QUANTIFICATION OF ODOROUS THIOLS IN WINE: AN EXTRACTIVE PENTAFLUOROBENZYL ALKYLATION FOLLOWED BY HEADSPACE SOLID-PHASE MICROEXTRACTION COUPLED TO GAS CHROMATOGRAPHY-ELECTRON IMPACT-MASS SPECTROMETRY

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ABSTRACT

A robust quantification method for polyfunctional thiols in wine is highly desirable due to their aromatic importance to many varietal wines. However, analytical determination of these compounds at levels near or below their ng/L sensory thresholds is difficult due to the complexity of a wine matrix, and poor detectability, poor chromatographic behavior, and high reactivity of the thiol functional group.

An improved method for the quantification of thiol contributors to wine aroma by headspace solid-phase microextraction (SPME) coupled to gas chromatography-electron impact ionization-mass spectrometry (GC-EI-MS) has been developed. Thiols are converted to their corresponding pentafluorobenzyl derivatives by extractive alkylation, the organic layer dried under nitrogen, and reconstituted in aqueous buffer prior to SPME analysis. Optimal extractive alkylation parameters (pH 12) and SPME parameters (70 °C, 60 minutes) were determined by response surface area modeling. Using 40 mL wine samples, achievable limits of detection for 4-methyl-4-mercapto-2-pentanone (4-MMP), 3-mercaptohexanol (3-MH), and 3-mercaptohexyl acetate (3-MHA) were 0.9 ng/L, 1 ng/L, and 17 ng/L, respectively. Standard addition of these thiols in a model wine system showed good linearity ($R^2 > 0.99$ for all thiols) over two orders of magnitude and applicability of method to a commercial wine matrix was confirmed through recovery (90-109%) and precision ($5.4\% < RSD < 11.1\%$) experiments. The method is scalable by pooling organic extracts over a sample size range of 10 to 160 mL, resulting in corresponding improvements in limits of detection. The method was validated using Riesling, Gewürztraminer, Cayuga White, Niagara, Rosé, and Sauvignon blanc wines from the Finger Lakes wine region in upstate New York. This method also permits measurement of low-molecular weight thiols responsible for certain aromatic defects in wine, such as H_2S and CH_3SH, in the same analysis.

BIOGRAPHICAL SKETCH

Lauren Musumeci is originally from Annandale, New Jersey. In May 2011, she received her Bachelors of Science in Chemistry from Rider University in Lawrenceville, New Jersey. At Rider University, she was a member and captain of the Women's Soccer team and president of Rider University's Chemistry Honors Society, Gamma Sigma Epsilon. While at Rider University, she also performed research in the area of organic synthesis, looking into the development of a novel synthetic pathway for stereospecific natural products shown to be active against tumor cells. She presented this research at an ACS Mid Atlantic Regional Meeting in May 2011. After an internship at ConAgra Foods, she decided that upon completing her Bachelors degree, she wanted to pursue a Masters of Science degree in the area of Food Science. In August 2011, she started her graduate studies in the Field of Food Science and Technology at Cornell University. While performing her graduate research in the area of wine flavor chemistry, she has been a member Cornell University's Food Science Club and an officer of the Student Association of the Geneva Experiment Station (SAGES). Additionally, she is an active member of the National and Western New York sections of the Institute of Food Technologists (IFT) and a participant in IFTSA & Mars, as well as, Disney-IFTSA product development competitions. While at Cornell University, she has also been a recipient of the WFFC Rita Flynn Memorial Fragrance Scholarship Award, Western NY IFT Scholarship, and AACT Walter Hopkins Memorial Scholarship. Upon completing her degree, she will begin work as an R&D Scientist at PepsiCo, Inc. in Valhalla, NY.

To my parents, mini-me, and Carlos. Without your infinite love, encouragement, and support, I would never be Ultimate.

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BACKGROUND

Thiol Importance to Aroma

Thiols are flavor compounds that play an important role in the aroma of many food products. The presence of thiols is widespread, having been identified in food and beverages such as coffee, meat, pan-roasted sesame seeds, grapefruit, passion fruit, green tea, onions, irradiated turkey, cheese, beer, and wine [1-12]. Despite often being present at low concentrations in foods and beverages, thiols can be critical to the aroma of foodstuffs due to their extremely low sensory thresholds, as low as ng/L level [13].

Flavor Properties for 3 Important Varietal Thiols in Wine

4-mercapto-4-methyl-2-pentanone (4-MMP), 3-mercaptohexanol (3-MH), and 3-mercaptohexylacetate (3-MHA), are three thiols that have been identified as important aromatic contributors to a wide range of varietal wines (Figure 1).

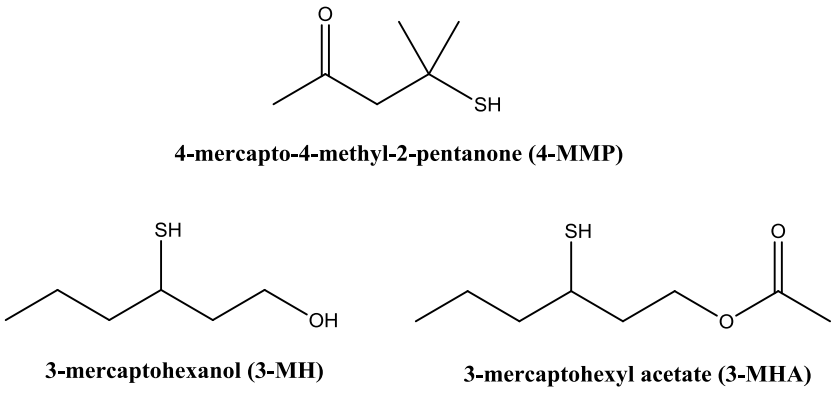

Figure 1. Chemical Structures of 4-MMP, 3-MH, and 3-MHA

Dating back many years, “boxwood” or “broom” has been used as descriptors for Sauvignon blanc aroma [14]. However, the compound responsible for that aroma, 4-MMP, was not identified in wine until the 1990s. Discovered in Sauvignon blanc wine via gas chromatography-olfactometry, 4-MMP was the first varietal thiol identified in wine [5]. 4-MMP was later identified in other varietal wines including Scheurebe, Maccabeo, Muscat, Gewürztraminer, Riesling, Pinot Gris and others [15, 16]. The presence of 3-MH and 3-MHA in varietal wines is more widespread than that of 4-MMP. In addition to their presence above threshold in most of the aforementioned wines, 3-MH and 3-MHA have also been identified in Petit Manseng, Petite Arvine, rosé and red wines produced from Merlot and Cabernet Sauvignon grapes, and others [9, 17-20].

4-MMP, 3-MH, and 3-MHA are able to contribute significantly to the aroma of many varietal wines due in part to their low sensory thresholds, which are down to the low ng/L level in model wine solutions (Table 1) [5, 13, 20]. The concentrations of 4-MMP, 3-MH and 3-MHA, particularly in Sauvignon blanc wines, can exceed concentrations of 10 ng/L, 5000 ng/L, and 60 ng/L, respectively [21].

Table 1. Organoleptic properties for 4-MMP, 3-MH, 3-MHA (ND = Not Determined)

	Detection Threshold in Water (ng/L)[a]	Detection Threshold in Model Wine* (ng/L)	Detection Threshold in White/Red Wine (ng/L)	Aroma Attributes
4-MMP	0.1	0.8[a]	3[b]	Box tree[a], Broom[a]
3-MH	17	60[c]	ND	Grape fruit[d], passion fruit[a]
3-MHA	2.3	4.2[c]	ND	Box tree[d] Passion fruit[a]

*Aqueous alcohol solution (12% v/v)
a: [13] d: [22]
b: [5]
c: [20]

Thiol Precursors

4-MMP, 3-MH, and 3-MHA are not detectable in grapes, but are instead released during fermentation. Both 4-MMP and 3-MH are believed to exist primarily as odorless S-cysteine conjugates in the juice [23]. During fermentation, it is thought that 4-MMP and 3-MH are released enzymatically, through cleavage of the C-S bond by β-lyase in the yeast (Figure 2) [23, 24]. In grapes, neither 3-MHA nor its cysteine precursor has been identified. This compound is believed to arise from the acetylation of 3-MH during fermentation [24, 25].

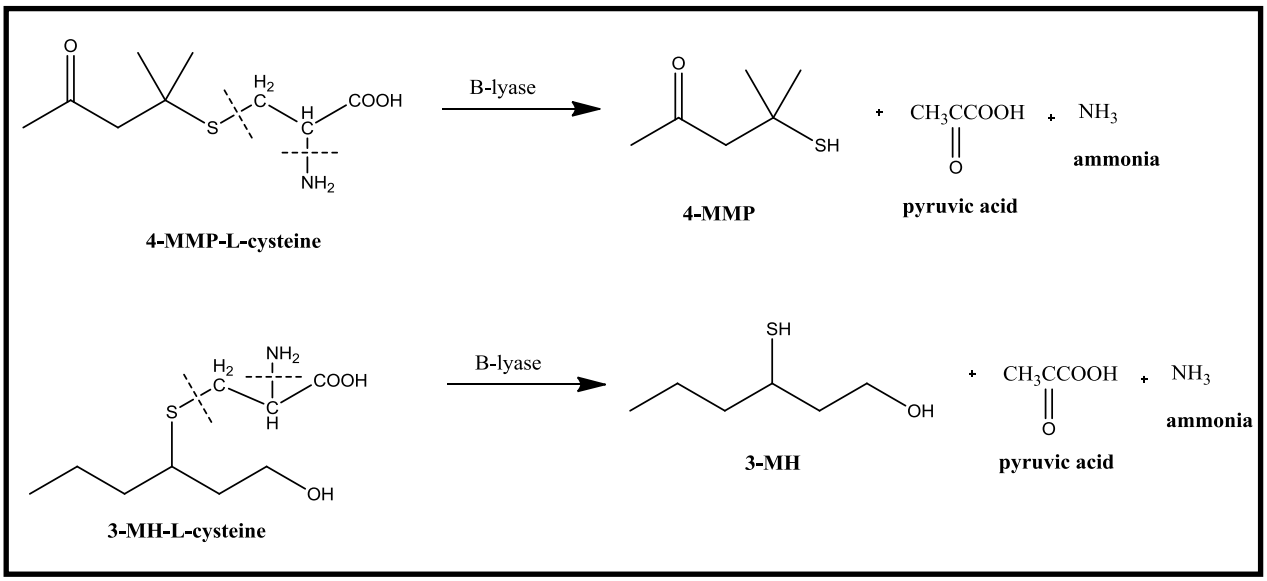

Figure 2. α,β Elimination reaction of S-cysteine conjugate β-lyase [14]

Since S-cysteine precursors are considered essential compounds for thiol production in wine, many groups have looked at what factors affect the concentration of these precursors in the grape. Both growing and post-harvest conditions are known to affect precursor concentrations in the juice. Viticulture practices can affect precursor concentration as environmental conditions during berry development including water, soil composition, and elevation have been found to

influence precursor concentrations [26, 27]. Harvest and post-harvest practices can also influence precursor concentrations. Early morning harvesting, rather than daytime harvesting, machine harvesting grapes, rather than hand harvesting, and transporting grapes, rather than processing immediately, were found to increase 3-MH precursor concentration [28, 29]. In regards to post-harvest practices, extended skin contact and higher contact temperatures during fermentation were found to increase 3-MH, and to a lesser degree, 4-MMP precursor concentrations [18, 30]. The differences in precursor extraction were theorized to arise due the presence of the 3-MH precursor in nearly equal amounts in the skin and juice, while the majority, approximately 80%, of the total 4-MMP precursors are located in the juice [18, 30]. Other enological conditions, including yeast selection and fermentation temperature, have also been shown to influence 4-MMP and 3-MH precursor concentrations [31, 32]. However, there has been difficulty in finding correlations between precursor concentrations in the juice and corresponding thiol concentrations in wine [33].

Thiol Contributors to Wine Off Aroma

Unlike polyfunctional thiols, such as 4-MMP, 3-MH, and 3-MHA, low-molecular weight thiols, including methanethiol, ethanethiol, and hydrogen sulfide, are associated with off aromas in wine [34, 35]. The odor of such compounds has been described as “cooked cabbage” and “rotten egg” [34, 36]. The sensory thresholds for these compounds are significantly higher than those of polyfunctional thiols, having thresholds in the low µg/L level (Table 2) [36]. Rather than emerging from grape precursors, the presence of low-molecular weight thiols in wine is a result of the degradation of sulfur containing pesticides or yeast metabolism of sulfur containing amino acids during fermentation [36].

