MASS SPECTROMETRY FOR THE HIGH-THROUGHPUT QUANTIFICATION AND MECHANISTIC INVESTIGATION OF ODOR-ACTIVE VOLATILES IN GRAPES AND WINE

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MASS SPECTROMETRY FOR THE HIGH-THROUGHPUT QUANTIFICATION AND MECHANISTIC INVESTIGATION OF ODOR-ACTIVE VOLATILES IN GRAPES AND WINE

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A novel mass spectrometry method was developed for the rapid quantification of odor-active volatiles in food matrices.

Quantitative analyses of trace-level volatile compounds are routinely performed as an objective measure of flavor in foodstuffs and raw materials. Standard analytical methods based on gas chromatography-mass spectrometry (GC-MS) usually require 30-60 minutes/sample. This low throughput is poorly suited for several emerging directions in flavor research or process control which can potentially create thousands of samples, e.g. characterization of breeding populations, as well as characterization of raw materials. Ambient ionization (AI) techniques like DART-MS are much faster, but with the drawback of lower sensitivity/ selectivity. A sorbent-coated mesh was developed for the extraction and pre-concentration of volatiles (Solid Phase Mesh Enhanced Sorption from Headspace, SPMESH), which could then be analyzed by Direct Analysis in Real Time (DART)-MS. The SPMESH coating material was optimized for the extraction of odor-active volatiles in complex food matrices. In combination with high resolution mass spectrometry (HRMS), SPMESH-DART could obtain detection limits below sensory thresholds for a variety of compounds with olfactory relevance in food matrices. The new method was validated in grape macerates, exhibiting excellent agreement with established GC-

MS methodology ($r^2 \geq 0.90$).

In a separate study, the mechanism for evolution of hydrogen sulfide during storage of wines fermented on elemental sulfur was investigated. Hydrogen sulfide (H_2S) is frequently found in faulted wines with sulfurous off-aromas. H_2S is reported to increase during bottle storage of some wines, and the identity of all potential precursors responsible for this latent H_2S is still not resolved. We have shown that elemental sulfur residues (S^0) on grapes can not only produce H_2S during fermentation, but also yield wine-soluble intermediates capable of generating more H_2S during storage. Through HPLC – high resolution mass spectrometry, we identified H_2S-releasing polythionates in the S^0 fermented wine sample that are absent in the control, and propose a mechanism for the formation of a supplementary S^0-derived source.

BIOGRAPHICAL SKETCH

The author grew up in Cherry Hill, NJ, and received her B.S. in Chemistry in 2013 from the University of Delaware. During her summer and winter breaks in college, she worked as assistant winemaker at Amalthea Cellars Winery and Vineyard in the Outer Coastal Plains AVA. In search of a career that combined her love of science with her love of wine, she googled “wine chemistry” and discovered Prof. Gavin Sacks. In Fall of 2013, she enrolled at Cornell University to pursue a PhD in Food Science with a concentration in Food Chemistry. Her time in the Sacks Lab has taught her a deep appreciation for the nuanced complexity of flavor and aroma chemistry, and the unique analytical challenges it presents. Following the completion of her degree, she will assume a position as analytical aroma chemist at Ava Winery in San Francisco, CA.

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CHAPTER 1

INTRODUCTION

Importance and challenges of high-throughput volatile analyses

Much of flavor perception is olfactory, and therefore, closely related to the aroma of a given foodstuff. The molecules responsible for the aroma sensory characteristics of a product exist in the volatile fraction, and measurement of these volatile molecules can therefore be used for objective flavor analysis, an area of great importance and increasing relevance in the food industry. For example, recent plant breeding trends emphasize breeding for flavor, as opposed to a traditional focus on yield and disease resistance, and consumer preference for "natural" products with fewer additives necessitates better flavor quality assessment of raw materials. While a given foodstuff may contain many hundreds of volatile compounds, Dunkel et al. estimate less than 3% of detectable volatiles contribute to aroma, and somewhere between 4 and 40 odorants are critical to duplicate the aroma of a given foodstuff.[1] Thus, for most foodstuffs, quantification of 10-20 targeted compounds will be sufficient to evaluate its odor quality.

Wine and wine grape flavor analysis represents an important application of odor quality assessment. Like most foodstuffs, wine and wine grapes have complex matrices with aroma molecules of high sensory impact existing at extremely low concentrations. Important wine odor-active volatiles may derive from the grapes, from fermentation, or storage conditions (e.g. extraction from oak barrels, aging). Aroma impact compounds can be grouped into several classes, including alcohols, esters, terpenes, methoxypyrazines, and volatile sulfur compounds.[2] These odorants may have sensory thresholds and concentrations ranging from mg/kg to ng/kg and possess a diverse range of functional groups. Given the low odor thresholds and concentrations of some compounds, minute differences in concentration can have profound

impacts on the sensory perception of the product.[3] Quantitation of these compounds is further complicated by the presence of other matrix components, necessitating a high degree of selectivity.

The most commonly employed universal approach to volatile quantification is gas chromatography - mass spectrometry (GC-MS).[4] GC-MS can achieve the necessary selectivity and sensitivity to quantify a broad range of odorant volatiles, and has enabled much advancement in our understanding of wine and flavor chemistry.[2] However, a major limitation to GC-MS is its low throughput – depending on the number of analytes to be quantified, chromatographic separation may require 30-60 minutes per sample, with additional time necessary for oven cooling and stabilization.[5-8] This low throughput is poorly suited for several emerging directions in flavor research or process control which can potentially create thousands (or tens of thousands) of samples, e.g. characterization of breeding populations to develop new high quality grains, fruits, or vegetables,[9-11] as well as characterization of raw materials.[12] Other approaches like FT-IR can achieve throughputs of <1 min per analysis, but typically have limits of detection of > 1 mg/kg, and thus are primarily used for indirect *fingerprinting* of samples rather than selective detection of critical odorants.[13] A cost-effective high-throughput method providing a quantitative odor-active volatile profile for complex matrices has the potential to improve the quality of plant products.

Several approaches for improving throughput while maintaining selectivity and sensitivity have been proposed. Fast GC-MS utilizes narrow bore columns to reduce run times up to 10-fold.[14, 15] However, these narrow columns have low sample capacity, causing problems for the broad range of concentrations represented in volatile flavor analyses,[16] and besides, ultimate improvement in throughput, accounting for sample preparation, is limited to about a factor of 3-

5. Fast GC with non-MS detectors, e.g. the commercially available Alpha MOS eNose ™ system, can produce volatile profiles for fruits and other foodstuffs, but lack selectivity for some compounds and are mainly used for fingerprinting and product differentiation.[13, 17]

Several commercial and literature approaches have reported improving throughput by eliminating chromatography and directly introducing volatiles into the MS, e.g. Atmospheric Pressure Chemical Ionization (APCI), Proton Transfer Reaction (PTR)-MS and the related technique Selected Ion Flow Tube (SIFT)-MS. Because these approaches directly sample the headspace, they are well suited for time-release studies, such as monitoring during processing or in-vivo flavor release, and are also appropriate for fingerprinting studies, where the goal is differentiation of e.g. treatments or cultivars by statistical analysis.[18] However, these direct introduction approaches suffer from poor specificity as they are typically coupled to unit mass resolution detectors,[19] and lack sensitivity due to the omission of a pre-concentration step - typically, detection limits are in the range of 0.1-1 mg/kg except for highly volatile compounds.[20, 21] Thus, these approaches are best suited as complementary approaches to GC-MS, and not as a high-throughput replacement.

Ambient ionization (AI) techniques, in which ionization of analytes occurs external to the mass spectrometer, allow for rapid analyses on the order of just seconds per sample. AI approaches related to APCI such as Direct Analysis in Real Time (DART) are best suited for analysis of small (<1 kDa) non-polar compounds, which encompasses most volatiles.[22] The DART mechanism consists of thermo-desorption of analytes followed by ionization by metastable species and detection by MS. DART-MS has been demonstrated for detection of pesticides, contaminants, additives, or other major components in food.[23] However, trace-level quantitative DART analyses must employ both sufficient sample preparation and high resolution

mass spectrometry (HRMS) to compensate for matrix and isobaric interferences introduced by the elimination of chromatographic separation. Headspace solid phase microextraction (SPME), which combines extraction and pre-concentration into one solvent-free step, is widely used in GC-MS on aroma compounds in foodstuffs (e.g. for grapes and wine).[24-26] The coupling of SPME to DART-MS has been reported for volatile analysis in beer.[27] However, SPME fiber is configured for a GC port and does not enable efficient desorption in commercial AI-MS systems like DART.[28] Alternative approaches for the direct coupling of SPME to AI-MS have been presented. Non-DART approaches include coupling to electrospray ionization (ESI) or dielectric barrier discharge ionization (DBDI) sources. Direct coupling of a SPME fiber to a DBDI source could achieve low ng/L detection limits for nonvolatile pesticides, in part due to more thorough and reproducible desorption from the SPME fiber.[29] However, the analysis of volatile compounds is not reported, and this format does not easily lend itself to automation. Direct ESI ionization involved an Open Port Probe (OPP) reported by Gomez-Rios et al., which achieved similar sensitivities for doping agents in biological matrices,[30] although we would not expect this method to fare well in the analysis of volatile compounds considering the ionization mechanism, which is poorly suited to low polarity compounds.[22] Atmospheric Pressure Chemical Ionization (APCI), the preferred ionization method for volatiles, could theoretically be coupled to SPME through the OPP, but this has not yet been demonstrated.[30] Alternatively, DART-related approaches can employ non-traditional SPME configurations that are more conducive to DART desorption. For example, Gomez-Rios et al. reported a C18-polyacrylonitrile coated stainless steel mesh, engineered specifically for coupling to Transition Mode (TM) DART.[31, 32] This method could achieve μg/kg detection limits for the quantification of drugs and pesticides in biological matrices, but involves direct immersion in liquid samples, which would likely result in

severe interferences for volatile analysis.[32]

This dissertation research aimed to develop a new extraction device appropriate for headspace extraction and pre-concentration of volatiles prior to AI-MS. Initial work focused on the development and characterization of polymer-coated mesh (SPMESH) that combines the sensitivity and reproducibility of TM-DART with the selectivity for volatiles inherent to traditional SPME (Chapter 2). This method was then optimized by improving the polymer coating and adding high resolution detection, followed by validation in real grape matrices (Chapter 3). Chapter 5 includes preliminary work towards the quantification of additional compounds and an automatable format.

Elemental sulfur-derived latent precursors to hydrogen sulfide in wine

A separate part of this dissertation research investigated the role elemental sulfur residues in the formation of precursors to hydrogen sulfide (H_2S) in wines. H_2S is a leading cause of reductive off-aromas in wines, which account for about 25% of reported wine faults.[33] H_2S can be produced during fermentation, either through S-amino acid metabolism,[34] or in some cases, through the reduction of elemental sulfur pesticide residues.[35] Much of this fermentation-derived H_2S will be lost due to CO_2 entrainment,[36] or otherwise remediated through aeration or Cu(II) treatments.[37, 38] However, H_2S may also increase during storage through poorly understood mechanisms, and is particularly problematic since remediation is less straightforward.[39] Recent work towards characterization of latent precursors to H_2S shows that H_2S can be complexed with metals and released under anaerobic conditions, but these complexes could not totally account for H_2S formed during accelerated aging.[40, 41] An additional class of precursors, reported by Kreitman et al., may exist as polysulfides and di- or mono-organopolysulfanes, which could

potentially be reduced to H_2S during storage.[42] Prior observations have demonstrated that an additional pathway towards H_2S precursors may result from S^0 substrate.[43] This research aimed to isolate and identify these S^0-dervied precursors to the formation of H_2S during storage through the use of semi-targeted HPLC-MS (Chapter 4).

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CHAPTER 2

Solid Phase Mesh Enhanced Sorption from Headspace (SPMESH) Coupled to DART-MS for Rapid Quantification of Trace-Level Volatiles

Abstract

Quantitation of trace-level (µg/L to ng/L) volatile compounds is routinely performed in a broad range of applications, including analyses of odorants, pesticide residues, or toxins in foodstuffs and related matrices. Conventional analyses based on gas chromatography-mass spectrometry (GC-MS) are limited by low throughput, and ambient approaches to sample introduction have typically had poor sensitivity. We prepared polydimethylsiloxane coated stainless steel meshes for extraction and pre-concentration of volatiles (Solid Phase Mesh Enhanced Sorption from Headspace, SPMESH), which could then be analyzed by Direct Analysis in Real Time (DART)-MS. The SPMESH cards were characterized by electron microscopy, and figures of merit for the approach were determined using two representative volatiles: 2-isobutyl-3-methoxypyrazine (IBMP) and linalool. Using DART-MS/MS and isotopically labelled internal standards, we achieved detection limits of 21 ng/L and 71 µg/L for IBMP and linalool in water. Good accuracy and precision could also be achieved for IBMP spikes in grape macerate, although accuracy for linalool was compromised by the presence of interferences. Detection limits could be further improved by an order of magnitude through use of high resolution (HR) MS. Because extraction can be performed inexpensively in parallel and because it requires short data acquisition times (<1 min) SPMESH-DART-MS may be appropriate for high throughput trace level volatile analyses.

Introduction

Quantitative analyses of volatile compounds are routinely performed on a range of analytes

(e.g. odorants, pesticides, toxins), often in complex matrices such as biological samples or foodstuffs.[1] Gas chromatography – mass spectrometry (GC-MS) is uniquely suited to achieve the necessary selectivity and sensitivity to quantify these volatiles, which may be present at ng/kg concentrations in the case of odorants in foods and beverages.[2]

However, a major limitation to GC-MS is its low throughput – depending on the number and types of analytes to be quantified, chromatographic separation may require 30-60 min per sample, with additional time necessary for oven cooling and stabilization.[3-6] These lengthy GC-MS run times are poorly suited for both routine quality control and emerging areas of research which generate large numbers of samples. For example, plant breeders create mapping populations with thousands of individuals to develop new high quality grains, fruits, or vegetables,[7-9] and GC-MS characterization can represent a bottleneck in population phenotyping.

Several approaches for improving throughput while maintaining acceptable selectivity and sensitivity have been proposed. Fast GC-MS utilizes narrow bore columns to reduce run times up to 10-fold.[10, 11] However, ultimate improvement in throughput is limited to about a factor of 3-5, and the low sample capacity of narrow-bore columns is not well suited to the broad concentration ranges encountered during volatile analyses of complex matrices.[12] Fast GC with non-MS detectors, e.g. certain commercially available electronic nose systems, can produce volatile profiles for foodstuffs in <5 min, but lack selectivity and sensitivity for some compounds and are mainly used for fingerprinting and product differentiation rather than trace-level quantification.[13]

Several commercial and literature approaches have reported improving throughput by eliminating chromatography and directly introducing volatiles into the MS, e.g. through use of Atmospheric Pressure Chemical Ionization (APCI), Proton Transfer Reaction (PTR)-MS or

Selected Ion Flow Tube (SIFT)-MS. Because these approaches directly sample the headspace, they are particularly well suited for time-resolved studies, such as process monitoring and *in vivo* flavor release, but have also found application in fingerprinting.[14, 15] However, these direct introduction approaches lack sensitivity due to the omission of a pre-concentration step - typically, detection limits are in the range of 0.1-1 mg/kg except for highly volatile compounds.[16, 17] They may also suffer from poor specificity as they are typically coupled to unit mass resolution detectors.[15] Thus, these approaches are best thought of as complementary approaches to GC-MS, and not as a high-throughput replacement.

A different approach to eliminating chromatography is ambient ionization (AI), in which ionization of analytes occurs external to the mass spectrometer. AI-MS allows for rapid analyses on the order of just seconds per sample. AI approaches with ionization mechanisms similar to APCI such as Direct Analysis in Real Time (DART) are best suited for analysis of small (<1 kDa) non-polar compounds, which encompasses most volatiles.[18] When combined with robotic positioning stages, DART-MS can achieve throughputs of 96 samples in 20 minutes.[19] The DART mechanism consists of thermal desorption of analytes followed by their ionization via metastable species and subsequent detection by MS. DART-MS has been utilized for detection of pesticides, contaminants, additives, or other components of food.[19] Detection limits as low as 2-10 µg/kg could be achieved for a range of pesticides on fruits by direct swabbing.[20, 21] However, these extraordinarily low detection limits are in part a consequence of the location of the pesticide at the fruit surface; detection limits in a bulk sample (e.g. a fruit macerate) are expected to be much lower due to dilution as well as matrix effects.[18]

The sensitivity of DART-MS for liquid samples can be improved by loading and pre-concentrating the sample on a stainless steel mesh. The analytes can then be thermally desorbed

prior to DART-MS. This approach is available commercially as transmission-mode (TM) DART (IonSense, 10 samples), X-Z Transmission DART (IonSense, 96 samples), and OpenSpot cards (IonSense, single sample). Detection limits of 300 µg/kg could be achieved for polar and semi-polar non-volatiles like imazalil,[22] but the appropriateness of mesh pre-concentration for non-polar volatiles is questionable. Headspace solid phase microextraction (SPME), which combines extraction and pre-concentration into one solvent-free step, is widely used for GC-MS analyses of aroma compounds in foodstuffs (e.g. for grapes and wine[23-25]). The coupling of SPME to DART-MS has been reported for fingerprinting in beer.[26] However, SPME fibers are configured for a GC port and do not enable efficient desorption in commercial AI-MS systems like DART. Stir-bar sorptive extraction (SBSE) offers another configuration for sample pre-concentration coupled to DART.[27, 28] However, similar to SPME, the sensitivity and throughput for SBSE is expected to be limited by its configuration. A C_{18}-polyacrylonitrile coated stainless steel mesh, engineered specifically for coupling to TM-DART was recently reported to achieve µg/kg detection limits for the quantification of cocaine and diazepam in urine and plasma,[29] but this coating was designed for extraction from liquid samples and was not demonstrated for volatiles.

We report the development and application of coated meshes specifically designed for headspace extraction and pre-concentration of volatiles (Solid Phase Mesh Enhanced Sorption from Headspace (SPMESH)) prior to DART-MS. The coated mesh configuration combines the speed and sensitivity of DART with the selective pre-concentration of volatiles inherent to traditional SPME.

