SIMULTANEOUS MEASUREMENT OF KEY ODORANTS AT THEIR SENSORY THRESHOLDS IN JUICE GRAPES

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science in Chemistry

by

Terry Lee Bates

May 2019

ABSTRACT

Methyl anthranilate (MA), *ortho*-aminoacetophenone (o-AAP), and furaneol contribute to the characteristic “native grape” and “cotton candy” aromas of *Vitis labruscana* grapes and their hybrids, e.g. ‘Concord’ and ‘Niagara’. Convenient measurement of these compounds is of interest to grape breeders who wish to either increase or decrease their concentrations in grape varieties. Previous approaches for measurement of these odorants either cannot measure all compounds in a single analysis, or else have detection limits above the sensory thresholds for the odorants (low µg/L). We evaluated the use of an optimized reverse phase solid phase extraction (SPE) approach prior to selected ion monitoring (SIM) GC-MS to achieve low µg/L detection limits for MA, o-AAP, and furaneol in a single analysis.

BIOGRAPHICAL SKETCH

Terry earned his B.S. in Molecular Biology with minors in Chemistry and Political Science from the University of Denver in Denver, CO. After teaching at the community college and high school levels, he pursued his M.S. in Chemistry at Cornell University in Ithaca, NY with research focused on characterization of volatile aromatic compounds in grapes. While at Cornell, Terry served as a teaching assistant for undergraduate chemistry lab courses and as a liaison for the Academic Excellence Workshops. Additionally, he joined the Leadership Alliance and attended their annual conference in 2018. Terry participates in outreach programs with the Cornell Center for Materials Research, working with middle and high school students to foster an appreciation and deep understanding of science. Terry also holds a leadership position in the club crew team at Cornell which seeks to make rowing more diverse and accessible to non-traditional students.

This work is dedicated to my father and grandmother who steadfastly provide me with support and love through all things. I cannot express in words how much you have inspired me.

ACKNOWLEDGMENTS

To my advisor, Dr. David B. Zax, thank you for your unwavering support and encouragement during my time at Cornell. Your witty responses to my frantic emails and unannounced office visits will be fondly remembered. I'm thankful that I will get to run into you in the gym for a couple more years, it seems like we meet there most often.

To my graduate school and Cornell mom, Pat Hine, thank you for always answering my questions about the program, deadlines, job applications, and life. Our chats gave me the push I needed to persevere during my first semester at Cornell, you are an inspiration. I am forever indebted to you.

To my friend, mentor, and former supervisor, Dr. Cynthia Kinsland, thank you for making my teaching experience great. I also thank you for being a true friend and mentor. Your kindness and sharp wit make our conversations amazing; sadly for you I can hardly pass your office door without coming in.

To my research advisor, Dr. Gavin Sacks, I cannot tell you how thankful I am that I randomly signed up for your wine chemistry course, your knowledge of chemistry, engineering, wine, and really everything else encourages me to dig deeper and always think critically. Thank you for providing me with a project that was fun and interesting, you've inspired me to stay and do more. Thank you for making me feel right at home in the Sacks lab from day one.

