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John Goodpaster
Chair

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Peter Kissinger

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Approved by: Eric Long 11/13/2013
Head of the Graduate Program Date

IDENTIFICATION OF TOBACCO-RELATED COMPOUNDS IN TOBACCO PRODUCTS AND HUMAN HAIR

A Dissertation

Submitted to the Faculty

of

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by

Christina L. Rainey

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This dissertation is dedicated to my father, Emerson Rainey.

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ABSTRACT

Rainey, Christina L. Ph.D., Purdue University, December 2013. Identification of Tobacco-Related Compounds in Tobacco Products and Human Hair. Major Professor: John V. Goodpaster.

Analyses of tobacco products and their usage are well-researched and have implications in analytical chemistry, forensic science, toxicology, and medicine. As such, analytical methods must be developed to extract compounds of interest from tobacco products and biological specimens in order to determine tobacco exposure.

In 2009, R.J. Reynolds Tobacco Co. released a line of dissolvable tobacco products that are marketed as a smoking alternative. The dissolvables were extracted and prepared by ultrasonic extractions, derivatization, and headspace solid phase microextraction (SPME) with analysis by gas chromatography-mass spectrometry (GC-MS). The results show that the compounds present are nicotine, flavoring compounds, humectants and binders.[1, 2]

Humectant concentrations vary among different tobacco types depending on the intended use. Humectants were quantified in various tobacco types by GC and “splitting” the column flow between a flame ionization detector (FID) and an MS using a microfluidic splitter in order to gain advantage from the MS’s selectivity. The results demonstrated excellent correlation between FID and MS

and show that MS provides a higher level of selectivity and ensures peak purity.[3] Chemometrics was also used to distinguish products by tobacco type.

Hair is a common type of evidence in forensic investigations, and it is often subjected to mitochondrial DNA (mtDNA) analysis. Preliminary data was gathered on potential “lifestyle” markers for smoking status as well as any indications of subject age, gender, or race by investigating the organic “waste” produced during a mtDNA extraction procedure. The normally discarded organic fractions were analyzed by GC-MS and various lipids and fatty acids were detected.

At this point, a total vaporization-SPME (TV-SPME) method was theorized, developed, and optimized for the specific determination of nicotine and its metabolite, cotinine. The theory of TV-SPME is to completely vaporize an organic extract which will eliminate the partitioning between the sample and the headspace, thereby simplifying the thermodynamic equilibrium. Parameters such as sample volume, incubation temperature, and extraction time were optimized to achieve the maximum analyte signal. Response surface methodology (RSM) is a statistical model that is very useful in predicting and determining optimum values for variables to ensure the ideal response. RSM was used to optimize the technique of TV-SPME for the analysis of nicotine and cotinine.

Lastly, quantitation of nicotine and cotinine in human hair typically requires large sample sizes and extensive extraction procedures. Hence, a method using small sample sizes and a simple alkaline digestion followed by TV-SPME-GC-MS has been developed. Hair samples were collected from anonymous volunteers

and nicotine and cotinine were identified and quantitated in the hair of tobacco users.

CHAPTER 1. CHEMICAL ANALYSIS OF DISSOLVABLE TOBACCO PRODUCTS

1.1 Introduction

The chemical composition of smokeless tobacco and its effect on health is a well-researched area. For example, several authors have discussed smokeless tobacco products such as moist snuff, with a particular emphasis on nicotine content [4-10] as well as the presence of tobacco-specific nitrosamines[5, 6, 8, 9, 11] and toxic metals.[12] A new development in the smokeless tobacco market occurred in 2009 when R.J. Reynolds Tobacco Company released a line of “dissolvable” tobacco products in test markets in Indianapolis, IN, Columbus, OH, and Portland, OR. In early 2010, a second release of the dissolvables was made in the same test markets. In late 2010, the dissolvable tobacco products were pulled from the shelves supposedly to allow the manufacturer to reformulate the tobacco products based on the results of the test market trial. The new and improved dissolvable tobacco products were released in Charlotte, NC and Denver, CO. Dissolvables released in the initial 2009 trial will be referred to hereafter as “first release of old dissolvables”; the dissolvables re-released in early 2010 will be referred to as “second-release of old dissolvables,” and the reformulated dissolvables re-released in late 2010 will be referred to as “new dissolvables.”

According to the manufacturer, dissolvable tobacco products are smokeless, spit-free, made from finely milled tobacco and come in three forms: Camel Orbs©, Camel Sticks©, and Camel Strips©. The dissolvables contain less moisture and salt than moist snuff, and therefore do not require the user to spit. Figure 1-1 shows the old dissolvables and their packaging. Orbs are small, brown/tan oval-shaped pellets that dissolve in ~15 minutes in the user's mouth. Sticks are brown toothpick-like rods which last ~10-30 minutes. Strips are flat brown rectangular tobacco strips, similar to breath freshening strips, that last ~3 minutes. The users of the dissolvable tobacco products are not supposed to swallow the Orb, Stick, or Strip, but to allow the tobacco product to dissolve in the mouth. Orbs are used by placing the pellet between the lip and gum, Sticks are to be held like a toothpick or broken into pieces and placed between the lip and gum, and Strips are to be placed on the tongue like a breath strip or placed between the lip and gum. Because the dissolvables are smokeless and spit-free, people can be discreet in their tobacco use in places where smoking is prohibited. In particular, the dissolvables gained popularity with women in the Charlotte and Denver test markets.[13]

Figure 1-1 Images of packaging and dissolvables. a) Mellow Orb b) Fresh Orb c) Fresh Strip d) Mellow Stick

Dissolvables were initially available in “mellow” and “fresh” flavors; the Orbs came in both flavors, the Sticks came in mellow flavor, and the Strips came in fresh flavor.[14] The dissolvables have since been reformulated so that they no longer have the mellow and fresh flavors – only “mint” flavor.[15] The packaging and number of dissolvables per package has also changed.

There is significant controversy about whether the dissolvable tobacco products are more or less harmful than cigarettes. Surveys indicate that the dissolvables are less harmful because they contain fewer toxins than cigarettes.[16, 17] Studies have shown that smokers had decreased cigarette use and increased desire to quit smoking while using smokeless tobacco products as a replacement.[13, 18, 19] On the other hand, while the manufacturer states that the products are marketed for adult consumption only,[15] studies explain that the packaging and shapes of the dissolvables may appeal to children, or get teens started on tobacco usage at a young age[14, 16, 17] A particular concern about some dissolvable products is the potential for harming children through unintentional poisoning.[17] For example, the packaging and design of the dissolvables may also appeal to children and some dissolvables like Orbs may be mistaken for candy.

There are concerns about dissolvable tobacco products just as with other smokeless tobacco. In particular, adverse complications in the oral cavity are of concern with prolonged use of dissolvable tobacco products. Product literature from the old dissolvables release indicate levels of nicotine as 1 mg/orb, 0.6 mg/strip, and 3.1 mg/stick.[17] One study examining the potential for unintentional child poisonings show 0.83 mg nicotine per Orb.[17] Product literature for the new

dissolvables release indicates levels of nicotine have changed (1.2 mg/orb, 1.3 mg/strip, and 2.4 mg/stick).[15] A study by Stepanov and others analyzing dissolvable tobacco has revealed the pH, moisture content, nicotine, and tobacco specific nitroamine (TSNA) levels in the old Mellow and Fresh Orbs, Mellow Sticks, and Fresh Strips. This study demonstrated that total TSNA and unprotonated nicotine levels were higher in the Camel dissolvables than Ariva and Stonewell tobacco lozenges.[20]

Nicotine adsorption by the user largely depends on the pH of the dissolvable tobacco product. With increasing pH, more free-base nicotine is present, and therefore more nicotine is absorbed by the user.[10] From measurement of pH and total nicotine concentration, the % un-ionized (free) nicotine can be calculated from the Henderson-Hasselbalch equation, where the pK_a of nicotine is 8.02:[21]

$$pH = pK_a + \log \frac{[B]}{[BH^+]} \qquad \text{(Equation 1-1)}$$

To date, there have not been any published research articles on mouth diseases that may result from use of the dissolvables, the effect of swallowing a dissolvable, or the effect of using a dissolvable in combination with smoking. One obstacle to research in this area is an overall lack of information on the chemical composition of the dissolvable tobacco products.

Furthermore, monitoring changes in smokeless tobacco products such as moist snuff and loose leaf tobacco are well documented in an effort to make consumers aware of the changes in tobacco formulations.[7, 8] As such, continued

surveillance of dissolvable tobacco is important to inform consumers of any modifications. In general, the new dissolvables are expected to have different chemical compositions, at least because of the change in flavors. Therefore, the dissolvables have been fully characterized using three sample preparation techniques together with gas chromatography-mass spectrometry (GC-MS) as well as pH measurements and nicotine determinations.

1.2 Experimental

1.2.1 Materials

Dissolvable tobacco products were purchased in January 2010 from gas stations and tobacco shops in Indianapolis, IN. In March 2010, a second release of dissolvables were marketed in the same test markets as “new and improved.” Dissolvables purchased prior to March 2010 will be referred to as the first release of the old dissolvables and dissolvables purchased after March 2010 will be referred to as the second release of the old dissolvables. The old Orb dissolvables came in packages of 15, the old Sticks came in packages of 10, and the old Strips came in packages of 20.

In late 2010, a reformulated line of dissolvables were released. The new dissolvables were purchased from various gas stations in Colorado Springs, CO. These dissolvables will be referred to as new dissolvables. The new dissolvables come in packages of 12, either with one of each type (orbs, strips or sticks) or a

variety pack containing all three forms. All samples were kept in their packaging at room temperature until needed for analysis.

Hexanes (99.7%), dichloromethane (99.9%), methanol (99.9%), acetone (99.8%), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), Trimethyl-Silyl (Tri-Sil), and chemical standards of nicotine (99+%), menthol (99%), ethyl citrate (99+%), palmitic acid (>99%), stearic acid (>99.5%), glycerol (lab grade), xylitol (>99%), sorbitol (99%), carvone (99+%), vanillin (99%), phytol (97+%), threitol (98+%) and coumarin (>99%) were purchased from either Sigma-Aldrich or Fisher Scientific.

1.2.2 Sample Preparation

The old dissolvables (Mellow and Fresh Orbs and Mellow Sticks) were ground to a powder with mortar and pestle. The old Fresh Strips were torn into pieces and broken up with a spatula into small pieces. All of the new dissolvables (Mint Orbs, Sticks, and Strips) were ground to a fine powder using a coffee grinder.

1.2.2.1 Ultrasonic Extractions

Approximately 200 mg of each product was weighed out and placed into glass test tubes and 2 mL of hexane, dichloromethane, acetone, or methanol (only acetone for the new dissolvables) was added to each test tube. The mixtures were then sonicated in an ultra-sonic bath for 60 min at room temperature, rotating the tube positions every ~10 min. After sonication, the

mixtures were filtered through 0.45 µm PTFE filters into autosampler vials and analyzed via GC/MS. Standard solutions were made by adding ~5 mg of the standard into a glass tube and adding 5 mL methanol. The samples were then sonicated using the same procedure as for the dissolvable tobacco samples.

1.2.2.2 Derivatization

5 mg of each ground tobacco product and 500 µL BSTFA or Tri-Sil derivatization agent (Tri-Sil only for new dissolvables) were added to 3 mL reaction vials. The vials were then incubated at 80°C for ~30 min. After cooling for ~5 min, the extracts were filtered through 0.45 µm PTFE filters. The samples were then transferred to autosampler vials and analyzed via GC/MS. Standard solutions were made by adding ~0.5 mg of the standard and 500 µL BSTFA or Tri-Sil reagent. The standard samples were incubated, cooled, and filtered using the same procedure as for the dissolvable tobacco samples.

1.2.2.3 Solid Phase Microextraction (SPME)

The method used for SPME analysis of the dissolvables is a modified version of the SPME methods previously reported for cigarette tobacco.[22, 23] In this method, 1 g of each ground tobacco sample was placed in a 20 mL SPME vial. Standard solutions (1 mg/mL) were analyzed in a similar fashion, where 1 µL of each solution was placed into a 20 mL SPME vial for analysis. All samples and standards were then analyzed via headspace SPME-GC/MS. Two different fiber chemistries were evaluated during the analysis of the old dissolvables; polydimethylsiloxane/ divinylbenzene (PDMS/DVB) and polyethylene glycol

(PEG). The SPME fiber was first conditioned for 30 min at 250°C (for the PDMS/DVB fiber) or at 240°C (for the PEG fiber). Prior to fiber absorption, each sample was incubated for 15 min at 100°C with agitation every 10 sec for 10 sec. The fiber was inserted into the vial and exposed to the headspace for 5 min and then the fiber was inserted into the GC column inlet with a desorption time of 1 min. After each injection, the fiber was conditioned for 2 min at 250°C (for the PDMS/DVB fiber) or at 240°C (for the PEG fiber).

1.2.3 GC/MS Analysis

1.2.3.1 GC/MS Analysis of Old Dissolvables

A 30 m x 0.25 mm x 0.25 µm capillary column in an Agilent 6890N GC with an Agilent 5975 mass selective detector was used for analysis. Helium carrier gas at a flow rate of 1 mL/min and a GC inlet temperature of 250°C were used. The mass spectrometer was scanned from m/z 50 to m/z 550. Solvent blanks and controls were also prepared and analyzed along with the tobacco samples.

For analysis of the solvent extracts, a split ratio of 20:1 was used. The GC oven temperature program began at 40°C, held for 1 min, then ramped at 20°C/min to 320°C. The total run time was 16 min. For analysis of the trimethylsilyl derivatized samples, a split ratio of 50:1 was used. The GC oven temperature program began at 100°C, held for 2 min, then ramped at 15°C/min to 325°C and held for 3 min. The total runtime was 20 min. For headspace SPME

analysis, splitless injection was used with flow rate of 0.8 mL/min. The GC oven temperature program began at 40°C, held for 3 min, then ramped at 6°C/min to 250°C and held for 3 min. The total runtime was 41 min.

1.2.3.2 GC/MS Analysis of New Dissolvables

For the acetone extractions and derivatized extractions, an Agilent 6890N GC with an Agilent 5975 mass spectrometer was used. Separations were completed on a 30 m x 0.25 mm x 0.25µm DB-5MS column. Helium carrier gas was used at a flow rate of 1 mL/min. The GC inlet temperature was 250°C. The mass spectrometer was scanned from m/z 50 to m/z 550. Compound identifications were made using NIST mass spectral library search and retention time to pure standards (nicotine, menthol, ethyl citrate, palmitic acid, stearic acid, glycerol, xylitol, sorbitol, carvone, vanillin, phytol, threitol and coumarin).

For the acetone extractions, a split ratio of 20:1 was used. The GC oven program was held at 40°C for 1 min then ramped at 20°C/min to 320°C for a total run time of 16 min. For the Tri-Sil derivatized samples, a split ratio of 50:1 was used. The GC oven program was held at 100°C for 2 min then ramped at 15°C/min to 325°C and held for 3 min for a total run time of 20 min.

Headspace SPME analysis was conducted on a Thermo Trace Ultra GC with a DSQ II mass spectrometer. Separations were completed on a 60 m x 0.25 mm x 0.25 µm ZB-5MS column. Helium carrier gas was used at a flow rate of 0.8 mL/min. The GC inlet temp was 240°C and samples were injected splitless with a splitless time of 1 min. The mass spectrometer was scanned from m/z 50 to m/z

650 with a 1 min solvent delay. The GC oven program began at 40°C for 3 min then ramped at 6°C/min to 250°C and held for 3 min for a total run time of 41 min.

1.2.4 pH Analysis

The pH measurement procedure was followed by the CDC.[21] Prior to tobacco analysis, the Accumet Basic AB15 digital pH meter was standardized with 4.00, 7.00, and 10.00 pH buffers. 2.0 g of each tobacco sample was placed in a 30 mL beaker. 20.0 mL deionized water was added via volumetric pipet. The mixture was magnetically stirred and the pH was measured every ~5 min for 60 min. Each tobacco sample was analyzed in triplicate. An average pH of each triplicate and an average of each tobacco product was then calculated.

pH was measured of all the dissolvables and % free nicotine was calculated based on the following equations, where the pK_a of nicotine is 8.02:[21]

$$pH = pK_a + \log\frac{[B]}{[BH^+]} \qquad \text{(Equation 1-1)}$$

Solving for $[B]/[BH^+]$,

$$\frac{[B]}{[BH^+]} = 10^{pH-pK_a} \qquad \text{(Equation 1-2)}$$

and substituting into the following equation gives the % free nicotine:[21]

$$\%\ \text{free nicotine} = \frac{\frac{[B]}{[BH^+]}}{\frac{[B]}{[BH^+]}+1} \times 100 \qquad \text{(Equation 1-3)}$$

1.2.5 Nicotine Quantification

1.2.5.1 Calibration Standards

The nicotine analysis procedure was adapted from the CDC procedure for nicotine analysis of smokeless tobacco[21] and from Stanfill, Jia, Ashley, and Watson.[10] First, a 40 mg/mL internal standard solution was prepared by adding 1.00 g quinoline to a 25 mL volumetric flask and diluting to the mark with methyl t-butyl ether (MTBE). A 0.4 mg/mL extraction solution was made by diluting 2.5 mL of the internal standard solution to 250 mL with MTBE. A 100 mg/mL nicotine stock solution was then prepared by diluting 2.50 g nicotine to 25 mL with MTBE in a volumetric flask. Then, 0.5 mL of the internal standard was added via auto-syringe to five 50 mL volumetric flasks. A 0.2 mg/mL nicotine standard was then prepared by adding 100 µL via auto-syringe of the nicotine stock solution to one flask and diluting to volume with MTBE. Standards with nicotine concentrations of 0.4 mg/mL, 0.6 mg/mL, and 0.8 mg/mL, and 1.0 mg/mL were made by adding 200 µL, 300 µL, 400 µL, and 500 µL, respectively, to the other flasks and diluting to volume with MTBE. Aliquots of each standard were transferred to autosampler vials and analyzed by GC/MS. A calibration curve was then made by plotting $Area_{nicotine}/Area_{IS}$ vs. nicotine concentration and a linear equation of the line was determined.

Recovery of nicotine was then determined by adding 5 mL of the 0.6 mg/mL nicotine standard to an amber tube containing 2.0 mL of 2N NaOH. These samples were made in triplicate. The tubes were then mixed on a vortex

for ~2 min. The tubes then sat to allow the phases to separate and an aliquot of the upper organic phase was transferred to autosampler vials. The three nicotine recovery samples were then analyzed using the same method as the nicotine standards. The concentration of nicotine of the recovery samples was then calculated from the calibration line equation. The recovery of nicotine was then calculated for each sample using the equation:[21]

$$\text{Recovery} = \text{Nicotine}_{\text{calculated}}/\text{Nicotine}_{\text{actual}} \qquad \text{(Equation 1-4)}$$

1.2.5.2 Standard Addition Assay

Standards addition assay was conducted for each tobacco product. 1.0000 g tobacco was added to an amber glass tube. This was repeated for a total of six samples. The first sample was not spiked with anything, but the remaining five samples were spiked with 10 µL, 20 µL, 30 µL, 40 µL, and 50 µL of the 100 mg/mL nicotine stock solution. The samples were then allowed to equilibrate for 10 min. Then, 2 mL of 2N NaOH was added to each sample. The tubes were swirled to allow the tobacco to be wet with the NaOH. After 15 min., 5.0 mL extraction solution (0.4 mg/mL quinoline/MTBE) was added to each sample. The tubes were then placed on a linear shaker table and shook at ~200 rpm for 2 hr. The tubes were then removed from the shaker table and the samples sat to allow the phases to separate. An aliquot of the upper organic layer of each sample was filtered through a 0.45 µm PTFE filter and transferred to an autosampler vial and analyzed by GC/MS using the same nicotine method. The $\text{Area}_{\text{nicotine}}/\text{Area}_{\text{IS}}$ of the blank was subtracted from the $\text{Area}_{\text{nicotine}}/\text{Area}_{\text{IS}}$ of

each of the standards. A calibration curve was then made by plotting the corrected $Area_{nicotine}/Area_{IS}$ vs. nicotine concentration of spiked amount.

Recovery of nicotine for the standard addition assay samples was then conducted by adding 30 µL of the nicotine stock to 2.0 mL 2N NaOH and 5.0 mL of the 0.4 mg/mL extraction solution in amber tube. This was repeated for a total of three samples. The tubes were then mixed on a vortex for ~2 min. After the phases were allowed to separate, an aliquot of the upper organic layer of each sample was transferred to autosampler vials and analyzed via GC/MS using the same nicotine method. The concentration of the recovery samples was then determined from the standard addition assay calibration equation. Recovery of the samples was then calculated from the recovery equation (Equation 1-4). The recovery of nicotine from the nicotine standards and the recovery of nicotine from the standards addition assay samples were compared to make sure the recovery values did not differ by more than 10%. This ensures the aqueous matrix is equivalent to the vegetable matrix of the tobacco product.[21]

1.2.5.3 Control Samples

Quality control samples at the low and high end of the expected nicotine concentration were prepared. The low control (0.3 mg/mL) was prepared by adding 15 µL nicotine stock to 2.0 mL 2N NaOH and 5.0 mL extraction solution. The high control (0.7 mg/mL) was prepared by adding 35 µL nicotine stock to 2.0 mL 2N NaOH and 5.0 mL extraction solution. The tubes were shaken on the shaker table with the standard addition assay samples. An aliquot of the upper

organic phase of each sample was filtered through a 0.45 µm PTFE filter and transferred to autosampler vials to be analyzed by GC/MS.

1.2.5.4 Tobacco Extraction

1.0000 g of the ground tobacco sample was added to an amber glass tube. This was done in triplicate. Then, 2 mL of 2N NaOH was added to each sample. The tubes were swirled to allow the tobacco to be wet with the NaOH. After 15 min, 5.0 mL extraction solution (0.4 mg/mL quinoline/MTBE) was added to each sample. The tubes were then placed on a linear shaker table and shook at ~200 rpm for 2 hr. The tubes were then removed from the shaker table and the samples sat to allow the phases to separate. An aliquot of the upper organic layer of each sample was filtered through a 0.45 µm PTFE filter and transferred to an autosampler vial and analyzed by GC/MS using the same nicotine method used for the nicotine standards, recovery samples, standards addition assay samples, and quality control samples.

1.2.5.5 GC/MS Quantification of Nicotine

The GC/MS method used is an established method for rapid and selective quantification of nicotine in tobacco.[10] A 25 m x 0.32 mm x 0.52 µm Agilent Ultra 2 capillary column in a Thermo Trace GC Ultra with a DSQ II mass selective detector was used for analysis. Helium carrier gas at a flow rate of 1.7 mL/min and a GC inlet temperature of 230°C were used. An injection volume of 1.0 µL and split ratio of 50:1 were used. The GC oven temperature program began at 175°C, held for 1 min, then ramped at 5°C/min to 180°C, then ramped at

35°C/min to 240°C. The total run time was 3.7 min. The mass spectrometer transfer line was held at 250°C with a 1.0 min solvent delay. Selected ion monitoring (SIM) was used as listed in Table 1-1. Two solvent blanks were analyzed before each sample.

Table 1-1 Selected ion monitoring (SIM) parameters used for quantification of nicotine

Analyte	Ion Type	*m/z*	Dwell Time (ms)
Quinoline	Quantification	102	10
	Confirmation	129	10
Nicotine	Quantification	133	10
	Confirmation	162	35
	Additional	161	35

1.3 Results and Discussion

1.3.1 Chemical Characterization

1.3.1.1 Solvent Extractions

Extracting the dissolvable tobacco products using the solvent polarity series of hexane, dichloromethane, acetone, and methanol was carried out to provide a comprehensive view of the extractables present in the products. Figure 1-2 shows chromatograms of the old Mellow Orb dissolvables extracted using

each of the solvents. A comparison of the results from the four different solvents determined that the less polar solvents (hexane and dichloromethane) extracted few, if any, sample components. In contrast, acetone allowed for more compounds to be seen in the chromatogram without extracting compounds such as carbohydrates which exhibit poor chromatographic behavior in the form of peak fronting. These highly polar compounds were present in the methanol extracts, however. Also note that the solvent extractions, derivatization, and SPME analyses are only qualitative; nicotine concentrations were determined using another procedure and the results are presented in section 1.3.3.

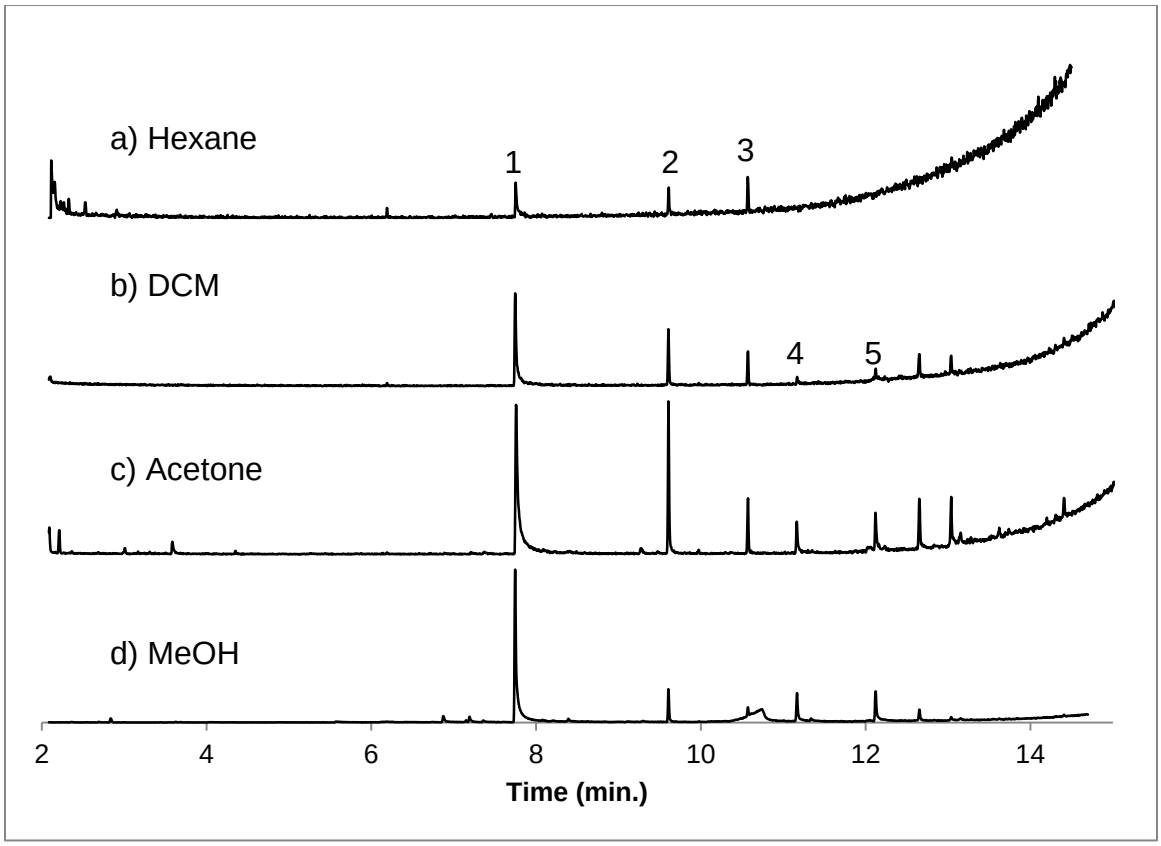

Figure 1-2 Chromatograms of solvent extractions from old Mellow Orbs.
a) hexanes b) dichloromethane (DCM) c) acetone and d) methanol (MeOH).
Peak labels: 1- nicotine 2- ethyl citrate 3- sorbitol 4- palmitic acid 5- stearic acid

1.3.1.1.1 Old Dissolvables

Figure 1-3 shows a comparison of the chromatograms of the four dissolvables in acetone. Nicotine, ethyl citrate, palmitic acid, and stearic acid were confirmed by mass spectral library search and by comparison to the retention times and mass spectra of authentic standards. As would be expected, all of the dissolvable products contain nicotine (peak 3) and those products denoted as “Fresh” flavor contain menthol (peak 2). Perhaps due to its tougher matrix, the extraction efficiency for the Mellow Stick was lower than that of the other products, as evidenced by the lower signal to noise in its chromatogram. It

should also be noted that ethyl citrate (peak 4), palmitic acid (peak 6) and stearic acid (peak 7) were only found in the Orb dissolvables. The peaks labeled with an asterisk (*) indicate a peak that was present in the chromatogram of a matrix-free control.

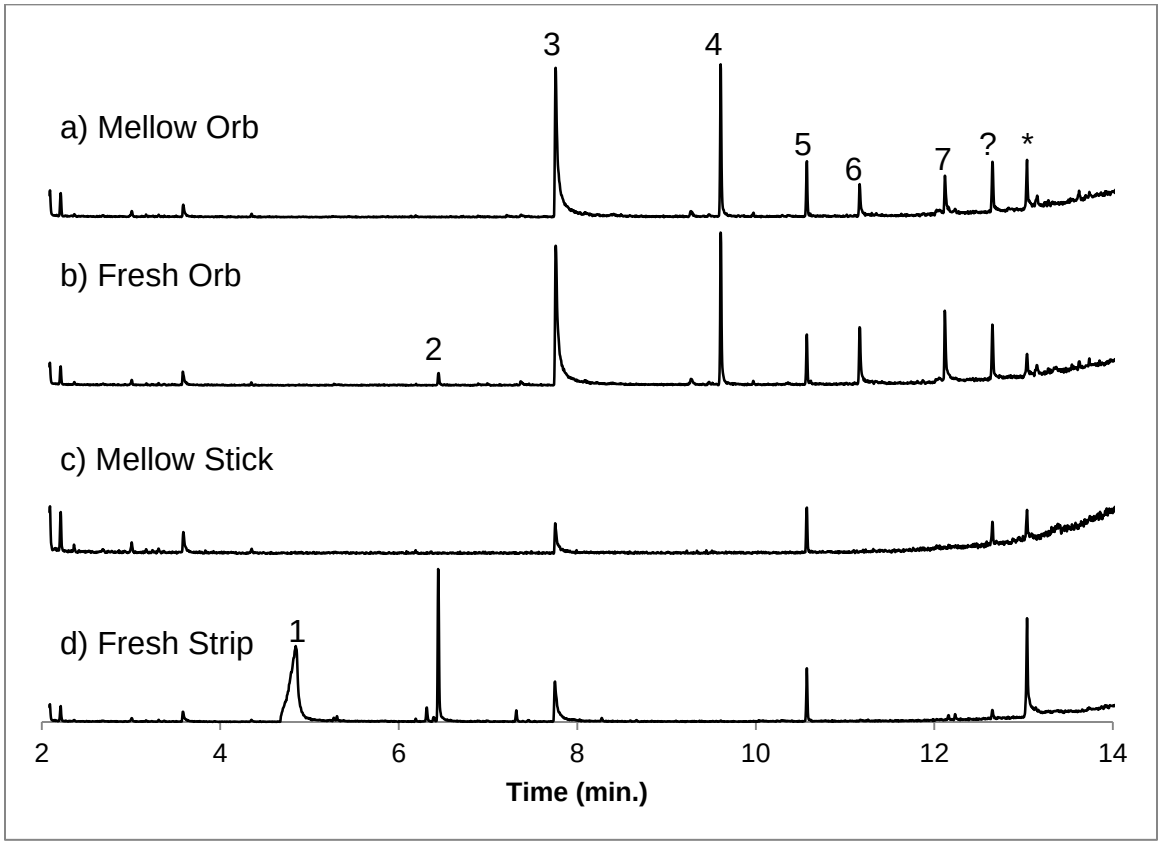

Figure 1-3 Chromatograms of acetone extracts of old dissolvables. a) Mellow Orb b) Fresh Orb c) Mellow Stick d) Fresh Strip Peak labels: 1- glycerol 2- menthol 3- nicotine 4- ethyl citrate 5- possible terpinoid 6- palmitic acid 7- stearic acid ?- unknown *- control

Peak 5 was found in all of the dissolvable products and it was identified as phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol; MW: 296.5 g/mol) by a mass spectral library search. However, the retention time of a standard solution of

phytol was significantly greater than that of peak 5 in the dissolvable tobacco chromatograms. Therefore, it is possible that this compound is in the same terpenoid structural class as phytol, but is of lower molecular weight. Additionally, the mass spectrum of phytol does not contain a molecular ion, making it difficult to identify the mass of this unknown compound. Finally, the peak at ~4.8 min (peak 1) in the Fresh Strip sample was identified as glycerol.

1.3.1.1.2 New Dissolvables

Figure 1-4 shows the chromatograms of the acetone extracts from the new dissolvables. The old mellow dissolvables (Orb and Stick) did not have menthol; however the new mint dissolvables all contain menthol, as expected given their flavor. The Orbs still contain ethyl citrate, palmitic acid, and stearic acid. While carvone and vanillin were identified in the old dissolvables only from the SPME extraction, the acetone extraction of the new dissolvables also identified these two compounds.

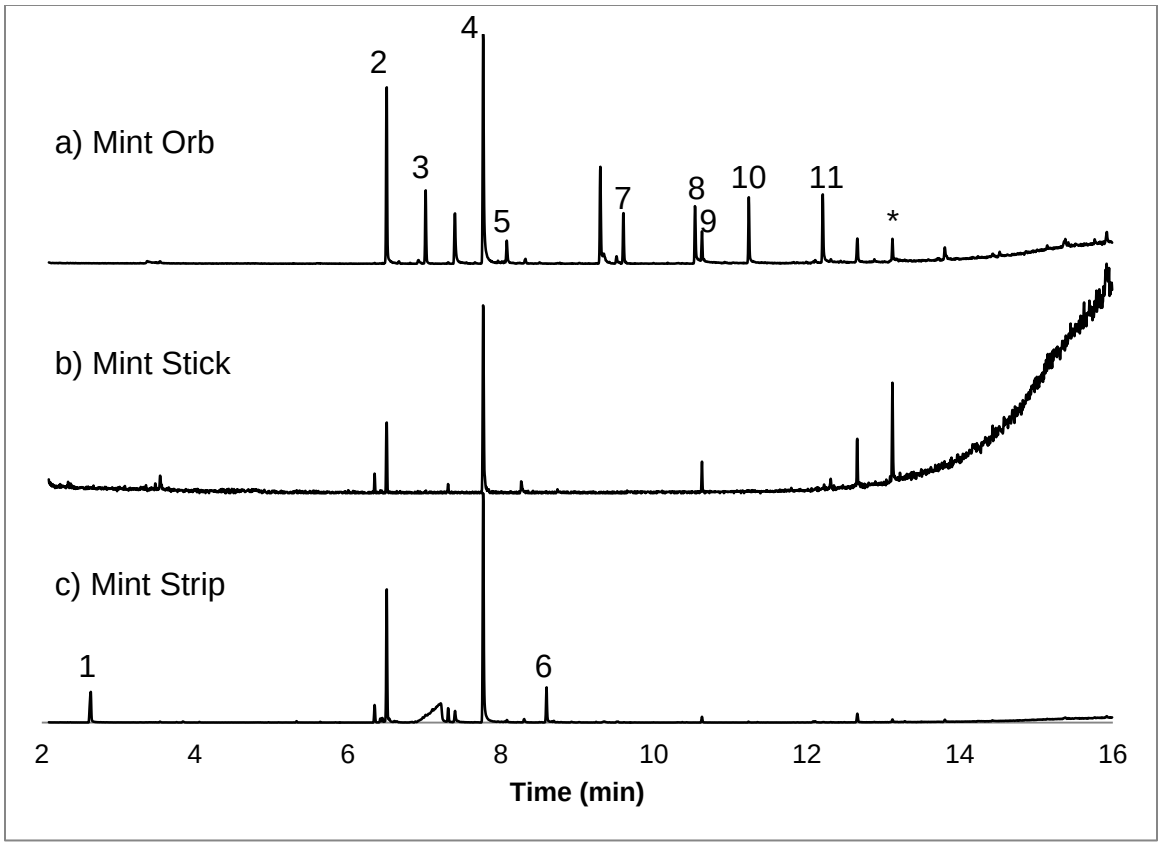

Figure 1-4 Chromatograms of acetone extractions of new dissolvables. a) Mint Orb b) Mint Stick c) Mint Strip Peak Labels: 1- glycerol 2- menthol 3- carvone 4- nicotine 5- vanillin 6- oxalic acid 7- ethyl citrate 8- menthyl acetate 9- possible terpenoid 10- palmitic acid 11- stearic acid *- control

1.3.1.2 Derivatization

Liquid chromatography has been used to profile carbohydrates such as glucose, fructose and sucrose in various types of tobacco.[24] In this work, derivatization was found to be particularly useful as any polar compound including sugars, alcohols, and amines can be analyzed by GC/MS.

1.3.1.2.1 Old Dissolvables

Figure 1-5 shows a comparison of the chromatograms of the four dissolvables analyzed by BSTFA derivatization. The identity of all derivatives was

confirmed by mass spectral library search and the retention times of derivatized standards of each compound.

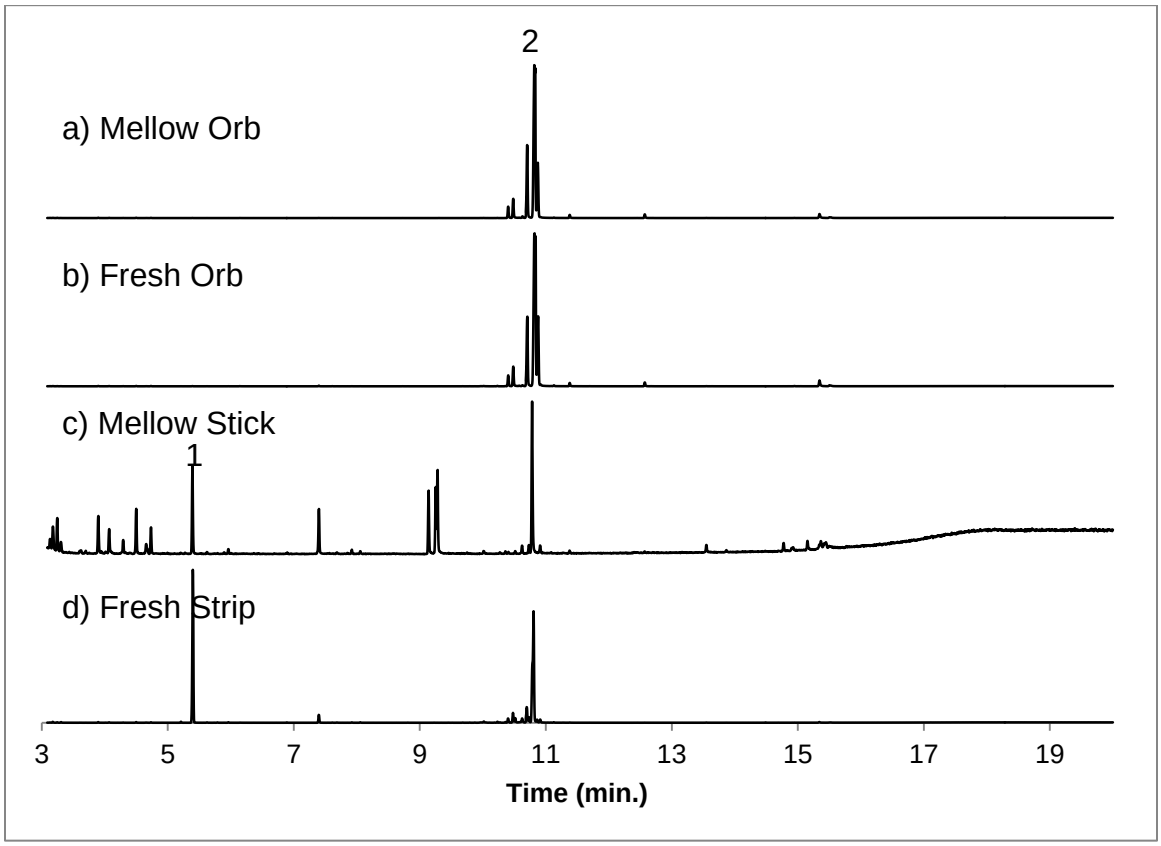

Figure 1-5 Chromatograms of BSFTA derivatization of old dissolvables. a) Mellow Orb b) Fresh Orb c) Mellow Stick d) Fresh Strip. Peak Labels: 1- glycerol 2- sorbitol

In addition to BSTFA derivatization, the old dissolvables were derivatized with Tri-Sil reagent, as shown in Figure 1-6. In contrast to conventional tobacco, significant amounts of sorbitol (peak 3), a sugar alcohol and widely used artificial sweetener, were identified in all products. The Mellow Stick was the only product found to contain the sugar alcohol xylitol (peak 2). Peak 4 was not identified by a

mass spectral library search, but appears to be a di- or tri- saccharide. Palmitic acid and stearic acid were also found as minor constituents in both the Mellow Orb and the Fresh Orb, consistent with the analysis of the acetone extracts. Threitol and malic acid were also found as minor compounds in all of the dissolvables (minor peaks not labelled).

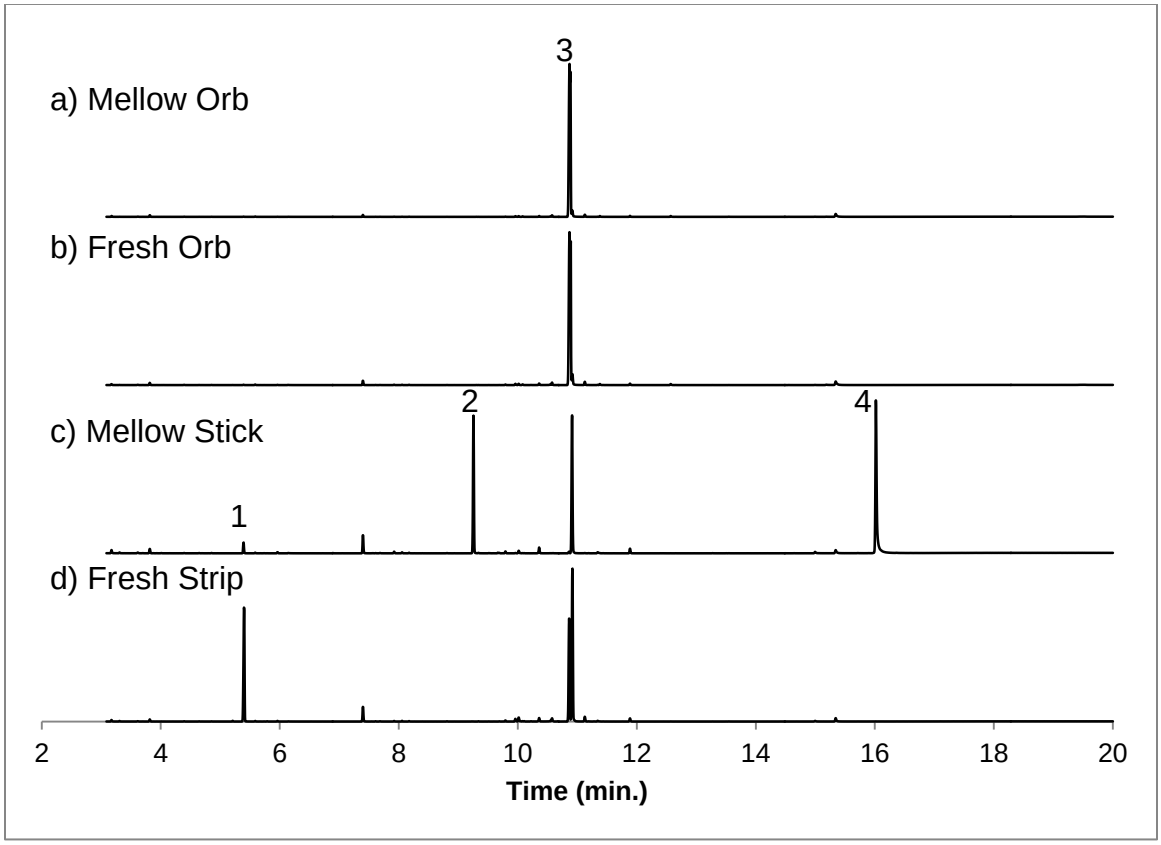

Figure 1-6 Chromatograms of Tri-Sil derivatization of old dissolvables. a) Mellow Orb b) Fresh Orb c) Mellow Stick d) Fresh Strip. Peak Labels: 1- glycerol 2- xylitol 3- sorbitol 4- di- or tri-saccharide

1.3.1.2.2 New Dissolvables

Figure 1-7 shows the chromatograms of derivatized samples from the new dissolvables. The chromatograms for the Orbs and Sticks do not show any major differences between the old and new dissolvables. Malic acid (peak 3) was identified in all new dissolvables as a minor component and was confirmed by mass spectral library search. There are some differences between the old and new Strips, namely that glycerol is no longer present in the new strips. The peak at 7.45 min (peak 4) was identified as a threitol based upon library search. Threitol was found in the old dissolvables as a minor constituent, therefore it is likely that the new dissolvables contain threitol instead of glycerol. Peak 7 was identified as malitol by library search.

