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Exhaled Breath Analysis of Smokers Using CMV-GC/MS

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EXHALED BREATH ANALYSIS OF SMOKERS USING CMV-GC/MS

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by

D'Nisha Darquise Hamblin

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This thesis, written by D'Nisha Darquise Hamblin, and entitled Exhaled Breath Analysis of Smokers Using CMV-GC/MS, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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Anthony Decaprio

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Anthony McGoron

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José Almirall, Major Professor

Date of Defense: May 24, 2016

The thesis of D'Nisha Darquise Hamblin is approved.

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College of Arts, Sciences and Education

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Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2016

DEDICATION

I dedicate this thesis to my loving family and friends. Their continuous encouragement and support throughout the journey of my graduate education kept me motivated and on track for the successful completion of this work.

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I am thankful to the university, my college and my department for my degree.

ABSTRACT OF THE THESIS

EXHALED BREATH ANALYSIS OF SMOKERS USING CMV-GC/MS

by

D'Nisha Darquise Hamblin

Florida International University, 2016

Miami, Florida

Professor José Almirall, Major Professor

The aim of this research was to demonstrate the potential of the novel pre-concentration device, capillary microextraction of volatiles (CMV), for breath analysis. The CMV offers dynamic sampling of volatile organic compounds with its simple coupling to a GC inlet for GC/MS analysis, avoiding expensive thermal desorption instrumentation needed for sorbent tubes, as well as an increased surface area over a single SPME fiber. CMV collectively identified 119 compounds in the breath of 13 self-reported smokers and 7 nonsmokers. The presence and intensity of twelve compounds were used to classify all the nonsmokers 100% of the time using Principal Component Analysis to elucidate the groupings. In some cases, nicotine was not detected in smokers and they were confused with the nonsmokers. Nicotine was detected in the breath of 69% of smokers with an average mass of 143 ± 31 pg for cigarette smokers from the approximate 5 L sample of breath collected. The successful use of the CMV sampler and preconcentration of breath to distinguish between smokers and nonsmokers served as a proof of concept for future applications of the CMV for detection of marijuana smokers' breath for impaired driver management.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
APCI	Atmospheric Pressure Chemical Ionization
AUC	Area Under the Curve
BCD	Breath Collection Device
CBN	Cannabinol
CMV	Capillary Microextraction of Volatiles
E-cig	Electronic Cigarette
EESI-LTQ	Electrospray Ionization coupled to Linear Trap Quadrupole
EI	Electron Impact Ionization
ELF	Epithelial Lining Fluid
ETS	Environmental Tobacco Smoke
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HS	Headspace
HSD	Honest Significant Difference
IMS	Ion Mobility Spectrometer
IRB	Institutional Review Board
MeOH	Methanol
MDGC	Multidimensional Gas Chromatography
MDL	Method Detection Limit
MS-O	Mass Spectrometry Olfactometry
MTBE	Methyl Tertiary Butyl Ether
MPT	Microwave Plasma Torch
MQL	Method Quantification Limit
MS	Mass Spectrometry

MSD	Mass Selective Detector
MW	Molecular Weight
NIC	Nicotine
NIST	National Institute of Standards and Technology
NVOCS	Nonvolatile organic compounds
PCA	Principal Component Analysis
PDMS	Polydimethylsiloxane
PM	Plant Material
PPB	Parts per billion
PPM	Parts per million
PPPO	Poly(2,6-diphenylphenylene oxide)
PSPME	Planar Solid Phase Microextraction
PTR	Proton Transfer Reaction
Q	Quadrupole
RSD	Relative Standard Deviation
RTL	Retention Time Locking
SD	Solvent Desorption
SIFT	Selected Ion Flow Tube
SIM	Select Ion Monitoring
SPI	Single Photon Ionization
SPME	Solid Phase Microextraction
TD	Thermal Desorption
THC	Δ^9-tetrahydrocannabinol
TIC	Total Ion Chromatogram
TOF	Time-of-Flight
TSP	Thermal Separation Probe
VOCs	Volatile Organic Compounds

CHAPTER 1: INTRODUCTION & RESEARCH MOTIVATION

1.0 Introduction to Breath Analysis

Exhaled breath analysis is a type of trace gas analysis where chemical compounds within a breath matrix are searched for information detailing the status of the body [1]. A breath matrix is composed of two fractions: gas phase volatile organic compounds (VOCs) and non-volatile compounds (NVOCs) carried in aerosol particles [2]. The aerosol fraction can consist of proteins, signaling molecules, dissolved inorganic compounds, bacteria, and viruses [2] which are expected to originate from the respiratory track lining fluid [3]. Collectively the gas and aerosol phase of the breath profile totals ~300-500 identified chemical compounds [4]. These compounds could be of endogenous or exogenous origin. Endogenous compounds are age, gender, ethnicity and health dependent [5] and are the result of normal metabolic activity or pathological disorders [6]. Exogenous compounds originate from ingestion of food or medicine, inhalation of ambient air contaminants from chemical environments as well as exercise and smoking activities [7]. One of the earliest recognized applications of breath analysis was blood alcohol concentration determination [8]. However, unlike alcohol, numerous other breath pollutants are expected to have concentrations orders of magnitude smaller, in the range of μmol/L to fmol/L (ppm/v to ppt/v) [9]. Measurement of endogenous compounds in breath can serve as a diagnostic tool for cancer and respiratory infections [10]. In exhaled breath analysis for disease diagnostics it is important to measure changes in

concentration, as disease onset is linked to concentration changed instead of the presence and absence of unique biomarkers [7]. As the concentration of compounds in breath are expected to be low, it is important to have necessary sampling methodologies to capture them.

2.0 Breath Sampling: Pre-concentration

The lack of a standard sampling procedure for exhaled breath analysis capable of sensitive detection, has sparked an onset of sampling techniques proposing resolution to the sensitivity issue. One technique, pre-concentration, operates on the bases of absorption processes. Pre-concentration sampling techniques permit portable sample collection, which is important for breath diagnostic testing studies; as onsite collection warrants improved sample integrity. Pre-concentration has been achieved through sorbent and fiber technology where VOCs were trapped within sorbents or absorbed to coated fibers. Activated charcoal, graphitized carbon, carbon molecular sieves, metal oxides and porous organic polymers have also been used for pre-concentration [11]. Not all of these pre-concentration technologies can be advantageous in the use of breath sampling as temperature limitations or high artefact levels may interfere with trace analysis [12]. Molecular sieves, graphite carbon and tenax® have high break through volumes, in that they do not retain absorption of molecules. Some sorbents may be hydrophilic and therefore not suitable for the sampling of water saturated breath samples [13]. Tenax® technology is least affected by water [12,14]. Solid phase microextraction (SPME) fibers have often

been deemed the “gold standard” in breath analysis [15], however the surface area of its single fiber limits the quantity of substances that can be absorbed [13]. It would be of interest to investigate a new technology that embodies the characteristics of a SPME fiber, but with increased surface area. Representation of a solution to the previously described sampling shortcomings have been exhibited by a novel sorbent technology known as the capillary microextraction of volatiles (CMV) device.

2.1 Pre-concentration Device: Capillary Microextraction of Volatiles

The CMV device used in the execution of this research was a novel sorbent filled mini capillary tube that was thermally desorbed through the coupling to a gas chromatographic instrument’s inlet. The CMV was manufactured in the Almirall research lab and consists of an open ended 2 cm long glass capillary glass tube packed with precut 2 mm wide by 2 cm long strips of a polydimethylsiloxane (PDMS) coated glass fiber filter called a planar solid phase microextraction (PSPME) device. The detailed chemical configuration of this device has been described elsewhere [16].

The PSPME device can be used as a pre-concentration filter for sample introduction into an Ion Mobility Spectrometer (IMS) for analysis. The PSPME has demonstrated its absorption capabilities of illicit drugs and explosives by dynamic sampling [17]. The restructure of the PSPME filter into the CMV design allowed for the exploration of other applications additional to explosives and

drugs. The CMV has been used to demonstrate absorption of volatiles in air [18], explosives [16], gunshot residue [19] and drugs [20].

The use of the CMV as a sampling device is advantageous because the design allows for simple collection and easy transport and storage of the device until analysis. The capillary glass tube design allows for the ability of the device to be reusable without contamination concern. Studies of explosive volatile headspace collection indicated the CMV devices' ability to retain at least 70% of analyte mass after storage at 23°C for more than 60 hours [16]. The PDMS sorbent coating of the CMV is reported to be ~0.05 m^2 thick with a calculated phase volume of 50 mm^3 [16]. An increased phase volume for absorption extraction allows for a quantitatively better extraction with the CMV than from any other device (i.e., SPME). The PDMS coating of the CMV encounters strong polar interactions through hydrogen bonding of the siloxane groups of the [-$Si(CH_3)_2$)O-] PDMS repeating unit as well as van der Waals interactions with samples containing alkyl groups [21]. The CMV device is thermally stable to at least 300°C [Patent US20140260974 A1] allowing for suitable desorption of high molecular weight compounds from the CMV coating.

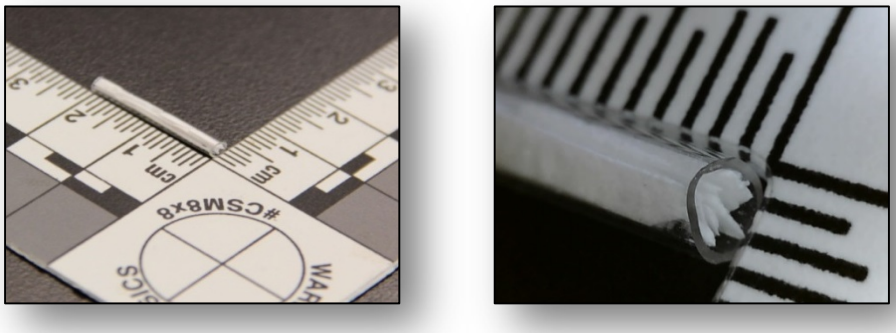

Figure 1: Illustrations of the novel CMV sorbent device.

3.0 Research Hypothesis & Goals

The focus of this research was to evaluate and describe the CMV's potential as a sampling device for exhaled breath analysis of gas phase volatiles and aerosol particulates by thermal desorption gas chromatography mass spectrometry (GC/MS). Evaluation focused on two aims:

1. The CMV's ability to identify individual compounds in breath samples of both exogenous and endogenous origin to establish a breath profile.
2. The CMV's ability to use the breath profiles to distinguish between two groups (smokers and nonsmokers) which are expected to have different profiles based on the presence or absence of significant biomarkers. Selected biomarker of this research is nicotine.

It was hypothesized that the CMV would capable of absorbing multiple analytes in order to obtain a breath profile, as well as to quantifiably detect low levels of significant biomarkers capable of distinguishing between smoker and nonsmoker exhale breath profiles.

4.0 Significance of Research

The CMV used as a sampling device would be able to perform indirect sampling and pre-concentration of a breath matrix. Success of this proof of concept idea using CMV as a part of a breath sampling device would be beneficial to breath analysis in clinical and forensic applications. The sensitivity of the device could be clinically relevant to cancer & medical condition diagnostics.

Forensically, the techniques' ability to detect narcotics would aid in the management and enforcement of drug impaired drivers in traffic patrols, or workplace drug testing; similarly, to the commercialization and utilization of breathalyzers for drunk drivers. The CMV's forensic application of drug detection can prove beneficial to address the concerns for managing wide spread marijuana legalization.

5.0 Overview of Research Study

The current study proposed an alternative sampling technique that would be applicable for the detection and tentative identification of both gas and aerosol phase exhaled breath. Nicotine, the addictive substance of tobacco products was chosen as the target analyte to access the sensitivity and capacity of the CMV to sample and pre-concentrate a range of low concentrations. Its selection as a significant biomarker was linked to its anticipated ability to distinguish between smoker and nonsmokers in this study. This thesis has thus introduced the concept of breath analysis (Chapter 1), and will later explain the fundamentals of the instrumentation used to execute the research (Chapter 2), describe the method development of operational parameters and breath sampling protocol (Chapter 3), discuss some applications of breath analysis (Chapter 4), analyze and statistically evaluate breath samples of volunteers of this research study (Chapter 5), review the limitations in the study's protocol and suggestions for future work (Chapter 6), evaluate an independent work investigating the headspace of marijuana plant material as it relates to future research in

marijuana breath detection (Chapter 7), and finally concluding with remarks and discussion of the CMV's overall performance (Chapter 8).

CHAPTER 2: INSTRUMENTATION BACKGROUND

The analytical process of analyzing volatiles and aerosols in exhaled breath involves sampling and sample preparation before introduction into instrumentation capable of separating and quantifying the complex mixture.

1.0 Indirect and Direct Analysis of Breath

Measurement techniques for breath are often categorized into two approaches: indirect and direct analysis. Direct analysis approaches, often referred to as online measuring techniques, are capable of real time analysis of breath samples. Indirect analysis approaches are referred to as offline measuring techniques, where breath samples are collected and trapped before being transferred to an analytical instrument for analysis [14, 22].

1.1 Online Instrumentation

Real time analysis of exhaled breath can be satisfactory for collective and continuous exhaled breath research studies. Real time devices such as proton transfer reaction time of flight mass spectrometry (PTR-TOF-MS) [23-25], selected ion flow tube mass spectrometry (SIFT-MS) [26], single photon ionization time of flight mass spectrometry (SPI-TOF-MS) [27] and microwave plasma torch time of flight mass spectrometry (MPT-TOF-MS) [28] are often used in breath analysis for monitoring breath volume and breathing rate. An example of a sensor based real time device used in exhaled breath analysis is called an electronic nose. These real time devices have been used in research studies

examining the exogenous compounds found in exhaled breath as a result of smoking activities. Two examples will be reviewed.

1.1.1 Proton Transfer Reaction Time of Flight Mass Spectrometry

Proton Transfer Reaction Time of Flight Mass Spectrometry is a one dimensional technique that operates under proton transfer reactions [23] and characterizes compounds by their mass [24]. After collection of the sample via a tedlar bag the mixed exhaled breath of 370 volunteers with a smoking history were analyzed by PTR-TOF-MS. In this study by Kushch and associates, seven significant VOCs of smokers were identified corresponding to m/z product ions 28, 42, 69, 79, 93, 97 and 123. Acetonitrile was one of the major VOCs of the smoker breath profile identified [25]. Another PTR-TOF-MS study by Kohl and associates confirmed acetonitrile as a marker in the exhaled breath of seventy-two women (26% smokers) over a 12-month period. The study revealed that acetonitrile and benzene unambiguously identify smokers from nonsmokers by their high fraction in the breath samples. Acetonitrile's concentration in breath has such a high fraction that it is detectable over a few days [29].

1.1.2 Electronic Nose

The electronic nose is a miniature portable device composed of a nanocomposite array of 32 organic polymer sensors [30]. It operates as a lab on a chip system and has also been applied to exhaled smoker breath analysis. In a research study by Cheng and associates, the breath of 15 smokers (46%) and nonsmokers (56%) were analyzed by the Cyranose 320 twelve hours after their

last cigarette was smoked. A significant difference between the two groups' breath profiles was determined with a high degree of accuracy, as determined by an algorithm that can differentiate mixtures [31].

1.1.3 Pros & Cons

Although these real time devices are capable of analyzing the constituents of a smokers' exhaled breath profile with high sensitivity and have the ability to distinguish between smokers and nonsmokers, the techniques have their drawbacks. The PTR-TOF-MS technique can be used as a standardized collection method for large screenings because it can use a single breath for repeated measurements in its short and highly reproducible analyses [23]. However, PTR-TOF-MS is not suitable for diagnostic identification of unknowns without prior calibration [24]. Online ionization methods such as PTR-TOF-MS have difficulties separating isobaric compounds, have contamination between successive measurements as a consequence of o-ring placement [25], and have difficulties measuring compounds with low proton affinity such as alkanes [23]. The main drawback of the electronic nose is its susceptibility to baseline drift and its lack of contribution to the identity of the compounds involved in its classification of smoker and nonsmokers [30]. A real time breath analysis techniques' ability to eliminate a sample pre-concentration step may not be as favorable as techniques that use sample pre-concentration.

1.2 Offline Instrumentation

The indirect analysis of exhaled breath often involves the collection and trapping of a breath sample onto a sorbent to pre-concentrate it followed by one of two sample extraction methods: solvent desorption (SD) or thermal desorption (TD). The use of these collection, trapping and extraction techniques will be reviewed in offline instrumentation techniques.

1.2.1 Collection

Examples of breath collection devices used to contain the exhaled breath sample before pre-concentration are the Bio-VOC sampler [9, 32, 33], and tedlar bags [34-36]. Depending on the size of the collection device, it may be designed to collect a sample of a single exhalation. The commercially available Bio-VOC sampler was designed for alveolar breath collection of a 100 ml sample [32]. The commercially available tedlar bags are available in varying volumes as well as in transparent and black films that provide UV-protection [37].

Collection of a sample into a collecting device followed by subsequent transfer to a pre-concentration device has potential faults. Indirect analysis processes are susceptible to sample volume restrictions, sample loss and sample contamination. The limitation of volume restrictions is observed with the use of the Bio-VOC sampler. Studies by Kwak and associates demonstrated that the sampler's actual volume capacity is 80% of what is reported [32]. Volume constrictions result in the action of continuous uses which are limited to 5 transfers for the Bio-VOC sampler [32]. Multiple transfers of the sample between

containers increases the chances of sample loss and compromises reproducibility during transfer [2]. In some research studies, the breath sample is transferred from tedlar bags via syringe into glass headspace vials before exposure to SPME fibers [2, 36, 38]. Sample loss may also occur as consequence of the adsorption and diffusion characteristics of the collection devices' surface material [22, 37]. Teflon has been identified as the material that can avoid adsorption of VOCs to the walls of the collection container [23]. Additionally, a collection containers' material may emit VOCs that can contaminate the exhaled breath sample. The plastic valves of the tedlar bags may emit VOCs of alkanes and plasticizers, subsequently causing lengthy cleaning processes of up to 2 hours with an inert gas before use [23, 39].

Suggested solutions to these potential faults are to bypass the collection step and directly pre-concentrate samples. A flow through sampling device would integrate sampling collection and pre-concentration into one step. Proposal of such a flow through system using the CMV will be discussed in a future chapter (Chapter 3, Section 4.1).

1.2.2 Trapping

Examples of sorbent materials used for pre-concentration of exhaled breath samples are activated carbon sorbents [8], SPME fibers [36, 38, 40, 41] and Tenax® sorbent tubes [33, 42] followed by analytical techniques such as GC/MS [8, 9, 35, 40, 41] or ion mobility spectrometry [42] analysis. During the early stages of exhaled breath analysis, researchers often collected breath into a

reservoir and then pumped the sample through an activated carbon sorbent for solvent desorption extraction [8]. As the scientific community began to transition away from SD extraction, alternative sorbent types had to be considered as the strength, hydrophilicity, and reactivity of charcoal based sorbents were incompatible with the newly established TD extraction technique [12]. The alternative sorbents of Tenax® sorbents and SPME fibers will be reviewed.

1.2.2.1 Solid Phase Microextraction Fibers

Solid phase microextraction fiber technology was first commercially introduced in 1998 by Supelco and is now the most widely used sampling method [5]. A SPME fiber follows non-exhaustive extraction fundamentals [5] and pre-concentrates breath constituents as the sample is statically extracted in a closed headspace system [22]. The microextraction process described by mass conservation is considered complete when analyte concentration reaches equilibrium in the sample matrix and the fiber coating [5]. The porous polymer fiber coating of the SPME is usually nonpolar PDMS because of its low cost and strong adhesion to the substrate [5]. The SPME fiber has small surface area of 9.4×10^{-6} m^2 and a phase volume of 0.612 mm^3 [17]. The SPME technology offers rapid sampling, high resolution, and low detection limits for breath samples. Samples can be stored on the fiber for 8 hours without any significant loss of analytes [15].

1.2.2.2 Tenax® sorbent tubes

Tenax® sorbent tubes contain porous polymer particles of a 60-80 mesh size [12] or other sorbents like chromosorb, porapak, or spherocarb [43]. A Tenax® sorbent tube pre-concentrates breath constituents as the sample is pumped from the collection device onto the tubes' column [22] following exhaustive extraction fundamentals [5]. Exhaustive techniques completely remove analytes from the matrix into the extraction phase. The porous polymer extraction phase dispersed on the supporting mesh material is usually Poly(2,6-diphenylphenylene oxide) (PPPO) [44]. When properly conditioned Tenax® sorbents have minimum background artefacts at 0.1-1.0 ng levels preventing the masking of detectable nanogram level breath markers [12]. The most favorable advantage of the use of Tenax® in breath analysis is its ability to repel water as breath samples can be vapor rich [14]. The sorbent provides suitable absorption and desorption for aliphatic hydrocarbons C7 to C10 and aromatics as well as for other high boilers [43]. The advertised surface area of an 80/100 mesh Tenax® tube is 35 m^2/g of mesh [44].

1.2.3 Extraction

Solvent desorption is carbon absorption followed by extraction via a versatile desorbing liquid [45]. Solvent desorption is compatible with high molecular mass and thermally unstable compounds and allows the pre-concentration of high volumes of air [11]. The adoption of increased sample volume is a consequence of the decreased method sensitivity caused by sample dilution [11, 45, 46]. Evaporative loss of the sample is observed during the

solvent reduction stage [46] and it is reported that only 1-5% of the sample is actually introduced into the GC system [47]. Solvent use is generally criticized because of toxic waste production, but additionally the solvent used in SD can cause analytical interference [46].

Thermal desorption extraction is a two stage extraction process using heat and a flow of inert gas [46]. Thermal desorption has been shown to be a more appropriate extraction technique than SD in studies by Ramirez and associates. In their study, of 90 industrial and urban environment VOCs, 18 out of 90 of the VOCs were found using SD extraction by carbon disulfide solvent desorption of charcoal tubes, while 50 out of 90 of the VOCs were found using TD extraction of Tenax® sorbent tubes [11]. The repeatability, recovery, detection, and quantification merit characteristics of TD were generally better than SD. A different air study of environmental pollutants was in agreement that tenax® TD has statistically better repeatability, recovery and temporal resolution than SD by an acetone and methyl tertiary butyl ether (MTBE) desorbing liquid [46].

Although SD has attractive properties, the advantages of the TD extraction technique are more suitable criteria for the analysis of exhaled breath because of its superior selectivity and sensitivity [5, 12]. Its pre-concentration does not require large volumes as SD extraction techniques would, preventing fatigue of subjects caused by the collection of large volumes of breath over an extended period [22, 23]. The advantages of TD extraction with Tenax® sorbents can be alternatively applied to the proposed CMV sorbent device.

2.0 Indirect Analysis of Breath using CMV-GC/MS

After evaluation of the available analytical approaches to breath analysis, GC/MS was selected as the appropriate technique relevant for the purposes of this thesis research, because of its adaptability to the TD of the CMV device and analysis of volatile, thermally stable species. Gas chromatography mass spectrometry is highly discriminative in its separation preventing overlap observed in online instrumentation and is capable of the identification of unknowns by mass spectrum comparisons to online computer libraries. Although GC/MS offers low detection limits and rapid sampling, the identification aspect of its analysis can be tedious and time consuming for complex matrices like breath [48]. The principles of instrumental operation of a GC/MS system for the analysis of exhaled breath using CMV will be described. A schematic of a basic GC system can be seen in Figure 2.

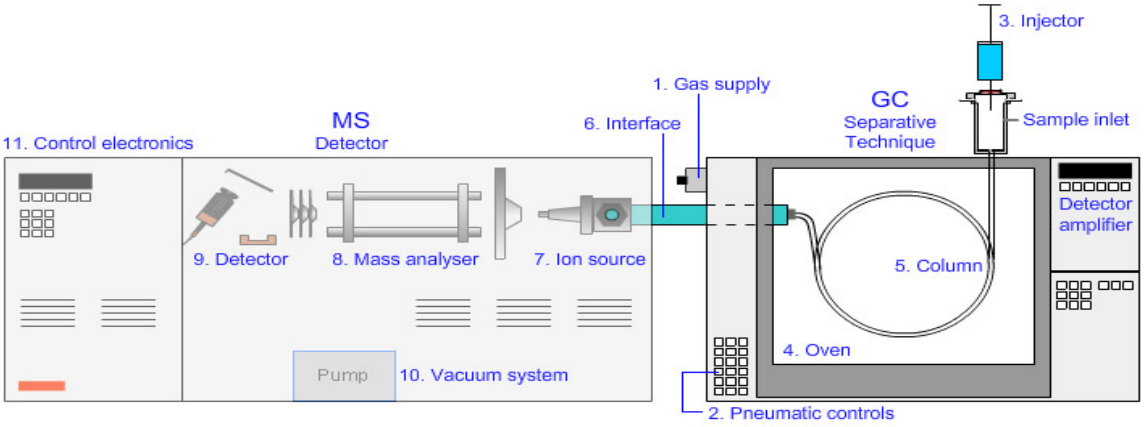

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Figure 2: Schematic of a GC/MS instrument.

2.1 Sample Introduction

Sample introduction into the gas chromatographic systems' inlet can be of liquid or gas phase. Liquid standard samples for calibration determination were introduced by an Agilent G4513A automatic liquid sampler (Santa Clara, CA) incorporated with a 10 µL Agilent gold standard syringe (Santa Clara, CA). Gas phase exhaled breath samples pre-concentrated on CMV devices were introduced into the inlet of the GC system via the coupled Agilent thermal separation probe (TSP) adapter (Santa Clara, CA) which facilitated sample transfer as seen in Figure 3. This apparatus was directly connected to the inlet compared to tubular/column connections of the bulky and expensive thermal desorption unit used for Tenax® sorbent tube analysis. During introduction, the molecular bonds of the analytes absorbed on the CMV were broken by heat applied in the inlet assisting the desorption process.

Figure 3: Thermal separation probe coupled to GC inlet.

A GC inlet operates under either a split or splitless injection mode. In split injection mode, the sample was contained within a splitless Restek ultra inert sky liner (Bellefonte, PA) and a percentage was carried to the column and the

remaining percentage was carried between the liner and the inlet body to the spilt line where the sample and helium carrier gas was released. The percent arriving at the columns' entrance was dependent on the split ratio and gas flow rate. In splitless injection mode the entire sample reached the column. The split operational modes affect peak shape of the resulting chromatogram during separation.

2.2 Gas Chromatography

After sample introduction, separation of the gaseous sample in an Agilent 7890A GC system (Santa Clara, CA) occurred on a HP-5ms capillary column (29.17 m x 0.25 mm x 0.25 μm) as the gaseous sample molecules were carried by the helium carrier gas. The temperature gradient applied to the column in a heated oven affected separation and elution of sample molecules depending on their volatility. The time-based separation is graphically illustrated by a chromatogram. The gas chromatographic technique only performs the separation of the complex breath samples, and needs to be coupled to a detection technique for identification of breath components.