TABLE OF CONTENTS

Chapter **Page**

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Methyl Anthranilate……MA

Ortho-Aminoacetophenone…….o-AAP

Limit of Detection……LOD

Limit of Quantitation……LOQ

Liquid-Liquid Extraction…….LLE

Gas Chromatography-Mass Spectrometry…….GCMS

Solid Phase Extraction……...SPE

Solid Phase Micro Extraction……..……..…SPME

Ultrasound Assisted Headspace Solid Phase Micro Extraction……..UAHSSPME

Voltage Root Mean Square….……V-RMS

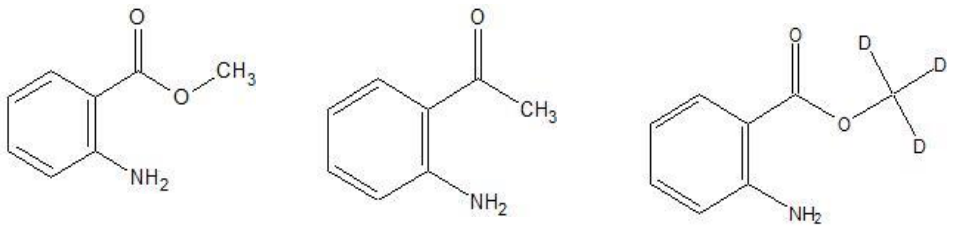
Partition Coefficient…….. K_{ow}

Total Ion Count……..……TIC

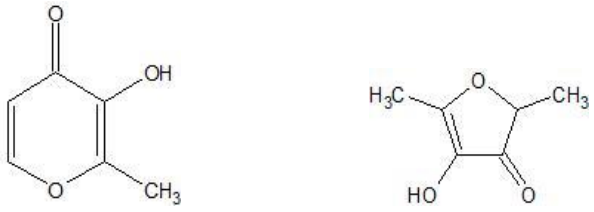
Single Ion Monitoring……..…….SIM

INTRODUCTION

Grape varieties exhibit distinct aroma profiles characterized by a small number of odorous volatile compounds with each having significant influences on the sensory perception of the nectar (Wang and Luca, 2005). Methyl anthranilate (MA), *ortho*-aminoacetophenone (o-AAP), and furaneol contribute to the characteristic "native grape" and "cotton candy" aromas of *Vitis labruscana* grapes and their hybrids, e.g. 'Concord' and 'Niagara' (Figure 1). Methyl anthranilate and 2'-aminoacetophenone, both derivatives of anthranilic acid, are important contributors to the organoleptic properties of the most commonly cultivated American grape variety, *Vitis labruscana* (Acree, et al, 1990). These compounds are the source of the characteristic grapy and foxy odors that are typical of *V. labrusca* nectars.



Methyl anthranilate ***o*-aminoacetophenone** **D3-Methyl anthranilate**



Maltol **Furaneol**

Figure 1. Structures of MA, o-AAP, deuterated MA, Maltol, and Furaneol.

The odor detection threshold of a compound is a value representing the lowest concentration perceptible by 50% of the human population. The odor detection thresholds for MA and 2-AAP are 300 µg/L and around 1 µg/L, respectively, and are highly dependent on the sample matrix (Perry and Hayes, 2016). Furaneol, a derivative of furan, also known as strawberry furanone, is described as having a sweet strawberry or pineapple odor at low concentrations and malodorous at high concentrations with an odor detection threshold of 21 µg/L (Buttery, et al, 1995).

In recent years, interspecies crosses between *Vitis vinifera* and other North American native varieties such as *V. labruscana* have produced cold hardy, disease resistant hybrid varieties providing a unique opportunity for locally grown and sustainable wine production in the humid climates of the Mid-Western and North Eastern United States and Canada (Slegers, et al, 2015). The aroma profiles of the initial interspecies hybrid cultivars are traditionally characterized by foxy compounds MA, o-AAP, and furaneol, compounds usually considered hallmarks of low quality wine. Modifications to interspecies crosses seek to reduce and/or eliminate these foxy odors and, in order to characterize the success of these new crosses, the levels of these compounds must be reliably and rapidly quantified (Slegers, et al, 2015).

The low concentration of these foxy compounds in complex matrices like grape juice and wine make rapid and successful quantification a challenging task. The nectar of common interspecies hybrid cultivars such as Clinton grapes (*V. labrusca* X *Vitis riparia*), contain concentrations of MA and o-AAP of 18.9 and 12.9 µg/L, respectively (Panighel, et al, 2010). Previous analytical methods using direct injection GC-MS acheive limit of detection (LOD) and quantification (LOQ) values as low as 23 µg/L and 93 µg/L, respectively

(Dutra, et al, 2018). Isolation and concentration of the compounds was achieved using liquid-liquid extraction (LLE) with ethyl acetate as the organic phase and a subsequent concentration step to further concentrate the sample prior to GC-MS analysis (Dutra, et al, 2018). Sample preparation protocols using LLE are labor intensive, require significant volumes of solvent and an added concentration step, and fail to effectively remove interfering compounds with high selectivity as compared to other extraction methods. In addition, desiccants such as anhydrous ammonium sulfate must often be used to remove residual water from the extract. Solid Phase Microextraction (SPME) can also be used to extract volatile compounds from samples prior to GC-MS analysis. Previous work found that this method is ineffective at extracting furaneol (Sun, et al, 2011). SPME is highly effective at extracting MA and o-AAP with detection limits reported at 9 µg/L and 7 µg/L, respectively. Extraction and quantification of MA, o-AAP, and furaneol in a single run makes high throughput analysis more efficient, meaning that this method would not be acceptable for characterization of the root of all foxy odors in a single run. Ultrasound assisted headspace solid phase microextraction (UAHSSPME) coupled to GC-MS demonstrates promising results with o-AAP yielding LODs of 0.1 µg/L. Unfortunately, this extraction method

rapidly destabilizes the SPME fiber, acting as a major cost barrier for high throughput analyses (Mihaljević, et al, 2015).

