Analysis of Human Scent

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

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DEDICATION

This is dedicated to my parents, Jaime and Carmen Escobar.

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Abstract

ANALYSIS OF HUMAN SCENT

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The science of extracting, analyzing, and identifying small molecule metabolites from a complex sample matrix is known as metabolomics. Volatilomics is specialized metabolomics, focused on volatile organic compounds (VOCs) emanating from biological samples such as exhaled breath and the skin. With potential applications in forensics, rapid medical diagnostics, and biothreat detection, volatilomics has recently become of great interest. In this MS thesis, four VOC analysis techniques were assessed to determine their impact on the derived human scent volatilome; 1) a localized “trap and contain” approach using a custom-built hand-held VOC entrapment device, which combined with solid-phase microextraction (SPME), traps and captures the VOCs emitted from the skin, 2) a “gauze swipe-SPME” approach to skin VOC collection, using a gauze sorbent pad and modified airtight jar custom designed to accommodate SPME, 3) a “gauze swipe-TD” approach, using a gauze sorbent pad and airtight jar custom designed to accommodate thermal desorption (TD) tubes, and 4) a “BodPod-TD” approach, using a whole-body chamber custom designed to entirely enclose a person and capture their emitted VOCs using TD. With each of these four techniques, the captured VOCs were analyzed via gas

chromatography (GC), coupled with either a flame ionization detector (GC-FID) or a mass spectrometer (GC-MS). Comparative analysis offers insight into important considerations for each technique and VOC analyses overall, and highlights the BodPod-TD technique as the best suited method of those tested for human scent analysis.

Introduction

The natural ability of canines to differentiate between odor profiles has been used for decades by law enforcement agencies tracking down fugitives or for the detection of narcotics and explosives [13-14]. Using canines, the threshold for detection of Volatile Organic Compounds (VOCs) reaches as low as one part per trillion (ppt) [13-14]. However, false-positives, variability in detection, and extensive training requirements for canine and handler have prompted researchers to develop gas chromatography (GC)-based electronic noses to emulate the detection capability of the canine [6,24,25]. The thesis research described herein examines the use of an electronic nose for the differentiation of human VOCs.

VOCs are organic molecules present in the gas phase at ambient temperature. Metabolomics, an "-omics science" concerned with the comprehensive analysis of molecules/metabolites in biological specimens, employs various analytical chemistry techniques for the purpose of detection, identification, quantification, and profiling. Volatilomics focuses attention specifically on VOCs. There are two broad strategies for a volatilomics investigation. One is an untargeted extraction of all VOCs, known and unknown, from a given sample, and is known as a "global analysis" [2]. In contrast, a targeted analysis involves the detection of specific (known) VOCs and is often used for

validation/confirmation studies or those focused on quantification of an analyte [10]. An untargeted analysis can encompass up to thousands of analytes, and thus allows the creation of VOC profiles/fingerprints. VOC fingerprinting has potential in supporting the development of diagnostic assays, in biomarker discovery, in detection of threat agents, and in differentiating humans based upon their scent profiles, akin to differentiation based on fingerprinting.

Human samples such as skin, exhaled breath, or fecal matter, are excellent targets for volatilomics analysis, and have the potential to expand our current knowledge on metabolic pathways/processes including pathophysiology/etiology of disease. For example, the human fecal VOC metabolome of alcoholics revealed a statistically significant association between alcohol consumption and gut microbiota composition, and metabolic changes affiliated with the detection of elevated oxidative stress biomarkers, decreased fatty alcohol content, decreased short chain fatty acid abundance, and decreased microbe decomposition products [1]. Additionally, GC-based exhaled breath analysis of firefighters has served as a means of documenting exposure to VOCs and polycyclic aromatic hydrocarbons [12]. A study of exhaled breath for precision medicine yielded identification of endogenous acetone and isoprene, which are derived from metabolic processes such as glycolysis and cholesterol biosynthesis, respectively [22]. Other VOCs identified were likely derived from bacterial emission or diet, such as hydrogen sulfide, dimethyl sulfide, and propionic acid [22]. Likewise, in light of a canine's ability to differentiate and track individuals, an untargeted, volatilomics analysis of human skin is expected to yield individual molecular profiles (i.e. scent prints). Over 100 compounds

have been associated with human skin, including various ketones, aldehydes, esters, carboxylic acids, and sulfur-containing compounds such as dimethylsulphone and benzothiazole [4,8]. The epidermis on the upper back has an increased concentration of eccrine glands as compared to the forearm, and thus may serve as a higher source of VOCs for analysis [5].

A variety of sampling methods and extraction procedures are available for an electronic nose based volatilomics analysis, each of which having their own advantages and disadvantages. Typically, methods of isolating VOCs involve the use of sorbent materials which are placed on or above the skin [4]. Headspace-solid-phase-microextraction (hSPME) involves the partitioning of VOCs from the headspace above a sample into a polymeric sorbent adhered to a fused silica rod [3]. Partitioning of VOCs utilizing the headspace above the skin has been shown to yield more VOCs than by using a skin contact SPME method [11]. By subsequently transitioning the SPME fibers into the GC, the analytes that are extracted are desorbed from the fiber and into the GC inlet. As the analytes travel through the GC column, chromatographic separation occurs. The separated compounds then reach the detector, which in this thesis work is either a flame ionization detector (FID) or a mass spectrometer (MS).

Solid phase microextraction is a sampling technique that is relatively inexpensive, requires no solvent, involves less sample preparation, and takes advantage of the free concentration of analytes in a biological sample [9]. However, one limitation in the use of SPME is that sorbent polarity dictates selectivity. An individual SPME fiber has a limited

range of compounds that it can extract, based upon its sorbent chemistry ("like-dissolves-like"). Though this may not present limitations in a targeted analysis, when the aim of a VOC analysis is a global survey of metabolites, more than one sorbent type needs to be used [1,3]. Another limitation with SPME, at least in the context of this thesis work, is that it is a passive sampling method. Passive sampling relies on the free flow of volatiles from the sample to the SPME fiber, which is time and concentration dependent [15-16]. Passive sampling methods have been used extensively in environmental studies which track change in targeted VOCs over the course of large spans of time, such as seasons [19]. In having to take into consideration extraction duration as well as maximizing VOC capture, an active sampling method may be preferred.

The process of active sampling uses a pump or vacuum to move the VOCs towards the sorbent [19]. With SPME fibers, the rate of adsorption of the analyte to the sorbent hinges on the fiber surface area and the passive movement of air in the headspace above the sample matrix. A limitation to this approach includes how the absolute recovery of analytes from the flow stream is low due to the placement of the SPME fibers above the sample matrix as well as the surface area of each SPME fiber. In contrast, the process of Thermal Desorption (TD) involves the use of a sorbent-filled tube to capture VOCs; the active movement of a sample through the tube drastically increases the surface area of sorbent that the VOCs are exposed to [26]. A multi-sorbent approach may also be used with TD, to address the diversity of metabolites found within human skin and breath. Whether SPME or TD is used, the captured analytes are often concentrated at the head of the GC column using a cryotrap in the GC [26].

VOC capture depends on several environmental and experimental factors for both SPME and TD, including polarity of the sorbent, nominal flow rate over or through the sorbent, length of interaction between the sorbent and analyte, and abundance of the sample in the sample matrix [20]. VOC capture can also be impacted by the flow of VOC emanating from sample matrix to sorbent. Longer interactions between analytes and sorbents favor higher recovery of VOCs, and thus increased signal intensities in the resulting gas chromatograph [20]. Fast sampling methods, by way of short extraction durations (hSPME) [1] or increased flow rate (TD) [20], typically decrease VOC extraction. Hence, to minimize analysis times yet still obtain sufficient analytes, extraction durations (hSPME) or flow rate (TD) should be optimized.

The Couch lab has demonstrated how enclosing a sample within a chamber traps VOCs and facilitates a volatilomics analysis [23]. Accordingly, four different techniques that build on the idea of preconcentration of analytes within a chamber were created for this thesis work and assessed for their ability to produce human VOC fingerprints. These techniques are the 1) localized “trap and contain”, 2) “gauze swipe-SPME”, 3) “gauze swipe-TD”, and 4) “BodPod-TD” approach. Some of these techniques focus on localized VOC detection, such as the “trap and contain” approach or both of the “gauze swipe” approaches, whereas the “BodPod-TD” approach is designed for whole body VOC analysis.

Research Aims

The two Specific Aims of this MS thesis research are as follows:

Specific Aim 1. Assess 4 different human VOC sampling methods to determine which is best suited for human volatilomics. The 4 methods are; 1) a localized "trap and contain" approach, 2) a "gauze swipe-SPME" approach, 3) a "gauze swipe-TD" approach, and 4) a "BodPod-TD" approach. Effort will focus on generating a human VOC fingerprint containing a numerous and diverse array of VOCs.

Specific Aim 2. Employ the ideal sampling technique identified in Specific Aim 1 and assess its ability to differentiate human study volunteers.

Methods and Experimental Design

In this study, methods of VOC capture involving the use of either SPME or TD coupled with GC-MS or GC-FID are assessed. The principles of GC-MS/FID are summarized in Appendix 1 of this thesis. An outline/overview of the VOC capture and analysis process used is shown in Figure 1.

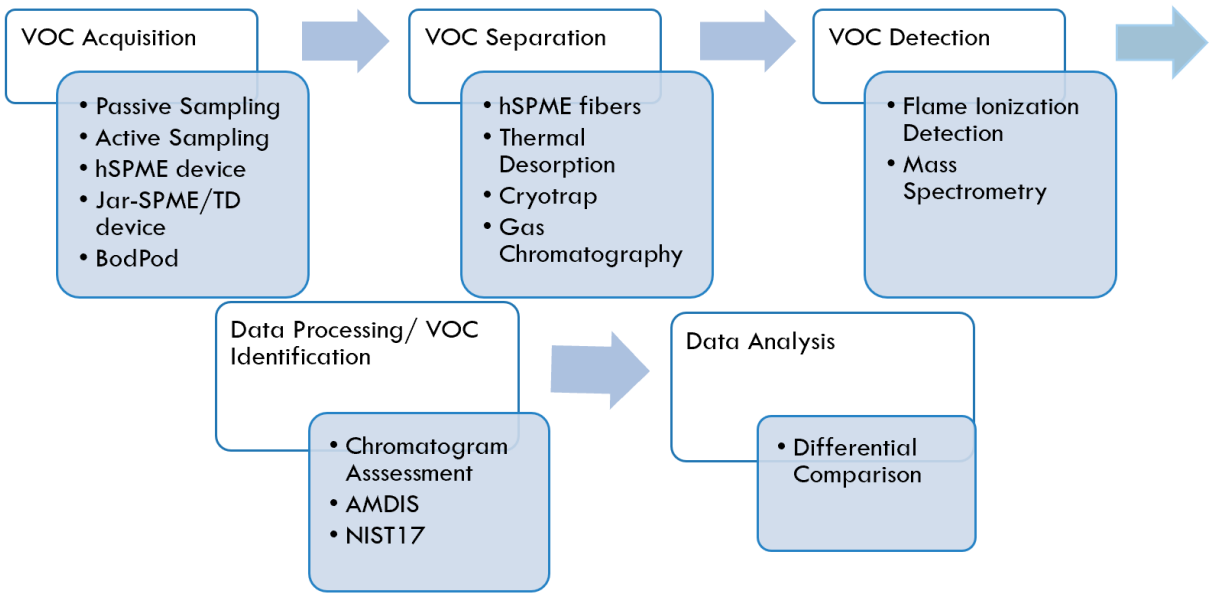


Figure 1. Metabolomics Workflow*. An untargeted VOC analysis follows a pipeline including VOC acquisition, VOC separation, and VOC detection, followed by data processing and analysis.*

For this investigation, VOCs were analyzed using an Agilent 7890A GC-FID, coupled with a DB5-MS capillary column (Agilent, Palo Alto, CA; 15 m length, 0.25 mm ID, 0.25 μm film thickness), an SIS Short Path Thermal Desorption attachment (Model TD-5), a 0.75 mm ID SPME injection port liner, and a flame ionization detector (FID). The Thermal Desorption port is set to 300 °C, which holds for 12.5 minutes, and has an injection duration of 1.5 minutes. The GC inlet is set to splitless mode which allows for the detection of low amounts of analytes in each sample matrix. Helium carrier gas flow rate is set to 1.5 mL/min. The GC oven is held initially at a temperature of 40 °C for 3.5 minutes, then ramped at 10 °C/min up to 325 °C for 30 minutes, for a total run time of 50 minutes. The cryogenic trap holds a temperature of -20 °C for 3.5 minutes before rapidly heating to 40 °C and releasing the analytes onto the column. The FID has a data acquisition rate of 20 Hz, the heater is set to 320 °C, H_2 flow is set to 40 mL/min, and the air flow is set to 450 mL/min.

VOCs were also analyzed using an Agilent 7890B GC-MS with a DB5-MS capillary column (Agilent, Palo Alto, CA; 30 m length, 0.25 mm, 0.25 um film thickness), SIS TD-5 short path system, and an Agilent 5977B MSD. The Thermal Desorption port is set to 250 °C for 16 minutes. The cryogenic trap is set to cool down to -20 °C for 12 minutes concurrently with the duration of the Thermal Desorption unit heating. The GC is set to splitless mode. Helium carrier gas flow rate is set to 1.5 mL/min. The GC oven is held initially at 30 °C then ramped at 20 °C/min up to 250 °C and held for 4 minutes. The total run time is 30 minutes. The MSD has a frequency of 2.9 scans/s and scan speed of 1.562 u/s.

