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GCMS method development of a volatiles analysis suite for Pinot noir

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Executive summary

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The aim of this project was to establish and implement a method to measure and profile wine aroma as part of Plant & Food Research's ongoing efforts to extend analytical capabilities to support wine research in Marlborough.

A quantitative method for volatile compounds known to influence wine flavour was developed. This report focuses on the optimisation of the analysis of C6 alcohols and aldehydes collectively known as green-leaf volatiles. The method procedure and method validation are described in detail. The validation, including assessing precision and accuracy, allowed us to identify and minimize variability, correct systematic errors, and ensure the method will be suitable for its intended purpose.

This work ensures that the method produced is reliable with accurate results and instils confidence for our research. Once the library standards are finalised, we will apply the method to assess differences between wines made with grapes derived from vineyards with different attributes and compare them with the potential of grape flavour.

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1 Introduction

Gas Chromatography – Mass Spectrometry (GCMS) is a high-value precision analytical technique for measuring trace aroma and flavour compounds. In the quest to continue development of Plant & Food's (PFR) Marlborough site's capability, this instrument and associated methodology will enable PFR to provide advanced analytical capability to support in-house and campus-wide research, the wider Marlborough wine industry, as well as other food and beverage industries within the Marlborough region.

Headspace-Solid Phase Micro-Extraction-Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS) is the technique of choice for wine volatile compound analysis. This is a fast and effective way to obtain qualitative and quantitative information on the volatile organic compounds (VOCs) present in wine.

Central to all HS-SPME-GC-MS procedures is the adsorption of VOCs from within the headspace of a sealed sample of wine onto a specific SPME fibre which resides within a hypodermic needle. Following exposure to the headspace VOCs, the needle and fibre are withdrawn from the sample headspace and the adsorbed VOCs are rapidly desorbed within the heated injection port of the GC-MS. Thereafter the VOCs undergo chromatographic separation within the GC's capillary column before entering the MS for qualitative and/or quantitative analysis.

The objective of this study was to develop an optimised HS-SPME-GC-MS method for quantifying VOCs in Pinot noir wine, using a SPME fibre and GC column set-up that could quantify a wide range of compounds across expected concentration ranges. A method that can accurately and reliably measure aroma compounds is needed to investigate the importance of these for the aroma in Pinot noir, and the influence on them due to different viticultural and winemaking practices.

1 Materials and methods

1.1 HS-SPME-GC-MS procedure

The method that was developed and optimised was based on protocols previously described for VOC analysis (Rodríguez-Bencomo et al. 2011; Brizuela et al. 2018), with some modifications. Wine samples were warmed in an incubator at 50°C for 5 min under constant stirring (500 rpm) before extraction. The extraction was performed with the exposure of a StableFlex™, 24 Ga, 50/30 µm Carboxen/DVB/PDMS fibre (Agilent technologies) to the headspace of the sample for 30 min at 50°C and under constant stirring (250 rpm). Following extraction, the fibre was removed from the sample vial and desorbed at 250°C in the GC injector port in splitless mode for 56 min. Prior to use, the fibre was conditioned following the manufacturer's instructions for 1 hour at 270°C.

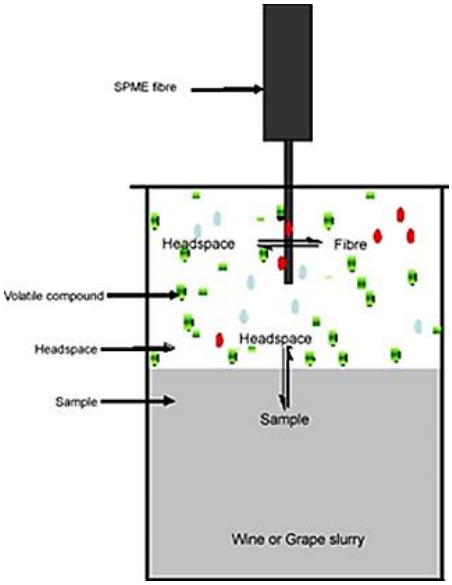

Figure 1. Headspace solid-phase microextraction (HS-SPME) extraction illustrating the equilibration of volatile compounds between the three phases: sample, headspace and SPME fibre.