Solid phase extraction (SPE) is a dynamic technique used to isolate and concentrate analytes from gas and liquid samples. Analytes are retained on an appropriate solid phase and removed using an appropriate eluent. This works to isolate analytes, concentrate samples for analysis, remove interfering compounds, and reduce sample storage volume (Poole, 2002). Normal Phase SPE is used to extract polar compounds with an appropriate polar solid phase. Ion exchange SPE is used to isolate analytes based on the charge of the species. Reverse-phase solid phase extraction using highly crosslinked non-polar ethylvinylbenzene-divinylbenzene copolymer cartridges is effective at extracting aromatic compounds containing hydrophobic regions from complex aqueous matrices based on hydrophobic interactions. This method is highly effective and can be performed rapidly with higher recoveries and better reproducibility than LLE (Nawaz, et al, 2014) and is capable of extracting MA, o-AAP, and furaneol in a single run. The versatility of analytical methods using SPE coupled to Gas chromatography-mass spectrometry (GC-MS) has applications that are potentially useful in food and beverage analysis, bio

analysis of blood and urine, pesticide analysis, various pharmaceutical applications, and environmental analysis (Chauhan, 2014).

GC-MS is a versatile analytical technique used in the qualitative and quantitative analysis of small, thermostable, and structurally diverse volatile compounds. This technique couples the separation and quantification power of gas chromatography with the detection capabilities of mass spectrometry and can be used to detect compounds at concentrations as low as the fg/L level (Fialkov, 2006).

In GC-MS, compounds in a complex sample matrix are separated on an appropriate solid phase column and quantified via gas chromatography. Separated compounds are subsequently fragmented into ions via electron, chemical, electrospray, field, laser, or matrix assisted laser desorption ionization methods. Ionized compounds are quantified based on relative abundance and compared with library databases to confidently identify compounds on the basis of mass (Sparkman, et al, 2011). GC-MS analysis can be tailored to rapidly and effectively analyze samples containing a broad range of compounds at low concentrations.

In this work we demonstrate that selective analyte extraction using reverse phase SPE coupled with GC-MS can considerably improve LOD

and LOQ values with acceptable recovery values. We also perform validation of this method by measuring concentrations of MA, o-AAP, and furaneol in wine and table grape nectar samples.

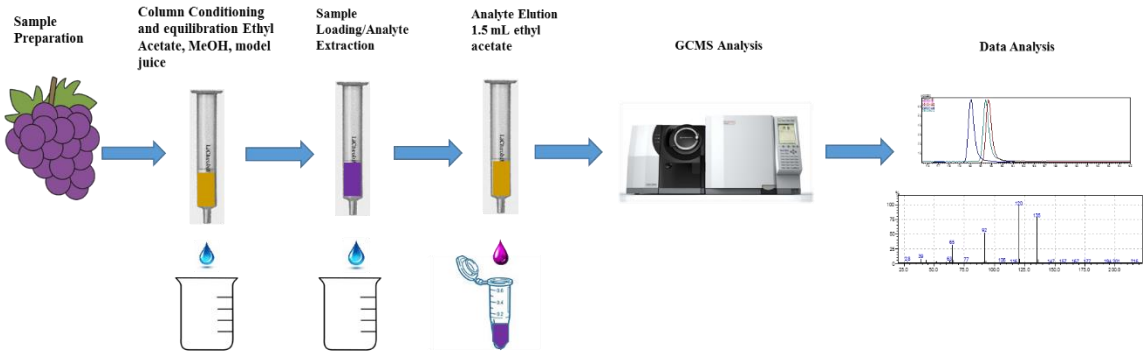


Figure 2. Schematic diagram of protocol workflow from crude sample to data analysis.

MATERIALS AND METHODS

Standards

Standards of MA, 2-AAP, furaneol, maltol, and deuterated MA were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethyl acetate and methanol were purchased from Fluka analytical (Mexico City, MX). Lichrolut EN 3 mL cartridges were obtained from Merck KGaA (Darmstadt, Germany). Dextrose and Citric Acid Monohydrate were

acquired through VWR chemical (Irving, TX, USA). D-Fructose was purchased from Fischer Chemical (Pittsburgh, PA, USA). Milli-Q water was acquired via a purification system at Cornell University (Ithaca, NY, USA).

Extraction of Aromatic Compounds

Figure 2 represents a general workflow for the protocol with an emphasis on the extraction portion of the method. The extraction of MA, 2'-AAP, and furaneol was performed via solid phase extraction in a protocol adapted from reference 16. 100 µL of 100 µL/L D_3-MA and maltol in ethyl acetate were added to 10 mL of all samples prior to analysis.