Technique 1: Localized “trap and contain” Approach

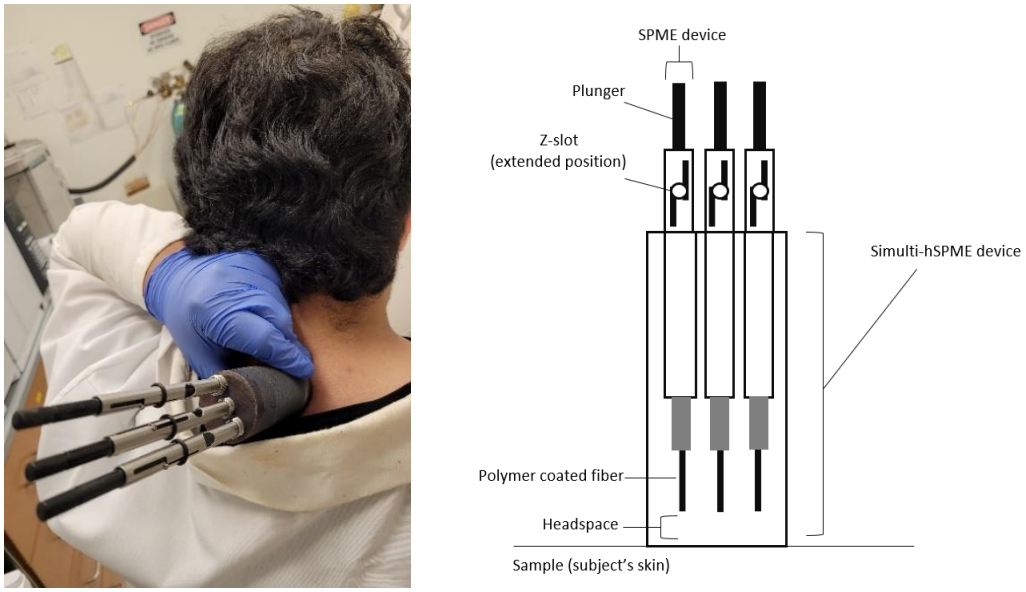


Figure 2. The localized “trap and contain” approach to VOC acquisition*. The simulti-hSPME device is a handheld entrapment device which can accommodate multiple SPME fibers for VOC extraction. The cylindrical, stainless steel device is pressed against the subject’s skin for 30 minutes as the fibers are fully extended, capturing the VOCs in the headspace above the skin. VOCs associate with the SPME fibers based upon solubilities (“like-dissolves-like”).*

The “trap and contain” device (Figure 2) is a custom-built stainless-steel cylinder that is open on one end and fitted with 5 SPME ports on the other. The device allows simultaneous multifiber (simulti-) headspace extraction of the emanating skin VOCs. The use of a simulti-hSPME device is incorporated into the workflow for collection of analytes to allow capture of the variety of polarities present in human skin. The fibers used are as follows: a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS fiber), a

polyacrylate fiber, and a Carbowax/Polyethylene Glycol fiber. After preconditioning each of the fibers in accordance with their manufacturer's guidelines, the SPME fibers are used to collect ambient VOCs in the room (30 minute extraction duration), which are then analyzed by GC-FID. Establishing the background (room) VOC profile allows for comparison to the subsequently derived human VOC metabolome. Analytes that would be found in the human VOC profile, but not in the room VOC profile, would be considered unique to that human VOC profile. Prior to the human VOC analysis, the fibers are preconditioned another time according to the manufacturer's directions, to avoid detection of residual analytes from the previous run. The hSPME device used for the "trap and contain" method is placed on the subject's upper back for 30 minutes. The SPME fibers are then analyzed by desorbing the collected VOCs into the GC-FID. Replicate runs are performed to ascertain reproducibility.

Technique 2: The "Gauze Swipe-SPME" Approach

Figure 3. A jar with modified lid fitted with ports allowing for insertion of SPME fibers. *The sample gauze pad, after having been wiped on the human skin, is placed inside the jar. Three SPME fibers insert into the ports of the modified lid, and capture the VOCs.*

The Gauze Swipe-SPME technique involves using multiple SPME fibers at the same time (simulti-hSPME) for sampling as a means of addressing the polarities of the VOCs present in the human skin. It also involves the use of a piece of gauze to swipe VOCs from a selected sampling site, which is then placed inside a jar in order to contain them.

The lid to the jar is modified to accommodate up to 4 SPME fibers at a time. The SPME fibers passively adsorb the VOCs present in the headspace above the gauze.

Technique 3: The "Gauze Swipe-TD" Approach

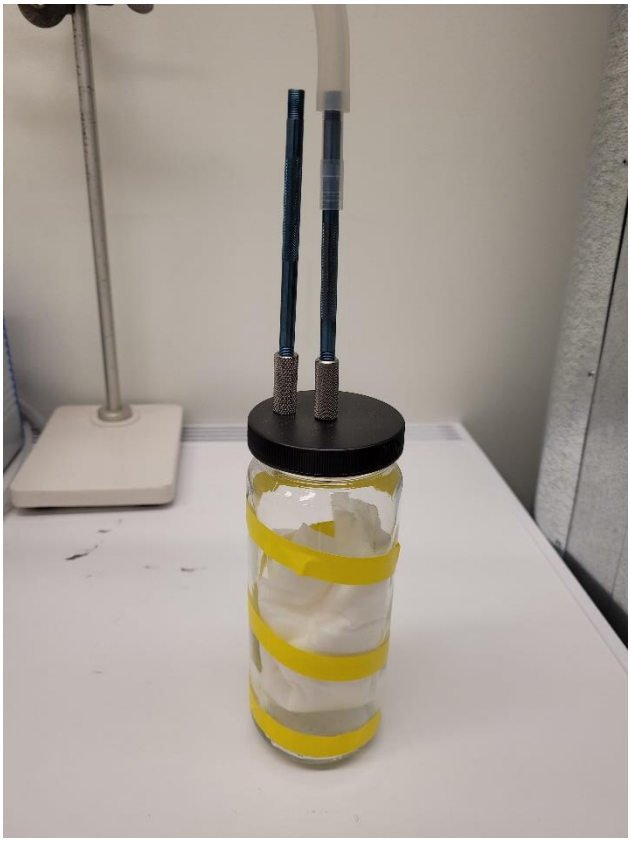


Figure 4. A Jar with Modified Lid for Thermal Desorption. *A gauze pad is used to wipe a sampling site on the subject skin and is placed in the jar. The modified lid allows the fitting of two thermal desorption tubes; one for active collection of the VOCs (via vacuum, pictured on the right), and the other to filter the incoming air entering the jar when the vacuum is applies (i.e. a "scrubber", pictured left).*

While SPME is passive sampling, TD is an active sampling method that utilizes a vacuum to create an active flow of analytes through a sorbent bed. The Gauze Swipe-TD

approach uses a jar with modified lid that can accommodate the TD tubes (Figure 4). To collect the VOCs, the gauze pad is wiped on the back of the subject's neck and then immediately placed into the jar. Once the lid is sealed, two thermal desorption tubes are affixed in place on the jar's lid. The first tube is used for VOC acquisition. Tubing is placed onto the first tube which itself is connected to a vacuum system. The vacuum pumps analytes from the gauze pad inside of the jar through the sorbent bed inside of the TD tube. The vacuum system is left on for 30 minutes as the analytes adhere to the sorbent. The second TD tube is used to trap exogenous VOCs before they enter into the jar, as negative pressure is created inside the jar when the vacuuming system is on. VOCs that are captured on the sampling TD tube are then eluted into the GC via thermal desorption.

Technique 4: The "BodPod-TD" Approach

A BodPod-TD approach was developed to capture "whole-body" VOCs emanating from a person sitting within a BodPod chamber (Figure 5). The BodPod, or air displacement plethysmograph, is a chamber usually used for determining body fat composition via measuring the displacement of the air within the chamber [21]. Our customized BodPod incorporates whole body VOC analysis by allowing analytes to concentrate within the BodPod before sampling. The volunteer sits in the BodPod for 30 minutes while VOCs from the skin and exhaled breath collect inside of the chamber. After 30 minutes, vacuum is used to actively pull a fraction of the air out of the BodPod chamber and into an airtight sampling bag.

Figure 5. The "BodPod-TD" Approach to human VOC capture. A) *The volunteer sits in the BodPod for 30 minutes as the exhaled breath and skin VOCs are contained.* ***B)*** *A port on the outside of the BodPod is used with vacuum to collect the VOCs into an airtight bag.* ***C)*** *The contents of the sampling bag are transferred through a TD tube. The flow is regulated at 0.5 L/min (Hilitand LZQ-2 air flow meter). Between each sampling, the chamber of the BodPod is washed with 70% EtOH*

solution and allowed to dry with the chamber door open so as to minimize contamination and carryover.

The contents of the sampling bag are then actively moved through a TD tube, using an external vacuum system (Figure 5C). The TD tube contains a homogenized resin mixture of Tenax TA (10 mg), Carbosieve (10 mg), Carbopack B (10 mg) and Carbopack C (10 mg). Once the sampling bag is empty, the TD tube is removed from the tubing and then placed in the Thermal Desorption system for analysis by GC-MS. Between each analysis, the BodPod is washed with 75% EtOH and left open to air dry. It is notable that study participants were informed to avoid using perfumes, fragranced hand soap, and to not eat immediately prior to entering the BodPod. Three individual human subjects were involved in the study and a total of ten samples were taken from each subject.

To account for temporal changes in VOC signature as well variations in signal intensity between each run, an internal standard was introduced into the BodPod. An internal standard is a known amount of compound that is distinct from the sample analytes and facilitates quantification. The use of an internal standard helps in normalization by reducing variations present in the chromatograms. When selecting an internal standard, certain factors should be taken into consideration: the polarity of the sample matrix, consistency of retention times, and consistency of signal intensity. Given the involvement of human subjects in this VOC analysis, the toxicity of the internal standard is also considered. Peppermint oil, an essential oil used for aromatherapy, meets these criteria as

it contains a variety of compounds which present themselves at different retention times along the chromatogram. These constituents include α-Pinene, β-Pinene, and α-Terpinene, which have been found in over 157 commercially available Peppermint oils analyzed by GC-MS [27]. One milliliter of internal standard is placed in an uncapped vial and positioned alongside the human subject inside of the BodPod for the 30 minute duration of sample preconcentration.

AMDIS and NIST Analyses

The spectral data produced by the GC-MS facilitates identification of analytes, and adds another dimension to the data set. Analytes were identified using National Institute of Standards and Technology (NIST17, version 2.3) molecular database coupled with the NIST Automated Mass Spectral Deconvolution and Identification System (AMDIS). The settings selected for AMDIS (build 149.31, version 2.73, National Institute of Standards and Technology, Gaithersburg, MD, USA) were found empirically and are listed in Appendix 3.

Data Filtering and Analysis

Each technique is analyzed by GC-FID. The technique that best demonstrates cohort separation between the blank and sample extractions is selected for GC-MS analysis. For the extractions analyzed by GC-MS, a total of 3 volunteer subjects are involved, with each volunteer having 10 replicate extractions performed. Each human sample run is preceded by a 'blank' or control run in which the VOCs of an empty, closed analysis vessel are analyzed. For GC-MS data, from the total number of peaks from each individual chromatogram, the list of peaks are reduced based upon their match criteria with NIST/AMDIS. A match score of 80% was chosen as the threshold. Analytes that meet the threshold were entered into an excel spreadsheet with the analyte identity and retention time being recorded. These entries are organized by human subject and their sample run number. The analytes found in the volunteer subject are compared to the set of analytes detected in the blank. Analytes that are found in both the human sample and in the blank are filtered out.

For both the GC-FID and the GC-MS analyses, a frequency filter cutoff was employed to ensure that only analytes of statistical significance contributed to the generation of dendrogram heatmaps. The threshold was kept at 60% presence within a given cohort. For the GC-MS analyses, ten replicate chromatograms were obtained for each study participant. The number of times a metabolite was identified out of the ten replicates

was labelled as that metabolite's frequency. Metabolites of low frequency occurrence within each cohort were noted but not included in the downstream data analysis.

Results and Discussion

Each of the 4 sampling techniques was assessed and the resulting VOC chromatograms were qualitatively compared using either an overlay or mirrored chromatogram display format. In a chromatogram, the peaks reflect the VOCs found in a given sample, and their retention times reflect their polarity (with more polar compounds eluting from the GC column first). When comparing two different chromatograms, if both share a peak at the same retention time, then it is possible that both samples share the same VOC in their molecular profile. GC-MS is advantageous in that it also reports m/z values for each of the VOCs present in the sample, giving further confidence that two samples have the same composition. Peak intensity reflects ionizability and correlates with VOC concentration a sample. Though retention indices can be used to determine compound identity, the purpose of the GC-FID based analyses was to qualitatively compare the chromatograms to determine if the given VOC analysis method will generate a human VOC fingerprint consisting of numerous peaks of significant intensity.

Technique 1: Localized "Trap and Contain" Approach Results

The localized "trap and contain" approach (method 1) is a passive sampling method involving the use of a stainless-steel hSPME device which functions as a chamber that can fit three differently coated hSPME fibers. The fibers sample the VOCs present in the headspace above the skin. For comparison, blank VOC extractions were also performed (sampling the room air). In total, 32 human VOC extractions and 32 room VOC extractions were performed. A representative pair of chromatograms are shown in Figure 6. Using ChemStation software (Appendix 2), a list of analytes and their associated retention times were obtained. The room VOC chromatogram has 20 detected analytes, while the human test subject had 68 analytes detected (Figure 6). The 20 analytes in the room VOC chromatogram matched the retention times of the analytes found in the human chromatogram, meaning that those analytes were VOCs found in both the room and human VOC profiles, and thus are not human-associated. On the other hand, 48 analytes detected in the human VOC chromatogram are unique to the human VOC profile, and not detected in the room VOC profile. Hence, the localized "trap and contain" approach was found to differentiate the room VOCs from the human VOC profile.

