouth further volatilizes the odorous compounds, making them detectable retronasally. Glindemann et al. (2006) supported the idea that the metallic odors accompanying ferrous solutions are created by reactions with skin cells. They found that contact between body skin, artificial sweat, and solid or ferrous solutions of iron resulted in the production of aldehydes and ketones, including *n*-hexanal (grassy odor) and 1-octen-3-one (mushroom odor), the latter of which accounted for about a third of the metallic odor emanating from the reaction site. It was deduced that sweat

corrodes solid metal to form ferrous iron, which quickly oxidizes to ferric iron. In the process, skin lipid peroxides, formed from skin lipids, are decomposed to odorous carbonyls. This is supported by an examination of increases in odor, which shows the odorous compounds increased predictably to a point at which the lipid peroxides were consumed. Many recognized lipid oxidation products have characteristic odors which could contribute metallic odor and, consequently, flavor. The odor thresholds of such are quite low (Table 4), so very small concentrations would be required to induce retronasal odor detection by most individuals.

Table 4 - Odor Thresholds in Air of Lipid Oxidation Products Detected in this Study

Compound	Odor Descriptor	Odor Threshold in Air ($\mu g/L_{air}$)
1-pentanol	plastic	0.153[a]
2,4-decadienal	fried, powerful , fatty, citrus	0.0023 [a]
2,4-nonadienal	nutty, fatty	0.0002 [a]
2-nonenal	grass	0.00009 [a]
2-octenal	fatty, walnuts	0.0027 [a]
2-pentylfuran	buttery, beany, rancid, metallic, vegetable	0.019 [a]
heptanal	soapy	0.023[b]
hexanal	green, fruity, bitter, almond, fatty, grassy	0.14[c]
nonanal	soapy, citrus, floral, orange, fatty, waxy	0.0134 [b]
octanal	almond, fat, soapy, fruity, citrus	0.0072 [b]
pentanal	fat, green, sharp, bitter, almond, woody, vanilla, fruity	0.022 [b]
propanal	sharp, pungent	0.065 [b]

[a]Yang et al., 2008; [b] Hyttinen et al., 2007; [c]Omur-Ozbek and Dietrich, 2011

Evidence indicates that odorous secondary lipid oxidation products play a role in the perception of metallic flavor. Historically, lipid oxidation has commonly been measured by the TBARS (thiobarbituac acid reactive substance) method, which colorimetrically quantifies malondialdehyde, an indicator of oxidation, in saliva. Increased metallic odor and a corresponding increase in oral lipid oxidation, as measured by TBARS analysis of saliva, were

demonstrated when subjects swished a ferrous iron solution in their mouth and expectorated (Mirlohi et al., 2011).

TBARS analysis takes significant time and effort in the laboratory and production of the amount of saliva needed can be difficult for both healthy and unhealthy subjects. Further, TBARS also measures protein oxidation products which may or may not play a role in producing metallic flavor. Because gaseous lipid oxidation products are produced in the oral cavity, breath may provide a simpler, more specific assay of the level of lipid oxidation in the mouth. This paper outlines the development of a method for comparing preconcentrator methods (SPME and μPCs) as a means of using breath to monitor iron catalyzed lipid oxidation in the oral cavity.

Methods

As with any novel approach, this procedure required a significant amount of method development. The methods described below represent the conditions (sample volumes, ferrous solution concentrations, loading times, etc.) in place at the completion of the project. Many of these were arrived at based on experiments conducted along the way. Appendix A includes schematics, which outline how such procedural specifics were arrived at, as applicable.

Oral Breath Sampling

Subjects were selected from among volunteer graduate students at Virginia Polytechnic Institute and State University and ranged in age from 22-34 years of age. The procedure was approved by the Institutional Review Board, and all subjects submitted signed informed consent releases. Two sample bags were designated for each subject: one control and one metal and were reused for the extent of sampling. Bags were in sealed mason jars between uses to prevent contamination from compounds found in ambient air.

Ferrous iron solution was made by dissolving $Fe_2(SO_4)_3$ (Fischer Scientific) in Aquafina brand bottled water. Aquafina is similar to distilled water as it is produced by reverse osmosis and no minerals are added. Solutions were created, 50 mL at a time, in plastic 50 mL centrifuge tubes. Solutions were made fresh daily, as the ferrous iron would oxidize to ferric iron. Experiments used a concentration of 80 mg/L as iron by adding Aquafina to 10 mg $Fe_2(SO_4)$ in a 50 mL centrifuge tube.

Figure 1 - Reduced Volume Sample Bag

Figure 2 - Reduced Volume Sample Bag from Side with Valve

To reduce its volume, an inert 250 ml Quintron breath collection bag was folded in half, width wise, and secured with binder clips along the crease. The large opening was easily accessible for the subject (Figure 1). A plastic valve (provided with the bags), in the closed position, was inserted into the smaller opening on the opposite side of the bag (Figure 2). This provided about a 150 ml volume which is roughly equivalent to the volume of the oral cavity.

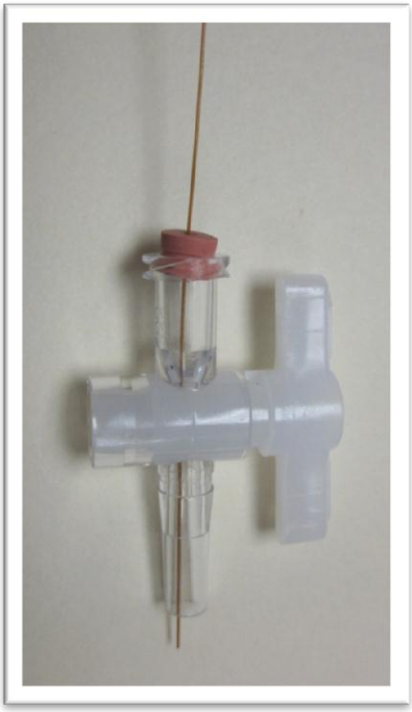


Figure 3 - µPC Capillary Tubing Inserted through Septum in Second Valve

The subject was provided with the modified bag (large blue cap removed), a nose clip, and a small cup containing 0.5 mL of 80 mg/L ferrous metallic solution (40 µg Fe^{2+}) or control (Aquafina water) sample. Prior to sampling, the procedure was explained and illustrated for the subject and any questions were answered. Before sample collection, the subject was asked to take several deep breaths and, when prepared, place the nose clip on his nose. When ready, s/he took the sample into his/her mouth, ideally while taking a deep breath, at which point he/she began holding his/her breath. Upon taking the sample from the cup, s/he was instructed to move it around his mouth with his tongue in an attempt to maximize its contact with the soft tissues of the oral cavity.

The subject was asked to hold his/her breath for one minute. At the conclusion of one minute, with nose clip still in place, the subject was asked to

breathe into the sample bag, through the larger hole, inflating the bag. The subject was asked to attempt to collect only the first portion of the breath, that from the oral cavity, in the bag. This may or may not entirely inflate the modified bag. The cap was then replaced on the large opening of the sample bag.

The sample was then drawn from the small valve on the opposite side of the bag. SPME samples were collected first. The tip of the SPME fiber assembly was inserted through a septum in the larger end of a *second* plastic valve (Figure 3). This valve was nested into the valve already in place allowed for easier concentration without loss or contamination of sample. Both plastic valves were opened, and the SPME fiber was inserted into the bag, where it was left to collect sample for 10 minutes before the sample was analyzed, as described below.

From the same bag, the sample was then concentrated using a micropreconcentrator (µPC). The capillary tubing from one end of the µPC was threaded through a septum* in the larger end of a *second* plastic valve (Figure 3). The tip of the capillary tubing was then removed with a ceramic cutter to ensure it was not clogged. This valve was nested into the valve already in place and allowed for easier concentration without loss or contamination of sample.

Figure 4 - µPC Capillary Tubing Inserted through Nested Valves, in Preparation for Sample Extraction from Bag

When prepared to concentrate the sample, the valve in the bag was opened, and the capillary tubing threaded through both valves, so the end of the tubing was close to the opening in the bag (Figure 4). The capillary tubing on the other end of the µPC was fed through a septum in a tube attached to a vacuum pump. The sample was drawn across the µPC, using the vacuum pump, until the bag was emptied. Knowing the flow rate of the pump and the amount of time this took to load the sample allowed for quantification of the volume collected. Once loaded, the sample was analyzed as described below.

In Vitro Oxidation of Linoleic Acid

Early in vitro sampling was conducted using modified 500 mL Erlenmeyer flaks. A 25 mL scintillation vial with screw-on rubber septum cap was annealed to the side of each flask (See Figure 5). Later trials were conducted in 60 mL amber glass vials with screw-on rubber septum caps.

Figure 5 - Modified Erlenmeyer Flasks Used for Some In Vitro Sample Collection

In an attempt to minimize oxidation, linoleic acid was stored in its original amber-colored glass container. Air in the container was always displaced with high purity (99.99%) liquid nitrogen before storage, and the bottle was stored at 4˚C. Three vials were prepared at a time, as follows. Through the septum in the cap of the vial were threaded two capillary tubes: one connected to a vacuum pump and the other an inert bag filled with high purity nitrogen. A volume of 0.5 mL of linoleic acid (Acros Organics) was added to a vial, the cap replaced, and the air pumped from it (and simultaneously replaced with high purity nitrogen). Vials were stored at 4˚ C until use.

During sample concentration, vials were placed in a water bath set at 40˚C. Control samples were taken from the headspace above the linoleic acid. SPME concentration was conducted first by placing the SPME fiber into the container's headspace, through the septum cap, for 10 minutes. The sample was then analyzed as described below. For µPC loading, two pieces of capillary tubing were inserted in the septum opening of the glassware. One was connected to a vacuum pump and the other an inert bag filled with high purity nitrogen, which replaced the sample gases in the glassware as they were drawn through the µPC. The sample was drawn across the µPC, for 10 minutes.

For experimental samples, iron solution was prepared as described above. After quickly uncapping the vial, 1 mL of solution was added to the linoleic acid. The cap was quickly replaced, and the headspace was then concentrated by SPME and µPC, respectively, as described above.

Some samples incorporated an internal standard as a reference. The standard chosen was an EPA 624 Surrogate Standard (Restek), containing fluorobenzene, pentafluorobenzene, and 1-

bromo-2-fluorobenzene. The standard was diluted from 2000 µg/L to 200 µg/L in methanol and stored in 25 mL GC vials with crimp caps, placed in a sealed mason jar at ~0 ˚C . Before each use, the standard was further diluted to 20 ng/L in Nanopure water, over heat.

The standard was loaded onto each concentrator from the aforementioned modified flasks. A volume of 0.5 mL was added to the flask, and the respective concentrator inserted through the flask's septum. The standard was then flash-heated with a hair dryer for one minute; the concentrator was left in for an additional four minutes to load the standard before the sample was analyzed.

Sample Analysis

Prior to sample concentration concentrators were conditioned to remove any compounds collected between uses. SPME fibers were conditioned at a temperature of 250°C for 30 minutes. Micropreconcentrators were heated to temperature of 200°C with carrier gas flowing through them and left to be conditioned for at least ten minutes.

Samples were analyzed using a gas-chromatography-mass-spectrometry (GC-MS) system with a DB- 17 MS 30 m fused-silica capillary column. SPME samples were desorbed in the GC injection port for 3 minutes. Samples was desorbed from the µPC on a ceramic heater at a temperature of 200°C, while carrier gas flowed through the µPC and then onto the column. The GC was programmed as follows: initial temperature of 33°C, held for six minutes, followed by a ramp rate of 2°C/min to a final temperature of 175°C, which was held for 2 minutes. The carrier gas flow was set to 1.2 mL/min.

Results

The MS software associated with the GC-MS (Xcalibur by Thermo Fisher Scientific) provided library search reports to aid in preliminary identification of compounds in samples. Library search reports contained lists of compounds, matched from the library, based on spectra for each identified peak. Three matches were listed for each peak and ranked by a value measuring the level of confidence of the match. Two confidence values were included for each match: the SI value is based on an examination of all spectra in the sample, while the RSI calculates the confidence of the match based solely on spectra found within the library; excluding unrecognized spectra often provides a higher confidence interval. Any compounds listed among the top three matches for each retention time were included.

Tentative identification, based on MS library search, revealed hundreds of compounds per report. Through a process of systematic data reduction, reports were reviewed to identify lipid oxidation products listed therein. A list of possible lipid oxidation products used for this search was compiled from the multiple tables (and sources cited) in Grosch (1987), a comprehensive review of products from hydroperoxide reactions. Because some lipid oxidation product categories in this reference (and the primary sources cited) were ambiguous stating, for example, that C3-C6 hydrocarbons were found as lipid oxidation products, branched compounds were included in the original data list if they contained the number of carbon atoms specified as being present in lipid oxidation products in the literature. Further, among the data were many branched alkanes with a low carbon number. Though Kneepkins reported in 1994 that "hydrocarbons implicated in lipid peroxidation are exclusively alkanes or alkenes with straight chains," individual literature searches were conducted on commonly found branched alkanes to determine if they had been identified as lipid oxidation products in any studies. If no relevant papers indicated they had been identified as lipid oxidation products, they were excluded from the data.

Lipid oxidation products made up a relatively small percentage ($\leq 20\%$) of compounds listed in these reports. Other compounds commonly found include aromatics (benzene, toluene, ethyl-methyl benzene), aforementioned branched alkanes, and long chain branched and linear alkanes (C >10). Though documented in the literature as being present in the breath (Phillips et al., 1999; Van den Velde et al., 2007), they were excluded from this analysis, as it focused solely on lipid oxidation products. From all library search reports reviewed, a total of 33 lipid oxidation products were found (Table 5); roughly 40% of these have odors described in the literature (Table 6). The majority of these compounds were identified qualitatively; however, hexanal, octanal, and nonanal were quantitatively analyzed by means of standard curves (see Figure 16). In the chromatograms that follow, each compound is labeled using the same number. These numbers are listed in Table 5. Though all compounds were detected at least once during this study, not all were present in the chromatograms included in this report; therefore, not every compound listed in Table 5 has a "Chromatogram Peak Label Number."

Table 5 - Lipid Oxidation Products Found in this Study and the Numbers Used to Label them in Chromatograms throughout this Report

Compound	Reference	Chromatogram Peak Label Number
1-butanol	a	
1-butanol, 2-methyl	b,c	
1-heptene	d	2
1-hexanol, 2-ethyl	e	10
1-pentanol	f,g,h	
2,4-decadienal	i	
2,4-nonadienal, (E,E)	j	19
2-decenal, (E)	k	13
2-heptanol	l	
2-heptanone, 4-methyl	k	
2-heptenal (E)	a,m	16
2-heptenal (Z)	a,g,h,i,m,n,o	23
2-nonenal (E)	m	
2-octenal, (E)	i,p	11
3-heptanone, 4-methyl	k	
3-heptanone, 6-methyl	k	
3-nonene	k	5
butanal, 2-methyl	b	7
cis-4-nonene	k	
Furan, 2-pentyl	h,k,p,o	18
Heptanal	k,f,g,p	17
heptane	k	3
hexanal	i	8
hexane	k,l	1
nonanal	k	20
nonanoic acid	p	
octanal	k,p	9
octane	k	4
pentanal	i	12
pentane	a,q	6
pentane, 2-methyl	k	
propanal	a,f,m	14

a)Horvat et al., 1965; b)Giri et al., 2011; c)Vasta et al., 2010; d)Jo and Ahn, 2000; e)Larick et al., 1992; f)Loury and Forney, 1968 a,b; g)Eriksson, 1970; h)Eriksson et al., 1973; i)Badings, 1970; j)Meynier et al., 1998; k)Selke et al., 1977; l)Loury et al., 1972; m)Ellis et al., 1968; n)Hoffman, 1962; o)Tressl et al., 1981; p)Horvat et al., 1969; q)Horvat et al., 1964

Table 6 - Odors of Lipid Oxidation Products Found in this Study

Compound	Odor
1-pentanol	plastic[7]
2,4-decadienal	citrus[2], fatty[2], fried[1], powerful[2]
2,4-nonadienal, (E,E)	nutty[7], fatty[7]
2-heptanol	earthy[2], oily[2]
2-heptenal (Z)	almond[4], bitter[4], fatty[1], green[2], putty[1]
2-nonenal (E)	grass[3]
2-octenal, (E)	fatty[1],walnuts[1]
Furan, 2-pentyl	beany[5],buttery[5], green bean[2], metallic[2], rancid[5], vegetable[2]
Heptanal	soapy[3]
hexanal	almond[6], bitter[6], fatty[2], fruity[6], grassy[2], green[2,6]
nonanal	citrus[2,3], fatty[2], floral[2], orange[2], rose[2], soapy[3], tallowy[1], waxy[2]
nonanoic acid	cheese[2], waxy[2]
octanal	almond[3], citrus[2], fat[2,3], fruity[6], soapy[6]
pentanal	almond[6], bitter[6], fat[3], fruity[2], green[3], sharp[4], vanilla[2], woody[2]
propanal	pungent[2], sharp[2]

[1] Badings, 1970; [2]Sigma Aldrich; [3]Malia et al., 2009; [4]Meijboom and Jogenotter, 1981; [5]Evans et al. 1971; [6]Schnabel, 1982; [7]Yang et al., 2008.

The goal of this study was to identify products resulting from metal-catalyzed lipid oxidation in the mouth. However, some lipid oxidation products are found in ambient air and there may be some level of background oxidation in subjects. Therefore, it was important to consider the differences between samples taken "Before" (control) and "After" metal dosage. Tables 5-9 quantitatively summarize these data. Each table lists the number of compounds in samples collected "Before" the addition of Fe^{2+}, "After" Fe^{2+} addition, and the number of compounds found *only* After" Fe^{2+} addition, not "Before." Because, often, compounds found "Before" were not always found After" Fe^{2+} addition, the value in the third column may not equal the difference between the other two. No matter the method of preconcentration or the source of the sample (breath or in vitro), most control samples contained at least a few lipid oxidation products. In most cases, though, there were more lipid oxidation products and at least one unique product measured after the addition of metal to the sample.

Table 7 shows a composite of all breath data combined. It highlights the superiority of µPCs over SPME in concentrating lipid oxidation products in breath samples. From the latter, only two lipid oxidation compounds were found in all breath trials. This performance disparity is easily seen in Figure 6, where the SPME chromatogram has only a few peaks, which are actually standards intentionally added to the sample. The DVB and Tenax µPCs appeared to

have roughly the same success at concentrating lipid oxidation products, though, as will be discussed below, they tend to concentrate different compounds.

Table 7 - Total Lipid Oxidation (LO) Products in all Subjects' Breath (Before and After Fe^{2+} Metal Exposure), Arranged by Method of Sample Preconcentration

Method	Matrix	# LO products BEFORE	# LO products AFTER	# unique LO products AFTER
DVB packed µPC	Breath	9	11	4
Tenax etched µPC	Breath	8	12	6
Tenax SPME	Breath	0	1	1
PDMS-DVB SPME	Breath	0	2	2

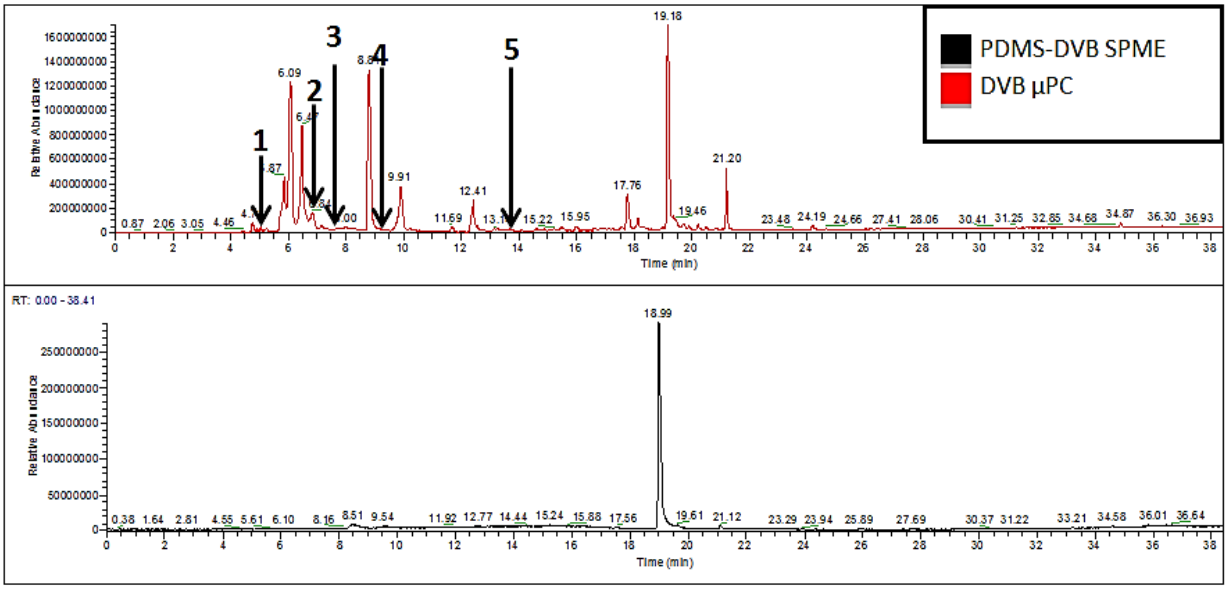


Figure 6 – Chromatograms of a Single "After Fe^{2+}" Breath Sample Concentrated by a PDMS-DVB SPME and a Packed DVB µPC
Lipid Oxidation Products (Retention time): µPC: [1]hexane (5.06); [2] 1-heptene (6.84); [3] heptane (7.36); [4] octane (9.40); [5] 3-nonene (13.66). SPME: none.

Table 8 summarizes composite data by subject. It should be noted that not all subjects provided the same number of samples, which could skew these data. This may explain why more lipid oxidation products were found in some individuals than others.