2.3 Mass Spectrometry

Analysis of the separated sample from the GC system occurred in a vacuum sealed Agilent 5975C Inert XL MSD mass spectrometer with triple axis detector (Santa Clara, CA). The MS can be composed of many combinations of ion sources, mass analyzers and detectors depending on their combined selectivity, sensitivity, and identification abilities. The MS in the present research

utilized electron impact ionization (EI) at 230°C, where neutral sample molecules were bombarded with 70eV electrons causing breakdown of the molecule into positive ions. The positive ions were filtered and separated by their mass according to an electromagnetic field produced in a single quadrupole mass analyzer. Electron impact ionization and the quadrupole are the most frequently used source and mass analyzer. The mass selective detector measured the filtered ions' characteristic mass-to-charge ratio and plots their distribution according to fragment intensities in a mass spectrum. Information was acquired in both scan mode and select ion monitoring (SIM) mode. The unity mass resolution of the MS has parts per billion (ppb) detection capability suitable for detection of trace exhaled breath volatile and aerosol concentrations.

CHAPTER 3: METHOD DEVELOPMENT

1.0 Parameter Programming

A detailed literature review of gas chromatographic temperature programs used in drug breath detection analysis was completed as a starting basis in the method development for this research. Studies reported lengthy comprehensive methods greater than 30 minutes [33, 36, 40, 41, 50], focusing separation of the lower molecular weight volatiles. For the purpose of the current research, the temperature program will reflect a shorter screening method specifically adjusted for detection and resolution of the target compounds.

The oven temperature was programmed for 2 min at 40˚C, then 25˚C/min to 260˚C, and finally at 260˚C for 10 min. The full chromatographic run totaled 20.8 minutes. The injector was operated in splitless mode at an initial temperature of 250˚C until optimization occurred. The transfer line was maintained at 280˚C. The constant GC column flow of helium was 1.2 ml/min. The mass spectrometer simultaneously collected total ion chromatogram (TIC) and SIM data over the acquisition range 40-340 m/z. The selected ion monitoring for nicotine were 84, 133, and 162 m/z. The selected ion monitoring for Δ9-tetrahydrocannabinol (THC) ions were 299, 271, 231, 314 m/z and were set to start at the 14-minute mark during the method. Since the SIM groups contain between 2-5 ions a dwell time of 50 msec was chosen allowing for 4.5 cycles/sec resulting in 17 SIM cycles per peak. Separate methods were essential for analysis of liquid standard solutions and CMV breath samples, as standard

solutions require a solvent delay of 4 minutes because solutions contain methanol (MeOH) solvent.

1.1 Optimization of Parameters

As a result of the differences in physical characteristics of the target analytes, the GC inlet temperatures were optimized for simultaneous detection to maximize instrument response. The literature review revealed that the GC inlet temperature ranged between 250˚C to 300˚C for nicotine and THC in air studies [51, 52]. The GC inlet temperature study examined temperatures 250˚C -300˚C in 10 degree increments for the method previously described (Chapter 3, Section 1.0). A 15 ppm nicotine and THC methanol solution mixture was used to spike 1 µL of solution onto a CMV before introduction into the GC system via a thermal desorption probe. Duplicate spikes of the solution were made for each of the six GC inlet temperatures. Data were analyzed for the peak area under the curve (AUC) and duplicate areas were averaged. Results of the peak area response comparison for individual inlet temperatures can be seen in Figure 4. Although 250˚C seems to be the optimum GC inlet temperature for nicotine alone, the temperature of 270˚C is the optimum temperature for simultaneous detection of both target analytes as they show a maximum response of peak area in their SIM data. All methods reflected this justified GC inlet temperature in successive experiments.

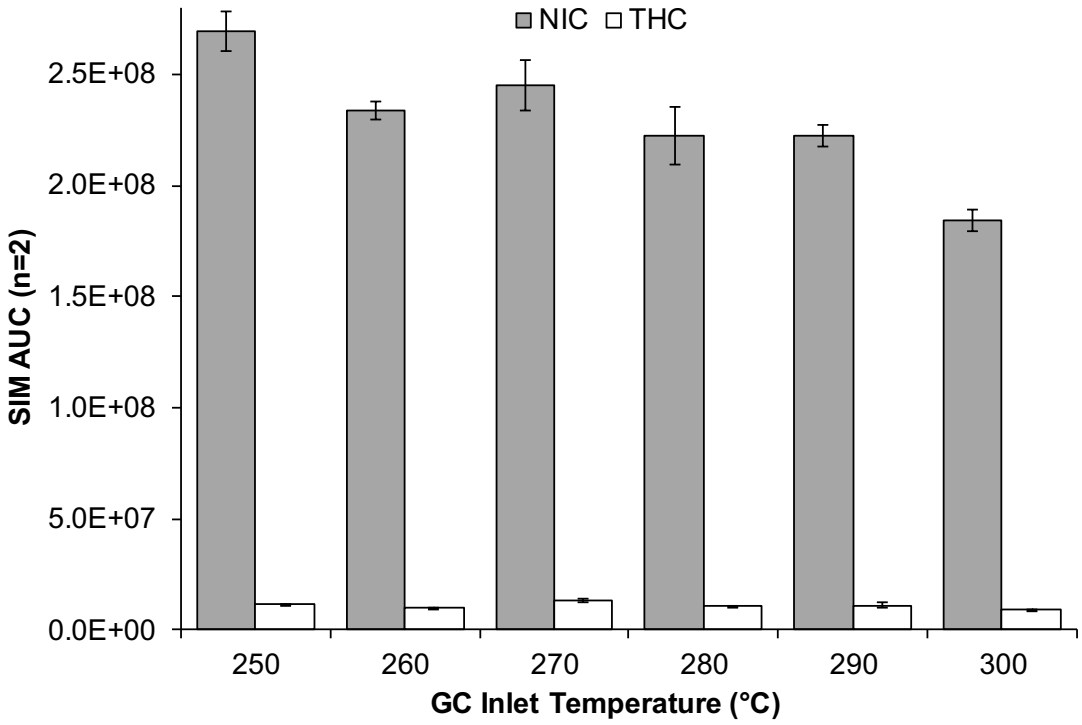

Figure 4: Peak area response of SIM data for target analytes NIC (grey) and THC (white) over inlet temperature ranges 250˚C - 300˚C.

1.2 Retention Time Locking

After optimization of the GC inlet temperature, the experimental method was locked for the retention times of the analytes of interest. The retention time locking (RTL) procedure in method development accounts for variation in peak shifts as the result of maintenance and chromatographic degradation. The procedure allows for a close match of retention times by making an adjustment to the inlet pressure at four predefined pressures ±10% and ±20% of the target pressure of the original method [53]. An automatic sampler was used to make the five direct injections of 1 µL of a 15 ppm nicotine MeOH solution into the GC/MS at the four predefined pressures and the target pressure. The software selects

and integrates the five nicotine peaks and plots their retention times with respect to the associated pressures in a calibration curve. The curve can then be used to correct the locked method to ensure proper confirmation and identification of the target analyte under circumstances of instrument drift. The retention times were 9.404, 8.906, 8.724, 8.485, and 8.282 minutes respective to pressures of negative percentage to positive percentage difference.

2.0 Analyte Calibration and Retention Time Determination

The target analytes were examined by their retention time for the correct identification of the compound in a biological sample. The target analytes for the proposed experiments were nicotine (NIC) and ∆9-tetrahydrocanabinnol (THC).

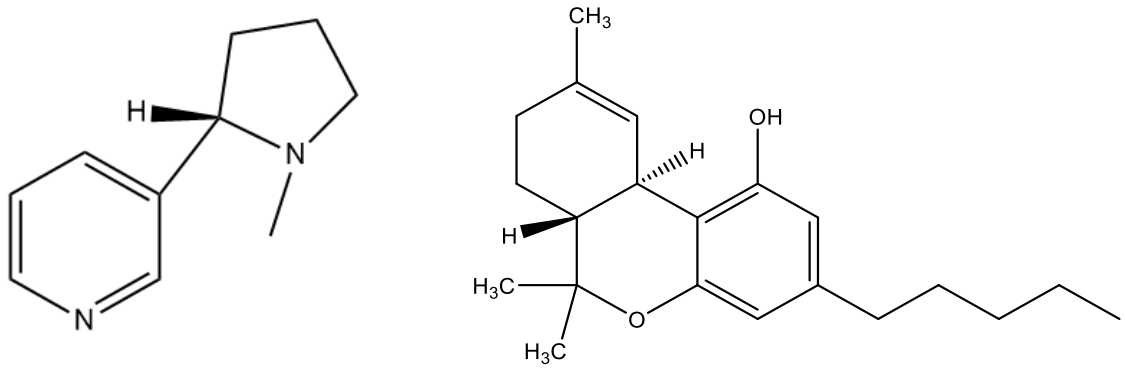

Figure 5: Chemical structure of Nicotine (left) & THC (right).

2.0.1 NIC

Experiments of 10 ppm nicotine MeOH solutions were used to examine the retention time of both a liquid and CMV method. The retention time of the direct liquid injection of the standard nicotine was 8.745 min over a range of 8.72-8.90 minutes for SIM mode. The retention time of the nicotine liquid standard spiked onto the CMV was 8.732 min over a range of 8.70-8.80 minutes for SIM

mode. Analyzing the data of the mass spectrum of a 10 ppm nicotine solution spiked onto a CMV seen in Figure 6 reveals a molecular ion, M+, of 162 with characteristic fragments of m/z 84 and 133.

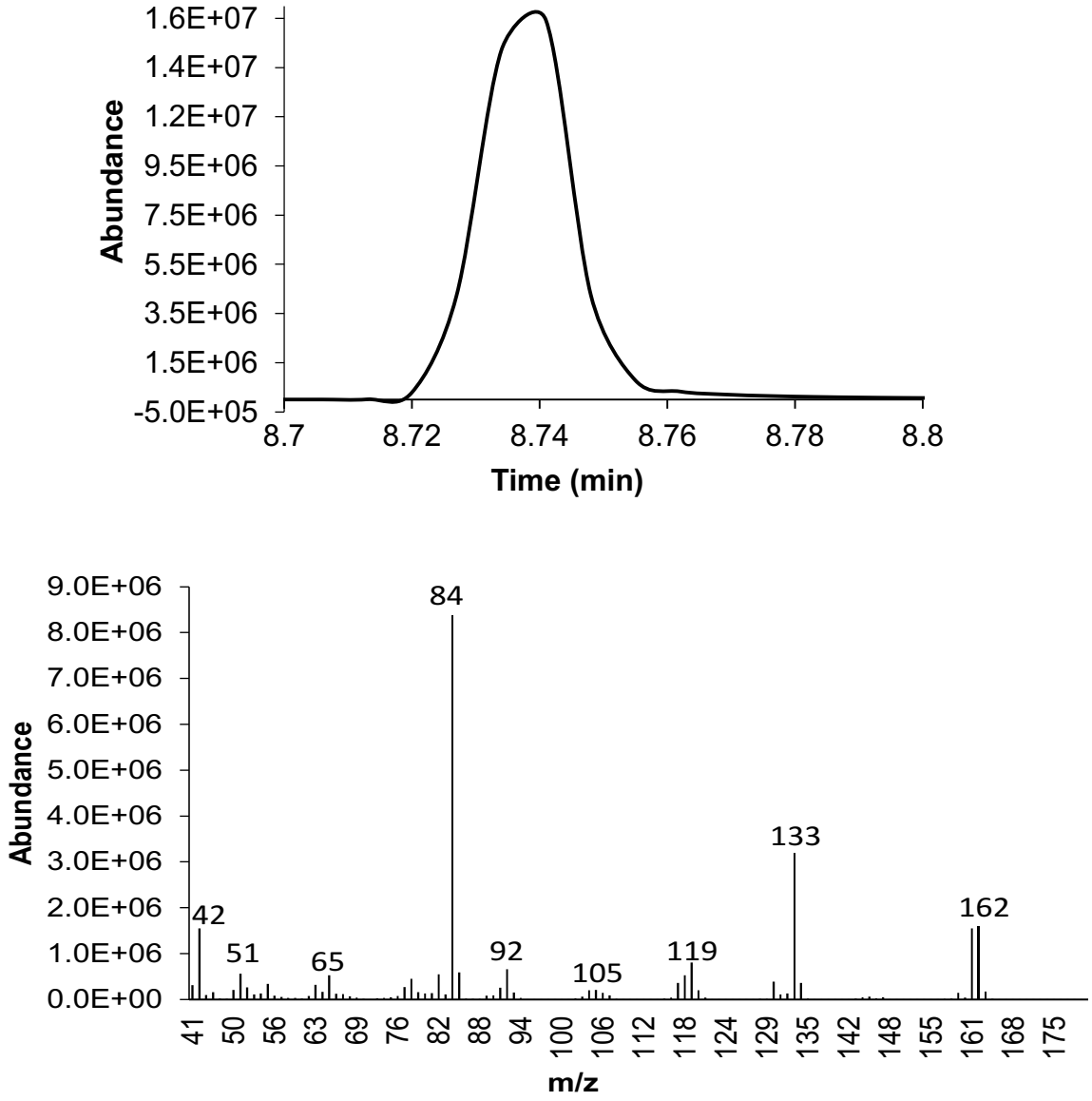

Figure 6: Chromatogram of SIM data (top) and mass spectrum (bottom) of a 10 ppm, 1 µL liquid nicotine solution spiked onto CMV.

The response of nicotine in the GC/MS was examined by calibration for both direct liquid injection and CMV spike introduction. The concentrations of

nicotine examined in these experiments reflected a range that would be representative of low levels that would be expected in exhaled breath samples [24, 33, 54]. Seven nicotine MeOH mixture calibration points were examined: 10, 50, 100, 200, 300, 400, 500 ppb. The seven calibration solutions were loaded into an auto sampler for a 1 µL direct liquid injection of each sample into the GC/MS for liquid calibration determination. For CMV calibration, the same solutions were used to spike 1 µL onto a CMV before manual introduction into the GC system via a thermal desorption probe. The CMV's spiked end was noted for correct orientation during introduction into the GC/MS system. For each calibration instance, triplicate chromatographic runs of the solutions were made for each of the seven calibration points. Data acquisition was done by collecting both SIM and fullscan data, however data analysis occurred for SIM data only. The three response replicates of the SIM peak AUC were averaged and plotted against concentration for a linearity examination. Upon examination of the nicotine curve, points 10 and 50 ppb were removed as they deviated from the linearity of the curve. The remaining five points of the nicotine curve gave a linear response for both liquid (R^2 = 0.9879) and CMV (R^2 = 0.9806) employed methods as shown in Figure 7. The relative standard deviations (RSDs) for the majority of all liquid calibration nicotine mixture concentrations were less than 5%, except for the 400 ppb solution which had 19% error. The RSDs for all CMV calibration nicotine mixture concentrations were less than 9%. The equations of the line and R-squared values for nicotine liquid and CMV calibration data are

presented in Table 1. Equations can be used for concentration determination of unknown nicotine presence in breath samples.

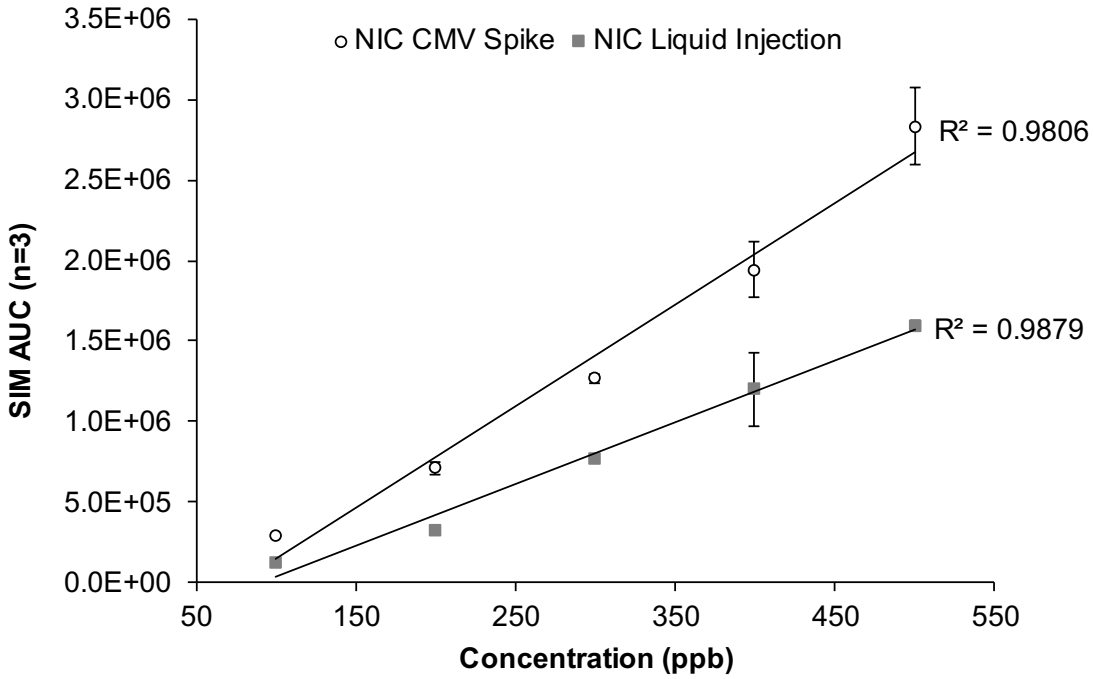

Figure 7: Calibration curves of 1 µL liquid nicotine standard MeOH solutions injected (square) and 1 µL liquid nicotine standard MeOH solution spiked onto CMV (circle).

2.0.2 THC

Experiments of 10 ppm THC MeOH solutions were used to examine the retention time of both a liquid and CMV method. The retention time of the direct liquid injection of the THC standard was 15.874 min over a range of 15.80-15.90 minutes for SIM mode. The retention time of the THC liquid standard spiked onto the CMV was 15.876 min over a range of 15.82-15.96 minutes for SIM mode. Analyzing the data of the mass spectrum of a 10 ppm THC solution spiked onto a

CMV seen in Figure 8 reveals a molecular ion, M+, of 314 with characteristic fragments of m/z 231, 271, and 299.

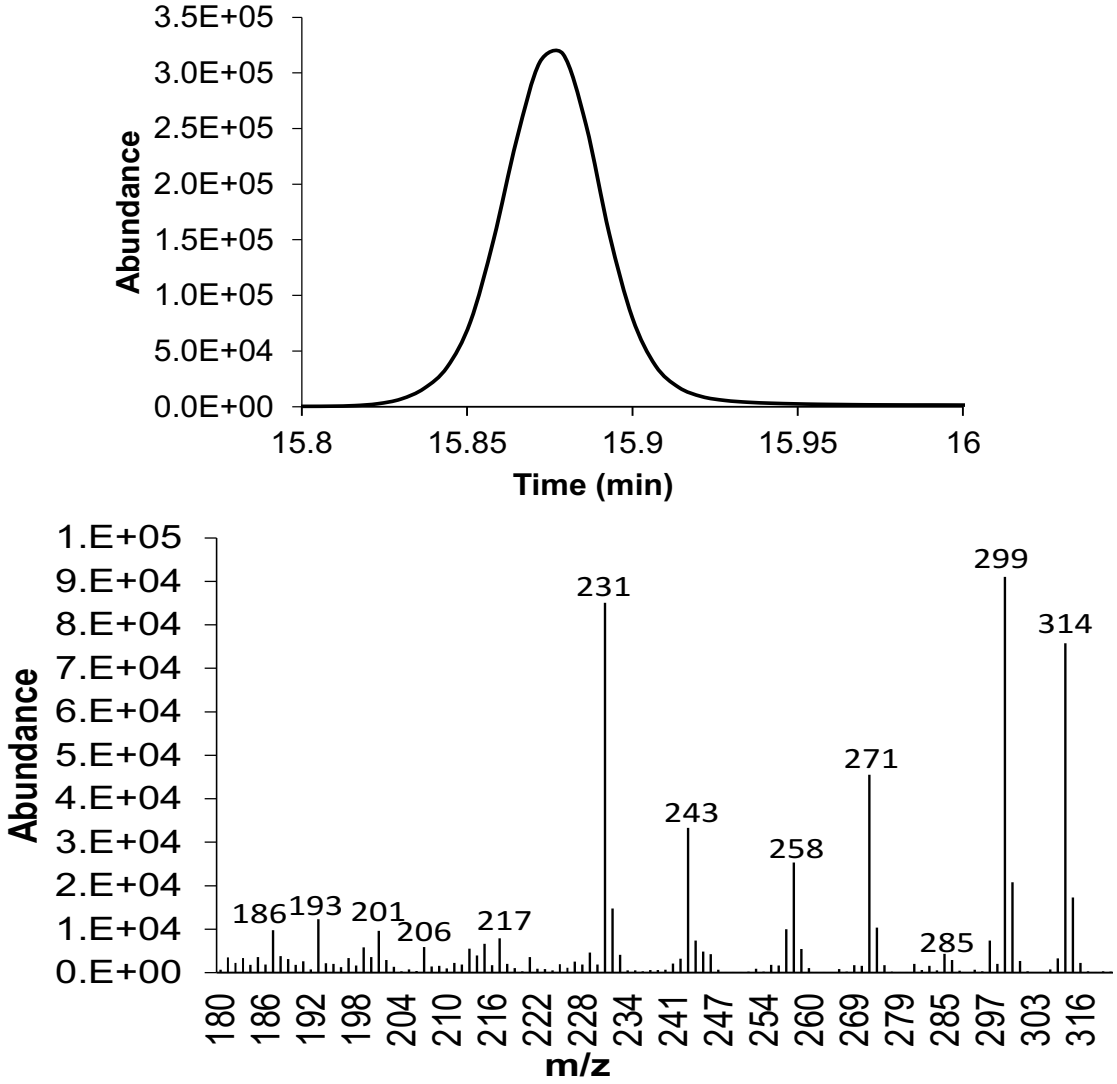

Figure 8: Chromatogram of SIM data (top) and mass spectrum (bottom) of a 10 ppm, 1 µL liquid THC solution spiked onto CMV.

The same procedures were used in the examination of the response of THC in a MeOH mixture for the following calibration points of 50, 100, 200, 300, 400, 500 ppb. The concentrations of THC examined in these experiments reflected a range that would be representative of the low levels that would be

expected in exhaled breath samples [55-58]. The six points gave a linear response for both liquid (R^2 =0.9865) and CMV (R^2 = 0.9798) employed methods as shown in Figure 9. The RSDs for all CMV calibration THC mixture concentrations were less than 10%. The RSDs for the majority of all liquid calibration THC mixture concentrations were less than 9%, except for the 400 ppb solution which had 20.4% error. The equations of the line and R-squared values for THC liquid and CMV calibration data are presented in Table 1. Equations can be used for concentration determination of unknown THC presence in breath samples or marijuana plant headspace samples.

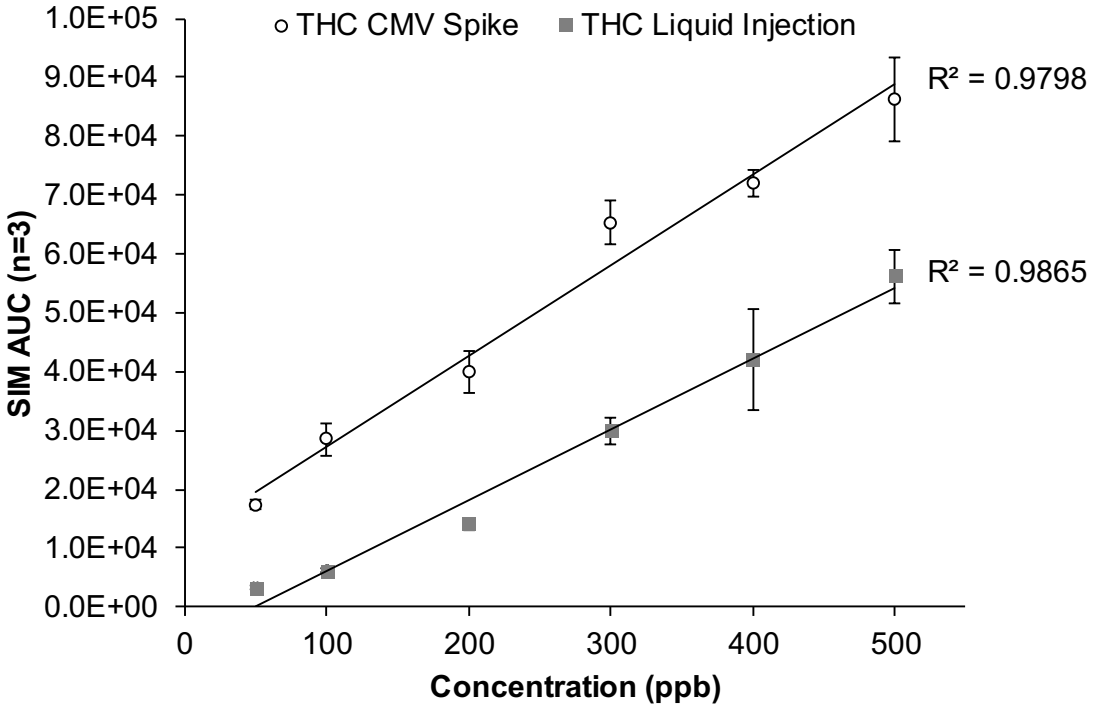

Figure 9: Calibration curves of 1 µL liquid THC standard MeOH solutions (square) and 1 µL liquid THC standard MeOH solution spiked onto CMV (circle).

2.1 Detection Limit Determination

The analytical method detection limit (MDL) and method quantification limit (MQL) of the analytes of interest response on the GC/MS were determined from the linear regression analysis. In the current research, MDL is defined as the lowest amount of target analyte that can be detected above background and the MQL as the statistically valid lowest amount of target analyte that can be detected in a standard free matrix. The MDL and MQL are determined from equations 1 and 2 respectively, where y_B is blank signal and $S_{y/x}$ is the standard deviation in the y-direction of the calculated regression line.

$$MDL = y_B + 3S_{y/x} \quad (1)$$

$$MQL = y_B + 10S_{y/x} \quad (2)$$

3.0 Conclusion

A short suitable method was developed for simultaneous detection of the analytes of interest. A standard calibration was performed for proper identification of unknown samples by comparison of retention time and mass spectra as summarized in Table 1. Further experimentation reflected the methodologies developed in this chapter.

Table 1: Summary of calibration data and figures of merit.