Materials and Methods

Materials. Lemberger grapes (V. vinifera) were harvested at commercial maturity from a Cornell University vineyard (Lansing, NY) in October 2014 and kept frozen at -20 °C. IonSense (Saugus, MA) OpenSpot (OS) sample cards were supplied by Gentech Scientific (Arcade, NY). Linalool, 2-isobutyl-3-methoxypyrazine (IBMP), methyltrimethoxysilane (MTMOS), hydrochloric acid (HCl), sodium hydroxide (NaOH), trifluoracetic acid (TFA), hydroxyl-terminated polydimethylsiloxane (OH-PDMS), sodium chloride (NaCl), and HPLC-grade methanol (MeOH) were purchased from Sigma Aldrich (St. Louis, MO). d_3-Linalool and d_3-IBMP were purchased from C/D/N Isotopes (Pointe-Claire, Quebec). Polymethylhydrosiloxane (PMHS) was purchased from Santa Cruz Biotechnology (Dallas, TX). Isopropyl Alcohol (IPA) was purchased from Fisher Scientific (Fair Lawn, NJ). Water was purified using a Milli-Q® Advantage A10 water purification system (EMD Millipore, Billerica, MA).

Preparation of PDMS coating on OpenSpot sample cards. OpenSpot cards were cut to facilitate the dipping of the stainless steel mesh into solutions. Mesh was prepared for sol-gel coating as described by Saraji et al., with a 2 h soak in 2 M NaOH, followed by a 30 min soak in 0.1 M HCl.[30] Sol-gel solutions were prepared in ratios described by Chong et al., as 4.2 mL of MTMOS, 2.5 mL PDMS-OH, 2.6 mL of TFA, and 420 µL of PMHS,[31] and thoroughly vortexed prior to use. This volume of solution was used for the simultaneous coating of 12 cards in welled plates. The mesh of each card was soaked in the sol-gel solution for 20 min, repeated three times, using a fresh solution each time. Afterwards, cards were allowed to dry for a minimum of 12 h before use in extraction experiments, described below.

Imaging of Coated Mesh by Field Emission Scanning Electron Microscope. To prepare the SPMESH sample for Field Emission Scanning Electron Microscopy (FESEM), a gold sputter-coating was applied to the PDMS-coated mesh for 30 s using a Denton Desk V sputter coater (Cornell Center for Materials Research, Ithaca, NY). The mesh was then cut with scissors to expose the uncoated cross-section. FESEM was performed using a Tescan Mira3 (Cornell Center for Materials Research) and secondary electron detection.

Preparation of Calibration Solutions. Stock solutions were prepared in MeOH or IPA. SPMESH-DART-MS/MS calibration solutions were prepared as aqueous 5 mL solutions in 20 mL amber SPME vials (Sigma-Aldrich, St. Louis, MO). For linalool, the concentrations were 0.1, 0.25, 0.5, 1, and 3 mg/L. For IBMP, the concentrations were 10, 25, 100, 250, 375, and 500 ng/L. Internal standards were d_3-linalool and d_3-IBMP. For the SPME-DART-HRMS IBMP calibration curve, the reduced sensitivity necessitated selection of higher concentrations, and those were 50 ng/L, 500 ng/L, 5 µg/L, and 10 µg/L.

Preparation of Grape Macerate. Partially defrosted berries were placed in a blender and blended on high for approximately 1 min. Five g of blended berry, 5 mL of water and 3 g NaCl were vortexed in a 20 mL SPME vial. Linalool was spiked in grape macerate at 5 mg/L and 500 µg/L, along with internal standard d_3-linalool; IBMP was added to result in 500 ng/L and 100 ng/L, along with internal standard d_3-IBMP. Quantification was performed in replicate (n=8) on 5 mL samples at each concentration.

Quantification of native linalool and IBMP in grapes by SPME-GC-MS. HS-SPME-GC-MS was performed on the grape macerate (prepared as described above). The instrument was a Shimadzu

TQ-8040 GCMS equipped with an AOC-5000 autosampler with HS-SPME attachment. The SPME fiber was a 2 cm, 50/30 µm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA). HS-SPME autosampler conditions were as follows: pre-incubation time of 60 sec, incubation temperature of 30 sec, extraction time of 1200 sec, and desorption time 120 sec. SPME injections were split with a desorption temperature of 240 °C. The column was a 30 m x 0.25 mm x 0.25 µm Rxi-5Sil-MS (Restek, Bellefonte, PA). Helium was used as the carrier gas at a constant flow rate of 1.54 mL/min. The MS ion source temperature and interface temperature were 240 °C. For linalool, a calibration curve was prepared in aqueous samples with isotopically labelled standard d_3-linalool over a range of 10 µg/L to 10 mg/L. The temperature program for linalool was as follows: the initial temperature was 50 °C, increased to 240 °C at a rate of 13 °C/min, and then held for 0.38 min for a total run time of 15.0 min. Data were collected in SIM mode, selecting *m/z* values of 71 and 154 for linalool, and 74 and 157 for d_3-linalool. For IBMP, a calibration curve was prepared in aqueous samples with internal standard d_3-IBMP over a range of 10 ng/L to 250 ng/L. The temperature program for IBMP was as follows: initial temperature of 50 °C, held for 0.5 min, then increased to 240 °C at a rate of 20 °C/min for a total run time of 10 min. MRM transitions were monitored from 124 to 94.1 and 81.1 for IBMP, and 127 to 95.1 and 83.1 for d_3-IBMP. Collision energy was 9.0 V and scans were run from 5.3-5.6 minutes for both compounds.

SPME-DART-MS Experiments. A 2 cm, 50/30 µm divinylbenzene-carboxen-polydimethylsiloxane (DVB/CARB/PDMS) fiber was used for HS-SPME experiments. The commercial SPME fiber was suspended in the headspace of a 20 mL SPME vial containing 5 mL of solution, and sealed with a vial cap. The vial was heated and magnetically stirred for 20 min

during volatile extraction. For MS analysis, the ion source was a DART-SVP (IonSense) coupled to an Exactive Orbitrap MS system (Thermo Scientific, Waltham, MA). Data were collected in full scan mode at a helium gas temperature of 150 °C.

SPMESH-DART-MS/MS Experiments. A coated SPMESH was suspended in the headspace of a 20 mL amber SPME vial containing 5 mL of solution, and sealed with a stopper. Coated meshes were not reused, and all experiments and replicates utilized a new coated mesh. Vials were heated and magnetically stirred for 20 minutes to allow volatiles to adsorb onto the coating. Following extraction, the cut sample cards containing the coated mesh were flattened and stapled prior to analysis by DART-MS. For instrumental analysis, the ion source was an IonSense ID-CUBE DART (Gentech Scientific) and the mass spectrometer was a Thermo Finnigan TSQ Discovery MAX (Gentech Scientific). Cards were placed in the ID-CUBE and analytes were desorbed, according to the standard protocol, for 30 s on the "low" electric current setting, using a helium gas flow. The monitored transitions were optimized by direct infusion with electrospray ionization in positive ion mode as follows: for IBMP, *m/z* = 167.1 to 125.0; for d_3-IBMP, *m/z* = 170.1 to 128.0; for linalool, *m/z* =137.1 to 81.0; and for d_3-linalool, *m/z* =140.1 to 83.0. Instrument settings were as follows: scan width of 0.10, scan time of 0.10 s, collision energy of 15V, Q1 width of 0.20, Q3 width of 0.70, and CID gas of 1.5.

Limit of Detection for Linalool and IBMP in Aqueous Matrix. Limits of detection (LOD) were determined according to the method of Pallesen[32] using six replicates at each concentration level of the calibration curve.

Storage Experiments. Extractions were performed as previously described for aqueous solutions. The test solutions contained 5 mg/L linalool and 10 µg/L IBMP. Cards were stored at 20 °C , 4 °C, -4 °C, or -18 °C for 3 or 5 days before analysis (four temperatures and two storage times in triplicate, for a total of 24 treated SPMESH cards). Control SPMESH cards that were not subjected to storage were also evaluated in triplicate. For statistical analysis, a full-factorial multi-way ANOVA was performed using JMP Pro 12.0 (SAS Institute, Cary, NC, USA), with time and temperature treated as nominal variables.

To evaluate the effects of humidity on SPMESH signal, cards were stored for 24 h following volatile extraction and placed back in the headspace of 5 mL of heated water for 20 min prior to analysis.

SPMESH-DART-SVP-HRMS Analysis. PDMS-coated SPMESH cards were prepared as before, with the additional step of puncturing 3-4 small holes in the coating to facilitate TM-DART in the SVP system. 5-mL aqueous solutions consisting of 100 µg/L linalool with internal standard d_3-linalool were prepared. Extraction was carried out as previously described. Desorption was performed using the same DART-SVP-Orbitrap system as for the SPME-DART-HRMS experiment. Data were collected in full scan mode at a helium gas temperature of 150 °C, and the selected ion chromatograms were generated for the quantification of ions 137.1325 (linalool) and 140.1513 (d_3-linalool) since the instrument did not possess MS/MS capabilities. The LOD for linalool was approximated using the U.S. EPA approach to calculate method detection limit (MDL) for 8 replicate samples.[32] The limit of quantitation (LOQ) was calculated as 3 × LOD.

Results and Discussion

Selection of Test Analytes. Two odor-active volatile compounds, linalool and IBMP, were selected for this study (Figure 2.1). These compounds were selected because they have low sensory thresholds, different functional groups, and are common contributors to food aroma. IBMP possesses a “green pepper” odor, with a sensory threshold in water reported as 2 ng/L.[33] Linalool possesses a fruity, floral aroma, with a sensory threshold in water reported as 6 µg/L.[34]

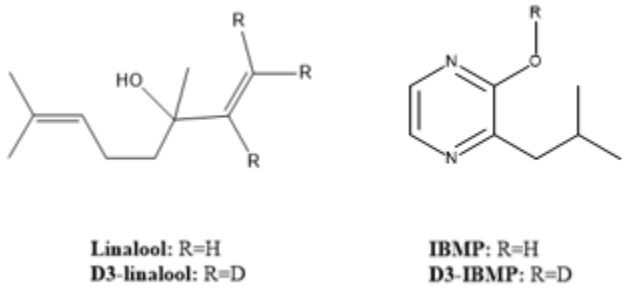

Figure 2.1. Structures of linalool and IBMP and their respective deuterated analogs.

SPME-DART-HRMS calibration curve. For comparison to SPMESH, a commercially available DVB/CARB/PDMS SPME fiber was first used to generate an IBMP calibration curve, coupled to DART-MS (Figure 2.2). The traditional SPME fiber configuration permits neither analysis in an ID-CUBE source nor replicable sample introduction. In our current work, we observed poor linearity (r^2=.83) and precision for SPME-DART-MS experiments, and limits of detection were estimated as >1 µg/L for IBMP due to the high variability of response, well above its sensory threshold. The poor sensitivity may arise either from the limited extraction capacity of SPME, or from challenges with desorption, ionization and introduction of volatiles from SPME into the DART source (as discussed later). These factors make HS-SPME-DART-MS ill-suited to

quantitative trace volatile analysis, and may explain why the single previous report on the approach focused on its use for fingerprinting (of beers) rather than quantitative analyses[26].

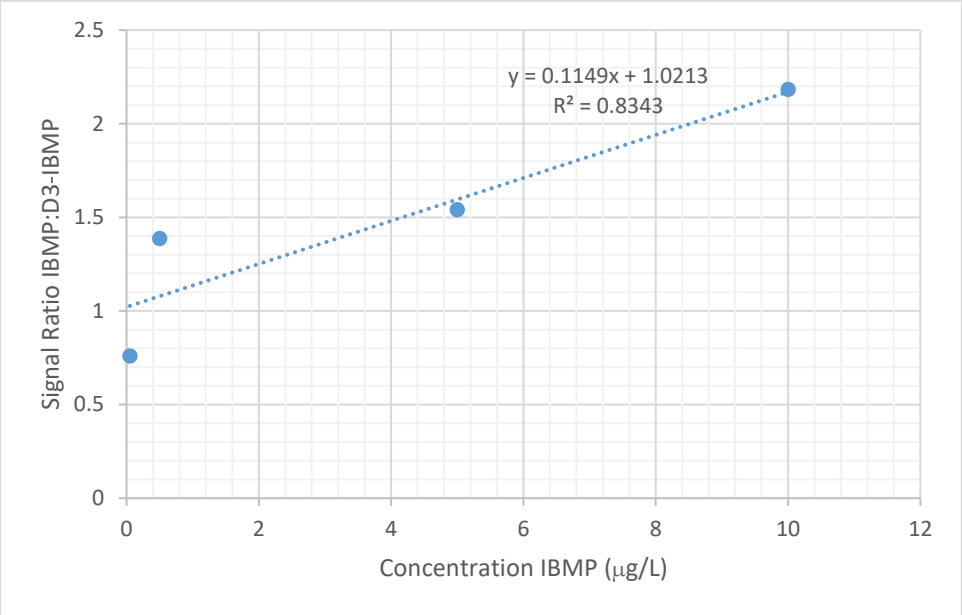

Figure 2.2. Calibration curve for SPME-DART-MS

Coating method and SPMESH geometry. To improve upon the SPME-DART results, a mesh configuration specifically designed for DART desorption (ID-CUBE Open Spot Sample Cards) was sol-gel coated with PDMS (Figure 2.3a,b). The sol-gel coating technique was chosen for being a cost-effective method requiring minimal special equipment and offering adequate thermal stability for coupling to DART desorption (Figure 2.3c) at temperatures below 200 °C. The stainless steel mesh of the OpenSpot cards was selected as the coating substrate because mesh is well established to yield good sensitivity for DART applications.[19] For ID-CUBE studies, it is important that the sample can be presented as an OpenSpot card (Figure 2.3d), since the instrument is not designed to fit other configurations. FESEM was used to characterize the

PDMS coating on the stainless steel mesh of the Open Spot sample cards (Figure 2.3e). The average coating thickness was 19.4 ±4.8 µm (n=20). This is in agreement with comparable sol-gel coating procedures, which report resulting thicknesses ranging from 10-30 µm .[30, 31]

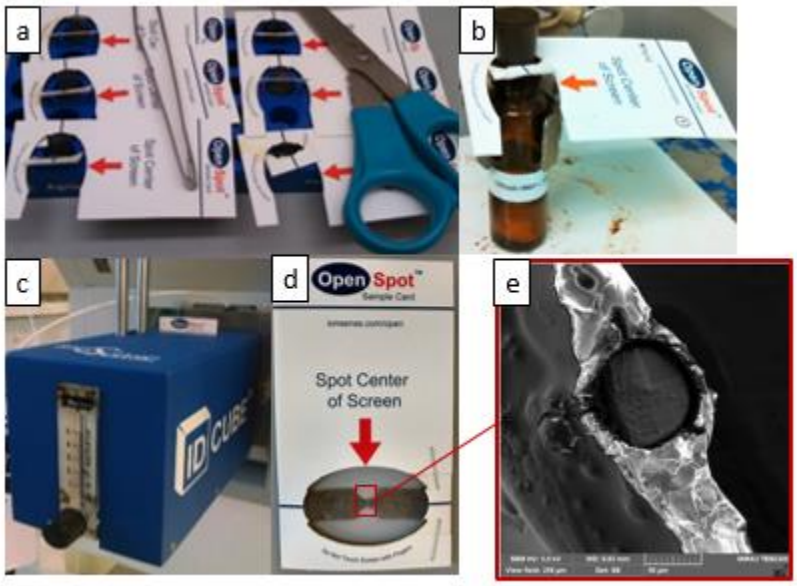

Figure 2.3. (a) dipping of mesh into sol-gel solution; (b) coated mesh suspended in headspace for extraction of volatiles; (c) desorption by ID-CUBE DART; (d) Coated SPMESH card stapled in place for analysis (e) cross-sectional view of PDMS-coated wire by FESEM

SPMESH-DART-MS/MS calibration curves and limit of detection for IBMP and linalool. Calibration curves were created for IBMP and linalool using SPMESH extraction followed by DART-MS/MS (Figure 2.4a,b). Figures of merit for these compounds are presented in Table 2.1.