The Lichrolut Columns were conditioned with 4 mL of ethyl acetate, 4 mL of methanol, and equilibrated with 4 mL of model juice (pH 3.14, 16 brix) under nitrogen gas generated by a Parker Balston Nitrogen Generator (Lancaster, NY, USA) at 0.25 Bar (linear flow rate 1 mL/min) in a Cerex SPE Varian positive pressure SPE processor (Baldwin Park, CA, USA). After conditioning, samples were added in five 2 mL increments and linear flow rate was carefully maintained at 1 mL/min.

After sample loading, pressure was increased to 1.7 bar and the column was dried for 20 minutes. Analytes were eluted using 1.5 mL of ethyl acetate under 0.1 Bar for 1 minute and then under gravity. 250 µL of eluent was transferred to 1.5 mL vials fitted with 250 uL glass spring inserts and remaining sample was stored at -15°C.

Instrumentation and Chromatographic Conditions

Analyses were acquired using a Shimadzu model GCMS TQ8040. Data analysis was performed using GCMSolution software (Kyoto, Japan). Splitless mode was used with a flow rate of 15.6 mL/min at 250°C. The mobile phase was helium gas (purity 5.0). The column used was a VF-Wax (Varian, Lake Forrest, CA, USA), (30m x 32mm x 0.5 µm) with a flow rate of 1.33 mL/min. The oven temperature was 40 °C (5 min), heating up at 20 °C/min to 100°C then 5 °C/min to 110°C and then 20°C/min to 210°C and held for 4 minutes, with a total run time of 24 minutes. The detector temperature was 200°C and the samples were performed using a hybrid of Selected Ion Monitoring/Total Ion Monitoring mode. The specific ions for MA were m/z 151 (molecular ion/ quantifier) and 119 (ion product/qualifier), 2-AAP m/z 135 (quantifier) and 119 (qualifier),

furaneol m/z 128 (quantifier) and 57 (qualifier), maltol m/z 126 (quantifier), and d_3 MA m/z 154 (quantifier) and 119 (qualifier). A representative chromatogram for the separation patterns is shown in figures 3 and 4 below.