		Equation of the Line	R^2 value	MDL	MQL
Nicotine	Liquid Injection	y = 3822x - 342754	0.9879	61 pg	202 pg
	CMV spike	y = 6326x - 488760	0.9806	77 pg	257 pg
THC	Liquid Injection	y = 120x - 5759	0.9865	68 pg	228 pg
	CMV spike	y = 153x + 12001	0.9798	84 pg	280 pg

4.0 Breath Sampling Analysis

Reliable analysis of exhaled breath samples is dependent upon their sampling materials and sampling protocol. Sampling must be thoroughly controlled.

4.1 Materials & Sampling Device Description

Two sampling methodologies were proposed for use in the current research. The first method directly collected the exhaled breath into a transparent 1 L Tedlar® bag purchased from Zefron International (Ocala, FL) by way of tubing connected to a disposable AlcoQuant mouthpiece purchased from West prime Healthcare (Chino, CA). The sample was dynamically drawn from the bag into the CMV using a Supelco MSA Elf escort sampling pump (Bellefonte, PA) at a set flow rate. The second method utilized a homemade flow through breath collection device (BCD) composed of the same AlcoQuant mouthpiece connected to a modified disposable polyethylene transfer pipette purchased from Fischer Scientific (Hudson, NH) directly coupled to the CMV. The AlcoQuant mouthpiece is equipped with a saliva trap to prevent contamination to the sample. Illustrations of the assembled devices are found in Figure 10.

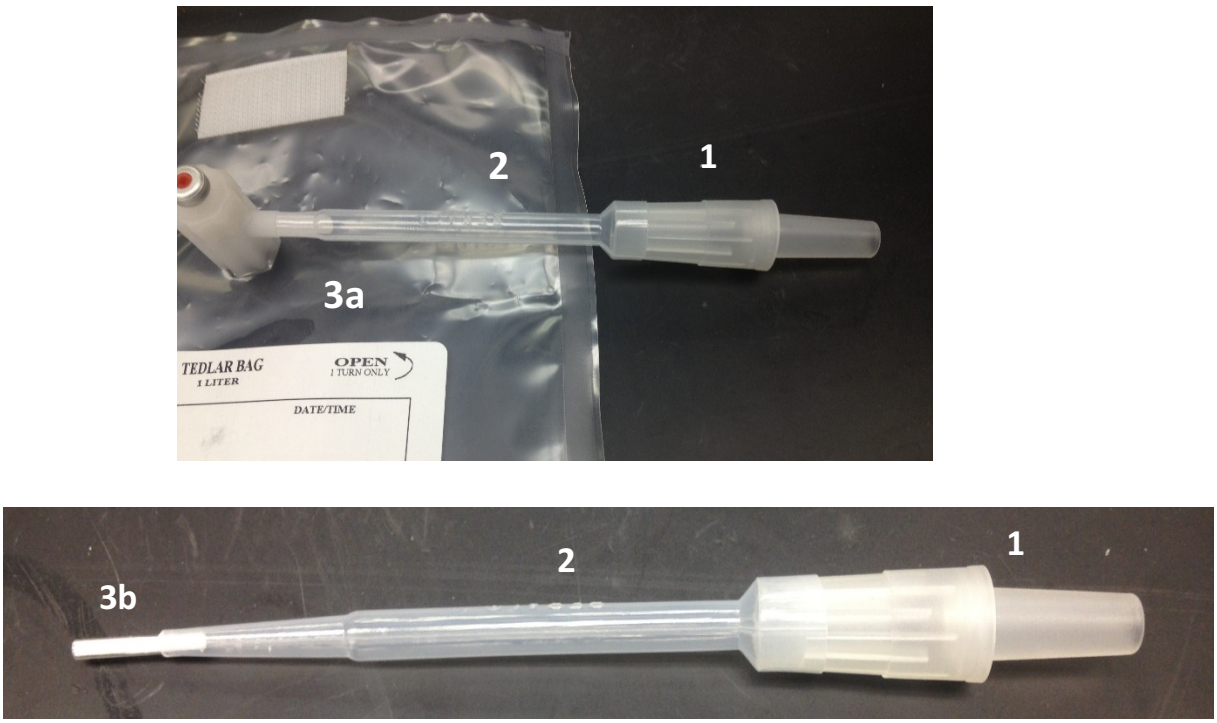

Figure 10: (Top) Bag sampling method (Bottom) BCD sampling method. Mouth piece (1), plastic tubing (2), tedlar bag (3a), and CMV (3b).

4.2 Sampling Protocol

Sampling protocol development explored the differences between collection device type and breath portion sampled on a small sample population of three subjects. Ethical approval for human studies was obtained from the Institutional Review Board (IRB) (No 2015/0157) at Florida International University (FIU). Upon completion of a single exhalation, the average adult with normal and healthy lung function exhales ~500 ml of air [9] which is composed of two portions: dead space and alveolar air. Dead space air exists in the upper airways and nasopharynx consisting of 150 ml of the totaled exhaled breath, while alveolar air from within the lung constitutes the remaining 350 ml portion of the breath [9]. Breath analysis can be performed with mixed breath consisting of

both exhaled breath portions or solely the alveolar portion depending on the expected sources of compounds detected. Since alveolar breath is generally used in research because of its sample reproducibility [22] alveolar samples were collected from the subjects directly into a CMV or into a tedlar bag followed by extraction via pump into a CMV.

During recruitment, subjects were allowed to make an appointment for sample collection in the laboratory. One female reported an age within the 26-35 age range and two males reported in the 18-25 age range were recruited. Details of the study were described to the individuals, stressing the importance of breath sampling occurrence within one hour of a completed smoking session. Any questions regarding research processes were answered and written consent was obtained after participation agreement. Subjects were given a copy of the consent form to keep for their records. Brief voluntary questionnaires were provided emphasizing their anonymity as they were de-identified by a unique code that linked the sample identification code to the collected breath sample but not the consent form. The unique code consisted of a number randomly generated which was coupled to an extension number distinguishing the nonsmoker controls (XX-00) from the smokers (XX-01). Questionnaire answers were used to determine if any documented factor accounted for any variation observed between samples. Sampling session time took approximately 15 minutes.

Before providing samples, subjects were briefly instructed on how the sampling devices worked. Breath sampling consisted of alveolar breath collection

into a 1 L tedlar bag followed by a consistent number of breaths through the homemade flow through sampling device directly connected to the CMV (Figure 10). Each subject was provided with a new device for each sample collection. The total volume of breath collected from each collection device was approximately one liter varying in 4-5 alveolar breaths from subjects. The volatile chemical components of the breath remained adsorbed to the CMV after sampling until analysis. The CMVs were wrapped in aluminum foil and sealed in labeled glass vials and tedlar bags were also labeled accordingly as illustrated in Figure 11. Packaged tubes and filled tedlar bags were stored on the bench top at room temperature only for a few hours (2-3 hours), since analysis could not be performed directly following sampling.

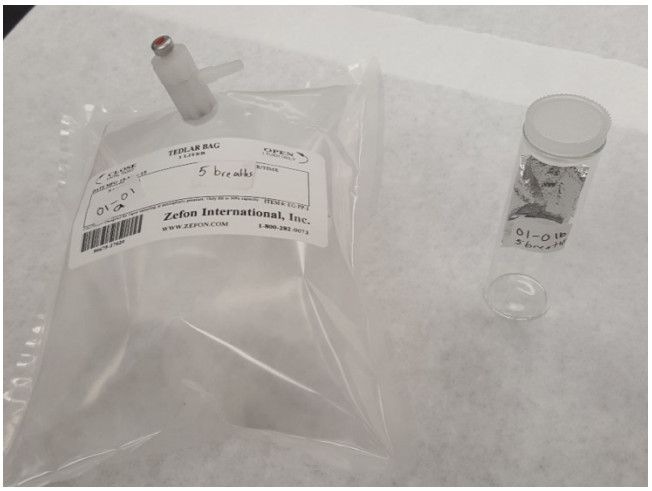

Figure 11: Storage of collected exhaled breath samples by both methods.

Before analysis by GC/MS, breath samples collected in the tedlar bags were extracted into a CMV by a vacuum pump as illustrated in Figure 12. The CMV tube was directly inserted into the GC using a thermal desorption probe.

The heat of the injection port desorbed the absorbed compounds off the CMV and the components were separated by gas chromatography and detected in the mass spectrometer. The peak associated with nicotine was quantitatively analyzed as well as concentration differences between the breath collection devices.

Figure 12: Collection of the exhaled breath Tedlar® bag sample with a CMV connected pump.

Table 2: Summary of the smokers' self-reported questionnaire responses on their smoking habits and summarized sampling protocol for each subject.

Subject ID	Smoke Frequency	Smoke Product	Time since Smoked	Number of breaths	Bag extraction Flow	Bag extraction Time
02-01	3 times/wk	Other	30 min	4	0.2 L/min	3 min
01-01	Daily	Marijuana Spliff + tobacco	15 min	5	0.8 L/min	1 min 15 sec
35-01	Twice Daily	American Spirit (Mellow)	15 min	5	0.8 L/min	1 min 15 sec

4.3 Results & Discussion

Although subjects 01-01 and 35-01 self-reported smoking a tobacco product within the expected 1-hour detection window, nicotine was not detected in either sample. Subject 02-01 self-reported smoking a non-tobacco product so nicotine was not expected to be detectable. Although the non-tobacco product consumed by subject 02-01 was not identified in the questionnaire, his breath profile was examined for THC which was not detected in the breath sample. The remaining compounds shown in the breath profile chromatograms were examined and tentatively identified using the NIST 2008 and WILEY 2008 libraries. An example overlay of a tedlar bag and BCD alveolar breath sample from subjects 35-01 and 01-01 are shown in Figures 13 and 14, respectively. The tentative compound identification of the breath samples is listed in Table 3. The breath profiles contained 30, 31, and 22 compounds collectively between both bag and BCD sampling for subjects 02-01, 35-01, and 01-01 respectively. The relative response of compounds in the tedlar bags were higher than those obtained with the BCD.

Two compounds of intense response were observed only in the tedlar bag samples. The compounds were N, N – dimethyl acetamide and phenol which have both been identified as tedlar bag contaminants [25]. N,N-dimethylacetamide is the solvent used to manufacture the tedlar film used in production of the bags, so it is expected that they will emit this compound [39]. Studies have shown that purging a bag decreases the contaminants

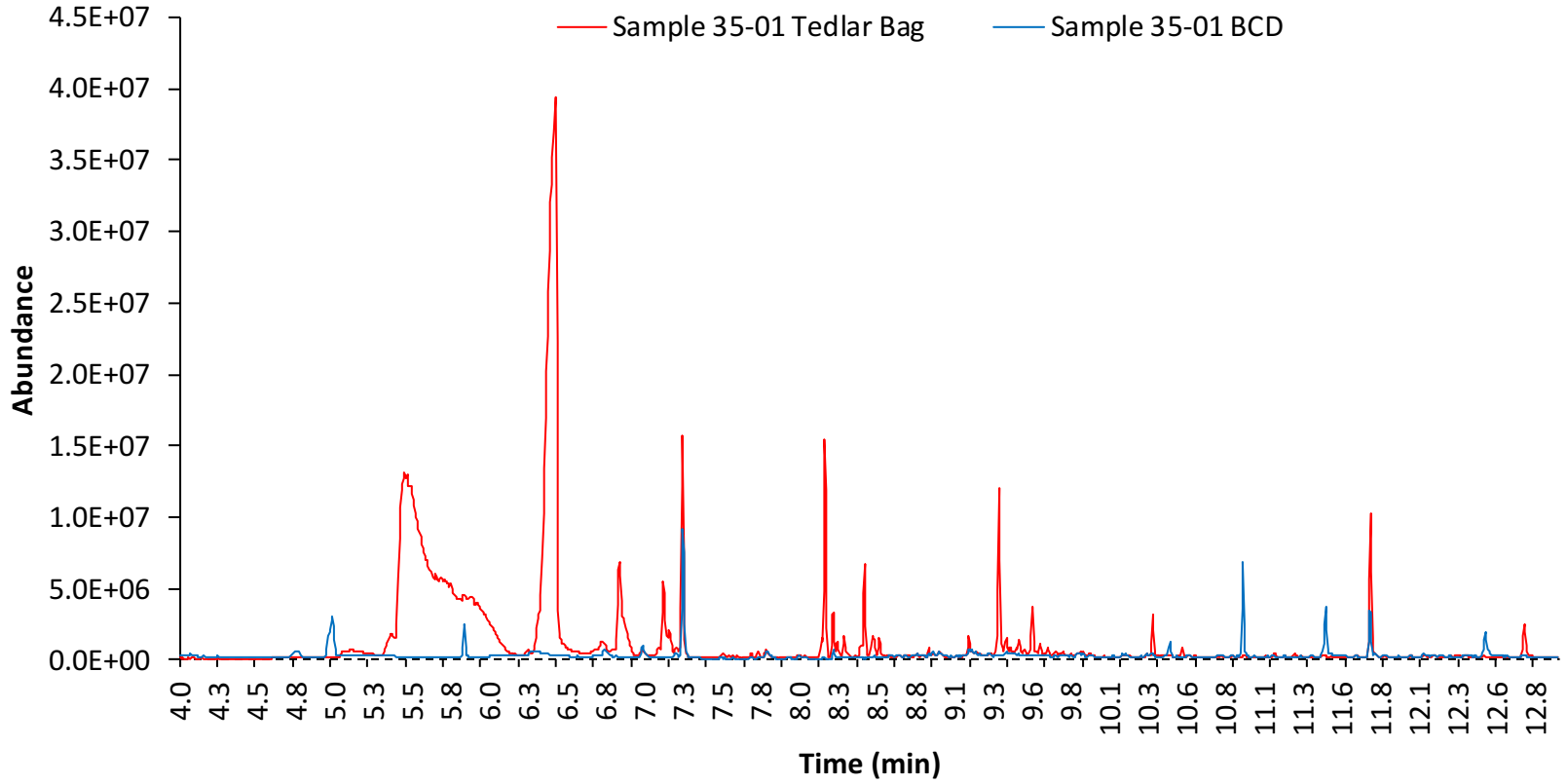

Figure 13: The overlay chromatogram of the exhaled breath profile from subject 35-01 from the bag sampling method (red) and the BCD sampling method (Blue).

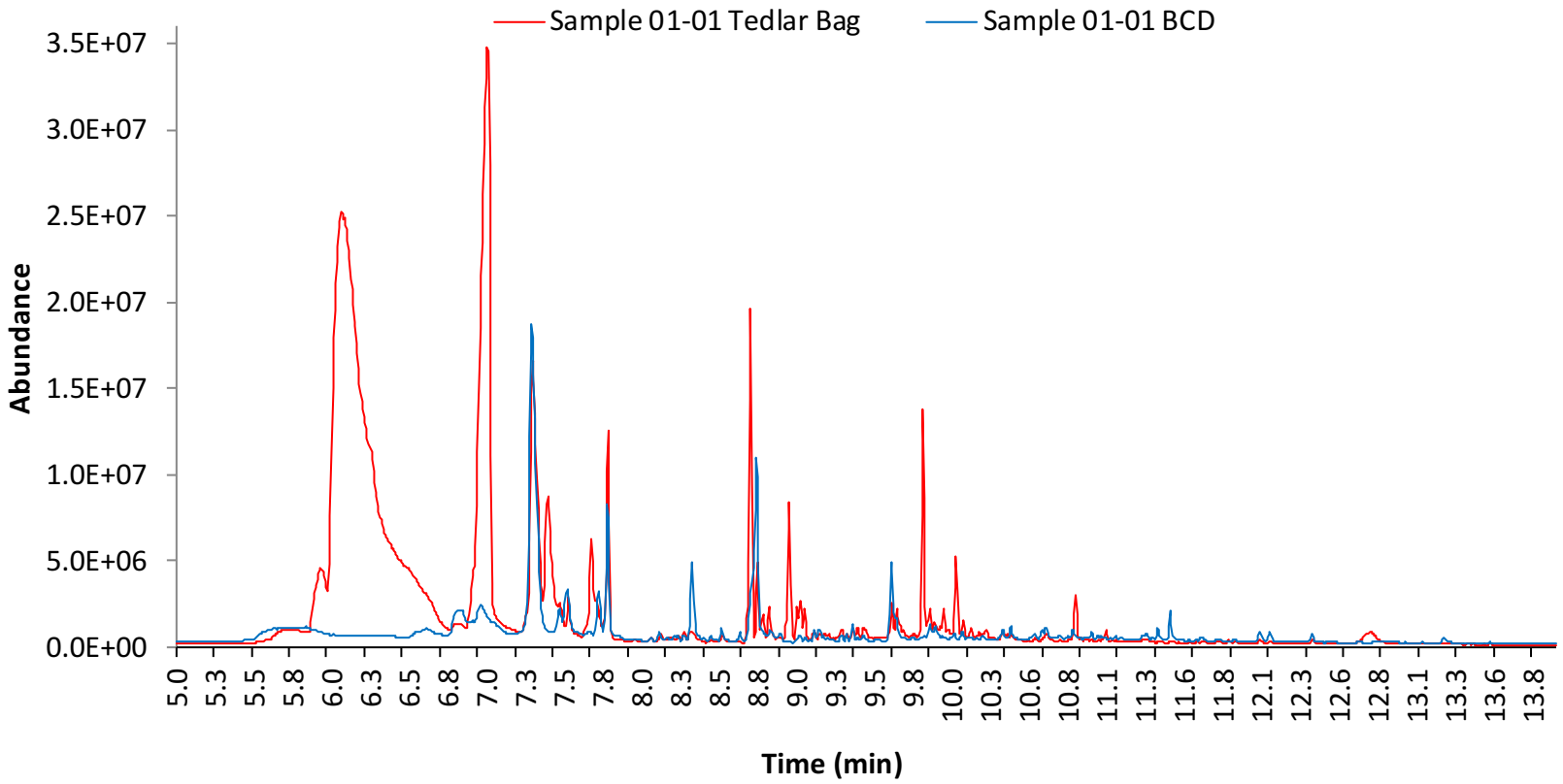

Figure 14: The overlay chromatogram of the exhaled breath profile from subject 01-01 from the bag sampling method (red) and the BCD sampling method (Blue).

by 2 times [39]. However, purging can prove to be a lengthy process as some studies have reported cleaning times up to 2 hours before use [23]. The carry over potential of breath samples in breath analysis using tedlar bags were examined by nitrogen gas flushing after sample extraction into the CMV. Figure 14 shows an overlay plot of the tedlar bag alveolar breath sample of subject 02-01 (Black), and two nitrogen flush occurrences (Red & Blue, consecutively). The first nitrogen flush consisted of a single filling and emptying of the bag into a CMV. The second nitrogen flush occurrence consisted of five consecutive fillings and emptying of the bag into a CMV on the 5^{th} empty cycle. There was a distinct decrease in response by a factor of 2.2 and 2.9 for the two respective contaminant compounds identified in the headspace of tedlar bags after multiple nitrogen flushes. There did not seem to be any significant carry over of the breath samples after cleaning with nitrogen. The bag sampling method may be suitable for reuse after proper cleaning.

4.4 Conclusions

To simplify the sampling protocol, the use of tedlar bags was excluded as their cost, reusability, and portability during sampling would prove problematic for current research. The presence of the two bag contaminants creates blind spots in the analysis because of their intensities [37]. Some studies suggest that contamination can be decreased 5-7 times by heating the bags to release the contaminants followed by subsequent gas purging [39]. However, these lengthy cleaning protocols [23] as well as the need for optimization before extraction

Table 3: Tentative identification of compounds in breath profiles of subjects in the sampling protocol study between the tedlar bag and the homemade CMV breath collection device.

No.	Compound	Subject 02-01		Subject 01-01		Subject 35-01	
		Bag	BCD	Bag	BCD	Bag	BCD
1	2,4 – dimethyl- heptane					✓	
2	N,N-dimethyl Acetamide	✓		✓		✓	
3	3-Phenylindole				✓		
4	α-pinene				✓		
5	phenol	✓		✓		✓	
6	D-Limonene			✓	✓	✓	✓
7	7,9-dimethyl hexadecane					✓	
8	1-methyl-4-(1-methylehtyl)-1,4-cyclohexadiene			✓	✓		
9	3,6 – dimethyl decane					✓	
10	Benzyl Alcohol	✓	✓				
11	undecane	✓	✓	✓			
12	nonanal	✓	✓		✓		
13	cyclododecane					✓	
14	Octanoic Acid	✓	✓				
15	2-(2-butoxyethoxy)-ethanol	✓					
16	dodecane	✓	✓	✓	✓	✓	
17	decanal	✓	✓		✓	✓	✓
18	napthalene					✓	✓
19	2-phenoxy-ethanol	✓	✓				
20	nonanoic acid	✓	✓		✓		
21	2,7,10-trimethyl-dodecane			✓			
22	caprolactam	✓	✓		✓		
23	tridecane	✓	✓	✓	✓		
24	2,6,11-trimethyl-dodecane			✓		✓	
25	2,2,4,4,6,8,8 - heptamethyl nonane			✓			
26	3,7-dimethyl decane					✓	
27	tetradecane	✓	✓	✓	✓	✓	✓
28	caryophyllene			✓	✓		
29	Hexadecane			✓	✓		
30	pristane					✓	

Table 3 (Continued on Next page)

Table 3 (Continued)							
31	3,7,11-trimethyl-1-dodecanol						✓
32	9-Eicosyne						✓
33	2,6,10,15-tetramethyl-heptadecane			✓			
34	(Z)-6,10-dimethyl-5,9-undecadien-2-one	✓	✓				
35	pentadecane	✓	✓				
36	butylated hydroxytoluene			✓			
37	Dodecanoic Acid	✓					
38	heptacosane			✓			
39	diethyl phthalate	✓	✓	✓	✓		
40	heptadecane	✓	✓	✓	✓		
41	Benzyl benzoate	✓	✓				
42	isopropyl myristate	✓	✓		✓		
43	Tetradecanoic Acid	✓					
44	Pentadecanoic Acid	✓					
45	nonadecane	✓	✓				
46	Cis-9-hexadecanoic acid	✓					
47	n-hexadecanoic acid	✓	✓	✓	✓	✓	✓
48	dibutyl phthalate				✓		
49	eicosane	✓	✓				
50	isopropyl palmitate	✓	✓	✓	✓	✓	✓
51	heneicosane	✓	✓			✓	✓
52	squalene			✓			
53	Docosane						
54	octadecanoic acid			✓	✓		✓
55	Bisphenol A					✓	✓
56	tricosane	✓				✓	✓
57	benzyl butyl phthalate				✓		

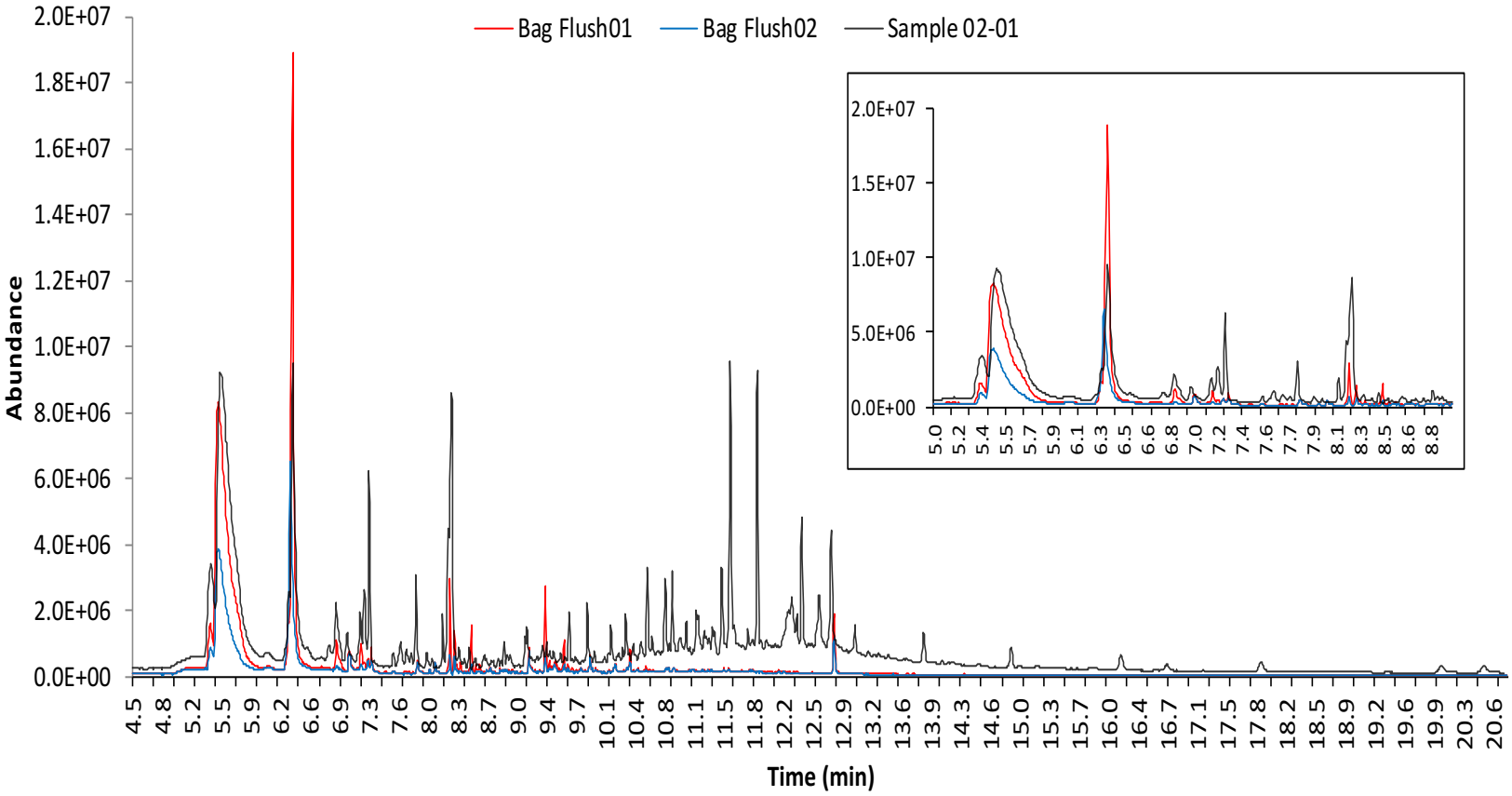

Figure 15: The overlay chromatogram of the exhaled breath profile from subject 02-01 from the bag sampling method (black) and Nitrogen flush #1 (red) and nitrogen flush #2 (blue). Inset of overlay at 5.0-9.0 minutes.

would not be feasible for the short and impromptu sampling periods of this research. The exclusion of the tedlar bag also lifts the restriction of the volume of breath collected. The sole use of the BCD can allow for increased volume and number of breaths which may be more appropriate for the detection of the target analytes. Further simplification of the sampling protocol excluded the mouth washing stage before sampling, as well as changing the breath portion collected. Research examining exogenous compounds are likely to use mixed breath, since air in the airways mostly resemble environmental condition exposures [23]. The breath of smokers is likely to contain more exogenous compounds, so the collection of mixed breath (whole breath including dead space and alveolar air) was preferred over alveolar space breath alone. In summary, the new sampling protocol consisted of multiple mixed whole breaths through the BCD approximating to a volume of 5 liters or 10 breaths according to a study that proved that 23% of total mass exhaled particles are detectable after 10 exhalations, suggesting a washout time [59, 60].