Table 8 - Total Lipid Oxidation (LO) Products in Different Subjects' Breath (Before and After Metal Exposure), Arranged by Subject and Preconcentration Method

Method	Subject	# LO products BEFORE	# LO products AFTER	# unique LO products AFTER
DVB packed µPC	A	9	12	5
DVB packed µPC	H	3	2	0
DVB packed µPC	J	4	6	2
Tenax etched µPC	B	6	4	1
Tenax etched µPC	H	4	10	6
Tenax etched µPC	J	6	4	1

Table 9 examines six individual breath samples, three each taken from two subjects. Roughly the same numbers of lipid oxidation products are found in each subject over the course of several samplings. An examination of specific compounds (below) is necessary to determine the true intra-individual reproducibility of the procedure.

Table 9 - Lipid Oxidation (LO) Products in Two Subjects' Breath (Before and After Metal Exposure) Over Three Separate Sampling Periods, Arranged by Sample

Method	Subject	# LO products BEFORE	# LO products AFTER	# unique LO products AFTER
DVB packed µPC	A	4	5	3
DVB packed µPC	A	4	3	2
DVB packed µPC	A	2	4	2
Tenax etched µPC	H	1	4	4
Tenax etched µPC	H	3	4	1
Tenax etched µPC	H	2	2	2

In vitro studies of lipid oxidation are summarized in Table 10. These studies were conducted in an attempt to control challenges with breath sampling, including the complex matrix of breath, collection, background oxidation, and inter-subject variation. It should be noted

that different numbers of samples were taken using each method, which could skew these data. SPME was more successful in in vitro than breath samples; however, the DVB µPC still outperformed SPME (Figure 7). Overall, there were more unique lipid oxidation products identified in in vitro studies than in breath studies. Despite extensive efforts to eliminate or control background oxidation in in vitro studies, it is apparent that there was still some level of oxidation before the addition of metal.

Table 10 - Lipid Oxidation (LO) Products from Linoleic Acid In Vitro Studies (Before and After Metal Exposure), Arranged by Preconcentration Method

Method	# LO products BEFORE	# LO products AFTER	# unique LO products AFTER
DVB packed µPC	7	16	11
Tenax packed µPC	N/A (leak)	5	5
Tenax etched µPC	2	4	3
PDMS-DVB SPME	5	10	6

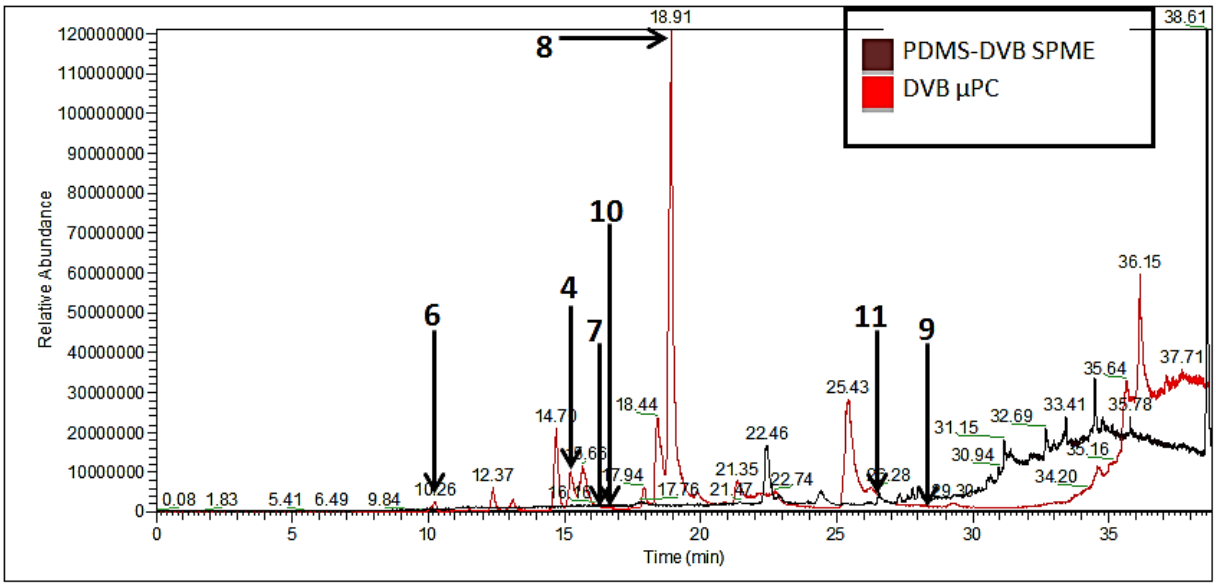


Figure 7 - Chromatogram Overlay of One "After Fe^{2+}" Linoleic Acid In vitro Sample Concentrated by a PDMS-DVB SPME and a Packed DVB µPC
Lipid Oxidation Products (Retention time): µPC: [6]pentane (10.23); [4] octane (15.21); [7] 2-methyl butanal (16.14); [8] hexanal (18.92); [9]octanal (28.14). SPME: [10] 2-ethyl-1-hexanal (16.48); [11] 2-octenal (E) (26.54)

To further ascertain data reproducibility and value, lipid oxidation data were organized based on sampling method (human breath or in vitro), concentrating mechanism (SPME, packed µPC, or etched µPC), and adsorbent (Tenax TA or PDMS) (Table 11). Human breath data were further broken down by subject to examine intra- and inter-subject variation (Tables 12 and 13). An "X" in a cell indicates that the compound was found, at least once, in the specific type of trial listed above. Highlighted "X's" represent compounds found in the "After" but not "Before" trials for that specific set of conditions. Raw data can be found in Appendices B and C.

Table 11 provides an overview of all in vitro (flask) and breath data collected for all trials and subjects. Of the 33 compounds listed in Table 5, 26 were found, at least once, exclusively "After" metal exposure; 13 of these were ***only*** found in "After" samples for all data collected. Several of these, however, were isolated findings, meaning they may not be reproducible across subjects and/or methods.

Table 11 – Composite Data of Lipid Oxidation Products Found by Different Sampling and Preconcentration Methods; highlighted cell indicates compound unique to after metal sample

Compound	DVB µPC				Tenax µPC						SPME					
	Packed				Packed		Etched				Tenax		PDMS			
	Breath		Linoleic Acid		Linoleic Acid		Linoleic Acid		Breath		Breath		Breath		Linoleic Acid	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1-butanol										X						
1-butanol, 2-methyl	X	X		X						X						
1-heptene	X	X														
1-hexanol, 2-ethyl	X	X	X	X				X	X	X						X
1-pentanol				X						X						X
2,4-decadienal				X											X	X
2,4-nonadienal, (E,E)				X											X	
2-decenal, (E)						X		X		X					X	X
2-heptanol	X															
2-heptanone, 4-methyl	X									X						
2-heptenal (E)			X	X												
2-heptenal (Z)		X		X					X							
2-nonenal (E)																X
2-octenal, (E)							X									X
3-heptanone, 4-methyl		X														
3-heptanone, 6-methyl		X														
3-nonene		X														
4-heptanal (E)										X						
butanal, 2-methyl				X		X										
cis-4-nonene		X														
Furan, 2-pentyl				X											X	X
Heptanal				X												
heptane	X	X	X	X					X	X						
hexanal			X			X	X	X	X	X					X	X
hexane	X	X	X													
nonanal									X	X						
nonanoic acid														X		
octanal				X				X	X	X		X				X
octane	X	X	X	X					X	X				X		X
pentanal				X		X										
pentane			x	X		X										
pentane, 2-methyl	X	X							X							
propanal				X												

Because this project aims to identify compounds responsible for metallic flavor and in vitro modeling may only mimic a portion of what occurs in the oral cavity, breath studies are particularly important. The first round of breath tests involved three different subjects (B, H, J) and used etched Tenax µPCs for preconcentration. Comparison tests revealed that DVB may be a more appropriate adsorbent for concentrating lipid oxidation products, as it produced more peaks of interest than either etched or packed Tenax µPCs (see Figure 8). Consequently, a second round of breath tests was conducted incorporating DVB-packed µPCs to study the breath

of three subjects (A,H,J). As previously mentioned, each subject was tested a different number of times. As described in the method, SPME preconcentration was typically conducted on all samples, along with µPC concentration; however, because they offered little contribution to breath data, SPME concentrated samples are not further examined beyond Table 11.

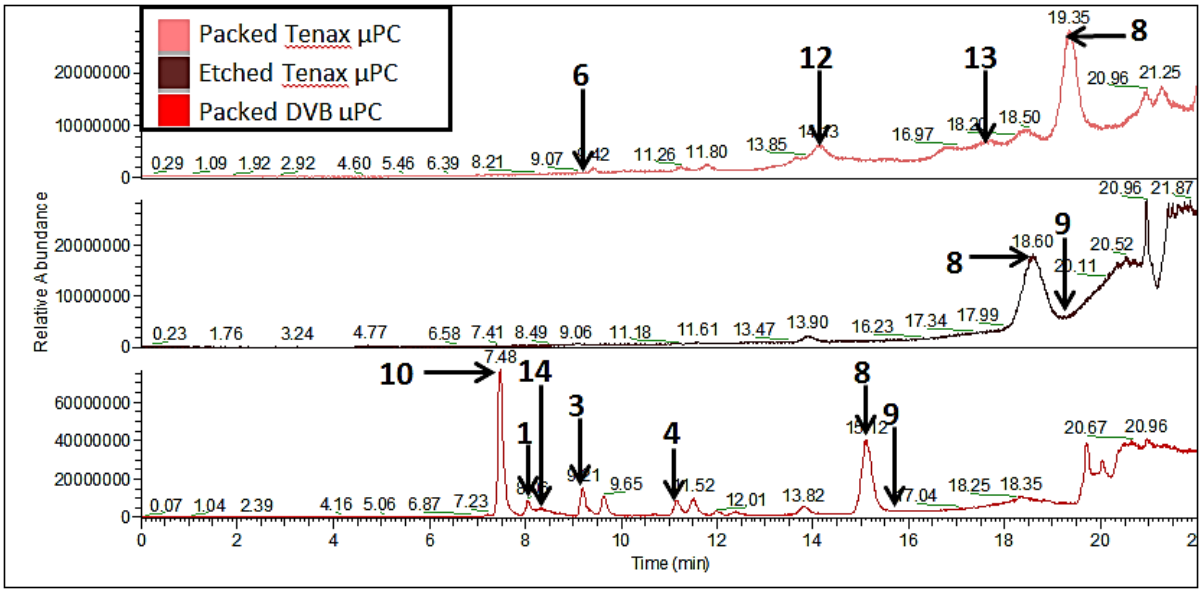


Figure 8 - Comparison of Packed Tenax, Etched Tenax, and Packed DVB µPCs, Concentrating "After Fe^{2+}" Linoleic Acid In vitro Samples

Lipid Oxidation Products (Retention time): Packed Tenax µPC: [6]pentane (9.39); [12] pentanal (14.16); [13] 2-decenal (E) (17.15); [8] hexanal (19.35). Etched Tenax µPC: [8] hexanal (18.50); [9] octanal (19.14). Packed DVB µPC: [10] 2-ethyl hexanal (7.48*); [1] hexane (8.06); [14] propanal (8.33); [3] heptane (9.20); [4] octane (11.15) ; [8] hexanal (15.12); [9] octanal (15.66).

In breath studies (Table 12), 14 different compounds were found (at least once) in the "After," but not "Before," samples for a given subject. Seven of these compounds were exclusively found in "After" samples for all breath data, but most were only found in one subject. Several compounds, including heptane, hexanal, octanal, and 2-methyl pentane were common in "Before" and "After" samples for most subjects. Overall, there is little consistency in data among different subjects.

Table 12 - Composite Breath Data of Lipid Oxidation Products Found, Organized by Preconcentration Method and Subject; highlighted cell indicates compound unique to after metal sample

Compound	Packed DVB µPC						Etched Tenax µPC					
	Subject A		Subject H		Subject J		Subject B		Subject H		Subject J	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1-butanol										X		
1-butanol, 2-methyl	X	X			X	X				X		
1-heptene	X	X										
1-hexanol, 2-ethyl	X	X					X			X	X	
1-pentanol										X		
2,4-decadienal												
2,4-nonadienal, (E,E)												
2-decenal, (E)												X
2-heptanol	X											
2-heptanone, 4-methyl	X									X		
2-heptenal (E)												
2-heptenal (Z)		X									X	
2-nonenal (E)												
2-octenal, (E)												
3-heptanone, 4-methyl		X	X	X		X						
3-heptanone, 6-methyl		X										
3-nonene		X										
4-heptanal (E)								X				
butanal, 2-methyl												
cis-4-nonene		X										
Furan, 2-pentyl												
Heptanal												
heptane	X	X	X		X	X	X	X	X	X	X	X
hexanal							X	X	X	X	X	X
hexane	X	X			X	X				X		
nonanal							X		X	X		
nonanoic acid												
octanal							X	X	X	X	X	
octane	X	X				X					X	X
pentanal												
pentane												
pentane, 2-methyl	X	X	X	X	X	X	X					
propanal												

Figures 9 and 10 show comparisons of two subjects' breath, concentrated and collected in the same manner, both before (Figure 9) and after (Figure 10) the addition of metal. Though the chromatograms appear similar, subject A had more different lipid oxidation products in both

samples (note difference in scales). This may have been due to the fact that breath samples from subject J had a lower volume and sat longer between collection and analysis.

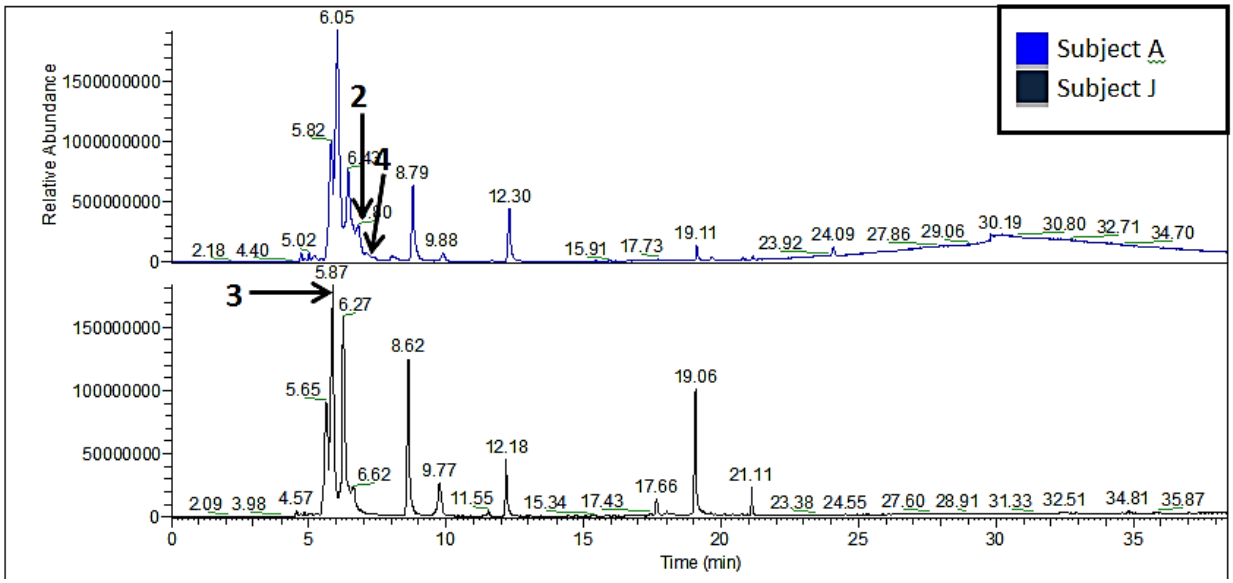


Figure 9 - Comparison of "Before" Breath from Subject A (top) and Subject J (bottom) Concentrated Using a Packed DVB µPC [Note difference in y-axis scale]

Lipid Oxidation Products (Retention time): Subject A: [2]1-heptene (6.80); [4] octane (7.11). Subject J: [3] heptane (5.87)

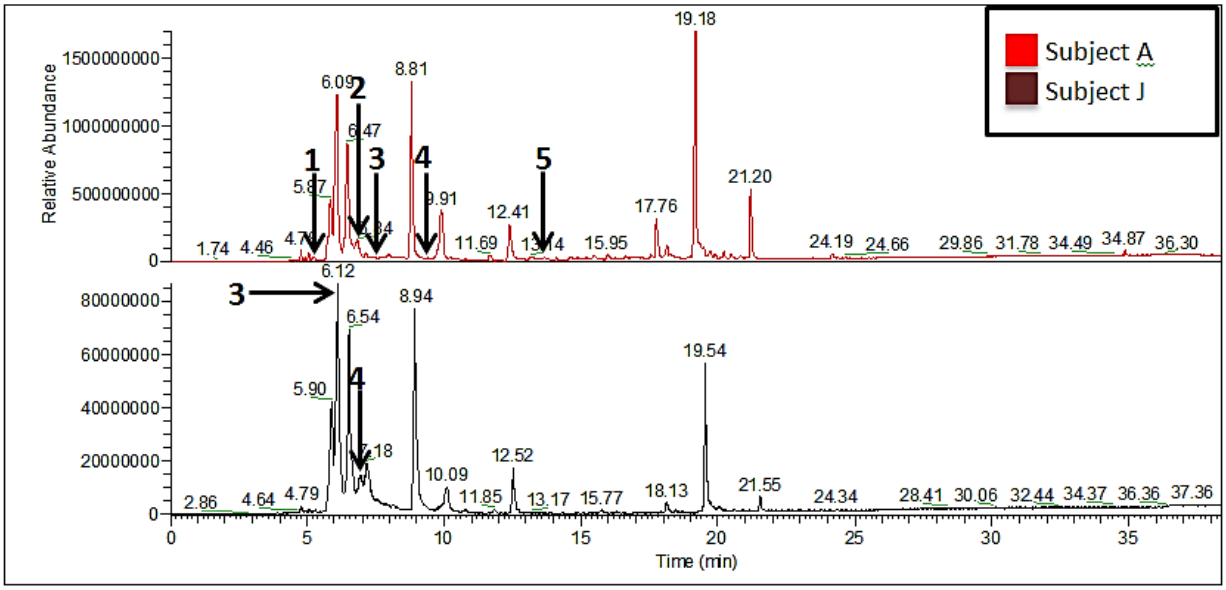


Figure 10 - Comparison of "After Fe^{2+}" Breath from Subject A (top) and Subject J (Bottom) Concentrated Using a Packed DVB µPC [Note difference in y-axis scale]

Lipid Oxidation Products (Retention time): Subject A: [1]hexane (5.06); [2] 1-heptene (6.84*); [3] heptane (7.36*); [4] octane (9.40); [5] 3-nonene (13.66). Subject J: [3] heptane (6.12); [4] octane (6.95).

***indicates was not ranked as a top match (by either the SI or RSI) but was among the top three possibilities in the library search report.**

Though differences among individuals were expected, there should be less variation from sample to sample for a single subject, as the researchers expected each subject to have a characteristic breath background profile. Table 13 summarizes data from two subjects, providing 3 samples each, to allow for examination of intra-subject variation. A few lipid oxidation products are common across both "Before" and "After" samples (subject A: heptane, hexane, octane; subject H: hexanal, octanal, nonanal). This indicates that Tenax may preferentially adsorb alkanals, while DVB effectively traps alkanes in breath. There are no other apparent trends in the data. At this time, the method does not appear to be exceptionally reproducible for lipid oxidation products within an individual.

Table 13 - Lipid Oxidation Products Found in Two Different Subjects during Three Separate Breath Sampling Events; highlighted cell indicates compound unique to after metal sample

Compound	DVB µPC						Tenax µPC					
	Subject A						Subject H					
	Sample 1		Sample 2		Sample 3		Sample 1		Sample 2		Sample 3	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1-butanol								X				
1-butanol, 2-methyl	X		X									
1-heptene	X	X										
1-hexanol, 2-ethyl	X			X						X		X
1-pentanol												
2,4-decadienal												
2,4-nonadienal, (E,E)												
2-decenal, (E)												
2-heptanol			X									
2-heptanone, 4-methyl												
2-heptenal (E)												
2-heptenal (Z)												
2-nonenal (E)												
2-octenal, (E)												
3-heptanone, 4-methyl												
3-heptanone, 6-methyl												
3-nonene		X										
4-heptanal (E)												
butanal, 2-methyl												
cis-4-nonene						X						
Furan, 2-pentyl												
Heptanal												
heptane		X	X	X	X	X	X				X	
hexanal								X	X	X	X	
hexane		X		X	X	X						X
nonanal								X	X	X		
nonanoic acid												
octanal								X	X	X		
octane	X	X	X			X						
pentanal												
pentane												
pentane, 2-methyl												
propanal												

Figures 11 and 12 show the day-to-day variability as chromatogram overlays. Some superficial differences may be attributable to shifts in retention times; however, as indicated in

Table 13, the lipid oxidation products found in a single subject vary from sample to sample. Comparing Figures 11 and 12, also indicates that the Tenax µPC (Figure 12) produces chromatograms with many more peaks than the DVB µPC (Figure 11). This may be due to a procedural difference, wherein samples shown in Figure 12 were of greater volumes than those shown in Figure 11 and, therefore, have a greater chance of being contaminated with air from areas other than the oral cavity. Further, as Table 14 indicates, the greater number of peaks in the Tenax µPC – concentrated samples did not necessarily translate to the collection of more lipid oxidation products of interest.