Table 2. Sensory Thresholds for 3 Low-Molecular Weight Thiols

	Sensory Detection Threshold[a]
Hydrogen Sulfide	1 ng/L – 150 µg/L[1]
Methanethiol	0.2-2 µg/L[2]
Ethanethiol	1.1 µg/L[1]

[1] in wine; [2] in water
a: [36]

Difficulties in Thiol Quantification

As mentioned above, several thiols in wines have low sensory thresholds, in the range of 1-1000 ng/L [18, 21, 22, 37]. To assess their aromatic importance to a wine, detection limits for quantification methods must be at or near the sensory threshold for the thiols of interest. Achieving these low detection limits has proven difficult for a few reasons. One is that most thiols are not easily detected by mass spectrometry [38]. Thiols do not have characteristic ions of high *m/z* that would differentiate them from other compounds in the complex wine matrix [38]. Second, the thiol functional group results in tailing peaks on a gas chromatograph due to adsorptive properties of the functional group with the chromatography column, resulting in loss of resolution, sensitivity, and precision [38]. Lastly, thiols are highly reactive and unstable compounds. The thiol functional group is prone to oxidation and can also readily form complexes with transition metals [38, 39].

Methods for Thiol Quantification in Wine

Acknowledging these difficulties, research groups have developed different methods to measure thiol concentrations in wine. The majority of these methods have been for polyfunctional thiol rather than low-molecular weight thiol measurement. The methods that

have been developed employ a range of different strategies. These strategies include Hg-based selective extraction, direct solid-phase microextraction and solid phase extraction, and derivatization.

Selective Extraction

Initial methods to isolate and quantify thiols in wine sought to purify these compounds via non-selective liquid-liquid extraction [16]. This inefficient selectivity gave rise to quantification methods that sought to identify and quantify volatile thiols by selective extraction. Research groups, including Tominaga et al. and Ferriera et al., have used the fact that the thiol functional group readily reacts with mercury containing salts to form complexes to isolate thiols from wine [5, 19, 20, 22, 37, 40]. When using this strategy for thiol isolation, a thiol-containing wine extract is exposed to an organomercury salt, to which the thiols bind [5, 20, 22]. A few different mercury sources have been explored, including *p*-hydroxymercuricbenzoic acid (*p*-HMB) and mercury ions fixed on Affi-Gel 501 column [5, 20, 37, 40]. Thiols are released from this mercury-thiol complex through the excess addition of another thiol that is not extractable by organic solvent, such as cysteine, and then quantified by gas chromatography coupled to mass spectrometry (GC-MS) or sulfur selective detectors (Figure 3) [5, 19, 21, 23, 37, 40]. More recent reports improve thiol selectivity and decrease non-thiol impurities by introducing an anion exchanger column after the mercury-based extraction or use solid phase extraction (SPE) with LiChrolut EN resin prior to extraction with the mercury source [22, 40]. When supplementing the selective extraction strategy with an anion exchange column step, the mercury-thiol complex attaches to the column resin and the non-bound compounds are eluted, ultimately increasing selectivity by eliminating non-thiol impurities from measurement [22]. Through the addition of SPE, fatty acids and other interferences are extracted, increasing

thiol selectivity by limiting the complexity of the matrix that is allowed to react with the mercury source [40]. The Hg-based selective extraction strategy excludes most non-sulfur containing organic acids, as well as other S-compounds like disulfides, sulfides, and thioesters, that will not strongly bind to mercury [5].

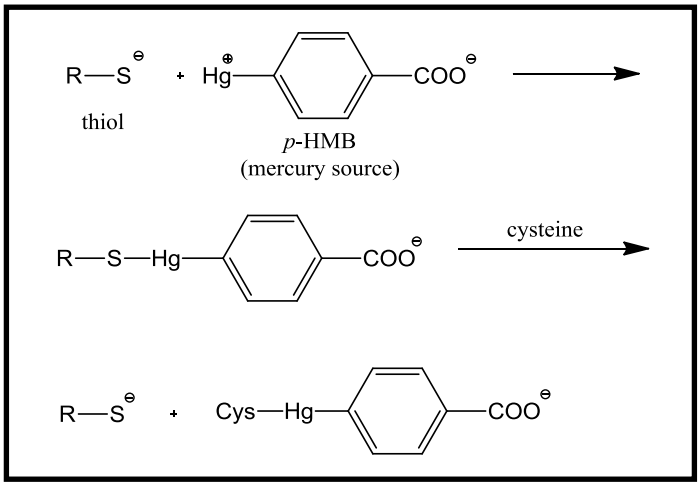

Figure 3. From Dubourdieu et al. 2006, the reversible reaction of thiols with *p*-HMB [24]

Implementation of this selective extraction strategy in wine has only been demonstrated for quantification of polyfunctional thiols. Also, none of the methods have addressed thiol instability. Ferriera et. al. reported oxidation of the thiol analytes during quantification using this strategy [38, 40]. Additionally, methods using selective extraction have required large volumes of wine, have long and complicated protocols, and have used mercury, which, even in low doses, has been shown to be toxic to humans [41].

Direct Headspace Solid-Phase Microextraction and Solid Phase Extraction

Solid-phase microextraction (SPME) separates compounds in a sample matrix based on their affinity for an extraction phase coating on a solid support, referred to as the fiber [42]. A compound's affinity for the extraction phase coating results in a transfer of analytes onto the fiber coating once it is exposed to the sample [42]. By using headspace SPME (HS-SPME),

additional selectivity is achieved by limiting non-volatile inferences and compounds with high molecular masses that may be present in the sample matrix [42]. In HS-SPME, the fiber is not in direct contact with the sample matrix, but is exposed to the headspace above it, requiring analytes to be in the gas phase for absorption onto the fiber [42].

In solid phase extraction (SPE), separation is based on the affinity for a solid (sorbent) phase, rather than an extraction phase coating as is used in SPME [43]. When using SPE, a liquid sample is passed through a vessel, known as the SPE cartridge, containing a solid phase [43]. This concentrates the analytes of interest, as not all compounds in the matrix will be retained in the stationary phase [43]. The retained analytes are then recovered by washing the sorbent with an appropriate solvent [43].

Simultaneous polyfunctional and low-molecular weight thiol measurement via this direct HS-SPME or SPE strategy has not been demonstrated. Fedrizzi et al. developed methods for quantifying polyfunctional thiols in wine using HS-SPME or SPE techniques directly on a wine sample [44]. The resulting techniques are faster and easier than the Hg-based approaches, and also avoid the use of toxic mercury [44]. However, the resulting detection limits are far above the sensory thresholds for these compounds in wine [44]. Fang et al. developed a method to quantify low-molecular weight thiols in wine where HS-SPME was performed directly on the wine sample [34]. With this fast and simple protocol that required no sample manipulation prior to quantification, acceptable LODs were achieved for many low-molecular weight thiols, although polyfunctional thiols were not considered [34]. Improved methods for low-molecular weight thiol quantification with this strategy employed extra precautions during sample preparation, such as the use of inert atmosphere glove bags or deactivated glass headspace sample vials [45].

Derivatization

Recent methods for thiol quantification have explored the derivatization of thiols prior to quantification [25, 38, 46-48]. Derivatization can improve stability, detectability by mass spectrometry, and chromatographic behavior by yielding compounds that can have mass spectral peaks at higher mass regions, where there is less potential for background interference, and compounds with better adsorption properties [49, 50]. As these improvements solve many of the quantification difficulties previously described for thiols, derivatization is the most suitable strategy for thiol quantification.

Hoffman et al. has suggested 4-vinylpyridine as a derivatizing agent and Ortin et al. has developed methodology for the use of this derivatizing agent for compounds in wine [38]. However, Mateo-Vivaracho et al. has noted the complicated chromatographic properties of these pyridine derivatives and only a small gain in detectability using this derivatizing agent [48].

The most utilized derivatizing agent for thiols in wine is pentafluorobenzyl bromide (PFBBr) [25, 38, 47, 48]. The first use of PFBBr as a derivatizing agent for thiol quantification was reported by Kawahara for thiol measurement in water [51]. With this derivatizing agent, the thiol proton is first removed by a base [49]. The negatively charged sulfur undergoes nucleophilic substitution with the PFBBr derivatizing agent, allowing for the concurrent loss of the bromine leaving group (Figure 4) [49]. This nucleophilic substitution with PFBBr is not exclusive for thiols, but can also form derivatives with phenols and organic acids [49, 51].

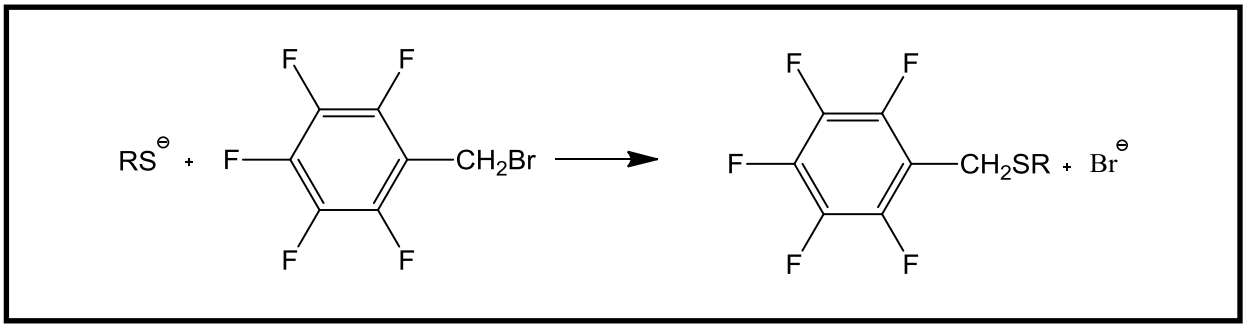

Figure 4. Derivatization of thiol with PFBBr reagent

Different methods have employed the PFBBr derivatization strategy for the quantification of polyfunctional thiols in wine. One method has involved a fully automated PFBBr derivatization directly on a SPME fiber [38]. This method noted acceptable analytical performance for only two thiols and limited linear ranges [38, 48]. Improved methods with acceptable limits of detection and linear ranges have utilized SPE steps prior to a PFBBr derivatization, which has been facilitated through the addition of the strong 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) base [46-48]. These improved methods have also carried out a methoximation step for 4-MMP prior to PFBBr derivatization to prevent intramolecular H-bonding of the thiol proton with the carbonyl group, increasing the molecule's availability for derivatization [46-48]. More recent methods have unified the SPE cartridges used for isolation and derivatization and have avoided the use of large volumes of toxic benzene solvent [46, 47]. Improvement in the limits of detection for some thiols has also been achieved through the incorporation of HS-SPME coupled to gas chromatography into the protocol, rather than liquid injection [46].

Quantification methods for low-molecular thiols in wine using a derivatization strategy have not been demonstrated. However, low-molecular weight thiol quantification using derivatization has been demonstrated in other matrices. Wang et al. was able to quantify

low-molecular thiols using N-phenylmaleimide as a derivatizing agent in various food matrices [52]. However, this method could not detect hydrogen sulfide, or any compound that contained two thiol groups [52]. Quantification of hydrogen sulfide using PFBBr as a derivatizing agent has been demonstrated in a blood matrix [53]. This method achieved an acceptable LOD for hydrogen sulfide, indicating the potential to extend the use of this derivatization agent for quantification of low-molecular weight thiols in wine [53].

Use of Deuterated Analogs as Internal Standards

In analytical derivatization, it is beneficial to introduce internal standards to correct for any losses that may occur during steps of the analysis and ensure consistent quantification results [49]. Initial methodology for thiol quantification in wine employed the addition of different thiol compounds as internal standards [19, 22, 38]. Linearity and matrix effect issues were reported when other thiols were used as internal standards, theorized to be a result of the behavior of the chosen internal standards not matching the behavior of the desired analytes [38]. These internal standards contained other functional groups that differed from those present in the thiols being quantified, aiding in the likelihood of inaccurate quantification [15, 21, 37]. Isotopic analogs, such as deuterium labeled analogs, are known to have similar physiochemical properties to the non-labeled compound, making them ideal internal standards for GC-MS analysis [49]. The use of deuterated analogs as internal standards for the quantification of thiols in wine was first reported by Schneider et al. [37]. A more recent report by Mateo-Vivaracho et al. that introduced deuterated analogs as internal standards has noted improvements in previously observed matrix effect issues [47].

In gas chromatography, changes in isotopic composition results in changes in retention time due to differing solute interactions with the stationary phase and changes in vapor pressure [54-56]. The introduction of a deuterium labeled analog can result in either a decrease or increase in retention time for the compound. In most cases, a decrease in retention time, termed an "inverse isotope effect" or "chromatographic isotope effect", is observed [56-58]. This results from the increased bond strength between C-D vs. C-H, yielding a decreased interaction between the deuterated analog and the stationary phase [55, 56, 59-61].

