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Identification Methods and Chemometric Analysis of Mammalian Volatile Biomarkers

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Identification methods and chemometric analysis of mammalian volatile biomarkers

By

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The ultimate goal of this research is to provide a low cost, efficient, reproducible, quantitative, non-invasive screening method to diagnose diseases at an early stage through identification of volatile biomarkers of disease. Progress has been made in the areas towards development of an analytical system that can provide a rapid and specific assay for above mentioned Volatile Organic Compounds (VOCs). (i) Methods have been designed for the collection, concentration, identification and quantification of volatile biomarkers. (ii) Advanced signal processing evaluation of data has tentatively identified key VOCs patterns with breath and body odor. (iii) Novel absorbent coatings have been studied for use with miniature chemical sensors that one day may be part of a portable analytical system.

Both breath and body odor contain a complex mixture of chemicals, which are influenced by many internal and external factors. Breath and skin odor samples were collected with minimum external contaminations using traditional SPME and active SPME GCMS techniques. Body odor from 65 human subjects was tested with and without selected scent removal products. Breath samples were collected from 21 canine

subjects. The VOCs profiles of these samples were determined and then statistically treated with principal component analysis, discriminant analysis, and tree regression techniques to simplify and interpret the complex mixtures.

While much of our work has utilized large bench-top equipment, our over-arching goal is to provide a portable device that can diagnose diseases at an early stage. Concurrent work was done to enhance the performance of a miniaturized detector for the detection of potential biomarkers. Two organic polymers mixed with conductive carbon nanoparticles were deposited between the microcapacitor plates of microsensors using ink-jet technology. Microsensors were also fabricated using conducting ionic liquids. The performances of the individual chemicapacitive sensors were characterized through exposure to different concentrations of varied volatile organic compounds with different functional groups in a climate-controlled vapor delivery system.

DEDICATION

I would like to dedicate this Doctoral dissertation to my parents, Jeewanada Dissanayake and Indrani Ujitha Dissanayake, grandmother, Gnanawathi Perera, uncle, Anil Kumara, sister, Sarojini Hemanthi, brothers, Lalith Sanjaya, Sandun Janaka, Chandana Deshapriya, all my teachers and my friends for their love and enormous support.

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LIST OF ABBREVIATIONS

VOCs	Volatile Organic Compounds
SPME	Solid phase micro extraction
PDMS	polydimethylsiloxane
OV275	polycyanopropyl siloxane
GCMS	gas chromatography mass spectrometry
PCA	principal component analysis
DA	discriminant analysis
IS	Internal standard
C	Control
S	Sample
D	Difference (Control – Sample)
P#	Product number
IL	Ionic liquid ((BMI)(PF6)) 1-Butyl-3-methylimidazolium hexafluorophosphate
CB	Carbon black nanoparticle
FTIR	Fourier transform infrared spectroscopy
SEM	Scanning electron microscopy
DRIFTS	Diffuse Reflectance Infrared Fourier Transform Spectroscopy

CHAPTER I

INTRODUCTION

Mammalian blood contains byproducts from numerous biochemical pathways.[1] In the lungs, volatile chemicals produced in these pathways diffuse into alveolar air and are released from the body with the exhaled air as a complex mixture of chemicals.[2-4] These byproducts can escape the body by diffusion through the skin or through numerous glands in the skin. The rate of diffusion depends on the concentration, size and the nature of the molecules.[4]

Exhale-breath constituents can be affected by diet, environmental exposure, health, activity, and numerous other complicated endogenous and exogenous factors.[5] Mammalian scent is also a complex mixture of chemicals, which can reflect internal and external stresses.[4-5] Further complicating analysis are bacterial populations that live on the skin and produce numerous compounds both from internal biology and through the breakdown of larger molecules found in skin gland secretions.[6]

Normal metabolic activities and their rates can be affected by certain health conditions such as cancers. These abnormal metabolic activities are expected to produce higher levels of metabolic byproducts than those found in the healthy subjects. Researchers have successfully identified many chemical compounds which may be related to diseases. These compounds are collectively known as biomarkers.[7]

Currently available biomarker screening processes in the medical field are invasive (blood, urine or stool samples), and require highly trained professionals.[8] Therefore, patients often avoid initiating the screening process until the disease symptoms advance to the point where the best chance of survival through early identification and treatments has passed. Hence, there is need for the implementation of a noninvasive, inexpensive, user-friendly, portable, and simple solution to point-of-care health assessment, which would enhance the survival rates through early diagnosis via frequent screenings.[9]

Identification of volatile biomarkers is an attractive health diagnosis tool due to its noninvasive nature. Exhaled breath reflects the condition of the blood due to the close association of exhale-breath with blood in lungs.[1, 10] In addition, collection of exhale-breath is more convenient and repeatable compared to collection of blood, urine, and stool samples.[2, 11]

Even though there are numerous publications related to the breath biomarker identification, there are significant discrepancies regarding lists of biomarkers.[12] We found no evidence for a single 'magic bullet' volatile compound which can be used to discriminate diseases at their early stages. However, evidence exists for the relationship between the quantitative differences of common volatile compounds observed in mammalian breath and the presence of disease.[7]

The ultimate goal of this research is to provide a low cost, efficient, reproducible, quantitative, non-invasive screening method to diagnose diseases at an early stage through the research and development of an analytical system that can provide a rapid and specific assay for Volatile Organic Compounds (VOCs). In order to achieve this

goal, there are three main requirements to be satisfied: 1) biomarker identification; 2) analytical methodology development; and 3) development of powerful signal processing methods to make sense of the complex chemical mixtures.

Many of the common breath VOCs are found only in trace amounts (ppb to ppt levels) therefore it is likely that any successful technique will require intense preconcentration for accurate analysis.[13] In order to preconcentrate large volumes containing breath or body odor VOCs, special sample collection strategies need to be applied which take into account the discomfort level of the subject. At the same time, sample collection methods need to be standardized – this requirement is addressed in Chapter 2 in detail.

There are several preconcentration strategies available for VOCSs. Solid phase micro extraction (SPME) techniques (Chapter 2) have several advantages over other techniques and can be operated in passive and active modes. In the passive mode (Figure 1) analytes[14] diffuse onto the fiber and in the active mode (Chapter 3) analytes are passed through the fiber material for efficient adsorption.

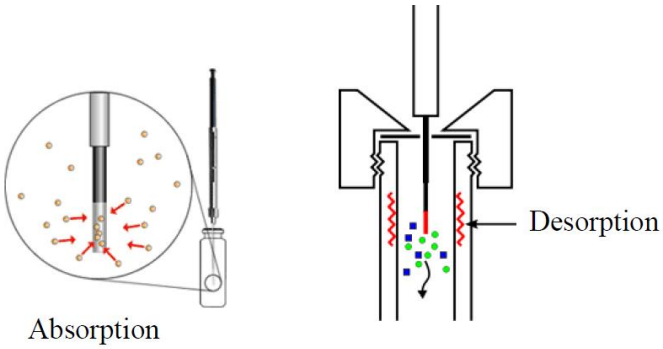


Figure 1.1 SPME fiber passive sampling and desorption at the injection port of a GC.[15]

Preconcentrated samples are thermally desorbed into gas chromatography (GC) columns for separation using the injection port heating unit. Samples are separated inside the GC column before passing into the mass spectrometer. A generated signal is recorded with retention time and the mass spectrum for each VOCs. This data is further processed through peak identification and peak area measuring algorithms in the software. Further processing of the complex data may require advanced statistical tools (Chapters 2, 3 and 4).

An electronic nose can also be used for the identification of analytes. Mimicking mammalian olfaction is a goal of this technology and often an array of sensors are used (Chapter 5) which play the role of epithelial cells in the nose. Complex signals generated from sensor arrays are processed using advanced pattern recognition software (Chapter 5) with the help of computers which mimic the role of the brain in the identification of odors.[7]

In this thesis, work related to biomarker identification paralleled work towards the development of portable instrumentation. Breath samples from canine subjects provided by the Mississippi State University Veterinary School were used to analyze and quantify the relative volatile biomarkers indicative of healthy and diseased subjects (Chapters 2 and 3). The identification and comparison of VOCS profiles produced by humans through the skin were also studied. Scent control products were used to evaluate our screening method and to analyze the efficacy of altering a human odor chemical profile (Chapter 4). Body odor from human subjects (65) were tested with and without the selected scent removal products. The VOCs profiles were then statistically treated with

principal component analysis, discriminant analysis, and tree regression techniques to simplify and interpret the complex results.

While much of the work has utilized large bench-top equipment, the over-arching goal is to provide a portable device that can diagnose diseases at an early stage as part of a visit to a health care provider. In order to achieve this goal, the portable device must allow for sample introduction followed by preconcentration, separation, and detection (Figure 2). Signal processing and pattern recognition software is also required in order to facilitate the disease diagnosis (Chapter 5).

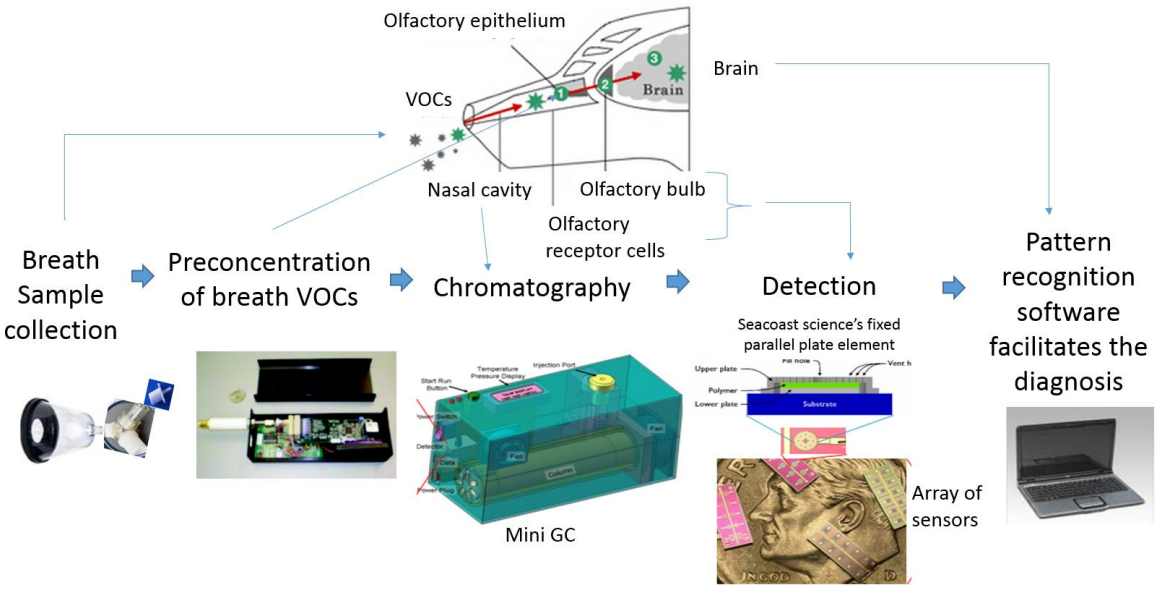


Figure 1.2 Portable hardware requirements for a portable analytical instrument.[16-17]

The ability to operate in air and the small size of chemicapacitive microsensors give them the potential to be utilized in portable analytical equipment. Sensitivity and selectivity enhancement of these chemicapacitive microsensors would have a significant

impact on complex VOCs mixture analysis. Higher selectivity would improve the accuracy of the pattern recognition software and may relax the requirement of baseline analyte chromatographic separations - reducing the total analysis time (Chapter 5).

CHAPTER II

NOVEL EXTRACTION OF VOLATILE BIOMARKERS FROM CANINE BREATH FOR GAS CHROMATOGRAPHY MASS SPECTROMETRY

Abstract

Here we describe an effective, reproducible, non-invasive volatile organic compound collection and analysis method for exhaled breath gas samples designed specifically for use with dogs. Conditions of the method were optimized, using a range of standard chemicals. This method utilizes a canine mask, two-way non re-breathing valve, Teflon connector, tubing and bag for sample collection. Collection is followed by condensation and head space solid phase microextraction (SPME) for sample concentration and gas chromatography-mass spectrometry for analysis. Custom made glassware, designed to hold the SPME fiber assembly, was cooled to -10 ºC and used for the collection of the condensate followed by 2 hours of headspace extraction at 37 ˚C. Standards show LOD of 0.6 – 16.8 ppbv, LOQ between 2.1- 55.8 ppbv, and good linearity with R^2 between 0.996-0.999 (RSD % 10-19). The method was verified with preliminary results from three dogs demonstrating that this technique is capable of collecting, identifying and quantifying volatile organic chemical constituents in different breath samples.

Introduction

It has been established that exhaled breath contains hundreds of volatile organic compounds (VOC) and that these chemicals may be indicative of the subjects state of health.[18] Specific breath chemicals have been identified as biomarkers of particular diseased states[19] and several of these biomarkers have been linked to both human and animal health.[20] The term biomarkers can be defined as molecules produced by the body that may indicate either normal or diseased processes in the body. Numerous studies have been completed on this topic but the literature is difficult to compare because variations in sample collection, concentration and analytical instrumentation preclude proper comparisons.[12]

Many analytical techniques are available for identification of VOC; however, sensitivities are not in the breath VOC concentration region without preconcentration or derivatization.[21] Some techniques achieve the required sensitivity level, but their detection capabilities are restricted to a limited number of compounds in breath, or require a derivatization step.[22] Sorbent trap gas chromatography mass spectrometry (GCMS)[23] and solid phase microextraction (SPME) GCMS[24] are successful techniques in identifying many compounds in breath through standard libraries followed by confirmation of tentatively identified compounds using standard chemical calibration.[25]

When key biomarkers in breath samples are present in trace levels, below the limits of detection, sample preconcentration is required before analysis. SPME preconcentration can be done by exposing SPME fiber devices to the exhaled breath air flow for 5-15 min time.[26] Even though this type of sample collection can be practical with

adult human applications it does present some difficulties in animal and infant applications where they are considered impractical without anesthesia.

Another breath preconcentration method involves condensation of breath VOC components followed by analysis. The exhaled breath condensate (EBC) method has been used to establish that valuable information concerning a subject's health can be found in the condensate.[12] Large sample volumes are often required in order to collect enough VOCs for successful detection with currently available techniques.[27] Common practice often requires at least 15 minutes of breath collection. Practically, this amount of breath sample cannot be collected from an animal without it being heavily sedated or anesthetized.[28] Even in adult humans, such a long period of sample collection has proven to be very uncomfortable for the patient.[29] Chamber studies (collection of breath samples while placing the animal inside a chamber) are comfortable for the animal; however, samples may be contaminated with VOCs from others parts of the body, and the larger dead space lowers the sensitivity of the technique and increase the time required for the analysis.[30]

In order to identify all VOCs present in a small breath sample volume, sample concentration and effective introduction into an analytical instrument is key. In an attempt to optimize trace breath chemical identification and quantification, a new method is presented here for effective breath sample collection, preconcentration and identification using exhaled breath condensation followed by headspace SPME GCMS.

Materials and methods

Standards

Gaseous calibration standards (ethyl alcohol, tert butyl methyl ether, 2-butanone, 4-penten-2-ol, 3-pentanone, methyl isobutyl ketone, 2-heptanone, 3-octanone) were prepared in 3 L Teflon bags filled with ultra-high purity nitrogen (99.995%). Corresponding amounts of liquid standards according to the target concentration are introduced with a constant amount of internal standard (2,3 hexanedione). The method has been optimized for VOC biomarkers with an approximate boiling point range of 50 ˚C to 180 ˚C. Several studies indicate that VOC with these properties cover a number of suspected biomarkers.[24, 31-32] The list is not meant to be exhaustive however most VOC that fall within this boiling point range will be concentrated and identified using the described method. Quantification of new biomarkers would require the generation of a new calibration data set by following the method calibration with standards.

Preconcentration

Carboxen/PDMS (polydimethylsiloxane) 85 µm stable flex SPME manual fiber assembly (Supelco Inc., Bellefonte, PA, USA) was selected for the sample preconcentration as Carboxen/PDMS fibers were shown to be the preferred material for trace level volatile compound analysis in the literature.[24] The preconcentration apparatus, Figure 2.1, was used to condense and then transfer VOC components to a SPME fiber for analysis. When using this assembly, septum is fixed with the vapor condensing glassware and flushed with helium at ambient temperatures for 10 min to dry. A cleaned empty Teflon bag is fixed inside the vacuum box where it can be inflated and deflated by controlling the box pressure. A Teflon tubing is used to connect the sample bag to the

glassware and the box is sealed for air tightness. A second sample bag containing standards or exhaled dog breath is connected to the other end of glassware through Teflon tubing.

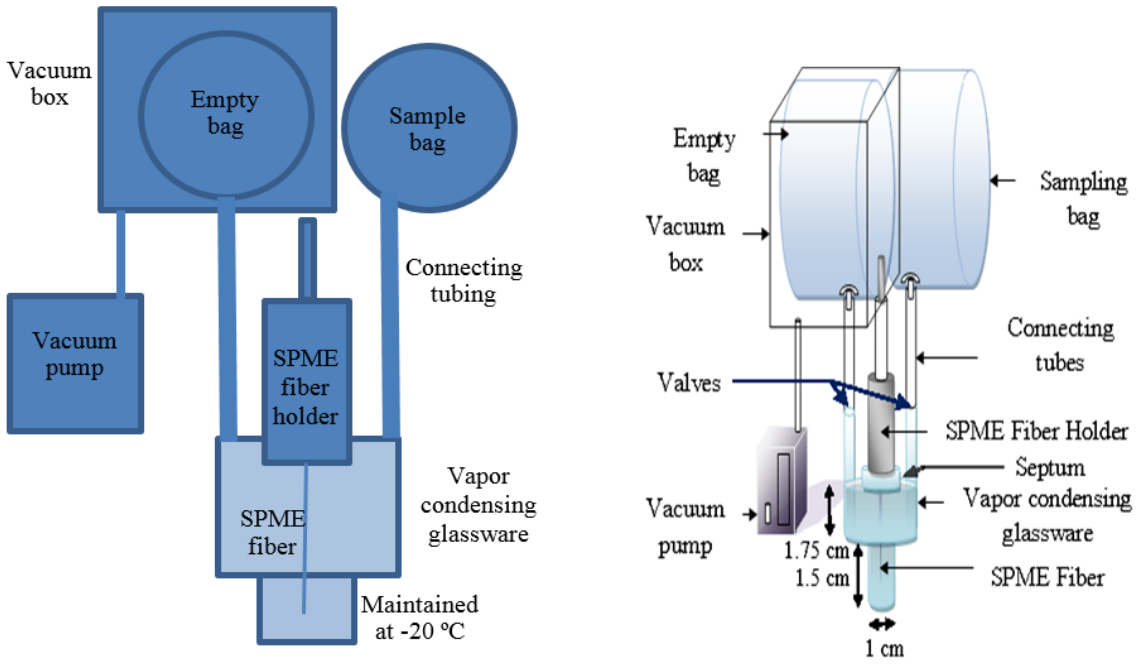


Figure 2.1 Breath sample preconcentrator assembly (not to scale).

Dual sample trapping strategies were employed starting with a cold trap EBC technique [27] followed by head space SPME. Once the assembly was complete, the vacuum box containing the empty bag was exposed to reduced pressure by action of the pump, this causes the bag to inflate and pull the test sample through the vapor condensing glassware. In this cold trap technique, the bottom vial of the glassware was dipped in antifreeze coolant at -20 ˚C (actual temperature on the fiber is -10 ˚C) for 10 min with a preconditioned SPME fiber fixed to the glassware through the septum. The vacuum box

universal sampling pump operated with a flow rate set at 200 mL/min. As it passes the cold zone, the SPME fiber is exposed to sample stream at -10 ˚C. A transparent vacuum box window material was selected for real time monitoring of the process.

Once the sample bag was totally deflated the flow was reversed by pumping air into the vacuum box. This second pass of the sample through the cold zone further reduces the amount of VOC remaining in the gas phase. As the final step, the exhale-breath condensate was concentrated onto the same SPME fiber by closing valves to reduce the fiber exposed volume to 5 mL and warming the sample to 37 °C for 2 h allowing breath constituents to reach the equilibrium inside the air tight glassware. The SPME fiber was then thermally desorbed of the collected VOCs into the GCMS at 220 °C for 3 min time. Samples were analyzed using Shimadzu QP2010S GC MS (Columbia, MD, USA) with a SHRXI-5MS column (30 m × 0.25 mm × 0.25 µm) (Bellefonte, PA, USA).

Collection of canine breath samples

3 dogs [a mixed breed with cancer (29 kg), a healthy rottweiler/german shepherd mix (21 kg), and a chihuahua with heart disease (4 kg)], were chosen for testing to demonstrate that the technique works on different sized animals and that the technique could be used to see variations in VOC profiles. With such a small sample size no attempts are made at this point to draw diagnostic conclusions from VOC profiles; only to show that different size dogs could successfully provide breath samples and that varied animals with varied states of health gave breath samples with different VOC profiles. In order to enhance the collection of VOC content in samples, a proper canine mask size was selected according to the subject size to minimize the dead volume. The mask and a

two-way non re-breathing valve were used for sample collection (3 L of breath within one minute). When collecting samples, special care was taken to keep animal stress low in order to protect the animal and to reduce the amount of stress related VOCs collected in to the bag. The experimental protocol was approved by the *Institutional Animal Care and Use Committee (IACUC) (*Appendix C, D*).* Connecting tubing with ¼ inch internal diameter was used to reduce the dead space. Inhalation and exhalation ports of the two-way non re-breathing valve facilitate a convenient inhalation process while eliminating the problem of mixing of exhaled breath air with inhaling air for more effective exhale-breath sample collection.

Results and discussion

Method calibration with standards

The breath vapor condensing glassware design (Figure 2.1) enhances the exposed surface area to the refrigerator coolant and the expanded bottom tip facilitates uninterrupted flow even when ice crystals form due to the high moisture content of breath samples. The high surface area of the fiber, which is cooled to approximately -2 ˚C, facilitates the condensation of higher amount of VOCs from the sample more than conventional, ambient temperature SPME sampling techniques. The reversed breath flow technique facilitates the recovery of remaining VOCs (missed on the first pass) onto the SPME fiber. Extraction of condensed volatile organic compounds on the glassware wall and dissolved VOCs in water (condensed breath moisture) on to the SPME fiber is facilitated by heating the glassware up to 37 ˚C (human body temperature) during the headspace SPME extraction.

Method calibration experiments were performed using standard chemicals including ketones, alcohols and ethers expected to be biomarkers of disease.[24, 31] Standard samples were prepared in concentrations from 1.1 to 44 ppbV by introducing 3 L of ultra-pure nitrogen, 3 µl of pure water, the appropriate amount of standard, and 1.0 µl of the internal standard diluted in methanol (55 µg/mL) into Teflon bags. For example 5.5 ppbV of 3-pentanone was prepared by adding 1.0 µL of the standard diluted in methanol (60 µg/mL) into the 3 L bag along with the internal standard, nitrogen and water. The 3 L sample bags were then loaded into the assembly (Figure 2.1) for sample concentration and transfer to the SPME fiber. The VOC components were then analyzed using the GCMS and the data was used to determine the standard to internal standard ratio. The peak area ratio calibration curve was plotted and the slope, intercept, and linearity of the curve were estimated using the linear regression method. An internal standard reduces error due to factors including age of the SPME fiber, ambient temperatures variations, GCMS variabilities, and time between sample collection and injection.

Teflon air sampling bags were employed because they have several advantages over other commercially available sampling bags. Advantages include thermal stability, chemical inertness and greater stability under intense cleaning temperatures (70 ºC overnight) and cleaning solvents (10 % acetone) resulting in a low chemical background for low ppb level analysis. The Teflon valve in the bag has a connector which can be fitted quickly with the tubing and a replaceable septum for extended lifetime. The valve and the bag were connected via a ¼" Teflon tube for uninterrupted flow. Another

advantage of the Teflon bag, Teflon connectors, and Teflon tubing is low surface energy which minimizes sample loss due to VOC adsorption.

Blank tests were carried out to estimate the method limit of detection. Eight blank samples were prepared by introducing 3 L of ultra-pure nitrogen, 3 µl of pure water, and the internal standard into Teflon bags followed by the concentration and analysis procedure described above. The standard deviation of the noise was determined to be 0.008. The LOD was calculated as 3 times the standard deviation of the blank/slope of the calibration curve and the LOQ as 10 times.[33] The results are summarized in table 2.1.

Table 2.1 Quantitative results of calibration standards.

Compound	Ret. Time /min	Conc range / ppbV	Calibration curve	R^2	LOD 3×(S/N)	LOQ 10×(S/N)	RSD %
ethyl alcohol	2.19	1.7 - 69	y = 0.006x + 0.020	0.998	0.6	2.1	19
tert butyl methyl ether	2.91	0.9 - 36	y = 0.007x + 0.003	0.926	14.0	46.7	14
2-butanone	3.24	1.1 - 44	y = 0.067x - 0.002	0.996	0.4	1.2	18
4-penten-2-ol	4.82	0.9 - 37	y = 0.074x + 0.489	0.993	6.9	22.8	10
3-pentanone	5.57	0.9 - 37	y = 0.092x + 0.232	0.990	3.3	10.9	16
methyl isobutyl ketone	7.47	0.8 - 32	y = 0.044x + 0.719	0.982	16.8	55.8	15
2-heptanone	14.39	0.7 - 28	y = 1.323x - 0.368	0.999	0.9	2.9	10
3-octanone	16.84	0.6 - 25	y = 2.43x - 3.846	0.998	1.8	5.8	15

Evidence suggests that quantitative analysis of biomarkers can be used for early disease detection if VOC analysis can be made in the low ppb range, for example disease related biomarkers are present in the 10 - 100 ppb for cancer subjects and 1 - 20 ppb for healthy subjects.[34-36] The limit of detection range for analyzed standards was 0.6 - 16.8 ppbV, with a limit of quantification range of 2.1 - 55.8 ppbV. The relative standard deviation range of the method (precision) for analyzed standards was 10 - 19. The results are summarized in Table 2.1. Results indicate different affinities of chemicals towards the

SPME fiber and careful selection of SPME fiber absorbent material may provide better results for specific applications.[37]

Preliminary animal subject tests

Preliminary animal subject tests were done to validate the method by ensuring that samples could be collected from different dogs and to demonstrate that tests could be done in the high humidity associated with breath samples. Use of the alveolar gradient can eliminate most of the background VOC concentrations which interferes with the real exhale-breath VOCs. Alveolar gradient (AG) was calculated according to the following equation and have been used for the calculation of quantitative composition of different breath samples.[38]

$$\text{Alveolar gradient (AG)} = \frac{\text{Vb}}{\text{Ib}} - \frac{\text{Va}}{\text{Ia}} \qquad (2.1)$$

Where,

Vb - peak area of a particular compound peak in breath sample

Ib - peak area of internal standard in breath sample

Va - peak area of the corresponding peak in normal air sample

Ia - peak area of internal standard in normal air sample

Table 2.2 Alveolar gradients of three dog breath samples.

Compound class	Alveolar Gradient*		
	Sample 1 /10^{-1}	Sample 2 /10^{-1}	Sample 3 /10^{-1}
Aromatic	1.23	1.08	0.696
Alkanes	6.95	3.88	3.86
Ketones	6.50	4.73	1.50
Aldehydes	13.3	1.08	2.56
Alcohols	3.05	4.17	1.13
Alkenes	2.30	0.0503	4.63
Ethers	0.110	0.237	0.0432
Esters	3.70	5.65	5.25
Acids	1.30	1.18	12.1
Hydrazines	0.00	0.0820	0.00
Amides	1.80	0.559	0.331
Amines	1.17	0.301	1.45
Azoles	0.898	0.465	0.223
Alkynes	0.294	0.793	0.326
Hydrazides	0.00	0.0290	0.125
Other	9.44	11.9	3.59

Notes: * Sample 1: Chihuahua, 12 year old female dog, 4 kg; Sample 2: Mixed breed, 13 year old female dog, 29 kg; Sample 3: Rottweiler/German Shepherd mix, 1.5 year old male dog, 21 kg. These results demonstrate that different VOC profiles can be quantified from different dogs. No disease diagnosis conclusions should be drawn from these results because of the small sample size.