Table 2.1. Figures of Merit for SPMESH-DART-MS/MS

	Linalool	IBMP
Calibration range	0.1-3 mg/L	25-500 ng/L
r^2	0.99	0.96
Mean %RSD	9.2	10.1
LOD	0.071 mg/L	21 ng/L
LOQ	0.21 mg/L	62 ng/L
Sensory Threshold[a]	0.006 mg/L[34]	2 ng/L[35]

[a]Sensory thresholds reported in water

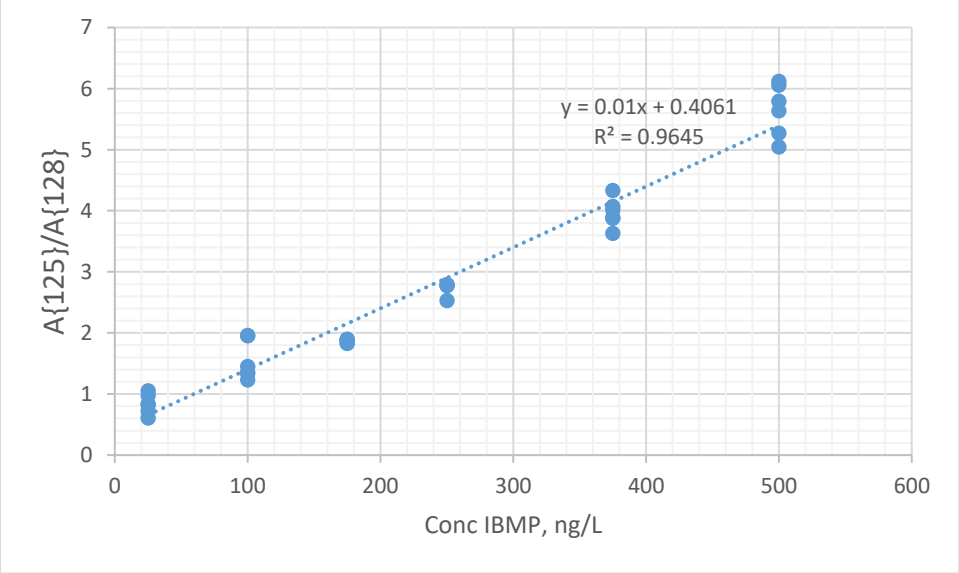

Figure 2.4a. Calibration curve for IBMP using SPMESH-DART-MS/MS

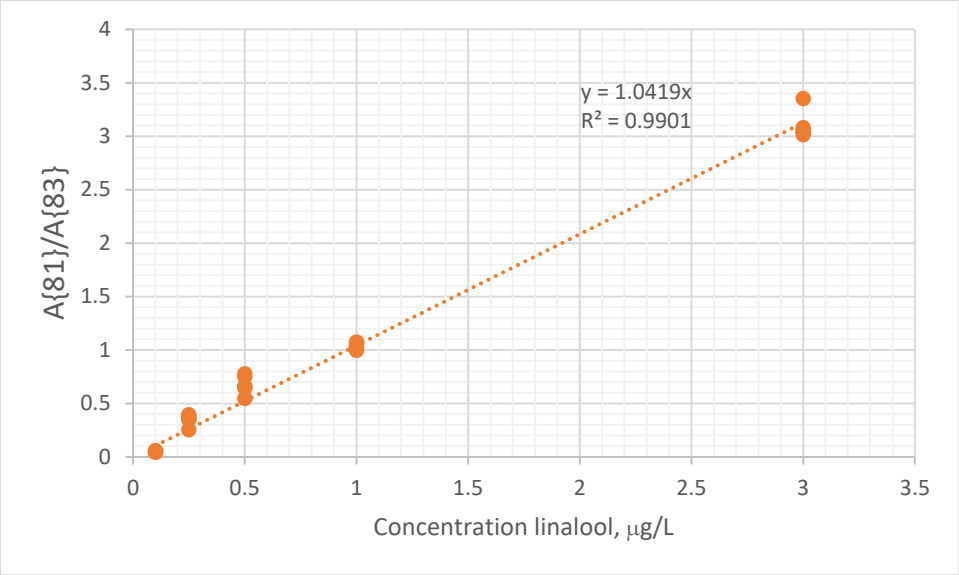

Figure 2.4b. Calibration Curve for Linalool using SPMESH-DART-MS/MS

By SPMESH-DART-MS/MS, we achieved good linearity (r^2=.96), detection limits (21 ng/L), and quantification limits (62 ng/L) for IBMP. For linalool, we achieved linearity of r^2=.99, detection limits of 71 µg/L, and quantification limits of 210 ng/L. The variation in coating thickness (19.4 ±4.8 µm) is expected to be a major contributor to variation in absolute signal (29.5-42.3% RSD) for SPMESH experiments. This level of variation in signal intensity is comparable to that observed in conventional SPME-GC-MS applications.[36] Other studies have reported that increasing the SPME fiber conditioning temperature can result in reduced variability.[31] It is expected that further development and/or automation of SPMESH card production will result in improved reproducibility. However, the use of isotopically labelled standards still permits reliable quantification (3.8-9.5% RSD, Table 2.1), and absolute signal variation should not be a major concern if these are employed.

SPMESH-DART-MS represents a considerable improvement over SPME-DART-MS studies. The lower detection limits likely arise from a combination of the greater surface area of the

sorbent material as well as the more reproducible sample introduction. Assuming for ID-CUBE experiments that one entire side of the mesh is desorbed in the center portion only, the available surface area is approximately 0.3 cm^2, more than 10 times that of a 2-cm SPME fiber with comparable coating thickness. Further improvements may have been realized by the greater extraction efficiency of the mesh configuration in a TM-DART ion source. The SPME fiber represents a much smaller target which, in our experiments, sometimes produced no signal for low concentration samples due to difficulty in positioning the fiber.

The detection limits achievable through SPMESH-DART-MS are a considerable improvement over chromatography free approaches that use no sample preconcentration, e.g APCI-MS, PTR-MS, and SIFT-MS. While these approaches are well suited for real time analyses, reported detection limits are usually in the range of 0.1-1 mg/kg except for highly volatile compounds[16, 17]. However, SPMESH-DART LODs must be improved by at least a factor of 10 to reach the sensory thresholds of the odorants investigated in our study (linalool, IBMP). Our data indicate that the sensitivity of SPMESH-DART-MS/MS was limited by a relatively high baseline noise (Figure 2.5b). Therefore, coupling to HRMS was expected to improve LODs by resolving interferences for the ions of interest. Initial results exploiting the advantages of HRMS are demonstrated later.

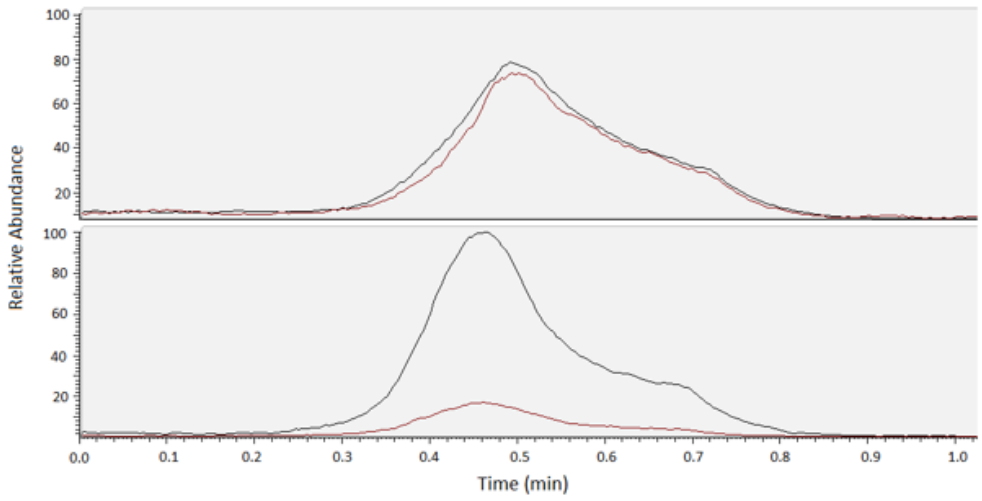

Figure 2.5a. Sample MS/MS chromatogram for SPMESH-DART analysis of IBMP (m/z 167.1 to 125.0) at 25 ng/L (top) and 500 ng/L (bottom), with internal standard d_3-IBMP (m/z 170.1 to 128.0). Desorption is occurring during ~0.3-0.6 min.

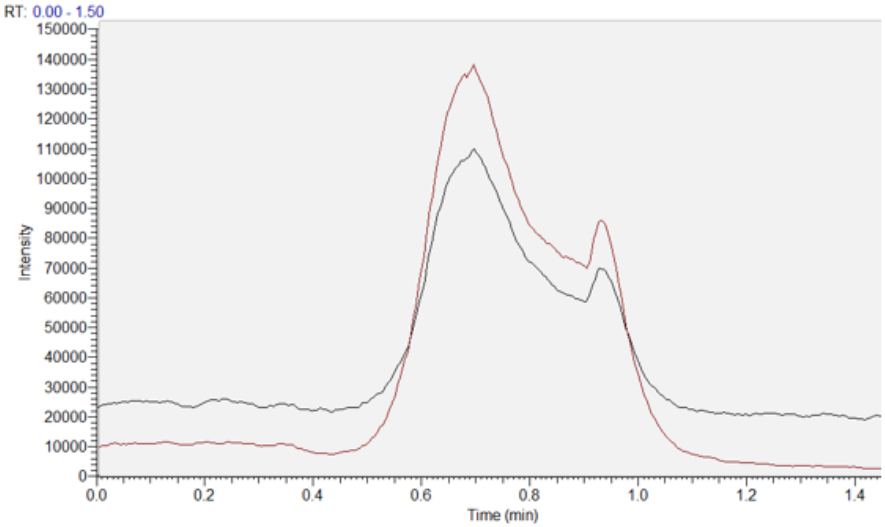

Figure 2.5b. Sample MS/MS chromatogram for SPMESH-DART analysis of linalool (m/z 137.1 to 81.1) at 25 ng/L (top) and 500 ng/L (bottom), with internal standard d_3-IBMP (m/z 140.1 to 83.0). Desorption is occurring during ~0.6-0.9 min.

Storage behavior of extracted volatiles on SPMESH. Unlike a commercial retractable SPME fiber, the coated mesh is not easily protected from the environment and is therefore potentially vulnerable to loss of analyte and an increase in detection limits. Following extraction, SPMESH cards were stored for 3 and 5 d at one of four temperatures. No significant change in the signal ratio of analyte to internal standard was observed over 5 days of storage at room temperature ($p>0.05$, data not shown), indicating that accuracy was not compromised by storage. However, the raw signal intensity for unlabeled IBMP and linalool was affected after 5 d at all storage temperatures (Figure 2.6), with an average decrease of 50% for both IBMP and linalool across all storage temperature and time combinations. The reason for this decrease – even at lower temperatures – was unclear. Potentially, the signal loss could have resulted from relatively rapid reaction of the analytes with active sites on the mesh or in the coating. Because DART ionization mechanisms can involve water[18] we evaluated if the decrease in signal resulting from sample storage was due to lower humidity by re-exposing SPMESH cards to humidified headspace prior to DART-MS/MS. However, no signal improvement was observed for the humidified card as compared to the control (data not shown). We also observed a significant time-temperature interaction term for both IBMP and linalool ($p<0.05$), which may result from volatilization losses at high temperatures and/or long storage times. In summary, although storage should not compromise accuracy due to concurrent losses of labeled and unlabeled compounds, samples should be stored cold to minimize analyte losses and a degradation of sensitivity.

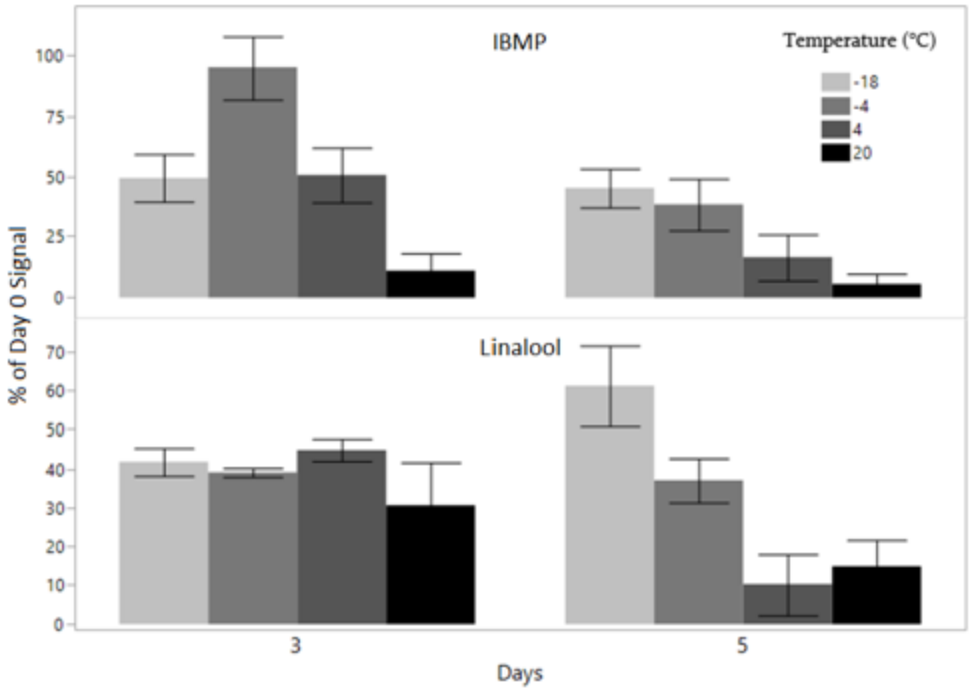

Figure 2.6. IBMP and linalool signal intensity (normalized to days = 0) versus days of storage at 4 different temperatures

Accuracy in real matrices. Accuracy by SPMESH-DART-MS/MS was evaluated for both linalool and IBMP using spikes in real samples (Lemberger grape macerate) for 8 replicates. These samples were selected because they had no detectable linalool or IBMP by SPME-GC-MS (<0.1 mg/L and <10 ng/L, respectively). Low and high recovery spike concentrations were selected to be approximately two-fold and ten-fold above the limits of quantification. Measured concentrations expressed as a percentage of expected concentrations and relative standard deviations are presented in Table 2.2. Recoveries for IBMP were excellent (96-102%) for both high and low concentration spikes (500 and 100 ng/L). Although reasonable accuracy could be achieved for the high linalool spike, the higher value at low linalool concentration (138% of

expected) is likely related to interferences present at the monitored transition for the unlabeled form (137.1→81.0).

Table 2.2. Accuracy and relative standard deviation for measurements of linalool and IBMP in a grape macerate

	Linalool[a]		IBMP[a]	
Expected concentration[a]	5 mg/L	0.5 mg/L	500 ng/L	100 ng/L
Accuracy[b]	87.2%	138.3%	96.0%	101.8%
%RSD	3.8	8.1	9.3	9.5

[a]Both compounds were below detection limits by SPME-GC-MS; expected concentration reflects the amount spiked into the macerate.

[b]Expressed as the ratio of measured concentration to expectation concentration × 100%.

Traditional SPME-GC-MS analyses of these compounds performed in biological matrices generally report lower LOD than our current SPMESH-DART-MS/MS method, but require longer run times. For example, IBMP can be detected by GC-MS down to low ng/L concentrations, with an oven cycle time (not including sample preparation and extraction) of approximately 30 minutes;[37] a different group reported an LOD for linalool of low µg/L, with an oven cycle time of approximately 45 minutes[38]. Because our preliminary work with SPMESH-DART-MS/MS indicated detection limits were likely compromised by high background noise,

particularly for linalool (Figure 2.5b), we investigated if high resolution mass spectrometry (HRMS) could offer improvements.

SPMESH-DART-SVP-HRMS. To evaluate if HRMS could improve LODs, as has been described elsewhere[18], SPMESH samples were prepared as usual and analyzed by DART-SVP-HRMS, using an Exactive Orbitrap as a detector. Sample preparation was similar to SPMESH-DART-MS/MS, except that holes were punctured in the SPMESH coating prior to extraction to facilitate TM-DART. A representative chromatogram of linalool acquired using SPMESH-DART-SVP-HRMS is shown in Figure 2.7, and an LOD for linalool using the HRMS detector of 6.4 µg/L (Figure 2.7) was achieved. This represents more than a factor of 10 improvement over MS/MS, and is comparable to sensory thresholds for linalool reported in water.[34]

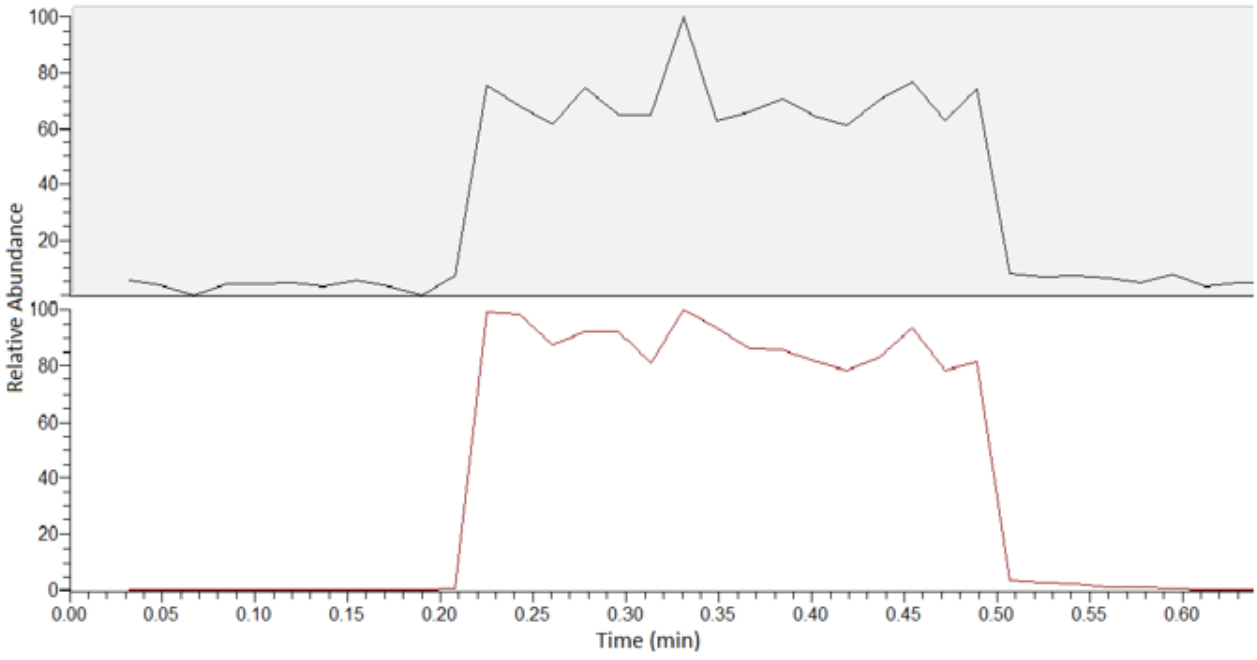

Figure 2.7. Sample chromatogram of linalool using SPMESH-DART-HRMS

Conclusions

We have demonstrated a novel approach to rapid trace level volatile analysis by developing coated mesh (SPMESH) cards for extraction and pre-concentration of volatiles prior to DART-MS analysis. When coupled to an MS/MS detector, detection limits in the µg/L – ng/L range could be achieved for volatiles, appropriate for measuring known potent odorants and considerably lower than limits reported for other chromatography-free approaches. Further improvements in sensitivity were demonstrated through use of HRMS as a detector. There are several potential areas for future improvement of the approach. Extraction could be performed in parallel using coated mesh panels instead of individual cards, and then desorbed sequentially on an appropriate positioning stage. A similar approach has been described for non-volatile analyses, and could result in processing of 100 or more samples per hour.[39, 40] Additionally, selectivity could be improved by slowly increasing the desorption temperature to allow for separation prior to ionization.[41]

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CHAPTER 3

Trace-Level Volatile Quantitation by DART-MS following Headspace Extraction – Optimization and Validation in Grapes

Abstract

Ambient Ionization – Mass Spectrometry (AI-MS) techniques like Direct Analysis in Real Time (DART) offer the potential for rapid quantitative analyses of trace volatiles in food matrices, but performance is generally limited by the lack of pre-concentration and extraction steps. The sensitivity and selectivity of AI-MS approaches can be improved through solid-phase microextraction (SPME) with appropriate thin-film geometries, e.g. solid phase mesh enhanced sorption from headspace (SPMESH). This work improves the SPMESH-DART-MS approach for use in food analyses, and validates the approach for trace volatile analysis in real samples (grape macerates). SPMESH units prepared with different sorbent coatings were evaluated for their ability to extract a range of odor-active volatiles, with polydimethylsiloxane/divinylbenzene giving the most satisfactory results. In combination with high-resolution mass spectrometry (HRMS), detection limits for SPMESH-DART-MS under 4 ng/L in less than 30 s acquisition times could be achieved for some volatiles (3-isobutyl-2-methoxypyrazine (IBMP), β-damascenone). A comparison of SPMESH-DART-MS and SPME-GC-MS quantitation of linalool and IBMP demonstrates excellent agreement between the two methods using real grape samples ($r^2 \geq 0.90$).