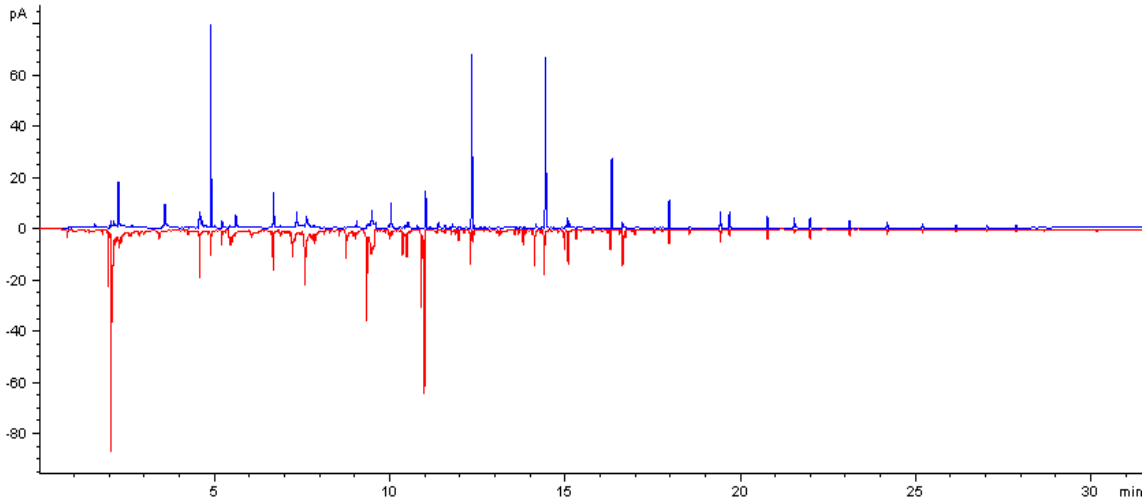


Figure 6. Mirror of chromatograms reflecting the VOC profiles of a 'blank' extraction (VOCs present in the room) and a human subject using the "trap and contain" approach. *Representative chromatograms are shown. The human VOC signature is shown in red. The room VOC profile is shown in blue. The x-axis displays retention time (in minutes) and the y-axis displays signal intensity (in pA).*

While retention times may be similar, the abundances, or intensity of the signals, are noticeably different. An all-encompassing comparison of the RT and signal amplitude from every sample and blank extraction was visualized through a dendrogram heatmap (Figure 7). To ensure that only analytes of statistical significance are included in analysis, one-offs and analytes of low frequency are disregarded. Only analytes that appear at least 60% of the time within a given cohort at a given retention time are included. The peak heights from each chromatogram were normalized by log10 transformation. Using the average abundances and standard deviations of the signal intensities at each given range for retention time, which was binned at increments of 0.1 minutes, the data was scaled via

pareto scaling. Using a custom R script, a dendrogram heatmap was generated in order to visualize the hierarchical clustering based on Euclidean distance. As observed in Figure 7, there was separation between the blank cohort and the human sample cohort. Though there were some extractions in the human cohort that were similar enough to cluster with the blank extractions, and vice versa, there appeared to be significant cohort separation.

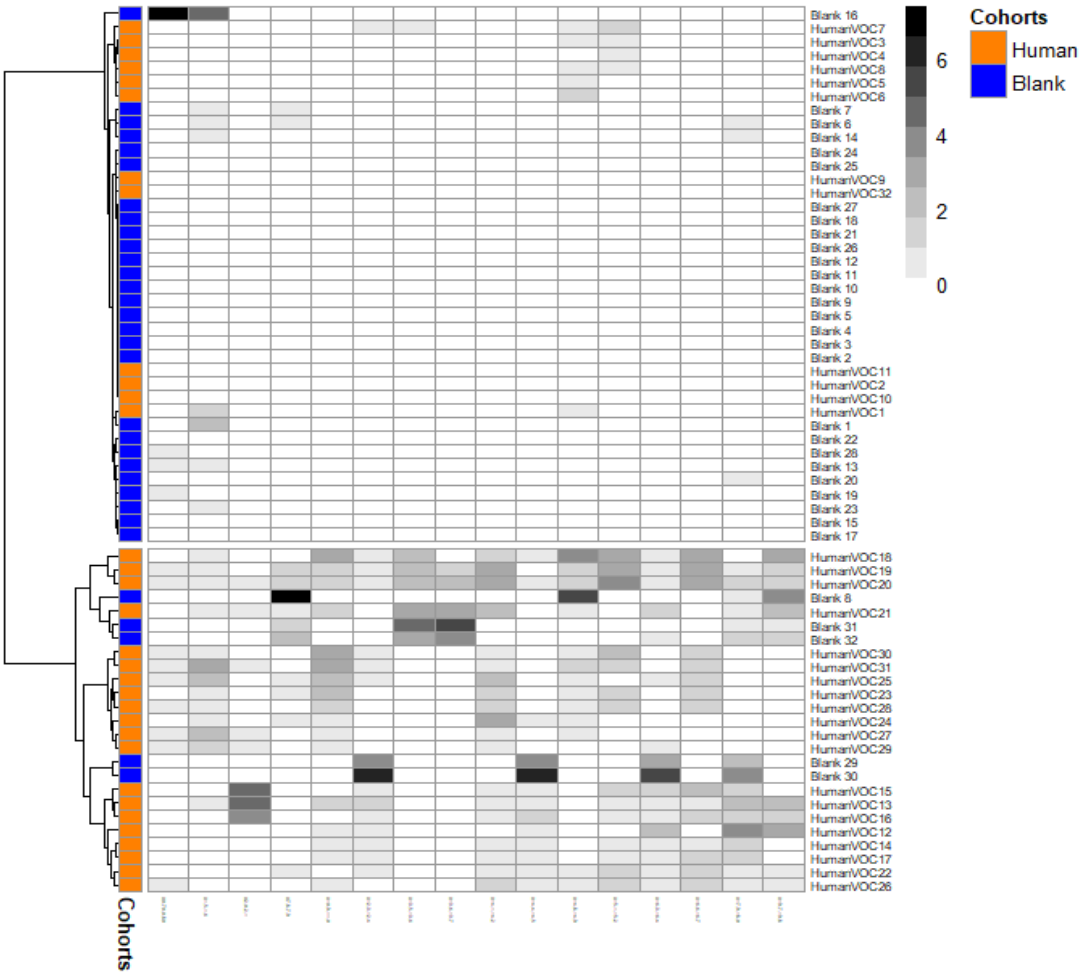


Figure 7. Dendrogram Heatmap of the sample and blank extractions performed using the localized "Trap and Contain" approach. *The x-axis represents the retention times, binned in increments of 0.1 minutes, that contain at least one feature. The y-axis consists of the labels of the individual extractions performed. Each node in the dendrogram represents two groups that have been clustered together based on similarity in terms of signal amplitude at the given retention time range. Only features that met the 60% frequency filter threshold were included. All of the extractions were sorted based on similarity, regardless of the order in which they are inputted. The shading of each square in the heatmap corresponds with amplitude of the signal. A white square means there were no features found for that given sample at that retention time while a black square signifies highest relative signal intensity. Cohorts are distinguished based on color with orange corresponding to human extractions and blue corresponding to blanks.*

An advantage of this technique is its ability to easily differentiate between room VOCs and human VOCs. The technique also had enough sensitivity to detect the human VOCs from the upper back region of the volunteer subject.

A possible disadvantage of this technique relates to the localized skin sampling area. As been reported, sampling site impacts the VOC profile generated using SPME [5]. VOCs found in the axillary region differ from those found in the forearm and upper back, due to the difference in the type of glands (the axillary region primarily having apocrine glands and the forearm and upper back primarily having sebaceous glands) [41]. Even between the upper back and forearm, the VOC profiles differ in terms of the presence of lipophilic salicylate [5]. Though this technique could lend itself toward a targeted analysis focusing on a specific site for sampling, this technique may not be conducive for a global analysis that does not discriminate based on sampling site.

Technique 2: The "Gauze Swipe-SPME" Approach Results

The "Gauze Swipe-SPME" approach is a passive sampling method involving the use of three differently coated hSPME fibers as well as a gauze pad which is used to swipe VOCs off a given site on the human skin before being placed inside of a jar for sampling. A total of 14 extractions, seven blanks and seven samples from one subject, were performed using this technique. A representative pair of GC-FID derived sample and blank chromatograms was chosen for differential comparison (Figure 8). The blank extraction refers to the VOC analysis of a clean piece of gauze while the sample extraction refers to that same piece of gauze after it was used to wipe the upper back region of the subject.

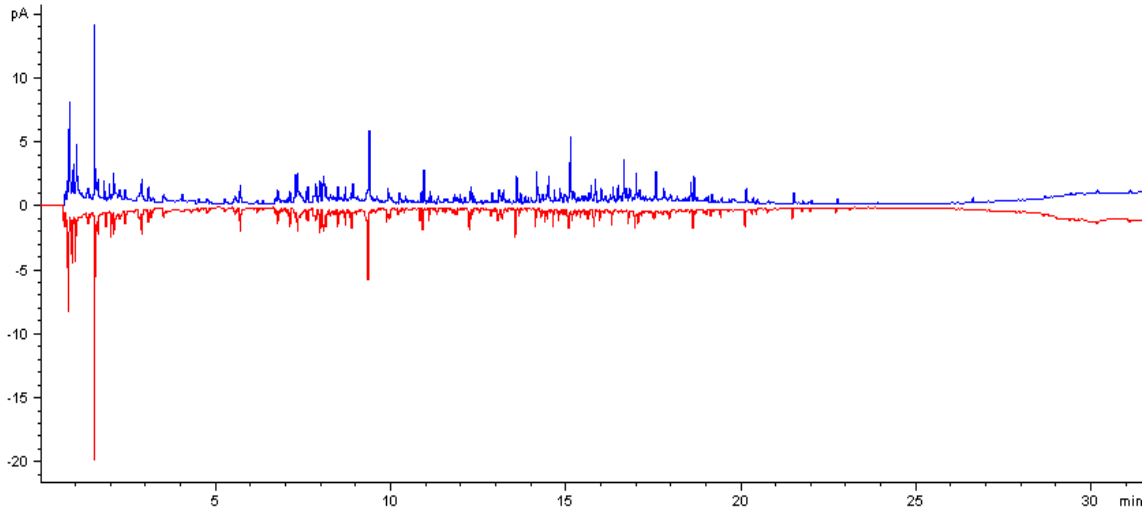


Figure 8. Mirrored chromatograms evaluating "Gauze Swipe-SPME" approach. *Two GC-FID chromatograms are displayed mirrored with the top chromatogram pertaining to human VOC signature (gauze that has swiped VOCs from upper neck region) and the bottom pertaining to a blank extraction (no human VOCs present at all). The x-axis displays retention time (in minutes) and the y-axis displays signal intensity (in pA). Each peak represents a potential analyte as detected*

by the FID. Peaks that have the same retention time and similar signal intensity in both chromatograms indicate that both samples have that same analyte.

The components involved in this comparison are based on the peak integration results performed by ChemStation software which outputs a list of peak signals which met the signal/noise ratio threshold (Appendix 2). The total number of analytes detected does not differ significantly between the sample and blank extraction: 23 components were detected for the sample chromatogram while 22 were detected for the blank chromatogram (Figure 9). Given the low amplitude of the signals in both chromatograms, only 22 and 23 components were detected respectively; some of the peaks visible on the chromatogram were of low enough abundance to not meet the threshold for being distinguished from noise and thus were not integrated as components of the chromatogram (Appendix 2). While the blank and sample chromatograms appear very similar, some notable differences are seen; for example the human VOC profile has peaks at retention times 0.267, 7.322, 15.119, 15.705, 15.831, 16.646 and 17.762 minutes which are not at all present in the blank extraction. The signals of greatest abundance in the human VOC chromatogram, those with a percentage area of greater than 5, were the analytes at retention times 0.805, 1.521, 9.377, 15.119 and 16.646 minutes. The most prominent peaks from the blank chromatogram (in red) were at 0.806, 1.528 and 9.375 minutes. Retention time variations between both chromatograms, for the retention times that matched, fit within 0.01 minutes, suggesting that they are the same analytes. However, there were two analytes of significant relative abundance that were detected in the human VOC chromatogram that were not detected in

the clean gauze VOC chromatogram: the analytes at retention times 15.119 and 16.646 minutes. Despite the number of analytes detected being similar, there was differentiation of VOC profile based on the retention times of the analytes, two of which were relatively abundant.

A dendrogram heatmap was generated in order to determine whether there was cohort separation between the sample and blank extractions. As observed in Figure 9, the human and blank extractions appeared to cluster together within separate clades with no indication of broader clustering within the whole human or whole blank cohort. The lack of complete cohort separation indicates the inability of the method to distinguish human VOC profiles from the blank (clean gauze) VOC profiles. The parameters for peak integrations, determining whether a peak is signal and not noise, are consistent between all of the approaches (Appendix 2), ruling it out as a possible source for variability.