CHAPTER 4: APPLICATIONS OF BREATH ANALYSIS

Recent interests in breath analysis have popularized applications in fields other than the medical field.

1.0 Breath Analysis of Narcotics

The analysis of breath has become an attractive technique for the medicinal screening of diseases such as lung cancer [36, 41] and has recently been considered as a potential tool in drug detection [61] considering its noninvasive approach to sample collection**.** Additional attractive advantages of breath analysis are its ease, ability to be performed anywhere**,** and low cost per sample. The conventional methods for drug testing involve sampling blood, urine, saliva, sweat and hair. Matrix selection for drug testing is dependent upon the desired result as differentiating detection windows are observed. Some matrices, like hair, can reveal a history of drug use, while others like breath reveal recent drug use. Studies have reported similarities in volatile compounds identified in urine and plasma to those in breath [35], however some drugs may be more readily measured in breath than in blood [7].

Various volatile signatures of drugs of abuse and their metabolites have been detectable in breath. Beck and associates examined amphetamine, methamphetamine, 3,4-methylenedioxy-methamphetamine, codeine, 6-acetylmorphine, cocaine, benzyolecgonine, methadone, buprenorphine, diazepam, oxazepam and THC [61]. Another study also examined cocaine and morphine, but additionally looked at fentanyl, sulfentanyl, naloxone, norfentanyl,

nicotine, and γ-butyrolactone [62]. Although multiple examples of drugs of abuse have been demonstrated as detectable in breath, the two drugs that will be further reviewed in detail are THC and nicotine.

1.1 THC Breath Detection

The first studies of marijuana detection in breath were conducted in the 1970's and have since then not been considered as a highly desirable noninvasive method of detection for marijuana usage [55, 58, 63]. Developments in the field of marijuana drug of abuse testing focused on drug detection in blood, urine and most recently oral fluid [64-66]. It was not until around 2010 that researchers at the Karolinska Insitutet began to further explore breath analysis for marijuana detection specifically. Several publications have expanded the available knowledge of THC and its metabolites' concentrations in both breath and air [51, 52] from smoking and passive smoking studies.

Previous studies have determined the relationship between THC breath concentrations and windows of detection after smoking. Research by Valentine and associates were the first to examine the presence of THC in breath after cannabis smoking in 1979. Using a polyethylene foam wafer breath entrapment device followed by solvent extraction and High Performance Liquid Chromatography – Mass Spectrometry (HPLC-MS) analysis an average concentration (n=6) of 11.2 ± 6.21 ng of THC in breath was detected 1 to 2 hours after smoking [58]. A 1983 study by Manolis and colleagues compared THC contaminated breath by Tenax® sorbent tube and solvent collection methods 20

minutes after cannabis smoking. The Tenax® desorption method by GC/MS obtained the highest concentration (n=1) of THC in breath at 8.1 ng before correction for losses, which was double that absorbed for the solvent method [55]. Despite the previously demonstrated benefits of thermal extraction using tenax® sorbent tubes, the more recent studies of cannabis user breath were achieved by solvent extraction of breath samples collected onto filter pads, followed by liquid chromatography analysis. In 2011, Beck and associates reported detectable levels of THC in breath following 1 (n=7) and 12 (n=1) hours after smoking at 180-773 pg and 90 pg, respectively. In 2013, Himes and associates classified detection windows by occasional and chronic marijuana smokers. Occasional marijuana smokers had a narrower detection time of 0.5-2 hrs with a median range (n=11) of 61.0 pg per filter pad of THC detected, compared to chronic marijuana smokers who had a detection window of 0.5-4 hrs with a median range (n=13) of 94.8 pg per filter pad of THC detected [57].

1.2 Nicotine Breath Detection

Studies of nicotine detection in urine [67], sweat and saliva [68] have been used for therapeutic applications of health insurance screening and smoking cessation programs [69]. Nicotine detection in breath has been considered in environmental applications investigating environmental tobacco smoke (ETS) and its impact on air quality of enclosed spaces [70]. The harmful constituents of tobacco smoke [71] and electronic cigarette (e-cig) vapor [72] are publicly reported and efforts measuring their concentrations for occupational workplace

ETS exposures [45, 73] have previously been examined using well developed sorbent based sampling and thermal desorption analytical techniques [43].

Environmental tobacco smoke pollution is the result of second hand and third hand smoking. Second hand smoking is characterized by mainstream smoke inhaled and subsequently exhaled by the smoker and sidestream smoke of the burning cigarette [70]. Third hand smoking is characterized by residue desorption of tobacco related VOCs absorbed into cushions, curtains, clothing or from a smoker's breath after smoking has ceased [34]. Popularization of alternative electronic smoking apparatus' has involved additional contributors to ETS, including e-cig vapor. These studies often research the differences in cigarette smoke inhalation and exhalation using nicotine as an exposure indicator for ETS. Feng and associates studied the Inhalation pattern of 16 smokers to determine the respiratory retention of nicotine while smoking 6 cigarettes each day for 4 days using Cambridge filter pads for collection and GC/MS analysis. The nicotine concentration of exhaled smoker breath after no smoke inhalation, normal smoke inhalation, and deep inhalation was respectively 1.015 ± 0.256 mg/cig, 0.019 ± 0.014 mg/cig, and 0.004 ± 0.002 mg/cig [54]. These investigations of nicotine concentrations in exhaled mainstream smoke [54, 74] are not to be confused with investigations of exhaled breath after smoking.

As smoking habits of individuals strongly influence their exhaled breath [28, 35, 75] have been able to demonstrate that nicotine is detectable in breath following tobacco smoking. Research by Ding and associates in 2009 analyzed 12 breath measurements of smokers' breath (n=2) one hour after smoking by

extractive electrospray ionization coupled to a linear trap quadrupole mass spectrometer (EESI-LTQ-MS) to determine nicotine concentrations of 5.8-7.6 pg/ml [76]. Research by Berchtold and colleagues investigating appropriate mass analyzers and ionization technology for narcotic breath detection were unable to confirm detectable nicotine levels with extractive electrospray ionization coupled to a quadrupole time of flight mass spectrometry (EESI-Q-TOF-MS) technology. However, analysis in this research of exhaled breath after smoking of a single cigarette by atmospheric pressure chemical ionization coupled to quadrupole time of flight mass spectrometry (APCI Q-TOF) was able to detect a concentration of 6.2 ± 0.9 pg/ml of nicotine after 70 minutes [62]. The more recent nicotine exhaled breath studies considered detection after smoking of low dose cigarettes as well as rechargeable e-cigarettes. Research by Marc and associates detected nicotine concentrations of 7 $\mu g/m^3$ and 1 $\mu g/m^3$ for cigarette and e-cig smokers respectively 30 minutes after smoking by tenax® sorbent tube sampling and GC/MS analysis [33]. Real time nicotine exhaled breath analysis by PTR-MS detected nicotine concentrations of 1150 ppb and 7 ppb for cigarette and e-cig respectively after a single exhalation [24, 77]. These research studies examining nicotine concentration during particular detection windows depend on the available amount of nicotine for exhalation.

2.0 Toxicokinetics of Nicotine in Breath Analysis

Breath analysis literature has demonstrated that inhaled drugs can be identified in exhaled breath within an appropriate detection window based on the

drugs' toxicokinetics. Toxicokinetics involves the processes of absorption, distribution, biotransformation and excretion. The toxicokinetic process of nicotine in breath analysis will be reviewed.

2.1 Absorption

Nicotine among many of the other 7,000 chemicals contained in cigarette smoke [70, 71] enter the body by route of the mouth when an individual smokes. The two-phase smoking action includes the drawing of cigarette mainstream smoke into the mouth during the puff of a cigarette and inhaling the smoke into lungs where it is held and mixed then exhaled [78]. The absorption and retention of nicotine as it is inhaled into the respiratory system undergoes two main mechanisms of deposition dependent on the chemical form of nicotine. The two main deposition mechanisms are evaporative gas deposition and particle deposition with evaporation from the vapor phase [79].

In tobacco smoke, nicotine is available in one of two forms depending on the pH of its solution [71, 80]. The unprotonated (lipophilic) free base form favored in basic conditions is semi-volatile and present in gas phase, while the protonated (hydrophilic) form favored in acidic conditions is nonvolatile and present in the particulate phase [71, 79]. Within the tobacco plant leaves, nicotine largely exists in its protonated form [71] and is transferred when inhaled into the body from mainstream smoke on tar droplets, identified as particulate matter [79, 81]. Any unprotonated nicotine in the particulate matter of the cigarette smoke can volatize out into the gas phase, where it undergoes rapid deposition in the

respiratory tract and readily passes into lipid membranes [71, 80]. Evaporation of nicotine from the particle to the vapor phase contributes to a higher percentage of unprotonated nicotine presence in the inhaled puff of cigarette smoke. However, evaporation has saturation limitations and not all nicotine in particulate matter can evaporate without dilution [79].

Nicotine retention in the lung and airways is additionally dependent on the particular smoking device and chemical concentration in the inhaled smoke [70]. Concentrations of commercial tobacco products range from 6-18 mg/g per cigarette [71, 81, 82]. Cigars have been reported to have half the concentration of cigarette tobacco [80]. While e-juice liquids can vary in nicotine solutions of 3-100 mg/ml concentrations.

Nicotine retention studies from cigarette smoking reported an 80-92% rate [74, 81]. Research on e-cig vapor absorption examined by inhalation and mouth hold patterns reported an 86% and >99% retention rate of nicotine delivered into the mouth and lung, respectively [83]. Nicotine mouth retention for a mild cigar was an average of 48%, and an average of 58% for a strong cigar [78].

2.2 Distribution

The distribution of a drug through the body occurs after absorption into the respiratory system. Respiratory system includes an upper and lower airway system and a gas exchange system [84]. As illustrated in Figure 16, the lower airway system is covered with a thin surface liquid, called the epithelial lining fluid (ELF). In the ELF, drugs must diffuse across the alveolar epithelium, the fluid in

the interstitial space and the capillary wall [84]. The drug can then enter the blood stream where it is further distributed. Factors that influence the passage of drugs into the ELF include protein binding, molecular weight, and lipophilicity [84]. The unprotonated form of nicotine is lipophilic and thus rapidly diffuses across the lung membranes for distribution into brain and heart tissues [79, 80, 82].

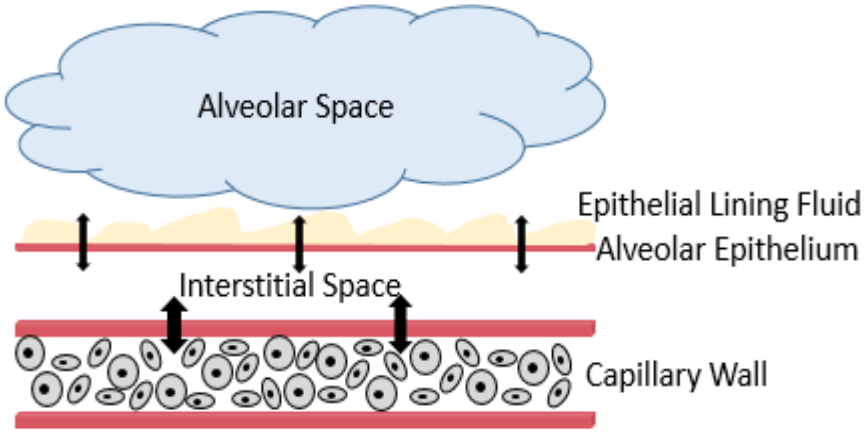

Figure 16: Schematic of a single alveoli of the alveolar capillary system showing the gas and molecule exchange.

2.3 Biotransformation

Metabolism after distribution of nicotine through the blood stream occurs primarily in the liver with secondary metabolism occurring in the lung and kidney [81, 82]. The predominant metabolite of nicotine is cotinine. In most people, 70% to 80% of nicotine is metabolized to cotinine by C-oxidation [70, 81].

2.4 Excretion

Although nicotine primarily undergoes renal excretion, it is also available for excretion through exhaled breath. Nicotine has a clearance half-life of 1.9 hrs from the trachea-bronchial region of the lung [78]. Nicotine is available for

excretion in exhaled breath because during the absorption process of nicotine diffusion into the surface fluid of the epithelium, some of the nicotine vapors are lost [79]. Given this information regarding the availability of nicotine in exhaled breath, studies were able to detect nicotine in breath 20 minutes [28] and even 70 minutes [62] after smoking dosages by cigarette. Even the metabolite cotinine has been reported as detectable in exhaled breath 1 hour after nicotine dosage by cigarette [76].

CHAPTER 5: BREATH ANALYSIS OF SMOKERS USING CMV-GC/MS

The experimental design implementing the newly proposed CMV method for exhaled breath collection for discrimination of smoker and nonsmoker breath by target analyte nicotine is described within this chapter.

1.0 Subjects

Ethical approval for human studies was obtained from the Institutional Review Board (IRB) (No 2015/0157) at Florida International University (FIU). The recruitment of study subjects on FIU campus was opened to individuals of ages 18 to 66 who participated in any type of smoking (i.e., cigarette, e-cigarette, cigar, hookah, etc.). Informational flyers posted around campus as well as in person soliciting assisted in the recruitment of participants. Upon completion of recruitment, 13 self-declared smokers and 7 nonsmokers control gave their written consent to participate in the study. It was determined that 84.6% of smokers were between the ages of 18-25 with the remaining 15.4% between the ages of 26-35 years old. 71.4% of the nonsmoking control participants were between the ages of 18-25 with the remaining 28.6% between the ages of 26-35 years old. To account for variation between subjects, participants were surveyed via a questionnaire to obtain demographic information, smoking habits as well as other potential volatile chemical exposure. Questionnaire responses are summarized in Table 4. No information on the health status of the participating individuals were included in the evaluation of their breath samples. In some

cases, food intake prior to sampling was noted, however volunteer diet was not considered as a variable in this study.

Table 4: Description of the demographics of participating subjects.

		Smoker	Nonsmoker
18-25 years	Male	8	2
	Female	3	3
26-35 years	Male	2	1
	Female	0	1
Total Subjects		13	7

2.0 Materials

The sampling method using the homemade BCD as described in Chapter 3, Section 4.1 was utilized in this case study of the exhaled breath collection of smokers and nonsmokers. Subjects were given new mouthpieces and previously conditioned CMVs. The CMVs were conditioned for 2 hours in an oven at 350˚C and blanked to record their baseline background in the instrument before sampling. The CMVs were wrapped in foil, labeled and stored in a sealed vial before until use. Minor discomfort was reported by some participants as a result of the resistance of the 7 mm opening of the BCD.

3.0 Standard Reagents

Separate experiments of standard compound solutions diluted with MeOH were ran under the CMV and liquid injection methods reported in Chapter 3, Section 1.0 to determine retention time and mass spectra confirmation of compounds identified in exhaled breath samples of smoker and nonsmoker control subjects.

A total of 32 standard compound solutions were available for retention time confirmation. Limonene, α-pinene, β-pinene, dibutyl phthalate, Bisphenol A, octadecane, tricosane, cinnamaldehyde, and octanol from Acros Organics in New Jersey. Nicotine, benzyl alcohol, pentadecane, 1-methylnapthalene, 2-methylnapthalene, naphthalene, phenol, undecane, tetradecane, dodecane, β-caryophyllene, α-humulene, benzaldehyde, nonanal, eicosane, docosane, nonadecane, heneicosane, pristane, and tridecane and from Sigma-Aldrich in Missouri. Linalool, indole, and propylene glycol were from Fluka, TCI America and Flavor Apprentice, respectively.

4.0 Sampling Protocol & Method

The sampling protocol of exhaled breath collection has been previously described in Chapter 3, Section 4.2. One different occurrence during recruitment of subjects were that the option of an onsite sampling session was offered in addition to the opportunity to make an appointment for a later date at the laboratory. Laboratory set up of appointment sampling is displayed in Figure 17, while the kit for onsite sampling is displayed in Figure 18. Breath sampling consisted of the subject performing up to ten prolonged mixed breaths through a homemade BCD (Chapter 3, Figure 10, bottom). To detect possible environmental contamination during onsite collection, field blank CMVs were opened in the sampling location to the environment for passive air sampling during the sampling period, then repackaged and analyzed along with other samples.

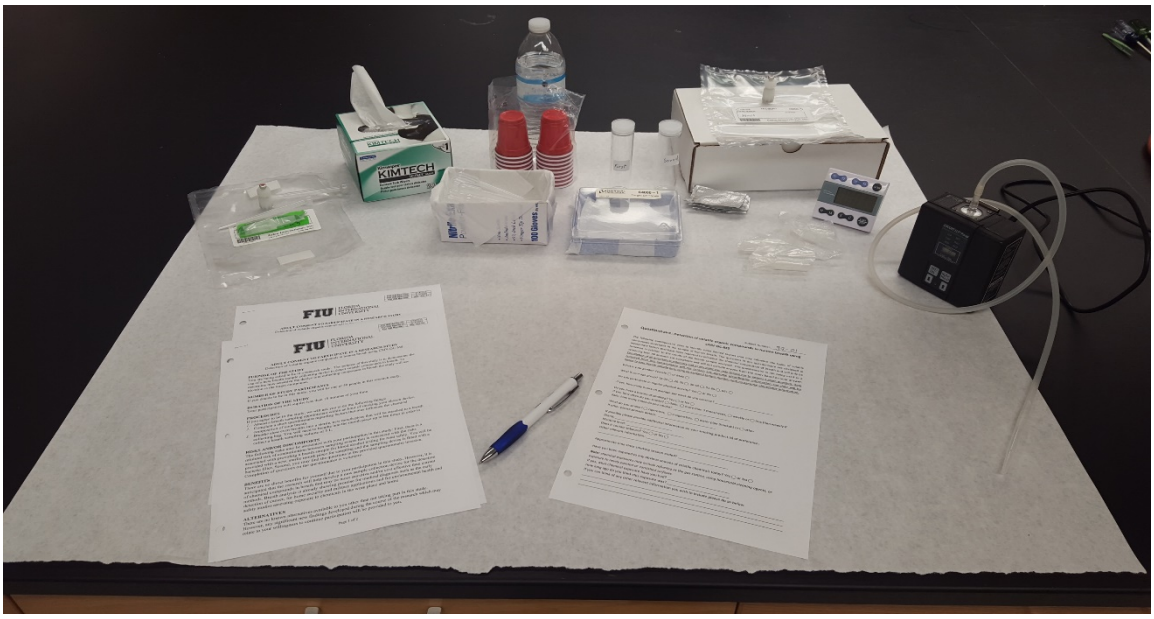

Figure 17: Laboratory set up of the sampling area for participants who made appointments. Pictured are consent forms, questionnaire, CMVs, tedlar sampling bags, pump, mouth piece, timer.

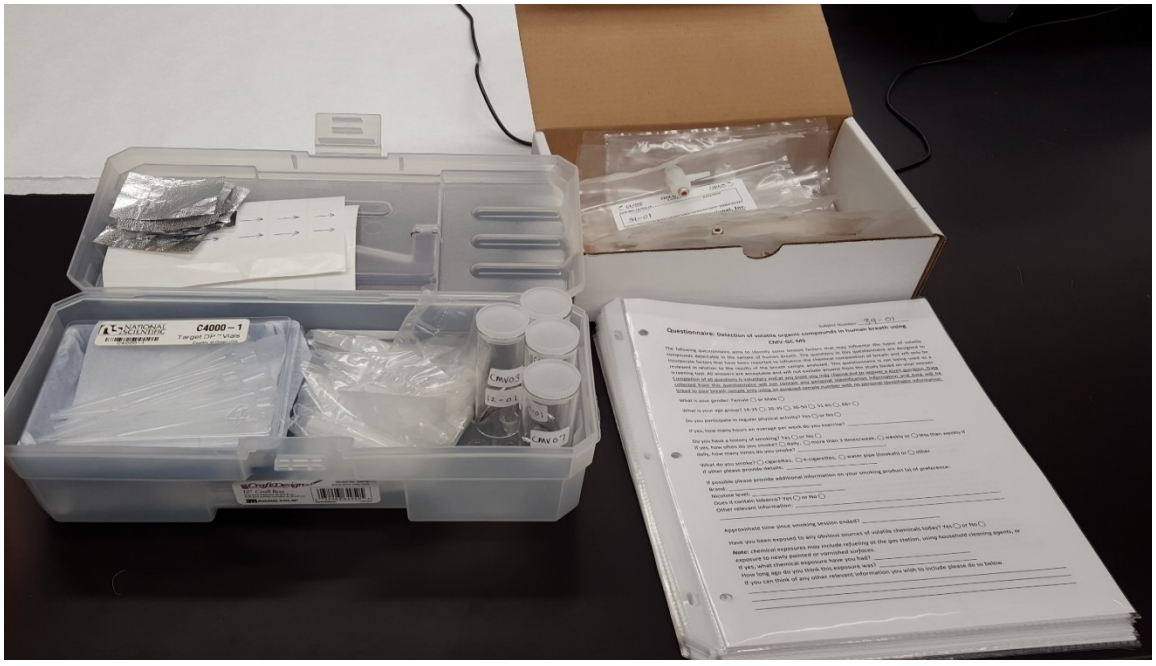

Figure 18: Sampling kit taken for onsite breath sample collection. Pictured are packets of consent forms and questionnaires. CMVs packaged in vials, mouth pieces, connection tubes, and kim wipes.

5.0 Results

5.1 Survey Results

Thirteen of the twenty-one subjects reported that they had a smoking history. Five smokers reported a singular smoking history of only smoking cigarettes in their past while the remaining eight smokers reported a history in at least two products (i.e., cigarettes, e-cigarettes, cigars, hookah, etc.). Eleven of the smokers reported that they smoked a cigarette before sampling, while two others reported smoking an e-cigarette and cigar, respectively. Eleven (84.6%) of the smokers reported that they smoked daily, one (7.7%) smoker reported that they smoked at least three times a week, and the remaining smoker (7.7%) reported that they smoked less than weekly. Those who stated that they smoked daily ranged in 1-15 cigarettes a day with an average of 6 ± 3.58 cigarettes. The following five brands of cigarettes as detailed in table 8 were reported as being the source of nicotine in the breath: Newport, L & M, Camel, American Spirit, and Marlboro.

5.2 Smoker and Nonsmoker Breath Analysis

Chromatographic profiles of each of the subjects exhaled breath were analyzed. Peaks were identified by NIST and WILEY mass spectra library matches as well as retention time and mass spectra comparison of a select number of standard solutions. The combined total of 119 compounds were identified between all subjects' (n=20) exhaled breath. Those 119 compounds consisted of aromatics (17%), alcohols (7%), alkanes (19%), alkenes (3%), aldehydes (4%), amines (2%), amides (1%), carboxylic acids (11%), furans (2%),

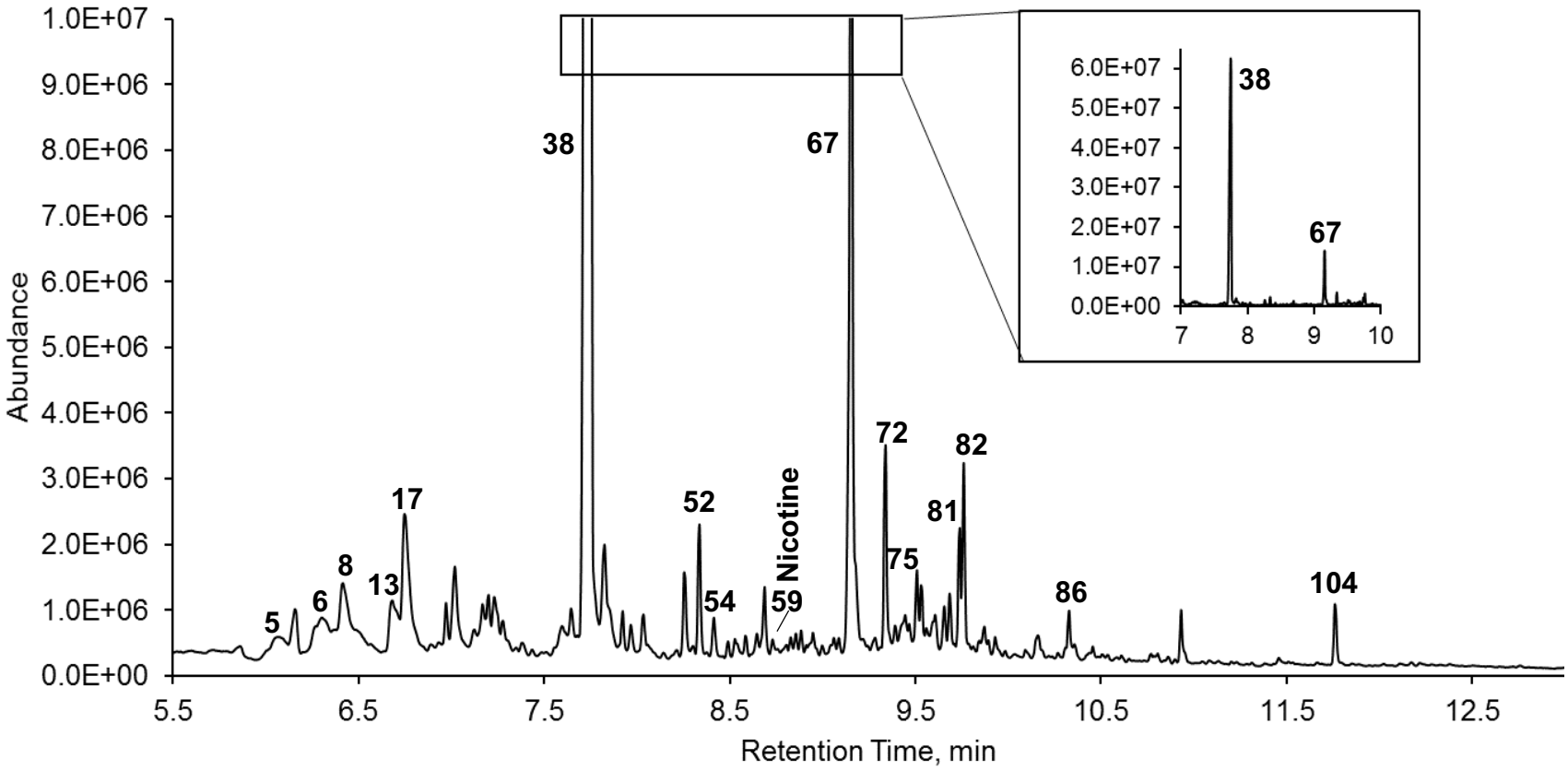

Figure 19: TIC of the exhaled breath profile of subject 27-01, a menthol cigarette smoker, with selected identified peaks numbered from Table 9.