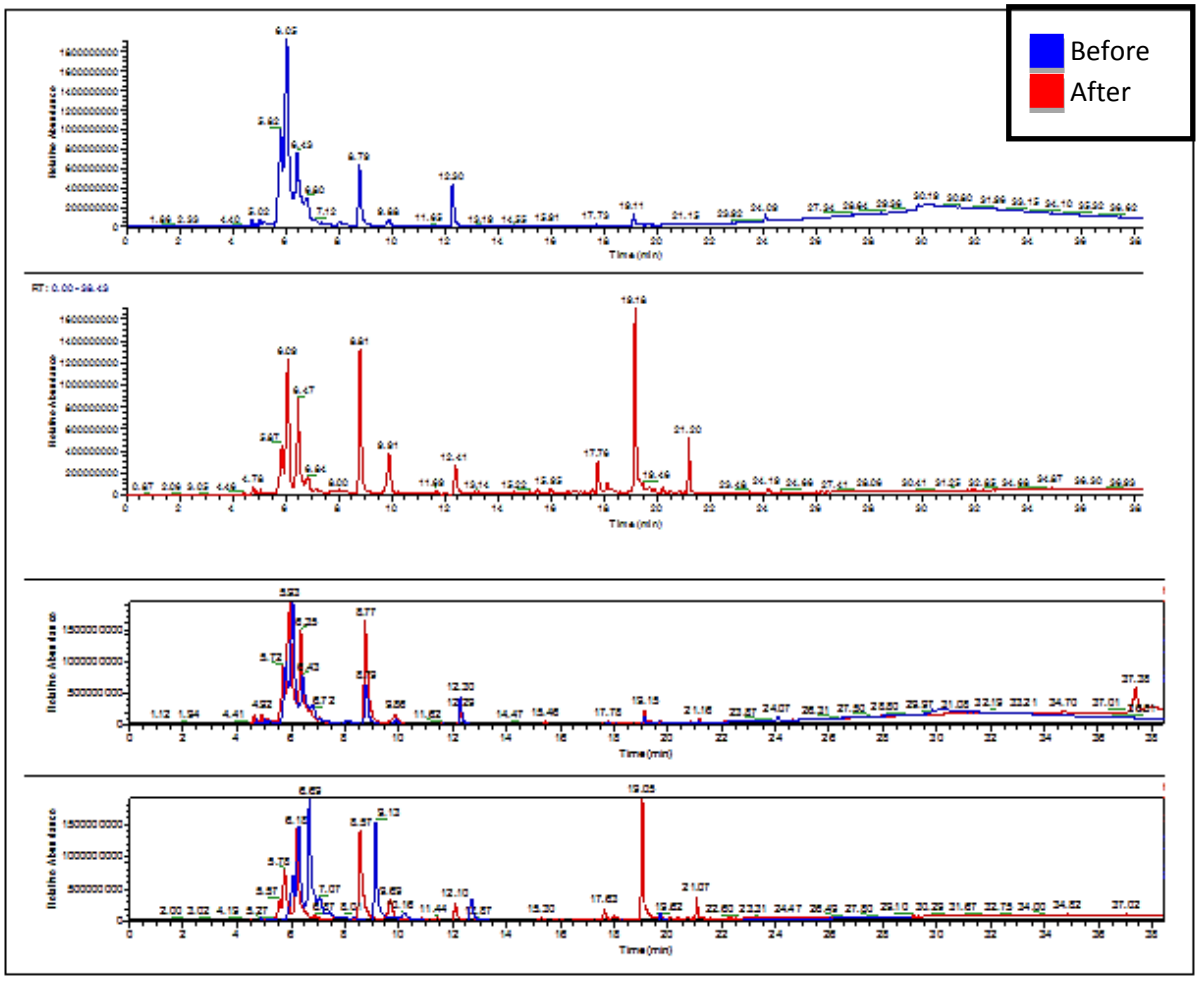


Figure 11 - Chromatogram Overlays ("Before" and "After Fe^{2+}") of Three Different Trials from Subject A, Breath Samples Concentrated with a Packed DVB µPC - See Table 13 for Lipid Oxidation Products

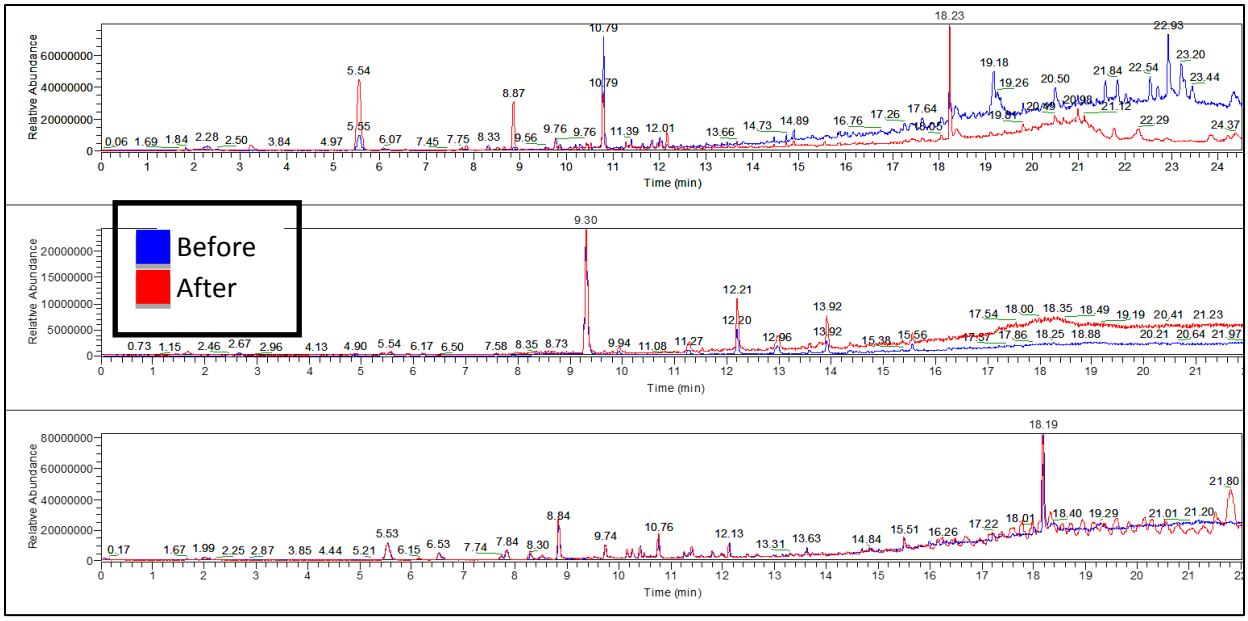


Figure 12 - Chromatogram Overlays ("Before" and "After Fe^{2+}") from Subject H, Breath Samples Concentrated with an Etched Tenax µPC [Note difference of x-axis scale in top chromatogram] - See Table 13 for Lipid Oxidation Products

As breath variation was expected, in vitro data were further examined to evaluate the reproducibility of the method. In vitro data are displayed on a sample by sample basis in Table 14; each column contains the results from a single sample collection. Eight compounds were found in "After" samples only for all trials. Several of these were found only in a single sample, but octanal was found in six and 1-pentanol and pentanal were found in three (of 13 total "After" samples). Hexanal, pentane, octane, and hexane commonly appeared in both "Before" and "After" samples. This is fitting, as alkanes, being nonpolar, are easier to detect using GC than polar compounds. It seems that the lipid oxidation products collected varied, according to adsorbent choice (as with breath), but this difference does not appear to be clearly generalizable by compound class (for example, alkanals vs. alkanones) or size. No other trends are apparent in the data.

Table 14 - Lipid Oxidation Products Found in Individual Linoleic Acid In vitro Sampling Events, Organized by Preconcentration Method; highlighted cell indicates compound unique to after metal sample

Compound	Flasks (In vitro)																														
	Before (Control)									After (metal)																					
	DVB Packed µPC					Etched Tenax µPC		PDMS-DVB SPME		DVB Packed µPC												Etched Tenax µPC		Packed Tenax µPC		SPME					
																										PDMS-DVB					Tenax
1-butanol																															
1-butanol, 2-methyl																	**X**														
1-heptene																															
1-hexanol, 2-ethyl					X													X		X	X	X						X		X	X	
1-pentanol														**X**	**X**											**X**						
2,4-decadienal								X						X												X						
2,4-nonadienal, (E,E)									X											X												
2-decenal, (E)								X														X			X	X						
2-heptanol																															
2-heptanone, 4-methyl																															
2-heptenal (E)				X																X												
2-heptenal (Z)						X														X												
2-nonenal (E)																													**X**			
2-octenal, (E)							X																						X	X		
3-heptanone, 4-methyl																															
3-heptanone, 6-methyl																															
3-nonene																															
4-heptanal (E)																															
butanal, 2-methyl																		**X**													
cis-4-nonene																															
Furan, 2-pentyl								X	X											X						X					X	
Heptanal																				**X**												
heptane	X		X	X										X	X	X				X												
hexanal	X			X	X		X	X						X	X	X	X	X	X	X	X	X	X		X	X	X				X	
hexane			X	X	X											X				X	X											
nonanal																															
nonanoic acid																															
octanal																**X**		**X**					**X**			**X**			**X**			**X**
octane		X	X	X	X							X	X	X	X	X		X	X	X	X					X						
pentanal																			**X**		**X**				**X**							
pentane			X	X	X					X	X			X	X		X	X		X	X			X	X							
pentane, 2-methyl																															
propanal																**X**																

Though great efforts were made to minimize background oxidation and standardize collection/concentration procedures, chromatograms indicate that these were persistent issues with in vitro samples. Figure 13 shows that a “Before” sample can have as many lipid oxidation products as an “After” sample. Though these samples were not taken from the same flask (such a pair of in vitro data were unavailable), the data show that it was not uncommon to find lipid oxidation products in “Before” samples that may or may not be in “After” samples. This indicates that oxidation is occurring before metal is added.

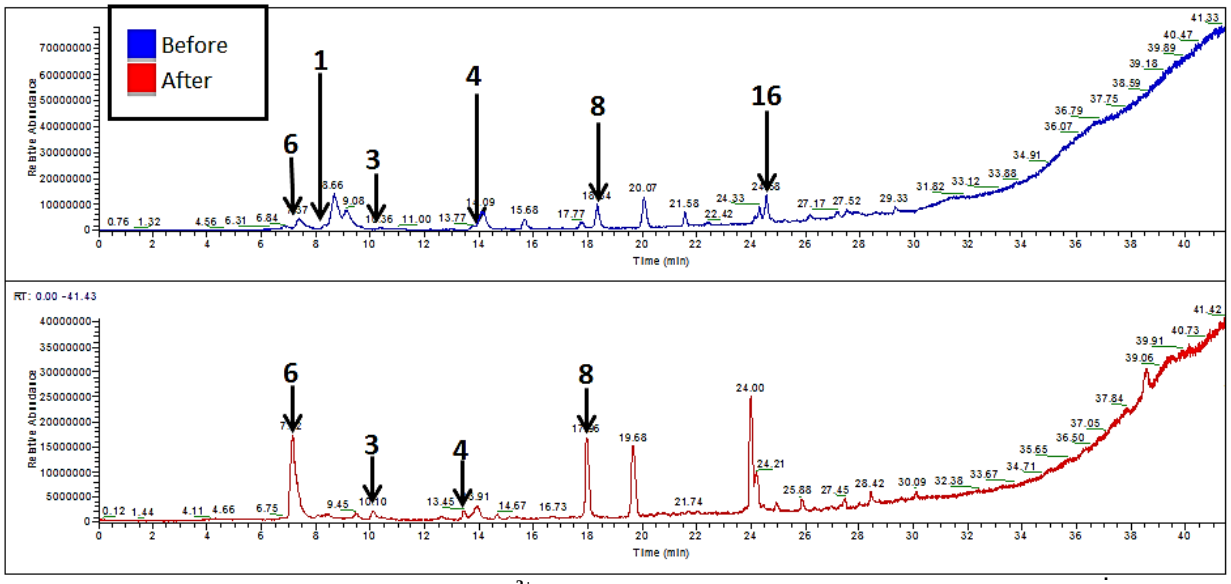


Figure 13 - "Before" and "After the addition of Fe^{2+}" Samples from In Vitro Studies of Linoleic Acid (from Different Trials), Concentrated with Same Packed DVB µPC

Lipid Oxidation Products : ^{6}pentane; ^{1}hexane; ^{3}heptane; ^{4}octane; ^{8}hexanal; ^{16}2-heptenal (E).

Each of the chromatograms in Figure 14 represents a single "After" run; all were completed using the same µPC and procedure. The top two were completed on the same day, while the bottom two were completed the next day. Despite controlling for all of these possible sources of variation in the procedure, these chromatograms are still quite different from one another; this further draws into question the reproducibility of the procedure and/or µPC. Inconsistent results may be due to procedural flaws, leaks in µPCs, changes in flow through packed µPC with each use, or the fact that compound concentrations are at or below the detection limit in many cases, especially in breath.

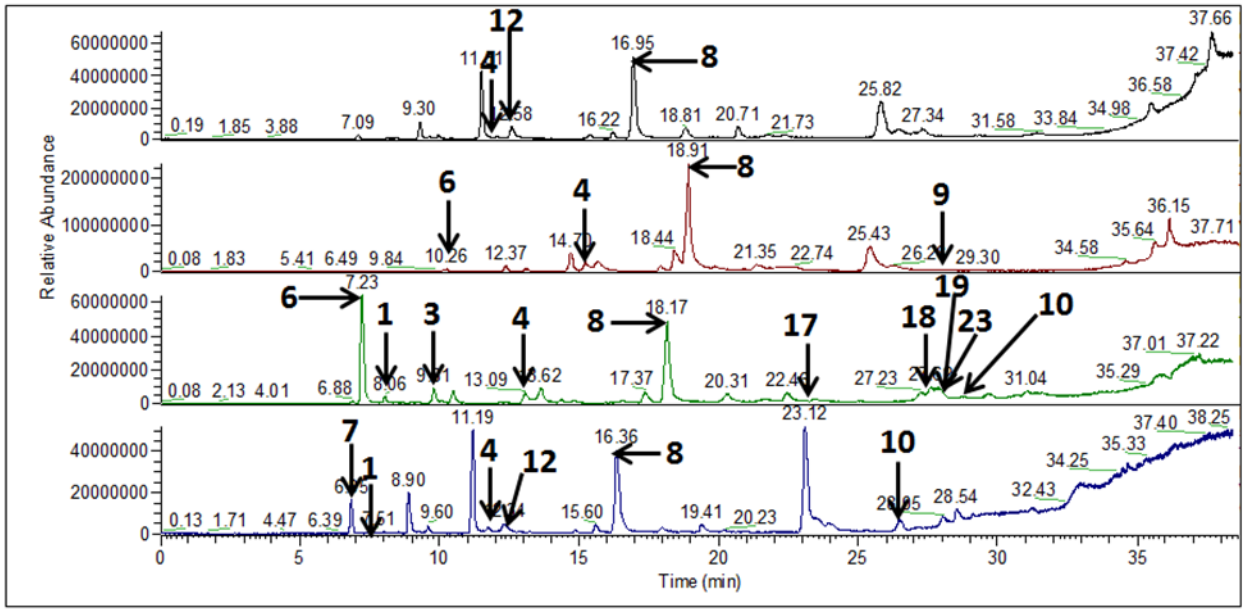


Figure 14 - Variation in Four "After Fe^{2+}" Linoleic Acid In Vitro Samples, Concentrated Using the Same Packed DVB µPC
Lipid Oxidation Products : [6]pentane; [1]hexane; [3] heptane; [4] octane; [12]pentanal; [7]2-methyl butanal; [8] hexanal; [17] heptanal; [10]2-ethyl-1-hexanol; [18]2-pentyl furan; [19] 2,4-nonadienal (E,E); [23] 2-heptenal (Z); [9]octanal.

One issue possibly affecting data is the presence of thousands of compounds, including lipid oxidation products, in ambient air. Such compounds may be introduced into glassware or sampling bags during the process of sampling or may simply be present in the subject's breath because they are being regularly inspired. Air from three locations (the lab in which this work was completed, an indoor room outside of the lab, and the outdoors) was sampled by drawing it directly across a Tenax µPC, using a vacuum pump. The analysis results depicted in Figure 15 show the presence of numerous compounds (many peaks), including several lipid oxidation products. A similar test with a DVB µPC would likely reveal other lipid oxidation products in the ambient air.

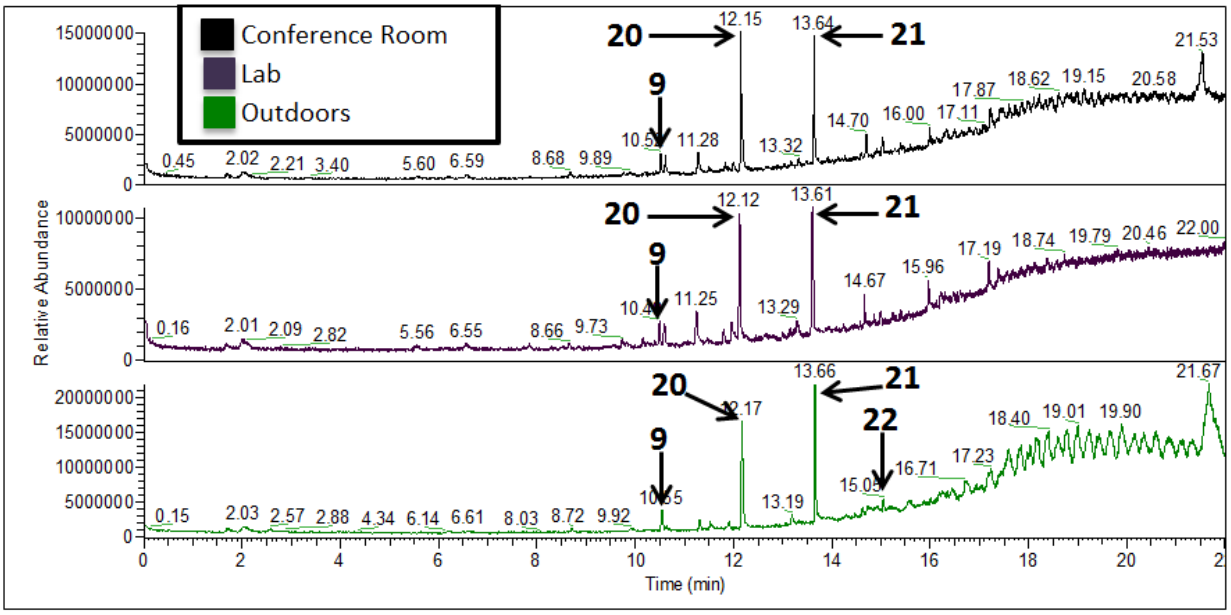
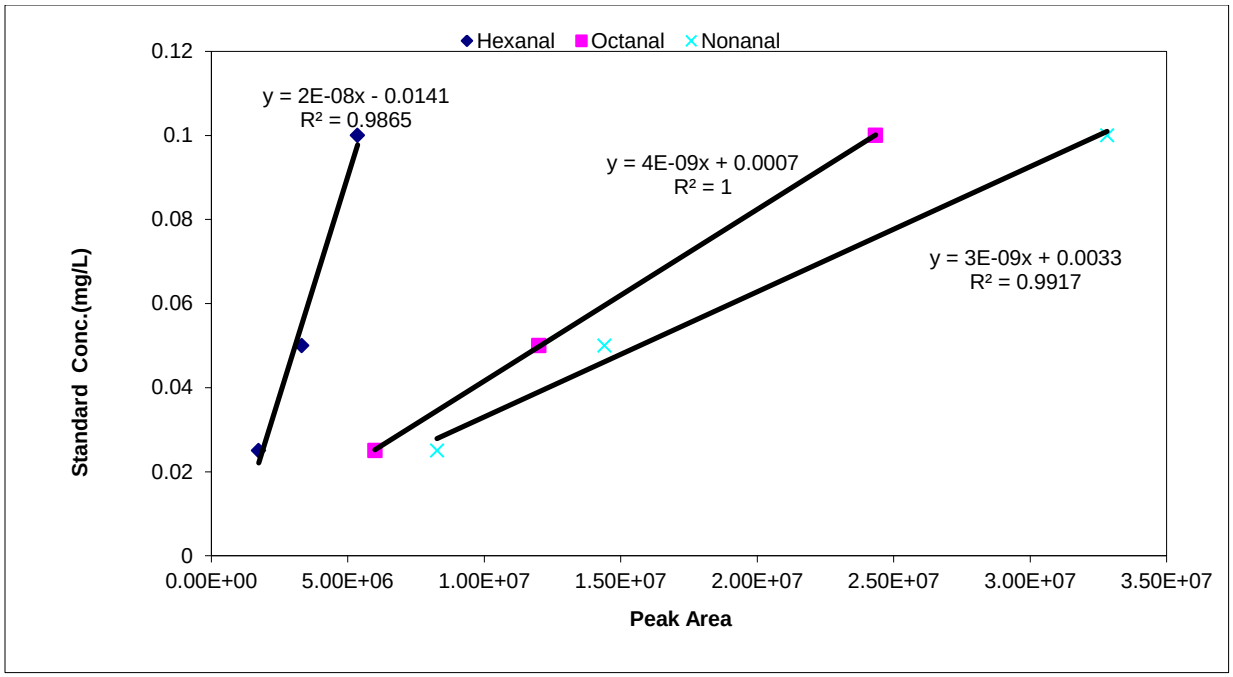


Figure 15 - Ambient Air from a Conference Room, Lab, and the Outdoors Concentrated Using an Etched Tenax µPC
Lipid Oxidation Products: [9]octanal; [20]nonanal; [21] decanal*; [22]undecanal*
***Lipid oxidation products present in ambient air, which were not present in other samples analyzed in this study.**

As with other breath studies, this one highlights the need to differentiate between endogenous and exogenous compounds, as well as between those present in "Before" breath and those that appear, or whose concentration is increased, in "After" breath. This would require quantification of lipid oxidation products present, using a constructed standard curve, the chromatogram peak area, and the volume of the sample loaded. For this project, standard curves were produced on the GC, using direct injection of hexanal, nonanal, and octanal standards. The standard curve and the data used to produce it are shown in Figure 16.