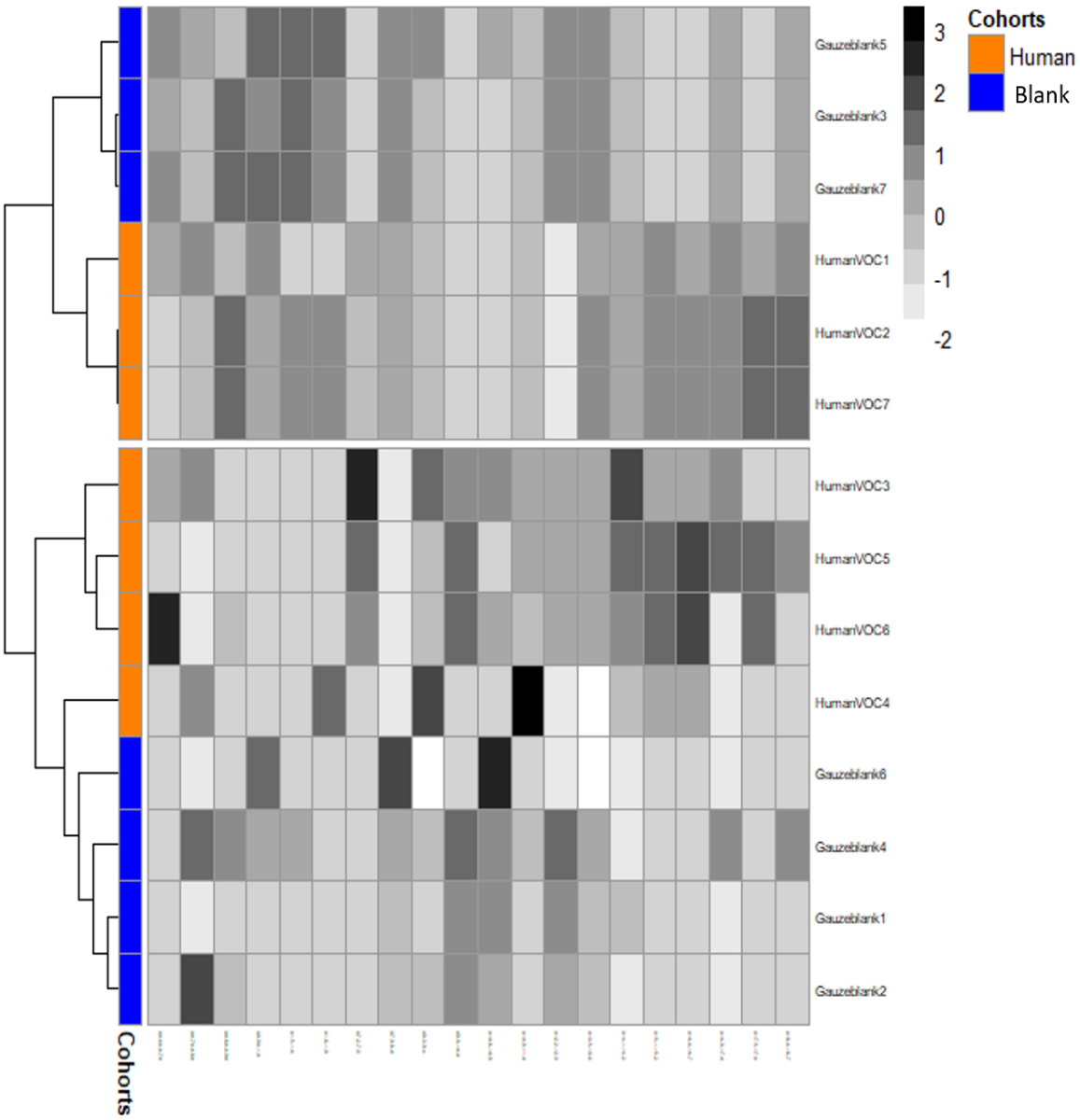


Figure 9. Dendrogram heatmap of the sample and blank extractions performed using Method 2. *The x-axis represents the retention times across the chromatogram, in increments of 0.1 minutes. The y-axis consists of the labels of the individual extractions performed. Peaks that were present in at least 60% of the extractions performed within each cohort at that given retention time were included in the heatmap. The shading of each square in the heatmap corresponds with area of the peak signal. A white square means there was no feature present at that retention time for that chromatogram. Meanwhile, a black square signifies highest relative signal intensity.*

An advantage of this technique includes the ease of use compared to the localized "trap and contain" approach. Whereas the localized "trap and contain" approach requires the researcher to keep the cylinder of the hSPME device pressed on the upper back of the volunteer subject for 30 minutes, method 2 only requires a single gauze swipe to be performed on the upper back before placing the gauze inside of a jar. Method 2 also yielded more sample-unique analytes than did method 1.

However, the main disadvantage is that this technique cannot ultimately distinguish sample VOC profile from the blank extractions, based on the dendrogram heatmap generated from the features found in each of the chromatograms. The threshold for what constitutes a signal was consistent between method 1 and method 2 (Appendix 2), ruling out integration parameter changes as a source for the difference in total analyte detection. Greater amounts of signal help facilitate downstream analysis of the VOCs so it is imperative for the method to yield as many components in the chromatogram as possible. Another disadvantage of this technique includes the lower average amplitude of the signal intensities. The most prominent peaks found in the method 1 chromatogram were of higher intensity than those found in the method 2 chromatogram. When coupled together, the low number of total analytes detected and the low signal intensity of the components relative to the first technique demonstrate that this method has lower usability with regards to detection of VOCs.

Technique 3: The "Gauze Swipe-TD" Approach Results

The "Gauze Swipe-TD" approach (method 3) is an active sampling method involving the use of a gauze pad which swipes VOCs off the upper back, a jar in which the gauze pad is placed, and thermal desorption tubes which have multiple sorbent types within them for the purpose of sampling the VOCs from the gauze pad. A vacuum system actively pumps air out of the jar so that VOCs collected on the gauze would flow out of that jar and onto the sorbent inside of the sampling TD tube. A total of 13 blank and sample extractions were performed; seven samples and six blanks. A representative pair of GC-FID chromatograms is chosen for differential comparison in Figure 10.

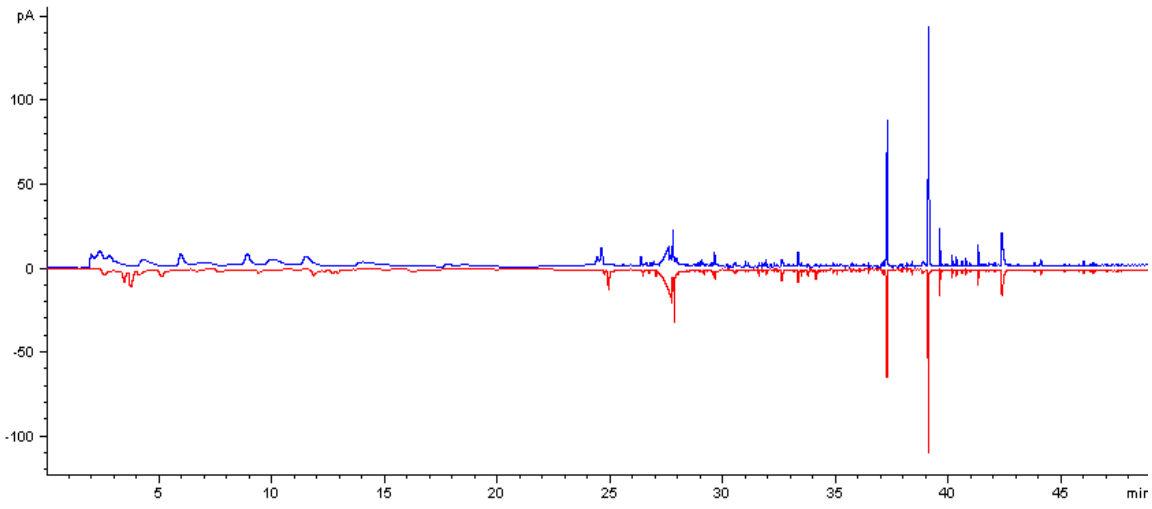


Figure 10. Comparison of GC-FID chromatograms evaluating the "gauze swipe-TD" approach. *The bottom chromatogram displays the signal detected from a new piece of gauze, serving as the blank extraction. The top chromatogram displays analytes detected from the same piece of gauze*

after it had been wiped on the back of the neck of a volunteer subject. The x-axis displays retention time (in minutes) and the y-axis displays signal intensity (in pA).

The human VOC chromatogram shows a total of 42 detected analytes while the blank shows a total of 49 detected analytes (Figure 10). For the purpose of determining variation of retention time of like peaks and determining the presence of distinguishing peaks, peaks that had a percentage area of greater than five in the human VOC were compared to those in the blank chromatogram. Prominent peaks in the human VOC chromatogram had retention times at 3.699, 27.712, 27.827, 37.264, 39.116 and 42.366 minutes. The blank chromatogram had prominent peaks at retention times 24.601, 37.281, 39.130, and 42.364 minutes. Retention time variations between prominent peaks with similar retention times in both chromatograms fit within 0.02 minutes, suggesting that these peaks represent the same analyte present in both extractions. There are components that distinguish the human VOC chromatogram from the blank; the components at retention times 3.699, 27.712, and 27.827 minutes. For the GC-FID extractions using Thermal Desorption, the cryogenic trapping stage takes place over the first 12 minutes of the chromatogram. Low boiling point solutes elute earlier in the chromatogram, meaning that the smaller, more volatile compounds would be the first components to elute. The peaks at 27.712 and 27.827 also contribute to the differentiation of the human VOC profile from the blank.

Just as with the previous methods, a dendrogram heatmap was generated (Figure 11) using the GC-FID data of retention time and signal amplitudes in order to determine whether there was cohort separation between the sample and blank extractions. Based on Figure 11, the human and blank cohorts had no indication of separation based on feature presence or signal amplitude, revealing that the technique was unable to distinguish sample human VOCs in the gauze from the clean gauze that served as the blanks. Peak integration parameters were consistent between method 3 and methods 1 and 2.

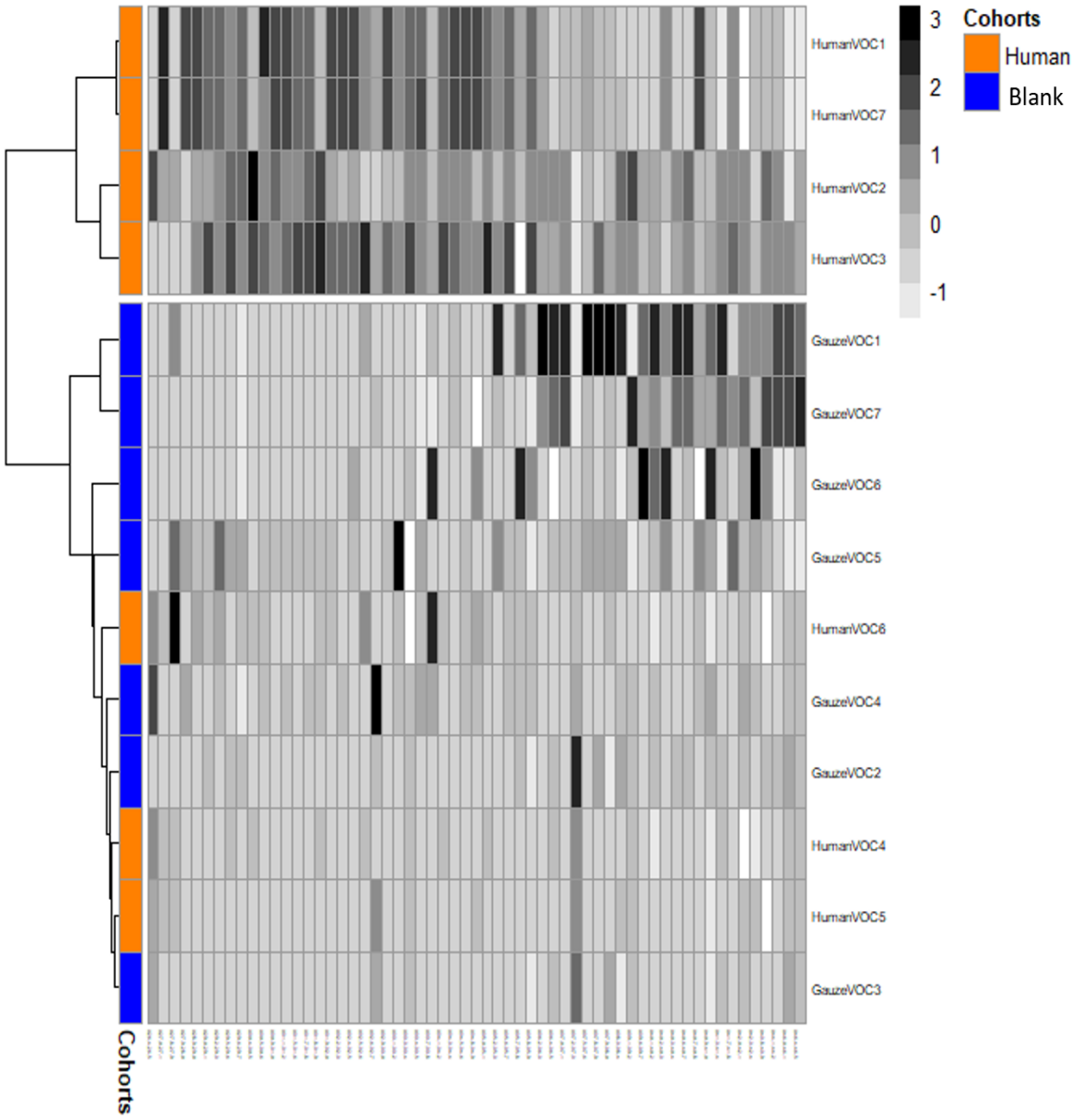


Figure 11. Dendrogram heatmap of the sample and blank extractions performed using Method 3. *The x-axis represents the retention time ranges across the chromatogram, in increments of 0.1 minutes, that contain features that met the 60% frequency filter cutoff. The y-axis consists of the labels of the individual extractions performed. The shading of each square in the heatmap corresponds with area of the signal. A white square means there were no features found at that retention time for that given extraction while a black square signifies highest relative signal area. The orange blocks correspond to the human cohort while the blue correspond to the blank extraction (clean gauze) cohort.*

An advantage of method 3 includes the higher number of total analytes detected relative to method 2. The integration parameters were consistent between these two methods, indicating that the difference in number of detected analytes can be attributed to method efficacy. Method 3 also achieved detection of slightly more differentiating analytes of high relative abundance (percentage area of greater than five). Two prominent peaks differentiated the human VOC extraction from the blank extraction in method 2, while three components distinguished the human VOC extraction from the blank extraction.

The main disadvantage of method 3 is that, similar to method 2, it did not demonstrate cohort separation between all of the human and blank VOC cohorts. The blank extractions, each of which consisted of a clean piece of gauze placed inside a jar for active sampling, were not found, as a whole, to be distinguishable from the human sample extractions, which consisted of that same piece gauze wiped across the upper back of the subject before being placed back in the jar for active sampling.

Technique 4: The "BodPod-TD" Approach Results

The "BodPod-TD" approach (method 4) is an active sampling method involving the use of an air-displacement plethysmograph, or 'BodPod', for preconcentration of analytes before sampling using thermal desorption tubes containing multiple sorbent types. Unlike the other methods, which are localized to the area of the body they are sampling, the "BodPod-TD" approach is a whole-body analysis. As the subject sits inside of the BodPod, Human VOCs collect within the chamber of the BodPod for 30 minutes before they are transferred into a 5-liter sampling bag via a vacuum pump. Next, the captured VOCs are pumped from the sampling bag through a TD tube (containing the sorbents). A total of 16 blank and companion sample extractions, eight for each, were performed. As seen in Figure 12, the empty BodPod results in a total of 40 detected analytes while the human in BodPod VOC extraction results in a total of 164 detected analytes.