Introduction

Ambient ionization mass spectrometry (AI-MS) techniques like direct analysis in real

time (DART)-MS can improve simplicity and throughput for a wide range of routine chemical analyses. DART relies on gas-phase proton-transfer Penning ionization to generate charged species prior to sampling into the MS via a vacuum interface, similar to atmospheric pressure chemical ionization (APCI).[1] In comparison to other popular AI approaches, e.g. desorption electrospray ionization (DESI), DART is better suited for volatile or semi-volatile low molecular weight compounds (<1 kDa).[1] To compensate for the absence of chromatography (and loss of selectivity) the performance of DART and related AI techniques can be improved by high resolution mass spectrometry (HRMS), such as quadrupole – time of flight (QTOF)-MS or Orbitrap-MS.[2] A wide range of DART-MS applications have been proposed[1, 3], including several related to agriculture and food science such as screening for fungicides on raw plant materials,[4] establishing olive oil authenticity,[5] profiling volatile organic compounds in lemon and onion.[6] and detecting contamination of foods following surface contact.[7] These DART-MS applications are typically qualitative or semi-quantitative, and many only involve surface characterization. Applications of DART-MS or related AI-MS techniques to trace-level (ng/L to mg/L) quantitative analyses of volatiles in bulk samples (e.g. whole fruit macerates) are less common, in spite of the widespread need for these analyses in food quality studies[8]. Currently the only method routinely employed for trace-level volatile quantitation is gas chromatography (GC)-MS, but typical GC-MS analyses require at least 30 min per sample, which can result in severe bottlenecks for sample characterization.[9] Although DART-MS or related approaches could expedite these analyses, the lack of a pre-concentration/extraction step in most reported DART-MS applications[4-7] will limit their sensitivity and selectivity[10]. Similarly, related direct-introduction approaches which rely on headspace sampling into an MS (e.g. Selected Ion Flow Tube-MS, Proton Transfer Reaction-MS) suffers from poor sensitivity due to a lack of pre-

concentration, resulting in detection limits in the range of 0.1-1 mg/kg for most odorants.[11, 12]

Recent publications have suggested that coupling a simple pre-concentration/extraction step with DART-MS could facilitate rapid trace level analyses of small molecules.[10, 13] For example, headspace - solid phase microextraction (HS-SPME) is widely used in GC-MS to preconcentrate headspace compounds and eliminate non-volatile matrix interferences,[14-16] and the coupling of HS-SPME and DART-MS has been described for analysis of beer volatiles.[17] Although useful for fingerprinting, the coupling of conventional HS-SPME with DART suffers from poor transfer efficiency, reproducibility, and sensitivity, and is not readily automated.[10] Current alternatives to DART for direct coupling of SPME to AI-MS include dielectric barrier discharge ionization (DBDI) and ESI interfaces. Mirabelli et al. proposed direct coupling of SPME to MS via a DBDI, ensuring a more thorough and reproducible desorption from the SPME fiber.[18] Low ng/L detection limits were reported for nonvolatile pesticides, but the analysis of volatile compounds is not reported, and the format is not expected to be easily automatable. Gomez-Rios et al. reported adapting an Open Port Probe (OPP) sampling interface for the direct coupling of SPME to ESI-MS, demonstrating excellent sensitivity (down to 100 ng/L) for quantitative determination of doping agents in biological matrices.[19] However, ESI is generally not very effective for the ionization of less polar compounds and thus this SPME-ESI approach is not ideal for most volatiles.

Another approach is to utilize non-traditional SPME geometries more conducive to DART desorption, e.g. thin-film microextraction, which is well-suited to transmission mode (TM) DART-MS.[20, 21] Gomez-Rios et al. reported a thin film "SPME-TM" device based on C_{18}-particles suspended in a polyacrylonitrile (PAN) support coated on steel mesh.[22] The authors have used SPME-TM-DART-MS for trace-level analysis of drugs in urine and plasma, and more

recently for the analysis of pesticides in food and environmental matrices and drugs of abuse in saliva samples.[13, 23] However, this approach relied on direct immersion of the SPME-TM in the sample,[23] which is expected to increase the risk of isobaric interferences or matrix suppression, making this technique inappropriate for trace-level volatile analyses.

We recently reported the development of Solid Phase Mesh Enhanced Sorption from Headspace (SPMESH), in which a polydimethylsiloxane (PDMS)-coated stainless steel mesh is suspended in a sample headspace to extract volatiles.[10] Although similar to conventional HS-SPME, the SPMESH approach is more cost-effective, has greater loading capacity, is better suited for TM-DART-MS, and potentially could be adapted to an automated well-plate format. Quantitation of two representative volatiles (linalool and 3-isobutyl-2-methoxypyrazine (IBMP)) by DART-MS/MS in model grape juice resulted in limits of detection (LODs) in the ng/L-µg/L range, but still approximately an order of magnitude above the sensory thresholds of the compounds.[10] Here, we demonstrate that an improved dual-phase coating incorporating PDMS and divinylbenzene (DVB), in combination with high-resolution Orbitrap-MS can improve LODs by more than an order of magnitude. The optimized SPMESH-DART-HRMS approach was then validated for odor-active volatiles in grape macerate.

MATERIALS AND METHODS

Materials. IonSense (Saugus, MA) OpenSpot (OS) sample cards were supplied by Gentech Scientific (Arcade, NY). Linalool, IBMP, 1-hexanol, β-damascenone, methyltrimethoxysilane (MTMOS), hydrochloric acid (HCl), sodium hydroxide (NaOH), trifluoracetic acid (TFA), hydroxyl-terminated PDMS (OH-PDMS), DVB, sodium chloride (NaCl), and HPLC-grade methanol (MeOH) were purchased from Sigma Aldrich (St. Louis, MO). d_3-Linalool and d_{11}-

hexanol were purchased from C/D/N Isotopes (Pointe-Claire, Quebec), and d_3-IBMP was purchased from Aroma Lab (Munich, Germany). Polymethylhydrosiloxane (PMHS) was purchased from Santa Cruz Biotechnology (Dallas, TX). Isopropyl alcohol (IPA) was purchased from Fisher Scientific (Fair Lawn, NJ). Water was purified using a Milli-Q® Advantage A10 water purification system (EMD Millipore, Billerica, MA). Cabernet Sauvignon, Muscat of Alexandria, Pinot noir, and Chardonnay grapes were sourced from E&J Gallo Winery (California) during the 2016 harvest, and Lemberger grapes were harvest locally in the Finger Lakes AVA (NY), also in 2016. All samples were kept frozen at -20 °C until analysis.

Preparation of SPMESH devices. PDMS-coated OS cards were prepared in-house as previously described.[10] Additional coated meshes of PDMS/DVB, PDMS/carboxen (PDMS/CARB), and PDMS/DVB/CARB for the optimization of multi-phase coating material were provided by Millipore Sigma (Bellefonte, PA). For all subsequent experiments including generation of calibration curves and the quantitation of native compounds in real grape macerate, PDMS/DVB coatings were prepared in-house as follows. Cards were cut to facilitate the dipping of the stainless steel mesh into solutions. Mesh was prepared for sol-gel coating as described by Saraji et al., with a 2 h soak in 2 M NaOH, followed by a 30 min soak in 0.1 M HCl. Sol-gel solutions were prepared as 4.2 mL of MTMOS, 2.5 mL PDMS-OH, 2.6 mL of TFA, and 420 µL of PMHS, 83 µL of DVB and thoroughly vortexed prior to use.[24-26] This volume of solution was used for the simultaneous coating of 12 cards in welled plates. The mesh of each card was soaked in the sol-gel solution for 20 min, repeated three times, using a fresh solution each time. Afterwards, cards were allowed to dry for a minimum of 12 h before use in extraction experiments, described below.

Preparation of calibration solutions. Stock solutions were prepared in 5 mL MeOH containing both the unlabeled standard compound and the isotopically labeled internal standard. Calibration solutions were prepared in 20 mL amber SPME vials (Sigma-Aldrich, St. Louis, MO) by spiking 100 µL of stock solution into 10 mL of Milli-Q water, using 3 concentrations for each compound, with 5 replicates per concentration. For linalool, concentrations were 10, 50, and 100 µg/L. For hexanol, concentrations were 5, 50, and 100 mg/L. For IBMP, concentrations were 20, 100, and 200 ng/L. For damascenone, concentrations were 0.1, 0.5, and 1 µg/L.

Preparation of grape macerate. Partially-thawed, destemmed berries (100 g) were placed in a chilled 250-mL stainless steel waring blender and blended on medium for 30 seconds and on high for 30 s. To a 20-mL amber SPME vial was added 5 g of the berry slurry, 3 g NaCl, 5 mL of a buffer solution consisting of 0.1 M sodium phosphate dibasic/0.1 M sodium phosphate monobasic, and 100 µL of the internal standard cocktail. The internal standard cocktail (25 µg/L d_3-IBMP, 5 mg/L d_3-linalool, and 30 mg/L d_{11}-hexanol) was prepared in MeOH. In the case of IBMP, we suspect there was inadvertent contamination of California samples, since concentrations were uncommonly high. Therefore, low concentrations of unlabeled compound were spiked into an IBMP-free variety (Lemberger) to demonstrate the capability of SPMESH-DART to detect these low-range concentrations.

Optimization of SPMESH coating by SPMESH-DART-MS/MS. The ion source was an IonSense ID-CUBE DART (Saugus, MA) and the mass spectrometer was a Thermo Finnigan TSQ Discovery MAX (Waltham, MA). Multi-phase coated SPMESH units were obtained from MilliporeSigma (St. Louis, MO). Absolute signal was monitored following headspace extraction of an aqueous solution containing the following compounds: dimethyl sulfide, furfural, hexanal, ethyl butyrate, linalool, methyl anthranilate, IBMP, β-damascenone.

SPMESH-DART-HRMS analysis conditions. The ion source was a DART-SVP (IonSense) coupled to an Orbitrap Elite MS system (Thermo Scientific, Waltham, MA), fitted with an Open Spot card holder to allow reproducible positioning of SPMESH cards. Data were collected in positive ion, full scan mode, over mass range *m/z* 50-200, with the helium gas temperature set to 200 °C. The quantifying ions were as follows: IBMP 167.1180, d_3-IBMP 170.1368, β-damascenone 191.1432, linalool 137.1323, d_3-linalool 140.1511, hexanol 85.1007, and d_{11}-hexanol 96.1679, with mass accuracy set to 5 ppm. Internal standards were the deuterated analogue of their respective unlabeled analytes in all cases besides β-damascenone, for which the internal standard was d_3-IBMP. SPMESH extractions were performed by using a rubber stopper to secure the coated mesh in the headspace of 20 mL amber SPME fibers containing 10 mL of calibration solution or wine grape macerate with internal standard, for a duration of 30 min and a temperature of 50 °C with magnetic stirring.

SPME-GC-MS/MS Analysis. The instrument was a Shimadzu TQ-8040 GCMS and AOC-5000 autosampler equipped with HS-SPME (Columbia, MD). The SPME fiber was a 2 cm, 50/30 µm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA). HS-SPME autosampler conditions were as follows: preincubation time of 60 s, incubation temperature of 50 °C, extraction time of 1800 s, and desorption time of 600 s. Injections were splitless with a desorption temperature of 240 °C. The column was a 30 m x 0.50 mm x 0.25 µm VF-WAXms (Agilent, Santa Clara, CA). Helium was used as the carrier gas at a constant flow rate of 1.54 mL/min. The MS ion source temperature and interface temperature were 240 °C. The temperature ramp was as follows: the initial temperature was 50 °C, increased to 100 °C at a rate of 20 °C/min, increased to 118 °C at a rate of 5 °C/min, increased to 125 °C at a rate of 1 °C/min, increased to 152 °C at a rate of 5 °C/min, and finally to 240 °C at a rate of 40 °C/min, followed by a 10 min hold time for a total

run time of 30.70 min. Data were collected in MRM mode, monitoring the following transitions: hexanol 69 to 53.1 (3-6.84 min), d_{11}-hexanol 76 to 58 (3-6.84 min), IBMP 124 to 95.1 (6.84-13.24 min), d_3-IBMP 127 to 95.1 (6.84-13.24 min), linalool 93 to 77.1 (6.84-13.24 min), d_3-linalool 96 to 77.1 (6.84-13.24 min), and β-damascenone 121 to 105.1 (13.24-30 min). Collision energy was 10.0 V.

Limits of Detection for odor-active volatiles in aqueous matrix. LODs were calculated according to the method of Pallesen[27] using six replicates at each concentration level of the calibration curve. The limit of quantitation (LOQ) was calculated as 3 x LOD.

SPMESH-DART-HRMS vs. SPME-GC-MS/MS comparison. Quantitation of linalool, IBMP, 1-hexanol, and β-damascenone was performed in wine grape samples harvested at commercial maturity from California (Central Valley AVA, CA) or New York State (Cayuga Lake AVA, NY). For linalool, a comparison was made across 16 samples, 8 Muscat and 8 non-Muscat. For IBMP, a comparison was made across 12 samples representing five different cultivars. Some IBMP samples had added unlabeled IBMP to create a range of IBMP concentrations. All samples were prepared and analyzed in analytical duplicate by both methods, and the methods compared by linear regression (1/x weighting factor).

Assessment of Matrix Effects. Data from the SPMESH-DART-MS study described above. To assess the effect of the grape matrix on DART-MS signal response of deuterated standards, a t-test was performed to compare mean signal response in aqueous samples. In the case of β-damascenone, 100 ng/L of standard was spiked into 10 mL of either water or grape macerate containing no detectable β-damascenone (n=3 for each).

RESULTS AND DISCUSSION

To assess if using coatings other than PDMS could improve SPMESH-DART-MS sensitivity, we tested the ability of four different phases on 8 odor-active compounds (dimethyl sulfide, furfural, hexanal, ethyl butyrate, linalool, methyl anthranilate, IBMP, and β-damascenone). These compounds represent a diverse range of functional groups and have olfactory relevance to multiple foodstuffs.[28] Results are displayed in Figure 3.1. PDMS generally gave the poorest response and in some cases (ethyl butyrate, furfural, hexanal) failed to yield any detectable signal. The incorporation of DVB and/or Carboxen improved sensitivity by 100 000-fold (5-log) or more in the case of ethyl butyrate. Large improvements in sensitivity (2-log or greater) as compared to PDMS were also noted for 4 other compounds: dimethylsulfide, ethyl butyrate, furfural, and hexanal. These results are consistent with those of analogous SPME-GC-MS studies for selection of optimal coatings.[29, 30]

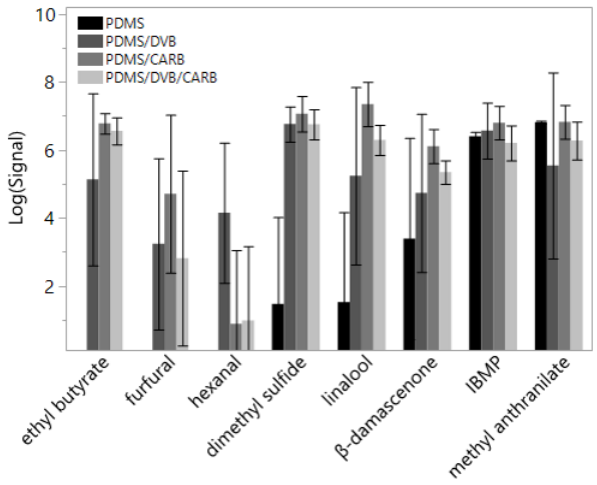

Figure 3.1. Comparison of response (absolute signal, log-transformed) for 8 compounds using four different SPMESH coating phases

Differences in sensitivity between PDMS/DVB and PDMS/CARB were more modest. However, PDMS/DVB gave a significantly higher signal for hexanal as compared to PDMS/CARB and

was therefore utilized as a mesh coating for subsequent experiments. PDMS/DVB/CARB had lower cumulative signal and worse precision than either PDMS/DVB or PDMS/CARB, and was also observed to easily flake from the stainless-steel substrate. Interestingly, we observed a much higher degree of run-to-run signal variability (standard deviation greater than an order of magnitude) for furfural and hexanal as compared to other compounds. The reason for the poor reproducibility associated with these aldehydes may be associated with their high reactivity, a problem which could potentially be overcome by derivatization prior to extraction, as has been demonstrated for SPME.[31]

Three volatiles from the initial evaluation (IBMP, linalool, β-damascenone) along with one additional odorant (hexanol) were prepared as calibration standards for evaluation of DVB/PDMS based-SPMESH coupled to DART-Orbitrap-MS. These four volatiles were selected because of their recognized importance in assessing grape quality.[32] Because of the number of target analytes we chose to operate the Orbitrap in full scan mode and generate single ion chromatograms (SIC) retrospectively, instead of performing multiple single ion monitoring (SIM) or single reaction monitoring (SRM) experiments, as has been suggested by other authors.[33]

