Industry allocated project number	PHI allocated project number

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FINAL REPORT
2017

1. PROGRAMME AND PROJECT LEADER INFORMATION

	Research Organisation Programme leader	ARC Research Team Manager	Project leader
Title, initials, surname	Dr AS Buica		Dr AS Buica Prof W du Toit
Present position	Researcher		Researcher Assoc Prof
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2. PROJECT INFORMATION

Research Organisation Project number	WW ASB 16-03
Project title	Development and implementation of GC-MS/MS method for the determination of volatile sulphur compounds (VSC) related to off-flavours in wine
Short title	VSC analysis by GC-MS/MS

Fruit kind(s)	grape		
Start date (mm/yyyy)	01/2016	**End date** (mm/yyyy)	12/2016

Key words	Volatile sulphur compounds (VSC); wine; GC-MS/MS analysis method

Approved by Research Organisation Programme leader (tick box) ☐

3. EXECUTIVE SUMMARY

Objectives & Rationale

The need for reliable data can be addressed by developing new analysis methods that make use of the technology available. Some methods are easy to implement because they make use of low-tech equipment, while others are highly specialized and require state-of-the-art instrumentation.
Organic sulphur compounds play a considerable role in the sensory characteristics of a wine, as they are frequently present among the character impact compounds. Volatile sulphur compounds (VSC) may determine quality and typical characteristics, not all of them pleasant, such as off-flavours resembling onions, garlic, cooked cabbage, rubber, and putrefaction. The difficulties to overcome when measuring these compounds are related to the required sensitivity of the analytical technique and to the instability, reactivity, and differences in volatility of reductive S compounds (RSC).

Methods

The determination of VSC was divided into two: (1) measurement of H_2S using a colorimetric reaction and (2) determination of RSC by GC-MS/MS. Both methods had to be tested before implementation.

Key Results

The two methods have proven to give reproducible results and are already implemented in our environment.

Conclusion/Discussion

Certain precautions have to be taken when working with either of the methods. The measurement of H_2S can be done *in situ* by wineries, given that the temperature and volume of the sample are kept constant and that the right volume tube is used. The sensitivity can be adapted based on the sample and on the type of tube chosen.
Even though the method is straightforward, the GC-MS/MS analysis of the RSC requires a skilled and experienced analyst.
By combining the two methods, a wide range of compounds can be measured and thus increase our knowledge regarding the RSC in wine. These methods also open the possibility for new projects and experiments that evaluate the impact that various winemaking techniques and products have on the final composition of wines.

4. PROBLEM IDENTIFICATION AND OBJECTIVES

Organic sulphur compounds play a considerable role in the sensory characteristics of a wine, as they are frequently present among the character impact compounds. Volatile sulphur compounds (VSC) may determine quality and typical characteristics, not all of them pleasant, such as off-flavours resembling onions, garlic, cooked cabbage, rubber, and putrefaction. The possible sources of these VSC (or RSC, reductive sulphur compounds) are numerous and varied and can occur at several stages during the winemaking process or storage of bottled wine. The relationships between the factors that affect the formation of RSC are complex and worth investigating to ensure that the final wine is of adequate quality.

From an analytical point of view, a limited number of methods is equivalent to limited knowledge of the subject matter. Therefore the goal of this project is to offer a sensitive and reliable tool for investigations, during which not only the already usual positive aromas are evaluated (esters, higher alcohols, terpenes, thiols), but also some of the negative ones, to obtain a more complex and complete picture of the phenomena occurring from winemaking stages up to the consumer.

RSC in wine are divided into 'light' (boiling point, b.p. <90°C) and 'heavy' (b.p. >90°C) compounds, indicating the difficulty of using a relevant common sampling and enrichment technique. The additional difficulties to overcome are related to the required sensitivity of the analytical technique and to the instability and differences in volatility of RSC. Several analytical approaches have been employed to quantify sulphur volatiles in wine, and headspace sampling coupled with solid-phase microextraction (HS-SPME) methods, combined with gas chromatography (GC) followed by either S-specific or mass spectrometry (MS) detection proved quite effective.

One approach makes use of S-specific detectors, such as chemiluminescence and (pulsed) flame photometric detector. While extremely sensitive towards S compounds, these detectors are non-standard in an analytical lab and, by extension, expensive. Also, because they can be used only for S compounds, this type of equipment is restrictive in the possible applications for which it can be used. Another approach makes use of a more standardized type of equipment, namely GC-MS or its more performant variant, GC-MS/MS (which offers increased selectivity and sensitivity).

Based on the state-of-the-art instrumentation available to us, we optimized and implemented a HS-SPME-GC-MS/MS method based on previous work done in New Zealand. This was coupled with a chemically sophisticated but low tech type of device for the measurement of H_2S.