An Agilent 7890A GC system (Agilent, Palo Alto, CA), with a split/splitless injector and interfaced with an Agilent 5975 C mass spectrometer, was used for the sample analyses. MassHunter Data Analysis software (version B.07.04.2260) was used to control the system. Volatile compounds were separated on an HP-Innowax capillary column (50 m × 0.2 mm diameter × 0.20 µm film thickness) from Agilent (J&W Scientific, Folsom, USA). Helium carrier gas was used at a flow rate of 1 mL min^{-1} for 1 minute and then increased to 1.5 mL min^{-1} for the rest of each sample run. The oven temperature was programmed as follows: an initial temperature of 40°C, which was maintained for 1 min, and then increased to 240°C (4°C/min) for 5 min. For the MS, the temperatures of the transfer line, quadrupole and ionisation source were 245, 150 and 230°C respectively. Electron impact mass spectra were recorded at an ionisation voltage of 70 Ev. Data acquisitions were performed in scan mode (from 33 to 300 amu). Peak identification was carried out by software comparison of mass spectra obtained with an extensive mass spectral library (National Institute of Standards and Technology, NIST, version 2.2), and with those from individual or mixtures of known reference compounds analysed under the same conditions.

1.2 Selection of reference standards

The following compounds were selected based on their known important role and influence on wine aroma and as being present in typical wine volatile profiles.

The initial compounds used and presented in this report are C6 alcohol and aldehyde standards including trans-2-hexenal (CAS No: 6728-26-3), 1-hexanol (Cas No: 111-27-3), trans-3-hexen-1-ol (Cas No: 928-97-2), cis-3-hexen-1-ol (CAS No: 928-96-1), trans-2-hexen-1-ol (Cas No:928-95-0). These compounds are often collectively referred to as green-leaf volatiles and have odours reminiscent of fresh cut and/or dried grass.

Further compounds and groups of compounds will continue to be added to the reference standard library including:

- Fatty acid standards: hexanoic acid (142-62-1), heptanoic acid (111-14-8), octanoic acid (124-07-2) and decanoic acid (334-48-5)
- Alcohols standards: isoamyl alcohol (123-51-3), phenylethyl alcohol (60-12-8) and benzyl alcohol (505-10-2)
- Ethyl ester standards: ethyl butanoate (105-54-4), ethyl hexanoate (123- 66-0), ethyl octanoate (106- 32-1) and ethyl decanoate (110-38-3)
- Acetate ester standards: isobutyl acetate (110-19-0), isopentyl acetate (123-92-2) and β-phenylethyl acetate (103-45-7)
- C13 Norisoprenoids standards: β-damascenone (23726-91-2) and β-ionone (79-77-6)
- Terpenes standards: linalool (78-70-6), α-terpineol (98-55-5), β-citronellol (106-22-9) and geraniol (106-24-1)
- Volatile phenols standards: 4- ethyl guaiacol (2785-89-9) and eugenol (97-53-0)

All aroma standards are purer than 96%, with all the solutions stored at 4°C.

1.3 Standards preparation

Individual stock standard solutions of trans-2-hexenal, 1-hexanol, trans-3-hexen-1-ol, cis-3-hexen-1-ol, trans-2-hexen-1-ol were prepared in HPLC grade Methanol solution. A composite standard was created using the stock solutions and diluted in a model wine solution before serial dilution to five levels of concentration of each aroma compound, covering the concentration ranges expected in wines.

A separate internal standard was prepared containing the compound 3-octanol.

To prepare working standards for each analysis run, the required volumes needed to prepare each working standard (0 to 1 mL) plus the required volume (1 to 0 mL) of model wine (13.24% aqueous ethanol at pH 3.5) were pipetted, along with 8.96 mL of acidified deionised water (pH 3.5), into 20 mL amber glass, screw cap vials, followed by 40 µL of the internal standard solution (3-octanol) and sealed with a TFE/silicone septum (Supelco, Bellefonte, PA).

Quantitative data were obtained by calculating the relative peak area of the target ion in relation to that of the internal standard used (3-octanol). Calibration curves of each compound were performed using

a model wine spiked with the commercial pure reference compounds at five levels of concentration tested in triplicate to ensure precision.

Table 1. Identification parameters for the five C6 alcohol/aldehyde compounds and the internal standard 3-octanol.