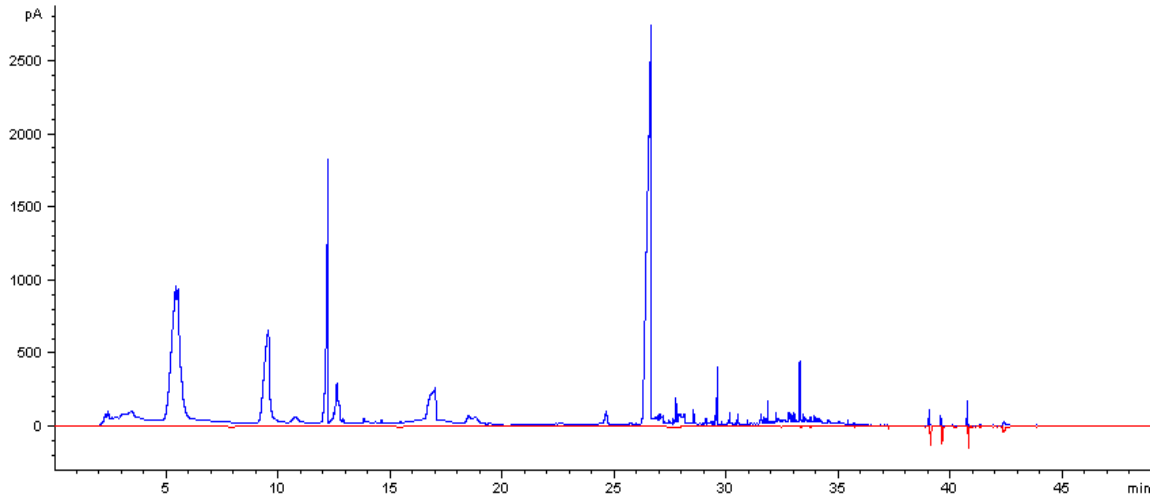


Figure 12. Mirrored chromatograms comparing a sample and blank VOC signature using the "BodPod-TD" approach. *Two GC-FID chromatograms are mirrored along their x-axis for differential comparison. The blue chromatogram shows a human VOC extraction (volunteer sitting in BodPod) while the red chromatogram shows a blank extraction (VOCs from an empty BodPod). The x-axis displays retention time (in minutes) and the y-axis displays absolute signal intensity (in pA). Given the difference in scale of the signal amplitudes between the human VOCs and the empty BodPod VOCs, the red chromatogram appears as if it is flat and almost featureless by comparison.*

In addition to the greater number of peaks detected for the human sample relative to the blank, the peaks for the human sample were also significantly greater in amplitude. Peak area considers the height and width of a peak and percentage area refers to how much that peak contributes to the total signal of a chromatogram, representing the relative abundance of that signal. For peaks with at least a 5% relative abundance (peaks that have 5% percentage area contribute 5% of signal to the total ion chromatogram), there was no overlap between the human VOC chromatogram and the blank chromatogram in terms of retention times, meaning that the prominent peaks in the human VOC chromatogram were

unique to the human VOC profile and that the technique was able to meaningfully differentiate the sample from the blank. Prominent peaks in the human VOC chromatogram include those at 5.390, 5.498, 9.538, 12.184, 16.987 and 26.681 minutes. Prominent peaks in the blank VOC chromatogram include those at 39.130, 39.623, 40.809 and 42.370 minutes. The prominent peaks in the human VOC chromatogram are not present at all in the blank VOC chromatogram, suggesting a high degree of differentiation of VOC profile between the sample and blank extraction. The retention time of the first detected analyte in the blank VOC chromatogram is 27.821 minutes. By that time on the human VOC chromatogram, there were already 50 analytes detected, 6 of which were of high relative abundance. Compared to the other techniques, the "BodPod-TD" technique demonstrates the greatest ability to differentiate between the blank and sample extractions, in terms of analytes detected. The BodPod was able to detect four times the number of analytes in the sample extraction compared to the blank extraction, with the majority of the analytes in the human VOC chromatogram not sharing the same retention times as those in the blank chromatogram, suggesting that this technique is the most successful in detection of human VOCs. Additionally, the amplitude of the peaks is also significantly increased, as the pA scale on the y-axis ranges in the low hundreds to the thousands for method 4, while the other methods range in the tens to the low hundreds. The blank extractions also have significantly lower numbers of total analytes detected than the sample extractions. The increased number of analytes detected coupled with the greater amplitude of the signals suggest increased usability of the method with regards to detecting human VOCs.

Of the eight sample extractions, four were performed on subject 1 while the other four were performed on subject 2. In order to assess whether subjects 1 and 2 can be differentiated, a chromatogram assessment involving a representative pair of GC-FID chromatograms was performed (Figure 13).

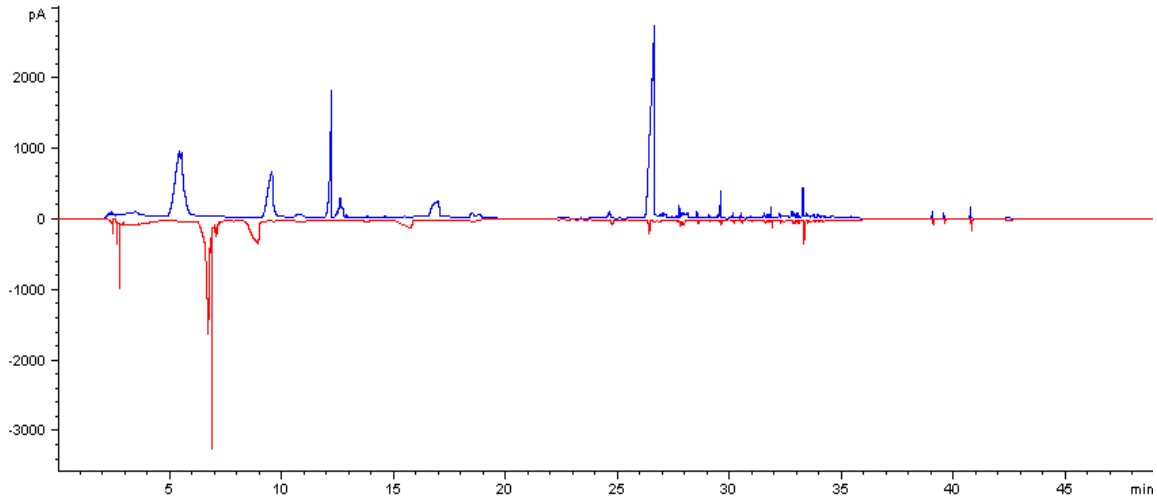


Figure 13. Mirrored chromatograms of two different individuals using the "BodPod-TD" approach. *Two different individuals were sampled using the BodPod-TD method, yielding two different chromatograms which are shown in mirrored format for differential comparison. The blue chromatogram represents volunteer subject 1 VOC extraction, while the red chromatogram shows volunteer subject 2 VOC extraction. The x-axis displays retention time (in minutes) and the y-axis displays signal intensity (in pA).*

Volunteer 1 had a total number of 164 detected analytes in the chromatogram while volunteer 2 had a total number of 146 detected analytes. Components with a percentage area of greater than 5% (contribute 5% of signal to the total amount of signal detected in the chromatogram) are considered to be prominent peaks in the chromatogram for the purposes of this analysis. The prominent peaks in volunteer 1 include those at retention times 5.390, 5.498, 9.538, 12.184, 16.987 and 26.681 minutes. The volunteer 2 chromatogram had prominent peaks at retention times 6.694, 6.869, 8.924 and 15.748 minutes. There was no overlap of retention times in terms of these prominent peaks. The subject 1 chromatogram also had 18 more analytes detected than subject 2. The prominent peaks all having different retention times coupled with the greater number of analytes detected for subject 1 suggest that there is the ability to differentiate human VOC profiles using this method.

In order to assess cohort separation between the sample extractions and blanks, a dendrogram heatmap was created using the GC-FID data from the eight sample runs and eight blanks (Figure 14). There were two volunteer subjects in the sample cohort. As can be observed in Figure 14, there was significant cohort separation between the blank extractions and the sample extractions of subjects 1 and 2. The clear distinction between the sample and blank cohorts suggests the usability of method 4 as a means of detecting human VOCs. Unlike the previous dendrogram heatmaps of the previous methods, which all utilized a 60% frequency filter cutoff but no higher, Figure 14 displays data that has met a frequency filter of 80%. Cohort separation between the sample and blank extractions was maintained, whether the cutoff was at 60% or at 80%, suggesting higher usability of the

data. The integration parameters were consistent as were the GC inlet and thermal desorption parameters with method 3, the other TD-based method. In addition to the clear distinction drawn between the blank and human sample cohorts, there was also separation observed between subjects 1 and 2.

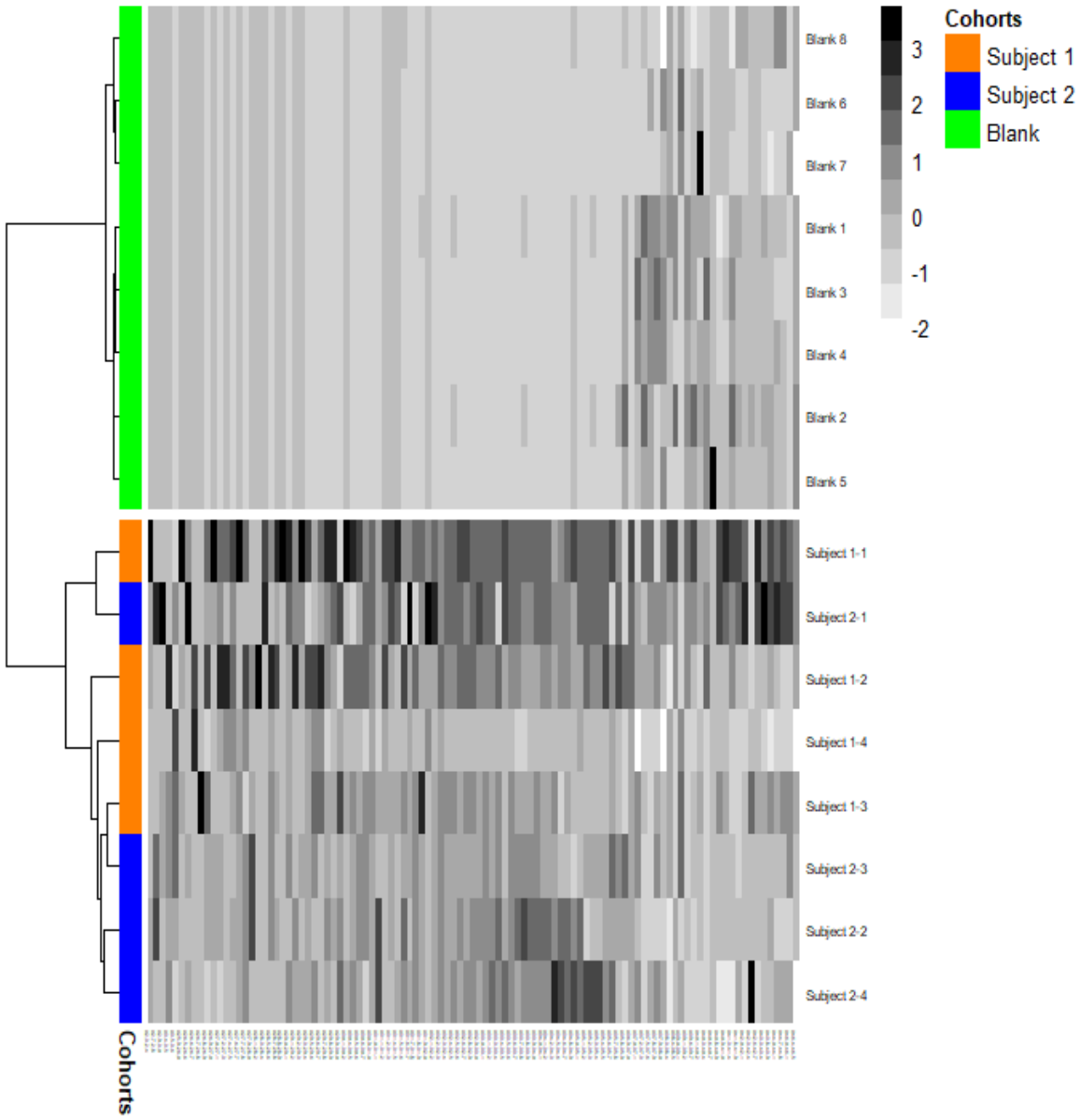


Figure 14. Dendrogram heatmap of sample and blank extractions analyzed by GC-FID using Method 4. *The x-axis represents the retention time ranges across the chromatogram, in increments of 0.1 minutes, that contain features which met an 80% frequency filter cutoff. The y-axis consists of the labels of the individual extractions performed within each cohort. The shading of each square in the heatmap corresponds with amplitude of the signal. A white square means there were no features found for that given sample at that retention time while a black square signifies highest relative signal intensity. There are three cohorts, subject 1, subject 2, and the blank. The blank cohort is represented in green while subject 1 is represented in orange and subject 2 in blue.*

The main advantage of this technique is the clear cohort separation between the sample and blanks indicating method 4's ability to detect and distinguish human VOCs from the sample matrix. Another advantage of method 4 is the significantly greater amplitude of the signals relative to the other methods. The peaks in the human VOC samples extracted from the BodPod are readily differentiated from the empty BodPod, as shown in Figure 13. This method also expands on the concept of preconcentration of VOCs by scaling up the sampling site to a whole-body analysis. Whereas the use of a gauze pad limits the sampling site to one region, the use of a whole-body chamber includes many different sampling sites at once, further facilitating untargeted VOC analysis.

Conclusions of GC-FID Results

Of the four methods evaluated, the "BodPod-TD" approach demonstrated the greatest differentiation of sample analytes from the blank analytes. The "BodPod-TD" approach yields the highest amplitude of signal and detects the greatest number of sample components, making it the best suited to facilitate human VOC analysis. Given that the amplitude of signal was greater using the BodPod, and that the chromatograms of the sample and blank cohorts were highly differentiated, this method was selected for further analysis by GC-MS.

