Summer 2024

Development of Vacuum-Assisted Headspace High-Capacity Solid-Phase Microextraction for the Determination of Semi-Volatile Compounds from Samples at Lower Temperatures

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DEVELOPMENT OF VACUUM-ASSISTED HEADSPACE HIGH-CAPACITY SOLID-PHASE MICROEXTRACTION FOR THE DETERMINATION OF SEMI-VOLATILE COMPOUNDS FROM SAMPLES AT LOWER TEMPERATURES

by

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DISSERTATION

Submitted in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

at The University of Texas at Arlington

August 2024

Arlington, Texas

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Acknowledgments

I would like to thank my research advisor, Dr. Kevin Schug, for piquing my interest in analytical chemistry. He connected me with Restek Corporation for funding as a research assistant and for internships. He was patient and encouraging during the research and writing process. There are so many possibilities available to me with the support of Dr. Schug. I would also like to give thanks to everyone in the Schug group. Everyone was there to support each other and to provide any insights and assistance as needed, of which, I am truly grateful.

I would also like to thank each of my committee members, Dr. Daniel Armstrong, Dr. Saiful Chowdhury, and Dr. Junha Jeon, for their advice and encouragement. I would like to express my gratitude to Restek Corporation for financial support throughout the course of my research, to Colton Myers for continued guidance and support, to Jason Herrington for his encouragement and support, and to the Restek family for welcoming me into the fold during my two internships. It was truly an unforgettable experience. I would also like to thank Prof. Elefteria Psillakis and ExtraTech Analytical Solutions SMPC for the provision and permission of use of the metal insert to seal the sampling vials.

Finally, I would like to give my appreciation to my family. To my husband, Samuel Thomas, for his support, encouragement, and love. To my children, Zackry, Warner, and Gilbert, for understanding the sacrifice and stepping up with chores without complaint. I am blessed to have such a loving family.

Abstract

Development of Vacuum-Assisted Headspace High-Capacity Solid-Phase Microextraction for the Determination of Semi-Volatile Compounds from Samples at Lower Temperatures

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Vacuum-assisted headspace solid-phase microextraction (Vac-HS-SPME) is a method used to increase solid-phase microextraction sampling of semi-volatile organic compounds. Three studies were conducted using Vac-HS-SPME via gas chromatography - mass spectrometry (GC-MS) methods for analysis of various sample types. First, a study was performed using Vac-HS-SPME combined with a high-capacity (HC) SPME Arrow to determine the operational fundamentals and workflow necessary to achieve optimal extraction of semi-volatile compounds from a model solid matrix (Ottawa sand). The fundamentals investigated included the seals necessary to create a leak-free sampling vial under vacuum conditions; the magnitude of the vacuum exerted, and the time needed to initiate the vacuum-assisted kinetics; and the order of sample vial preparation. Results for sample vial preparation methods demonstrated an ideal workflow requires the solid sample to be spiked before sealing the vial, rest the sample overnight, then apply vacuum to the rested sample at a pressure of -677 mbar (out of -789 mbar maximum possible vacuum with the compressor used), applied on the vial for 90 seconds.

Second, an application study was conducted using Vac-HS-HC-SPME coupled with GC-MS to compare the volatile and semi-volatile compounds of five psilocybin mushrooms

(*Psilocybe cubensis*), along with three non-psilocybin mushroom species. Using an untargeted approach, the common volatiles detected included acids, alcohols, aldehydes, ketones, and hydrocarbons. Vac-HS-HC-SPME and HS-HC-SPME were initially compared providing 2 times increase in compound response as well as the extraction of 8 additional compounds undetected by HS-HC-SPME. The compounds unique to psilocybin mushrooms included 2-methylbutanal, valeraldehyde, benzaldehyde, 3-octen-2-one, 2-methyl-dodecane, and 2-butyl-2-octenal. The compounds unique to non-psilocybin mushrooms consisted of 2-methyl-pyrazine, 2,3-butanediol, butyric acid, butyrolactone, benzyl alcohol, 2-pyrrolidinone, and estragole. The commonly shared mushroom alcohol, 1-octen-3-ol, was determined to have a higher compound response among the psilocybin mushroom species.

Finally, a third study was conducted to develop a novel Vac-HS-HC-SPME method to derivatize in-situ fatty acids into fatty acid methyl esters from the headspace of olive oil using sulfonated poly(divinylbenzene) microspheres. The application of Vac-HS-HC-SPME combined with in-situ derivatization could potentially provide a simpler and greener method for the derivatization of oils.

Abbreviations

BTO	Bleed and Temperature Optimized
DVB	Divinylbenzene
FTIR	Fourier Transform infrared spectroscopy
GC	Gas Chromatography
HC	High-capacity
HS	Headspace
K_H	Henry's law constant
MS	Mass spectrometry
PAHs	Polycyclic aromatic hydrocarbons
PDMS	Polydimethylsiloxane
PDVB	Poly(divinylbenzene)
RSD	Relative standard deviation
SE	SPME evaluation
SI	Supplemental information
SPME	Solid-phase microextraction
SVOCs	Semi-volatile organic compounds
Vac	Vacuum-assisted
VPM	Vial preparation method

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CHAPTER ONE

Introduction to Dissertation

Developed by Janusz Pawliszyn in 1990,[1] solid-phase microextraction (SPME) is a solvent-free sampling technique that directly extracts analytes from a sample with an extraction phase coated on a small-diameter fused silica fiber. The phase coated fiber can be used for direct immersion extraction (DI-SPME) in a liquid sample or for headspace sampling (HS-SPME), where the fiber is exposed to the gas phase above a sample. There are many advantages to SPME sampling, including field portability and reduction of sample handling, sample volume, extraction time, and solvent consumption. SPME sampling combines both sample preparation and sample introduction into one device which enables fully automated analysis for increased throughput using commercial autosampler systems and common analytical instruments.

HS-SPME has been used for the extraction of volatile analytes in the headspace for applications such as food, drug, and environmental analysis.[2,3] There are four stages in the HS-SPME sampling process: 1) Pre-conditioning; 2) incubation; 3) extraction; and 4) desorption. Pre-conditioning (1) cleans and prepares the SPME fiber for analyte extraction. The incubation (2) stage is the time allowed for the sample to equilibrate with the headspace. Once equilibrium is established, the SPME fiber is inserted into the sample vial for analyte extraction (3). The fiber is exposed to the headspace to allow for analytes from the gas phase to partition into the stationary phase. The extraction of volatile analytes transpires faster than for semi-volatile analytes. Methods used to shorten the equilibration time for SVOCs during the incubation and

extraction steps include the addition of salt to a liquid sample to disrupt analyte hydration,[4] agitation of the sample, and/or heating to higher temperatures.[5] The final stage is thermal desorption (4) of analytes from the phase into a GC for analyte separation. GC can be coupled to a multitude of different detectors to achieve more selective or universal detection of the extracted and separated analytes, as desired. To achieve shorter extraction times of SVOCs at reduced temperatures, Psillakis et al.[6] developed a method, coined vacuum-assisted HS-SPME (Vac-HS-SPME), which reduced pressure in the sampling vial by applying vacuum.

Chapter 2 provides a comprehensive discussion of the history of Vac-HS-SPME and its various applications to date. With the aim of contributing to the ever-growing field of Vac-HS-SPME, Chapter 3 provides an exploration into the workflow necessary for the preparation of solid samples. This includes the operational fundamentals of vial preparation for Vac-HS-SPME sampling from a solid sample matrix using only high capacity (HC-SPME) Arrow sampling fibers, and the optimal conditions needed for GC-MS analysis of SVOCs.

Chapter 4 gives an application to the fundamentals of Vac-HS-HC-SPME for solid samples. It provides a characterization of fragrance and flavor profiles for both psilocybin and non-psilocybin mushrooms. Chapter 5 focuses on a novel method for derivatization of fatty acid methyl esters (FAMEs) in olive oil by combining in-situ derivatization using poly(divinylbenzene) microspheres with Vac-HS-HC-SPME sampling.

Lastly, chapter 6 summarizes this work. Potential future studies include expanding the application of Vac-HS-HC-SPME to various biological samples and tissues, optimizing fiber coatings, and conducting targeted and untargeted metabolomics. The potential applications in

food quality control, authenticity testing, and biomarker discovery in clinical diagnostics are also highlighted.

CHAPTER TWO

VACUUM-ASSISTED HEADSPACE SOLID-PHASE MICROEXTRACTION SAMPLING METHOD FOR THE EXTRACTION OF SEMI-VOLATILE COMPOUNDS: AN OVERVIEW

Thomas, S.L., Schug, K. A., Vacuum-assisted headspace solid-phase microextraction sampling for the extraction of semi-volatile compounds: an overview. *LCGCNA: Advances in Sample Preparation.* 2023; 41:25-27. https://doi.org/10.56530/lcgc.na.ny8976g7 [7]

Vacuum-Assisted Headspace Solid-Phase Microextraction Sampling for the Extraction of Semi-Volatile Compounds: An Overview

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2.1 Abstract

Vacuum-assisted headspace solid-phase microextraction (Vac-HS-SPME) is an emerging sampling technique that enhances the extraction of semi-volatile compounds. The one extra step of pulling vacuum from the sampling vial pre-equilibrium increases the concentration of semi-volatiles in the headspace and allows for faster extraction times. This overview highlights the timeline, applications, and fundamentals of Vac-HS-SPME.

2.2 Introduction

Microextraction is an analytical extraction technique where the volume of the extraction phase is substantially smaller than the volume of the sample to be extracted. Because very small amounts of chemical compounds are extracted during sampling, microextraction allows multiple extractions of a sample with minimal change in the sample composition.[8] Solid-phase microextraction (SPME) was pioneered by Janusz Pawliszyn in 1990.[1] This widely-used sampling technique was designed for fast, convenient sample preparation with greatly-reduced volumes of solvents applied. It also reduced needed sample volumes, sample handling, and extraction times. Traditional SPME uses a small-diameter fused

silica fiber, coated with a small volume of extraction phase for the direct extraction of analytes from a sample. Sampling is typically conducted by direct immersion (DI-SPME) in a liquid sample or by headspace extraction (HS-SPME). In DI-SPME, the fiber is placed directly in the sample and analytes are extracted from the sample matrix. In HS-SPME, the fiber is exposed to the gas phase above a sample, where analytes partition into the fiber phase until equilibrium is reached.[9]

The extracted sample components in the fiber are most often sampled using thermal desorption in the injection port of a gas chromatograph (GC). For liquid chromatography (LC), introduction of extracted compounds can be achieved through solvent desorption.[10] SPME sampling offers completely automated analysis for increased throughput using commercial autosampler systems and customary analytical instruments.

HS-SPME is used for the extraction of volatile analytes in the headspace. Partitioning equilibration times are dependent on analyte volatility, sample matrix, and composition of the extraction phase. Due to semi-volatile analytes having a low affinity for the gas-phase, equilibration times for semi-volatiles (higher boiling point, lower vapor pressure) are longer than for volatiles (lower boiling point, higher vapor pressure). In order to shorten the equilibration time and enrich the fraction of semi-volatiles available for sampling in the headspace, salt can be added to a liquid sample to disturb analyte hydration,[4] and the sample can be heated and/or agitated.[11] Higher temperatures are known to cause sample decomposition and/or the formation of by-products.

The drawbacks related with long equilibration times and high extraction temperatures to improve HS-SPME sampling of semi-volatile analytes brought about the development of vacuum-assisted HS-SPME. This method consists of an extra step where the pressure in the sample vial is reduced by applying a vacuum, prior to equilibration for sampling. Reducing pressure in the sampling vial assists analytes with longer equilibration times under standard atmospheric pressure to increase their concentration in the headspace. For a deep dive into the theory behind vacuum-assisted (Vac) HS-SPME, Psillakis[12] provides an exhaustive tutorial for Vac-HS-SPME sampling with a focus on liquid samples, and Yiantzi et al.[4] gives a detailed procedure for recovering PAHs from solid matrices. This article gives an overview of the development, applications, and fundamentals of Vac-HS-SPME sampling.

2.3 Timeline and Applications

Table 2-1. Vac-HS-SPME timeline of applications including analytes of interest and sample matrix.

Analyte(s) of Interest	**Sample Matrix**	**Year and Author(s)**
Aroma-contributing compounds	Cooked and raw turkey breast	2001; Brunton et al.[13]
Ethylated derivatives of butyl- and phenyltin compounds	Sodium ethanoate/ethanoic acid buffer	2005; Darrouzes et al.[14]
Organophosphorus compounds	Glass surface	2011; Groenewold et al.[15]
Polycyclic aromatic hydrocarbons (PAHs)	Water	2012; Psillakis et al.[6]
Aromatic amines	Water; polyamide spoons	2014; Rubio et al.[3]
PAHs	Sand	2015; Yiantzi et al.[4]
Aromatic compounds	Mulberry juice; tobacco leaf	2015; Lee et al.[16]
Earthy-musty odor compounds	Water	2016; Glykioti et al.[17]
Free fatty acids and phenols	Milk; milk derivatives	2017; Trujillo-Rodriguez et al.[18]
Dimethylhydrazine	Aqueous extracts from soil	2018; Orazbayeva et al.[19]
BTEX	Polluted soil	2018; Ghiasvand et al.[20]
Nicotine	Hair; tobacco	2018; Ghiasvand et al.[21]
Haloanisoles	Wine	2019; Vakinti et al.[22]
Terpenoids	Frankincense resins	2020; Capetti et al.[23]
Aromatic compounds	Extra-virgin olive oil	2020; Mascrez et al.[24]

Butanoic acid	Hard cheeses	2020; Sykora et al.[25]
Fish volatiles	Frozen fish	2021; Delbecque et al.[26]
Steranes and pentacyclic terpanes	Oil-bearing source rock	2022; Pollo et al.[27]
Terpenoids and cannabinoids	Hemp inflorescences	2022; Capetti et al.[28]
Mushroom volatiles	Psilocybin and non-psilocybin mushrooms	2023; Thomas et al.[29]

Table 2-1 outlines the development and applications of Vac-HS-SPME. In 2001, Brunton et al.[13] reported benefits of reduced pressure HS-SPME sampling of food aroma volatiles from cooked and raw turkey breast. These positive effects were later confirmed by both Darrouzes et al.[14] and Groenewold et al.[15] In 2012, Psillakis et al.[6] reduced the pressure in the sampling headspace by evacuating the air from a sample container prior to introducing the sample. They presented the theoretical model for pressure dependence and coined the technique as Vac-HS-SPME. Studies using Vac-HS-SPME sampling for liquid samples have included extraction of polycyclic aromatic hydrocarbons (PAHs) from water samples,[30] aromatic amines in water, aromatic compounds in mulberry juice, earthy-musty odor compounds in water, free fatty acids and phenols found in milk,[18] haloanisoles in wine,[22] and a temperature study with extra virgin olive oil.[24] Researchers have also explored Vac-HS-SPME sampling for solid samples, such as PAHs from sand, BTEX from polluted soil, nicotine in hair and tobacco, terpenoids from frankincense resins, butanoic acids in hard cheeses, greener sampling techniques for oil bearing source-rock analysis,[27] volatiles from raw fish at sub-ambient temperatures,[26] analyzing terpenoids and cannabinoids in hemp inflorescences in one combined method,[28] and comparing volatiles among psilocybin and non-psilocybin mushrooms.[26]

2.4 Vac-HS-SPME Sampling

Vac-HS-SPME sampling involves the evacuation of a sample vial before equilibration for sampling and extraction. There are several things to consider when preparing a sample for extraction, including the type of vessel, how to create a gas-tight seal, the device used to pull vacuum, and the order of vial preparation, which is dependent on sample type.

2.4.1 Sample Containers

Vac-HS-SPME sampling requires a vessel, which has a gas-tight seal and maintains vacuum for at least 24 hours, and ultimately, a vial that is used for common autosamplers. Psillakis et al.[12] have explored variations of sample containers and seals. Starting with a 1000-mL gas-sampling bulb, this container maintained vacuum up to 150 min but lacked the ability to be agitated.[6] This led next to investigating 500-mL and 1000-mL custom-made glass sample vessels.[31] These samplers were difficult to heat evenly and awkward to use. Plus, the volume of headspace compared to sample volume reduced the amount of analyte extracted by the fiber. To reduce the headspace volume, a 22-mL container was made from a standard 20-mL headspace vial with the addition of two gas-tight ports and able to accommodate solid samples.[4,31] This container still lacked the ability to be automated. Psillakis et al.[18,32] explored several versions of seals that would accommodate a standard 20-mL headspace vial. Now a stainless-steel insert (created by Prof. Elefteria Psillakis, ExtraTech Analytical Solutions SMPC, Chania, Greece) combined with a Thermogreen® LB-1 septum with half-hole (Supelco, Bellefonte, PA), can be placed in the vial opening of a 20 mL headspace vial to create a gas-tight seal and used on a HS-SPME autosampler. This insert has been provided recently to researchers

for Vac-HS-SPME studies, including measurements of volatiles and semivolatiles in hemp[28] and in edible vs. psychedelic mushrooms.[29]

2.4.2 Evacuation Process

Once a gas-tight seal has been created, one must consider how to evacuate the vial. A gas-tight syringe can be used by hand.[15,25] The drawback is the seal could be compromised for the number of times needed to remove the air from the vial. Also, the rate at which one pulls the syringe can be inconsistent and be cause for variability. This method is cost effective and has the possibility to be automated. A more effective and commonly used approach is to use a vacuum pump. A typical setup, shown in Figure 2-1, would be a diaphragm vacuum pump (generating ultimate vacuum to approximately 7 mbar) connected via metal tubing to an open/close valve. The metal tubing prevents collapsing, and the open/close valve allows the shut off pressure from the vacuum pump when desired. A digital pressure gauge can be used to determine the amount of vacuum in the sampling vial, as well as to detect any leaks via pressure decay. A T-joint can be used to connect a digital pressure gauge with tubing on both sides. The other side of the T-joint would connect to tubing with a Luer lock attachment for a Luer lock side-port gas needle.

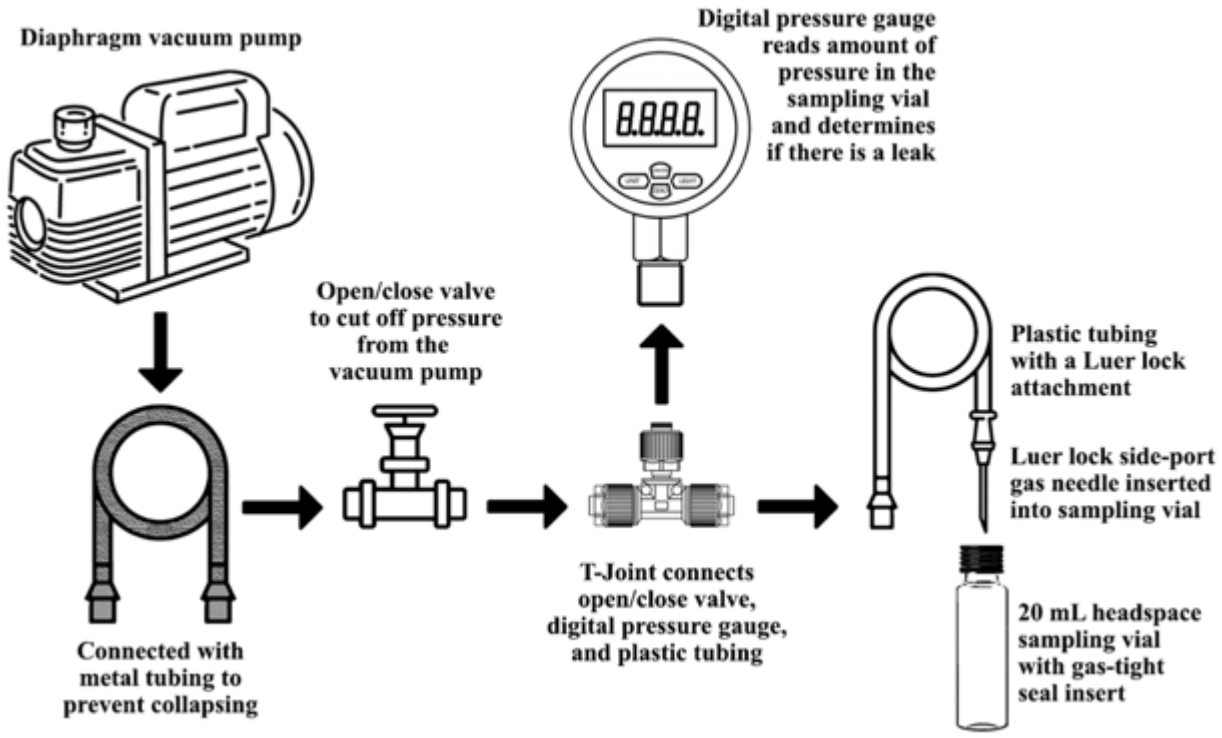

Figure 2-1. Vac-HS-SPME sampling setup

The steps to evacuate the prepared sampling vial are as follows. Turn on the vacuum pump. Insert the side-port gas needle through the septum of the sealed vial. Vacuum time will be dependent on the type of vacuum pump used and the size of the vial. This can be determined with the use of the attached pressure gauge. Remove the needle while the vacuum pump is still running to ensure maximum vacuum. Only use the open/close valve before sample preparation to check for leaks and to verify vial pressure. The sample vial is now ready for equilibration and HS-SPME extraction.

2.4.3 Vial Preparation

When preparing a liquid sample, the liquid can be introduced before or after the air has been evacuated from the vial.[17,30,32] If introduced before pulling vacuum, one needs to

consider possible removal of some highly volatile compounds during the evacuation process. One could analyze the highly volatile first by performing traditional HS-SPME before pulling vacuum from the vial, then proceed with Vac-HS-SPME to sample the semi-volatiles, which have lower affinity for the headspace. When vacuum is pulled from the vial first, a syringe is used to introduce the liquid sample. Due to the reduced pressure in the vial, the liquid will be pulled quickly from the syringe and spray on the vial walls. Depending on the properties of the liquid sample, this order of vial preparation could cause variability.

Solid samples need to be placed in the vial prior to air evacuation unless a special device is made, such as used by Ghiasvand et al.[21] Again, this means that highly volatile compounds could possibly be removed during evacuation. Steps can be made to ensure the least amount of volatile loss. A smaller vessel, such as a 20-mL headspace vial, requires less time to evacuate. If using a vacuum pump, one could attach a pressure gauge, as shown in Figure 2-1, to determine when evacuation is complete to minimize vacuum time and loss of volatiles. One could also freeze the sample immediately after placing it in the vial, as demonstrated by Capetti et al.[23] This would prevent the concentration of volatiles in the headspace and minimize volatile loss during vacuum.

2.5 Effects of Temperature and Extraction Time

Temperature is a key parameter used during HS-SPME sampling.[9] At room temperature, volatiles will reach headspace equilibrium in a short amount of time. Semi-volatiles will require more time to reach equilibrium and will be at low concentrations in the headspace. Increased temperature speeds up equilibration time and increases the

concentration of semi-volatiles. For Vac-HS-SPME, studies have shown that increased extraction temperatures reduced extraction efficiency of semi-volatile analytes.[33] One explanation is that increased temperature increases the vapor pressure of analytes, thus increasing the total pressure in the sampling vial and minimizing the effects of Vac-HS-SPME.[34] Thus, lower sampling temperatures can be used in Vac-HS-SPME to avoid sample degradation or unwanted by-products, and these conditions can be advantageous for increased extraction yield for many analytes.

Extraction times for semi-volatile analytes under atmospheric pressure take much longer than volatile analytes. It may take only a minute for volatiles to reach equilibrium compared to an hour or more for some semi-volatiles. Reducing the pressure in the sampling vial accelerates equilibration time and increases concentration of semi-volatiles in the headspace. This makes the overall extraction time shorter. Studies have commonly reported shorter extraction times for semi-volatile analytes, as much as half the time, for Vac-HS-SPME compared to traditional HS-SPME.[14,18,22,29,30]

2.6 Conclusions

Vac-HS-SPME sampling is an advantageous sampling technique for the extraction of semi-volatile compounds. This method can be used for both liquid and solid samples. With the ability to extract at low temperatures, Vac-HS-SPME has the potential for many applications, which involve thermally labile compounds and samples that degrade with increased temperature. The progression of seals designed for standard 20-mL headspace vials combined

with the ability to pull vacuum with a gas-tight syringe, opens the door for this sampling method to be fully automated in the coming future.

Acknowledgements:

The authors would like to thank Restek Corporation for research and research materials support.

CHAPTER THREE

INVESTIGATION OF OPERATIONAL FUNDAMENTALS FOR VACUUM-ASSISTED HEADSPACE HIGH-CAPACITY SOLID-PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHIC ANALYSIS OF SEMIVOLATILE COMPOUNDS FROM A MODEL SOLID SAMPLE

Thomas, S. L., Myers, C., Herrington, J. S., Schug, K. A., Investigation of operational fundamentals for vacuum-assisted headspace high-capacity solid-phase microextraction and gas chromatographic analysis of semivolatile compounds from a model solid sample. *J Sep Sci.* 2024; 47:2300779. https://doi.org/10.1002/jssc.202300779[35]

Investigation of Operational Fundamentals for Vacuum-Assisted Headspace High-Capacity Solid-Phase Microextraction and Gas Chromatographic Analysis of Semivolatile Compounds from a Model Solid Sample

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Abbreviations: divinyl benzene (DVB), GC, high-capacity (HC), headspace (HS), Henry's law constant (K_H), MS, polycyclic aromatic hydrocarbons (PAHs), polydimethylsiloxane (PDMS), RSD, SPME Evaluation (SE), supplemental information (SI), semi-volatile organic compounds (SVOCs), SPME, vacuum-assisted (Vac), vial preparation method (VPM)

Keywords: gas chromatography – mass spectrometry, headspace solid-phase microextraction, sample preparation, solid matrix, vacuum-assisted

3.1 Abstract

Vacuum-assisted headspace solid-phase microextraction is a technique used to enhance solid-phase microextraction sampling of semi-volatile organic compounds. Here, it was combined with a high-capacity solid-phase microextraction Arrow, which features a larger volume of extraction phase and a more rugged configuration than traditional extraction fibers. An in-depth assessment of the critical parameters was conducted to achieve optimal extraction of representative compounds from a model solid sample matrix (Ottawa sand). Operational

fundamentals investigated included: Types of seals needed to create a leak-free environment under vacuum conditions; magnitude of the vacuum applied and time needed to activate the vacuum-assisted kinetics; order of sample vial preparation methods; and other standard variables associated with extract analysis by gas chromatography – mass spectrometry. When exploring the limits of sample vial preparation methods, results indicated an ideal workflow requires the solid sample to be spiked before sealing the vial, allow the sample to rest overnight, then apply vacuum at a pressure of -677 mbar (out of -789 mbar maximum possible vacuum with pump and compressor used), exerted on the vial for 90 seconds. This work provides the necessary workflow for the optimization of vacuum-assisted headspace solid-phase microextraction sampling of analytes from solid matrices.

3.2 Introduction

Microextraction refers to the condition where the volume of an extraction phase is small relative to the volume of the sample to be extracted. Since very small amounts of the sample are extracted, microextraction can often be performed multiple times on a sample without significant change in the sample composition. For microextraction, when equilibrium for the partitioning of analytes between the extraction phase and the sample phase is reached, the number of moles of a chemical compound extracted can be correlated with its concentration in the sample using standard calibration procedures for quantitative analysis. Microextraction phases can be conceived from a variety of liquid- and solid-phase configurations and formats [8]. Microextraction procedures are considered to be a more environmentally friendly technology than traditional extraction techniques due to the largely-reduced volumes of solvents and sorbents utilized.

Developed by Janusz Pawliszyn in 1990 [1], solid-phase microextraction (SPME) is a widely-used, solvent-free sampling technique which enables direct extraction of analytes from a sample by using a phase-coated, small-diameter fused silica fiber. HS-SPME is used for the extraction of volatile analytes in a headspace. Depending on analyte volatility, sample matrix, and makeup of the extraction phase, partitioning equilibration times can vary from minutes to hours. Equilibration times for semi-volatile organic compounds (SVOCs) are longer than for volatiles. To shorten the equilibration time for SVOCs during the incubation and extraction steps, the sample can be agitated, and/or it can be heated to higher temperatures [5]. Higher temperatures pose problems such as decreased extraction efficiency due to desorption in the sampling vial, sample degradation, and/or the formation of by-products.

The disadvantages associated with using high incubation/extraction temperatures to improve HS-SPME sampling prompted the development of vacuum-assisted HS-SPME, as an alternative method to hasten the kinetics involved with extraction of SVOCs and to allow use of lower temperatures. In this approach, pressure is reduced in the sample vial by applying a vacuum. In 2001, Brunton et al. [13] reported positive effects of low-pressure HS-SPME sampling of food aroma volatiles from raw turkey. In 2012, Psillakis et al. [6] evacuated the air from the sampling container prior to introducing the sample to reduce the pressure in the headspace and designated the method as vacuum-assisted headspace solid-phase microextraction (Vac-HS-SPME).

By reducing the pressure in the sample vial as a pre-equilibration step, Vac-HS-SPME increases the degree of headspace partitioning for SVOCs. For water-containing samples, the pressure dependence of HS-SPME sampling methods prior to equilibration was experimentally

proven by a theoretical model posited by Psillakis et al., [31] with respect to partition coefficients/Henry's law constant, K_H. K_H is the ratio of the partial pressure of an analyte to its aqueous concentration. This theory showed, for those analytes present in water and characterized by a low K_H, reduced pressure in the vial prior to sample introduction resulted in improved extraction efficiency with faster extraction times at room temperature. Analytes present in solid samples have even greater resistance to volatilization than liquid samples. They can be present as adsorbed, dissolved, and/or in gaseous phases within the solid matrix. Using a modified form of Fick's law of diffusion [36], Yiantzi et al. [4] stated if the total pressure is reduced, the vapor flux of chemicals at the surface of a solid will increase, which accelerates the volatilization rates of analytes and thus, shifts the equilibrium toward a higher concentration of analyte in the headspace.

To date, a few studies have focused on using Vac-HS-SPME sampling for liquid samples, which include free fatty acids and phenols found in milk [18], haloanisoles in wine [22], a temperature study with extra virgin olive oil [24], and extraction of polycyclic aromatic hydrocarbons (PAHs) from water samples [30]. Most recently, researchers have begun to explore Vac-HS-SPME sampling for solid samples. These have included extraction of PAHs from sand [4], BTEX from polluted soil [20], terpenoids from frankincense resins [23], butanoic acids in hard cheeses [25], volatiles from raw fish at sub-ambient temperatures [26], greener sampling methods for oil bearing source-rock analysis [27], one combined technique for analyzing terpenoids and cannabinoids in hemp [28], and flavor and fragrance components in psilocybin and non-psilocybin mushrooms [29].