Ionization Methods for Mass Spectrometry

Both electron impact ionization (EI) and negative ion chemical ionization (NICI) conditions have been used for methods that have quantified thiols in wine through their pentafluorobenzyl (PFBn) derivatives [25, 38, 46-48]. In EI mass spectrometry (EI-MS), the mass spectrum is produced from the bombardment of electrons onto the neutral molecule of interest [62]. A collision between an electron and the molecule results in an excited, positively-charged molecule [62]. Fragmentation of this excited molecule occurs, and positively charged fragments are detected to produce the mass spectrum [62]. In NICI mass spectrometry (NICI-MS), a collision between the molecule of interest and another gaseous molecule results in the ionization of molecule of interest [63]. With this collision, there is little energy transfer to the desired molecule resulting in less fragmentation [63]. The detection of negative ion fragments produces a less fragmented mass spectrum for NICI [63].

For the detection of PFBn derivatives, NICI is the more selective and sensitive type of ionization, since it favors electrophilic atoms, such as fluorine, that readily form negative ions [38, 64]. The limit of detection (LOD) for PFBn derivatives when employing NICI-MS, as

compared to EI-MS, has improvements of 1 to 5 orders of magnitude [65-68]. These significant differences in sensitivity are evident in the previous methods that have quantified thiol PFBn derivatives in wine. When NICI-MS was employed, acceptable LODs were achieved with low starting wine volumes for multiple thiols [46-48]. Using the more widely available EI-MS, Capone et al. was able to achieve an acceptable LOD for only one thiol and required a large volume of starting wine [25].

Goals of Project

Despite improvements with methods for quantifying polyfunctional thiols in wine, there are still many problems that make these methods inapplicable for usage in industrial laboratories (Table 3).

Table 3. Summary of previous thiol quantification methods in wine

Reference	Analytical Strategy	Downfalls of Method
Darriet et al. 1995 Tominaga et al. 1998 Tominaga et al. 2000 Schneider et al. 2003 Ferriera et al. 2007	Selective Extraction with Mercury	Use of toxic mercuric reagent, does not address thiol instability, poor chromatographic behavior, poor detectability
Fedrizzi et al. 2007	Direct HS-SPME; Direct SPE	Unacceptable detection limits, does not address thiol instability, poor chromatographic behavior, poor detectability
Mateo-Vivaracho et al. 2006	On Fiber PFBBr derivatization	Linearity problems, acceptable LODs for only two thiols, use NICI mass spectrometry
Mateo-Vivaracho et al. 2007	PFBBr derivatization	Use NICI mass spectrometry, use strong DBU base
Mateo-Vivaracho et al. 2008	PFBBr derivatization	Use NICI mass spectrometry, use strong DBU base
Rodriguez-Bencomo et al. 2009	PFBBr derivatization	Use NICI mass spectrometry, use strong DBU base
Capone et al. 2011	PFBBr derivatization	Only quantified one thiol, method likely unacceptable for thiols with lower thresholds

The goal of this project was to develop an improved method for the quantification of three important varietal thiols in wine, 4-MMP, 3-MH, and 3-MHA. Specifically, the aims of this new method would

i) achieve detection limits at or near the sensory threshold for each thiol

ii) Use EI-MS, due to its wider availability

iii) Minimize use of toxic reagents, particularly mercury

iv) Use modest volumes of wine (< 100 mL)

This method would then be implemented for use in the quantification of wines from the Finger Lakes wine region in upstate New York. Additionally, as there have been no reports of a quantification method that can measure polyfunctional and low-molecular weight thiols in wine in the same run, one last goal was to test this new method for its ability to measure low-molecular weight and polyfunctional thiols simultaneously.

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CHAPTER 2

QUANTIFICATION OF ODOROUS THIOLS IN WINE: AN EXTRACTIVE PENTAFLUOROBENZYL ALKYLATION FOLLOWED BY HEADSPACE SOLID-PHASE MICROEXTRACTION COUPLED TO GAS CHROMATOGRAPHY-ELECTRON IMPACT-MASS SPECTROMETRY

Abstract

An improved method for the quantification of thiol contributors to wine aroma by headspace solid-phase microextraction (SPME) coupled to gas chromatography-electron impact ionization-mass spectrometry (GC-EI-MS) has been developed. Thiols are converted to their corresponding pentafluorobenzyl derivatives by extractive alkylation, the organic layer dried under nitrogen, and reconstituted in aqueous buffer prior to SPME analysis. Optimal extractive alkylation parameters (pH 12) and SPME parameters (70 °C, 60 minutes) were determined by response surface area modeling. Using 40 mL wine samples, achievable limits of detection for 4-methyl-4-mercapto-2-pentanone (4-MMP), 3-mercaptohexanol (3-MH), and 3-mercaptohexyl acetate (3-MHA) were 0.9 ng/L, 1 ng/L, and 17 ng/L, respectively. Standard addition of these thiols in a model wine system showed good linearity ($R^2 > 0.99$ for all thiols) over two orders of magnitude and applicability of method to a commercial wine matrix was confirmed through recovery (90-109%) and precision (5.4% < RSD < 11.1%) experiments. The method is scalable by pooling organic extracts over a sample size range of 10 to 160 mL, resulting in corresponding improvements in limits of detection. The method was validated using Riesling, Gewürztraminer, Cayuga White, Niagara, Rosé, and Sauvignon blanc wines from the Finger Lakes wine region in upstate New York. Additionally, this method permits measurement of derivatives of low-molecular weight thiols, such as H_2S and CH_3SH, in the same analysis.

Introduction

Thiols are sulfur containing compounds known for their strong odors and extremely low sensory detection thresholds. These flavor compounds play an important role in the aroma of many food and beverage products, such as coffee, meat, pan-roasted sesame seeds, grapefruit, passion fruit, green tea, onions, irradiated meat, cheese, and beer [1-10]. They are of particular importance in wine aroma [11-14]. In wine, low-molecular weight thiols are often associated with off-aromas, arising as byproducts of yeast metabolism of sulfur containing amino acids [13-15]. In contrast, polyfunctional thiols provide important and desirable varietal aromas, arising from precursors in the grape as a result of fermentation [16]. Three polyfunctional thiols that have been determined to be of particular importance to varietal wine aroma are 4-mercapto-4-methyl-2-pentanone (4-MMP), 3-mercaptohexanol (3-MH), and 3-mercaptohexyl acetate (3-MHA), and are described as having box tree, grapefruit, and passion fruit aroma, respectively [17, 18]. These thiols have been identified as important contributors to the aroma of many different varietal wines, including Sauvignon Blanc, Riesling, Gewürztraminer, and others [11, 12, 18-24].

The sensory detection thresholds for 4-MMP, 3-MH, and 3-MHA are 0.8 ng/L, 60 ng/L, and 4.2 ng/L, respectively [18, 24]. Trying to quantify these thiols at levels near or below their sensory detection threshold is a challenge. Thiols have low detectability as a result of non-characteristic ions of high *m/z*. Additionally, thiols exhibit poor chromatographic behavior and have a high potential for oxidation during handling [25, 26].

There have been a few different approaches for analytical methods that seek to quantify thiols in wine at these low levels. One such approach, explored by Darriet et al. and Tominaga et al., was quantification through selective extraction with a mercury containing salt

[11, 17, 18, 22, 27, 28]. However, the use of a toxic substrate and no consideration for the detectability, chromatographic issues, and instability of the thiol functional group plague this strategy. Additionally, to achieve acceptable limits of detection, large volumes (1-2 L) of wine must be used. A second strategy by Fedrizzi et al. explored performing solid phase extraction (SPE) and solid-phase microextraction (SPME) directly on a wine sample [29]. However, the resulting detection limits were far above the sensory thresholds of the thiols being measured. This strategy also failed to address the detectability and chromatographic issues that trouble thiol quantification. A third strategy that has been explored by Mateo-Vivaracho et al. and others and has shown to improve detectability and chromatographic behavior in thiol analysis is derivatization of the thiol prior to quantification [25, 30-33]. Derivatization with pentafluorobenzyl bromide (PFBBr) has been the most commonly employed derivatizing agent. Resulting derivatized adducts are easily detected by mass spectrometry and disassemble the thiol functional group to produce more stable adducts with better chromatographic behavior. However, acceptable detection limits for previous methods require the use of negative chemical ionization mass spectrometry (NICI-MS), which is not available in many commercial laboratories [25, 30-33]. The more widely used type of ionization is electron impact ionization (EI). However, EI-mass spectrometry (EI-MS) achieves limits of detection (LOD) that are 1 to 5 orders of magnitude higher than those of NICI-MS [34-37].

This paper proposes an improved method to quantify 3 polyfunctional thiols, 4-MMP, 3-MH, and 3-MHA, achieving method detection limits at or near their respective sensory thresholds using the less sensitive EI-MS detection. Validation of this method is achieved through thiol measurement in several varietal wines from the Finger Lakes region of upstate New

York. Additionally, this method is tested for its versatility for both low- and high-molecular weight thiol analysis.

Materials and Methods

Chemical Reagents and Standards

Sodium chloride (NaCl), sodium hydroxide (NaOH), pentane, 2-propanol, diethyl ether, ethanol, and the unlabeled standards, 3-MH, 4-MMP, and 3-MHA, were purchased from Sigma Aldrich (Allentown, PA). The deuterated internal standards, $^{8}[H_2]$-3-mercaptohexanol (d_8-3-MH), $^{10}[H_2]$-4-mercapto-4-methyl-2-pentanone (d_{10}-4-MMP), and $^{5}[H_2]$-3-mercaptohexyl acetate (d_5-3-MHA), were synthesized by adapting the protocols described by Kotseridis et al. and Pardon et al. [38, 39]. Stock solutions of the undeuterated and deuterated standards were prepared volumetrically in 2-propanol and stored at -4 °C until required. Model wine consisted of 12% v/v aqueous ethanol solution containing 7 g/L tartaric acid and the pH adjusted to 3.4 with 2 M NaOH. Commercial wine samples used for analysis were obtained from retail outlets. Water was purified by Milli-Q system from Millipore (Bedford, MA. USA).

Assessment of Method Parameters

All optimization experiments were performed in a model wine matrix with LODs for each sample calculated as 3 standard deviations x sample noise (height of the baseline near the peak of interest). The description and parameter range of each factor are listed in Table 1.

Derivatization

Six different derivatization parameters were investigated: agitation time, buffer reconstitution volume, organic solvent volume, sample pH, sample volume, and concentration of the 18-crown-6 ether phase transfer catalyst. Optimal parameters were either determined using a

center composite face-centered (CCF) model or individually. Optimization of three factors (sample pH, sample volume, phase transfer catalyst [PTC] concentration) was performed using a CCF model with the goal of minimizing the LOD for each thiol. The remaining three factors, agitation time, reconstitution volume, and solvent volume were optimized individually. Significant differences between LODs for individual parameters were determined by Tukey's test. Optimal reconstitution volume was determined individually as this factor does not affect the yield of the extractive alkylation. The optimal solvent volume was determined individually due to the complexity it would add to the CCF model, as variable solvent volume results in variable solvent dry down times. Optimal agitation time for the extractive alkylation was determined individually because too many factors in one CCF model could lead to too much of the intermediate region uninvestigated compromising the calculated significance for each parameter [40]. The conditions for each derivatization optimization experiment are summarized in Table 2.

HS-SPME

Two HS-SPME factors were examined: extraction time, and extraction temperature. These factors were examined by a CCF model with the goal of minimizing the LOD for each thiol. Conditions of the optimization experiment are summarized in Table 2.

Statistical Analysis

Statistical analysis was performed by JMP version 9 (SAS Institute, Cary, NC) using Tukey's test and least squares model.