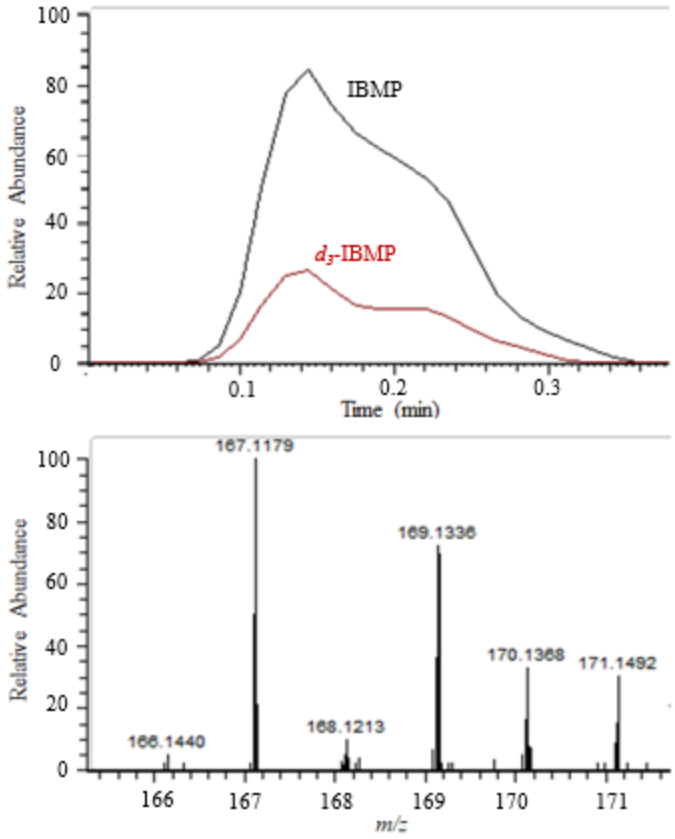

Figure 3.2. Representative mass spectrum and SIC for IBMP (*m/z* 167.1179) and d_3-IBMP (*m/z* 170.1368) in aqueous sample

A representative mass spectrum along with SIC for IBMP and d_3-IBMP are shown in Figure 3.2. Figures of merit for the SPMESH-DART-Orbitrap-MS study are displayed in Table 3.1. Data acqusition required 30 s or less per analysis (Figure 3.2), as compared to 30 min for a typical GC-MS analysis. We observed excellent linearity and precision ($r^2 \geq 0.98$ and RSD < 6%) for all analytes, and LODs were below or comparable to the sensory thresholds for all compounds. The lowest LOD was achieved for β-damascenone (1.9 ng/L), below its sensory threshold in water of 2 ng/L.[34] The LOD achieved for IBMP (3.8 ng/L) was 5-fold better than what was previously reported using PDMS based SPMESH-DART-MS/MS. Furthermore, this value is comparable to LODs typically achieved for IBMP using SPME-GC-MS.[35]

Table 3.1. Figures of Merit for SPMESH-DART-HRMS

	Linalool	IBMP	β-damascenone	1-Hexanol
Calibration Range	10-100 µg/L	20-200 ng/L	0.1-1 µg/L	5-100 mg/L
r^2	0.99	0.99	0.98	0.99
Mean %RSD	3.6	2.9	1.7	5.7
LOD	2.1 µg/L	3.8 ng/L	1.9 ng/L	1.1 mg/L
LOQ	6.5 µg/L	11.3 ng/L	5.6 ng/L	3.3 mg/L
Sensory Threshold	6 µg/L[49]	2 ng/L[50]	2 ng/L[34]	2.5 mg/L[51]

Similarly, the LOD for linalool (2.1 µg/L) was 30-fold better than what was previously reported using PDMS based SPMESH-DART-MS/MS. This considerable improvement is attributable to the high background observed at the MRM transition for unlabeled linalool in our previous work, which can be resolved with exact mass.[10] The 5-fold and 30-fold improvements in LOD for IBMP and linalool, respectively, are within the range of what other groups have seen for comparison of MS/MS and HRMS.[36] The highest LOD (1.1 mg/L) was observed for hexanol. The relatively poor performance for hexanol is potentially related to the ionization pattern, which did not produce a clearly dominant ion for quantitation, as well as the many interferences present at lower *m/z* values.

Our SPMESH approach compares favorably to other approaches to coupling SPME-type extraction to AI-MS. Gomez-Rios et al. reported coupling thin-film microextraction to DART-MS, in which the sorbent-coated mesh ("SPME-TM") was coated with HLB particles with PAN

as a binder, making it appropriate for extraction of nonvolatiles by direct immersion in biological matrices.[13] This approach led to LODs comparable to those of SPMESH-DART-MS, but it is unclear how the approach would fare for volatile extractions. Alternative approaches for direct coupling of conventional (needle-based) SPME to MS, for instance via nano-ESI,[37] ESI,[19] or DBDI,[18] have also been proposed. These approaches have been successfully employed for quantitation of e.g. trace-level pesticides (LOD 0.3-100 ng/L), but as yet there is no comparative case for detection of volatile compounds. ESI and related approaches are generally poorly suited to the ionization of lower polarity compounds – a category which includes compounds in this study and many other odorants – and thus are not expected to improve upon our current method. The Open Port Probe proposed by Gomez-Rios et al. was used to couple SPME to ESI, but also has the potential for direct coupling of SPME to APCI,[19] which would make for an interesting head-to-head comparison with SPMESH-DART-MS.

Validation of SPMESH-DART-HRMS Against SPME-GC-MS/MS in a Biological Matrix. For method validation, compounds were quantified in wine grapes by the optimized SPMESH-DART-HRMS method. Neither β-damascenone nor hexanol were detected in any grape samples. Our inability to detect hexanol was likely because the typical concentration of this compound in grape macerate is often below our methodological detection limit.[38] Although hexanol was below sensory threshold in all samples, it would still be of interest to improve detection limits, since the compound may be used as a quality marker.[39] Prior studies suggested PMDS/CARB can outperform PDMS/DVB or PDMS alone,[30] although this was not evaluated in our current work. Our observation that β-damascenone was not observed in any samples was somewhat surprising, since our detection limit was <1.9 ng/L, but the compound is often reported to be present in grape juice at μg/L concentrations[40]. A potential explanation is that β-damascenone was not

effectively extracted by SPMESH due to matrix effects. As discussed later in this manuscript, there is little evidence that matrix effects could reduce β-damascenone by several orders of magnitude. Alternatively, the presence of detectable β-damascenone in most literature GC-MS reports is speculated to arise as an analytical artifact (e.g. via degradation of precursors during extraction or in a hot GC injector).[40] DART-MS using polymer coated meshes is reported to minimize thermal artifacts, which may explain our non-detectable concentrations.[13]

Both IBMP and linalool could be measured by SPMESH-DART-MS in some grape samples, and concentrations were validated by comparison against conventional SPME-GC-MS/MS.

IBMP. IBMP ("green pepper" aroma) is proposed to be a marker of red wine grape quality.[32] IBMP possesses a very low odor threshold in water (2 ng/L), and its concentration in certain cultivars (e.g. Cabernet Sauvignon, Sauvignon blanc) is reported to range from 100 ng/kg to less than 10 ng/kg during grape ripening,[41] but can exceed 600 ng/kg in immature fruit.[42] In our work, 12 grape samples at commercial maturity were used, with some containing exogenous IBMP to generate a range of IBMP concentrations (from 25 ng/L up to 1400 ng/L based on SPME-GC-MS/MS) comparable to the range observed in mature and immature fruit (Figure 3). Representative SPMESH-DART-Orbitrap-MS chromatograms for IBMP samples are shown in Figure 3.2. A strong agreement was observed between SPMESH-DART-MS and SPME-GC-MS (Figure 3). The average error was 14% across all samples. A linear regression of results achieved with each method had a slope of near unity (1.1) and excellent linearity (r^2 = 0.97, 1/x weighting, plot not shown). Thus, SPMESH-DART-MS should be suited to ng/L quantitative measurements of IBMP in a fraction of the time of a typical SPME-GC-MS analysis (30 s vs. 30 min).

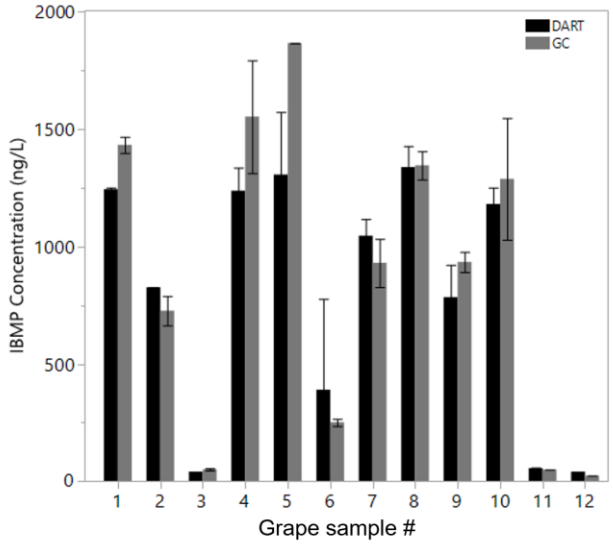

Figure 3.3. Comparison of IBMP concentrations in 12 grape samples as quantified by SPMESH-DART-HRMS vs. SPME-GC-MS/MS.

Linalool. Linalool, along with other closely related monoterpenes, is responsible for the "floral, fruity" aroma of Muscat-type grapes.[43] Linalool may exist at concentrations approaching 500 µg/L in wine, well in excess of sensory threshold, and has been proposed as a marker for the intensity of Muscat aromas.[44] For validation experiments on linalool, half of the grape samples (n=8) were of the Muscat-type cultivars, and the other half (n=8) were from non-Muscat cultivars. The range of linalool concentrations measured in Muscat-type cultivars (69 to 237 µg/L) by SPME-GC-MS/MS (Figure 3.4) was within the range reported in the literature.[45] Linalool concentrations in non-Muscat cultivars were below GC-MS detection limits (~20 µg/L). An excellent correlation was observed between linalool concentrations measured by GC-MS vs. DART-MS (r^2=0.90, 1/x weighting, Figure 3.4). However, the best-fit line had a slope of 0.19, as opposed to unity. Additionally, non-Muscat cultivars had undetectable levels of linalool by GC-MS, but had detectable linalool by DART-MS. This is likely because of isobaric interferences

from other closely related monoterpene alcohols, (e.g. geraniol, nerol) which may be present at equal or higher concentrations. These compounds are structural isomers of linalool, and likely could form the same quantifying ion via protonation and dehydration ($[C_{10}H_{17}]^+$, *m/z* 137.132). Alternatively, grapes can possess higher concentrations of linalyl glycosides (so-called "bound" forms) in addition to free linalool.[43] These bound forms could potentially release free linalool during DART-MS analysis, although recent work with SPME-DART-MS suggests that the approach is not highly susceptible to thermal artifacts.[13] The strong correlation between the SPMESH-DART-MS and SPME-GC-MS approaches is likely because increases in free linalool are concurrent with increases in other free monoterpenes and linalyl glycosides, and suggests that measurement of *m/z* 137.132 by SPMESH-DART-MS should provide a good proxy for linalool concentration of grapes in spite of interferences.

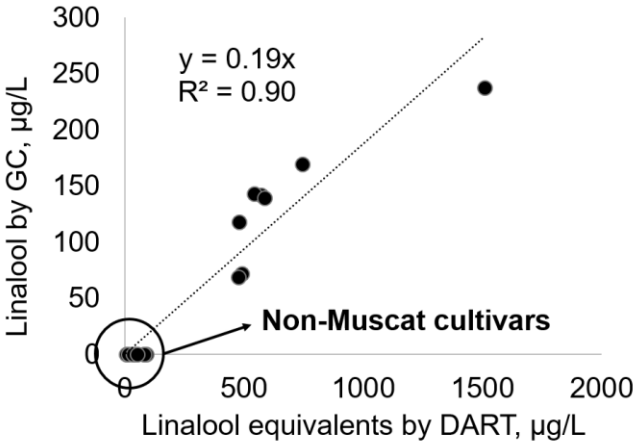

Figure 3.4. Correlation between linalool measured by SPMESH-DART-HRMS and SPME-GC-MS/MS

Matrix effects on signal. The use of internal standards appears to be critical to reliable quantitation in SPMESH-DART-MS, as raw signal could vary up to 2-fold from run to run, even in the same matrix (Figure 3.5). This behavior is likely attributable to variations among coated

meshes, e.g. previous work showed that coating thickness varied by ±25%, which should affect extraction capacity.[10] However, as mentioned earlier, normalizing signal against internal standards resulted in excellent precision, (RSD <6% for aqueous calibration standards). Furthermore, signals among isotopically labeled standards were well correlated among samples (r^2>0.84 for all pairwise comparisons, data not shown) suggesting that using a single internal standard may be acceptable for simple aqueous matrices.

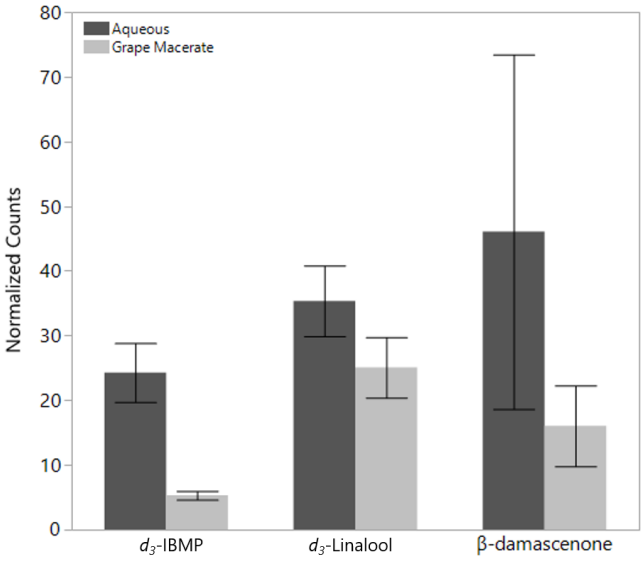

Figure 3.5. Comparison of counts for *d_3-IBMP*, *d_3*-linalool, and β-damascenone (normalized to a maximum signal of 100) in aqueous sample and grape macerate. Bars represent standard errors.

However, compound specific matrix effects were apparent in grape macerates. The raw signal for *d_3*-IBMP decreased by almost 5-fold in grape macerate (Figure 3.5, $p<0.05$). In contrast, a negligible and non-significant decrease in raw *d_3*-linalool signal was observed in grape macerate matrix as observed to aqueous. A non-significant decrease was also observed for β-damascenone, although this comparison was hindered by much greater run-to-run variability. The presence of compound specific matrix effects in SPME is well-established, either as a result of changes in

volatility or competition on the SPME coating.[46] The greater matrix effect on IBMP than on linalool could be explained by pi-pi interactions involving the former compound and polyphenols in the grape matrix.[47] Regardless of the reason, these results indicate that quantitative analyses by SPMESH should use well-matched internal standards (e.g. isotopically labeled), recovery spikes, or other strategies developed for SPME quantitation to avoid matrix effects.[46]

In summary, we have demonstrated that PDMS/DVB coated meshes in combination with Orbitrap HRMS can result in detection limits to <4 ng/L for certain volatiles – approaching the sensory threshold of the most potent naturally occurring odorants, and comparable to the performance of conventional GC-MS methods. The strong correlations observed for two volatiles across multiple grape samples between SPME-GC-MS and SPMESH-DART-MS suggest that the latter could be used as a rapid alternative (~30 s per sample) to conventional GC-MS analyses (~30 min per sample). Future work is necessary to adapt the current "one-at-a-time" SPMESH analyses to a higher throughput approach, e.g. automated positioning stages handling multi-spot meshes as has been described for non-volatile DART-MS analyses.[48]

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CHAPTER 4

Role of Elemental Sulfur in Formation of Wine-soluble H2S Precurosrs and Tetrathionate during Fermentation

ABSTRACT

Hydrogen sulfide (H_2S) can increase during abiotic storage of wines, and potential latent sources of H_2S are still under investigation. We demonstrate that elemental sulfur residues (S^0) on grapes can not only produce H_2S during fermentation, but also form precursors capable of generating additional H_2S during three months bottle storage. H_2S could be released from S^0-derived precursors by addition of a reducing agent (TCEP), but not by addition of strong brine. The TCEP-releasable pool varied among yeast strain. Using the TCEP assay, multiple polar S^0-derived precursors were detected following normal-phase preparative chromatography. Using reversed-phase liquid chromatography high resolution mass spectrometry (HPLC-HRMS) we detected an increase in glutathione trisulfane (GSSSG) and glutathione disulfide (GSSG) in S^0-fermented red wine and an increase in glutathione *S*-sulfonate ($GSSO_3^-$) and tetrathionate ($S_4O_6^{2-}$) in S^0-fermented white wine as compared to controls. GSSSG, but not $S_4O_6^{2-}$, was shown to evolve H_2S in the presence of TCEP. A mechanism for the formation of GSSSG, GSSG, $GSSO_3^-$, and $S_4O_6^{2-}$ from S^0 is proposed.

INTRODUCTION

The phenomenon of sulfurous-like off-aromas (SLOs) accounts for roughly one-fourth of faults detected in commercial wines.[1] Although several compounds have been historically implicated to contribute to SLOs in wine, recent work has emphasized the importance of hydrogen sulfide (H_2S, "rotten egg aroma") as a common marker or cause of SLOs due to its frequent appearance in faulty wines in excess of its sensory threshold (> 1 µg/L).[2] H_2S formation during fermentation is well-studied, with a major source being S-amino acid metabolism. For example, through the sulfur reduction sequence pathway, yeast produce (S^{2-}) as an intermediate during cysteine and methionine formation, which may subsequently diffuse to form H_2S.[3] Enzymatic catabolism of cysteine can also generate H_2S.[4] Another well-established pathway is the reduction of elemental sulfur (S^0) pesticide residues.[5, 6] This reaction can proceed through non-enzymatic reduction in the presence of glutathione, a well-known yeast metabolite,[7] although recent work suggests that the reaction may be enzymatic in real winemaking systems.[7, 8] Because H_2S is highly volatile, the majority of H_2S formed during fermentation is lost to CO_2 entrainment.[9] As a result, H_2S concentrations at the end of fermentation are often < 5 µg/L even though total H_2S production may be greater than 1 mg/L.[10] During cellaring, winemakers can further decrease H_2S by aeration of the wine (resulting in H_2S loss due to sparging or due to formation of adducts with polyphenol quinones) or by addition of Cu(II) salts to make non-volatile complexes.[11-13] Caveats associated with the effects of Cu(II) treatment on H_2S in wine are discussed below.