Since the inception of SPME devices, numerous formats of coated substrates have been developed for the purpose of analyte extraction. Of those devices created, the SPME device most commonly used is the format described by Belardi et al. [37], which consists of a coated, cylindrical fused silica fiber. With the expiration of some intellectual property protection, new devices have since emerged, with one of the most notable being the high-capacity (HC) SPME Arrow. The SPME Arrow, developed by CTC Analytics AG, is a redesigned SPME fiber that has enhanced mechanical robustness and a larger phase volume to improve sensitivity [38]. Herrington et al. [39] published an in-depth review of the SPME Arrow, showing direct comparisons of the traditional SPME fiber to the SPME Arrow. Major advantages of the SPME Arrow include greater analyte sensitivity due to the larger phase volume, and improved mechanical robustness due to the larger outer diameter of the device. The 1.5 mm SPME Arrow has a phase volume of 11.8 µL compared to a traditional SPME fiber of 0.600 µL, as well as a phase diameter of 0.912 mm compared to 0.285 mm, respectively. Disadvantages of the SPME Arrow include that it requires the installation of a modified GC inlet weldment and septum nut, and that only specific rail-style autosamplers can be used for automation of the process.

With the greater volume of the SPME Arrow fiber phase, it is a common misconception that this leads to longer extraction times. For a consistent surface area to volume ratio (e.g., 100 um polydimethylsiloxane (PDMS)), a SPME Arrow extraction curve showed equivalent rates for reaching extraction equilibration as observed for a traditional SPME fiber; the main difference was the larger analyte amount extracted by the SPME Arrow [40]. Overall, with improved sensitivity and greater mechanical robustness, the SPME Arrow represents an improved tool for faster sample extraction and introduction, relative to traditional SPME.

Psillakis [12] has published a tutorial for Vac-HS-SPME sampling with a focus on liquid samples, and Yiantzi et al. [4] has investigated the theoretical and experimental procedure for recovering PAHs from solid matrices. With the aim of contributing to their work and to the growing use of Vac-HS-SPME, this study provides an exploration into the workflow necessary for the preparation of solid samples. The aim of this study was to explore the operational fundamentals for vial preparation methods involved in Vac-HS-SPME sampling from solid matrices. This body of work uses only high-capacity Arrow (HC-SPME) sampling fibers. To conduct the experiments, a custom SPME evaluation (SE) mix, which consisted of a range of analytes with different boiling points and functional groups, was spiked on a model solid sample matrix, Ottawa sand. The operational fundamentals investigated included the types of seals needed to create a gas-tight environment, the order and timing of steps for sample vial preparation, the magnitude of the vacuum applied, and the time needed under vacuum to maximize the enrichment of SVOCs in the headspace. The overarching goal of this study was to delineate important considerations for optimal Vac-HS-HC-SPME sampling for the subsequent GC-MS analysis of SVOCs from a solid sample matrix.

3.3 Materials and Methods

3.3.1 *Instrumentation*

For all experiments involving the analysis of SE mix on Ottawa sand, an Agilent 7890B gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA), equipped with a PAL3 RTC autosampler system (CTC Analytics AG, Zwingen, Switzerland), and coupled to an Agilent 5977B mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA), was used. The column was an

Rxi-624Sil MS (30 m x 0.25 mm x 1.4 µm) from Restek Corporation (Bellefonte, PA). The instrument was operated in constant flow mode (1.4 mL min^{-1}) using helium carrier gas. Sample extraction was carried out using a HC-SPME Arrow with a PDMS/divinyl benzene (PDMS/DVB) biphasic extraction phase (Restek). A Topaz 1.8 mm ID Straight/SPME inlet liner from Restek was used for all applications. For consistency, the HC-SPME sampling parameters, as well as the gas chromatograph and mass spectrometer settings remained the same throughout the experiments, as shown in Table 3-1. Note that a 10:1 split ratio was used to improve chromatography while maintaining a high response.

Table 3-1. HC-SPME sampling parameters and GC-MS program settings

CTC RTC Parameters		**Agilent 7890B/5977A GC-MS Parameters**	
Vac-HS-HC-SPME		**Inlet**	
Tool	SPME Arrow	280 °C	
Agitator Speed	250 rpm	Split 10:1	
Agitator Temperature	40 °C	Topaz 1.8 mm ID Straight/SPME Inlet Liner	
Incubation Time	300 s		
Heatex Stirrer Speed	250 rpm	**Column**	
Heatex Stirrer Temperature	40 °C	Rxi-624Sil MS, 30 m, 0.25 mm ID, 1.4 µm	
Extraction Time	480 s		
Vial Penetration Depth	45 mm	**Oven**	
Injector Penetration Depth	50 mm	50 °C (hold 1.0 min) to 150 °C at 15 °C min^{-1}, to 280 °C at 25 °C min^{-1} (hold 5.0 min)	
Desorption Time	60 s		
Pre-Conditioning	True	**Carrier Gas**	
Post Conditioning	False	Type	Helium
Conditioning Time	60 s	Mode	Constant Flow
Conditioning Temperature	280 °C	Flow Rate	1.40 mL min^{-1}
		Detector	
		Type	Single Quadrupole MS
		Mode	Scan (35 to 350 m/z)
		Transfer Line Temp.	280 °C
		Source Temp.	325 °C
		Quad Temp.	200 °C
		Electron Energy	70 eV
		Tune Type	DFTPP
		Ionization Mode	EI

3.3.2 *Sample preparation*

A 1000 µg mL^{-1} per compound SE mix in methanol was prepared and diluted to 10 µg mL^{-1} as a standard for fortification of the sample material. The SE mix was a custom standard mix from Restek; methanol (ULTRA RESI-ANALYZED®, J.T. Baker) was from VWR International, LLC, Radnor, PA, USA. The SE mix contained 15 compounds with a variety of boiling points and functional groups. A list of these compounds is given in Table 3-2. Ottawa sand (Restek) was measured at 2.5 g and placed in a 20 mL headspace screwcap vial (Restek). A 50 µL spike of the diluted SE mix was introduced onto the Ottawa sand via a 100 µL SGE gas tight syringe (Restek). A stainless-steel insert (provided by Prof. Elefteria Psillakis, ExtraTech Analytical Solutions SMPC, Chania, Greece), with a hole to allow for a Thermogreen® LB-1 septum with half-hole (Supelco, Bellefonte, PA), was placed in the vial opening to create a gas-tight seal.

Table 3-2. SE Mix List of Compounds

Elution Order	Compound	Formula	Molecular Weight (g mol^{-1})	Boiling Point (°C)	K_H (atm-m^3 mol^{-1})[a]
1	*n*-pentane	C_5H_{12}	72.149	36	1.25
2	chloroform	$CHCl_3$	119.378	61	3.67E-03
3	methyl isobutyl ketone (MIBK)	$C_6H_{12}O$	100.159	116	1.38E-04
4	2-picoline	C_6H_7N	93.127	129	9.96E-06
8	N,N-dimethylaniline	$C_8H_{11}N$	121.180	193	5.68E-05
6	1-octanol	$C_8H_{18}O$	130.228	195	2.45E-05
7	linalool	$C_{10}H_{18}O$	154.249	199	2.15E-05
9	2,6-dimethylphenol	$C_8H_{10}O$	122.164	201	6.65E-06
10	hexachlorobutadiene	C_4Cl_6	260.761	215	1.03E-02
5	1,4-butanediol	$C_4H_{10}O_2$	90.121	230	2.3E-07
12	dicyclohexylamine	$C_{12}H_{23}N$	181.318	256	5.5E-05
13	butylhydroxytoluene (BHT)	$C_{15}H_{24}O$	220.351	265	3.38E-06
14	acenaphthylene	$C_{12}H_8$	152.192	280	1.25E-04
11	sulfolane	$C_4H_8O_2S$	120.170	285	4.85E-06
15	*n*-docosane	$C_{22}H_{46}$	310.601	369	1.59E+02

3.4 Results and Discussion

Although the investigation of seals and septa were not considered part of the standard workflow, they were investigated due to their importance for creation of a gas-tight seal. This investigation compared black and green o-rings for durability and contamination. Black o-rings were more durable compared to green o-rings, which would deteriorate within two to five uses. Thermogreen® LB-1 septa were compared to BTO septa for bleed, carryover, and a gas-tight seal. The Thermogreen® LB-1 septum has a high off-gassing of siloxanes compared to a BTO septum with no off-gassing. Both types of septa have the potential for carryover of analytes. The most important factor being the gas-tight seal, the Thermogreen® LB-1 septum maintained a gas-tight seal and exhibited a vacuum reproducibility of 1% RSD. Details of this study can be found in the Supplementary Information (SI) document.

Operational fundamentals of Vac-HS-HC-SPME (section 3.1) were investigated to establish the order in which to prepare the sample vial for Vac-HS-HC-SPME sampling, including vial preparation methods (VPMs). Section 3.2 explores the basic setup for Vac-HS-HC-SPME sampling, as well as the amount of time vacuum should be applied to the sampling vial, and the magnitude of applied vacuum needed. After those steps, the sampling vial was ready to be placed in the autosampler for HS-HC-SPME sampling. It is worth noting that both *n*-pentane and *n*-docosane have K_H values greater than 1 compared to the other analytes, as seen in Table 3-2. Both analytes show little to no detection in the following experiments.

3.4.1 *Operational fundamentals of vacuum-assisted headspace-SPME sampling*

The operational fundamentals cover the steps required to prepare the sample vial starting from the first step of adding solid sample material to the headspace vial and ending with the air-evacuated vial ready for the autosampler. Figure 3-1 shows the workflow for this study, with the parameters explored and the final obtained parameters for the enrichment of the SVOCs.

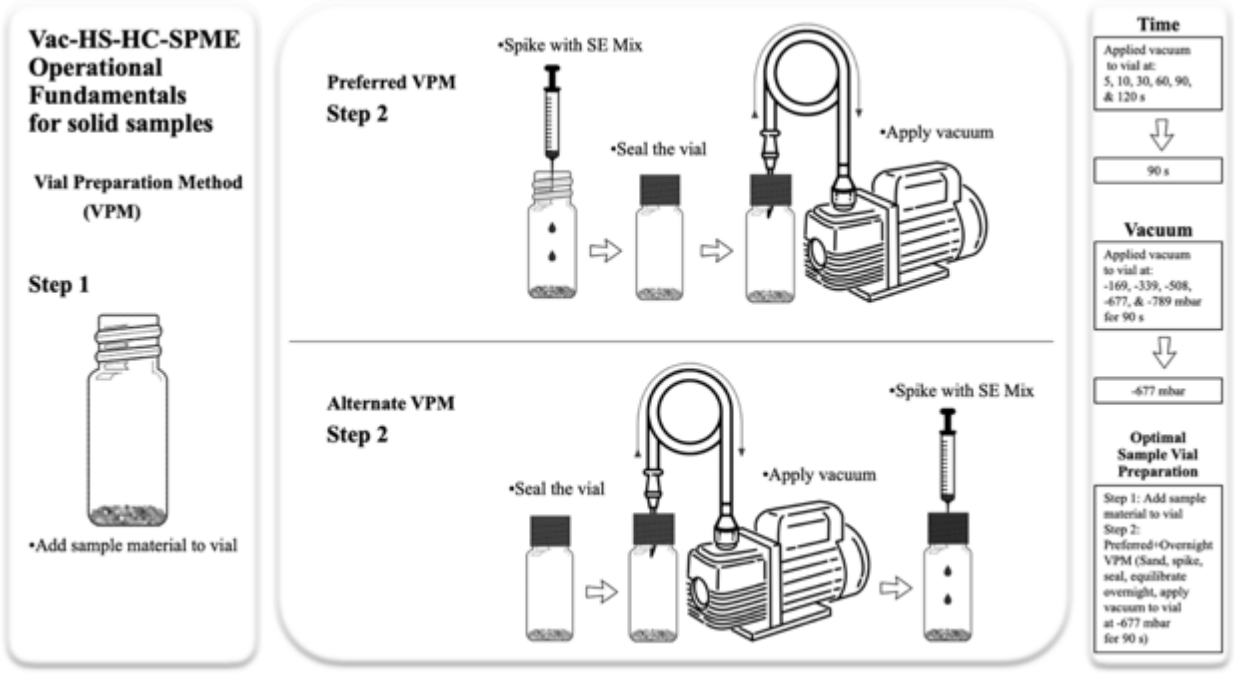

Figure 3-1. Vac-HS-HC-SPME operational fundamentals workflow for sample vial preparation of solid samples to be spiked with a standard solution.

The first step in the workflow for sample vial preparation was to place the measured solid sample into the headspace vial. The second step in the workflow was split into two sections consisting of 1) a study of the VPM in terms of the order of vial preparation steps, and 2) a study of the duration of time that vacuum was applied, and the magnitude of vacuum applied, to the vial. Analytes need time to equilibrate after spiking onto the solid matrix.

Studies were conducted to explore the effects of spiking the sample before and after applying vacuum, as well as to show the importance of equilibration time.

3.4.1.1 *Vial preparation methods; Order of preparation*

Vac-HS-SPME sampling of liquids is a straightforward preparation method. The liquid could be added before or after the vacuum is applied. In the literature, researchers tended to add liquid after vacuum was applied. A solid, on the other hand, must be introduced to the vial before pulling vacuum. If one intends to then spike a standard solution onto the solid material, the order of vial preparation needs to be determined. Order of preparation could be placement of the solid material into the vial, spiking with standard, sealing the vial, and then pulling vacuum. Alternatively, one could consider, addition of the solid material into the vial, sealing the vial, pulling vacuum, then spiking with standard. Another consideration is the time to wait before evacuation once the sample has been spiked with standard and the vial sealed with the vacuum insert. Previous Vac-HS-SPME studies involving solids have spiked, sealed, and rested anywhere from one hour to overnight in refrigeration, before evacuation of the sample vial [4,26,28]. This study explored the order of vial preparation and compared resting times ranging from 5 to 60 min and resting overnight. The goal was to reveal the relationship between volatiles and pressure and the importance of vial preparation order. All experiments were performed in triplicate.

3.4.1.1.1 *Vacuum-assisted headspace high-capacity SPME pre-spike vial preparation method*

In a 20-mL headspace vial, Ottawa sand was spiked with SE mix (500 ppb), sealed, then allowed to rest at ambient temperature for various times (5, 15, 30, 45, and 60 min) before applying vacuum. The different resting times produced similar responses for the analytes and

were not significantly different. This experiment indicated that longer time is needed for equilibration of the spiked sample. Thus, this Pre-Spike VPM was combined with an overnight resting time (Pre-Spike+Overnight VPM) to further investigate the need for longer resting times in a spiked solid sample when pressure is reduced.

The suggested procedure (Overnight VPM) from Yiantzi's work [4] would be the following: Ottawa sand spiked with SE mix, sealed, refrigerated overnight, and then vacuum applied. This method was compared to the Pre-Spike VPM (no resting overnight). Figure 3-2 shows that the Pre-Spike VPM returned measurements with a reasonable precision, with less than 20% RSD, and had a 2-fold greater response than the Pre-Spike+Overnight VPM.

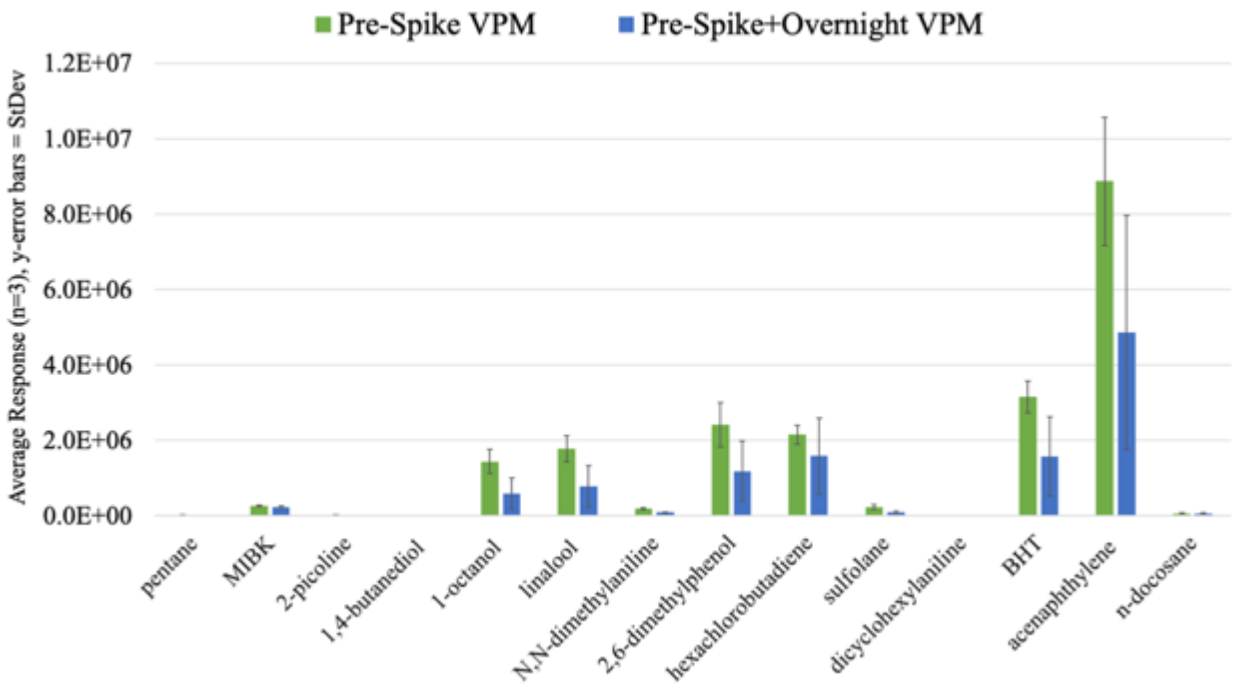

Figure 3-2. Pre-Spike VPM (sand, spike, seal, vacuum) compared to Pre-Spike+Overnight VPM with the order of 2.5 g Ottawa sand spiked with 50 µL SE mix, sealed, refrigerated overnight, then applied vacuum at -677 mbar for 90 s.

As expected, the volatile compound 1,4-butanediol exhibited a low response using the Pre-Spike VPM but showed no response for the Pre-Spike+Overnight VPM. A zoomed-in view is shown in Figure 3-S1 of the SI document. Given the time to rest overnight, 1,4-butanediol had two possible outcomes. Either 1,4-butanediol was evacuated during the headspace vial vacuum process, or 1,4-butanediol was bound so tightly to the sand to not exert significant concentration in the headspace.

To further examine this query, Figure 3-3 compares Pre-Spike+Overnight VPM with an overnight spiked sample analyzed using traditional HS-SPME sampling at atmospheric pressure. The volatiles *n*-pentane, chloroform, and 2-picoline produced a greater response using the no-Vac method, with a p-value less than 0.05. The SVOCs produced a greater response using the Pre-Spike+Overnight VPM, with a p-value less than 0.05. A comparison of performance, denoted as Vac/no-Vac ratios, can be calculated by dividing the compound response from Vac-HS-HC-SPME sampling by the compound response from HS-SPME without vacuum assistance. The Vac/no-Vac ratios consisted of 1-octanol at 20.6, linalool at 19.0, 2,6-dimethylphenol at 10.8, hexachlorobutadiene at 3.8, sulfolane at 6.4, BHT at 10.1, and acenaphthylene at 6.2.

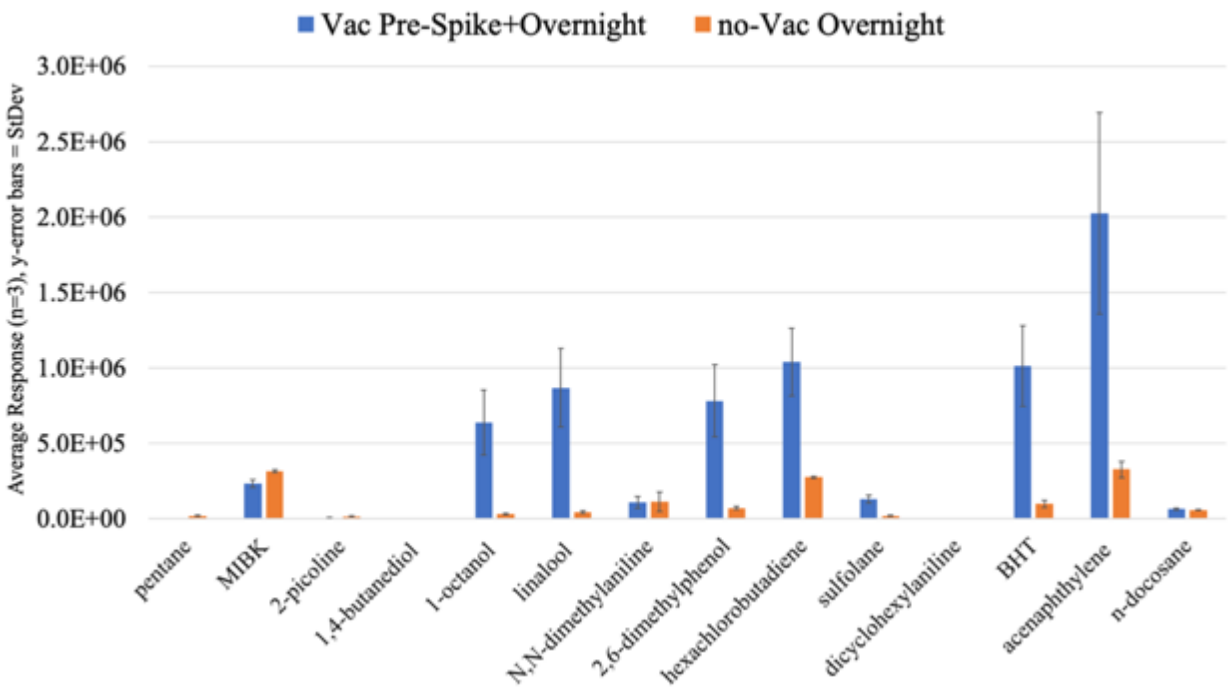

Figure 3-3. Pre-Spike+Overnight VPM with the order of 2.5 g Ottawa sand spiked with 50 µL SE mix, sealed, refrigerated overnight, then applied vacuum at -677 mbar for 90 s compared to a no-Vac overnight spiked sample.

1,4-butanediol was not extracted in either method. When no vacuum is pulled, there is still no response for 1,4-butanediol. This shows that 1,4-butanediol was present in the vial (on the sand) but not in the headspace to be extracted. Therefore, it is retained by the sand after resting overnight. This is a good example why resting overnight is needed in this study to imitate a real-world sample. In practice, with real samples, it would be important to spike internal standards onto the sample material and allow the mixture to equilibrate overnight before vacuum-assisted extraction.

The lower response given by the Pre-Spike+Overnight method is due to the time given for the larger compounds to adsorb to the surface of the sand while resting overnight. Due to the variability of solid samples and the analytes adsorbed to the solid material, these results

will not be consistent across all sample types. Thus, a higher compound response is not the goal when considering the fortification of a sample with standards to approximate the conditions needed for extraction of real analytes. For example, the reduced response obtained with resting overnight of the spike with the material would be the better way to introduce an internal standard that was going to mimic an adsorbed analyte. Some more complex matrices, such as soils may require even longer periods for equilibration. When considering the order of vial preparation for resting overnight, the ideal order should be: Addition of sample; spike; seal; rest overnight; then apply vacuum and allow for equilibration.

3.4.1.1.2 *Vacuum-assisted headspace high-capacity SPME post-spike vial preparation method*

The Post-Spike VPM (Ottawa sand sealed in the vial, vacuum applied, then spiked with SE mix) was investigated to show the variability that can occur if standards are applied after the vacuum is pulled. The Pre-Spike VPM (solid sample, spike, seal, vacuum) was compared to the Post-Spike VPM (solid sample, seal, vacuum, spike), as exhibited in Figure 3-1. These samples were not rested overnight to explore the relationship between volatiles, SVOCs, and application of vacuum. The Pre-Spike VPM allows one to homogenize the sample after the spiked standards are applied at atmospheric pressure. Whereas, the Post-Spike VPM could have losses because, as the syringe is applied through the metal insert to maintain vacuum, this causes the solution to spray from the syringe needle onto the vial walls, in addition to onto the sand. In the Post-Spike VPM, the spike occurs after the vacuum is applied. As the syringe is inserted through the septum, there is potential for leaks which would allow air to enter the vial and displace the vacuum. An increase in the pressure in the sample vial would reduce the benefits of vacuum assistance and could result in greater variability, as well as worsened recoveries. Applying vacuum before spiking

the sand (Post-Spike VPM) did not provide enrichment of SVOCs in the headspace and produced similar results as that of HS-SPME methods under atmospheric pressure, as seen in Figure 3-4.

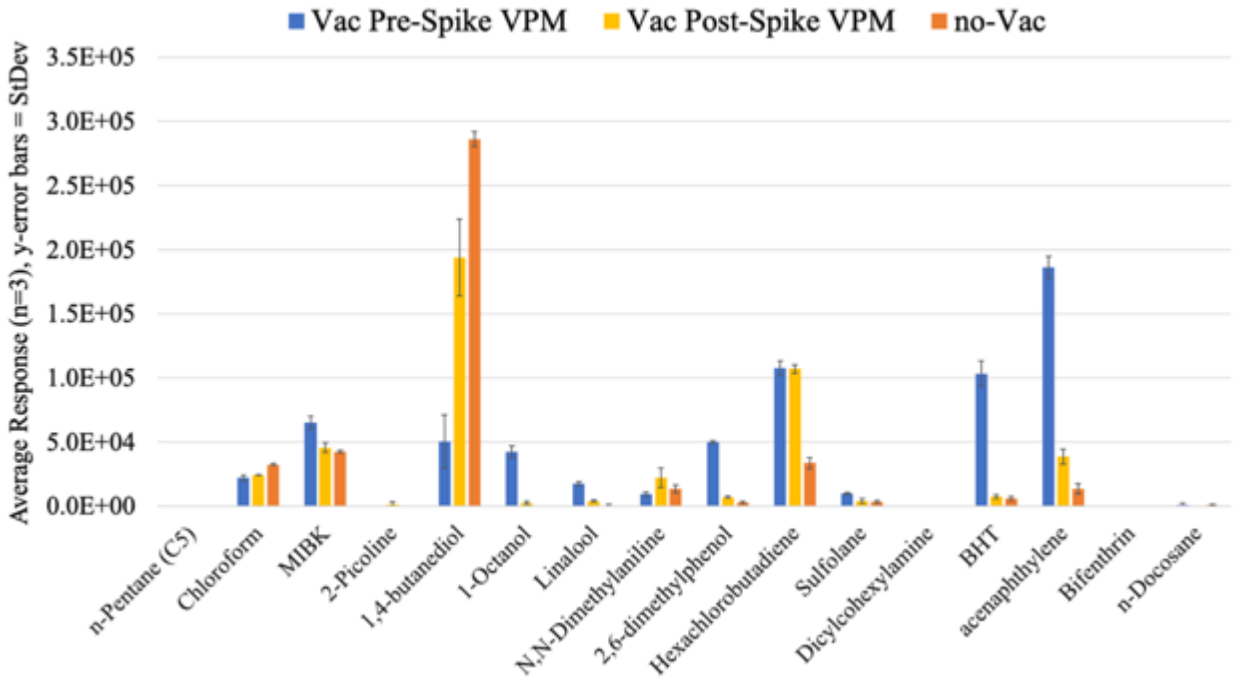

Figure 3-4. Vac-HS-HC-SPME using the Pre-Spike vial preparation method (VPM) (50 µL spike of SE Mix, seal, then vacuum at -677 mbar for 90 s) and the Post-Spike VPM (seal, vacuum at -677 mbar for 90 s, then spike 50 µL of SE Mix) compared to HS-SPME with no vacuum assistance (no-Vac), including Vac/no-Vac response ratios to judge relative performance.

The Pre-Spike VPM provided Vac/no-Vac response ratios for 1,4-butanediol, 2,6-dimethylphenol, BHT, and acenaphthylene to be 0.7, 18.2, 17.8, and 13.7, respectively, with a closer view provided in Figure 3-S2 of the SI document. The average response for all compounds can be found in Figure 3-S4 of the SI document. The results, as seen in Figure 3-4, show that Pre-Spike VPM is effectively removing the highly volatile analytes from the headspace prior to extraction and reducing the competition for headspace with the SVOCs compared to the high response of volatiles in the no-Vac method. This could introduce variability from sample to

sample if the preparation times are not consistent, including time after sealing for volatiles to equilibrate and time applying vacuum. This variability leads to the need for precise weights of sample material that have been homogenized thoroughly by processes such as milling or cryomilling.

3.4.2 *Vacuum-assisted headspace high-capacity SPME sampling apparatus*

The apparatus shown in Figure 3-5 was constructed to reduce pressure in a sample vial for Vac-HS-HC-SPME sampling; it included a diaphragm vacuum pump and compressor (Model: WP6111560, MilliporeSigma, Burlington, MA; maximum achievable vacuum was -789 mbar with this pumping system). The vacuum pump was connected by metal tubing to an open/close RAVE+ valve (Restek). The metal tubing prevented collapsing and the open/close valve was used to cut off pressure from the vacuum pump. A digital pressure gauge (Type 2074, Ashcroft Inc., Stratford, CT) was used to measure the amount of vacuum in the sampling vial, as well as to detect any leaks during pressure loss. A T-joint was used to connect the digital pressure gauge with both sides of tubing. The T-joint connected the other side to plastic tubing with a Luer lock attachment and a Luer lock side-port gas needle (23 gauge; H pt. style, Restek).