		Peak Area		
Standards (mg/L)	Standards (pg/µL*)	Hexanal	Octanal	Nonanal
0.025	25	1.73E+06	6.00E+06	8.27E+06
0.05	50	3.31E+06	1.20E+07	1.44E+07
0.1	100	5.36E+06	2.43E+07	3.28E+07

*Standard curves were produced from 1µL injections of standard compounds

Figure 16 - Standard Curves for Hexanal, Octanal, and Nonanal, and the Values used to Produce Them

Though these aldehydes are not present in every sample, the standard curves were used to estimate their concentrations in several, as shown in Table 15. This is not intended to be a thorough review of the differences between ambient, "Before," and "After" concentrations (though that is recommended for further study in this area) but instead to provide a general idea of the concentrations of compounds observed in these tests. Concentrations of hexanal, nonanal, and octanal in these samples were in the nanogram per liter (ng/L) range. Concentrations in breath, in vitro samples, and ambient air are similar, typically within the same order of magnitude.

Table 15 - Peak Areas and Calculated Concentrations of C6, C8, and C9 Aldehydes in Select Samples

		Hexanal		Nonanal		Octanal	
	Volume (mL)	Peak Area	Concentration (ng/L)	Peak Area	Concentration (ng/L)	Peak Area	Concentration (ng/L)
"Before" breath (Subject B)	60	N/A	N/A	N/A	N/A	N/A	N/A
"After" breath (Subject B)	60	1.99E+06	0.428	N/A	N/A	N/A	N/A
"Before" breath (Subject H)	90	1.76E+07	3.754	1.40E+07	0.630	3.01E+06	0.137
"After" breath (Subject H)	60	2.34E+07	7.565	1.70E+07	1.145	8.07E+06	0.459
"Before" breath (Subject H)	60	N/A	N/A	N/A	N/A	N/A	N/A
"After" breath (Subject H)	60	1.67E+07	5.332	N/A	N/A	N/A	N/A
"Before" breath (Subject H)	60	N/A	N/A	N/A	N/A	N/A	N/A
"After" breath (Subject H)	60	6.29E+06	1.862	1.74E+07	1.172	6.24E+06	0.367
"Before" breath (Subject H)	60	N/A	N/A	N/A	N/A	N/A	N/A
"After" breath (Subject H)	60	3.06E+07	9.965	4.14E+07	2.772	1.85E+07	0.980
"Before" breath (Subject H)	60	N/A	N/A	N/A	N/A	N/A	N/A
"After" breath (Subject H)	60	7.36E+06	2.218	1.49E+07	1.005	8.16E+06	0.463
"Before" breath (Subject H)	60	N/A	N/A	N/A	N/A	N/A	N/A
"After" breath (Subject H)	60	3.94E+06	1.078	2.16E+07	1.452	8.37E+06	0.474
"Before" In-vitro	60	8.34E+07	27.565	N/A	N/A	N/A	N/A
"After" In-vitro	60	4.89E+07	16.065	N/A	N/A	N/A	N/A
Ambient Air - Conference Room	180	N/A	N/A	3.35E+07	0.748	4.47E+06	0.093
Ambient Air - Lab	180	N/A	N/A	1.88E+07	0.422	3.69E+06	0.080
Ambient Air - Outdoor	180	N/A	N/A	3.11E+07	0.695	6.28E+06	0.123

Table 16 relates the concentrations of the select alkanals in two breath samples to the odor thresholds for those compounds. While odor thresholds in existing literature are determined based on orthonasal ("sniff") intake of odors, concentrations in breath sampled in this study would have been detected retronasally. Retronasal odor detection may have different threshold values; however, such detection limits are not available in the literature.

Table 16 - Odor Thresholds of Select Lipid Oxidation Products and the Concentrations at which they were Detected in Breath

	"Before" Breath Retronasal Concentration (µg/L)	µg in "Before" Breath of Oral Cavity*	"After Fe^{2+}" Breath Retronasal Concentration (µg/L)	µg in "After" Breath of Oral Cavity*	Orthonasal Odor Threshold in Air ($\mu g/L_{air}$)
Hexanal	0.003754	0.0005631	0.007565	0.00113475	0.14
Nonanal	0.000630	9.45E-5	0.001145	0.00017175	0.0134
Octanal	0.000137	2.055E-5	0.000459	6.885E-5	0.0072

*Based on an average oral cavity volume of 150mL

Discussion

The µPCs were shown to be superior to SPME when concentrating lipid oxidation products, both from breath and in vitro samples. Actively drawing samples across the µPC increased contact between the adsorbent and the sample. Further, the design of the µPC optimized adsorbent surface area and gas flow to further enhance adsorption. SPME fibers were used, as designed, to passively load samples. Especially when samples were not heated (as was the case with breath samples), SPME failed to adsorb detectable amounts of the low-concentration analytes. This may be why many breath studies involving SPME incorporate on-fiber derivitization to achieve the necessary detection limits in the nanomolar range (Poli et al., 2010; Svensson et al., 2007; Fuchs et al., 2009).

More standard curves are needed to verify the concentrations of lipid oxidation products and detection limits for methods used in this project. However, concentrations of C6, C8, and C9 alkanals appear be present in the ng/L range. This does not exceed the odor thresholds for these compounds (fractions of a µg/L, as seen in Table 16). Subjects reported metallic flavor

upon removal of the nose clips, suggesting that lipid oxidation products were present in detectable concentrations; however, subjects in this study were detecting odors retronasally. Little study has been completed in the area of retronasal odor thresholds, so they may differ from the orthonasal thresholds reported in the literature (as shown in Table 16). This discrepancy may also be related to the small volume of breath sampled in this study and the relatively small volume of the oral cavity (150mL, as compared to 750ml, the volume of a full breath.

Other studies measuring lipid oxidation products in breath have found aldehydes and alkanes in lower concentrations, typically in the low nanomolar to picomolar range (Fuchs et al., 2009; Kanoh et al., 2005; Larsted et al., 2002; Van den Velde et al., 2007b). Methods to produce these results, which include sorbent trapping with (Larsted et al., 2002; Van den Velde et al., 2007b) and without cryofocusing (Kanoh et al., 2005) and on-fiber SPME derivitization (Fuchs et al., 2009), have picomolar range detection limits. Though the concentrations of compounds in this study seem relatively high, these alkanals do tend to have relatively sizable peaks in chromatograms when compared with other lipid oxidation products' peaks. Therefore, other compounds may be found in lower concentrations. The developers of the µPC, similar to the etched Tenax version used in this study, illustrated its ability to concentrate a complex alkane standard of 1ppb (~1ng/L) concentration (Alfeeli and Agah, 2011). Clearly, more standards must be run to quantify the concentrations of other lipid oxidation products in samples and determine the limits of detection for this method.

Most breath studies, including those mentioned above, analyzed alveolar or mixed air, which prevents a direct comparison with this study. Such studies collect much larger volumes of air from several consecutive breaths (Aghdassi et al., 2000; Kanoh et al., 2005; Qin et al., 2006), thereby increasing the opportunity to adsorb and detect very low concentration analytes. Studies on oral breath are rare and those analyzing lipid oxidation products are nearly nonexistent. Van den Velde et al. (2007b) examined oral breath for malodor, and they measured propanal, a lipid oxidation product. However, it is difficult even to compare this study because the pervasiveness of the compounds responsible for malodor allowed those investigators to draw three consecutive samples of oral breath, again, providing a much larger volume of sample to work with (compared to this study) and increasing the chance of finding low concentration analytes therein.

The low volumes (<60 mL) of samples in this study are just one factor that can make it difficult to achieve consistent results. As mentioned, µPCs surpassed SPME for concentrating samples, but the former was still not notably reproducible across breath or in vitro sampling events. Loading this relatively small sample volume through the µPC may not allow adequate adsorption of analyte concentrations consistently detectable by the GC-MS. Interference from other endogenous and exogenous compounds may also mask or overpower these low concentration analytes. For some compounds, ambient air concentrations may be the same or greater than endogenous concentrations (Larsted et al., 2006; Kneepkins et al., 1994; Qin et al., 2006). Many breath studies "flush" subjects' lungs with hydrocarbon free air to help eliminate such contamination (Agdhassi et al., 2000; Kanoh et al., 2005). Flushing the oral cavity may be an option for future studies of metallic flavor VOC production in the mouth, especially because oral air tends to have even more compounds than alveolar air (Van den Velde et al., 2007). This may also help to reduce the presence of lipid oxidation products in "Before" samples or allow for a better quantification of background oxidation levels with which to compare "After" samples. Quantification of analyte concentrations, using standard curves, will enhance researchers' ability to compare changes between "Before" and "After" samples, even when there is some level of background oxidation, as was noted in this study. Ambient air concentrations could then also be subtracted from sample concentrations of relevant analytes, allowing researchers to determine if compounds are endogenous or exogenous based on a calculated gradient (Van den Velde et al,. 2007; Phillips et al., 1999)

Inconsistent results may also stem from a lack of knowledge about how the lipid oxidation reaction occurs in the mouth. In this study, subjects were asked to hold their breath for up to one minute before providing a sample, under the assumption that this would provide time for the products of interest to accumulate in a greater concentration. However, because temperature of the mouth is quite high and lipid oxidation products are volatile, this may be counterproductive. Omur-Ozbek and Dietrich (2011) reported that detection of metallic flavor of ferrous iron was instantaneous, meaning lipid oxidation products responsible for this flavor were present immediately after the addition of metal. Copper flavor, they reported, took about 10-15 seconds to develop; a flavor that Hong et al. (2010) found to peak at 20-40 seconds. If iron flavor develops more quickly than copper, perhaps a quicker collection of oral breath, even directly following metal addition, is more appropriate. It should be noted, however, that in vitro

samples were drawn quickly after the addition of metal and did not show much more reproducibility than breath samples.

One other factor that may be considered for the improvement of consistency among samples is that of µPC design. The two adsorbents used in this study, Tenax TA and DVB, adsorbed different lipid oxidation products. Therefore, a hybrid µPC, containing both adsorbents, may be more efficient at capturing a variety of products. It is not uncommon for breath studies examining oxidative stress to incorporate multiple adsorbents in the preconcentration step (Larsted et al., 2002; Van den Velde et al., 2007b). Also, though some µPCs in this study were sealed, using epoxy, it appears that annealing the Pyrex and silicon wafers is crucial for avoiding leaks and ensuring robustness over repeated uses. If lipid oxidation products of interest exhibit a large enough difference in boiling points, future studies may also explore the application of staged trapping, which has proven effective in reducing sample humidity and honing in on compounds of interest, while removing other, unnecessary, background compounds (Cho et al., 2005; Alfeeli et al., 2009; Tian et al., 2005)

Conclusion

In this study, thirty three different lipid oxidation products were found in breath and/or in vitro samples concentrated on µPCs and/or SPME fibers prior to GC-MS analysis. Many of these compounds have known odors which could contribute to the retronasal sensation that induces metallic flavor upon drinking water containing the ferrous ion. It is estimated that these compounds are present in ng/L amounts in breath, which is below their sensory thresholds. In many cases, the number of odorous compounds increased after samples were treated with iron, supporting that they are likely contributors to metallic flavor. Further quantification of compounds present and refinement of methods should be explored to enhance the reproducibility of this procedure in consistently detecting lipid oxidation products.

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Part II: The Possibilities Will Take Your Breath Away: Breath Analysis for Assessing Environmental Exposure

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The Possibilities Will Take Your Breath Away: Breath Analysis for Assessing Environmental Exposure

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A BREATH OF LIFE

Breathe in: it happens every few seconds. Protective barriers are bypassed, allowing your external environment to exchange with your internal environment at the blood−air barrier within the alveoli of the lungs. Exogenous compounds from recent environmental exposures diffuse into your blood, allowing them to contact virtually every tissue in your body.

Breathe out: endogenous compounds reflecting internal bodily conditions diffuse from the blood into the breath. Alveolar air (Figure 1, Table 1) contains important information regarding compounds within your body. Data show that chemicals of concern, including benzene,[1] toluene,[2] and disinfection byproducts,[3] are found in the people's breath. As indoor air pollution becomes a prevalent concern, the fears about unhealthy exposures can be disconcerting.

Breathe easy: breath is recognized as a medium through which doses of chemicals from environmental and occupational exposures, as well as clinical conditions, can be quantified. Sampling breath is less invasive than drawing blood, more convenient than obtaining urine, and shows promise as an inexpensive method with fast turnaround time and minimal biohazard waste. Basic studies date back to 1758 when Lavoisier measured carbon dioxide in breath. By 1971, Pauling et al.[4] quantified 250 compounds in breath. Owing to the advancement of gas chromatography, complex gas mixtures can be analyzed even at concentrations of parts per billion (ppb) or less.

BREATH AS AN INDICATOR OF EXPOSURE AND DOSE

Since the late 1970s, analysis of gaseous components in human breath has confirmed occupational/indoor exposures to benzene,[5] toluene,[6,7] xylenes,[7,8] ethylbenzene,[7] butanone,[9] naphthalene,[5] and trihalomethanes[10] and correlations with concentrations in blood or urine. Increasingly, sampling enhancements have improved breath's reliability as an exposure indicator[6,7,10] or a means to monitor disease conditions, such as lung cancer,[11] diabetes,[12] and liver cirrhosis.[13]

Breath is comprised of hundreds of compounds, but knowledge of which compounds and what concentrations are "normal" has yet to be obtained. Typical exhaled gases (e.g., CO_2, N_2, H_2O) are found in percent concentrations. Constituents present at ppb concentrations or less include volatile organic compounds (VOCs) associated with normal metabolism (e.g., ethanol, isoprene, propanone, methanethiol),[14] products of oxidative stress (e.g., propanedial, pentane),[15] and compounds associated with environmental and occupational exposure (e.g., trichloromethane,[3] benzene).[5]

Table 1 defines terms associated with air and breath. Though breath studies, particularly those involving flavor, may focus on oral cavity breath, alveolar breath is the preferred indicator of internal conditions. It maximizes diffusion of compounds at the blood−breath interface and minimizes contamination from the surroundings, nasal passages, and oral cavity.[16] Table 2 presents a summary of a few recent studies that successfully implemented breath analysis to monitor environmental exposures and doses. Particulate matter is likewise an inhalable hazard of concern, but this article focuses on gaseous agents.

The studies in Table 2 illustrate the diverse applications of breath analysis for examining environmental exposures and doses, from those in the typical home[3] to domestic exposures influenced by nearby businesses,[18] to those outside the home, which may have several potential agent sources.[2,17] Further, various compounds are successfully found and quantified at quite low (roughly nanomolar) concentrations. Compound measurements in breath are significantly correlated to blood and/or ambient air concentrations. Urine, which often requires the identification of metabolites of the compound of interest, showed inconsistent results for correlating with breath.[17,18] One study found a temporal correlation between exposure and dose, as tetrachloroethene levels in subjects living above a dry cleaning establishment were higher in the morning, reflecting dosage accumulated through the night while sleeping. Diversity of applications, low detection limits, and correlations among matrices, as well as temporal dose-detection correlations all contribute to the usefulness of breath analysis for quantifying doses of environmental agents.

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BREATH ANALYSIS

Current Sample Collection, Preconcentration, and Chromatography. Qualitative and quantitative analysis of parts per trillion (ppt) to ppb concentrations of gaseous target analytes is always challenging. The task is further complicated by complex matrices which may interfere with the anlaytes of interest. In addition to the target contaminants from environmental exposure, breath samples contain background levels of water vapor, permanent gases, and organic chemicals from normal metabolism. Thus, target analytes must be separated from the background matrix and concentrated into a form compatible with analytical instrumentation. Breath is typically collected during exhalation, either directly into a storage container (glass tube[18] or inert bag)[20] or by means of a sampling device.[2,19,21] Sample preparation is then usually performed in a laboratory setting by trained personnel.[22]

After, or sometimes during, collection, target analytes are enriched and the background matrix is reduced in a step known as preconcentration. Preconcentration is achieved by trapping target analyte(s) over a period of time until the desired concentration is attained. Then, analytes are released in the form of a narrow, highly concentrated plug for subsequent chemical analysis, which is most commonly capillary gas chromatographic (GC) separation and mass spectrometric (MS) detection.[23,24]

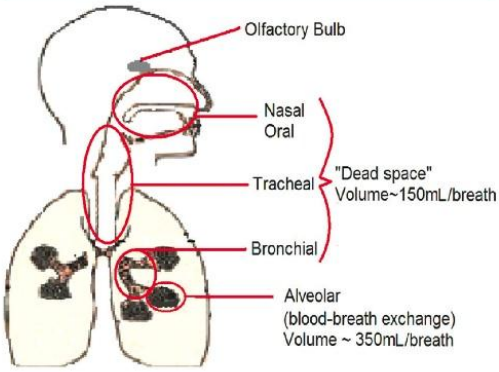

Figure 1. Respiratory system and components of breath.

Two main preconcentration methods are widely used: cryogenic and sorption trapping. Cryogenic trapping relies on condensation of analytes on a cooled bed of glass beads at low temperatures, for example liquid nitrogen (−196 °C) or dry ice (carbon dioxide, −78 °C).[25] Cryotrapping requires special apparati for sample acquisition.

Preconcentration by sorption trapping relies on sorption phenomena, which include adsorption, absorption, chemical adsorption, capillary condensation, and dissolution in solid or liquid sorbents.[26] Two common techniques are sorbent tubes and solid phase micro extraction (SPME). The choice of technique and sorbent type depends on many factors, such as target analytes, concentration range, analysis complexity, cost, time, and whether qualitative or quantitative analysis is desired. Sorbent tubes, initially developed in the 1970s for air sampling applications,[27−29] consist of one or multiple adsorbent materials packed in stainless steel or glass tubes. Typically, these tubes are 1−10 cm in length. A specialized apparatus thermally desorbs the trapped analytes from the sorbent tube into a chromatographic system.

Developed in 1989,[30] SPME consists of an approximately 1-cm polymer fiber coated with a sorbent and attached to a plunger in a holder similar to a syringe. The SPME fiber is inserted directly into the heated injector port of a gas chromatograph for thermal desorption of analytes. SPME combines collection, preconcentration, and sample introduction into a single solvent-free step.[22] SPME is an accepted, standard VOCs analysis procedure in a variety of industries.[31] Several breath studies incorporated SPME,[11,12,32−35] either by passive sampling of breath in a bag or by direct insertion into the subject's mouth.[36]

Following preconcentration and thermal desorption, samples are processed in the laboratory using capillary gas chromatography (GC) to separate mixtures of analytes into individual components in time and space. Capillary GC has been a favored and sensitive method of separation and analysis of organic compounds for decades. A variety of detectors may be paired with the GC. Flame ionization detectors (FID) are effective for detecting organic molecules, though sample identification

Table 1. Definitions of Terms Common in Breath Studies

Alveolar Air	Found in the alveoli of the lungs, where it interfaces with capillary blood; 350ml of the 500ml total volume of each breath; also called end-exhaled breath.
Ambient Air	Found in the surroundings; may be outdoor or indoor, depending on the location of focus.
Breath	Air that originates from within the respiratory tract of a subject; it may include dead space and/or alveolar air.
Dead Space Air	Includes air from subject's nasal, oral, tracheal, and bronchial cavities, in which no gas exchange takes place; about 150ml of the 500ml total volume of each breath.
Indoor Air	Found within enclosed spaces, including buildings and vehicles.
Oral Air	Found in the oral cavity of a subject; less than 100ml of the 500ml total volume of each breath.
Outdoor Air	Found outside of enclosed spaces, including buildings and vehicles.
Personal Air	Found within close proximity to a subject, often collected by a sampler worn by the subject. Personal exposure is a measure of compound concentrations in personal air.
Units	ppm = part per million = mg/m^3 x 24.45/MW at 25 °C and 1 atm. ppb = part per billion = $\mu g/m^3$ x 24.45/MW at 25 °C and 1 atm.

Table 2. Studies Illustrating the Usefulness of Breath As a Biomarker of Environmental Exposure and Dose

Study	Breath Methods	Matrix	Compounds and Concentrations ($\mu g/m^3$)[a,b]					Findings
Non-industrial exposure of residents in a mountain village[a,17]	• Collection: glass vials • Sampling: headspace • Analysis: GC-MS		1,3-Butadiene	2,5-Dimethylfuran	Benzene			• Significant correlations between ambient air, breath, and blood for all compounds • Urine only significantly correlated with breath and blood for butadiene • All compound concentrations higher in smokers than nonsmokers
		Breath	1.2	0.5	5.7			
		Blood	2.2	2.5	62.3			
		Urine	1.1	51.8	63.4			
Indoor exposure of housewives to chlorinated tap water[b,3]	• Collection: aluminum bag to adsorbent tube • Sampling: thermal desorption, cold trap concentration • Analysis: GC-PDECD		Trichloromethane	Bromodichloro-methane	Dichloro-acetonitrile			• Significant correlations between ambient air and breath for all three compounds • Ambient air concentrations significantly higher in winter than summer • In winter, trichloromethane dose is most significant from airborne exposure; summer exposure mainly due to showering
		Ambient air (winter)	1.505	0.139	0.047			
		Ambient air (summer)	0.156	0.020	BDL			
		Breath (winter)	0.316	0.024	0.059			
		Breath (summer)	0.117	0.011	BDL			
Exposure of residents	• Collection: glass tubes		Tetrachloroethene (Perc)					• Significant correlations for personal air, ambient air, breath,
living near dry-cleaning facilities to tetrachloro-ethene[a,18]	• Sampling: direct injection • Analysis: GC-ECD	Ambient Air	620 (day); 205 (night)					and blood • Urine not highly correlated with other matrices; Perc sometimes absent in urine, even when in other matrices • Perc levels in residents living near dry cleaner higher than national averages • Ambient and personal air Perc levels were higher during the day but remained high even after the dry cleaner ceased to operate; blood and breath concentrations were higher in the morning, reflecting exposure through the night
		Personal Air	403 (day); 206 (night)					
		Breath	186 (day); 231 (night)					
		Blood	4950 (day); 5750 (night)					
		Urine	2.85 µg/g creatine (day); 0.77 µg/g creatine (night)					
School children exposed to VOCs from nearby industries[2]	• Collection: stainless steel canister[19] • Sampling: thermal desorption • Analysis: GC-FID		Benzene	Toluene	m, p-Xylene	o-Xylene	MTBE	• Significant correlation between personal air and breath • Toluene and xylene compounds higher in children attending school nearer an industrial complex • Toluene and MTBE higher in children attending school near higher traffic density • Benzene derivatives higher in children living with smoker
		Personal air and breath (range)	2.6-8.1	25-183	6.3-40.4	2.5-8.7	1.7-4.9	

[a] Median concentrations shown. [b] Mean concentrations shown. Abbreviations: GC = Gas Chromatograph; MS = Mass Spectrometry; PDECD = Pulsed Discharge Electron Capture Detector; ECD = Electron Capture Detector; FID = Flame Ionization Detector; BDL = below detection limit; MTBE = methyl-*t*-butyl ether.

requires the comparison of analyte retention times with those of standards. Mass spectrometry (MS) is often preferred for environmental samples because it provides unique information that can be used to identify unknown analytes by means of a library search feature.