Compounds were tentatively identified using the 2005 NIST library for the Shimadzu GCMS and were categorized into compound groups based on literature [39]. Quantitative data were generated using the equation number (1) for each category and the quantitative composition of different breath samples were illustrated in Table 2.2. Different chemical groups show variation between our subjects demonstrating that the different animals gave breath samples with different VOC profiles. Alveolar gradients of aromatics, ketones, and alcohols (Table 2.2) of the 3 animal test show that the different subjects produced samples with different VOC. This information could become part of a larger study where multiple samples are tested to determine trends and compared with literature findings.[31, 35, 39]

Conclusion

A new method for the concentration of VOC from breath has been developed. This method utilizes smaller sample volumes (approximately 3 liters), an internal standard and a dual pass sample concentration step followed by transfer to SPME fibers at 37 °C for GCMS analysis. Results with selected common breath chemicals standards show limits of detection in the 0.6 – 16.8 ppbV, with a limit of quantification range of 2.1- 55.8 ppbV. The described method has the advantage of employing a reusable, low cost experimental setup with small sample sizes for decreased subject stress, and low limits of detection which are required for breath VOC analysis. Tests with dogs demonstrated that samples could be collected from small medium and large dogs and those different dogs produced samples with different VOC profiles.

CHAPTER III

IDENTIFICATION OF VOLATILE BREATH BIOMARKER PROFILES USING ACTIVE SPME GCMS

Abstract

The ultimate aim of this work is to develop a low cost, efficient, reproducible, quantitative, non-invasive screening method to diagnose diseases at an early stage. This requires research and development of an analytical system that can provide a rapid and specific assay for diseases by analyzing breath biomarkers. Various challenges associated with the collection and analysis of breath samples with parts per billion concentrations of volatile organic markers at high humidities were successfully addressed with the introduction of the novel techniques described here. Exhaled breath gas samples from canine were collected using a canine mask, two way non rebreathing valve, Teflon connector, Teflon connecting tubes and a Teflon bag. The collected volatile organic compounds (VOCs), and water vapor of breath samples were preconcentrated using an Entech 7150 concentrator and a consistent sample volume. Three different cold traps [tenax (lighter VOC trap), solid phase micro extraction (SPME) (heavier VOC trap), and silonite coated (water trap)] (liquid nitrogen cooled to fixed temperatures) were employed in the preconcentration step. Water is removed from further analysis by collection on a water trap followed by heating and flushing before a Tenax refocusing step. The Tenax trap was then heated allowing the VOC's to be refocused onto a cold SPME trap. Finally,

all the traps were heated and the VOC's released in a narrow slug onto the GC column for chromatographic separation followed by MS analysis. As a control, a similar procedure was used for clinical room air. Alveolar gradient calculations were used for quantitative analysis. Instrument performance was calibrated using standards known to be biomarkers of human lung cancer. The described method has been used successfully to detect the chemical profiles of control (healthy) and diseased animals.

Introduction

It has been established that normal metabolism in all humans results in the generation of several volatile organic compounds (VOCs) being excreted in the breath that are indicative of the subject's state of health. An alternative approach to lung disease diagnosis and monitoring could involve breath analysis to track the abundance of these VOC health biomarkers. The focus of this study was to provide an efficient, reproducible, quantitative, non-invasive screening method to identify and quantify breath VOC's in order to diagnose diseases at an early stage. Various challenges associated with the collection and analysis of breath samples with parts per billion VOC concentrations and very high humidities were successfully addressed with the introduction of the novel techniques described here. Although the recently developed EBV EBC SPME GCMS method[40] was cost effective and successful, the technique was time consuming and required intense cleaning procedures prior to each analysis. In addition complications with SPME fiber cleaning steps (conditioning), SPME fiber consistency and air tight assemblies encouraged the development of a more advanced analytical technique.

The combination of Active SPME (Entech) preconcentration and GCMS (Agilent) has resulted in improved breath sample analysis. Analysis time has been

reduced (~ 1 h) and the enhanced efficiency of the SPME trap bake out has eliminated the time consuming manual fiber conditioning step. In addition this method has enhanced reproducibility through precise and consistent breath sample volume analysis while reducing introduction of human errors through incorporation of fully automated steps. The overall performance enhancement due to the ability to precisely control the tuning of each step including sample trapping, recovery, and injection, and improvements due to water management (to protect the MS detector and improve VOC analysis) is presented. A schematic diagram of the Entech Active SPME system is available in Figure 3.1. The direction of the flow is controlled by the Dean switch which balances the pressure on each side to direct the flow. Dean switch is a tool which has electronic pressure controlling ability on each side and able to control the flow according to the requirement. An auto sampler and the preconcentrator are coupled to the GCMS according to the diagram in Figure 3.2.

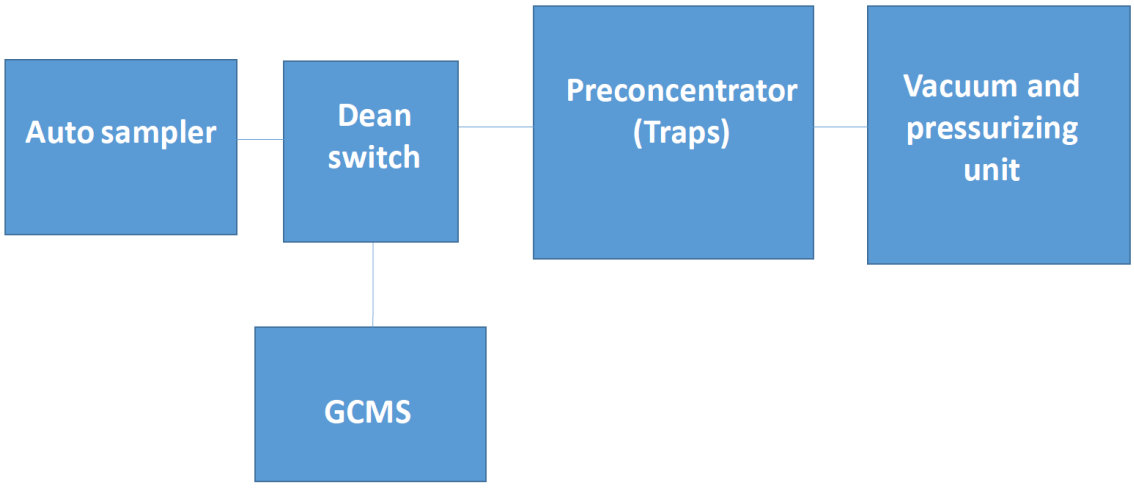


Figure 3.1 Schematic diagram of the auto sampler, preconcentrator, and the GCMS system.[41]

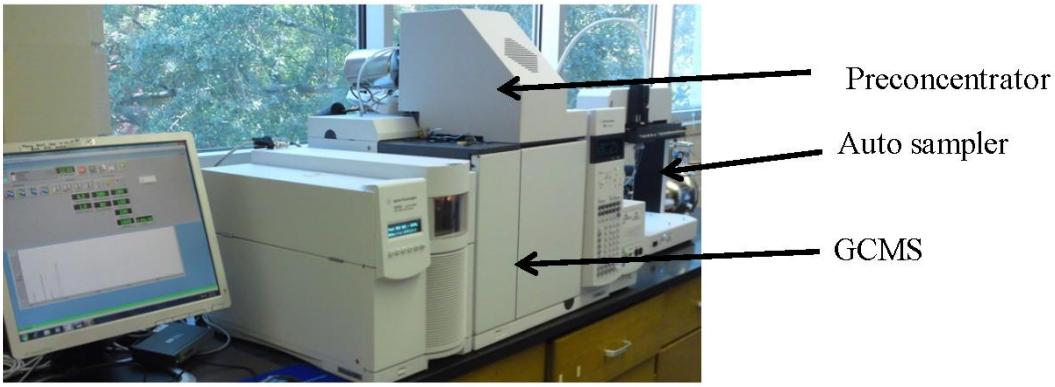


Figure 3.2 Auto sampler and preconcentrator is coupled to the GCMS according to the diagram.

Materials and methods

Sample collection

Exhaled breath gas samples from canine were collected using a canine mask, two way non rebreathing valve, Teflon connector, Teflon connecting tubes and a Teflon bag (Figure 3.3). Attempts were made to collect samples under non-stressful conditions to protect the animal and to reduce the production of stress related VOCs. This experimental protocol was approved by the Case Institutional Animal Care and Use Committee (IACUC) (See appendix A, B, C). All the samples were collected between 10 AM and 3 PM keeping sampling devices well above the mouth which helps to avoid saliva collection.[12]

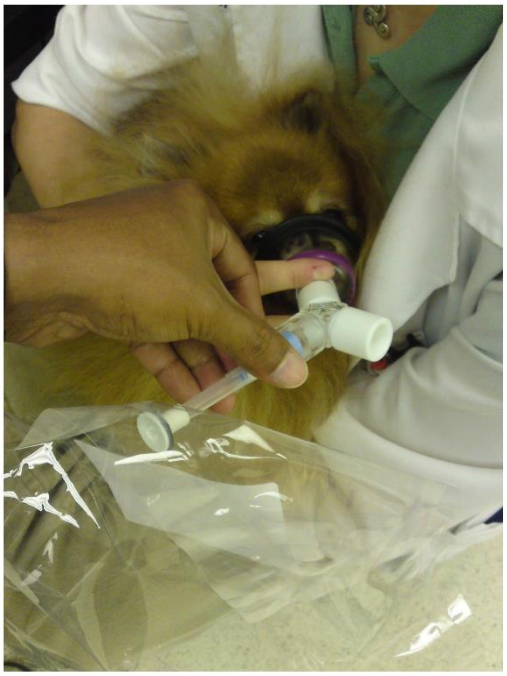


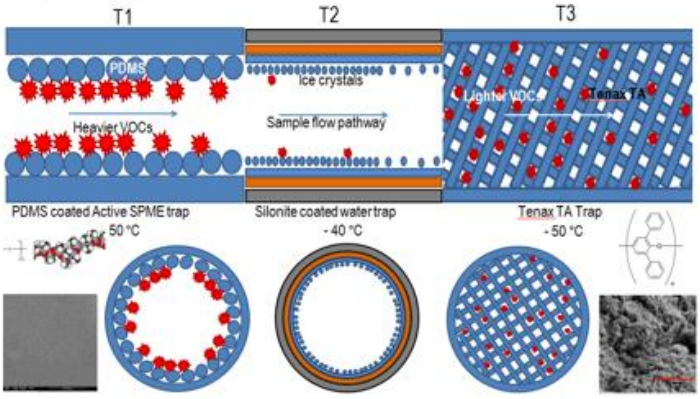
Figure 3.3 Canine breath sample collection.

Sample analysis

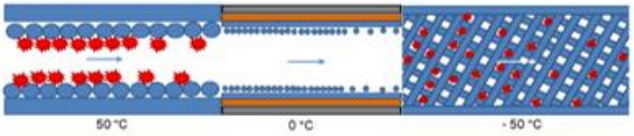
Volatile compounds (VOCs), and water vapor of breath samples were preconcentrated by an Entech 7150 concentrator using a sample volume of 850 (±0.5) mL measured automatically using the pressure and volume relationship in the reservoir. Three different cold traps [Tenax (2,6-diphenylene oxide) (lighter VOC trap) (15 cm, outer diameter 3 mm, packed), solid phase micro extraction (SPME) (heavier VOC trap) (PDMS - polydimethylsiloxane) (2 m with a 3 µm film (open tubular), diameter 3 mm, and silonite coated open tubular (water trap)] (length 4 cm, outer diameter 3 mm) (liquid nitrogen cooled) were employed in the preconcentration step. Following collection, the water trap was heated and flushed before the Tenax refocusing step to remove water. The Tenax trap was heated and refocused on to the cold SPME

trap. Finally, all the traps are heated and the VOC's released onto the GC column for the chromatographic separation followed by MS analysis. The major sample preconcentration steps of the technique are summarized in Figure 3.4.

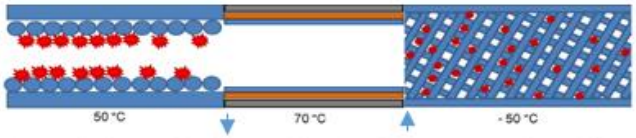
1. VOCs in breath samples are trapped by three specific traps (T1, T2, T3) during the sample trapping step while taking precise (±0.5) mL volume measurements.



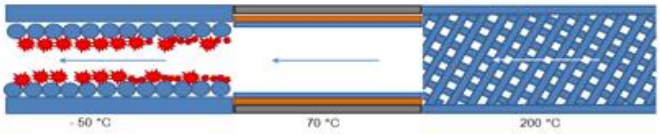
2. Traps are flushed with He for a short time while warming the T2 trap to recover adsorbed VOCs on ice crystals.



3. T2 trap is heated and flushed to remove collected water to protect the GC column and MS filament while improving the chromatographic peak shape.



4. T3 trap is heated while cooling the T1 trap to refocus lighter VOCs on to the T1 trap to improve the chromatographic peak shapes.



5. All T1, T2, T3 traps are immediately heated and flushed with pressurized Helium flow to introduce a small plug of VOCs into the GC column to have a better peak resolution in Total ion chromatogram.

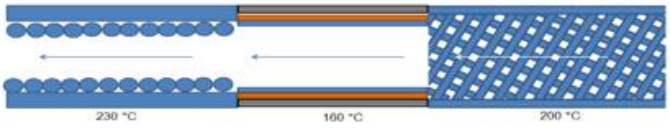


Figure 3.4 The role of each trap for each of the 5 major steps used in the preconcentration strategy.

Instrument parameters

Animal breath samples were concentrated, purified and injected following the Table 3.1 temperature and time profiles.

Table 3.1 Instrument trap time and temperature conditions for the major preconcentration steps.

Step	Event	Time (min)	T1(°C)	T2(°C)	T3(°C)	Average flow rate (mL/min)
1	Trapping	15	50	-40	-50	55
2	Recover	2	50	0	-50	10
3	Bake Out	2	-52	70	-50	25
4	Refocus	2.8	-52	50	200	25
5	Injection	6	230	160	200	2

Preconcentration was followed by Agilent 7890A GC separation (Temperature program: 35 ºC for 5 min, 4 ºC/min to 110ºC then hold for 0.1 min, 15 ºC/min to 220 ºC for 5 min) with an Agilent DB1 column (1 µm, 0.32 mm × 60 m) and mass spectrometric analysis with a 5975C triple axis mass detector with a scan speed of 4.3 Hz at a 45 m/z to 206 m/z mass range. The interface temperature was maintained at 180 °C and ion source, and quadrupole were maintained at 230 °C and 150 °C, respectively.

Data analysis techniques

Healthy and unhealthy canine breath sample chromatograms were processed using Agilent Chemstation data analysis software which gives height and area of each peak in a chromatograph. The tentative identity of each peak was determined (and assigned a confidence factor) by Chemstation software using mass spectrometer data with the help of the NIST 2008 GCMS compound library. Retention times and peak areas

were standardized using four internal standards including IS1 (bromochlorobenzene), IS2 (1,4-difluorobenzene), IS3 (chlorobenzene-d5), and IS4 (1-bromo-4-fluorobenezene).

Results and discussion

System calibration

Instrument performance was tested using the standards thought to be biomarkers of human lung cancer (Table 3.2) for high throughput screening.[42-45] Calibration results are available in Table 3.3.

Table 3.2 List of standard compounds (biomarkers) – name, structure, CAS number, molecular weight, boiling point.

Compound	CAS number	Molecular weight (g/mol)	Boiling point/ ºC
isopropyl alcohol	67-63-0	60.1	82.5
pentane	109-66-0	72.15	36.1
2-butanone	78-93-3	72.12	79.6
benzene	71-43-2	78.11	80.1
4-pentene-2-ol	625-31-0	86.13	115
toluene	108-88-3	92.14	110
hexanal	66-25-1	100.16	131
octane	111-65-9	114.23	125
ethylbenzene	100-41-4	106.16	136
p-xylene	106-42-3	106.17	138
o-xylene	95-47-6	106.17	144

Table 3.3 Calibration results for the biomarkers.

Compound	Retention time (min)	Concentration range/ppbV	Calibration curve	R^2	MDL	MLQ	RSD
isopropyl alcohol	7.071	0.67-53.87	y=0.071x+0.001	0.998	0.373	1.242	11
pentane	7.482	0.67-53.68	y=1.321x+0.081	0.997	0.039	0.128	22
2-butanone	9.367	0.65-51.80	y=5.617x-0.478	0.999	0.007	0.023	25
benzene	11.747	0.65-51.92	y=8.674x+0.211	0.999	0.003	0.008	21
4-pentene-2-ol	11.914	0.66-52.87	y=0.025x-0.001	0.997	1.076	3.586	23
toluene	16.079	0.67-53.24	y=20.848x-1.006	0.998	0.001	0.004	19
hexanal	17.122	0.63-50.34	y=0.010x+0.001	0.999	1.542	5.139	14
octane	18.103	0.63-50.77	y=0.733x+0.001	0.999	0.016	0.055	19
ethylbenzene	20.496	0.63-50.27	y=10.011x-0.26	0.999	0.011	0.038	23
p-xylene	20.883	0.64-51.22	y=5.530x-0.249	0.997	0.024	0.079	24
o-xylene	21.957	0.63-50.18	y=9.965x+0.105	0.996	0.007	0.022	18

A similar procedure was applied for clinical room air and alveolar gradient was used for quantitative analysis of animal breath samples.[40] Alveolar gradient (AG) was calculated according to the following equation and have been used for the calculation of quantitative composition of different breath samples.[38]

$$\text{Alveolar gradient (AG)} = Vb/Ib - Va/Ia \quad (3.1)$$

(Vb-peak area of a particular compound peak in breath sample, Ib – peak area of internal standard in breath sample, Va – peak area of the corresponding peak in normal air sample, Ia – peak area of internal standard in normal air sample).

Preliminary canine study

The described active SPME method has been used successfully to detect the chemical profiles of control (healthy) and diseased (lung related and not related) canines. The diagnosis and the weight of each animal was recorded (Table 3.4). HDG represents healthy dogs and UHD represents unhealthy dogs. UHD 07 and 08 were diagnosed as

lung cancer patients and UHD 06, UHD 11, UHD 18 were diagnosed as lung related diseases. UHD 12, UHD 13, UHD 17 were diagnosed as non-lung related diseases. Preliminary animal subject tests were done to validate the method by ensuring that samples could be collected from different dogs and to demonstrate that tests could be done in the high humidity associated with breath samples. No disease diagnosis conclusions should be drawn from these results because of the small sample size however we present trends that have emerged from the preliminary data.

Table 3.4 The MSU College of Veterinarian Medicine diagnosis and the weight of each animal used for the study.

ID	Diagnosis	weight (lb)
HDG01	Healthy	96
HDG02	Healthy	71
HDG03	Healthy	27
HDG04	Healthy	34
HDG05	Healthy	45
HDG09	Healthy	53
HDG10	Healthy	60
HDG14	Healthy	40
HDG15	Healthy	86
HDG16	Healthy	80
UHD06	Collapsing trachea	11
UHD07	Lung mass (lung cancer)	50
UHD08	Adenorcarcinoma (lung cancer)	52
UHD11	Eosinophilic inflamation	65
UHD12	Rodenticide toxicity	29
UHD13	Kidney and heart failure	8
UHD17	Ligment rupture	16
UHD18	Pulmonary bullae	60

Healthy and unhealthy canine alveolar gradient comparisons are illustrated in Figure 3.5. Canine UHD 11 (eosinophilic inflammation), had very high chemical VOC concentration compared to the other animals and UHD 11 was removed from the Figure

3.5 for the clarity. Quantitative differences of the healthy and unhealthy group are more pronounced in lower retention time compounds.

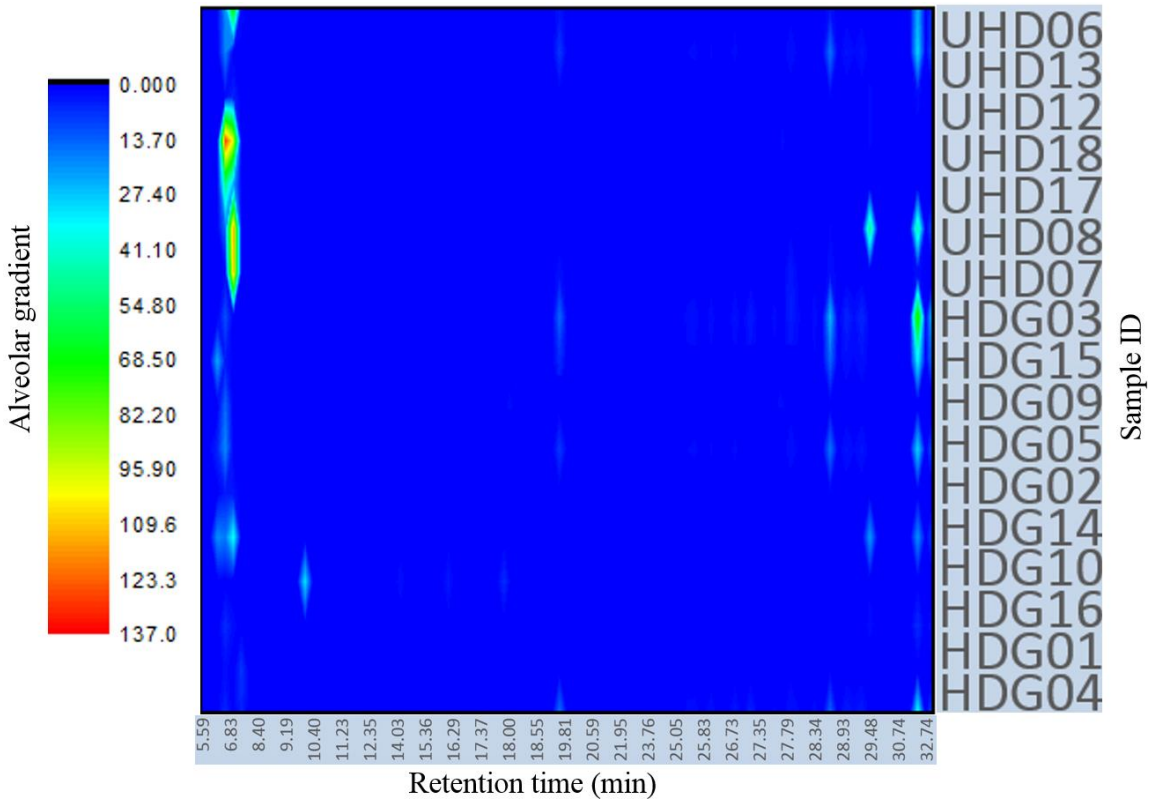


Figure 3.5 Healthy (HDG) and unhealthy (UHD) canine alveolar gradient comparison. (UHD 11 was removed for clarity).

Each peak of each analyzed sample was tentatively identified using the NIST library. Tentatively identified compounds in the cancer breath samples were compare to other samples in Table 3.5 which focuses on several lung cancer biomarkers (according to the literature).[42, 44, 3, 46]

Table 3.5 Tentatively identified compounds in the cancer breath samples compare to other samples.

Retention time (min)	Compound	Comment
6.93	Isopropyl alcohol[4, 6]	Found in all collected samples, significantly higher in cancer patients (UHD07 & 08) than HDG group
8.61	Pentane, 2-methyl-[8]	Only found in animals diagnosed with cancer (UHD07 & 08)
10.19	1-Pentene, 2-methyl-	Only found in animals diagnosed with cancer (UHD07 & 08)
16.60	Heptane, 4-methyl-[8]	Found in all collected samples, significantly higher in cancer patients (UHD07 & 08) than HDG group
18.89	Hexane, 3-ethyl-[9]	Only found in animals diagnosed with cancer (UHD07 & 08)
20.03	2,4-Dimethyl-1-heptene[8-9]	Found in all collected samples, significantly higher in cancer patients (UHD07 & 08) than HDG group
21.06	Octane, 4-methyl-	Found in all collected samples, significantly higher in cancer patients (UHD07 & 08) than HDG group

Concentrations (ppb) of selected compounds (used for the calibration (Table 3.3)) of healthy and unhealthy dogs are available in Table 3.6 and 3.7. Quantitative differences between healthy and unhealthy groups were compared in Table 3.8.

Table 3.6 Concentrations of selected compounds* of healthy samples in ppb.

Ret. Time (min)	Compound	HDG04	HDG01	HDG16	HDG10	HDG14	HDG15	HDG05	HDG09	HDG02	HDG03
7.54	pentane	4	5.97	0.16	0	0	0	1.1	0	0.71	1.01
6.94	isopropyl alcohol	0	0	29.3	0	527	48.3	0	0	0	0
16.05	toluene	0.07	0.07	0.05	0.22	0.06	0.05	0.07	0.06	0.07	0.07
17.14	hexanal	50.8	9.46	0	0	0	0	41.8	0	41.6	60.4
20.89	p-xylene	0.22	0.15	0.05	0.05	0.05	0.05	0.16	0.05	0.19	0.22
11.72	benzene	0	0	0	0	0	0	0	0	0	0
20.48	ethyl benzene	0.05	0.06	0.03	0.03	0.03	0.03	0.05	0.03	0.05	0.05
9.30	2-butanone	0.09	0.11	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
21.95	o-xylene	0	0	0	0	0	0	0	0	0	0
18.10	octane	0.02	0.02	0.05	0	0.52	0.04	0.02	0	0.02	0
11.89	4-pentene-2-ol	0.66	0.64	0.04	0.04	0.04	0.04	1.23	0.85	1.44	0.57

Notes: *(used for the calibration)

Table 3.7 Concentrations of selected compounds* of unhealthy samples in ppb.

Ret. Time (min)	Compound	UHD07	UHD08	UHD17	UHD18	UHD11	UHD12	UHD13	UHD06
7.54	pentane	0.65	0.37	0.41	0.47	1279	0.04	0.71	1.36
6.94	isopropyl alcohol	1597	1680	328	1398	13850	76.4	0	1043
16.05	toluene	0.11	0.06	0.06	0.05	0.07	0.05	0.07	0.07
17.14	hexanal	13	0	0	0.32	0	0.27	41.8	52.7
20.89	p-xylene	0.11	0.05	0.05	0.05	0.05	0.05	0.07	0.11
11.72	benzene	0	0	0	0	0	0	0	0
20.48	ethyl benzene	0.04	0.03	0.03	0.03	0.03	0.03	0.05	0.04
9.30	2-butanone	0.13	0.09	0.1	0.09	0.09	0.09	0.09	0.09
21.95	o-xylene	0	0	0	0	0	0	0	0
18.10	octane	0.06	0.01	0	0.1	0	0.05	0	0.03
11.89	4-pentene-2-ol	2.06	0.04	0.04	0.04	0.04	0.04	1.23	0.35

Notes: *(used for the calibration)

Table 3.8 Comparison of healthy and unhealthy groups with and without outliers.

		Healthy					Unhealthy				
Ret. Time (min)	Compound	Avg	STD	Outliers	Avg*	STD*	Avg	STD	Outliers	Avg*	STD*
7.54	pentane	1.3	1.94	N/A	1.3	2.04	160	423	UHD11	0.57	0.41
6.935	isopropyl alcohol	60.5	156	HDG14	8.63	17.8	2497	4337	UHD11	875	727
16.05	toluene	0.08	0.05	HDG10	0.06	0.01	0.07	0.02	UHD07	0.06	0.01
17.141	hexanal	20.4	23.7	N/A	20.4	25	13.5	20.1	N/A	13.5	21.5
20.887	p-xylene	0.12	0.07	N/A	0.12	0.08	0.07	0.03	N/A	0.07	0.03
11.72	benzene	0	0	N/A	0	0	0	0	N/A	0	0
20.48	ethyl benzene	0.04	0.01	N/A	0.04	0.01	0.04	0.01	N/A	0.04	0.01
9.301	2-butanone	0.09	0.01	HDG01	0.09	0	0.1	0.01	UHD07	0.09	0
21.946	o-xylene	0	0	N/A	0	0	0	0	N/A	0	0
18.097	octane	0.07	0.15	HDG14	0.02	0.02	0.03	0.03	N/A	0.03	0.04
11.891	4-pentene-2-ol	0.56	0.49	N/A	0.56	0.52	0.48	0.71	N/A	0.48	0.76

Notes: *Without outliers (outliers were determined using Grubb's test)

Conclusion

An Active SPME GCMS technique and method was used for standard compounds believed to be biomarkers of disease and then applied to several dog collected breath samples (with varied states of health). This method was used to tentative identify important compounds from healthy and unhealthy canine breath samples in a preliminary study involving 18 dogs to validate the method. Some of the target compounds were found at elevated levels in lung cancer samples compare to the healthy subjects and those compounds were also found in lung cancer cell cultures according to the literature. Further work will be done using this promising method in studies focused on matching disease diagnosis with breath VOCs.