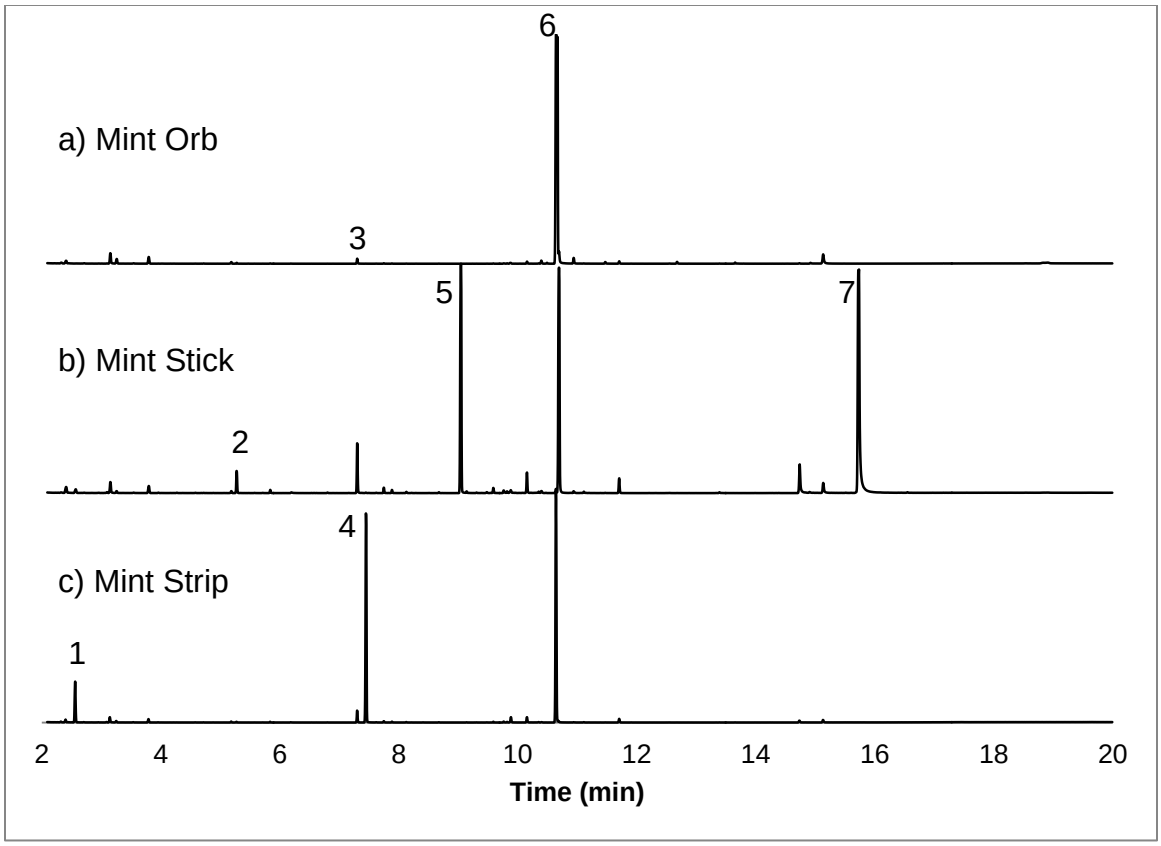

Figure 1-7 Chromatograms of Tri-Sil derivatization of new dissolvables. a) Mint Orb b) Mint Stick c) Mint Strip. Peak Labels: 1- propylene glycol 2- glycerol 3- malic acid 4- threitol 5- xylitol 6- sorbitol 7- malitol

1.3.1.3 Solid Phase Microextraction (SPME)

Solid phase microextraction (SPME) is a sampling technique in which a fiber is exposed to the headspace of a sample inside a vial. Fiber chemistry can be selected based on the composition of the compounds of interest. In SPME analysis, a sample can be heated to allow volatile components to be present in the headspace. The SPME fiber is then exposed to the headspace where volatile components absorb onto the SPME fiber. The fiber is then desorbed into the inlet of a gas chromatograph and the sample is analyzed. A more detailed description of SPME will be discussed in Chapter 4.

SPME analysis was conducted using PDMS/DVB and PEG fibers. The PEG fiber was found to be better suited for the volatile components in the dissolvables because it is polar while the PDMS/DVB fiber is non-polar and better suited for adsorption of neutral components and hydrocarbons.

1.3.1.3.1 Old Dissolvables

Figure 1-8 and Figure 1-9 show the chromatograms of the old dissolvables analyzed with the PDMS/DVB and PEG fiber, respectively. Each of the chromatograms is clearly dominated by nicotine (peak 4 in both figures) as it has appreciable vapor pressure in free base form. Also, and as was seen previously, ethyl citrate was identified in the Orbs and menthol was identified in products with fresh flavors. Other compounds that were identified include carvone (peak 2 in both figures), cinnamaldehyde (peak 3 in both figures), vanillin (peak 5 in Figure 1-9), and coumarin (peak 5 in Figure 1-8 and peak 6 in Figure 1-9). It should also be noted that vanillin was only found in the Orbs and the Mellow Stick and carvone was only found in the fresh flavor dissolvables (Orb and Strip). Peak 7 in Figure 1-8 (peak 8 in Figure 1-9) was identified as phytol from mass spectral library search but its retention time does not match that of a standard solution of phytol. Hence, as was stated previously, it is suggested that this compound is in the same terpenoid structural class as phytol, but is of lower molecular weight.

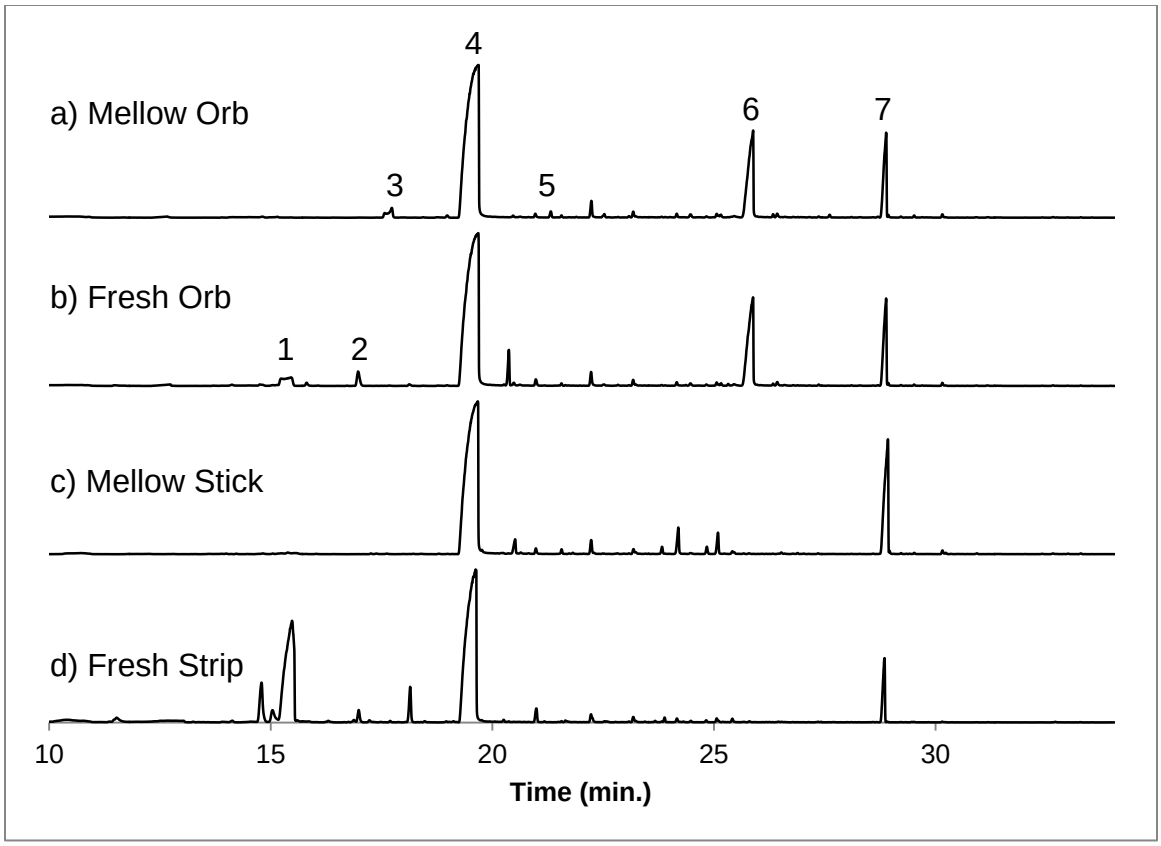

Figure 1-8 Chromatograms of SPME analysis of old dissolvables using a PDMS/DVB fiber. a) Mellow Orb b) Fresh Orb c) Mellow Stick d) Fresh Strip. Peak Labels: 1- menthol 2- carvone 3- cinnamaldehyde 4- nicotine 5- coumarin 6- ethyl citrate 7- possible terpenoid

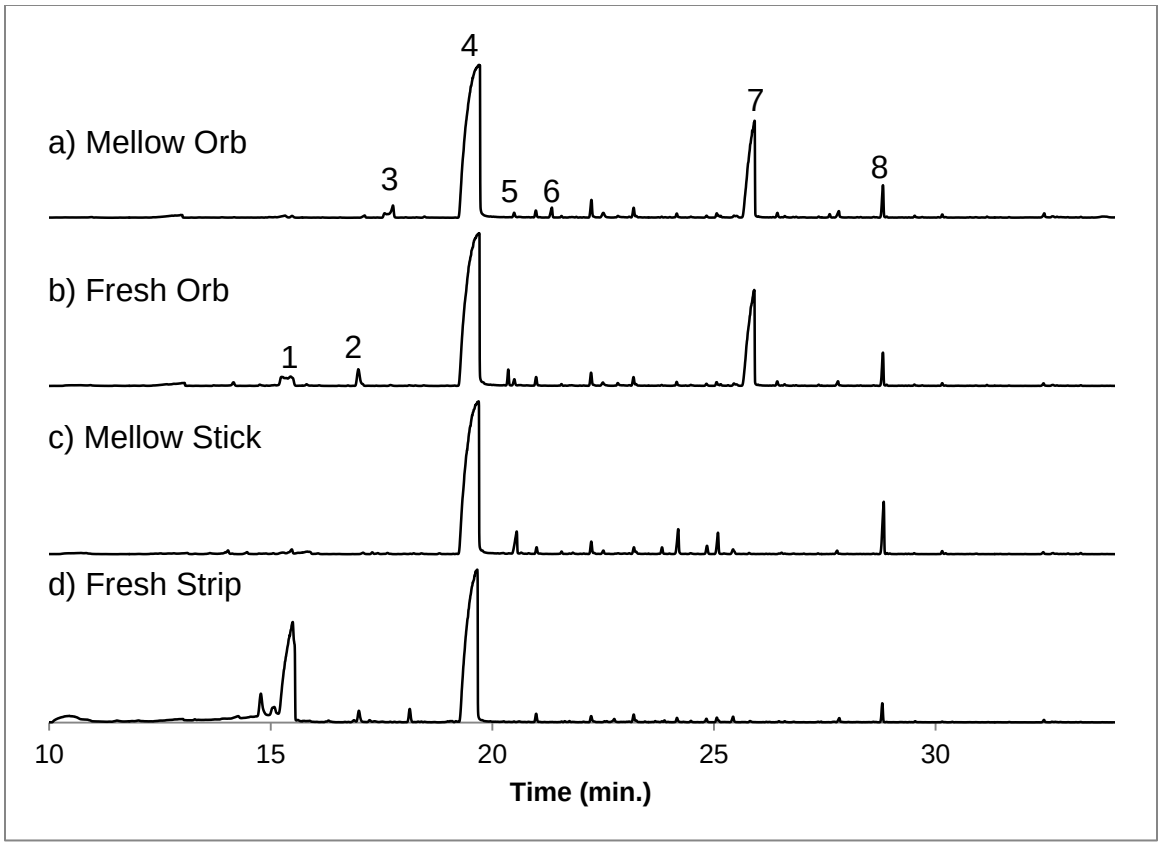

Figure 1-9 Chromatograms of SPME analysis of old dissolvables using a PEG fiber. a) Mellow Orb b) Fresh Orb c) Mellow Stick d) Fresh Strip. Peak Labels: 1- menthol 2- carvone 3- cinnamaldehyde 4- nicotine 5- vanillin 6- coumarin 7- ethyl citrate 8- possible terpenoid

1.3.1.3.2 New Dissolvables

Figure 1-10 shows the SPME chromatograms of the new dissolvables analyzed with a PEG fiber. As mentioned previously, menthol is identified in all the new dissolvables, unlike the old dissolvables in which menthol was only found in the fresh flavors. Furthermore, ethyl citrate was identified in the new Orbs but not in the new Sticks or Strips. This is similar to the old dissolvables in which only Orbs contained ethyl citrate. Carvone was also detected in all the new

dissolvables but only in the fresh old dissolvables, consistent with flavors of fresh or mint.

Comparison of the old fresh Orbs and Strips to the new mint Orbs and Strips do not show any notable differences by SPME. There are, however, many chromatographic differences between the old mellow Sticks and the new mint Sticks. Menthol was identified in the new Sticks, which was not present in the old Sticks. In addition, many compounds were identified as flavors/fragrances having similar structure to menthol. These compounds were identified by mass spectral library search and were determined to be menthone, methyl salicylate, ethyl salicylate, menthyl acetate, and menthol crotonate (Figure 1-10 peaks 1, 3, 5, 6, and 8, respectively).

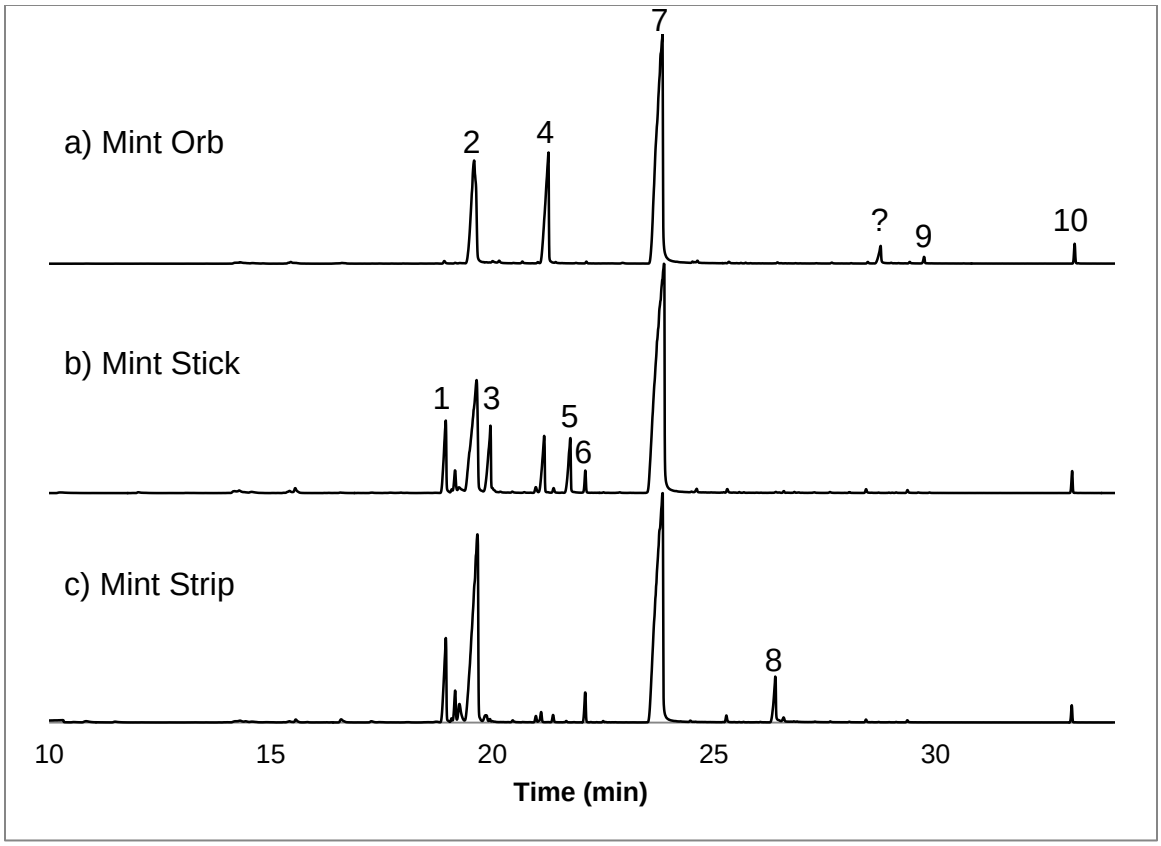

Figure 1-10 Chromatograms of SPME analysis of new dissolvables using a PEG fiber. a) Mint Orb b) Mint Stick c) Mint Strip. Peak Labels: 1- menthone 2- menthol 3- methyl salicylate 4- carvone 5- ethyl salicylate 6- menthyl acetate 7- nicotine 8- menthol crotonate 9- ethyl citrate 10- possible terpenoid ?- Unknown

1.3.1.4 Summary of Chemical Characterization of Old and New Dissolvables

A complete chemical characterization was conducted for both releases of the old dissolvable tobacco products. Upon comparison of the major compounds detected via acetone extraction, derivatization, and SPME, there was no difference between the two releases of the old dissolvables. There are, however, differences between the old and new dissolvables.

Table 2 summarizes the major compounds identified in the dissolvables via acetone extraction, Tri-Sil derivatization, and SPME and also compares the

chemical composition of the old and new dissolvables. Overall, most of the apparent differences can be attributed to the fact that the old dissolvables had mellow and fresh flavors while the new dissolvables only have mint flavor. Unless otherwise noted, all compounds were identified by mass spectral library search and retention time to a high purity standard. From the acetone extractions, derivatization, and SPME, the following compounds have been identified in the new dissolvable tobacco products: nicotine, menthol, ethyl citrate, palmitic acid, stearic acid, glycerol, xylitol, threitol, sorbitol, carvone, and vanillin. Aside from nicotine, the compounds identified in the dissolvables serve as sweeteners, flavors, binders, or humectants.

Table 1-2 Major compounds identified by GC/MS using each extraction method for old and new dissolvables. Mellow Orb (MO), Fresh Orb (FO), Mellow Stick (MS), and Fresh Strip (FS).

Compound	Old Dissolvables												New Dissolvables								
	Acetone			Tri-Sil					SPME				Acetone			Tri-Sil			SPME		
	MO	FO	MS	FS	MO	FO	MS	FS	MO	FO	MS	FS	orb	stick	strip	orb	stick	strip	orb	stick	strip
menthol		x		x						x		x	x	x	x				x	x	x
nicotine	x	x	x	x					x	x	x	x	x	x	x				x	x	x
ethyl citrate	x	x							x	x			x						x		
palmitic acid	x	x											x								
stearic acid	x	x											x								
glycerol							x	x							x		x				
xylitol							x							x							
sorbitol					x	x	x	x								x	x	x			
threitol																		x			
carvone										x		x	x						x		
cinnamaldehyde									x												
vanillin									x	x	x		x								
coumarin									x												

1.3.2 pH and % Free Nicotine in Dissolvable Tobacco Products

Nicotine can be either in the “free-base” or an ionized form depending on the pH of the environment. As free-base nicotine is better able to diffuse across cell membranes than ionized nicotine, it is important to know how much of the total nicotine is in the free-base form and available to be absorbed by the user. Knowing the total nicotine concentration, the pH of the dissolvables, and the pK_a of nicotine, the amount of free-base nicotine can be calculated from the Henderson-Hasselbalch equation.[21]

Table 1-3 shows the pH and % free nicotine of the old and new dissolvables. These results demonstrate that the % free nicotine in the first release of the old dissolvables ranges from 24-50%, in the second release of the old dissolvables ranges from 23-29%, and in the new dissolvables ranges from 21-37%. A two-tailed t-test (assuming equal variances at 95% confidence) indicates that there is no difference in the % free nicotine present in old and new Sticks and Strips. However, there is a significant increase in % free nicotine in the new Orbs. A difference in pH of 0.29 between the old and new Orbs results in a difference of 13.9% free nicotine. This ultimately results in a difference of 100 µg of nicotine per orb.

In addition to the pH of the dissolvables having an effect on absorption of nicotine, the pH of the oral cavity will affect nicotine absorption. In a study monitoring nicotine absorption of tablets in acidic and alkaline oral environments, the rate of nicotine absorption was significantly higher in alkaline environments.[25]

Therefore, further research examining the effect of dissolvables on pH of the oral cavity would be beneficial.

1.3.3 Nicotine Quantification of Dissolvable Tobacco Products

Nicotine was quantified using quinoline as an internal standard. The quantification results of the dissolvables can be found in Table 1-4 and a typical chromatogram of a dissolvable tobacco product can be seen in Figure 1-11. The concentration of free nicotine was calculated by multiplying the total nicotine concentration by the % free nicotine. Calibration curves for nicotine standards and the standard addition assay were performed with each dissolvable product analyzed. All of the calibration curves had R^2 values greater than 0.99, indicating excellent linearity of the data. A typical calibration curve is shown in Figure 1-12. Recovery of nicotine ranged from 92-96% with recovery of nicotine from the standard addition assay always within ±5%. This indicates that there were minimal matrix effects in the analysis.

Table 1-3 pH and % free nicotine in old and new dissolvables

	First Release Old Dissolvables		Second Release Old Dissolvables		New Dissolvables		
Dissolvable	pH	% Free Nicotine	pH	% Free Nicotine	Dissolvable	pH	% Free Nicotine
MO	7.82 ± 0.04	38.5%	7.50 ± 0.03	23.2%	Orb	7.79 ± 0.03	37.1%
FO	7.61 ± 0.17	28.0%	7.50 ± 0.03	23.2%			
MS	7.51 ± 0.07	23.5%	7.64 ± 0.07	29.4%	Stick	7.70 ± 0.02	32.5%
FS	8.02 ± 0.06	50.2%	7.53 ± 0.20	24.6%	Strip	7.44 ± 0.01	20.8%

Table 1-4 Nicotine concentrations in old and new dissolvables. n=3.
Mellow Orb (MO), Fresh Orb (FO), Mellow Stick (MS), Fresh Strip (FS)

Second Release Old Dissolvables			New Dissolvables		
Dissolvable	Total Nicotine (mg/g)	Free Nicotine (mg/g)	Dissolvable	Total Nicotine (mg/g)	Free Nicotine (mg/g)
MO	3.65 ± 0.08	0.847 ± 0.20	Mint Orb	3.35 ± 0.03	1.24 ± 0.01
FO	3.42 ± 0.1	0.750 ± 0.20			
MS	1.78 ± 0.12	0.490 ± 0.30	Mint Stick	2.60 ± 0.05	0.85 ± 0.02
FS	2.12 ± 0.02	0.580 ± 0.05	Mint Strip	2.74 ± 0.40	0.57 ± 0.08

Total nicotine concentrations were statistically indistinguishable for fresh Orbs and mint Orbs as well as for fresh Strips and mint Strips. In contrast, total nicotine was significantly lower from mellow Orbs to mint Orbs, whereas it was significantly higher from mellow Sticks to mint Sticks. In addition, differences in free nicotine concentration were statistically similar for Strips, but free nicotine concentrations were significantly higher in Orbs and Sticks.

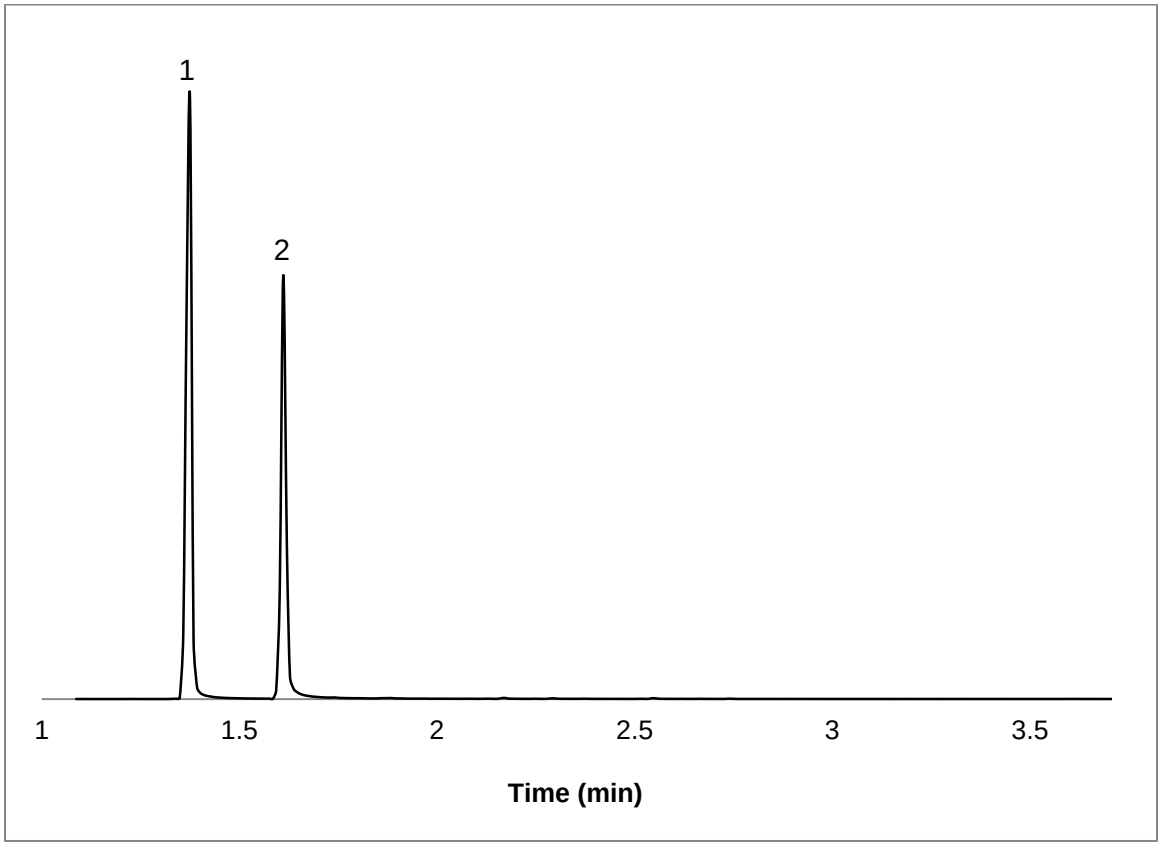

Figure 1-11 Nicotine extraction chromatogram of new Orb. Peak Labels:
1- quinoline (IS) 2- nicotine

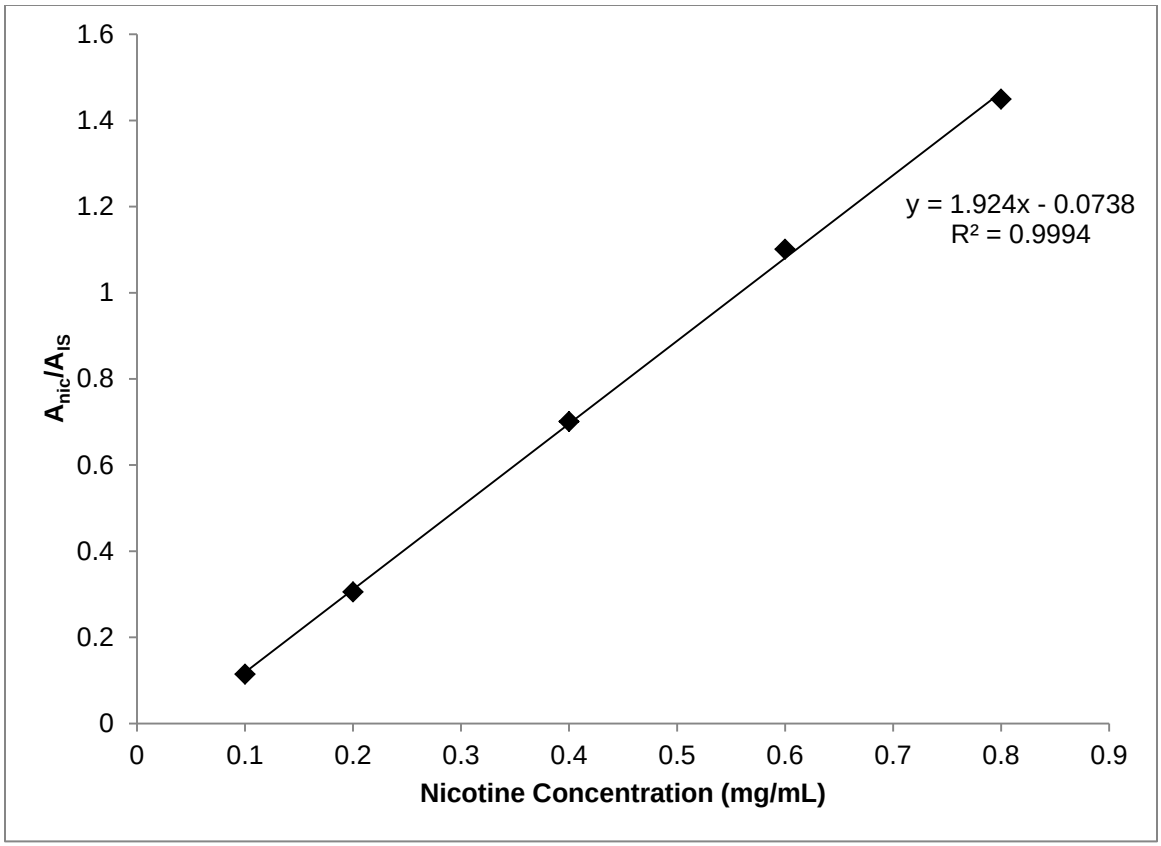

Figure 1-12 Calibration curve for nicotine analysis

1.3.4 Toxicology of Dissolvable Tobacco Products

The dissolvable tobacco products have the potential to cause mouth diseases and complications in the users of these products. It is therefore important to understand the toxicological effects of some of the ingredients of these products, particularly nicotine.

Sorbitol and xylitol are sugar alcohols that are commonly used as a sucrose substitute in foods such as sugar-free chewing gums. Frequently, both sorbitol and xylitol are added together as a sweetener because xylitol is expensive but allegedly has better health effects than sorbitol alone.[26] Frequent

exposure to sorbitol can lead to an increase in tooth demineralization and can be potentially carcinogenic when regularly used by people with low salivary secretions.[27] Xylitol has not shown any harmful effects in the oral cavity, but contrary to popular belief, it also has not been shown to be beneficial. Consumption of xylitol for long periods of time may result in selection of xylitol-resistant *Streptococcus mutans* (micro-organisms in dental plaque), leading to increased oral bacteria.[28]

The toxicity of nicotine has been extensively researched and the complications associated with nicotine are widely accepted. Nicotine is a tertiary amine and can be converted into *N*-nitrosamines within the body. Tobacco specific *N*-nitrosamines (TSNAs) are carcinogenic, particularly 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosonornicotine (NNN). Once TSNAs are metabolically activated they can form DNA adducts which can eventually lead to cancer.[29] Nicotine also has adverse effects on the oral cavity such as inhibited gingival fibroblast growth and collagen production.[30] Nicotine can also inhibit mineralization of human dental pulp cells and inhibit apoptosis in oral cancer cells.[31-33]

It is important to monitor the concentration of nicotine in the dissolvables to understand the potential toxicity of nicotine in humans. The route of administration of nicotine, such as orally, dermally, intravenously, etc., is also very important. Exposure of nicotine is largely dependent on how the user consumes the nicotine containing product. Rapid injection of nicotine leads to the highest blood and brain concentrations at the lowest doses of nicotine. However,

oral administration requires higher doses of nicotine to produce the same toxic effects. As mentioned above, there have been some concerns regarding the potential toxicity of dissolvable tobacco products to children who mistake it for candy.[17] However these concerns would not apply to an average 70 kg (~155 lbs.) person, as it has been estimated that an oral dose of 5 mg/kg (2.3 mg/lbs.) of nicotine is lethal.[34] This equates to approximately 425 old Orbs, 385 old Sticks, and 1580 old Strips. It is also assumed that ingestion of a total dose of nicotine between 40-60 mg is lethal to humans.[34] Even so, this would equate to approximately 50-70 old Orbs, 45-65 old Sticks, and 180-280 old Strips.

1.4 Conclusions

The chemical characterization, pH measurements, and nicotine determination of the dissolvables generate valuable information about these tobacco products. Although there are thousands of known compounds in tobacco products, this study was designed to characterize easily extractable compounds such as flavors, binders, and humectants that may have been added to tobacco during the manufacture of this processed form of tobacco.

Overall, one of the most significant differences between the old and new dissolvables are that the new dissolvables all come in mint flavor rather than mellow or fresh. This is apparent by the differences in flavoring compounds (i.e., addition of menthol to all dissolvables and removal of cinnamaldehyde and

coumarin). Another noteworthy difference is the use of threitol in place of glycerol. Additionally, % free nicotine was found to be statistically higher between old and new Orbs, with no significant difference between old and new Sticks and Strips. Overall, total nicotine concentrations were found to be lower between mellow and mint Orbs, higher between mellow and mint Sticks, and unchanged between fresh Orbs and fresh Strips and their mint flavored counterparts.

CHAPTER 2. QUANTITATIVE ANALYSIS OF HUMECTANTS IN TOBACCO PRODUCTS

2.1 Introduction

Humectants such as glycerol, propylene glycol, and triethylene glycol have been added to tobacco products for many years to retain moisture and increase shelf life.[35-39] Humectant concentrations vary greatly among different tobacco product types (cigarettes, hookah, etc.). For example, humectants in products such as cigarettes and pipe tobacco are added at levels that maintain moisture content without compromising the burn characteristics of the tobacco.[36]

In 1963 Friedman and Raab described a method for determining glycerol, diethylene glycol, and propylene glycol by gas chromatography (GC).[40] The multistep sample preparation involved Soxhlet extraction, reflux in acetone, evaporation, reconstitution in methanol, and filtration. The extracts were analyzed via GC with a 6 foot stainless steel DB-Wax packed column.

A collaborative study established in 1970 used GC with either thermal conductivity or flame ionization detection (FID) for the determination of glycerol, propylene glycol, and triethylene glycol.[41] Anethole was used as an internal standard. In contrast to the method developed in 1963, sample preparation was simple and involved shaking the tobacco in methanol and injecting the

supernatant onto the GC. As written, the method described in the 1970 collaborative study was applicable to tobaccos containing 1 to 3.5% of a given humectant.[41] In 1971, the collaborative study was continued using a modified method where 1,3-butanediol replaced anethole as the internal standard.[42] This modification addressed coelution problems reported in the 1970 collaboration between the internal standard and triethylene glycol. Overall, the results of the 1971 study showed improvements in precision for the determination of propylene glycol and glycerol relative to the 1970 collaborative study.

It appears that there was no further research on analytical methods for the determination of humectants in tobacco products until Health Canada imposed regulations in 1999[43] and a CORESTA subgroup studied sample preparation, extraction procedures, and analytical parameters between 1993-1999.[44] In the Health Canada Official Method T-304, humectants were determined by analyzing methanolic extracts of tobacco via GC with FID. This method uses a DB-Wax fused silica column with 1,3 butanediol as the internal standard. Some improvement was shown over previous methods concerning the separation of propylene glycol, glycerol, and triethylene glycol being completed in less than 10 minutes. However, the new chromatographic conditions of the Health Canada method resulted in inadequate separation of the glycerol and triethylene glycol peaks (6.119 min and 6.220 min, respectively). Such poor resolution is particularly challenging when one of the humectants in question is formulated at a significantly higher concentration than the other. At best, the resulting chromatography would produce a shoulder peak for the less prominent

humectant. Less favorably, the smaller peak could be completely assimilated into the larger peak.

In 2011, CORESTA updated the recommended method for determining propylene glycol and glycerol in tobacco by gas chromatography.[44] This method was similar to the Health Canada method concerning extraction procedures and analysis; however, this method did not include detection of triethylene glycol. Although triethylene glycol is not used as frequently as in the past, it can still be detected in tobacco products, and therefore should be evaluated using analytical methods. Additionally, if a tobacco sample contains triethylene glycol, the amount of glycerol detected using the CORESTA method may have limitations. As such, it is important to have a method that can provide chromatographic separation and selective detection of humectants.

Although FID is a sensitive detection technique, it is not selective and relies on chromatographic retention time to differentiate analytes. As was discussed in reference to Health Canada Official Method T-304, difficulties can arise in data analysis when peaks are not well resolved. MS has the advantage of mass selectivity, which allows for peak identification that is not dependent on chromatographic resolution unless the component masses cannot be resolved. This chapter describes a comparison of MS and FID for GC analysis of humectants by post-column splitting of the column effluent prior to detection. The combination of MS and FID with GC provides a rapid, sensitive and selective method for determination of humectants in tobacco products.

2.2 Experimental

2.2.1 Materials

Glycerol, propylene glycol, triethylene glycol, 1,3-butanediol, and methanol were purchased from Sigma-Aldrich. Three roll-your-own (RYO), thirteen cigar, eleven cigarette, ten moist snuff, and seven hookah tobacco products were purchased from Tobacconists in Laurel, Maryland.

2.2.2 Calibration Standards

Standards of humectants were prepared according to the Health Canada method.[43] Standards containing glycerol, propylene glycol, and triethylene glycol were prepared by dissolving the humectants in extraction solution (methanol containing 2.0 mg/mL 1,3-butanediol). Diluting from stock solutions, working standards were prepared containing glycerol (0.8, 1.6, 2.8, and 4.0 mg/mL) and both propylene glycol and triethylene glycol with concentrations of 0.4, 0.8, 1.4, and 2.0 mg/mL. Linear dynamic range and limits of detection and quantitation were determined using the calibrant solutions.

2.2.3 Tobacco Extraction

Four grams of each tobacco product were extracted with 50 mL of extraction solution and shaken for 1 hr on a Burrell model 75 wrist action shaker. After the samples settled for ~30 min, the extracts were filtered through Whatman

30 µm filter paper. Since hookah tobacco contains as much as 65% humectants by weight, these samples were further diluted by a factor of 50 with extraction solution before injection.[45] An aliquot of each extract was transferred to an autosampler vial and analyzed by GC-MS-FID. Each tobacco sample was extracted twice (replicates) and each extract was analyzed three times.

2.2.4 Instrumental Parameters

Tobacco extracts were analyzed using an Agilent 6890N GC with a split/splitless inlet. Simultaneous detection was achieved with an Agilent 5975 inert XL mass selective detector and flame ionization detector. Chromatographic parameters were chosen to mimic the Health Canada method for the determination of humectants in tobacco.[43] Extracts were analyzed by splitless injection of 1 µL at 250°C. Analytes were separated on a 15 m x 0.53 mm x 1 µm DB-Wax column with helium carrier gas at constant pressure of 14.5 psi. The GC oven was held at 120°C for 2 minutes, then ramped at 15°C/minute to 180°C and held for 4 minutes (total run time of 10 minutes).

Flow from the analytical column was split using a microfluidic splitter with a 1 m x 0.32 mm uncoated deactivated fused-silica (UCDFS) restrictor tube at 12.5 psi to the FID and a 2 m X 0.18 mm UCDFS restrictor tube at 2 psi to the MS. The FID was run at 300°C with 30 mL/min hydrogen flow, 400 mL/min air flow, and 10 mL/min makeup flow. The MS transfer line was maintained at 280°C, MS source at 230°C, and MS quadrupole at 150°C. The MS was run in scan mode with mass range between m/z 30-300.

A post run that included reversing the flow from the electronic pneumatics control (EPC) at 20 psi was conducted at the end of each analysis for 5 minutes at an oven temperature of 220°C. This backflush was to prevent carryover and to allow any retained analytes to exit the column through the split vent of the inlet. Agilent ChemStation software (D.02) was used for data acquisition and data analysis.

2.2.5 Chemometrics

The raw humectant concentration data was normalized for tobacco sample mass and solution volume. Using XLSTAT (add-on in Microscoft Excel), discriminant analysis (DA) was performed on the products labeled as RYO, cigar, and cigarette. Principal component analysis (PCA) was also performed on this data; however DA was not computed from the principal components due to the low number of variables.

2.3 Results and Discussion

2.3.1 Separation of Humectants via GC/MS/FID

The purpose of this study was to modify an existing method for the determination of humectants in tobacco to provide sufficient selectivity and sensitivity to resolve analytes of interest. Figure 2-1 shows typical chromatograms of a standard solution using MS and FID detection. Similar to

results observed using Health Canada Official Method T-304[43], the data in Figure 2-1 show marginal chromatographic resolution of the glycerol and triethylene glycol peaks (R=1.03).While peak overlap in Figure 2-1b appears to be minor, low resolution can convolute quantitative results particularly when using non-selective detection techniques such as FID. It is important to note that previous method development[41-44, 46] focused on the quantitation of humectants in cigarette tobacco, which are relatively low in total humectant concentration. This is reflected in the scope of application for the Health Canada humectants method[43], which describes the expected range of individual humectants to be 0.5% to 4.0% on an “as received” basis. This range is applicable to cigarettes, roll-your-own, and most conventional pipe tobaccos. However, the levels observed in hookah-type tobaccos are substantially higher, as shown in the results presented here. The effect of substantially increased levels of humectants on the analysis by GC-FID has been observed in this study. Difficulties arose when a tobacco product contained a large amount of glycerol, which produced a broad peak around 9 minutes (results not shown). Since the method used FID, it was impossible to determine if it was glycerol, triethylene glycol, or a combination of glycerol and triethylene glycol. Ultimately, the sample was diluted substantially to bring the level of humectant(s) within the range of the method. As a result, the peak was identified as glycerol but, given the lack of specificity in the detection technique (FID) and the level of dilution, this experiment was unable to determine if a minor level of triethylene glycol was present in the undiluted sample. Although chromatographic resolution could possibly be improved by options such

as using different columns, the purposes of this study were to enhance current methodology and to compare detection by FID with detection by MS. Mass spectrometry provides the mass selectivity to distinguish glycerol and triethylene glycol and the broad peak around 9 minutes could have been deconvoluted through the use of extracted ion chromatograms (EIC) as seen in Figure 2-2. Using MS detection, poor chromatographic resolution is nullified as a limitation of the method.