Internal Standard Extractions

In order to control for variation of signal within the human VOC sample extractions for GC-MS analysis, internal standard extractions were next evaluated using the "BodPod-TD" method. An internal standard is a compound of known quantity and identity that is added to the sample for the purpose of standardization and normalization. Factors that would affect the absolute signal intensity of the sample are controlled for, as they would also affect the internal standard signal. Criteria for selection of internal standards include consistent retention times, similar polarity to the sample analytes and consistent signal intensity. Another criterion is safety, as the internal standard should not be toxic to the volunteer subject during analyte preconcentration and extraction. Hence, peppermint oil was selected as an internal standard for this investigation. A total of ten internal standard extractions were performed using the "BodPod-TD" method without a subject in the chamber. A vial containing 1 ml of peppermint oil was placed inside a closed BodPod and allowed to incubate for 30 minutes before each extraction. A chromatogram overlaying all ten of the internal standard extractions analyzed by GC-MS is displayed in Figure 15.

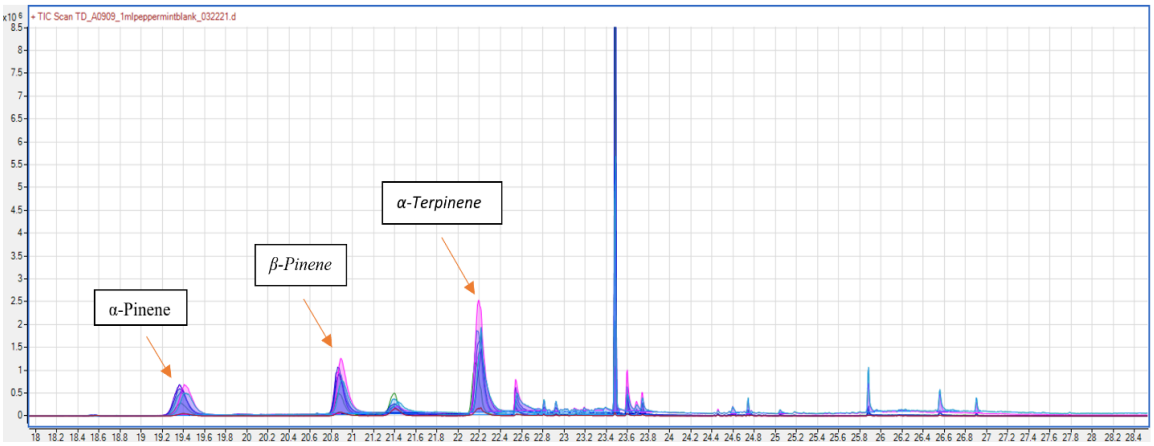


Figure 15. Overlay of internal standard GC-MS chromatograms. *The peaks at retention times ~19.4, ~20.9, and ~22.2 minutes are associated with α-Pinene, β-Pinene, and α-Terpinene, respectively. The most prominent peak at retention time 23.5 minutes is siloxane, a product of GC column bleed. The retention times and spectra associated with these peaks were analyzed using AMDIS with the NIST database and were present in all ten extractions.*

The signal intensities of three peppermint oil-associated compounds, identified by AMDIS and the NIST molecular library (Appendix 3), were determined from the chromatograms and examined using a box and whisker plot (Figure 16). The standard deviations for the signal intensities of α-Pinene, β-Pinene, and α-Terpinene were $1.62 \times 10^5 \pm 25.18\%$, $2.88 \times 10^5 \pm 28.96\%$, and $6.03 \times 10^5 \pm 31.96\%$ respectively. The retention time averages and relative standard deviations for α-Pinene, β-Pinene, and α-Terpinene were $19.42 \pm 16.29\%$ minutes, $20.89 \pm 8.04\%$ minutes, and $22.21 \pm 6.46\%$ minutes, respectively. Hence, there is a relatively high variation for the signal intensities of the three compounds, while the relative deviations for the retention times of these compounds are lower. Signal intensity variation of the internal standard can be normalized, and as long as the sample

VOC signals also fluctuate at the same rate as the internal standard, they too can be normalized proportionally. Variation is corrected for as long as the ratio of the sample peaks remain consistent with the internal standard peaks. The retention times of the peppermint oil compounds demonstrated more consistency, fitting the retention time consistency criteria for internal standard selection. The polarity of the internal standard includes hydrocarbons, which are compounds expected to be found in exhaled breath and the human skin based on the literature [4,8,12].

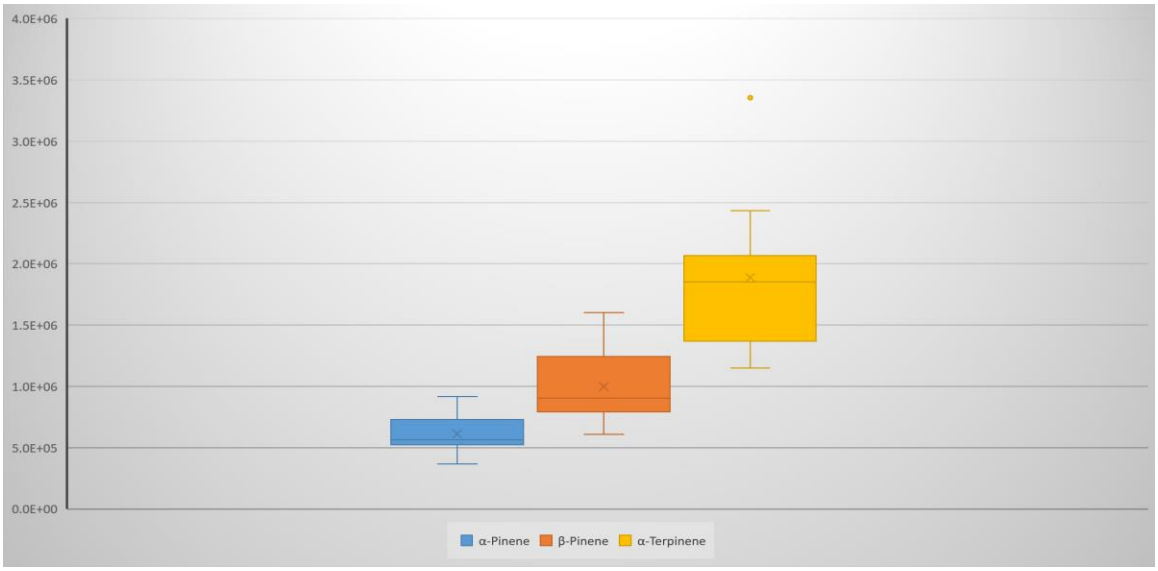
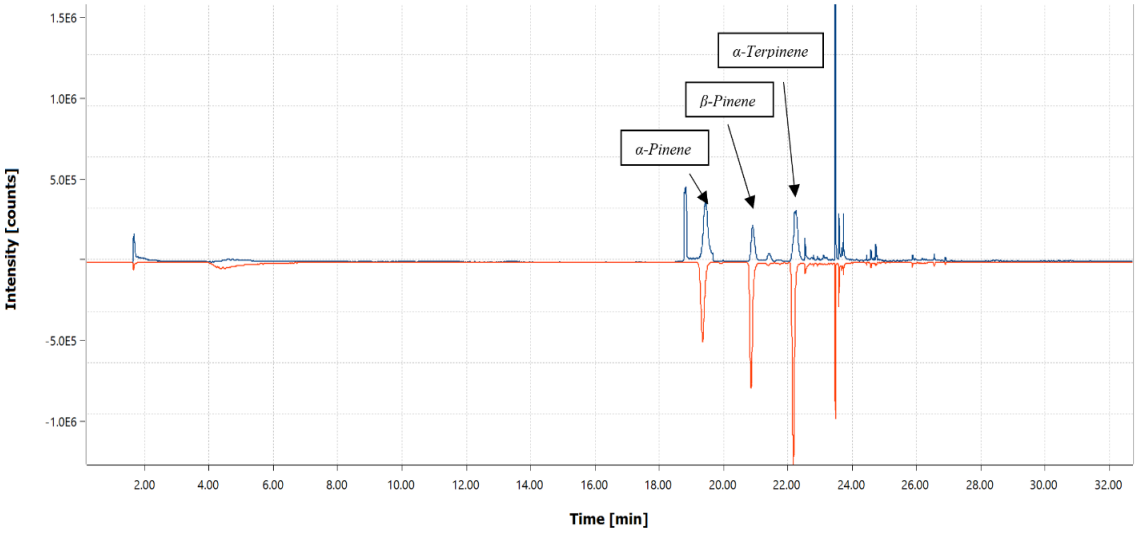


Figure 16. Peak Intensity Variability of 1ml Peppermint Oil using "BodPod-TD" approach. *The y-axis displays absolute signal intensity (measured in counts per second).*

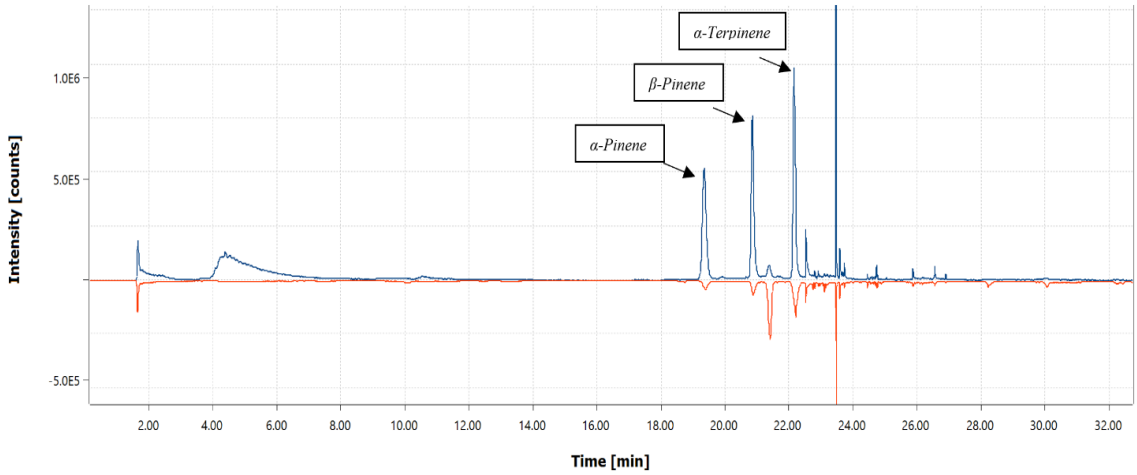
GC-MS Analysis Results: "BodPod-TD" Extractions

The analyses carried out using the GC-FID were performed primarily to assess whether each of the 4 methods of interest could yield chromatograms that differentiate a blank from a human VOC fingerprint, and if so, to ascertain whether they can detect differences in VOC profiles between three different individuals. The "BodPod-TD" approach was the method found most successful in achieving differentiation of VOC profiles in terms of quantity of analytes and qualitative differences in chromatograms. This was further evidenced in Figure 15 which showed clear separation between the sample and blank cohorts. The next step then, was to perform 10 replicate sample and blank extractions for each of three study participants. A total of 60 analyses were performed using GC-MS, which included 30 samples (10 for each subject) and 30 blanks. The GC-MS was used for this analysis because, in addition to retention time, the GC-MS also produces spectral data which can permit identification of analytes when using deconvolution software coupled with a reference database, such as AMDIS and NIST17. The blank extraction consisted of 1 ml of internal standard (peppermint oil) in a vial placed inside an otherwise empty, clean BodPod for 30 minutes before sampling and analysis by GC-MS. The human VOC extraction was performed the same, but with the 1 ml of internal standard placed in the BodPod alongside a human volunteer. A total of ten extractions were performed for each of the three individual subjects. The demographic backgrounds of the volunteer subjects were as follows: Subject 1 was a Hispanic male in his early 20's, Subject 2 was a Caucasian female in her early 30s, and Subject 3 was an East African male in his early 30's.

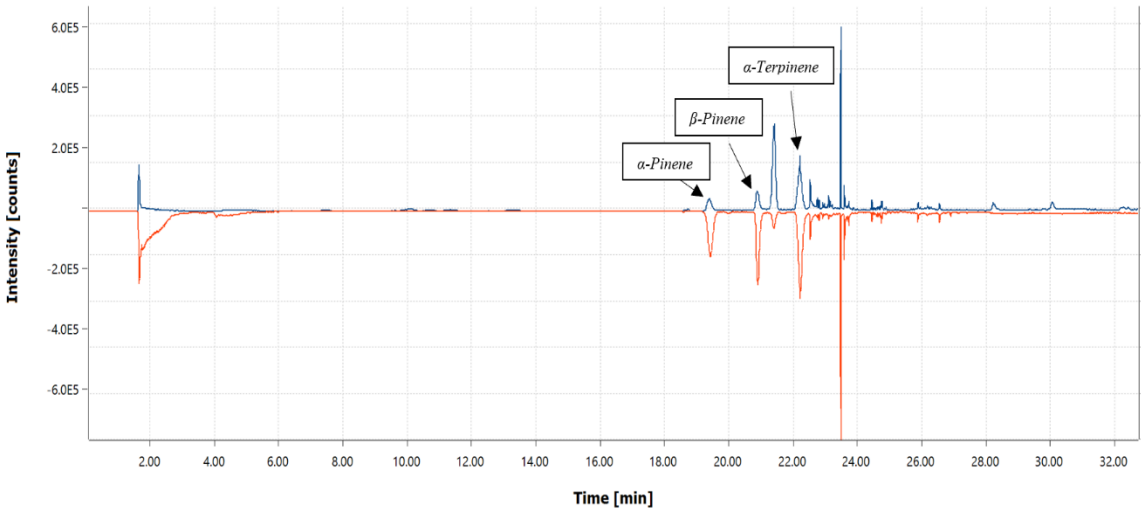
Representative chromatograms comparing the volunteer subjects to each other are displayed in Figure 17.