CHAPTER IV

ECOLOGICALLY IMPORTANT HUMAN SCENT DISCOVERY VIA ACTIVE SPME GCMS

Abstract

Intra and interspecies chemical communication is well-known in the animal kingdom. Marking territory, finding a mate and prey/predator interactions can all involve identifying chemicals released by another animal. The aim of this work was to identify likely chemicals that deer (prey) associate with humans (predator). Human scent is a complex mixture of chemicals and its composition continuously changes due to many internal and external factors. Numerous scent elimination products are available that operate using a range of mechanisms to reduce odor. Four such products targeted for deer hunters were used to identify likely volatile organic chemicals (VOC) associated with humans that initiate the flight response in deer. A method to identify human body odor chemicals with minimum non-skin odor contaminations and subject discomfort has been developed. The method includes precise sample collection, active solid phase micro extraction gas chromatography mass spectrometry (SPME GCMS) analysis and advanced signal processing algorithms to identify and compare VOC profiles produced by test subjects. The human subjects (65 volunteers) were tested with and without the selected scent elimination products using our novel collection/analysis techniques. The resulting data was used to rank suspected chemicals of importance in human recognition based on

their abundance, occurrence and reduction following scent elimination product use. Selected highly ranked chemicals were then statistically treated with principal component analysis (PCA), discriminant analysis (DA), and decision tree techniques to simplify the complex outcomes associated with the scent elimination mechanisms for each product used in this study. PCA and DA plots were used to illustrate the complexity of the mechanisms of each product and decision tree results emphasize the key compounds which are suspected to be important in ecological chemical communication. Based on our results, likely chemicals used in human/deer chemical communication include acetone, isopropyl alcohol, tetradecane, 1-ethyl-3-methyl-benzene, alpha-pinene, nonanal, 2-methyl-1,3-butadiene, trimethylbenzene, toluene, and hexadecane.

Introduction

Human scent consists of a complex mixture of volatile organic compounds (VOCs).[5, 47] Each chemical is generated from skin bacterial fauna or from biochemical processes associated with life.[48] Each human produces a unique VOC profile according to a range of biochemical, dietary, and environmental factors.[47, 49] Primary odors are considered to be the compounds available in human scent profiles which are stable throughout time while secondary odors are the compounds which can be affected by diet and environmental factors.[5] Exogenous factors (personal-care products) called tertiary odor compounds can add complexity to odor profiles.[5] Diet and genetics influence apocrine, sebaceous, and sebum gland secretions while associated bacterial activity adds complexity to a subject's odor profile.[6, 50 51]

Removal of this entire VOC profile would be the ideal case for human scent elimination products, however this is not possible because humans continuously produce

more chemicals.[52] In addition, bacterial action on non-volatile chemicals, travelling with shedding dead skin cell rafts, can continue the production of human associated volatile compounds even without the subject present.[51, 53] Commercially available human scent elimination products claim to have odor removal or suppression capabilities, which specifically target VOCs that trigger unwanted animal behavior such as flight. Four possible scent elimination mechanisms have been hypothesized, including: 1. odor masking[54]; 2. bactericidal effects [55-56]; 3. binding odor-causing compounds[57-58]; and 4. conversion of volatile compounds into non volatiles.[59] Odor masking refers to a product which dominates a human VOC profile with a highly volatile different chemical profile. These dominating chemicals hide the human odor profile and thus confound the animal.

Chemical communication is prevalent and varied in the animal kingdom. Dogs have a remarkably sensitive olfactory system that can identify chemicals at very low concentrations and have been successfully trained to identify human odor tracks in forensic studies.[60] Tarsal hair rafts are used as the chemical communication media between black tailed deer.[61] Hyena species use scent markers as early warning signals for potential intruders.[62] The analysis of the scent-marking of great cats has recently been reviewed.[63] Identification of ecologically important VOCs related to humans can fill the gap in the understanding of predator/prey ecological chemical communication. The methods developed here could be applied to study and further classify animal odor, urine and breath VOCs for other applications including medical diagnosis.

Chemicals generated by bacteria are a potential source of human odor. Diversity of associated bacterial populations causes individual humans to smell differently. A person's clothing can be contaminated with different strains of bacteria, and will also

contribute characteristic odors unique to a person. Volatile odor-causing compounds can bind with non-volatile compounds reducing their vapor pressure.[57-58] These non-covalent binding events reduce the VOC profiles of particular odor samples. Odorous volatile organic compounds can also be converted into semi or non-volatile compounds through the covalent bonding of chemicals with odor molecules [59].

Developing a method which extracts maximum body odor with minimum non-skin odor contaminants is an essential challenge in identification of VOCs associated with odor. Current human scent sampling techniques can be divided into two categories; contact sampling methods and non-contact sampling methods. Human olfactory based odor rating techniques are available for the discrimination of collected samples [64-66]. Mass spectrometry[5, 47, 67] and electrochemical sensor array based e-nose techniques[68-69] have been established to reduce the biasness and to enhance the reliability of the odor analysis techniques.[70] Scent samples can be directly collected from the skin using SPME stir bars [49], cotton swabs, or gauze pads in contact methods.[71-73] Non-contact methods use active flow techniques which pass air that has been in contact with skin through cotton or other fibrous adsorbent materials (for example SPME fibers and related solid phase absorbents) using vacuum pumps[67, 74] or passive techniques.[48, 75-76]

Contact methods are not suitable to evaluate volatile odor profile changes associated with scent elimination products because they also extract non-volatile chemicals and microorganisms, which can complicate analysis.[57-58] Current GCMS sensitivity levels require extensive pre-concentration steps to detect trace level VOCs.[40, 67] Time consuming fiber conditioning and lengthy sample extraction times of non-contact methods are not attractive in human clinical trials, which necessitate minimum

discomfort levels.[65] The non-contact method developed here is designed to improve the sensitivity, efficiency, and comfort of the subject and reduce the contamination of samples with non-skin odors.

The aim of this study was to develop methods to identify and quantify the VOC profile produced by humans and to determine the effect of four different commercial scent control products on this profile in order to identify ecologically important VOCs. The scent elimination products used in this study are marketed towards deer hunters and all advertise that they can eliminate odors that initiate deer flight. As part of this study, the authors make two assumptions: 1. That deer respond to human odor, and 2. The action of the scent elimination product reduces deer response to human odor. This project was funded in part by a commercial manufacturer of these products which asked that no product names be used in this manuscript. Extensive anecdotal evidence exists that supports the efficacy of these products and the authors – working with the assumptions listed above, sought to determine key VOCs that trigger the flight response in deer. Our method was developed to be able to extract maximum body odor with minimum non-skin odor contaminations and with minimum subject discomfort.

Materials and methods

Scent elimination products

Four scent elimination products (P#1,2,3,4) targeted to deer hunters were used in this study. All claim to be able to remove human scent and supply anecdotal evidence. None of the products used supplied a complete list of ingredients. (One of the sponsors of this study supplied the four commercial scent elimination products and asked that specific

products remain anonymous. The focus of this study is on identification of likely ecologically important human odors that trigger the deer flight response).

Sample collection.

New T-shirts were machine washed twice with unscented washing powder and then dried for 3 h. The T-shirts were placed in a vacuum-oven for 10 h and the cleaned T-shirts were stored in airtight bags prior to the treatments. One set of cleaned T-shirts were treated with deionized water as the control and the other set was treated similarly with one of the four scent elimination products as samples. Volatile organic compound (VOC) profiles of cleaned untreated T-shirts were tested for their cleanliness and exhibited no significant VOC profile.

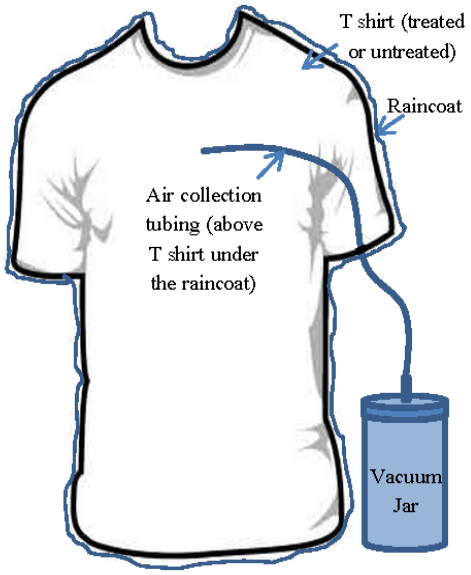


Figure 4.1 Human odor sample collection.

T-shirts were sprayed evenly using the original sprayer of the product to distribute active ingredients homogeneously. Weight measurements were taken to maintain the consistency between samples. Approximately 40 g of each product were required to cover the entire surface of the T-shirt according to the application methods on each product's label. The same amount of distilled water was sprayed onto control T-shirts. Treated T-shirts were allowed to dry for 2 h prior to human contact. Treatment and sampling sessions were restricted to a single product to avoid any contamination and to minimize the interactions between products.

Human scent samples were collected from 65 volunteer individuals who were randomly assigned into four different product groups. The sample collection method was approved by the IRB (institutional review board) (Appendix D, E, F). Two T-shirts were given to each individual (one untreated and one treated with the assigned product) with the instructions to wear each T-shirt for 2 h under a light disposable rain coat cover. A 1 L glass container at reduced pressure (<0.1 torr) equipped with a valve and a 0.6 meter ¼ inch copper tube was used for odor sample collection. After 2 h the metal tube was inserted in the T-shirt/raincoat gap (Figure 4.1) and a gas sample was collected for the Active SPME GCMS analysis. The process was repeated for the second T-shirt.

Subjects were asked not to use any personal care products or perfumes on the sampling day. Four subjects were arranged in a conference room maintaining an approximate distance of 5 feet between the subjects. The room was well separated from other compartments of the building except for the building air-conditioning system. The room was maintained under normal lighting conditions with fluorescent light. The temperature (70 – 80 °F), and humidity levels (40 – 60 %) were monitored throughout the

sampling time. Our test protocol required the collection of control (untreated, 2 h) and sample (treated, 2 h) odor contaminated air from each human subject. Therefore, subjects were asked to participate for 4-5 consecutive hours. The sequence of control and sample collection has been equally randomized for each product.

Age and sex were recorded prior to sample collection and test subjects were discouraged to have food during the sample collection time to avoid any possible food odorants. All the other possible odor sources were removed from the test area which was restricted for public access to minimize possible contaminants. Sampling time and conditions were optimized for the developed method in order to minimize the discomfort on human individuals while collecting enough odor producing chemicals. Both 2 h and 6 h of sampling times were evaluated. Longer sampling times resulted in higher concentrations of VOCs collected but also resulted in reported higher discomfort levels. Both 2 h and 6 h of sampling times showed relative peak area (to the internal standard) difference in selected compounds (>0.1% difference) between controls and samples. Therefore, the 2 h sampling time was used for our testing.

Analysis technique

Active Solid Phase MicroExtraction (Active SPME) gas chromatograph mass spectrometry (GCMS) was chosen for sample analysis over passive SPME GCMS due to its shorter analysis time, higher reproducibility, more efficient collection of smaller analytes and more convenient internal standard introduction methods. Automated sample introduction using the Entech 7410 autosampler and water removal using the Entech 7150 preconcentrator are advantages with the selection of the Active SPME technique. Three different cold traps including 1) a Tenax VOC trap (T3), 2) a SPME PDMS

(polydimethylsiloxane) VOC (heavier volatile organic compound) trap (T1); and 3) a Silonite (deactivated silica) coated water trap (T2) were employed in the preconcentration step. The traps were cooled to below ambient temperatures using liquid nitrogen under a set program. There are five major steps in the preconcentration of the collected chemicals (see Table 4.1).

Table 4.1 Temperature profile for active SPME sample preconcentration

Step	Event	Time (min)	T1(°C)	T2(°C)	T3(°C)
1	Trapping	15	50	-40	-50
2	Recover	2	50	0	-50
3	Bake Out	2	-52	70	-50
4	Refocus	2.8	-52	50	200
5	Injection	6	230	160	200

Preconcentration was followed by Agilent 7890A gas chromatographic separation with an Agilent DB1 column (1 µm, 0.32 mm × 60 m) and mass spectrometric analysis with a 5975C triple axis mass detector with a scan speed of 4.3 Hz at a 45 m/z to 206 m/z mass range. (Temperature program: 35 ºC for 5 min, 4 ºC/min to 110 ºC then hold for 0.1 min, 15 ºC/min to 220 ºC for 5 min). The interface temperature was maintained at 180 °C, the ion source, and quadruple were maintained at 230 °C and 150 °C, respectively. Therefore, the optimized method (active SPME GCMS analysis of 400 mL of sampled air for 2 h) was used to test 65 individuals with 4 different scent elimination products.

Data analysis techniques

Controls and samples (130) chromatograms were processed using Agilent Chemstation data analysis software which gives height and area of each peak in a chromatograph. The tentative identity of each peak was determined (and assigned a confidence factor) by Chemstation software using mass spectrometer data with the help of the NIST 2008 GCMS compound library. Retention times and peak areas were

standardized using four internal standards including IS1 (bromochlorobenzene), IS2 (1,4-difluorobenzene), IS3 (chlorobenzene-d5), and IS4 (1-bromo-4-fluorobenzene).

Each subject produced 200 to 300 quantifiable chemicals. Many chemicals were found in most of the subjects however each of the 65 subjects produced a unique set of chemicals. In order to determine the extent of odor reduction each identified compound was given a rank score based on the original size of the peak in the control [larger peak = lower number score (1 through 50)], decreased percentage when comparing the control to the sample [larger decrease = lower number score (1 through 50)], frequency of occurrence in individual subjects [higher frequency = lower number score (1 through 16)]. The ranks were multiplied to determine the final ***impact score*** (a lower score equates to a greater overall impact (***high impact compounds***) for each compound).

The final set of high impact compounds were narrowed down to 29 by considering the magnitude of the relative peak area (>10000 arbitrary peak area units) and the frequency of occurrence (found in at least 60 % of the control samples). These 29 compounds were used for the statistical analysis (data matrix 65 × 29). Gaussian distribution of the relative peak areas of the controls and samples were analyzed. The retention index of selected compounds was also calculated comparing their retention times with analyzed standard linear chain hydrocarbons using the equation 1.1 .[77]

$$I_A = 100\,N + 100\,\frac{log\ t'R(A) - log\ t'R(N)}{\log t'R(N+1) - \log t'R(N)} \qquad (4.1)$$

Where, $\mathbf{I_A}$ is the retention index of component A, t′R(A) is the adjusted retention time of component A, and t′R(N) and t′R(N + 1) are the adjusted retention times of the n-

hydrocarbons with carbon number N and N + 1 respectively. N is chosen such that it is the highest carbon number n-hydrocarbon which elutes earlier than component **A.**

Discriminant analysis (DA) was used to determine if the 29 high impact compounds could be used to statistically distinguish the products. Canonical discriminant analysis was used on the sample matrix to evaluate the separation of each product group using statistical software SAS 9.3. Finally, decision tree[78] and principal component analysis (PCA) were used to compute the reduction of scent profile of each subject using statistical software R 3.0.1.

Initial data treatment

Analyte retention times from each GCMS analysis were adjusted in order to match internal standards. This resulted in a slight shifting of retention times because the deviations in internal standard retention times generally were within a few seconds. Adjusted retention times were used to aid in tentative compound identification using NIST 2008 GCMS compound library. The four internal standard peak areas were normalized and then relative peak areas were calculated for each detected chemical. Silicon containing compounds (produced from GC components) and internal standard impurities were identified and removed from further consideration.

Chemometric analysis: discriminant and principal component analysis

We tested for equal variance (homogeneity) of the 29 high impact compound's relative peak area (in treated samples) within the product covariance matrices of the four groups (products), using a 5% significance level using SAS 9.3.[79-80] Since variances of the data from the different samples are equal, the variances were pooled and linear

discriminant analysis (LDA) was performed on the 29 high impact compounds for classification using SAS 9.3. An important LDA step is calculation of the Mahalanobis squared distance (relative difference of each peak area from the corresponding mean) [81-82] given by equation 4.2,

$$D_{ij} = (x_{ij} - \mu_i)S^{-1}(x_{ij} - \mu_i)' \quad (4.2)$$

where, i(=1,2,3,4) represents the four product groups, x_i is the predictor matrix (relative peak area), μ_i is the mean matrix and S represents the pooled variance-covariance matrix. The average Mahalanobis distances were compared with an average value of data points in the four groups. Finally, a group was assigned to each subject if the Mahalanobis distance was similar to the average value.

It is equivalent to say that each subject (x_0) was classified to a group "i" if (inequality 4.3),

$$(x_0 - \mu_i)^T S(x_0 - \mu_i) < (x_0 - \mu_j)^T S^{-1}(x_0 - \mu_j)\ i,j = 1,2,3,4\ and\ i \neq j \quad (4.3)$$

The classification was further validated using the cross validation re-substitution method.

We compared each subject's control profile with the sample profile using principal component analysis (PCA).[83] The 130 observations (65 controls and 65 samples) were divided into four groups (products). The data were standardized (scaled) using the internal standards; therefore, to centralize the data we subtracted the mean of each column (co-variate) (compound) (Z score calculation) within each group from the scaled data. Finally the principal component (PC) scores were obtained from the centralized data using the statistical software R 3.0.1. The first three PC scores in the

control contributed more than 80% of the total variation. The first three PC scores in the sample contributed more than 88% of the variation within each group, where Product number 1(P#1)-94.16%, Product number 2(P#2)-97.73%, Product number 3(P#3)-88.42%, and Product number 4(P#4)-97.13%. Each subject's norm in the control was compared with its norm in the sample. The norm (distance from the origin) of 3-dimensional space is given by equation 4.4,

$$\|P\| = \sqrt{\sum_{i=1}^{3} PC_i^2} \tag{4.4}$$

where P is a point given by $(PC_1(principal\ component\ \#1), PC_2, PC_3)$ in the 3-d space. A lower P value corresponds to a lower peak area of the high impact compounds. We computed the norm of each subject's control and sample, then calculated the fraction of reduction R_j for each subject as given by equation 4.5,

$$R_j = \left(1 - \frac{\|P_j\|_s}{\|P_j\|_c}\right) \tag{4.5}$$

where j (=1,2,…,n_i) is the subject, i (=1,2,3,4) represent the group/product, n_i is the number of subjects in each product (n_1=n_2=n_4=16, n_3=17) and $\|P_j\|_s$, $\|P_j\|_c$ represent the norm of j^{th} subject in sample and control respectively. In some cases, R_j was negative, which implies that the profile was increased in the sample compared to the control profile. A summary of the steps followed during the analysis can be seen in Figure 4.2.

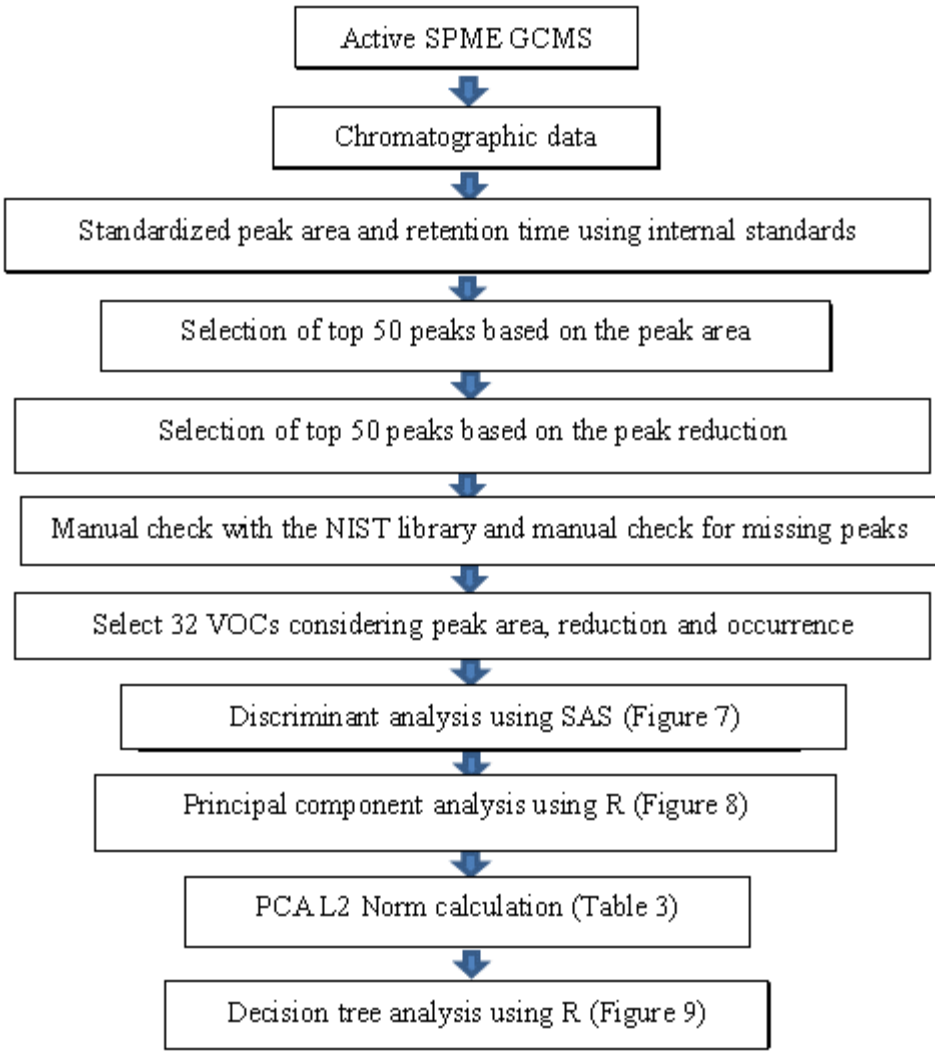


Figure 4.2 Summary of steps followed during the analysis.

Results and discussion

All 130 collected samples were analyzed using active SPME GCMS. Human scent is a mixture of volatile compounds. The complexity of the mixture can be visualized by the typical chromatogram in Figure 4.3. IS1, IS2, IS3 and IS4 in this figure are the four internal standards introduced into each sample. The horizontal axis represents the retention time: the length of the time which a particular compound is retained in the

gas chromatographic column before reaching the detector. Each peak represents a different chemical and a larger peak indicates higher concentration of that specific chemical. A typical data set from a single sample would contain more than 200 different chemicals. None of the four products had a significant VOC profile that could overshadow the general human scent VOC profile (odor masking).

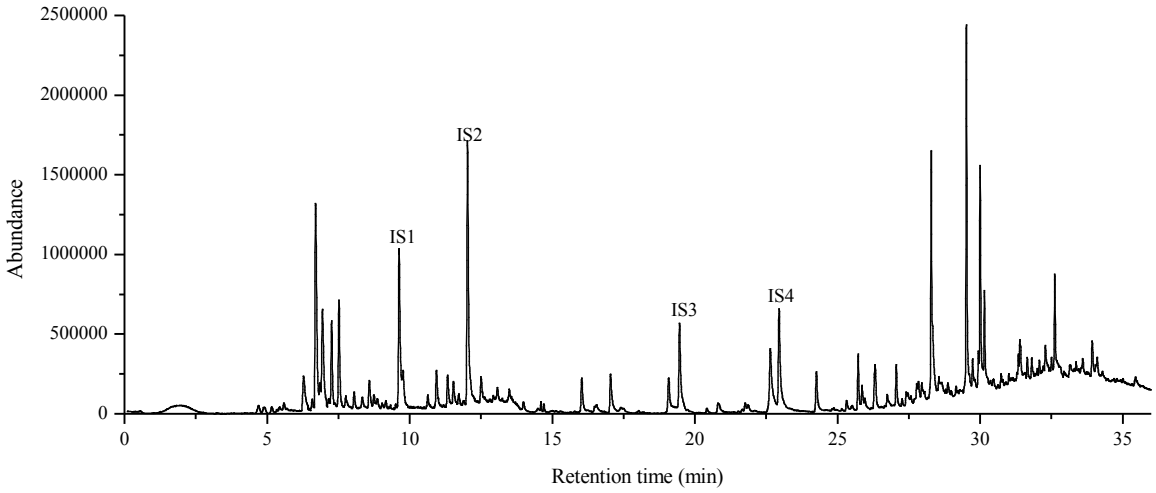


Figure 4.3 Typical GCMS chromatogram of human scent. (x axis – chromatographic retention time in minutes, y axis – Abundance (arbitrary units).

Note : IS 1-4 represents the four internal standards used to aid in peak standardization.

The potential exists for the reduction of odors produced from bacteria if the product being tested contains bactericidal agents. If chemical odorants are being reduced through the killing of bacteria, we would expect to see a reduction in certain peaks in our chromatographs. Reduction of volatile organic compounds may also be due to the binding effect of different agents available in the scent elimination products. Overlapped control and sample chromatograms can be seen in Figure 4.4, where the effect of bactericidal agents or the effect of odor binding agents can be observed. Many of the

early eluting peaks are smaller in the blue trace, ***sample,*** (with the product) than the black trace, ***control***, (without the product).

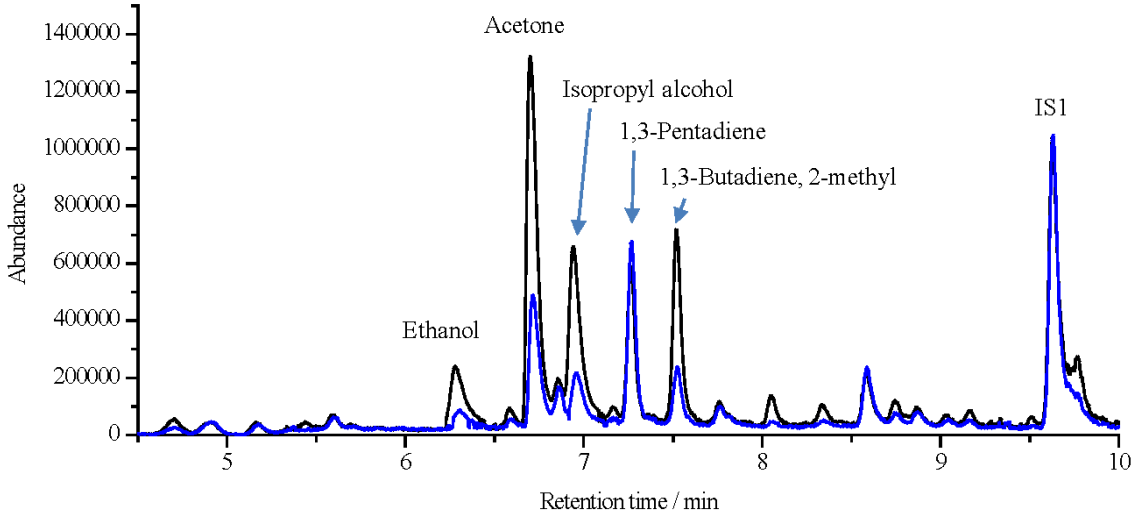


Figure 4.4 Comparison of control (untreated) (Black line) and sample (treated) (Blue line) overlapped chromatograms.

Note: Typically a reduction in magnitude in some chemicals was observed in the samples (with product) when compared with the controls.

Total relative peak areas (cumulative total of all the relative peak areas) from the GCMS data of controls (C), samples (S) and the difference (D) (Control – Sample) of all four products (P#1,2,3,4) of selected compounds are compared in Figure 4.5 and the distribution of data is illustrated next to each box plot. (Whiskers extend from 5% to 95%, while the box plot represents 25% to 75%, the middle line represents the median and the small square represents the mean of the entire data set). All samples have a lower average total relative peak area compared to controls. Thus each product tested, on average, resulted in a reduced total VOC profile.