Table 1. Description and range of optimized derivatization and HS-SPME parameters

Parameters	Values Used	Descriptions
Derivatization Parameters		
Agitation Time[a]	10, 25, 40 min	The amount of time the sample is agitated during the derivatization step
Solvent Volume[a]	8, 12, 16 mL	The amount of organic solvent used for the extractive alkylation
Reconstitution Volume[a]	0, 5, 10 mL	The amount of aqueous buffer added to the dried down SPME vial prior to HS-SPME analysis
pH[b]	6, 9.5, 12	Sample's pH adjusted with 2 M NaOH solution
Catalyst[b]	0, 0.05, 0.1 g	The amount of phase transfer catalyst (18-Crown-6 ether) in a solution containing 0.1 mL PFBBr in 5 mL 2-propanol
Volume[b]	10, 25, 40 mL	The initial volume of wine sample used
SPME Parameters		
Time[b]	10, 30, 60 min	Sample extraction time in the heating block after exposure of the SPME fiber
Temperature[b]	50, 70, 90 °C	The setting for the incubation and extraction temperature on the heating block

[a]Optimized value determined individually
[b]Optimized value determined by CCF modeling

Table 2. Conditions used for derivatization and HS-SPME optimization experiments

Parameter(s)	Model Wine Volume	Thiol Concentration	No. of replicates
Agitation Time	20 mL	200 ng/L	3
Solvent Volume	40 mL	3000 ng/L	3
Reconstitution Volume	40 mL	200 ng/L	2
pH, Volume, Catalyst	--	2000 ng/L	2
SPME Time, Temperature	40 mL	3000 ng/L	2

Optimized Method

Derivatization - Extractive Alkylation

In a 60 mL screw cap glass vial, 40 mL of wine was spiked with 80 µL of a 270 ppb isopropanolic solution of $^{10}[H_2]$-4-mercapto-4-methyl-2-pentanone, 80 µL of a 220 ppb isopropanolic solution of $^{8}[H_2]$-3-mercaptohexanol, and 80 µL of a 200 ppb isopropanolic solution of $^{5}[H_2]$-3-mercaptohexylacetate. The pH of the mixture was then adjusted using a 2 M solution of NaOH to a final pH of 12. After this, 30 µL of the derivatizing agent (100 µL of 100% pentafluorobenzyl bromide (PFBBr) in 5 mL of isopropanol) was added. Finally, 9 mL of an organic solvent mixture (1:3 v/v pentane:diethyl ether) was added. The vial was placed in an agitator (The Belly Dancer® Hybridization Water Bath, Stovall Life Science, Greensboro, NC, U.S.A.) and agitated for 10 minutes at room temperature. To break the resulting emulsion, the mixture was transferred to a centrifuge tube and loaded into a centrifuge (Sorvall® RC 6™ Plus Centrifuge, Thermo Scientific, Asheville, NC, U.S.A.). The mixture was centrifuged for 5 minutes at 13,000 rpm. With the organic and aqueous layers separated, the organic layer was transferred to a 20 mL SPME autosampler vial. The organic layer was dried down completely under nitrogen at room temperature. The vial was then reconstituted with 10 mL of H_2O and 2 g of NaCl.

HS-SPME Analysis

HS-SPME analysis was performed using an automatic CombiPal system (Cohesive Technologies, Alpharetta, GA, U.S.A.). A 1 cm, 65 µm, SPME fiber (PDMS-DVB) was used for all experiments (Supleco, Bellafonte, PA, U.S.A.). The sample was extracted for 60 minutes at 70 °C and the compounds were then desorbed from the fiber directly in the GC injector in splitless mode for 5 minutes at 250 °C.

GC-EI-MS Analysis

The quantification of the derivatized thiols was carried out by gas chromatography-electron impact-mass spectrometry (GC-EI-MS). Samples were analyzed with an Agilent 6890N coupled to an Agilent 5973N mass spectrometer (Santa Clara, CA, U.S.A.). The GC operated at a constant pressure of 10 psi and helium was used as a carrier gas with a starting flow rate of 1.2 mL/min. The temperature program was: Starting temperature of 50 °C with an initial ramp of 5 °C/min up to 225 °C; then 20 °C/min ramp to 250 °C, 5 minute hold. The MS data was collected using selective ion monitoring for specific time intervals. Data processing was carried out by Enhanced ChemStation software. The quantification ion and qualification ions for each compound and its corresponding internal standard along with their retention times is listed in Table 3. Figure 1 shows a typical chromatogram for each compound in the conditions reported.

Table 3. Retention times and quantification and qualification ions for thiol-pentafluorobenzyl derivatives and their internal standards via HS-SPME coupled to GC-EI-MS

Compound	**Retention Time (min)**	**Quantifying ion**	**Qualifying ions**
4-MMP	26.0	*m/z:* 312	*m/z:* 181
d_{10}-4-MMP	25.8	*m/z:* 322	*m/z:* 181
3-MH	31.7	*m/z:* 314	*m/z:* 181
d_8-3-MH	31.5	*m/z:* 322	*m/z:* 181, 229
3-MHA	28.7	*m/z:* 227	*m/z:* *
d_5-3-MHA	28.55	*m/z:* 255	*m/z:* 117

*lack of qualifier ion due to interferences for most *m/z* fragments of compound

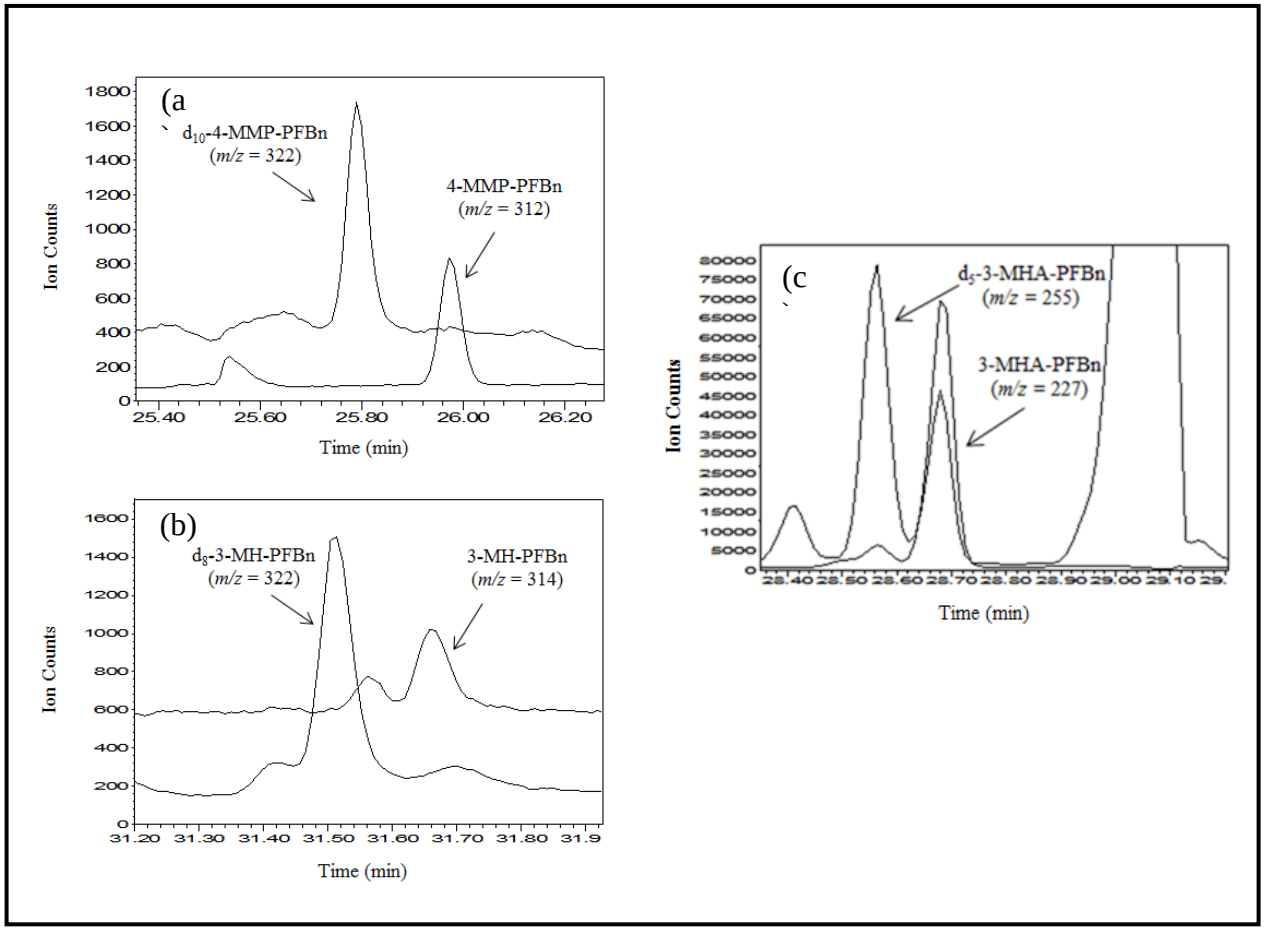

Figure 1. Typical chromatogram obtained in SIM mode. (a) Model wine spiked with 100 ng/L 4-MMP and 270 ng/L d_{10}-4-MMP; (b) Model wine spiked with 100 ng/L 3-MH and 220 ng/L d_8-3-MH; (c) Model wine spiked with 630 ng/L 3-MHA and 400 ng/L d_5-3-MHA

Method Validation

Linearity

Calibration curves were generated by a 8-point standard addition for 4-MMP, 3-MH, and 3-MHA over a range of 6-20,000 ng/L in a model wine solution. Samples were prepared in duplicate according to the optimized method described above. Weighted linear regressions (1/x) of [312]/[322] ions vs. 4-MMP concentration, [314]/[322] ions vs. 3-MH concentration, and [227]/[255] ions vs. 3-MHA concentration were determined.

Limits of Detection

Using the regression data, LODs were calculated by the method of Pallesen [41].

Recovery Experiments

Recovery measurements were performed using white wine (2011 Flip Flop® Pinot Grigio, California, U.S.A.) samples spiked at a low (60 ng/L) and high (500 ng/L) concentration. Measurements at each concentration level were performed in duplicate.

Precision Experiments

Precision measurements were performed using white wine (2011 Flip Flop® Pinot Grigio, California, U.S.A.) spiked at two different concentrations (n=5 for each concentration). Percent relative standard deviation (%RSD) was calculated at 10 ng/L and 100 ng/L for 4-MMP, 25 ng/L and 200 ng/L for 3-MH, and 20 ng/L and 200 ng/L for 3-MHA.

Samples for Thiol Quantification of Commercial Wines

The validated linear regressions were used for quantification of wine samples. A total of 30 wines were tests, 5 wines from 6 varietals (Riesling, Sauvignon blanc, Gewürztraminer, Rosé, Cayuga White, Niagara) were analyzed in duplicate. All the commercial wines were young, ranging in vintages from 2007 to 2012, and were obtained from retail outlets in upstate New York.

Results and Discussion

Extractive PFBBr Alkylation for Thiol Derivatization

Transformation of thiols into their corresponding pentafluorobenzyl (PFBn) derivatives yields stable extracts that possess better chromatographic behavior and detectability than their corresponding thiols. The formation of PFBn derivatives is not exclusive to thiols, but has also

been reported for the derivatization of organic acids and phenols [42, 43]. Due to the promiscuity of this derivatization reaction and the presence of organic acids and phenols in wine, previous reports that employed PFBBr derivatization for thiol measurement in wine have added steps in order to derivatize the thiols in more selective conditions. These selective conditions have been achieved through the addition of SPE or liquid-liquid extraction steps prior to the derivatization step [31, 33]. However, these additional steps pose an oxidation risk for the underivatized thiol as well as add length and complexity to sample preparation. This improved method lowers the risk of thiol oxidation as well as decreases the length and complexity of sample preparation by limiting the amount of wine sample handling prior to derivatization, while still maintaining selective derivatization conditions. The proposed method eliminates all extraction steps prior to derivatization of the thiols by coupling extraction and the derivatization reaction into one step via an extractive PFBBr alkylation reaction. There has been one other reported unsuccessful attempt at an extractive PFBBr alkylation for thiol derivatization in wine, but since minimal details were provided, it is not possible to evaluate why this was unsuccessful [33].

Under the experimental extractive alkylation conditions, the thiolate is believed to be transferred from the aqueous wine sample phase into the organic phase, where it then reacts with PFBBr to form the thiol-PFBn derivative (Figure 2). In order to facilitate a PFBBr derivatization reaction, the use of strongly basic conditions in addition to heat and/or catalysts has previously been employed [31-33, 42, 43]. As heating to high temperatures cannot be used due to the likely vaporization of the volatile thiols, the use of 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) as a catalyst has most often been employed [31, 33]. Because of the potential toxicity of DBU, initial work while developing this method utilized an alternative catalyst, 18-crown-6 ether. The

18-crown-6 ether, which has been used to facilitate PFBBr derivatization reactions in water, is theorized to function by complexing with the sodium cation, increasing the nucleophilicity of the thiolate [44].