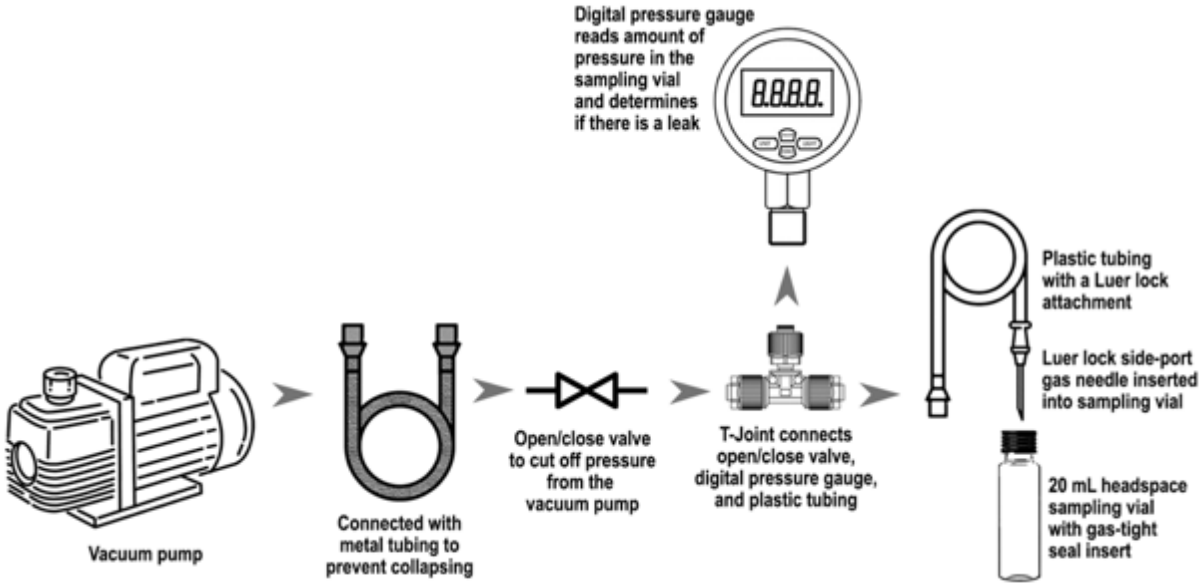

Figure 3-5. Apparatus for applying vacuum to headspace vials.

Steps to ensure enough vacuum has been pulled from the prepared (Pre-Spike+Overnight VPM) sampling vial are as follows: Turn on the vacuum pump. Make sure the gauge reading is at approximately -677 mbar. Insert the needle through the septum of the metal insert. For maximum vacuum, remove the needle after 90 seconds while the vacuum pump is still running. The sample is now ready to be incubated and then sampled for analysis. Only use the open/close valve on an empty sample vial before sample preparation for verification of no leaks in your vacuum setup and production of desired vial pressure. Every vacuum pump is going to operate at different pressures. It is important note that pressure and vacuum time can change responses; consistent results across different vacuum-assistance set-ups should not be assumed [7].

3.4.2.1 *Sampling vial vacuum level and time*

Using vacuum-assisted sampling, an experiment was conducted to determine the minimum magnitude of vacuum applied, and the time needed to maximize the enrichment of

certain compounds in the headspace. The vacuum gauge for the diaphragm vacuum pump was set to different vacuum pressures (-169, -339, -508, -677, and -789 mbar) and vacuum was applied for different times (5, 10, 30, 60, 90, and 120 s). All experiments were performed in triplicate.

Results were compared with HS-SPME sampling where the vial was at ambient pressure. The optimal amount of time needed to apply vacuum was determined to be 90 s. As shown in Figure 3-6, responses for the SVOCs generally increase up to 90 s, then a slight drop in response was observed at 120 s.

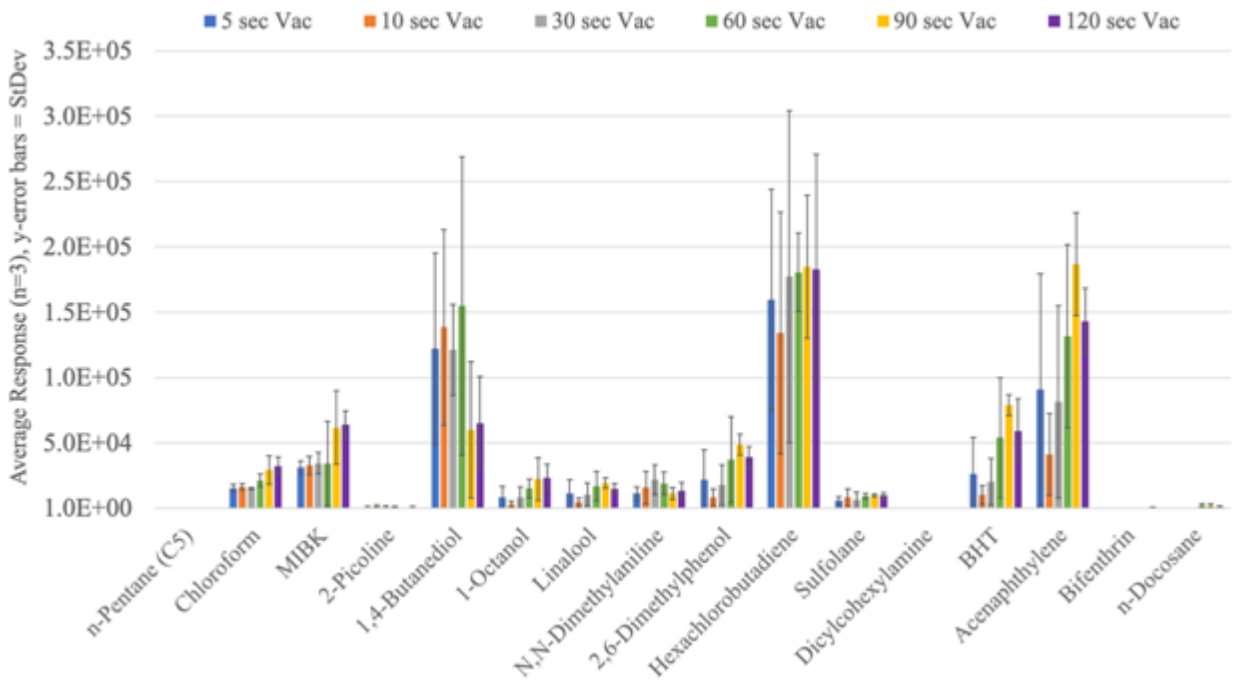

Figure 3-6. Average compound response when vacuum (-677 mbar) was applied to the headspace vial for varied times: 5, 10, 30, 60 90, and 120 s.

There is no significant difference in the responses between 60 s and 120 s, with a p-value greater than 0.05. The vacuum time of 90 s produced the highest response and the best

reproducibility with RSD ≤ 20 % for SVOCs linalool, 2,6-dimethylphenol, sulfolane, BHT, and acenaphthylene. Figure 3-S3 in the SI document gives the % RSD values for all compounds. There is no need to continue pulling vacuum after 90 s as a drop in response was observed at 120 s. Shown in Figure 3-S4, 1,4-butanediol is noticeably reduced after 60 s, showing the removal of highly volatile compounds during the vacuum process.

Vacuum was then applied for 90 s at the pressures -169, -339, -508, -677, and -789 mbar. As shown in Figure 3-7, both -169 and -339 mbar produced similar results as atmospheric HS-SPME. At -508 mbar, the effect of the vacuum was apparent, but the improvement was not substantial.

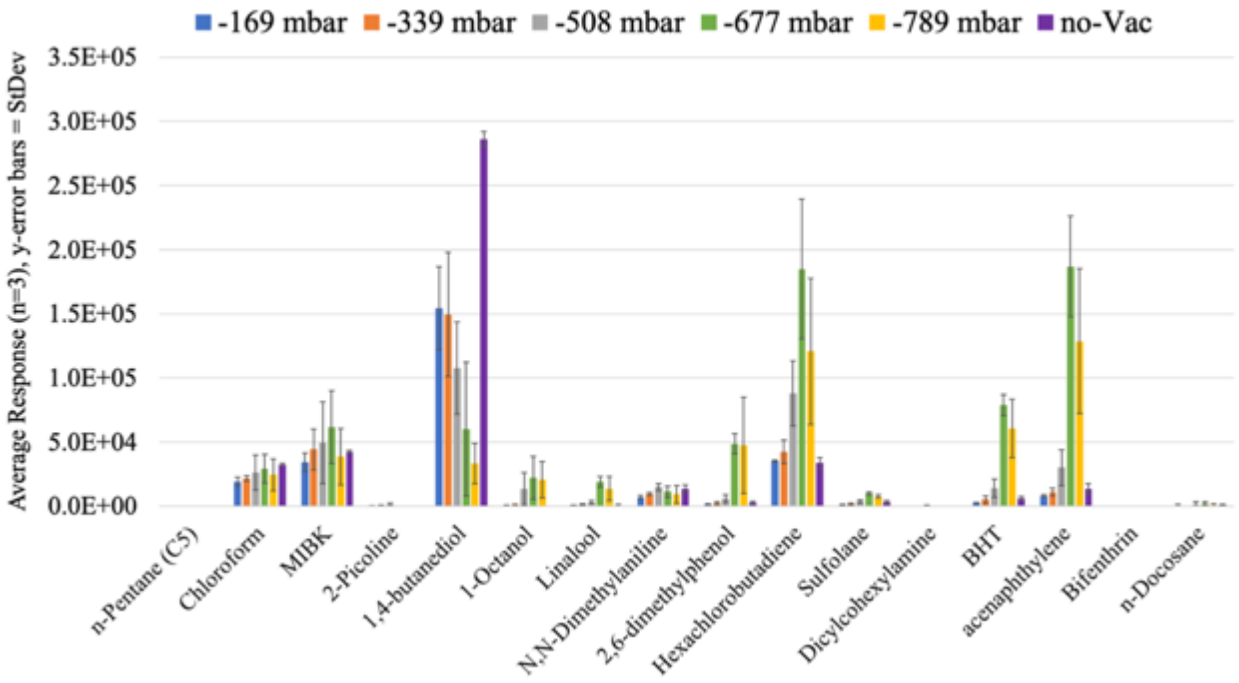

Figure 3-7. Vacuum applied at -169, -339, -508, -677, and -789 mbar to sampling vial for 90 s compared to no-Vac with Vac/no-Vac ratios.

There was a significant difference between results obtained with evacuation at -508 mbar and at -677 mbar, with a p-value less than 0.05. Both -677 mbar and -789 mbar (the maximum achievable for the vacuum pump used) provided a 17.7- and 17.3-fold increase, respectively, as shown in Figure 3-S5 in the SI document, in response for 2,6-dimethylphenol compared to HS-SPME with no vacuum assistance. Representing the trend of volatiles, 1,4-butanediol shows the lack of benefit Vac-HS-HC-SPME provides for volatile analytes alone. Worthy to note, although there was no significant difference in responses (p-value greater than 0.05), -677 mbar was deemed as the better condition on account of reproducibility, as previously shown in Figure 3-S3, with RSD ≤ 20 % for SVOCs linalool, 2,6-dimethylphenol, sulfolane, BHT, and acenaphthylene compared to -789 mbar, the maximum allowable pressure using this pump. It is possible that using the pump at its maximum recommended pressure induces more strain on the pump and causes more variability in the resultant pressure established, sample vial to sample vial, relative to the slightly lower pressure setting.

3.4.3 *Optimized results*

The benefit of Vac-HS-HC-SPME was assessed by Vac/no-Vac ratios, using the optimized sampling parameters. Figure 3-8 shows boiling point (°C) versus order of elution, and the size of the points represent the magnitude of the measured Vac/no-Vac response ratios. Pentane would be the first to elute, but it was not detected, possibly due to its high K_H value as stated earlier. As the boiling point of target analytes increased, Vac/no-Vac response ratios also generally increased. Interesting to note, the nitrogen-containing compounds that eluted later did not follow the same trend, possibly due to a stronger affinity for the sand. It should be emphasized that this is a model system, and every system will exhibit different sets of analyte-

sample interactions, which will cause deviations from strictly vapor pressure-based predictions of analyte response.

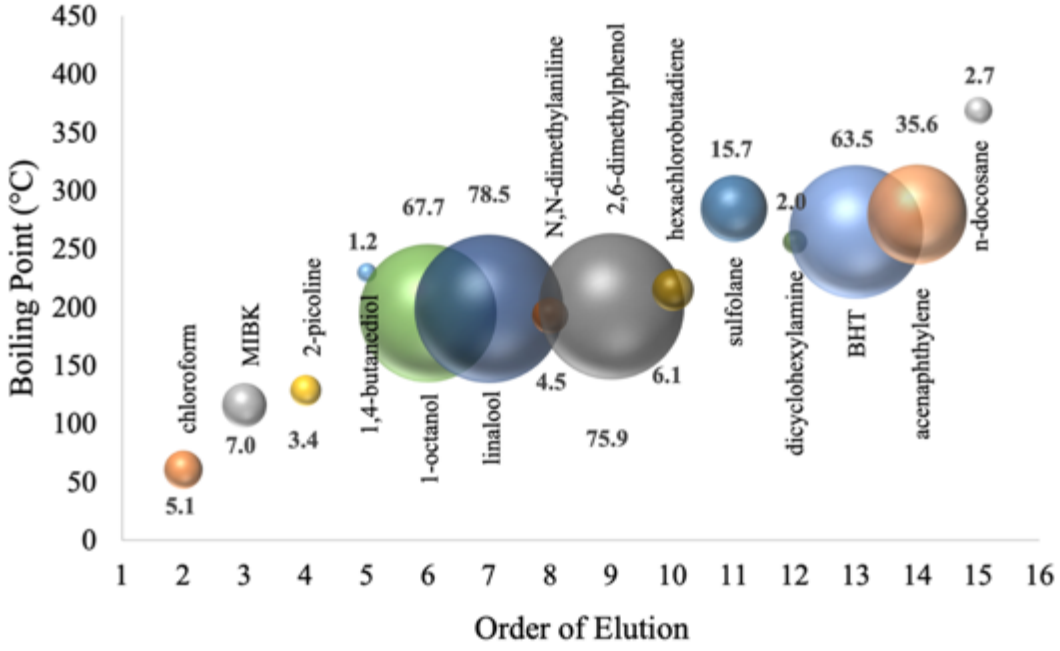

Figure 3-8. Benefit of Vac-HS-HC-SPME for SVOCs compared to HS-SPME at standard atmospheric pressure. Boiling point (°C) versus order of elution where the size of the points represent magnitude of Vac/no-Vac ratios. Each point shows the name of the compound and the average Vac/no-Vac ratio calculation, n=3.

3.5 Concluding Remarks

The goal of this work was to investigate the operational fundamentals necessary for optimal Vac-HS-HC-SPME sampling of solid samples. Operational fundamentals of sample vial preparation methods for an ideal workflow included evacuation at -677 mbar for 90 seconds. A user with a limited budget can use a relatively inexpensive diaphragm vacuum pump to achieve

the desired effects of Vac-HS-SPME sampling. Unfortunately, the process of applying the vacuum to sample vials to assist headspace sampling is not yet automated; a configuration to accommodate automated vacuum-assistance at sufficient pressures using rail-based autosampler configurations is a logical next advancement to consider.

Available septa were tested (SI document), and the current need to replace each septum for each analysis is certainly a limitation of the technique, as it currently stands. It may not be necessary to replace the septum for every experiment in every application. It will depend on the compounds being analyzed and the sample type, since carryover can be variable. Still, septa are not particularly high priced, but this cost could become substantial for large sample sets. Due to the cost and environmental impact of having to replace the septa for each analysis, ideally, new materials would be available in the future that would ameliorate this need and make the technique greener.

There are many possibilities for applying Vac-HS-HC-SPME sampling to other types of solid samples. Ottawa sand was chosen as a model matrix due to its porosity, uniform particle size, and dispersibility. Quantification of analytes should be investigated in future studies. Considerations need to be made for other sample types, such as solid samples that can be milled into a semi-fine powder, and some solids need the addition of dry ice or liquid nitrogen to maintain their solid state. The effects of extended resting times together with the incorporation of standards with various solid sample types requires further investigation and is likely to vary with sample type.

Vac-HS-HC-SPME sampling provides the ability to extract SVOCs at lower temperatures, which provides possibilities for investigation of solid matrices, including food and environmental samples, while avoiding thermal degradation. Perishable foods can remain at refrigerated temperatures for sample preparation and still deliver the necessary flavor and odor compounds in the Vac-HS-HC-SPME sampling process, as shown by Delbecque et al [26].

Some headspace partition coefficients can be found in the literature for different analytes of interest. Both the volatility and the nature of the interactions between the analyte and a solid material will alter these partition coefficients. However, a future consideration would be to use vacuum-assistance to investigate headspace partition coefficients, to maximize discrimination between abundant volatile compounds and less volatile SVOCs. While a solid matrix can be extracted in standard headspace conditions, adding vacuum-assistance offers a new avenue for detection of SVOCs that were once difficult to analyze from solid material.

Data Availability Statement

Data will be made available upon reasonable written request to the corresponding author.

Acknowledgements

The authors would like to thank Prof. Elefteria Psillakis for valuable contributions in the preparation of this manuscript. Additional thanks is given to ExtraTech Analytical Solutions SMPC for providing the metal inserts. SLT and KAS thank Restek Corporation for materials and monetary support needed to complete this study.

Conflict of Interest

The authors CM and JH are employees of Restek Corporation. Restek Corporation is a supplier of Vac-SPME and SPME Arrow materials. Restek provided funding to SLT and KAS for performance of this research. KAS has a potential research conflict of interest due to a financial interest with VUV Analytics, Inc. A management plan has been created to preserve objectivity in research in accordance with University of Texas at Arlington policy.

3.6 Supporting Information

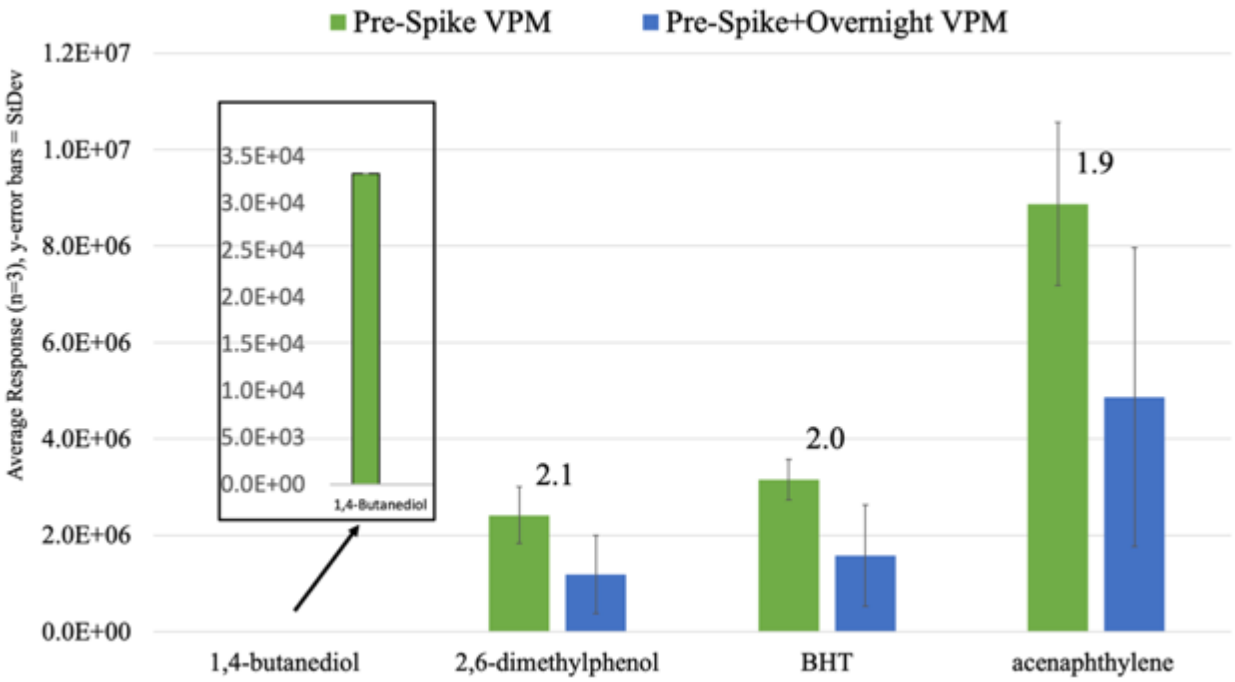

Figure 3-S1. Preferred VPM (sand, spike, seal, vacuum) compared to Preferred+Overnight VPM with the order of 2.5 g Ottawa sand spiked with 50 µL SE mix, sealed, equilibrate overnight, then applied vacuum at -677 mbar (-20 inHg) for 90 s.

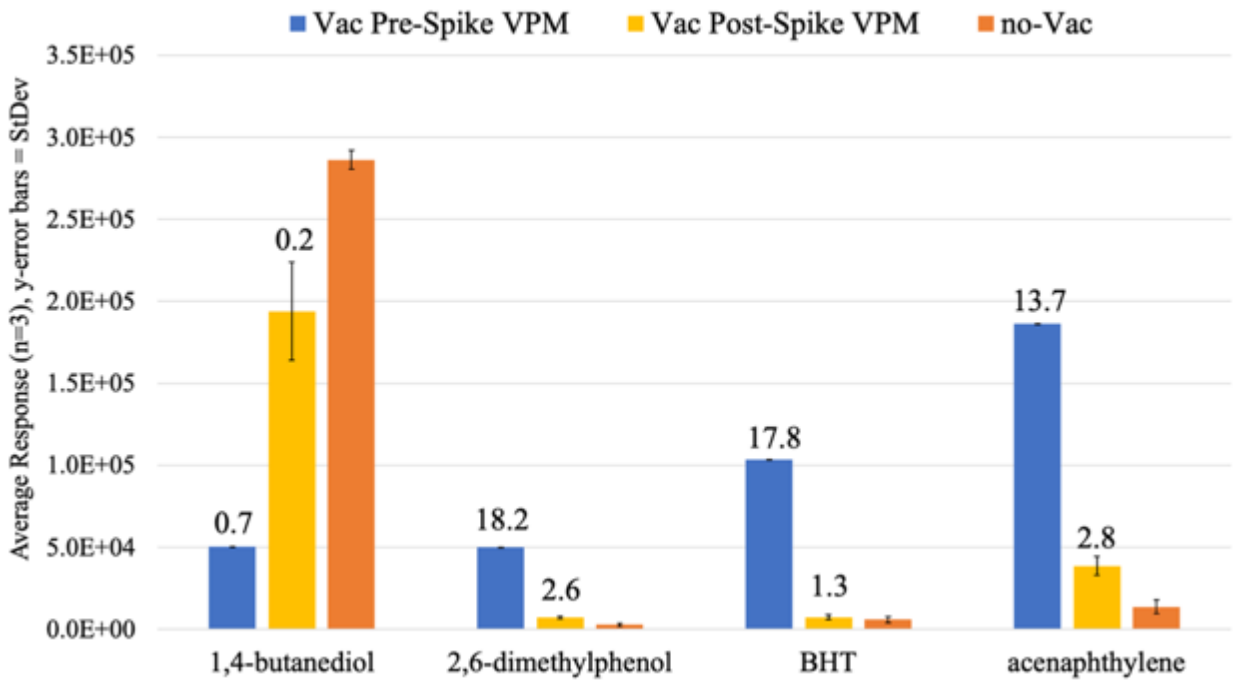

Figure 3-S2. Vac-HS-HC-SPME Preferred vial preparation method (VPM) (spike, seal, vacuum) and Alternate VPM (seal, vacuum, spike) compared to no-Vac.

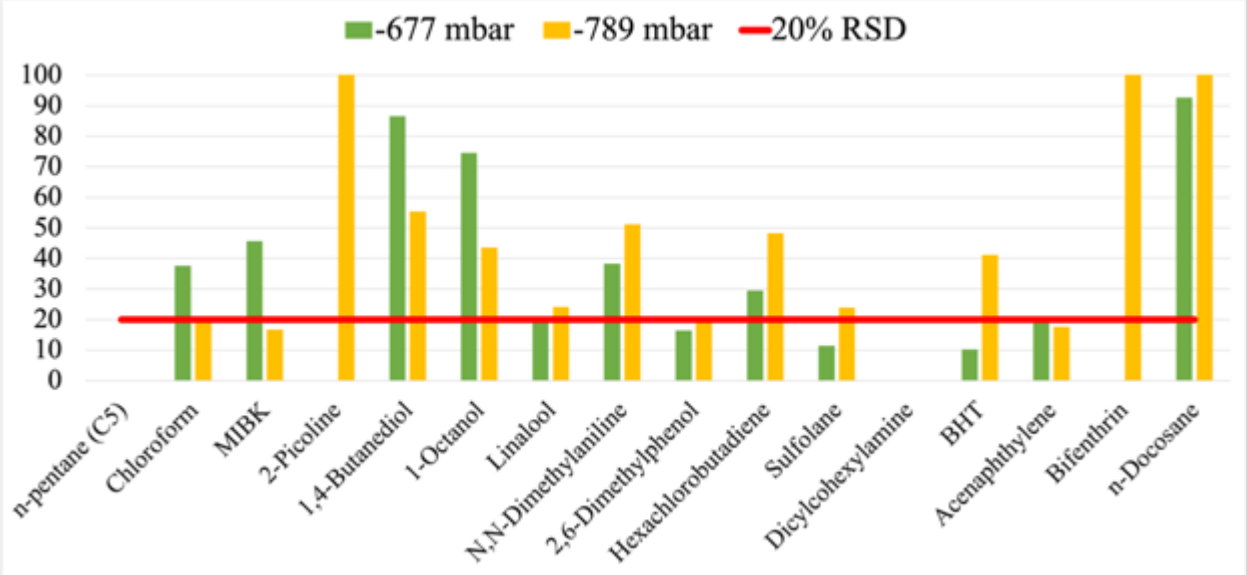

Figure 3-S3. RSD values (%) for Vac Pre-Spike VPM at -677 mbar and -789 mbar.

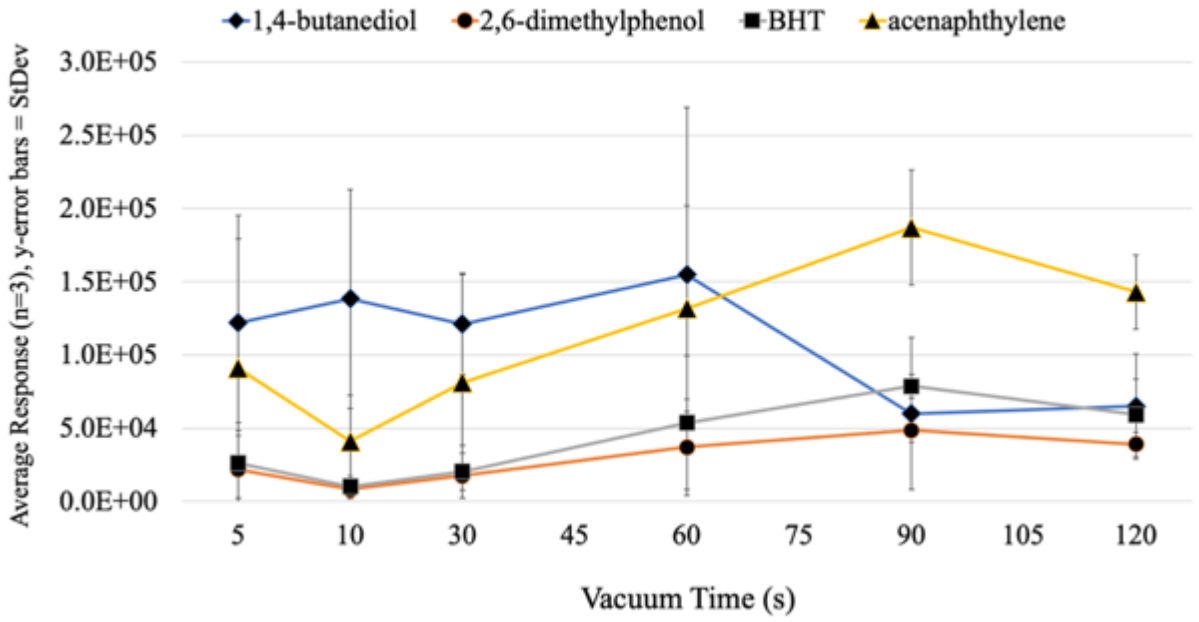

Figure 3-S4. Vacuum (-677 mbar) applied to sampling vial for varied times: 5, 10, 30, 60 90, and 120 s.

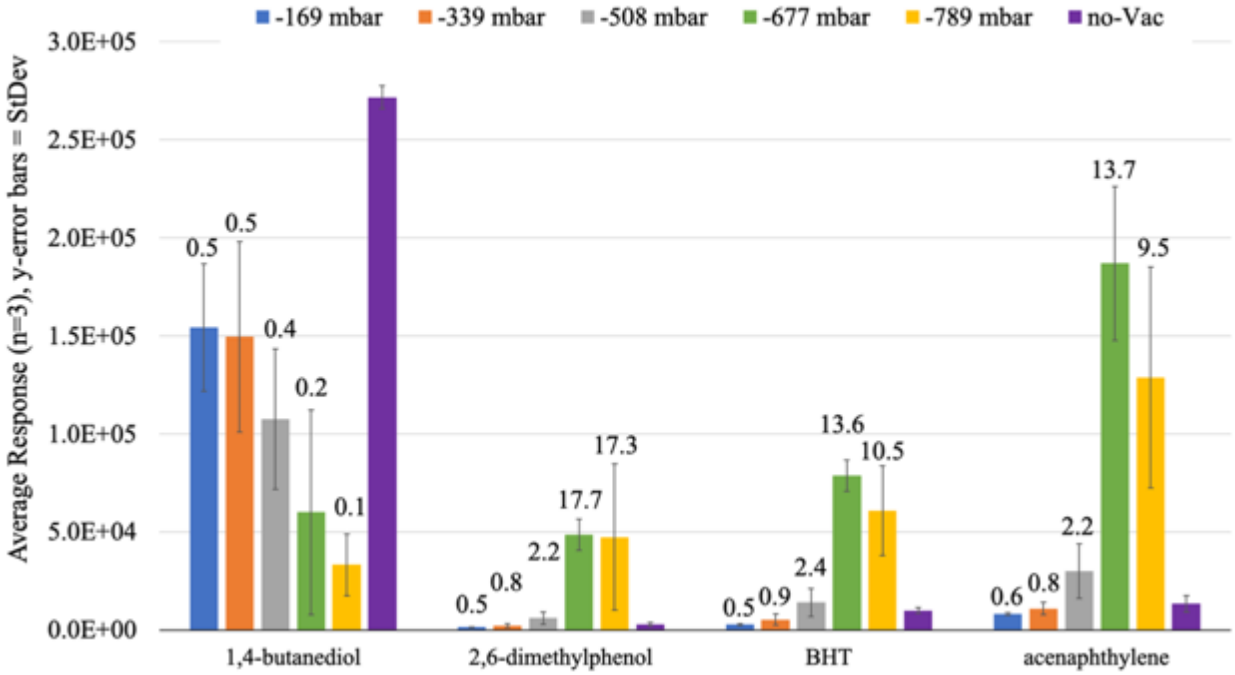

Figure 3-S5. Comparison of pressures at -169, -339, -508, -677, and -789 mbar applied to sampling vial for 90 s.