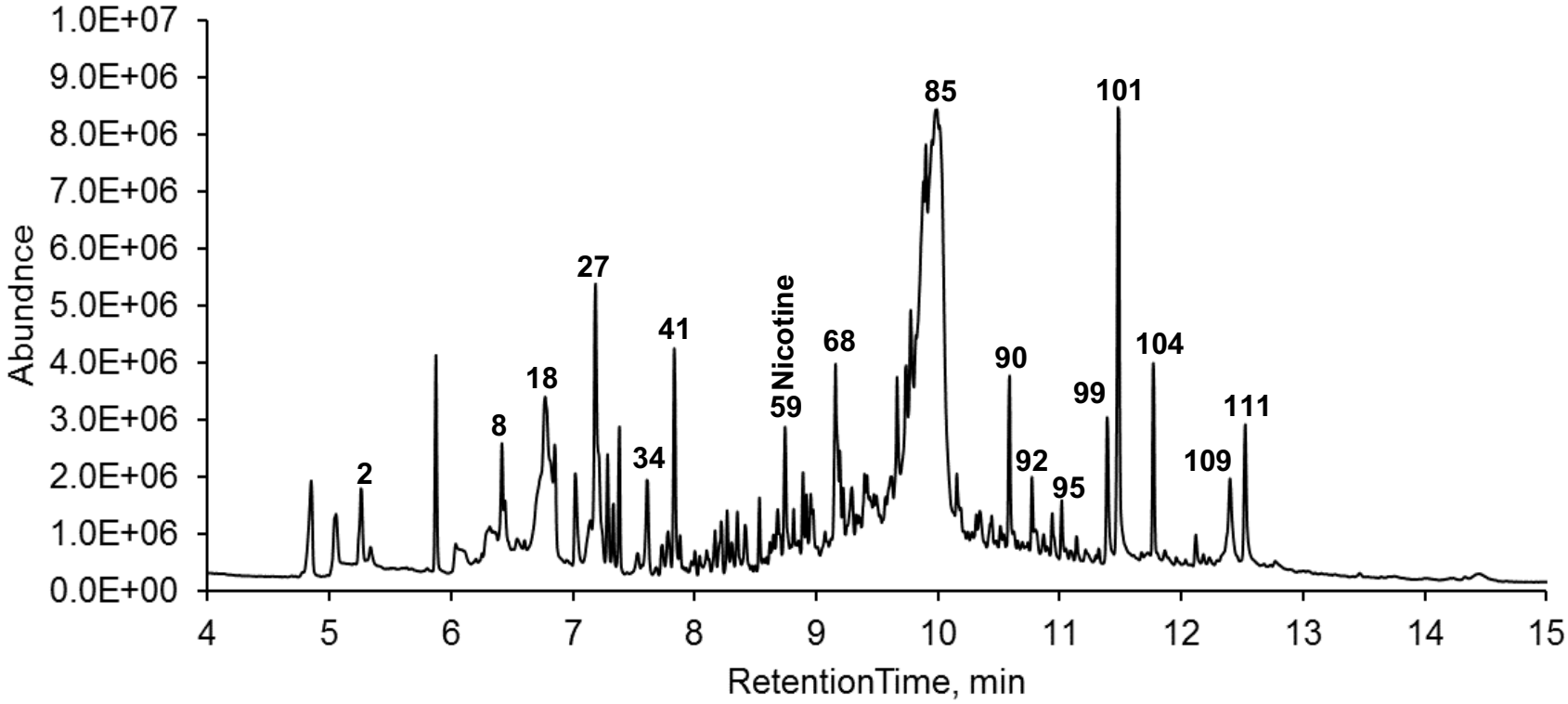

Figure 20: TIC of the exhaled breath profile of subject 36-01, the e-cigarette smoker, with selected identified peaks numbered from Table 9.

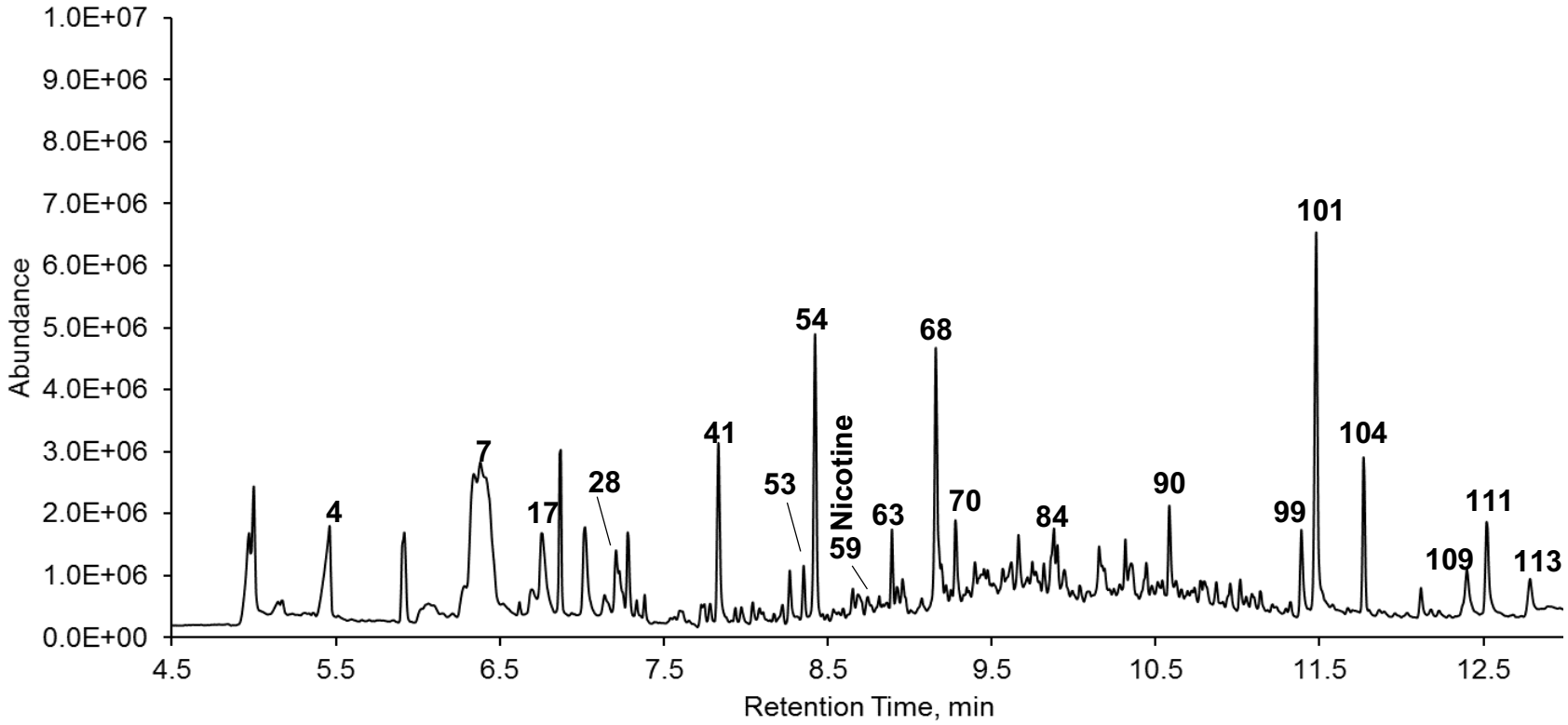

Figure 21: TIC of the exhaled breath profile of subject 44-01, the cigar smoker, with selected identified peaks numbered from Table 9.

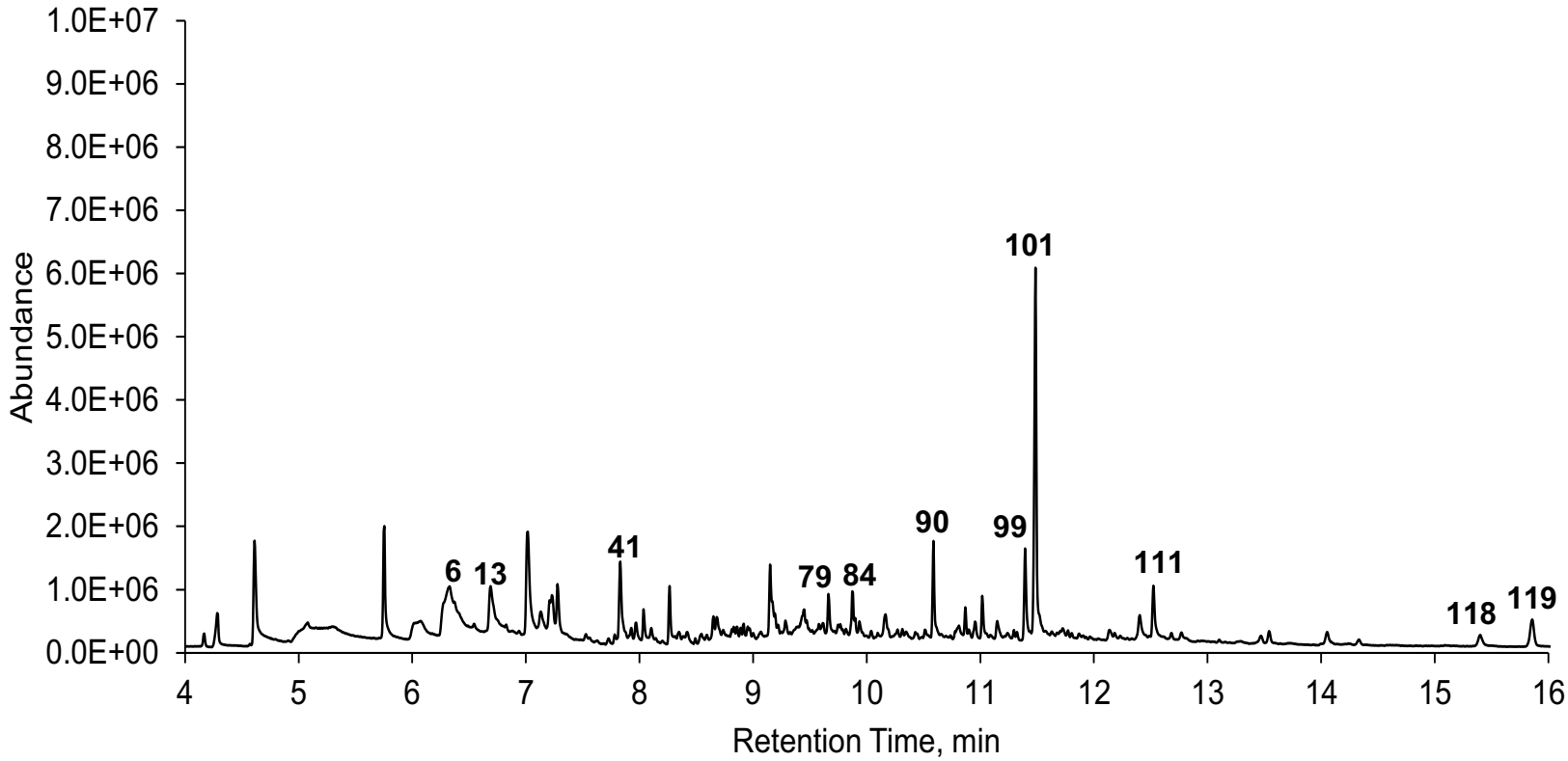

Figure 22: TIC of the exhaled breath profile of subject 28-00, a nonsmoker, with selected identified peaks numbered from Table 9.

ketones (4%), monoterpenes (12%), sesquiterpenes (8%) and triterpenes (1%) which are described in table 7. Cigarette smokers (n=11) collectively totaled 104 compounds, the e-cigarette smoker (n=1) totaled of 32 compounds, the cigar smoker (n=1) totaled 50 compounds and the nonsmokers (n=7) collectively totaled 62 compounds in exhaled breath samples. There were more compounds found in smokers than nonsmokers suggesting that smoking contributes a greater amount of exogenous compounds to the breath profile. The mean total number of compounds identified per smoker subject (i.e., Cigarette, e-cig, cigar) was 42 (RSD ±8, range 28 to 52). The mean total number of compounds identified per nonsmoker subject was 33 (RSD ±4, range 28 to 40). The exact numbers of compounds found per subject are described in table 5. Example total ion chromatograms (TIC) of each smoker device type (i.e., cigarette, e-cigarette, cigar) and a nonsmoker showing distinct peak patterns are shown in Figures 19, 20, 21 and 22 respectively plotted on the same scale. The TIC of the cigarette smoker exhaled breath was more complex, because of the increased chemical components expected in tobacco products as a result of combustion [85, 86]. The varying chemical compositions of the exhaled breath from cigarette smokers depend on the differences in tobacco blend of the cigarette brands used [71].

Total of 119 compounds (32 confirmed by standards) were found and compared across subjects and between samples with 14 of them present at least once in each of the three smoker categories and the nonsmokers. The 14 identified compounds response in each group are shown in Figure 23.

E-cigarette smokers were generally more concentrated in eleven of the ubiquitous compounds: nonanal, dodecane, decanal, tetradecane, 3-ethyl-5-(2-ethylbutyl)-octadecane, tetradecanoic acid, z-7 hexadecanoic acid, n-hexadecanoic acid, isopropyl palmitate, 1-octadecene, and oleic acid. While the cigar smoker was more concentrated in indole, and the nonsmokers were more concentrated in octadecanoic acid, and isooctyl phthalate.

Table 5: Total number of compounds found per exhaled breath subject sample.

Subject	Identifier	Number of Compounds
07-01	S-1	52
38-01	S-2	48
08-01	S-3	45
39-01	S-4	28
12-01	S-5	36
29-01	S-6	37
34-01	S-7	44
06-01	S-8	41
27-01	M-1	44
47-01	M-2	39
25-01	M-3	52
36-01	E-1	32
44-01	C-1	50
26-00	N-1	40
46-00	N-2	34
30-00	N-3	28
48-00	N-4	31
21-00	N-5	35
28-00	N-6	34
09-00	N-7	32

S= Cigarette Smoker, M=menthol cigarette Smoker, E= electronic cigarette smoker, C=cigar smoker, N= nonsmoker

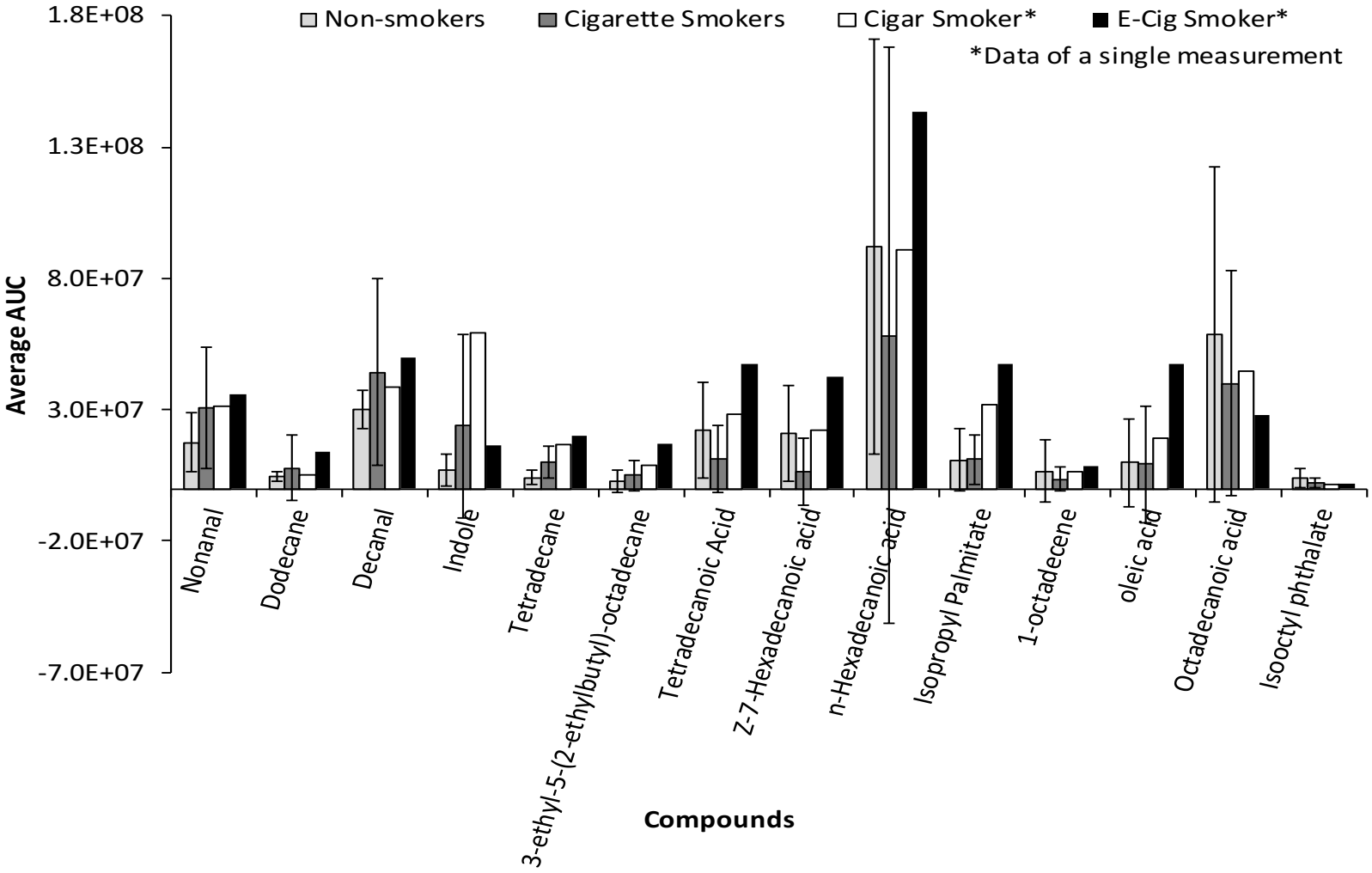

Figure 23: Visual representation of the 14 ubiquitous compounds found at least once in the four groups: Cigarette smokers (dark grey), cigar smokers (white), e-cigarette smokers (black), and nonsmokers (light grey).

5.2.1 Smoker Breath Literature Comparison

Breath analysis research investigating the exhaled breath of smokers and nonsmokers have been able to successfully distinguish between the two groups as a result of key smoking biomarkers. The significant biomarkers associated with cigarette smoking reported in literature are listed in table 6. A few of those listed compounds (i.e., acetone, acetonitrile, isoprene, benzene and toluene) found in a smokers' breath are also found in nonsmokers' breath, but at smaller concentrations [34, 36, 37]. A few of the main VOCs identified in healthy breath are as follows: methanol, acetaldehyde, acetone, isoprene [37, 41] and ethanol [87]. These compounds are identified in both groups as they are endogenous compounds resulting from production within the body. For example, isoprene is a product of the mevalonic acid pathway of cholesterol synthesis and acetone is a product of glucose metabolism [6]. However, the VOCs common to smokers only are likely a result of incomplete combustion of organic matter in tobacco products [71].

Previously reported literature focused on the detection and identification of VOCs in exhaled breath and thus reported lists of low molecular weight compounds with boiling points between 50-150°C. The current research study focused on the specific detection of nicotine in exhaled breath of smokers, and thus the experimental method was out of range to detect the majority of previously reported compounds in breath including the significant compounds associated with smoking. In this research, 11 compounds were found to be consistent with literature reporting exhaled breath profiles of smokers.

Table 6: List of compounds significant to exhaled breath of smokers identified in literature.

Chemical Class	Compound	Literature Source
Aromatic Hydrocarbons	Benzene	[25, 27, 34, 36, 41, 88]
	Toluene	[25, 27, 34, 36, 40, 41]
	Xylene isomers	[27, 36, 40]
	1,3 - cyclohexadiene	[41, 90]
	1,3 - cyclopentadiene	[27, 41, 90]
	Styrene	[36, 90]
	ethylbenzene	[36, 90]
Furan derivatives	2,5 - dimethylfuran	[27, 36, 40, 41, 88, 89]
	Furan	[25, 36, 41, 88]
	2 – methylfuran	[25, 41 88]
	3 - methylfuran	[25, 36, 41, 88]
Saturated Hydrocarbons	2-methyl-1-butene	[41]
	1,4 – pentadiene	[41]
	Butadiene	[27]
	Pentene	[27]
	isoprene	[25, 27, 88]
Unsaturated Hydrocarbons	Butane	[27]
	Octane	[36]
	Decane	[36]
Nitrile	Acetonitrile	[25, 36, 34, 41, 88, 90]
Ketone	Acetone	[27, 34, 90]
Alcohol	2-propanol	[25]

Those compounds of the current study identified in smokers are as follows: propanoic acid [91], propylene glycol [33], α-pinene [91, 92], β-pinene [91, 92], octanal [91], eucalyptol [91, 92], undecane [91, 92], menthone [92], nicotine [8, 33, 35, 54], pentadecane [9] and butylated hydroxytoluene [9]. In this research, 17 compounds were reported in literature as being consistent with exhaled breath profiles of both smokers and nonsmokers. Those compounds were phenol [9, 35], limonene [8, 9, 35], γ-terpinene [9, 91], nonanol [8, 9, 33], dodecane [8, 9], decanal [8, 9, 33], naphthalene [8, 9], 2-phenoxy-ethanol [9, 91], nananoic acid [33], tridecane [8, 9], indole [9], tetradecane [8, 9], (Z)-6,10-dimethyl-5,9-undecadien-2-one [9], diethyl phthalate [9], pristine [8], octadecane [8], and heneicosane [8].

Table 7: Statistical Mann Whitney test results of only the tentatively identified compounds found in >9% of exhaled breath profiles of at least one of the groups: cigarette smokers (n=11) and nonsmokers (n=7).

Class	CAS No.	Compound	Mean Peak Area		P-value
			Cigarette Smokers (n=11)	Nonsmokers (n=7)	Cigarette Versus Non- Smokers
Alcohols	57-55-6	Propylene glycol	0	0	-
	111-90-0	2-(2-ethoxyethoxy)-ethanol	4.46E+06	2.78E+06	0.53601
	100-51-6	benzyl alcohol	0	4.87E+06	0.65899
	4217-66-7	di-2-phenyl-,1,2-propanediol	4.03E+06	0	0.59615
	617-94-7	α-α-dimethyl benzenemethanol	2.08E+06	8.94E+06	0.15089
	122-99-6	2-phenoxy-ethanol	2.29E+06	4.40E+06	0.32830
	2136-72-3	2-(octadecloxy)-ethanol	6.35E+06	3.34E+06	0.86010
Aldehydes	124-19-6	Nonanal	3.08E+07	1.76E+07	0.47894
	124-13-0	Octanal	2.08E+06	0	0.59615
	112-31-2	Decanal	4.43E+07	2.99E+07	0.59615
	104-55-2	cinnamaldehyde	0	0	-
	629-90-3	3-heptadecanal	1.16E+07	4.50E+06	0.10419
	629-90-3	heptadecenal	1.48E+05	5.92E+04	0.86010
Alkanes	1120-21-4	Undecane	4.85E+06	0	0.79141
	1632-70-8	5-methyl-undecane	6.46E+05	0	0.79141
	930-02-9	1-(ethenyloxy)-octadecane	1.26E+06	0	0.59615
	112-40-3	Dodecane	7.84E+06	4.35E+06	0.72423
	629-50-5	tridecane	7.89E+06	3.38E+06	0.21091
	4390-04-9	2,2,4,4,6,8,8-heptamethyl-nonane	2.16E+06	1.87E+06	0.42515
	629-59-4	***Tetradecane***	***9.98E+06***	***4.07E+06***	***0.01137***
	19780-34-8	3-methylene-tridecane	3.48E+06	1.93E+06	0.42515
	55282-12-7	3-ethyl-5-(2-ethylbutyl)-octadecane	5.01E+06	2.66E+06	0.53601
	629-62-9	pentadecane	4.37E+06	0	0.12594
	17312-55-9	3,8-dimethyl-decane	4.91E+06	0	0.37493
	544-76-3	hexadecane	4.81E+06	0	0.59615
	629-78-7	heptadecane	2.07E+06	1.16E+06	0.86010

Table 7 (Continued)

Class	CAS No.	Compound	Mean Peak Area		P-value
			Cigarette Smokers (n=11)	Nonsmokers (n=7)	Cigarette Versus Non- Smokers
Alkanes (cont.)	1921-70-6	pristane	6.25E+06	3.49E+06	1
	593-45-3	octadecane	6.46E+05	6.56E+05	0.92980
	295-65-8	cyclohexadecane	1.19E+07	0	0.12594
	629-92-5	nonadecane	8.31E+05	1.60E+06	0.72423
	112-95-8	eicosane	1.17E+05	0	0.79141
	629-94-7	heneicosane	1.06E+06	4.78E+06	0.86010
	629-97-0	Docosane	5.76E+05	7.30E+06	0.42515
	638-67-5	***Tricosane***	***6.29E+05***	***6.74E+06***	***0.01539***
	646-313-1	***tetracosane***	***5.45E+05***	***6.23E+06***	***0.02677***
	629-99-2	***pentacosane***	***8.70E+05***	***7.37E+06***	***0.04411***
Alkenes	34303-81-6	3-hexadecene	1.11E+07	1.31E+06	0.21091
	872-05-9	1-decene	4.32E+06	7.58E+06	0.42515
	112-88-9	1-octadecene	3.58E+06	6.54E+06	0.37493
Amides	301-02-0	9-octadecenamide	5.18E+05	1.42E+06	0.59615
Amines	**54-11-5**	***Nicotine***	***5.46E+06***	***0***	***0.00829***
	7378-99-6	N,N-dimethyloctylamine	2.47E+06	1.78E+05	0.59615
Aromatics	108-95-2	Phenol	2.95E+07	3.54E+07	0.65899
	106-46-7	1,4-dichloro-benzene	7.02E+06	0	0.21091
	100-45-8	4-cyanocyclohexene	2.82E+07	2.00E+07	0.37493
	141-93-5	1,3-diethyl benzene	6.81E+06	0	0.79141
	150-76-5	Mequinol	0	0	-
	94-71-3	2-ethoxy-phenol	8.07E+05	0	0.79141
	150-78-7	1,4-dimethoxy-benzene	5.53E+06	0	0.06925
	89-78-1	Menthol	6.94E+08	0	0.37493
	3623-52-7	Isomenthol	2.12E+06	1.49E+06	0.53601
	91-20-3	Napthalene	2.53E+06	7.94E+05	0.59615
	104-45-0	1-methoxy-4-propyl-benzene	0	0	-
	95-16-9	Benzothiazole	3.19E+05	1.11E+06	0.86010