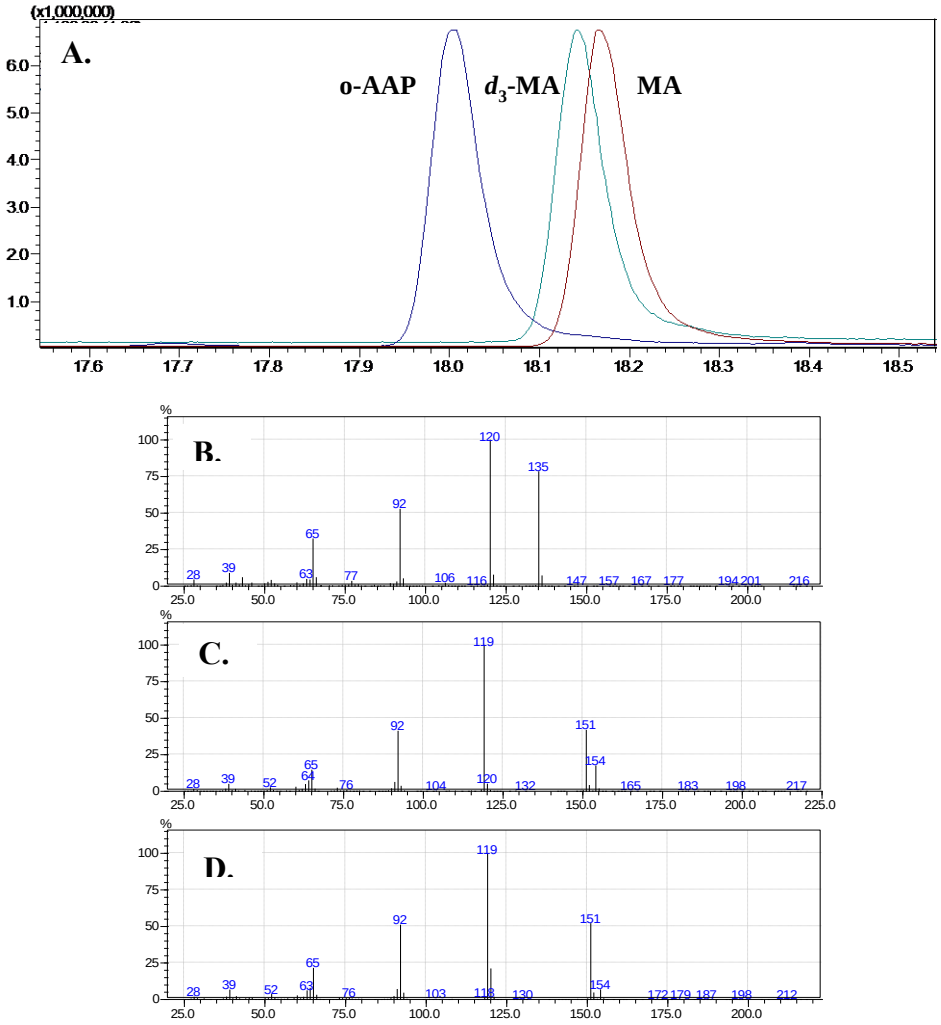


Figure 3. Representative 1D chromatogram (A) and mass spectrum for o-AAP (*m/z* 135), d_3-3 MA (m/z 154) and MA (*m/z* 151) in model juice sample (B, C, and D, respectively). The chromatogram shows peaks collected in SIM mode based on quantifying ions for MA, d_3-MA, and o-AAP, respectively. The spectra (B,C, and D) show the corresponding compound fragments collected at each peak on the 1D chromatogram.

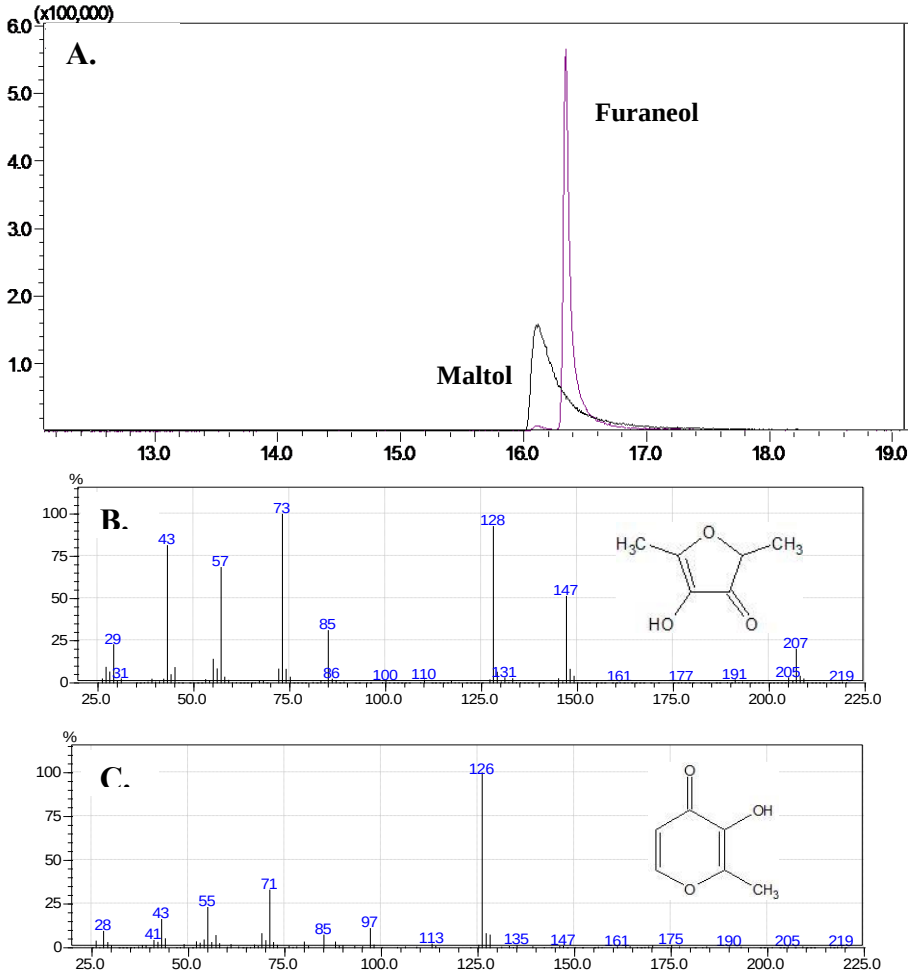


Figure 4. Representative 1D chromatogram (A) and mass spectrum for furaneol (*m/z* 128) and maltol (*m/z* 126) in model juice sample (B and C). The chromatogram shows peaks based on SIM data collected for quantifying ions of maltol and furaneol, respectively. The spectra (B and C) show the corresponding compound fragments collected at each peak on the 1D chromatogram.