For experiments investigating seals and septa, a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Scientific Instruments, Inc., Columbia MD) equipped with an AOC-6000 auto sampler system (Shimadzu Scientific Instruments, Inc., Columbia MD), and coupled to a VGA-101 detector (VUV Analytics, Inc., Cedar Park TX), was used. The column was an Rxi-1301Sil MS (30 m x 0.25 mm x 0.25 µm) from Restek. The instrument was operated in constant linear velocity mode (42.7 cm sec^{-1}) using helium carrier gas. Sample extraction was carried out using a HC-SPME Arrow with a polydimethylsiloxane/divinyl benzene (PDMS/DVB) biphasic extraction phase (Restek). A Topaz 1.8 mm ID Straight/SPME Inlet Liner from Restek was used.

3.6.1 O-Rings, Seals, and Septa

Due to the makeup of o-rings and septa, along with their exposure to the headspace and heat, studies were conducted to minimize outgassing of siloxanes, contamination, and carryover of analytes, as well as to optimize the reproducibility/durability of the gas-tight seal. To create a gas-tight environment within the headspace vial, o-rings were placed around the section of the metal insert that interface with the opening of the glass vial. A septum was inserted into the cavity of the metal insert to allow for needle insertion and HS-SPME sampling whilst maintaining a gas-tight seal. Ruggedness, bleed, carryover, and the ability for the o-rings and septa to create and maintain a gas-tight seal were all explored. All experiments were performed in triplicate.

3.6.1.1 O-Rings

When considering o-ring selection, the focus was on durability, as well as the potential for contamination during the extraction phase. Black o-rings consisting of nitrile were the best option due to higher durability, whereas the green o-rings, consisting of hydrogenated nitrile,

degraded significantly after two to five uses. Pieces of the green o-rings would deteriorate, as seen on Figure 3-S6.

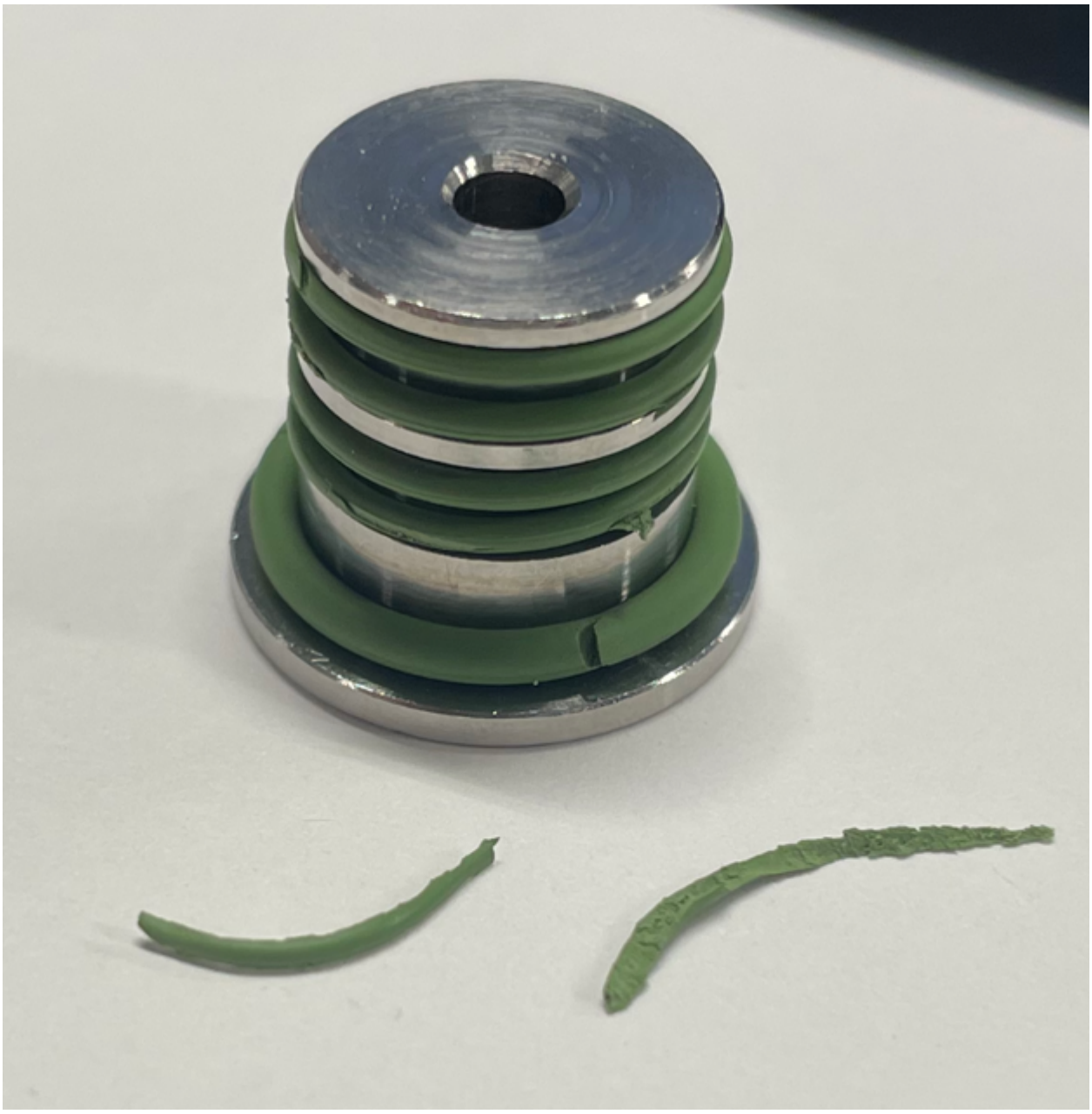

Figure 3-S6. Degradation of the green o-rings consisting of hydrogenated nitrile after two to five uses. Pieces of the green o-rings would tear off and eventually rip apart.

Representative chromatograms are shown in Figure 3-S7, when black and green o-rings were placed in separate vials to test for contamination using HS-SPME methods at atmospheric

pressure. Using a VUV detector for GC, no contamination was detected in the black or green o-rings. Due to the lack of sensitivity for the VUV detector, there could be other contaminant species detectable by more sensitive detectors, such as MS. The position of the o-rings is not in direct contact with the headspace, thus they are less likely to cause contamination.

3.6.1.2 Septa

During the extraction phase, a septum in the vacuum insert was pierced by a SPME Arrow, exposed to temperatures ranging from 30 °C to 80 °C, and directly exposed to the headspace being analyzed. Thermal stability, outgassing of siloxanes, contamination, and carryover need consideration when choosing the ideal septum. Using a VGA-101 detector, Thermogreen® silicone septa were placed in a headspace vial and tested for contamination using HS-SPME methods at atmospheric pressure. As shown in Figure 3-S7, Thermogreen® septa yielded intense siloxane peaks, as well as other contaminants. In the case of untargeted experiments, the unwanted peaks are difficult to eliminate and interfere with the detection of desired analytes.[29] An alternative, BTO (bleed and temperature optimized) septa consist of a platinum cured silicone, which produced no outgassing of siloxanes or other contaminants. The suggested procedure reported by ExtraTech guide for Vac-HS-SPME and Arrow-SPME states to treat the septum at 150 °C for 16 hours. This suggested treatment and others at higher temperatures (200 °C, 250 °C, and 300 °C) have been tested. Higher temperatures degraded the structure of the septa and could not hold a vacuum seal. The suggested procedure reduced response but did not eliminate siloxane contamination, which poses problems when conducting untargeted experiments. This is not a problem when performing a targeted experiment using GC-MS that can select for specific ions.

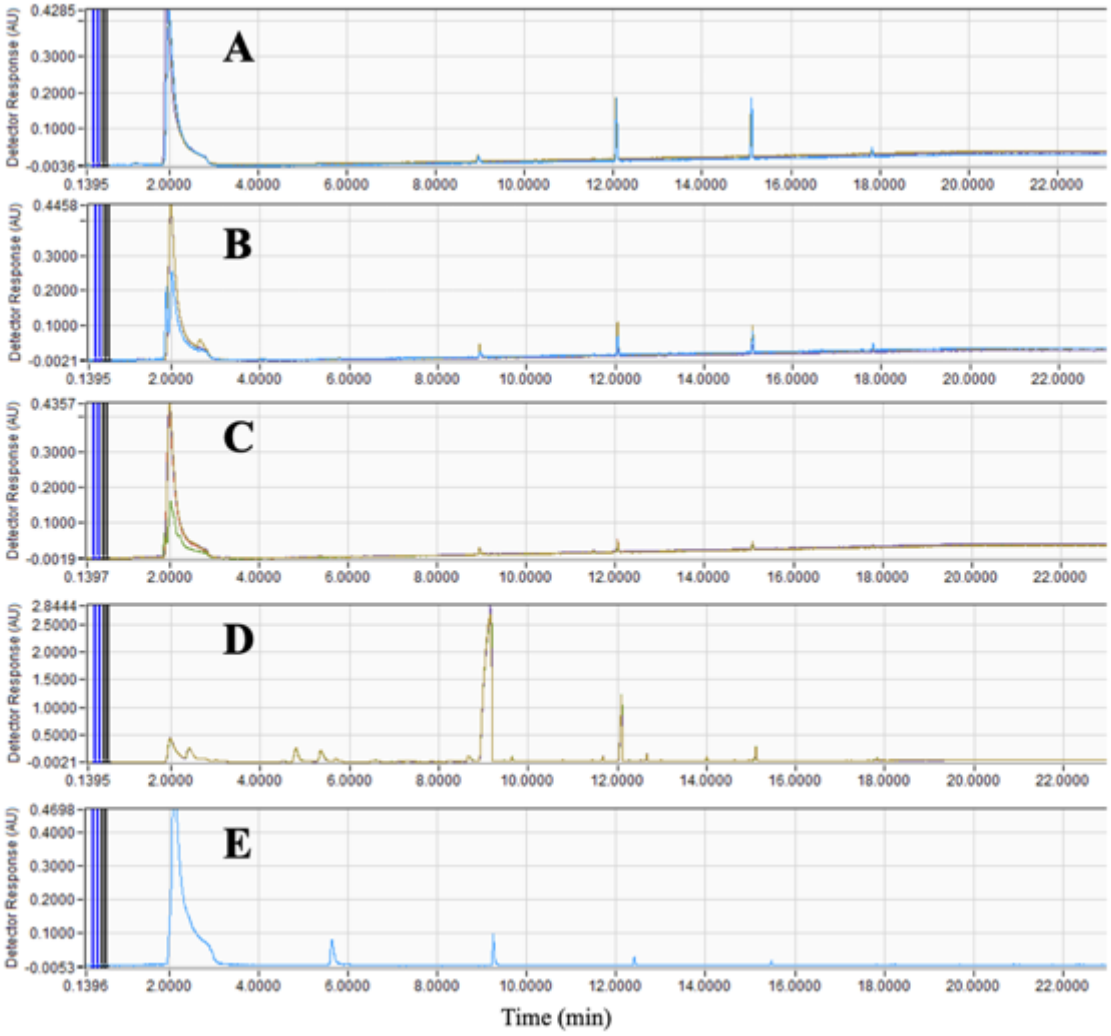

Figure 3-S7. Test for outgassing of siloxanes and other contaminants using gas chromatography with a vacuum ultraviolet detector; data is displayed for absorption from 125 – 160 nm. A.) Blank with only air in the vial; B.) Black o-rings; C.) Green o-rings; D.) ThermoGreen® septa; E.) BTO septa.

Although septa can be used repeatedly and pierced 50 to 100 times and still maintain the gas-tight seal, carryover of analytes can occur even when washing with isopropanol. In Figure 3-S8, the results of a brief study investigating carryover is shown where terpenoids from hemp were tested using GC-VUV. Figure 3-S8A shows a chromatogram of volatiles and semivolatiles extracted from a 50 mg sample of hemp using Vac-HS-HC-SPME. The conditions

show a saturation of the detector for some compounds. Figure 3-S8B shows a subsequent analysis of a used (previously had contact with terpenes in the headspace) BTO septum in the vial, using the same GC method. Shown in Figure 3-S8C is the analysis of a used (previously had contact with terpenes in the headspace) Thermogreen® septum in the vial. The chromatograms of the used septa show carryover of beta-pinene, beta-myrcene, and limonene following hemp analysis. Due to this occurrence, it may be necessary to consider changing the septum for each separate analytical run, depending on the nature of the compounds being analyzed. Not all compounds will produce carryover. Carryover may also be minimized by reducing the amount of solid material extracted. When investigating a new solid sample, once a septum has been used, it should be cleaned and tested prior to its potential for a second use, to check for contamination. Although it is not ideal to have to change septa for each analysis from an environmental standpoint, this may be necessary until a septum that does not produce carryover can be implemented, or sampling conditions can be established that show carryover is minimal, for a given application.

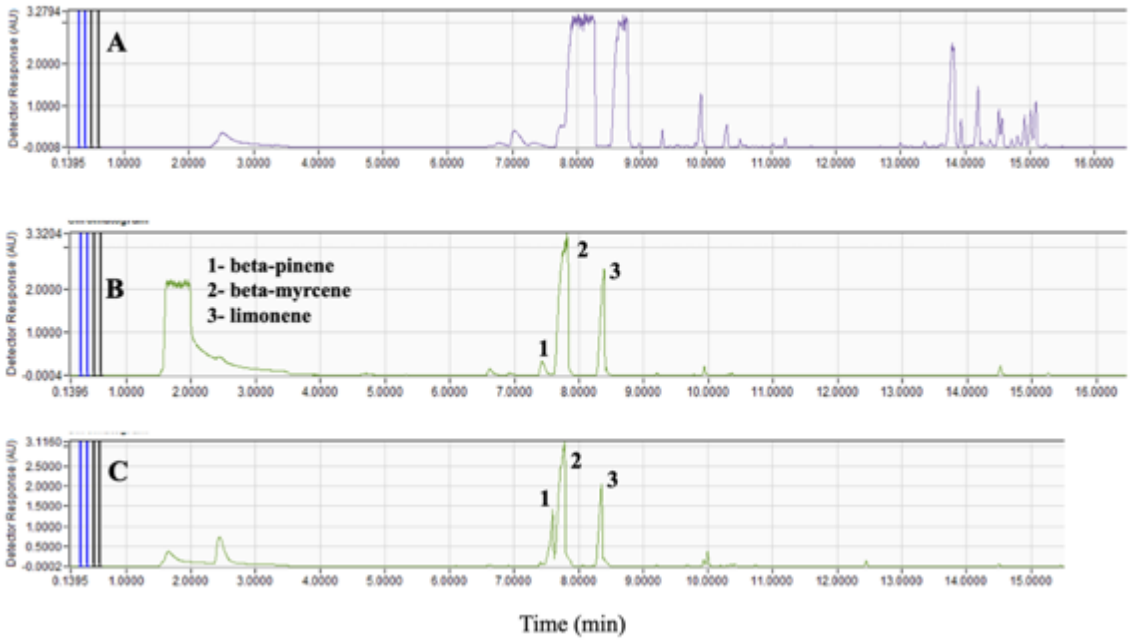

Figure 3-S8. Test for carryover of terpenoids from hemp using GC-VUV, showing absorption from 125 – 160 nm. A.) Chromatogram of terpenes from hemp using Vac-HS-HC-SPME; B.) Analyzed by same GC method with used BTO septum in the vial; C.) Analyzed by same GC method with used Thermogreen® septum in the vial.

3.6.1.3 Gas-tight Seal in the Sample Vial

An experiment was conducted to determine the reproducibility of the amount of air evacuated from the vial and the degree to which the gas-tight seal holds. The vacuum pressure gauge was set to -677 mbar. Air was evacuated from the vial for 90 s. A syringe with 20 mL of deionized water was then inserted into the septum of the vial and the volume of water pulled into the vial was recorded. Vials sealed with Thermogreen® septa pulled an average of 15.5 ± 0.1 mL (1% RSD), while those sealed with BTO septa pulled an average of 13.1 ± 2.0 mL (18% RSD). The Thermogreen® septa created the best seal and provided a more reproducible seal for

evacuated vials. Table 3-S1 displays the complete list of measurements associated with these results.

Table 3-S1. Vac-HS-HC-SPME sampling applied -677 mbar (-20 inHg) for 90 s. Tested sample vial pressure by measuring amount of DI water (mL) pulled from syringe.

Attempt	**Thermogreen® Septa DI Water (mL)**	**BTO Septa DI Water (mL)**
1	15.6±0.1	14.4±1.3
2	15.5±0.0	15.4±2.3
3	15.6±0.1	10.6±2.5
4	15.5±0.0	14.8±1.7
5	15.5±0.0	15.0±1.9
6	15.4±0.1	15.3±2.2
7	15.6±0.1	14.0±0.9
8	15.8±0.3	12.0±1.1
9	15.6±0.1	8.0±5.1
10	15.2±0.3	11.7±1.4
Average±absSD (mL)	**15.5±0.1**	**13.1±2.0**
RSD (%)	**1**	**18**

Although BTO septa removed the issue of initial contamination, it produced carryover and does not provide the proper seal for reproducible results. The seal provided by the Thermogreen® septum is more important to Vac-HC-HS-SPME sampling than the hassle of off-gassing siloxanes. The user needs to be aware of the numerous siloxane peaks that must be subtracted when performing untargeted work.[29] At the moment, this particular Thermogreen® septa is only made in the LB-1 material. The Thermogreen® LB-2 material is more heat resistant and prevents off-gassing similar to the BTO septa. Thermogreen® LB-2 septa exist as GC injection port septa but not currently in the cylindrical half-hole design needed for this insert.

CHAPTER FOUR

COMPARISON OF FRAGRANCE AND FLAVOR COMPONENTS IN NON-PSILOCYBIN AND PSILOCYBIN MUSHROOMS USING VACUUM-ASSISTED HEADSPACE HIGH-CAPACITY SOLID-PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY – MASS SPECTROMETRY

Thomas, S. L., Myers, C., Schug, K. A., Comparison of fragrance and flavor components in non-psilocybin and psilocybin mushrooms using vacuum-assisted headspace high-capacity solid-phase microextraction and gas chromatography – mass spectrometry. *J. Sampre.* 2023; 8:100090. https://doi.org/10.1016/j.sampre.2023.100090[29]

Comparison of Fragrance and Flavor Components in Non-Psilocybin and Psilocybin Mushrooms using Vacuum-Assisted Headspace High-Capacity Solid-Phase Microextraction and Gas Chromatography – Mass Spectrometry

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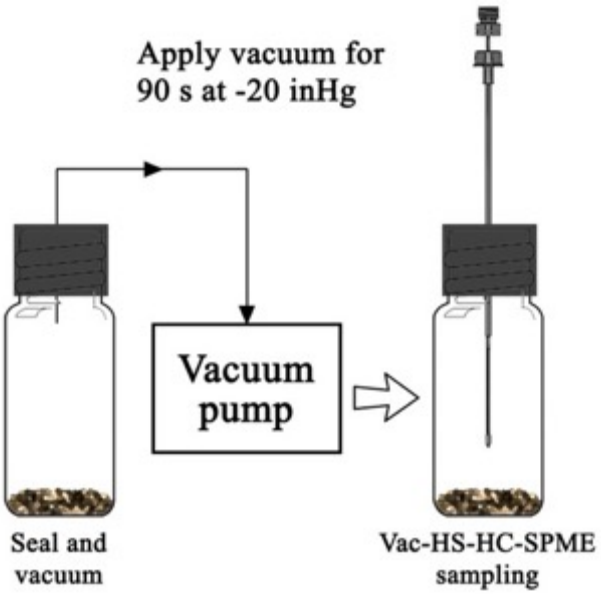

Figure 4-graphical abstract

4.1 Abstract

Vacuum-assisted headspace high capacity solid-phase microextraction (Vac-HS-HC-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) was used to compare the volatile compounds that make up the volatile and semi-volatile components of five psilocybin mushrooms (*Psilocybe cubensis*), as well as three non-psilocybin mushroom species. Using an

untargeted analysis, common volatiles detected consisted of acids, alcohols, aldehydes, ketones, and hydrocarbons. The initial comparison of Vac-HS-HC-SPME and HS-HC-SPME conditions showed 2 times increase in compound response as well as the detection of 8 additional compounds undetected by HS-HC-SPME. Compounds unique to psilocybin mushrooms were 2-methylbutanal, valeraldehyde, benzaldehyde, 3-octen-2-one, 2-methyl-dodecane, and 2-butyl-2-octenal. Compounds unique to non-psilocybin mushrooms were 2-methyl-pyrazine, 2,3-butanediol, butyric acid, butyrolactone, benzyl alcohol, 2-pyrrolidinone, and estragole. The commonly shared compound, 1-octen-3-ol, was shown to have a higher compound response among the psilocybin mushroom species.

Keywords: Vac-SPME, semi-volatiles, untargeted, sample preparation, and psychedelic

4.2 Introduction

Edible mushrooms have been enjoyed by many for the fragrance and flavor they provide to a meal. Mushrooms have been reported to have medicinal properties, such as antioxidant,[41] antiviral,[42] and prebiotic[43] attributes, mainly conferred by the presence of polysaccharides and different volatile compounds. Mushrooms are also a good source of essential fatty acids.[44] Volatile compounds are key components to their flavor and fragrance and different species can potentially be differentiated and classified by the various and variable chemical markers detected. Different genetic strains contain significant variability in their volatile content. Edible mushrooms also have distinct fragrance and flavor components. The mushroom alcohol, 1-octen-3-ol, gives mushrooms a distinct flavor, and this compound was also demonstrated to attract pests.[45] Acids such as acetic acid, isovaleric acid, butanoic acid,

and propionic acid have been shown to be responsible for the variations of flavors among mushroom species.[46]

Psilocybin mushrooms (*Psilocybe cubensis*), also known as magic mushrooms, are a group of fungi that contain psilocybin, a phosphorylated tryptamine precursor to the psychoactive substance psilocin. Psilocybin converts readily into psilocin in the presence of moisture and following ingestion.[47] Psilocybin mushrooms have been used for spiritual rituals, recreational drugs, and most recently for micro-dosing and psychedelic therapy. The primary interest in *P. cubensis* studies reported previously have primarily revolved around characterization of psilocybin, psilocin, and other potentially bioactive tryptamine compounds.[48] An investigation of volatile components in *P. cubensis* has not been previously reported.

Studies have been conducted to identify volatile compounds in edible mushrooms. These compounds consist of a variety of alcohols, aldehydes, ketones, and esters.[46] Analyses were performed using gas chromatography-mass spectrometry (GC-MS). The sample preparation of edible mushrooms varied. Ground pine-mushrooms were extracted by dichloromethane, filtered, and separated using high-vacuum sublimation, then dehydrated. The liquid was injected into the GC-MS.[49] Petrovic et al.[50] extracted ground mushrooms with methanol, acetone, and dichloromethane over a 24-hour period, then filtered. This was repeated two more times for a total of three days. A solid-phase microextraction (SPME) fiber was exposed to the sample then directly injected to the GC-MS. Tian et al.[46] ground and sealed mushrooms in a vial, then incubated at 60 °C for 20 minutes, exposed a SPME fiber to the headspace (HS) for 30 minutes, then inserted into the injector for 20 minutes for desorption

and analysis of the volatile compounds. They discovered nonanoic acid, 9-oxo-methyl ester, 2-pentyl-furan and 5,6-dihydro-2-pyranone in the species *C. yunnanensis* for the first time.

Heating a solid sample such as mushrooms can change its volatile composition due to various chemical reactions. These can be avoided using lower temperatures and vacuum-assisted HS-SPME (Vac-HS-SPME).[35] This sampling method involves a reduction in pressure of the sample vial pre-equilibrium by applying a vacuum, which accelerates the kinetics involved in the extraction of semi-volatile compounds. The Vac-HS-SPME technique was coined in 2012 by Psillakis et al.[6] For Vac-HS-SPME of liquid samples, the pressure in the vial is reduced in the sampling headspace by evacuating the air from a sample container before introducing the sample. For solid samples, the sample must be present in the vial prior to applying vacuum. Vac-HS-SPME sampling for solids samples reported in the literature have included analysis of the volatiles from raw fish at sub-ambient temperatures,[26] green sampling techniques for oil bearing source-rock analysis,[27] and a combined method for analysis of terpenoids and cannabinoids in hemp.[28]

The aim of this untargeted study was to compare the volatile compounds that make up the aromatic and flavor components of five psilocybin mushroom and three non-psilocybin mushroom varietals using Vac-HS-high capacity (HC)-SPME coupled with GC-MS. High capacity refers to the SPME Arrow, developed by CTC Analytics AG, which is a redesigned SPME fiber that has increased mechanical robustness and provides greater sensitivity due to a larger phase volume.[38,39]

4.3 Experimental

4.3.1 *Instrumentation*

Instrumentation and sample preparation parameters are detailed in Table 4-1. A Shimadzu GC-2010 Plus gas chromatograph equipped with an AOC-6000 auto sampler system, was coupled to a GCMS-TQ8030 mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia MD) for mushroom volatiles analysis. The column used was an Rxi-1301Sil MS (30 m x 0.25 mm x 0.25 µm) from Restek Corporation (Bellefonte, PA). The instrument was operated in linear velocity mode (42.9 cm/sec) using helium carrier gas. Sample introduction was carried out using a SPME Arrow with a divinyl benzene/polydimethylsiloxane (DVB/PDMS) biphasic extraction coating (Restek Corporation, Bellefonte, PA). The DVB/PDMS is a mid- to semi-volatile range phase coating which has been shown to provide similar extractions of different compound classes.[51] The biphasic phase also tends to provide more reproducible extractions as indicated in a prior study by Zanella et al.[52] A Topaz 1.8 mm ID Straight/SPME Inlet Liner from Restek Corporation was used for all applications. An initial experiment with a ramp rate of 3 °C/min up to 310 °C was conducted initially to investigate the need for a fast versus slow oven temperature rate. This lower temperature ramp rate was found not to provide significant benefit for compound detection.

Peaks considered for identification had a signal to noise (S/N) ratio greater than five. Identification of compounds from mass spectra was determined using Shimadzu LabSolutions Postrun Analysis with the NIST17-1 library. Compounds were accepted as positive identification when at least 2/3 replicates had a match factor greater than 80%. The retention index was not

used to verify the identification of compounds, thus identified compounds are considered tentative.

Table 4-1. SPME Arrow sampling parameters, gas chromatograph (GC) and mass spectrometer (MS) programming parameters

[a]**AOC-6000 Sampling Parameters**		[b]**GC-2010 Plus/GCMS-TQ8030 GC-MS Parameters**	
[c]**Vac-HS-HC-SPME**		**Inlet**	
Tool	SPME Arrow	280 °C	
Agitator Speed	500 rpm	Split 10:1	
Agitator Temp.	30 °C	[d] Topaz 1.8 mm ID Straight/SPME Inlet Liner	
Incubation Time	60 s		
Heatex Stirrer Speed	500 rpm	[d]**Column**	
Heatex Stirrer Temp.	30 °C	Rxi-1301Sil MS, 30 m, 0.25 mm ID, 0.25 µm	
Extraction Time	600 s		
Vial Penetration Depth	45 mm	**Oven**	
Injector Penetration Depth	45 mm	35 °C (hold 2.0 min) to 160 °C at 15 °C min^{-1} (hold 1.0 min) to 180 °C at 5 °C min^{-1} to 275 °C at 30 °C min^{-1} (hold 2.0 min)	
Desorption Time	60 s		
Pre-Conditioning	True	**Carrier Gas**	
Post Conditioning	False	Type	Helium
Conditioning Time	60 s	Mode	Linear Velocity
Conditioning Temp.	280 °C	Linear Velocity	42.9 cm/sec
		Flow Rate	1.42 mL min^{-1}
		[b]**Detector**	
		Type	Triple Quadrupole MS
		Mode	Scan (20 to 500 m/z)
		Ion Source Temp.	230 °C
		Interface Temp.	250 °C
		Electron Energy	70 eV

[a] SPME autosampler: AOC-6000 (Shimadzu Scientific Instruments, Inc., Columbia MD)

[b] Gas chromatograph – GC-2010 Plus, Mass spectrometer (MS) – GCMS-TQ8030: Shimadzu Scientific Instruments, Inc., Columbia MD

[c] SPME in Vac-HS-HC-SPME mode

[d] Restek Corporation, Bellefonte, PA, USA

4.3.2 *Sample preparation*

As shown in Figure 4-1, samples from five types of dried (8 – 10% moisture by weight) psilocybin mushrooms were provided by the Scottsdale Research Institute (Scottsdale, AZ) under Drug Enforcement Agency schedule 1 licensure (Thai Cubensis, Blue Meanie, Texas Yellow, Creeper, and B+). Three types of dried non-psilocybin mushrooms (button, wild forest, and shiitake) were acquired from local grocers. All samples were independently milled using a Fritsch Pulverisette 11 blade mill (Fritsch Milling and Sizing, Inc., Pittsboro, NC).

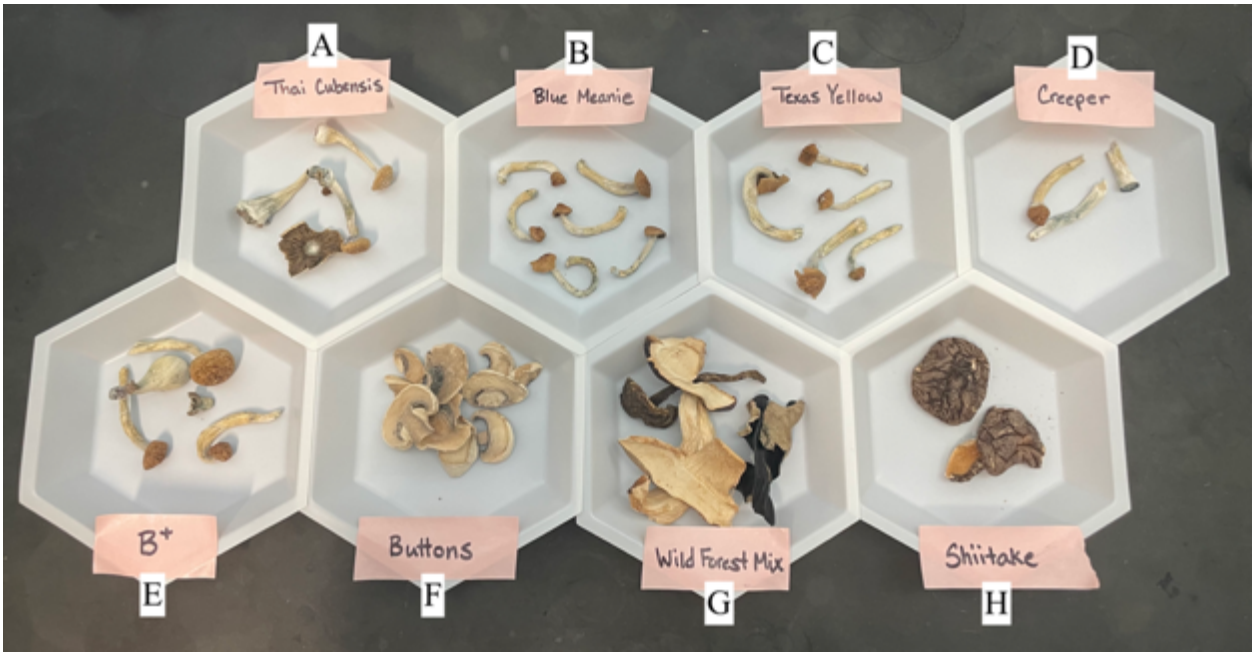

Figure 4-1. Psilocybin mushrooms (*Psilocybe cubensis*): A. Thai Cubensis, B. Blue Meanie, C. Texas Yellow, D. Creeper, and E. B+; Non-psilocybin mushrooms: F. Button, G. Wild Forest (mix of shiitake, oyster, porcini, and black fungus), H. Shiitake

Volatile constituents in mushrooms can be lost through the milling process due to heat exposure from contact with the blade and/or the motor overheating. This is a result of high

speeds (> 10,000 rpm) and/or extended homogenization periods (> 10 s) without pauses. To avoid this loss, the milling parameters, as listed in Table 4-2, were set at lower speeds (5,000 rpm) and used short bursts (8 s). Failure to optimize these conditions could produce poor recoveries and reproducibility of the volatile constituents in many samples, including mushrooms.