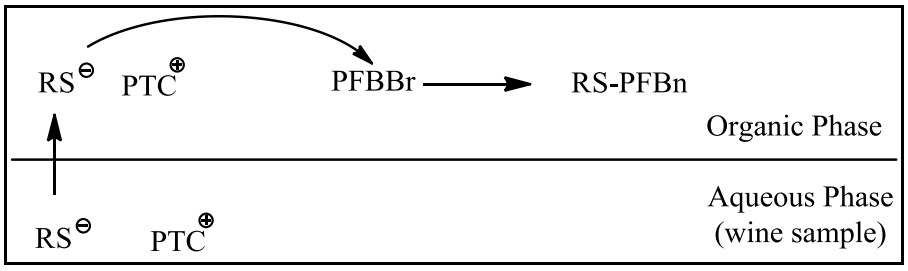

Figure 2. Theorized reaction scheme for extractive pentafluorobenzyl alkylation, Fiamegos and Stalikas [45]

Internal Standards

Initially, investigations of method reproducibility looked at the introduction of two deuterated analogs: d_8-3-MH and d_{10}-4-MMP. Deuterated analogs were chosen as they can correct for any losses that may occur during steps of the analysis and ensure consistent quantification results due to their similar physiological properties to the non-labeled compounds [46]. Standard addition experiments confirmed reproducibility improvement for 4-MMP and 3-MH through the introduction of d_{10}-4-MMP and d_8-3-MH, respectively. However, neither of these deuterated compounds was able to account for the variability observed in the 3-MHA absolute peak area between runs, confirming the necessity for each analyte to have its own deuterated analog as an internal standard. Thus, the current protocol employs d_5-3-MHA along with d_8-3-MH and d_{10}-4-MMP as internal standards for their corresponding non-deuterated thiol.

Optimization

Derivatization Parameters

Agitation Time

The effect of the length of the extractive alkylation reaction on the formation of the thiol-PFBn derivatives via sample agitation time was investigated. The absolute signal for each thiol generally increased with increasing agitation time, however, the noise of the chromatographic baseline also increased proportionally. Thus, no significant differences in LODs for 4-MMP and 3-MHA were obtained for reaction times of 10, 25, and 40 minutes (Figure 3). While a significant decrease ($p < 0.05$) was observed for the LOD for 3-MH by increasing the agitation time from 10 to 40 mL, an agitation time of 10 minutes was chosen for the optimized method. Since a LOD below the sensory detection threshold for 3-MH could be achieved with a 10 minute agitation time, an increase in agitation time to 40 min was deemed unnecessary.

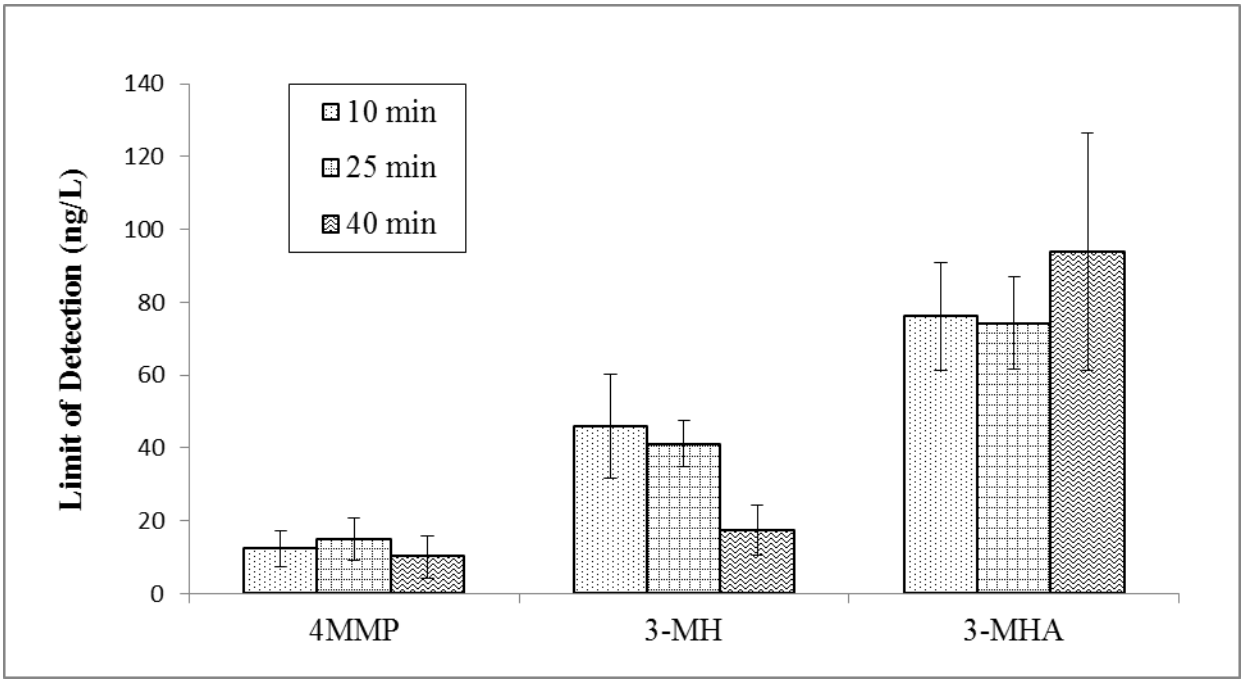

Figure 3. Effect of agitation time for the extractive alkylation: LODs for 3 different agitation times (10, 25, 40 min) for 4-MMP (*m/z*: 312), 3-MH (*m/z*: 314), and 3-MHA (*m/z*: 227). Error bars represent ± 1 standard deviation for 3 replicates.

Volume of Organic Solvent

Various volumes of organic solvent were investigated to determine if increases in volume would affect the amount of analyte extracted from the wine. Organic solvent (1:3 pentane:diethyl ether %v/v) volumes of 8, 12, and 16 mL were compared (Figure 4). The signal for each analyte did not improve by increasing the amount of organic solvent used. This resulted in no significant differences in LODs for the derivatized thiols when the amount of solvent used for extraction is increased. A volume of 9 mL was used for the optimized protocol for convenience; however, less organic solvent could have been used.

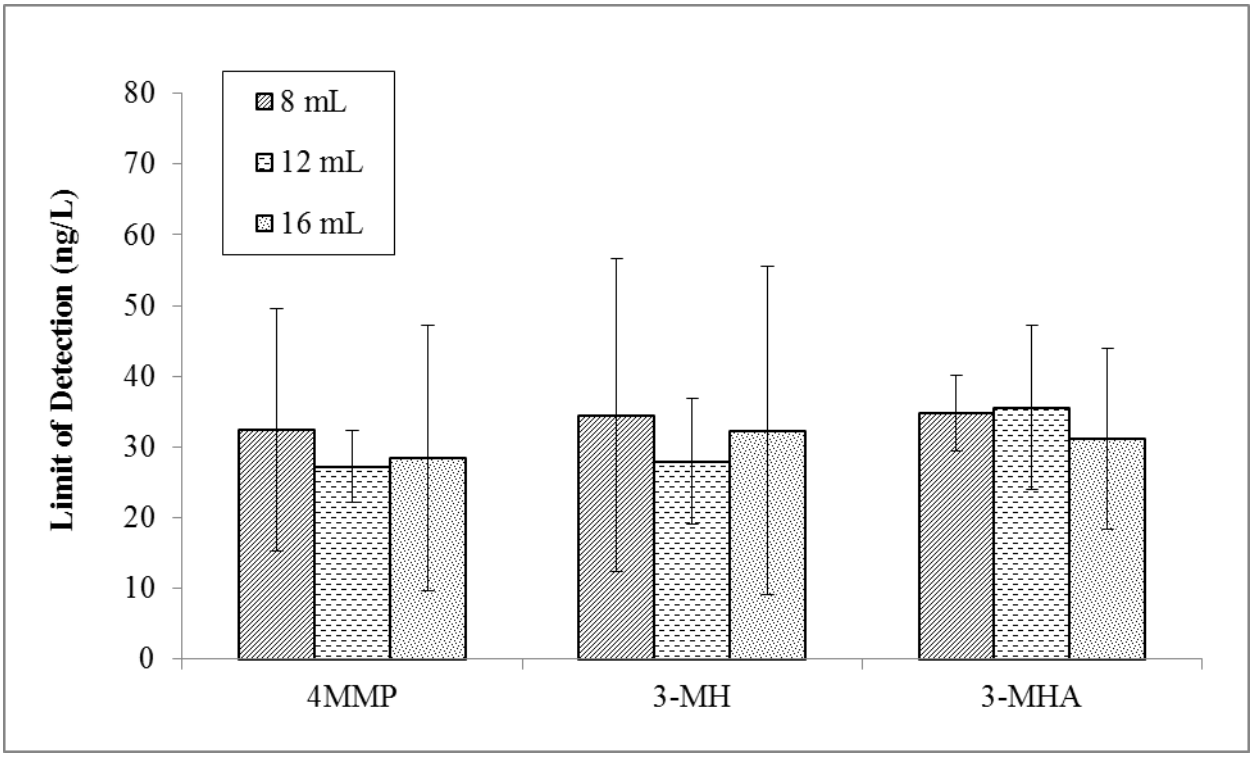

Figure 4. Effect of volume of organic solvent: LODs obtained for 3 different volumes of organic solvent (8, 12, 16 mL) for 4-MMP (*m/z*: 312), 3-MH (*m/z*: 314), and 3-MHA (*m/z*: 227). Error bars represent ± 1 standard deviation for 3 replicates.

Buffer Reconstitution Volume

In the proposed method, the organic extract from the extractive alkylation is completely dried down to remove potential absorption competition from the solvent and then reconstituted with aqueous buffer. This idea of reconstitution of dried down extracts for HS-SPME analysis has also been reported for pesticide quantification in soil analysis [47]. For the proposed method, reconstituting the dried down vial with an aqueous buffer prior to HS-SPME analysis was found to have a significant effect on the LOD for the derivatized thiols ($p < 0.1$ for dry vial vs. 10 mL buffer) as a result of increased absolute signal for the derivatized thiols (Figure 5). Other reports using HS-SPME analysis on aqueous matrices have also found an increased analyte response as the headspace:sample volume ratio decreased [47, 48]. One explanation for the scaling of signal with reconstitution volume is the presence of active sites on the SPME vial in the headspace.

Increasing the volume of buffer in the vial would decrease the number of available active sites on the vial, due to decreased vial surface area exposed in the headspace. This would make more analytes available for absorption onto the SPME fiber. Additionally, reconstitution could potentially alleviate hot spots of the SPME vial during extraction, preventing thermal degradation of the analytes.

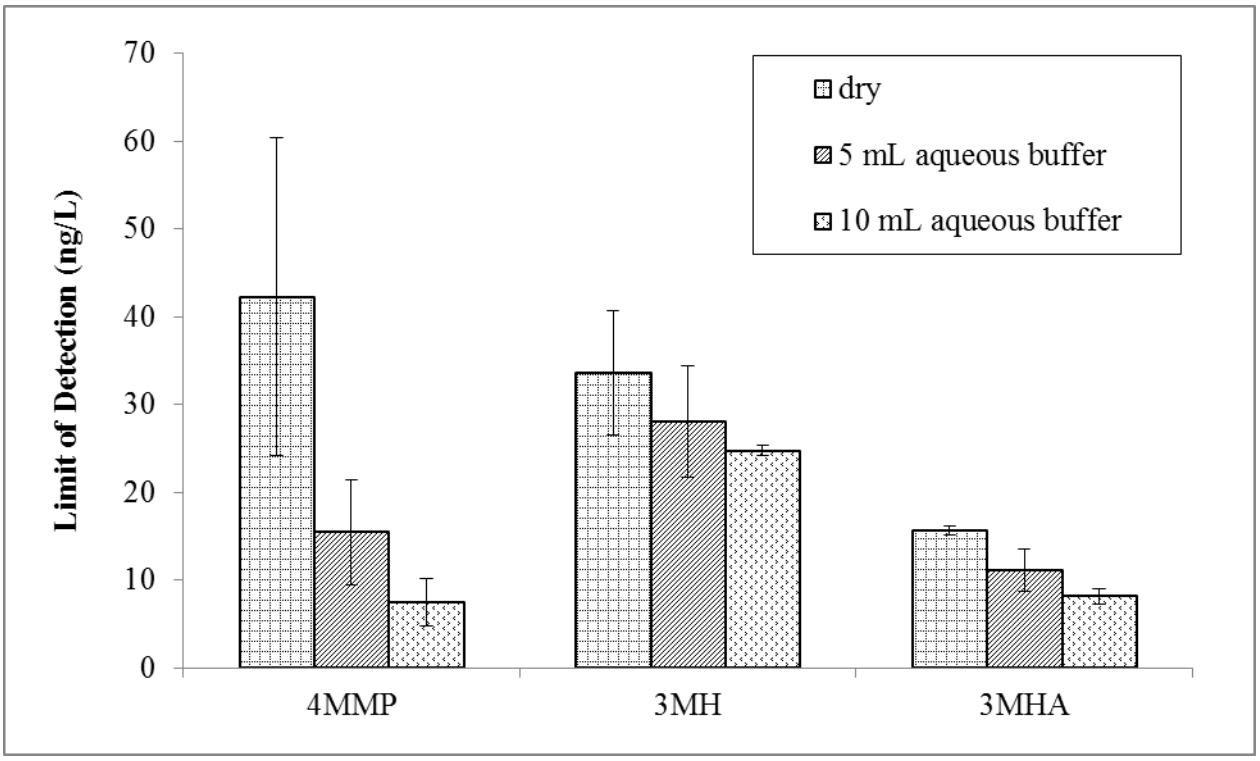

Figure 5. Effect of reconstituting dried SPME vial with aqueous buffer: LODs obtained for dried vials and vials reconstituted with 5 and 10 mL of aqueous buffer for 4-MMP (*m/z*: 312), 3-MH (*m/z*: 314), and 3-MHA (*m/z*: 227). Error bars represent ± 1 standard deviation for 2 replicates.