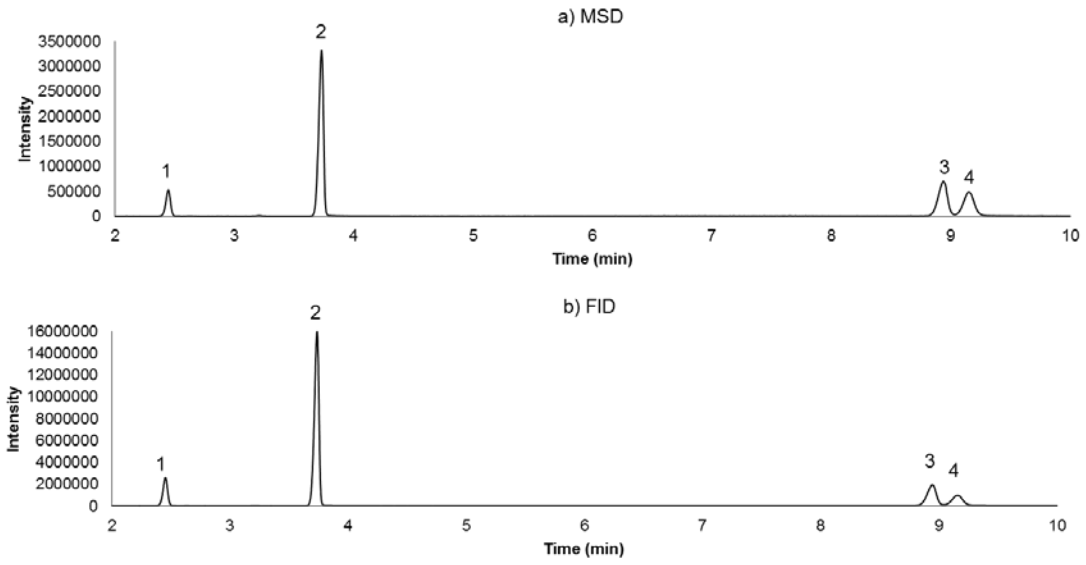

Figure 2-1 Comparison of chromatograms using a) MS and b) FID of a humectant standard. Peak labels 1- propylene glycol, 2- 1,3-butanediol (IS), 3- glycerol, 4- triethylene glycol

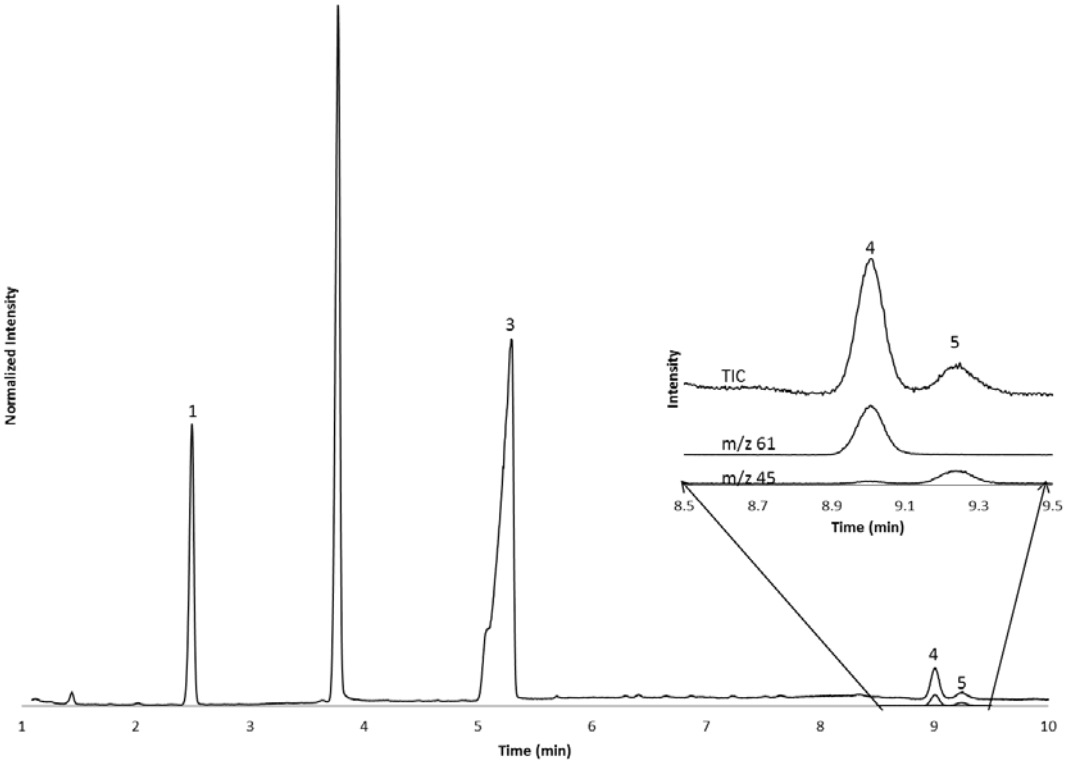

Figure 2-2 Chromatogram of tobacco sample #5. Inset demonstrates the added benefit of using MS to ensure chromatographic separation of glycerol and triethylene glycol. Peak labels 1- propylene glycol, 2- 1,3-butanediol (IS), 3- nicotine, 4- glycerol, 5- triethylene glycol

2.3.2 Analytical Parameters

Once the method was developed, linearity, linear range, limit of detection, and carryover were evaluated. Calibration standards of glycerol, propylene glycol, and triethylene glycol were analyzed. Table 2-1 shows the limit of detection, linear range, and correlation coefficient (R^2) determined using the calibration curves taken from MS and FID data. These parameters were determined based on the injected concentrations. The amount of analyte that actually reached each detector was dependent on the microfluidic splitter, which provides a split ratio of approximately 15:1 with the majority of the column effluent going the FID.

Table 2-1 Retention time, limit of detection, linear range, and calibration curve R^2 values for humectants.

Humectant	RT (min)	Limit of Detection (µg/mL)		Linear Range (µg/mL)		R^2	
		FID	MS	FID	MS	FID	MS
Propylene Glycol	2.4	0.5	2	2-2000	20-2000	0.9998	1.0000
Glycerol	8.9	0.25	4	1-4000	40-4000	0.9999	1.0000
Triethylene Glycol	9.1	0.5	2	2-2000	20-2000	1.0000	0.9999

Carryover was evaluated as a potential source of error and was eliminated by implementing a 5 minute post-run backflush. The post-run conditions involved an increase in the oven temperature to 220°C (40°C hotter than the ending temperature of the GC method) and a pressure from the EPC of 20 psi, keeping the inlet pressure at 14.5 psi. This reverses column flow, which allows any retained analytes to exit the column through the split vent of the inlet. Use of this post-run step eliminated any carryover from the previous tobacco sample, as evidenced in Figure 2-3 where vanillin and ethyl vanillin were identified by spectral matching with the NIST mass spectral library.

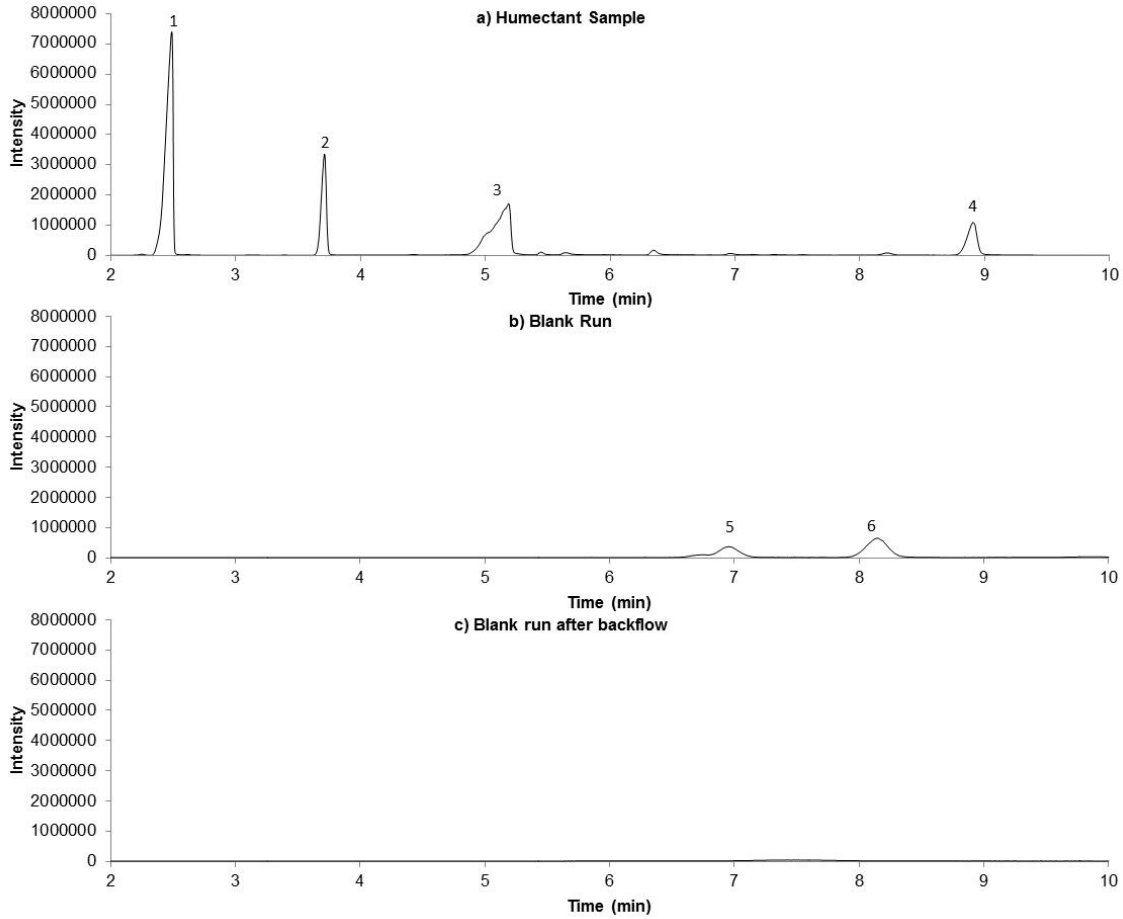

Figure 2-3 Evaluation of a backflush post-run. a) chromatogram of a tobacco sample b) chromatogram of a methanol blank run after the tobacco sample with no post-run c) chromatogram of a methanol blank run after a tobacco sample with post-run. Peak labels 1- propylene glycol 2- 1,3-butanediol (IS) 3- nicotine 4- ethyl vanillin 5- vanillin 6- glycerol

2.3.3 Quantitative Analysis of Tobacco

Figure 2-4 shows chromatograms that are characteristic of each tobacco type and Table 2-2 lists a description of each tobacco sample. The humectants were confirmed by retention time and mass spectra. These chromatograms show that, in general RYO, cigarettes, cigars and moist snuff contain relatively low levels of glycerol. It is also evident from Figure 2-4 that hookah tobaccos contain

a large amount of glycerol. In such cases, the mass spectral data was useful in demonstrating that there was no co-elution of glycerol and triethylene glycol in the FID results. It should be noted that triethylene glycol was found in six of the 44 tobacco product samples analyzed. In all six of these samples, it was possible to resolve the triethylene glycol and glycerol peaks in the FID chromatograms. This is because, in each case, both peaks were small and did not have sufficient peak width to interfere significantly with one another. The detection of triethylene glycol in the six samples was further confirmed using extracted ion chromatograms as demonstrated in Figure 2-2.

The concentration of humectants in each sample was quantified using data from MS and FID. The average concentrations (percent by weight) from three injections of two extractions of each tobacco sample are presented in Table 2-3. These concentrations are based on “as received” weight for all tobacco products. Results shown in these tables were calculated from both MS and FID data.

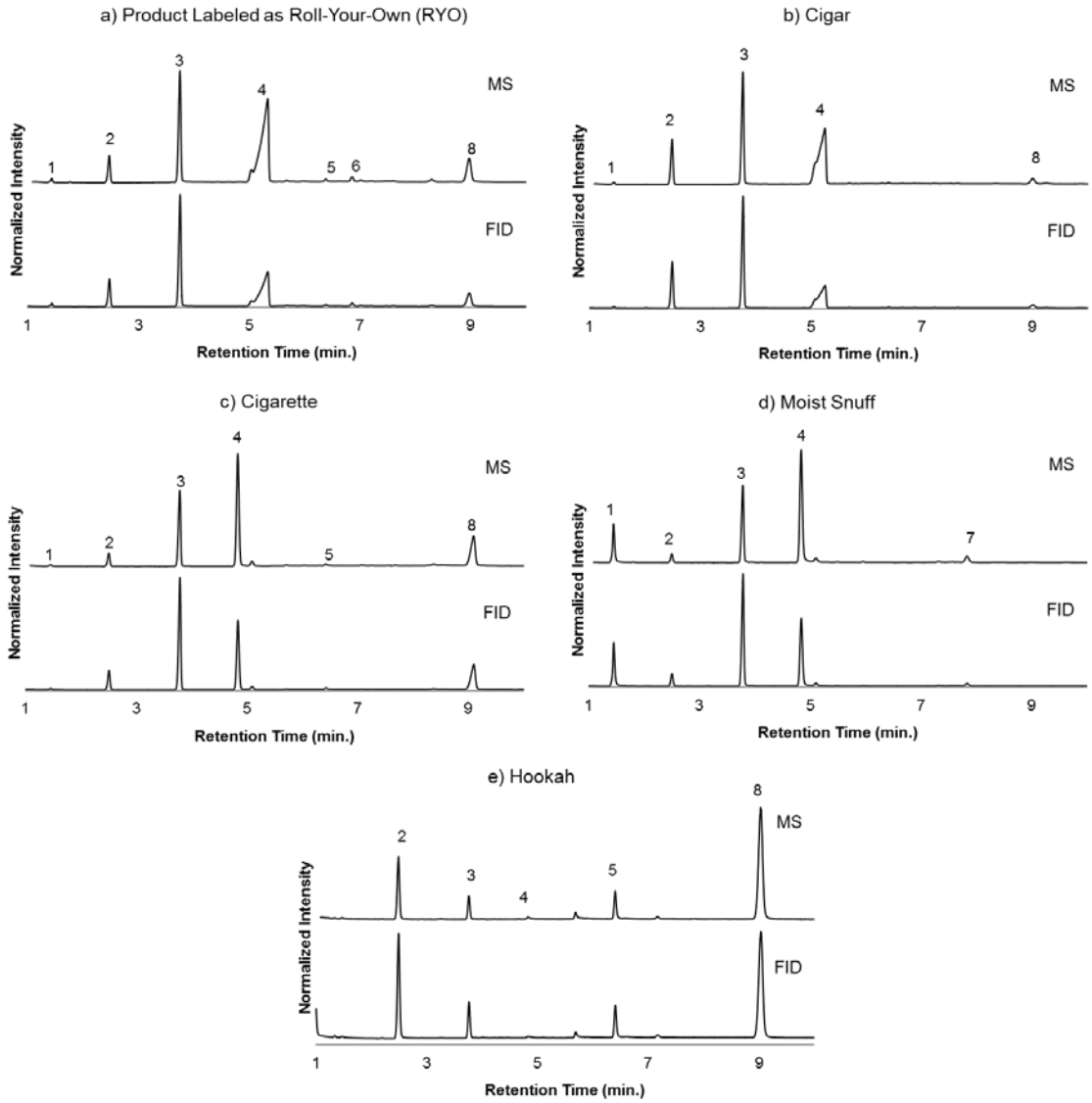

Figure 2-4 MS and FID chromatograms of tobacco samples analyzed. a) product labeled as RYO b) cigar c) cigarette d) moist snuff e) hookah. Peak labels: 1- acetic acid 2- propylene glycol 3- 1,3-butanediol 4- nicotine 5- dihydroxyacetone 6- sorbic acid 7- piperonal 8- glycerol

Table 2-2 List of tobacco products

Tobacco Type	Tobacco Name	ID #	Tobacco Type	Tobacco Name	ID #
RYO	American Gambler Menthol Flavor	1	Cigarette	Marlboro Filter	23
	American Gambler Light Flavor	2		Parliament Recessed Filter	24
	American Gambler Full Flavor	3		Salem Refreshing Menthol	25
Cigar	American Gambler Menthol Flavor 100's Cigars	4		Newport	26
	American Gambler Full Flavor 100's Cigars	5		Merit Lights	27
	American Gambler Light Flavor 100's Cigars	6	Moist Snuff	Skoal Long Cut Berry Blend	28
	Hav-A-Tampa Sweet Filter Tipped	7		Skoal Long Cut Cherry	29
	Hav-A-Tampa Jewels Sweet	8		Skoal Long Cut Straight	30
	Hav-A-Tampa Jewels Vanilla	9		Skoal Long Cut Citrus Blend	31
	Black & Mild Fast Break Apple	10		Skoal Long Cut Vanilla Blend	32
	Butch Masters Vanilla Cigarillos	11		Grizzly Fine Cut Natural	33
	Blackstone Pipe Tobacco Cigars Vanilla	12		Grizzly Long Cut Wintergreen	34
	Swisher Sweets Peach Flavor Little Cigars	13		Kodiak Premium Wintergreen	35
	Swisher Sweets Mini Cigarillos	14		Kayak Long Cut Straight	36
	Swisher Sweets Mini Cigarillos Grape	15		Longhorn Long Cut Straight	37
	Al Capone Slims Rum Dipped	16	Hookah	Pharaoh's Watermelon Splash	38
Cigarette	Camel Filters Turkish Domestic Blend	17		Pharaoh's Fruitopia	39
	Benson & Hedges 100's Premium Filter	18		Pharaoh's Blue Berry	40
	Doral Full Flavor	19		Rosetta Double Apple	41
	L & M Bold 100's Menthol	20		Rosetta Peach Passion	42
	Kool Filter Kings True Menthol	21		Rosetta Vanilla Spice	43
	Winston Red 100’s	22		Rosetta Double Apple	44
			Cigarette	Reference Cigarette	3R4F

Table 2-3 Humectant concentrations detected by GC with MS and FID. BQL and ND indicate results that were below quantitation limit or below the limit of detection. n=2, three injections per replicate

Tobacco Type	ID	Humectants (% wt/wt) (n=2)							
		MS				FID			
		Propylene Glycol	Glycerol	Triethylene Glycol	Total	Propylene Glycol	Glycerol	Triethylene Glycol	Total
RYO	1	1.23 ± 0.03	1.30 ± 0.05	ND	2.53 ± 0.03	1.22 ± 0.04	1.26 ± 0.04	ND	2.48 ± 0.03
	2	0.83 ± 0.01	1.04 ± 0.05	ND	1.87 ± 0.04	0.82 ± 0.01	1.01 ± 0.02	ND	1.83 ± 0.02
	3	0.87 ± 0.01	1.45 ± 0.03	ND	2.32 ± 0.03	0.858 ± 0.003	1.39 ± 0.03	ND	2.25 ± 0.03
Cigar	4	1.59 ± 0.01	0.274 ± 0.005	ND	1.86 ± 0.02	1.56 ± 0.01	0.274 ± 0.005	ND	1.83 ± 0.01
	5	1.33 ± 0.01	0.275 ± 0.007	BQL	1.63 ± 0.01	1.30 ± 0.01	0.27 ± 0.01	0.050 ± 0.001	1.62 ± 0.01
	6	1.40 ± 0.02	0.277 ± 0.001	ND	1.68 ± 0.02	1.37 ± 0.02	0.28 ± 0.01	0.040 ± 0.001	1.69 ± 0.02
	7	0.9 ± 0.4	0.16 ± 0.04	ND	1.1 ± 0.5	0.9 ± 0.4	0.18 ± 0.04	ND	1.1 ± 0.4
	8	0.77 ± 0.02	0.112 ± 0.004	0.117 ± 0.002	1.00 ± 0.01	0.75 ± 0.02	0.13 ± 0.01	0.140 ± 0.006	1.02 ± 0.01
	9	0.30 ± 0.03	0.092 ± 0.001	0.109 ± 0.004	0.50 ± 0.03	0.32 ± 0.02	0.111 ± 0.001	0.140 ± 0.004	0.57 ± 0.03
	10	2.1 ± 0.1	1.31 ± 0.03	ND	3.4 ± 0.2	2.1 ± 0.1	1.28 ± 0.03	ND	3.4 ± 0.2
	11	0.96 ± 0.06	0.134 ± 0.001	ND	1.09 ± 0.06	0.94 ± 0.06	0.151 ± 0.001	ND	1.09 ± 0.06
	12	1.08 ± 0.01	1.71 ± 0.02	ND	2.79 ± 0.03	1.08 ± 0.01	1.63 ± 0.02	ND	2.71 ± 0.02
	13	1.14 ± 0.01	0.247 ± 0.001	ND	1.39 ± 0.01	1.12 ± 0.01	0.258 ± 0.001	ND	1.38 ± 0.01
	14	1.05 ± 0.02	0.118 ± 0.003	0.39 ± 0.01	1.56 ± 0.01	1.02 ± 0.02	0.140 ± 0.005	0.390 ± 0.004	1.55 ± 0.01
	15	1.70 ± 0.06	0.129 ± 0.002	0.37 ± 0.01	2.20 ± 0.04	1.67 ± 0.06	0.153 ± 0.001	0.37 ± 0.01	2.19 ± 0.04
	16	0.96 ± 0.02	0.158 ± 0.002	ND	1.12 ± 0.02	0.96 ± 0.02	0.183 ± 0.001	ND	1.14 ± 0.02
Cigarette	17	1.00 ± 0.01	2.61 ± 0.04	ND	3.61 ± 0.03	0.989 ± 0.005	2.49 ± 0.04	ND	3.48 ± 0.03
	18	0.91 ± 0.01	1.83 ± 0.02	ND	2.74 ± 0.03	0.90 ± 0.01	1.73 ± 0.02	ND	2.63 ± 0.03
	19	0.61 ± 0.01	2.15 ± 0.07	ND	2.76 ± 0.08	0.607 ± 0.003	2.03 ± 0.07	ND	2.64 ± 0.07
	20	0.646 ± 0.004	1.61 ± 0.02	ND	2.26 ± 0.03	0.641 ± 0.003	1.51 ± 0.02	ND	2.15 ± 0.02
	21	1.049 ± 0.005	3.66 ± 0.05	ND	4.71 ± 0.06	1.031 ± 0.004	3.54 ± 0.06	ND	4.57 ± 0.06
	22	ND	0.134 ± 0.002	ND	0.134 ± 0.002	ND	0.152 ± 0.002	ND	0.152 ± 0.002

Table 2-3 Continued

Tobacco Type	ID	Humectants (% wt/wt) (n=2)							
		MS				FID			
		Propylene Glycol	Glycerol	Triethylene Glycol	Total	Propylene Glycol	Glycerol	Triethylene Glycol	Total
Cigarette	23	1.238 ± 0.004	1.819 ± 0.009	ND	3.06 ± 0.01	1.219 ± 0.003	1.712 ± 0.006	ND	2.931 ± 0.007
	24	1.314 ± 0.005	1.922 ± 0.006	ND	3.24 ± 0.01	1.293 ± 0.005	1.812 ± 0.006	ND	3.105 ± 0.007
	25	0.292 ± 0.002	2.75 ± 0.09	ND	3.04 ± 0.09	0.310 ± 0.001	2.63 ± 0.09	ND	2.94 ± 0.09
	26	0.732 ± 0.002	2.66 ± 0.02	ND	3.39 ± 0.02	0.736 ± 0.004	2.554 ± 0.009	ND	3.290 ± 0.006
	27	0.96 ± 0.01	1.37 ± 0.03	ND	2.33 ± 0.04	0.95 ± 0.01	1.29 ± 0.03	ND	2.24 ± 0.04
	3R4F	BQL	2.52 ± 0.02	ND	2.52 ± 0.02	0.04 ± 0.02	2.40 ± 0.02	ND	2.44 ± 0.03
Moist Snuff	28	0.181 ± 0.001	0.01 ± 0.03	ND	0.19 ± 0.03	0.200 ± 0.002	ND	ND	0.200 ± 0.002
	29	0.344 ± 0.002	0.03 ± 0.04	ND	0.37 ± 0.03	0.357 ± 0.002	ND	ND	0.357 ± 0.002
	30	0.002 ± 0.003	ND	ND	ND	ND	ND	ND	ND
	31	0.354 ± 0.001	ND	ND	0.354 ± 0.001	0.370 ± 0.003	ND	ND	0.370 ± 0.003
	32	0.384 ± 0.001	ND	ND	0.384 ± 0.001	0.410 ± 0.002	ND	ND	0.410 ± 0.002
	33	ND	ND	ND	ND	ND	ND	ND	ND
	34	BQL	ND	ND	BQL	ND	ND	ND	ND
	35	BQL	ND	ND	BQL	ND	ND	ND	ND
	36	BQL	4.19 ± 0.03	ND	4.19 ± 0.03	ND	4.11 ± 0.02	ND	4.11 ± 0.02
	37	ND	ND	ND	ND	ND	ND	ND	ND
Hookah	38	ND	40.2 ± 0.5	ND	40.2 ± 0.5	ND	40.2 ± 0.6	ND	40.2 ± 0.6
	39	1.56 ± 0.03	43.3 ± 0.9	ND	44.9 ± 0.9	1.69 ±0.003	43.3 ± 0.9	ND	45 ± 1
	40	3.2 ± 0.1	34 ± 2	ND	37 ± 2	3.3 ± 0.01	34 ± 2	ND	37 ± 2
	41	9.37 ± 0.04	19.5 ± 0.2	ND	28.9 ± 0.2	9.62 ± 0.06	19.3 ± 0.2	ND	28.9 ± 0.2
	42	10.35 ± 0.06	21.2 ± 0.2	ND	31.6 ± 0.2	10.69 ± 0.02	21.2 ± 0.1	ND	31.9 ± 0.1
	43	9.7 ± 0.2	23.5 ± 0.4	ND	33.2 ± 0.5	10.10 ± 0.03	23.6 ± 0.3	ND	33.7 ± 0.2
	44	10.1 ± 0.1	20.4 ± 0.3	ND	30.5 ± 0.4	10.31 ± 0.09	20.2 ± 0.1	ND	30.5 ± 0.2

Correlation between MS and FID data was also evaluated by plotting concentration of humectants (% by weight) from MS results versus FID results as seen in Figure 2-5. An R^2 of 0.9999 was calculated from linear regression analysis and demonstrates a high degree of linearity between the results from FID and MS. Figure 2-6 focuses on the results for RYO, cigarette, cigar, and moist snuff tobaccos and shows that, although there is some clustering of data, these tobacco products overlap with respect to humectant content. As was discussed previously, hookah tobaccos contain significantly greater levels of humectants. This is observable in both Table 2-3 and Figure 2-4.

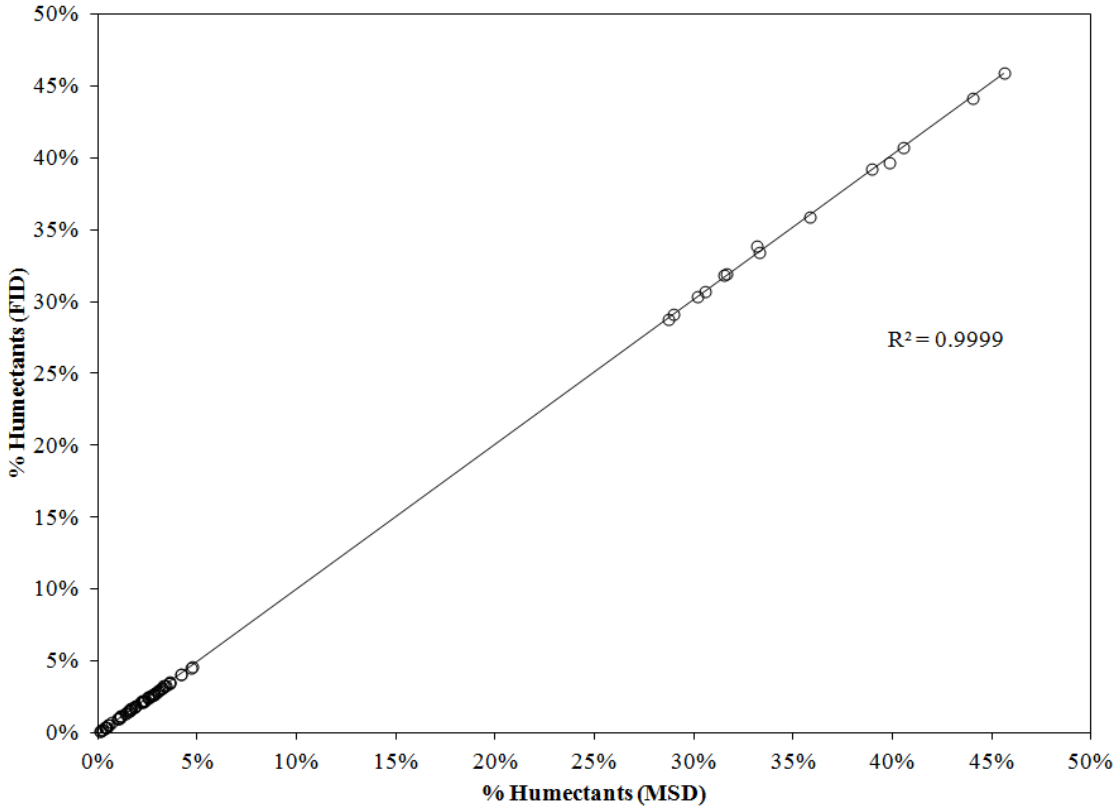

Figure 2-5 Correlation of total % humectants by GC-FID vs. total % humectants by GC-MS measured in various tobacco products

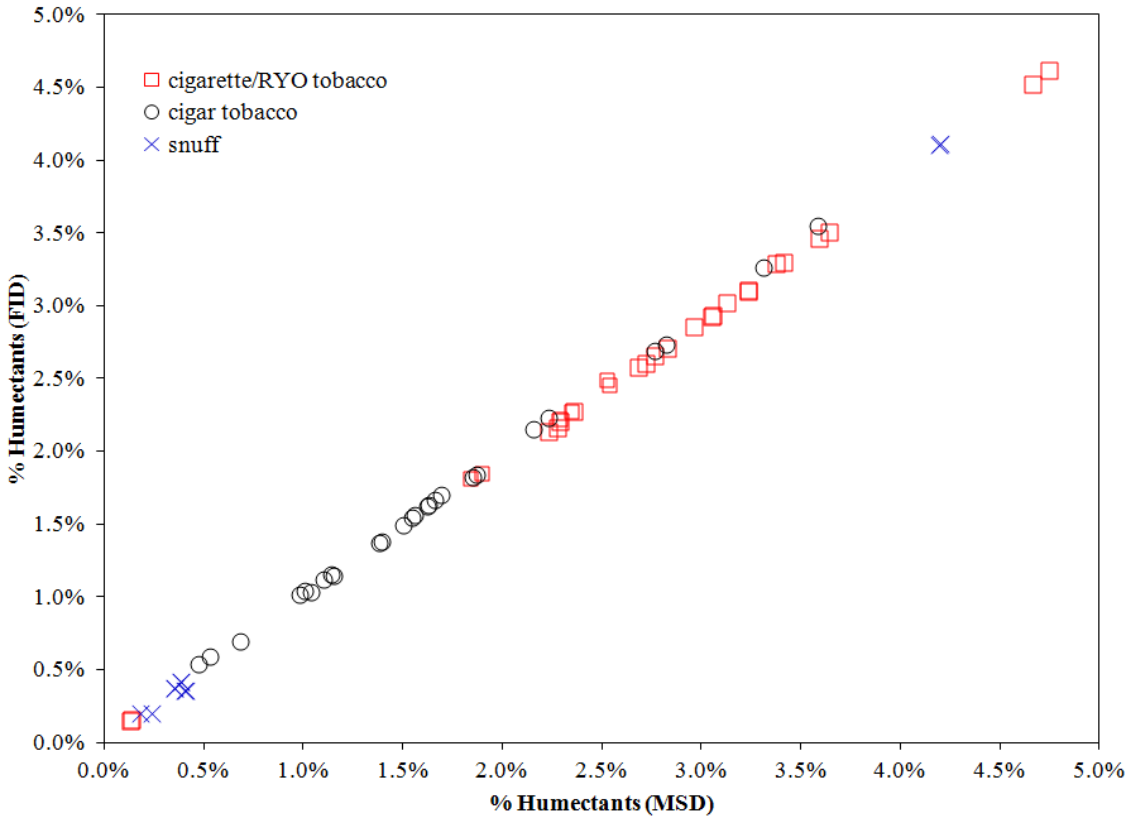

Figure 2-6 Correlation of total % humectants by GC-FID vs. total % humectants by GC-MS measured in RYO, cigarette, cigar, and moist snuff tobaccos

2.4 Chemometrics

Chemometric techniques were used to further compare and discriminate the different tobacco products. Specifically, discriminant analysis (DA) was utilized. Since products labeled as RYO, cigar, and cigarette tobaccos can be visually discriminated from moist snuff and hookah tobaccos, chemometrics would be irrelevant to distinguish all samples by tobacco type. However, chemometrics is important in discriminating tobacco products labeled as RYO, cigar, and cigarette.

Discriminant analysis is a statistical technique used to describe the relationship between categorical classes based upon multi-variate data. DA takes multi-variate data and creates new variables (called canonical variates) that maximize inter-group variance and minimize intra-group variance.[47, 48] DA produces multiple results; such as a variables plot, an observations plot, and a cross-validation confusion matrix. A variables plot demonstrates how each variable classifies the samples. Figure 2-7 is the variables plot for the DA of the tobacco samples labeled as RYO, cigar, and cigarette. The variables plot can be used to visually see which variable has the most influence on the samples in an observations plot. The observations plot uses the new variables to plot each sample to allow the similarities/ differences between samples to be visualized. Samples that are similar will be grouped together into classes, and samples that are dissimilar will not be plotted together. Ellipses are drawn to show the 95% confidence limit of each class. In addition to the variables and observations plots, a cross-validation confusion matrix can show how well the sample classes are distinguished. In the cross-validation, each sample is removed from the data set, the classification model is rebuilt, and the sample is placed into a class as if it were an unknown sample.

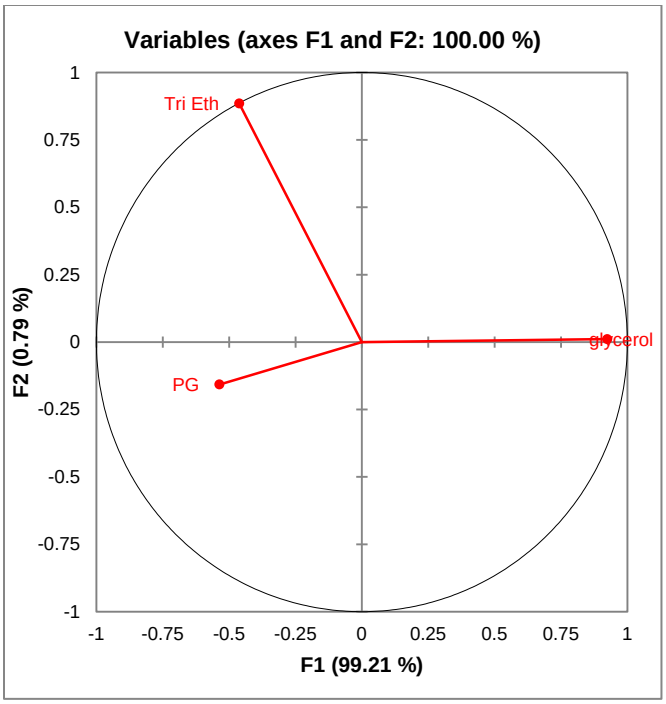

Figure 2-7 Variables plot for discriminant analysis of products labeled as RYO, cigar and cigarette showing the correlations between the variables (propylene glycol, glycerol, and triethylene glycol) and the canonical variates (F1 and F2)

Figure 2-8 shows how products labeled as RYO, cigar, and cigarettes are discriminated by tobacco brand. This is an observations plot for DA where each tobacco brand/flavor was considered as its own class. From this, it can be seen that products labeled as RYO, cigar, and cigarette could be clustered into their own classes (green box=RYO, red box=cigar, blue box=cigarette). Table 2-4 is the cross-validation confusion matrix demonstrating how well the discriminant analysis classifies each tobacco sample into a specific class.

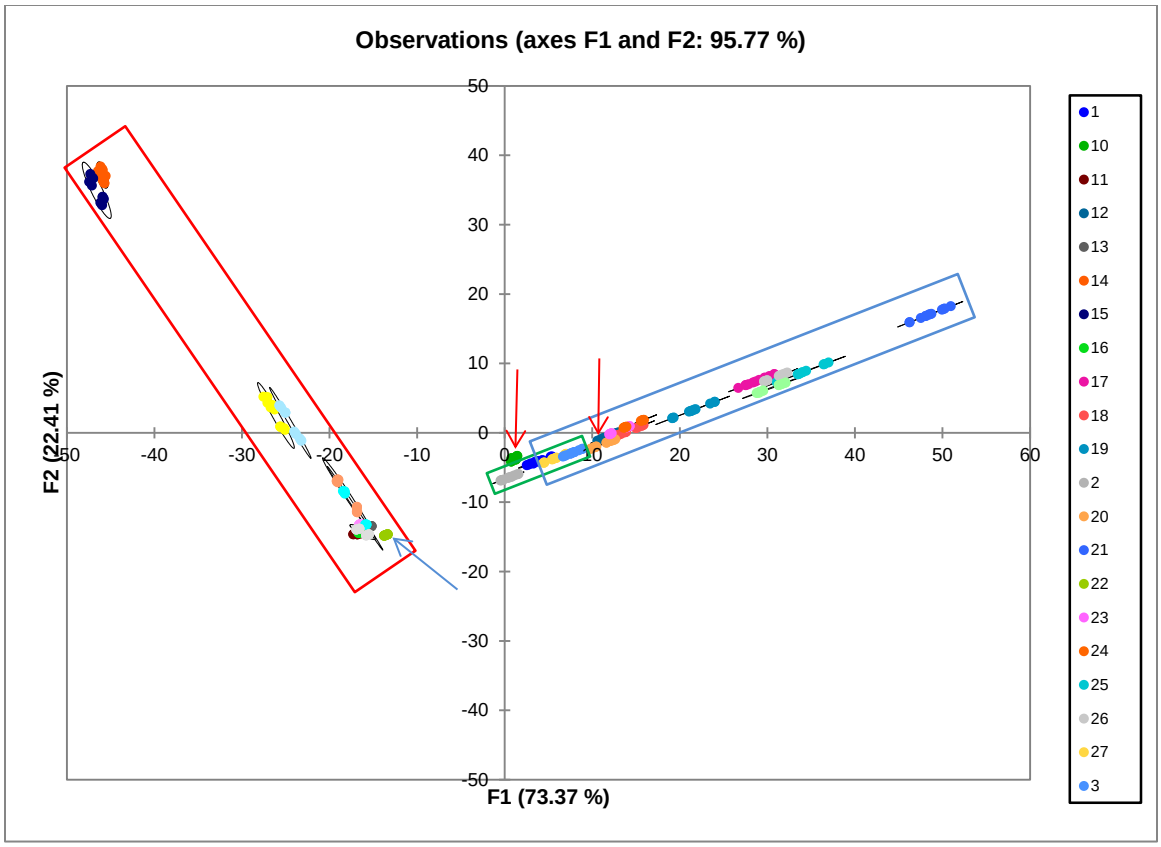

Figure 2-8 Observations plots from DA for products labeled as RYO, cigar, and cigarette discriminated by brand/flavor. Green box represents products labeled as RYO, red box represents cigars, and blue box represents cigarettes. Red and blue arrows point to tobacco samples that should be grouped with cigars and cigarettes, respectively.

Table 2-4 Cross-validation confusion matrix for DA of RYO, cigar, and cigarette tobacco samples classifying each by tobacco brand/flavor

from \ to	1	10	11	12	13	14	15	16	17	18	19	2	20	21	22	23	24	25	26	27	3	3R4F	4	5	6	7	8	9	Total	% correct
1	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
10	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
11	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	12	75.00%
12	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
13	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
14	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
15	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
16	0	0	2	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	12	58.33%
17	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
18	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
19	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
2	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
20	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
21	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	12	100.00%
23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	2	0	0	0	0	0	0	0	0	0	0	0	12	83.33%
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	9	0	0	0	0	0	0	0	0	0	0	0	12	75.00%
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	12	100.00%
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	10	100.00%
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	2	0	0	0	0	0	0	0	12	83.33%
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	12	100.00%
3R4F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	12	100.00%
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	12	100.00%
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	6	0	0	0	12	50.00%
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	6	0	0	0	0	12	0.00%
7	0	0	3	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	12	25.00%
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	12	100.00%
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	12	100.00%
Total	12	12	14	12	18	12	12	7	12	12	12	12	12	12	12	13	11	12	10	10	14	12	18	12	6	9	12	12	334	87.43%

Figure 2-9 is an observations plot and Table 2-5 is the cross-validation confusion matrix for DA where each tobacco type (RYO, cigar, and cigarette) was considered as its own class. From this, cigars and cigarettes can be distinguished by class. However, no products labeled as RYO were classified into the correct class; 61% of the products labeled as RYO were classified as cigars.