Emerging Technologies for Hand-Held Breath Analyzers. Current sorbent-trapping/desorption-GC-MS breath analysis methods are laboratory-based with limited portability for direct use in environmental or occupational settings.[37] Thus, there is a need for easy-to-use and portable hand-held systems capable of ppt−ppb detection of analytes in complex matrices. Although the concept of GC miniaturization was introduced in late 1970s,[38] 21st century advancements in microfabrication technologies have enabled the realization of micro GCs (μGC) as sophisticated hand-held qualitative and quantitative analyzers that eliminate sample transport and storage. Like conventional GC systems, μGC systems for gaseous analytes consist of three main components. The preconcentrator (Figure 2) provides sample collection and injection; it typically uses the same sorbents applied in sorption trapping. The second component is a microcolumn to separate desired components from the mixture, and the last component is a single detector or array of detectors, which identify the eluted analytes. The utilization of silicon micromachining technology has allowed for the development[39] of preconcentrators,[40−53] separation columns,[54−64] and detectors (including micro thermal conductivity detectors, micro flame ionization detectors, surface acoustic waves, chemiresistors, and micro differential mobility spectrometers).[65−72] These μGCs have demonstrated improved analysis performance in terms of analysis time, sample volume, and consumables, such as solvents and reagents. Their low power consumption makes them sustainable and desirable for environmental analyses.

Emerging Sensors. Chemical sensors, frequently termed "electronic noses," are modeled on the unsurpassed sensitivity of the mammalian olfactory system in identifying differences among chemical compound signatures. Two recent developments of note incorporate nanoscale technology to identify target analytes in breath. Semiconducting carbon nanotubes, coated with different sequences of single-stranded DNA respond to the chemical identity of odorants based on changes in measured transconductance. The instrument is capable of differentiating among different aldehydes, including octanal, nonanal, and decanal, all of which are commonly found in breath.[73]

Specially coated gold nanoparticles were developed and applied to detect ppb concentrations of compounds in breath from healthy subjects and lung cancer patients. Of the 42 total VOCs identified in this study, nine were found solely in lung cancer patients. These include 2-ethyl-1-hexanol, 2,3,4-trimethyl-pentane, and 3-ethyl-3-methyl-2-pentanone, which are known lipid oxidation products of fatty acids. Though profiling biomarkers in breath of afflicted subjects is a common application, the ability to diagnose based on such biomarkers has not been realized. The gold nanoparticle sensor eliminated the need for dehumidification and preconcentration of samples and is inexpensive and portable, compared to lab bench scale methods previously discussed.[74]

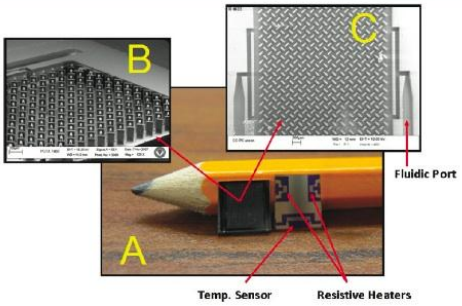

Figure 2. Micropreconcentrators (μPCs) are used to concentrate gaseous analytes. (A) The front (left) and back (right) view of a μPC next to a pencil. The back of the device includes heaters to volatize samples for GC analysis. (B) and (C)Magnifications of the front of the device, including the adsorbent covered 3-D microstructures within the central microcavity (B) used to increase adsorptive surface area for better sample concentration and the ports on either side of the device through where carrier gas is introduced during analysis (C).

Data Analysis. When attempting to quantify the subject's body burden for a particular chemical, which is often the focus of environmental studies, it is important to distinguish between exposure and dose. An agent (chemical) emanating from a source may come in contact with a person without being fully assimilated into the body. The contact between an agent and a person (exposure) may or may not result in the person actually receiving a dose of the agent. The dose depends on the amount of agent entering the person by crossing a contact boundary (i.e., the blood−air barrier).[75] The amount of agent that is able to cross the contact boundary depends on characteristics of the agent, including size, reactivity, and solubility,[76,77] as well as the subject's distance from the source (Figure 3) .

In breath studies, the calculation of actual dose may be confounded by the substantial ambient concentrations of many compounds of interest; background correction is necessary. However, there is still no consensus on whether this should be done by flushing the subject's lungs with pure air,[3] subtracting ambient air concentrations from breath concentrations,[21] or by some other means. As with other procedures, this one requires standardization for data to be meaningful and comparable across studies.

■ ENVIRONMENTAL SOURCES

A few large-scale studies are representative of attempts to qualitatively and quantitatively evaluate environmental agent doses in the general population. The EPA TEAM (Total Exposure Assessment Methodology) data were collected from 1979 to 1985.[78] Roughly 5000 samples, including outdoor air, personal air, drinking water, and breath were collected from a

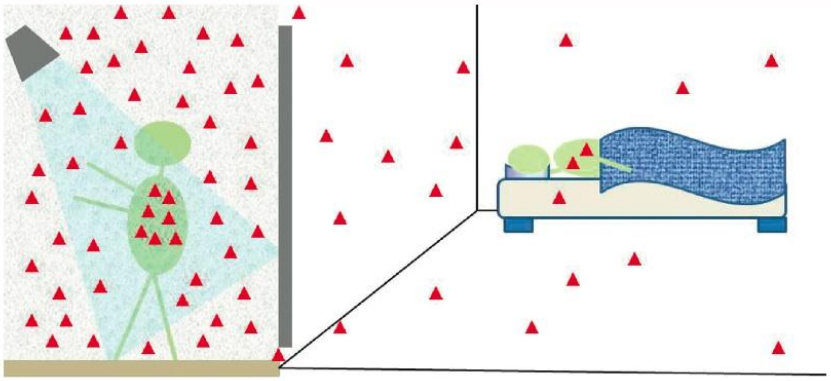

Figure 3. Hot showers are a source of trichloromethane, a volatile disinfection byproduct in tap water and the agent in the above scenario (represented by red triangles). The individual on the left is proximal to the source and experiences a relatively high exposure (triangles in the air) and consequent dose (triangles in the body) of the chemical. Though the trichloromethane will diffuse around the bathroom door and into the adjacent room (right), the sleeping individual is farther from the source, where the agent is more diffuse. This results in a lower exposure and a smaller dose of the agent, relative to the individual in the shower.

representative section of the population in three selected U.S. cities. The study examined 20 total VOCs, finding trichloromethane, *o*-, *m*-, and *p*-xylenes, 1,1,1-tricholoroethane, benzene, tetrachloroethene, styrene, *p*-dichlorobenzene, ethylbenzene, and tetrachloromethane consistently in air and breath sampled at all locations. Results also indicated that indoor sources of VOCs constituted a major source of exposure, showing a strong correlation between breath concentrations and personal exposure and explaining why personal exposures often exceeded outdoor concentrations, sometimes by as much as ten times.[78]

The European Indoor Air Monitoring and Exposure Assessment (AIRMEX) study, conducted between 2003 and 2008, aimed to examine relationships between outdoor and indoor concentrations of chemicals, as well as consequent human exposure levels. Roughly 1150 samples were taken from homes, public buildings, and schools in 11 representative European cities. Statistical analysis revealed that most VOCs, with the exception of aromatic hydrocarbons, were of predominantly indoor origin. When formaldehyde and benzene were specifically examined, data revealed that personal exposures exceeded indoor concentrations, and concentrations in the home were much greater than those in public buildings and schools.[79,80]

Americans now spend roughly 90% of their time indoors every day,[81] thus increasing their exposure to VOCs and SVOCs emanating from both building materials and occupants themselves. New consumer and building materials can introduce carcinogens,[17] suspected endocrine disrupters,[83] and other toxic substances. Common examples include aldehydes and terpenoids from composite wood products in building structures and furniture,[84,85] di-2-ethylhexyl phthalate (DEHP) from vinyl flooring,[86] odorants, antioxidants, and plasticizers from polyethylene pipe,[87−89] 3-hydroxy-2,2,4-trimethylpentyl-1-isobutyrate from latex paints,[90] unsaturated organic compounds, such as D-limonene, from air fresheners,[91,92] phthalates from personal care products,[93] alkylphenol ethoxylates (nonionic surfactants) from cleaning products,[94] and tetrachloroethene from dry-cleaned clothes.[78]

Health Effects. Recognized acute health effects stemming from air contaminant exposures include mucous membrane irritation, tightness in the chest, fatigue, headaches, and cardiovascular disease.[95] When such symptoms can be linked with timely exposure to a particular building, they are characterized collectively as Sick Building Syndrome. The condition is exacerbated by the amount of time people spend inside, as well as the more "sealed" nature of buildings due to energy efficiency and increased use of air conditioners.[96] Total VOCs (TVOCs), often used to measure possible detriment to occupants, can be more than 50−100 times greater in new buildings than outdoors. It is difficult, however, to link the symptoms of such illnesses with their causes, due to the low (ppb) concentrations of contaminants and the lack of meaningful biomarkers to link human doses with ambient air concentrations.[82] Studies are complicated by the fact that odor thresholds for many compounds are 1−4 orders of magnitude lower than sensory irritation thresholds.[97] Breath analysis may be the missing piece of the puzzle. It can be used to proactively monitor occupants' doses of contaminants and help to identify a health threat before widespread symptoms arise.

Current Status of Exposure Assessments. Exposure assessments rely heavily on the measurement of ambient air levels. However, indoor concentrations of contaminants are continually altered by inflow from outdoors, ventilation, filtration, adsorption to surfaces, reactions, and nearness of the human to contaminant source. Thus, measurements of ambient air alone may not be sufficient to determine human exposure and dosage.[98] Additionally, sampling in a variety of locations can quickly become costly and time-consuming. Modeling could provide answers, but there is relatively little knowledge on concentrations and behavior of indoor contaminants, which makes breath analysis an accurate means of assessing human exposure and dosage.[98]

Challenges for Broad Implementation. Though breath analysis as a means of dose measurement shows promise for large scale utilization, there are obstacles to overcome. First, compounds and associated concentration ranges that are typical of "normal" breath must be determined. Second, environmental exposure-related compounds must be identified in breath, their environmental sources identified, and toxicokinetics[79,99] understood. Once in the body, compounds may react or be stored in a way that would affect the inhalation−exhalation ratio predicted by simple models. Therefore, each agent may have to be studied individually to effectively use breath concentrations to predict dose. Progress is slowly being made in this area,[79,100] but a plethora of contaminants have yet to identified, studied, and prioritized. Ohara et al.[20] found intersubject variations in blood−breath ratios of compounds and suggested that for clinical applications, the change from a patient's baseline breath profile is a more important indicator than the actual concentration present. This is likely not the case for environmental exposures because all individuals in an area (home, office building, etc.) have some level of exposure, thereby preventing the comparison of pre- and post-exposure concentration. Therefore, relationships between breath and blood concentrations must be assessed and partition coefficients calculated so that accurate body burdens may be deduced from measured breath concentrations. Perhaps, as with medical blood work, a normal range of concentrations can be developed for the most common or potentially offensive breath constituents. Also, temporal studies should be conducted to determine the most appropriate time, relative to exposure, to sample subjects. Because subjects may be exposed to agents from a variety of sources at different times during the day, the ability to identify a single source from which the agent is emanating may be particularly challenging.

INDOOR AIR REGULATIONS

Although the capabilities to quantify compounds in indoor air exist, the drive to extensively measure and regulate indoor pollutants, except industrial occupational exposures, has been slow to develop. Outdoor levels of air pollutants have been regulated in the United States since the 1960s.[93] Experts recommend that indoor air standards not exceed 50% of outdoor National Ambient Air Quality Standards set forth by the EPA.[82] Existing publications tend to assess the current situation and provide recommendations for reducing exposures, as opposed to suggesting levels below which indoor concentrations should be maintained. The INDEX study in Europe sought to prioritize indoor air pollution needs by identifying exposures, describing toxicokinetics, and assessing risk of twelve pollutants, selected based on their prevalence in European buildings and their adverse health effects.[79] Similarly, the World Health Organization (WHO) published guidelines for what they have identified as nine of the most important indoor pollutants, globally. The report is intended for public health professionals, as well as those responsible for the design and maintenance of materials and

buildings. However, WHO suggests that it may be used as a foundation for "legally enforceable standards".[100] In the United States, the EPA's Tools for Schools voluntary program shows schools how to reduce indoor air pollution by addressing issues with ventilation, maintaining cleanliness, choosing appropriate cleaning materials, and pest control. About 40% of the schools surveyed were implementing the plan.[101]

LOOKING AHEAD: NEEDS FOR ENVIRONMENTAL BREATH ANALYSIS

Potential mainstream applications of breath analysis to measuring doses from environmental exposures abound, but for the field to progress and diversify, several areas must be expanded. Many studies have looked into the popular analytes, such as the benzene derivatives,[5−8,34] while studies of emerging contaminants and those that may be harder to measure, such as SVOCs and polar compounds, have yet to gain popularity.

Current analysis methods either qualitatively determine the presence of many VOCs or quantitatively identify a few. Method standardization, especially in collection, is essential. While the compounds of interest likely drive the choice of analysis and detector, retention times vary with carrier gas, temperature program, and GC column, making it difficult to compare data that have been collected by different means. Current studies also rely on instrumentation that requires substantial time and significant skill to utilize. Moreover, current GC-MS systems are moderately expensive, large, table-top instruments with high power consumptions.[102] These factors have limited the use of breath analysis in field settings.[37] All these factors combined have given rise to the need for portable, easy-to-use screening systems to facilitate breath analysis in physicians' offices or in the field operation.[103]

A variety of compounds have been identified in significant quantities in our homes, workplaces, and outdoor surroundings. The sources of some chemicals are well-known and extensively studied in the literature, while others simply appear in the complex chemical soup that surrounds us with no identified, or several potential, sources. Because they are present in a gaseous form, exposure is obligatory, as no one can refuse to breathe. The ability to quantify dose is crucial for determining the extent of detriment caused. However, as discussed, ambient concentrations are constantly changing. Monitoring of breath presents the needed solution for identifying and quantifying the risk associated with exposure to environmental contaminants. No matter the origin of contaminants or changes they undergo in the environment, their assimilation into the body can be assessed by measuring them in the breath of affected individuals. The breath presents a composite of all doses, providing information about exogenous compounds absorbed from the surroundings, as well as changes in endogenous compounds which may result from such exposures.

Achieving such goals will allow for the advancement of breath analysis as a means of quantifying environmental exposures and doses, as well as useful data which can eventually be used to limit individuals' exposure to harmful contaminants. We will be waiting with bated breath.

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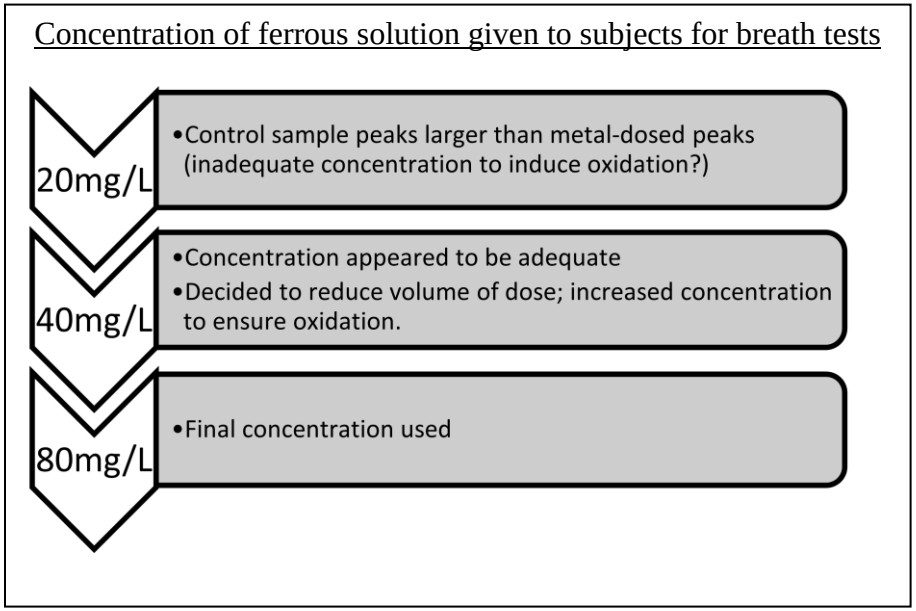
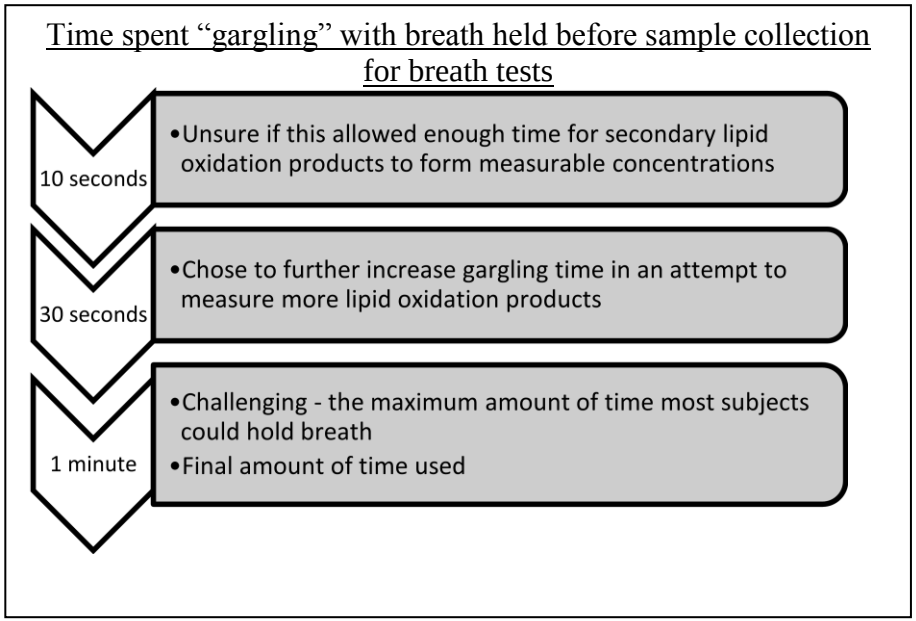
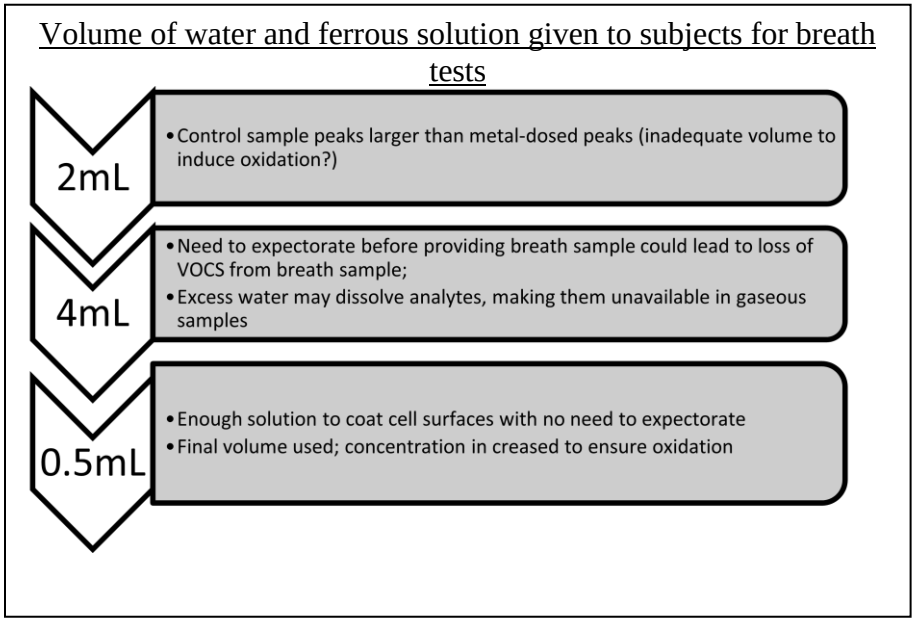
Appendix A: Method Development Schematics

These diagrams illustrate the process of method development for several aspects of the procedure. The conditions listed in the white arrows on the left show the progression of variables from early in testing (uppermost arrow) to the final condition set for the variable (bottom arrow). Information in the gray boxes to the right explains why a condition was changed or found to be satisfactory.