5. DETAILED REPORT

a. PERFORMANCE CHART (for the duration of the project)

Milestone	Target Date	Extension Date	Date completed
1. Familiarisation with the GC-MS method (Auckland, NZ)	June 2015	n/a	
2. Training post-doc fellow on method specifics	Jan 2016	n/a	n/a
3. Method adaptation and optimisation for GC-MS/MS (CAF)	Jan-May 2016	Jan-June 2017	done
4. Method validation	July 2016	June-July 2017	done
5. Sample analysis	Aug-Nov 2016	July-Aug 2017	done
6. Training of technical officers	July 2016	Jan-July 2017	done
7. Reports	Dec 2016	Aug 2017	done

6. Journal publication(s) – final milestone One popular One peer-reviewed	Feb 2019 Mid-2019		

b) WORKPLAN (MATERIALS AND METHODS)

1. Familiarisation with the GC-MS method (Auckland, NZ)

As mentioned above, we had the opportunity to collaborate with the researchers that set up the original GC-MS method. It was a chance to learn first-hand from their experience about the practicalities of the method, and especially its issues seeing that the RSC are difficult compounds to handle and analyse.
The experience was of extraordinary value for our work regarding all the aspects of the analysis, from sample handling to instrumental set-up.

2. Training post-doc fellow on method specifics

As stated in the risk assessment for the project, finding a suitable post-doc fellow for this work has proven difficult. In the end, the people working on the method were a PhD student (S Vannevel, whose PhD project relies partly on the measurement of the RSC), a technical officer from Oenology/IGWS (A Schulze) and research staff from CAF, where the GC-MS/MS part of the method is implemented.

3. Method adaptation and optimisation for GC-MS/MS (CAF)
4. Method validation
5. Sample analysis

The project followed the analytical method stages. In order to be implemented in our environment, the method was adapted to our specific instrumentation. During this stage, extensive optimization took place (detailed in the Results&Discussion section below). The method was validated for synthetic, white, and red wine to make sure that it is reliable, precise, and accurate. As the method was already earmarked for some of the projects currently running in our environment, the initial application took place with samples from one of these projects.

6. Training of technical officers

The training took place as the method was validated and implemented.

c) RESULTS AND DISCUSSION

The original method had some features that set it apart and made it less straightforward to implement in our environment. The issues, their implications, and the solutions we found are presented here, before discussing the results in more detail.

- H_2S posed problems in the original method due to the compound's extremely high volatility. The lab in Auckland partly solved the problem by using in their setup a cryotrap (cooled on-line with liquid Nitrogen), but they still encountered problems with a lot of their samples when trying to determine H_2S.
  The GC-MS/MS at CAF doesn't have the cryotrap feature, therefore we cannot measure H_2S with this instrument, but the solution came from a low-tech device. Its characteristics and measurement procedure will be described below, in the section ***Measuring H_2S***.
- The NZ instrumental setup includes two capillary columns with different chemistries of the stationary phase connected in series. Using two columns increases the length and time during which the separation of similar compounds can take place, and the two different chemistries assist by offering different mechanisms through which separation can be achieved. The disadvantage is that this type of column setup is very unusual and cannot be used for regular applications. Which means, in turn, that every single time the RSC analysis is run, the column has to be changed to the two-column setup and back to the original

configuration, which for any MS instrument means long down times. Or that a GC-MS has to be dedicated solely to this type of analysis, which is excessive.
Plus, being able to align two capillary columns (internal diameter 0.25 mm) without losing performance is extremely difficult, and the setup is very delicate and challenging to handle, especially if the columns have to be taken out and re-installed whenever the analysis is done. In a word, impractical.
The solution on our side came from the performance of the instrument we used. The issues with separation and sensitivity required by the RSC were solved by MS/MS detection. The use of tandem Mass Spectrometer results in an increase of separation ***within*** the detector, additional to the separation in the column (no need to use two columns).
The details of the method and the results for validation and application are presented in the section below ***Measuring RSC by GC-MS/MS***

Measuring H_2S

S and S-containing compounds react selectively with a variety of reagents. This chemical property can be used for the determination of S-containing compounds. For example, the reaction with Hg was used in the original Tominaga method for the analysis of thiols in wines. Some of the reactions produce coloured compounds and these can be used to monitor and measure S compounds. Some reagents are selective to only one compound, like in our case H_2S.
A commercial device that can be used for this purpose is available for a couple of years. Detector tubes are thin glass tubes with calibration scales printed on them to directly read concentrations of the substances to be measured. Each tube contains detecting reagents that are especially sensitive to the target substance and quickly produce a distinct layer colour change. The amount of reagent in a tube is calibrated in such a way that there are different ranges for H_2S, depending on the application. The reaction is irreversible and progressive, starting from level 0 and continuing until all the H_2S present in the headspace has reacted. The tubes can be used until the reaction level reaches the top of the tube.