Table 4-2. Fritsch Pulverisette 11 blade mill settings

Step	RPM	Run (s)	Stop (s)	Repetition	Rotation
1	5000	8	2	8	Right
2	5000	8	2	8	Left
3	5000	8	3	8	Right

A 20 mL headspace screwcap vial (Restek Corporation, Bellefonte, PA) was filled with 200 mg of homogenized mushroom samples. To create a gas-tight seal, a stainless-steal insert (provided by Prof. Elefteria Psillakis, ExtraTech Analytical Solutions SMPC) with a hole to allow for a Thermogreen® LB-1 septum with half-hole (Supelco, Bellefonte, PA) was placed in the vial opening. For Vac-HS-HC-SPME sampling, shown in Figure 4-2, a Luer lock side-port gas needle (23 g/H pt. style, Restek Corporation, Bellefonte, PA) attached to a diaphragm vacuum pump and compressor (Model: DOA-P704-AA, GAST Manufacturing, Inc., Benton Harbor, MI) was inserted into the vial. Vacuum was pulled for 90 s at -20 inHg.

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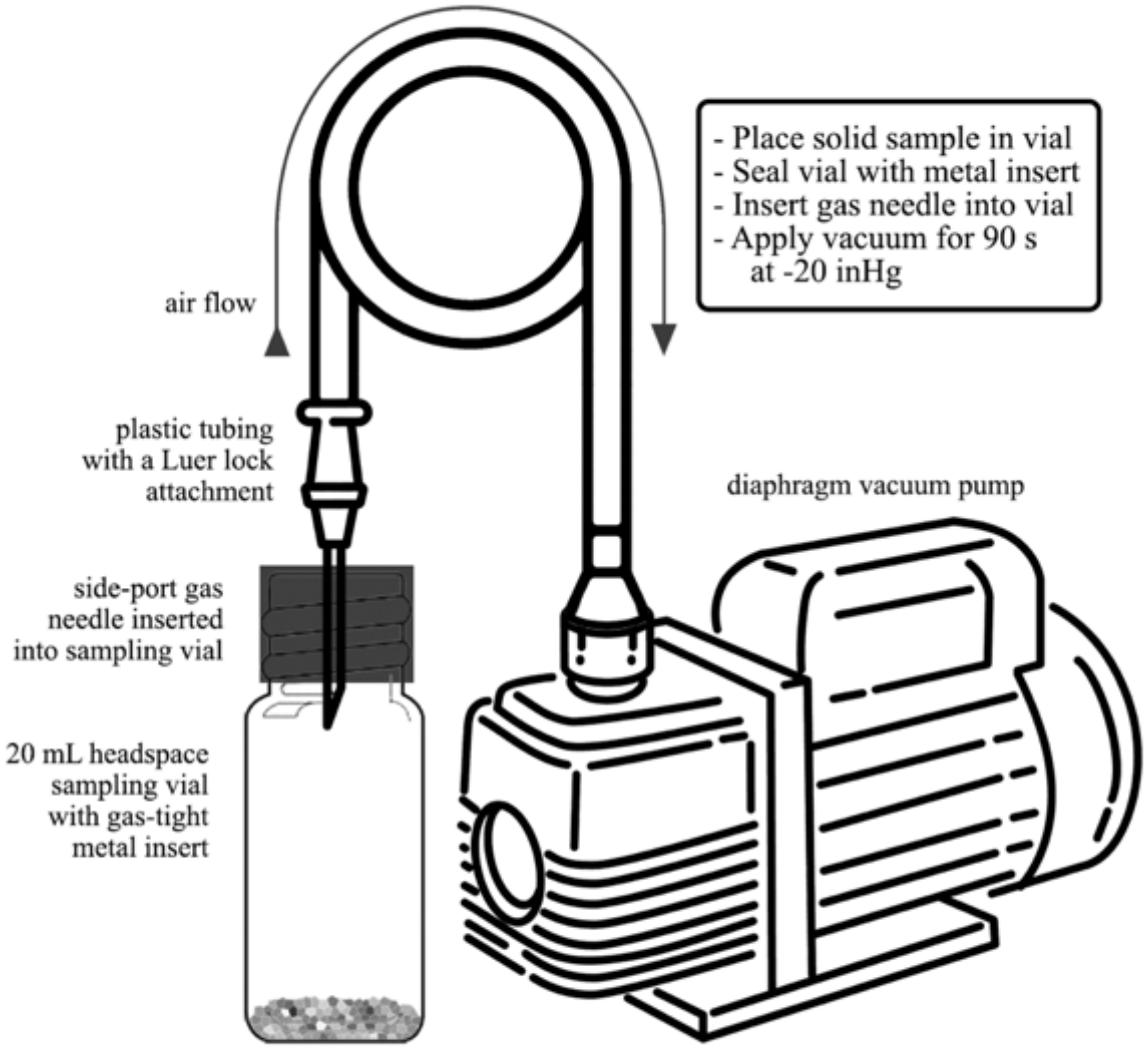

Figure 4-2. Vac-HS-HC-SPME Sampling Setup

All samples were prepared and analyzed in triplicate, including evacuated air blanks for evaluation of background contaminants.

4.4 Results and Discussion

Vac-HS-SPME increases the degree of analyte partitioning by shifting the equilibrium toward a higher headspace concentration for semi-volatile analytes through a reduction of the pressure in the sample vial. This phenomenon enables the Vac-HS-SPME approach to evaluate a wider range of volatiles, particularly the addition of semi-volatiles compared to HS-SPME. Solid samples can be analyzed in a minimally unadulterated form using Vac-HS-SPME with simple milling of the sample and no solvents needed. Mushroom samples were homogenized, placed in the vial, air evacuated, and incubation and extraction steps were be performed at 30 °C. Utilizing the SPME Arrow, Vac-HS-HC-SPME was used to compare the volatiles and semi-volatile constituents present in psilocybin and non-psilocybin mushrooms.

4.4.1 *Comparison of Vac-HS-HC-SPME and HS-HC-SPME*

A study was conducted to validate the use of Vac-HS-HC-SPME sampling as a reasonable method for detecting odor and flavor compounds of various mushrooms. Thai Cubensis was examined at 30 °C and 40 °C using both Vac-HS-HC-SPME sampling and HS-HC-SPME sampling at standard atmospheric pressure. As shown in Figure 4-3, Vac-HS-HC-SPME at 30 °C (A) has a two-times increase in response intensity compared to both 30 °C (B) and 40 °C (D) HS-HC-SPME. Vac-HS-HC-SPME provides a marginal improvement with respect to semi-volatile compounds.

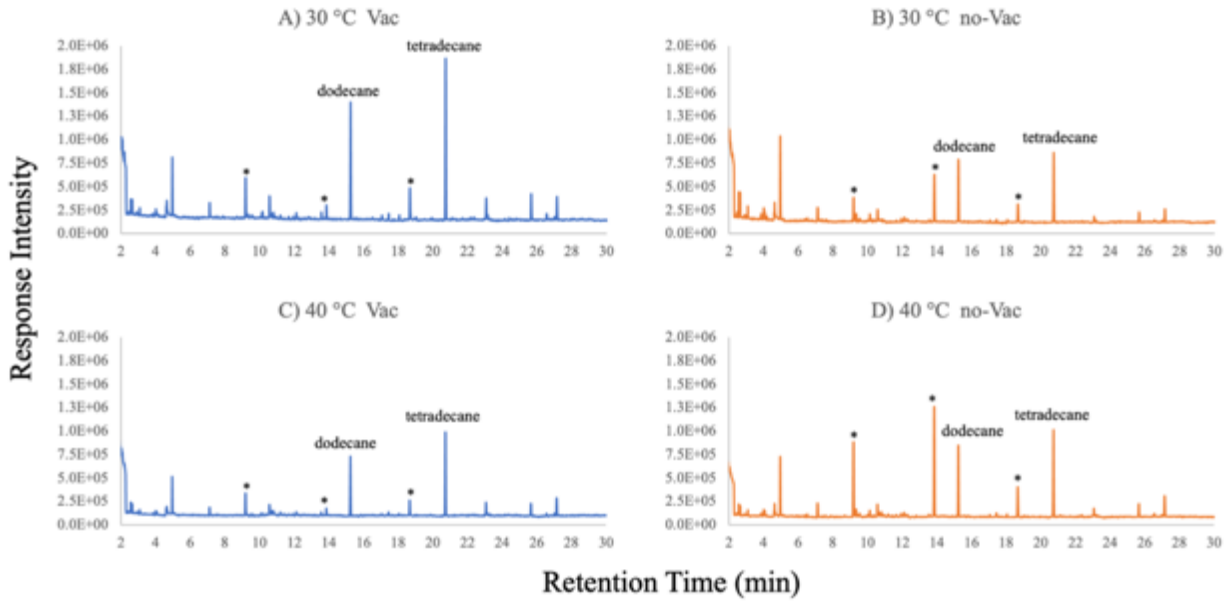

Figure 4-3. Comparison of Vac-HS-HC-SPME (Vac) and HS-HC-SPME (no-Vac) sampling at 30 °C and 40 °C of the Thai Cubensis psilocybin mushroom. The symbol (*) represents siloxane peaks.

Figure 4-4 further exhibits that at 30 °C, Vac-HS-HC-SPME detects eight more semi-volatile compounds and a two-times increase in peak area for the semi-volatiles compared to HS-HC-SPME. The volatile compounds methyl-cyclopentane, trimethyl-silanol, and 2-methyl-1-butanol were likely largely removed from the headspace during the vacuum process.

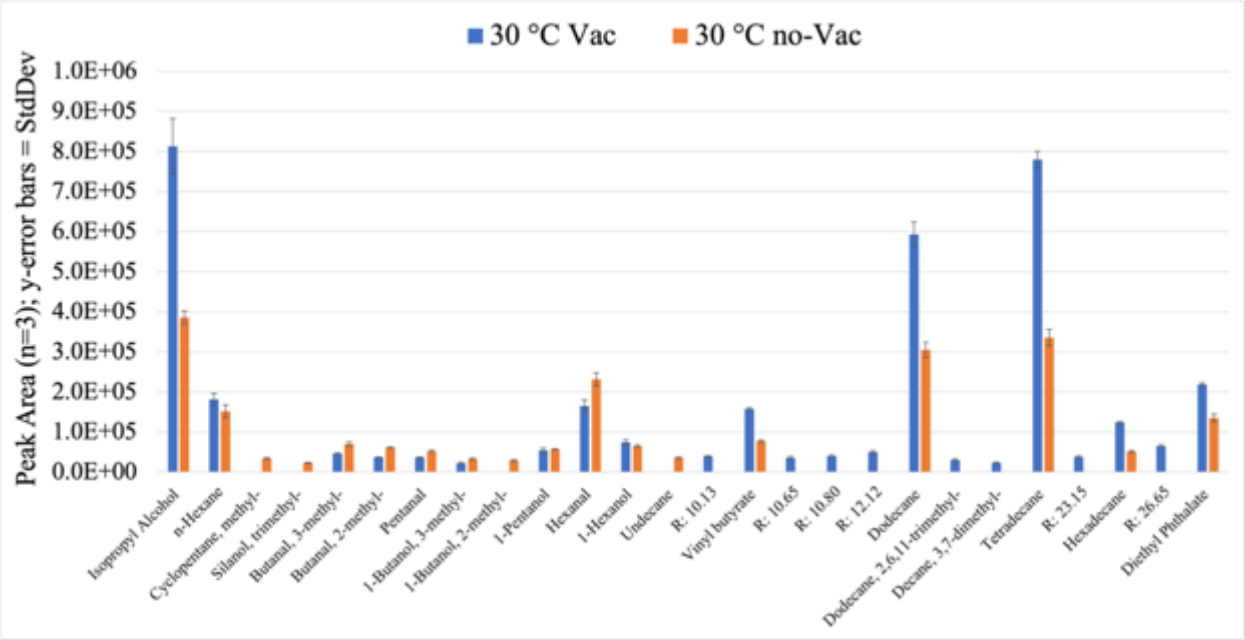

Figure 4-4. Comparison of Vac-HS-HC-SPME (Vac) and HS-HC-SPME (no-Vac) at 30 °C of the Thai Cubensis psilocybin mushroom. Vac-HS-HC-SPME outperforms no-Vac sampling with a two-times increase in peak area and detection of eight more semi-volatile compounds.

4.4.2 *Psilocybin Mushrooms*

For the psilocybin mushrooms, from the 278 peaks detected, 45 compounds met the threshold for detection (S/N > 5) and 34 of those compounds were identified. All relevant information for the compounds identified in the psilocybin mushroom samples can be seen in Table 4-3.

Table 4-3. Chemical compounds identified in psilocybin mushrooms, including their peak number, retention time, name, formula, molecular weight (MW), characteristic m/z, boiling point (°C), and flavor/fragrance properties.

Peak #	Retention Time (min)	Compound Name	Formula	MW (g/mol)	m/z	Boiling Point (°C)	Flavor/Fragrance
1	2.82	Isovaleraldehyde	$C_5H_{10}O$	86.13	58	92.5	Warm, herbaceous, fruit- & nut-like taste; apple-like odor
2	2.91	2-Methylbutanal	$C_5H_{10}O$	86.13	29	92.5	Unpleasant odor
3	3.18	1-Butanol	$C_4H_{10}O$	74.12	31	117	Whiskey characteristic, mild alcoholic odor
4	3.36	Valeraldehyde	$C_5H_{10}O$	86.13	44	103	Warm, fruity & nut-like taste; chocolate aroma
5	4.04	2-Methylpentanal	$C_6H_{12}O$	100.16	43	117	Woody, oily ham taste & smell
6	4.16	Isoamyl alcohol	$C_5H_{12}O$	88.15	55	132.5	Pungent repulsive taste & disagreeable odor
7	4.20	2-Methyl-1-butanol	$C_5H_{12}O$	88.15	57	129	Cooked roasted aroma with alcoholic undertones
8	4.59	1-Pentanol	$C_5H_{12}O$	88.15	70	137	Burning tast with a fusel-like odor
9	4.79	Caproaldehyde	$C_6H_{12}O$	100.16	56	130	Fatty-green pungent odor with a green, woody, fruity taste
10	5.41	2,3-Dimethyl-1-butanol	$C_6H_{14}O$	102.17	71	142	Mild alcoholic odor
11	5.68	Nonane	C_9H_{20}	128.25	85	150	Gasoline-like odor
12	5.78	Diacetone alcohol	$C_6H_{12}O_2$	116.16	59	168	Faint, pleasant odor
13	5.94	1-Hexanol	$C_6H_{14}O$	102.17	56	157	Fatty, fruity taste with a sweet alcoholic odor
14	6.19	Heptanal	$C_7H_{14}O$	114.19	70	153	Fatty, fruity taste & odor
15	6.95	Decane	$C_{10}H_{22}$	142.28	57	174	Gasoline-like odor
16	7.02	2-Pentyl-furan	$C_9H_{14}O$	138.21	81	65	Fruity aroma
17	7.09	Benzaldehyde	C_7H_6O	106.12	77	179	Bitter almond oil taste & odor
18	7.16	1-Octen-3-one	$C_8H_{14}O$	126.2	55	60	Mushroom odor
19	7.28	1-Octen-3-ol	$C_8H_{16}O$	128.21	57	84	Sweet, earthy, herbaceous odor
20	7.41	Vinyl butyrate	$C_6H_{10}O$	114.14	71	119	Coffee odor
21	7.54	Gamma-Valerolactone	$C_5H_8O_2$	100.12	85	207	Warm, sweet, herbaceous odor
22	7.63	2-Methyl-octane	C_9H_{20}	128.26	57	150	Gasoline-like odor
23	7.98	3-Octen-2-one	$C_8H_{14}O$	126.2	111	100	Earthy, fruity blueberry flavor with a pleasant odor
24	8.12	(Z)-6-Octen-2-one	$C_8H_{14}O$	126.2	68	173	Earthy, fruity odor
25	9.17	Dodecane	$C_{12}H_{26}$	170.33	57	216	Aliphatic hydrocarbon odor
26	9.81	2-Methyl-dodecane	$C_{13}H_{28}$	184.36	43	227	Gasoline-like odor
27	9.96	2,6,11-Trimethyl-dodecane	$C_{15}H_{32}$	212.41	71	248	Gasoline-like odor
28	10.16	Tridecane	$C_{13}H_{28}$	184.36	57	235	Oily with a hydrocarbon odor
29	10.53	2-Undecanone	$C_{11}H_{22}O$	170.29	58	232	Oily with a citrus, fatty odor
30	11.17	Tetradecane	$C_{14}H_{30}$	198.39	57	254	Hydrocarbon odor
31	11.42	2-Butyl-2-octenal	$C_{12}H_{22}O$	182.3	41	262	Green, herbaceous, fruity, fatty flavor & odor
32	12.01	Gamma-Nonalactone	$C_9H_{16}O$	156.22	85	134	Fatty, oily, coconut-like flavor & odor
33	13.84	Hexadecane	$C_{16}H_{34}$	226.44	57	287	Odorless
34	14.90	Diethyl Phthalate	$C_{12}H_{14}O_4$	222.24	149	295	Bitter taste; oily with a slight aromatic odor

It is worth comparing the HS-SPME method conducted by Tian et al.[46] to this Vac-HS-HC-SPME sampling method. Tian incubated the samples at 60 °C for 20 min, extracted for 20 min, and desorbed for 20 min. In this study, using vacuum-assisted with a high-capacity SPME Arrow, the samples were incubated at 30 °C for 1 min, extracted for 10 min, and desorbed for 1 min. This combination saved almost an hour of sampling time. One of the compounds that was first discovered by Tian, 2-pentyl-furan, was also detected in Thai Cubensis, Texas Yellow, and B+. There were many common compounds detected including the lactones, except for gamma-nonalactone (Table 4-3, Peak 32) which was detected in Blue Meanie by Vac-HS-HC-SPME. This compound offers a fatty, coconut-like flavor and odor. Other unique detects by Vac-HS-HC-SPME include 2-methyl-dodecane, 2,6,11-trimethyl-dodecane, and 2-butyl-2-octenal.

As shown in Figure 4-5, the compounds identified in the psilocybin mushrooms were classified as acids, hydrocarbons, alcohols, aldehydes, esters, and ketones.

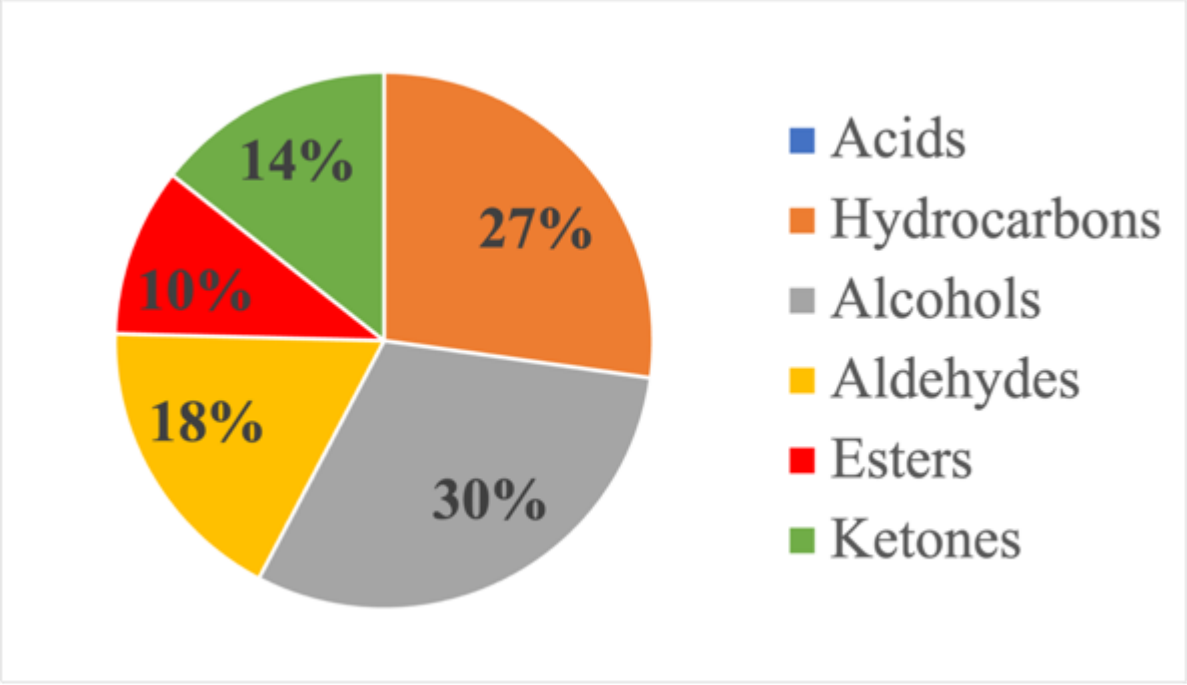

Figure 4-5. Distribution of the classes among all of the psilocybin mushrooms: Acids (0%), Hydrocarbons (27%), Alcohols (30%), Aldehydes (18%), Esters (10%), and Ketones (14%)

Of the identified compounds, 30% were alcohols. Diacetone alcohol, 1-hexanol, and 1-octen-3-ol were identified in all of the psilocybin mushrooms. The mushroom alcohol, 1-octen-3-ol, is the key compound that gives raw mushrooms its distinct aroma.[53] Of the hydrocarbons identified, all of the psilocybin species contained the compounds dodecane, 2,6,11-trimethyl-docecane, tetradecane, and hexadecane. Tetradecane and hexadecane have been shown to provide antimicrobial activity among mushrooms.[54] Caproaldehyde, known to have a fatty-green, grassy taste and odor,[55] was the only common identified compound of the aldehydes. The psilocybin mushrooms consisted of 14% ketones, none of which were common. The only common ester was the phthalate ester, diethyl phthalate, known to easily penetrate soil and contaminate ground water.[56] It is used in the manufacture of plastics. All dried mushrooms were stored in plastic containers, and this is a possible reason for contamination. No acids were identified in the psilocybin mushrooms.

The chemical class distribution for each species of psilocybin mushroom is presented in Figure 4-6 as follows: A. Thai Cubensis; B. Blue Meanie; C. Texas Yellow; D. Creeper; and E. B+.

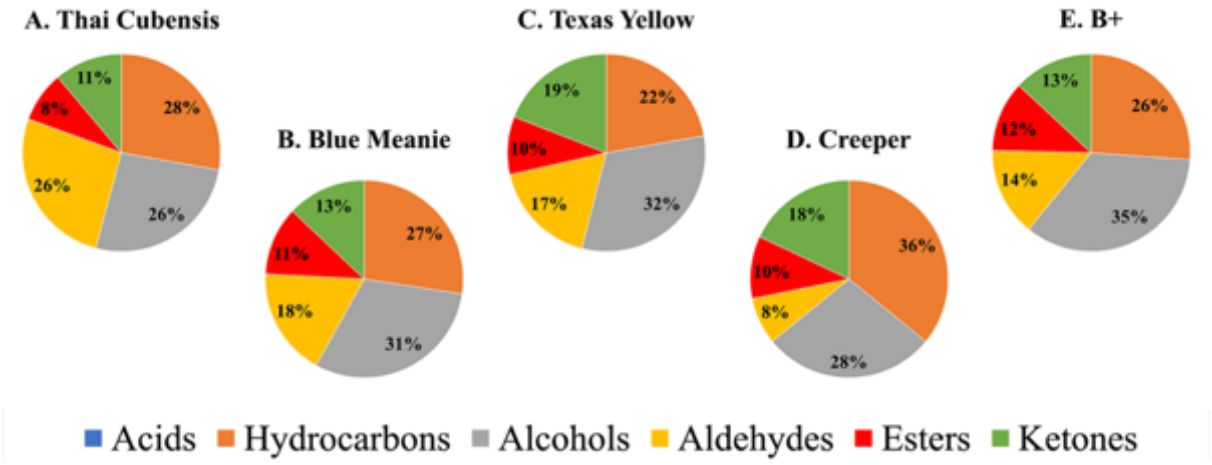

Figure 4-6. Classification of compounds among psilocybin mushrooms: A. Thai Cubensis; B. Blue Meanie; C. Texas Yellow; D. Creeper; and E. B+

Compounds of interest identified in Thai Cubensis (Figure 4-6A) included 1-butanol, which has a whiskey-like odor and is known for anti-bacterial properties,[57,58] and 2-methylpentanal which can be found in shiitake mushrooms.[57] Blue Meanie (Figure 4-6B) was the only psilocybin mushroom with the hydrocarbon, nonane, identified. Gamma-valerolactone, a cyclic ester, has a sweet, herbaceous odor and was only detected in Blue Meanie and B+ (Figure 4-6E). The predominant compound detected in Texas Yellow (Figure 4-6C) was the aldehyde, 2-butyl-2-octenal, that has been detected in chanterelle mushrooms.[59] The ketone, (Z)-6-octen-2-one, was only detected in Texas Yellow and Creeper (Figure 4-6D). The predominant compound found in B+ was the alcohol, 2,3-dimethyl-1-butanol.

Figure 4-7 shows sample chromatograms of two psilocybin mushrooms: A) Thai Cubensis and B) Texas Yellow.

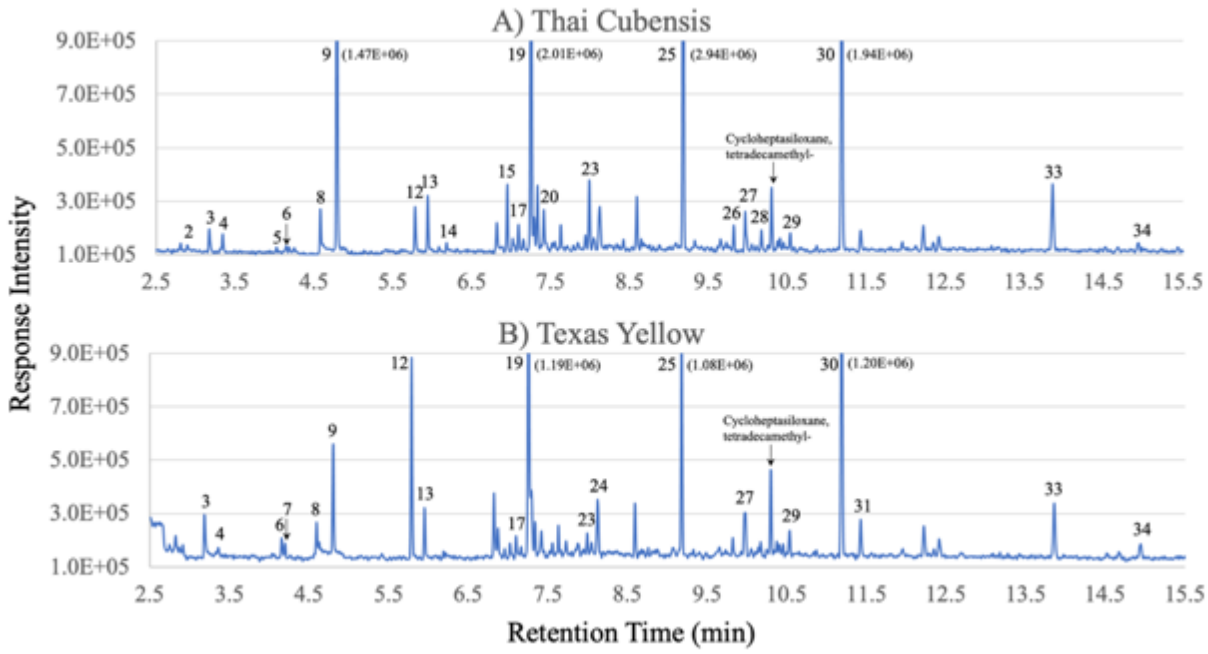

Figure 4-7. Sample chromatograms of psilocybin mushrooms: A) Thai Cubensis had 146 peaks detected with 35 peaks at S/N ratio greater than 5; B) Texas Yellow had 255 peaks detected with 30 peaks at S/N ratio greater than 5. Peak numbers are identified in Table 4-3.

The Thai Cubensis (A) strain had 146 peaks detected, with 35 of those peaks at a detection limit of S/N > 5. The Texas Yellow (B) strain had 255 peaks with 30 peaks at S/N > 5. The peak numbers correspond with the identified compounds in Table 4-3. Note that Thai Cubensis has peaks 9 (Caproaldehyde), 19 (1-octen-3-ol), 25 (docecane), and 30 (Tetradecane) that are off-scaling, making them the most abundant compounds. Texas Yellow is also most abundant with the latter three compounds.

Due to the use of a septum in the metal insert, off-gassing of siloxanes occurs in the headspace. To identify the siloxane peaks as background noise, a sample vial sealed with a metal insert and septum was evacuated then analyzed. Nine siloxane peaks were identified due

to off-gassing. As seen in Figure 4-5, cycloheptasiloxane, tetradecamethyl- has a higher concentration than some of the other compounds. Treatment of the septa is still being explored. Additional chromatograms for the other psilocybin mushrooms and evacuated blank can be found in the Supplementary Information (SI) document.

4.4.3 *Non-Psilocybin Mushrooms*

In total, 274 peaks were detected with 40 peaks above the detection limit (S/N > 5). There were 33 compounds identified among the non-psilocybin mushrooms. All relevant information for the compounds identified in the non-psilocybin mushroom samples can be seen in Table 4-4.