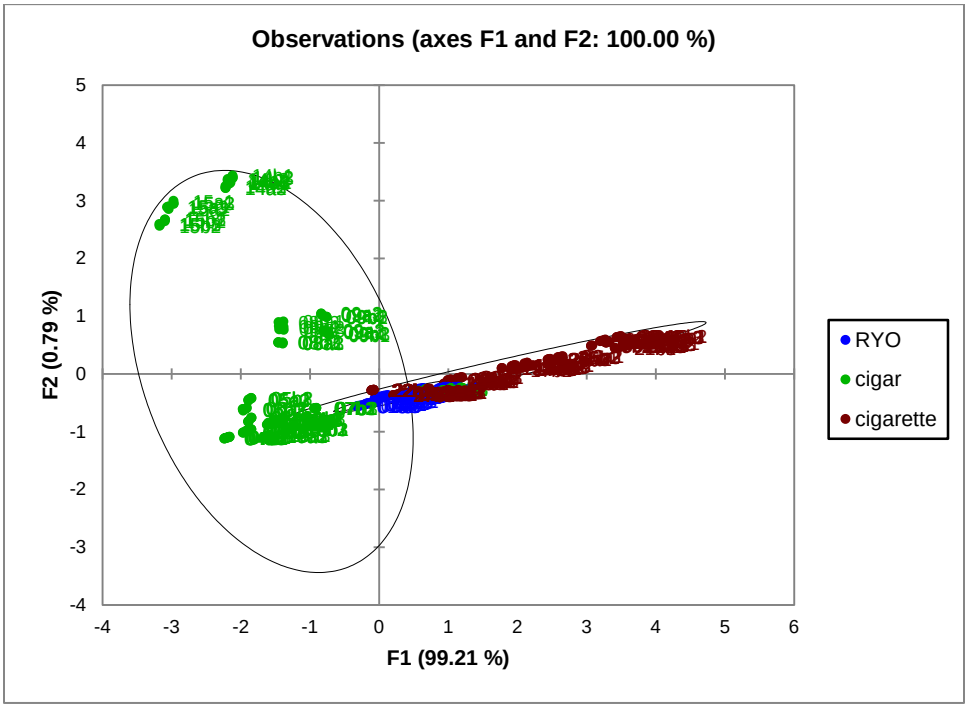

Figure 2-9 Observations plots from DA for products labeled as RYO, cigar, and cigarette discriminated by tobacco type

Table 2-5 Cross-validation confusion matrix for DA of RYO, cigar, and cigarette tobacco samples classifying each tobacco sample by type.

from \ to	RYO	cigar	cigarette	Total	% correct
RYO	0	22	14	36	0.00%
cigar	0	144	12	156	92.31%
cigarette	0	12	130	142	91.55%
Total	0	178	156	334	82.04%

Figure 2-10 and Table 2-6 demonstrate that hookah samples can be discriminated by tobacco brand and flavor using chemometrics. The cross-validation correctly classified 96% of the tobacco samples. Only one tobacco brand/flavor was incorrectly classified, and it was classified into a different flavor of the same brand. This shows that hookah tobaccos are chemically distinguished from one another according to the amounts of the humectants. It should be noted that the three hookah samples along the positive x-axis are Pharaoh's brand and samples along the negative x-axis are Rosetta brand. The two brands are different from each other in that one brand contains more glycerol while the other brand contains more propylene glycol.

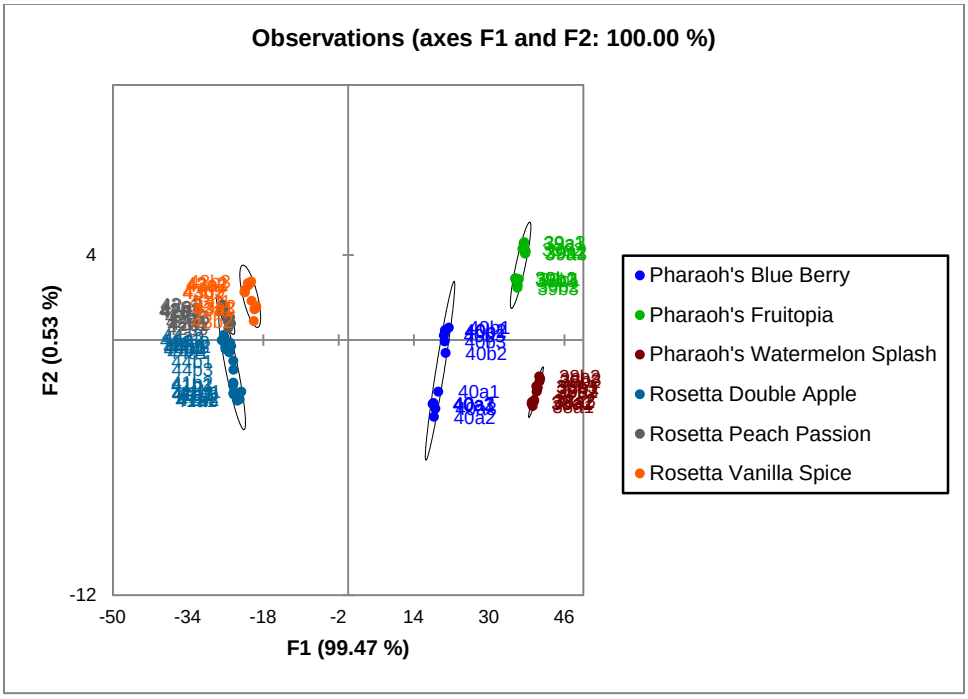

Figure 2-10 Observations plot for DA of hookah samples by brand and flavor

Table 2-6 Cross-validation confusion matrix for DA of hookah samples by brand and flavor

from \ to	Pharaoh's Blue Berry	Pharaoh's Fruitopia	Pharaoh's Watermelon Splash	Rosetta Double Apple	Rosetta Peach Passion	Rosetta Vanilla Spice	Total	% correct
Pharaoh's Blue Berry	12	0	0	0	0	0	12	100.00%
Pharaoh's Fruitopia	0	12	0	0	0	0	12	100.00%
Pharaoh's Watermelon Splash	0	0	12	0	0	0	12	100.00%
Rosetta Double Apple	0	0	0	21	3	0	24	87.50%
Rosetta Peach Passion	0	0	0	0	12	0	12	100.00%
Rosetta Vanilla Spice	0	0	0	0	0	12	12	100.00%
Total	12	12	12	21	15	12	84	96.43%

2.5 Conclusions

In this study, the results for the MS and FID were very similar in accuracy and precision. The use of mass spectrometric detection in these analyses provide selectively not provided by FID detection. It should be noted that, on comparison to the current MS results (in scan mode), FID detection does have the advantage of lower LOD's and extended calibration range to lower concentrations. The detection limits for the individual humectants by MS are in the 2 to 4 ppm range while the detection limits by FID are in the 0.25 to 0.5 ppm range. Even though the MS has slightly higher LODs than the FID, MS is well suited for measurement of humectants in the products of interest and provides confirmation of the chemical identity of the humectant compounds measured. In a head-to-head comparison, this study shows that a GC/MS method is comparable to GC/FID approach in the accuracy/precision of measured values and offers adequate detection limits and chemical specificity not offered by the GC/FID approach. This study demonstrates that the GC/MS method presented here provides the appropriate calibration range, accuracy and chemical specificity needed for measuring % humectants in a wide range of tobacco product types. In addition, the determination of humectants was improved using a post-run backflush to eliminate carry-over and late eluting compounds.

Statistical analysis of the results show good correlation between % humectants calculated from FID and MS. The high degree of correlation between the data sets might suggest that the added resolving power of the MS is

unnecessary. Such a conclusion is unwarranted based on the limited scope of the product analysis. In samples containing triethylene glycol, the levels of humectants were so low that the separation of the triethylene glycol and glycerol peaks was sufficient for quantitation. Since triethylene glycol was not found in samples that contained high levels of glycerol, chromatographic resolution under these conditions could not be investigated. It is important to point out that, without MS, verification that triethylene glycol was absent from samples with high glycerol content would have been difficult. It is certain that application of the existing method[43] to hookah-type products will produce ambiguous results if the samples contain large amounts of glycerol. For the analyst attempting to identify the humectants present in the product using FID alone, dilution may be required to resolve glycerol and triethylene glycol, if one or both are thought to be present in the sample. Given that a minor component could be diluted to a level below the LOD, a limitation to the application of the existing method[43] is exposed.

Regardless of the product analyses shown here, mass spectrometry is the detection method of choice when using the GC conditions described to provide the chemical selectivity not offered by flame ionization detection. Furthermore, utilization of selective ion monitoring further enhances the sensitivity of MS and potentially erases the apparent sensitivity advantage of FID.

CHAPTER 3. EXTRACTION AND IDENTIFICATION OF "LIFESTYLE MARKERS" IN MTDNA ANALYSIS OF HUMAN HAIR

3.1 Introduction

This research links the genetic analysis of hair with chemical analysis. The premise is that discarded fractions from a typical protocol for mitochondrial DNA (mtDNA) sequencing can be subjected to chemical analysis with results providing information about the lifestyle of the subject. Such "lifestyle markers" may include cosmetic modifications to the hair, use of tobacco, and demographic data such as age and gender. In particular, it is hypothesized that small organic compounds incorporated in the hair (e.g., hair dyes, nicotine/cotinine) will be released into the organic layer during liquid-liquid extractions.[49]

During a microscopic examination, hairs containing the hair root can be selected for nuclear (nDNA) analysis. Hairs removed during the anagen phase of hair growth are likely to yield nDNA profiles, while naturally shed hairs (telogen phase) are less likely to yield DNA profiles. In instances when the hair root is not present or nDNA cannot be analyzed, mtDNA may be utilized.

This project is focused on the extraction of hairs for surface and integral components. Using literature methods for analytes of interest, the protocols have been recreated and shown to be reliable using known standards. The published

methods have been scaled down to milligram quantities of hair and extrapolated to the level of a typical mtDNA analysis (2 cm hair strand).

This procedure seeks to open up new possibilities for information that can be obtained from a hair sample, to include factors such as age (youth versus adult), tobacco use and hair dyes. This could also eliminate people at the level of a microscopic exam as well as distinguish between people with the same mtDNA profiles (from the same maternal line). These methods could be applied during a typical extraction protocol for mtDNA and would result in an unknown sample being more fully characterized, while a comparison of a questioned and known sample would be more probative.

3.2 Experimental

3.2.1 Materials

Dichloromethane, proteinase K, dithiothreitol (DTT), Terg-A-Zyme, and phenol/chloroform/isoamyl alcohol (25:24:1) were purchased from Fisher Scientific. Ethanol, palmitic acid, stearic acid, and palmityl palmitate were purchased from Sigma Aldrich. Squalene and cholesterol were purchased from VWR. Stain extraction buffer was purchased from Teknova.

3.2.2 Lipid Standard

A lipid standard was created containing ~1.0 mg/mL palmitic acid, stearic acid, squalene, cholesterol, and palmityl palmitate in dichloromethane.

3.2.3 Hair Extractions

Hair (10mg, 1mg, and one strand) was subjected to mtDNA analysis following an extraction procedure used at the Minnesota Bureau of Criminal Apprehension Forensic Science Laboratory, which is outlined in Figure 3-1.

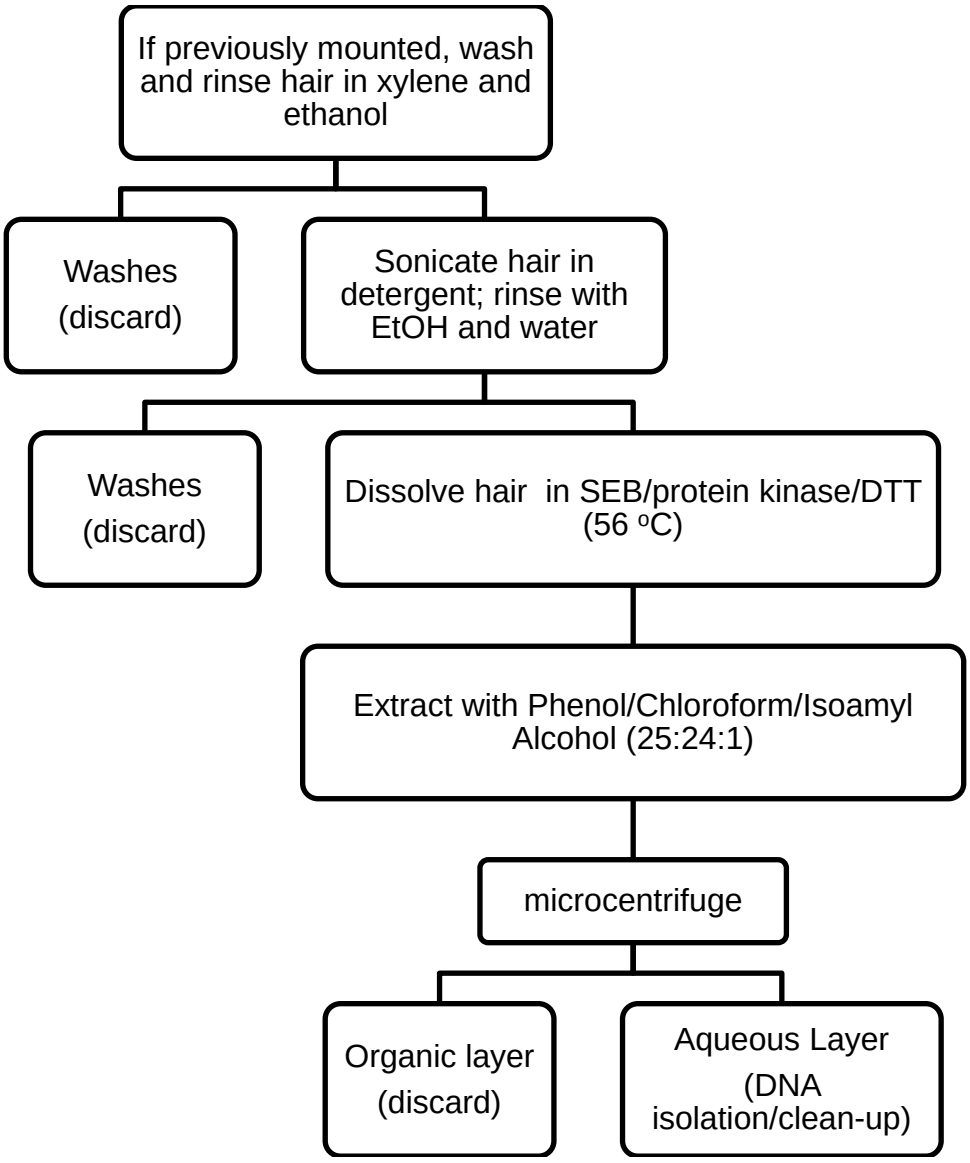

Figure 3-1 Protocol for mtDNA extract from hair

The hair was first washed with ~1 mL xylenes and then with ~1mL ethanol. Next, the hair was sonicated for 20 min in 1 mL 5% Terg-A-Zyme twice. The hair was then rinsed with ~1 mL portions of ethanol then water to rinse out the detergent. The hair was then digested in 500 µL stain extraction buffer (SEB), 7.5 µL proteinase K (20 mg/mL), and 20 µL dithiolthreitol (DTT). The samples

were then sonicated with heat until the hair had dissolved. Next, 500 µL PCIA (Phenol:Chloroform:Isoamyl alcohol 25:24:1) was added and the sample was vortexed. The extracts were then microcentrifuged to obtain layer separation. The organic layer was then separated from the aqueous layer. All organic washes and extracts (xylenes and ethanol washes and PCIA extract) were evaporated under nitrogen stream and heated to dryness. The PICA extracts did not fully evaporate, but were still treated in the same manner as the washes. All extracts were then reconstituted into 300 µL dichloromethane and analyzed by GC/MS.

3.2.4 Instrumental Parameters

The hair extracts were analyzed on an Agilent 6890N GC with an Agilent 5975 mass spectrometer. Analytes were separated on a 30 m x 0.25 mm x 0.25 µm DB-5MS column. Helium carrier gas at a flow rate of 1 mL/min and inlet temperature of 250°C were used. Sample volumes of 2 µL were injected under splitless conditions. The oven started at 155°C and held for 1 min; ramped at 15°C/min to 325°C and was held for 8 min. The MS transfer line was at 250°C with a 2.5 min solvent delay and mass scan from m/z 50 to 550.

3.3 Results and Discussion

3.3.1 Lipid Standards and Xylenes Sonications

A standard lipid mixture containing palmitic acid, stearic acid, squalene, cholesterol and palmityl palmitate was analyzed with excellent chromatographic separation (Figure 3-2). Hair samples (100mg, 10mg, 1mg, and a 2 cm hair strand) were sonicated with xylenes for 30 minutes and the extract was analyzed by GC/MS. All analytes were detected in the 100 mg portion of hair (Figure 3-3); while, only squalene, cholesterol, and palmityl palmitate were detected in the 10 mg and 1 mg portions and only squalene was detected in the hair strand.

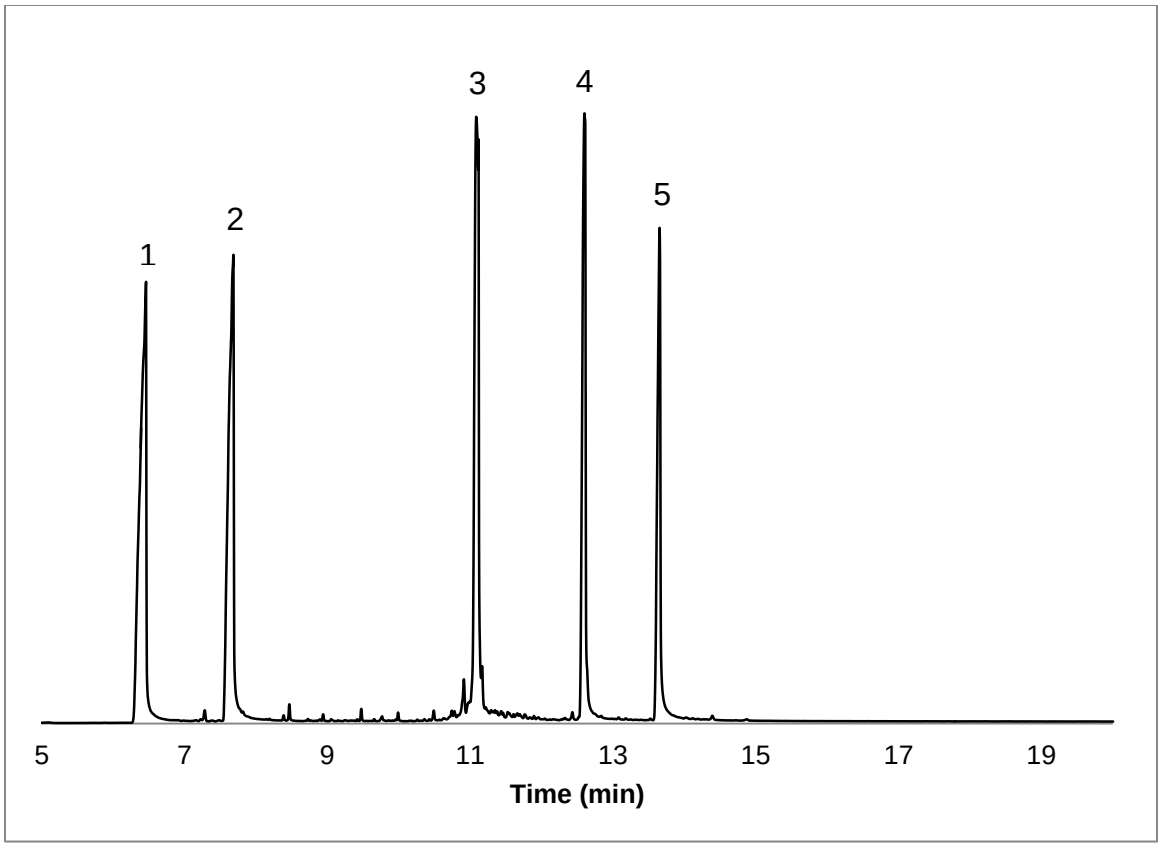

Figure 3-2 Chromatogram of a lipid mixture. Peak labels: 1- palmitic acid 2- stearic acid 3- squalene 4- cholesterol 5- palmityl palmitate

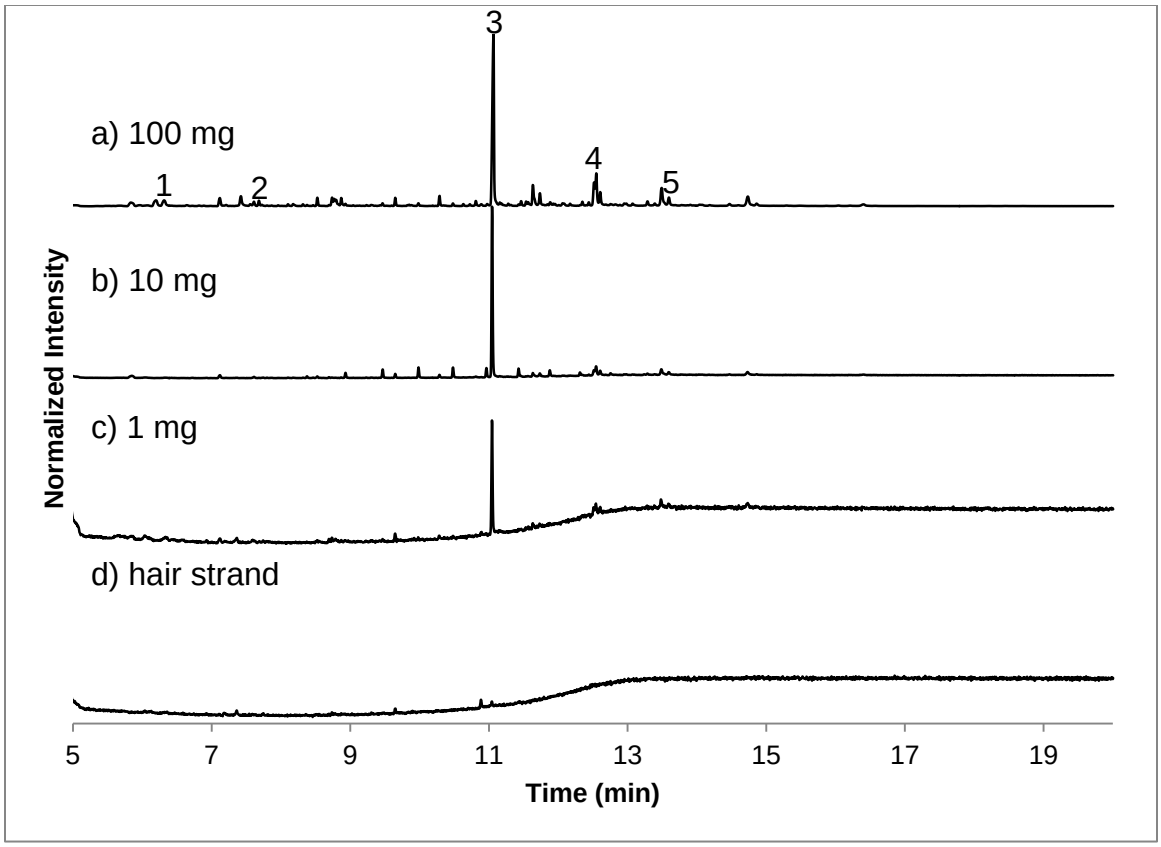

Figure 3-3 Chromatograms of xylenes sonications. a) 100 mg hair b) 10 mg hair c) 1 mg hair d) 2 cm hair strand. Peak labels: 1- palmitic acid 2- stearic acid 3- squalene 4- cholesterol 5- palmityl palmitate

3.3.2 mtDNA Hair Extractions

Hair samples (10 mg, 1mg, and a 2 cm hair strand) were subjected to the mtDNA extraction method and all organic extracts were analyzed by GC/MS. Summed extracted ion chromatograms (m/z 256, 284, 69, 386, 257 for palmitic acid, stearic acid, squalene, cholesterol, and palmityl palmitate, respectively) are shown for the xylenes wash, ethanol wash, and PCIA extract (Figures 3-4, 3-5, and 3-6, respectively). Squalene, cholesterol, and palmityl palmitate were successfully detected in 10 mg hair samples while only squalene and palmityl palmitate were detected in the 1 mg sample. Unfortunately, the washes of the

hair strand were not as successful as the larger hair portions, with only palmityl palmitate being detected in the xylenes wash. Unlike the xylenes and ethanol washes, the PCIA extracts contained palmitic and stearic acid. Additionally, squalene, cholesterol, and palmityl palmitate were detected in the 10 mg PCIA extract, while squalene and cholesterol were detected in the 1 mg hair sample and squalene was detected in the hair strand.

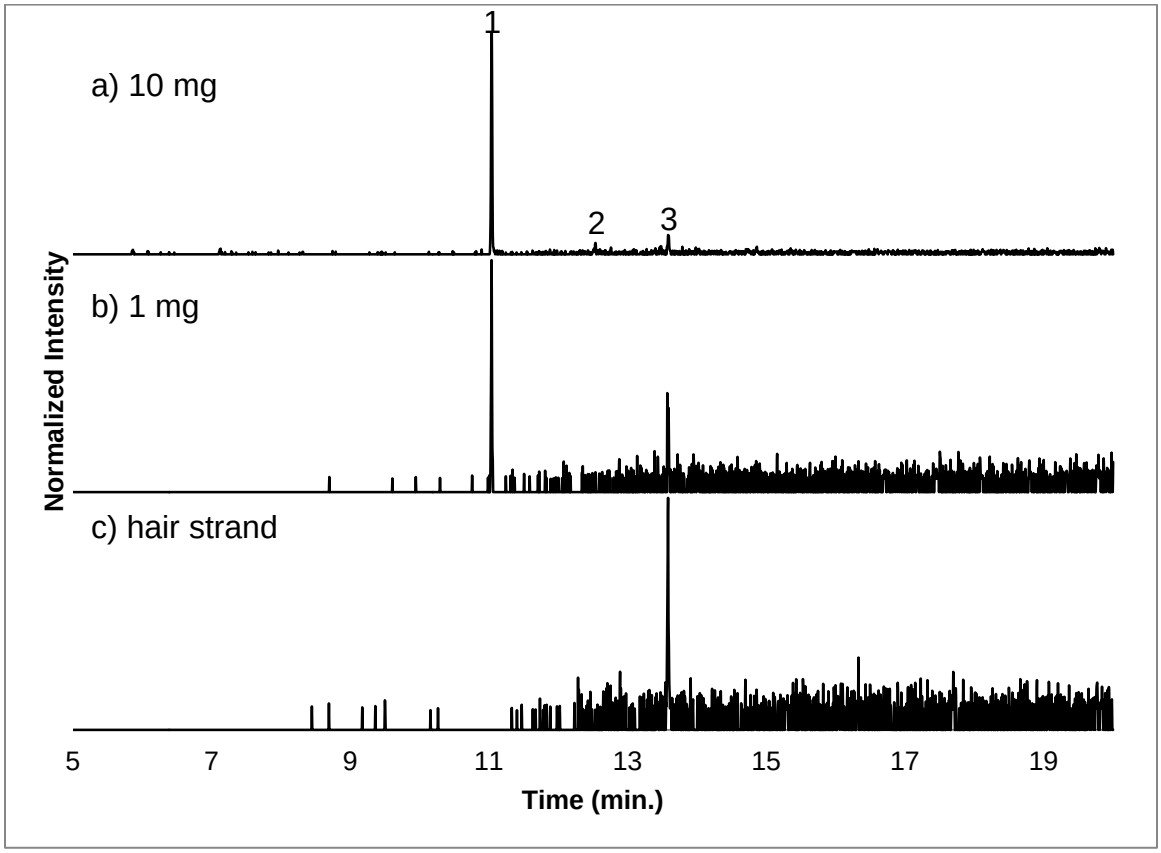

Figure 3-4 Chromatograms of xylenes washes. a) 10 mg b) 1 mg c) 2 cm hair strand. Peak labels: 1- squalene 2- cholesterol 3- palmityl palmitate

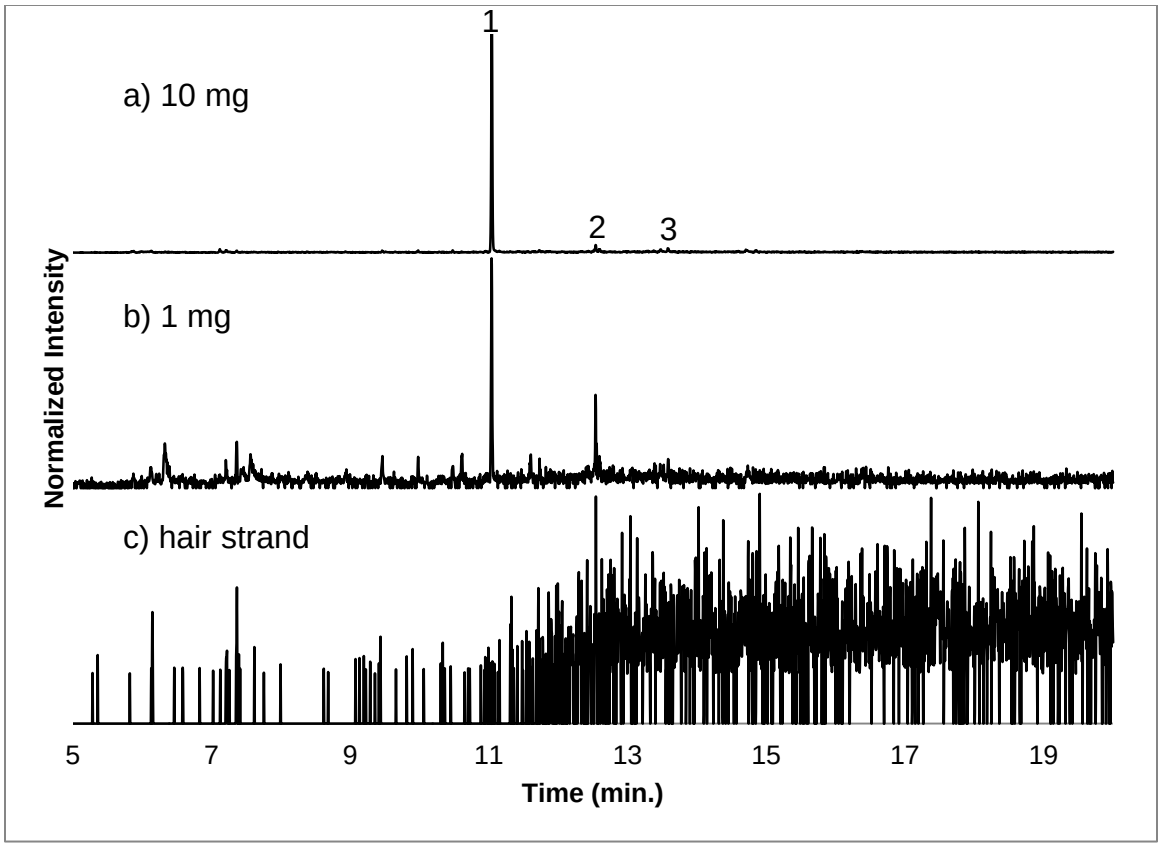

Figure 3-5 Chromatograms of ethanol washes. a) 10 mg b) 1 mg c) 2 cm hair strand. Peak labels: 1- squalene 2- cholesterol 3- palmityl palmitate

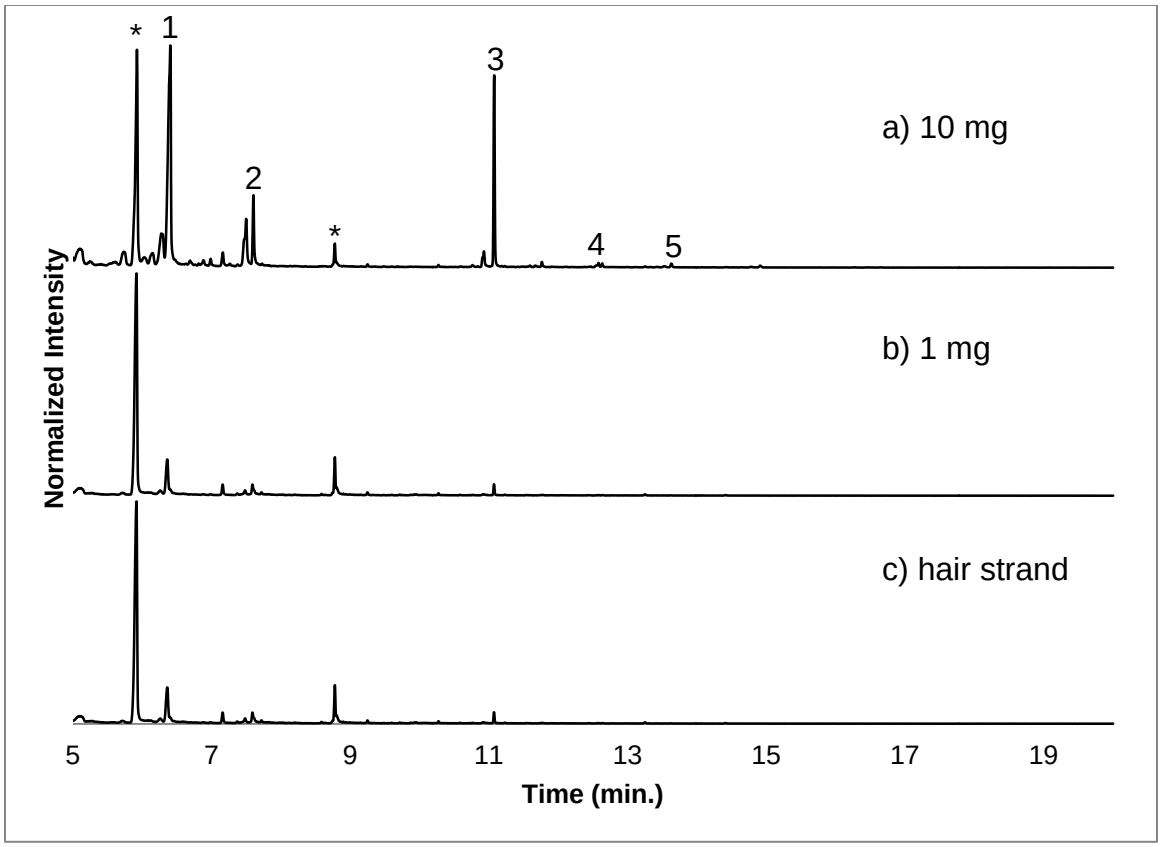

Figure 3-6 Chromatograms of PCIA extracts. a) 10mg b) 1mg c) 2 cm hair strand. Peak labels:1- palmitic acid 2- stearic acid 3- squalene 4- cholesterol 5- palmityl palmitate *- control

Table 3-1 summarizes the presence of lipids from the xylenes washes, ethanol washes, and PCIA extractions for the various hair portions. From these results, it is feasible to perform a mtDNA extraction of a 2 cm hair strand and successfully detect palmitic acid, stearic acid, squalene, and palmityl palmitate in various organic fractions that would normally be discarded.

Table 3-1 Results of xylenes wash, ethanol wash, and PCIA extraction of 10 mg, 1 mg and hair strand

		Palmitic Acid	Stearic Acid	Squalene	Cholesterol	Palmityl Palmitate
Xylenes Washes	10 mg			X	X	X
	1 mg			X		X
	Strand					X
Ethanol Washes	10 mg			X	X	X
	1 mg			X	X	
	Strand					
PCIA Extracts	10 mg	X	X	X	X	X
	1 mg	X	X	X	X	
	Strand	X	X	X		

3.4 Conclusions

These results demonstrate how “waste” from a mtDNA extraction procedure can be utilized to discover additional information about the individual with which the hair sample originated. Although the signal to noise was too low in the xylenes and ethanol washes to accurately detect the lipids, the detection from the PCIA extract shows promising results. The major limitation to this project is sample size. As the goal is to use “waste” from a mtDNA extraction, the amount of hair used and the protocol cannot be changed. With proper improvements to the analytical methods, this project could open up possibilities for detecting analytes and making conclusions that would otherwise be disregarded. Possible improvements to the methods will be discussed in Chapter 6.

CHAPTER 4. DEVELOPMENT AND OPTIMIZATION OF TOTAL VAPORIZATION-SOLID PHASE MICROEXTRACTION (TV-SPME)

4.1 Introduction

4.1.1 TV-SPME Theory

Solid phase microextraction (SPME) is a pre-concentration sampling technique in which components are adsorbed and/or absorbed onto a fiber and then subsequently desorbed in the inlet of a gas or liquid chromatograph.[50-53] The thermodynamics of headspace SPME are largely dependent on distribution of the analyte between the liquid (or solid) sample, the headspace, and the fiber (Figure 4-1A). The amount of analyte on a SPME fiber positioned in the headspace above a liquid/solid sample can be described by the following equation:[50]

$$n = \frac{K_{fs} V_s V_f C_0}{K_{fs} V_f + K_{hs} V_h + V_s} \qquad \text{(Equation 4-1)}$$

where n is the mass of analyte on the fiber, K_{fs} is the distribution coefficient between the fiber and the sample, V_s is the volume of sample, V_f is the volume of fiber coating, C_0 is the initial concentration of analyte (in the sample), K_{hs} is the

distribution coefficient between the headspace and the sample, and V_h is the volume of the headspace.

The thermodynamics of immersion SPME are largely dependent on analyte distributing between a liquid sample and the SPME fiber (Figure 4-1B). The amount of analyte on a SPME fiber that is directly immersed into a liquid sample can be described by the following equation:[50]

$$n = \frac{K_{fs}V_sV_fC_0}{K_{fs}V_f + V_s} \approx K_{fs}V_fC_0 \qquad \text{(Equation 4-2)}$$

Since the sample volume is much greater than the fiber volume ($V_s \gg V_f$), this equation can be simplified so that n depends solely on K_{fs}, V_f, and C_0.

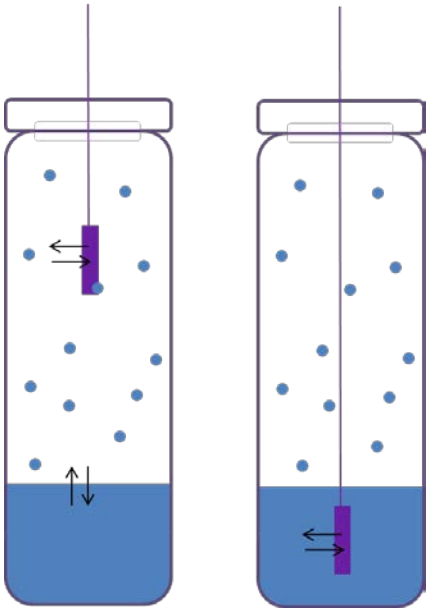

Figure 4-1 Depiction of headspace SPME (A) and immersion SPME (B)

Total vaporization (TV) is a technique that is typically utilized in simple headspace sampling.[54] Matrix effects in headspace sampling are of particular concern and one way to eliminate matrix effects is to completely evaporate both the analyte and its matrix. In this case, the temperature must be high enough to completely vaporize the solvent as well as any volatile or semi-volatile analytes that are present (Figure 4-2B).

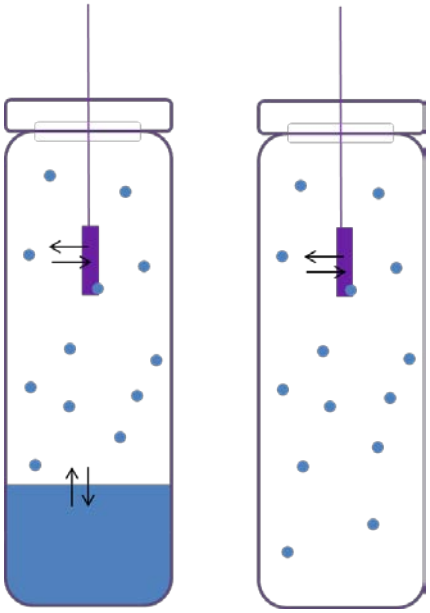

Figure 4-2 Depiction of headspace SPME of a liquid sample (A) and of a totally vaporized sample (B)

The partitioning of an analyte between a liquid sample and gas phase can be described by the partition coefficient, defined as:[54]

$$K_{sg} = \frac{n_s}{n_g} \cdot \beta \qquad \text{(Equation 4-3)}$$

where $K_{sg}$is the partition coefficient between the sample and gas phase, n_s is the amount of analyte in the sample (moles), $n_g$is the amount of analyte in the gas phase (moles), and β is the phase ratio. The phase ratio can be defined as:

$$\beta = \frac{V_g}{V_s} = \frac{V_v - V_s}{V_s} \qquad \text{(Equation 4-4)}$$

where $V_g$is the volume of gas, V_s is the volume of liquid sample, and V_v is the volume of the vial. Reducing the sample volume will result in an increase in the phase ratio. At a given temperature, the partition coefficient is constant, so an increase in phase ratio would require the $\frac{n_s}{n_g}$ term in (Equation 4-3) to decrease in the same proportion. Since $n_s + n_g$ remains constant, this can only be achieved by an increase in n_g and a subsequent decrease in n_s.

When the entire liquid sample is vaporized, there is no longer a headspace and condensed phase and therefore no analyte partitioning between a gas and liquid phase. The gaseous analyte concentration in the vial is then dependent on the amount of analyte in the original sample and the volume of the vial.

The technique of total vaporization is being proposed for use with SPME. By completely vaporizing the sample, the partitioning between the sample and the headspace is eliminated, thus simplifying the thermodynamic equilibria (Figure 4-2B). The theoretical equation describing TV-SPME utilizes (Equation 4-2), with slight modification:

$$n \approx K_{fs} V_f C_v \qquad \text{(Equation 4-5)}$$

where C_v is the concentration of analyte in the vial. This assumes that the analyte will be completely vaporized at the given volume and temperature that is sufficient to vaporize the solvent. The amount of analyte that can be totally vaporized will depend on its vapor pressure and temperature.

Combining total vaporization with the ability of SPME to pre-concentrate analytes onto the fiber greatly increases sensitivity and has several other distinct advantages:

1) In liquid injection, selectivity in the GC inlet is mostly based on the boiling point of the analytes and their solvent. SPME adds more selectivity because fibers of different chemistries can be used based upon the analytes of interest.

2) In GC, injection volumes are usually limited to several microliters, therefore only a small fraction of the sample extract is injected. Using TV-SPME, lower analyte concentrations can be easily detected because a larger fraction of the extract (>100 µL) is introduced.

3) In TV-SPME, samples do not need to be filtered and minimal sample preparation is required. Any solids or non-volatile compounds that may be present in a sample extract will remain on the surface of the vial. This can greatly reduce the amount of buildup and contamination that may occur in the inlet and GC column.

4) The distribution of the analyte in TV-SPME occurs at a vapor/solid interface. Hence, the kinetics should be rapid and the system will reach

equilibrium very quickly. This would indicate that extraction times will not be a major factor in recovery of the analytes.

The key parameters that must be considered when designing and optimizing a TV-SPME method are selecting an appropriate combination of sample volume and extraction temperature. The amount of liquid sample that can be completely vaporized inside a vial can be estimated using the ideal gas law:

$$n = \frac{PV}{RT} \qquad \text{(Equation 4-6)}$$

where n is the number of moles of liquid sample, P is the vapor pressure of the liquid (bar), V is the volume of the vial (L), R is the ideal gas constant (8.3145×10^{-2} L bar/K mol), and T is temperature (K).

The maximum volume of sample that can be placed into the vial can be calculated from the number of moles:

$$V_s = \frac{n \times M}{\rho} \qquad \text{(Equation 4-7)}$$

where V_s is the volume of sample (mL), M is the molar mass (g/mol), and ρ is the density at room temperature (g/mL). If (Equation 4-6) is substituted into (Equation 4-7), the volume of sample that will be totally vaporized can be defined as:

$$V_s = \left(\frac{PV}{RT}\right)\left(\frac{M}{\rho}\right) \qquad \text{(Equation 4-8)}$$

As vapor pressure of the solvent is dependent on temperature, the Antoine equation can be used for pressure:

$$\log_{10} P = A - \frac{B}{T + C} \qquad \text{(Equation 4-9)}$$

where A, B, and C are Antoine constants for the solvent.