CCF Model for Sample pH, Sample Volume, PTC Concentration

To determine the impact of pH, sample volume, and PTC concentration on method sensitivity, a CCF model was developed with outputs from 32 runs fit using Partial Least Squares (PLS) modeling. A 2D profiler plot depicting the optimal extractive alkylation parameters for each thiol is shown in Figure 6. The responses were well modeled by sample pH ($p < 0.01$ for all

thiols) and starting wine volume ($p < 0.01$ for 4-MMP and 3-MH; $p < 0.1$ for 3-MHA). The lowest LODs were achieved with a starting volume of 40 mL and a sample pH of 12. Surprisingly, the presence of the phase transfer catalyst, 18-crown-6 ether, was found to provide no improvement to LODs for any of the thiols. As a result, 18-crown-6 ether was completely removed from the optimized protocol.

One of the roles of the phase transfer catalyst is to help facilitate the nucleophilic substitution for derivatization. However, not all reports require the use of such catalysts to facilitate thiol derivatization with PFBBr in wine. While Mateo-Vivaracho et al. noted the necessity for a non-nucleophilic base to facilitate this reaction and that an inorganic base, such as NaOH, could not be used as an alternative, Capone et al. was able to perform this derivatization reaction without the aid of a catalyst [30, 32, 33]. The differences in necessity for a catalyst can be explained by the phase in which the derivatization reaction is taking place. The protocol by Capone et al. has the PFBBr derivatization taking place in an aqueous extract, while the protocol of Mateo-Vivaracho et al. has the derivatization taking place in an organic extract [30, 32, 33]. When performed in the aqueous phase, the sufficient basicity of aqueous phase stabilizes the thiolate and allows the reaction to occur without the need for a catalyst. But, when being performed in an organic phase, a catalyst is required to make the thiolate more available to undergo the derivatization reaction. Thus, the insignificance of the addition of a phase transfer catalyst is likely due to the derivatization reaction occurring in the aqueous phase during the extractive alkylation, followed by the extraction of the derivative into the organic solvent layer.

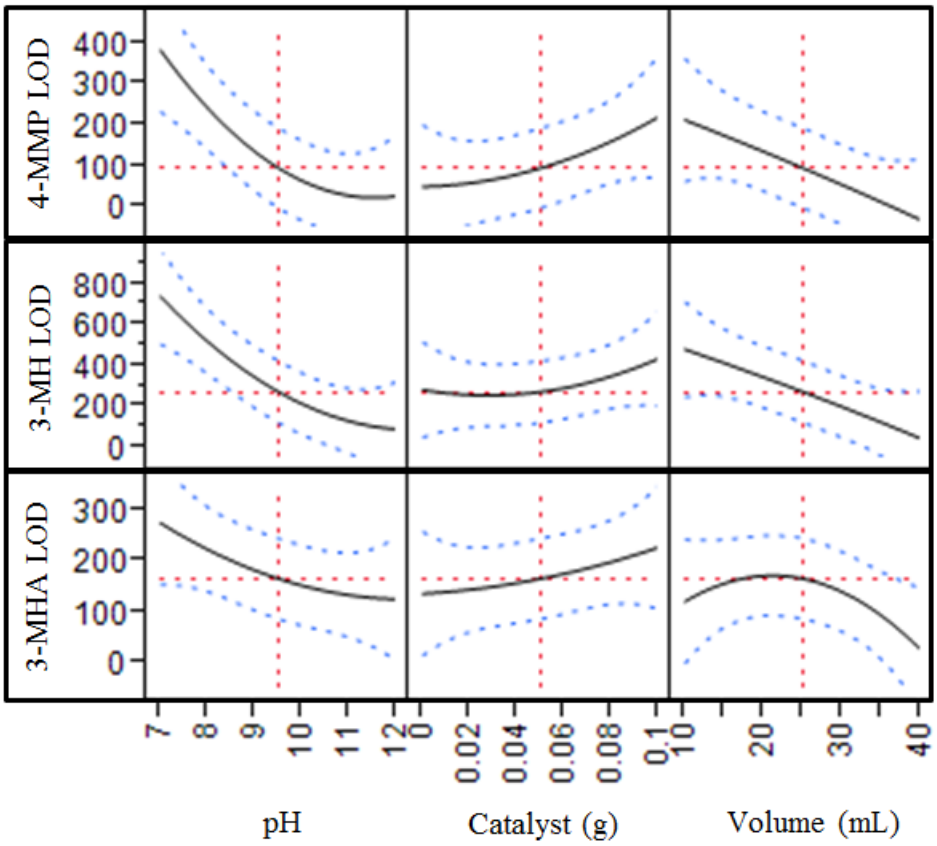

Figure 6. Effect of pH, catalyst concentration, and starting volume on LOD for 4-MMP (*m/z*: 312), 3-MH (*m/z*: 314), and 3-MHA (*m/z*: 227)

SPME parameters

Studies using a SPME fiber for extraction of thiol-PFBn derivatives employ either a two-phase (PDMS/DVB) fiber or three-phase (PDMS/DVB/CARB) fiber [25, 30, 31]. There has been only one report of fiber optimization for the extraction of thiol-PFBn derivatives, which noted higher responses as well as a consistently better peak shape using the two-phase PDMS/DVB fiber rather than the three-phase PDMS/DVB/CARB fiber [30]. As a 2 cm PDMS/DVB fiber is not commercially available, a 1 cm PDMS/DVB fiber was chosen for the optimization study.

CCF Model for SPME Extraction Time and Extraction Temperature

To determine the impact of extraction parameters on method sensitivity, the outputs from 20 runs were fit by PLS modeling. A 2D profiler plot depicting the optimal SPME parameters for each thiol is shown in Figure 7. SPME extraction time was significant ($p < 0.01$) with an optimal extraction time of 60 minutes for all thiols. Extraction temperature was also significant for the three thiols ($p < 0.1$ for 4-MMP and 3-MHA; $p < 0.01$ for 3-MH). However, optimal extraction temperatures were not consistent across each of the thiols, with minimum LODs for 4-MMP and 3-MHA achieved with an extraction temperature of around 70 °C, while the optimal extraction temperature for 3-MH was 90 °C. The extraction temperature chosen for the optimized method was 70 °C, as it was optimal temperature for the two thiols with the lowest targeted method detection limits.

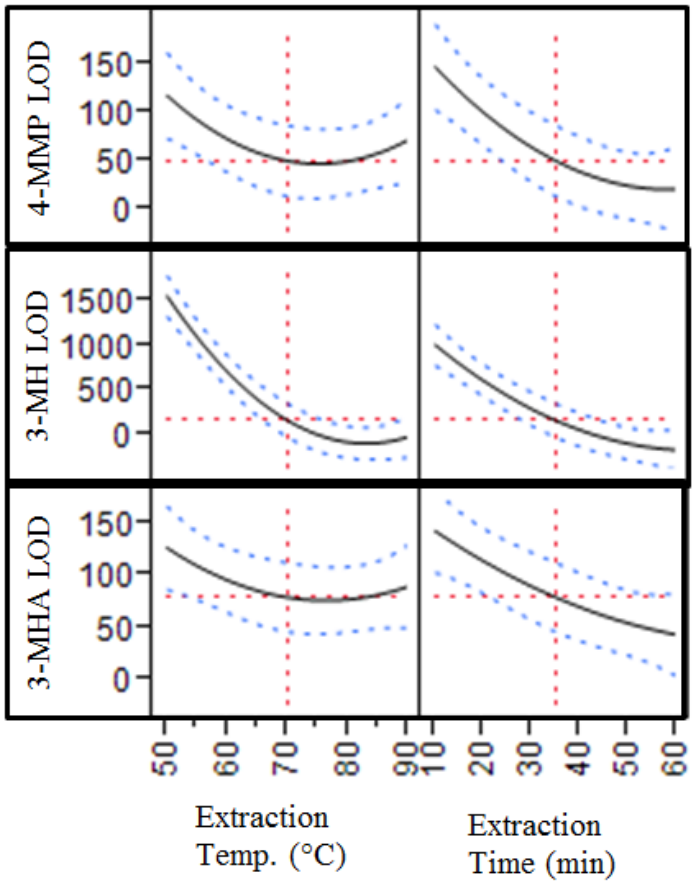

Figure 7. Effect of extraction temperature and extraction time on LOD for 4-MMP (*m/z*: 312), 3-MH (*m/z*: 314), and 3-MHA (*m/z*: 227)

Column Type

Initially, all samples were run on a DB-5 column. However, once the application of the method was switched from a model wine matrix to a commercial wine matrix, interferences were observed at the retention time where the 4-MMP-PFBn derivative eluted. Therefore, samples were run on DB-FFAP to avoid this interference. Switching to a DB-FFAP column also afforded better peak shape for all analytes by reducing the amount of tailing behavior, allowing for easier quantification. Figure 8a-d compares chromatograms of 4-MMP and 3-MH derivatives on the DB-5 and DB-FFAP columns.

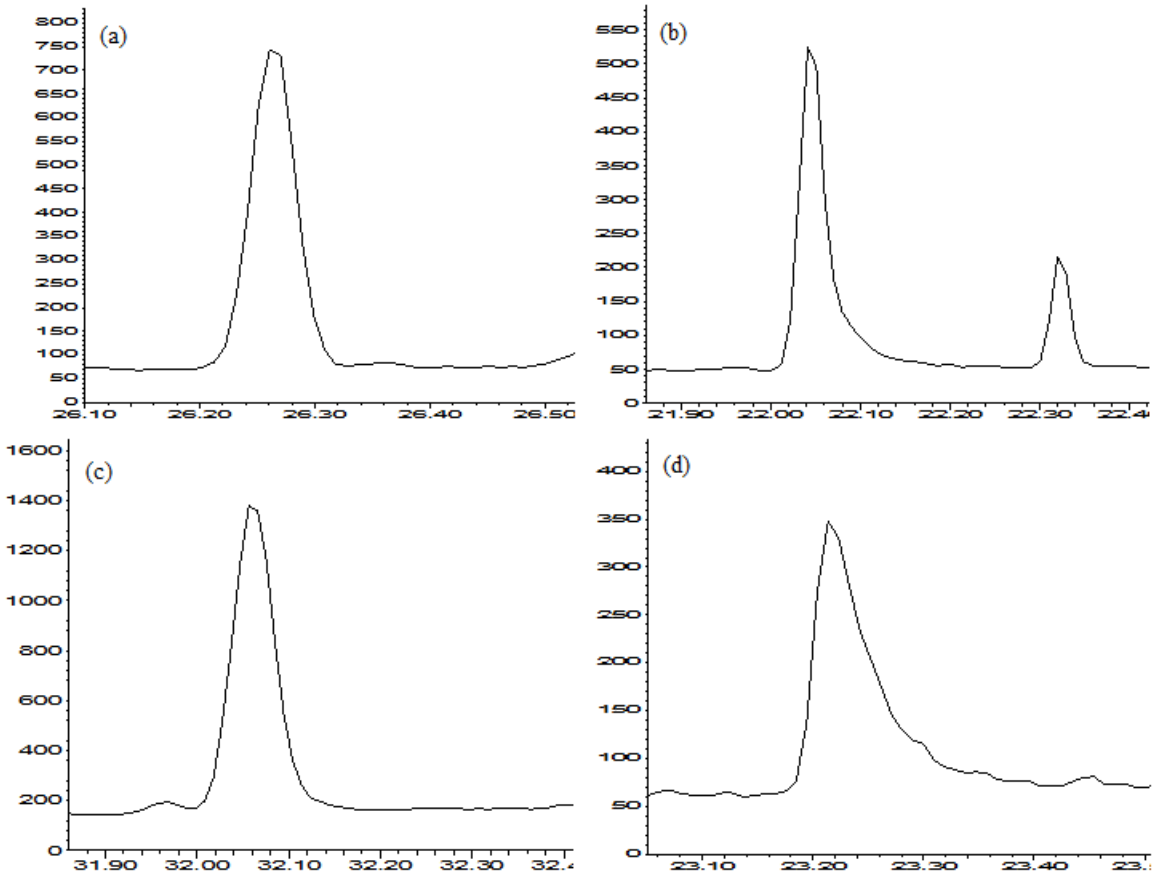

Figure 8. Comparison of Peak Shape on DB-5 and DB-FFAP GC columns . Model wine spiked with 200 ng/L of each thiol (a) 4-MMP-PFBn (*m/z*: 312) on DB-FFAP column;
(b) 4-MMP-PFBn (*m/z*: 312) on DB-5 column; (c) 3-MH-PFBn (*m/z*: 314) on DB-FFAP column;
(d) 3-MH-PFBn (*m/z*: 314) on DB-5 column

Figures of Merit

Regression curves were generated for plots of *m/z*: [312]/[322] vs. 4-MMP addition, *m/z*: [314]/[322] vs. 3-MH addition, and *m/z*: [227]/[255] vs. 3-MHA addition. For 4-MMP and 3-MH, eight standard addition levels were used, ranging from 6-20,000 ng/L. For 3-MHA, there were five standard addition levels, due to the higher method detection limit for the thiol and quantification issues with a 20,000 ng/L spike as the absolute area for d_5-3-MHA could not be measured because of abundant sensitivity (overlap with non-deuterated peak). Each standard addition level was performed in duplicate, for a total of 18 standard addition experiments for

4-MMP and 3-MH, and 10 standard addition experiments for 3-MHA. The regression parameters (slope, intercept, R^2) are shown in table (Table 4). The observed linearity was excellent, with all regression coefficients, R^2, > 0.99.