A



B



C

Figure 17. Representative GC-MS chromatograms comparing VOC profiles from different individuals. *The most prominent peak at ~23.5 minutes, which is present in all of the extractions, is associated with a polysiloxane, as confirmed by NIST. Siloxanes are not endemic to the empty BodPod, the internal standard, or volunteer subject and can be disregarded.* ***A) Subject 1 vs Subject 2:*** *An overlay of total ion chromatograms from individuals 1 and 2. Based solely on peaks, it can be observed that there is a lot similarity between subjects 1 (red) and 2 (blue) outside of the internal standard peaks at ~19.4 minutes, ~20.9 minutes, and ~22.2 minutes. There is one distinguishing peak at ~18.8 minutes for the subject 2 chromatogram.* ***B) Subject 1 vs Subject 3:*** *An overlay of total ion chromatograms comparing subjects 1 (red) and 3 (blue) showing overall increased intensity of signal in subject 3 relative to subject 1.* ***C) Subject 2 vs Subject 3:*** *An overlay of total ion chromatograms comparing subjects 2 (red) and 3 (blue). The early eluting compounds within the first three minutes of the chromatogram show higher absolute intensity in subject 2 than in subject 3. The peak to the right of the β-Pinene peak for subject 3 (blue) was identified as a heptane and was found in both the blank (empty bodpod) and sample (human VOC) extractions, indicating that it was not a human-associated analyte.*

For GC-MS chromatograms, in addition to retention time, identification of analytes is dependent on the deconvolution of acquired spectral data, a process performed by AMDIS (see Appendix 2 and Appendix 3 for details). Once the 30 extractions were

performed, the raw data files were deconvoluted using AMDIS and the analytes were identified by spectral matching with the NIST17 molecular database (Appendix 3). A threshold of 80% match between a feature in the extracted chromatograms and a molecule in NIST17 was chosen for inclusion. Analytes found in both the blank and sample extractions were filtered out, leaving a list of only human-associated VOCs. Table 1 displays the list of the identified human VOCs associated with subjects 1-3 and their associated frequencies.

Table 1. Metabolites identified from each volunteer subject. *Each metabolite in this table has met the 80% match score cutoff with NIST database. The frequency of the metabolite among the 10 replicate extractions is displayed in parentheses. For example, in subject 1, 'Acetone (80)' means that Acetone is a metabolite present in eight out of the ten replicate extractions. The metabolites are arranged in descending order with the most frequently detected at the top.*

Human Subject Metabolites		
Subject 1	**Subject 2**	**Subject 3**
• Acetone (80) • 2,4-Hexadiene, 2,5-dimethyl- (70) • 2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone (50) • Methanethiol (40) • Octane, 2,3,6,7-tetramethyl (40) • Decane, 2,3,5,8-tetramethyl (40) • Bicyclo[4.1.0]heptane, 3,7,7-trimethyl-, [1S-(1α,3α,6α)]- (40) • 1,3-Butadiyne (30) • Cyclohexene, 1-methyl-4-(1-methylethylidene) (30) • Hexane, 2,3,5-trimethyl (30) • Sulfurous acid, 2-ethylhexyl nonyl ester (30) • N-ethyl Ethanamine (20) • 2-methyl Decane (20) • 1,1-Dimethyl-2-propenyl acetate (20) • (Z)-3-Octene (20) • 2,6,7 trimethyldecane (20)	• Oxalic acid, allyl butyl ester (80) • Undecane, 4,7-dimethyl (50) • N-ethyl Ethanamine (50) • Bicyclo[4.1.0]heptane, 3,7,7-trimethyl (50) • 1H-Tetrazol-5-amine (50) • 3-Octene, (E)- (40) • Dimethyl Diazine (40) • Sulfurous acid, 2-ethylhexyl hexyl ester (40) • Pentane, 2,2,3,4-tetramethyl- (40) • 2,4-Hexadiene, 2,5-dimethyl (30) • 2,3,3-Trimethyl-1-hexene (30) • Undecane, 5,7-dimethyl (30) • Propanoic acid, 2-methyl-, 2-propenyl ester (30) • Octane, 2,7-dimethyl- (30) • Cyclobutanone, 2,3,3-trimethyl- (30) • Sulfurous acid, hexyl octyl ester (20) • 1,2,3-Trimethyldiaziridine (20) • 1,3-Propanediamine (20) • 1-Propanol, 2,2-dimethyl- (20) • 2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone (20) • Acetone (20) • Furan, 2,5-diethyltetrahydro- (20) • Nonadecane (20) • Hexyl octyl ether (20) • 1-Propanol, 2,2-dimethyl- (20)	• Acetone (80) • Methanethiol (80) • Sulfurous acid, 2-ethylhexyl hexyl ester (80) • Methyl glyoxal (60) • Undecane, 5,7-dimethyl- (60) • Carbonic acid, nonyl vinyl ester (60) • Decane, 2,3,5,8-tetramethyl- (60) • 1,4-Hexadiene, 2,3-dimethyl- (60) • Undecane, 3-methyl- (50) • Heptane, 2,2-dimethyl- (40) • Octane, 4-methyl- (40) • Ethanamine, 2-(methylthio)- (40) • Cyclohexanone, 5-methyl-2-(1-methylethyl)-, trans- (40) • 1,3-Butadiyne (40) • 11-Methyldodecanol (40) • 2,4-Dimethyl-1-heptene (40)

• 4-(E)-Octene (20) • Hydroxylamine, O-decyl (20) • Methyl glyoxal (20) • Dimethyl sulfide (10) • Phenylurea (10)	• 5H-Tetrazol-5-amine (10) • Hydroxylamine, O-(3-methylbutyl)- (10) • Phenylmethanesulfonylacetic acid (10)	• 2,4-Hexadiene, 2,5-dimethyl- (40) • Acetic Acid (30) • Oxalic acid, allyl butyl ester (30) • Propanoic acid, 2-methyl-, 2-propenyl ester (30) • Tridecane (30) • Dodecane (30) • N-(2-Methoxyethyl)-N-ethylnitrosamine (30) • 2-Propanone, 1-phenyl-, oxime (30) • Acetamide, N-ethenyl-N-methyl- (30) • 3-Methylheptacosane (30) • 2,6,10-Trimethyltridecane (30) • 1H-Pyrazol-3-amine, 4-methyl- (20) • 1H-Tetrazol-5-amine (20) • Heptacosane (20) • Urea (20) • 1,2-Propanediamine, N,N'-dimethyl-, (S)- (20) • l-Alanine, N-benzyloxycarbonyl-, ethyl ester (10) • 1H-Pyrrole, 2-methyl- (10)

The metabolites in Table 1 were listed in a descending order in terms of frequency of detection. A couple of metabolites were found to be frequent within their respective

cohorts, while one was found to be conserved between cohorts. For subjects 1 and 3, Acetone was found to be present in 80% of the extractions. Acetone is an extensively documented biomarker of ketosis in healthy and diabetic patients in exhaled breath analyses [32]. Though excessive acetone (>100 ppm) is known to be correlative with diabetic ketoacidosis, there is also a basal level of acetone (<2 ppm) in the breath due to its volatility and it being an endogenous by-product of fat metabolism [32]. Presence of acetone in the breath, on its own, is not indicative of disease, however. Acetone cannot be concluded to be a distinct metabolite to either subject 1 or 3, rather it is conserved between them. The second highest frequency metabolites found in Subject 1 extractions include the hydrocarbon 2,5-dimethyl-2,4-Hexadiene and the sulfone 2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone, the latter of which was also found in subject 2, though at a lower frequency.

The highest frequency metabolite found for subject 2 was allyl butyl ester oxalic acid, a microbial secondary metabolite. An analysis of the volatile compounds produced by lactic acid bacteria *L. lactis sp.* found that it would yield this oxalic acid and that it would serve different purposes during the stationary phase and decline phase [33]. Changes in the skin microbiome can confer changes in metabolites present on the skin, which could lead to variability within cohorts and between them. Presence of oxalic acids could also be indicative of ingestion of oxalate-rich foods such as leafy greens like spinach and rhubarb [34]. The guidelines for the volunteers prior to entering the BodPod for sampling included not eating right before entry into the BodPod. The frequent presence of this oxalic acid

could be indicative of regular consumption of the foods that have high concentrations of this metabolite, such that its presence could be detected even when not recently consumed.

Subject 3 had two metabolites that were found in high frequency: methanethiol and sulfurous acid, 2-ethylhexyl hexyl ester. Methanethiol is a compound which has its origins in the gut microbiome. Free methionine is converted to methanethiol by two different metabolic pathways. One way is through transamination of methionine, forming 4-methylthio-2-oxybutyric acid, followed by formation of 3-methylthiopropionic acid. Another way involves two-step degradation pathway which has been demonstrated in Lactococcus bacteria [35]. Other bacteria, such as *Staphylococcus hemolyticus*, *Fusobacterium nucleatum,* and *Porphyromonas gingivalis*, which are present on the human skin or in the oral cavity, have been observed to have enzymes which convert methionine into methanethiol [35-37]. The human skin and oral cavity could serve as sites of origin for the methanethiol detected in subject 3 as result of methionine metabolism. Sulfurous acid, 2-ethylhexyl hexyl ester is a volatile compound identified during fermentation of African oil bean seeds [38]. The presence of this metabolite can be considered associated with regular consumption of foods that contain this fermentation product.

Despite guidelines which were enacted to control for any intra- and inter-individual variability, there are still factors which can affect the variability of metabolites. Dietary changes appear to be a significant contributor to metabolite variability based on the background information of the most frequently identified metabolites. Dietary routines can also be affected by culture as certain customary foods can have unique metabolites not

endogenous to foods elsewhere. The presence of 2-ethylhexyl hexyl ester sulfurous acid, a metabolite associated with African oil bean seed, was found in subject 3, a male within the ages of 18-35 who is of Central Ethiopian ethnicity. Low frequency metabolites and one-offs can either be attributed to transient changes in the metabolic pathways of the skin or exhaled breath metabolome, or can be attributed to exogenous VOC sources which still need to be controlled for with stricter protocols.

Conclusion

In this proof-of-concept study, 4 different techniques were assessed for their ability to detect human VOCs: the localized "trap and contain" approach, the "gauze swipe-SPME" approach, the "gauze swipe-TD" approach, and the "BodPod-TD" approach. The technique found to achieve the highest degree of differentiation between the sample and blank extraction as well as between two different individuals was the "BodPod-TD" technique. This technique employed the concept of preconcentration of analytes in a whole-body chamber prior to adsorption of analytes onto the sorbent within a TD tube. Ten replicate extractions with 3 test subjects and an empty BodPod blank were performed using a selected internal standard and the "BodPod-TD" technique. Using the VOC signature of the blank extractions for comparison, metabolites that were found in the subject extractions but not in the blank extractions were deemed to be human-associated. However, some variability within the replicate extractions highlight the need for additional method refinement.

There are limitations consistent with the scope of a proof-of-concept study which should be addressed in future studies. Increasing the sample size can further facilitate this VOC analysis, as different population cohorts of differing demographics can be compared rather than just individuals. Further method optimization can include fine-tuning the GC-MS conditions as well as further modifications to the BodPod which can allow improved

preconcentration of VOCs prior to sample adsorption. For example, a modification to the BodPod (eg. making it out of stainless steel instead of fiberglass) may help facilitate GC-MS analysis, as this could minimize VOC presence in the blank extractions (fiberglass-associated VOC emissions). A stainless-steel chamber does not present exogenous VOCs and minimizes adherence of sample VOCs to its walls. Changes to the volunteer guidelines and protocols can also limit exogenous VOCs and give a clearer snapshot of human-associated VOC fingerprints. Mandating use of odorless soaps and that the volunteer change into hospital gowns prior to sampling could serve to further minimize exogenous VOCs. In addition to qualitative analyses, a future study could also quantitatively compare exhaled-breath acetone levels between a healthy and diseased cohort using the "BodPod-TD" approach. Diabetic patients are expected to have a 50-100-fold difference in exhaled-breath acetone levels relative to healthy patients [32].

Further data will be needed to validate the "BodPod-TD" active sampling method. Much like how a fingerprint does not consist of only one or two data points, a VOC fingerprint would also require more metabolites which are distinct in order to establish a clearer, more robust VOC profile. However, through this work, the "BodPod-TD" approach has demonstrated the ability to detect distinct human metabolites. From rapid diagnostics, to forensics, to biothreat detection, the "BodPod-TD" technique could serve as a useful tool. The preconcentration of analytes, coupled with active sampling, is shown to be a particularly effective analytical technique. Though the field of volatilomics is still relatively new, there are potentials for breakthroughs from the further development of the techniques assessed in this work.

Appendix 1

Flame Ionization Detection (FID) detects sample with the use of a flame which burns organic compounds in order to generate a current. The carrier gas used for this technique typically includes hydrogen [18]. The GC-FID also incorporates use of air gas and oxygen in order to create an environment for the flame to spark. The flame is produced from the positive electrode end while the negative electrode sits above the flame. The current generated by the negative ions hitting the negative electrode plate is measured in picoamps (pA).

The GCMS is an analytical tool which couples gas chromatography with mass spectrometry. Gas chromatography separates the compounds while mass spectrometry allows for identification and quantification. GCMS ionizes the compounds and separates based on mass/charge ratio wherein each mass charge ratio unit is measured in m/z. Electron impact (EI) ionization, or 'hard ionization', is the most commonly used method for ionizing the sample compounds and works by directly hitting the outer shell, high energy electron of the target molecule, in order to produce a cation [17,42]. This process of knocking out electrons to produce radical cations is also known as fragmentation. Inert gasses for the mobile phases of GCMS systems typically include helium or nitrogen. The stationary phase is contained within a capillary column. The volatile sample is carried past the column by the inert gas. Once the sample volatiles exit the GC column, it is fragmented

and sorted based on mass. Each fragmentation pattern for a given sample is unique and contributes to molecular fingerprinting. The fragmentation patterns are referred as spectra. The signal intensities per spectrum is plotted vs retention time and summed up in order to yield a Total Ion Chromatogram (TIC) [42].

Appendix 2

ChemStation software integrates signals in a chromatogram through a four step process. The software identifies a start and end time for each peak, marking each point with tick marks. Then, the apex of each peak is found, a baseline is constructed, and, finally, the area, height and width for each peak is calculated [40]. The continuous tracking and updating of the baseline also distinguish noise from signal. Based on linear regression, which is calculated using all the data points within a given time range, the noise is determined using the formula:

$$N = 6 \times Std.$$

N refers to noise based on six times the standard deviation method and Std. is the standard deviation of the calculated data points in the time range. When printing a report that lists all of the detected analytes and their associated retention times, ChemStation software filters out signals that fit within six times the standard deviation of the linear regression, considering them to be noise.