Linearity

A linear calibration curve was obtained via SPE extractions of MA, 2-AAP, and furaneol in a series of model juice samples at five concentrations, data not shown. The compounds were diluted in model juice, vortexed for 30 seconds, and extracted using the previously outlined SPE method. The model juice was a solution of 8 g/L fructose, 8 g/L dextrose at pH 3.14 in milli Q ultra-pure water. 2.5 µL injections were analyzed using the analysis method above. Concentration was plotted against MA:D3, 2-AAP:D3, and Furaneol:Maltol and was verified using a least squares regression method, with R^2>0.995. All analyses were performed in triplicate.

Recovery

Recovery was calculated by comparing samples fortified with the specific compound to identical untreated controls. Differences between concentration values for the fortified samples were compared to concentration values for the initial untreated samples to establish recovery values. Recovery analyses were performed in triplicate.

Limit of Detection and Limit of Quantitation Values

Limit of detection values and quantitation values were estimated using a V RMS signal to noise ratio calculation of 3:1 for LOD and 10:1 for LOQ.

RESULTS AND DISCUSSION

In preliminary work, the use of ethyl acetate as an eluent demonstrated the best recovery values in comparison to methanol and acetonitrile. These results were expected as the elutropic strength was predicted to exceed that of methanol and acetonitrile based on the respective partition coefficient values (K_{ow}) (Patel and Jefferies, 1987). The K_{ow} value of ethyl acetate (0.7) verifies that this compound is the least polar eluent of the previously mentioned series. Dichloromethane (K_{ow} 1.25) may prove to be a more efficient eluent, however, the volatility and health risks associated with the use of this compound make it a less desirable option.

Linearity was achieved by diluting five concentrations of MA, 2-AAP, and Furaneol (0.21, 0.630, 1.89, 5.67, 16.7 µg/L) in model juice with internal standards at 1 µg/L (Table 1). We chose d_3-MA as an internal standard for MA and o-AAP based on structural similarity. Slightly earlier retention times were noted for D_3 MA as compared to MA due to weaker interactions with the column. We chose maltol as an internal standard for furaneol based on structural similarity. Isotopically labelled furaneol is a preferable internal standard, however, the price of the labelled compound was prohibitive. The internal standards were added at 1 mg/L to all samples and were effective at validating individual samples by illuminating signal suppression and enhancement. Each standard was run in triplicate and returned acceptable RSD values (2.15-2.03%), as evidenced in table 1.

Table 1. Calibration and recovery data for extractions from model juice.

	MA	o-AAP	Furaneol
Calibration Range	0.21-16.7 mg/L	0.21-16.7 mg/L	0.17-13.5 mg/L
Coefficient of Determination r^2	0.99	0.99	0.99
Mean % RSD	2.54	3.02	2.15
LOD	4.8 µg/L	7.3 µg/L	4.8 µg/L
LOQ	15.9 µg/L	24.3 µg/L	16.6 µg/L
Recovery %	85.6	81.4	--

In order to improve recovery, linear flow rate was optimized and maintained at 2 mL/min during the conditioning and equilibration steps and at 1 mL/min during sample loading. One minute of low pressure was applied during the elution step to increase linear flow rate and prevent evaporation as elution under gravity alone required over 40 minutes of elution time.

The SPE method worked to efficiently concentrate and extract MA, o-AAP, and furaneol both from model juice and various grape nectars. This method removed impurities and other interfering compounds that tend to remain in the extract using methods such as LLE. The sensitivity of the analysis was further increased by using a hybridized TIC/SIM analysis mode and confirming the presence of each compound using the aforementioned quantifying and qualifying ions.

The recovery results for each compound ranged from 81.4 to 84.6% which are similar to recovery values in previously published work using LLE, SPME, and SPE extraction methods (Zulj 2015, lyer 2012, Bertrand 1995). The LOD and LOQ values obtained in this work evidence the

efficacy of this method and are the lowest values for an analysis covering all three compounds in a single run. The LOD values for MA, 2-AAP, and Furaneol were established to be 4.8 ug/L, 7.3 ug/L, and 4.8 ug/L, respectively, as evidenced in table 1. The LOQ values for MA, 2-AAP, and Furaneol were established to be 15.9 ug/L, 24.3 ug/L, and 16.6 ug/L, respectively (Table 1). These values were lower than previously reported direct injection values and are well below the detection thresholds for MA and furaneol. The detection threshold of o-AAP is as low as 1 ug/L, therefore, increases in detectability are necessary to quantify this compound in trace quantities.