Table 7 (Continued)

Class	CAS No.	Compound	Mean Peak Area		P-value
			Cigarette Smokers (n=11)	Nonsmokers (n=7)	Cigarette Versus Non- Smokers
Aromatics (cont.)	120-72-9	Indole	2.38E+07	6.91E+06	0.15089
	90-12-0	***1-methyl-napthalene***	***2.76E+06***	***8.09E+05***	***0.01539***
	91-57-6	2-methyl-napthalene	3.89E+06	1.79E+06	0.24629
	128-37-0	Butylated hydroxytoluene	0	0	-
	88-29-9	7-acetyl-6-ethyl-1,1,4-tetramethyletralin	3.32E+05	0	0.79141
	599-64-4	4-(1-methyl-1-phenylethyl)- phenol	1.22E+06	0	0.79141
	80-05-7	Bisphenol A	2.23E+06	8.34E+05	0.86010
	2882-20-4	2-methyl-3(methylthio)-pyrazine	0	0	-
Carboxylic Acids	79-09-4	Propanoic Acid	0	0	-
	107-92-6	Butanoic acid	0	1.00E+06	0.65899
	79-31-2	2-methyl-2-propenoic acid	0	0	-
	124-07-2	Octanoic acid	1.98E+06	3.70E+06	0.86010
	112-05-0	nonanoic acid	3.63E+06	5.80E+06	0.37493
	143-07-7	Dodecanoic Acid	1.47E+07	1.89E+07	0.72423
	544-63-8	Tetradecanoic Acid	1.11E+07	2.21E+07	0.17911
	1002-84-2	Pentadecanoic acid	3.87E+06	7.67E+06	0.42515
	2416-19-5	Z-7-Hexadecanoic acid	6.40E+06	2.07E+07	0.06925
	57-10-3	n-Hexadecanoic acid	5.83E+07	9.22E+07	0.12594
	112-80-1	oleic acid	9.25E+06	9.72E+06	1
	693-72-1	Vaccenic acid	0	1.43E+07	0.37493
	57-11-4	Octadecanoic acid	2.81E+07	4.01E+07	0.21091
Esters	**103-11-7**	***2-ethylhexyl acrylate***	***1.04E+07***	***5.65E+05***	***0.02042***
	109-21-7	Butyl butylate	1.35E+06	0	0.37493
	84-66-2	Diethyl Phthalate	2.40E+07	1.87E+07	0.53601
	120-51-4	benzyl benzoate	2.75E+06	0	0.37493
	110-27-0	***Isopropyl Mysitate***	***1.81E+06***	***6.31E+06***	***0.02677***
	84-74-2	dibutyl phthalate	3.81E+06	1.98E+06	0.24629
	142-91-6	Isopropyl Palmitate	1.09E+07	1.09E+07	0.72423

Table 7 (Continued)

Class	CAS No.	Compound	Mean Peak Area		P-value
			Cigarette Smokers (n=11)	Nonsmokers (n=7)	Cigarette Versus Non- Smokers
Esters (cont.)	27554-26-3	Isooctyl phthalate	2.05E+06	4.08E+06	0.21091
	5444-75-7	2-ethylhexyl ester benzoic acid	3.51E+06	0	0.59615
	102-20-5	2-phenylethyl ester Benzeneacetic acid	5.13E+05	0	0.79141
	5466-77-3	Octyl methoxy cinnamate	5.95E+05	0	0.59615
Furans	494-90-6	Menthofuran	1.04E+06	0	0.79141
	632-15-5	3,4-diethyl-thiopene	0	0	-
Ketones	409-02-9	6-methyl-5-hepten-2-one	9.43E+06	4.51E+06	0.59615
	10458-14-7	Menthone	1.00E+06	0	0.79141
	1937-54-8	***Solanone***	***1.16E+07***	***0***	***0.00829***
	104-67-6	5-heptyldihydro-2(3H)-Furanone	0	6.04E+05	0.65899
	3796-70-1	(Z)-6,10-dimethyl - 5,9-undecadien-2-one	2.98E+07	1.75E+07	0.65899
Monoterpenes	80-56-8	α-pinene	1.14E+07	0	0.37493
	123-35-3	***β-myrcene***	***4.68E+07***	***0***	***0.00829***
	127-91-3	β-pinene	9.03E+06	0	0.37493
	99-83-2	α-phellandrene	2.51E+06	0	0.59615
	527-84-4	o-cymene	3.07E+06	0	0.59615
	138-86-3	***Limonene***	***2.38E+08***	***7.85E+06***	***0.00119***
	470-82-6	Eucalyptol	4.56E+07	0	0.59615
	99-85-4	γ-Terpinene	2.19E+07	1.33E+06	0.32830
	99-86-5	α-Terpinene	3.90E+06	0	0.21091
	78-70-6	Linalool	0	0	-
	673-84-7	(4E,6Z) – allo-ocimene	1.47E+06	0	0.59615
	76-22-2	Menthacamphor	4.24E+05	0	0.79141
	106-22-9	Citronellol	4.44E+06	0	0.79141
	5392-40-5	***Citral***	***5.47E+06***	***0***	***0.00829***
	2623-23-6	Menthyl Acetate	4.91E+07	0	0.59615
Sesquiterpenes	515-69-5	α-bisabolol	2.74E+05	0	0.79141
	512-61-8	α-Santalene	1.06E+06	0	0.59615

Table 7 (Continued)

Class	Identifier	Compound	Mean Peak Area		P-value
			Cigarette Smokers (n=11)	Nonsmokers (n=7)	Cigarette Versus Non- Smokers
Sesquiterpenes (Cont.)	87-44-5	β-Caryophyllene	2.11E+07	4.39E+06	0.86010
	18797-84-8	β-Farnesene	1.67E+06	0	0.79141
	6753-98-6	α-humulene	5.60E+06	0	0.59615
	109119-91-7	Aromadedrene	1.52E+06	0	0.79141
	177066-67-0	β-Selinene	2.54E+06	0	0.59615
	88-84-6	β - guaiene	7.96E+05	0	0.79141
	489-29-2	β-Maaliene	1.22E+06	0	0.79141
	6813-21-4	Selina-3,7(11)-diene	3.78E+06	3.66E+05	0.86010
Triterpenes	7683-64-9	Squalene	0	0	-

Compounds are organized by their chemical class.
P-values of significant compounds are bolded and italicized.

5.3 Nicotine Quantitation in Smoker Breath Samples

Peaks tentatively identified as nicotine in the exhaled breath profiles of smokers were similar in retention time (8.732 min) and mass spectra to those observed in the chromatograms of nicotine liquid standards spiked on the CMV. The target ion 84 m/z and 133 and 162 m/z qualifier ions of nicotine were selected for ion monitoring of exhaled breath samples as observed in sample of subject 27-01 in Figure 24. Nicotine in breath was quantified from the linear standard curve (R^2= 0.9806) as seen in Figure 7 of Section 2.0.1 in Chapter 3. The nicotine mass extracted from the exhaled breath of the smokers are reported in table 8. Nicotine was detected in 9/13 (69%) of smoker exhaled breath. The relationship between nicotine concentration smoke frequency and sampling time since smoking session ended was not considered in this study.

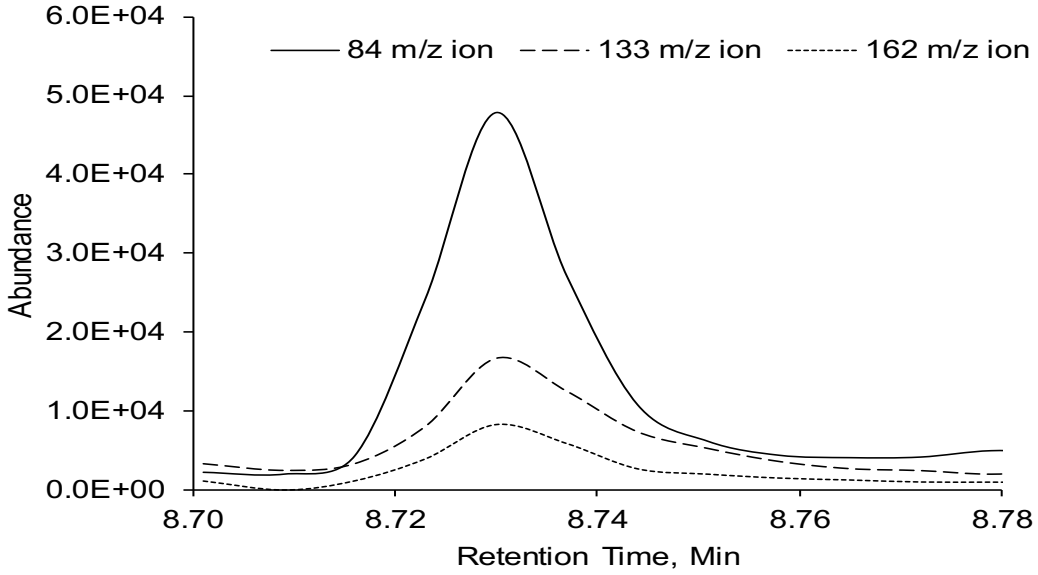

Figure 24: Chromatogram of the extracted ions of nicotine identified in the exhaled breath sample of subject 27-01 at RT= 8.730 min. Presence of m/z ions 84 (line), 133 (dashed line), and 162 (dotted line) confirm nicotine in the sample.

Table 8: Smokers self-reported questionnaire responses describing smoking habits and quantified nicotine response.

Subject ID	Smoke Frequency	Smoke Product	Time since Smoked	Absolute Mass Extracted
39-01	Less than Weekly	Newport cigarette	4 min	n.d.‡
38-01	8 times daily	L&M cigarette	15 min	130 pg
12-01	4 times daily	Camel cigarette	10 min	n.d.‡
36-01	7-10 times daily	E-cigarette	20 min	552 pg
27-01	4 times daily	American Spirit Menthol cigarette	5 min	208 pg
06-01	More than 3 times a week	Marlboro cigarette	60 min	142 pg
08-01	10-15 times daily	Newport cigarette	10 min	131 pg
29-01	Twice daily	Marlboro Red cigarette	10 min	n.d.‡
07-01	7 times daily	Marlboro Red cigarette	5 min	149 pg
34-01	8 times daily	Marlboro Gold Light cigarette	20 min	131 pg
25-01	Once a day	Newport cigarette	2 min	113 pg
44-01	3 times a day	Drew Estate Cigar	30 min	201 pg
47-01	7 times a day	Newport Red cigarette	3 min	n.d.‡

n.d. ‡ = nicotine not detected

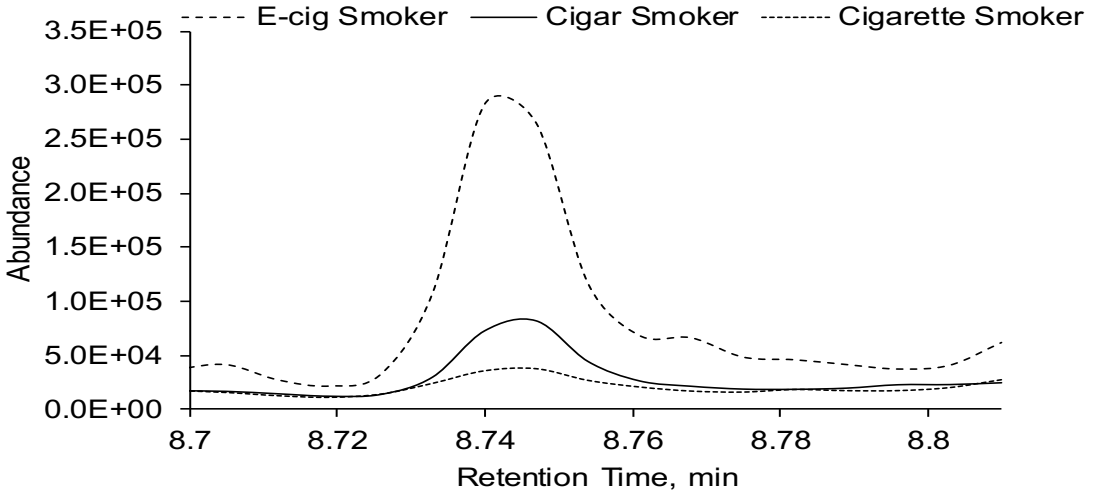

Figure 25: Overlay SIM chromatograms over the retention range 8.7-8.8 minutes for e-cigarette smoker subject 36-01(dashed line), cigar smoker subject 44-01(line), and cigarette smoker subject 08-01 (dotted line). Peak of nicotine in the respective exhaled breath samples shown at 8.745 min.

For cigarette smokers the average amount of nicotine detected was 143 ± 31 pg, compared to 201 pg for a cigar and 552 pg from the e-cigarette. An overlay of the nicotine SIM TIC peaks of an e-cig, cigar and cigarette response are shown in Figure 25. Comparison of the detected nicotine concentrations in the tobacco cigarettes and e-cigarette are not in agreement with previous literature. It is expected that nicotine emissions from tobacco based products should be more than those of e-cigs [24, 77, 86, 93]. Nicotine emission differs by e-cig brand and solution concentrations. The e-cig user of this study smoked from an open system e-cig device that can be refilled, however the solution concentration used was not disclosed in the self-reported survey. Provided the heavily concentrated nicotine e-cig solutions available, it seems fair to assume that there could be an instance where the nicotine emission of an e-cig would be higher than emissions of a cigarette given a high enough concentration.

5.4 Statistical Analysis of Breath Samples

Multiple statistical analyses were applied to the data. The statistical difference within the treatment groups of the three smoker device types cigarette, e-cigarette and cigar was examined using the Freidman test and peak areas, where the devices were significant at a p value of 7.517E-06 for an α value of 0.05. The Freidman test is a non-parametric test equivalent to the parametric repeated measures one-way ANOVA, which is used to detect difference in treatments across multiple test attempts. This significance between smoker

device type is expected as different brands and devices will have different ingredients.

Although there was a significant difference within smoker devices, the Freidman test of the smoker types versus the control only determined significant differences between the cigarette smoker and nonsmoker breath profiles and not the cigar or e-cigarette smokers. Of the cigarette device type there were possibilities of regular cigarettes as well as menthol flavored cigarettes. Using the Wilcoxon Rank test no significant difference was observed between the two at a p value of 0.4026 for an α value of 0.05.

Determination of statistically significant compounds in the exhaled breath samples of the smoker to the nonsmokers were examined by the Mann-Whitney Exact test. Mann-Whitey Exact test is the non-parametric equivalent of the independent samples t test. Smoker device types of e-cigarette and cigar were excluded from the analysis because of limited sample size for the statistical test. Each cigar and e-cigarette smoker groups only had one subject, which would result in a Mann-Whitney Exact test of low statistical power making it difficult to conclude any significant differences. A 9% rule was applied to the cigarette smokers where only compounds present in at least 9% of one of the groups (i.e., cigarette smoker or nonsmoker) were used in the statistical analysis. After applying the rule, the data set contained 18 samples (11 cigarette smokers and 7 nonsmokers) and 108 compounds of the 119 total. A P value < 0.05 was considered significant. P values of the Mann-Whitney exact test identifying significant compounds between cigarette smokers and nonsmokers are reported

in Table 7. Twelve compounds were determined to be significant between cigarette smokers and nonsmokers as follows: β-myrcene, limonene, 2-ethylhexyl acrylate, citral, 1-methyl-napthalene, solanone, nicotine, tetradecane, isopropyl mysitate, tricosane, pentacosane, and tetracosane. These compounds suggest significance for a smoking habit as some have been identified as common tobacco flavoring agents and some pyrolysis products.

5.5 Principal Component Analysis of Breath Samples

Visualization of the compounds found in the exhaled breath samples of all subjects was statistically analyzed by principal component analysis (PCA). The data set of the PCA consisted of 20 samples and a collective integrated peak area of 119 compounds from TICs as listed in Table 9. Not all the compounds were present in all of the measured samples, and thus the original data consisted of many zero values. JMP (Cary, NC, USA) software were used for the statistical analysis. In PCA the first principle component is constructed in the direction of the highest variance in the data. The closer points are to one another, the more similar profile they have. The 3D PCA score plot in Figure 26 (top) shows a summary of all data points between all subjects for the comparisons of all smokers to nonsmokers. Generalized groupings were manually drawn by colored circles. The single e-cigarette smoker (□) was distinguishable from the non-menthol flavored tobacco cigarette smokers (△), and cigar smoker (○) indicated by yellow circle. The menthol flavored cigarette smoker (▲) had a distinct separation from the traditional cigarette smokers indicated by a black circle.

Table 9: Summary of the tentative identification of the VOCs and NVOCs in the exhaled breath samples of 20 total subjects: 13 smokers and 7 nonsmokers listed in their elution order. Thirty-two compounds labeled “S” have been identified by certified standard solutions and library match, while the remaining compounds label “L” are identified by library match only. Compounds are numbered as identified in exhaled breath sample chromatograms.

No.	Compound	Proportion of Cigarette Smokers	Proportion of E-Cigarette Smokers	Proportion of Cigar Smokers	Proportion of Nonsmokers	Confirmation Method	Match Quality %
1	Propanoic Acid	0/11	0/1	1/1	0/7	L	46%
2	Propylene glycol	0/11	1/1	0/1	0/7	S	72%
3	Butanoic acid	0/11	1/1	0/1	1/7	L	43%
4	2-methyl-2-propenoic acid	0/11	0/1	1/1	0/7	L	81%
5	α-pinene	6/11	0/1	0/1	0/7	S	80%
6	phenol	9/11	0/1	1/1	5/7	S	86%
7	6-methyl-5-hepten-2-one	5/11	0/1	1/1	2/7	L	86%
8	B-myrcene	8/11	0/1	1/1	0/7	L	90%
9	β-pinene	3/11	0/1	0/1	0/7	S	97%
10	2-(2-ethoxyethoxy)-ethanol	4/11	0/1	0/1	1/7	L	80%
11	octanal	2/11	0/1	0/1	0/7	L	45%
12	α-phellandrene	2/11	0/1	0/1	0/7	L	86%
13	1,4-dichloro-benzene	4/11	0/1	0/1	0/7	L	98%
14	4-cyanocyclohexene	4/11	0/1	1/1	6/7	L	96%
15	o-cymene	2/11	0/1	0/1	0/7	L	95%
16	1,3-diethyl benzene	1/11	0/1	0/1	0/7	L	9%
17	Limonene	11/11	0/1	1/1	2/7	S	80%
18	benzyl alcohol	0/11	1/1	0/1	1/7	S	98%
19	Eucalyptol	2/11	0/1	0/1	0/7	L	96%
20	γ-Terpinene	5/11	0/1	0/1	2/7	L	90%
21	α-Terpinene	4/11	0/1	0/1	0/7	L	86%
22	di-2-phenyl-,1,2-propanediol	2/11	0/1	0/1	0/7	L	72%
23	α-α-dimethyl benzenemethanol	1/11	0/1	0/1	4/7	L	93%
24	Mequinol	0/11	1/1	1/1	0/7	L	93%

Table 9 (Continued)

No.	Compound	Proportion of Cigarette Smokers	Proportion of E-Cigarette Smokers	Proportion of Cigar Smokers	Proportion of Nonsmokers	Confirmation Method	Match Quality %
25	undecane	1/11	0/1	0/1	0/7	S	95%
26	5-methyl-undecane	1/11	0/1	0/1	0/7	L	45%
27	Linalool	0/11	1/1	0/1	0/7	S	91%
28	Nonanal	11/11	1/1	1/1	6/7	S	97%
29	(4E,6Z) – allo-ocimene	2/11	0/1	0/1	0/7	L	92%
30	2-ethoxy-phenol	1/11	1/1	0/1	0/7	L	55%
31	Octanoic acid	3/11	0/1	0/1	2/7	L	76%
32	1-(ethenyloxy)-octadecane	2/11	0/1	0/1	0/7	L	72%
33	Menthone	1/11	0/1	1/1	0/7	L	96%
34	1,4-dimethoxy-benzene	6/11	0/1	0/1	0/7	L	94%
35	Menthofuran	1/11	0/1	0/1	0/7	L	87%
36	Menthacamphor	1/11	0/1	0/1	0/7	L	70%
37	2-methyl-3(methylthio)-pyrazine	0/11	1/1	0/1	0/7	L	95%
38	Menthol	3/11	0/1	0/1	0/7	L	91%
39	Isomenthol	5/11	0/1	1/1	2/7	L	91%
40	Dodecane	8/11	1/1	1/1	7/7	S	96%
41	Decanal	9/11	1/1	1/1	7/7	L	81%
42	Napthalene	3/11	0/1	0/1	1/7	S	95%
43	1-methoxy-4-propyl-benzene	0/11	1/1	1/1	0/7	L	76%
44	2-ethylhexyl acrylate	8/11	0/1	1/1	1/7	L	86%
45	6-octen-1-ol, 3,7-dimethyl	1/11	0/1	0/1	0/7	L	96%
46	2-phenoxy-ethanol	5/11	0/1	1/1	4/7	L	90%
47	3,4-diethyl-thiopene	0/11	1/1	0/1	0/7	L	72%
48	Benzothiazole	1/11	0/1	1/1	1/7	L	15%
49	nonanoic acid	2/11	0/1	1/1	4/7	L	72%
50	citral	8/11	0/1	0/1	0/7	L	94%
51	cinnamaldehyde	0/11	1/1	0/1	0/7	S	98%
52	tridecane	9/11	1/1	0/1	5/7	S	92%
53	Menthyl Acetate	2/11	0/1	1/1	0/7	L	91%
54	Indole	9/11	1/1	1/1	6/7	S	95%
55	1-methyl-napthalene	10/11	0/1	0/1	5/7	S	95%

Table 9 (Continued)

No.	Compound	Proportion of Cigarette Smokers	Proportion of E-Cigarette Smokers	Proportion of Cigar Smokers	Proportion of Nonsmokers	Confirmation Method	Match Quality %
56	2,2,4,4,6,8,8-heptamethyl-nonane	5/11	1/1	0/1	1/7	L	58%
57	2-methyl-napthalene	9/11	0/1	0/1	5/7	S	91%
58	Solanone	8/11	0/1	1/1	0/7	L	95%
59	Nicotine	7/11	1/1	1/1	0/7	S	93%
60	2(3H)-Furanone, 5-heptyldihydro-	0/11	1/1	0/1	1/7	L	78%
61	butyl ester butanoic acid	3/11	0/1	0/1	0/7	L	59%
62	3-methylene-tridecane	5/11	1/1	0/1	1/7	L	78%
63	Tetradecane	11/11	1/1	1/1	7/7	S	97%
64	3-ethyl-5-(2-ethylbutyl)-octadecane	6/11	1/1	1/1	3/7	L	58%
65	3-heptadecanal	10/11	0/1	1/1	5/7	L	62%
66	α-Santalene	2/11	0/1	0/1	0/7	L	78%
67	β-Caryophyllene	2/11	0/1	0/1	1/7	S	99%
68	(Z)-6,10-dimethyl - 5,9-undecadien-2-one	6/11	0/1	1/1	5/7	L	90%
69	β-Farnesene	1/11	0/1	0/1	0/7	L	94%
70	3-hexadecene	6/11	0/1	1/1	2/7	L	98%
71	1-decene	3/11	0/1	0/1	4/7	L	86%
72	α-humulene	2/11	0/1	0/1	0/7	S	97%
73	pentadecane	5/11	0/1	1/1	0/7	S	80%
74	Butylated hydroxytoluene	0/11	0/1	1/1	0/7	L	91%
75	Aromadedrene	1/11	0/1	0/1	0/7	L	92%
76	β-Selinene	2/11	0/1	0/1	0/7	L	98%
77	2-(octadecloxy)-ethanol	3/11	0/1	1/1	2/7	L	93%
78	β - guaiene	1/11	0/1	0/1	0/7	L	70%
79	Dodecanoic Acid	6/11	0/1	0/1	7/7	L	96%
80	β-Maaliene	1/11	0/1	0/1	0/7	L	74%
81	3,8-dimethyl-decane	3/11	0/1	1/1	0/7	L	70%
82	Selina-3,7(11)-diene	2/11	0/1	0/1	1/7	L	99%
83	hexadecane	2/11	0/1	0/1	0/7	S	70%
84	Diethyl Phthalate	9/11	0/1	1/1	7/7	L	98%
85	Squalene	0/11	1/1	0/1	0/7	L	94%

Table 9 (Continued)

No.	Compound	Proportion of Cigarette Smokers	Proportion of E-Cigarette Smokers	Proportion of Cigar Smokers	Proportion of Nonsmokers	Confirmation Method	Match Quality %
86	heptadecane	2/11	0/1	0/1	2/7	S	91%
87	pristane	4/11	0/1	1/1	4/7	S	91%
88	α-bisabolol	1/11	0/1	0/1	0/7	L	87%
89	2-ethylhexyl ester benzoic acid	2/11	0/1	1/1	0/7	L	64%
90	Tetradecanoic Acid	8/11	1/1	1/1	7/7	L	98%
91	benzyl benzoate	3/11	1/1	0/1	0/7	L	98%
92	octadecane	2/11	0/1	1/1	1/7	S	64%
93	Isopropyl Mysitate	5/11	0/1	0/1	7/7	L	64%
94	cyclohexadecane	5/11	0/1	0/1	0/7	L	98%
95	Pentadecanoic acid	7/11	0/1	1/1	5/7	L	99%
96	7-acetyl-6-ethyl-1,1,4-tetramethyletralin	1/11	0/1	0/1	0/7	L	99%
97	phenol, 4-(1-methyl-1-phenylethyl)-	1/11	0/1	0/1	0/7	L	94%
98	nonadecane	2/11	0/1	0/1	2/7	S	98%
99	Z-7-Hexadecanoic acid	7/11	1/1	1/1	7/7	L	99%
100	Benzeneacetic acid, 2-phenylethyl ester	1/11	0/1	0/1	0/7	L	78%
101	n-Hexadecanoic acid	10/11	1/1	1/1	7/7	L	99%
102	dibutyl phthalate	7/11	0/1	0/1	2/7	S	55%
103	eicosane	1/11	0/1	0/1	0/7	S	95%
104	Isopropyl Palmitate	11/11	1/1	1/1	6/7	L	91%
105	heptadecenal	2/11	0/1	0/1	1/7	L	59%
106	1-octadecene	9/11	1/1	1/1	3/7	L	98%
107	heneicosane	7/11	0/1	1/1	4/7	S	98%
108	N,N-dimethyloctylamine	3/11	0/1	1/1	1/7	L	80%
109	oleic acid	8/11	1/1	1/1	4/7	L	99%
110	Vaccenic acid	0/11	0/1	0/1	2/7	L	98%
111	Octadecanoic acid	10/11	1/1	1/1	7/7	L	99%
112	Docosane	5/11	1/1	0/1	4/7	S	97%
113	Bisphenol A	2/11	0/1	1/1	1/7	S	98%
114	Tricosane	5/11	0/1	1/1	7/7	S	95%