Compound	Target Ion m/z	Purity (%)	CAS No
trans-2-hexenal	**41**, 83, 69	96	6728-26-3
1-hexanol	**56**, 43, 69	98	111-27-3
trans-3-hexen-1-ol	**41**, 67, 82	97	928-97-2
cis-3-hexen-1-ol	**67**, 41, 82	98	928-96-1
trans-2-hexen-1-ol	**57**, 41, 82	97	928-95-0
3-octanol	**59**, 83, 101	99	589-98-0

Quantifying ion is indicated in bold.

1.4 Wine sample preparation

All wine samples were diluted immediately prior to analysis. This sample dilution involved pipetting 1 mL of wine and 8.96 mL of acidified deionised water (pH 3.5) into 20 mL amber glass, screw cap vials, followed by 40 µL of the internal standard solution (3-octanol). The volumes used were equivalent to a 10-fold dilution of the wine sample. Following sample dilution and internal standard addition, the initial procedure was as follows: NaCl (3 g) was added to the SPME vial and vials were tightly capped and vortexed for 10 seconds. Samples were then incubated initially for 5 minutes at 50°C during which time the vial was agitated at 500 rpm. After 5 minutes, the SPME fibre was exposed to the headspace of the vial for a period of 30 minutes at 50°C, during which time the headspace volatiles were adsorbed onto the fibre with constant agitation at 250 rpm. All samples were analysed five times to look at the precision and accuracy of the method.

Wine samples, spiked with a small amount of composite standard stock solution, were also prepared following the same protocol. All spiked samples were analysed five times.

2 Results

2.1 Compounds calibration

Calibration curves are graphical representations of the relationship between the concentration of a substance and the peak area of the target ion response generated by the GCMS. Calibration curves are commonly used to determine the concentration of unknown samples based on the GCMS's response. To create a calibration curve, a set of known standard solutions with varying concentrations of the composite standard stock solution is used to measure the relative peak area of the target ion for each standard solution. These standards should cover a range of concentrations that span the expected concentration range of the unknown samples. Once the standard peak areas are obtained for the range of concentrations analysed, they are plotted against the corresponding concentrations. This results in a calibration curve, which is typically a linear relationship. To determine the concentration of an unknown sample using the calibration curve, the response of the unknown sample is measured using the same protocol. Then, the unknown sample's response is plotted on the calibration curve and determine its corresponding concentration.

All the standard samples have been integrated and analysed. An example of chromatogram is shown on Figure 2. Compounds were identified by comparison of their retention time and spectral data with those of pure standard compounds.

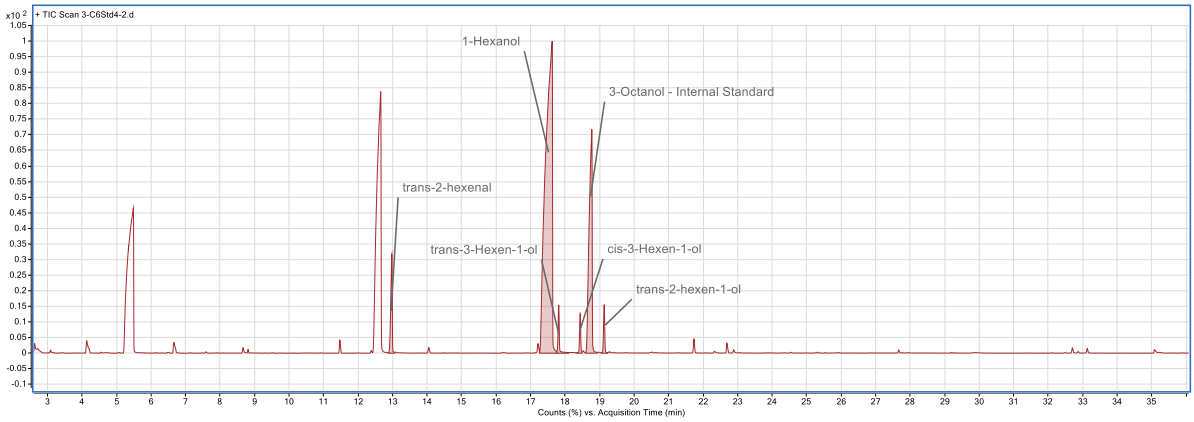

Figure 2. Chromatogram obtained of a composite standard dilution.