The method was validated and successful at characterizing each compound in samples of *V. labrusca* and several interspecies hybrid nectars. These samples were characterized, however, only MA was found at appreciable concentrations, as expected in this variety. Figure 5 is a representative chromatogram for an actual juice sample, o-AAP and furaneol were omitted as concentrations were below LOQ values.

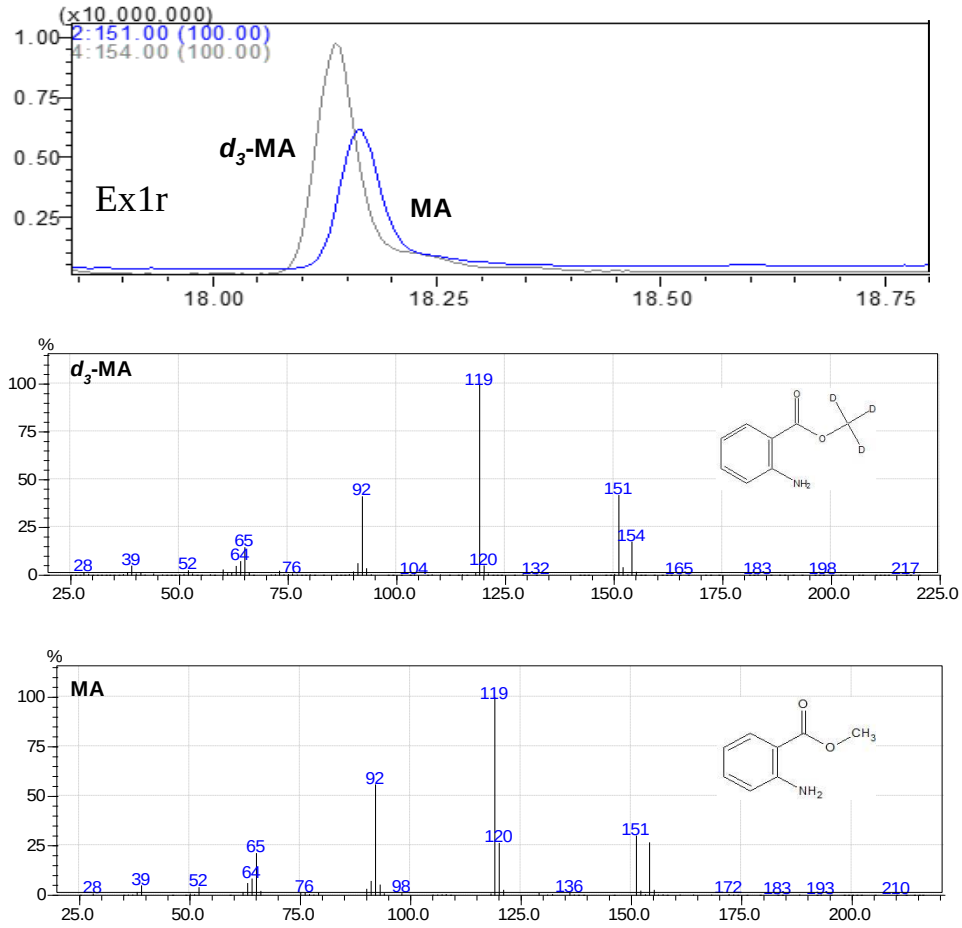


Figure 5. 1D chromatogram and mass spectrum for d_3-MA (*m/z* 154) and MA (m/z 151) in a grape juice sample.

CONCLUSIONS

The SPE extraction method and GCMS analysis method presented in our work successfully extracts and quantitates MA, 2-AAP, and furaneol at trace levels. This protocol provides an analytical method that is versatile and effective at characterizing the compounds in various matrices.

Future research should focus on attempts to lower LOD and LOQ values for MA and o-AAP by employing separate ion exchange SPE protocols to isolate o-AAP/ MA and furaneol. This could lower LOD and LOQ values by removing interfering compounds and improving signal to noise ratios, however, it would also decrease throughput and increase cost. Perhaps a more simple remedy would be to adjust the sample pH to ensure that all compounds are neutral prior to reverse phase SPE and analyze eluent using GCMS-MS. GCMS-MS could reduce increase sensitivity and confidence of identification by providing daughter spectra that are highly specific to each compound, only monitoring fragments that

are specific to the compound of interest. This technique could lower the LOD and LOQ values considerably.