As compared to formation during fermentation, formation of H_2S during wine storage is not as well understood. In-bottle H_2S formation is most often observed under anaerobic storage conditions, e.g. in wines bottled with low levels of oxygen and in packages with low levels of

oxygen transmission.[11, 14, 15] H_2S produced during bottle storage is particularly problematic, since there is no straightforward means to its remediation once formed, and thus understanding potential pathways or mechanisms responsible for this abiotic formation is of considerable recent interest.[16] Ferreira and colleagues have demonstrated that H_2S can be released by dilution of wine with brine.[17, 18] This brine-releasable fraction appears be composed of soluble transition metal – sulfide complexes, particularly copper sulfide, [17] which can release H_2S under anaerobic environments through an unknown mechanism. Copper-sulfide complexes can be formed by addition of Cu(II) salts to wines containing H_2S, which (in contradiction to many winemaking texts) do not necessarily precipitate.[18, 19] Under accelerated aging conditions (anaerobic storage, 3 wks, 50 °C), an average of 90% of H_2S formed in reds and 58% of H_2S in whites and roses could be credited to the brine-releasable fraction.[18] H_2S formation during accelerated aging also correlated with H_2S formation during longer-term (379 d) storage at ambient temperatures, although the proportion of H_2S formed due to the brine-releasable precursor under ambient conditions was not determined.[20]

An additional class of latent H_2S precursors was suggested by Kreitman et al., who demonstrated that H_2S and other sulfhydryls (e.g. glutathione) can be oxidized in the presence of Cu(II) to yield polysulfides (HS_nH) monoorganopolysulfanes (RS_nH) and diorganopolysulfanes (RS_nR').[21] These S-S containing species can putatively be reduced to reform H_2S and sulfhydryls during wine storage, and the analogous release of ethanethiol from the simple symmetrical disulfide (diethyl disulfide) has been demonstrated in model wine.[22] Treatment of model systems containing putative mono/diorganopolysulfanes (produced by metal catalyzed oxidation of H_2S and thiols) with tris(2-carboxyethyl)phosphine (TCEP) and cysteine to simulate reductive storage conditions resulted in partial recovery of initial H_2S.[16] TCEP addition can also release H_2S from

copper sulfide complexes, although less efficiently than brine addition.[23] TCEP-releasable H_2S is greater than brine-releasable H_2S in some commercial wines, suggesting that mono/diorganopolysulfanes (or related S-S containing compounds) may be of importance as latent H_2S sources.[23]

Other precursors of H_2S during wine storage have been suggested.[16] Cysteine and related aminothiols (e.g. glutathione) have been proposed to serve as latent H_2S precursors, either through metal catalyzed degradation,[11, 14] or through a Strecker-type degradation via reaction with dicarbonyl species.[16, 24] However, conclusive evidence of the former pathway is still lacking, and the oxidizing conditions associated with formation of dicarbonyls in the latter pathway make it unlikely to be relevant to anaerobic wine storage. Bisulfite has also been proposed to serve as a potential H_2S precursor during wine storage, although no evidence has been presented to support this claim.[15]

In this paper, we present evidence that S^0 can form wine soluble degradation products during fermentation that are capable of releasing H_2S during anaerobic storage. We also provide HPLC-MS evidence for likely pathways for S^0 degradation.

MATERIALS AND METHODS

Materials and Chemical Reagents. Gastec 4LT gas detection tubes for H_2S were purchased from Nexteq (Tampa, FL). A "wettable" S^0-based fungicide (Yellow Jacket, 90% elemental sulfur) was purchased from Georgia Gulf Sulfur Corp. (Valdosta, GA). Methanol (MeOH), acetonitrile (MeCN), dichloromethane (DCM), formic acid, tris(2-carboxyethyl)phosphine (TCEP), glutathione disulfide (GSSG), elemental sulfur (S^0), potassium metabisulfite (PMBS), diammonium phosphate (DAP), potassium tetrathionate, and ammonium carbonate, were purchased at ≥99% purity from Sigma-Aldrich (St. Louis, MO). Alka-Seltzer® tablets (Bayer

Healthcare, Morristown, NJ), red organic grape juice (15 °Brix, Cascadian Farms, Skagit Valley, WA), and white organic grape juice (17 °Brix, Wegmans Food Markets, Inc., Rochester, NY) were purchased locally. Distilled de-ionized water was used for all experiments.

Glutathione polysulfanes (GS_nG) were prepared according to a literature method (Moutiez et al., 1994). Glutathione disulfide (GSSG, 500 mg (0.8 mmol) was added to a solution of 261 mg (8 mmol) elemental sulfur (S^0) in $EtOH/CHCl_3/CS_2/NH_4OH$ (45/5/2/2). The reaction was stirred at 30 °C for 2.5 h, and acidified to pH 2 with concentrated HCl. The solvent mixture was removed by rotary evaporation, at 30 °C under vacuum, and the solute was reconstituted in distilled water. The resulting mixture was filtered with Whatman Qualitative filter paper (Maidstone, England) to remove S^0, followed by filtering in a 0.2 µm PTFE membrane filter.

Quantitation of Free H_2S in Wines. Free H_2S was quantified by adapting a protocol developed for quantifying S^0 residues.[25] An aliquot of 30 mL of wine was measured into a plastic squeeze bottle and two Alka-Seltzer® tablets were added to deaerate the sample. The cap, fitted with a 4LT H_2S detection stick, was immediately replaced tightly on the bottle. Once bubbling had ceased, the length of color change on the H_2S detection tube was measured, and H_2S quantified based on the length of the stain against a calibration curve (1.1 µg/L - 92 µg/L), as described elsewhere.[25]

Quantification of H_2S Precursors in Wines. TCEP-releasable H_2S was quantified using a protocol similar to that described elsewhere.[23] Briefly, 30 mL of sample were N_2-sparged as described above, and TCEP was added allowed to react for 5-10 min. A second Alka-Seltzer® tablet was added, capped immediately with a Gastec 4LT gas detection tube, and the resulting color change used to quantify TCEP-releasable H_2S, as describe for free H_2S measurement.

Brine-releasable H_2S was determined by a similar protocol, except that 50 mL NaCl brine

(35% w/v) was added to the wine sample in place of the TCEP reducing agent prior to colorimetric detection with 4LT gas detection tube.

Effects of S^0 Concentration and Yeast Strain and Production of H_2S Precursors during Wine Fermentation and Free H_2S after Storage. For initial evaluation of the ability of S^0-treated fermentations to form H_2S during storage, red grape juice was spiked with 50 mg/L potassium metabisulfite, as well as wettable sulfur fungicide (0, 20, and 100 mg/L) prepared in triplicate, in 1 L Erlenmyer flasks fitted with airlocks through a silicone bung. Samples were inoculated with 0.3g of Lalvin W15 yeast (Scott Laboratories, Petaluma, CA) rehydrated in 5 mL of water at 40 °C and supplemented with 1 g/L DAP. Samples were fermented at ambient temperature in a fume hood until dry, as determined by a hydrometer density measurement. Upon completion, samples were racked once, and sparged until free H_2S was not detectable by 4LT gas detection tubes (typically 20 min). At this point, wines were also assessed for levels of TCEP-releasable and brine-releasable H_2S, as described below. Potassium metabisulfite was again added at a rate of 50 mg/L. Wines were bottled under nitrogen in 187 mL glass bottles, and sealed with Astir oxygen scavenging pry-off crown caps (Attiki, Greece). Dissolved oxygen of the wine was monitored during bottling using a Hach LDO meter (Loveland, CO) and did not exceed 5% of saturation. Wines were stored for 3 months at ambient temperature prior to measuring free H_2S as described above.

To assess the effect of yeast strain effect on formation of TCEP-releasable H_2S from S^0, the same protocol was followed except that the fermentation volume was 100 mL and only two S^0 -treatment levels were used (0 and 100 mg/L). Five yeast strains were compared (Alchemy I, EC1118, Viti Levure 58W3, BRL97, and CY3079) (Scott Laboratories, Petaluma, CA), with

each yeast + S^0 combination performed in triplicate. TCEP-releasable H_2S was assessed as described below in analytical replicate for each treatment, for a total of 60 analyses. Data was analyzed using Multiple Analysis of Variance (mANOVA) with Tukey HSD (JMP Pro 13; SAS Institute, Cary, NC).

Fractionation of TCEP-Releasing Precursors in Wine by Flash Chromatography

S^0-treated red wines was prepared as described above using Lalvin W15 yeast and 100 mg/L S^0. Flash chromatography was performed on wines using a Combiflash RF75 system (Teledyne Isco, Lincoln, NE) approximately one month after fermentation. Two methods were evaluated.

1) Reversed-phase: The column was a 5.5 g C18 "Gold" column (20-40 µm particle size, Teledyne Isco, Lincoln, NE). Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in MeOH, and the gradient was as follows: 5% B, held for 2.5 min linear gradient to 95% B for 10 min, 95% B held for 10 min. The flow rate was 18 mL/min and the equilibration volume was 28.7 mL. Thirty mL of wine (control or 100 mg/L S^0 treatment) were concentrated to ~2 mL on a rotary evaporator under vacuum at 70 °C and the entire volume injected onto the equilibrated column. Absorbance was monitored at 214 nm and 280 nm. Fractions (n=18) were collected and assayed for free and TCEP-releasable H_2S.
2) Normal phase: Fifty mL of wine (control or 100 mg/L S^0 treatment) was mixed with acid-washed Celite, dried on a rotary evaporator, and packed into an empty cartridge for chromatography. The column was a 24 g silica "Gold" (20-40 µm, 60 Å) (Teledyne Isco, Lincoln, NE). Solvent A was DCM and solvent B was MeOH. The flow rate was 35 mL/min and the equilibration volume was 252 mL. The gradient was as follows: 2.5% B, held for 2 min, linear gradient to 80% B for 10.5 min, and held at 80% B for 1.5 min.

Absorbance was monitored at 214 nm and 280 nm. Fractions (n=30) were collected and assayed for free and TCEP-releasable H_2S.

Characterization of Potential TCEP-Releasing Precursors by Liquid Chromatography – Mass Spectrometry (LC-MS). Red wine and white wine samples were prepared from red and white juices, respectively, using the same winemaking protocol as described earlier. The instrument used was an Orbitrap Elite mass spectrometer coupled to a Thermo UltiMate2000 Rapid Separation HPLC system, operated in both negative and positive ESI modes, using methods adapted from Arapitsas et al. (negative ion mode), and Kreitman et al. (positive ion mode).[21, 26] Injection volume was 10 µL of either undiluted wine or standard solution. Orbitrap Elite conditions were optimized using the GS_nG standard. Samples run were control wines (0 mg/L wettable sulfur fungicide) and treatment wines (100 mg/L wettable sulfur), with 3 fermentation replicates and 2 analytical replicates per treatment, for a total of 12 samples. Analysis was performed on the GS_nG standard mixture and on a 5 mg/L (16 µM) potassium tetrathionate solution. Full scan MS data was collected at resolving power 120000 in profile mode, with five MS/MS data-dependent scans. Data analysis was performed using XCalibur 2.2 software. Time from fermentation start to positive mode analysis was 2 weeks, and time from fermentation start to negative mode analysis was 3 weeks.

1) Positive ESI mode analysis: The column was a Jupiter C18 5 µm 300Å column (150 mm x 2 mm i.d., Phenomenex, Inc., Torrance, CA, USA). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in 95% MeCN. The flow rate was 200 µL/min. The gradient was as follows: starting solvent 3% B held for 2 min, increased to 45% B over 8 min, increased to 75% B over 2 min, held for 5.5 min, and returned to 3% B, followed by 6 min equilibration. The instrument was operated in ESI

positive ion mode, scanning from m/z 350-750, with the following optimized operating conditions: voltage 4.0 kV, sheath gas 58, auxiliary gas 10, sweep gas 0, source temperature 350 °C, and S-lens RF level 45%. Data dependent MS^2 data were collected for precursor ions m/z 613.1598, 645.1319, and 677.1039 (GSS_nG, n=1, 2, 3, respectively).

2) Negative ESI mode analysis: The column was an Acquity 1.7 µm C18 130Å column (150mm x 2.1 mm i.d., Waters Corporation, Milford, MA). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in MeOH. The flow rate was 170 µL/min with a column temperature of 45 °C. The gradient was as follows: 0% B held for 2 min, increased to 2% B for 6 min, increased to 40% B for 10 min, increased to 100% for 2 min, held for 3 min, and decreased back to 0% B for a 6 min equilibration. The instrument was operated in ESI negative ion mode, scanning from m/z 200-1000, with the following optimized operating conditions: voltage -4.5 kV, sheath gas 64, auxiliary gas 2, sweep gas 0, source temperature 350 °C, and S-lens RF level 50%. Data dependent MS^2 data were collected for precursor ions 386.0328, 418.0049, and 449.9769 ($GS_nSO_3^-$, n=1, 2, 3, respectively); and for precursor ions 306.0759, 338.04806, and 370.0201 (GS_n^-, S=1, 2, 3, respectively).

Due to the noisiness of the HRMS data, chromatograms were plotted with 15-point Gaussian peak smoothing.

Evaluating Potential of Glutathione Polysulfanes or Tetrathionate as TCEP-Releasable Precursor. The previously synthesized glutathione polysulfane standard (30 mL) or potassium tetrathionate (30 mL, 3 µM in 12% EtOH model wine) were evaluated separately for their ability to release H_2S in the presence of TCEP. The previously described TCEP assay was used, with

the exception that Gastec 4LL tubes were used in place of 4LT tubes for the tetrathionate analyses due to the unavailability of the latter.

RESULTS AND DISCUSSION

S^0 concentration and yeast strain on production of free H_2S and TCEP-releasable precursors. In initial experiments, we evaluated the effects of added S^0 (0, 20, and 100 mg/L) on H_2S during fermentation, using a single yeast strain (Lavlin W15). The concentrations of 20 and 100 mg/L were chosen because they bracket the upper range of residues observed on grapes sprayed with S^0-containing fungicides within 8 days of harvest, and thus represent a worst-case scenario.[6] Prior to bottling, wines were sparged with inert gas to decrease free H_2S to below detection limits (<0.1 mg/L). After three months anaerobic storage, we observed a significant increase ($p < 0.05$) in free H_2S in both the 20 and 100 mg/L treatments, and no detectable free H_2S could be observed in the control (Table 4.1). The amount of H_2S formed during storage of the 100 mg/L treatment (10 µg/L) represented ~0.01% of the original S^0 addition, with a similar relationship observed for the 20 mg/L treatment (2.4 µg/L H_2S formed during storage). The presence of S^0 residues in grape must are well known to result in H_2S formation during fermentation.[5, 6, 27] However, to our knowledge, this is the first report to show that S^0 residues can result in precursors capable of continued formation of H_2S formation during storage.

Table 4.1. H_2S released under reductive storage conditions and TCEP-releasable "latent" H_2S for wines fermented with added S^0. Values represent biological replicates (n = 3) using Lalvin W15 yeast. Errors represent standard deviation.

	H_2S (µg/L)			
S^0 added pre-fermentation (mg/L)[a]	Free, at bottling	TCEP releasable at bottling	Brine releasable at bottling	Free, after 3 months storage
0	nd	nd	nd	nd
20	nd	14.5(±3.5)	nd	2.4(±0.2)
100	nd	42.0(±7.1)	nd	10.0(±0.9)

[a] Added as wettable sulfur (90% S^0)

Recent studies have proposed that likely precursors of H_2S during anaerobic storage are transition metal - sulfide complexes, particularly copper sulfides[17-19] or di/monoorganopolysulfanes.[21] Copper sulfide complexes in wines are thought to largely arise through intentional addition of cupric salts during winemaking to remediate wines with high levels of H_2S or other malodorous sulfhydryls.[17] No copper additions were performed in our current work, but because trace amounts of Cu can be found in some grape musts (~0.5-1 mg/L), copper sulfides or related metal complexes could potentially be formed as a consequence of high H_2S production. To evaluate this possibility, we used a brine-dilution assay to evaluate for latent H_2S precursors, since concentrated NaCl brine is reported to release H_2S from metal-sulfide complexes.[17, 18, 23] However, we observed no brine-releasable H_2S in either the control or treated wines (Table 4.1), suggesting that the precursors of H_2S in the S^0-fermented wines are not copper sulfide complexes.

Polysulfides and organopolysulfanes are also suggested to be a latent of source of H_2S in bottled wines. To evaluate if the S^0-derived precursors belonged to these classes of compounds, wine samples were treated with TCEP, a reagent well known for its ability to reduce S-S bonds.[28] TCEP addition (alone or in combination with other reagents) has been proposed as means to release sulfhydryls from polysulfides and organopolysulfanes,[21] and TCEP appears to release H_2S from different precursors than brine addition.[23] We observed significant increases in TCEP-releasable H_2S from both the 100 and 20 mg/L S^0 treatments (42 and 10 µg/L), as compared to undetectable TCEP-releasable H_2S in the untreated control (Table 4.1). This pool of TCEP-releasable H_2S was about four-fold greater than the amount of H_2S released during the three month storage, and thus represents a plausible reservoir.