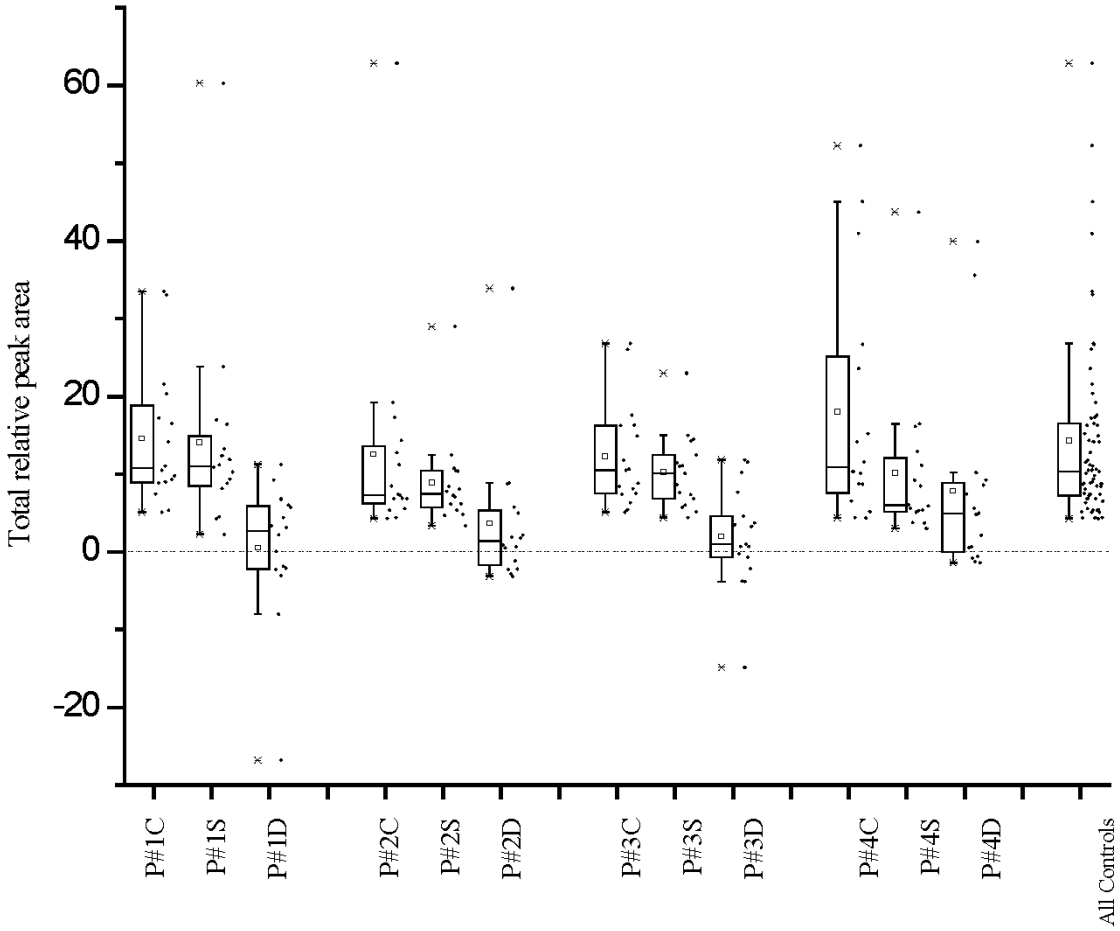


Figure 4.5 Total relative peak areas of controls, samples and the difference of all four products. (C – Control, S – Sample, D – Difference).

Further analysis was done to determine the extent of odorant reduction. Compounds which appeared in at least half of the chromatograms (8 or more individuals) and decreased more than 50% with application of scent elimination product were identified. Lower scores were given to chemicals that were 1) higher in abundance, 2) were produced by greater number of subjects and 3) were more significantly reduced through the action of the scent removal product. For example, (in test subject # 50 (P#3)) acetone had high peak area; rank = 1 (out of the top 50 compounds), was significantly

reduced in sample; rank = 11 (out of 50 most reduced compounds by %) and was found in many samples in the P#3 group; rank = 1 (out of 32 high impact chemicals). Thus the acetone impact score = 11 for this individual (114 average for all samples). In the same individual butanal, 3-methyl-, in contrast, had lower peak area; rank = 36, was not significantly reduced in samples; rank = 20; and was found in less subjects; rank = 23 with an overall impact score of 16560 (21653 average for all samples). Table 4.2 orders the 32 high impact chemicals by retention index, with their corresponding impact score and rank.

Table 4.2 Retention index and average impact score of the most abundant and most common VOCs in human scent.

Compound Identification #	Compound Name	Retention Index	Impact Score* (and Rank)
1	Ethanol	460	3469 (11)
2	2-Propenal (acrolein)	471	18361 (26)
3	Acetone	475	114 (1)
4	Isopropyl Alcohol	484	372 (2)
5	2-Methyl-1,3-butadiene (Isoprene)	495	1587 (7)
6	Methacrolein	534	10436 (17)
7	2-Pentene, (*Z*)-	548	14123 (23)
8	3-Methyl-butanal	618	21653 (29)
9	2-Methyl-butanal	628	21544 (28)
10	Benzene	633	11241 (19)
11	Cyclohexane	642	18737 (27)
12	3-Methyl-hexane	657	17639 (25)
13	Heptane	700	25910 (31)
14	Methyl-cyclohexane	714	7298 (14)
15	Toluene	747	2683 (9)
16	Hexanal	772	4985 (13)
17	o-Xylene	867	29085 (32)
18	Heptanal	909	17418 (24)
19	1R-.Alpha.-Pinene	951	873 (5)
20	6-Methyl-5-hepten-2-one	986	13463 (22)
21	Octanal	992	21949 (30)
22	1,2,3-Trimethyl-benzene	1006	1779 (8)
23	1-Ethyl-3-methyl-benzene	1038	577 (4)
24	1-Octanol	1065	11489 (20)
25	Nonanal	1092	1494 (6)
26	Naphthalene	1193	10858 (18)
27	Decanal	1196	4094 (12)
28	Dodecane	1200	7489 (15)
29	Tridecane	1300	8815 (16)
30	3-Methyl-tridecane,	1379	12570 (21)
31	Tetradecane	1400	453 (3)
32	Hexadecane	1600	2900 (10)

Notes: *Average product ranks from peak area (1-50), reduction % (1-50) and occurrence in subjects (1-16) for all products.

Figure 4.6 represents the impact score for each individual scent elimination product. Both acetone (#3) and isopropyl alcohol (#4) have very low bars due to their relatively high abundance and occurrence (smallest bars) and all scent elimination products resulted in a reduction of these chemicals. Compound #19 (1*R*-.alpha.-pinene),

#22 (1,2,3-trimethyl-benzene1,2,3-trimethyl-benzene), #23 (benzene, 1-ethyl-3-methyl-), #5 (1,3-butadiene, 2-methyl- (isoprene)), #31 (tetradecane), and #32 (hexadecane) were also reduced by all products. In addition, compound #14 (cyclohexane, methyl-), #15 (toluene), and #16 (hexanal) show less reduction with all products when compared to Compound #s 3 and 4. All the chemicals are identified according to numbers assigned in Table 4.2.

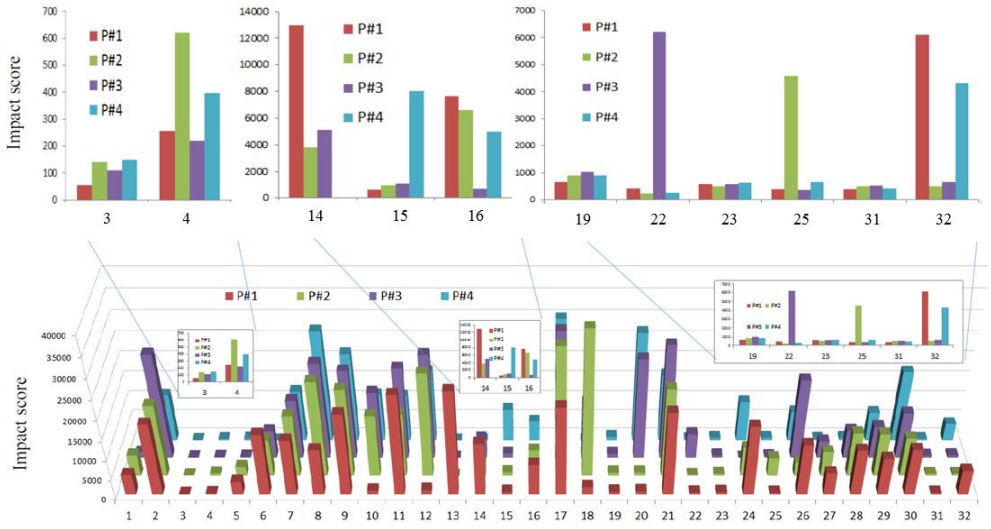


Figure 4.6 Comparison of abundance, occurrence, and reduction of VOCs in human scent among different scent elimination products.

Notes: Smaller values are given to most abundant, most occurrence and most reduction from the scent product. (High impact compounds are magnified for clarification). Tentative compound names of corresponding numbers are available in Table 4.2.

The Table 4.2 compounds can be produced through different routes. Acetone is one of the major byproducts of human metabolism and is produced by decarboxylation of compounds derived from lipid peroxidation.[84] alpha.-pinene, 1,2,3-trimethyl-benzene, 1-

ethyl-3-methyl-benzene, isoprene, tetradecane, hexadecane, toluene, and hexanal are produced on skin due to the microbial activity and are found in live and decaying human bodies.[5, 85-86]

Changes in VOC profiles may be due to bactericidal action from the scent control products. Numerous bacterial species are known to degrade non-volatile organic compounds, live on the secretions of skin glands and are able to cleave molecules at different sites depending on the availability of their enzymes.[87-89] Odor controlling products often employ bactericidal agents. Applying these products will reduce bacterial populations and thus alter VOC profiles.[90] In addition, different odor binding and neutralizing agents uniquely available in different products can make significant changes in VOC profile.[57-58]

DA and PCA analysis of control and samples

Homogeneity of variance within products were tested (p-value <0.0001) and covariance matrices were used for the discriminant function. The re-substitution method classified the subjects without any misclassification while the cross validation reported some misclassification. The canonical discrimination plot of all four products is available in Figure 4.7. Each symbol is dedicated to a product and the four groups distributed in three dimensional space represent the difference in functionality of each product. Raw Canonical Coefficients of considered compounds are available in the appendix (Table G.1).

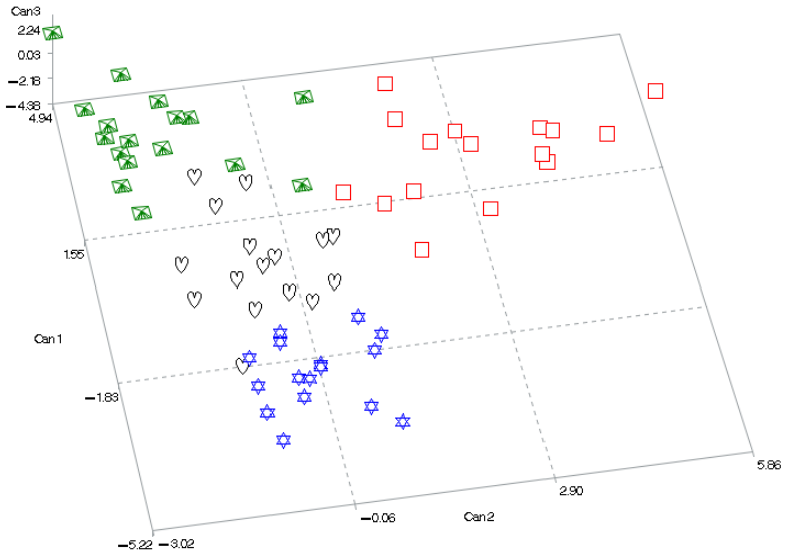


Figure 4.7 Three dimensional canonical discriminant plot of all four products (red square-P#1, black heart-P#2, green pyramids-P#3, blue star-P#4).

Three-dimensional and two-dimensional PC plots for each product are available in Figure 4.8. Each dot in the three-dimensional plot represents the (x,y,z) relative location from the origin and the magnitude of each point corresponds to a scaled relative peak area of the compounds in each subject. We believe that control dots (in black) should have higher magnitudes compared to the corresponding samples (in red triangles), as we expect to have a lower quantitative VOC profile for samples compared to controls due to the effect of the scent elimination products. The controls (black dots) were scattered more compared to the samples (red triangles), and samples tend to converge to a specific domain in each product which can be illustrated as the overall effect of different scent control products. The 3-D representation shows that each set of test subjects had at least one individual that drastically deviated from normal trends. The 2-D plots are shown without the outliers to better show the normal trends. The top five principal component

compounds (top five compounds which are mostly contributed to the variation of each axis) for each product are available in the appendix (Table H.1).

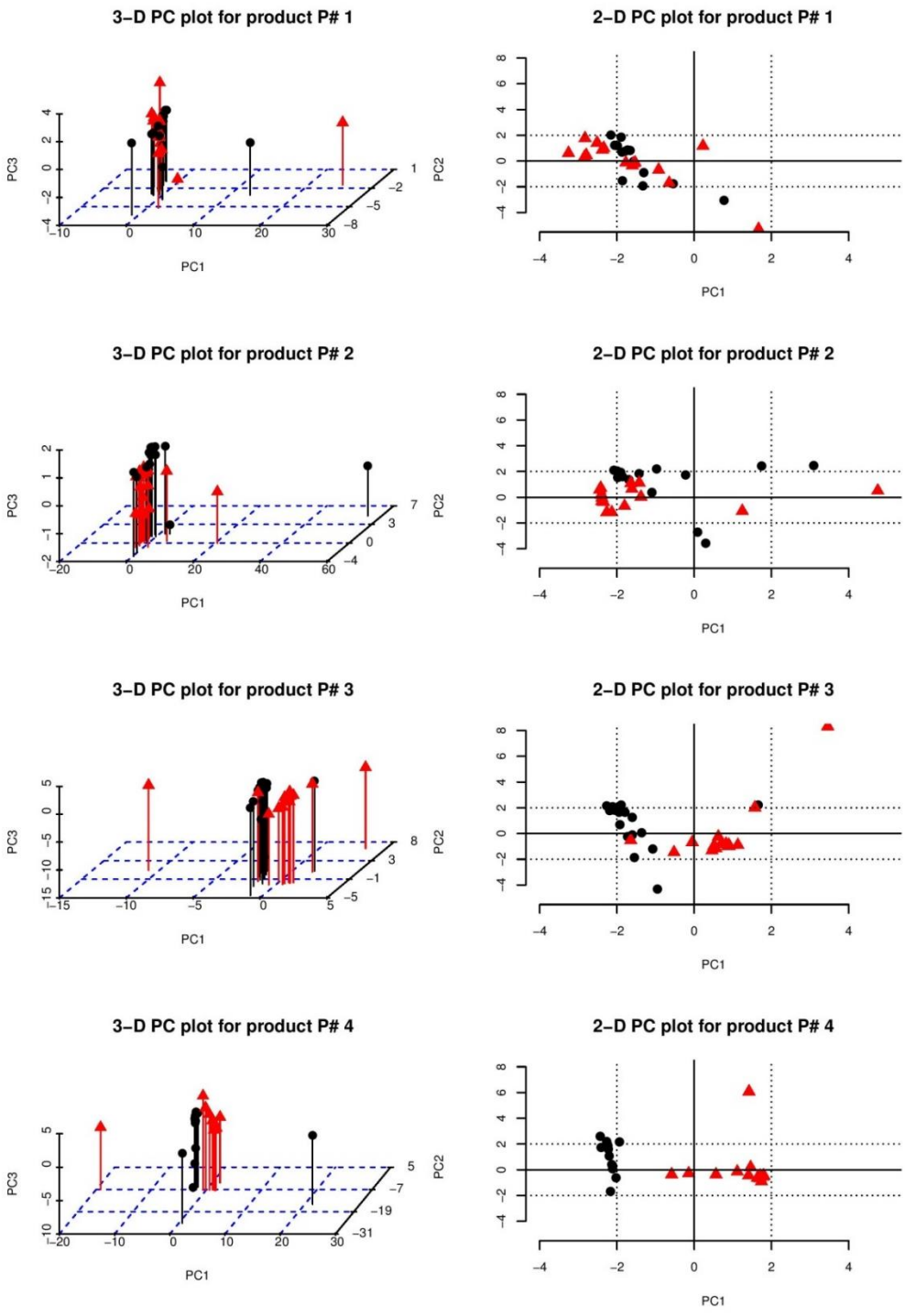


Figure 4.8 PCA plots for controls and samples of each product. (Black dots –Controls, Red triangles – Samples).

First, second and third principal components are plotted for controls and samples in each three-dimensional plot. Two-dimensional plots are plotted for the corresponding first and second principal components. Control and sample points are located in clearly distinguishable clusters (with some outliers) and scattered control dots and converged sample dots illustrate the influence of scent elimination products on control samples. The calculated percentage reduction values using the equation 4.4 and 4.5 of the principle components for each subject with the corresponding product are available in Table 4.3.

Table 4.3 The norm principal component distance from the origin (p*) for controls and samples and the reduction fraction (r_j**) for each subject.

Product # 1

Subject	Control (P)	Sample (P)	Reduction (R_j)
1	2.47	3.66	-0.48
2	2.06	3.01	-0.46
3	2.92	3.1	-0.06
4	1.73	2.61	-0.51
5	1.91	3.05	-0.6
6	1.84	1.85	-0.01
7	2.67	3.12	-0.17
8	2.14	1.67	0.22
9	3.51	4.02	-0.15
10	1.47	1.56	-0.06
11	2.19	1.79	0.18
12	1.75	1.8	-0.03
13	7.3	5.63	0.23
14	1.77	3.34	-0.88
15	13.56	25.11	-0.85
16	1.36	1.97	-0.45

Product # 2

Subject	Control (P)	Sample (P)	Reduction (R_j
1	1.07	2.49	-1.34
2	3.43	1.66	0.52
3	2.75	2.59	0.06
4	2.32	2.39	-0.03
5	2.75	2.41	0.12
6	2.46	2.04	0.17
7	2.19	2.66	-0.21
8	2.8	2.02	0.28
9	2.01	1.79	0.11
10	3.72	1.7	0.54
11	2.58	2.03	0.21
12	4.56	4.81	-0.05
13	56.65	20.65	0.64
14	2.8	2.55	0.09
15	2.31	2.45	-0.06
16	2.91	2.59	0.11

Product 3

Subject	Control (P)	Sample (P)	Reduction (R_j)
1	11.28	2.81	0.75
2	2.77	1.27	0.54
3	3.03	1.59	0.48
4	2	1.53	0.24
5	1.41	1.94	-0.37
6	1.87	11.09	-4.93
7	4.37	1.33	0.7
8	2.45	1.3	0.47
9	2.8	1.35	0.52
10	6.08	9.02	-0.48
11	2.35	1.7	0.28
12	1.78	1.84	-0.03
13	2.82	2.66	0.06
14	2.52	1.81	0.28
15	2.2	1.17	0.47
16	3.23	1.94	0.4
17	1.39	1.15	0.17

Product 4

Subject	Control (P)	Sample (P)	Reduction (R_j
1	1.91	1.7	0.11
2	30.12	1.78	0.94
3	2.01	0.48	0.76
4	3.13	1.74	0.44
5	4.76	1.71	0.64
6	3.07	1.77	0.42
7	6.96	6.15	0.12
8	3.07	1.71	0.44
9	3.06	1.52	0.5
10	2.9	1.7	0.41
11	2.64	1.04	0.61
12	2.17	0.96	0.56
13	15.27	10.78	0.29
14	1.85	2.1	-0.13
15	9.94	3.98	0.6
16	1.78	1.66	0.07

Notes: * Calculated from equation 4.4
** Calculated from equation 4.5

Calculated overall average reduced (Control > Sample) and increased (Control < Sample) percentages for the 32 high impact compounds and efficiencies (% ratio of the number of reduced subjects out of the total number of subjects analyzed in each product

group) from the principal component analysis norm calculations are available in Table 4.4.

Table 4.4 Calculated overall reduced and increased percentages.

Product	Average reduction (%)*	Average Increase (%)*	Efficiency (%)**
P#1	17.1	42.1	44
P#2	24.9	36.4	81
P#3	42.8	161.8	82
P#4	40.4	5.2	94

Notes: *Average reduction % when Control>Sample and average increase % when Control<Sample for the P values listed in Table 4.3.
** Percent ratio of the number of reduced subjects out of the total number of subjects analyzed in each product group. R_j values found in Table 3.

The perfect scent elimination product would eradicate any volatile chemical signature. However, all products tested resulted in a reduction of some chemicals and an increase in others. P#1 showed a larger increase in the profile than reduction, and also showed the lowest efficiency rate. Both the reduction and increase due to application of P#1 is illustrated in Figure 4.8 which shows a larger deviance of sample points (red triangles) from the origin. Most of the points in the sample are not closer to the origin according to the 2D and 3D PC plots and that represents the poor performance of P#1 regarding scent elimination. Moreover, that observation is evident from Table 4.4 P#1 average reduction and increase values. However, this could be the strategy of P#1 which tries to mislead the animal by conveying a confused message with an altered odor profile. The P#2 sample points seems to be converging to a point shifted from the origin (PC1=-2, PC2=0), but it has a high efficiency rate. P#3 and P#4 show good results and the sample points for both are closer to the origin (notice most of the sample points are bounded between ±2 in PC1 and PC2). However, P#3 showed the highest reduction of all

products in some compounds but, according to Table 4.3, it also showed the highest increase in others. This was mainly due to one subject where the product increased the subject's scent profile by 4 times compared to the control. P#4 shows a comparable reduction to P#3 and the highest reduction efficiency with the lowest increase of scent profile. The number of scattered points in the sample of P#4 is lower compared to P#3 which is evident in Figure 4.8. (P#4 sample points are congested along the zero of PC2 axis and highly deviated from control dots).

The decision tree statistical technique was used to determine high impact chemicals which are important in categorizing controls and samples. If the condition is satisfied tree is progressed through the true branch otherwise through the false branch. For example in product number 1 decision tree, if the ethanol content is lower than 0.2 relative peak area units subjects are categorized through the true branch otherwise categorize through the false branch. In product #1 most of the controls are categorized into the higher ethanol content branch and most of the samples are categorized into the lower ethanol branch. If the nonanal content is smaller in subjects (in this case is controls) than 0.94 relative peak area units they are categorized through the true branch. If the acetone content is less than 0.58 subjects (in this case samples) are categorized into the true branch otherwise into the false branch.

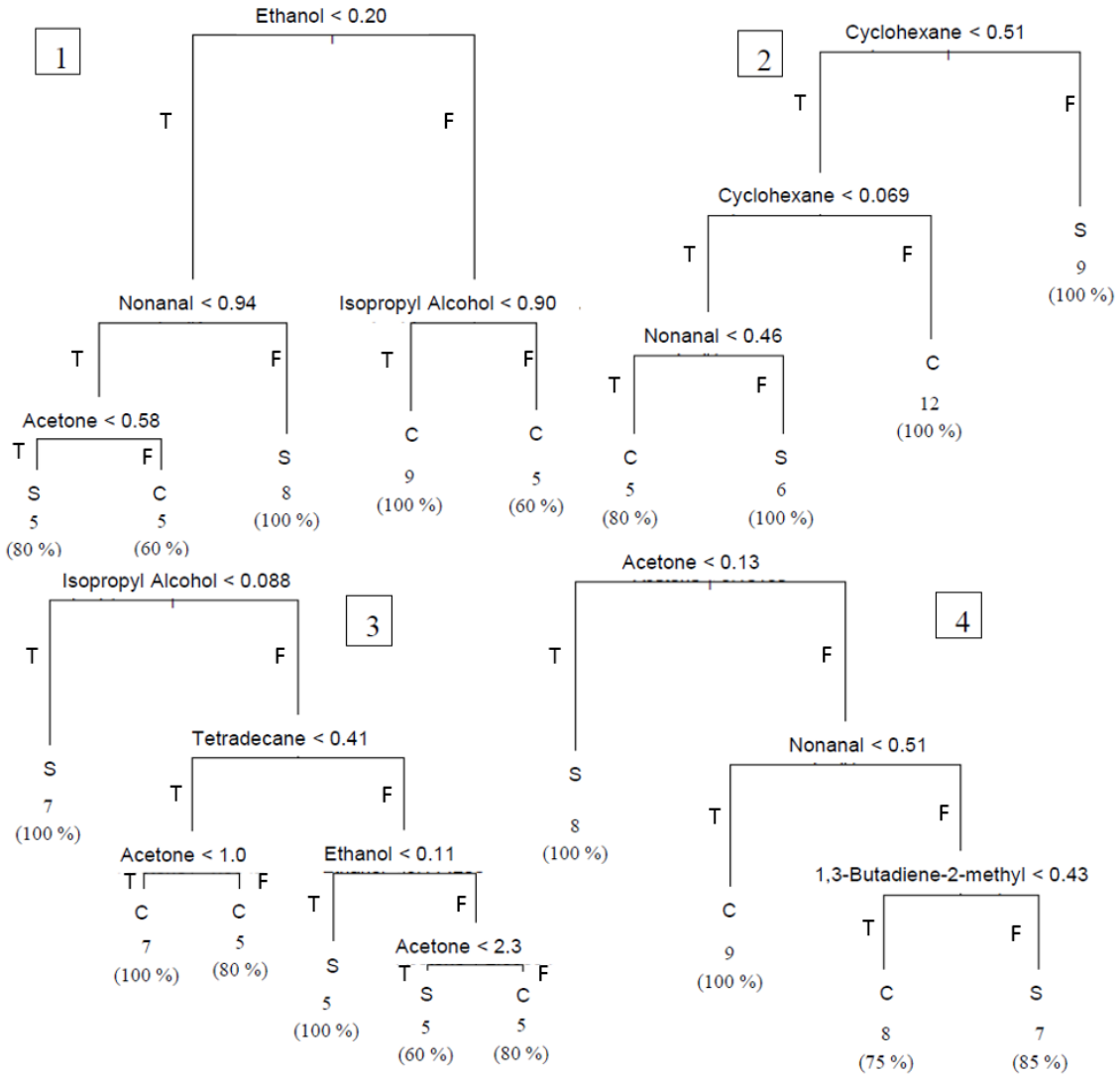


Figure 4.9 Decision trees for controls and samples of all four products. (1- P#1, 2 - P#2, 3 - P#3, 4 – P#4) (C – control, S – sample) (T – true, F – false) (relative peak area of each compound is compared).

As discussed earlier, VOC profile can be influenced by the mortality rate of different bacterial species due to the effect of human odor controlling products. At the same time VOCs can be converted into semi-VOCs or non-VOCs thus producing chemicals that are not readily transported from the human. According to Figure 4.9,

Product #1 controls and samples are mainly discriminated via ethanol, nonanal, isopropyl alcohol and acetone. According to the decision tree output, the majority of treated samples have reduced levels of ethanol, acetone and elevated levels of nonanal. Product #2 controls and samples are mainly discriminated through cyclohexane, nonanal. Nine out of 16 samples are categorized based on the elevated cyclohexane levels and 12 out of 16 controls are categorized based on intermediate levels of cyclohexane. Remaining samples are categorized based on the elevated levels of nonanal. Ethanol and acetone are produced in humans due to the metabolic pyruvate and Acetyl-CoA degradation and due to microbial activity[91], hence available in elevated levels in untreated controls.

In Product #3, controls and samples are mainly discriminated through isopropyl alcohol, tetradecane, ethanol and acetone. Seven out of 17 samples are categorized based on reduced levels of isopropyl alcohol and the remaining 10 samples out of 17 are categorized based on elevated tetradecane and reduced ethanol and acetone levels. nonanal, cyclohexane and tetradecane levels may have increased due to the enhanced activity of certain microbial species because the lack of competition with other microorganisms may cause imbalance in the impact of scent elimination products.[86] Product #4 controls and samples are discriminated via acetone, nonanal, 1,3-butadiene-2-methyl. Reduced levels of acetone in samples indicated the influence of P#4. 1,3-butadiene-2-methyl is one of the metabolic by-products of humans and is commonly known as isoprene.[92] Isoprene also can be produced by bacterial activity.[93] The elevated level of isoprene with P#4 may indicate the enhanced activity of a bacterial species unaffected by P#4 agents in addition to the metabolic contribution.

Some levels of human odor VOCs increased upon addition of scent elimination products. This may convey a confused ecological signal to the prey animal about their human predator. For example, nonanal, benzene and decanal have all been found in deer and dog decaying bodies.[94] Interaction of scent elimination products on microbial growth and metabolic influences may explain increases in 6 and 9 carbon aldehydes (hexanal and nonanal) and 8 carbon alcohols (octanol) through lipoxigenase activity.[95] Different chemicals also can affect positively[96] and negatively[97] on microbial activities. Another possibility for volatile level fluctuation may be the imbalance of free radical activity on different polyunsaturated fatty acids on the cell membrane due to the influence of scent elimination products.[86]

Conclusion

Likely VOCs candidates that play a role in chemical communication were tentatively recognized based on abundance, reduction and occurrence. These high impact compounds were then subjected to PCA and decision tree analysis aimed at discriminating between controls and subjects using human scent removal products. Thus three disparate approaches allow for the ranking of these high impact compounds associated with human odor (Table 4.5). Detection of ecologically important human odor is a challenging task which requires a combination of expertise in different fields. The human body produces hundreds of chemicals, and every person generates a unique set that is continuously changing due to variations in diet, activity levels and a range of other factors. We know of no study that has identified specific human chemicals that initiate the flight response in deer.

Table 4.5 Most likely VOCS associated with human/deer ecological chemical communication.