Table 4. Regression parameters (slope, intercept, R^2)

Analyte	Slope of Regresssion	Regresssion Intercept	R^2
4-MMP-PFBn	0.0018498	0.0047	0.996
3-MH-PFBn	0.0022422	0.0046	0.998
3-MHA-PFBn	0.0025454	-0.039	0.999

Method detection limits were determined by standard addition of thiols to a model wine. Rather than calculating LODs based on the noise resulting from variation in the chromatographic baseline, which has been the routine reported previously, limits of detection were calculated based on the error of the entire analysis to more accurately reflect the limitations of the method [31, 41]. Signal independent noise was calculated by regression analysis of total variance vs. thiol concentration (Pallesen's Method) with a 1/x weighting factor. The limit of detection was calculated as $3^* \sigma_i$. Acceptable LODs were achieved for 4-MMP and 3-MH, having detection limits of 0.9 ng/L and 1 ng/L, respectively, while the LOD for 3-MHA was higher than its detection threshold at 17 ng/L (Table 5). The high LOD of 3-MHA in comparison to those achieved for 4-MMP and 3-MH was due in part to a non-existent M^+ peak for 3-MHA-PFBn. As a result of the molecular ion not being visible on the derivative's mass spectrum, the use of an ion of lower *m/z* was required for 3-MHA quantification. Many ion fragments of the 3-MHA derivative could not be used to quantify due to chromatographic interferences, including *m/z:* 175, which was the ion used in many previous reports for 3-MHA-PFBn quantification

[31-33]. The presence of chromatographic interferences at *m/z* < 300 also lead to difficulty in establishing proper qualifier ions for each of the three thiols and their deuterated internal standards.

Table 5. Coefficients of variation (%CV) at varying levels of 4-MMP, 3-MH, and 3-MHA resulting from standard addition

Spike Level	ng/L of 4-MMP	ng/L of 3-MH	ng/L of 3-MHA
1	8.0 (4.0%)	4.1 (2.9%)	72 (10.4%)
2	19 (4.1%)	19 (12%)	178 (7.5%)
3	50 (7.4%)	66 (5.9%)	589 (5.7%)
4	179 (8.3%)	202 (6.8%)	2080 (2.5%)
5	525 (12%)	722 (1.0%)	6301 (4.3%)
6	1792 (9.1%)	2245 (0.9%)	--
7	5848 (4.6%)	6335 (1.7%)	--
8	20826 (15%)	19652 (1.2%)	--
σ_i, ng/L	0.31	0.33	5.8
LOD, ng/L	0.9	1.0	17.3

%CV is the relative standard deviation of normalized ratios of replicate measurements (n=2) at the stated concentration. Abbreviations: σ_i = signal independent background noise, LOD = Limit of detection

However, improvements in the LOD for 3-MHA using this method can be achieved by simply increasing the starting amount of wine to a volume greater than the 40 mL sample used in this protocol. It was observed that by increasing the starting sample volume, one can achieve an increase in signal for the analyte without an incremental increase in chromatographic noise for the sample (Figure 9).

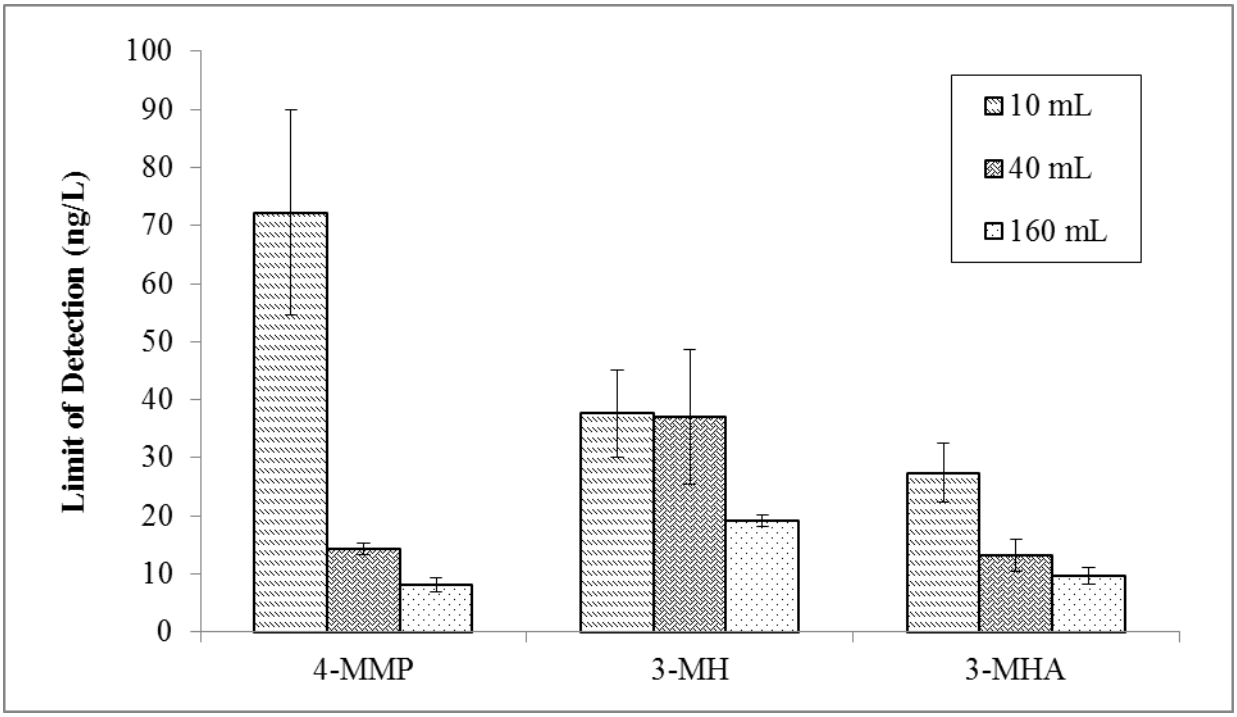

Figure 9. Scalability of LOD with starting wine volume. LODs were determined for 3 starting wine volumes (10, 40, 160 mL) for 4-MMP (*m/z*: 312), 3-MH (*m/z*: 314), and 3-MHA (*m/z*: 227). For the 160 mL sample, the organic extracts of four 40 mL extracts were pooled together. Error bars represent ± 1 standard deviation of 3 replicates.

The robustness of the method was confirmed through precision experiments and recovery spikes performed in a commercial Pinot Grigio wine. This type of wine was chosen for these experiments as Pinot Grigio wines are known to have relatively low native thiol content [22]. Reproducibility was within the acceptable range for precision experiments (Table 6). Acceptable recovery using regression curves produced from standard additions in model wine indicate that the concentration of 4-MMP, 3-MH, and 3-MHA can be calculated from a calibration curve developed in model wine despite the complexity differences between a commercial wine system and a model wine system.

Table 6. Recovery and Precision Experiments

Analyte	Recovery (%)		RSD (%)	
	Low level	High level	Low level	High level
4-MMP	104.9	108.7	9.8	6.6
3-MH	102.6	90.5	6.9	5.4
3-MHA	90.2	100.5	11.1	5.6

Recovery: samples spiked at low level (60 ng/L 4-MMP, 3-MH, 3-MHA); high level (500 ng/L 4-MMP, 3-MH, 3-MHA)
RSD: relative standard deviation of 5 samples spiked at low level (10 ng/L 4-MMP; 25 ng/L 3-MH; 25 ng/L 3-MHA); high level (100 ng/L 4-MMP; 200 ng/L 3-MH; 100 ng/L 3-MHA)

Comparison of Proposed Method to Previous Methods

The performance of this optimized method was compared to that of previous reports for thiol quantification in wine using PFBBr derivatization.

The first report of PFBBr derivatization for thiol quantification in wine was by Mateo-Vivaracho et al. in 2006 through an automated method using on-fiber derivatization. The Mateo-Vivaracho group developed later improvements on their own quantification method that allowed for acceptable detection limits for a range of thiols found in wine. The group's most sensitive method achieved LODs for 4-MMP, 3-MH, and 3-MHA of 0.1 ng/L, 2 ng/L, and 0.3 ng/L, respectively [32]. The LODs for 4-MMP and 3-MHA achieved by the Mateo-Vivaracho group were lower than those achieved for the method reported here. These differences can be attributed to the use of NICI-MS for detection rather than the EI-MS used for this protocol. As noted in other reports, GC-NICI-MS has noted improvements in LODs of 1 to 5 orders of magnitude compared to GC-EI-MS for detection of PFBn derivatives [34-37]. However, despite using EI-MS, the method proposed here achieves an LOD for 3-MH that is lower than that achieved by the Mateo-Vivaracho group. This is likely the result of the use of

smaller starting wine volumes (6 mL vs. 40 mL) and/or the use of liquid injection rather than the more sensitive HS-SPME by the Mateo-Vivaracho group.

Rodriguez-Bencomo et al. improved upon the method proposed by the Mateo-Vivaracho group through the addition of HS-SPME to their reported protocol. The introduction of HS-SPME improved method sensitivity, achieving LODs for 4-MMP, 3-MH, and 3-MHA of 0.03 ng/L, 1.29 ng/L, and 0.25 ng/L, respectively [31]. Again, the sensitivity differences between this thiol quantification method and the method proposed here can be attributed to the use of NICI-MS detection rather than EI-MS detection. The comparable LODs for 3-MH despite the use NICI-MS by Rodriguez-Bencomo et al. can be explained by their use of a smaller volume of starting wine (6 mL vs. 40 mL).

There has only been one previous report, by Capone et al., that uses EI-MS detection for thiol-PFBn derivatives in a wine matrix. The method proposed here achieves significantly lower LODs than those reported by Capone et al. (1 ng/L vs. 30 ng/L) [30]. The method proposed here was able to achieve this lower LOD despite using less starting wine (40 mL vs. 200 mL). Several differences in the protocol by the Capone group and the one reported here can contribute to the LOD differences observed. Capone et al. performed extractions and washes prior to derivatization, serving as a potential for product loss. Additionally, their protocol used a SPME extraction time of 30 minutes, half the time used in this protocol. As was reported in this paper, notable improvements in signal for thiol-PFBn derivatives can be achieved by increasing extraction time. Lastly, unlike in the proposed protocol, salt was not added to the sample prior to HS-SPME extraction in the protocol by Capone et al. In general, the presence of an electrolyte can influence absorption by decreasing the solubility of compounds in the aqueous phase [49].

Thiol Measurement of Finger Lakes Wines

Thiol measurement in wines from upstate New York has not yet been reported. Using the proposed method, a total of 30 wines (5 wines each for six different varietals) produced in the Finger Lakes wine region of upstate New York were analyzed for their 4-MMP, 3-MH, and 3-MHA content (Table 7). Of the six varietals tested, this is the first report of thiol measurement for Niagara and Cayuga White wines, two hybrid varieties commonly produced in the Finger Lakes region. The 4-MMP derivative was detectable in 4 out of the 6 wine varieties tested and 3-MH was detected in all the wines tested. The 3-MHA derivative was only detectable in 4 out of the 30 wines tested; 2 Niagara wines, 1 Rosé wine, and 1 Sauvignon Blanc wine. The concentration of 3-MHA is generally reported as being no greater than 10% (although, normally much less) of the concentration of 3-MH in a particular wine, as 3-MHA is formed from the acetylation of 3-MH during fermentation [12, 22, 50, 51]. As the range of average 3-MH concentrations for the varietals tested in the Finger Lakes was 195 to 569 ng/L, it is not surprising that 3-MHA concentrations would be less than the method detection limit in a majority of the wines.