Appendix 3

AMDIS (Version 2.7) analysis of raw GC-MS data files is compartmentalized into four steps: noise analysis, component perception, spectral deconvolution, and compound identification. The first step distinguishes noise from signal in order to correctly identify valid peaks in a chromatogram. Noise determination is first achieved by calculation of noise factor (N_f). A selected segment of the chromatogram is only considered to be noise when the number of times the signal crosses over the mean is greater than half the number of points in a segment of the chromatogram. The median deviation from the mean within this segment is determined within this given segment of the chromatogram. The average random deviation of this segment is divided by the square root of the average signal abundance within that segment.

$$N_f = \frac{average\ random\ deviation}{\sqrt{average\ signal\ in\ segment}}$$

Equation 1. Noise factor calculation. *This calculation is performed by AMDIS. The median of all the specific N_f values is determined as the characteristic N_f for the data file.*

The second step is component perception, in which individual components are extracted from the chromatogram. Peaks that could potentially be extracted must reach a threshold above noise level in order to be classified as a peak. The peak must also be larger

than five noise units, which refer to the square root of the signal multiplied by median N_f values of the total ion chromatogram.

The third step, deconvolution, takes the noise and neighboring peaks away from the selected, potentially identified peak. AMDIS removes other ions of different peak shape, model, and retention time. The ions that remain are then grouped together and based on any similarity with regards to peak shape and retention time.

The final step, compound identification, takes place once the ions have been adequately extracted into their specific extracted ion chromatograms (EIC). For each EIC resulting from more than one component, the previous deconvolution step calculates the contribution of each component to that EIC. These components are considered identified compounds if the spectra match NIST database by a score of >80% or higher. This cutoff was determined empirically as raising it to 90% yielded too few analytes to be able to analyze, while lowering it to 70% is too low of a threshold to be reliable as multiple isomers can be considered the same compound based off one peak [27-31].

After AMDIS is opened, a raw data file containing the chromatogram is selected for analysis. Under the “Analyze” tab, “Analyze GC/MS Data” is selected and a prompt appears as shown in Figure 4.

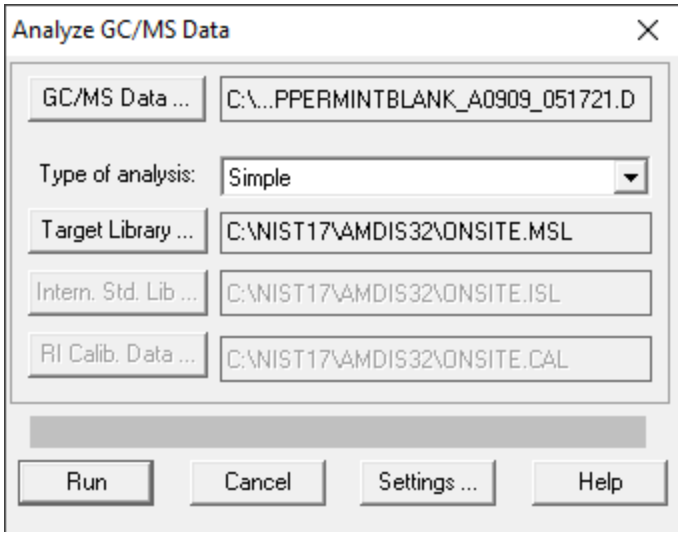


Figure 4. AMDIS analysis initial parameters. *The "simple" analysis type refers to how only mass spectra are used to determine a matched target, whereas other options include retention time, retention index, or a combination of these.*

The parameters needed for analysis of the chromatogram are displayed in figure 5. The identification tab allows the user to set limits such as a what the minimum match factor can be, indicating the lowest possible value at which a target can be given an identity from the NIST library. For this analysis, an 80 min match factor is chosen as the threshold, as displayed in figure 5A. This is equivalent to an 80% match with the spectra in the NIST molecular library. In figure 5B, the user selects the data file and instrument types. The m/z range boxes that dictate the low and high m/z are auto-selected, meaning that they default to the minimum and maximum m/z values found in the raw data. "High to Low" scan direction indicates that the peaks from the raw data file are acquired from the highest to lowest mass [30]. The signal threshold value used in the chromatogram

analysis is from the data file if the threshold option is “Off”. For the data file format, “Agilent Files” are selected as these are the files that are outputted by the GCMS with MassHunter software. Under the “Instrument Type” drop down window, “Quadripole” is selected. Figure 5C is the deconvolution tab. The component width is defaulted at 12 and refers to the number of scans AMDIS performs on a peak before spectra are extracted. Adjacent peak subtraction is set to “One” and means that the ion that interferes the most with the target peak will be subtracted out. Resolution determines separation between the peaks. The sensitivity parameter distinguishes peak width and noise. Shape requirements determine how much importance peak shape has for analysis. The Libraries tab in figure 5D allows for the user to select the database which is to be used as comparison for the target analytes. Figure 5E, the Quality Control tab, is how the user checks if the instrument functions properly. The Scan Sets tab in 5F determines the m/z ranges from which the raw data is to be gathered from. The Filter tab, not shown, does not have any bearing on target identification but is used to remove components that have overlapping characteristics or noisy spectra. This can also be useful for removal of unidentified components for targeted analyses [30].

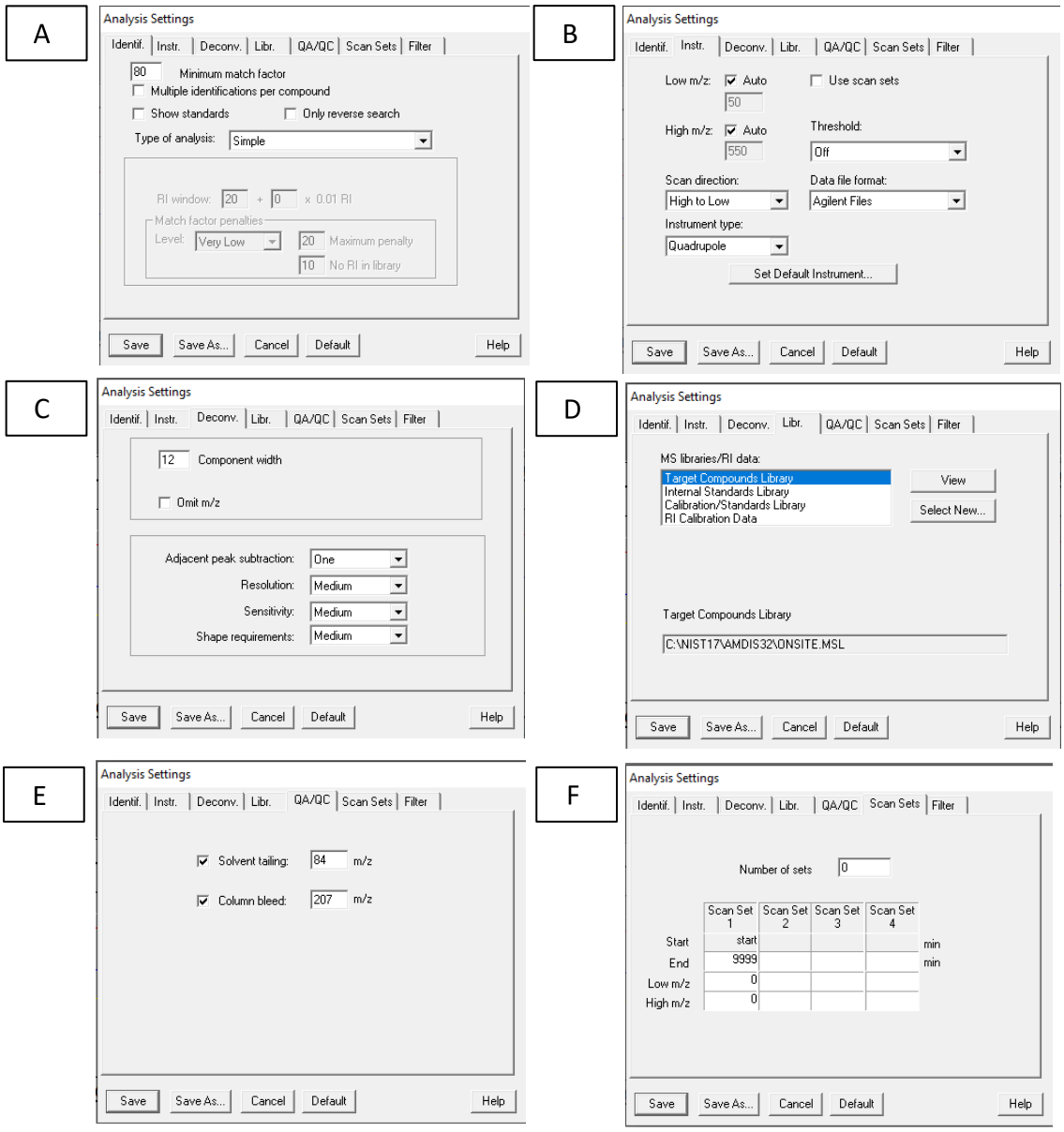
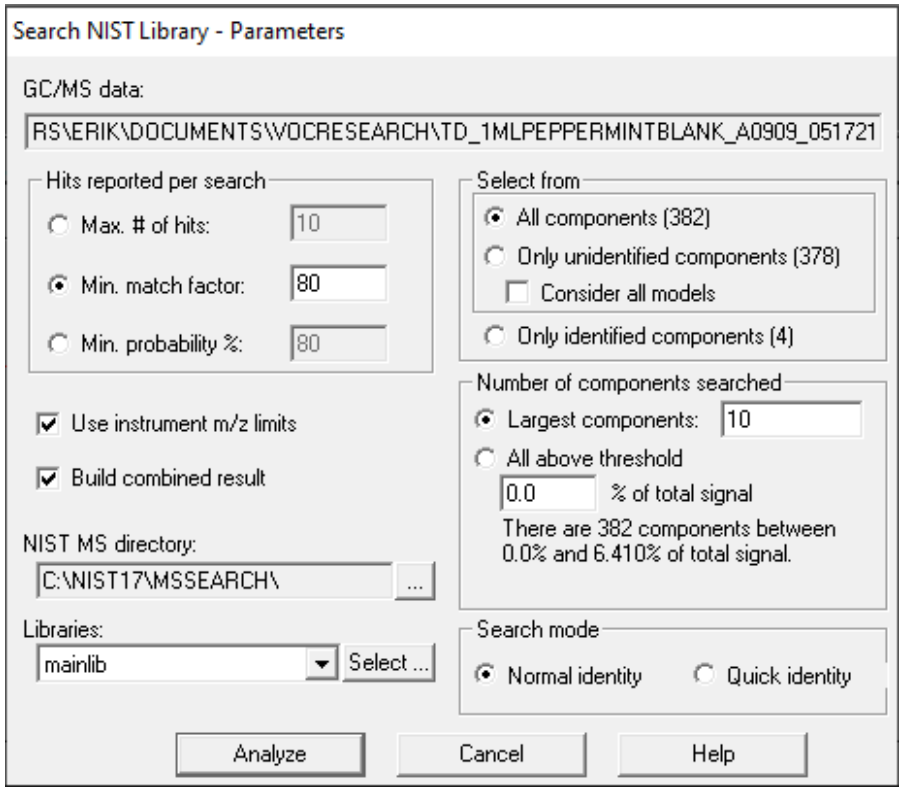


Figure 5. AMDIS settings for chromatogram analysis. *A) Identification tab B) Instrument tab C) Deconvolution tab D) Library tab E) QA/QC tab F) Scan sets tab.*

Figure 6 displays the parameters for NIST database software used for compound identification. In figure 6A, an 80 match factor is selected, which is equivalent to an 80% match with NIST database. Figure 6B allows for the user to save NIST analysis results to a specific output file.

A



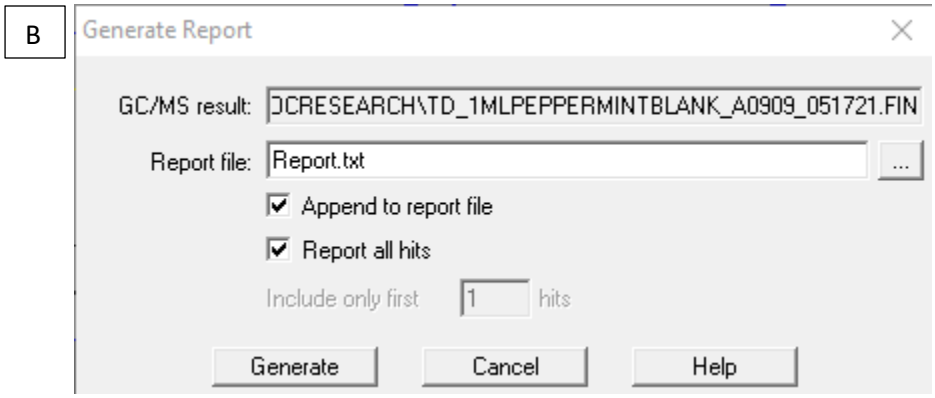


Figure 6. NIST analysis settings. *A) NIST match factor parameters B) report generated as a text file.*

Appendix 4

Solid-phase microextraction, SPME, is a passive sampling technique which utilizes coated polymer fibers that eject out of a syringe to acquire VOCs in the headspace above a sample matrix. Headspace SPME (hSPME) extraction is a technique that involves the insertion of a fiber above the sample matrix and relies on the passive emanation of VOCs from the sample matrix. SPME fibers may also be in used in immersion for Liquid Chromatography-Mass Spectrometry. The coupling of SPME with chromatographic techniques has applicability as an analytical technique [39].

Appendix 5

Thermal Desorption involves the use of tubes which carry sorbent and functions by a two-stage process in which the sample analytes are focused onto a narrow band of gas: tube desorption followed by trap desorption. For tube desorption, the sorbent tube is heated and the analytes are swept onto a focusing trap. For trap desorption, the focusing trap is heated and the analytes are transferred onto a GC column, allowing for faster sampling relative passive sampling methods and for higher sensitivity [26]. TD tubes can accommodate multiple sorbents to allow for acquisition of a range of analytes.

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Biography

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