Time spent "gargling" with breath held before sample collection for breath tests
10 seconds
•Unsure if this allowed enough time for secondary lipid oxidation products to form measurable concentrations
30 seconds
•Chose to further increase gargling time in an attempt to measure more lipid oxidation products
1 minute
•Challenging - the maximum amount of time most subjects could hold breath
•Final amount of time used


Volume of water and ferrous solution given to subjects for breath tests
2mL
•Control sample peaks larger than metal-dosed peaks (inadequate volume to induce oxidation?)
4mL
•Need to expectorate before providing breath sample could lead to loss of VOCS from breath sample;
•Excess water may dissolve analytes, making them unavailable in gaseous samples
0.5mL
•Enough solution to coat cell surfaces with no need to expectorate
•Final volume used; concentration in creased to ensure oxidation

Appendix B: Lipid Oxidation Raw Data - Combined

	In Vitro Samples (µPC)[1]	Tenax Etched µPC – Breath Samples[2]	DVB µPC – Breath Samples[3]	SPME[4]
Before (control)	1-hexanol, 2-ethyl (DVB) 2-heptenal, (E)- (DVB) 2-octenal, (E)- (Tenax etched) heptane (DVB) hexanal (DVB; Tenax etched) hexane (DVB) octane (DVB) pentane (DVB)	1-hexanol, 2-ethyl (B,J) 2-heptenal (Z)- (J) heptane (B,H,J) hexanal (B,H,J) nonanal (B,H) octanal (B,H,J) octane (J) pentane, 2-methyl (B)	1-butanol, 2-methyl- (A,J) 1-heptene (A) 1-hexanol, 2-ethyl (A) 2-heptanol (A) 3-heptanone, 4-methyl (A,H) heptane (A,H,J) hexane (A,J) octane (A) pentane, 2-methyl (A,H,J)	2, 4-nonadienal, (E,E)- (PDMS, flask) 2,4-decadienal (PDMS, flask) 2-decenal, (E)- (PDMS, flask) furan, 2-pentyl (PDMS, flask) hexanal (PDMS, flask)
After (metal)	1-butanol, 2-methyl (DVB) 1-hexanol, 2-ethyl (DVB; Tenax Etched) 1-pentanol (DVB) 2,4-decadienal (DVB) 2,4-nonadienal, (E,E)- (DVB) 2-decenal,(E)- (Tenax etched; Tenax packed) 2-heptenal (E)- (DVB) 2-heptenal (Z)- (DVB) butanal, 2-methyl (DVB; Tenax packed) Furan, 2-pentyl (DVB) heptanal (DVB) heptane (DVB) hexanal (DVB; Tenax etched; Tenax packed) hexane (DVB) octanal (DVB; Tenax etched) octane (DVB) pentanal (DVB; Tenax packed) pentane(DVB; Tenax packed) propanal (DVB)	1-butanol (H) 1-butanol, 2-methyl (H) 1-hexanol, 2-ethyl (H) 1-pentanol (H) 2-decenal, (E)- (J) 2-heptanone, 4-methyl (H) 4-heptanal, (E), (B) heptane (B,H,J) hexanal (B, H,J) hexane (H) nonanal (H) octanal (B,H) octane (J)	1-butanol, 2-methyl (A,J) 1-heptene (A) 1-hexanol, 2-ethyl (A) 2-heptenal, (Z)- (A) 3-heptanone, 4-methyl (A,H,J) 3-heptanone, 6-methyl (A) 3-nonene (A) cis-4-nonene (A) heptane (A,J) hexane (A,J) octane (A,J) pentane, 2-methyl (A,H,J)	1-hexanol, 2-ethyl (PDMS, flask) 1-pentanol (PDMS, flask) 2,4-decadienal (PDMS, flask) 2-decenal, (E) – ((PDMS, flask) 2-nonenal , (E)- (PDMS, flask) 2-octenal, (E)- (PDMS, flask) furan, 2-pentyl (PDMS, flask) hexanal (PDMS, flask) nonanoic acid (PDMS, breath) octanal (PDMS, flask; Tenax, flask) octane (PDMS, breath; PDMS, flask)

[1]In parentheses: type of µPC used to concentrate sample in which listed lipid oxidation product was found

[2]In parentheses: subject in whose breath listed lipid oxidation product was found; three subjects in this portion of the study: B, H, J

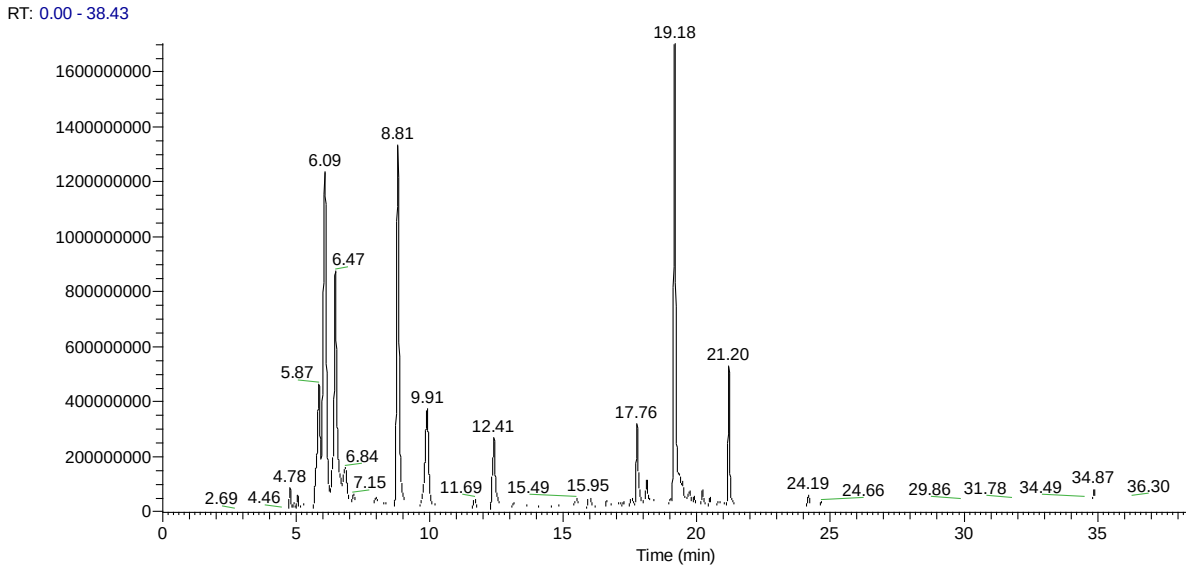
[3]In parentheses: subject in whose breath listed lipid oxidation product was found; three subjects in this portion of the study: A, H, J

[4]In parentheses: method (breath or in vitro (flask)) and SPME adsorbent used to collect and concentrate sample in which listed lipid oxidation product was found

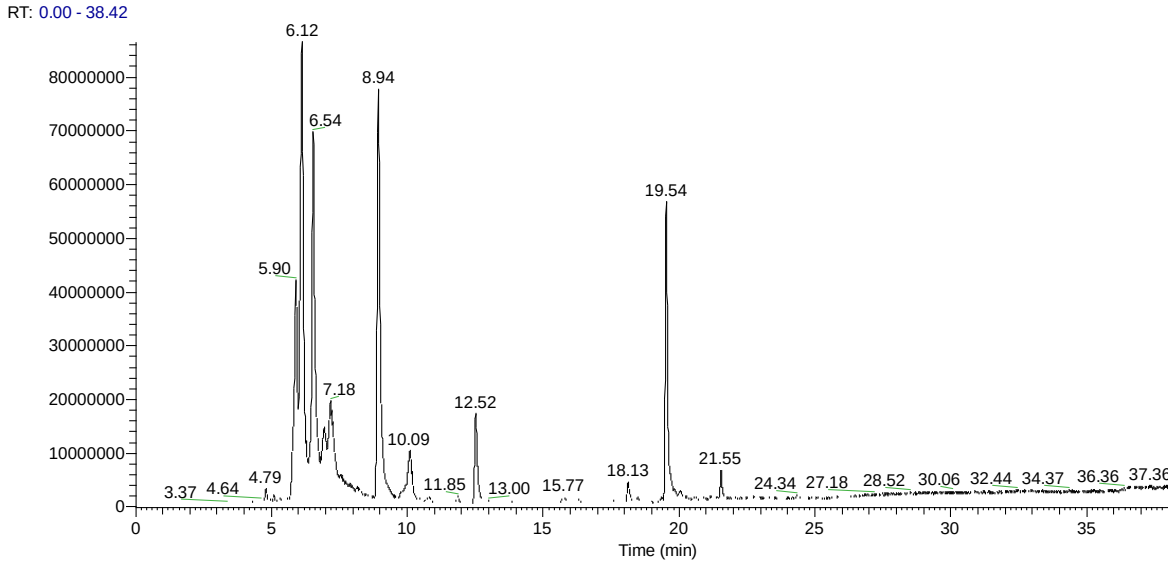
Appendix C: Select Individual Chromatograms

From Figure 10: Comparison of "After Fe2+" Breath from Subject A (top) and Subject J (Bottom) Concentrated Using a Packed DVB µPC

Subject A

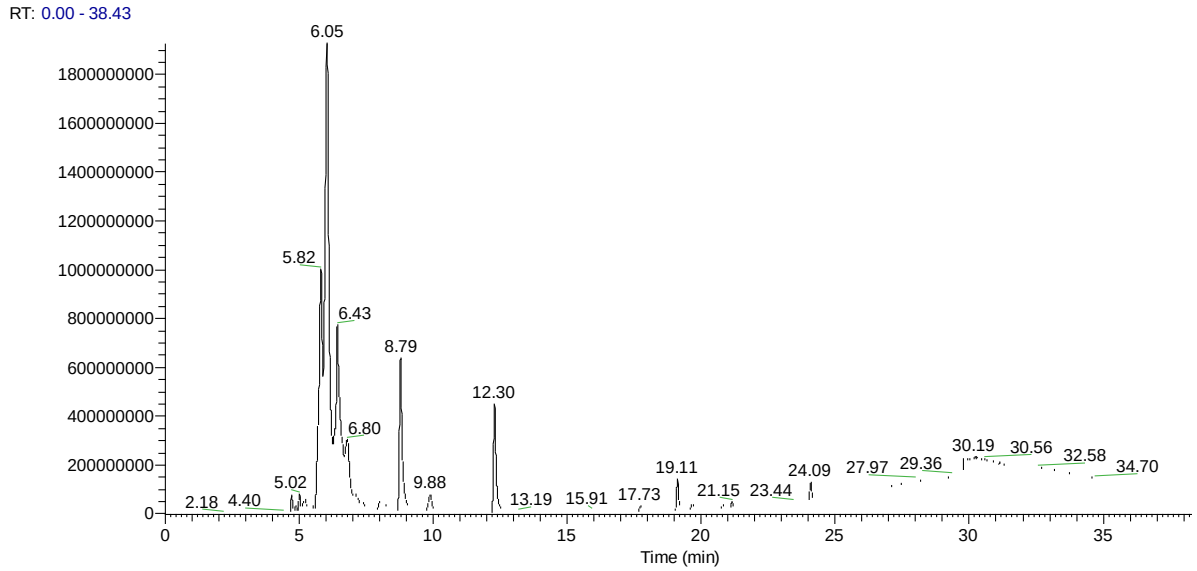


Subject J

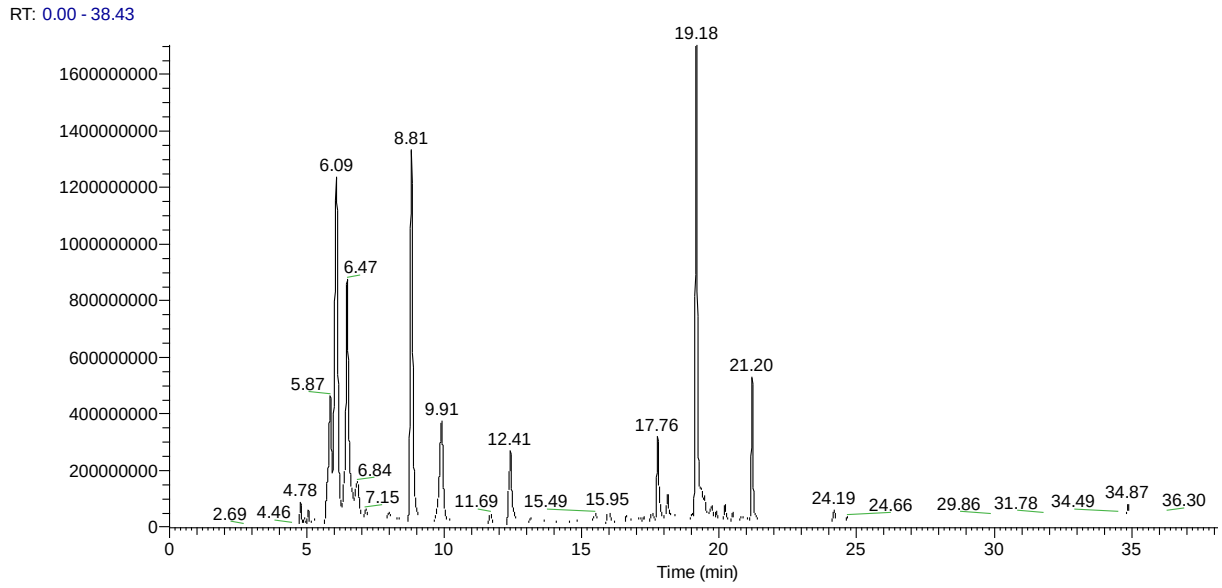


From Figure 11: "Before" and "After Fe^{2+}" Chromatograms of Three Different Trials from Subject A, Breath Samples Concentrated with a packed DVB µPC