Table 2. C6 alcohol/aldehyde calibration results

Compound	Retention time (min)	Calibration Range (µg/L)	Standard curve (R^2)	Regression equation f(x)	Perception threshold (µg/L)
trans-2-hexenal	12.959	0-250	0.9878	y=677.81x-5.4043	300
1-hexanol	17.435	0-5,000	0.9932	y=1567.7x-74.107	8000
trans-3-hexen-1-ol	17.728	0-250	0.983	y=1760.4x-3.0771	1000
cis-3-hexen-1-ol	18.397	0-250	0.9843	y=2393.6x-6.7144	400
trans-2-hexen-1-ol	19.123	0-250	0.9878	y=1800.3x-6.4642	

The slope (a) and intercept (b) and linearity, shown in Table 2 column 5, were calculated using the following equation: y = ax + b, with “y” representing the relative concentration of the compound in the sample (concentration compound/concentration internal standard) and “x” representing the relative area (area compound / area internal standard).

The Table also shows the calibration range and the determination coefficient (R^2), which is the estimator of the adequacy of the regression model. As can be seen on the table, calibrations of the studied aroma compounds all showed an R^2 greater than 0.98.

2.2 Method validation

Method validation involves demonstrating that the method is suitable for its intended purpose and provides reliable and accurate results. Precise and accurate measurements help establish the method's performance characteristics, such as linearity, range, sensitivity, specificity, limit of detection, and limit of quantification.

Precision and accuracy provide information about the reliability and correctness of the measurements obtained from the method.

Precision refers to the consistency and reproducibility of results. In the context of a new method, it is crucial to assess precision to determine the method's reliability and the degree of variation between replicate measurements. High precision indicates that the method produces consistent and reliable results, while low precision suggests that there is significant variation or random error in the measurements. By understanding the precision, we can identify and minimise sources of variability, improve the method, and estimate the level of confidence in the obtained results.

Repeatability or precision was evaluated by analysing five replicates of a red wine, Pinot noir and spiked wine samples using the same method and instrument within the same working day.

Accuracy refers to the closeness of measured values to the true or accepted values. When developing a new method, it is essential to evaluate its accuracy to ensure that the measurements are correct and free from systematic errors. Systematic errors can arise from various sources, such as instrument bias, methodological limitations, or sample preparation issues. By assessing accuracy, we can identify and correct systematic errors, adjust improve the method's performance, and ensure the reliability and validity of the obtained results.

Accuracy was assessed with the same wine, spiked with 0.25 ml of composite standard stock solution to increase the quantity of 1-hexanol in the wine to 2356 µg/L and the other compounds to around 60 µg/L.

Recovery refers to the measurement of how well a method can accurately determine the amount or concentration of a substance in a sample. It is a measure of the method's ability to recover the known amount or concentration of the substance added to a sample. Recovery studies are performed to evaluate any potential matrix effects, sample losses, or interferences that may affect the measurement accuracy.

Table 3. C6 alcohol aldehyde accuracy and precision results.

Compound	Repeatability (% RSD)	Recovery (%)	% error
trans-2-hexenal	3.9	104.2	4.2
1-hexanol	3.2	85.5	14.5
trans-3-hexen-1-ol	3.9	93.8	6.2
cis-3-hexen-1-ol	6.6	95.4	4.6
trans-2-hexen-1-ol	3.7	103.9	3.9

The results indicate that the method developed is accurate and precise with relative standard deviation consistently below 7%. The recovery of spiked sample is between 85.5% to 104.2% which is within acceptable range for trace analysis of compounds utilising the HS-SPME technique and instrumentation used.

3 Conclusion

This method development and validation study has included the determination of response linearity, precision (repeatability) and accuracy (recovery studies). Under the experimental conditions used in this study and the results obtained, the method has been demonstrated to be very robust and reliable for the analysis of volatile C6 alcohols and aldehydes in wines.

The work has been very involved as this type of instrumentation is very complex and requires a lot of expertise that has been learnt through the year. Ongoing work will increase the standards library and the range of compounds able to be quantified with our method.

However, we are now in a position to measure and assess differences green aromas between Pinot noir wines made with grapes derived from viticultural or winemaking experiments.

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