Rank in importance	Abundance, Reduction and Occurrence	PCA**	Decision Tree Analysis***
1	Acetone	Trimethylbenzene	Acetone
2	Isopropyl alcohol	Acetone	Isopropyl alcohol
3	Tetradecane	Isopropyl alcohol	Ethanol
4	1-Ethyl-3-methyl-benzene	Isoprene	Cyclohexane
5	Alpha-pinene	Dodecane	Nonanal
6	Nonanal	Nonanal	Tetradecane
7	Isoprene	Cyclohexane	Isoprene

Notes: * From Table 4.5
** Compounds are selected based on the average rank of the first principle component of the 4 products
***Key chemicals identified in decision tree analysis to discriminate samples from controls (Figure 4.2) (first column rank is not related to the decision tree)

It is difficult to draw definite conclusions regarding which of the products performed the best. But in summary, it appears likely that the four products tested work through a combination of three mechanisms**.** 1) Reducing bacteria: Each product tested showed a significant decrease in the production of VOCs in human-related bacteria and this could be due to the killing of VOC producing bacteria. 2) Binding chemicals: It is possible that animals key on specific combinations of chemicals that are uniquely human. If this is true, it is only necessary to remove or alter amounts of these key volatile chemicals. The decision tree has isolated key compounds which have the greatest effect from each product. 3) Conversion of volatile chemicals into non-volatiles: Another mechanism for eliminating key chemicals is converting them into new chemicals that are less volatile. Complexity of the resistance of microorganisms for different agents in scent

control products might confuse the observations regarding conversion effects. Volatiles common to both human and animals (deer, dog) have shown enhancement in levels with the product treatments while reducing the other volatile compounds. That may confuse the prey who is not warned by regular quantitatively unique predator scent. Future work could include a biological response study to confirm chemical patterns important for prey response.

The sample collection, standardization and analysis developed here for scent elimination products could be directly used to identify other ecologically important chemicals. Diseases like cancer or diabetes have been shown to produce unique odor profiles and the methods describe here are currently being studied for these applications.

CHAPTER V

CONDUCTING ABSORBENT COMPOSITE FOR PARALLEL PLATE CHEMICAPACITIVE MICROSENSORS WITH IMPROVED SELECTIVITY

Abstract

Conducting absorbent composites were prepared using two organic polymers (polar and nonpolar) mixed with conductive carbon nanoparticles and an ionic liquid ((BMI)(PF6)). The mixture was deposited between the microcapacitor plates of chemicapacitive microsensors using ink-jet technology. Different coatings were characterized using SEM and DRIFTS techniques. The response magnitude for each sensor depends on numerous phenomenon but changes in permittivity of the analyte and polymer swelling dominate. The performance of individual chemicapacitive sensors were characterized through exposure to concentrations of varied volatile organic compounds with different functional groups in a climate controlled vapor delivery system. Sensitivity, selectivity and limits of detection of each prepared sensors were compared and the discrimination power was evaluated using quadratic discriminant analysis. Ionic liquid doped polymers were able to enhance the sensitivity and the selectivity of parallel plate capacitive sensors. Improved analyte classification was achieved with the IL doped polymers (97% accuracy) over the pure polymers.

Introduction

Among chemical sensing techniques, thin absorbent polymer films with a sensitive transducer are well suited for low power, low-cost and portable applications.[98-100] Polymers are selected based on their ability to form stronger reversible hydrogen bonds, van der Waals bonds, and dipole-dipole interactions with some analytes over others.[98] Using multiple sensors in the array can mitigate cross-sensitivities resulting in improved selectivity and reduce requirements of chromatography. Parallel plate chemicapacitive microsensors with absorbent polymer coatings have been successfully used to detect a wide range of volatile organic chemicals.[98, 101] These microsensors have been employed as the detector in commercially available mini gas chromatographs because of their small size and ability to operate in air.[102] Typically individual parallel plate capacitors are filled or partially filled with selectively absorbing polymers. When exposed to volatile analytes, absorption of the chemical into the polymer film alters the permittivity of the polymers resulting in changes in the capacitance of the sensor elements. Dozens of polymers have been studied for use with these systems in order to achieve improved sensitivity and selectivity.[98-100, 103-104]

The response magnitude for each sensor element upon analyte exposure depends on a combination of different phenomenon such as dielectric chemical structure modification from reversible weak interactions (hydrogen, dipole, van der Waals) with the analyte, the amount of analyte that absorbs, the dielectric constant of both the polymer and analyte and polymer swelling. The swelling effect in sensors has been utilized to enhance the sensitivity of chemresistors.[105-106] This is typically done by combining a conductor (carbon black (CB) particles) with a selectively absorbent

polymer.[105, 107] The applicability of conducting materials in enhancing the sensitivity and selectivity of a polymer matrix in parallel plate chemicapacitor sensors has not been thoroughly investigated and is the focus of this work (Figure 5.1). A charge to voltage converting circuit was used to extract the capacitance signal of the chemicapacitor sensors through the use of an embedded microcontroller.[98]

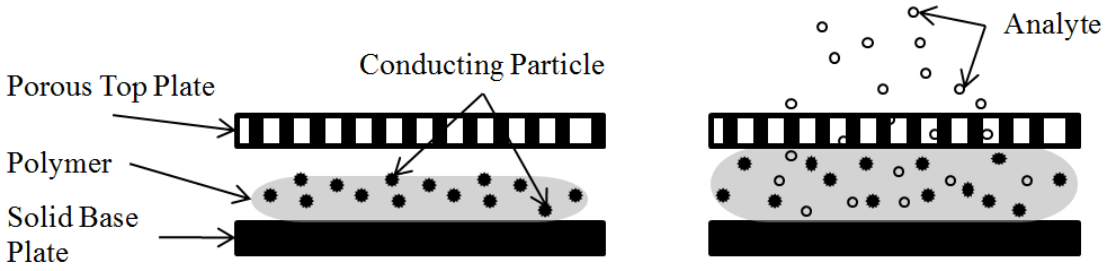


Figure 5.1 Conducting particles are mixed with functionalized polymers and deposited between the microcapacitor plates.

Note: The polymer swells as it absorbs the analyte resulting in a significant change in capacitance and a larger sensor response when compared to pure polymer.

Nonlinear contributions from swelling effects following analyte absorption can make the interpretation of capacitance change complex.[100, 107] Analyte adsorption phenomena on a polymer surface can also play a major role in sensor response and is related to polymer film thickness.[100] Sensor responses are related to dielectric chemical structure modifications due to different chemical and physical interactions, swelling effects, and the amount and the permittivity of the analyte absorbed or adsorbed to the polymer.[108] When the polymer matrix swells during exposure, the effective polymer volume (density of dipole moments) between the capacitor plates decrease, lowering the sensor response.[98] The swelling effect can be converted into an advantage by

incorporating conducting particles in to the polymer.[105] The polymer matrix will swell during exposure to analyte, increasing the average distance between conducting particles. This can increase the charge holding capacity of the sensor leading to enhanced capacitance changes and a more sensitive system.

Carbon black nanoparticles and ionic liquids are mixed with polymers to enhance the sensitivity and selectivity of microsensors. Carbon black particles may get aggregated and the average size of particles may vary from the original (manufacturer specified). But ionic liquid may have a better distribution in the matrix due to various type of interactions with the polymer matrix. Selection of ionic liquid is based on several factors. Density, viscosity, volatility and the level of interactions with VOCs is depend on the chain length of the cation and gel like ionic liquids are easy to coat on microsensors and the level of interactions are higher. $BMIPF_6$ is an economical and commonly available ionic liquid and used in quartz crystal microbalance applications.[109] Butyl chain on the cation has determined unique properties of $BMIPF_6$ and selected in this application.

Background

Polymer sensors

Solubility-prediction systems such as the Hansen solubility parameters[110-112] or the Linear Solvation Energy Relationship (LSER)[113] have been used to predict the amount of VOCs absorbed into a functionalized polymer. The extent of the solubility will depend not only on the ambient concentration of the VOCs, but also on the chemical properties of the VOCs and polymer. Hansen solubility parameters consider the dispersion, dipolar, and hydrogen-bonding strength of numerous organic compounds.[110] LSER considers chemical properties of materials, combining factors such as

polarizability, dipolarity, hydrogen bond-acidity, and hydrogen bond-basicity, to determine the gas-liquid partition coefficient (K_p). Other considerations include polymer physical properties (viscous liquids tend to be the best physical property for high absorption) and chemical stability. The chemistry of polymer-solvent interactions has been studied in detail with these tools, with the intent of producing highly selective coatings for sensors. Successful polymers for use in sensor applications require similar properties to those used in gas chromatography. Two polymers extensively studied in sensor and chromatography applications, polydimethyl siloxane (PDMS) and polycyanopropyl siloxane (OV275) have been used in this study.

Polymer-based sensors can be used to detect most volatile and semi-volatile organic compounds with a boiling point in the range of 40 to 200 °C. Highly volatile chemicals that boil below 40 °C do not partition well into polymers and those that boil above 200 °C tend to have vapor pressures that are too low for vapor phase detection. Other materials have been used to broaden the range of detectable chemicals including high surface area functionalized sol-gels used in the detection of carbon dioxide[114] although such materials often have to be heated to achieve optimal performance.[98]

A number of microfabricated transducers have been developed that utilize polymers to selectively absorb VOCs. Examples include those that measure polymer swelling including resistive sensors[115] and cantilever stress sensors,[116] resonating cantilevers, surface acoustic wave (SAW) devices[117], quartz crystal microsensors (QCM),[118] and flexural plate wave (FPW) sensors[119] that measure mass and viscoelasticity changes, and capacitive sensors[120] (used in this study) which measure changes in polymer permittivity.

Chemicapacitive sensors

Chemicapacitors use two basic geometries, *interdigitated electrodes and parallel-plate configurations. Interdigitated electrodes* employ single layers of metal either slightly elevated[121] or deposited on a substrate to form meshed combs. The absorbent material is then deposited onto the electrodes. *Parallel-plate sensors*[122] (this study) consist of a layer of metal deposited on a substrate, a layer of polymer and a second, porous layer of metal above the polymer. Details on the specific capacitive transducer used in this study can be found in the section below.

Materials and methods

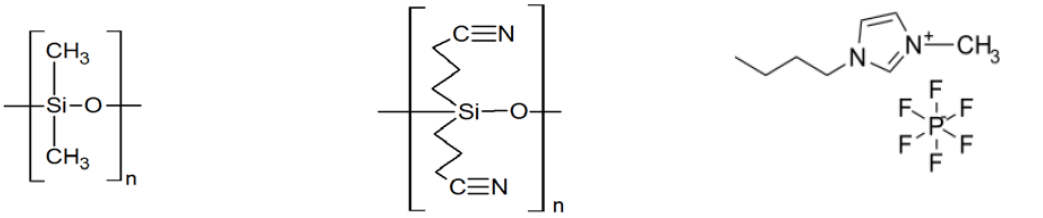
Sensor element

The sensor chips used in this study were fabricated using the Multi-User MEMS Process[16] (MUMPs; JDS Uniphase, Research Triangle Park, NC). The capacitive sensors were designed and donated by Seacoast Science. Each capacitor is a 300 µm square and has a perforated top plate suspended over a solid bottom plate, with a 0.75 µm gap between the plates with a base capacitance of ~1 pF. The gap is filled with a polymer that is injected through a porous top plate. All sensor chips measured 3 mm × 2 mm and had 3 parallel-plate capacitors. The capacitor plates were made of conductive polycrystalline silicon consisting of a 0.5 µm-thick bottom plate resting on the substrate, an air gap that was filled with polymer subsequent to the MEMS fabrication process, and a 2 µm-thick ventilated top plate. The top plate was anchored to the substrate with posts at approximately 60 µm intervals in order to minimize flexing when the polymer absorbed VOCs and swelled. The top plate also had 10 µm x 10 µm square holes separated by

about 10 μm. These holes were required for removal of a sacrificial silicon oxide layer during fabrication but also allow analyte vapors to pass through to the sorbent polymer.

Polymer materials

Polydimethyl siloxane (PDMS), poly (cyanopropyl siloxane) (OV275), 1-Butyl-3-methylimidazolium hexafluorophosphate ((BMIPF$_6$)) (ionic liquid), and carbon black nanoparticles (~50 nm) were purchased from Sigma Aldrich. Polymer/carbon black (CB) nanocomposites were prepared in different proportions to determine optimum CB to PDMS and OV275 ratios. These mixtures proved to be problematic because clumping of the nanoparticles cause difficulties in the ink jet coating process. Thus only one mixture of each was successfully prepared. Ionic liquid/polymer mixtures mixed well and coated sensors without problems. BMIPF$_6$ and polymer structures are illustrated in Figure 5.2.



Poly (dimethylsiloxane) (PDMS)

Poly (cyanopropyl siloxane) (OV275)

1-Butyl-3-methylimidazolium hexafluorophosphate (BMIPF$_6$)

Figure 5.2 Ionic liquid and polymers used to prepare composites.

Polymers and conductors/ preparation

Polymer nanocomposite mixtures were prepared according to the Table 5.1 weight ratios. Polymer nanocomposites were diluted to approximately 0.1 wt% with the proper solvents and then were introduced into the gap between the sensor plates using the

ink jet technology. Sensor outputs were recorded throughout the coating process. The coating process was determined to be complete once the sensor output ceased to change with the addition of more polymer composite. Sensors were then dried under nitrogen flow to remove remaining solvent molecules.

Table 5.1 Prepared polymer nanocomposites and Ionic liquid polymer composites mixtures.

Non-polar polymer	**Polar polymer**	**Ionic liquid/polymer**
PDMS	OV275	$BMIPF_6$
PDMS/20% CB	OV275/30% CB	OV275/10% $BMIPF_6$
		PDMS/10% $BMIPF_6$

Coatings were characterized using the diffuse reflectance infrared fourier transform spectroscopy (DRIFTS) techniques. Carbon black nanocomposites were further characterized using scanning electron microscopy (SEM) technique.

Coating process

The gap between the plates was filled with polymer and composites introduced through the etch holes of the top plate. Introduction of nanocomposite coatings into the microsensor plates is a challenging task due to its small dimensions (Figure 5.3). Polymers are applied in dilute solutions to the sensors with an inkjet head similar to that used in printers. The head, which is mounted on a translation stage, has an 80 μm diameter nozzle that expels droplets of a polymer solution. Each drop is typically a few tens of picoliters in volume and 30-100 μm in diameter.

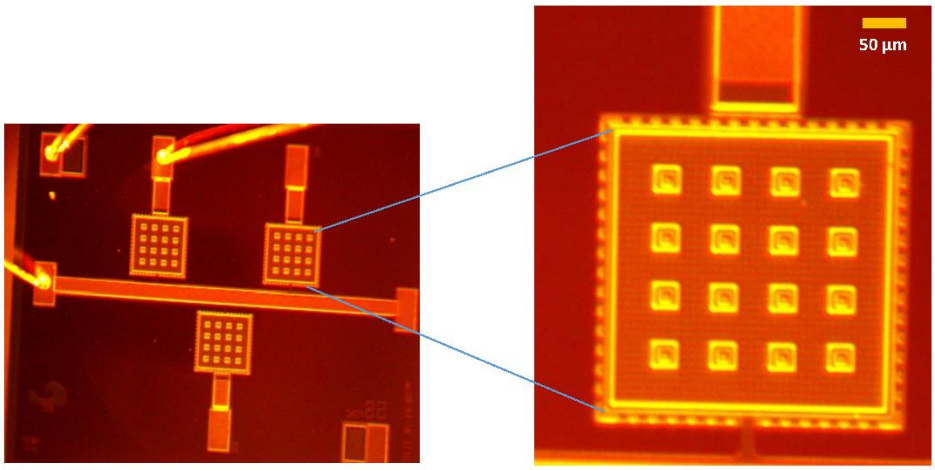


Figure 5.3 Micrographs of the chemicapacitive microsensors used in this study.

Sensor testing

All of the prepared sensors were tested in the assembled vapor delivery system (schematic diagram of the vapor delivery system is available in Figure 5.4). Ultra high purity (UHP) nitrogen (dilution gas in the diagram) was passed through temperature controlled liquid analyte bubbler under control flow conditions using mass flow controllers (MFC) (MKS instruments). Analyte flow was diluted by combining the analyte channel with another UHP nitrogen channel in a chamber (manifold) to introduce the desired concentration to the sensors. Acetone, 2-butanone, ethanol, ethyl acetate, hexane, toluene (Sigma Aldrich) were used as analytes for this study.

Typically chemicapacitive microsensors will need a preconcentration step for trace chemicals because of the platform's low sensitivity.[16] Concentrations were selected to produce a significant sensor response in a reasonable time in order to compare coating selectivities.[16] Sensors were exposed to 0 ppm, 1500 ppm, 2500 ppm, 4000 ppm, 6000 ppm, and 8000 ppm concentrations maintained 100 cc/min total flow rate in a climate

controlled (0 % humidity, 25 ºC temperature) environmental chamber (sensor flow chamber in the diagram). Each part of the flow system including mass flow controllers (MFC), valves (V), and sensor outputs were connected to the laptop through analog to digital interfaces (National Instruments) (NI 9201, NI 9263, NI 9472, NI cDAQ 9174) and the system was controlled by our custom built control panel programmed using the Labview software (National Instruments).

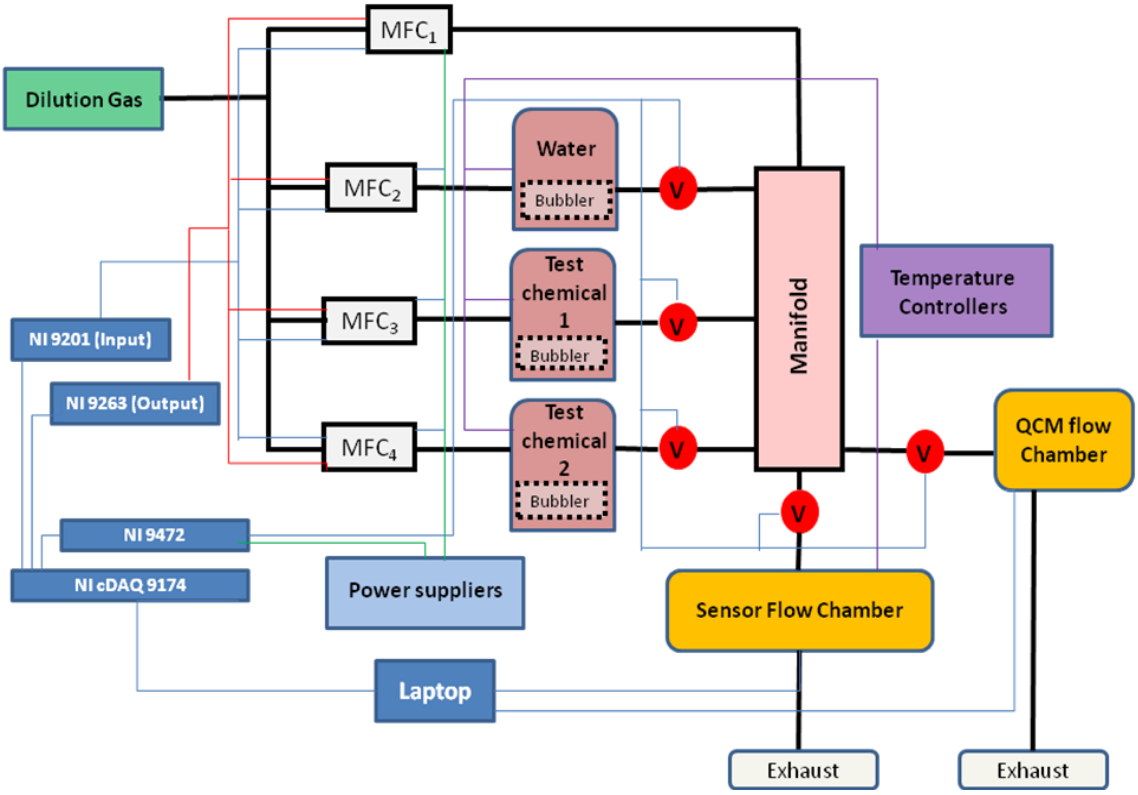


Figure 5.4 Schematic diagram of the vapor delivery system.

Notes: MFC –mass flow controllers, V – valves, NI cDAQ 9174 – National instrument chassis for analog to digital controllers (NI 9201 (input), NI 9263 (output), NI 9472 (valve control).

Data analysis

Microsensor analyte exposure data was collected, standardized and statistically treated with discriminant analysis (DA) technique using SAS 9.3 to assess the discrimination power of the prepared sensors. Responses were tested for equal variance (homogeneity) in different sensors for different compounds (for a given sensor or a pair of sensors, the variation between six selected compounds), using a 5% significance level using SAS 9.3.[79-80] Since the variation between different compounds were not similar, within-group covariance matrices were used and quadratic discriminant analysis (QDA) was performed for the classification using SAS 9.3. The Mahalanobis squared distance (relative difference of each sensor response from the corresponding mean)[81-82] were calculated using SAS 9.3 and the average Mahalanobis distances were compared with an average value of data points in each individual compound group. Finally, a group was assigned to each compound with the corresponding compound name if the Mahalanobis distance was similar to the average value. The classification was further validated using the cross validation method using SAS 9.3.

Results and discussion

Composite characterization

In this study the performance of the parallel plate chemicapacitive microsensors coated with carbon black-polymer nanocomposites and mixtures with ionic liquid were evaluated. Only the 20% CB doped PDMS and the 30 % CB doped OV275 sensors were usable out of several prepared sensors CB doped sensors. Performances of lower percentages of CB doped sensors were similar to the pure polymer coated sensors. Thus, due to the coating irregularities, we do not know the percent of the CB that made it

between the capacitor plates. Only that the sensor baseline capacitances were significantly changed and that is a clear indication of deposition of unknown amount of CB between the sensor plates. The sensors made from nanocomposites of CB concentrations over 30 % for OV275 and over 20 % for PDMS were abandoned due to noisy baseline which may be due to short circuiting of the two plates. As the concentration of CB increases, the average distance between the conducting CB particles gets smaller and charges (electrons) may find easier pathways between the two plates due to narrower tunneling gaps[123] while reducing the charge holding capacity. The sensitivity also can drop with increasing proportions of CB due to the reduction of the analyte absorbing polymer portion.[124]

Pure polymer and polymer composite functional group characterization was carried out using DRIFTS. Functional groups are important for selectivity of the technique through specific interactions with the desired volatile organic compounds. Introduction of conducting particles should have a minimum effect on the polymer matrix functional groups thus preserving the original selectivity of the polymer towards the desired marker molecules. DRIFTS analysis show that the functional groups in OV275 were not affected with carbon loadings (Figure 5.5). OV275 usually has Si-C (767 cm^{-1}), Si-O (1071 cm^{-1}), CH_2 (1432 cm^{-1}), C-N (2242 cm^{-1}), C-H (2920 cm^{-1}) bonds. Carbon loadings have not significantly shifted for these functional groups.

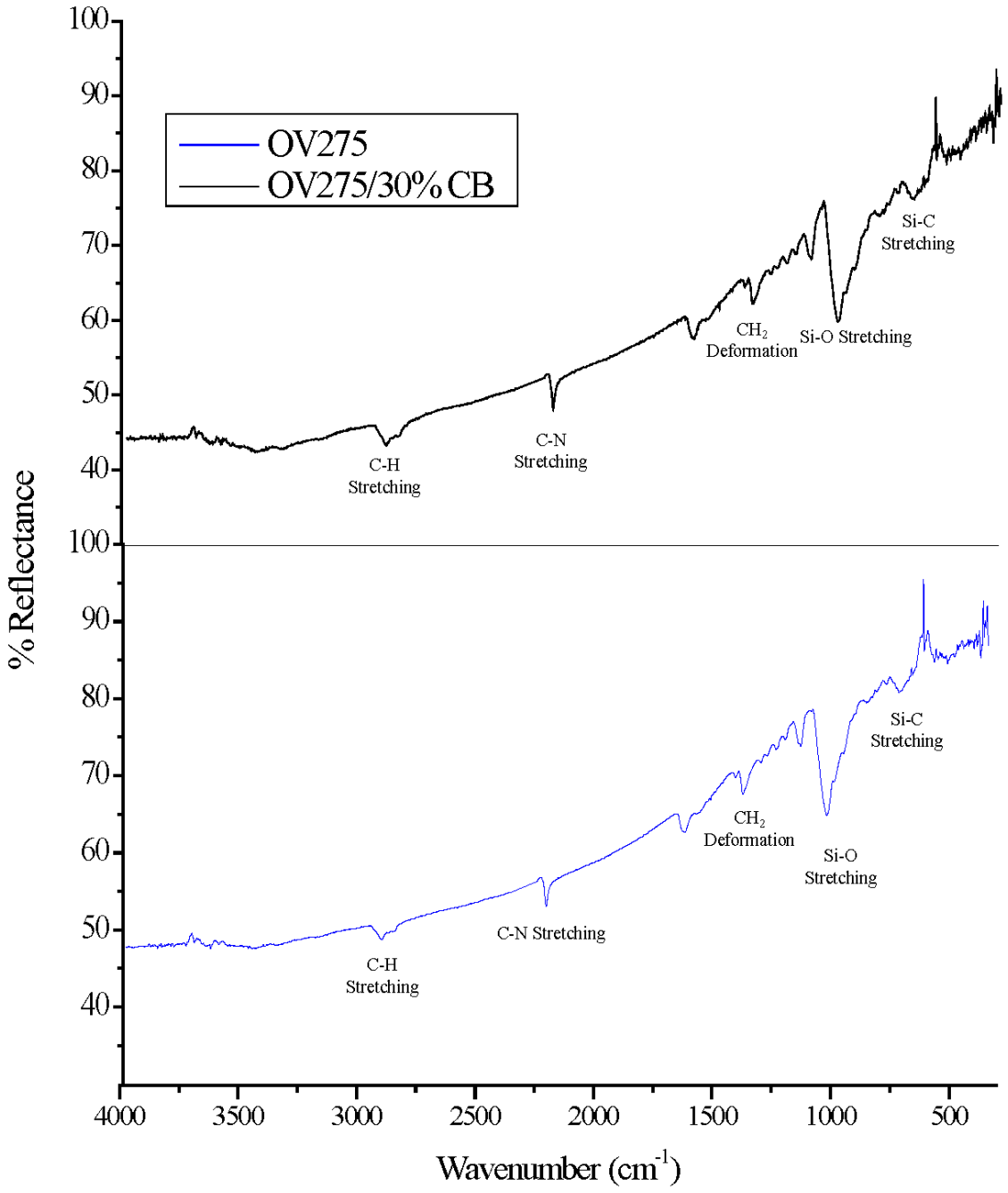


Figure 5.5 FTIR characterization of pure OV275 and OV275/ 30%CB.

Functional groups of pure PDMS and composites were also characterized with DRIFTS and the characteristic Si-C (789), Si-O (1011), Si-CH_3 (1261), C-H (2910) peaks

were found (Figure 5.6). Again carbon loadings did not result in a shift of the functional groups of PDMS.