Table 7. Application of optimized method to six different types of wine produced in Finger Lakes wine region (upstate NY)

Wine Varietal	Calculated Concentrations (± 1σ)		
	4-MMP (ng/L)	3-MH (ng/L)	3-MHA (ng/L)
Riesling (n=5)	2.6 (2.0)	569 (334)	N.D.
Gewürztraminer (n=5)	1.5, 10 (n = 2)	373 (134)	N.D.
Cayuga White (n=5)	N.D.	195 (39)	N.D.
Niagara (n=5)	17 (13)	230 (159)	22, 41 (n = 2)
Rosé (n=5)	N.D.	296 (116)	59 (n =1)
Sauvignon blanc (n=5)	25 (8)	528 (224)	41 (n =1)

For wines where >50% of samples had detectable amounts, means and standard deviations were calculated by assigning non-detectable samples a concentration of LOD/2.

With no other reports regarding thiol content in Finger Lakes wines available, the results of this study were compared to thiol concentrations of wines of the same varietal from other regions (Table 8). As has been previously reported, a high amount of variation in thiol concentrations among wines of the same varietal, regardless of the actual varietal, was observed. The concentrations of 4-MMP in the varietals of the Finger Lakes wines tested were comparable to the concentrations reported for those varietals in other regions. However, notably lower concentrations of 3-MH were observed for Finger Lakes wines, particularly in the Sauvignon blanc and Gewurztraminer varieties. One explanation for this is differences in 3-MH precursor concentrations in grapes from the Finger Lakes compared to those from other regions, contributing to the differing concentrations of 3-MH found in the finished wines. One factor that has been shown to cause differences in precursor concentrations is differences in processing practices. Capone and Jeffery noted that hand harvesting grapes resulted in lower 3-MH

precursor concentrations as compared to machine harvesting [52]. Since many vineyards in the Finger Lakes are likely to hand harvest grapes rather than California and Australian vineyards, whose grapes are most often machine harvested, this may, at least in part, explain differences in final 3-MH concentration. But, it is important to note that correlations between thiol precursor concentrations in the juice and corresponding thiol concentrations in wine are still rather poor [53].

Table 8. Comparison of thiol content in Finger Lakes wine compared to content in wines from other regions

Wine	**Region**	**Calculated Concentrations (1σ)**		
		4-MMP (ng/L)	3-MH (ng/L)	3-MHA (ng/L)
Riesling (n=5)	Finger Lakes	2.6 (2.0)	569 (334)	N.D.
Riesling (n=5)[a]	Alsace	2.2 (3.8)	648 (213)	2.0 (2.8)
Gewürztraminer (n=5)	Finger Lakes	1.5, 10 (n=2)	373 (134)	N.D.
Gewürztraminer (n=5)[a]	Alsace	5.8 (6.3)	2119 (748)	3.2 (2.2)
Cayuga White (n=5)	Finger Lakes	N.D.	195 (39)	N.D.
Niagara (n=5)	Finger Lakes	18 (13)	230 (159)	22, 41 (n = 2)
Rosé (n=5)	Finger Lakes	N.D.	296 (116)	59 (n =1)
Cabernet Sauvignon/Merlot[c]	Bordeaux	--	10-5000	1-200
Sauvignon blanc (n=5)	Finger Lakes	25 (8)	528 (224)	41 (n =1)
Sauvignon blanc (n=7)[b]	Australia	8.2 (3.4)	7080 (5567)	65 (45)
Sauvignon blanc (n=5)[b]	California	5.7 (2.1)	2094 (1628)	45 (18)

a: [22] b: [50] c: [12]
For wines where >50% of samples had detectable amounts, means and standard deviations were calculated by assigning non-detectable samples a concentration of LOD/2.

Measurement of Low- and High-MW Thiols in the Same Run

There have been no reports of a method for simultaneous low-molecular weight and polyfunctional thiol measurement in wine. As both of these types of thiols can be present in wine, a unified method of measurement would be highly desirable. The proposed method was investigated for its potential application of low-molecular weight thiol measurement. The ability of this method to successfully derivatize methyl mercaptan (CH_3SH) and hydrogen sulfide (H_2S) to their corresponding PFBn derivatives (CH_3S-PFBn; S-$(PFBn)_2$) was confirmed by mass spectrum analysis (Figure 10a-b). As there are two hydrogens available in hydrogen sulfide to undergo the derivatization reaction, the derivatized adduct of hydrogen sulfur was found to be doubly derivatized. However, a small portion of the singly derivatized product was observed. For the derivatized analogs of H_2S and CH_3SH, the molecular ion was observed (*m/z*: 228 for CH_3S-PFBn and *m/z*: 394 for S-$(PFBn)_2$) as well as *m/z*: 181, which is the characteristic ion for the derivatized portion of a PFBn analog.

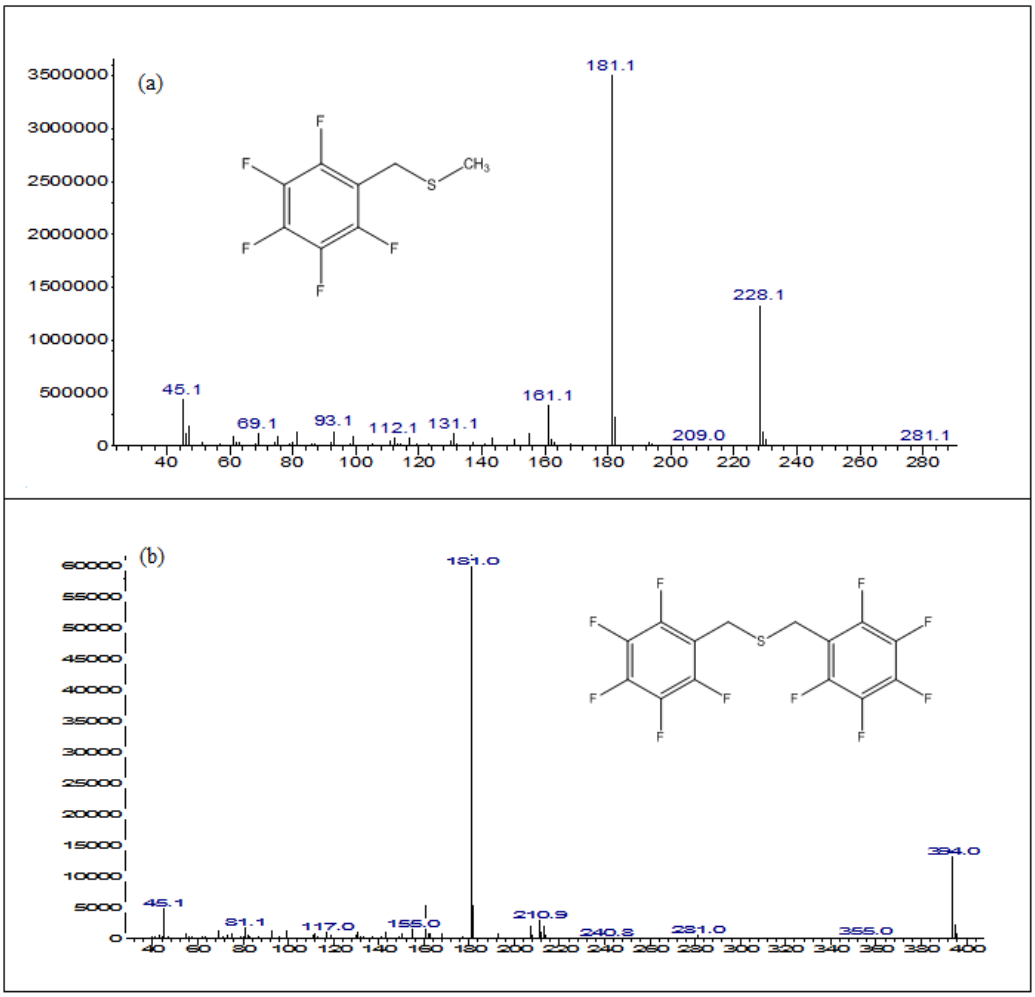

Figure 10. Mass spectrums for (a) CH_3S-PFBn; (b) S-$(PFBn)_2$

The proposed method was then applied to model wine sample containing spikes of both low-molecular weight (H_2S and CH_3SH) and high-molecular weight (4-MMP, 3-MH, and 3-MHA) thiols providing the first reported chromatogram demonstrating the ability to measure low- and high-molecular weight thiols simultaneously (Figure 11). As low-molecular weight thiols have sensory thresholds much higher than the high-molecular weight thiols, in the µg/L level for hydrogen sulfide and methyl mercaptan, this proposed method should be able to be applied to low molecular weight thiol measurement and achieve detection limits near or below

the sensory thresholds for those thiols [15]. Low molecular weight thiol analysis is likely to require isotopically labeled standards that correspond to each of the thiols being quantified in order to account for variability that can occur during derivatization and HS-SPME extraction.

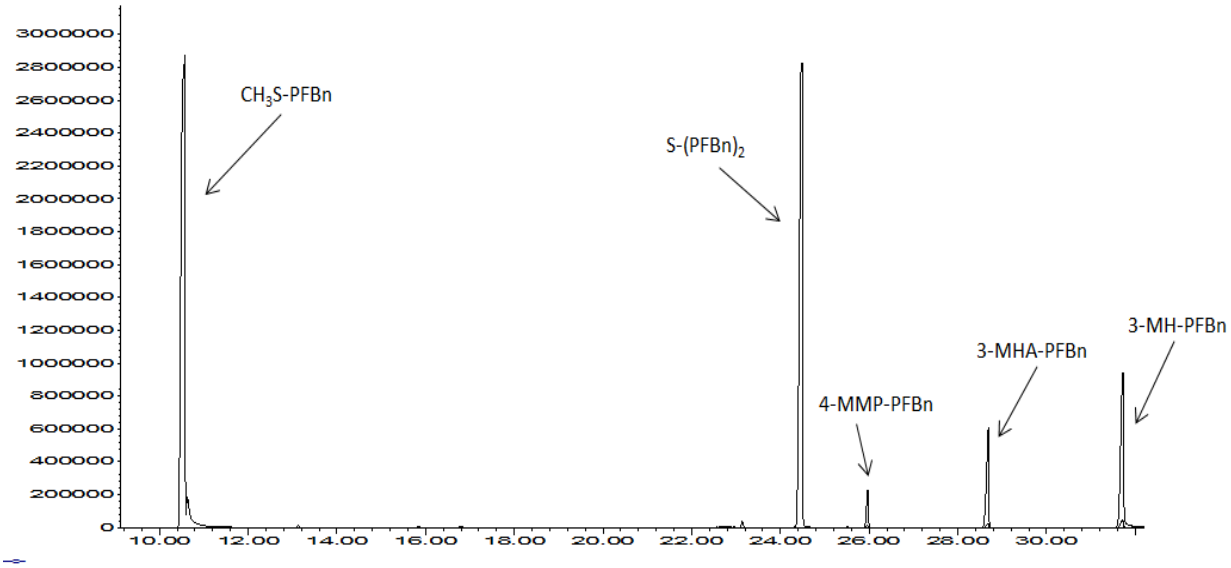

Figure 11. Gas chromatogram of pentafluorobenzyl derivatives for 4-MMP, 3-MH, 3-MHA, H_2S, and CH_3SH obtained using optimized protocol

Conclusion

The proposed method allows for the fast and simple measurement of 4-MMP, 3-MH, and 3-MHA in wine through the quantification of their corresponding PFBn derivatives using EI-MS detection. Method detection limits for 4-MMP and 3-MH were acceptably near the sensory thresholds for those thiols. While the method detection limit for 3-MHA was slightly higher than the sensory threshold for the thiol, acceptable limits of detection for the thiol can be achieved by simply increasing the starting volume of wines used for analysis. The LOD for each of the thiols scales with the volume of wine used for analysis.

This study reports the first measurement of thiol concentrations in wines produced in the Finger Lakes region of upstate New York. The concentrations of 4-MMP for each varietal were comparable to concentrations reported in those varietals from other regions. However, notably lower 3-MH and 3-MHA concentrations were observed in Finger Lakes wines compared to other regions, possibly a result of differing precursor concentrations due to different grape processing practices. This study also reports the first observation of thiol content in Niagara and Cayuga White varietals.

Lastly, this study marks the first report of simultaneous low- and high-molecular weight thiol analysis. With the introduction of isotopically labeled internal standards for all thiols being measured, this method shows promise for its applicability for low- and high-molecular weight thiol measurement in the same run.

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