Table 4-4. Chemical compounds identified in non-psilocybin mushrooms, including their peak number, retention time, name, formula, molecular weight (MW), characteristic m/z, boiling point (°C), and flavor/fragrance properties.

Peak #	Retention Time (min)	Compound Name	Formula	MW (g/mol)	m/z	Boiling Point (°C)	Flavor/Fragrance
1	2.82	Isovaleraldehyde	$C_5H_{10}O$	86.13	58	92.5	Warm, herbaceous, fruit- & nut-like taste; apple-like odor
2	3.18	1-Butanol	$C_4H_{10}O$	74.12	31	117	Whiskey characteristic, mild alcoholic odor
3	4.04	2-Methylpentanal	$C_6H_{12}O$	100.16	43	117	Woody, oily ham taste & smell
4	4.16	Isoamyl alcohol	$C_5H_{12}O$	88.15	55	132.5	Pungent repulsive taste & disagreeable odor
5	4.20	2-Methyl-1-butanol	$C_5H_{12}O$	88.15	57	129	Cooked roasted aroma with alcoholic undertones
6	4.59	1-Pentanol	$C_5H_{12}O$	88.15	70	137	Burning tast with a fusel-like odor
7	4.79	Caproaldehyde	$C_6H_{12}O$	100.16	56	130	Fatty-green pungent odor with a green, woody, fruity taste
8	5.07	2-Methyl-pyrazine	$C_5H_6N_2$	94.11	94	135	Nutty, cocoa-like odor
9	5.38	2,3-Butanediol	$C_4H_{10}O_2$	90.12	45	182	Sweet taste & fruity, creamy, buttery odor
10	5.48	Butyric acid	$C_4H_8O_2$	88.11	60	164	Oily with a rancid, butter-like taste & odor
11	5.68	Nonane	C_9H_{20}	128.25	85	150	Gasoline-like odor
12	5.78	Diacetone alcohol	$C_6H_{12}O_2$	116.16	59	168	Faint, pleasant odor
13	5.94	1-Hexanol	$C_6H_{14}O$	102.17	56	157	Fatty, fruity taste with a sweet alcoholic odor
14	6.19	Heptanal	$C_7H_{14}O$	114.19	70	153	Fatty, fruity taste & odor
15	6.95	Decane	$C_{10}H_{22}$	142.28	57	174	Gasoline-like odor
16	7.02	2-Pentyl-furan	$C_9H_{14}O$	138.21	81	65	Fruity, green, earthy, musty taste & odor
17	7.09	Butyrolactone	$C_4H_6O_2$	86.09	42	204	Oily with a sweet, caramel-like odor
18	7.28	1-Octen-3-ol	$C_8H_{16}O$	128.21	57	84	Sweet, earthy, herbaceous odor
19	7.41	Vinyl butyrate	$C_6H_{10}O$	114.14	71	119	Coffee odor
20	7.54	Gamma-Valerolactone	$C_5H_8O_2$	100.12	85	207	Warm, sweet, herbaceous odor
21	7.63	2-Methyl-octane	C_9H_{20}	128.26	57	150	Gasoline-like odor
22	8.12	(Z)-6-Octen-2-one	$C_8H_{14}O$	126.2	68	173	Earthy, fruity odor
23	8.23	Benzyl alcohol	C_7H_8O	108.14	79	205	Fruity, balsamic, bitter taste & odor
24	8.88	2-Pyrrolidinone	C_4H_7NO	85.10	85	245	Cocoa-like odor
25	9.17	Dodecane	$C_{12}H_{26}$	170.33	57	216	Aliphatic hydrocarbon odor
26	9.53	Estragole	$C_{10}H_{12}O$	148.2	148	216	Sweet, anise-like taste & odor
27	9.96	2,6,11-Trimethyl-dodecane	$C_{15}H_{32}$	212.41	71	248	Gasoline-like odor
28	10.53	2-Undecanone	$C_{11}H_{22}O$	170.29	58	232	Oily with a citrus, fatty odor
29	11.17	Tetradecane	$C_{14}H_{30}$	198.39	57	254	Hydrocarbon odor
30	11.42	2-Butyl-2-octenal	$C_{12}H_{22}O$	182.3	41	262	Green, herbaceous, fruity, fatty flavor & odor
31	12.01	Gamma-Nonalactone	$C_9H_{16}O$	156.22	85	134	Fatty, oily, coconut-like flavor & odor
32	13.84	Hexadecane	$C_{16}H_{34}$	226.44	57	287	Odorless
33	14.90	Diethyl Phthalate	$C_{12}H_{14}O_4$	222.24	149	295	Bitter taste; oily with a slight aromatic odor

As shown in Figure 4-8, the compounds identified in the non-psilocybin mushrooms were classified as acids, hydrocarbons, alcohols, aldehydes, esters, and ketones.

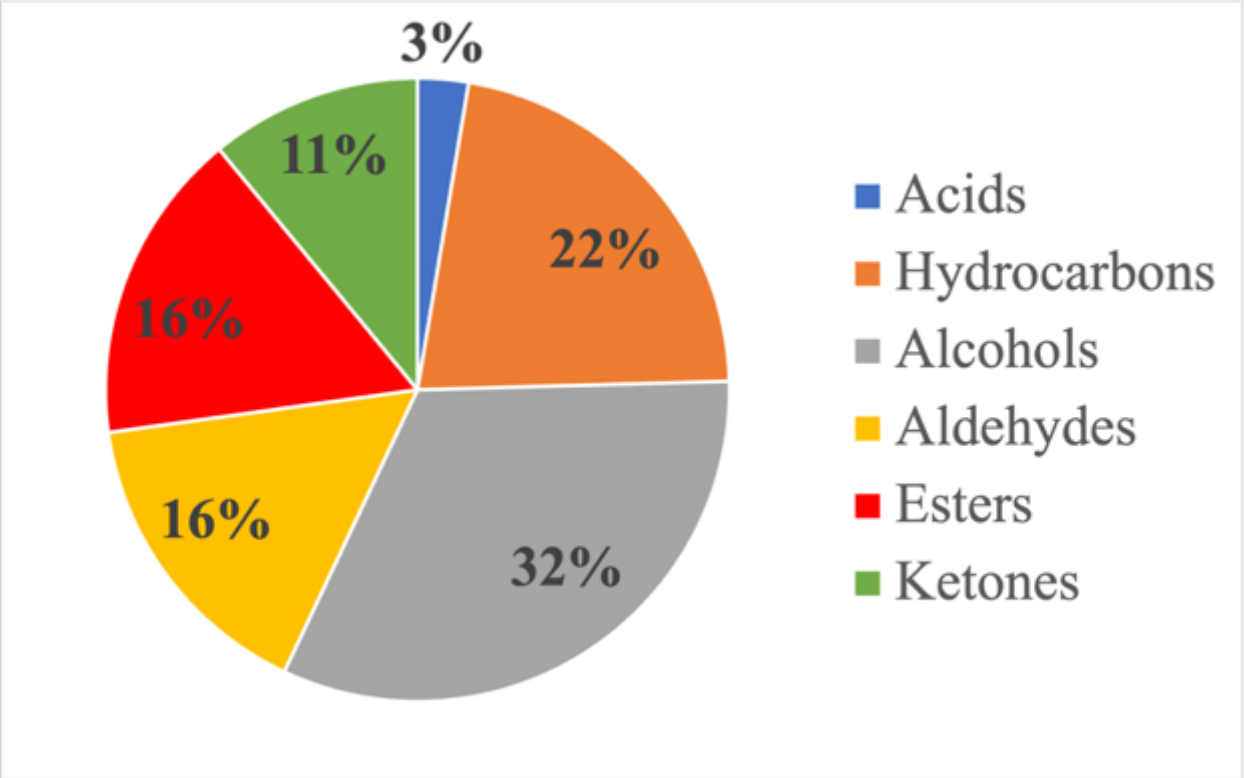

Figure 4-8. Distribution of the classes among all of the non-psilocybin mushrooms: Acids (3%), Hydrocarbons (22%), Alcohols (32%), Aldehydes (16%), Esters (16%), and Ketones (11%)

Of the identified compounds, 32% were alcohols. 2-methyl-1-butanol, 2,3-butanediol, 1-hexanol, and 1-octen-3-ol were identified in all of the non-psilocybin mushrooms. 2,3-butanediol acts as a bacterial metabolite.[60] Of the compounds identified, 16% were aldehydes such as caproaldehyde and heptanal. Heptanal has a fatty-fruity odor and taste and was identified as a common compound among wild edible Nordic mushrooms.[61] Butyrolactone and gamma-valerolactone were among the esters identified, contributing to a sweet odor. These lactones were also identified in the Tian et al.[46] study. Butyric acid was the only identified acid detected

in button and shiitake mushrooms. Further investigation is needed to determine if Vac-HS-HC-SPME is useful in detecting acids.

The distribution of the chemical classes for each non-psilocybin mushroom is presented in Figure 4-9 as follows: A. Button; B. Wild Forest; and C. Shiitake.

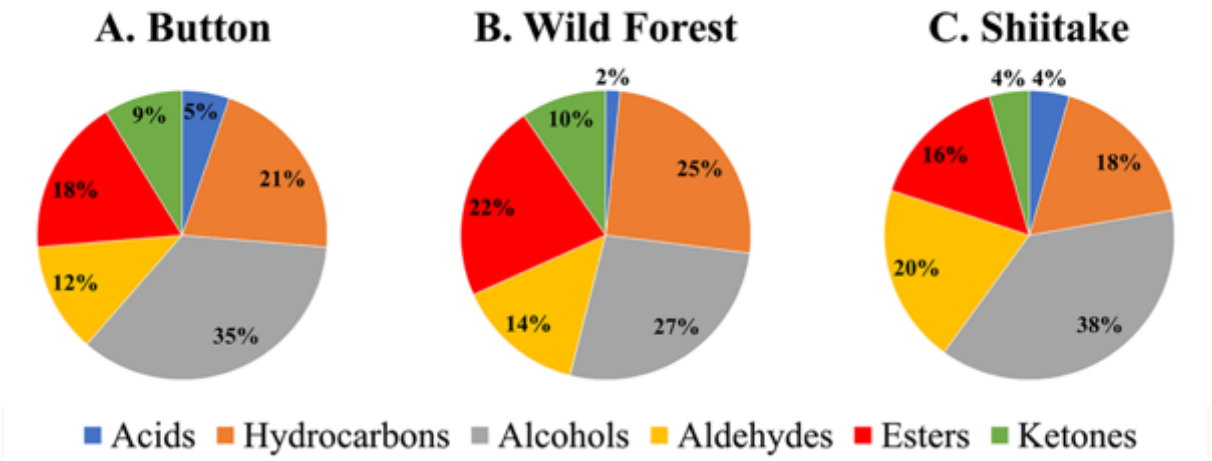

Figure 4-9. Classification of compounds among non-psilocybin mushrooms: A. Button; B. Wild Forest; and C. Shiitake

Compounds of interest identified in button mushrooms (Figure 4-9A) included benzyl alcohol, which has a fruity, balsamic, bitter taste and odor (The Good Scents Company), and estragole, a phenylpropanoid. Estragole is known to prevent gastric ulcers due to its antioxidant, anti-inflammatory, and vasorelaxant activity.[62] The previously mentioned 2-pentyl-furan was detected in Wild Forest mushrooms (Figure 4-9B). Furans were shown to increase in concentration in shiitake mushrooms with an increase in roasting temperatures.[57] The aldehyde, 2-methylpentanal was detected in the shiitake mushrooms, verifying the study by Hwang et al.[57]

Figure 4-10 shows a sample chromatogram of the Button mushrooms. All chromatograms of the non-psilocybin mushrooms can be found in the SI document.

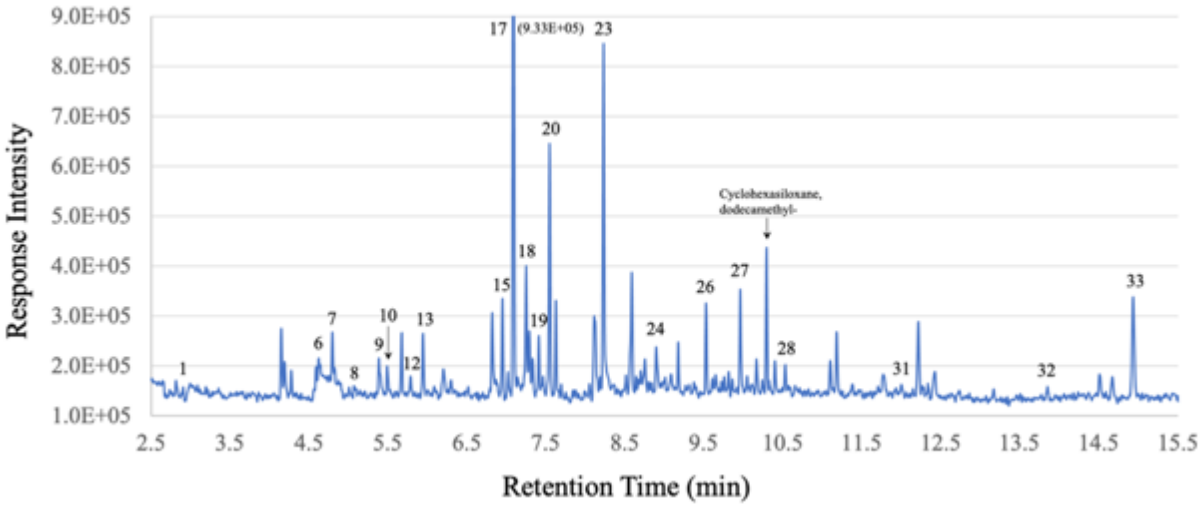

Figure 4-10. Sample chromatograms of non-psilocybin mushrooms: button mushrooms had 274 peaks detected with 51 peaks at S/N ratio greater than 5. Peak numbers are identified in Table 4-4.

Button mushrooms had 274 peaks detected, with 51 of those peaks at a detection limit of S/N > 5. The peak numbers correspond with the identified compounds in Table 4-4. Note that peak 17 (Butyrolactone) was off-scaling, making it the most abundant compound. Cyclohexasiloxane, dodecamethyl- was the most abundant compound caused by the off-gassing from the septum.

4.4.4 *Comparison of Psilocybin and Non-Psilocybin Mushrooms*

Figure 4-11 gives an overall comparison between the relative abundance of chemical classes found in psilocybin and non-psilocybin mushrooms.

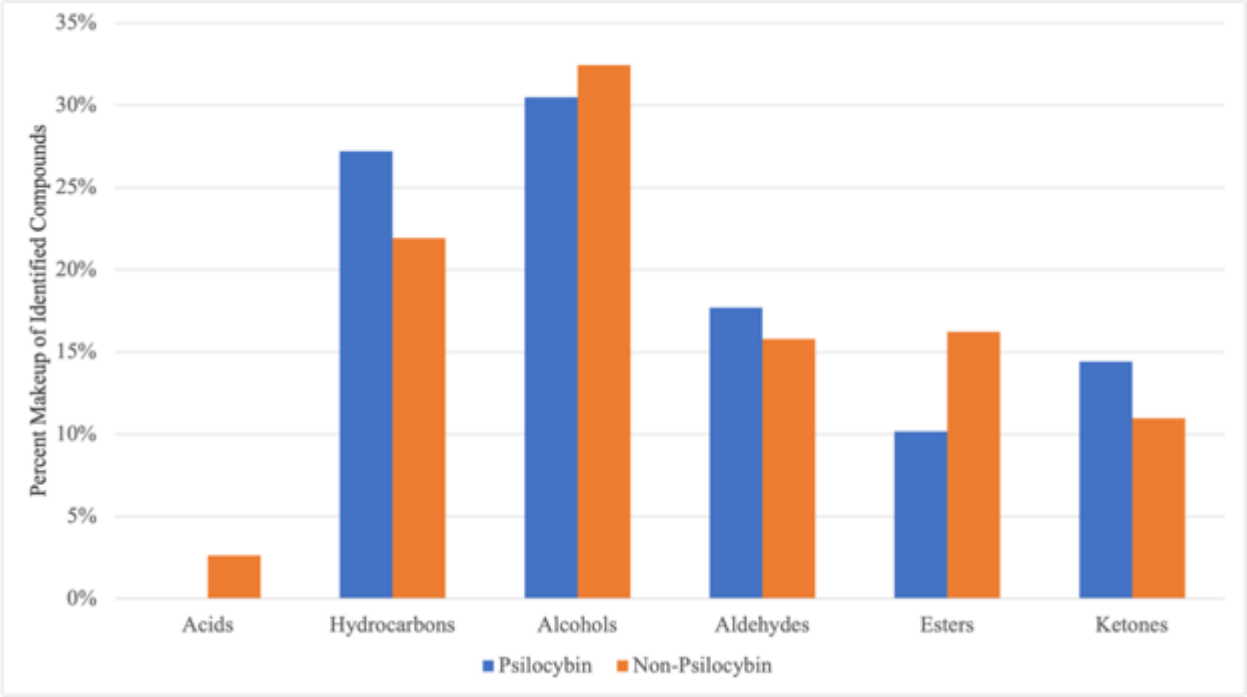

Figure 4-11. Comparison of chemical components between psilocybin and non-psilocybin mushrooms

The identified compounds that were only detected in psilocybin mushrooms included 2-methylbutanal, valeraldehyde, benzaldehyde, 3-octen-2-one, 2-methyl-dodecane, and 2-butyl-2-octenal. The bitter almond taste[55] of benzaldehyde possibly contributes to the unpleasant taste of psilocybin mushrooms. The identified compounds that were only detected in non-psilocybin mushrooms included 2-methyl-pyrazine, 2,3-butanediol, butyric acid, butyrolactone, benzyl alcohol, 2-pyrrolidinone, and estragole. Studies show that pyrazines increase in concentration with the rise of roasting temperatures[57] and across longer storage durations.[61] These studies suggest a variety of methods used to dry the mushrooms, and variances in the duration of grocery store shelf-life that the non-psilocybin mushrooms experienced. Additional evidence of variances in drying techniques is the greater peak area of 1-

octen-3-ol in the psilocybin mushrooms, as shown in Figure 4-12. Psilocybin mushrooms possibly have more moisture. It has also been shown that heat decreases the concentration of 1-octen-3-ol.[53]

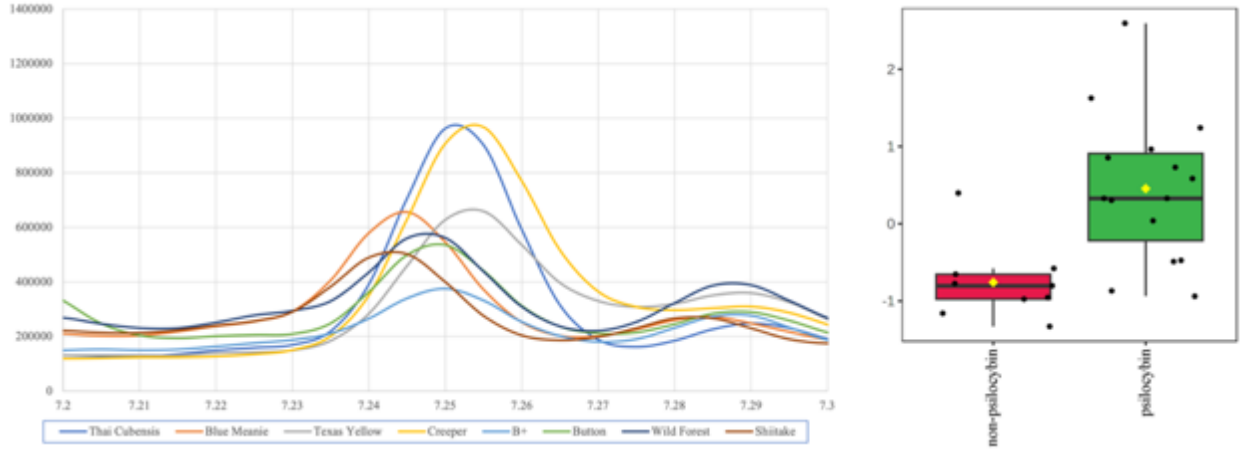

Figure 4-12. Chromatogram of 1-octen-3-ol (left) and normalized peak area box plot

4.5 Conclusion

Vac-HS-SPME sampling, with the addition of a high-capacity SPME Arrow, increases the range of volatiles analyzed compared to HS-HC-SPME by shifting the equilibrium toward a higher headspace concentration for semi-volatile analytes. The initial comparison of Vac versus no-Vac conditions showed marginal improvements with two-times increase in compound response as well as the detection of 8 additional compounds not detected by HS-SPME. For example, the Tian study of mushroom volatiles, using HS-SPME at higher temperatures, detected comparable compounds as the Vac-HS-HC-SPME sampling method. However, Vac-HS-HC-SPME was able to detect the 2-pentyl-furan at lower temperatures and in less time. Also, Vac-HS-HC-SPME detected gamma-nonalactone that was not detected in HS-HC-SPME. The advantages of Vac-HS-HC-SPME include incubation and extraction at lower temperatures and in

less time. The combination of Vac-HS-SPME with the addition of the HC-SPME Arrow saved almost an hour of sampling time. In addition, solid samples can be homogenized by simple milling with no solvents needed in sample preparation.

Identified compounds that were unique to psilocybin mushrooms included 2-methylbutanal, valeraldehyde, benzaldehyde, 3-octen-2-one, 2-methyl-dodecane, and 2-butyl-2-octenal. The compounds 2-methyl-pyrazine, 2,3-butanediol, butyric acid, butyrolactone, benzyl alcohol, 2-pyrrolidinone, and estragole were only detected in non-psilocybin mushrooms. The common mushroom alcohol, 1-octen-3-ol, was shown to have higher responses among the psilocybin mushroom species. Further investigation is recommended to identify peaks, such as coupling to a VUV detector (GC-MS/VUV) as well as implementing reference standards to verify both retention times and ion matching. A study manipulating the parameters of temperature and time under vacuum-assisted conditions can be conducted to determine the best response with the most compounds detected.

Conflict of Interest

CM is an employee of Restek Corporation, the company that supported this research.

Acknowledgements

The authors would like to thank Prof. Elefteria Psillakis and ExtraTech Analytical Solutions SMPC for providing the metal insert. The authors also acknowledge research support provided by Restek Corporation.

4.6 Supplementary Materials

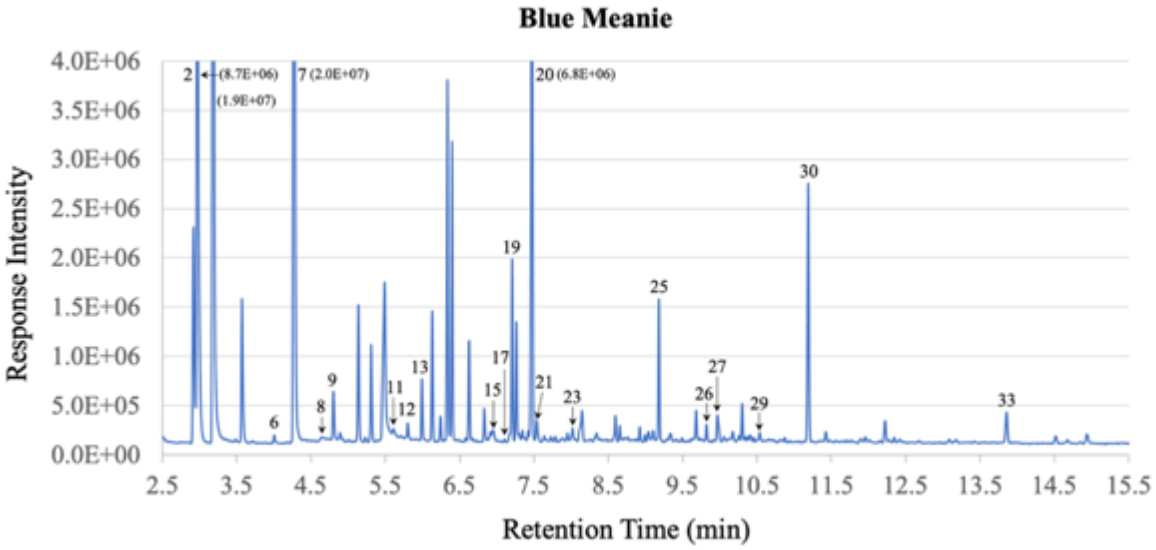

Figure 4-S1. Chromatograms of psilocybin mushroom: Blue Meanie had 223 peaks detected with 43 peaks at S/N ratio greater than 5. Peak numbers are identified in Table 4-3.

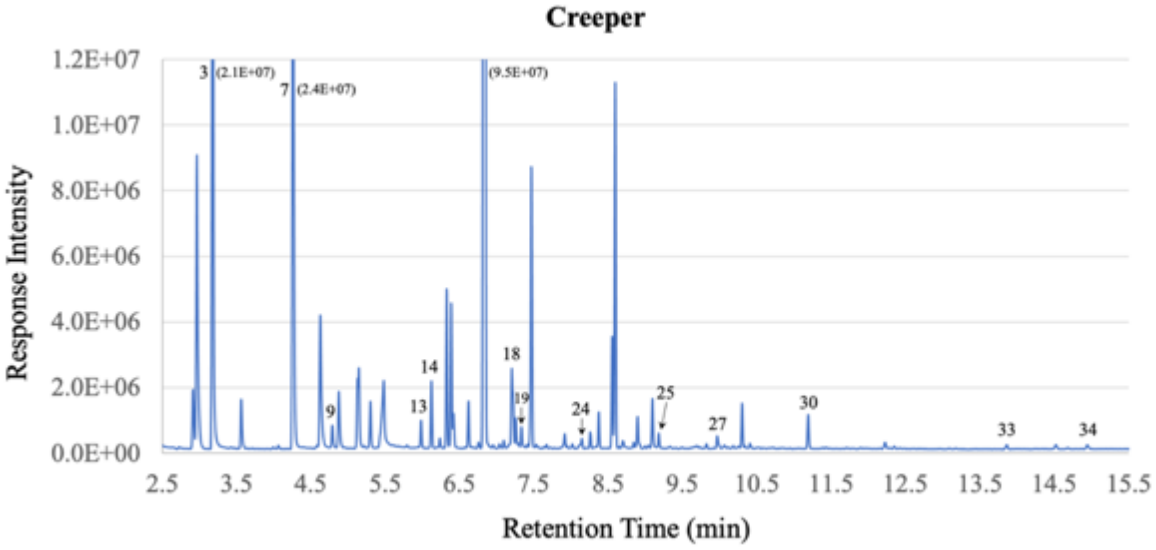

Figure 4-S2. Chromatogram of psilocybin mushroom: Creeper had 39 peaks detected with 36 peaks at S/N ratio greater than 5. Peak numbers are identified in Table 4-3.

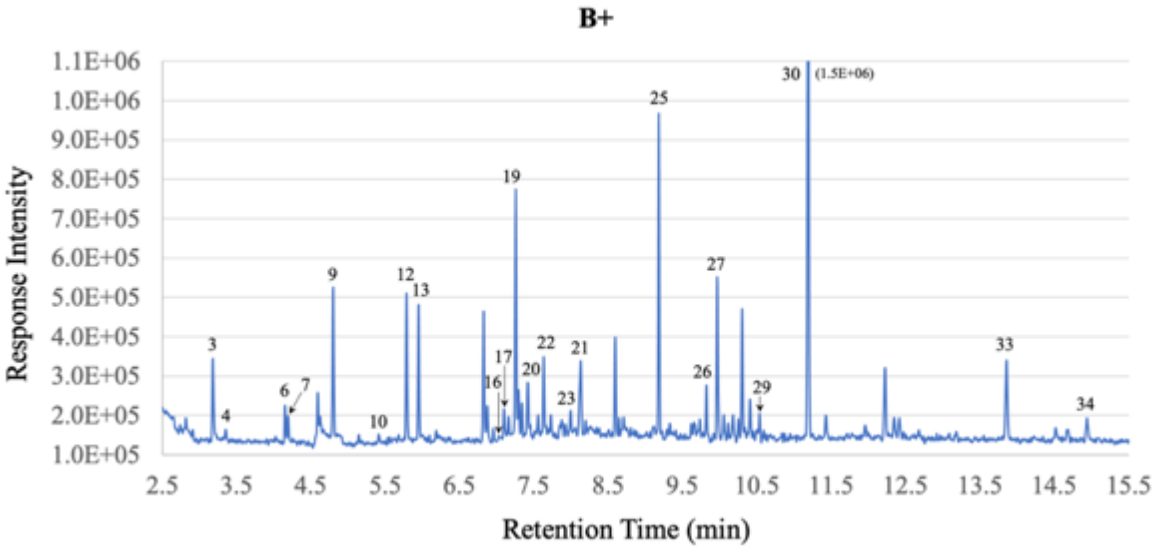

Figure 4-S3. Chromatogram of psilocybin mushroom: B+ had 266 peaks detected with 40 peaks at S/N ratio greater than 5. Peak numbers are identified in Table 4-3.

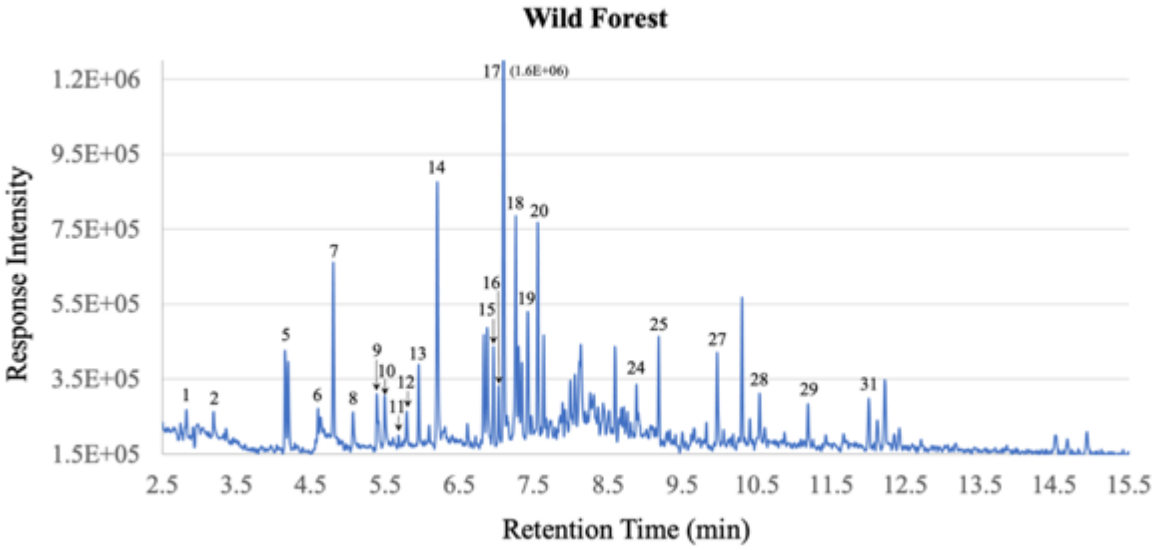

Figure 4-S4. Chromatograms of non-psilocybin mushrooms: Wild Forest had 273 peaks detected with 46 peaks at S/N ratio greater than 5. Peak numbers are identified in Table 4-4.