Table 9 (Continued)

No.	Compound	Proportion of Cigarette Smokers	Proportion of E-Cigarette Smokers	Proportion of Cigar Smokers	Proportion of Nonsmokers	Confirmation Method	Match Quality %
115	2-propenoic acid, 3-(4-methoxyphenyl)- 2-ethylhexyl ester	2/11	0/1	0/1	0/7	L	98%
116	9-octadecenamide	2/11	0/1	0/1	2/7	L	91%
117	tetracosane	5/11	0/1	1/1	7/7	L	98%
118	pentacosane	6/11	0/1	1/1	7/7	L	35%
119	Isooctyl phthalate	9/11	1/1	1/1	7/7	L	59%

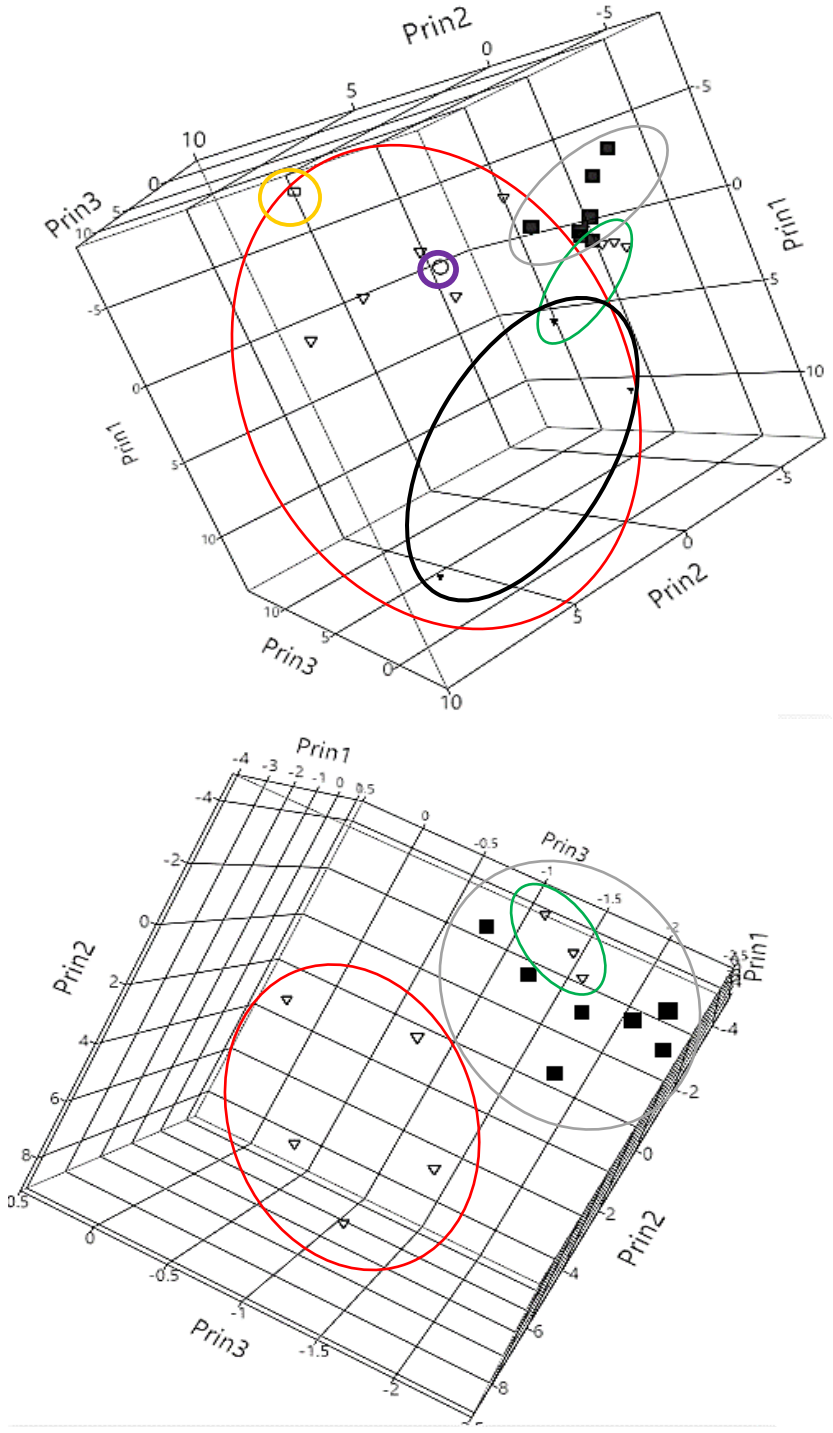

Figure 26: PCAs of the 119 compounds from the 20 exhaled breath profiles. Symbols represented as follows: e-cigarette smoker (▯),cigarette smokers (△),menthol cigarette smoker (▲), cigar smoker (O) and Nonsmokers (■). (Top) n=20 subjects (Bottom) n =15 subjects.

Nonsmokers (■) were tightly clustered together with some overlap from smokers where nicotine was not detected indicated by green circle. An additional 3D score plot of 15 samples of only non-menthol flavored cigarette smokers and nonsmokers is shown in Figure 26 (bottom) displaying a more apparent separation of the groupings. The misclassification of smokers as nonsmokers when nicotine was not detected, demonstrated the possibility of false negatives. In addition to the lack of the detection of nicotine, the three smokers misclassified also did not detect six of the other significant smoker compounds as follows: β-myrcene, citral, solanone, tricosane, pentacosane, and tetracosane.

6.0 Discussion

The analyses of the compounds of the exhaled breath profiles in this study were interpreted without consideration of inspired air contamination. Some breath analysis studies consider the alveolar gradient principle in their analysis. Researcher Michael Phillips describes the alveolar gradient principle as subtracting the AUC of VOCs in the air from the AUC of VOCs in the breath [8]. By this principle positive gradients would indicate endogenous compounds while negative gradients would indicate compounds derived from the environment [9, 50]. The sporadic onsite sampling protocol did not allow for consistent calculation of an alveolar gradient because of the imbalance of prepared sampling CMVs and available study subjects during a sampling period. To increase recruitment opportunities priority of the use of prepared CMVs was given to subject samples instead of field blanks if needed. Therefore, field blanks were not consistently

taken during sampling. Sampling of the 20 subjects occurred over many sampling sessions and in many locations. Differences between locations were not considered for this research.

Field blanks were only taken in three instances. Similar compounds were compared between blanks and samples where a small amount of compounds found in air were also found in breath. Two examples are shown in Figures 27 and 28. Figure 27 represents a situation where if the alveolar gradient principle was applied, the compounds would all have positive gradients in the samples as the AUC response for the field blank was higher than that in the samples. Figure 28 represents a situation where if the alveolar gradient principle was applied, the compounds would all have negative gradients in the samples as the AUC response for the field blank was lower than that in the samples. Although efforts were made to sample away from the environments with mainstream smoke exposure, it is possible that the ambient air may have been contaminated with compounds from residual cigarette mainstream smoke as the collection occurred near the vicinity of the initial smoking area. These uncontrolled flaws contributed to the decision to collectively not apply the alveolar gradient principle in this research.

A lack of alveolar gradient could contribute to exogenous contamination of the sample from the contaminated air inhaled. Additional exogenous contamination could be a result of contamination from the sampling device or from food intake which was not considered in this study. A few of the speculated contaminated compounds tentatively identified in the breath samples of this

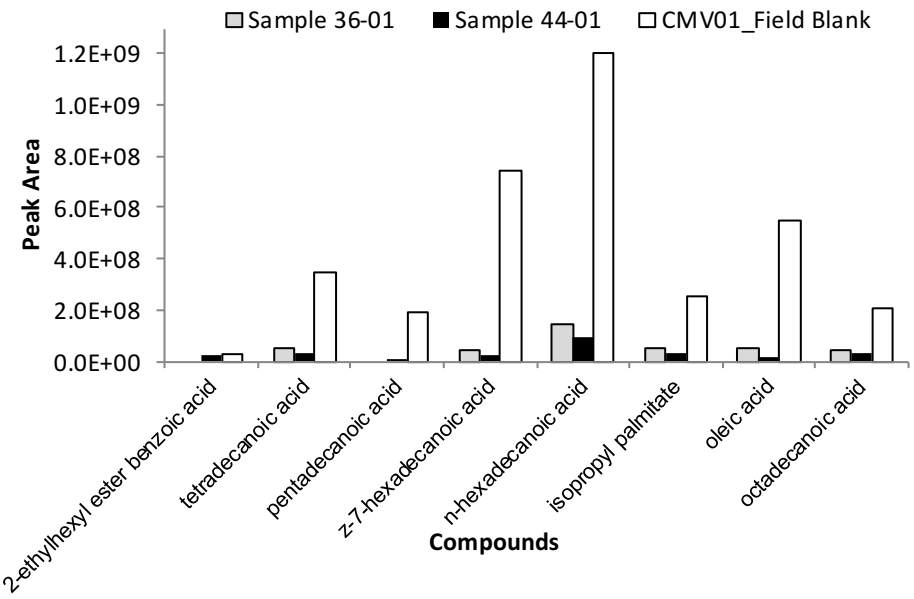

Figure 27: Visual illustration of the comparison of the AUC for the field blank (white) taken during the sampling session of subjects 36-01 (light grey) and 44-01 (black).

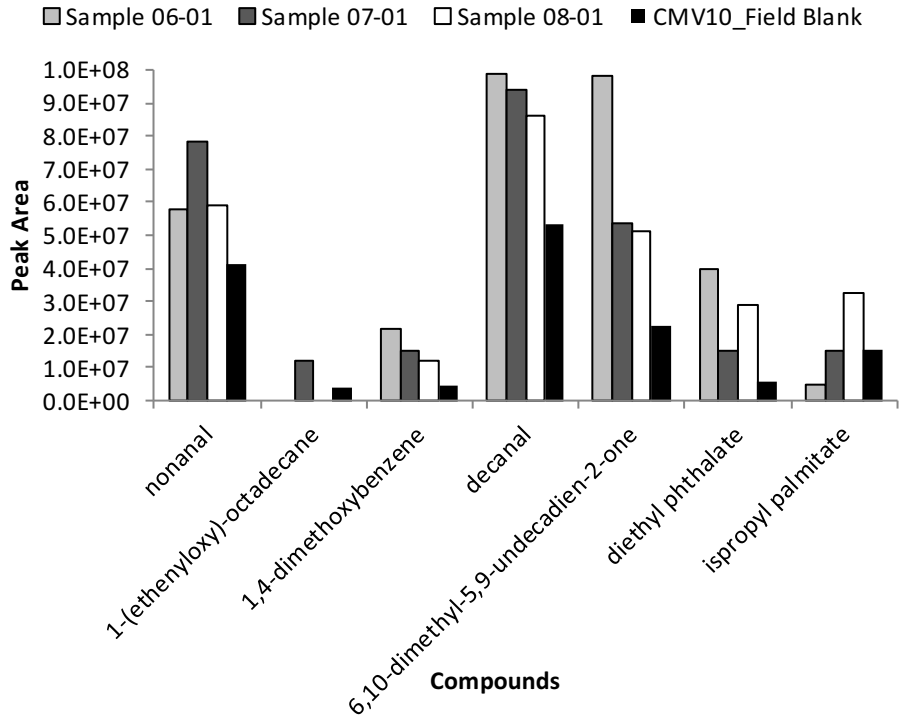

Figure 28: Visual illustration of the comparison of the AUC for the field blank (black) taken during the sampling session of subjects 06-01 (light grey), 07-01 (dark grey) and 08-01 (white).

research are limonene, cinnamaldehyde, diethyl phthalates, dibutyl phthalate and isooctyl phthalates and bisphenol A.

Limonene and cinnamaldehyde are flavoring agents and could have originated from previously ingested food. Phthalates and Bisphenol A are associated with plastics and could originate from particles in the air or vapors emitted from the BCD. Considering the ranges of exogenous contamination sources it is important to note that some of the tentatively identified compounds may not be relevant to that of the exhaled breath of healthy people or smokers.

7.0 Conclusion

The present study was aimed at demonstrating the potential of a new sorbent device, CMV, for the collection of exhaled breath volatiles and aerosol particulates of smokers and nonsmokers. For this purpose, GC/MS coupled to a thermal desorption probe was applied for use of the CMV device. The CMV was capable of identifying individual compounds in breath samples of both exogenous and endogenous origin totaling 119 compounds collectively over the 20 subjects. The identified compounds consisted of various chemical classes. Twelve compounds were identified as significant between cigarette treatment group and the nonsmoker control group. Nicotine, one of the significantly identified compounds and target analyte was detected in 9/13 smoker subjects averaging a nicotine concentration of 143 ± 31 pg of cigarette smokers for an approximate volume of five liters of breath. The collected breath profiles were distinguishable

between smoker and nonsmoker groups when nicotine was detected, resulting in zero false positives, but four false negatives.

CHAPTER 6: LIMITATIONS & FUTURE WORK

Exhaled breath research has proved to be complex in the execution of its sample collection, data analysis and interpretation. Each methodological approach to executing this research presents limitations as well as provides insight into solutions toward them. The limitations to the sample collection, data analysis and interpretation of the research in this study will be reviewed followed by suggestions for improvement to the study for future work.

1.0 Sample Collection Limitations

The sample collection limitations of this research stem from the lack of preparation of the subjects before sampling and the sampling device itself. This research did not conduct sampling protocol under the necessary controlled conditions to limit the inter-individual variability between subjects. The subjects were not asked to submit to any unusual requests in preparation of sample collection nor were they monitored in the period before sample collection. In this respect, the breath profile and concentration of nicotine from the subject could vary based on their smoking preferences and habits. The smoking habits of a smoker reflect their style of smoking. Each subject may have differing nicotine tolerances contributing toward their choice of cigarette brand, puff volume and duration, depth of inhalation, puff hold and cigarette length smoked. If this study were to be repeated, it is suggested that more variables are controlled such as cigarette brand and smoking style. A more controlled study can help to decrease

the inter-individual variability, although other factors such as metabolic processing within the body cannot be controlled.

The sampling device used in this research also contributed to sample collection limitations. The sampling device must support standardized collection of breath volumes. The flow through BCD used in this study was unable to offer the recommended minimum resistance needed in a breath device for comfortable collection of controlled breath sample volumes from a volunteer. The diameter of the BCD is 7 mm compared to wide tubing of up to 1 inch in diameter reported in other studies [50]. Resistance in the BCD could contribute to varying exhaled flow rates which are directly connected to exhaled concentration because of the relationship of flow rate and contact time of breath with the mucosa of the airway before expiration [23]. If this study were to be repeated, efforts would be made to hold the BCD at an appropriate temperature (~45 C) during collection to prevent condensation of water vapor from the breath where VOCs could partition into the aqueous phase. A redesign of the BCD would be suggested to incorporate a vacuum to assist in decreasing resistance as well as a meter to observe breath flow for inter-individual adjustments for constant volume collection.

2.0 Data Analysis Limitations

The main limitation of the data analysis of exhaled breath samples were the observed contaminations of background interference from the sorbent material used in the CMV which have potential to interfere with target analyte identification. Some of the common background siloxane interferences observed

in the CMV are m/z ions 73, 207, 267, 327, 281, 193. Peaks at m/z 207 and 281 are hexamethyl-cyclotrisiloxane and octamethyl cyclotertrasiloxane, respectively. Proper manufacturing of the CMV device along with proper conditioning before use minimized the interferences during analysis. Background contamination became problematic for low concentrations as seen in Figure 29.

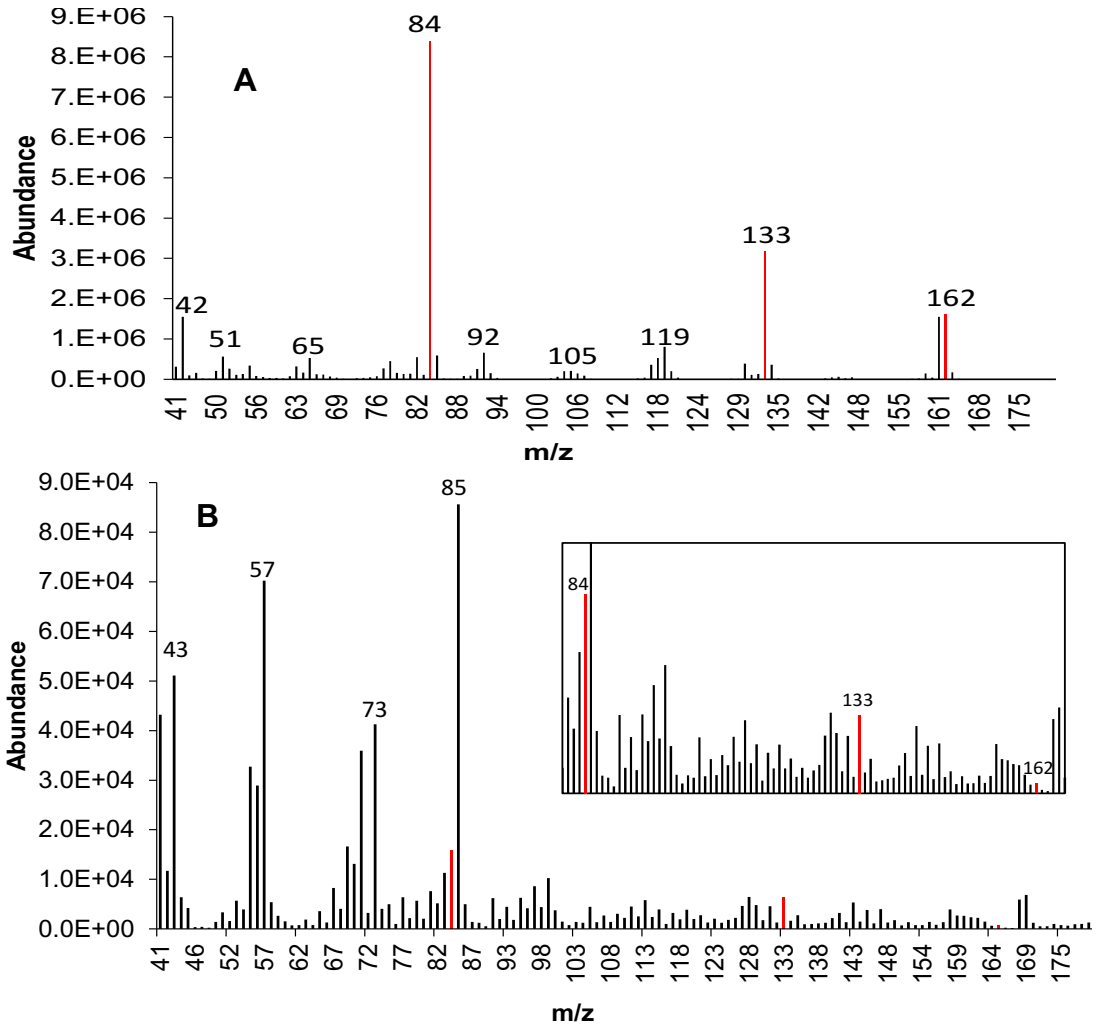

Figure 29: (A) Mass spectrum of a 1 µL 500 ppb solution of nicotine spiked onto a CMV and (B) mass spectrum of 131 ppb nicotine peak in breath sample of subject 34-01.

3.0 Data Interpretation Limitations

Data interpretation of exhaled breath profiles are limited when proper action of background elimination is not controlled. Exogenous background contamination in exhaled breath can originate from saliva, mouth air or contaminated ambient inspired air. Suppression of inspired air contamination post sample collection can be achieved by applying the alveolar gradient principle to data analysis where the environment is sampled and subtracted from the breath sample as described in research by Michael Philips [50]. A pre sample collection prevention measure for inspired air contamination is to supply subjects with ultrapure breathing air before sampling as demonstrated in protocols of previous research studies [35, 50]. A study by Van den Velede and associates identified a significant amount of compounds found in mouth air [9] which could be contributed by mouth bacterial conditions such as halitosis [94]. Provisions for this contamination source can be instilled in the protocol by implementing an oral hygiene mouth rinse before sampling.

4.0 Future Work

In addition to accounting for all of the previously suggested improvements on limitations in this preliminary research, efforts to increase subject recruitment should be considered in future work. This research was conducted on a small scale, limiting the power of statistical interpretation as well as implying a lack of confidence in the results. Increased participation would present clarification on any previously identified significant conclusions.

Provided the initial promising results of breath collection by CMV, investigations into the improvement of the devices' sampling ability should be considered in future work. Aspects under consideration are chemical and analytical sensitivity involving new sorbent coatings for the CMV and cryogenic focused sample introduction.

CHAPTER 7: FUTURE WORK IN MARIJUANA BREATH DETECTION

The literature review thus far in this thesis has demonstrated that the exhaled breath profile of cigarette smokers can be distinguished from nonsmokers based on the quantitative and qualitative investigation of VOCS and NVOCS which prove to be statistically significant between the groups. Theoretically, under this same principle the exhaled breath profile of marijuana smokers should be able to be distinguished from nonsmokers. This distinction is dependent on the VOCS and NVOCS observed in the exhaled breath samples and the capability and sensitivity of an analytical technique in their measurement.

1.0 Preliminary Study: Headspace Analysis of Seized Marijuana Plant Material by CMV-GC/MS

Chapter 5 of this thesis has demonstrated the CMV's ability to detect constituents of cigarette smoke by way of absorption and excretion from the lung airways in exhaled breath. In attempts to demonstrate the CMV's potential to detect constituents in marijuana smoke by way of absorption and excretion from the lung airways in exhaled breath, preliminary studies examining the headspace vapor of marijuana plant material (PM) was used to demonstrate detectable fractions by dynamic CMV sampling. The observed fractions were expected to be analogous with some in marijuana smoke, and therefore expected to be available to be inhaled and subsequently exhaled in breath. Description of this experiment will follow.

1.1 Introduction to Marijuana Analysis

Trained canines are routinely employed in the detection of marijuana plants by associating the presence of the volatile compounds emitted by the plant. Alternative analytical methods to the use of canines have been recently proposed for use by law enforcement. The headspace of marijuana is classified into four categories (i.e., fractions) on the basis of the compounds' physical properties. The classifications are termed fractions and are described as follows: (I) volatiles (bp 20-80˚C; MW<100 g/mol), (II) intermediate volatiles (bp 150-198˚C; MW>100 g/mol), (III) less volatiles (bp>198˚C; MW>200 g/mol), and (IV) non volatiles (bp>200˚C; MW>300 g/mol) [95]. The identified fractions of a particular marijuana sample are dependent upon the operational conditions of the analytical technique as well as its sample preparation procedure. The following previous studies have investigated the examination of marijuana headspace where variable results were produced.

Traditional headspace analysis of marijuana using a marijuana standard (grown from Mexican seed containing 1.9% THC content) was first demonstrated by Hood and associates, implementing gas tight syringes to extract volatiles for direct GC/MS analysis. The analysis of one gram of a marijuana standard sample heated at 65˚C for one-hour equilibrium time, resulted in the detection of 20 compounds [96]. A decade later Osman and colleagues, demonstrated the benefits of the preconcentration of volatiles onto Tenax® sorbent tubes in comparison to traditional headspace analysis using gas tight syringes. Their examination of the β-caryophyllene volatile in a 100 mg sample of 10-year-old

cannabis resin heated to 100˚C demonstrated that Tenax® tubes for TD-GC/MS resulted in much improved detection limits over HS-GC/MS with gas tight syringes at 3 mg and 10 mg, respectively [97].

With the introduction of SPME technology, Illias and associates focused on the examination of the cannabinoids considered the “nonvolatile fraction” in marijuana headspace according to these authors. Ten cannabinoids were extracted from 60 mg of powdered marijuana plants heated to 150˚C, to distinguish between marijuana grown in different regions [98]. Continuing in the direction of pre-concentration techniques, Rothschild and associates, used a Porapak Q mesh adsorbent to pre-concentrate volatiles of flowering male and female cannabis plant pollen, before solvent extraction followed by GC/MS analysis. Marijuana pollen and plant volatiles were distinguished from each other, as well as 68 compounds were identified [99].

As researchers such as Wu & Chang demonstrated that thermal desorption methods statistically demonstrated better repeatability and recovery than the classic solvent extraction method in ambient air volatile analysis [46], new sorbent technologies were developed. One such technology was the CMV whose physical and chemical characteristics have been previously described in Chapter 1, Section 2.1. In addition to its forensic applications in explosive detection [16] and gunshot residue detection [18], the CMV has also been used to detect the volatiles of drugs including marijuana [20]. The CMV-GC/MS

marijuana headspace studies of a single 25 g packaged marijuana sample kept at equilibrium for 3 hours resulted in the identification of 26 compounds [20].

In this study, the CMV explored the headspace profile of seized marijuana PM. In addition, the potential of the CMV device for the detection of marijuana plants by association of the VOCs detected, was evaluated. Finally, the analytical figures of merit for the detection of the VOCs are also reported.

1.2 Instrumentation

Analysis was performed on an Agilent 7890A Gas Chromatograph (GC) and an Agilent 5975C Inert XL MSD mass spectrometer (Santa Clara, CA). Chromatographic separation occurred on a HP-5ms capillary column (29.17 m x 0.25 mm x 0.25 μm). The oven temperature was programmed for 2 min at 40˚C, then 25˚C/min to 260˚C, and finally in 260˚C for 10 min. Injector was operated in splitless mode at 270˚C and the transfer line was 280˚C. The constant GC column flow of helium was 1.2 ml/min. The mass spectrometer simultaneously collected TIC and SIM data. The THC ions selected for ion monitoring were target ion 299 m/z and qualifier ions 271, 231, 314 m/z over the acquisition range 40-340 m/z.

1.3 Materials

A description of the assembly of the CMV device used for the headspace analysis have been previously described [16]. Before sampling, the CMV devices were conditioned for 2 hours in an oven at 250˚C. After conditioning, the CMVs were blanked to record their baseline background in the GC/MS via coupling with

the Agilent TSP (Santa Clara, CA). The CMVs have been reported to withstand several extraction and desorption cycles and were wrapped in aluminum foil and labeled for storage and transport between sampling uses.

1.3.1 Reagents

Separate experiments of standard compounds diluted with GC grade methanol (Fischer Scientific, Waltham, MA) were analyzed to confirm retention time and mass spectra by comparison of compounds identified in marijuana headspace profile. Compounds limonene, α-pinene, octadecane and β-pinene were from Acros Organics (Morris Plains, NJ), nicotine, β-caryophyllene, α-humulene, dodecane, tetradecane, tridecane, benzyl alcohol, benzaldehyde, nonanal, eicosane, heneicosane, tricosane, and THC from Sigma-Aldrich (St. Louis, MO), and linalool from Fluka (Buchs, Switzerland).