To determine if production of TCEP-releasable H_2S varied among yeasts, fermentations were performed again with a different juice using one of five yeast strains and one of two S^0 levels (0 or 100 mg/L). Results are shown in Figure 4.1. Yeast strain had a significant effect on TCEP-releasable H_2S (mANOVA, $p<0.0001$), with the highest concentration observed in wines fermented with CY3079 (89 µg/L). Assuming that the TCEP-releasable pool could be entirely converted to H_2S during storage, then the maximum tolerable S^0 residue concentration in grape must should be set at 1.1 mg/L to keep the amount of H_2S released less than its reported sensory threshold (~1 µg/L).[2] Interestingly, this maximum tolerable limit is near-identical to the limit based on the minimum amount of S^0 necessary to increase H_2S formation during fermentation.[6]

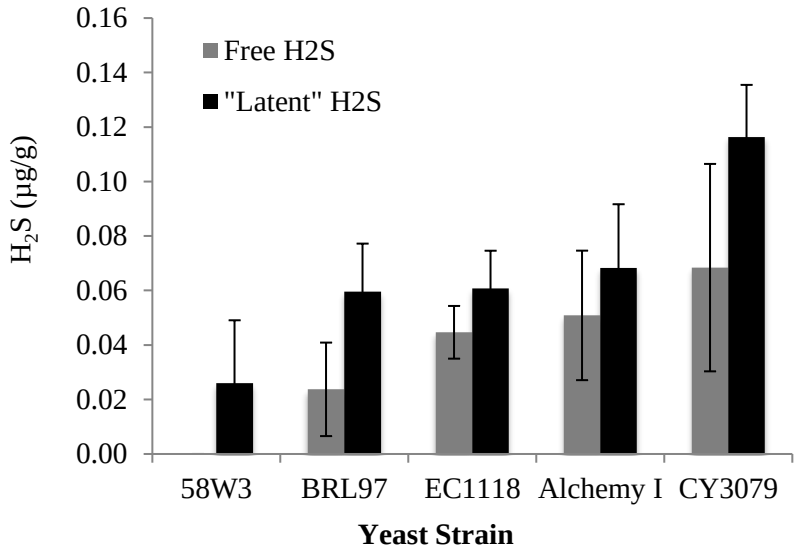


Figure 4.1. Free and TCEP-releaseable H_2S across 5 different yeast strains, corrected for H_2S observed in controls of the same yeast strain. Error bars represent standard error of the mean.

Fractionation of TCEP-releasable H_2S precursors by preparative chromatography

To identify the TCEP-releasable precursor generated by fermentation in the presence of S^0, a treated must was fermented and the resulting wine fractionated by preparative (flash) chromatography. The total amount of TCEP-releasable H_2S just prior to chromatography (6.7 µg/L) was lower than in the previous section, presumably because of degradation of some of the precursors during the one month gap between the end of fermentation and preparative chromatography. Fractions were assayed by TCEP release followed by H_2S measurement. We initially evaluated fractionation on a reversed-phase C18 stationary phase, but we were unable to retain any TCEP-releasable H_2S on this phase, with similar results observed for C18-based solid phase extraction (data not shown). We then evaluated normal phase silica flash chromatography, which necessitated drying of wine samples under vacuum prior to column loading. Under these conditions, we observed an increase in total TCEP-releasable H_2S in both control and treated

samples (from 6.7 to 20.1 µg/L), suggesting that some artefactual formation of TCEP-releasable forms occurred during drying (Table 4.2). However, the difference in TCEP-releasable H_2S between control and S^0 treated wines was the same before and after drying (6.7 µg/L). The increase in TCEP-releasable H_2S precursors specific to the S^0 treatment only with high levels of polar solvent (40-100% MeOH in DCM).

Table 4.2. TCEP-releasable H_2S following fractionation by preparative chromatography using a normal phase silica column

	H_2S (µg/L)		
%MeOH	Treatment	Control	Difference
0-30	0	0	0
30-40	4.0	4.0	0
40-50	4.7	2.7	2.0
50-60	4.7	2.0	2.7
60-85	5.4	4.7	0.7
85-100	1.3	0	1.3
100	0	0	0
Total of all fractions	20.1	13.4	6.7
Total pre-fractionation	6.7	0	6.7

Characterization of Potential TCEP-Releasing Precursors by LC-MS.

Our flash chromatography work suggested i) that multiple forms of TCEP-releasable H_2S precursors likely exist in S^0-treated wines and ii) these precursors are polar, and thus unlikely to be highly non-polar S^0. Recent work has shown that H_2S can combine with other sulfhydryls (e.g. GSH, Cys) to form symmetric and asymmetric organopolysulfanes, and that these forms can likely be reduced by TCEP to release the original sulfhydryls.[21] Because the major sulfhydryl species in wine is GSH (mean value of 12.5 mg/L or ~40 µM, in Sauvignon blanc wines)[29] we hypothesized that some of the unknown H_2S precursor(s) took the form of GSH diorganopolysulfanes or monoorganopolysulfanes (GSS_nSG, GSS_nSH, $n \geq 1$).

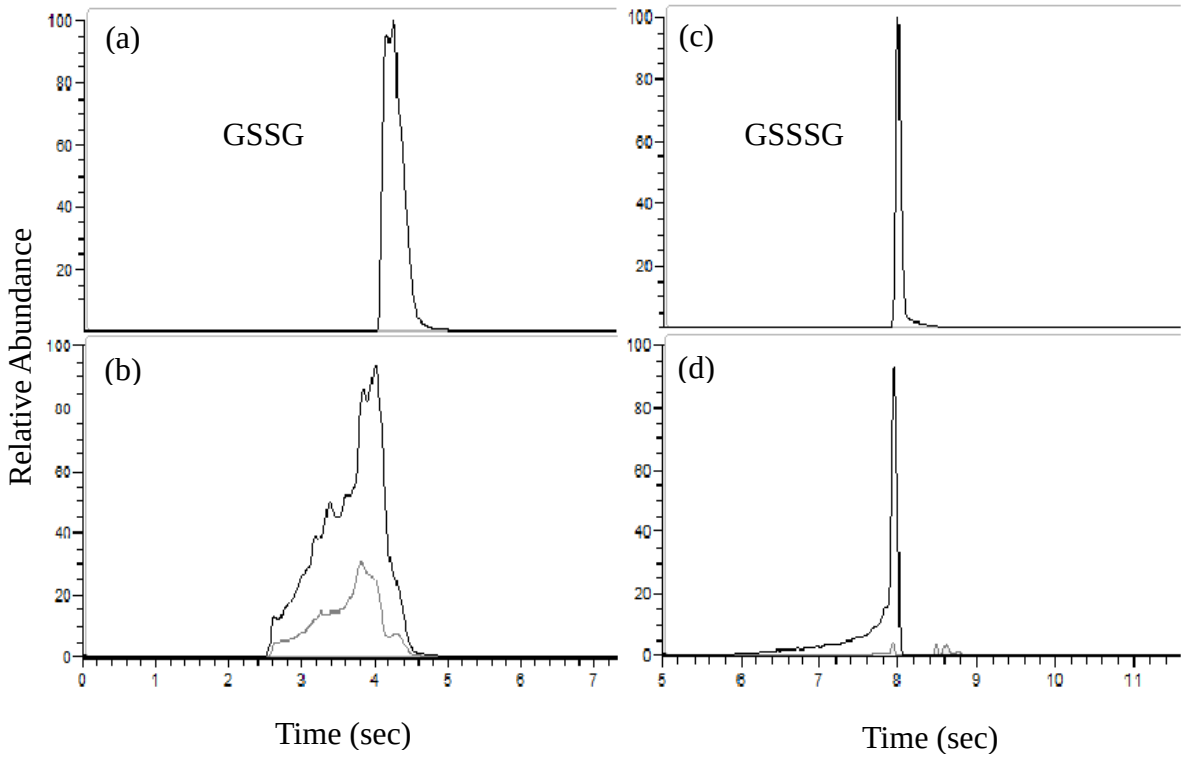


Figure 4.2. *Left*: Extracted Ion Chromatograms (EIC) for glutathione disulfide, m/z 613.1598 (5 ppm mass accuracy), corresponding to the exact mass of $[GSSG+H]^+$ in 3 samples: (a) standard solution in water, max counts 2.35×10^7 (b) red control wine fermented on 0 mg/L wettable

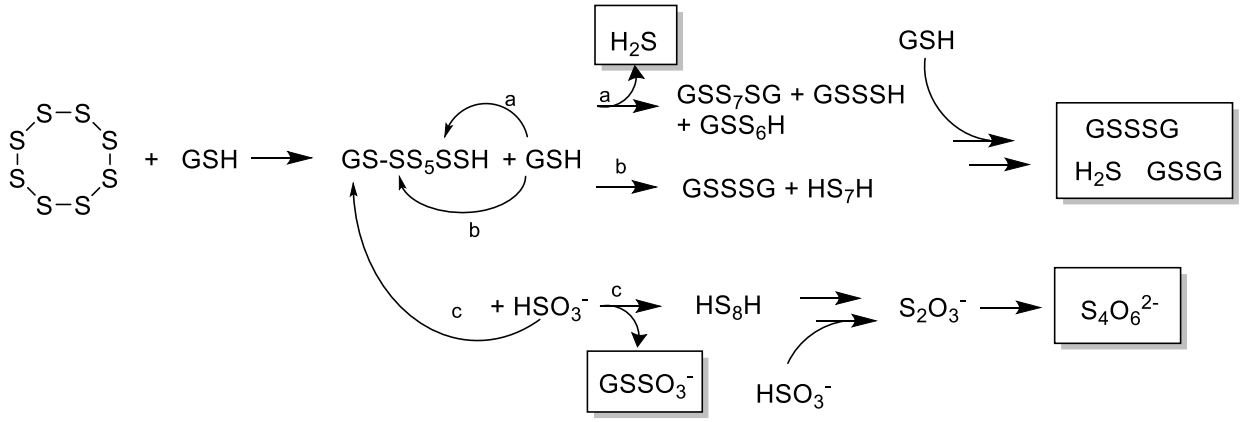
sulfur, max counts 3.77×10^5, overlaid with red treatment wine fermented on 100 mg/L wettable sulfur, max counts 1.09×10^6

Right: EIC for glutathione trisulfane, m/z 645.1319 (5 ppm mass accuracy), corresponding to the exact mass of $[GSSSG+H]^+$ in 3 samples (c)glutathione polysulfane standard mixture solution in water, max counts 1.08×10^7 (d) red control wine fermented on 0 mg/L wettable sulfur, max counts 2.72×10^3, overlaid with red treatment wine fermented on 100 mg/L wettable sulfur, max counts 5.56×10^4

To investigate if GSS_nSG could be formed in a wine fermented in the presence of S^0, we adapted a reversed phase HPLC-MS method from the literature. Normal-phase HPLC was not used, since it required sample drying and generated artefacts (see previous section). Using positive ion mode ESI-HRMS, we detected glutathione trisulfane (GSSSG) in the S^0 treatment wine, and the peak identity was confirmed by comparison to a synthesized standard (*m/z* = 645.13187, Figure 4.2). No GSSSG was detected in the control wines (Figure 4.2). GSSSG has been previously identified in a model wine system containing H_2S, GSH, and Cu(II).[21] Because the other GSS_nG compounds were present along with GSSSG in the standard, quantification of GSSSG in wine was not attempted. Our current work represents the first report of formation of GSSSG in a real wine, although previous authors have reported formation of a mixed methylglutathionyl trisulfane following oxidation of wine containing H_2S, GSH, methanethiol, and Cu(II). No higher-order GSS_nSG with $n\geq2$ were detectable in either treatment or the control (data not shown). However, GSSG was 3-fold higher in the S^0 treatment as compared to the control (Figure 4.2).

The pathway by which GSSSG and GSSG could be formed from S^0 is unclear. One

possibility is that GSH produced by yeast reacts with S^0 through a nucleophilic ring-opening reaction to generate an intermediate monoorganopolysulfane (GSS_6SH, Scheme 4.1). This intermediate could then undergo nucleophilic substitution with additional GSH equivalents in a manner analogous to well-known thiol-disulfide reactions to eventually yield GSSSG and GSSG.[30] This pathway would also release H_2S (whose appearance during fermentation in the presence of S^0 is well-established),[5] mono-organopolysulfanes (GSS_nSH) and/or inorganic polysulfanes (HSS_nSH) intermediates. These last two classes (RSS_nSH and HSS_nSH) have been detected in model wine systems containing Fe(III), Cu(II) and high H_2S concentrations along with thiols. However, these putative intermediates were not detected by HPLC-Orbitrap-MS in our current study, although HSS_nSH with $n \leq 4$ would have been outside the mass range of the MS. Potentially, these species rapidly undergo further substitution reactions with GSH or other sulfhydryls (e.g. Cys) in real wines. These results suggest that GSSSG may be more stable than other organopolysulfanes, although the reason for this is unclear.



Scheme 4.1. Proposed mechanism for formation of GSSSG, glutathione *S*-sulfonate, tetrathionate, and H_2S in wines fermented in presence of S^0. All pathways commence through ring opening of S^0 by GSH to yield a monoorganopolysulfane (GSS_nSH). Pathways a and b

proceed under low sulfite conditions, through GSH-monoorganopolysulfane exchange to yield smaller monoorganopolysulfanes, GSSSG, H_2S, and/or inorganic polysulfanes (HSS_nSH). In the presence of sufficient sulfite, pathway c would result in the formation of glutathione *S*-sulfonate and inorganic polysulfanes, which would subsequently undergo sulfitolysis to yield thiosulfate and its oxidation product tetrathionate.

An alternative "bottom-up" pathway to form glutathione polysulfanes could involve reaction of H_2S (formed in high concentrations in the S^0-treated wine) with Cu(II) and GSH, which could preferentially form GSSG and GSSSG over longer-chain GSS_nSG in model wines.[16] However, this reaction required oxidizing conditions and added Cu(II), neither of which were present in our system.

As a caveat, the pathways proposed in Scheme 4.1 are non-enzymatic. Previous work has shown that GSH can partially release H_2S from S^0.[7, 25] However, other authors have reported that at GSH concentrations more comparable to those in juice or wine (30 µM), no H_2S release from S^0 is detected.[8] Based on these results, the authors propose that release of H_2S from S^0 is most likely enzymatic, although an alternate explanation is that reductive fermentation conditions help regenerate GSH. Assuming that the reaction is indeed enzymatic, the pathways shown in Scheme 4.1 may still be valid, although other unexpected intermediates could also be involved.

To assess the validity of GSSSG as a precursor to H_2S, the gas detection tube protocol for TCEP-releasable H_2S was performed on an aqueous solution of synthesized di-organopolysulfanes. We have tentatively ascertained that H_2S can be released from these di-organopolysulfanes, with the caveat that residual S^0 substrate could have contributed to a false

positive signal. However, given the steps taken to filter the reaction mixture and the low solubility of S^0 in water, a false positive seems unlikely.

We then repeated the experiment using a white grape juice treated with S^0 prior to fermentation, which was subsequently evaluated by positive ion mode HPLC-MS. Surprisingly, GSSSG and GSSG were undetectable in both treated and control white wine samples. Previous reports indicate that GSSG can undergo sulfitolysis to form the glutathione *S*-sulfonate adducts,[26, 31] and we hypothesized that both GSSG and GSSSG could have been lost through this pathway. Although the red and white wines were made using identical procedures, including SO_2 addition rate, red wines typically have a much lower concentration of "true" free SO_2 due to anthocyanin binding.[32]

To test for the presence of *S*-sulfonated adducts in the white wines, samples were re-run in negative-ion mode by HPLC-MS. Glutathione *S*-sulfonate (*m/z*=386.0328) was tentatively identified in both treatment and control samples with confirmation by MS2 spectra, and had on average 5-fold greater response in treatment samples compared to the control (Figure 4.3). Glutathione *S*-sulfonate was previously reported in wines produced under low oxygen conditions using a similar high resolution HPLC-MS method.[26] We detected no longer-chain *S*-sulfonates ($GS_nSO_3^-$, $n \geq 2$) in either sample (data not shown).

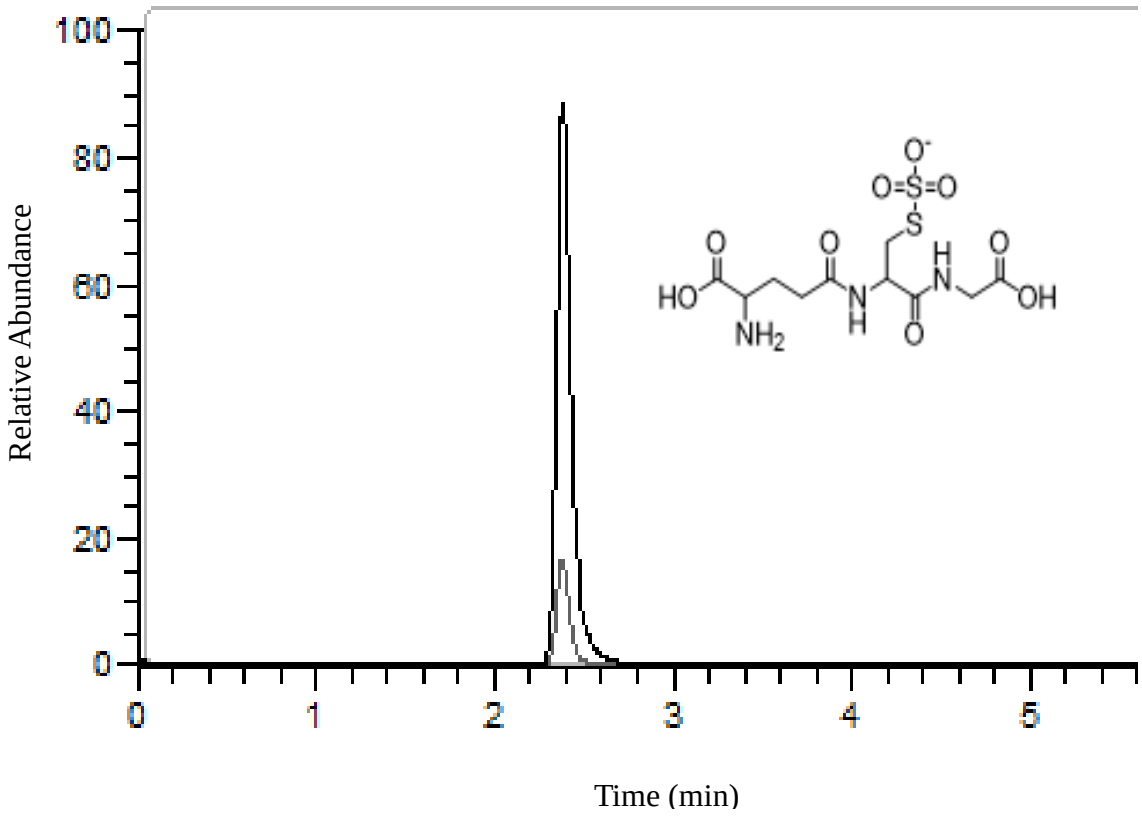


Figure 4.3. Overlaid Extracted Ion Chromatogram of $[GS\text{-}SO_3]^-$ (m/z 386.0328) (10 ppm mass accuracy) in control (gray, max counts 1.04×10^6) and S^0-treated (black, max counts 5.63×10^6) white wines, confirmed by MS2 data

The increase in $GSSO_3^-$ in treatment wines could be explained by nucleophilic ring opening of S^0 by GSH to form the monoorganopolysulfane derivative ($GS\text{-}S_n\text{-}SH$),[33] which could subsequently undergo sulfitolysis to yield $GSSO_3^-$ and polysulfanes ($HS\text{-}S_n\text{-}SH$) (Scheme 4.1, pathway c). The polysulfanes could undergo successive sulfitolysis to yield H_2S, explaining the increase observed during storage for these wines. The alternate sulfitolysis pathway in the scenario would yield $GSS_nSO_3^-$ and H_2S. The former was not detected in any of the samples using HPLC-Orbitrap-MS, potentially because these compounds could undergo additional sulfitolysis to form

polythionates ($[SO_3\text{-}S_n\text{-}SO_3]^{2-}$, n = 1 to 6) by nucleophilic addition of HSO_3^- to an *S*-sulfonate. The negative ion HPLC-Orbitrap-MS data were retrospectively analyzed for the presence of these polythionates. The protonated form of tetrathionate ($[HS_4O_6]^-$, *m/z*=224.8656) was detected in the S^0 treatments, but was undetectable in control wine (Figure 4.4). The identity was confirmed by analysis of an authentic tetrathionate standard (Figure 4.4). To our knowledge, this is the first report of this compound (or any polythionate) in wines. We observed no other higher polythionates ($[SO_3\text{-}S_n\text{-}SO_3]^{2-}$, n = 3-6) by HPLC-MS. The reason for the appearance of only $S_4O_6^{2-}$ and no other polythionates is unclear. One possibility is that sulfitolysis intermediates are not immediately converted to polythionates. Instead, they may be degraded by sulfitolysis to form thiosulfate ($S_2O_3^-$), a well-known reaction product of S^0 and sulfite, which could subsequently oxidize to form $S_4O_6^{2-}$.[34] Potentially, $S_2O_3^-$ (and hence $S_4O_6^{2-}$) could be formed from sulfitolysis of organic or inorganic polysulfanes, too (Scheme 1, pathway c).