In this work we optimized a reverse phase SPE extraction method and a GCMS analytical program developing a cost effective, high-throughput analytical technique to quantitate the foxy compounds, MA, o-AAP, and furaneol with high precision and low LOD and LOQ values. It is evidenced in this work that MA, 2-AAP, and Furaneol can be effectively extracted using reverse phase SPE and analyzed using GCMS to characterize the aroma profile of a real juice samples.

REFERENCES

1. Wang, J. and Luca, V. D. (2005), The biosynthesis and regulation of biosynthesis of Concord grape fruit esters, including 'foxy' methylanthranilate. The Plant Journal, 44: 606-619. doi:10.1111/j.1365-313X.2005.02552.x

2. Acree, T. E., E. H. Lavin, R. Nishida, and S. Watanabe. 1990. The serendipitous discovery of ortho-amino acetophenone as the 'foxy' smelling component of Labruscana grapes. Chem. and Eng. News 9:80.

3. Perry, D.M.; Hayes, J.E. Effects of Matrix Composition on Detection Threshold Estimates for Methyl Anthranilate and 2-Aminoacetophenone. *Foods* **2016**, *5*, 35.

4. Buttery, R., Takeoka, G.R., and L.C. Ling. *Journal of Agricultural and Food Chemistry* **1995** *43* (6), 1638-1640.

5. Slegers, A.; Angers, P.; Ouellet, É.; Truchon, T.; Pedneault, K. Volatile Compounds from Grape Skin, Juice and Wine from Five Interspecific Hybrid Grape Cultivars Grown in Québec (Canada) for Wine Production. *Molecules* **2015**, *20*, 10980-11016.)

6. Panighel, A. , Vedova, A. D., De Rosso, M. , Gardiman, M. and Flamini, R. (2010), A solid-phase microextraction gas chromatography/ion trap tandem mass spectrometry method for simultaneous determination of 'foxy smelling

compounds' and 3-alkyl-2-methoxypyrazines in grape juice. Rapid Commun. Mass Spectrom., 24: 2023-2029. doi:10.1002/rcm.4611.

7. Prudêncio Dutra, Maria da Conceição; de Souza, Joyce Fagundes; Viana, Arão Cardoso; de Oliveira, Débora; Pereira, Giuliano Elias; Dos Santos Lima, Marcos M. Rapid determination of the aromatic compounds methyl-anthranilate, 2'-aminoacetophenone and furaneol by GC-MS: Method validation and characterization of grape derivatives. *Food research international* **2018**, 107,613-618.

8. Qun Sun, Matthew J. Gates, Edward H. Lavin, Terry E. Acree, and Gavin L. Sacks. *Journal of Agricultural and Food Chemistry* **2011** *59* (19), 10657-10664.

9. Mihaljević Žulj, M., Maslov, L., Tomaz, I. et al. *J Anal Chem* (**2015**) 70: 814. https://doi.org/10.1134/S1061934815070102.

10. Poole, Colin. Principles and practice of solid phase extraction. *Comprehensive Analytical Chemistry **(2002)*** 37, 341-387.

11. Md. Saddam Nawaz, Farhana Khanam Ferdousi, Mohammad Arifur Rahman, and A. M. Shafiqul Alam, "Reversed Phase SPE and GC-MS Study of Polycyclic Aromatic Hydrocarbons in Water Samples from the River Buriganga, Bangladesh," *International Scholarly Research Notices*, vol. **2014**, https://doi.org/10.1155/2014/234092).

12. Chauhan A, Goyal MK, Chauhan P. GC-MS Technique and its Analytical Applications in Science and Technology. *J Anal Bioanal Tech.* **2014.** 5:222. doi: 10.4172/2155-9872.1000222.

13. Fialkov, Alexander B. et al. "Sensitivity and noise in GC – MS : Achieving low limits of detection for difficult analytes. International Journal of Mass Spectrometry. **2006**. doi10.1016/j.ijms.2006.07.002)

14. Sparkman, David; Penton, Zelda; and Fulton Kitson. Gas Chromatography and Mass Spectrometry: A Practical Guide 2nd Edition. Academic Press. **2011**.

15. Sun, Qun et al. Comparison of Odor-Active Compounds in Grapes and Wines from *Vitis vinifera* and Non-foxy American Grape Species. J Agricultural and Food Chemistry. **2011**. 59:10657-10664.

16. H. B. Patel, T. M. Jefferies. Eluotropic strength of solvents : Prediction and use in reversed-phase high-performance liquid chromatography. Journal of Chromatography. **389**, 21 (1987)