1.3.2 Samples

Confirmed marijuana plant samples were analyzed within a local forensic laboratory as a result of drug seizures. The eight marijuana samples analyzed in this study are described in Table 10.

1.4 Sample Collection Methods

Marijuana sample seizures consisted of various quantities of marijuana bags. Only a grab sample consisting of an individual marijuana bag was analyzed from each marijuana evidence seizure, except for sample 2, where the entire sample seizure was analyzed as a whole. The headspace directly above the PM inside the ziplock bags was taken for all samples except for sample two.

For sample two, the headspace of a paper bag containing loose tobacco leaves and sealed marijuana baggies was taken. Before sampling of the first marijuana seizure began, a blank of the laboratory air and the air in the fume hood, where samples would be handled, were taken to ensure no contamination of the sample. Sampling did not occur under optimized conditions because of limited access to samples. Headspace sampling was performed by affixing a CMV to the end of a Supelco MSA Elf air sampling pump (Bellefonte, PA) and hovering the CMV in the opening of the opened ziplock baggies for 1 minute at an extraction flow rate of 1 L/min. Duplicate samples of each marijuana seizure was taken with a 1-minute waiting period between sampling with the zip opening closed. After each sampling the CMV was wrapped in foil and the sampling end of the CMV was noted for proper insertion orientation during GC/MS analysis. A trip blank CMV was taken as a control. CMVs were transported back to lab under an icepack to prevent loss. GC/MS analysis was performed immediately on fume hood and laboratory air blanks and the first replicate of all the marijuana sampled CMVs. The trip blank and the second replicates of the marijuana sampled CMVs were individually sealed in glass vials and refrigerated overnight for next day analysis.

1.5 Results and Discussion

The headspace of numerous seizure samples of PM suspected to be marijuana were examined and similar headspace profiles were obtained. Acquisition of chromatographic data was performed using Agilent Chemstation

Table 10: Description of the seized marijuana samples provided from a local forensic laboratory.

Sample Number	Sample description	Seizure Date (MM/YYYY)	Dimensions of packaging (mm x mm)	Weight of sample (g)
1	Clear plastic ziplock w/ compressed plant material	09/2014	50 x 63	4.084
2	Brown lunch bag w/ loose tobacco leaves and sandwich bag of ziplock baggies w/ plant material	01/2015	-	37
3	Clear plastic ziplock w/ compressed plant material	10/2014	50 x 63	5.483
4	Blue plastic ziplock w/ compressed plant material	04/2015	32 x 33	0.741
5	Clear plastic vials w/ loose plant material	03/2014	40 x 18	3.394
6	Clear plastic ziplock w/ compressed plant material	05/2013	50 x 63	6.496
7	Clear plastic ziplock w/ compressed plant material	03/2014	38 x 43	1.641
8	Clear plastic ziplock w/ compressed plant material	04/2015	50 x 53	3.136

software and computer mass spectrum reference libraries NIST 2008 and WILEY 2008 were applied for identification reporting the match quality percent of those generally higher than 60%. Where standards were available, identification was further confirmed by standard solution retention time and mass spectra of specific compounds indicated in Table 11. In this study, 44 compounds were identified as a part of the marijuana headspace profile between the eight marijuana seizures, with fourteen confirmed by a standard solution (Table 11). The samples (1-8 in Table 10) individually contained 29, 28, 33, 37, 17, 34, 34, and 36 compounds, respectively. All sample seizure varieties contained

α-pinene, α-terpinolene, β–linalool, borneol, dodecane, tridecane, tetradecane, valencene, eicosane, and tricosane. Thirty-five compounds, as follows, were non-inclusively consistent with those reported from multiple literature sources: α-pinene [96, 99-106], benzaldehyde [104, 105], β-myrcene [20, 96, 99-106], β-pinene [20, 96, 99, 101-106], 3-carene [20,96, 99, 102, 104-106], 2-ethylhexanol [104, 105], limonene [20, 96, 99-106], benzyl alcohol [99, 104-106], β–ocimene [96, 99, 101-103, 106], γ-terpinene [20, 96, 104-106], α-terpinolene [96, 99, 102, 104-106], β-linalool [20, 96, 99, 102, 104-106], nonanal [104-106], exo-fenchol [96, 99, 104-106], borneol [104,106], dodecane [104, 106], tridecane [104-106], ylangene [99, 106], 4,11–selinadiene [106], α–zingiberene [99], α-bergamotene [96, 99, 106], α-santalene [106], β-caryophyllene [96, 97, 99, 106], α-humulene [20, 96, 99, 102-106], α-guaiene [20, 104-106], β-guiaene [20], α-gurjunene [99, 104, 105], valencene [20, 103-105], 3,7(11)–selinadiene [20, 103, 106], β–maaliene [103], guaiol [99, 103, 106], α–bisabolol [99, 104-106], octadecane [106], THC [98] and cannabinol (CBN) [98].

Twelve compounds were consistent with those reported in previous CMV-GC/MS marijuana studies [20]. The following 20 compounds identified in this study are consistent with other literature sources, but not previously reported by CMV detection until this study: α-pinene, benzaldehyde, benzyl alcohol, β–ocimene, α-terpinolene, nonanal, exo-fenchol, borneol, dodecane, tridecane, ylangene, α–zingiberene, α-bergamotene, β-caryophyllene, α-gurjunene, β–maaliene, guaiol, α–bisabolol, THC, and CBN. It is of interest to note that the

Table 11: Qualitative analysis of compounds identified to be emitted from headspace of marijuana samples captured by CMV after 1 min dynamic sampling at 1 L/min extraction flow.

No.	Retention Time (min)	Compound	Confirmation	Match Quality Percent	VOC sample Frequency (n=8)
1	6.079	α-pinene	L	76%	8/8
2	6.283	Benzaldehyde	S	90%	2/8
3	6.304	β–myrcene	L	80%	5/8
4	6.430	β–pinene	S	89%	3/8
5	6.619	3-carene	L	83%	1/8
6	6.683	2-ethylhexanol	L	72%	7/8
7	6.746	Limonene	S	91%	3/8
8	6.767	Benzyl Alcohol	S	96%	5/8
9	6.823	B-ocimene	L	96%	2/8
10	6.942	γ-terpinene	L	97%	5/8
11	7.131	α-terpinolene	L	96%	8/8
12	7.181	β-linalool	S	96%	8/8
13	7.209	Nonanal	S	93%	6/8
14	7.536	Allo-Ocimene	L	97%	4/8
15	7.391	Exo-Fenchol	L	96%	7/8
16	7.728	Borneol	L	91%	8/8
17	7.777	Dodecane	S	96%	8/8
18	8.352	Tridecane	S	98%	8/8
-	8.738	Nicotine*	S	96%	1/8
19	8.857	Ylangene	L	99%	5/8
20	8.885	Tetradecane	S	91%	8/8
21	8.920	Surfynol	L	83%	5/8
22	8.962	α-zingiberene	L	72%	2/8
23	9.060	α-bergamotene	L	96%	3/8
24	9.095	α- santalene	L	99%	4/8
25	9.166	β-caryophyllene	L	99%	7/8
26	9.271	cyclododecane	L	94%	2/8
27	9.348	α-humulene	L	97%	7/8
28	9.474	4,11–selinadiene	L	96%	6/8
29	9.516	Seychellene	L	93%	7/8
30	9.544	α- guiaene	L	93%	7/8
31	9.664	β-guaiene	L	62%	7/8
32	9.699	α- gurjunene	L	96%	5/8
33	9.748	Valencene	L	90%	8/8
34	9.776	3,7,(11)-selinadiene	L	98%	6/8
35	9.846	β–maaliene	L	92%	2/8

Table 11 (Continued)

No.	Retention Time (min)	Compound	Confirmation	Match Quality Percent	VOC sample Frequency (n=8)
36	9.993	Guaiol	L	99%	6/8
37	10.330	α–campholene aldehyde	L	52%	7/8
38	10.365	α–bisabolol	L	90%	6/8
39	11.779	Octadecane	L	86%	7/8
40	11.663	Eicosane	S	92%	8/8
41	12.175	Heneicosane	S	90%	7/8
42	13.465	Tricosane	S	94%	8/8
43	15.862	THC	S	*SIM	6/8
44	16.754	CBN	L	46%	3/8

***** Nicotine is not associated with the headspace profile of marijuana but of the tobacco leaves present in that particular sample.
Compound identification confirmed by retention time of standard solutions (S) or mass spectrum in library (L).
THC was confirmed by the following selected ions 299, 231, 271, 314 m/z.

following nine compounds found by CMV have not previously been reported as constituents of marijuana headspace in any literature: allo-ocimene, surfynol, tetradecane, cyclododecane, seychellene, α-campholene aldehyde, eicosane, heneicosane, and tricosane.

Sample seizures numbers four, eight, and one respectively showed great intensity in their chromatographic response for the monoterpenes such as α-pinene, β-myrcene, and limonene and sesquiterpenes such as β-caryophyllene, α-humulene, and α-guaiene. An example chromatogram showing the profile of marijuana headspace is shown in Figure 31B. As seen from the chromatogram and list of compounds in Table 11 the chromatographic method and sampling in this study has only identified compounds in the second fraction (monoterpenes), third fraction (sesquiterpenes) and the fourth fraction (cannabinoids). The oxygenated compounds of the first fraction are commonly identified in most plant vapors [96] and are not particularly indicative of marijuana specifically. The sesquiterpene, β-caryophyllene, is usually found to be more concentrated and odorous than other compounds [105]. This can be seen in Figure 31 comparing the unpackaged (B) to the packaged (C) marijuana headspace profiles. The packaged marijuana sample (seizure #2) contained a minimum of 75% of the same compounds identified in the other seven unpackaged sample seizures. The compounds are present, but just at different concentrations. The SPME-MDGC/MS-O studies by Rice & Koziel of one gram of packaged and unpackaged air-dried marijuana revealed that packaging was not significant in volatile detection. Sample 5 had the least amount of compounds identified, and was also

the only sample that did not contain β-caryophyllene. It is hypothesized that, that particular PM was older than the others, since it did not contain the most odorous compound known to be present in the marijuana. This hypothesis was additionally supported by the visual appearance of the PM as it was brown and appeared to be dried out, compared to the green appearance of the other samples.

Each replicate of each sample seizure was analyzed separately and the detected compounds between each were combined for a total representation of the sample as a whole. Compounds present between duplicate sampling of each seizure were generally consistent with each other. Compound identification of the marijuana headspace profile did not include compounds consistent with the contaminants found in the trip, fume hood and laboratory air blanks. Some of the contaminants identified in the blanks were dibutyl phthalate, diethyl phthalate, 2-phenoxyethanol and 1-phenoxypropan-2-ol. A comparison of the chromatogram of the laboratory air blank and an example chromatogram of a marijuana headspace profile is shown in Figure 31A and Figure 31B.

Figure 30, illustrates the identification of the THC in the chromatogram of marijuana headspace sample #8, with the confirmation by mass spectrum (data not shown). It is of interest to note the peculiarity of the identification of cannabinoids THC and CBN in the marijuana samples occurred under the room temperature sampling conditions as described in this study. Cannabinoids occur mainly in their carboxylic acid derivative form in the plant and are not usually released until the sample is heated since they decarboxylate slowly at room

temperature [98]. Since the direct detection of cannabinoids are unlikely at room temperature based on their low vapor pressure, it is hypothesized that THC and CBN entered the CMV absorbed on particles (possibly marijuana pollen particles). Rothschild and associates, reported that benzyl alcohol was exclusively found in marijuana pollen. Of the six occurrences of THC identified in the marijuana profile, three of those also identified benzyl alcohol in samples three, six and seven, respectively.

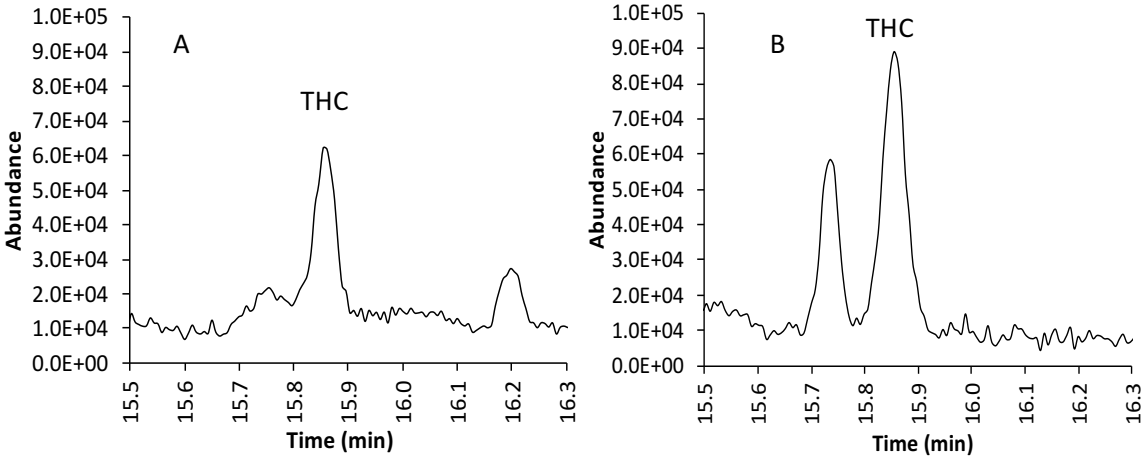

Figure 30: (A) Total ion chromatogram of the VOC profile of marijuana headspace sample #8, emphasizing THC peak at 15.855 min and (B) the direct spike response of 1 ppm THC in a MeOH solution on CMV; peak at 15.855 min.

A calibration curve of liquid THC solutions spiked onto CMVs, showed linearity (R^2=0.99) over concentration range of 0.5-20 ppm (0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0 ppm). The average of three replicates over the 5-20 ppm concentration range showed relative standard deviations of ≤12%, while 0.5-2.5 ppm concentration range showed relative standard deviations of ≤26%. The limit of detection of THC on CMV is 1.0 ng. An approximation of the mass of THC collected on the CMV from marijuana sample #8, was determined to be 1.8 ng

after integration of the THC peak in the sample and use of calibration curve [Chapter 3, Section 2.0, Figure 9].

Statistical analysis was used to examine the relationship of packaged and unpackaged marijuana headspace profiles. One-way analysis of variance (ANOVA) and Tukey's honest significant difference test (Tukey HSD) was performed on the eight marijuana samples using the data from the peak area under the curve of the identified compounds in order to determine if the samples headspace profiles are different from each other. P values of <0.05 were considered as significant for one-way ANOVA. One-way ANOVA resulted in p-value of 0.000284 ($p<0.05$) which indicated that there was a significant difference between one or more of the eight marijuana samples. A comparison between each sample was examined using the Tukey HSD test to identify which pairs were significantly different from each other. Results are summarized in Table 12. There was a significant difference between sample four and all other samples except samples 1 and 8. It is hypothesized that samples 4 and 8 are not significantly different because they were most recently seized on the same date of 4/2015. The significance of samples 4,8, and 1 could be a result of their difference in concentration as they were more concentrated in certain monoterpenes and sesquiterpenes. Just as represented in the Rice & Koziel studies the headspace profile through the packaged sample #2 and the remaining unpackaged headspace marijuana profiles were not significantly different.

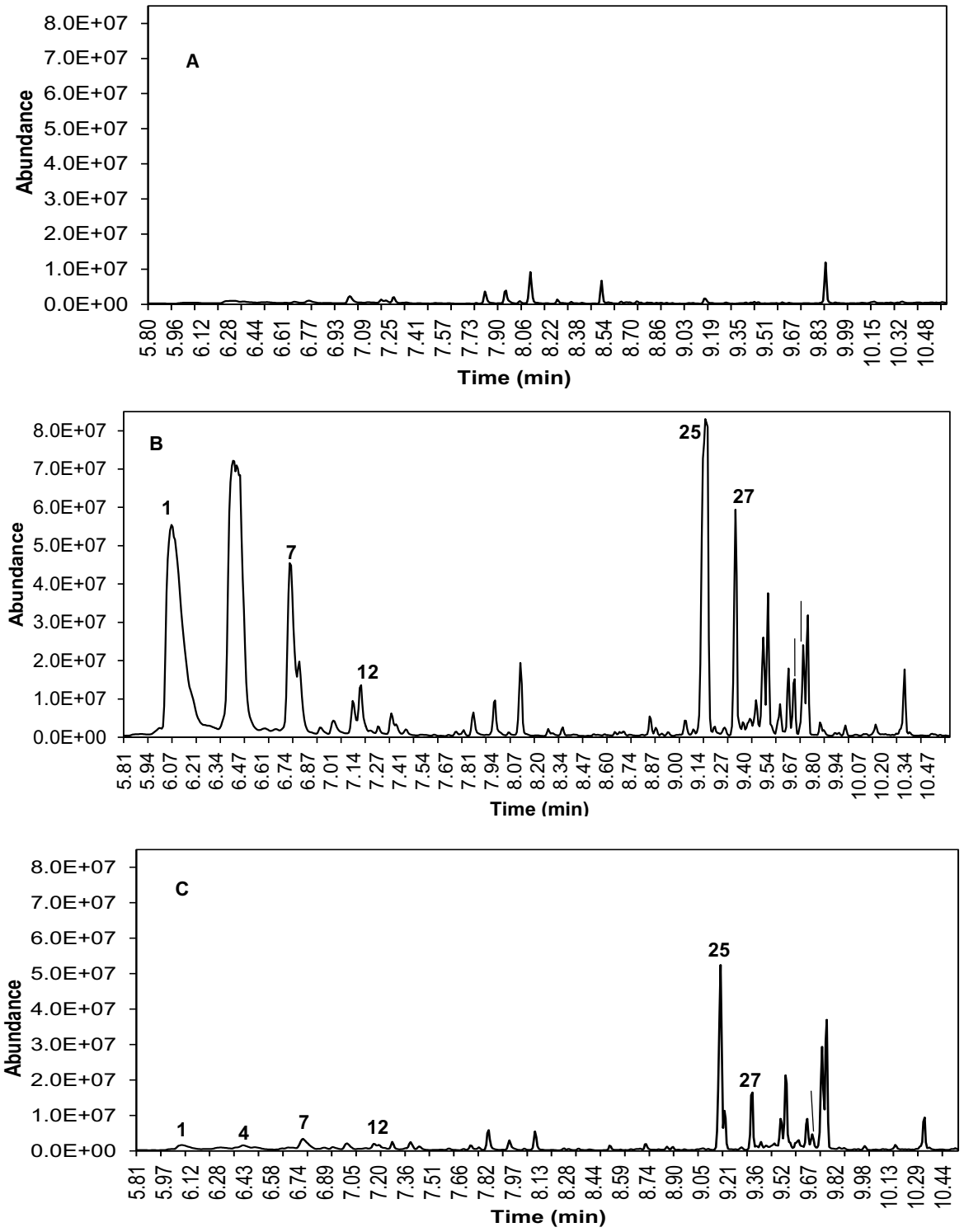

Figure 31: Total ion chromatogram of laboratory room air (A), profile of headspace over open marijuana sample #4 (B), and profile of headspace through packaging of marijuana sample #2 (C). Characteristic peak identification of marijuana sample identified according to compounds in Table 11.

Table 12: Statistical analysis of the eight marijuana sample seizures using Tukey HSD test.

Sample Comparison	P-value	Significance
Sample 1 vs Sample 2	0.8999	N.S.
Sample 1 vs Sample 3	0.8999	N.S.
Sample 1 vs Sample 4	0.1168	N.S.
Sample 1 vs Sample 5	0.7933	N.S.
Sample 1 vs Sample 6	0.8999	N.S.
Sample 1 vs Sample 7	0.8673	N.S.
Sample 1 vs Sample 8	0.8999	N.S.
Sample 2 vs Sample 3	0.8999	N.S.
Sample 2 vs Sample 4	0.0093	P<0.01
Sample 2 vs Sample 5	0.8999	N.S.
Sample 2 vs Sample 6	0.8999	N.S.
Sample 2 vs Sample 7	0.8999	N.S.
Sample 2 vs Sample 8	0.8999	N.S.
Sample 3 vs Sample 4	0.0020	P<0.01
Sample 3 vs Sample 5	0.8999	N.S.
Sample 3 vs Sample 6	0.8999	N.S.
Sample 3 vs Sample 7	0.8999	N.S.
Sample 3 vs Sample 8	0.7563	N.S.
Sample 4 vs Sample 5	0.0010	p<0.01
Sample 4 vs Sample 6	0.0022	P<0.01
Sample 4 vs Sample 7	0.0013	P<0.01
Sample 4 vs Sample 8	0.2228	N.S.
Sample 5 vs Sample 6	0.8999	N.S.
Sample 5 vs Sample 7	0.8999	N.S.
Sample 5 vs Sample 8	0.6234	N.S.
Sample 6 vs Sample 7	0.8999	N.S.
Sample 6 vs Sample 8	0.7710	N.S.
Sample 7 vs Sample 8	0.6974	N.S.

***Results were obtained using an online calculator at**
http://statistica.mooo.com/OneWay_Anova_with_TukeyHSD_result

1.6 Conclusions

This study demonstrated a method for analysis of the volatile headspace of multiple seized marijuana samples from a local forensic laboratory employing CMV adsorption/desorption analysis. The sorption technology of the CMV sufficiently identified 44 compounds in the headspace of marijuana, 35 of which are consistent with eleven previously published studies. The remaining nine compounds allo-ocimene, surfynol, tetradecane, cyclododecane, seychellene, α-campholene aldehyde, eicosane, heneicosane, and tricosane have not previously been reported in the marijuana headspace profile. The CMV's simplicity showed its usefulness for the forensic application of identifying unknown PM as marijuana by its capability of identifying its volatiles. Although the reported method is different from those in previous studies, specific compounds and overall similarity in the composition of the marijuana headspace profile has been demonstrated.

2.0 Implications of Preliminary Study for Future Research

The methodological sampling approach of the CMV has demonstrated its ability to detect the chemical fractions available in marijuana PM. Studies have demonstrated the similarity of VOCs present in the headspace of marijuana PM and marijuana plant extract toward the VOCs present in marijuana smoke condensate [100, 107]. It is expected that additional compounds will be observed in marijuana smoke as the burning of marijuana creates pyrolysis and

combustion byproducts. Studies have also demonstrated the chemical similarities of cigarette smoke and marijuana smoke [52, 108].

Provided their similarities it would not be unreasonable to suggest that the CMV would be capable of detecting marijuana smoke by way of absorption and excretion from the lung airways in exhaled breath for purposes of distinguishing between marijuana smokers and nonsmokers. One burdening objective to this project would be the identification of significant VOCs necessary for the distinction of the groups. Differences in marijuana smoke and cigarette smoke reveal multiple classes of terpenes and cannabinoids which are only found in marijuana smoke [95]. Contingent upon their quantitative representation in the smoke, they should be theoretically found in exhaled breath after smoking marijuana. Exhaled breath research of marijuana smokers have focused on the detection of THC and its metabolites [See Chapter 4, Section 1.1] and not any other compounds associated with other fractions of marijuana plant. Evaluation of THC detection by CMV, using nicotine detection by CMV as a proxy will be discussed.

2.1 Nicotine as a proxy for THC

Nicotine was a convenient surrogate model for studying the exhalation of drugs administered through smoke inhalation, as it was legally accessible in comparison to the legal constraints placed around the research of THC and marijuana in the state of Florida. However, the chemical and physical properties of the drug must be reviewed for comparison and evaluation of the

appropriateness of nicotine as its surrogate. Some chemical and physical properties of nicotine and THC are listed in Table 13.

Table 13: Physical and chemical properties of nicotine and THC [84].

	THC	**Nicotine**
Vapor Pressure	4.63 x 10^{-8} mm Hg @ 25˚C	3.8 x 10^{-2} mm Hg @ 25˚C
Boiling Point	157˚C	247.57˚C
Flash Point	149.3˚C	101˚C
Henry constant	1.56 x 10^{-8} atm*m^3/mol	3.47 x 10^{-8} atm*m^3/mol
Log P	6	1.1
MW	314.5 g/mol	162.2 g/mol

Breath analysis reviews have suggested that compounds found in breath need a sufficient vapor pressure to be released [84]. Nicotine is semi-volatile in its free base form, but THC is nonvolatile. Under these assumptions it would be assumed that THC would not be detectable in breath, however that statement is inconsistent with published literature [See Chapter 4, Section 1.1]. Research clarifying the elimination conditions of THC in breath has not been well established. However, it is hypothesized that THC molecules are carried in breath adhered on aerosol particles. Hence the importance of capturing particles as well as capturing gas phase volatiles.

2.2 Evaluation of the CMV's Potential for THC Detection

The long pentyl side chain of the THC structure permits adequate Van der Waal force interactions with the siloxane groups of the PDMS coating of the CMV. The limit of detection of THC on CMV as reported in Table 1 of Chapter 3 is 84 pg. Considering the detectable concentration ranges of THC in the literature, it is

plausible that CMV technology would be capable of detecting THC in breath of marijuana smokers under appropriate detection windows.

Successful detection of THC by CMV would provide recent research analyzing THC in breath by a direct sampling and desorption extraction method, as solvent extraction is the current popular approach of THC detection in breath. It would demonstrate the application of CMV as an appropriate breath analysis confirmatory method for active ingredients of marijuana in the breath of recent marijuana smokers to reveal recent drug use. This technique has the capability to be made portable, which could aid law enforcement management of drug impaired drivers in traffic patrols, similar to breathalyzers for drunk drivers

CHAPTER 8: OVERALL CONCLUSIONS

The current study has demonstrated a clear potential of the CMV device as a non-invasive alternative sampling technique to current Tenax® sorbent tubes or SPME fibers for the detection and tentative identification of compounds in exhaled breath by thermal desorption GC/MS.

Within the limits of the experimental set up of the GC/MS method, it was concluded that the CMV can individually identify the endogenous and exogenous compounds in exhaled breath samples of cigarette smoker and nonsmoker volunteers. The resulting profile was not 100% diagnostic of a breath profile, but characteristic enough to distinguish between the two groups. Twelve compounds, including nicotine were statistically significant between the groups' breath profiles. Nicotine was detectable in 69% of smokers with a limit of detection in the lower pictogram range (absolute mass detected). Preliminary experiments on the headspace of marijuana PM offered insight on the expectations of CMV breath detection of marijuana smokers using THC as a target analyte.

This research exploring the idea of the CMV as a breath sampling device for nicotine detection acted as a proof of concept, and suggests sufficient reasoning for continued research and development of this application toward the detection of THC in the exhaled breath of marijuana smokers.

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