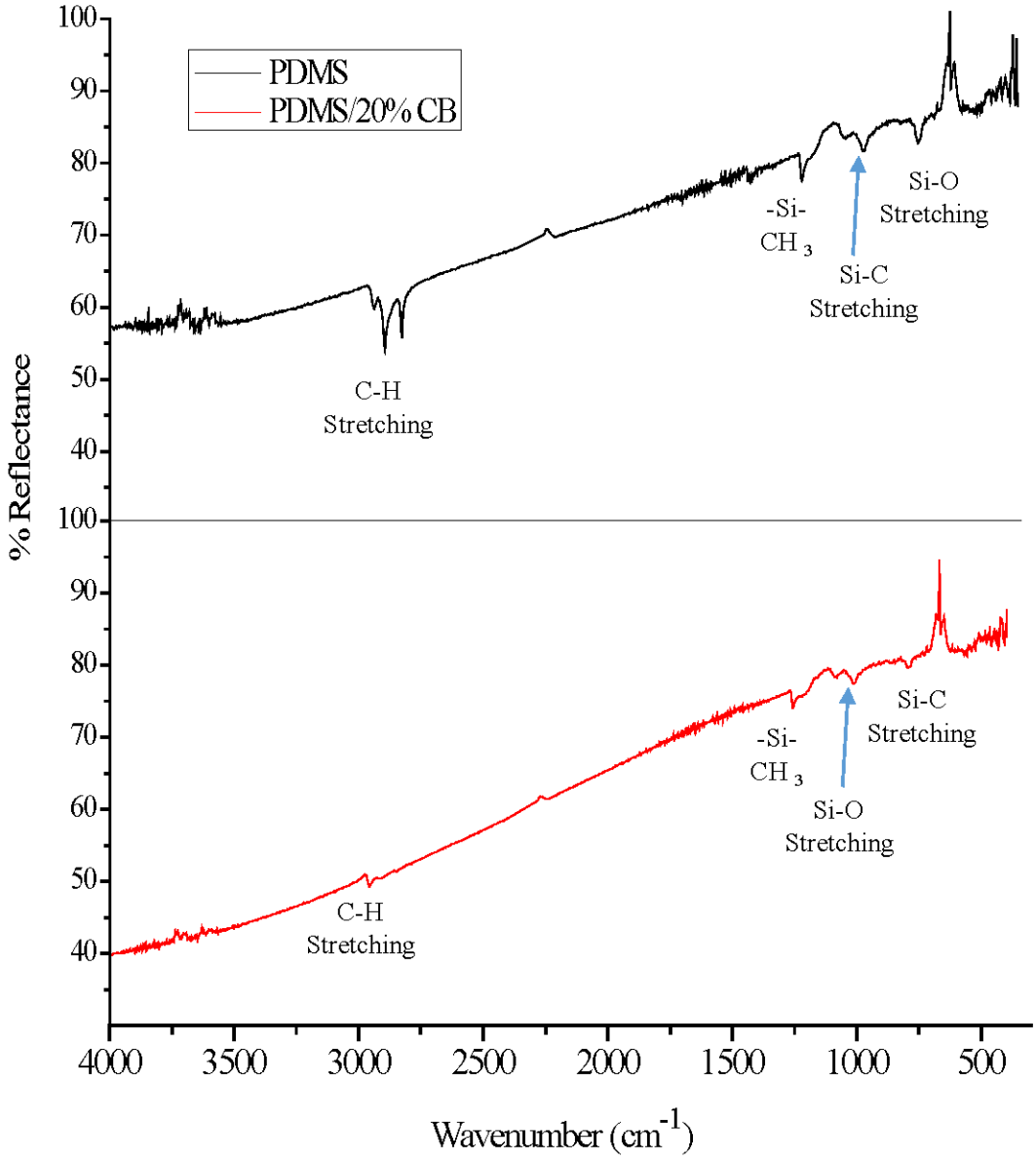


Figure 5.6 FTIR characterization of pure PDMS, PDMS 20% CB.

Polymer ionic liquid mixtures were also analyzed using FTIR (Figure 5.7). The PDMS mixed with 10% IL did not have a significant difference spectra from the FTIR of pure PDMS. This implies the minimal effect of the IL on PDMS confirming literature findings.[125] Similar results were observed with OV275.[126]

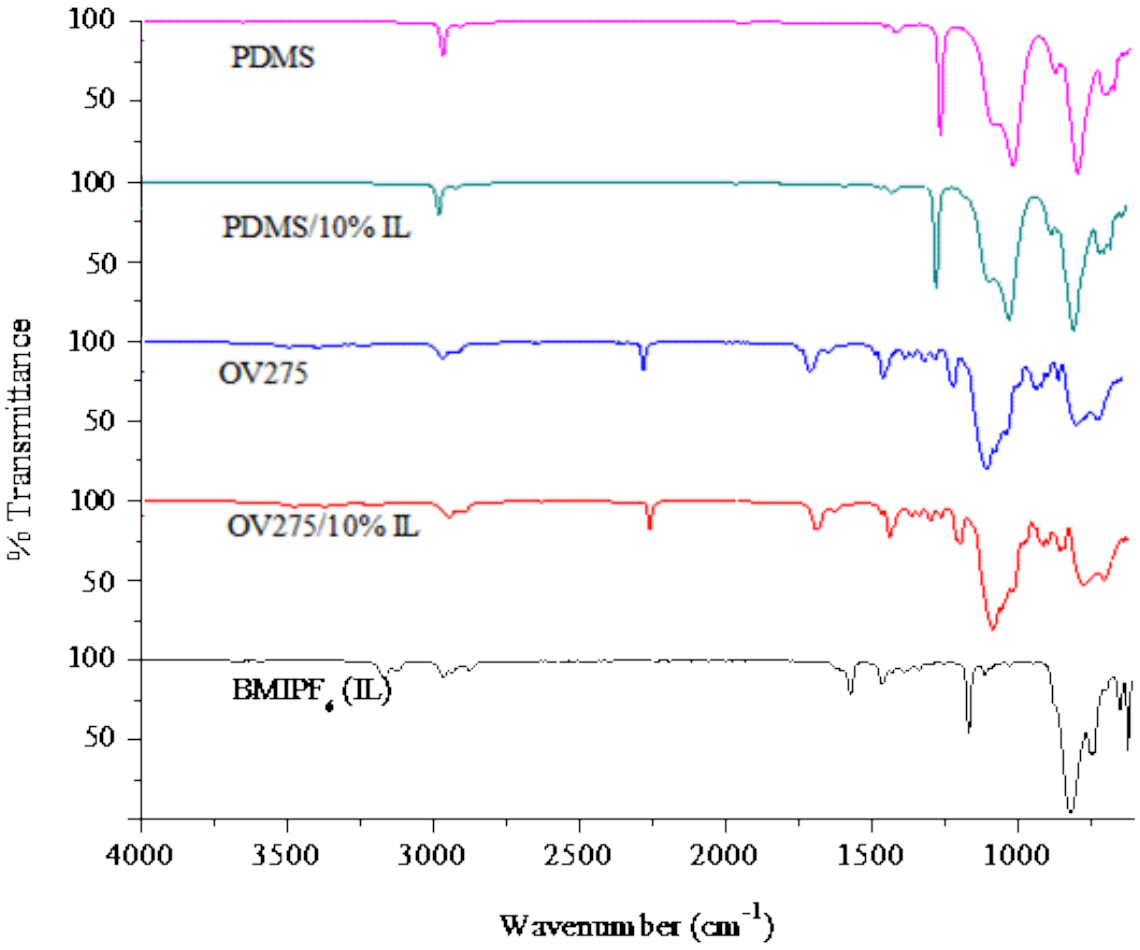


Figure 5.7 Comparison of FTIR spectrums of polymers, ionic liquid, and polymer ionic liquid mixtures.

Nanoparticle distribution studies of nanocomposites were carried out using SEM. More wettability was observed with PDMS compared to OV275 regarding carbon

nanoparticles (Figure 5.8). Clumping sizes of CB in PDMS was between 150 - 300 nm and for OV275 was between 50 - 150 nm.

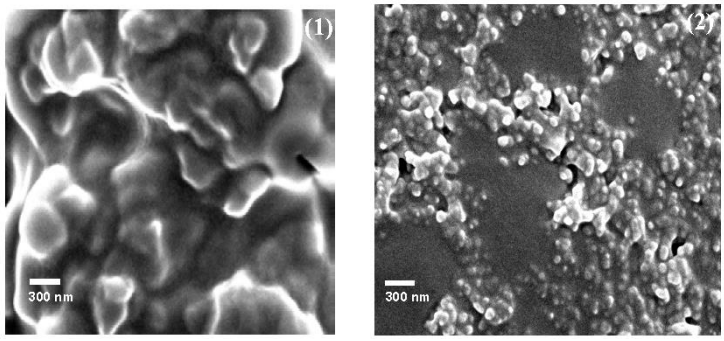


Figure 5.8 SEM images of PDMS/20% CB (Image 1), OV275/30% CB (Image 2).

Sensor response

Figure 5.9 shows a typical raw data set for an analyte (ethanol) exposure to a polymer (OV275/30%CB). In this data four pulses of ethanol lasting approximately 600 seconds were delivered to the sensor. Upon exposure to the analyte the capacitance changed quickly to about 95% of its max response before drifting slowly up. Pure nitrogen was used to purge the system between pulses which caused the sensor to return to its original baseline. Each vapor exposure was repeated at least 3 times in order to determine standard deviation of response magnitudes.

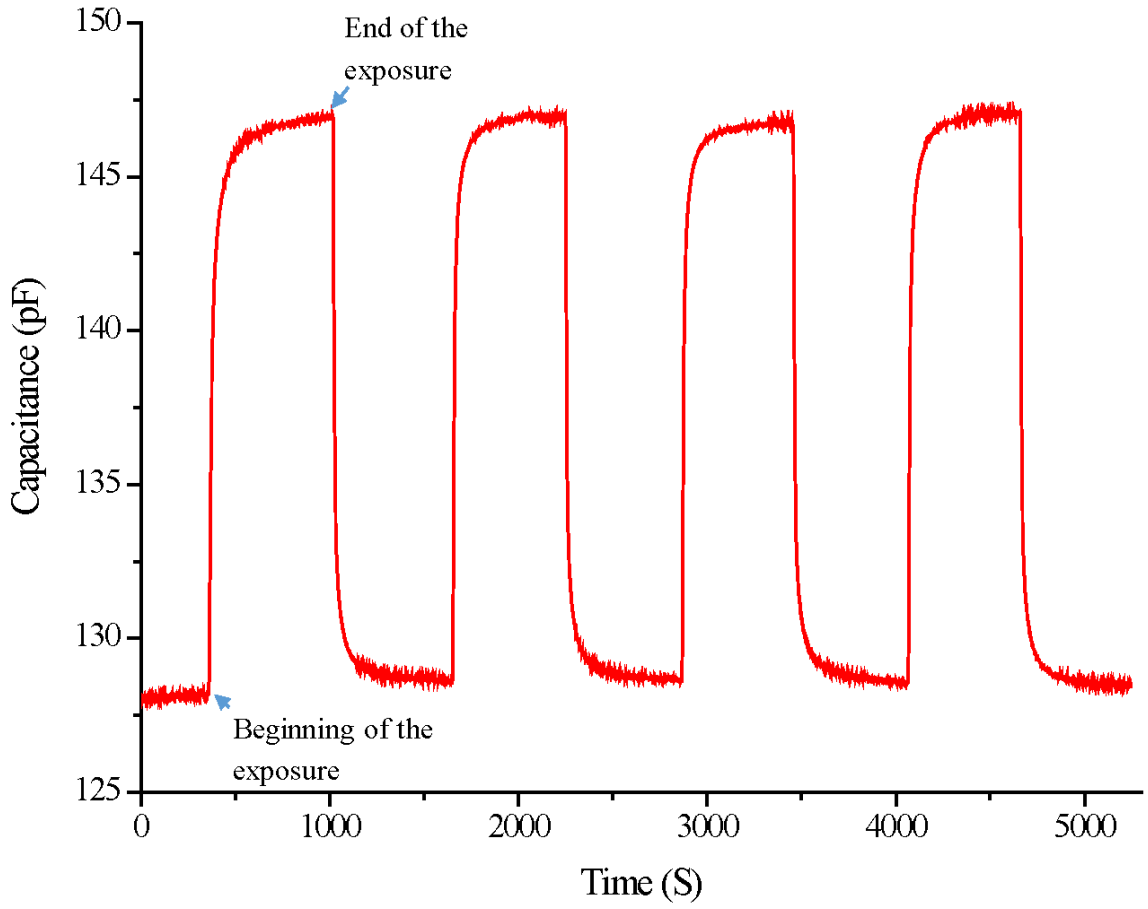


Figure 5.9 Raw sensor response plot of OV275/ 30% CB exposed to ethanol at the same concentration (1500 ppm) for four consecutive exposures.

Responses of different sensors for the same concentration of acetone can be found in Table 5.2. As discussed above, different sensors have different responses depending on the intrinsic permittivity of each coating and the solubility of each analyte in the polymer. The permittivity of the analyte and swelling effects also affect individual sensor response variation. Therefore the overall sensor response depends on a combination of factors and a simple model may not be able to completely describe the sensor mechanism due to the complexity of the combination of phenomena.

Table 5.2 Responses of different sensors for acetone (1033 ppm) at 25ºC and 0% RH.

Sensor	Average Sensor Response	SD±	%RSD
OV275	43.170	0.007	1.66%
OV275 30%CB	138.350	0.008	0.61%
PDMS	3.133	0.0005	1.47%
PDMS 20%CB	6.299	0.0008	1.26%
BMIPF6	523.600	0.1	2.28%
OV275 10% BMIPF6	97.730	0.007	0.71%
PDMS 10% BMIPF6	10.937	0.0009	0.85%

The neat ionic liquid has shown the highest sensor response but the lowest response repeatability as it had a higher relative standard deviation. Both the ionic liquid and CB doped sensors had higher capacitance signals compared to the pure polymer coated sensors. Sensors were exposed to four or five different concentrations inside the vapor delivery system and their concentration ranges, temperature, and humidity conditions are available in Table 5.3.

Table 5.3 Analytes and the concentrations used in this study*.

Analyte	Concentration range (ppm)
Acetone	0 - 4670
Hexane	0 - 5240
Toluene	0 - 3520
Ethanol	0 - 4180
2-butanone	0 - 3910
Ethyl acetate	0 - 6000

Notes: * All exposures were done at 25 ºC at 0% relative humidity.

The performance of the chemicapacitive sensors are characterized through exposure to different concentrations of varied analytes (Table 5.3) using a vapor delivery

system. The magnitude of the sensor response to exposure of the chemicals at 2000 ppm is available in Figure 5.10.

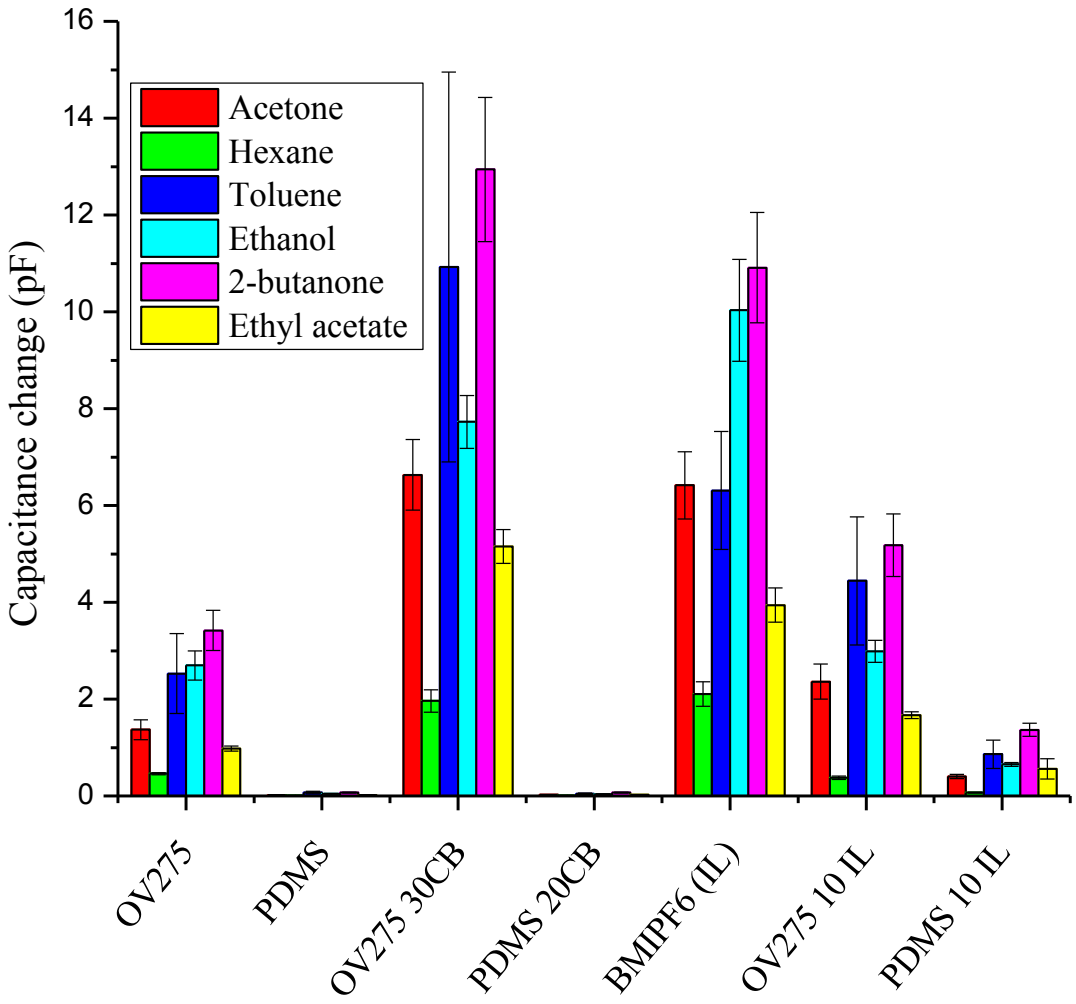


Figure 5.10 Sensor responses of 7 differently coated capacitance sensors upon exposure to 6 different chemicals at 2000 ppm, 25 ºC, 0% RH.

Note: Analyte exposure to coated sensors was repeated 3 times. Error bars – instrument variation.

The highest capacitance changes were found with OV275 / 30 % CB and the ionic liquid coated sensors for the selected compounds. The selectivity of OV275 / 30 % CB sensor for toluene and ethanol was significantly different compared to the ionic liquid

coated sensor. Introduction of CB into the OV275 polymer significantly enhanced the sensor performance at the same time the standard deviation of the sensor response also increased. In each group toluene has shown the highest variation (standard deviation). In contrast hexane displayed the lowest variation (standard deviation).

According to results displayed in Figure 5.10, toluene and 2-butanone have shown the highest sensitivity out of tested analytes with OV275 30% CB. This may be due to the attractive match of polar 2-butanone and slightly polar toluene with the polar OV275. Increased attraction results in increased solubility which leads to polymer swelling. This increases the average distance between carbon nanoparticles further enhancing the sensitivity. OV275 has a larger intrinsic capacitance response due to its polarity and responses are larger upon exposure to analytes compared to the nonpolar PDMS.

PDMS has lower sensitivity towards analytes but this diverse response pattern can aid in distinguishing analytes with pattern recognition programs. Incorporation of IL into the polar polymer OV275 has enhanced the sensitivity to acetone, toluene, 2-butanone, and ethyl acetate while reducing the sensitivity to ethanol. The sensitivity for hexane has not changed considerably. Similarly 10% IL incorporated PDMS has shown a dramatic increase in sensitivity which is illustrated in Figure 5.11.

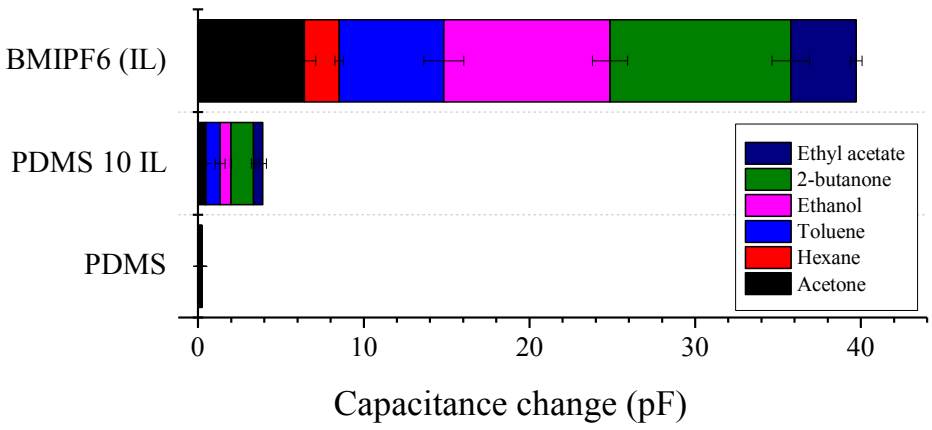


Figure 5.11 Stacked response (3 repeated exposures) comparison of IL, PDMS and PDMS doped with IL sensors to the six analytes.

Note: For all analytes the response magnitude followed the same pattern IL>PDMS 10 IL>PDMS.

The normalized (largest response set to1.0) selectivity of PDMS, PDMS /10 % IL and IL sensors were compared and results are available in Figure 5.12. PDMS /10 % IL sensor is more selective for 2-butanone compared to other sensors. With toluene (large error bars) as the only exception it appears that the selectivity of the PDMS/IL mixture has more similarities to the selectivity of the PDMS (90%) than the IL (10%).

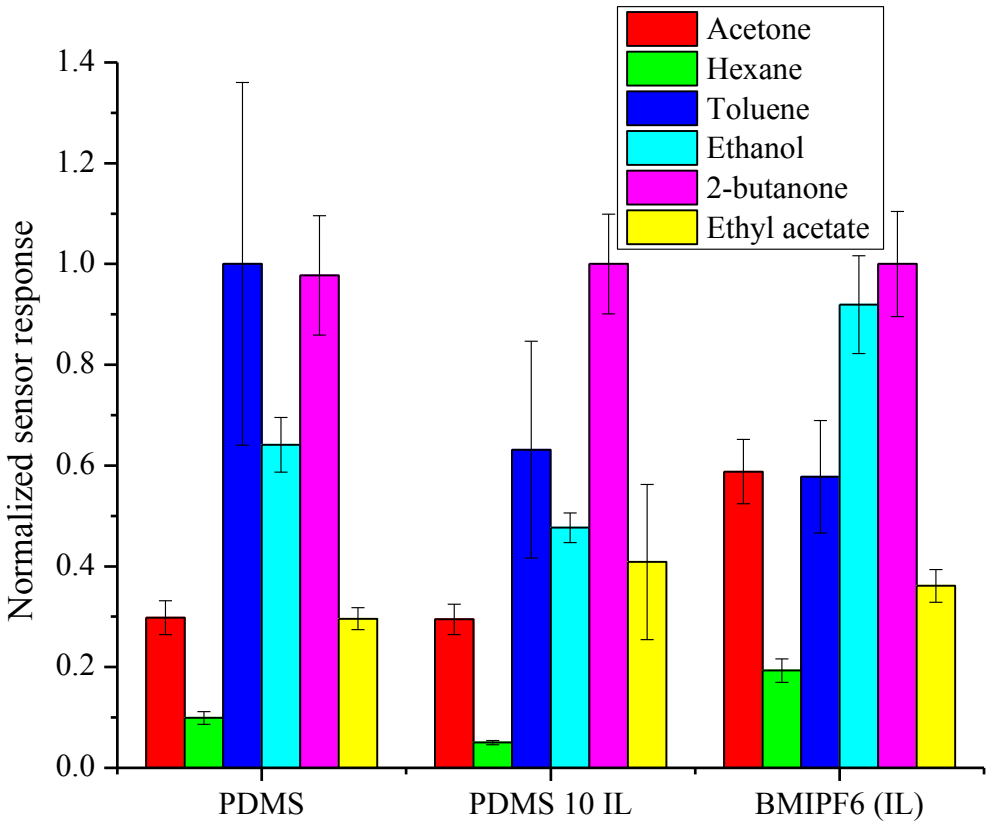


Figure 5.12 Normalized sensor response showing the selectivity of PDMS, PDMS /10 % IL, and IL for selected analytes (3 repeated exposures).

No consistent trend was observed in the normalized selectivity with the introduction of ionic liquid into the OV275 (Figure 5.13). The OV275 10 IL selectivity for acetone and ethyl acetate appear relative to 2-butanone fall in between the selectivity of OV275 and IL. The relative selectivity for the OV275 10 IL coated sensor towards toluene (large error bars) was enhanced while ethanol and hexane selectivity decreased.

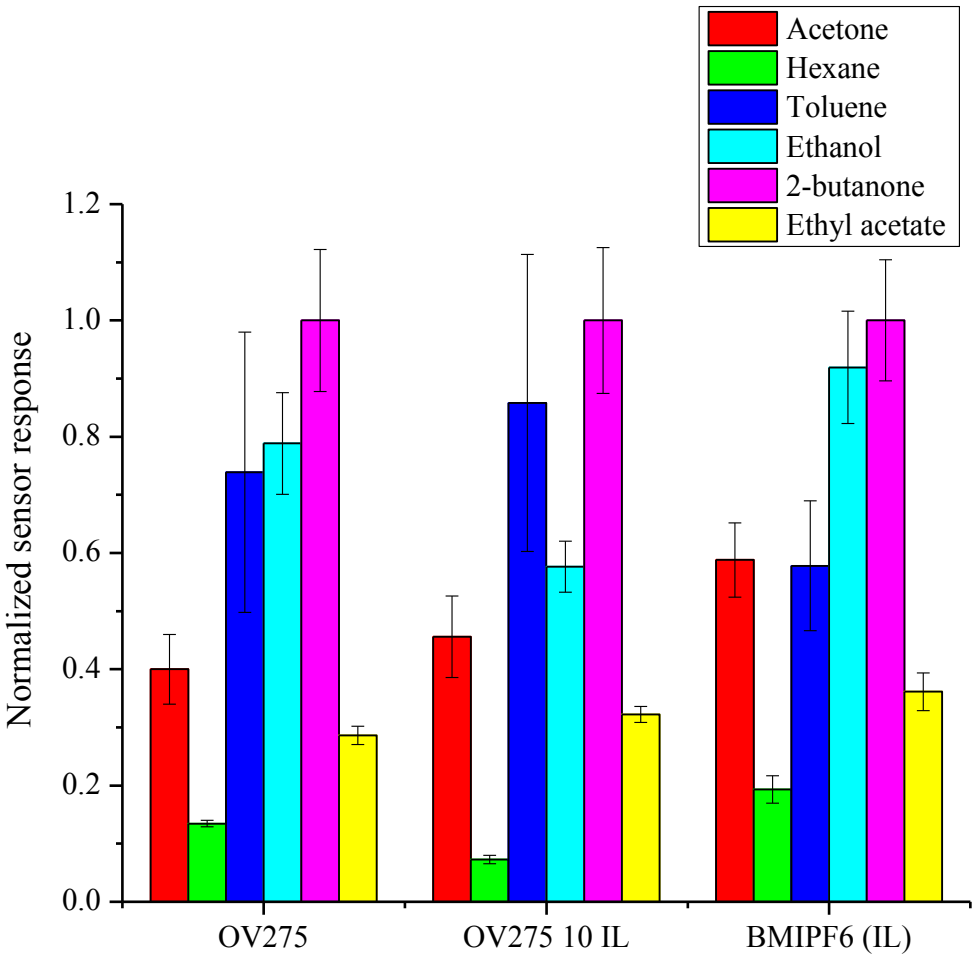


Figure 5.13 Normalized responses showing selectivity of OV275, OV275 /10 % IL, and IL coated sensors for selected analytes.

Limits of detection (3×S/N = (3 × Standard deviation of the baseline/ slope of the calibration curve) (standard deviation of the baseline was determined by calculating the standard deviation of baseline over one minute without analytes (after 1 h of any exposure)), for the analyte coating combinations were calculated using sensor response and baseline noise (Table 5.4). The OV275/ 30 % CB coated microsensor had the lowest LOD (except for ethanol) because of its combination of high sensitivity and low noise.

The ionic liquid coated sensor displayed a larger response to analyte, however, it had poorer limits of detection due to larger baseline noise levels.

Table 5.4 LODs (ppm) of prepared sensors.

Polymer	Acetone	Hexane	Toluene	Ethanol	2-butanone	Ethyl acetate
OV275	10.5	42.1	3.7	4.0	3.4	14.0
OV275 30CB	6.2	22.3	2.1	5.5	2.9	8.9
PDMS	94.2	313.9	13.5	31.4	23.5	94.2
PDMS 20CB	129.4	323.6	64.7	129.4	64.7	129.4
$BMIPF_6$ (IL)	65.0	235.6	40.1	44.9	36.2	110.9
OV275 10 IL	13.4	100.4	5.1	12.6	6.3	20.1
PDMS 10 IL	25.8	171.9	6.4	17.2	6.4	25.8

Hexane had the highest limits of detection while toluene and 2-butanone had the lowest. Ionic liquid incorporated PDMS has a better LOD compare to the pure PDMS for all analytes tested. In contrast, the ionic liquid incorporated OV275 sensor has a slightly poorer LOD compare to the pure OV275. This may be due to the higher conductivity of the ionic liquid resulting in larger noise. Carbon black nanoparticles doped PDMS sensors have not shown any improvement in LOD values compared to the pure PDMS. With ethanol as the exception, all analytes tested have shown an improved LOD with carbon black in OV275 polymer nanocomposites.

Baseline noise levels (standard deviation (instrument variation) of the baseline of 5 measurements) of the conducting particles doped and ionic liquid doped sensors are higher than the pure polymer coated sensors due to their higher conductivity. This phenomenon is most prominent in the pure ionic liquid coated sensors (Figure 5.14).