Substituting (Equation 4-9) into (Equation 4-8) describes the volume of sample that can be totally vaporized as a function of temperature:

$$V_s = \left(\frac{\left(10^{A-\frac{B}{T+C}}\right) V}{RT} \right) \left(\frac{M}{\rho}\right) \qquad \text{(Equation 4-10)}$$

This relationship can be seen in Figure 4-3 using chloroform as the solvent in a 20 mL SPME vial.

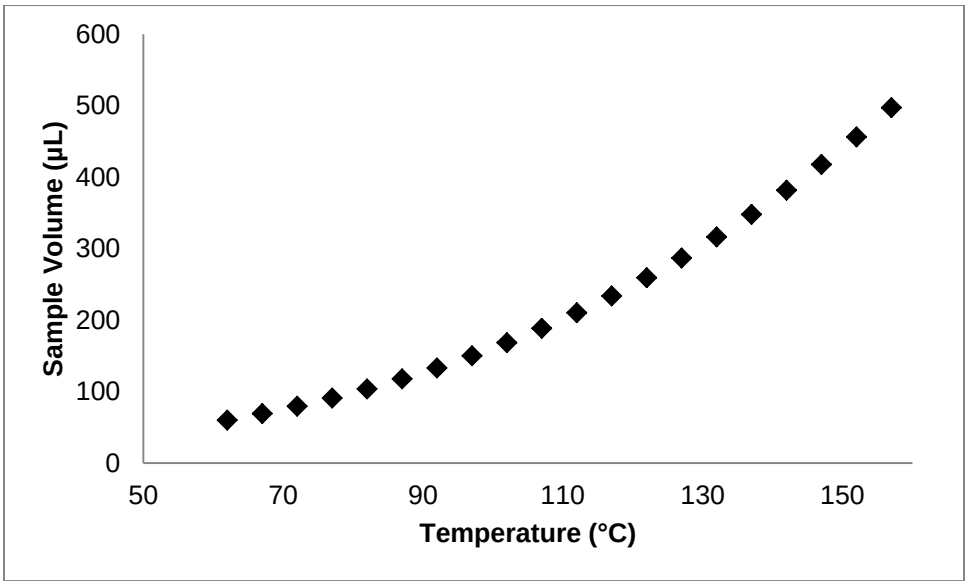

Figure 4-3 Maximum volume of chloroform that can be totally vaporized in a 20 mL headspace vial as a function of temperature

Temperature will also have a major effect on the ability of a SPME fiber to adsorb/absorb analytes. An increase in incubation temperature will aid in the volatilization of the analytes as well as decreasing the equilibration time. Increasing the temperature from T_0 to T of the SPME system (sample,

headspace, and fiber) will cause the partition coefficient between the fiber and sample to change as follows:[50]

$$K_{fs} = K_0 exp \frac{-\Delta H}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right) \qquad \text{(Equation 4-11)}$$

where ΔH is the analyte change in enthalpy and R is the gas constant.[50] When the partition coefficient is greater than 1, the potential energy of the analyte is greater in the sample than the fiber coating, which means that partitioning into the fiber is exothermic and $\frac{-\Delta H}{R}$ is positive (for typical SPME experiments, ΔH is considered to remain constant). This ultimately demonstrates that an increase in temperature will result in a decrease in the partition coefficient. This would imply that increasing the temperature will decrease the amount of analyte that will absorb onto the fiber. However, in total vaporization, increasing the temperature allows more sample to be vaporized, and therefore more analyte that can absorb onto the fiber. This is because temperature has a greater influence on vapor pressure than it does on the partition coefficient.

4.1.1.1 TV-SPME Limitations

An important concern with total vaporization of a liquid sample in a headspace vial is exceeding the pressure limitations of the vial. When a solvent is totally vaporized, the pressure within the vial will greatly increase. Much care must be taken to ensure that pressures inside the vial do not cause the vial to burst. Special “pressure release” caps are made for headspace vials that will release the internal pressure when 3 bar is exceeded.[55] Some headspace vials have been manufactured which can withstand pressures of 4-8 bar.[56] It is

therefore assumed that unless high-pressure vials are used, the pressure in the vial when using TV-SPME should not exceed 3 bar. The pressure in the vial can be described by the sum of partial pressures of air in the vial and of the solvent:

$$P_{vial} = P_{air} + P_{solvent} \qquad \text{Equation 4-12}$$

Using the ideal gas law (Equation 4-6) to determine the partial pressure of air and using the Antoine equation (Equation 4-9) to determine the partial pressure of solvent, the equation becomes:

$$P_{vial} = \frac{nRT}{V} + 10^{A-\frac{B}{T+C}} \qquad \text{Equation 4-13}$$

For chloroform as the solvent, the pressure within a 20 mL headspace vial can be calculated and the relationship is seen in Figure 4-4.

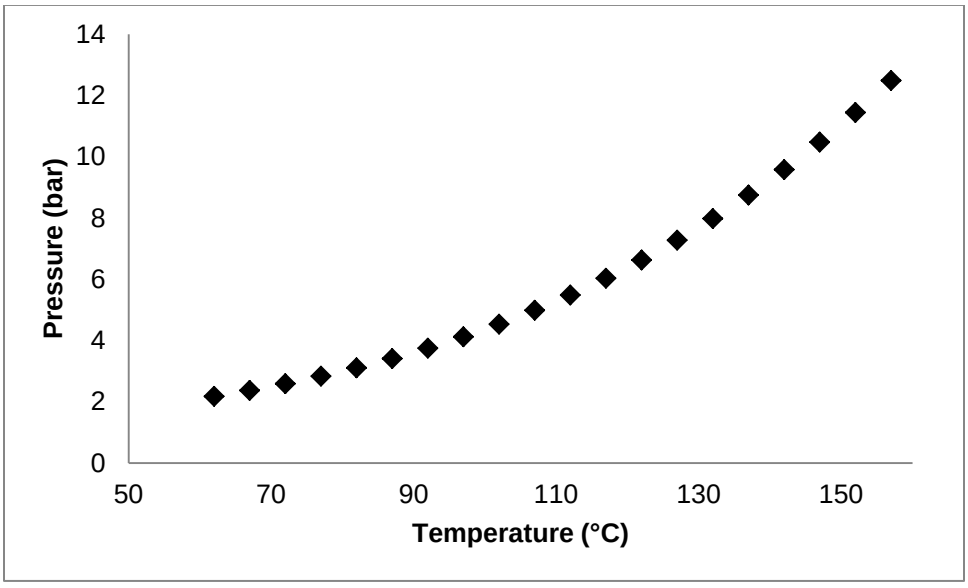

Figure 4-4 Pressure in a 20 mL headspace vial with total vaporization of chloroform as a function of temperature

Another important concern when using organic solvents with SPME is fiber swelling.[51] When a SPME fiber is exposed to a solvent, the fiber will absorb the solvent and therefore the diameter of the fiber will increase. This is of particular concern because the swelled fiber coating could strip off when the fiber is retracted into the SPME needle. While this is a concern, the concept of SPME fiber swelling can be used to its advantage. As the fiber absorbs some of the solvent vapor and becomes swelled, it has a greater capacity for analyte absorption and therefore greater analyte response. Although extraction times may be longer, a thicker fiber coating ultimately could allow for more analyte to be absorbed and therefore increase sensitivity.

4.1.2 TV-SPME Optimization

There are many parameters that must be optimized in developing a TV-SPME method, including SPME fiber type, incubation temperature, incubation time, extraction time, desorption time, and sample volume. Using a statistical experimental design is the best way to determine the optimal parameters without performing every possible variable combination, or a “vary one parameter at a time” method.

Response surface methodology (RSM) and central composite design (CCD) are commonly used techniques to optimize parameters (variables) in analytical chemistry.[57-59] RSM uses mathematical and statistical techniques to model and analyze responses which are dependent on many variables with the ultimate goal being to optimize the response.[60] When there are multiple

responses, it becomes important to find the best compromise of the variables so that all responses are optimized.[61] RSM is useful to understand changes in response by adjusting the design variables. RSM generally follows first or second order models. First order models would describe a linear function of independent variables. Second order models contain quadratic and interaction terms and they are used when the first order model surface is curved:[57, 60]

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \sum_{i<j} \beta_{ij} x_i x_j + \in \quad \text{(Equation 4-14)}$$

where y is the response, β_0 is a constant, β_i is the coefficient of the linear term, x_i is the linear variable, β_{ii} is the coefficient of the square term, x_i^2 is the square variable, β_{ij} is the coefficient of the interaction terms, $x_i x_j$ is the interaction variable term, $\in$ is the error in the response, and k is the number of variables. The variable terms are coded on a scale from -1 to +1 to represent the low and high value variables.[60]

In order to get the most efficient approximation of the polynomials, a proper experimental design must be used to collect the data.[61] Many different experimental designs can be used to fit response surfaces and CCD is the most popular design.[60] CCD generally contains 2^k factorial runs, 2k axial runs, and n_c center runs, where k is the number of variables. A CCD for k=2 variables is shown in Figure 4-5. Table 4-1 represents an experimental design using coded variables for k=2 demonstrating four factorial runs, four axial runs, and one center point.

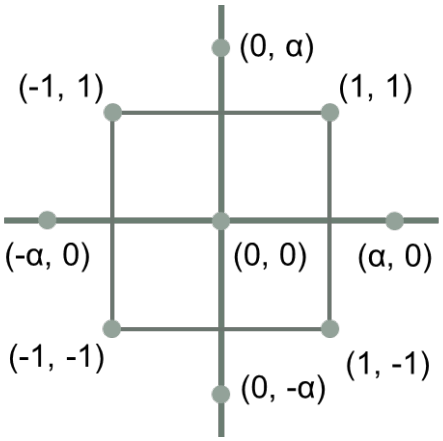

Figure 4-5 Central composite design for 2 variables (figure adapted from Montgomery[60])

Table 4-1 CCD matrix using coded variables for a 2 variable design

Run type	x_1	x_2
Factorial design points	1	1
	1	-1
	-1	1
	-1	-1
Axial points	α	0
	-α	0
	0	α
	0	-α
Center point	0	0

In CCD, two parameters can be chosen which will determine the design for fitting the model: α, which is the distance of the axial points from the center and n_c. The number of center points, n_c, is chosen so as to give enough experimental runs to provide good variance of the predicted response, typically two to five. A CCD design can be rotatable by defining $\alpha=(2^k)^{1/4}$.[60] Rotatability of a design can ensure equal precision in all directions (Figure 4-6).

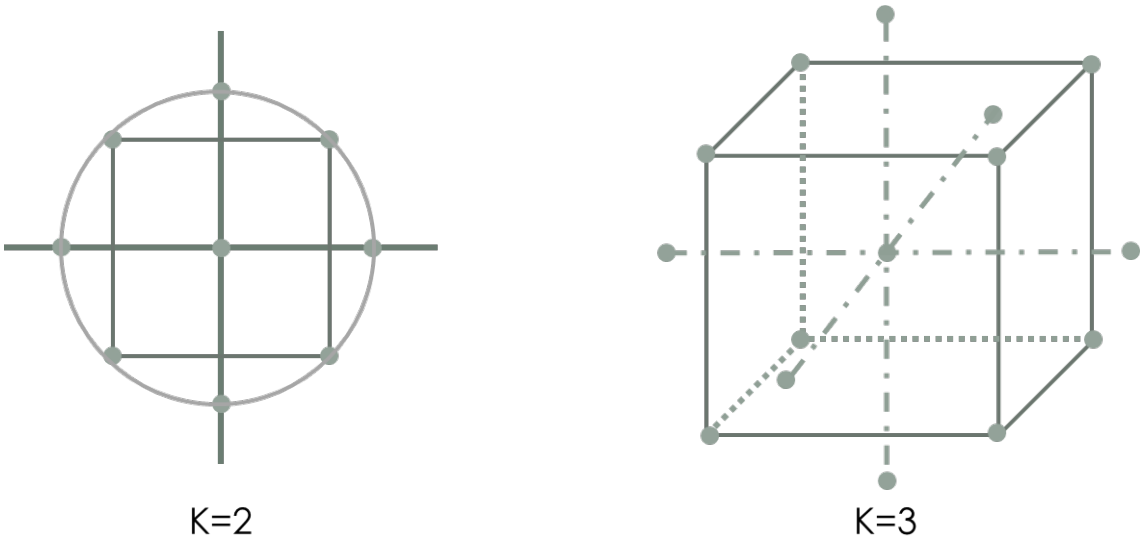

Figure 4-6 Rotatable central composite design for k=2 and k=3 (figure adapted from Montgomery[60])

In many situations, however, the region of interest is cuboidal rather than spherical and a face-centered CCD with α=1 can be used (Figure 4-7).

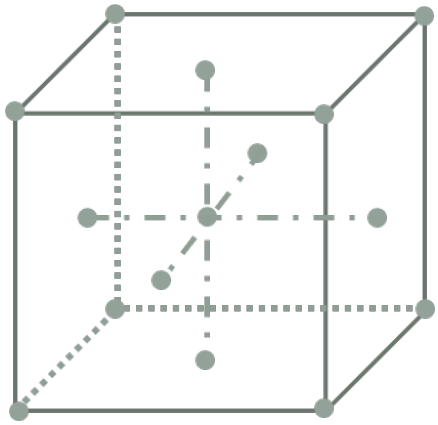

Figure 4-7 Face-centered central composite design for k=3 (figure adapted from Montgomery[60])

4.2 Experimental

4.2.1 Materials

Nicotine (99%), cotinine (98%), quinoline (99%), and sodium hydroxide were purchased from Fisher Scientific. Chloroform and polyacrylate SPME fibers were purchased from Sigma Aldrich.

4.2.2 Theory of TV-SPME

To test the theory that analyte response will increase with increasing volume, but begin to decrease when the sample is no longer being totally vaporized, standards containing differing volumes of 5 ppm of nicotine and cotinine and 2 ppm of quinoline were sampled. Volumes of 130 µL, 150 µL, 170 µL, 190 µL, and 210 µL of the standards were placed into 20 mL headspace SPME vials and analyzed via GC/MS.

4.2.2.1 Instrumental Parameters

Standards of varying volume were analyzed on an Agilent 6890N GC with a 5975 MS. Samples were incubated at 115°C for 5 min. A polydimethylsiloxane/ divinylbenzene (PDMS/DVB) fiber was then exposed inside the SPME vial for 60 min. After sample extraction, the SPME fiber was inserted into the inlet of the GC and desorbed for 1 min. The GC inlet was held at 250°C with a splitless injection of 1 min to correspond to the SPME desorption time. After desorption, the SPME fiber was then conditioned for 2 min at 250°C. Analytes were separated on a DB-

5MS column (30 m x 0.25 mm x 0.25 µm) using helium as the carrier gas at a flow of 1.5 mL/min. The oven program began at 50°C for 1 min, ramped at 20°C/min to 300°C and held for 1.5 min. The transfer line to the MS was held at 280°C. Full scan (m/z 50-550) and selected ion monitoring (SIM) were used for analyte detection (Table 4-2).

Table 4-2 Selected ion monitoring parameters for evaluating sample volume

Analyte	m/z	Dwell Time (ms)
Quinoline	102	100
	129	100
Nicotine	133	100
	161	100
	162	100
Cotinine	98	100
	176	100

4.2.3 SPME Optimization

Minitab 16 was used to set-up the experimental design. A response surface methodology with a central composite design was utilized. For the optimization of nicotine and cotinine, three SPME parameters were optimized: incubation temperature (70-120°), sample volume (80-120 µL), and extraction time (20-60 min). A face-centered CCD (α=1) was created and the parameters for each experimental run are listed in Table 4-3. This experimental design resulted in 20 experiments: eight factorial runs, six axial runs, and six center point runs. The sample volume and incubation temperature combinations used for the experimental design can be compared to the theoretical maximum volume

of chloroform that can be totally vaporized in a 20 mL headspace vial in Figure 4-8.

Table 4-3 Face centered central composite design for optimizing response to nicotine and cotinine

Run Order	Incubation Temperature (°C)	Extraction Time (min.)	Volume (µL)
1	95	40	100
2	95	40	100
3	95	40	80
4	70	40	100
5	70	60	120
6	120	60	80
7	95	40	100
8	95	40	100
9	95	20	100
10	120	20	80
11	70	60	80
12	120	20	120
13	95	40	120
14	70	20	80
15	95	40	100
16	70	20	120
17	95	60	100
18	120	60	120
19	120	40	100
20	95	40	100

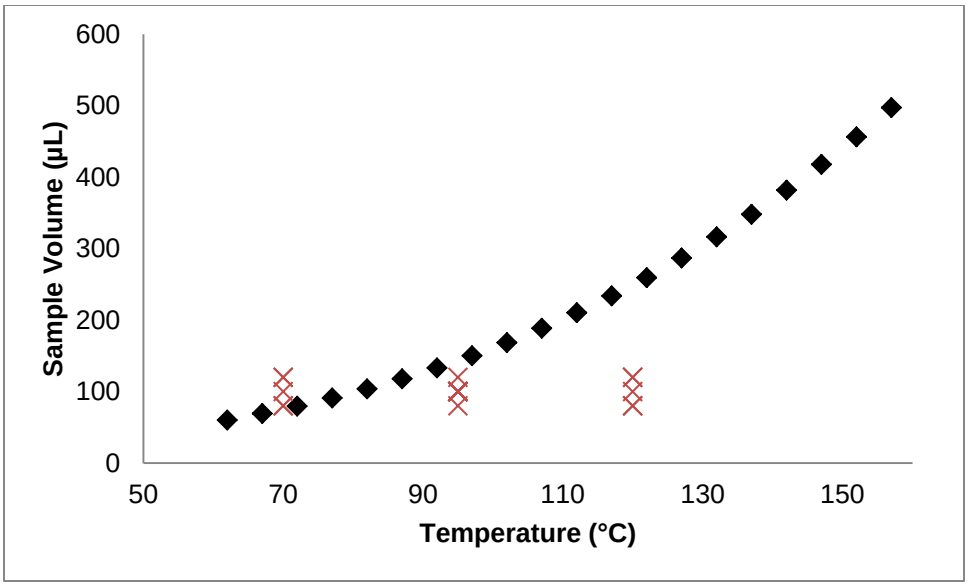

Figure 4-8 Locations of the optimization runs (red x) relative to the maximum volume of chloroform (black diamond) that can be totally vaporized in a 20 mL headspace vial

4.2.3.1 Instrumental Parameters

For the optimization of nicotine and cotinine, a 1 ppm solution containing each analyte in chloroform was analyzed on a Thermo Ultra II GC with a DSQ II mass spectrometer. Varying volumes (determined by the experimental design; see Table 4-3) were placed into 20 mL SPME vials and incubated at varying temperatures (determined by the experimental design; see Table 4-3) for 5 min. A polyacrylate (PA) fiber was then exposed inside the SPME vial for varying times (determined by the experimental design; see Table 4-3). After sample extraction, the SPME fiber was inserted into the inlet of the GC and desorbed for 1 min. The GC inlet was held at 250°C with a splitless injection of 1 min to correspond to the SPME desorption time. After desorption, the SPME fiber was then conditioned for 2 min at 280°C. Analytes were separated on a ZB-5MS

column (60 m x 0.25 mm x 0.25 µm) using hydrogen as the carrier gas at a flow of 2 mL/min and an oven program starting at 100°C for 1 min and ramping at 25°C/min to 300°C and held for 1 min. The transfer line to the MS was held at 280°C. Selected ion monitoring (SIM) was used for analyte detection (Table 4-4).

Table 4-4 Selected ion monitoring parameters for nicotine and cotinine

Analyte	m/z	Dwell Time (ms)
Nicotine	161	35
	162	35
Cotinine	98	10
	176	10

Summed peak areas for nicotine (m/z 161 + 162) and cotinine (m/z 98 + 176) were used to analyze and optimize the experimental design within Minitab 16. The optimized parameters were determined for nicotine and cotinine individually, as well as combined, using the maximum peak area total as the target data response.

4.2.4 Nicotine and Cotinine Calibration Standards

4.2.4.1 Instrumental Parameters

Calibration standards were prepared containing 0.1-2 ppm nicotine and cotinine (with 0.5 ppm quinoline) and analyzed using liquid injection and SPME on an Agilent 6890N GC with a 5975 MS. For liquid injection, 2.0 µL were injected into the GC inlet held at 250°C with a splitless time of 1.0 min. For SPME analysis, 120 µL of each standard was placed in a SPME vial. Samples were

incubated at 95°C for 5 min (as determined from TV-SPME optimization). A polyacrylate (PA) fiber was then exposed inside the SPME vial for 20 min (as determined from TV-SPME optimization). After sample extraction, the SPME fiber was inserted into the inlet of the GC and desorbed for 1 min. The GC inlet was held at 250°C with a splitless injection of 1 min to correspond to the SPME desorption time. After desorption, the SPME fiber was then conditioned for 2 min at 250°C. For both liquid and SPME analysis, analytes were separated on a DB-5MS column (30 m x 0.25 mm x 0.25 µm) using helium as the carrier gas at a flow of 1.5 mL/min. The oven program began at 50°C for 1 min, ramped at 20°C/min to 300°C and held for 1.5 min. The transfer line to the MS was held at 280°C. Full scan (m/z 50-550) and selected ion monitoring (Table 4-5) were used for analyte detection.

Table 4-5 Selected ion monitoring parameters for liquid and SPME injections

Analyte	m/z	Dwell Time (ms)
Quinoline	102	100
	129	100
Nicotine	161	100
	162	100
Cotinine	98	100
	176	100

4.3 Results and Discussion

4.3.1 Theory of TV-SPME

In practice, if the volume of sample placed in a headspace vial exceeds the maximum volume for a given temperature, the sample is no longer being totally vaporized. This will cause the analyte to partition back into the solvent and analyte response will decrease. This can be seen in Figures 4-9, 4-10, and 4-11 where nicotine, cotinine, and quinoline analyte response are plotted as a function of volume. According to Equation 4 10, the theoretical maximum volume of chloroform that can be totally vaporized in a 20 mL headspace vial incubated at 115°C would be 224 µL. However, the signal for all three analytes begins decreasing at volumes larger than 170-190 µL. Clearly, there are several additional factors which ultimately dictate the analyte response. As stated previously, the amount of analyte that will absorb onto the SPME fiber will depend on the distribution coefficient, fiber film thickness, and the concentration of analyte in the sample. As sample volume increases, the amount of vapor increases. The fiber coating will absorb the organic solvent and the chemical changes of the two phases will change the distribution coefficient as well as the film thickness of the fiber (see below). Additionally, for this experiment, the concentration of analyte in the liquid sample was the same, but since the volume of extract in the vial was increased, the amount of analyte in the vapor phase also increased.

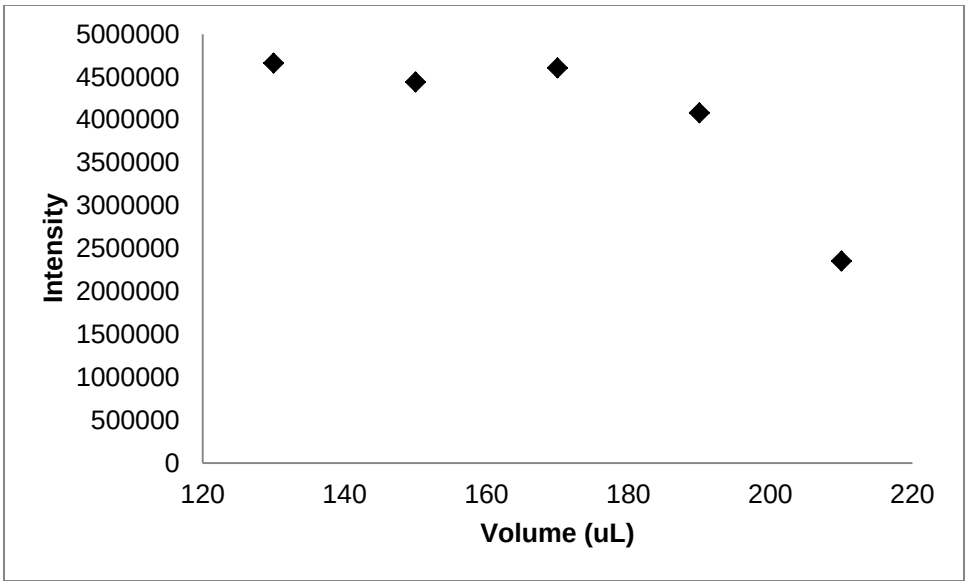

Figure 4-9 Nicotine (5 ppm; m/z 133) signal as a function of sample volume

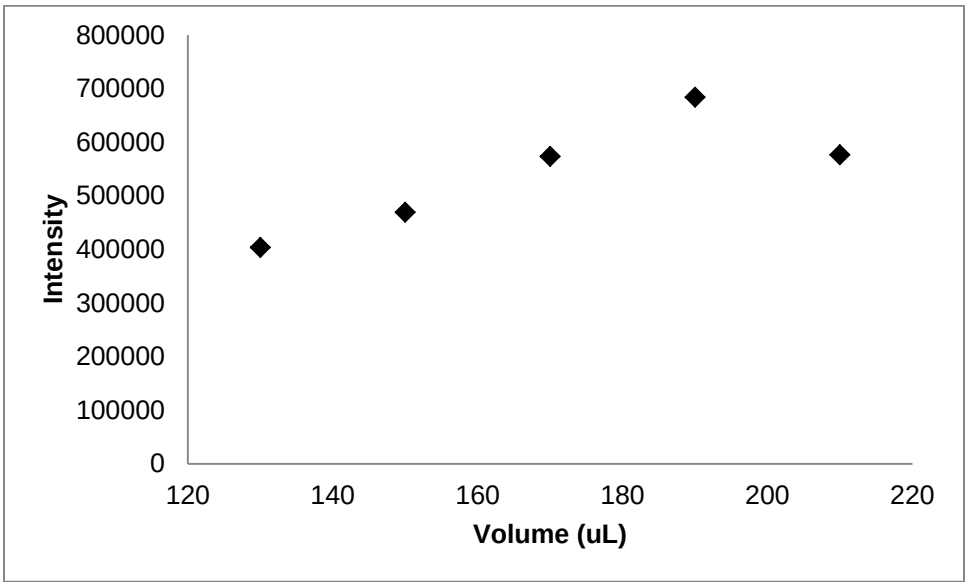

Figure 4-10 Cotinine (5 ppm; m/z 176) signal as a function of sample volume

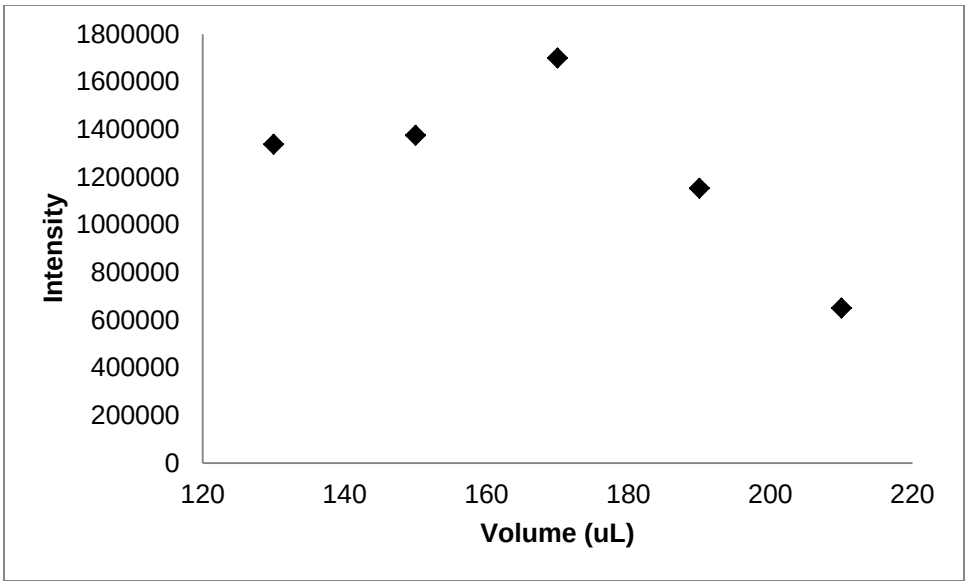

Figure 4-11 Quinoline (2 ppm; m/z 102) signal as a function of sample volume

4.3.2 SPME Optimization

To determine the optimal parameters for TV-SPME, an experimental design and response surface methodology were used to optimize sample volume, incubation temperature, and extraction time. Contour plots can be used to visualize and determine the interactions between two parameters while holding all other parameters constant. For the optimization of nicotine and cotinine, the contour plots of volume vs. incubation temperature can be seen in Figure 4-12 and Figure 4-13, respectively. The contour plot for nicotine shows that there is higher analyte response with higher sample volumes and lower incubation temperatures. The contour plot for cotinine also shows increased analyte response with higher sample volumes, but with higher incubation temperatures.

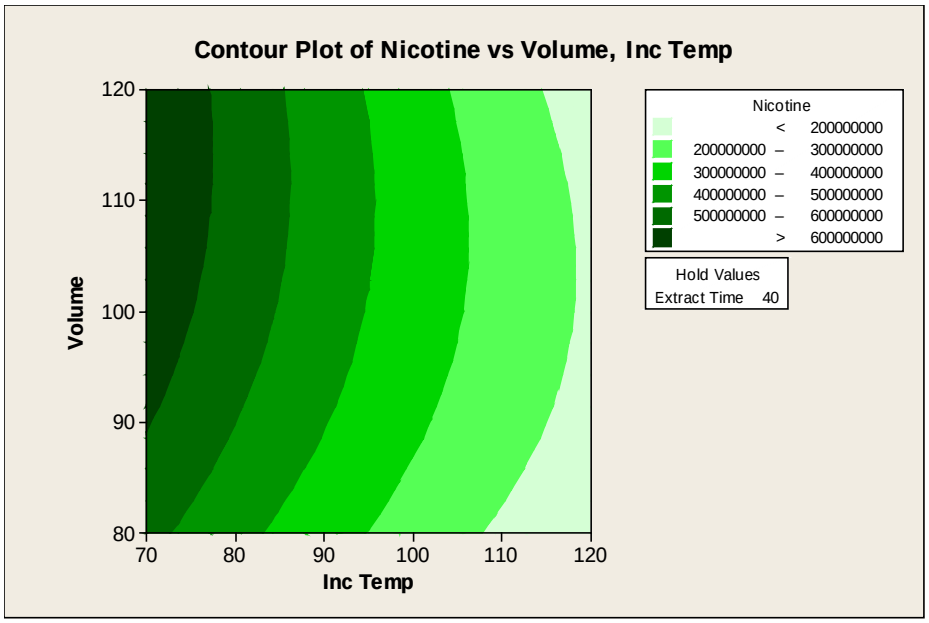

Figure 4-12 Contour plot of volume vs. temperature with extraction time held constant at 40 min. for nicotine

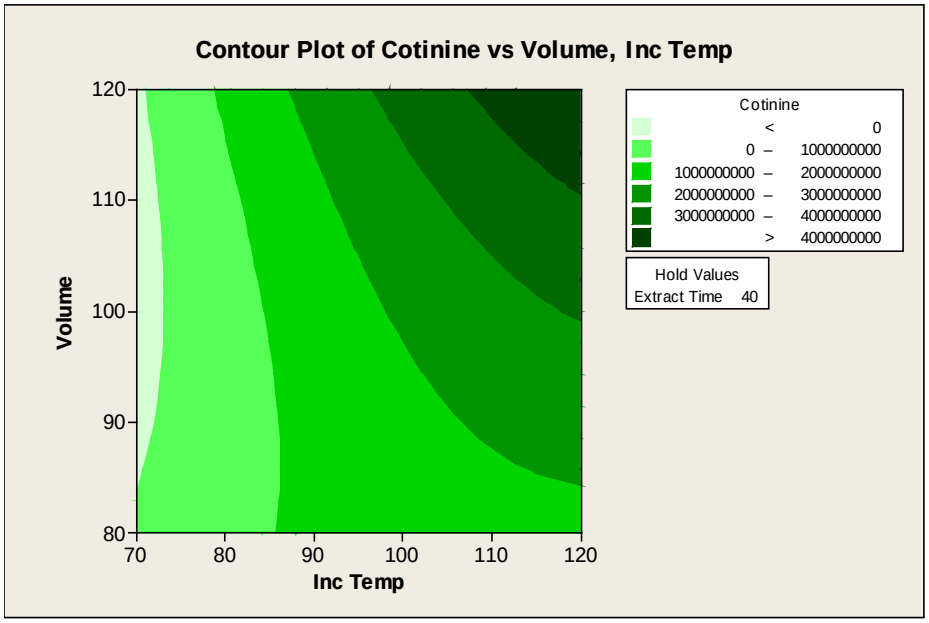

Figure 4-13 Contour plot of volume vs. temperature with extraction time held constant at 40 min. for cotinine

Response surface methodology can be used to describe the predicted analyte response for each response (analyte) depending on the various parameters (described by (Equation 4-14). The estimated coefficients for each variable are listed in Table 4-6. R^2 values for the regression equations are also calculated and demonstrate how well the estimated model fits the data.[61] In this case, an R^2 of 0.91 was determined for nicotine and an R^2 of 0.87 was determined for cotinine.

Table 4-6 Coefficients to describe the response model using coded units

		Coefficient	
Term	Variable	Nicotine	Cotinine
Incubation Temperature	x_t	-2.36 x10^8	1.69 x10^9
Extraction Time	x_e	4.30 x10^7	7.39 x10^8
Volume	x_v	4.80 x10^7	7.36 x10^8
Incubation Temperature^2	x_t^2	2.70 x10^7	-4.15 x10^8
Extraction Time^2	x_e^2	-2.25 x10^7	1.98 x10^8
Volume^2	x_v^2	-4.99 x10^7	3.07 x10^8
Temperature*Time	$x_t x_e$	-3.13 x10^7	7.45 x10^8
Temp*Volume	$x_t x_v$	-3.25 x10^7	8.68 x10^8
Time*Volume	$x_e x_v$	2.47 x10^7	8.49 x10^8
Constant		3.96 x10^8	1.79 x10^9

The relative impact of the three parameters was examined. Because the coefficient for extraction time (x_e) and the coefficient for the squared extraction time term (x_e^2) have the lowest absolute value, it can be concluded that extraction time is not as important as the other parameters. Based on the regression, an increase in extraction time from 20 min. to 60 min. would only increase analyte response by ~25% when using the parameters coded as zero for volume and temperature. Additionally, the F values calculated for the terms containing extraction time were lowest for the linear, square and interaction terms. Therefore, an extraction time of 20 min. was used because the additional time required for higher analyte response was not worth the extra time.

The optimal values for incubation temperature were different for nicotine and cotinine. The response optimizer calculated 70°C as the best temperature for nicotine and calculated 120°C as the best temperature for cotinine. The calculated optimum volume was 111 µL and 120 µL for nicotine and cotinine, respectively. In addition to providing the optimal values for the variables, the response optimizer calculates a desirability value. The desirability indicates how desirable the calculated parameters are in ensuring optimum response. When the response optimizer was used to optimize nicotine response only, the desirability was calculated to be 0.999. For cotinine only, the desirability was calculated to be 0.856.

Optimizing for multiple responses can become complicated because a compromise must be made. In this case, the optimized values for both nicotine and cotinine response were determined to be 96°C and 120 µL with a composite

desirability of 0.551. Although the composite desirability is low, this is the best compromise to ensure acceptable response for nicotine and cotinine. Table 4-7 summarizes the results from the RSM optimization.

Table 4-7 Optimum value for parameters, desirability, and R^2 for the optimization of nicotine and cotinine

		Optimum			Desirability			R^2	
Parameter	Range	nicotine	cotinine	both	nicotine	cotinine	both	nicotine	cotinine
Temp (°C)	70-120	70	120	96	0.999	0.856	0.551	0.91	0.87
Volume (µL)	80-120	111	120	120					

4.3.3 Comparison of Liquid Injection and SPME

Calibration curves were constructed for nicotine and cotinine after analysis by GC/MS using liquid injection and SPME injection. Figures 4-14, 4-15, 4-16, and 4-17 show the calibration curves for nicotine by liquid injection, cotinine by liquid injection, nicotine by SPME, and cotinine by SPME, respectively. Although all four calibration curves have non-linear response below 1 ppm, there is linearity from 1-2 ppm. Slopes and R^2 values were calculated for all four calibrations in the 3-point concentration range of 1-2 ppm (Table 4-8). The slopes of the 3-point calibration curves were much greater for both nicotine and cotinine using SPME; demonstrating that SPME is more sensitive. Signal-to-noise was

calculated for the 2.0 ppm standard for the summed ions of 161 + 162 for nicotine and 98 + 176 for cotinine. In addition to the analytical sensitivity, SPME is preferred over liquid injection due to its increased fiber selectivity and its ability to pre-concentrate analytes without having to filter or perform extensive clean-up steps prior to analysis.

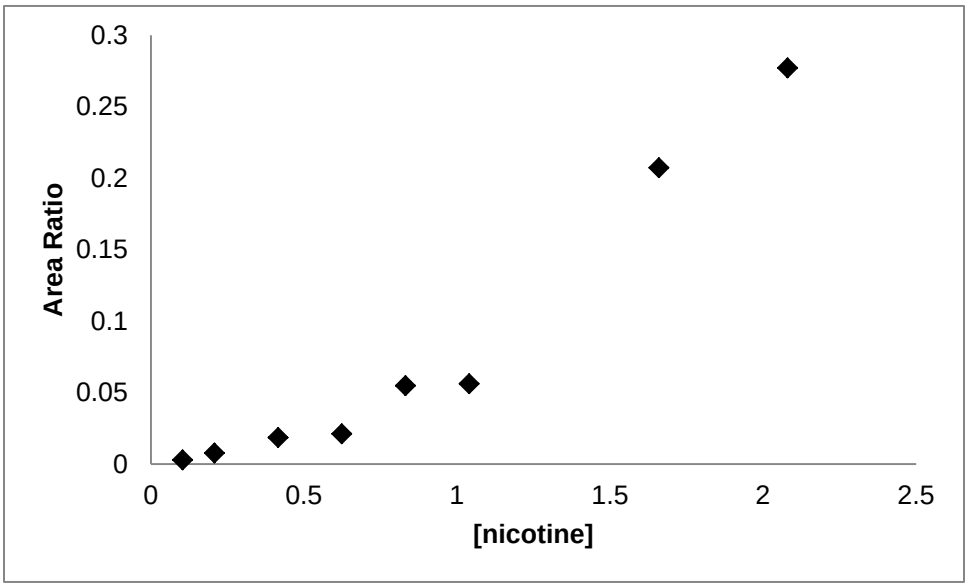

Figure 4-14 Nicotine calibration curve from liquid injection

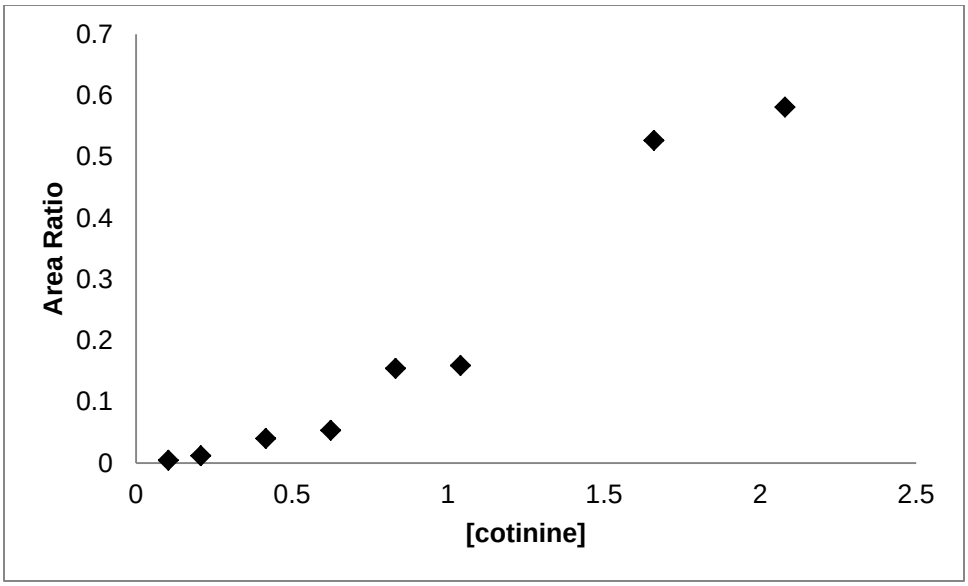

Figure 4-15 Cotinine calibration curve from liquid injection

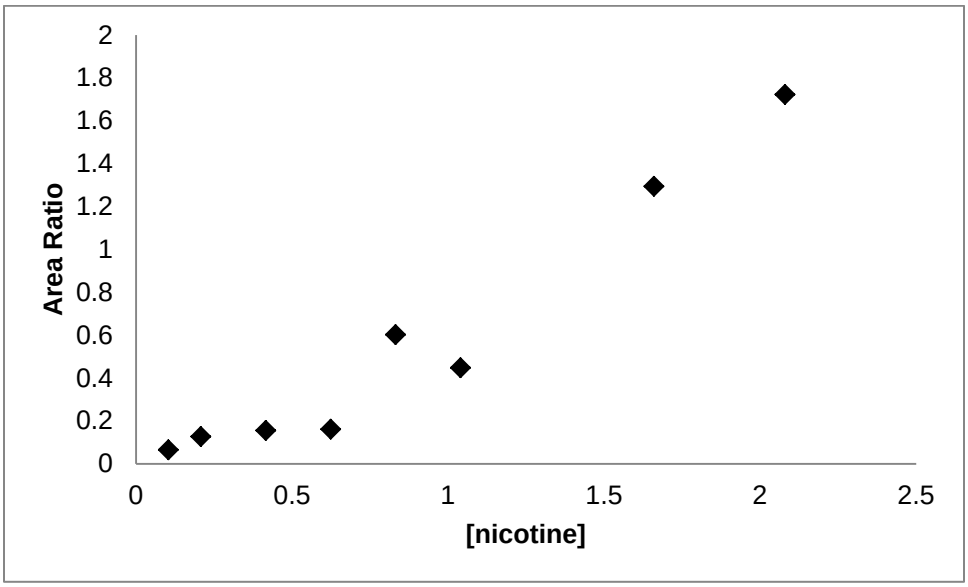

Figure 4-16 Nicotine calibration curve from SPME

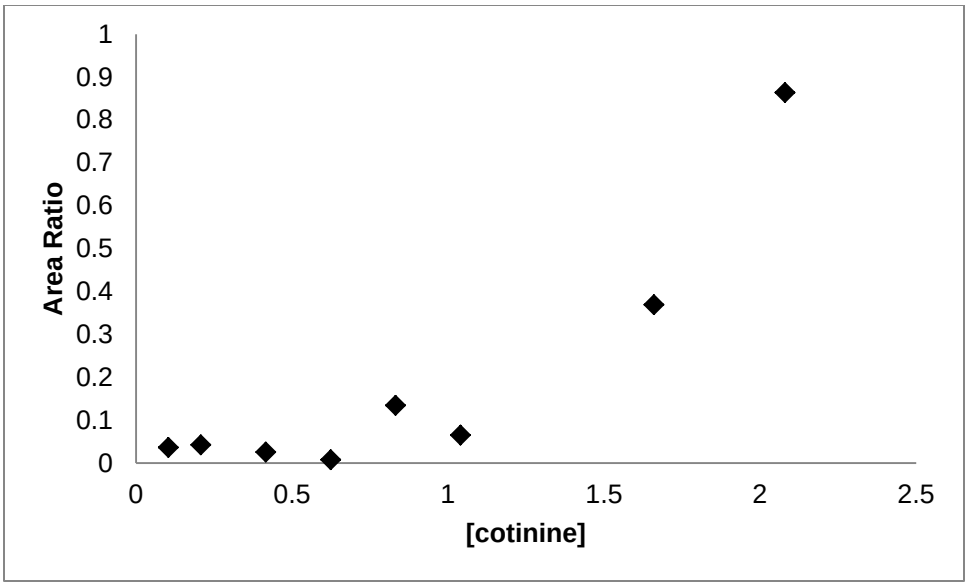

Figure 4-17 Cotinine calibration curve from SPME

Table 4-8 Analytical parameters for nicotine and cotinine by liquid and SPME injection methods

Injection Method	Nicotine			Cotinine		
	Slope	R^2	S/N	Slope	R^2	S/N
Liquid	0.2148	0.9903	3528	0.4198	0.9160	14863
SPME	1.2361	0.9942	6866	0.7472	0.9401	17486

4.4 Conclusions

A TV-SPME method was theorized, developed, and optimized for the analysis of nicotine and cotinine in chloroform. In this work, sample volume, incubation temperature, and extraction time of the TV-SPME method were optimized for the detection of nicotine and cotinine. It was determined that extraction time was the least important factor compared to sample volume and incubation temperature, therefore an extraction time of 20 min was deemed

appropriate. A compromise in the response of nicotine and cotinine was made in order to find the optimal sample volume (120 µL) and incubation temperature (95°C). Additionally, sensitivity was compared between liquid injection and SPME, and based on the slopes of the calibration curves, SPME is more sensitive.