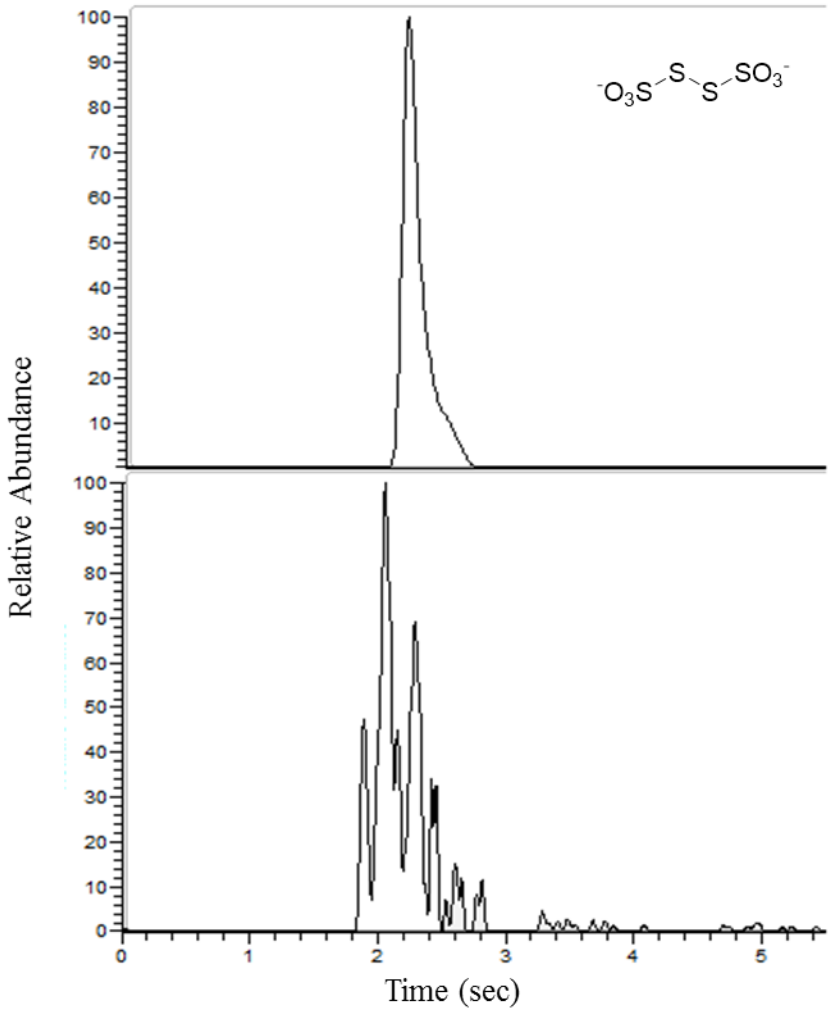


Figure 4.4. EIC for hydrogen tetrathionate, m/z 224.8656 (10 ppm mass accuracy), corresponding to the exact mass of $[S_4O_6H]^-$ in 3 samples. *Top*: 5 mg/L potassium tetrathionate standard solution in water, max counts 1.06×10^7. *Bottom*: Treatment wine fermented on 100 mg/L wettable sulfur (max counts 2.68×10^3). The control wine had no detectable signal (max counts = 0).

Finally, we investigated if tetrathionate could serve as a TCEP-releasable precursor, but no H_2S release could be detected (data not shown). Based on the detection limit of the method (10 µg/L, or 0.3 µM) and the concentration of tetrathionate (6 µM H_2S equivalents assuming only the

interior sulfur atoms can be released), accelerated reducing conditions are capable of converting no more than 5% of tetrathionate to H_2S. Thus, tetrathionate is unlikely to act as additional latent H_2S precursor, and along with GS-SO_3^-, tetrathionate may serve as a stable end-product of S^0 degradation in wines,.

In summary, wines fermented in the presence of S^0 can continue to form H_2S even after fermentation is completed. These S^0 treated wines have increased concentrations of H_2S precursors which can be released by addition of TCEP reducing agent. Based on the TCEP-release test, the maximum tolerable limit for S^0 on grapes is estimated to be 1.1 mg/L – above this value, latent H_2S may exceed the sensory threshold for free H_2S. Thus, S^0 on grapes presents a hazard to wine quality not only by generating excess H_2S during fermentation (as is previously established), but also by producing latent H_2S sources. In one treated red wine, multiple polar precursors appeared to be formed, one of which was identified as glutathione trisulfane (GSSSG). However, no evidence of GSSSG was found in a treated white wine, but glutathione *S*-sulfonate (GS-SO_3^-) and tetrathionate ($S_4O_6^{2-}$) were increased, suggesting that a portion of S^0 and/or its degradation products may undergo sulfitolysis if free SO_2 is sufficiently high. Future work is necessary to determine the kinetics and pathways associated with the degradation of S^0 derived precursors, and to quantify their importance to appearance of H_2S during storage of commercial wines.

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CHAPTER 5

Practical Considerations towards Implementation of SPMESH-DART-MS: matrix effects and Spatial Resolution

Abstract

SPMESH-DART-MS can achieve sub-sensory threshold limits of detection for select odor-active compounds, demonstrating strong quantitative correlations with GC-MS data. SPMESH-DART-MS is advantageous because it requires < 30 sec/sample, whereas GC-MS may require >30 min/sample. However, there still exist some barriers to the immediate implementation of the new method. For example, prior work indicates that the sub-sensory threshold limits of detection (LODs) achieved by SPMESH-DART-MS suffer from matrix effects in real matrices, resulting in up to 5-fold reduction in sensitivity. These matrix effects can have additional implications for quantification when the method is dependent upon use of internal standards. Methods and preliminary work towards improved sensitivity and reproducibility in real samples are discussed, including the use of improved coating materials and the potential for temperature ramping. Furthermore, the success of this method of high-throughput performance is dependent upon an automated format, with parallel SPMESH extractions. Challenges and preliminary work towards automation are presented.

Introduction

SPMESH-DART-MS can obtain GC-MS-like data in less than 30 seconds per sample for odor-active volatile compounds (chapter 3). However, interferences and ion suppression from matrix components could reduce sensitivity for some compounds in real samples. One limitation to the

current SPMESH method is the variation in coating thickness, which may vary up to 25% (chapter 2).[1] This variation, in combination with other variables (e.g. ionization/desorption in the DART-MS), may result in raw signal variation up to 2-fold (chapter 3). Hence, sensitivity is often lost due to lower quality SPMESH units. An improved, more reproducible unit would not only help compensate for sensitivity losses in real matrices, but could reduce the currently high dependence upon internal standards by stabilizing raw signal, as demonstrated in related work for nonvolatiles.[2] In this chapter, we present preliminary work towards improved SPMESH materials for improved sensitivity and repeatability. An additional consideration for the updated SPMESH device is that it should be amenable to a high-throughput, automated format (i.e. headspace extraction over a welled plate). A final parameter that might be explored to improve selectivity/sensitivity, temperature ramping, is also discussed.

Improved SPMESH materials for reproducibility and efficiency of desorption. One limitation of the current SPMESH-DART-MS approach has been the reproducibility of the SPMESH coating, as discussed in chapter 3. Coating thickness, as assessed by scanning electron microscopy, varied by up to 25% (chapter 2). While the use of isotopically labelled internal standards permitted reliable quantification (RSD<6% in all cases), raw signal could vary by up to 2-fold (chapter 3). This could result in reduced sensitivity for some SPMESH devices. Furthermore, the current SPMESH device requires manually puncturing a hole through the coating to enable transmission of Helium gas during DART desorption, a step that introduces more variability. Similar methods were employed by Gomez-Rios et al. for their C18 SPME-TM unit,[3] but a greater sensitivity could be achieved using a device with individually-coated strands of mesh,[2] presumably due to the greater available surface area available and improved transfer

efficiency into the MS. In preliminary work, we have designed an improved SPMESH device with a truer "mesh"-like configuration, in which squares were etched out of a PMDS-sheet to form a grid ("etched-SPMESH" or eSPMESH). This is expected to correct poor reproducibility due to coating thickness/quality, reducing the dependence upon internal standards. Furthermore, the new configuration is hypothesized to improve sensitivity by making more efficient use of available surface area for extraction/desorption.

Spatial Resolution for parallel SPMESH extractions in a welled-plate. The practical success of this method for industry implementation is dependent upon an automatable, high-throughput format that will employ parallel extraction and automated desorption. Figure 5.1. depicts the proposed automated format, which makes use of an existing DART module (X-Y positioner) for the analysis of 96 samples in less than 60 minutes, comparable to existing methods for analysis of non-volatiles.[4, 5] In the first step, samples are loaded into a welled-plate. A SPMESH sheet is placed over the plate and covered to seal the samples from the atmosphere. Samples are agitated and heated for extraction, followed by DART desorption/ionization and MS detection using the X-Y positioner.

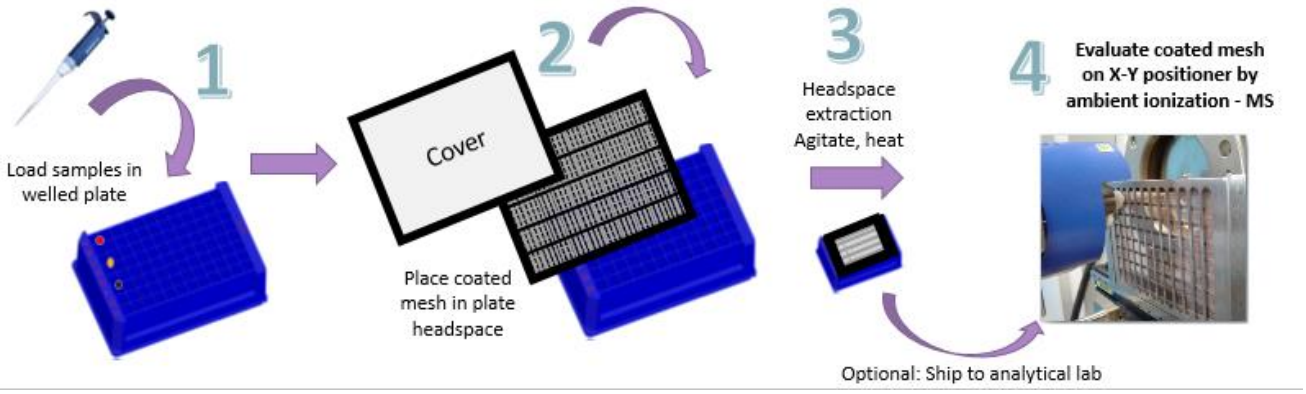

Figure 5.1. Proposed high-throughput format for SPMESH-DART-MS

An important consideration for the parallel extraction step is that individual wells must be isolated sufficiently to prevent cross-talk between sample headspaces. Preliminary work demonstrates that headspace isolation is possible through use of stainless steel washers to create a seal in a 24-well plate (Figure 5.2). A well was filled with an aqueous solution of 250 µg/L linalool and 500 ng/L IBMP, and an adjacent well contained a blank (water). SPMESH extraction was performed in an incubator for 1 hour, and the individual SPMESH units were analyzed by DART-MS/MS. Figure 5.2 shows that there was no detectable cross-talk between the blank and spiked wells. Future work would require spatial resolution across the entire well plate. The fully spatially-resolved format could theoretically be implemented for volatile mapping over a surface for the study of flavor distribution of foodstuffs as well as volatile components in biological systems.

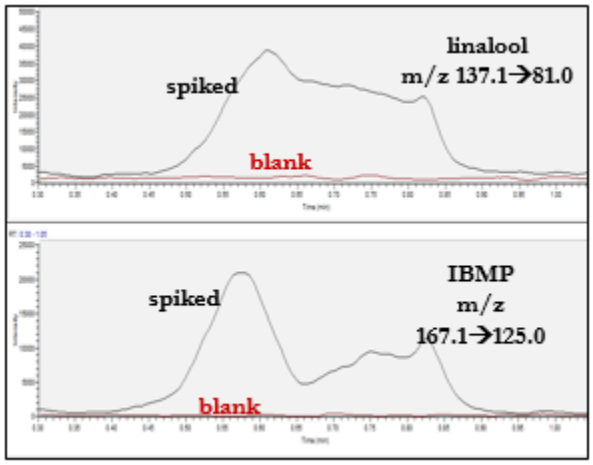

Figure 5.2. Chromatogram demonstrating spatial resolution between adjacent wells in a 24-well plate

Crude chromatography through temperature ramping in DART-MS.

Prior work has demonstrated that the use of fast temperature ramping during DART

desorption should facilitate separation of isobaric compounds based on differences in volatility,[6, 7] which is expected to improve both selectivity and sensitivity. Figure 5.3 illustrates that even without temperatures ramping, the slight differences in boiling point for linalool and IBMP (198 and 214 °C, respectively), cause an offset in retention time (0.43 vs. 0.50). This disparity could be increased with use of temperature ramping as a form of crude chromatography that would add an additional parameter of separation. In future work, helium gas flow for desorption of the analyte could be varied across a range of temperatures (i.e. 50-200 °C), in order to design an optimized temperature ramp. The implementation of these experiments would be straightforward following the development of a well-plate format, since existing IonSense software can facilitate automatic temperature optimization. Small differences in boiling point are expected to correspond to smaller thermal separations, but deconvolution software (e.g. AMDIS by NIST) can be employed to separate isobaric peaks that are not baseline resolved. Temperature ramping is expected to take slightly longer than fixed temperature analyses, but should preserve high throughput capabilities. As a caveat, the peak capacity achieved by ramping will be much less than by GC, and will not circumvent the need for HRMS or MS/MS.

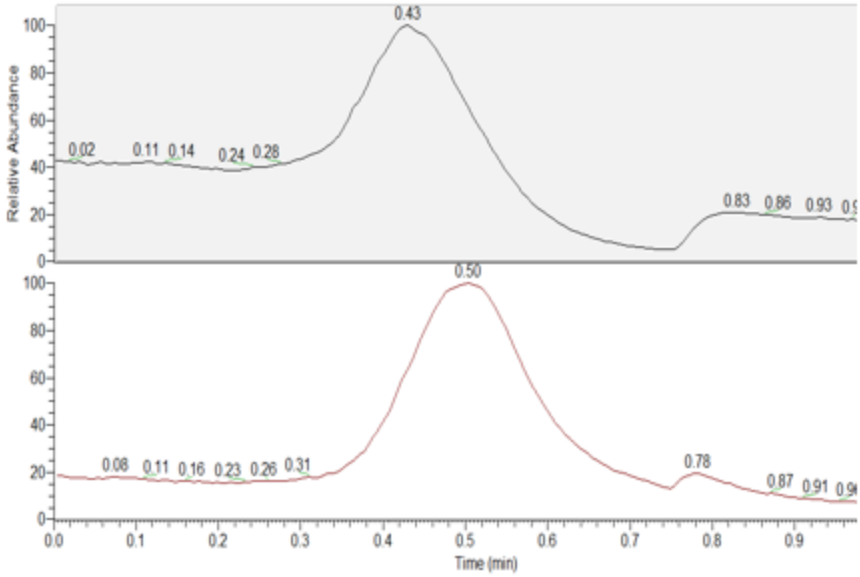

Figure 5.3. Sample chromatogram demonstrating retention time disparity between linalool and IBMP without ramping

Conclusions

Current obstacles to the immediate implementation of SPMESH-DART-MS for volatile analysis include sensitivity loss due to matrix suppression, as well as a lack of automatable format. Two methods are proposed to compensate for sensitivity losses. First, an improved SPMESH material could ensure consistent and efficient extraction/desorption, by increasing available surface area. A recently fabricated eSPMESH device, in which a grid is laser-cut from a thin PDMS sheet, provides this increase in surface area which will theoretically improve sensitivity. An added advantage of eSPMESH is that its fabrication enables an easily reproducible format, which should help reduce the requirement for internal standards. Temperature ramping may add an additional dimension for selectivity and, consequently, sensitivity. Finally, the eSPMESH can be constructed in sheets designed to fit over a standard

welled plate. Preliminary experiments show that headspace isolation in individual wells can be achieved, enabling parallel extractions with spatial resolution. The eSPMESH sheet can then be analyzed using the DART X-Z module in an automated run.

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