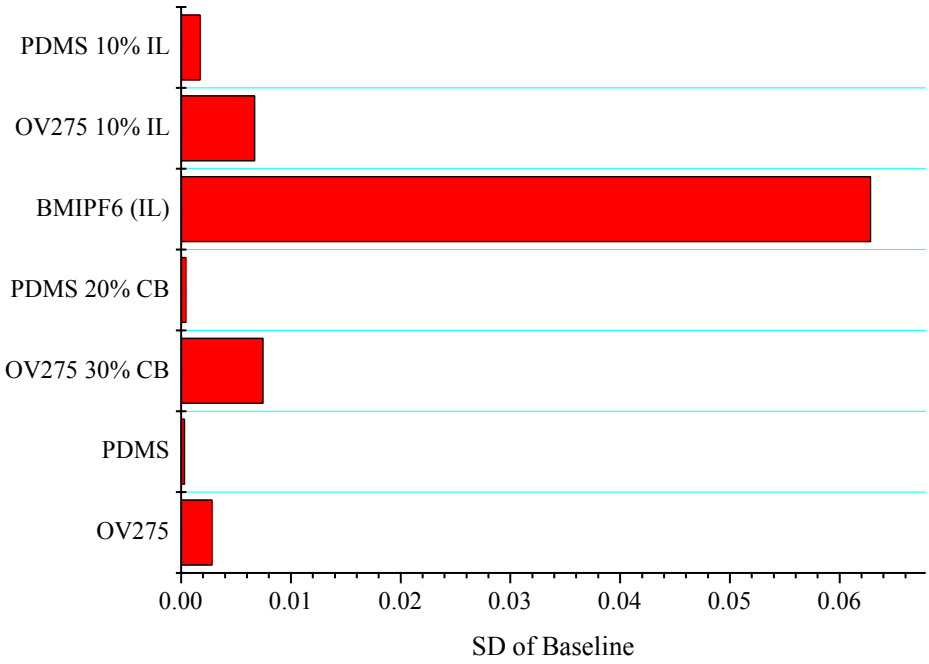


Figure 5.14 Noise (standard deviation of the baseline) comparison of the prepared sensors.

Permittivity of the sensing layer can be drastically changed by polarizable analytes which are rich in electrons, such as toluene, leading to a larger signal compared to analytes with lower polarizability. Figure 5.15 shows that the different sensors have different responses to the analytes providing a fingerprint response pattern that can be used for identification. These multivariate data can be treated with statistical techniques such as discriminant analysis to assess the efficiency of those sensors in terms of selectivity.

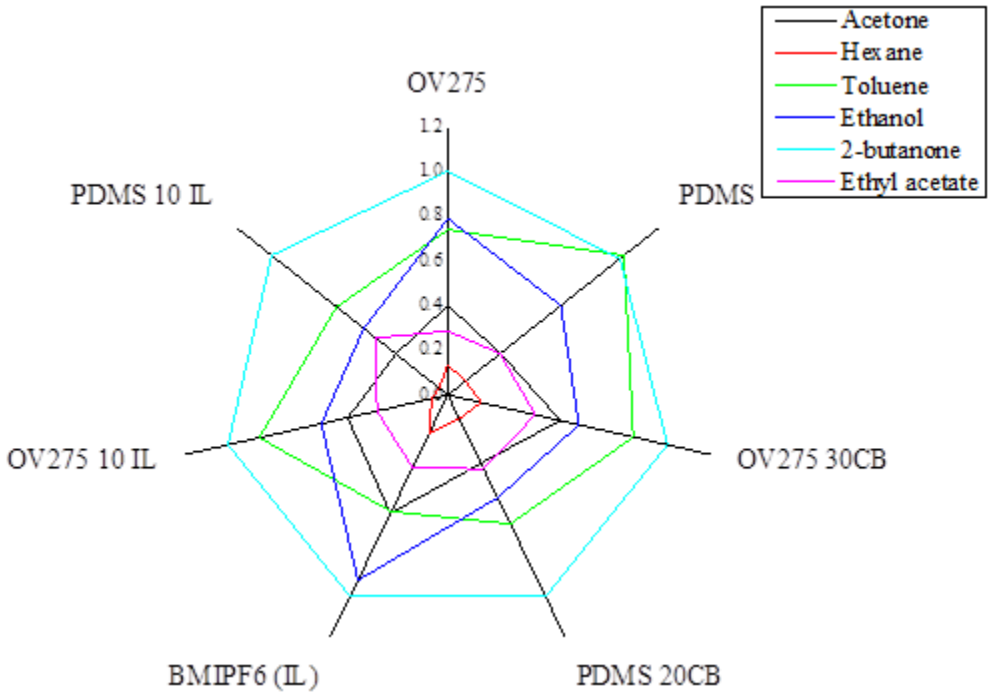


Figure 5.15 Normalized response patterns of tested analytes exposed to microsensors coated with OV275 and PDMS polymers and those polymers doped with carbon black and (BMI)(PF6).

The diverse response patterns can be used to discriminate the volatile chemicals from each other. Canonical discriminant plots are available in Figures 16, 17 and 18. Pure polymer coated sensors have a 10 % error in classification of the 6 analytes tested. Individual concentration clusters are illustrated in Figure 5.16 and the classification accuracy of concentration (tested) for a given compound is 100%.

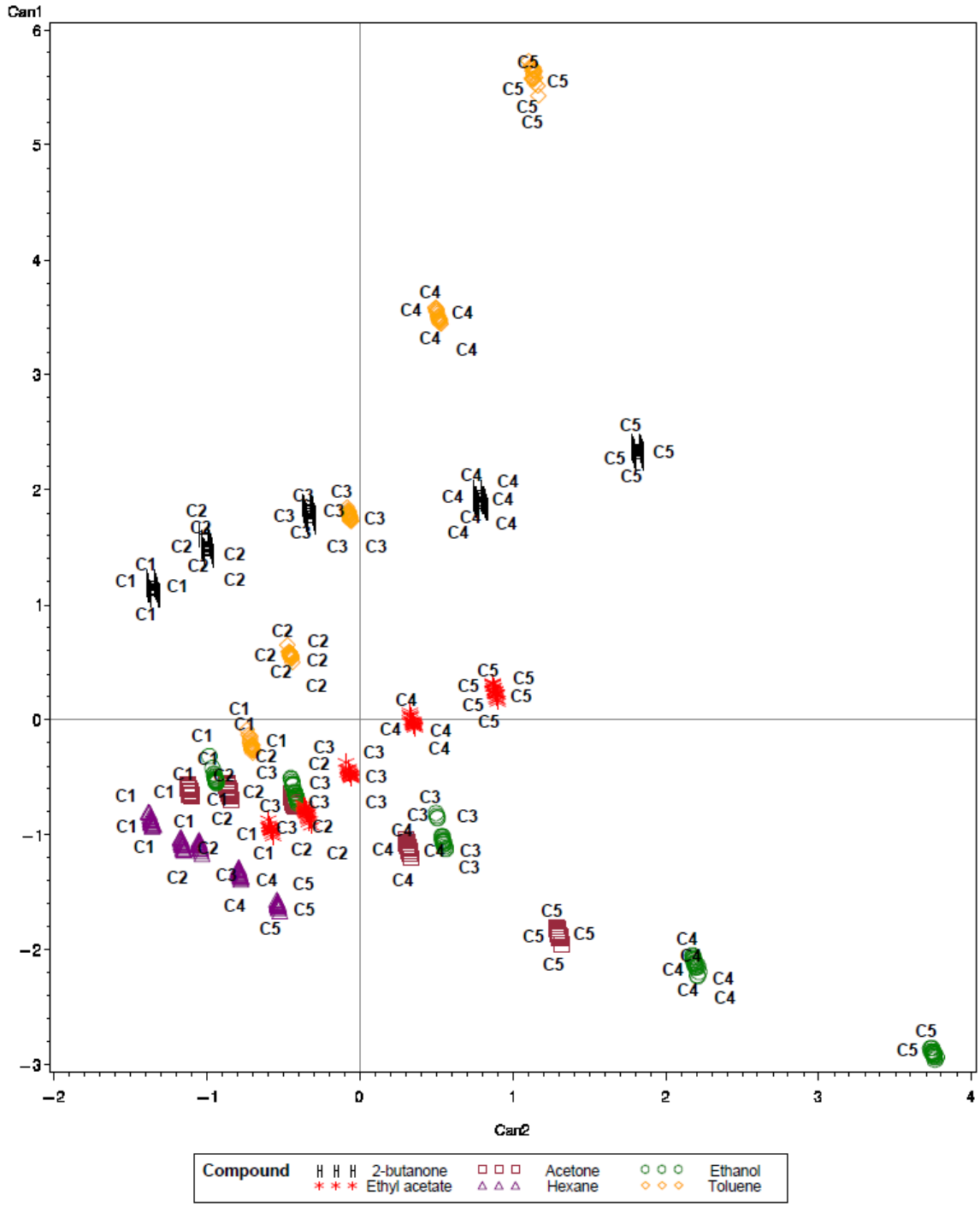


Figure 5.16 Canonical discriminant plot of the pure OV275 and PDMS for selected biomarkers.

According to the cross validation method classification accuracy evaluations, carbon black nanoparticle doped polymer coated sensor category has a 16% error in discrimination of individual compounds (Figure 5.17). Individual concentration clusters are illustrated in Figure 5.17 and the classification accuracy of concentration (tested) for a given compound is 100%.

Higher noise levels can be observed (individual concentration spots are not exactly overlapping each other compared to Figure 5.18) in Figure 5.17 which is related to the carbon doping.

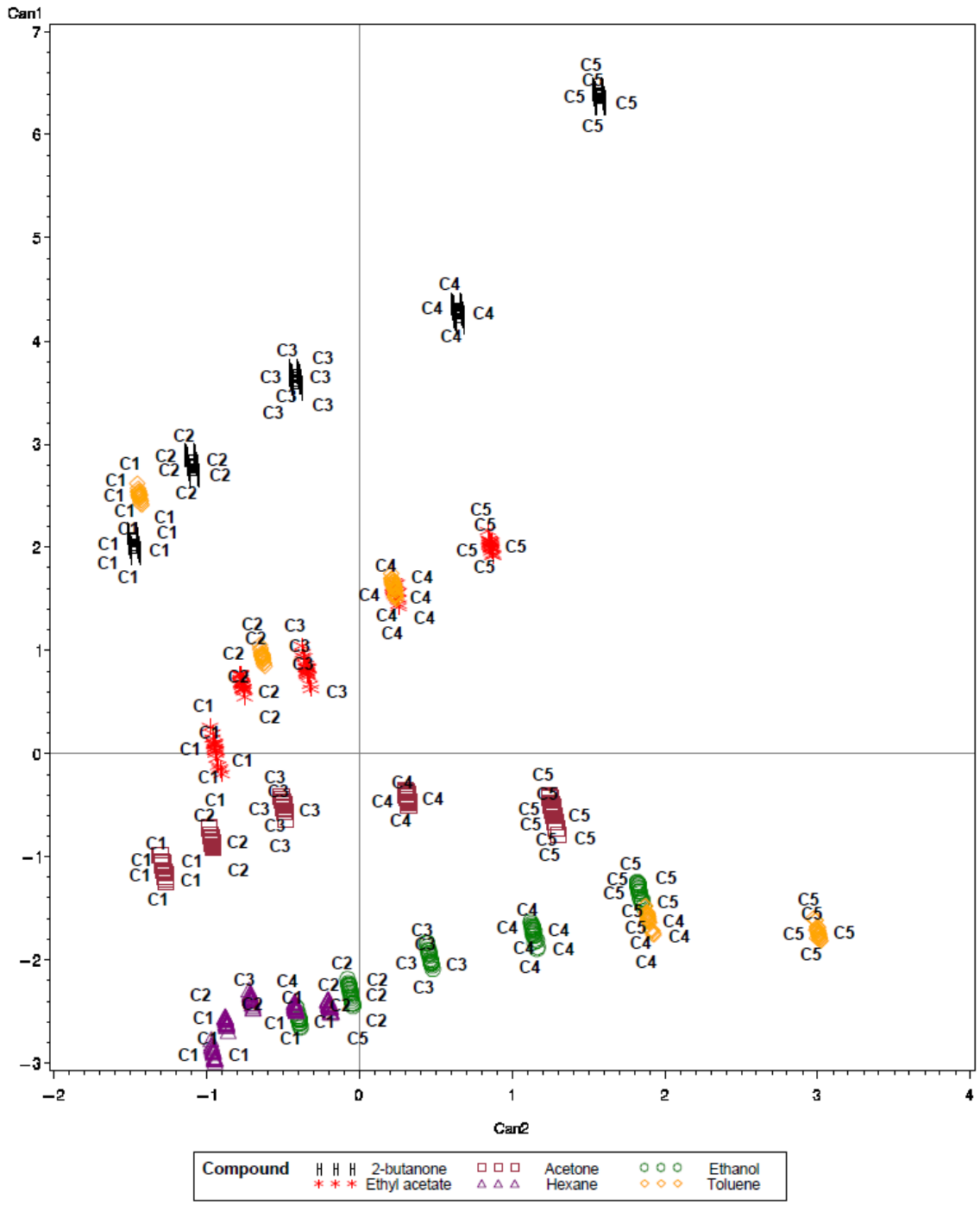


Figure 5.17 Canonical discriminant plot of the CB group (OV275 30%/CB, PDMS 20% CB) for selected biomarkers.

The discrimination power of the ionic liquid doped polymer coated sensors were investigated and is shown in Figure 5.18. Individual compounds can be distinguished with a 97 % classification accuracy according to the cross validation evaluation method. Individual concentration clusters are illustrated in Figure 5.18 and the classification accuracy of concentration (tested) for a given compound is 100%.

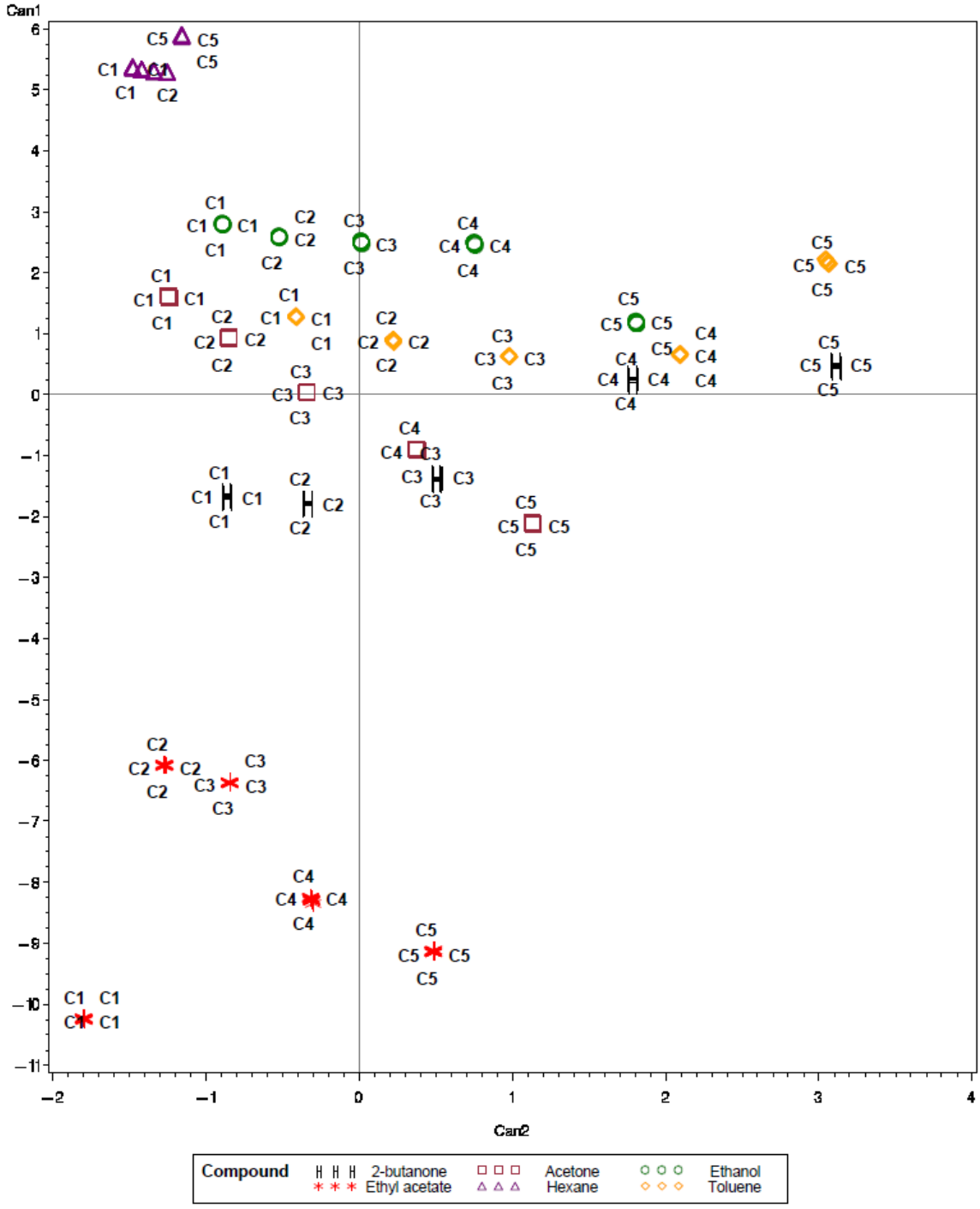


Figure 5.18 Canonical discriminant plot of the OV275/ 10% IL and PDMS /10 % IL for selected biomarkers.

Conclusion

Several sorbent polymer/conducting material composites were prepared without significant changes to functional groups according to DRIFTS analysis. Difficulties arose with application of carbon black/polymer coatings through the etch holes of the capacitor sensors used in this study. Application of the ionic liquid doped polymers was much more consistent and can be used to enhance the sensitivity and the selectivity of parallel plate capacitive sensors. In general the addition of the conducting material increased the sensor response magnitude but also increased the baseline noise of the sensor. The combined effect, however, was an improvement of limits of detection. Improved analyte classification was achieved with the IL doped polymers (97% accuracy) over the pure polymers and classification accuracy of concentration (tested) for a given compound is 100%.

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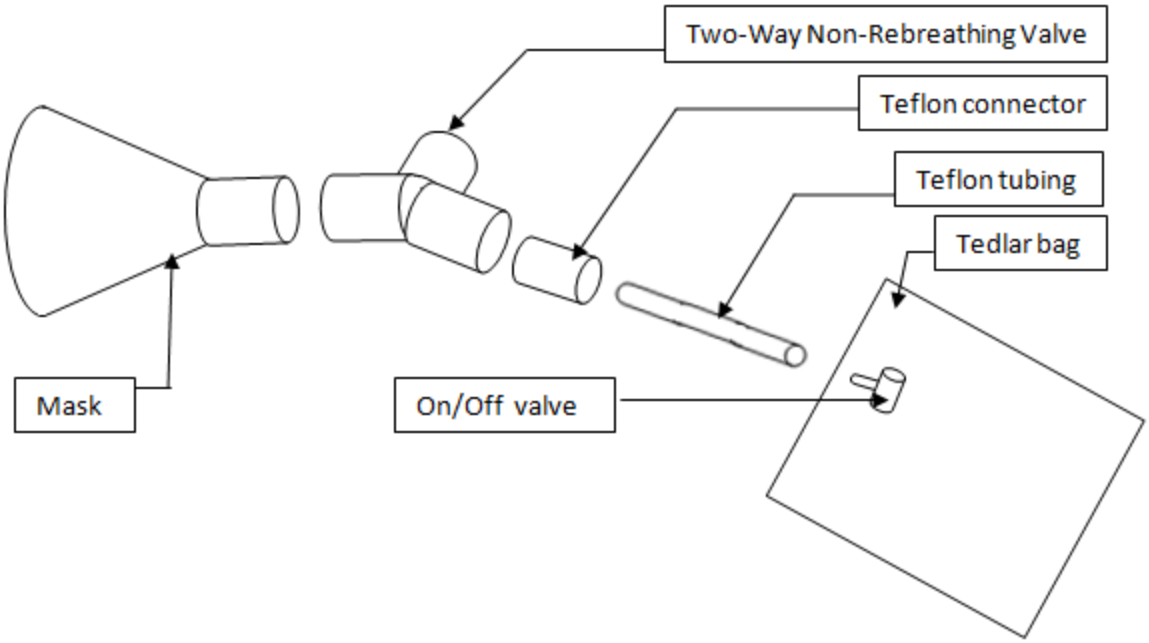
APPENDIX A

BREATH-COLLECTION APPARATUS



APPENDIX B

CANINE MASK WITH DIAPHRAGM

Picture from:
http://www.myneurolab.com/myneurolab/Products/ProductDetail.aspx/Stereotaxic+and+Surgical+Products/Gas+Anesthesia+Equipment/Masks+and+Replacement+Parts/Diaphragm+Large+Canine+Mask/2/12/557/39418548

APPENDIX C

CLIENT CONSENT FORM FOR CLINICAL STUDY

Client Consent Form for Clinical Study

Study:
Breath Sample Analysis in Dogs

Purpose of Study:
The purpose of our study is to determine whether there are any chemicals in the breath of dogs with lung disease that can be used to help us diagnose lung disease in dogs.

Principal Investigators: Drs. Patty Lathan and Andrew Mackin (Mississippi State University College of Veterinary Medicine), Drs. Todd Mlsna and Ed Lewis (College of Arts and Sciences), Drs. Lori Bruce and Oliver Myers (College of Engineering)

Description of Study:
People with lung cancer have chemicals in their breath that can be detected with equipment available to most research chemistry laboratories. Thus, breath sample analysis represents a non-invasive method of diagnosis of lung cancer in people. Breath samples have been used to evaluate lung, liver, and gastrointestinal function in dogs. However, studies have not yet evaluated the use of breath samples for diagnosis of naturally-occurring lung disease in dogs.

The purpose of this study is to identify chemicals present in the breath of normal dogs, dogs with lung disease (including cancer), and dogs with diseases not directly affecting the lung. Breath samples will be collected from patients in the MSU-CVM Animal Health Center, and these samples will be analyzed by collaborators in the Chemistry department. If patterns of chemicals can be identified in the breath samples of patients with lung disease, this information can be used to help design medical diagnostic instrumentation.

Your dog will donate a breath sample for analysis. He/she will breathe into a mask (the same type of mask used for oxygen delivery) for up to one minute, until a collection bag is full. The content of this sample will then be analyzed. The findings will be recorded and used to help us determine whether any specific chemicals present in breath samples can help us diagnose lung disease.

Risks:
We do not anticipate any problems associated with breath collection from your dog. We will not perform the procedure in patients if we feel that it will cause distress; furthermore, if it does appear to cause distress, it will be discontinued. In patients with trouble breathing, respiratory distress can make the breathing more labored.

Voluntary Participation:
Participation in this study is voluntary. You will not be penalized in any way if you elect not to participate.

Confidentiality of Records:
Although information gained from this investigation may be published and used for educational or regulatory purposes, your identity and your animal's identity will remain confidential to the extent provided by law.

Financial Obligation, Withdrawal from Study:

Participation in this study does not require any financial obligation related to breath sample collection or analysis. However, you will still be financially responsible for any other diagnostics or treatment provided for your dog while he/she is hospitalized.

☐ **I agree to the previously listed guidelines, and want to enter my pet into this study. I understand that there will be no extra cost to me, and that all samples and information derived from the study will belong to the study sponsor. I also understand that the investigators may terminate my animal's participation in the study if continuation is not in the best interest of my animal.**

Owner Name: Pet Name:

Owner Signature: Date:

Clinician Signature: Date:

Thank you for agreeing to enter your dog into this study. If you have any questions about this study, please contact Drs. Patty Lathan or Andrew Mackin at 662-325-3432.

APPENDIX D

PROTOCOL REVIEW FORM

IACUC Submission #: _____
IACUC Approval #: _____
Date of Approval: _____

Mississippi State University
Institutional Animal Care and Use Committee

Protocol Review Form

Name of Principal Investigator(s): Drs. Patty Lathan and Andrew Mackin
(At least one PI must be a faculty member at Mississippi State University)
Department MSU-CVM Clinical Sciences Mailstop 9825
Contact Person at Office Gail Bishop Telephone 325-1266
Title of Project: Pilot Study: Breath Sample Analysis in Dogs

NOTE: This form must be completed in detail for protocols involving the use of vertebrate animals in teaching, testing, or research and subject to IACUC jurisdiction.
Animals must not be obtained (unless in resident herd or colony) or used on this project until the IACUC has granted full approval of the work intended.

Place a checkmark by the items below to verify tasks were completed.
Failure to perform any one of the following will result in your protocol being returned to you without review.

☒ Consulted with an IACUC representative other than ULAV concerning protocol form content and composition. Representative: initial & date here after pre-review: _____
☒ Ensured that numbers of animals stated are consistent in Parts II 3. and II 4. B.
☒ OHSP Health Evaluation form AND OHSP Risk Inventory form are current for each person listed on this protocol.

ALSO NOTE: The committee invites investigators to attend the IACUC meeting to discuss the protocol review form. Do you wish to attend the meeting of the IACUC to present this project?
☐ Yes ☒ No. If yes, contact the Office of Regulatory Compliance at 5-0994 to schedule a time to be present at the meeting.

Certification
I (the PI(s)) certify that the information furnished herein is complete and correct to the best of my knowledge. I accept the obligation to observe these animals frequently and report any abnormalities or concerns to the Office of Regulatory Compliance [5-0994]. I also certify that I accept the responsibility for the proper care and use of animals as stated in this protocol.

I certify that the activities described in this protocol do not unnecessarily duplicate previous experiments.

I certify that procedures described in this protocol are congruent with those described in the related proposal(s) for funding.

I certify that persons using animals are qualified to do so, have been trained to reduce risk inherent in this study, and have attended a basic training session given by the ULAV within the last 4 years.

I certify that all personnel have been informed as to the content of this proposal and have agreed to participate as written.

Principal Investigator(s)_____ Date _____

Part I
Project Description

I. 1. Other Personnel:

Name all other investigators and technical personnel responsible for any aspect of animal use in teaching, research, or testing. Please state by each name if those persons are Co-Investigators (CI), Technicians (T), or Students (S) and whether or not (Y/N) they have taken the basic laboratory animal users training class.

Derrick Moore T Y	Joyce Billow T Y	Lisa Crestman T Y	Matthew Raby T Y
John Thomason CI Y	Christine Bryan CI Y	Kari Lunsford CI Y	Todd Archer CI Y

I. 2. Project Information: ☒**Initial Submission** ☐**Revision**_____ ☐**Renewal** _____
(Protocol #) (Protocol #)

Type of protocol you are submitting: ☒Research ☐Teaching ☐Other _____

Note: The IACUC cannot approve a protocol beyond a 3 year period without a new protocol being submitted.

Project Period: from 10/15/10 to 10/15/12 Time Animals Present: from 10/15/10 to 10/15/12

External Supporting Agency _____ SPA Log# _____
MSU Account # (if applicable) _____

I. 3. Project Summary:

In lay terms, understandable by a non-scientist and written from a 4th grade education perspective, give a **general** description of the project, including its objectives. Note: **A technical description is requested in Part II.5.A. below.**

Diagnosis of lung disease in people and animals can be time-consuming and expensive, and often involves invasive diagnostics, including lung biopsy. These factors contribute to the delay of diagnosis. Treatment of lung disease, particularly lung cancer, in the early stages results in significantly improved survival rates. Thus, the development of less invasive, less expensive, and more rapid diagnostic methods will likely improve the prognosis of patients with lung disease.

People with lung cancer have chemicals in their breath that can be detected with equipment available to most research chemistry laboratories. Thus, breath sample analysis represents a non-invasive method of diagnosis of lung cancer in people. Breath samples have been used to evaluate lung, liver, and gastrointestinal function in dogs. However, studies have not yet evaluated the use of breath samples for diagnosis of naturally-occurring lung disease in dogs.

The overall purpose of this study is to identify chemicals present in the breath of normal dogs, dogs with lung disease (including cancer), and dogs with diseases not directly affecting the lung. Breath samples will be collected from patients in the Mississippi State University Animal Health Center, and these samples will be analyzed by collaborators in the MSU Chemistry department. If patterns of chemicals can be identified in the breath samples of patients with lung disease, this information can be used to help design medical diagnostic instrumentation for use in both dogs and people.

In this pilot study, the technique of breath sample collection and handling will be optimized. Optimal sample preparation and analysis techniques will also be determined. Identification of chemicals in patients with lung disease will then allow for design of a study with sufficient numbers to establish the usefulness of breath analysis for detecting different kinds of lung disease in dogs.

PART II

ANIMAL CARE & USE

II. 1. **Hazards:**

Are *in vivo* aspects of this project under the auspices of the committees below? ☐ Yes ☒ No
(If so, please mark which committee(s).)

☐ Institutional Biosafety Committee
☐ Chemical, Radiological, and Lab Safety Committee
☐ Hazardous Waste Committee
☐ Institutional Review Board for the Protection of Human Subjects in Research

If you have answered yes to this question, you must provide an approval statement from the appropriate committee. IACUC will not approve this protocol until said approval statement is received.