CHAPTER 5. TOTAL VAPORIZATION-SOLID PHASE MICROEXTRACTION (TV-SPME) OF NICOTINE AND COTININE IN HUMAN HAIR

5.1 Introduction

The use of and exposure to tobacco products has been monitored through the analysis of various biological specimens such as blood, urine, saliva, and hair.[62-76] Evaluation of tobacco exposure is difficult because the half-life of nicotine is ~2 hours and the half-life of nicotine's metabolite, cotinine, is ~16 hours.[62] Although blood, urine, and saliva can give an excellent evaluation of current or recent tobacco exposure, hair is a better alternative for determining long-term exposure. Hair is also a viable biological matrix because it can be easily collected with minimal pain and inconvenience to the subject and can be easily stored without deterioration of the hair or loss of analytes.[74, 77]

Hair has been used as a matrix for many applications in regards to forensic science and toxicology.[77, 78] Just as drugs and their metabolites can be detected in blood and urine, drugs can also be found in hair. Drugs and metabolites are incorporated into hair through the bloodstream, sweat, and sebum. One of the major advantages of using hair over matrices such as blood and urine is that a toxicological time profile can be constructed. As hair grows ~1 cm/month, a chronological profile can be used to determine when someone

was using a drug based on a toxicological analysis of the hair.[62, 74, 77] This can be useful in drug compliance testing and to determine previous drug use despite current usage. It can also be of particular use in tobacco cessation programs. In addition, passive and active exposure of drugs can be determined by comparing compounds present on the outside of the hair to what is present inside the matrix of the hair.[70]

There are many different sources of error that must be considered when conducting analyses of hair.[77] The hair matrix is not homogeneous and therefore collection and pre-treatment of hair samples is of great importance. Additionally, the degree to which a drug is incorporated into the hair will depend on the chemical properties of the drug, the binding affinity of the drug to hair components, the metabolism of the drug, and cosmetic modifications to the hair. Cosmetic products and hair treatments such as shampoos, hair dyes, and hair perms can damage the keratin structure of the hair or make modifications to the hair which make drug detection complicated.[74, 79] Additionally, pigmentation of hair will have an effect on the binding of nicotine and cotinine to the hair. Melanin binds to nicotine and cotinine, and therefore, darker hair will bind more nicotine and cotinine than lighter hair will.[62, 80]

Solid phase microextraction (SPME) has been used to analyze nicotine in tobacco products,[23, 81] tobacco smoke,[82, 83] and urine,[84] but has not been used as extensively for detecting nicotine and cotinine in human hair.[73, 85] Current methods not using SPME for determining tobacco exposure from hair require large sample sizes and extensive extraction procedures. This can be costly and

very time consuming, and therefore SPME is an excellent alternative sampling technique. Two papers have reported using headspace SPME for the detection of nicotine from human hair.[73, 85] Both studies used a polyacrylate (PA) SPME fiber to detect nicotine, but neither successfully detected cotinine, with one paper citing that cotinine must be too hydrophilic for headspace SPME analysis.[73, 85] The previous chapter of this dissertation detailed the development and optimization of a total vaporization SPME (TV-SPME) method for quantitating nicotine and cotinine. This technique was then applied to analyze nicotine and cotinine in chloroform extracts of alkaline digests of human head hair.

5.2 Experimental

5.2.1 Hair Collection

Hair samples were collected from anonymous volunteers on the IUPUI campus as approved by the Human Subjects Internal Review Board (IRB study number 1301010349). Subjects were asked to complete a questionnaire (Figure 5-1) and consent form prior to hair collection. Loose hair was collected by having the subject comb through their hair and the loose hair was placed in a small manila envelope. A small tuft of hair was then cut close to the scalp in an unobtrusive location on the back of the head and placed into another manila envelope. Both manila envelopes for each subject were then stored in a plastic zipped bag.

Hair Volunteer Questionnaire

Age: _____ Subject #_____

Gender:

☐ Male ☐ Female

Ethnicity:

☐ Hispanic ☐ non-Hispanic

Race (check all that apply):

☐ White ☐ Black or African American

☐ Asian ☐ Native Hawaiian or Pacific Islander

☐ Other; please specify_____

Tobacco Usage:

☐ Never used tobacco

Are you exposed to tobacco smoke? ☐ Yes ☐ No

If yes, how often and for how long are you exposed to smoke? _____

☐ Quit using tobacco

What kind of tobacco? (check all that apply)

☐ cigarettes ☐ smokeless tobacco ☐ other _____

When did you start using tobacco? _____

How often did you use tobacco? _____ per day week month

When did you quit using tobacco? _____

☐ Currently use tobacco

What kind of tobacco? (check all that apply)

☐ cigarettes ☐ smokeless tobacco ☐ other _____

When did you start using tobacco? _____

How often do you use tobacco? _____ per day week month

Figure 5-1 Questionnaire filled out by hair volunteers

5.2.2 Hair Digestion and Extraction

Hair was cut into small pieces ~2 cm in length and 25 mg (or the complete sample in cases where 25 mg was not available) was weighed into sample vials. Replicates were prepared when enough hair was available. Hair was then washed by adding 2 mL chloroform to the vial and vortexing the sample for ~10 sec. The chloroform wash was then removed via disposable pipet and the hair was dried in an oven at ~85°C for ~1 hr. Hair was digested by sonication in 5 mL 2M NaOH for 80 min. Hair was then vortexed to ensure all hair was digested. The digested hair was then extracted by adding 500 µL chloroform (containing

0.5 ppm diphenylamine) to the digest, vortexing for ~10 sec, and allowing the phases to separate. The organic layer was separated and placed into a new sample vial. Samples were prepared for SPME analysis by placing 120 µL of the chloroform extract into 20 mL SPME vials (volume determined from TV-SPME optimization in previous chapter).

Recovery of nicotine and cotinine was also evaluated. Three recovery samples were made by adding 500 µL of a standard containing 3 ppm of nicotine and cotinine (with 0.5 ppm diphenylamine) in chloroform to 5 mL 2M NaOH. The samples were then vortexed for ~10 sec and the organic layer was removed. Recovery samples were then prepared for SPME analysis by placing 120 µL of the chloroform extract into 20 mL SPME vials.

5.2.2.1 GC/MS Parameters

Hair extracts, calibration standards (0.5-5 ppm nicotine and cotinine with 0.5 ppm diphenylamine), and recovery standards were analyzed on an Agilent 6890N GC with a 5975 MS. Samples were incubated at 95°C for 5 min (as determined from TV-SPME optimization in the previous chapter). A polyacrylate (PA) fiber was conditioned for 2 min at 280°C prior to sample extraction. The fiber was then exposed inside the SPME vial for 20 min (as determined from TV-SPME optimization in the previous chapter). After sample extraction, the SPME fiber was inserted into the inlet of the GC and desorbed for 1 min. The GC inlet was held at 280°C with a splitless injection of 1 min to correspond to the SPME desorption time. After desorption, the SPME fiber was then conditioned for 2 min

at 280°C. Analytes were separated on a DB-5MS column (30 m x 0.25 mm x 0.25 µm) using helium as the carrier gas at a flow of 1.5 mL/min. The oven program began at 50°C for 1 min, ramped at 20°C/min to 300°C and held for 1.5 min. The transfer line to the MS was held at 280°C. Full scan (m/z 50-550) and selected ion monitoring (SIM) were used for analyte detection (Table 5-1). Summed peak areas (161+162 and 98+176 for nicotine and cotinine, respectively) were used for quantitation.

Table 5-1 Selected ion monitoring parameters for nicotine and cotinine (0.88 cycles/sec combined for SIM and scan)

Analyte	m/z	Dwell Time (ms)
Diphenylamine	169	200
Nicotine	161	200
	162	200
Cotinine	98	200
	176	200

5.3 Results and Discussion

Recovery of nicotine and cotinine were evaluated by analyzing chloroform extracts of a standard solution that had been vortexed with 2M NaOH. The results indicate excellent recovery of both analytes from the alkaline matrix, with a recovery of nicotine at 96% and a recovery of cotinine at 102%.

Prior to the analysis of the hair samples collected in the study, bulk hair samples were used to test the extraction technique and TV-SPME analysis. Bulk hair was collected from a known female smoker and a known male non-smoker.

Hair was subjected to the extraction procedures described in the experimental section. Figure 5-2 shows the full scan and SIM chromatograms for the female smoker. SIM was used to quantitate nicotine and cotinine and there were no interferences with these peaks from the control. Many of the peaks in the hair extract chromatograms were identified as siloxanes and were present in the control samples and blanks. Siloxanes could arise from the GC column, the inlet septum, vial septum, and/or from the SPME fiber. In addition to nicotine and cotinine, 1,4-benzenediamine, phenacetin, and homosalate were identified by mass spectral library search. 1,4-Benzenediamine is used as a dye precursor in hair dyes.[86] Phenacetin is known as an analgesic but has also been used as a hydrogen peroxide stabilizer in hair-bleaching products.[87] Homosalate is a UV filter additive in sunscreens and cosmetic products.[88, 89] Nicotine was quantitated and the hair from the female smoker was found to contain 17 ng/mg nicotine. Cotinine was detected, but below the linear range of the calibration curve, and therefore contains less than ~50 ng/mg cotinine in the hair.

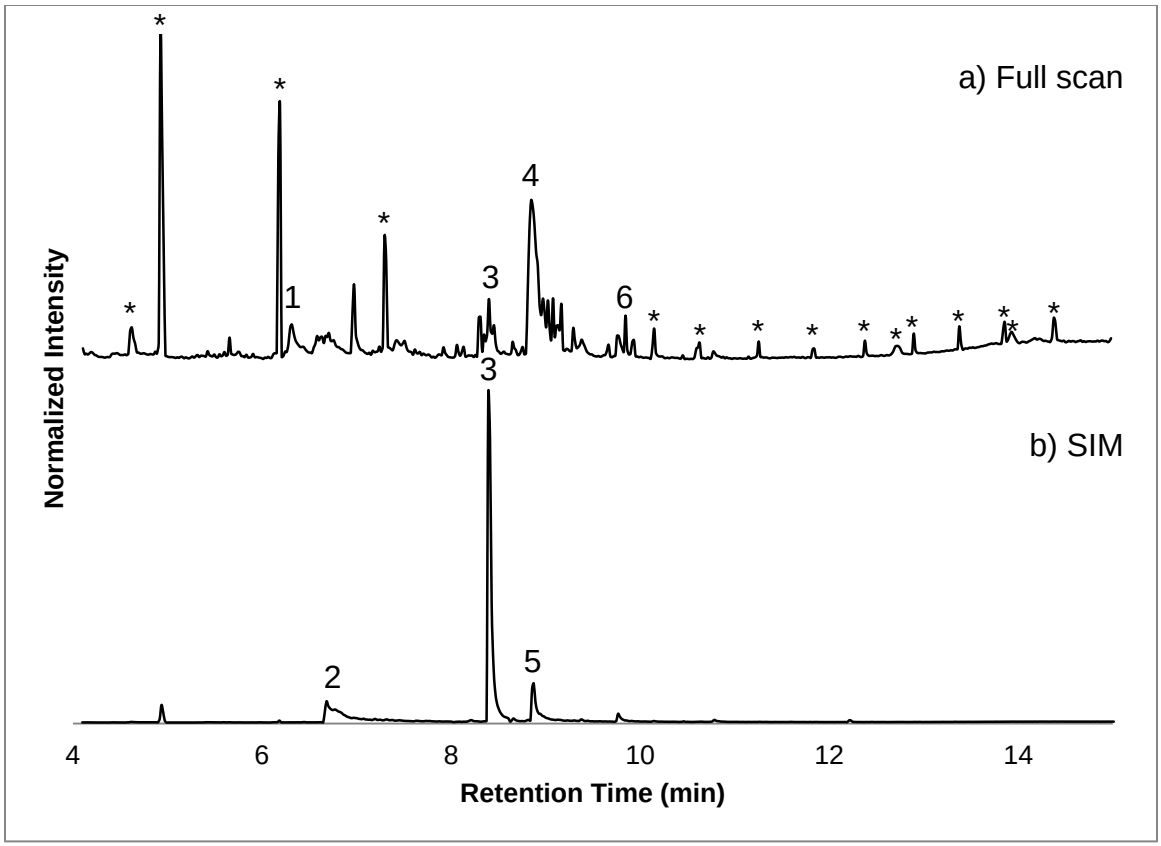

Figure 5-2 Chloroform extract from a NaOH digestion of 25 mg hair from a female smoker. (a) full scan chromatogram; (b) SIM m/z 161, 162, 168, 169, 98, 176. Peak labels: 1- 1,4-benzenediamine 2- nicotine 3- diphenylamine (IS) 4- phenacetin 5- cotinine 6- homosalate *- control.

Figure 5-3 shows the full scan and SIM chromatograms for a male non-smoker. Nicotine and cotinine were not detected in this hair sample, as would be expected. Benzophenone and homosalate (both compounds are found in sunscreens) were detected and identified by mass spectral library search.

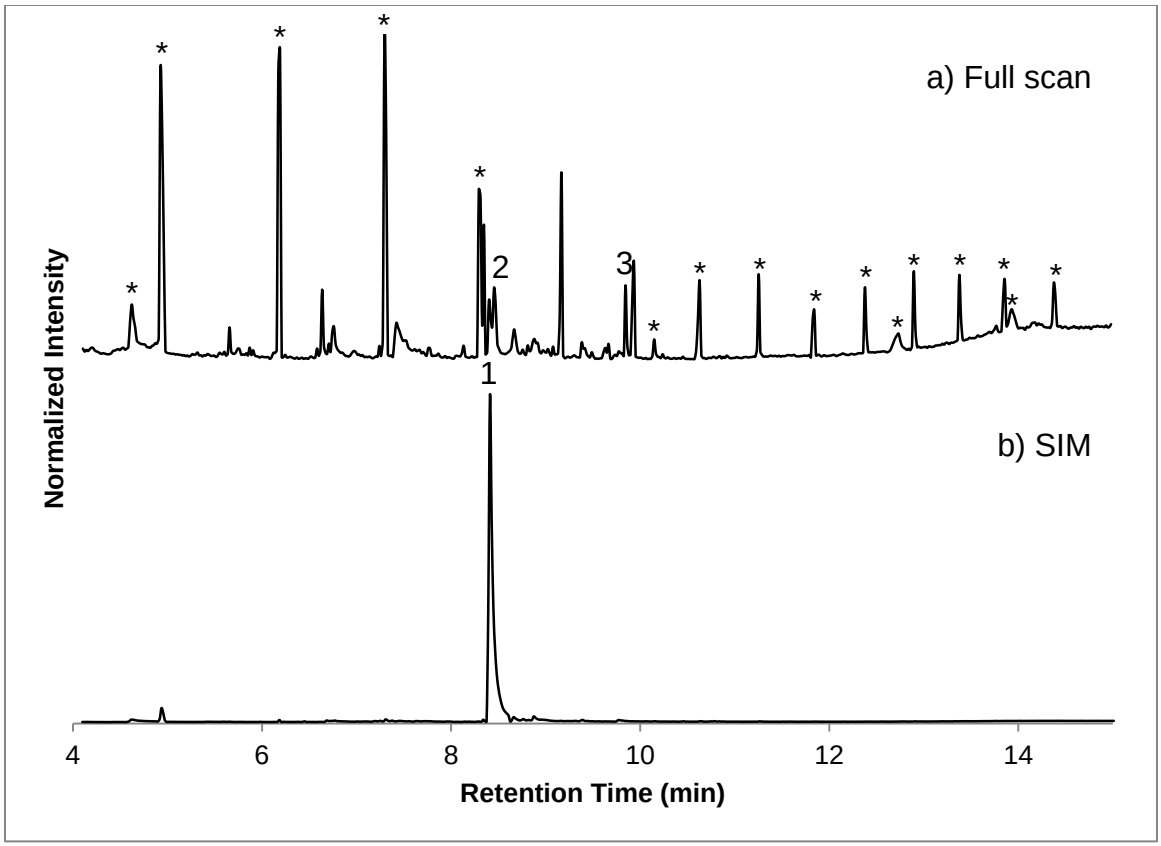

Figure 5-3 Chloroform extract from a NaOH digestion of 25 mg hair from a male non- smoker. (a) full scan chromatogram; (b) SIM m/z 161, 162, 168, 169, 98, 176. Peak labels: 1- diphenylamine (IS) 2- benzophenone 3- homosalate *- control

Hair samples were collected from 47 individuals and were extracted and analyzed as previously described. Of these individuals, only six reported being a current user of tobacco products; four of which were cigarette users (Table 5-2). Nicotine and cotinine were detected in four hair samples from three individuals. The quantitative results can be seen in Table 5-3.

Table 5-2 Responses to the questionnaire from hair volunteers

ID #	Age	Sex	Ethnicity	Race	Tobacco Usage
1	30	F	non-Hispanic	White	4 pks cigarettes/week for 12 years
2	23	F	non-Hispanic	White	never used; not exposed
3	25	F	non-Hispanic	White	never used; not exposed
4	24	M	non-Hispanic	White	3-4 cigars/month for 5 years
5	26	F	non-Hispanic	White	2 pks cigarettes/week for 11 years
6	33	M	non-Hispanic	White	never used; not exposed
7	25	M	non-Hispanic	White	1 cigar/day for 5 years
8	36	F	non-Hispanic	White	never used; not exposed
9	25	M	non-Hispanic	White	never used; not exposed
10	24	F	non-Hispanic	White	never used; not exposed
11	34	F	non-Hispanic	White	3-4 cigarettes/day for 19 years
12	22	M	non-Hispanic	White	never used; not exposed
13	23	F	non-Hispanic	White	never used; not exposed
14	22	F	non-Hispanic	White	never used
15	21	F	non-Hispanic	White	never used; not exposed
16	20	F	non-Hispanic	White	never used; exposed once/month
17	21	F	non-Hispanic	White; Black	quit; 1 cigarette/day for 3 years
18	22	F	non-Hispanic	White	never used; exposed occasionally
19	37	M	non-Hispanic	White; Asian	quit; 5 smokeless tobacco/day from age 17-20
20	39	M	non-Hispanic	White	never used; not exposed
21	33	M	non-Hispanic	White	quit; 1-2 pack/day from age 14-27
22		F	non-Hispanic	White	never used
23	29	M	non-Hispanic	White	quit; 1 pack/month from age 24-26
24	28	M	non-Hispanic	White	never used
25	51	M		White	never used; exposed
26	21	F	non-Hispanic	White	never used
38	42	M	non-Hispanic	White	never used; not exposed
39	18	F	non-Hispanic	White	never used; not exposed
40	21	F	non-Hispanic	Black	never used; exposed occasionally
41	20	F	Hispanic		never used; not exposed
42	21	M	non-Hispanic	White	never used; not exposed
43	57	F	non-Hispanic	White	never used; not exposed
44	43	F	non-Hispanic	White	never used; not exposed
45	20	F	non-Hispanic	White	never used; not exposed
46	20	F	Hispanic		never used
47	19	F	non-Hispanic	White	never used; not exposed
48	19	F	non-Hispanic	White	never used; not exposed
49	25	F		White; Black	1 pk cigarettes/day from age 18-23; E-cig since age 23
53	20	F	non-Hispanic	White	never used; not exposed
54	18	M	non-Hispanic	Black	never used; not exposed
55	19	F	non-Hispanic	White	never used; not exposed
59	22	M	non-Hispanic	White	never used; not exposed
60	24	M		Black	never used
61	19	F	non-Hispanic	White	used once 2-3 weeks ago
62	19	F	non-Hispanic	White	never used; not exposed
65	33	M	non-Hispanic	White	never used; not exposed
68	21	F	non-Hispanic	White	quit; 5 cigarettes/day from age 17-20

Table 5-3 Nicotine and cotinine concentrations from hair extractions of self-reported tobacco users. ND indicates that the analyte was not detected. ID#'s with A and B indicate replicates. One pack of cigarettes contains 20 cigarettes.

ID#	Tobacco usage	Hair mass (mg)	[nicotine] (ng/mg hair)	[cotinine] (ng/mg hair)
1A	4 packs cigarettes/week	25.0	16.0	<60
1B		10.8	30.1	<139
4	3-4 cigars/month	13.0	21.9	<115
5A	2 packs cigarettes/week	21.5	ND	ND
5B		11.1	27.7	<135
7	1 cigar/day	7.1	ND	ND
11	3-4 cigarettes/day	12	ND	ND
49	1 pack cigarettes/day; E-cig	4.8	ND	ND

In addition to nicotine and cotinine, several other compounds were identified in the full scan total ion chromatograms (TICs) by mass spectral library searches (Table 5-4). Squalene, a natural lipid, was identified in almost every hair sample. Compounds known to be found in hair care products and cosmetics were also identified (2-phenoxy-ethanol, 1,4-benzenediamine, galaxolide and aminoacetophenone). Additionally, compounds consistent with cyclic hydrocarbons (ie. cyclododecane, cyclotetradecane, cyclohexadecane) and fatty alcohols (ie. pentadecanol, nonadecanol, hexadecanol) were detected in most of the hair extracts. These compounds were identified by mass spectral library searches, but are not definitively identified because the retention times of the peaks and mass spectra were not compared to known standards.

Table 5-4 Compounds detected in total ion chromatograms of hair extracts

ID #	Compounds detected in TIC
1	squalene, 2-phenoxy-ethanol
2	squalene
3	squalene
4	squalene
5	squalene, 2-methyl-1,4-benzenediamine
7	squalene
8	squalene, 2-phenoxy-ethanol
9	squalene
10	squalene
11	squalene
12	squalene
14	squalene, 2-phenoxy-ethanol
15	squalene, aminoacetophenone, 2-phenoxy-ethanol, galaxolide
16	squalene, 2-methyl-1,4-benzenediamine, 2-phenoxy-ethanol, galaxolide
17	squalene, 2-methyl-1,4-benzenediamine, 2-phenoxy-ethanol, galaxolide
18	squalene
20	squalene
25	squalene
26	squalene
38	squalene
39	squalene, galaxolide
40	squalene
41	squalene, galaxolide
42	squalene
43	squalene
44	galaxolide
45	squalene
46	squalene
47	squalene, 2-phenoxy-ethanol
48	squalene, 2-phenoxy-ethanol, galaxolide
49	squalene, 2-phenoxy-ethanol
53	squalene, 2-phenoxy-ethanol, galaxolide
54	squalene
55	squalene, 2-phenoxy-ethanol
59	squalene
60	squalene
61	squalene, 2-phenoxy-ethanol
62	squalene, 2-phenoxy-ethanol
65	squalene
68	squalene, 2-phenoxy-ethanol, galaxolide

5.4 Conclusions

Hair samples were collected from 47 individuals and the hair was subjected to an alkaline digestion followed by a chloroform extraction and subsequent analysis by TV-SPME GC/MS. Six of the subjects reported tobacco use, and nicotine and cotinine were detected in four hair samples from three subjects. Concentrations of nicotine ranged from 16.0-30.1 ng/mg in hair, which is similar to concentrations reported in hair from several other studies.[63, 68, 69, 71] Even though the level of cotinine could not be accurately quantitated, it was detected in all of the samples which also contained nicotine. Using full scan mode in addition to SIM allows other compounds to be detected in the hair, providing additional information, such as components of hair care products and natural lipids and fatty acids. This work is informative and with more research into the theory and optimization of TV-SPME and improvements to the method, this procedure shows promise for the toxicological analysis of nicotine and cotinine in human hair.

CHAPTER 6. FUTURE DIRECTIONS

6.1 Dissolvable Tobacco

According to Camel's website as of September 1, 2013, dissolvable tobacco products are still being marketed in Colorado and Charlotte, NC.[90] As these products are still being sold in select cities, it is important to continue research into the complexity of the dissolvables. Although many compounds were identified in the chemical characterization, it would be beneficial to quantitate the amount of these compounds in the dissolvables. In addition, tobacco specific nitrosamines and other tobacco alkaloids should be evaluated and quantitated.

Further research is needed in order to evaluate the relationship between dissolvable tobacco and potential health concerns, particularly oral diseases and cancers. An informative but more complex experiment would be to evaluate the compounds present in saliva after a dissolvable product is used; these compounds and their relative concentrations could shed light on potential harm to the oral cavity. Direct immersion SPME has been used to detect drugs of abuse in saliva samples and could easily be adapted for analyzing oral fluids of smokeless tobacco users.[91] Immersion SPME could be used to identify the

compounds present in saliva by directly exposing a SPME fiber to saliva samples taken before, during, and after dissolvable tobacco product use.

Additionally, the pH of the oral cavity (average pH 6.5-7.5) will largely dictate the amount of free-base nicotine available to be absorbed by the user.[92] Saliva has a natural buffering ability to prevent the pH from dropping too low which causes acid erosion. The buffering capacity in the oral cavity is largely dictated by the concentration of bicarbonate, which generally buffers the saliva in a pH range of 6-7.75.[93] The buffering capacity varies from person to person and will also be affected by the use of tobacco products. An informative experiment would be to measure the pH and buffering capacity of oral cavities before, during, and after dissolvable tobacco usage in order to quantitate the amount of free-base nicotine that can penetrate into cell walls of the oral cavity and to understand other possible oral diseases that may occur through use of these products. Nicotine absorption is very rapid, and it would be very important to understand how the dissolvables affect the pH of the oral cavity.

6.2 Humectants in Tobacco Products

A gas chromatographic method with flame ionization detection and mass spectrometry was used to quantitate humectants in various tobacco products. The study utilized an established chromatographic method with the goal to improve the method by using mass spectrometry. The results indicate excellent

correlation between FID and MS detection methods. This study could be further investigated by analyzing a larger set of tobacco samples. This would also benefit the chemometric analysis by having more samples within each tobacco type. Additional quantitative analysis of other tobacco compounds (alkaloids, tobacco-specific nitrosamines, etc.) could be added to the chemometric analysis to try to better separate the groupings within the data. Alkaloids such as nornicotine, anatabine, and anabasine have different levels in different types of tobacco products; nornicotine is highest in cigars and anatabine and anabasine are lowest in oral tobacco products.[62, 94]

6.3 mtDNA Extraction of Human Hair

The chromatographic separation and signal to noise from the PCIA extracts were excellent and can possibly be improved to achieve lower detection limits of the lipids. The lipids in the current project were detected using full scan mass spectrometric detection. The use of selected ion monitoring (SIM) would increase the selectivity of detecting the lipids. Additionally, large volume injections of the extracts would greatly improve the detection of low concentration lipids. Typical GC injection volumes are around 1-2 µL, which allows 1-2 ng of analyte to be injected for a 1 ppm standard solution. Use of programed temperature vaporization (PTV) injection ports allow large volumes of solvent to be injected. A 100 µL injection volume could easily be used; increasing the amount of analyte

by 100-fold in comparison to typical GC injection volumes. At this injection volume, it is speculated that low concentrations of lipids could be detected. Another possible route to explore in order to improve the detection of low concentration lipids is improving the protocol for preparing the hair extracts. Since the entire premise of this project was to take “waste” from an existing mtDNA extraction procedure, the mtDNA protocol must remain the same. In this project, the washes and extracts were pre-concentrated by evaporating to dryness and reconstituting into 300 µL of solvent. A lower volume could be used to reconstitute, therefore increasing the analyte concentration in solution.

Once the analytical method is improved and can accurately detect low levels of integral lipids from a mtDNA extraction of a 2 cm hair strand, additional questions will need to be answered. In particular, can the concentrations in the hair samples be quantitated? If so, it could be possible to determine age, gender, race, and certain diseases based on the concentration of certain analytes in the hair.[95-98] Additionally, chemometric techniques can be used to differentiate hair samples based upon their integral lipid profiles.

6.4 TV-SPME Theory and Optimization

A TV-SPME method was theorized, developed, and optimized for the analysis of nicotine and cotinine in chloroform. Although the method was optimized using response surface methodology, there are many parameters that

can be further optimized and other experiments that can be performed to improve the methods.

The chemistry of a SPME fiber is chosen based on the polarity and volatility of the analytes of interest.[50] For the optimization of nicotine and cotinine, a polyacrylate SPME fiber was used. This fiber seemed suitable for its purpose; however, an interfering compound (identified as 2,4-diisocyanato-1-methylbenzene by mass spectral library search) was eluting at the same retention time as nicotine. This compound does not have mass fragments at m/z 161 or 162, so it did not interfere with the quantitative analysis, but efforts should be made to eliminate this as a possible issue. This compound is a contaminant from the SPME fiber.[99] It was also apparent that the intensity of 2,4-diisocyanato-1-methylbenzene decreased with use of the SPME fiber. This compound was also identified with use of a polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber, but other fibers may not have this interference and should therefore be investigated.

Additional investigations into the theory and applicability of TV-SPME are required before recommending this method for the analysis of volatile and semi-volatile compounds. An experiment to evaluate analyte response while varying sample volume but keeping the net mass of analyte constant would be beneficial to investigate. Generally, the analyte response should remain the same because the amount of analyte vaporized in the vial remains the same. However, the volume of solvent will affect the thickness of the fiber which ultimately dictates

the amount of analyte absorbed by the fiber. The concentration of solvent in the vial will also affect the partition coefficient.

Another major concern with the applicability of TV-SPME is the degradation of SPME fibers. SPME fibers have a finite life-time and the use of abrasive chemicals will inevitably shorten the practical use of the fiber. In TV-SPME, a large volume (>100 µL) is completely vaporized inside the SPME vial. The SPME fiber then absorbs the solvent and swells. Continually forcing a SPME fiber to swell and contract may cause a fiber to break and/or degrade. This hypothesis was briefly investigated by taking microscopic images at various procedural steps of a polyacrylate fiber used to extract 120 µL of chloroform. Figure 6-1 shows a PA fiber prior to fiber conditioning or use. No damage can be seen in these images. After fiber conditioning for one hour at 280°C (time and temperature recommended by the manufacturer), it can be seen that the fiber is beginning to turn brown and become discolored (Figure 6-2). Images were taken of the same fiber after being used for several TV-SPME experiments using 120 µL of a chloroform hair extract (Figure 6-3). The fiber has become dramatically discolored and several cracks in the fiber are visible. Some fiber breakage was even macroscopically observed.

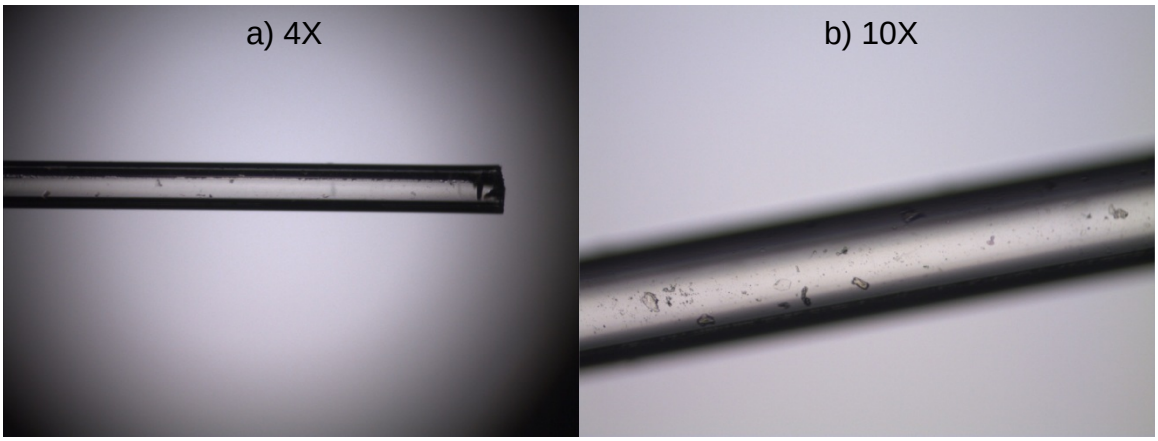

Figure 6-1 Microscopic images of a polyacrylate fiber prior to conditioning or use. a) image taken at 4X b) image taken at 10X

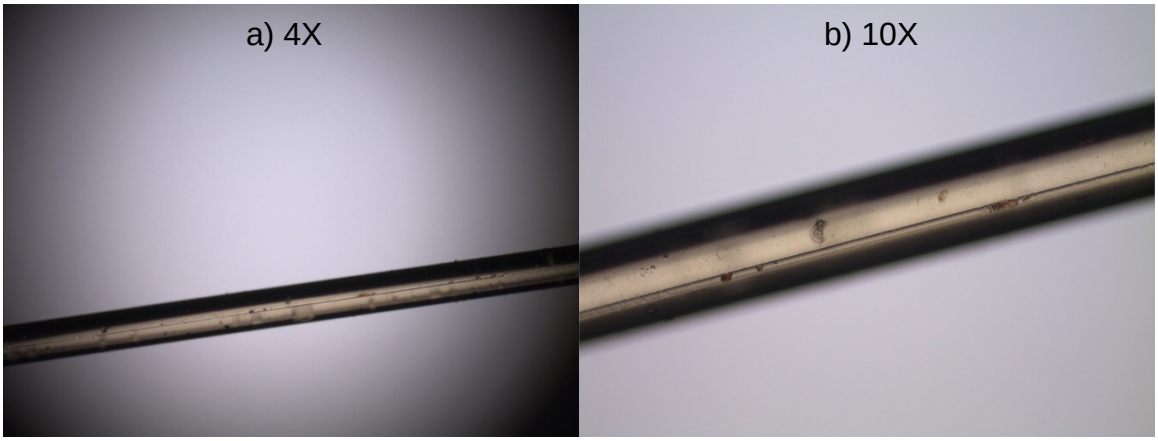

Figure 6-2 Microscopic images of a polyacrylate fiber after conditioning at 280°C for 1 hr. a) image taken at 4X b) image taken at 10X

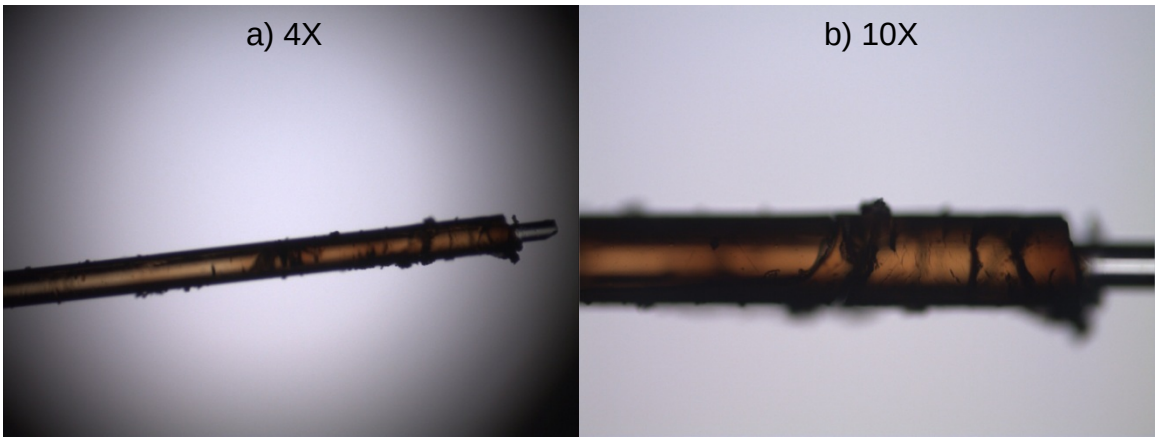

Figure 6-3 Microscopic images of a polyacrylate fiber after ~20 uses of TV-SPME of 120 µL chloroform hair extracts. a) image taken at 4X b) image taken at 10X

These images make it apparent that the lifetime of a SPME fiber when using total vaporization is very limited. Further work should investigate the fiber stability of different fiber chemistries with different organic solvents and with different solvent volumes. For example, a polyethylene glycol (PEG) fiber has been used several times using TV-SPME with 50 µL of dichloromethane with no obvious signs of degradation (results not presented here). (Equation 4-10 can be used to determine the maximal volume of a solvent that will vaporize inside a SPME vial and this should be used for setting up this experiment. An experimental design could be used to set-up and evaluate these experiments.

6.5 TV-SPME of Nicotine and Cotinine in Human Hair

In addition to understanding the theory and applicability of TV-SPME, several suggestions can be made for the analysis of nicotine and cotinine in hair.

One of the major limitations of this study was sample size. When the hair samples were originally collected, they were going to be used with the mtDNA hair extraction procedure, so a small sample size was sufficient. This became problematic for the alkaline digestion/chloroform extraction procedure as it called for 25 mg of hair and many of the samples collected contained much less than this. Also, it would have been beneficial to have duplicate or triplicate samples for all study subjects. Some hair samples were analyzed in replicate when sufficient hair sample was available, but 34 of the 47 subjects did not have enough for replicate analysis.

Efforts should be made to decrease the limit of linearity for both nicotine and cotinine. Of the hair samples where nicotine and cotinine were detected, nicotine was within the linear range of the calibration curve, but cotinine was not. Additionally, the concentration of nicotine was very close to the limit of linearity. Although sample volume, incubation temperature, and extraction time were optimized for the TV-SPME method, the temperature was a compromise between optimizing nicotine and cotinine. Other SPME fiber chemistries may also be more sensitive to nicotine and cotinine and should be investigated.