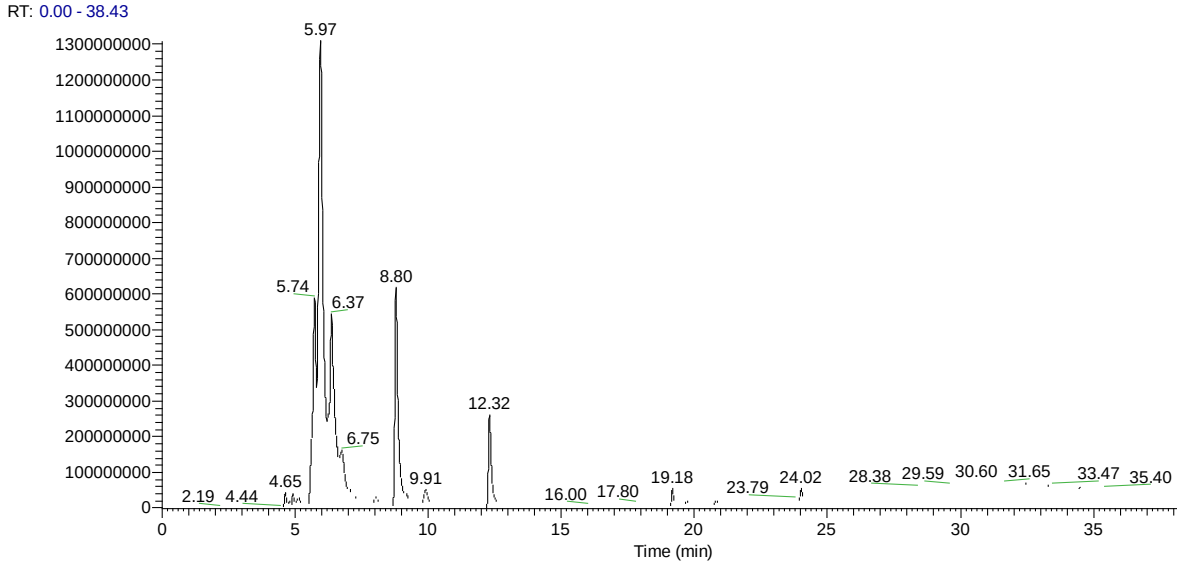
"Before" #1



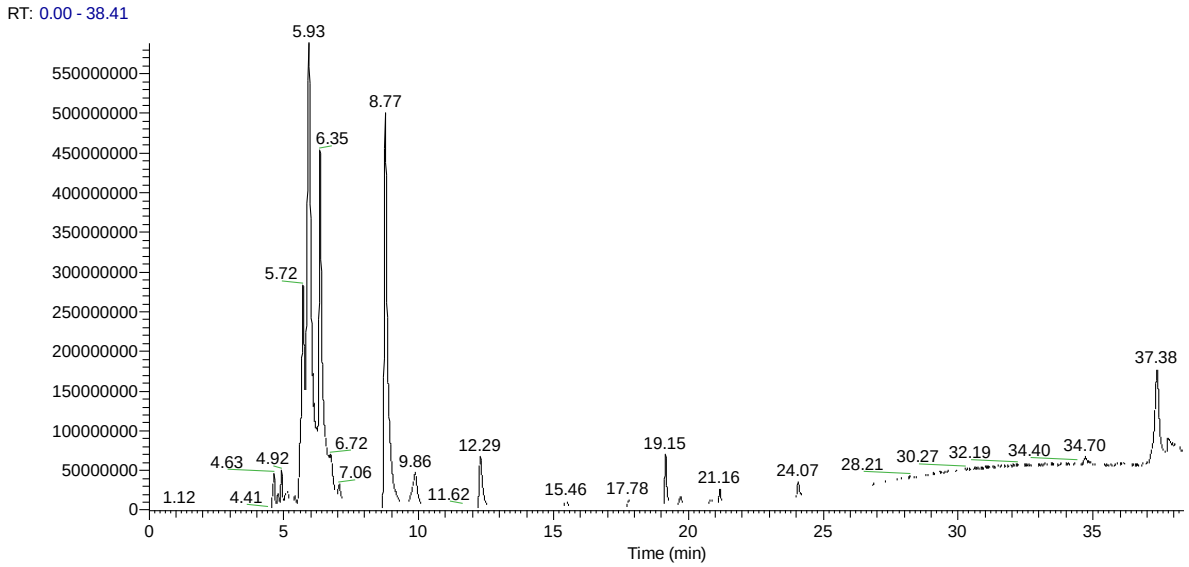
"After" #1



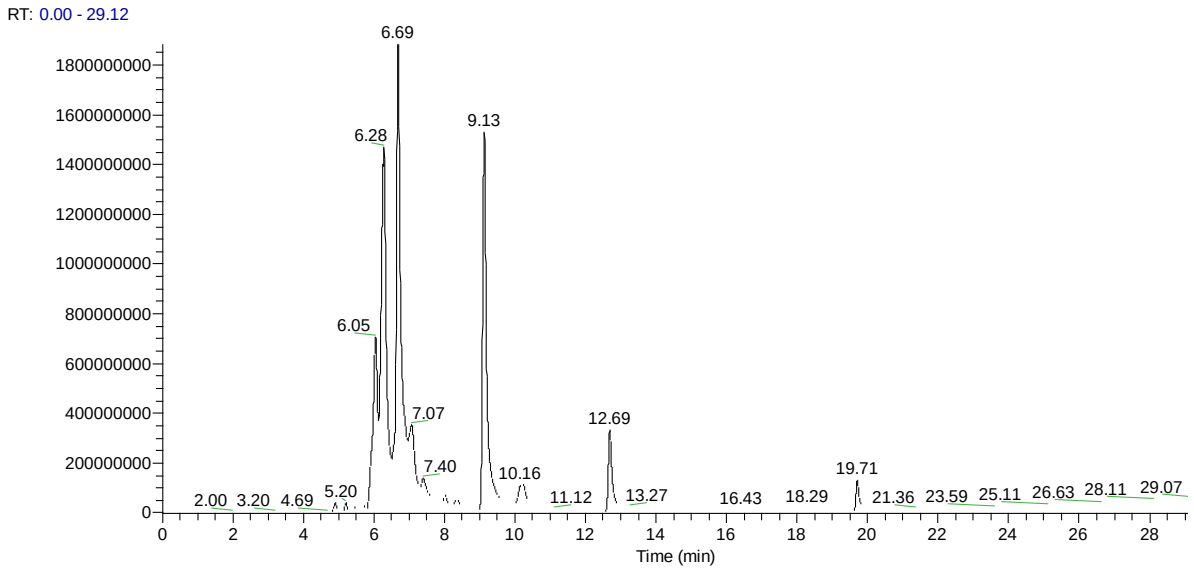
“Before” #2



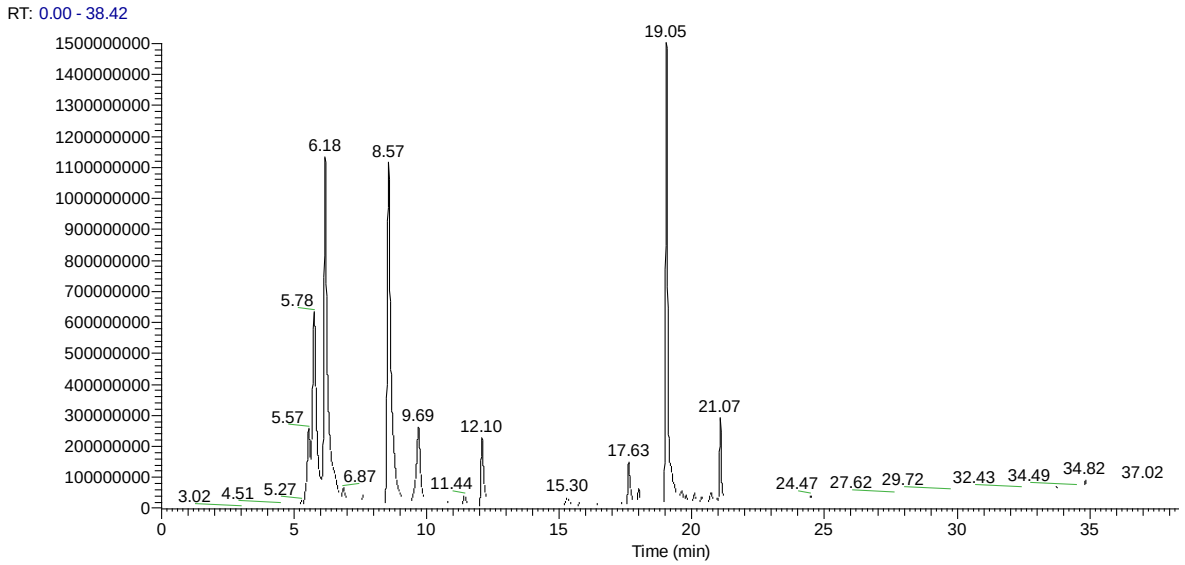
“After” #2



“Before” #3

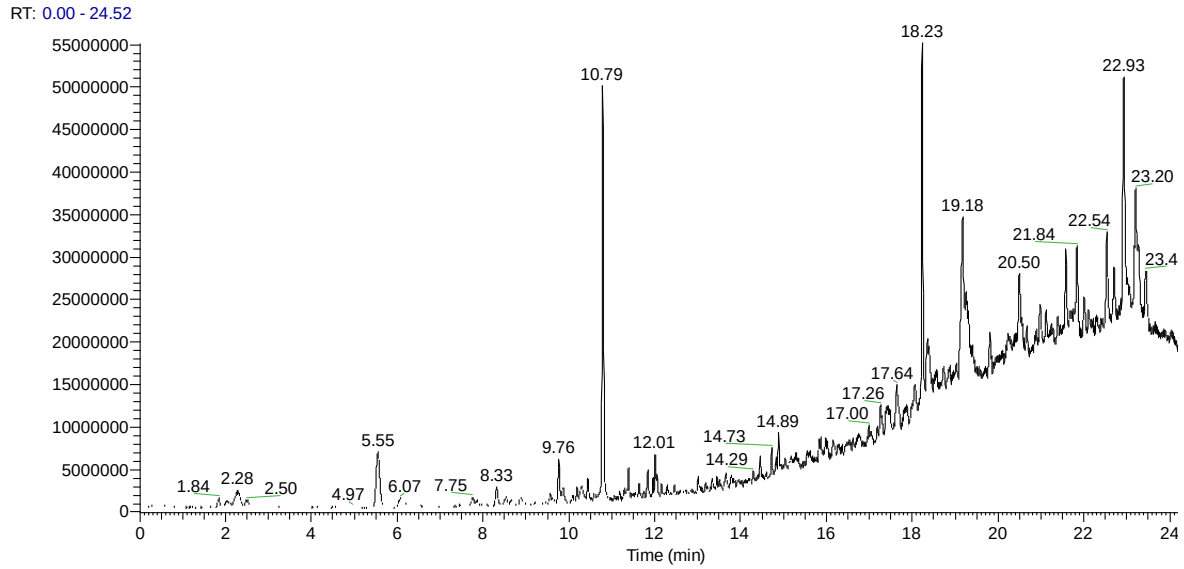


“After” #3

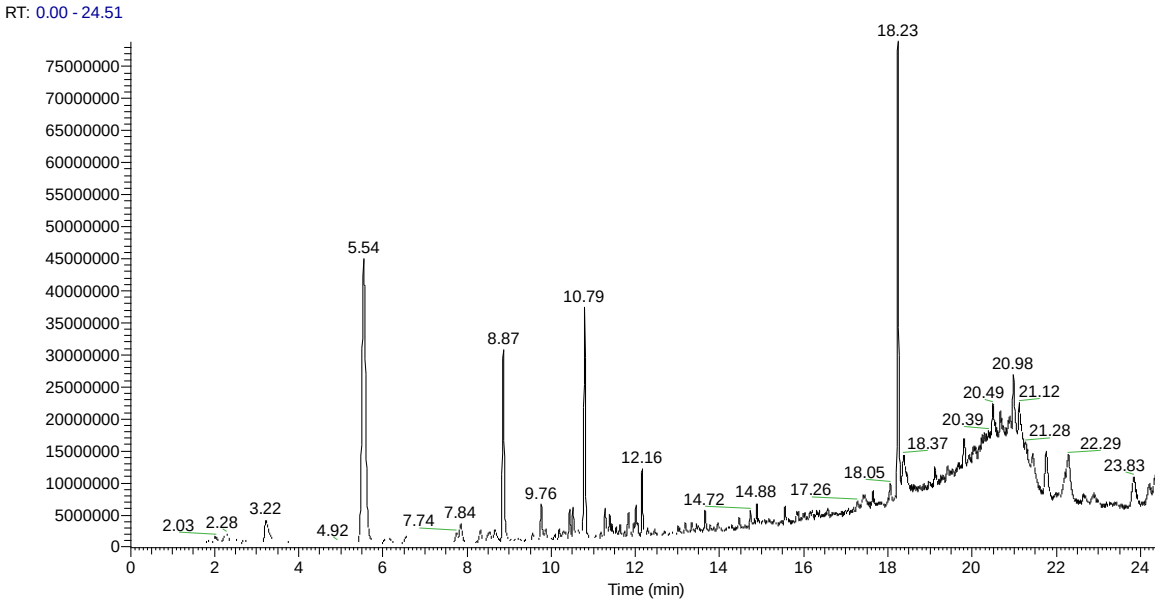


From Figure 12: “Before” and “After Fe2+” Chromatograms of Three Different Trials from Subject H, Breath Samples Concentrated with etched Tenax µPC

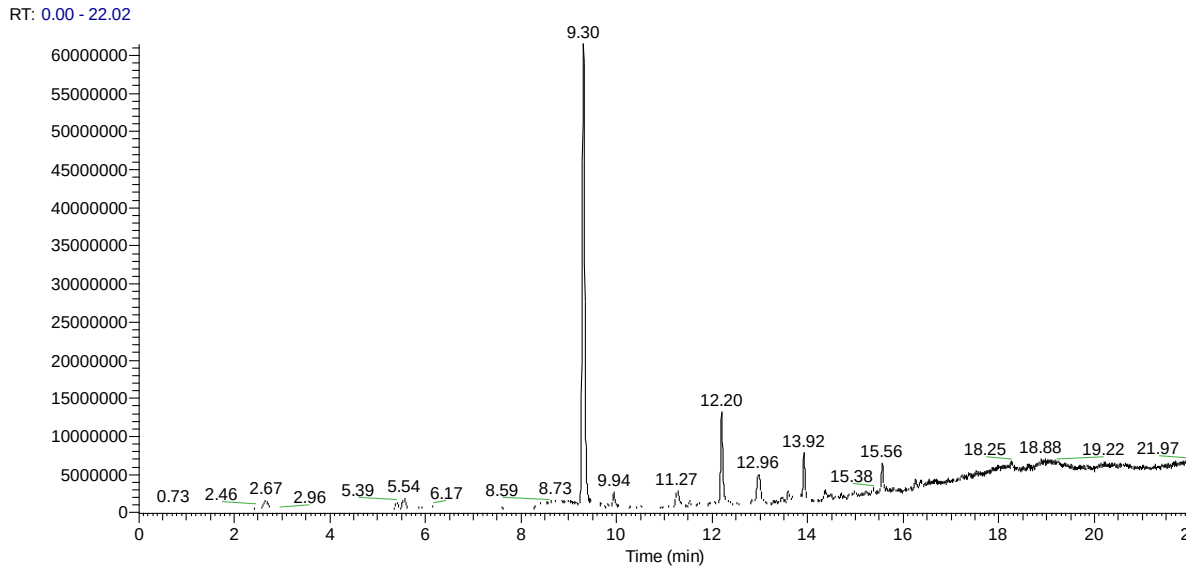
“Before” #1



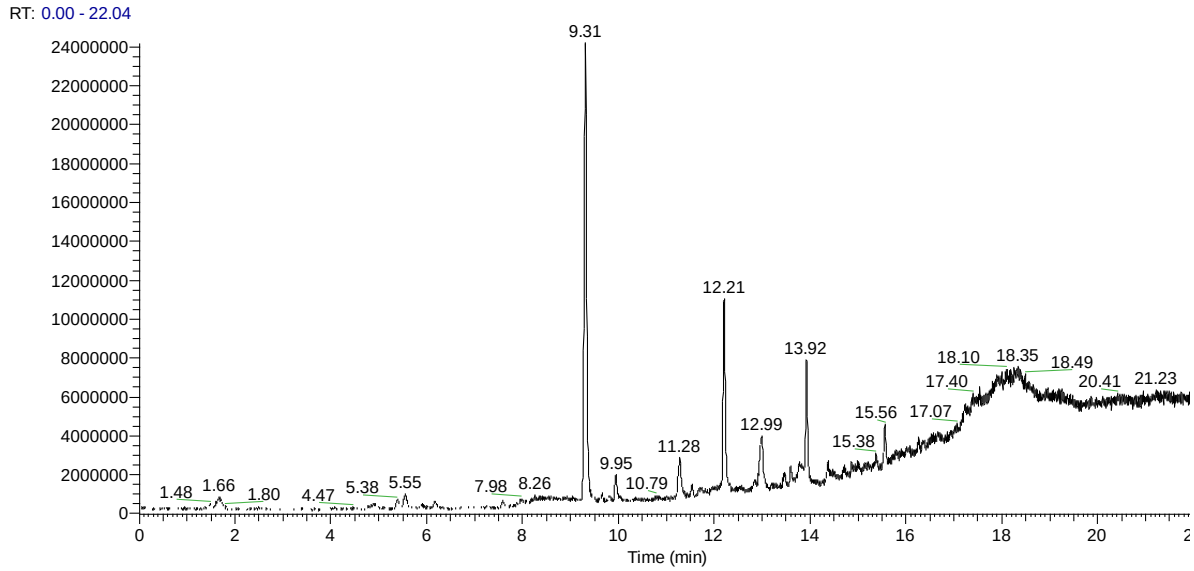
“After” #1



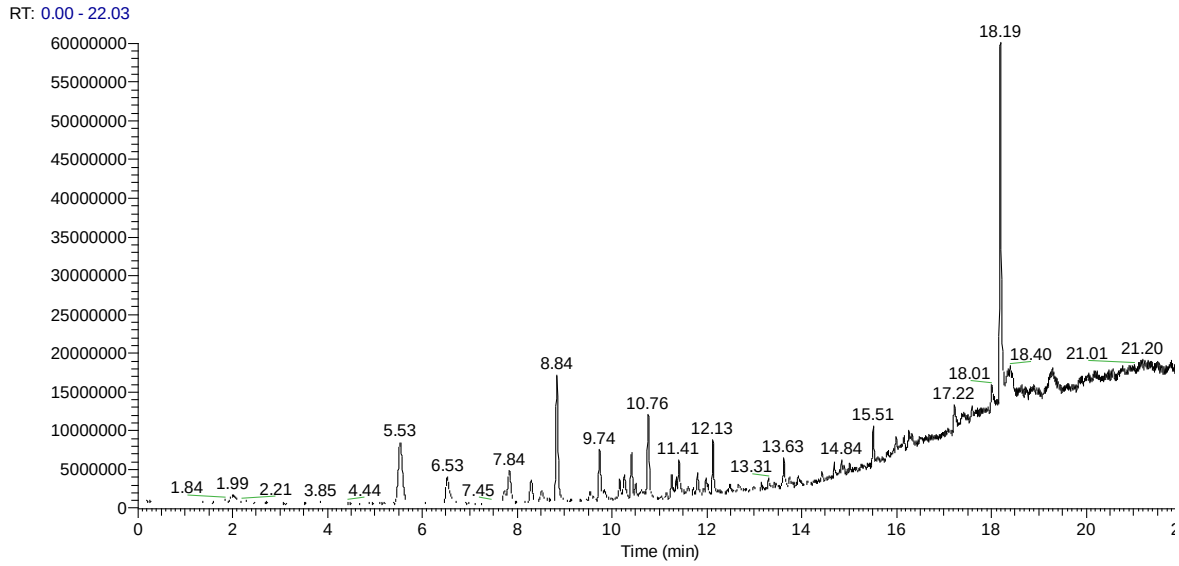
“Before” #2



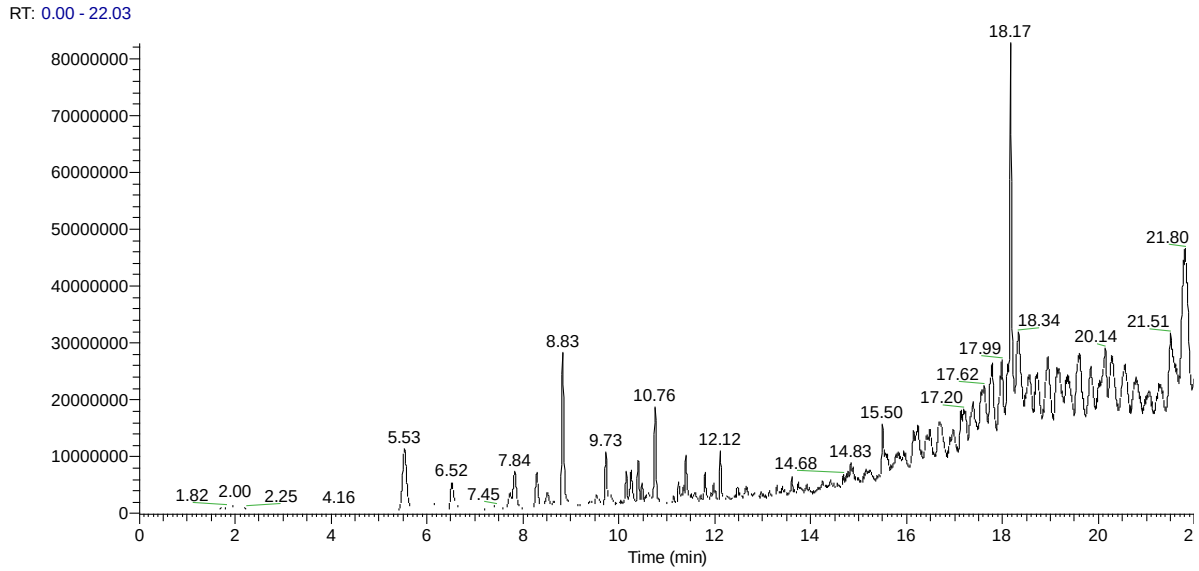
“After” #2



“Before” #3

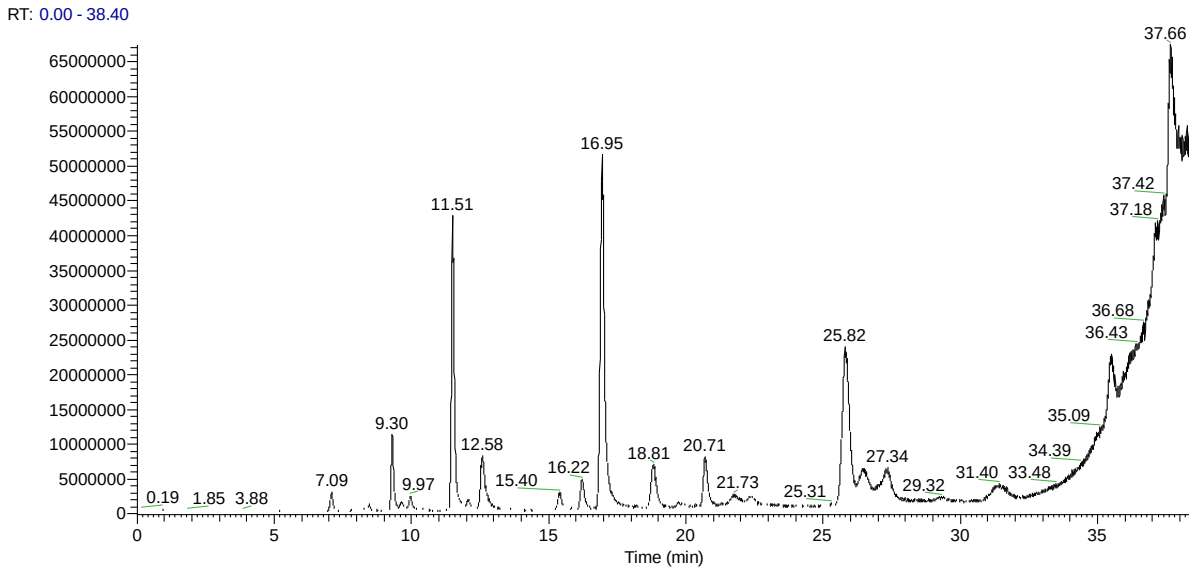


“After” #3

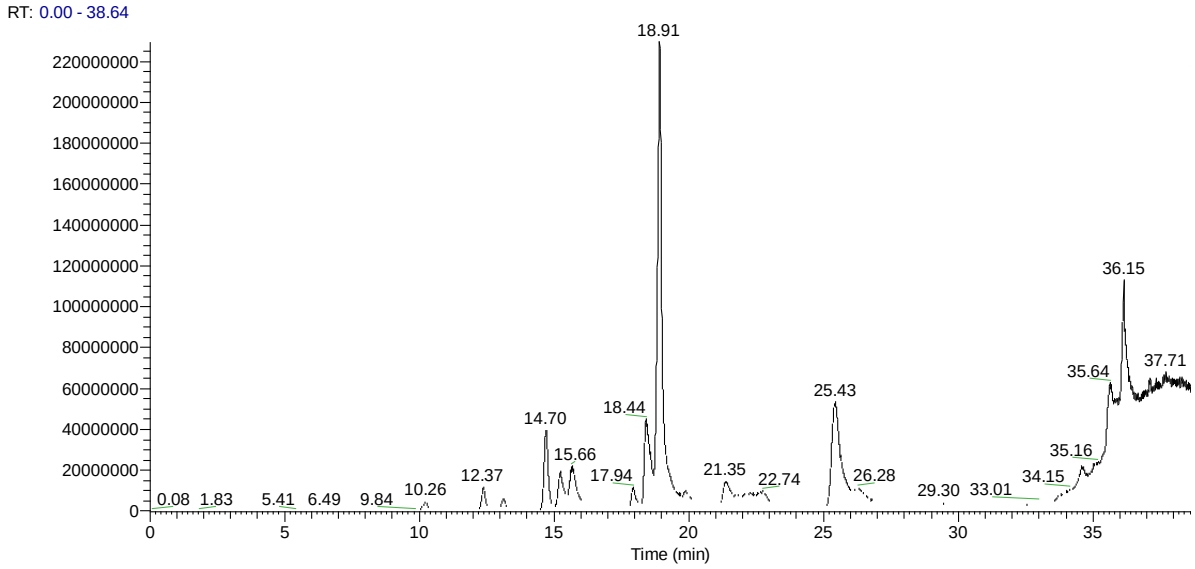


From Figure 14: “After Metal Addition” Linoleic Acid Samples Concentrated Using the Same Packed DVB µPC