We tested tubes from two different manufacturers, Gastec and Honeywell, to determine their suitability for use in our type of application.

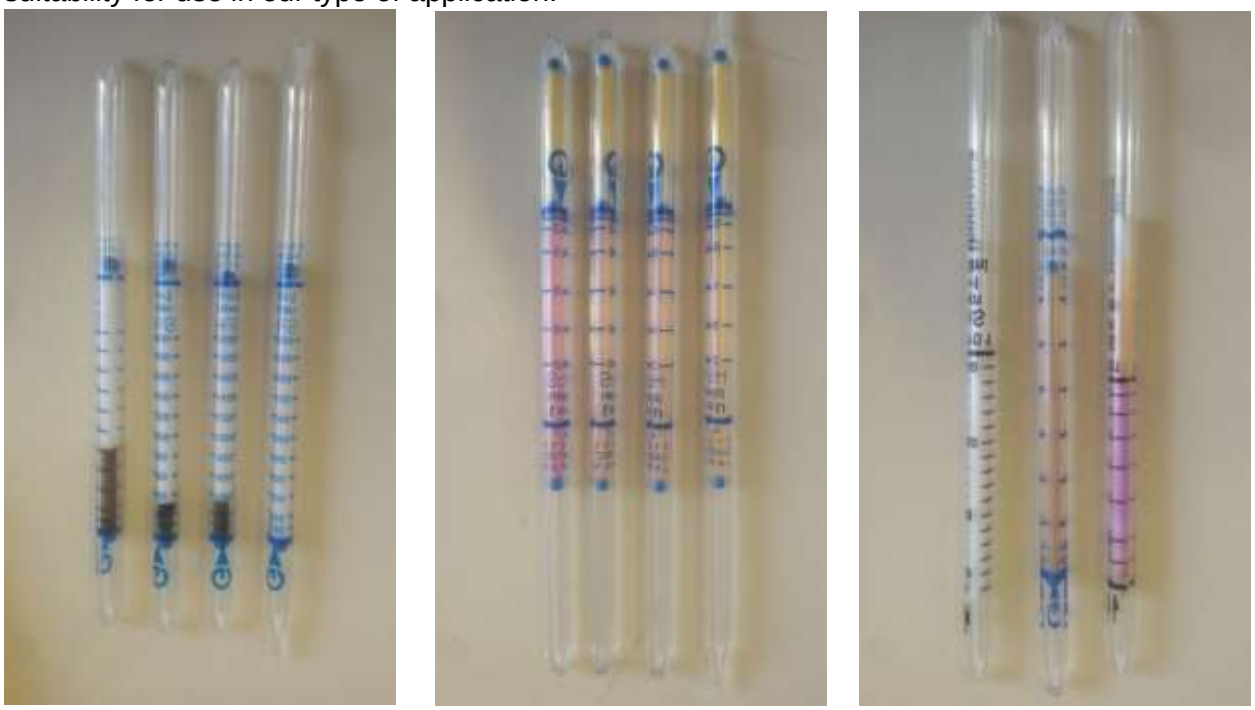

Figure 1: The types of tubes chosen and their colour change after exposure to H_2S. Left: Gastec, colour change white to dark brown/black. Middle: Honeywell, colour change white to

pink, yellow colouration due to exposure to water vapours. Right: different types of tubes after exposure to water vapours.

Things to be taken into account when using the tubes are: temperature, atmospheric pressure (depending on how the measurements are done), relative humidity, the volume of sample tested, and the volume of gas sampled. The tubes chosen were tested for repeatability and robustness in the lab working conditions. At the end, ten samples from an ongoing project were also measured.
The sampling can be done with a gas sampling pump, which is commercially available. After considering various possibilities, including using the pump, we have decided on the following setup, with the idea that the inert gas we use displaces the H_2S present in the solution (Figure 2). This setup was chosen because it avoids issues related to the atmospheric pressure adjustment, and only the volume and the temperature of the liquid have to be controlled.

Figure 2: In-house setup for the H_2S measurements

What has been tested:
1) Wine spiked with ammonium sulphide (AS) 3.1 mg/L, sparged for 30 min with Suremix (mixture of N2 and CO2) and measured on Honeywell 10-103-04 tube (0.2-3 ppmv)
Response: 0.4ppmv H_2S (relative humidity issues)

2) Wine spiked with 3.1 mg/L AS, sparged for 30 min with Suremix, 2 tubes used
Gastec 4LT: excess after 5 sec
Gastec 4LL: excess after 10 sec
Both tubes too sensitive for this level of spiking, to be considered for lower levels of H_2S present in solution.