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APPENDIX

APPENDIX. GC/FID/MS CHROMATOGRAMS OF TOBACCO SAMPLES FOR HUMECTANT ANALYSIS

Table A-1 List of compounds labeled in Appendix A. All compounds were identified by mass spectral library search, except propylene glycol, 1,3-butanediol, glycerol, and triethylene glycol were also confirmed by retention time with a known standard

Peak Label	RT (min)	Compound
1	1.417	Acetic acid
2	2.041	Propanoic acid
3	2.057	Benzaldehyde
4	2.45	Propylene glycol
5	2.871	Menthol
6	3.728	1,3-Butanediol
7	4.064	Methyl salicylate
8	4.306	Ethyl salicylate
9	4.444	Anethole
10	4.846	Benzyl alcohol
11	4.852	Nicotine
12	6.366	Dihydroxyacetone
13	6.846	Sorbic acid
14	7.833	Piperonal
15	7.951	Methyl anthranilate
16	8.951	Glycerol
17	9.239	Triethylene glycol

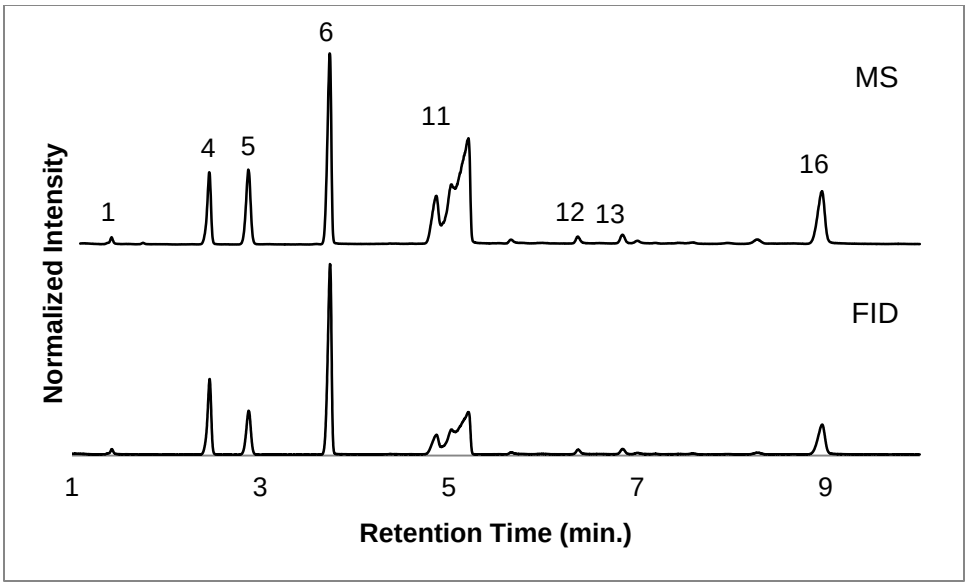

Figure A-1 American Gambler Menthol Flavor RYO

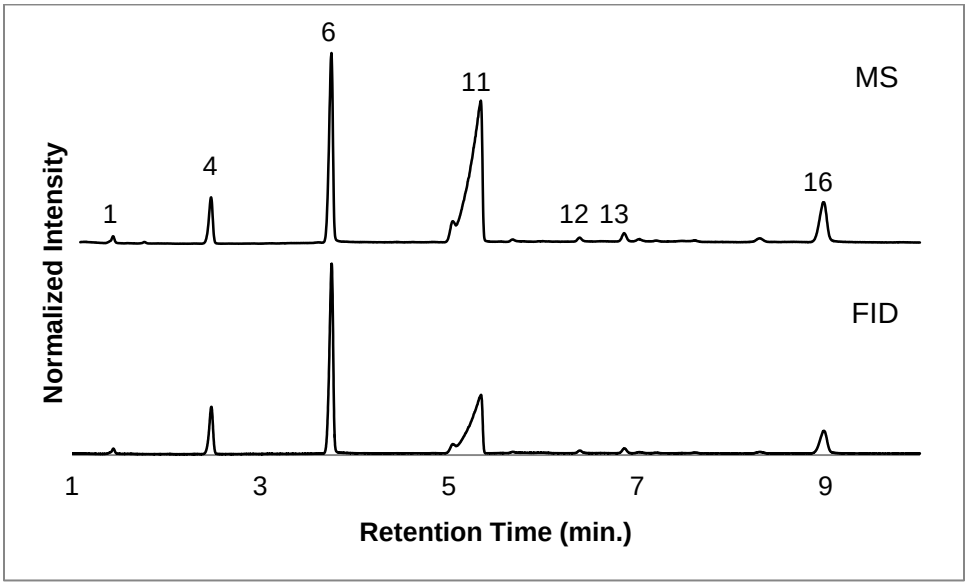

Figure A-2 American Gambler Light Flavor RYO

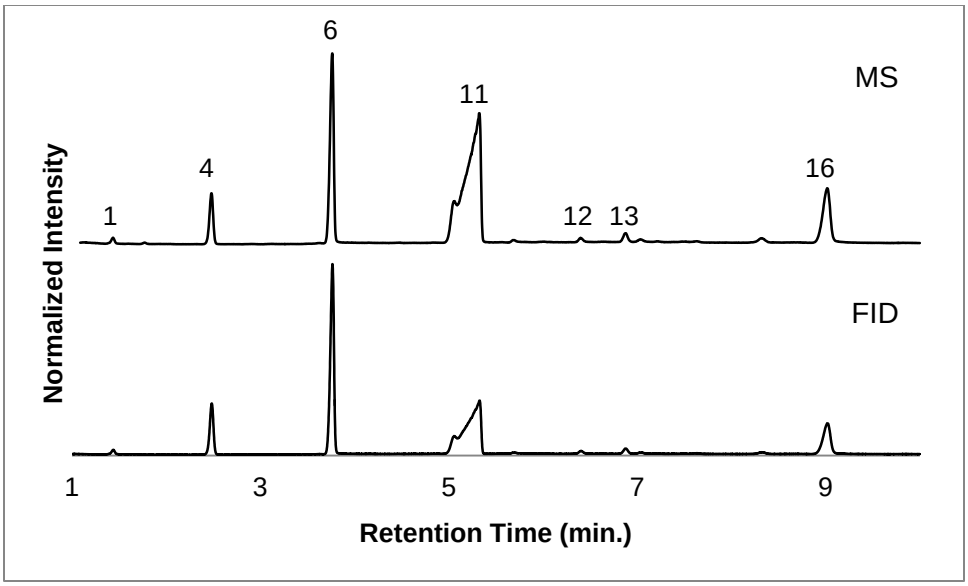

Figure A-3 American Gambler Full Flavor RYO

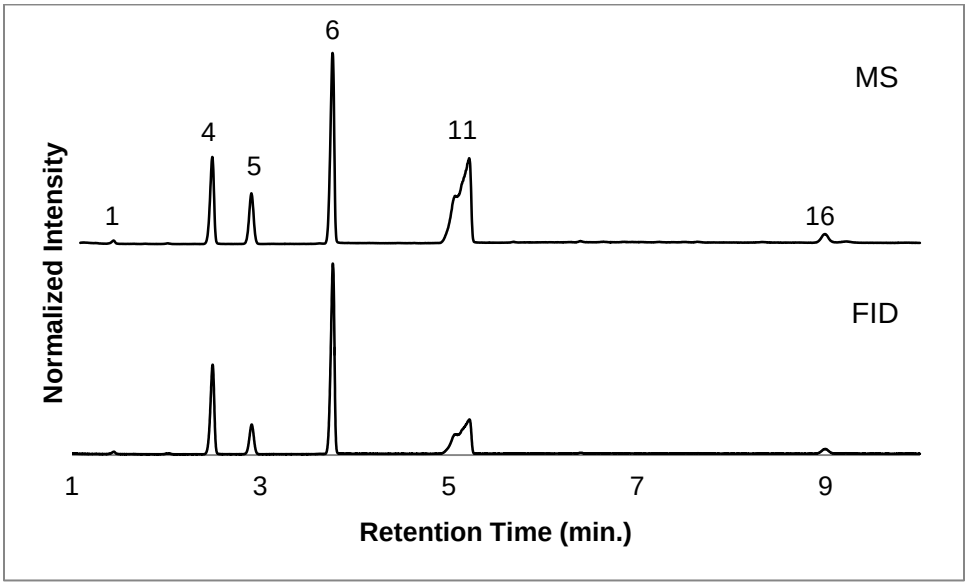

Figure A-4 American Gambler Menthol Flavor 100's Cigars

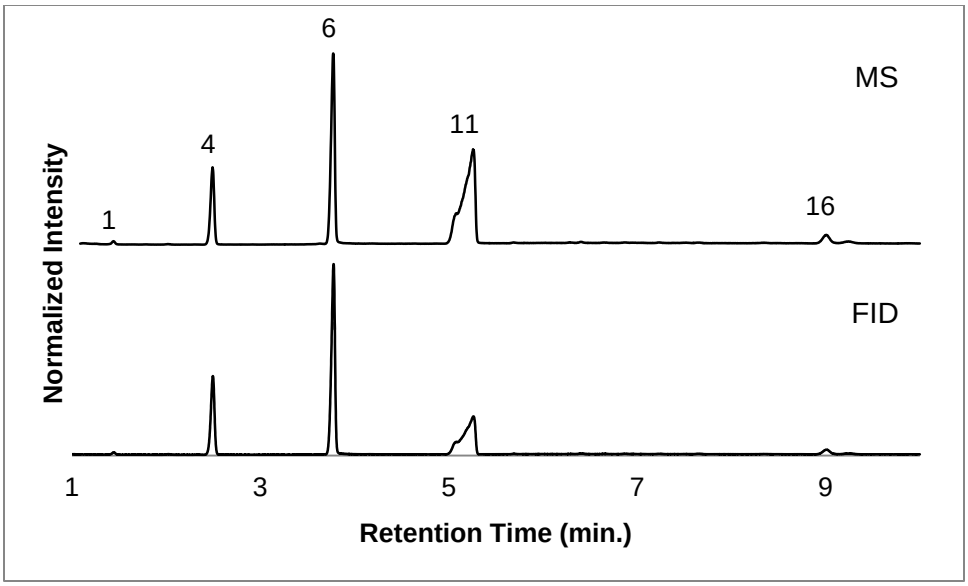

Figure A-5 American Gambler Full Flavor 100's Cigars

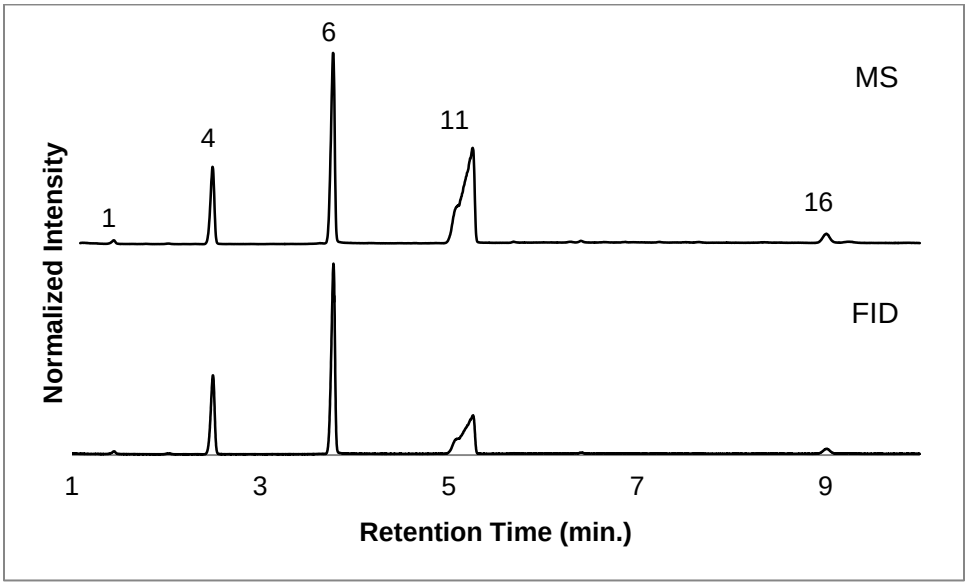

Figure A-6 American Gambler Light Flavor 100's Cigars

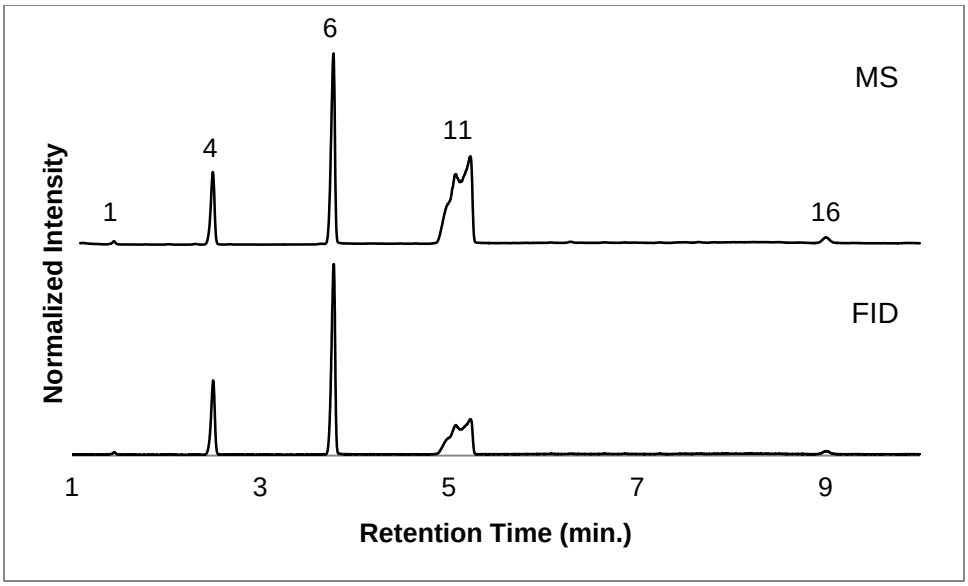

Figure A-7 Hav-A-Tampa Sweet Filter Tipped Cigars

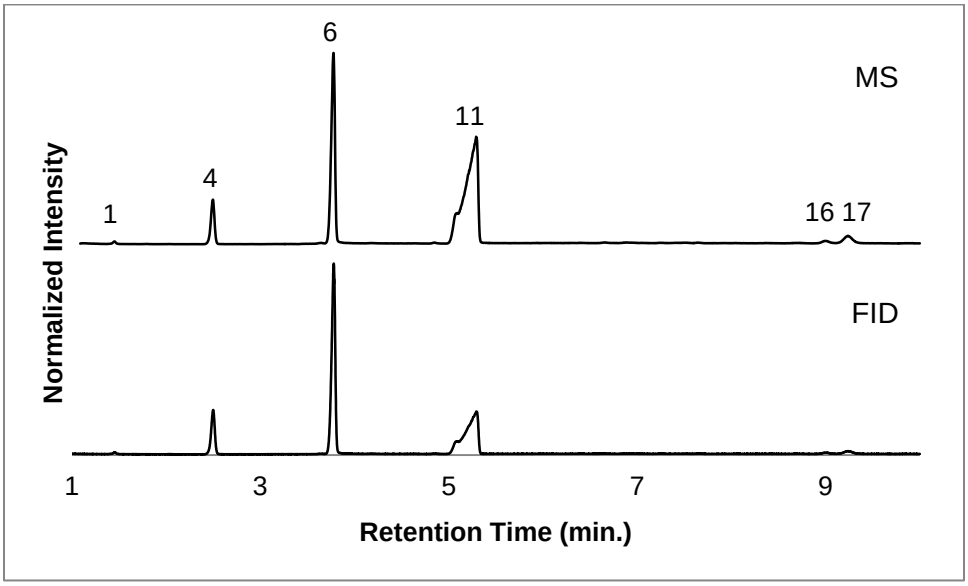

Figure A-8 Hav-A-Tampa Jewels Sweet Cigars

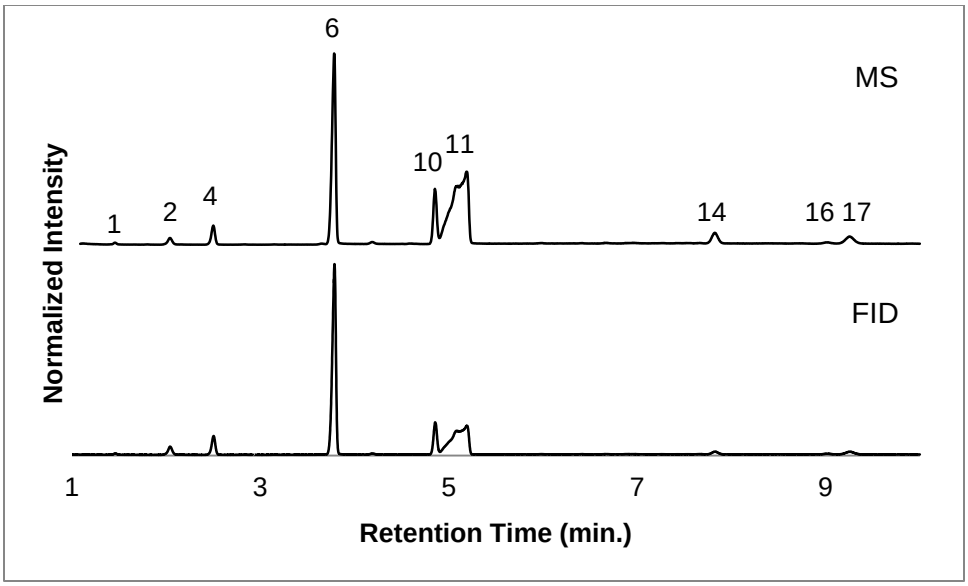

Figure A-9 Hav-A-Tampa Jewels Vanilla Cigars

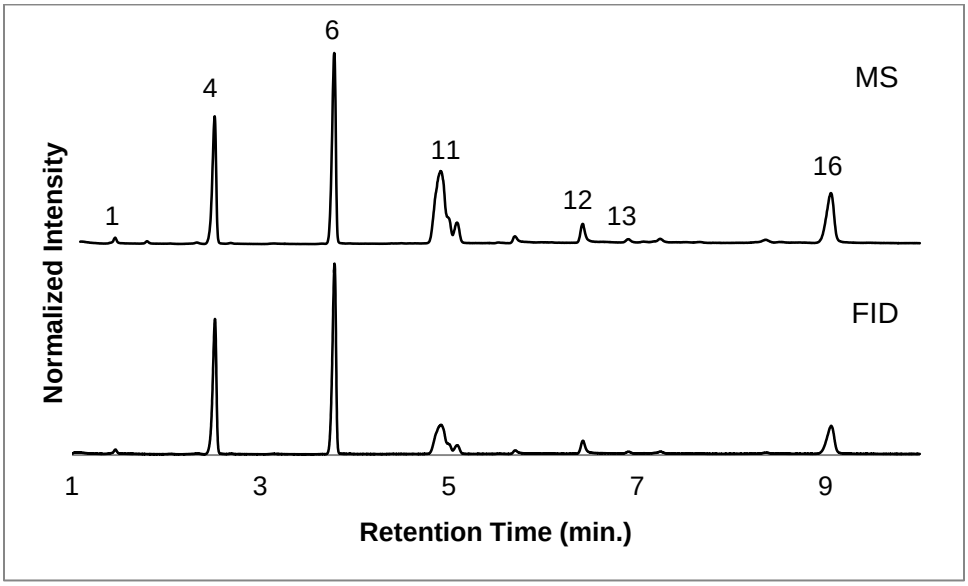

Figure A-10 Black & Mild Fast Break Apple Cigars

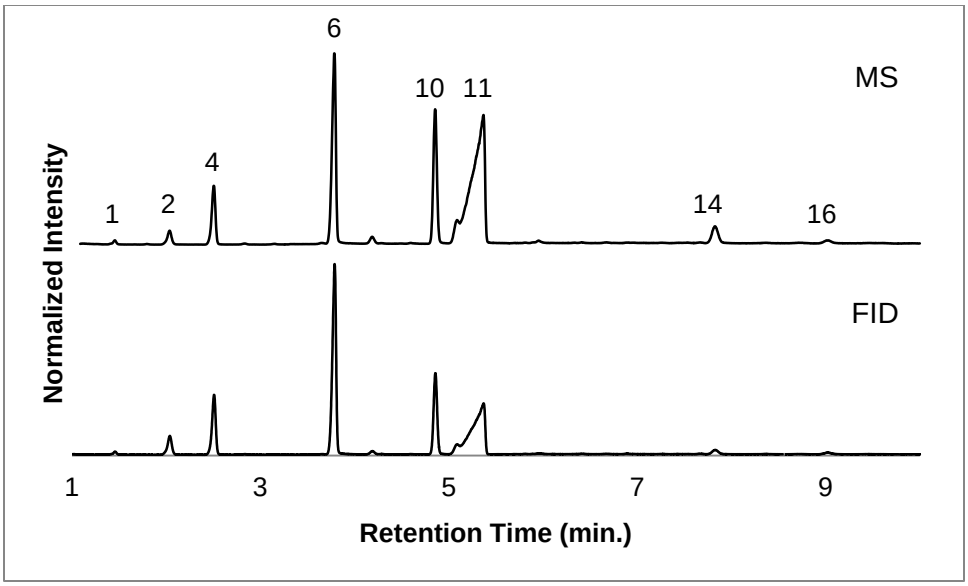

Figure A-11 Butch Masters Vanilla Cigarillos

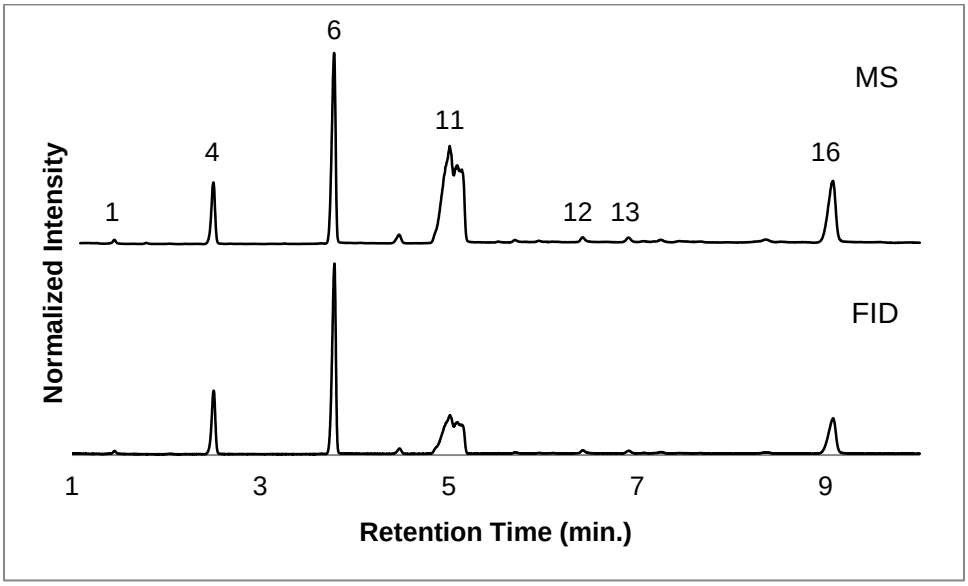

Figure A-12 Blackstone Pipe Tobacco Vanilla Cigars

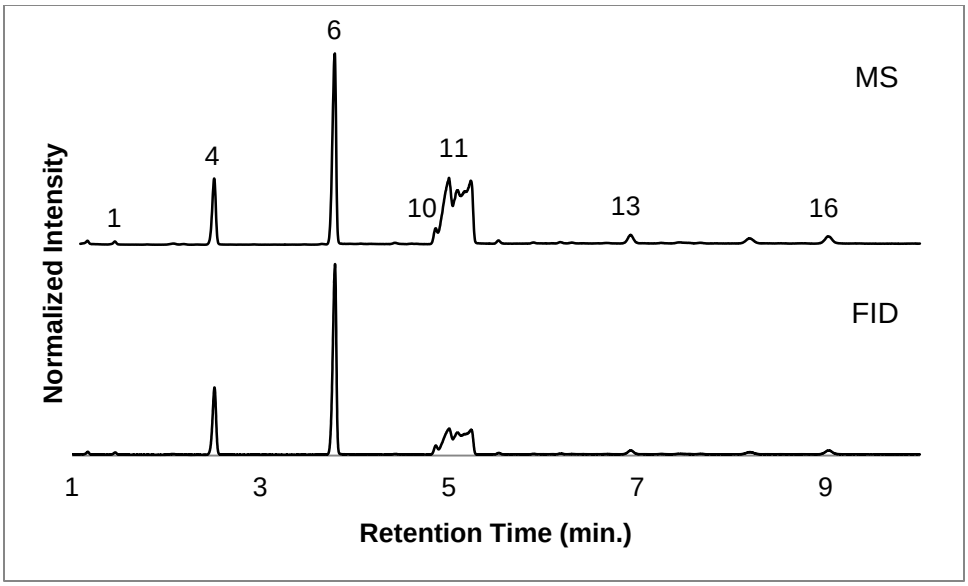

Figure A-13 Swisher Sweets Peach Flavor Little Cigars

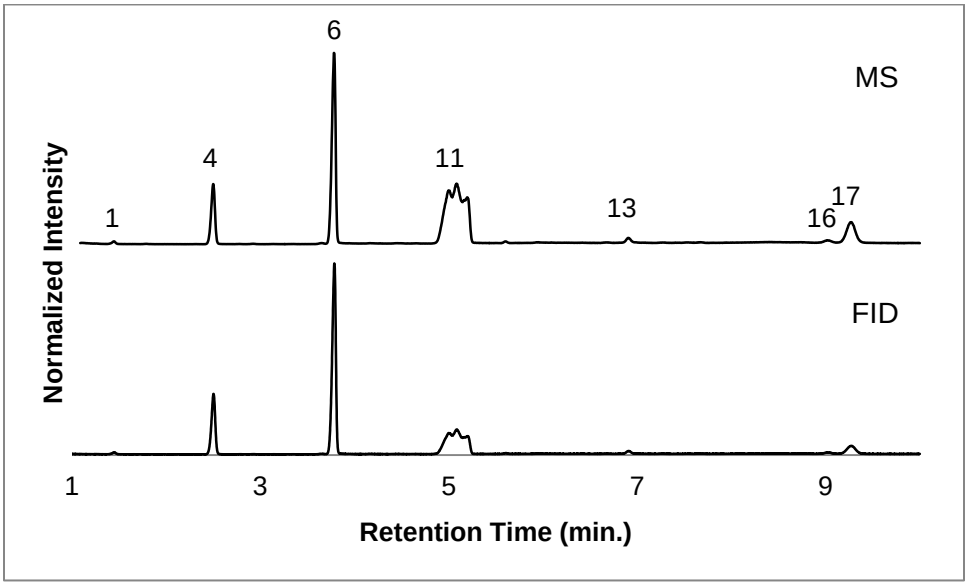

Figure A-14 Swisher Sweets Mini Cigarillos

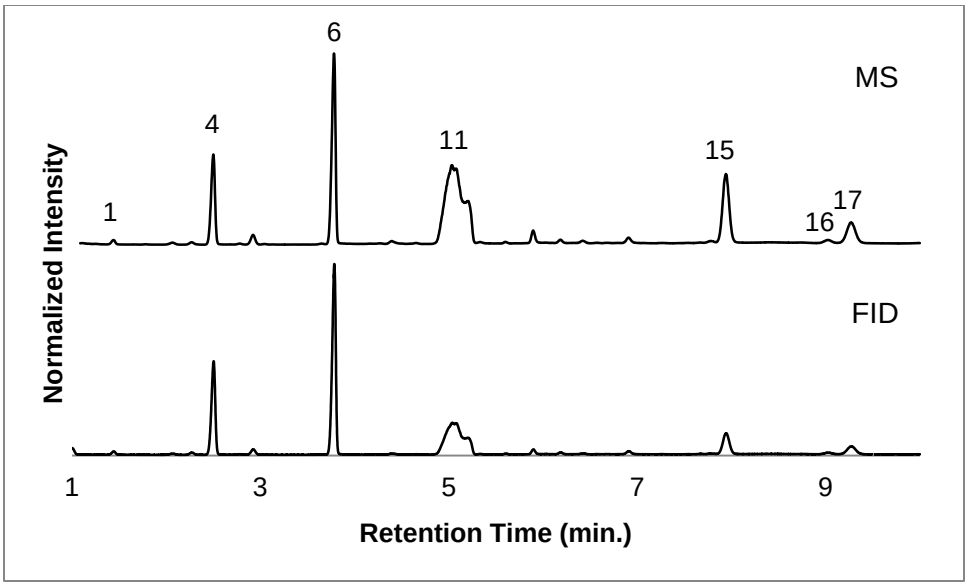

Figure A-15 Swisher Sweets Grape Mini Cigarillos

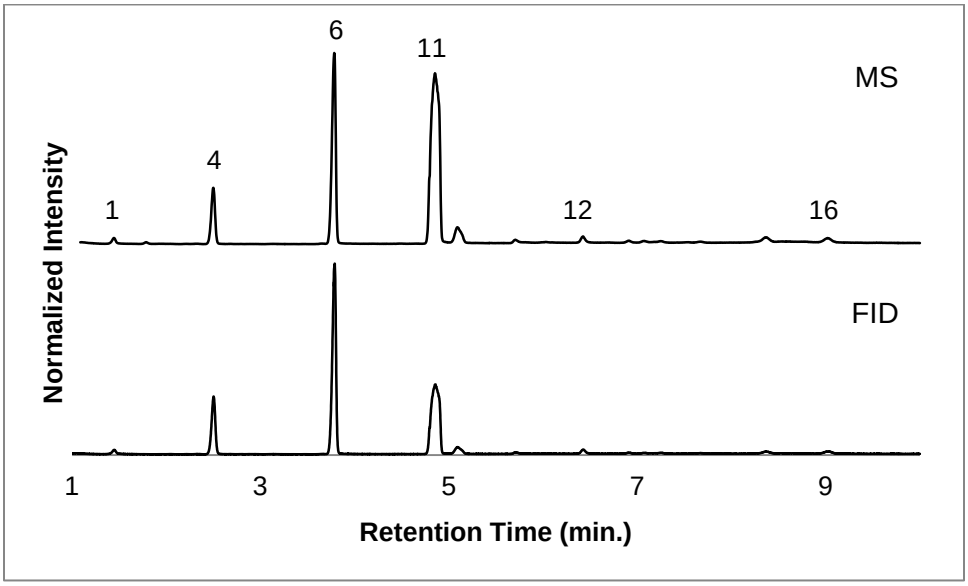

Figure A-16 Al Capone Slims Rum Dipped Cigars

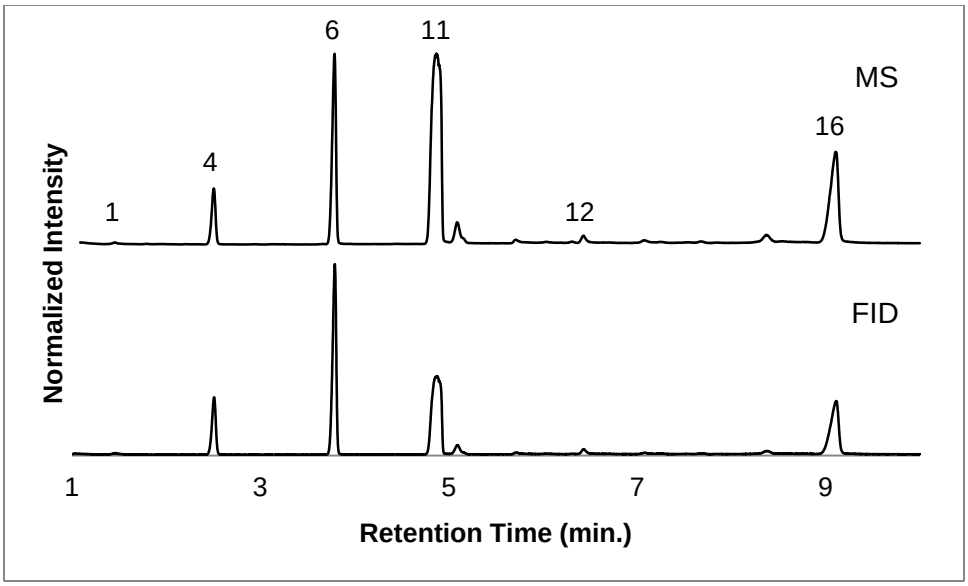

Figure A-17 Camel Filters Turkish Domestic Blend Cigarettes

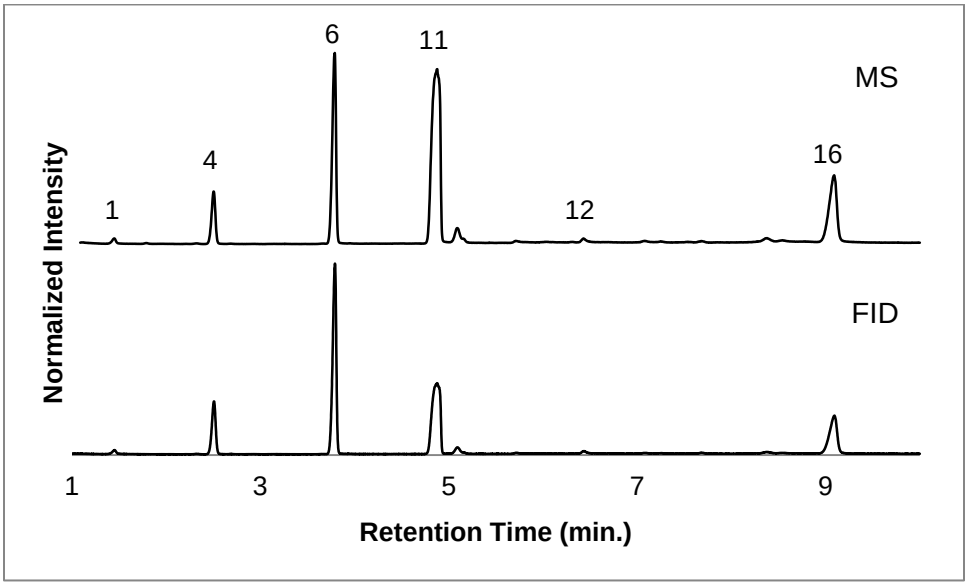

Figure A-18 Benson & Hedges 100's Premium Filter Cigarettes

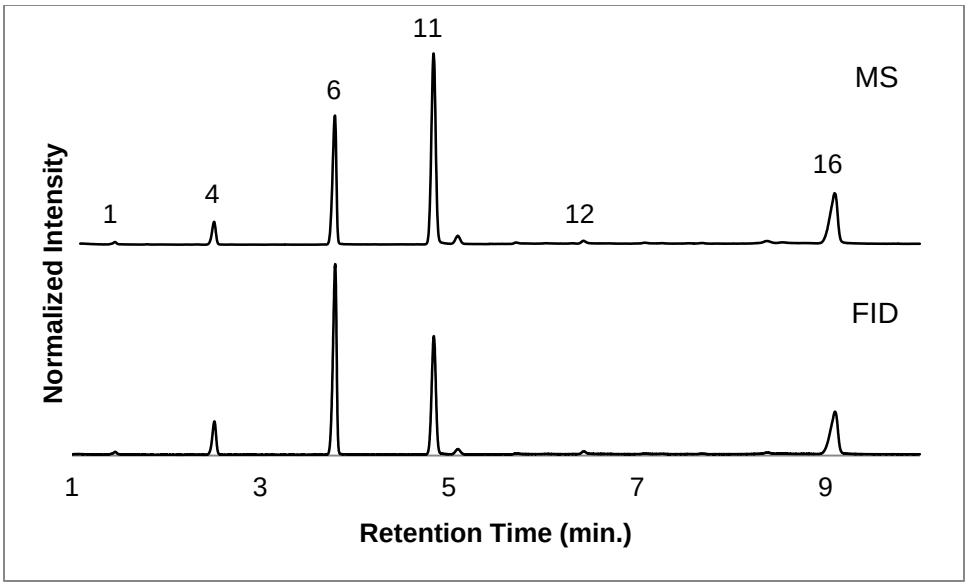

Figure A-19 Doral Full Flavor Cigarettes

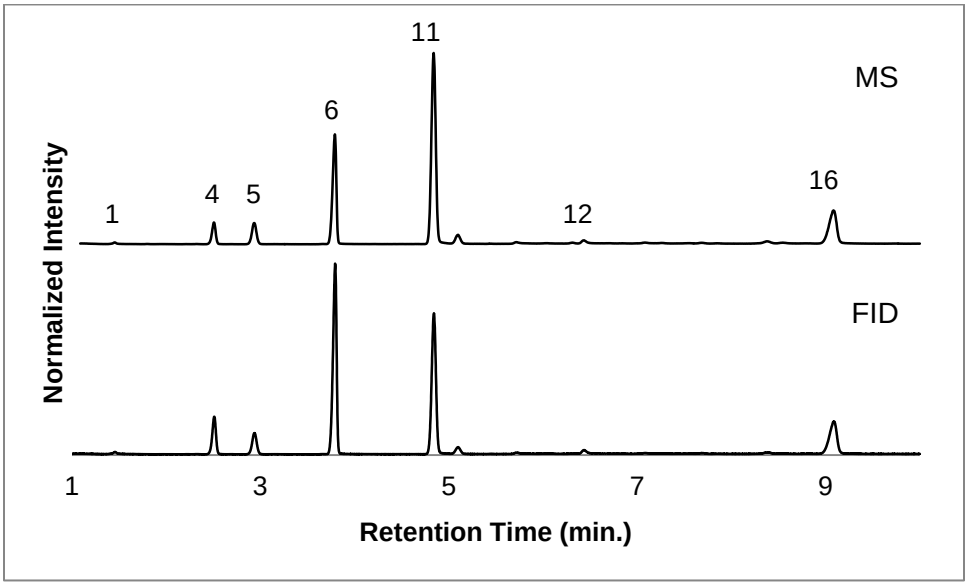

Figure A-20 L & M Bold 100's Menthol Cigarettes

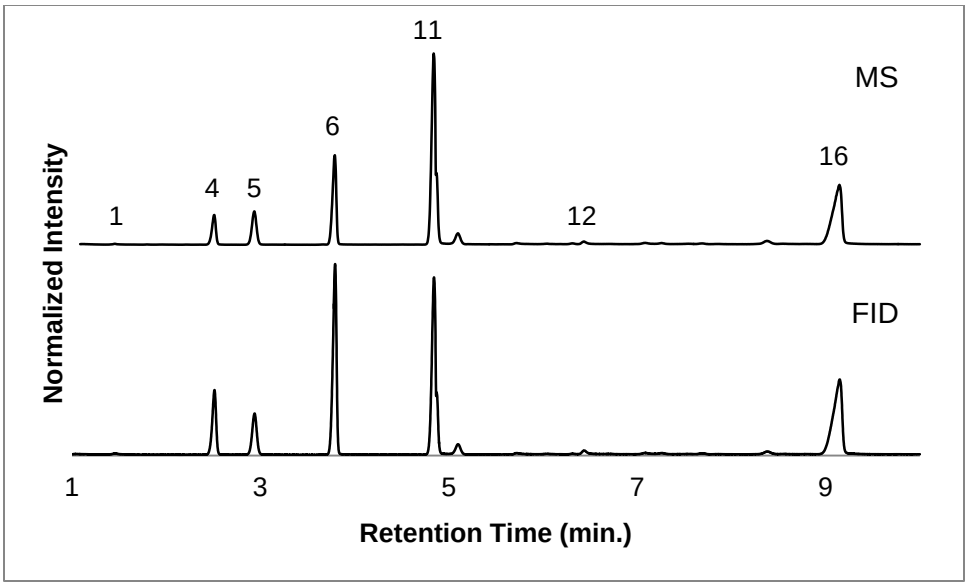

Figure A-21 Kool Filter Kings True Menthol Cigarettes

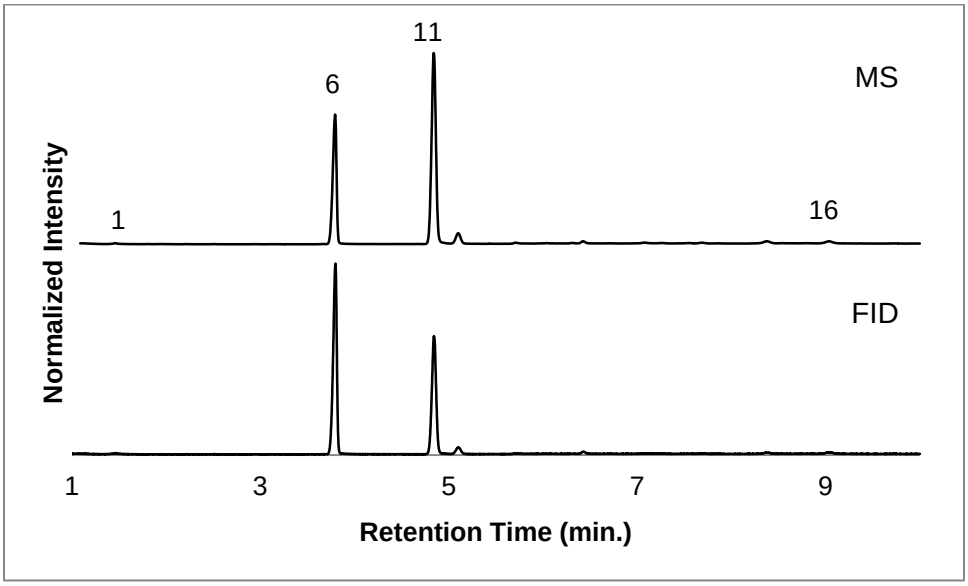

Figure A-22 Winston Red 100's Cigarettes

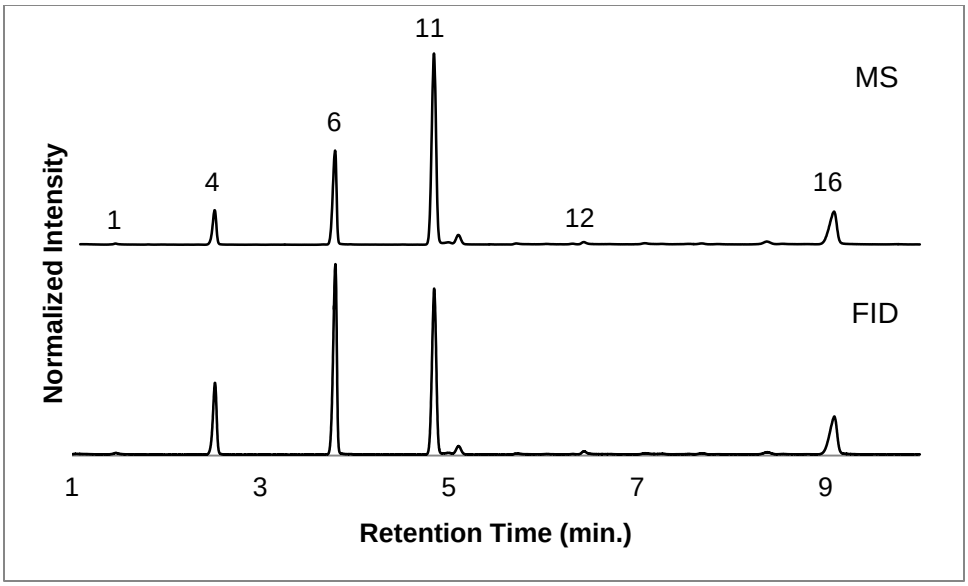

Figure A-23 Marlboro Filter Cigarettes

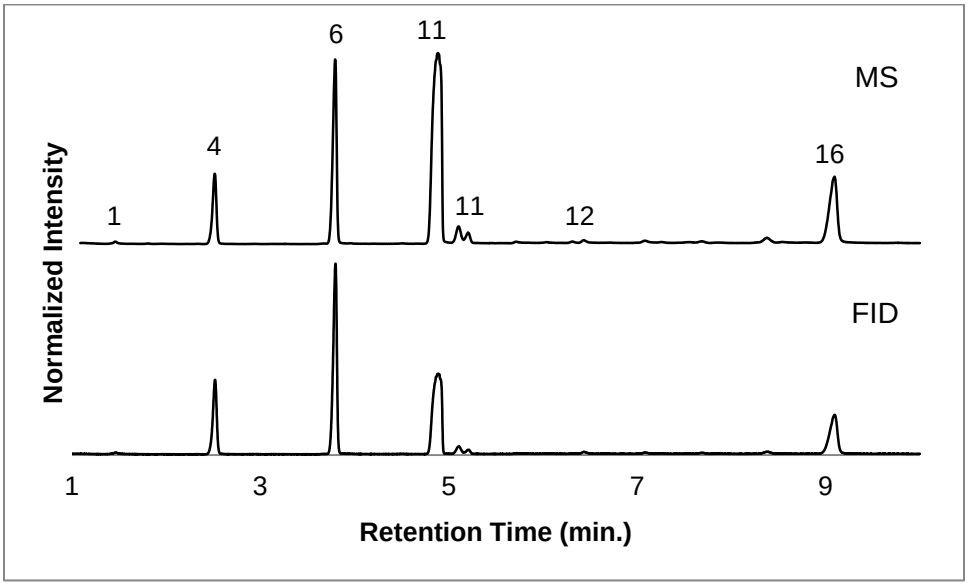

Figure A-24 Parliament Recessed Filter Cigarettes

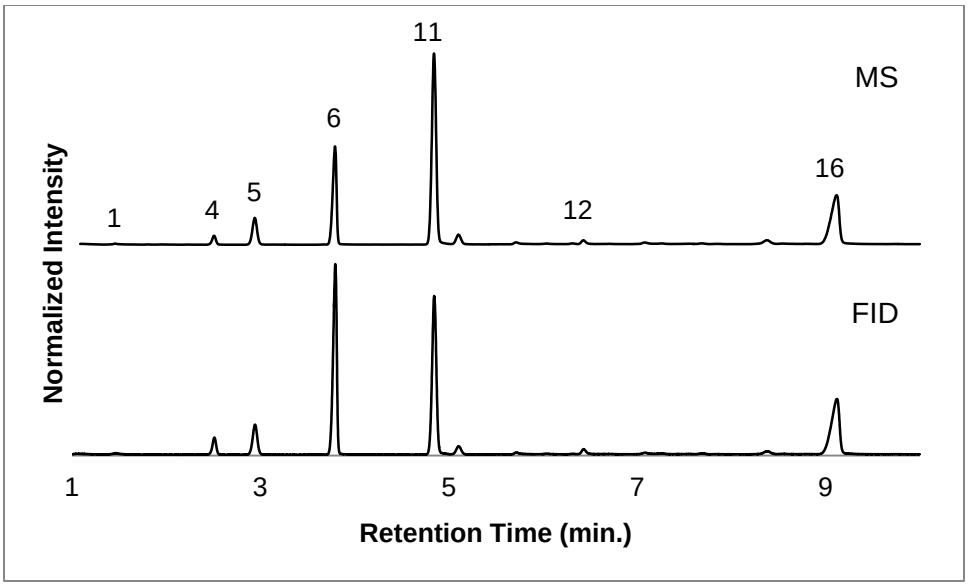

Figure A-25 Salem Refreshing Menthol Cigarettes

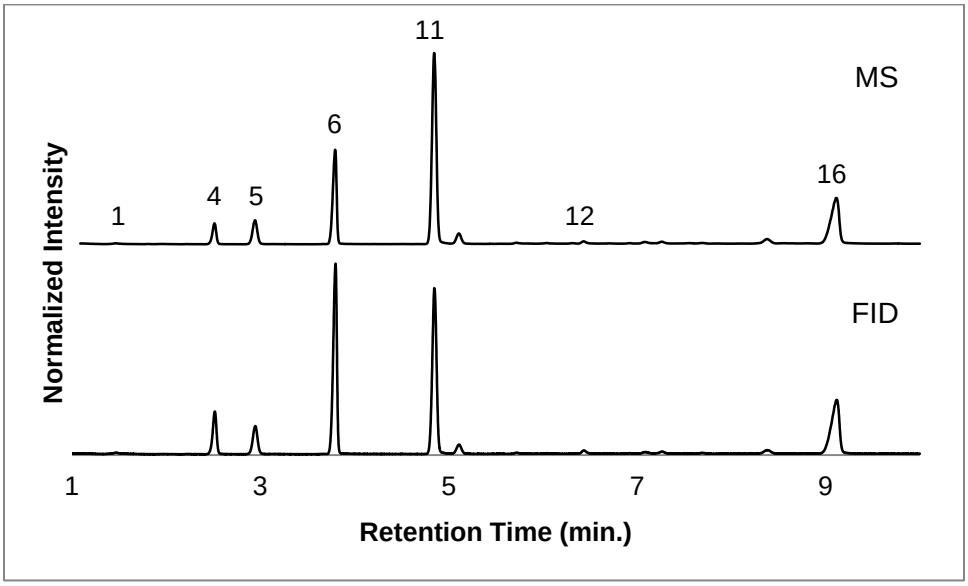

Figure A-26 Newport Cigarettes

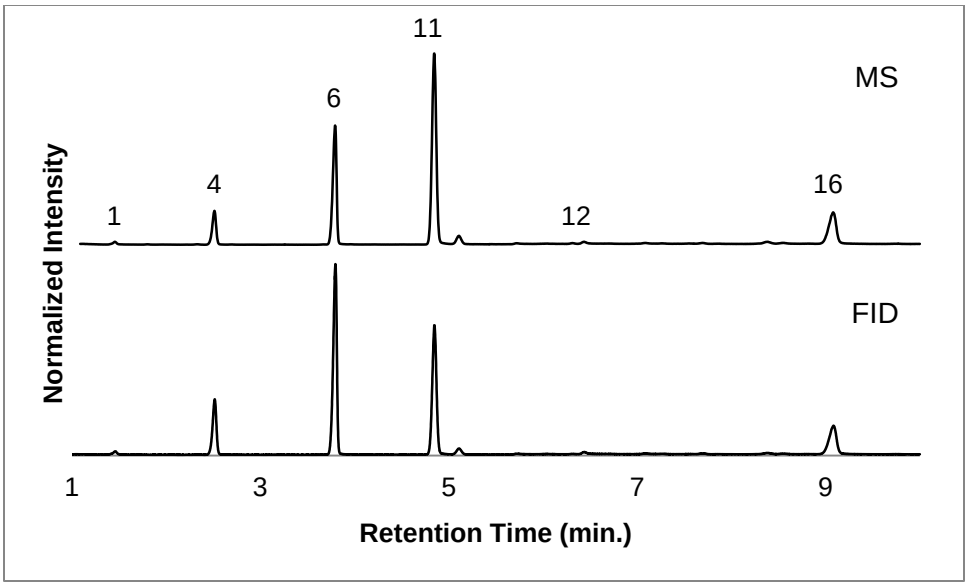

Figure A-27 Merit Lights Cigarettes

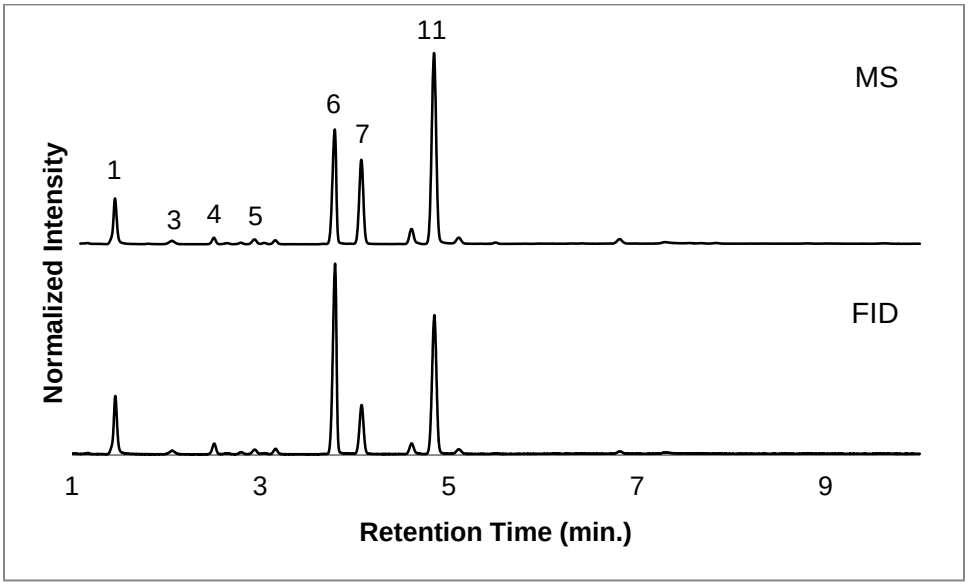

Figure A-28 Skoal Long Cut Berry Blend Moist Snuff

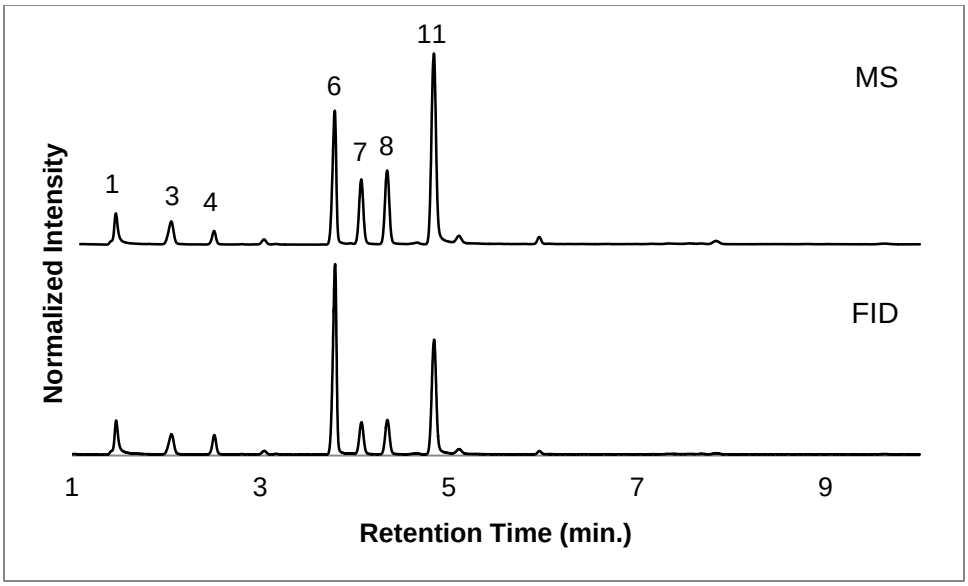

Figure A-29 Skoal Long Cut Cherry Moist Snuff

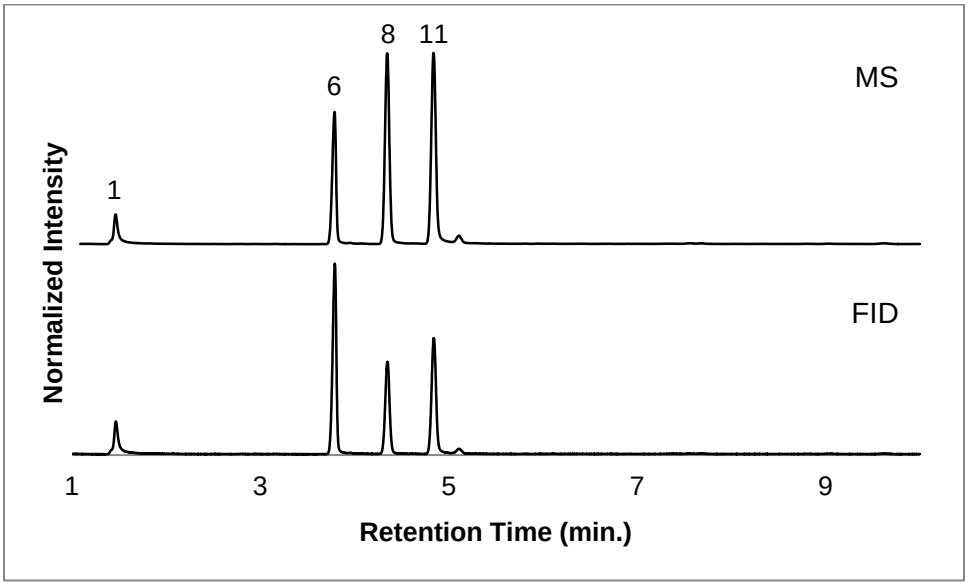

Figure A-30 Skoal Long Cut Straight Moist Snuff

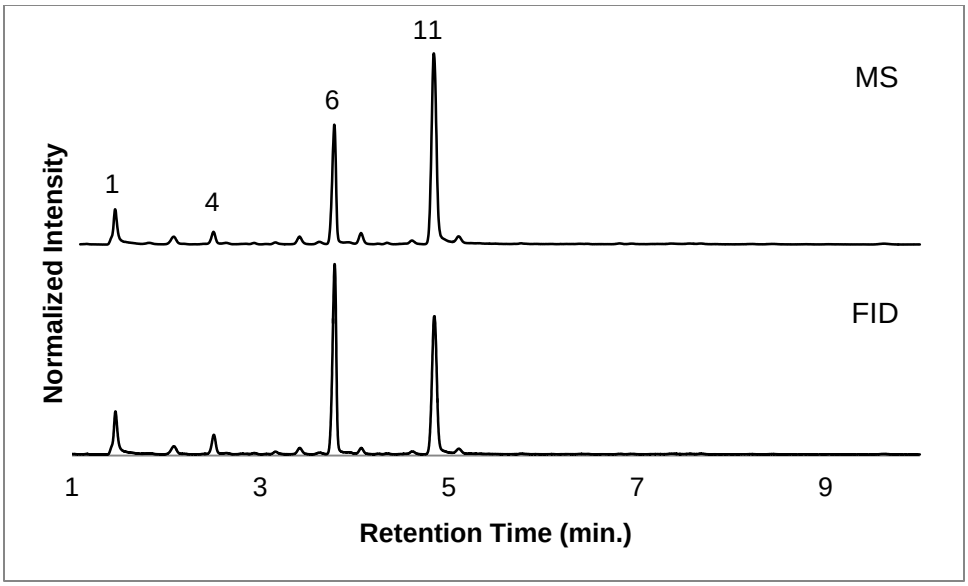

Figure A-31 Skoal Long Cut Citrus Blend Moist Snuff

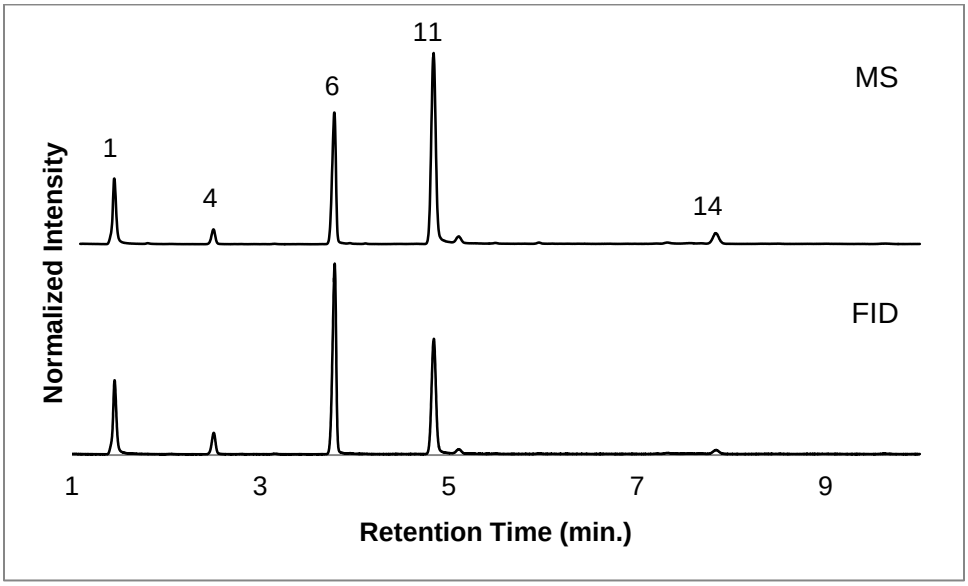

Figure A-32 Skoal Long Cut Vanilla Blend Moist Snuff

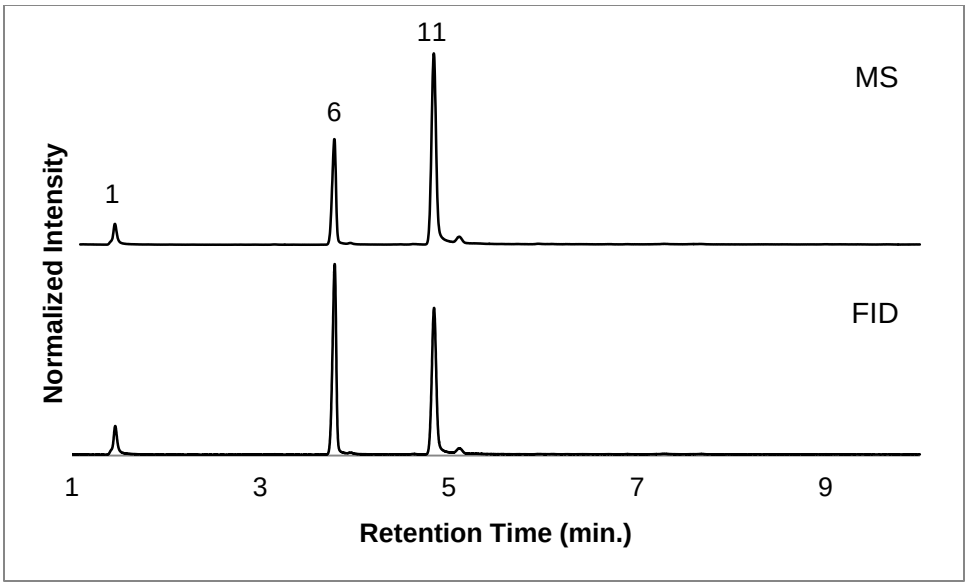

Figure A-33 Grizzly Fine Cut Natural Moist Snuff

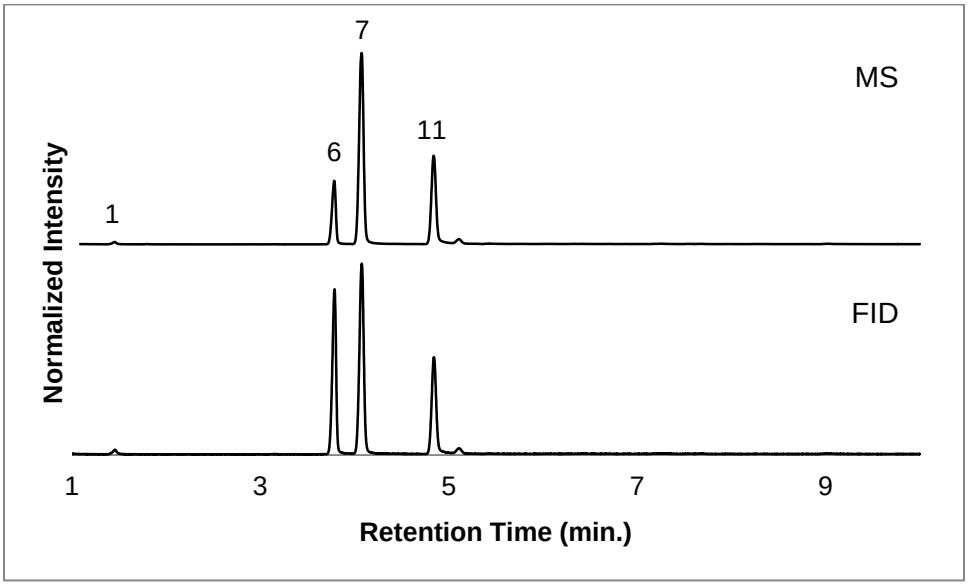

Figure A-34 Grizzly Long Cut Wintergreen Moist Snuff

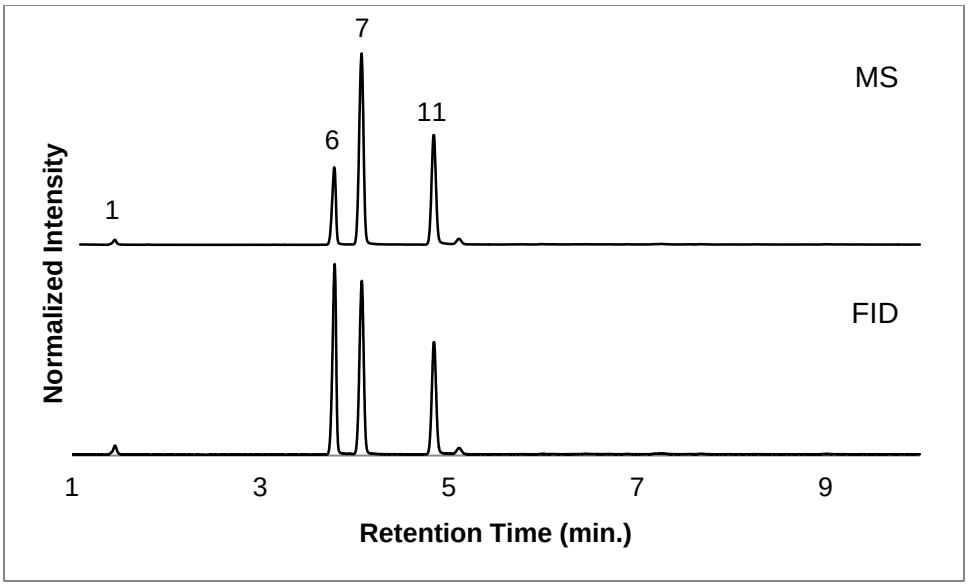

Figure A-35 Kodiak Premium Wintergreen Moist Snuff

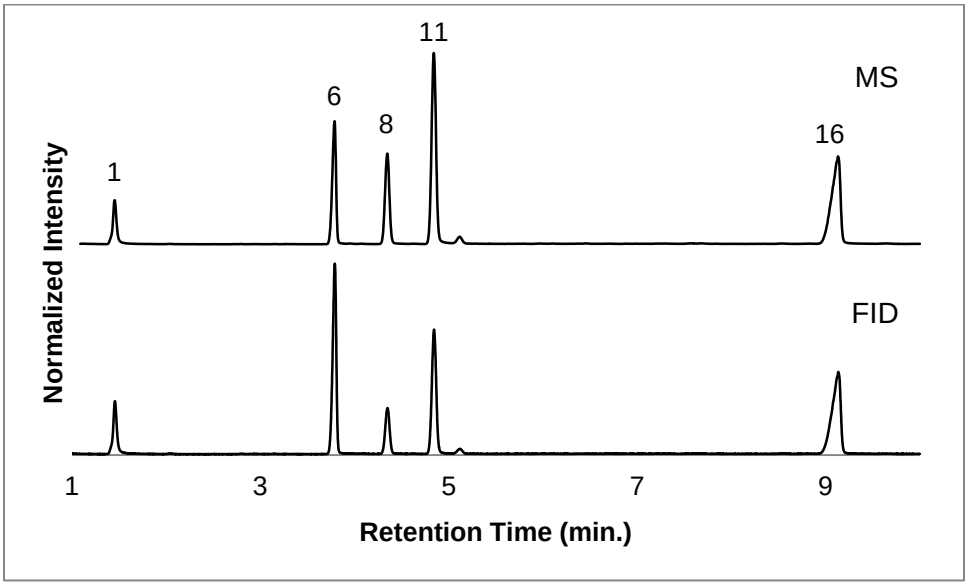

Figure A-36 Kayak Long Cut Straight Moist Snuff

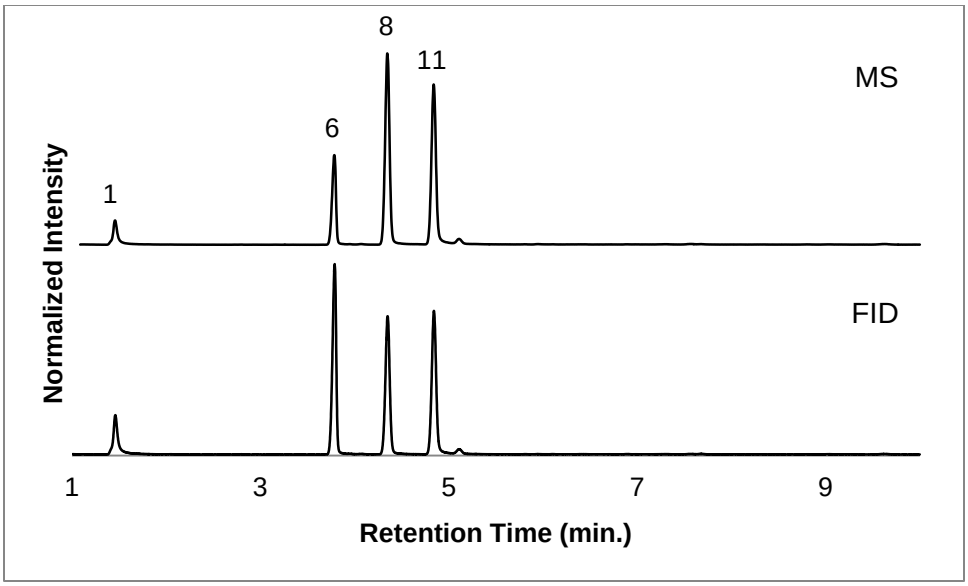

Figure A-37 Longhorn Long Cut Straight Moist Snuff

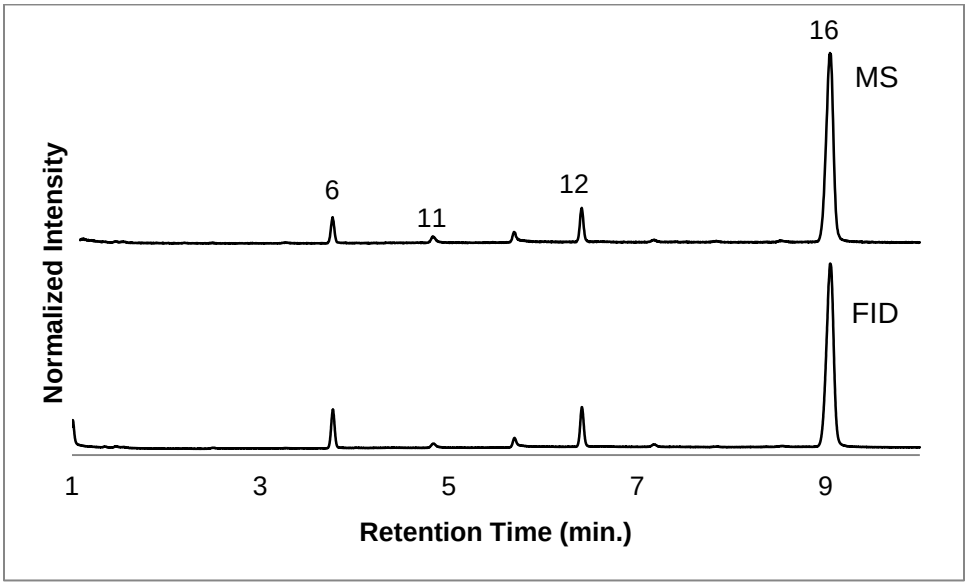

Figure A-38 Pharaoh's Watermelon Splash Hookah

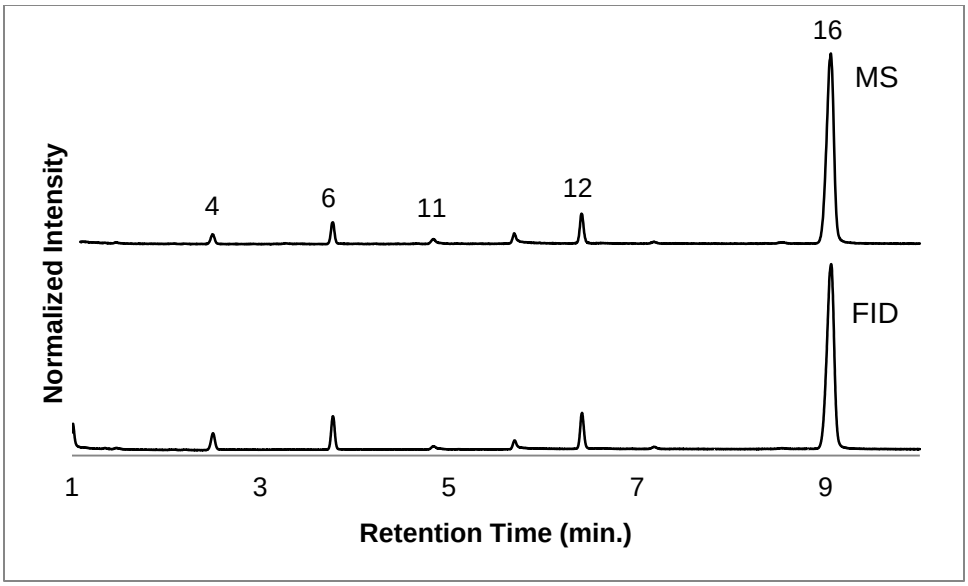

Figure A-39 Pharaoh's Fruitopia Hookah

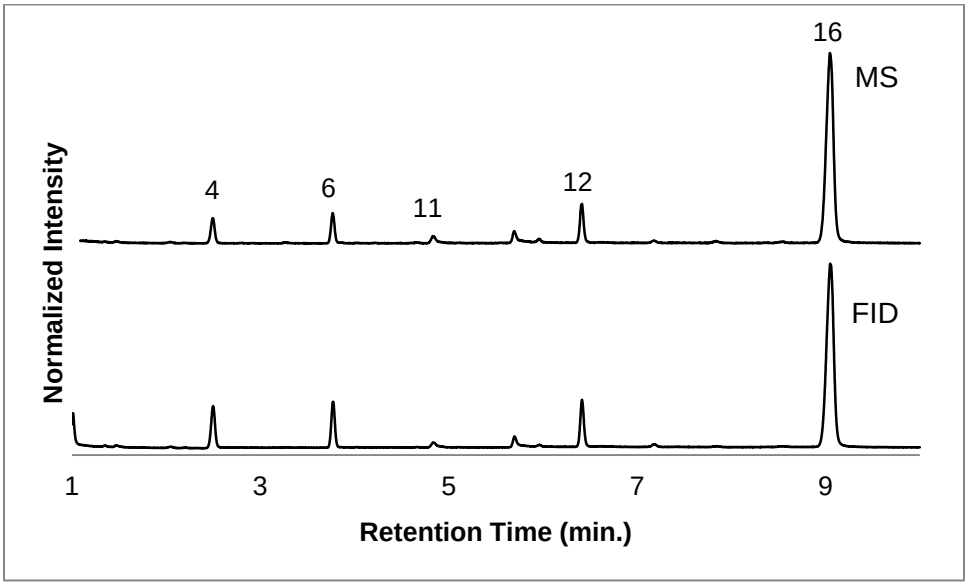

Figure A-40 Pharaoh's Blue Berry Hookah

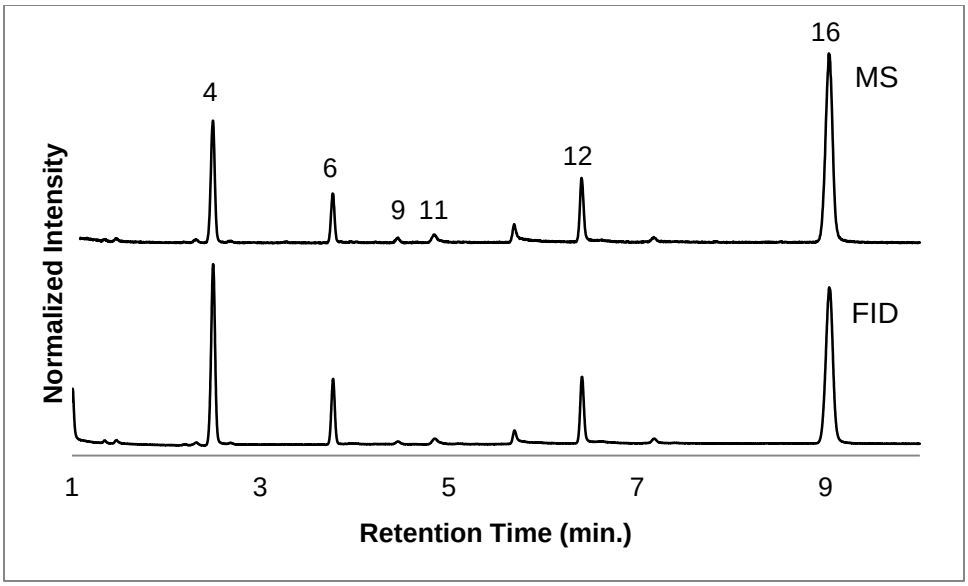

Figure A-41 Rosetta Double Apple Hookah

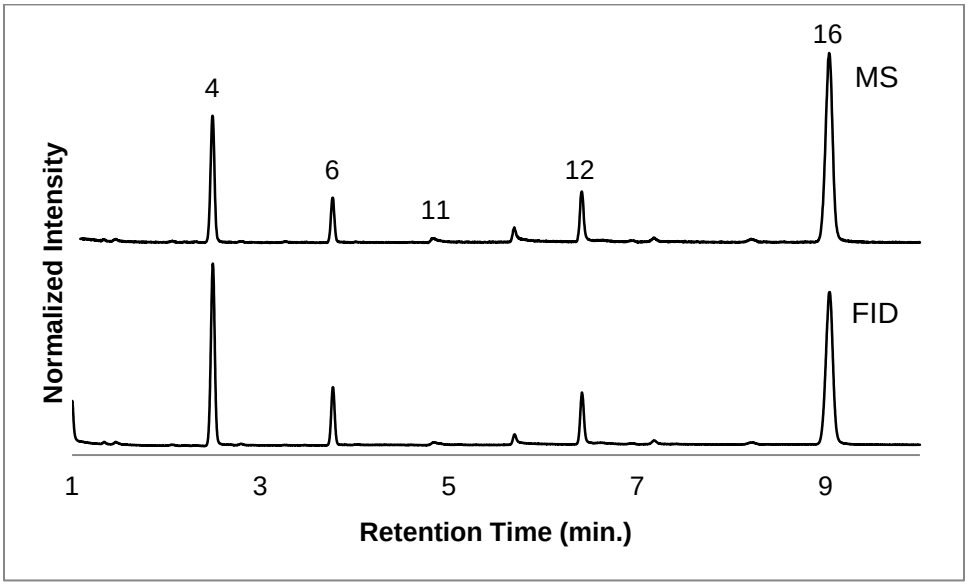

Figure A-42 Rosetta Peach Passion Hookah

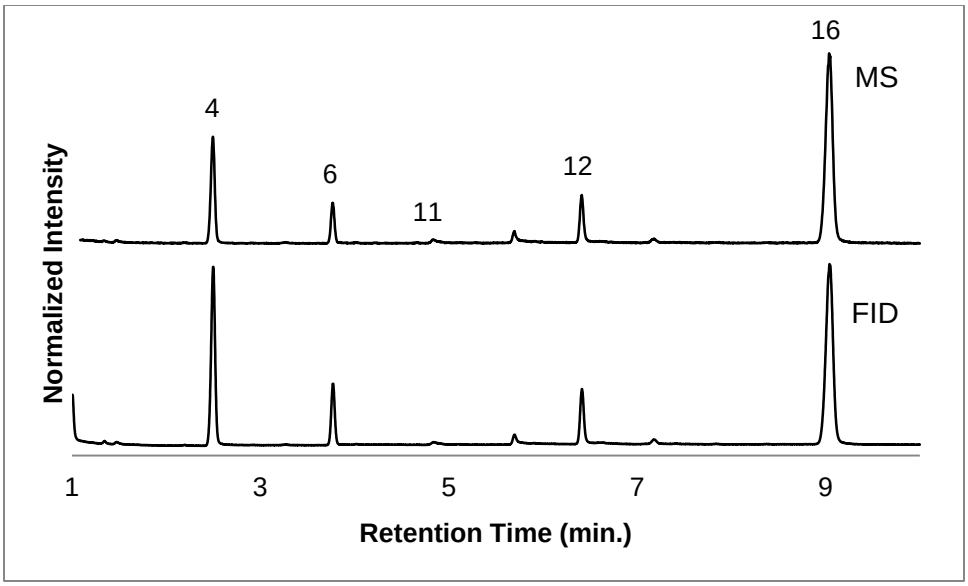

Figure A-43 Rosetta Vanilla Spice Hookah

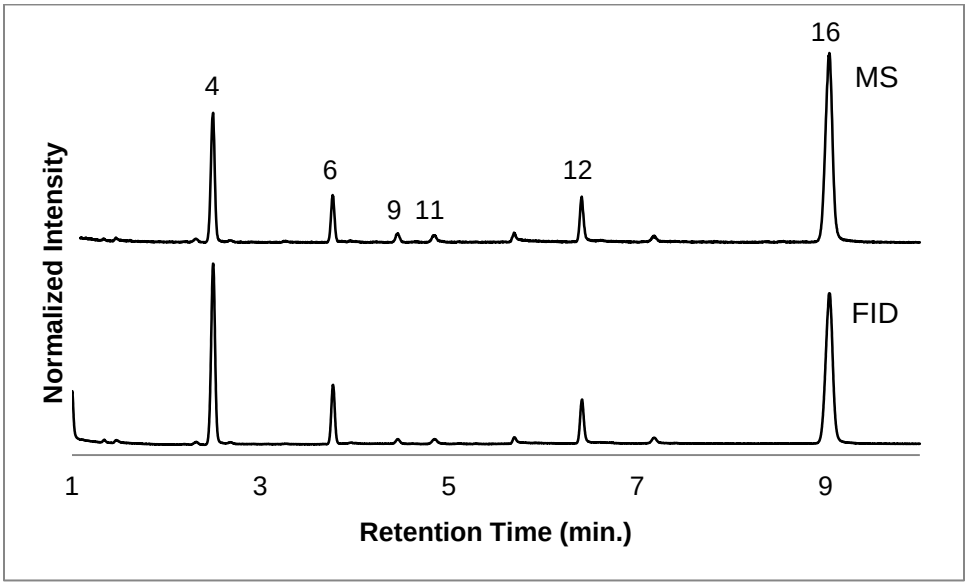

Figure A-44 Rosetta Double Apple Hookah

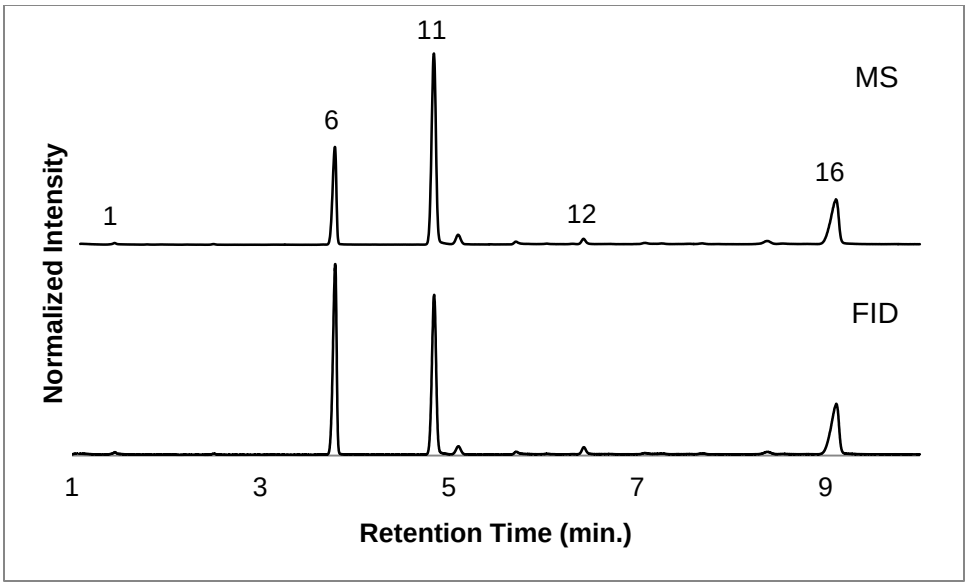

Figure A-45 Reference Cigarette 3R4F

VITA

VITA

Christina Rainey, Ph.D.
Purdue University

Education

Ph.D., Analytical Chemistry, Purdue University, Indianapolis, IN 2013
- Advisor: John V. Goodpaster, Ph.D.
- Dissertation: Identification of Tobacco Related Compounds in Tobacco Products and Human Hair

B.S., Chemistry, Wright State University, Dayton, OH 2009
- Biological Sciences minor
- General Studies Honors Scholar, *cum laude*

Research Experience

Graduate Research Assistant 2009-present
Department of Chemistry and Chemical Biology, Indiana University-Purdue University Indianapolis, Indianapolis, IN
Research advisor: John V. Goodpaster, Ph.D.