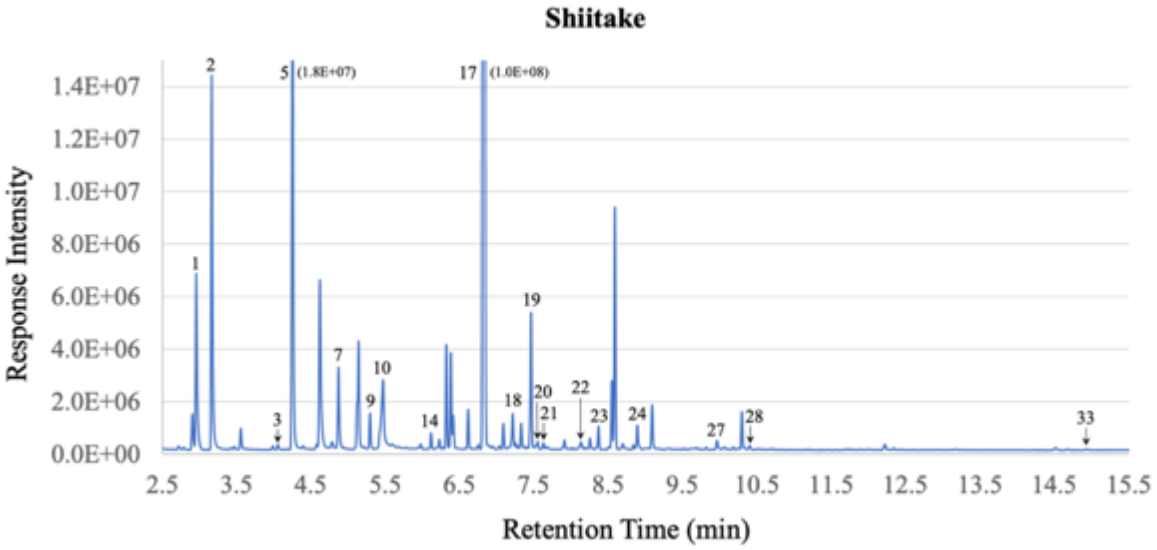

Figure 4-S5. Chromatograms of non-psilocybin mushrooms: shiitake mushrooms had 42 peaks detected with 35 peaks at S/N ratio greater than 5. Peak numbers are identified in Table 4-4.

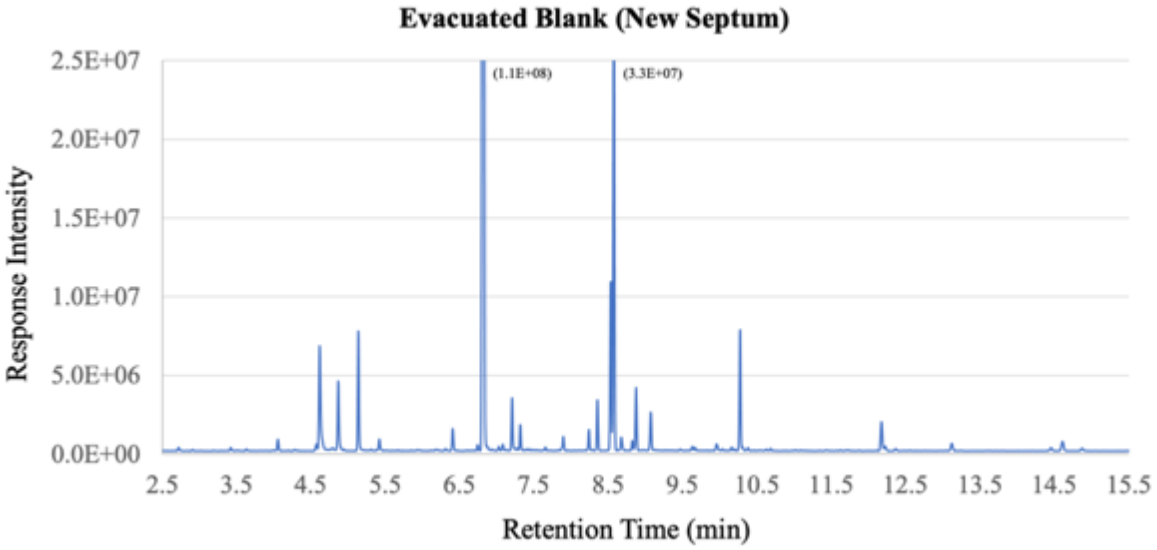

Figure 4-S6. Sample chromatogram of a blank by evacuating the air out of a vial and using a new septum for the metal insert

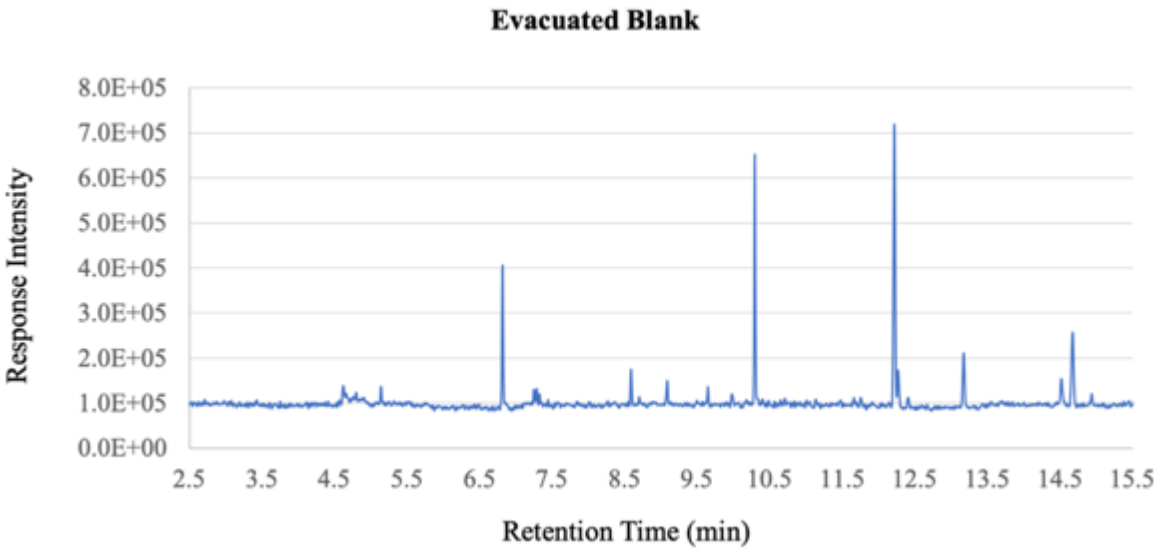

Figure 4-S7. Sample chromatogram of a blank by evacuating the air out of a vial and using a used septum for the metal insert

CHAPTER FIVE

IN-SITU DERIVATIZATION OF FATTY ACIDS INTO FATTY ACID METHYL ESTERS IN OLIVE OIL USING SULFONATED POLY(DIVINYLBENZENE) MICROSPHERES COMBINED WITH VACUUM-ASSISTED HEADSPACE HIGH-CAPACITY SOLID-PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Thomas, S. L., Miller, R. L., Pabley, A., Foss, F.W., Schug, K. A.

In-Situ Derivatization of Fatty Acids into Fatty Acid Methyl Esters in Olive Oil using Sulfonated Poly(Divinylbenzene) Microspheres Combined with Vacuum-Assisted Headspace High-Capacity Solid-Phase Microextraction and Gas Chromatography-Mass Spectrometry

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5.1 Abstract

In this study, we present a novel method for the derivatization of fatty acids into fatty acid methyl esters (FAMEs) in olive oil via in-situ vacuum-assisted headspace solid-phase high-capacity microextraction (Vac-HS-HC-SPME) using sulfonated poly(divinylbenzene) microspheres, followed by analysis with gas chromatography-mass spectrometry (GC-MS). This approach leverages the benefits of vacuum-assisted sampling to enhance the extraction efficiency and sensitivity of semi-volatile compounds.

Comparative experiments between vacuum-assisted and non-vacuum-assisted conditions demonstrated that the application of vacuum resulted in significantly greater chromatographic areas for the analytes, indicating improved extraction efficiency. Specifically, the vacuum-assisted method detected 46 peaks that were not observed under non-vacuum-assisted

conditions, and of the nine methyl esters identified, vacuum-assisted sampling detected five additional FAMEs with two showing greater peak areas compared to non-vacuum sampling.

The method successfully converted fatty acids in olive oil to FAMEs, and its applicability was further validated by testing other oils. The results showed successful conversion to FAMEs in sesame, avocado, canola, coconut, and vegetable shortening oils, with the detection of numerous new peaks in the derivatized samples compared to their respective untreated samples.

This research represents a novel approach for the Vac-HS-HC-SPME in-situ sulfonated PDVB derivatization and GC-MS analysis of fatty acids, offering a simple, greener method for the profiling of lipid compounds in various oil matrices.

Key Words: derivatization, FAMEs, headspace, in-situ, olive oil, poly(divinylbenzene), SPME, sulfonation, vacuum-assisted

Abbreviations: DVB, FAMEs, FTIR, HC, HS, PDMS, PDVB, Vac-HS-HC-SPME

5.2 Introduction

The derivatization of fatty acids to fatty acid methyl esters (FAMEs) is a crucial analytical step for the accurate analysis of lipids, particularly in complex matrices like olive oil. Derivatization improves the volatility and stability of fatty acids, which facilitates their analysis by gas chromatography-mass spectrometry (GC-MS). Common derivatization methods often involve liquid-liquid extraction and direct derivatization in solution, which can be time-consuming and require large amounts of solvents. Previous studies on derivatization of fatty

acids have used various catalysts and conditions to achieve efficient conversion to FAMEs. Traditional methods often involve acid or base-catalyzed esterification using reagents like methanolic HCl or BF3-methanol [63, 64]. While effective, these methods require careful handling of corrosive reagents and extensive sample preparation.

Esterification of fatty acids utilizing Lewis and Bronsted acids is well understood in the solution phase [65-68]; however, utilizing a solid support for esterification is a developing area of research [68-70] for various reasons such as biodiesel production and analysis of food products. To perform this reaction on a solid polymer support, the polymer must be first functionalized using acidic conditions. Polymer supports need to have high physical strength, be resistant to hydrolysis or chemical oxidation, and tolerate high temperatures to withstand the sulfonation process [71]. Using a polymer with monomers that have known methods for chemical modification, such as the sulfonation of benzene utilizing sulfuric acid [73], is an attractive starting point for initial studies towards a polymer capable of performing solid supported esterification of fatty acids. The aromatic ring of the polymer undergoes electrophilic aromatic substitution to allow for the inclusion of the functional group involved in the esterification process. These functionalized polymers act as solid cation-exchange resins for heterogenous catalysis of the esterification reaction instead of relying on bulk acid in the standard solution phase reactions [72, 74].

Recent advancements have focused on in-situ derivatization techniques that streamline sample preparation and reduce solvent usage. One such method involves the use of sulfonated poly(divinylbenzene) microspheres for in-situ derivatization. These microspheres provide a high surface area and acidic sites that facilitate the esterification reaction, offering a more

environmentally friendly and efficient alternative to traditional liquid-phase derivatization methods [69].

Vacuum-assisted headspace solid-phase microextraction (Vac-HS-SPME) has also gained attention for its ability to improve the extraction of volatile and semi-volatile compounds by reducing the pressure in the sample vial. This reduction in pressure increases the analyte concentration in the headspace, improving the sensitivity and selectivity of the extraction process. Studies have demonstrated the effectiveness of Vac-HS-SPME in extracting a wide range of analytes from complex matrices, including food products and environmental samples [4,6,12,17-19,22,26].

Combining in-situ derivatization with Vac-HS-SPME offers a novel approach for the efficient and sensitive analysis of FAMEs from olive oil. This study aims to optimize the derivatization conditions using sulfonated poly(divinylbenzene) microspheres and to evaluate the performance of Vac-HS-SPME with a high-capacity (HC) SPME Arrow for extracting FAMEs, coupled with GC-MS analysis.

5.3 Experimental

5.3.1 *Chemicals and Reagents*

Oils were purchased at a local grocery store. All standards were provided by Restek Corporation (Bellefonte, PA). All reagents and solvents were used as purchased unless otherwise stated. All reagents and solvents for polymer beads preparation were purchased from Sigma Aldrich, Alfa Aesar, and Fisher Scientific. All solvents for Vac-HS-HC-SPME sampling

were purchased through MilliporeSigma (Burlington, MA) or Thermo Fisher Scientific (Waltham, MA).

5.3.2 *Sample Preparation*

5.3.2.1 *Polymerization of poly(divinylbenzene)*

In a 250 mL round bottom flask equipped with a magnetic stir bar, aqueous phase was prepared by heating 100 mL of a 1% polyvinyl alcohol in water to 80 ^{o}C. Organic phase was prepared with 8 mL isooctane, 30 mL divinylbenzene monomer, and 1.8 g benzoyl peroxide and subsequently added to aqueous mixture and stirred vigorously for 2 hours or until solid poly(divinylbenzene) (PDVB) microspheres form. PDVB microspheres are filtered utilizing a coarse fritted filter, washed with water, and dried utilizing reduced pressure.

5.3.2.2 *Sulfonation of poly(divinylbenzene)*

In a 100 mL round bottom flask equipped with a magnetic stir bar, 3.6 g PDVB microspheres are added to 20 mL of dichloromethane and stirred gently for 1 h and subsequently filtered and used directly for the next step. To the PDVB microspheres, 10 mL concentrated sulfuric acid was added dropwise to a reaction vessel and then refluxed at 80 ^{o}C for 24 h. The mixture was filtered and washed with copious amounts of deionized water and then dried. The resultant sulfonated PDVB microspheres were analyzed utilizing Fourier Transform infrared spectroscopy (FTIR) in the 4000-400 cm^{-1} region with 16 scans. Figure 5-1 shows the sulfonated product resulted with a band from 3600-3000 cm^{-1} due to -OH stretching and bands from 1300-1100 cm^{-1} due to -SO_2 asymmetric and symmetric stretching. This

confirms the success of the sulfonation and suggests that the sulfonic acid group was added onto the PDVB microspheres

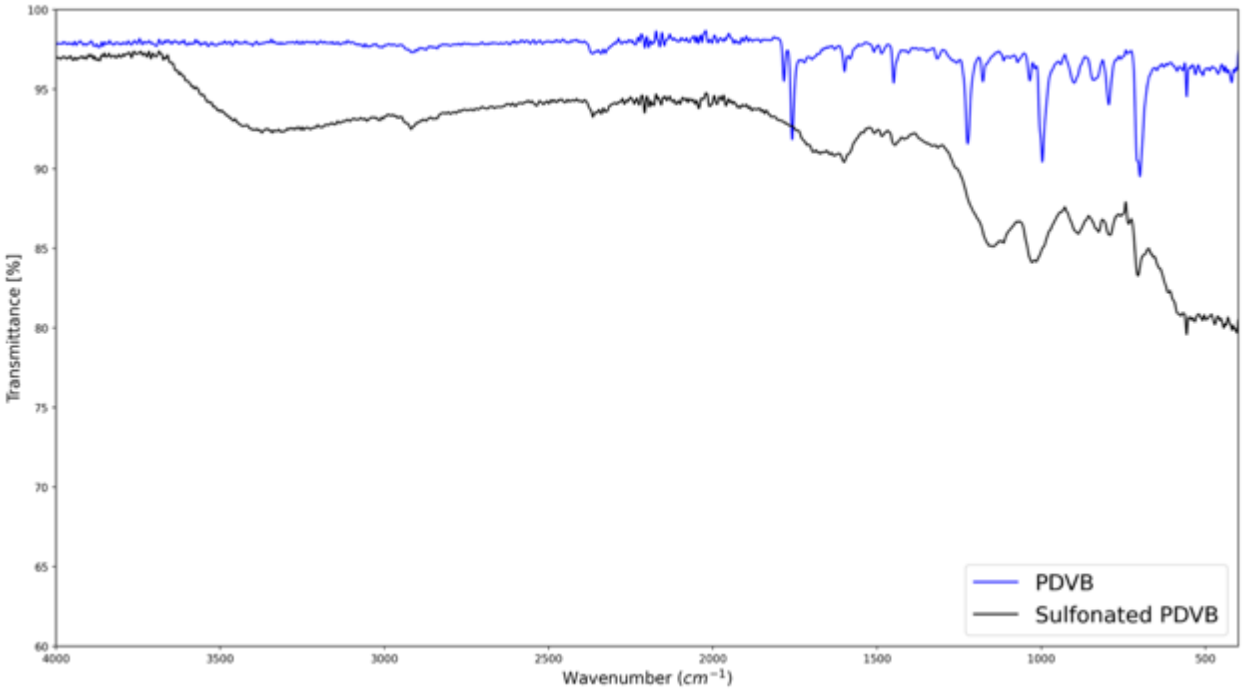

Figure 5-1. FTIR spectrum of PDVB microspheres before and after sulfonation method

5.3.2.3 *Vacuum-Assisted Headspace High-Capacity Solid-Phase Microextraction Sampling*

Sulfonated DVB polymer beads (115 mg), 50 µL olive oil, and 1 mL methanol (MeOH) were placed into 20 mL headspace vials. The SO_4 group on the polymer beads interact with the fatty acids, which undergo esterification to create fatty acid methyl esters. The MeOH is needed to complete the reaction. The vials were then sealed with a stainless-steel insert (created and provided by Prof. Elefteria Psillakis, ExtraTech Analytical Solutions SMPC, Chania, Greece) combined with a Thermogreen® LB-2 septum with half-hole (Supelco, Bellefonte, PA). A Trajan SGE 50 mL gas-tight syringe (provided by Restek Corporation, Bellefonte, PA) was inserted into the septum and pulled air from the vial at an approximate volume of 20 mL. Air could be pulled

immediately after sealing because the FAMEs are not yet present in the headspace. At room temperature, vials were set aside overnight to complete the esterification and equilibration processes.

5.3.3 *Instrumentation*

IR spectra were recorded in a Bruker Alpha-P FT-IR Spectrometer by attenuated total reflectance on a diamond sample plate. Instrumentation and sample preparation parameters are detailed in Table 5-1. A Shimadzu GC-2010 Plus gas chromatograph equipped with an AOC-6000 auto sampler system for automated Vac-HS-HC-SPME extraction and desorption, was coupled to a GCMS-TQ8030 triple quadrupole mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia MD) for FAMEs analysis. Separation was achieved using non-polar Rxi-5Sil MS column (30 m x 0.25 mm x 0.25 µm) from Restek Corporation (Bellefonte, PA). The instrument was operated in linear velocity mode (47.6 cm/sec) using helium carrier gas. Sample extraction and desorption was carried out using a 1.10 mm SPME Arrow with a divinyl benzene/polydimethylsiloxane (DVB/PDMS) biphasic extraction coating with a thickness of 120 µm (Restek Corporation, Bellefonte, PA). A Topaz 1.8 mm ID Straight/SPME Inlet Liner from Restek Corporation was used for all applications.

Table 5-1. SPME Arrow sampling parameters, gas chromatograph (GC) and mass spectrometer (MS) programming parameters

[a]**AOC-6000 Sampling Parameters**		[b]**GC-2010 Plus/GCMS-TQ8030 GC-MS Parameters**	
[c]**Vac-HS-HC-SPME**		**Inlet**	
Tool	SPME Arrow	280 °C	
Agitator Speed	500 rpm	Split 10:1	
Agitator Temp.	60 °C	[d] Topaz 1.8 mm ID Straight/SPME Inlet Liner	
Incubation Time	1 min		
Heatex Stirrer Speed	500 rpm	[d]**Column**	
Heatex Stirrer Temp.	60 °C	Rxi-5Sil MS, 30 m, 0.25 mm ID, 0.25 µm	
Extraction Time	5 min		
Vial Penetration Depth	45 mm	**Oven**	
Injector Penetration Depth	45 mm	60 °C (hold 2.0 min) to 200 °C at 10 °C min^{-1} (hold 0.0 min) to 300 °C at 5 °C min^{-1} (hold 5.0 min)	
Desorption Time	5 min		
Pre-Conditioning	True	**Carrier Gas**	
Post Conditioning	False	Type	Helium
Conditioning Time	10 min	Mode	Linear Velocity
Conditioning Temp.	280 °C	Linear Velocity	47.6 cm/sec
		Flow Rate	1.70 mL min^{-1}
		[b]**Detector**	
		Type	Triple Quadrupole MS
		Mode	Q3 Scan (35 to 350 m/z)
		Ion Source Temp.	250 °C
		Interface Temp.	200 °C
		Electron Energy	70 eV

[a] SPME autosampler: AOC-6000 (Shimadzu Scientific Instruments, Inc., Columbia MD)

[b] Gas chromatograph – GC-2010 Plus, Mass spectrometer (MS) – GCMS-TQ8030: Shimadzu Scientific Instruments, Inc., Columbia MD

[c] SPME in Vac-HS-HC-SPME mode

[d] Restek Corporation, Bellefonte, PA, USA

5.4 Results and Discussion

5.4.1 FAMEs verification

To verify the formation of fatty acid methyl esters (FAMEs) from olive oil, chromatograms of an untreated olive oil (no derivatization) with vacuum-assisted derivatized olive oil were compared. As shown in Figure 5-2 and Figure 5-3, the initial peak in both chromatograms was identical, confirming that the base matrix of the olive oil was consistent across both samples.

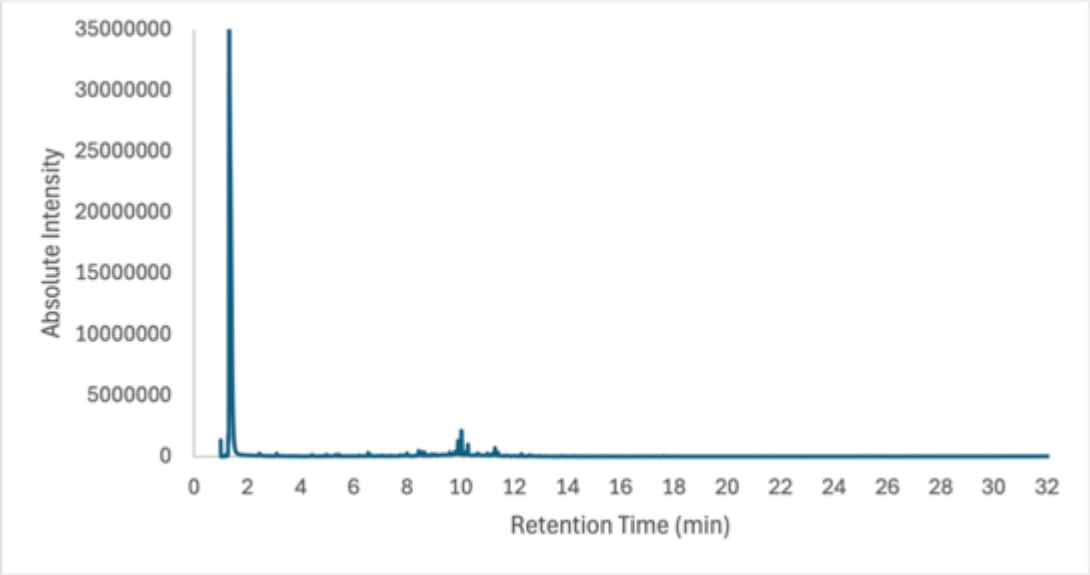

Figure 5-2. Chromatogram of untreated olive oil

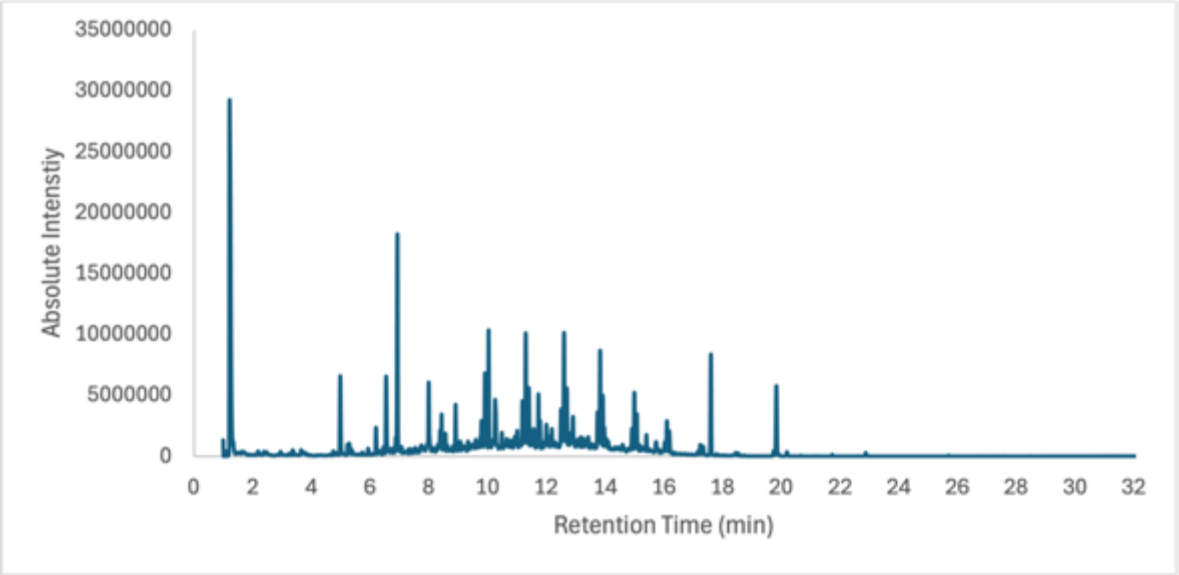

Figure 5-3. Chromatogram of in-situ derivatization of olive oil with sulfonated poly(divinylbenzene) microspheres and methanol using Vac-HS-HC-SPME sampling

However, the derivatized chromatogram (Figure 5-3) exhibited approximately 70 new peaks, indicating successful derivatization and the presence of additional compounds not seen in the untreated sample. Notably, nine of these peaks were identified as fatty acid methyl esters through comparison with the Shimadzu NIST-17 library. This significant increase in detectable compounds, specifically FAMEs, demonstrates the effectiveness of the vacuum-assisted in-situ derivatization method in converting fatty acids within olive oil to their methyl ester counterparts, thereby enhancing the sample's analytical profile for GC-MS analysis.

5.4.2 Vacuum-assisted and non-Vacuum-assisted Comparison

Figure 5-3 (above) and Figure 5-4, show the comparison of the chromatographic results of vacuum-assisted versus non-vacuum derivatization of olive oil. The vacuum-assisted method outperformed the non-vacuum approach in several key aspects.

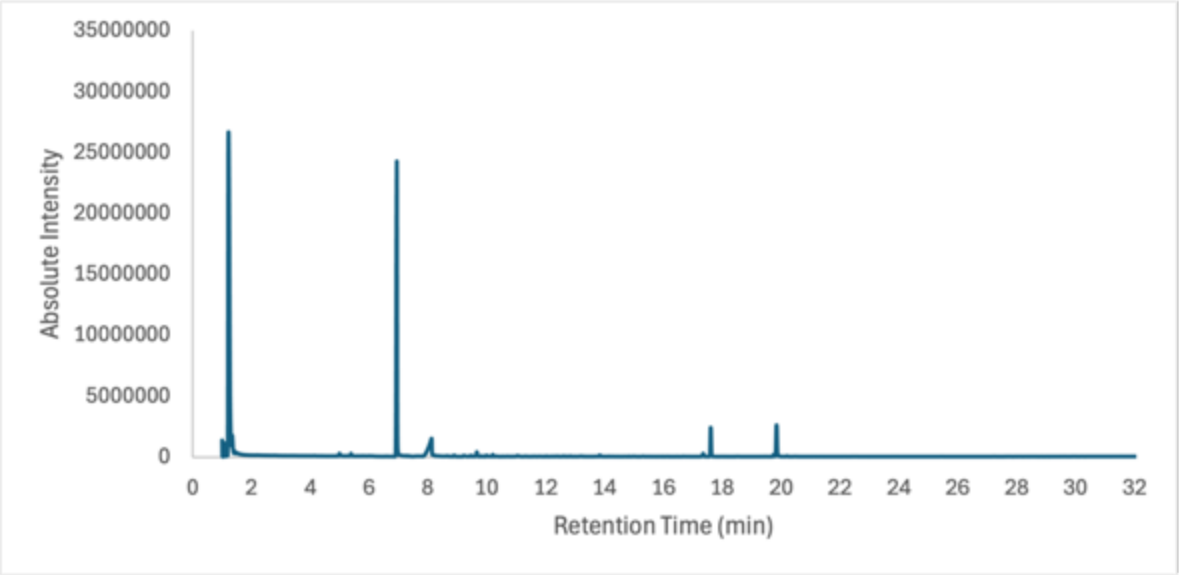

Figure 5-4. Chromatogram of in-situ derivatization of olive oil with sulfonated poly(divinylbenzene) microspheres and methanol using HS-HC-SPME sampling at atmospheric pressure (non-vacuum)

Firstly, the vacuum-assisted chromatogram revealed 46 unique peaks that were not detected in the non-vacuum chromatogram, indicating a superior extraction capability under vacuum conditions. Of the nine FAMEs identified in the vacuum-assisted sample, only four were detected in the non-vacuum sample. Furthermore, two of these four FAMEs exhibited greater peak areas in the vacuum-assisted chromatogram, underscoring the enhanced sensitivity and efficiency of the vacuum-assisted derivatization process.

These findings demonstrate that vacuum-assisted in-situ derivatization not only increases the number of detectable compounds but also improves the detection sensitivity of specific FAMEs in olive oil.

5.4.3 Application to Different Oil Varieties

To demonstrate the versatility and broad applicability of the in-situ sulfonated PDVB derivatization method using Vac-HS-HC-SPME, this study was extended to include various food oils, as listed below (Figures located in Supplementary Material):

- Sesame oil untreated (Figure 5-S1) and vacuum derivatized sesame oil (Figure 5-S2)
- Avocado oil untreated (Figure 5-S3) and vacuum derivatized avocado oil (Figure 5-S4)
- Canola oil untreated (Figure 5-S5) and vacuum derivatized avocado oil (Figure 5-S6)
- Coconut oil untreated (Figure 5-S7) and vacuum derivatized coconut oil (Figure 5-S8)
- Vegetable shortening untreated (Figure 5-S9) and vacuum derivatized coconut oil (Figure 5-S10)

The results showed that this method is effective across different types of oils, as summarized in Table 5-2.