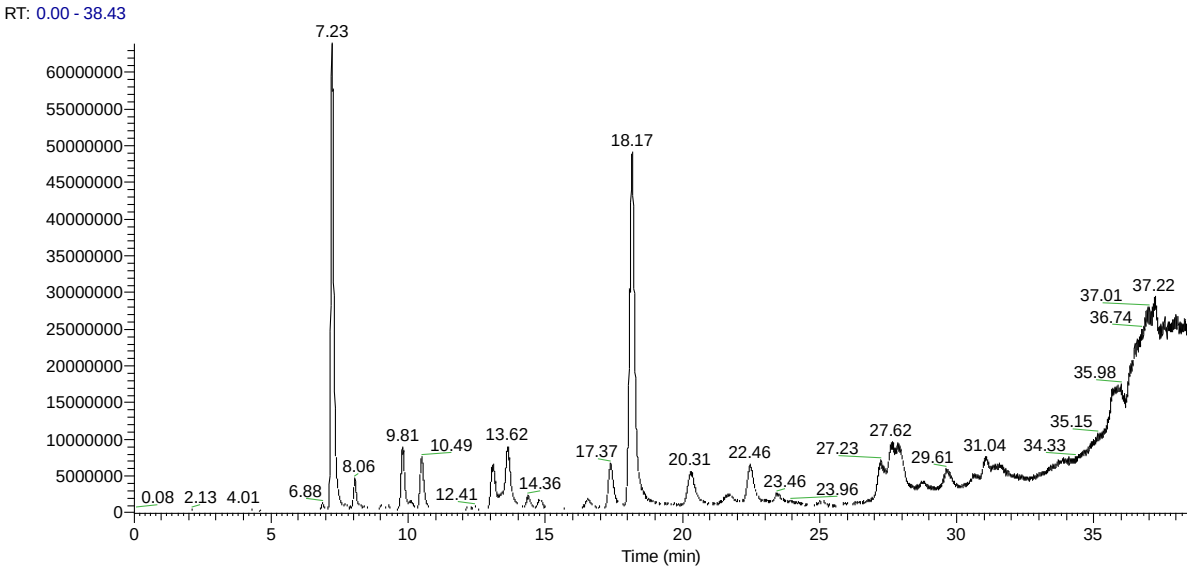
Sample 1, Day 1



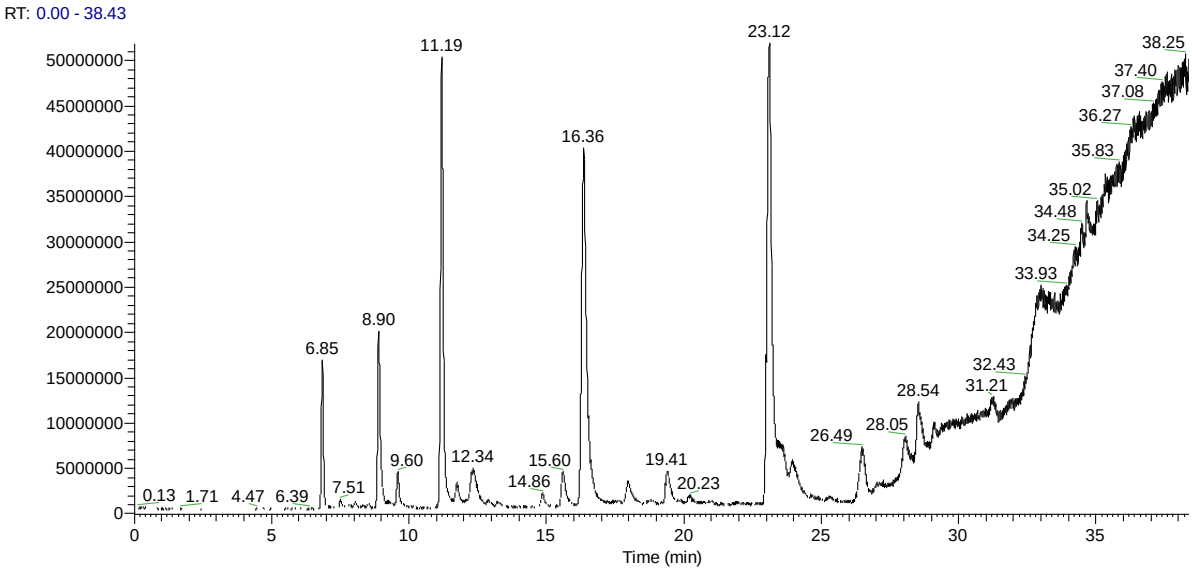
Sample 2, Day 1



Sample 1, Day 2

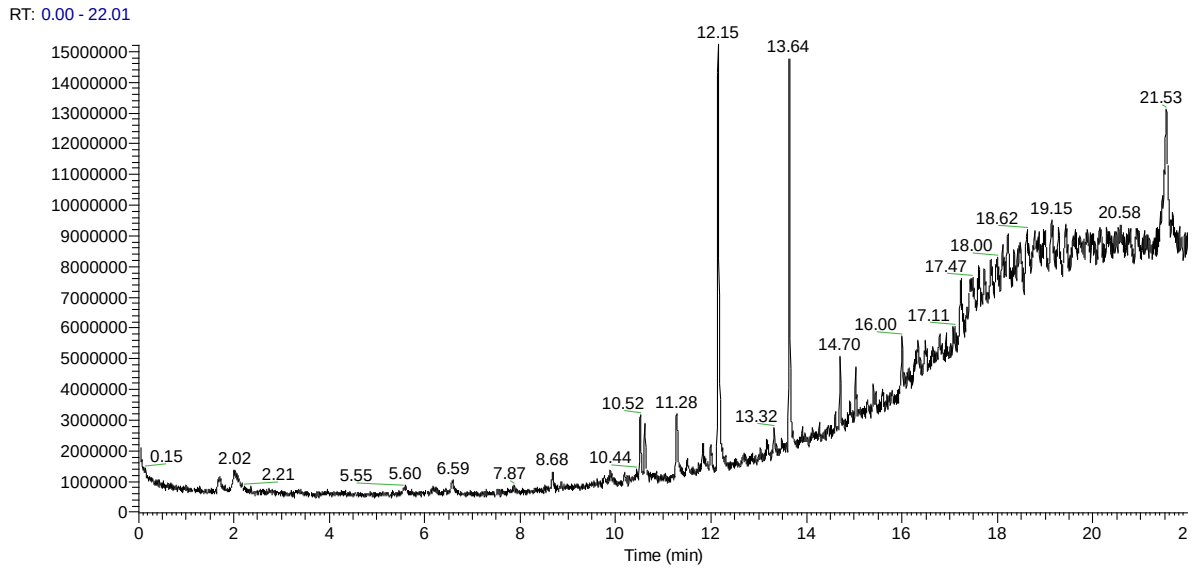


Sample 2, Day 2

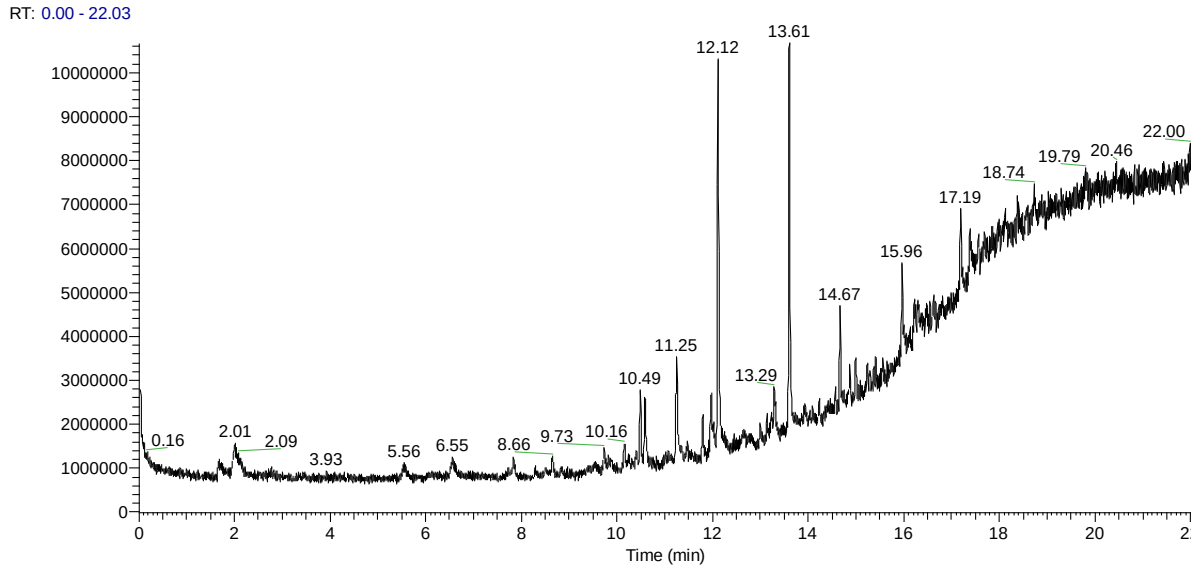


From Figure 15: Ambient Air from a Conference Room, Lab, and the Outdoors, Concentrated Using an Etched Tenax µPC

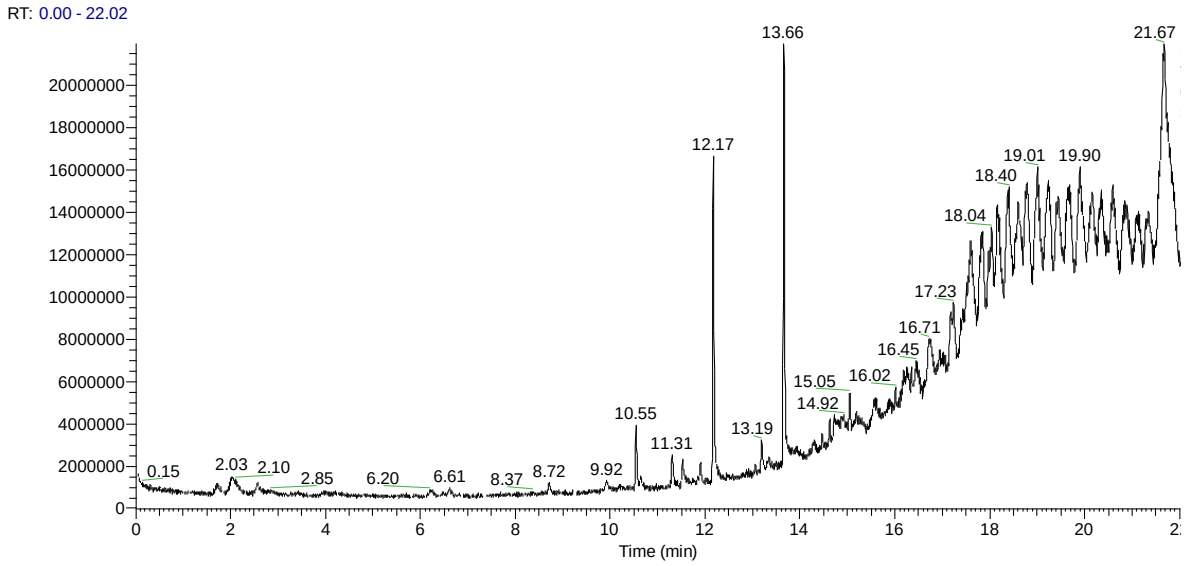
Conference Room Air



Lab Air



Outdoor Air


RT: 0.00 - 22.02
20000000
18000000
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
0.15
2.03
2.10
2.85
6.20
6.61
8.37
8.72
9.92
10.55
11.31
12.17
13.19
13.66
14.92
15.05
16.02
16.45
16.71
17.23
18.04
18.40
19.01
19.90
21.67
0
2
4
6
8
10
12
14
16
18
20
Time (min)

Appendix D: High Density Polyethylene (HDPE) Pipe Data

Purpose

In addition to the scope of the above-described project, further studies were pursued to illustrate the functionality of the µPC as a means of analyzing the potential for other environmental exposure, specifically the effects of residual chlorine in drinking water on HDPE pipe. The DVB packed µPC was used to concentrate the gases from within HDPE pipe and from the headspace above water, which had been stored in HDPE pipe.

Methods

HDPE pipe with an internal diameter of 1.6 cm was cut to lengths of 99 cm to achieve a volume of 200 cm^3 per pipe section. Two water solutions were prepared: a control solution of non-chlorinated simulated tap water, and a solution of highly chlorinated simulated tap water. The latter was made fresh daily, using chlorine bleach, at a concentration of 250 mg/L ± 5% chlorine; the concentration was verified using the average of three measurements taken with a colorimetric total chlorine test kit (Hach).

Silicon stoppers were wrapped in Teflon tape. One stopper was inserted in the end of a pipe section, and the pipe was filled, until overflowing, with the appropriate water sample. Two pipes were prepared at a time, one with non-chlorinated water and the other with chlorinated water. Once filled, the other end of each pipe was capped with a Teflon tape-wrapped silicon stopper. Stoppers were secured with tape to prevent leakage. Both pipes were then inserted into a large piece of PVC pipe, which was capped on both ends and stored in a 38˚C room for 24 hours.

After 24 hours, the PVC pipe was removed from the warm room and HDPE pipes removed from therein. One stopper was removed from the end of a HDPE pipe section, and the water within poured into a modified 500 mL Erlenmeyer flaks (onto which a 25 mL scintillation vial with screw-on rubber septum cap was annealed to the side). The flask receiving the chlorinated water already contained 11 mL of hydrogen peroxide (as a dechlorinating agent) before the water from the pipe was emptied into the flask. The flask stoppers were quickly secured and sealed with parafilm. Stoppers were also quickly replaced in HDPE pipe.

If sampling could not be achieved immediately, sealed flasks containing sample water were stored at 2˚C. Pipes, with silicon stoppers replaced, were stored in the capped PVC pipe at 38˚C.

Sampling was done from both the headspace above both water samples and the space from within each HDPE pipe section. All four samples per trial were concentrated both with a PDMS DVB SPME fiber and a DVB packed µPC. During sampling of the water headspace, the flask was heated to 40˚C in a water bath. SPME concentration was conducted first by placing the SPME fiber into the flask's headspace, through the septum cap, for 10 minutes. The sample was then analyzed as described below. For µPC loading, two pieces of capillary tubing were inserted in the septum opening of the glassware. One was connected to a vacuum pump and the other an inert bag filled with high purity nitrogen, which replaced the sample gases in the glassware as they were drawn through the µPC. The sample was drawn across the µPC, for 10 minutes. Sampling of the gases from within HDPE pipe sections was conducted as described above, through a septum in a bored-out, Teflon tape wrapped silicon stopper.

Some samples incorporated an internal standard as a reference. The standard chosen was an EPA 624 Surrogate Standard (Restek), containing fluorobenzene, pentafluorobenzene, and 1-bromo-2-fluorobenzene. The standard was diluted from 2000 µg/L to 200 µg/L in methanol and stored in 25 mL GC vials with crimp caps, placed in a sealed mason jar at ~0 ˚C . Before each use, the standard was further diluted to 20 ng/L in Nanopure water, over heat. Standard was loaded from the above described flask (containing 0.5 mL of standard) for 5 minutes per sample.

Prior to sample concentration concentrators were conditioned to remove any compounds collected between uses. SPME fibers were conditioned at a temperature of 250°C for 30 minutes. Micropreconcentrators were heated to temperature of 200°C with carrier gas flowing through them and left to be conditioned for at least ten minutes.

Samples were analyzed using a gas-chromatography-mass-spectrometry (GC-MS) system with a DB- 17 MS 30 m fused-silica capillary column. SPME samples were desorbed in the GC injection port for 3 minutes. Samples was desorbed from the µPC on a ceramic heater at a temperature of 200°C, while carrier gas flowed through the µPC and then onto the column. The

GC was programmed as follows: initial temperature of 33°C, held for six minutes, followed by a ramp rate of 2°C/min to a final temperature of 175°C, which was held for 2 minutes. The carrier gas flow was set to 1.2 mL/min.

Data

Compounds of interest were not readily found in either the pipe or the headspace above the water stored in the pipe. Results from non-chlorinated and chlorinated water were similar in most cases. SPME was outperformed by µPCs as a means of sample preconcentration.

Table AD1 – Numbers Used to Label Respective Compounds in Chromatograms Below

Number	Compound
1	pentafluorobenzene (external standard)
2	fluorobenzene (external standard)
3	1-bromo-3-fluorobenzene (external standard)
4	methylene chloride
5	trichloromethane

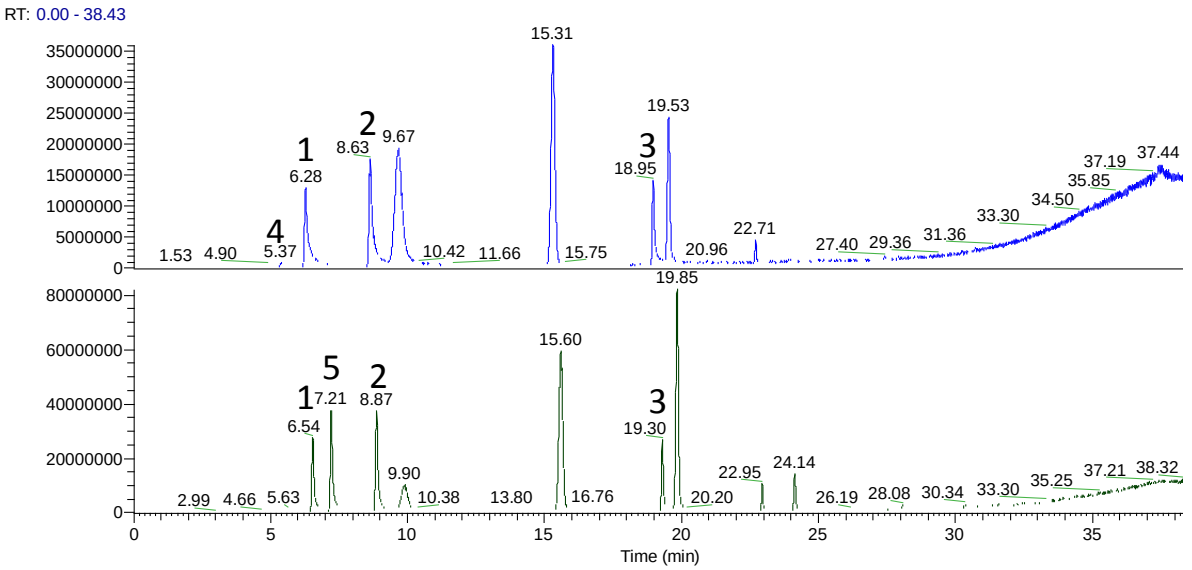


Figure AD1 – Chromatograms of Headspace above Non-chlorinated (top) and Chlorinated (bottom) Water Stored in HDPE Pipes; Samples Concentrated using a Packed DVB µPC

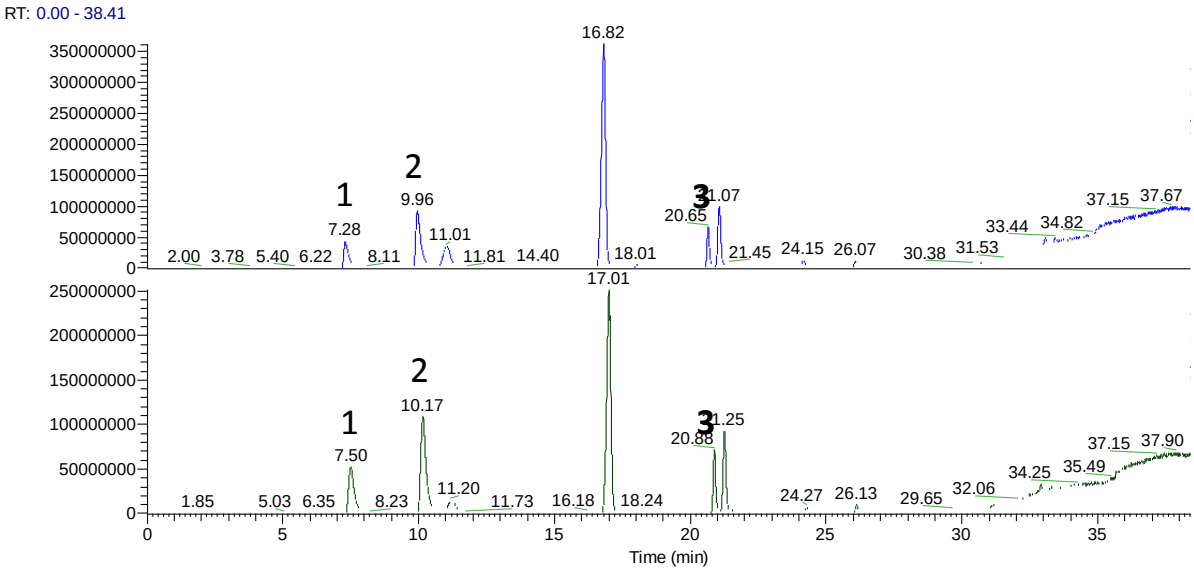


Figure AD2 – Chromatograms of Air Sampled from within HDPE Pipes, which Stored Non-chlorinated (top) and Chlorinated (bottom); Samples Concentrated using a Packed DVB µPC

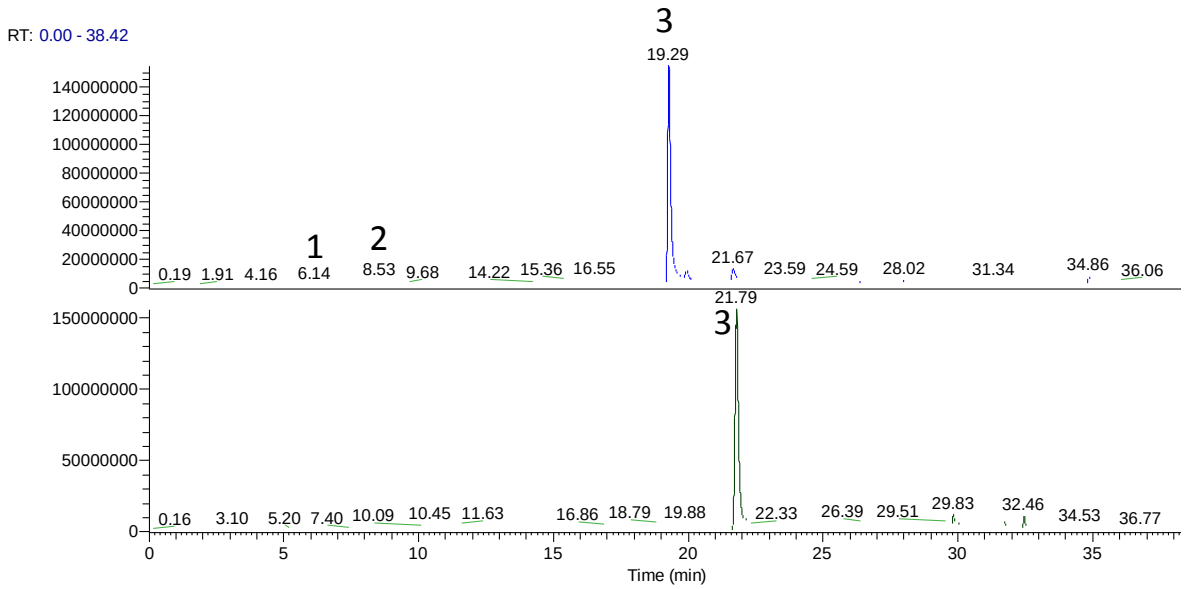


Figure AD3 – Chromatograms of Headspace above Non-chlorinated (top) and Chlorinated (bottom) Water Stored in HDPE Pipes; Samples Concentrated using a PDMS DVB SPME Fiber

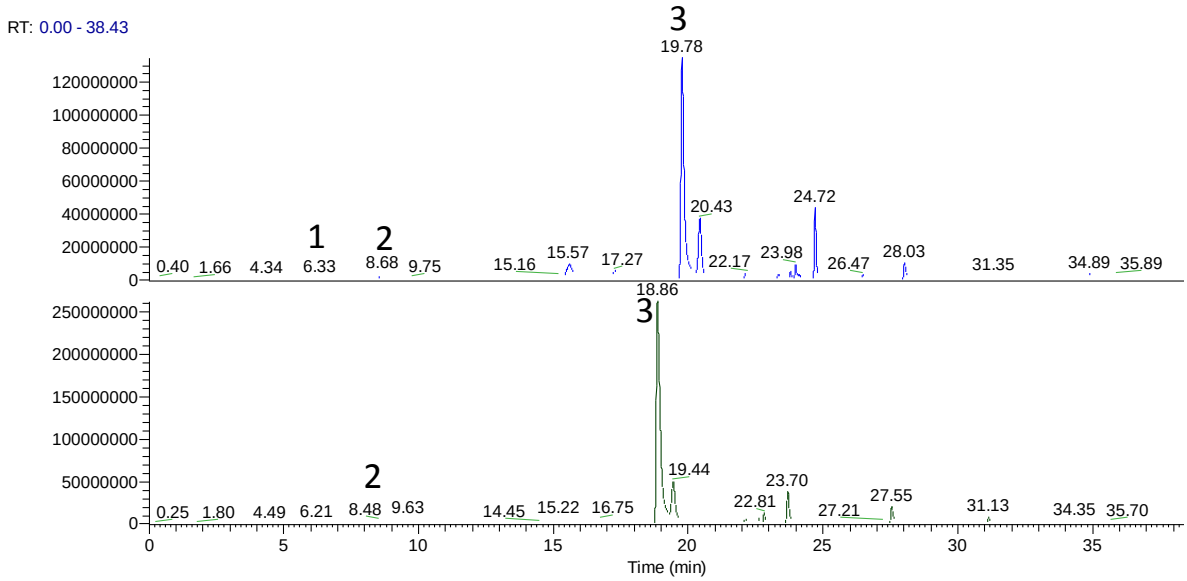


Figure AD4 – Chromatograms of Air Sampled from within HDPE Pipes, which Stored Non-chlorinated (top) and Chlorinated (bottom); Samples Concentrated using a PDMS DVB SPME fiber