- Extracted dissolvable tobacco products using solvent extraction, derivatization, and solid phase microextraction (SPME) and subsequent analysis by GC/MS
- Extracted and identified lipids and fatty acids present in human hair after mtDNA analysis by GC/MS
- Developed total vaporization SPME GC/MS method for the analysis of nicotine and cotinine in human hair

Graduate Research Intern 2011
Tobacco Laboratory, Alcohol and Tobacco Tax and Trade Bureau, Beltsville, MD
Research advisor: Dawit Bezabeh, Ph.D.
- Developed a GC/FID/MS method for quantitating humectants in various tobacco products
- Developed a GC/MS method for quantitating alkaloids in various tobacco products

Undergraduate Research Intern 2008-2009
Wright Patterson Air Force Base, Southwestern Ohio Council for Higher Education, Dayton, OH
Research advisor: Max Alexander, M.S.

- Synthesized magnetic nanoparticles and characterized the nanoparticles using X-ray diffraction, TEM and SEM/EDX

Undergraduate Research Intern 2007-2008
Wright Patterson Air Force Base, Southwestern Ohio Council for Higher Education, Dayton, OH
Research advisor: Loon-Seng Tan, Ph.D.

- Synthesized heterocyclic ring compounds and characterized the optical chromophores using NMR, FTIR, UV-VIS, and Fluorescence

Undergraduate Research Assistant 2008-2009
Department of Chemistry, Wright State University, Dayton, OH
Research advisor: Kenneth Turnbull, Ph.D.

- Synthesized 3-phenylsydnone derivatives and characterized the derivatives by FTIR

Teaching Experience

Graduate Teaching Assistant 2009-2013
Department of Chemistry and Chemical Biology, Indiana University-Purdue University Indianapolis, Indianapolis, IN

- Lab instructor for general chemistry, analytical chemistry, and instrumental analysis

Supplemental Instructor 2008-2009
Department of Chemistry, Wright State University, Dayton, OH

- Tutor for general chemistry lecture course

Undergraduate Teaching Assistant 2008
Department of Biological Sciences, Wright State University

- Lab assistant for freshman biology laboratory

Publications

C. Rainey, J. Berry, J.V. Goodpaster. “Monitoring Changes in the Chemical Composition of Dissolvable Tobacco Products,” *Analytical Methods,* 2013, 5 (13); 3216-3221.

C. Rainey, J. Shifflett, J.V. Goodpaster, D.Z. Bezabeh, “Quantitative Analysis of Humectants in Tobacco Products Using Gas Chromatography (GC) with Simultaneous Mass Spectrometry (MS) and Flame Ionization Detection (FID),” *Beiträge zur Tabakforschung International/ Contributions to Tobacco Research*, 25 (6); 576-585.

C. Rainey, P. Conder, J.V. Goodpaster; “Chemical Characterization of Dissolvable Tobacco Products Promoted to Reduce Harm,” *Journal of Agricultural and Food Chemistry*, 2011, 59 (6); 2745-2751.

Presentations

“Solid Phase Microextraction (SPME) Optimization for the Analysis of Nicotine and Cotinine in Human Hair,” American Chemical Society National Meeting, Indianapolis, IN, 2013, Poster.

“Quantitative Analysis of Humectants in Tobacco Products using Gas Chromatography with Simultaneous Mass Selective and Flame Ionization Detection,” Department of Chemistry and Chemical Biology Annual Poster Session, IUPUI, Indianapolis, IN, 2012, Poster.

“Quantitative Analysis of Humectants in Tobacco Products using Gas Chromatography with Simultaneous Mass Selective and Flame Ionization Detection,” PittCon Analytical Conference, Orlando, FL, 2012, Poster.

“Quantitative Analysis of Humectants in Tobacco Products using Gas Chromatography with Simultaneous Mass Selective and Flame Ionization Detection,” American Chemical Society National Meeting, San Diego, CA, 2012, Oral.

“Extraction and Identification of ‘Lifestyle Markers’ During mtDNA Extraction of Human Hair,” American Academy of Forensic Sciences National Meeting, Atlanta, GA, 2012, Oral.

“Nicotine Quantification of Dissolvable Tobacco Products Using Solid Phase Microextraction (SPME) GC/MS,” American Chemical Society Central Regional Meeting, Indianapolis, IN, 2011. Oral.

“Nicotine Analysis of Dissolvable Tobacco Products Promoted to Reduce Harm,” Society for Research on Nicotine and Tobacco Conference, Toronto, ON, Canada, 2011, Poster.

“Chemical Characterization of Dissolvable Tobacco Products Promoted to Reduce Harm,” Department of Chemistry and Chemical Biology Annual Poster Session, IUPUI, Indianapolis, IN, 2010, Poster.

"Chemical Characterization of Dissolvable Tobacco Products Promoted to Reduce Harm," Turkey Run Analytical Chemistry Conference, Turkey Run, IN, 2010, Poster.

Professional Organizations

- American Academy of Forensic Sciences, Student Affiliate, 2011-present
- Midwestern Association of Forensic Scientists, Student Member, 2010-2012
- Society for Research on Nicotine and Tobacco, Student Member, 2011
- American Chemical Society, Student Member, 2009-present

Honors and Awards

- Student Travel Grant, Forensic Science Foundation, 2012
- Graduate Travel Grant, Educational Enhancement Grant, Student Development Funding Committee, 2012
- Outstanding Student Researcher Award, Southwestern Council for Higher Education, 2009
- Dean's List, Wright State University, 2005-2009

Volunteer Work

- Organizer for high school competition "build-it-day," National Science Olympiad, 2011-2012
- Student blog writer, University College, Wright State University, 2008-2009
- Student Advisory Board, College of Science and Mathematics, Wright State University, 2007-2009
- Secretary, Chemistry Club, Wright State University, 2008-2009, member 2006-2009