Table 5-2. List of oils Vac-HS-HC-SPME sulfonated PDVB derivatized in-situ oils described by the number of new peaks and fatty acid methyl esters (FAMEs) detected

OIL VARIETY	# OF NEW PEAKS	# OF FAMES
SESAME OIL	19	9
AVOCADO OIL	24	14
CANOLA OIL	77	19
COCONUT OIL	52	14

VEGETABLE SHORTENING	66	27

These results confirm that the Vac-HS-HC-SPME method effectively enhances the detection of FAMEs and other compounds across a variety of food oils. The increased number of peaks in the derivatized samples compared to their respective untreated samples highlights the method's ability to extract and identify a broader range of components, proving its potential for broader applications in lipid analysis and food quality assessment.

5.5 Conclusion

This study demonstrated that combining in-situ derivatization with Vac-HS-SPME is a novel and powerful approach for the efficient and sensitive analysis of FAMEs from olive oil. By optimizing the derivatization conditions using sulfonated poly(divinylbenzene) microspheres and employing a high-capacity (HC) SPME Arrow, we achieved superior extraction capabilities, as evidenced by significantly greater chromatographic areas under vacuum conditions compared to non-vacuum conditions. Specifically, the vacuum-assisted method detected 46 unique peaks and identified nine FAMEs, with two showing greater peak areas compared to non-vacuum sampling.

The versatility of this method was further demonstrated by applying it to other oils, including sesame, avocado, canola, coconut, and vegetable shortening. The vacuum-assisted derivatization resulted in the detection of numerous new peaks and successful conversion of fatty acids to FAMEs in all tested oils.

Further studies should focus on method validation and quantification, investigation of matrix effects, and comparison with conventional methods. Future studies could further explore the application of this novel vacuum-assisted in-situ GC-MS analysis method to a wider range of food products and biological samples. Investigations into the effects of different fiber coatings and derivatization reagents could optimize the method for specific classes of metabolites and biomolecules. Additionally, extending this method to untargeted metabolomics could provide comprehensive profiling of complex matrices, leading to new insights in food science, nutrition, and biomedical research.

Acknowledgements

The authors would like to thank Restek Corporation for providing financial support during the course of this research.

5.6 Supplementary Material

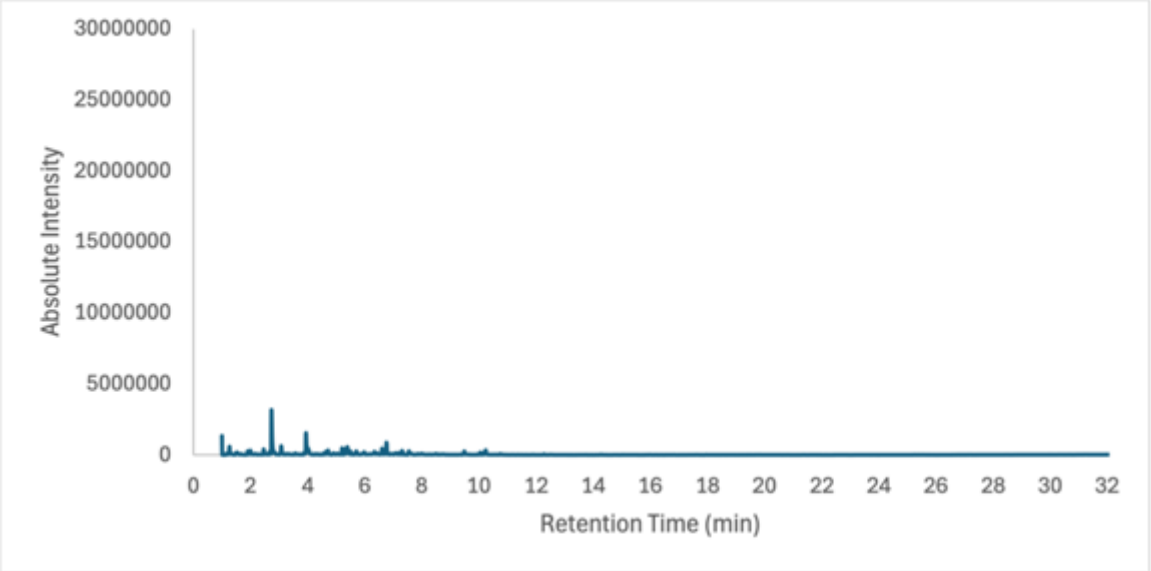

Figure 5-S1. Chromatogram of untreated sesame oil

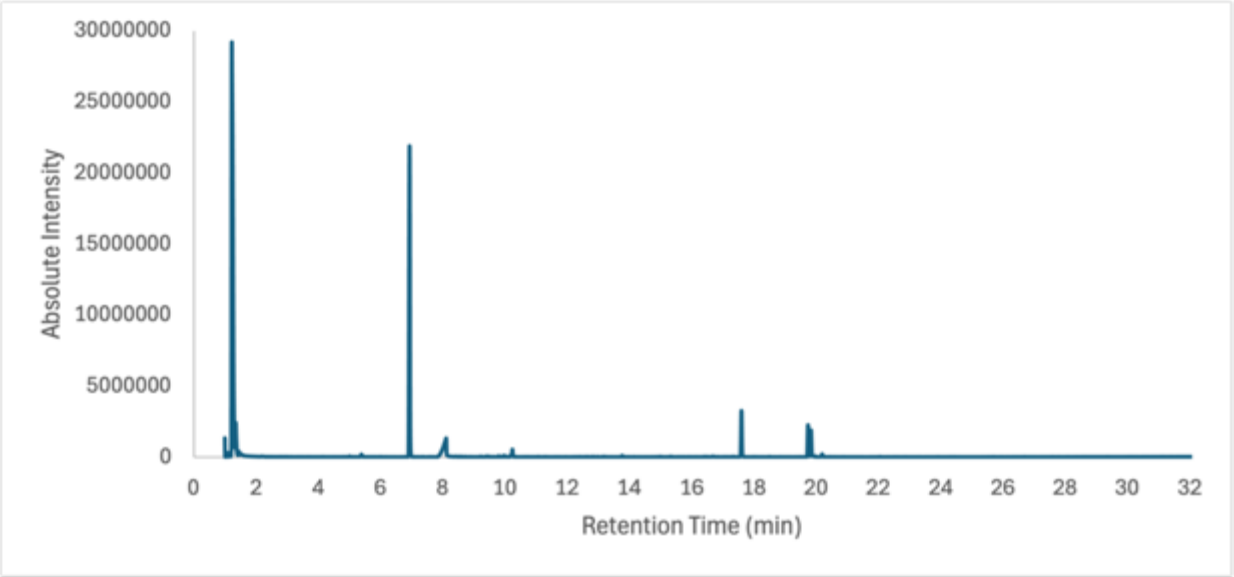

Figure 5-S2. Chromatogram of in-situ derivatization of sesame oil with sulfonated poly(divinylbenzene) microspheres and methanol using Vac-HS-HC-SPME sampling

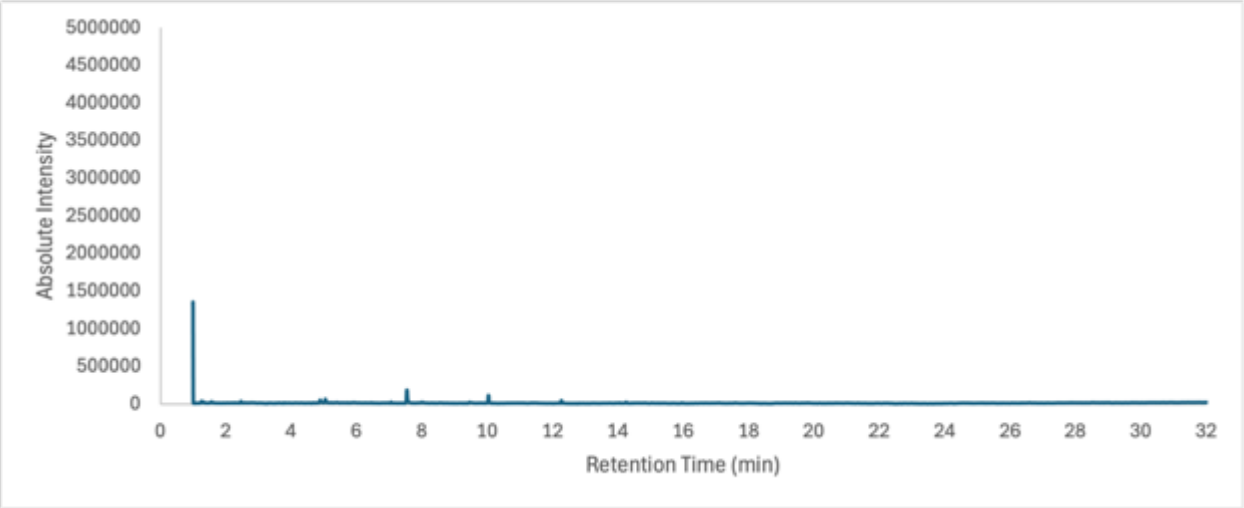

Figure 5-S3. Chromatogram of untreated avocado oil

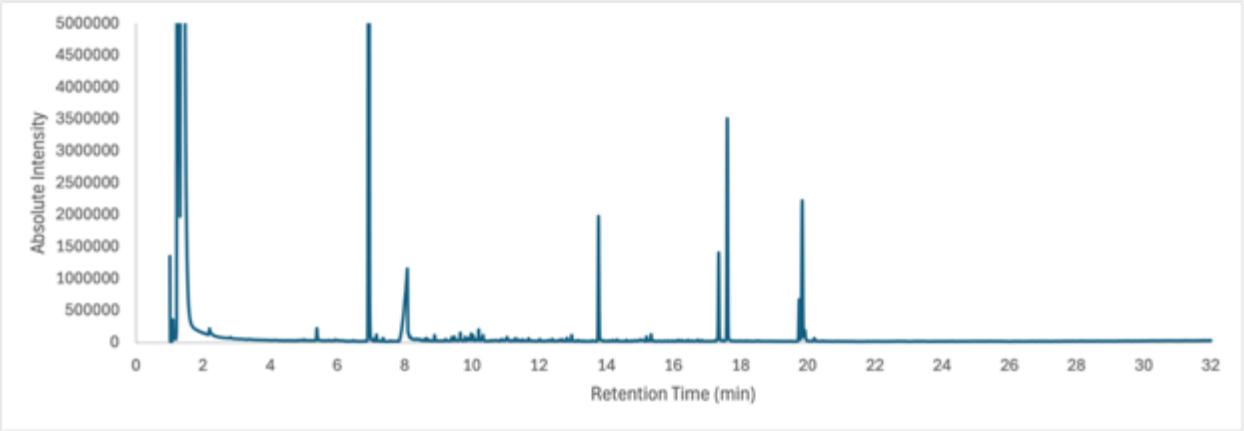

Figure 5-S4. Chromatogram of in-situ derivatization of avocado oil with sulfonated poly(divinylbenzene) microspheres and methanol using Vac-HS-HC-SPME sampling

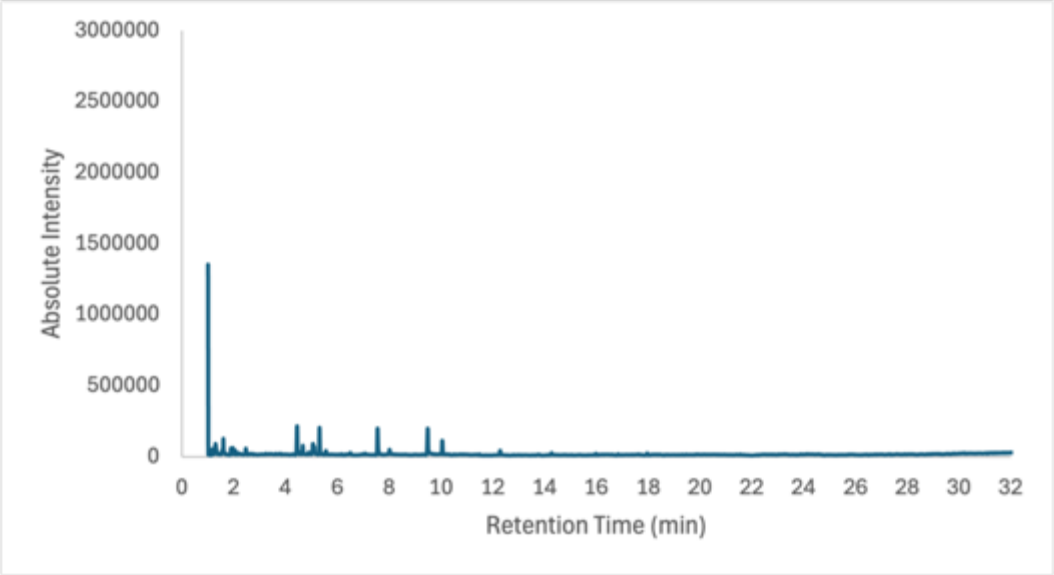

Figure 5-S5. Chromatogram of untreated canola oil

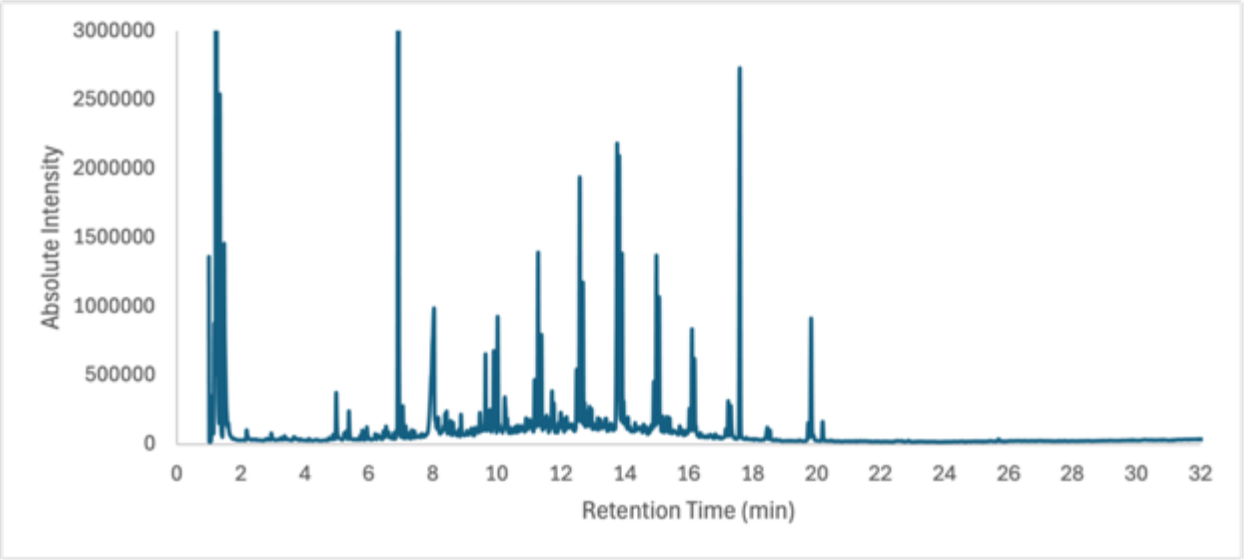

Figure 5-S6. Chromatogram of in-situ derivatization of canola oil with sulfonated poly(divinylbenzene) microspheres and methanol using Vac-HS-HC-SPME sampling

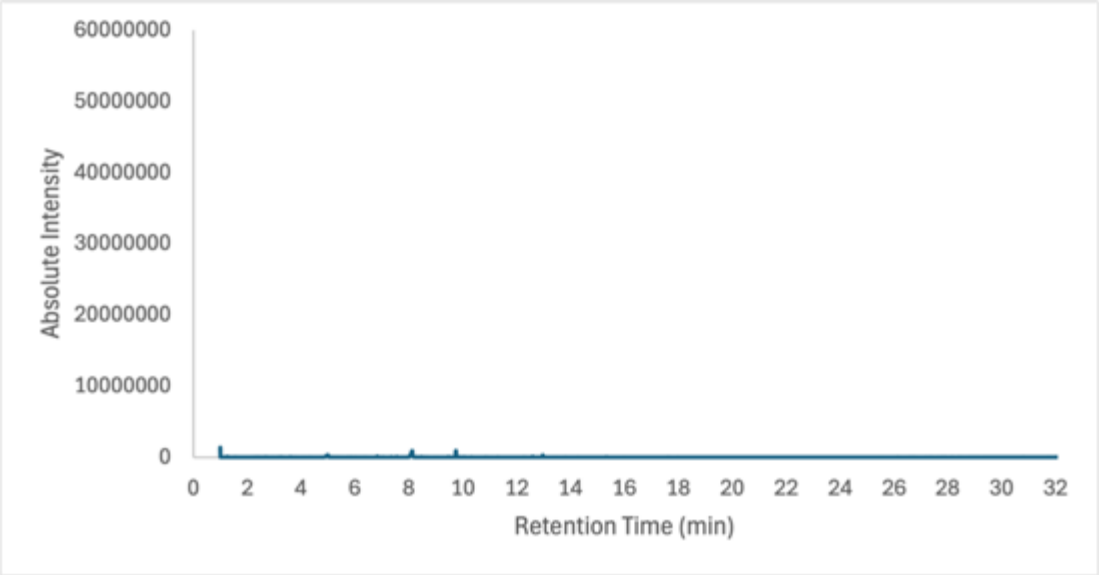

Figure 5-S7. Chromatogram of untreated coconut oil

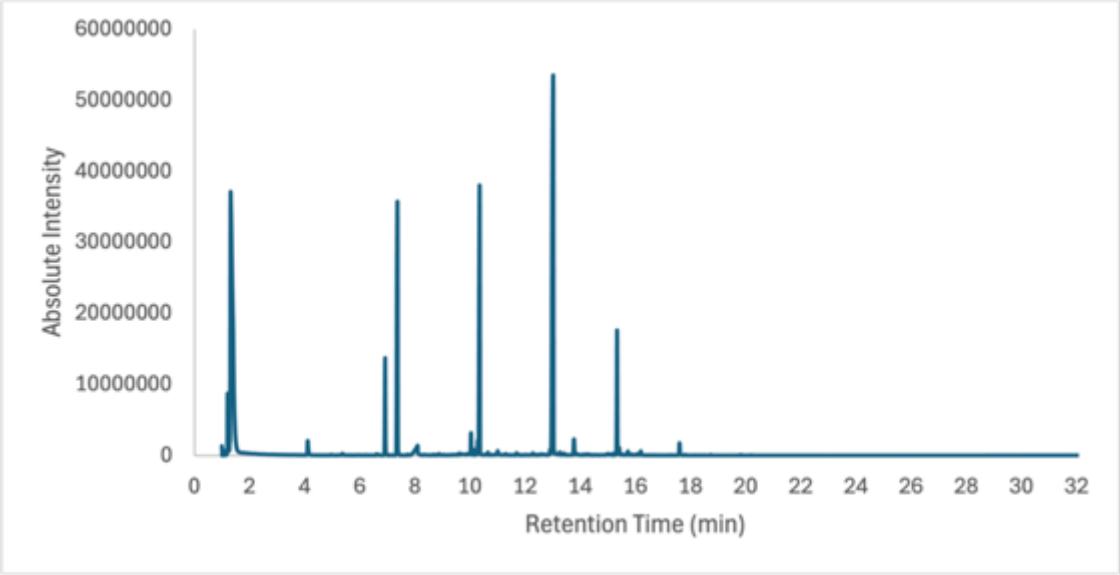

Figure 5-S8. Chromatogram of in-situ derivatization of coconut oil with sulfonated poly(divinylbenzene) microspheres and methanol using Vac-HS-HC-SPME sampling

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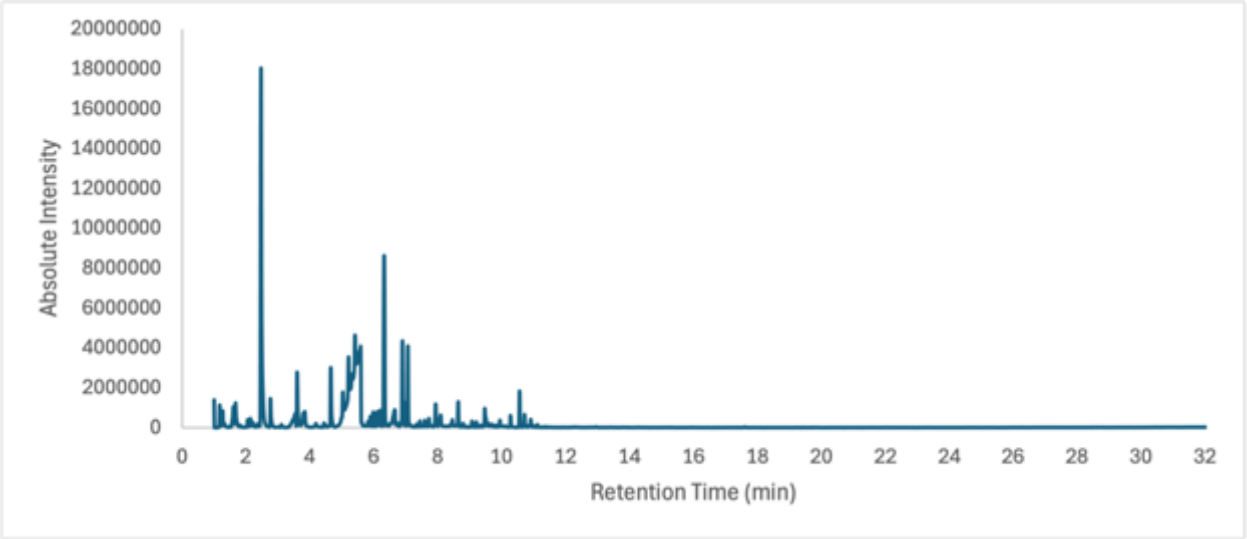

Figure 5-S9. Chromatogram of untreated vegetable shortening

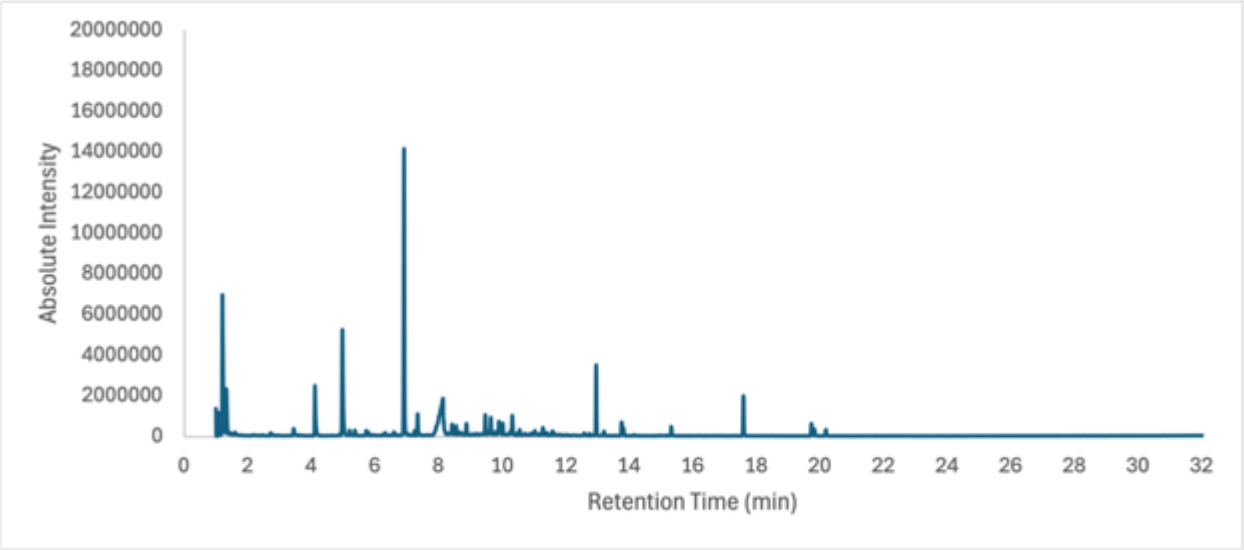

Figure 5-S10. Chromatogram of in-situ derivatization of vegetable shortening with sulfonated poly(divinylbenzene) microspheres and methanol using Vac-HS-HC-SPME sampling

CHAPTER SIX

Summary and Future Work

Vac-HS-HC-SPME sampling is an advantageous technique that allows lower temperature extraction from both liquid and solid samples, preserving the integrity of perishable samples and enhancing the detection of SVOCs. This method shows promise for diverse solid samples. Ottawa sand was used as a model sample material due to its favorable properties. Key findings include optimal sample vial preparation at -677 mbar for 90 seconds, with significant potential for automation. Current limitations involve the need to replace septa for each analysis, impacting cost and environmental sustainability. Thermogreen® LB-2 materials could mitigate this issue due to their heat resistance and prevention of off-gassing. Quantification and headspace partition coefficients require further study. Comparison studies of psilocybin and non-psilocybin mushrooms demonstrated increased compound response and the detection of additional compounds using Vac-HS-HC-SPME. Unique compounds were identified in psilocybin mushrooms, with further investigation recommended to optimize and verify findings. Combining in-situ derivatization with Vac-HS-SPME proved to be a novel and powerful method for the efficient and sensitive analysis of fatty acid methyl esters (FAMEs) from olive oil, achieving superior extraction capabilities and greater chromatographic areas under vacuum conditions. This approach also demonstrated versatility in converting fatty acids in various oils to FAMEs, indicating its potential for broader applications in lipid analysis.

Specifically focusing on in-situ derivatization of fatty acids to fatty acid methyl esters (FAMEs) from olive oil using sulfonated PDVB polymer microspheres, several future experiments

using Vac-HS-HC-SPME sampling could be conducted to expand and deepen the research. These experiments would include method validation and quantification, studying matrix effects, comparing with conventional methods, and applying the method to different types of olive oil and other oils. The proof of concept has been established, but the method needs validation for precision, accuracy, and linearity. Future experiments could investigate the impact of olive oil matrix components on derivatization efficiency and analytical performance. A study could be designed to compare the performance of Vac-HS-HC-SPME with traditional methods such as liquid-liquid extraction and direct injection for FAMEs analysis. In-situ derivatization can be applied to analyze fatty acid profiles from different olive oil varietals and geographical origins to assess the method's applicability and potential for authenticity testing. Additionally, the relationship between fatty acid composition and olive oil quality/freshness, including the effects of storage conditions, could be studied. This method could then be applied to other types of oils.

Pursuing these experiments could significantly advance the understanding and application of Vac-HS-HC-SPME sampling for the analysis of fatty acids in olive oil and potentially other oils, contributing to practical applications in the food industry.

Vac-HS-HC-SPME sampling has potential for many applications, including profiling biological tissues and systems. Future research could focus on method development and optimization for biological samples, optimization of fiber coatings specific to metabolites and biomolecules from biological tissues, targeted and untargeted metabolomics, sample stability, and applications to specific biological systems.

A Vac-HS-HC-SPME sampling method could be developed for tissue sample preparation. Protocols would be optimized for preparing biological tissues, including homogenization, cryomilling, or enzymatic digestion. On-fiber derivatization conditions could be optimized specifically for metabolites and biomarkers in biological tissues, exploring different derivatization reagents and conditions specific to biological matrices. Various SPME fiber coatings should be investigated to enhance the selective extraction of specific metabolites or biomolecules from biological tissues, including biocompatible coatings that minimize the degradation of sensitive biological compounds.

An experiment could be developed for targeted profiling of specific classes of metabolites, such as amino acids, lipids, and fatty acids in biological tissues. Untargeted metabolomics approaches could also be applied to identify and quantify a wide range of metabolites in biological systems using Vac-HS-HC-SPME coupled with GC-MS or other advanced analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy, for comprehensive profiling. Other applications could include profiling metabolites in specific organs or tissues, such as liver, brain, or muscle, to study organ-specific metabolism and function, and to identify and validate biomarkers for specific diseases such as cancer or metabolic disorders in biological tissues.

These experiments could significantly advance the application of Vac-HS-HC-SPME sampling for profiling biological systems and tissues, contributing to our understanding of biological processes, disease mechanisms, and biomarker discovery, with potential applications in research and clinical settings.

In conclusion, developing and optimizing Vac-HS-HC-SPME sampling hold significant promise for enhancing the analysis of both food and biological samples. When addressing current limitations and exploring new applications, this technique can further a more detailed understanding of various biological systems, improve quality control in the food industry, and assist in the discovery of biomarkers for disease, ultimately advancing scientific research and clinical diagnostics.

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BIOGRAPHICAL INFORMATION

Shannon L. Thomas received her Bachelor of Arts degree in Kinesiology from the University of Texas at Arlington in 2001. She was the head of the math department and a coach for 10 years at Trinity Christian School in Cedar Hill, Texas. Shannon returned to the University of Texas at Arlington to pursue a Bachelor of Science in Biological Chemistry in 2018. She began her Ph.D. journey in the Fall semester of 2020. Shannon joined Dr. Kevin Schug's Analytical Chemistry lab in 2019, where she pursued her interest in protein analysis of biological samples. Once funded by Restek Corporation, her dissertation research has focused on sample preparation of solid samples using vacuum-assisted headspace high-capacity solid-phase microextraction and analysis using gas chromatography and mass spectrometry. She completed two internships with Restek Corporation in Bellefonte, PA. She graduates with her Ph.D. in Chemistry from the University of Texas at Arlington in August of 2024 and plans to start a career in the health and nutrition industry.

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CHAPTER 2 – Vacuum-Assisted Headspace Solid-Phase Microextraction Sampling for the Extraction of Semi-Volatile Compounds: An Overview

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Permission given by Caroline Hroncich, Associate Editorial Director, on July 7, 2024.

CHAPTER 3 – Investigation of Operational Fundamentals for Vacuum-Assisted Headspace High-Capacity Solid-Phase Microextraction and Gas Chromatographic Analysis of Semivolatile Compounds from a Model Solid Sample

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Title of new work	Development of Vacuum-Assisted Headspace High-Capacity Solid-Phase Microextraction for the Determination of Semi-Volatile Compounds from Samples at Lower Temperatures
Institution name	University of Texas At Arlington
Expected presentation date	Aug 2024
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CHAPTER 4 – Comparison of Fragrance and Flavor Components in Non-Psilocybin and Psilocybin Mushrooms using Vacuum-Assisted Headspace High-Capacity Solid-Phase Microextraction and Gas Chromatography – Mass Spectrometry

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