II. 2. Proposed Animals:

A. Species Dogs
B. Source

☐ Purchased from USDA licensed dealer. If dogs or cats; ☐ dealer-raised or ☐ random source.
☐ Purchased from a non-licensed individual or entity.
☐ Donated from a non-licensed private or public source.
☐ Obtained by collection in the wild.
☐ Obtained from MSU breeding populations.
☐ Transferred from another MSU project. Give protocol number of that project. _____
☐ MSU-owned animals from non-MSU site. From where? _____
☐ Non-MSU owned animals from MSU site. From where? _____
☒ Other Client-owned

C. Breed/Strain Variable
D. Health status required Healthy dogs and dogs with pulmonary and non-pulmonary disease
E. Age/size/weight Male and Female; intact and neutered
F. Sex Variable
G. Needed by (date) 10/15/10
H. To be housed in which building or area? MSU-AHC Internal Medicine dog cages, runs, or ICU
Are the animals housed according to space requirements? ☒ Yes ☐ No. If no, request exemption of the ILAR Guide or the Ag Guide.
Is this area visited routinely by the IACUC? ☒ Yes ☐ No ☐ Don't Know

I. Special requirements for maintaining the animals:

a. Temperature range (°C) 18-26 humidity (%) 30-70 light cycle: 12:12
b. Caging/pen type: cage size: standard filter tops? N/A changes/week:N/A
c. Bedding/litter type: noneautoclaved? _____ bedding changes/week: _____
d. Type of water (e.g., sterile, deionized, acidified, tap): tap
e. Diet and feeding requirements: Ad libitum
If other than *ad libitum* feed and water, state quantities: _____
f. Other special equipment and/or instructions for animal care and veterinary staff: None

J. What specialized laboratories will be needed to conduct this study?

☒ None
☐ Surgical Suite Where? _____
☐ Necropsy Room Where? _____
☐ Other Where?_____

K. Will animals admitted to the Animal Health Center, CVM, be used in this project? ☒ Yes ☐ No. If yes, you must attach a Client Consent Form as evidence that permission to use the animals will be obtained.

L. Is this a field study (animals living in the wild)? ☐ Yes ☒ No
If animals in the wild will be used, describe how they will be obtained?_____
Are Federal or State permits required to use these animals? ☐ Yes ☒ No. If yes, have such permits been obtained? ☐ Yes ☐ No. If permit(s) have been obtained, please attach a copy to the protocol. If permit(s) have not been obtained, by what date will you have the permit(s). _____

II. 3. **Numbers of Animals:**

Maximum **number** of animals (by species) to be used in this study per level of pain and/or distress. You **must not exceed** this number unless you amend the protocol to add additional animals.

A._____ Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery **but not yet used for such purposes**. **(Note: Holding animals without a specific, intended use is unacceptable for longer than a few months.)**

B1.30 Animals to be used in teaching, research, (includes euthanasia) or tests **involving no or short-term, single occurrence, minor distress or pain.**

B2._____ Animals to be used in teaching, research, or tests **involving repeated but minor instances of distress or pain.**

C1._____ Animals to be used in teaching, research, or testing **that may cause distress or pain to animals for which anesthetic, analgesic, or tranquilizing drugs are used** (includes drugs used for restraint and terminal or survival surgery) and for which the minimal distress or pain following the procedure is alleviated by drugs and/or other means.

C2._____ Animals to be used in studies that may cause distress or pain for which anesthetic, analgesic, or tranquilizing drugs are used **but in which there is an associated chronic period of distress or pain following recovery from the procedure that will not be alleviated by drugs.**

D._____ Animals to be used in teaching, experiments, research, surgery or tests that may cause distress or pain to animals and for which **the use of appropriate anesthetic, analgesic, or tranquilizing drugs would adversely affect the procedures, results, or interpretation of the teaching or research. This use of animals in this category must be scientifically justified in the space below.** _____

II. 4. **Experimental Design:**

A. Give a rationale for involving animals in this study and the justification for using the species selected.
The purpose of this study is to evaluate the contents of breath samples in dogs. Although there is some data concerning breath content in other species, it is unknown whether the content will be the same in dogs.

B. Give a complete description of the experimental design emphasizing the number of treatment groups and the number of animals per treatment group. Note: The total must match those stated in Part II. 3. above. (**Describe technical procedures in 5 A. below**.)

Dogs seen by the MSU-CVM Animal Health Center will be recruited for this pilot study. All owners will sign a Client Consent Form. 10 healthy dogs will be recruited, as will 10 dogs with respiratory disease and 10 dogs with non-respiratory illness. Breath samples will be collected as detailed in II.5.A., and collection, storage, and transport technique will be optimized.

The breath samples will be stored in specialized (Tedlar) bags, and analyzed by collaborators in the MSU Chemistry department. Briefly, samples will be pre-concentrated through Exhale Breath Condensate Solid Phase Micro Extraction methods and analyzed with Gas Chromatography Mass Spectrometry (GC MS). GC MS analysis results will be used to determine the pattern of volatile biomarkers in breath samples for lung diseases. Optimal sample preconcentration, optimal sample extraction, optimal sample introduction and optimal temperature programs will be determined for the analysis of volatile biomarkers. The identity and the quantity of each volatile compound will be determined with the use of standard compounds. Symptoms and diagnosis will be recorded for corresponding animals and relationship between the data and results of breath sample analysis will be determined.

Identification of potential biomarkers in patients with lung disease will then allow for design of a study with sufficient numbers to establish sensitivity and specificity of the biomarker.

C. Pertaining to the number of animals to be used, select and complete the following statement that applies **(Note: Using others' names without their signature is unacceptable.)**

☒ The numbers of animals to be used were determined by the principal investigator(s) and are based on the following technical or statistical criteria below.

☐ The numbers of animals to be used were determined by the principal investigator(s) after consultation with the following biostatistician: (method of number determination is provided below)

Name: _____

Signature (if local): _____ Date:_____
(if statistician was used a signature or email from them must be provided to the IACUC office)

☐ Other process and/or persons determining numbers (*specify*):

Explanation: Please provide method of number determination:

The number of animals in each group in this pilot study was selected based on the number of humans in each group in a recent very comparable study evaluating volatile biomarkers in people with lung cancer (Deng C, Zhang X, Li N, J of Chromatography B, 808(2004), 269-277). The study resulted in the optimization of collection and testing methods, and identification of two biomarkers for lung cancer in humans.

II. 5. **Care and Use of the Animals:**

A. **Technical Procedures:**

Describe in detail what will be done to the animals.

Please see Appendix A for a diagram of the breath collection apparatus.

In summary, the dog will be gently restrained in a standing, sitting or sternally or laterally recumbent position, and allowed to breathe normally into a mask that is attached to the breath collection apparatus.

1. An appropriately-sized mask will be chosen so that the dog's nose and mouth can fit comfortably inside it while the diaphragm prevents loss of breath sample. *Please see Appendix B.*
2. The mask will be placed comfortably over the dog's nose and mouth. The dog will then be allowed to breathe normally.
2. Three full breaths will be allowed for acclimatization and to allow the dead space in the collection apparatus to fill prior to opening the valve to the collection bag ("Tedlar bag").
3. The valve to the collection bag will be opened, and the mask will be left in place for as long as it takes to fill a 1-L bag with the dog's breath, or one minute, whichever is shorter.
4. If the procedure appears to be causing undue stress to the patient, as determined by veterinary personnel named in I.1., it will be aborted.
5. Dogs will not be recruited for the study if this procedure is anticipated to cause undue stress or exacerbation of clinical signs.
6. Client consent forms will be signed by owners of all dogs used in this study *(see Appendix C)*.

B. The following questions are intended to help the committee to assess pain, stress, or distress to the animals:

Pain category from Part II, 3 above B1.

1. Restraint - Does proposed use involve restraint of animals? ☒ Yes ☐ No

a. If yes, explain type and duration of restraint (including manual restraint).

The patient will be restrained in standing, sitting or sternally or laterally recumbent position, according to patient preference. The patient's nose will be placed in the mask, and the mask will be held on while holding the back of the patient's head to stabilize the mask.

b. Describe any preconditioning to the restraint procedure before the actual study begins.

Three full breaths will be allowed for acclimatization prior to opening the valve to the collection bag.

c. Describe how and how often animals will be monitored for negative effects from the device or apparatus during and immediately after prolonged or stressful restraint.

Normal patients will be monitored for 3-5 minutes after the procedure to ensure that there are no signs of respiratory difficulty. Patients with illness will be monitored as long as deemed necessary by veterinary personnel. Monitoring parameters will include respiratory rate and effort.

and mucous membrane color; pulse oximetry will be used if necessary. This procedure should not result in negative effects in the dogs.

2. **Distress without pain** - Will the animal(s) endure prolonged, noncompensated distress without pain in this study (e.g., close confinement, proximity to predators, exposure to constant light)?
☐ Yes ☒ No
If yes, describe the nature of the non-painful distress.

3. **Minor Invasive Manipulations** - Will minor invasive manipulations be performed? (e.g., blood collection, catheterization, intubation, skin biopsy) ☐ Yes ☒ No

   a. **Minor pain and/or distress alleviation** - Will anesthetics, analgesics or tranquilizers be used to reduce minor pain or distress in these animals? ☐ Yes ☒ No. If yes, please provide the information below:

Drug	Route	Dose (mg/kg)	Schedule (times/day etc.)

   b. **Adjuvant Use** - Does proposed project involve use of adjuvants? ☐ Yes ☒ No. If yes, list type of adjuvant(s), volume, site, and frequency of injections.

4. **Substance Administration**

   a. Indicate if drugs or compounds given to the animals in this protocol are cleared/approved for use in this species (i.e., specifically indicated on the label or package insert for use in the species involved). If any drug or compound is not cleared/approved for use in the species listed in this protocol, provide justification for use of the drug or compound. Describe the known or anticipated consequences (e.g., discomfort, distress, pain) for the animals following administration of the substance(s).
   N/A

   b. Will proprietary compounds be used? ☐ Yes ☒ No. If yes, provide written assurance that these compounds, at levels used, are not toxic or otherwise harmful to animals/humans involved in this research and will not interact adversely with other compounds administered. If effects on animal well being are anticipated, describe those effects.

5. Nutrient Restriction

a. Does the study involve food or water restriction of any type? ☐ Yes ☒ No. If yes, describe in detail and justify the restriction.

b. Are diets formulated and fed to meet or exceed all NRC or equivalent recommendations? ☒ Yes ☐ No. If no, describe in detail and justify.

6. Surgery - Does proposed use involve surgical procedures? ☐ Yes ☒ No. If yes, answer the following:

a. Will the procedure penetrate a body cavity, involve the head, perineum, or joints, or cause permanent disability? ☐ Yes ☐ No.

b. Describe the qualifications of personnel performing surgery. (Attach documentation describing training and experience in surgery on the species in this study.)

c. Before surgery, will the animals be kept off: food? ☐ Yes ☐ No. How long? _____
water? ☐ Yes ☐ No. How long? _____

d. Will multiple, invasive (major), survival surgical procedures be conducted on the same animal? ☐ Yes ☐ No. If yes, describe and justify the procedures and how the procedures are related to the goals of the study.

e. If anesthetics, analgesics, or tranquilizers will be used in these animals to alleviate significant pain or distress, please provide the information below.

Drug	Route	Dose (mg/kg)	Schedule (times/day etc.)

f. If anesthetics, analgesics, or tranquilizers are not used, explain why and give proposed alternative methods to alleviate or control pain or distress.

g. Describe presurgical planning and preparation:

h. Describe surgical procedure planned:

i. Site of surgery: Room _____ Building _____
Where will anesthetic and surgery records be filed? _____

j. Are neuromuscular blocking agents to be used? ☐ Yes ☐ No.

1. If yes, provide justification.
_____

2. If neuromuscular blocking agents are used in conjunction with anesthesia, what physiological parameters are monitored during the procedure to assess adequacy of anesthesia?
_____

k. Recovery from anesthesia and postoperative care: (Survival procedures only)

1. Post-surgical recovery site: Room _____ Building _____
2. Describe post surgical and/or post anesthesia care procedures: e.g., duration and frequency of monitoring, parameters monitored.)_____
3. Who will be responsible for post-surgical care? _____

7. Prior Use of Animals:

Describe any distressful or painful procedures known to you that have been conducted by anyone on these animals before their use in this project.

No distressful or painful procedures other than those associated with treatment and diagnostics in the MSU-CVM AHC will be performed. These diagnostics will be performed based on clinician recommendation and with owner permission, and will be performed independently of this study.

We do not expect for any of these patients to have had painful or distressful procedures performed on them prior to inclusion in this study. However, since these are client-owned dogs with variable clinical histories, it is impossible for us to rule this out completely.

8. Unintended Consequences:
Even in well-established procedures, animals sometimes experience unanticipated or unintended pain or distress. Describe such possible outcomes in this study, how you would monitor for those possible outcomes, and how you would intervene in a timely fashion.

Collection of breath from dogs for procedures such as breath hydrogen analysis has been performed for many decades in veterinary practice using the equipment and techniques that will be used in this study, and the procedure has been established to be benign and very well tolerated. In patients with difficulty breathing, however, stress from restraint could result in exacerbation of that breathing difficulty. Clinicians will therefore not enroll patients in this study if this result is anticipated. Patients will be visually monitored by veterinary personnel (technician, veterinarian, or veterinary student) following the procedure. If dyspnea (difficulty breathing) occurs in a patient in which it is not expected, oxygen administration and/or sedation may be administered. Potential sedatives include, but are not limited to, acepromazine, 0.01 - 0.03 mg/kg intravenously, and diazepam, 0.1 - 0.3 mg/kg intravenously. These will be used only in consultation with the dog's primary clinician.

9. Euthanasia and Disposal

a. If any animals are to be euthanatized as part of or at the end of this protocol, describe the method to be used (Consult the most current Report of the AVMA Panel on Euthanasia). If a conditionally acceptable method is proposed for use, please request an exemption for its use and reasons why. State the names of drugs to be used, including dosage and route of application. Describe how the person performing euthanasia will be trained to conduct the intended procedure(s).

Euthanasia will not be performed as part of or at the end of this protocol. Clinical patients will only be euthanized at the request of their owners due to suffering caused by or prognosis associated with their clinical condition. AHC clnicians (licensed veterinarians) will be responsible for this procedure.

b. Provide specific criteria used to determine a humane endpoint before reaching physical impairment or morbidity.
N/A

c. What will be done with animals remaining alive following the completion of this project?

They will be returned to their owners.

10. Medical Care of Animals: Name who will provide veterinary care for animals, if needed, under the oversight of a university laboratory animal veterinarian.

All patients will be under the care of a veterinarian employed by the Animal Health Center. In the event of a complication due to this study, Drs. Lathan and/or Mackin will oversee veterinary care.

11. Reduction, Refinement, Replacement, and Literature Searches:

If this project involves,
Live, vertebrate animals, including farm animals, used in biomedical research, teaching or testing **OR Any mammal usually found in the wild, you must provide the following information.**
(Note: Animals used in agricultural production research, teaching, or testing are not required to answer this question.)

Provide a written narrative of your database search results that includes:

1. Names of databases searched: *EBSCODiscovery Service, Academic Search Premier, CAB Abstracts, MEDLINE*
2. Date(s) search was performed: *9/23, 9/24, 9/27, 2010*
3. Period (dates) covered by search: *For entire period covered by databases*
4. Key words and/or search strategy used: *breath, dog, dogs, canine, canines*
5. Provide a written narrative that include the following:
   a. Any alternatives identified in the search
   b. Justify why you selected the method(s) described in this protocol and why alternatives were unacceptable.
   c. Methods that use non-animal systems or less sentient animal species that may partially or fully (i) REPLACE animals (e.g., use of an in vitro or insect model to

replace a vertebrate animal model), methods that (ii) REDUCE the number of animals to the minimum required to obtain scientifically valid data, and methods that (iii) REFINE animal use by lessening or eliminating pain or distress, and thereby enhance well being. If these descriptions are stated elsewhere in this protocol, identify the page and section involved.

Consult the training manual "Animal Users' Policies and Procedures" for further details on alternatives and the "3 R's" – replacement, reduction, and refinement.

Replacement: *Since our goal is to identify the presence of volatile compounds in breath samples of dogs, and vertebrate and invertebrate metabolism differ significantly, no viable alternatives to using dogs was found.*

Reduction: *Reduction in sample number is not possible, as it will not permit the evaluation of enough healthy animals for determination of specificity of a novel biomarker.*

Refinement: *The method for breath sample collection we will be using is non-invasive and unlikely to result in pain or distress. A less invasive method of breath sample collection is unknown.*

c. Veterinary Review

This protocol must be reviewed by a university laboratory animal veterinarian prior to submission. Review can be documented by obtaining signature below or attaching relevant email/correspondence.

Signature _____ Date:_____.
University Laboratory Animal Veterinarian or Designee

Comments by veterinarian (if not in email): _____

PART III
PERSONNEL TRAINING

1. **Personnel Qualifications**

Federal regulations require that the personnel listed (Part I, 1.) are qualified to perform their tasks in the proposed project. Therefore use the following format to state the qualifications of each individual involved in the animal use aspect of the project. You must insure that the study is conducted by trained or supervised persons with due concern for personnel health and safety as described in this proposal. **Make certain also that all named in this protocol form have attended one of the training sessions on the essentials of animal care and use before their role in the project begins.**

Name:

Patty Lathan
Andrew Mackin
Lisa Chrestman
Leslie Reed
Derrick Moore
Joyce Billow
Matthew Raby
Kari Lunsford
Todd Archer
Christine Bryan
John Thomason

Procedure(s) this person will perform: Breath sample collection
Species on which this person will perform stated procedure(s): Dogs
How was this individual trained (i.e., hands-on assistance, direct supervision, coursework)? Direct Supervision
Who trained this individual? Veterinarians and technicians at a veterinary teaching hospital
How much experience has this individual had performing the procedure(s) (e.g., "x" years, "y" animals)?

Note: Roughly the same procedure will be used for breath sample collection as is usually used for oxygen administration by mask; the number of procedures listed below is the estimated number of times the person has administered oxygen by mask.

Patty Lathan - 14 years with hundreds of procedures
Andrew Mackin - over 20 years with hundreds of procedures
Lisa Chrestman - over 20 years with hundreds of procedures
Joyce Billow - 15 years with hundreds of procedures
Leslie Reed - 4 years with hundreds of procedures
Derrick Moore - 1 year with >50 procedures
Matthew Raby - 2 years with at least 100 procedures
Christine Bryan - 6 years with hundreds of procedures
John Thomason - 6 years with hundreds of procedures
Todd Archer - 6 years with hundreds of procedures
Kari Lunsford - 12 years with hundreds of procedures

2. **Occupational Health & Safety Program (OHSP)**

The Regulatory Compliance Office and The John C. Longest Student Health Center have developed an Occupational Health & Safety Program intended to serve the needs of those at risk from animal studies conducted at Mississippi State University.

Enrollment

All persons listed on this protocol are required to enroll in the program at some level. Enrolling means that contact information is provided and the person may either

1. choose to submit medical information for assessment, or
2. choose to decline further participation

http://www.orc.msstate.edu/animals/ohsp.php

Steps

1. If you have submitted the OHSP forms electronically, you do not need to resubmit, unless
   a. your MSU work assignment changes.
   b. your personal health status changes.
   c. your environmental risk conditions change.
   d. the species you work with changes.
2. If you have not filed the OHSP forms electronically, you should complete this process prior to working with animals involved in research. Failure to do so could result in suspension of your ability to work on an animal protocol or in an animal facility.

If you have questions about the process, please contact the Office of Regulatory Compliance at 325-3294.
The Student Health Center will review the health evaluations in conjunction with the risk assessment and make recommendations to the individual. At no time will the medical information be shared with the Regulatory Compliance Office or the committees. This medical information is private and will be maintained confidentially by the Student Health Center.

IACUC USE ONLY

With due regard for the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, other regulations, and relevant University policy, the Institutional Animal Care and Use Committee of Mississippi State University has reviewed and approved the care and use of vertebrate animals in this project.

_____ Date _____
Chairman, MSU Institutional Animal Care and Use Committee

_____ Date _____
University Laboratory Animal Veterinarian

APPENDIX E

TEXT FOR VOLUNTEER RECRUITING

IRB Requirement: Text for Volunteer Recruiting

Human Scent project

Dear All,

We are conducting a study to determine if commercial products can reduce or eliminate human odors and we are looking for volunteers. The four hour test will take place in Hand Lab. Upon arriving at Hand, volunteers will be given a clean t-shirt and a disposable rain coat to wear for up to two hours. During the last few minutes of the test an air sample will be collected under the raincoat and above the t-shirt. The test will then be repeated with a second t-shirt. One of the t-shirts will have been treated with one of four scent control products and allowed to dry before being given to the volunteers.

During the four hour test volunteers will be asked to wait in the small seminar room (see picture below). Volunteers are encouraged to bring a laptop - water and snacks will be provided. All volunteers will be given gift basket.

If interested please contact Dr. Todd Mlsna (tmlsna@chemistry.msstate.edu) or **Shamitha Dissanayake (**sad157@msstate.edu).

APPENDIX F

INFORMED CONSENT FORM

Mississippi State University
Informed Consent Form for Participation in Research

Title of Research Study: Determination of the Effectiveness of (Deleted)

Study Site: Hand Lab

Researchers: Todd Mlsna, Bronson Strickland and Steve Demarais

Purpose
Human odor samples will be collected from volunteers to validate human odor controlling products. Volunteers will be deemed acceptable for this study if they have no known allergies to ingredients used in 4 commercial scent control products and are willing to participate in the study that can take up to 4 hours. These products have a combined ingredient list that includes: Silver XP particles, water, fermented vegetable enzyme, preservative.

Procedures
A maximum total of 200 male and female volunteers ranging from 18 – 65 years will be participating in the study.

Volunteers will be asked to arrive at Hand laboratory prepared to commit 4 hours of time to participate in this study. Upon arrival volunteers will be given a t-shirt and a raincoat (outer layer) to wear over their clothing. After 1:45 minutes a plastic tube will be inserted under the raincoat and above the t-shirt for 15 minutes of sample collection. Then the test will be repeated with another t-shirt. In this study both t-shirts will be freshly laundered and one of the t-shirts will be treated with one of four commercial products containing some combination of the following listed ingredients: Silver XP particles, water, fermented vegetable enzyme, and preservative.

Treated t-shirts will be prepared by following instructions on the commercial product labels before arrival of the volunteers. T-shirts will be placed on hangers and spritzed with one of four commercial products and allowed to dry before being given to volunteers.

Water and snacks will be provided and movies will be shown to help volunteers pass the time.

Risks or Discomforts
There is a small chance of an allergic reaction to the products.

Ingredients of products and allergic information (Please indicate if you are allergic to any of the products listed below)

Commercial human odor controlling product ingredients: Silver XP particles, water, fermented vegetable enzyme, preservative.

Benefits
This study will result in the following benefits:

1. Human odor identification method development.
2. The identification of chemicals associated with human odor.
3. The evaluation of human odor control products.

Incentive to participate
Deleted

Confidentiality
All participants data and results will remain confidential. Please note that these records will be held by a state entity and therefore are subject to disclosure if required by law. Research information may be shared with the MSU Institutional Review Board (IRB) and the Office for Human Research Protections (OHRP). The sponsor of this study (Deleted) may also have access to the records of the research ***but personal information will remain confidential***.

Questions
If you have any questions about this research project, please feel free to contact ***Dr. Todd MIsna of the Chemistry Department*** 662 325 6744.

For questions regarding your rights as a research participant, or to express concerns or complaints, please feel free to contact the MSU Regulatory Compliance Office by phone at 662-325-3994, by e-mail at irb@research.msstate.edu, or on the web at http://orc.msstate.edu/participant/.

Research-related injuries
(Deleted) has not provided for any payment to you or for your treatment if you are harmed as a result of taking part in this study.

In addition to reporting an injury to ***Dr. Todd MIsna of the Chemistry Department***

662-325-6744 and to the Regulatory Compliance Office at 662-325-3994, you may be able to obtain limited compensation from the State of Mississippi if the injury was caused by the negligent act of a state employee where the damage is a result of an act for which payment may be made under §11-46-1, et seq. Mississippi Code Annotated 1972. To obtain a claim form, contact the University Police Department at *MSU UNIVERSITY POLICE DEPARTMENT, Williams Building, Mississippi State, MS 39762, (662) 325-2121.*

Voluntary Participation

Please understand that your **participation is voluntary.** Your **refusal to participate will involve no penalty or loss** of benefits to which you are otherwise entitled. You **may discontinue your participation** at any time without penalty or loss of benefits.

Please take all the time you need to read through this document and decide whether you would like to participate in this research study.

If you agree to participate in this research study, please sign below. You will be given a copy of this form for your records.

_____ _____

Participant Signature

_____

Investigator Signature Date

APPENDIX G

CANONICAL COEFFICIENTS OF MOST COMMON VOCS

Table G.1 Raw Canonical Coefficients of most abundant and most common VOCs.

Compound Name	Can1	Can2	Can3
Acetone	-0.8784	0.097683	0.524681
Isopropyl_Alcohol	0.558887	-0.30124	-0.1659
1R-.alpha.-Pinene	-0.65454	1.114258	-1.09456
Benzene, 1-ethyl-3-methyl-	0.358826	-0.89837	-0.17364
Tetradecane	0.188028	-0.48489	0.04701
Toluene	-1.16813	-0.35621	0.504037
Nonanal	-0.12098	-0.39555	-0.5408
1,3-Butadiene, 2-methyl-	-0.19446	0.550444	-0.18171
Hexanal	0.505046	0.257737	-0.27288
Hexadecane	0.050393	0.414344	0.516853
Decanal	-0.25023	0.478147	0.681819
Benzene	1.00886	0.131775	-0.24414
Ethanol	-0.07282	-0.42224	0.310094
Dodecane	0.230434	-0.04324	0.174699
Hexane, 3-methyl-	0.010638	0.499734	0.529223
Octanal	-0.9023	-0.29983	-0.10053
5-Hepten-2-one, 6-methyl-	-0.42659	0.685688	-0.31135
Benzene, 1,2,3-trimethyl-	-0.15628	-0.00719	-0.23677
Tridecane, 3-methyl-	0.259486	-0.86976	1.329226
2-Pentene, (Z)-	-0.39468	-0.01689	-0.36042
Tridecane	0.1703	-0.19151	-0.18323
Cyclohexane	1.46804	-0.02216	-0.65448
Butanal, 3-methyl-	0.124326	-0.44999	-0.27343
Naphthalene	0.298919	0.004605	-0.1901
Butanal, 2-methyl-	-0.48752	0.294187	-0.2012
1-octanol	-0.23492	0.159669	-0.33026
Methacrolein	1.615831	-0.29897	0.772492
2-Propenal	-0.4084	0.028814	0.39475
o-Xylene	0.720179	0.681798	0.267519

APPENDIX H

CONTRIBUTION OF COMPOUNDS FOR EACH PRINCIPAL COMPONENT

Table H.1 Top five compounds contributed to the each principal component regarding each product.

P#2		
PC1	PC2	PC3
Benzene, 1,2,3-trimethyl-	Acetone	Nonanal
Cyclohexane	1,3-Butadiene-2-methyl	Naphthalene
Acetone	Isopropyl Alcohol	Hexanal
1,3-Butadiene-2-methyl	Cyclohexane	Acetone
Hexanal	Benzene, 1-ethyl-3-methyl-	1-octanol

P#1		
PC1	PC2	PC3
Benzene, 1,2,3-trimethyl-	Acetone	Nonanal
Acetone	Benzene, 1,2,3-trimethyl-	Decanal
1,3-Butadiene-2-methyl	Decanal	1R-alpha-Pinene
Nonanal	Nonanal	Benzene, 1,2,3-trimethyl-
Isopropyl Alcohol	Isopropyl Alcohol	Tridecane-3-methyl

P#3		
PC1	PC2	PC3
Benzene, 1,2,3-trimethyl-	Dodecane	Acetone
Dodecane	Benzene, 1,2,3-trimethyl-	Ethanol
Acetone	Acetone	Isopropyl Alcohol
Nonanal	Nonanal	Cyclohexane
Isopropyl Alcohol	Ethanol	Octanal

P#4		
PC1	PC2	PC3
Acetone	Tetradecane	Ethanol
Isopropyl Alcohol	Hexadecane	Isopropyl Alcohol
Hexanal	1-octanol	Nonanal
Ethanol	1R-alpha-Pinene	Acetone
Cyclohexane	Ethanol	Cyclohexane