3) Wine spiked with AS 3.1 mg/L, sparged for 30 min and an additional 30 min with Suremix, two Gastec 4H tubes used for repeatability.

Response 1: 350 ppmv after 30 min, no further change after 1 hr
Response 2: 325 ppmv after 30 min, no further change after 1 hr
Repeatability is acceptable for a colorimetric reaction.

4) Wine spiked with AS 6.2 mg/L, sparged for 30 min and an additional 30 min with Suremix, Gastec 4H tube used.
Response: 750 ppmv
The response is proportional to the concentration, response is double compared to (3), in this case the concentration of the spike was also double compared to (3).

5) Wine samples with Gastec 4LT, sparged 30 min
NO RESPONSE FOR ANY OF THE 10 SAMPLES.
This tube is the most sensitive one, used for the lowest concentrations of H_2S, therefore there was no H_2S present in the samples.

The tubes are convenient from a cost point of view, too, Honeywell tubes are R94.04 ea and Gastec tubes R61.05 ea.
Besides having to take care that the conditions in which the measurement take place are controlled, choosing the right tube for the right sample taking into account the concentration of H_2S is the only major issue with this measurement. This method is currently being implemented in our lab for routine analyses, and will be adapted for fermenting media.

Measuring RSC by GC-MS/MS
The list of analytes measured with this method is given in Table 1. It is a comprehensive list, including compounds from various classes and with a variety of volatilities. This means that not all of the compounds will contribute to wine aroma in normal circumstances. Nevertheless, it is important to be able to measure them, since they can be involved in many processes occurring in wine and might be key to elucidating metabolic pathways, for example.
Since there are many classes of S compounds included in the method, each class uses its own deuterated internal standard for normalization of measurement. These internal standards are commercially available, therefore not a limiting factor.

The sample preparation procedure is straightforward: 10 mL wine are added in a headspace GC vials containing 2.5 g $MgSO_4$ and the vial is sealed. Due to the nature of the internal standards used, their solution is added through the septum of the cap, to ensure that no compounds are lost in the process. The vials are put in the instrument and the analysis follows using headspace solid phase microextraction (HS-SPME) for sampling and injection.

During the method optimization stage, an HP-1MS column was used. Each standard and internal standard were run separately to determine retention time and to optimize the MS conditions to ensure the best signal-to-noise ratio. Some compounds were detected in selected ion monitoring mode (SIM, based on mass/charge ratios in single MS) and others in selected reaction monitoring mode (SRM transitions in MS/MS). During validation is was observed that the compounds with the highest volatility, methane thiol, CS_2, and ethane thiol, couldn't be measured accurately. This posed a big problem, since these compounds contribute most to the reductive wine aroma exactly because they are the most volatile.
The solution to this problem was changing the column. The second column used was HP-InnoWax. The standards and internal standards had to be re-analysed one by one, as the different chemical nature of the stationary phase meant that the retention times of the various compounds changed. But it also meant that the problematic compounds could now be measured and the validation of the method could proceed.

The method was tested for linearity (6-10 point calibration), limit of detection (LOD), limit of quantitation (LOQ), and repeatability at two different concentrations. A summary of the method

validation parameters is presented in Table 1. All calibrations are linear in the range tested, with R^2 values of at least 0.9 and in most cases at least 0.99.
The method is considered suitable for the analysis of RSC in both red and white wines.

Table 1: Summary of parameters tested for the RSC method

Compound	***class***	***R^2***	***LOD, µg/L***	***LOQ, µg/L***	***linearity range tested, µg/L***	***repeatability low level, %RSD***	***repeatability high level, %RSD***
Methanethiol	alkylthiol	0.9482	0.06	0.02	0.04-8.84	12.17	19.65
Carbon disulfide	disulfide	0.9908	0.06	0.02	0.25-50.32	14.11	11.83
Ethanethiol	alkylthiol	0.9985	0.21	0.06	0.62-124.91	11.46	28.37
Dimethyl sulfide	sulfide	0.9947			3.13-625.35	5.65	11.16
Diethyl sulfide	sulfide	0.9971	0.07	0.02	0.3-59.76	3.26	6.78
Methylthioacetate	thioacetate	0.9963	2.71	0.81	0.39-78.92	1.38	5.25
Dimethyldisulfide	disulfide	0.9973	0.06	0.02	0.31-61.56	6.46	10.84
S-ethyl thioacetate	thioacetate	0.9976	0.18	0.05	0.28-56.32	10.87	14.47
Diethyl disulfide	disulfide	0.9962	0.02	0.01	0.82-164.63	5.56	6.46
4-methylthiazole	thiazole	0.9982	0.48	0.14	0.37-74.91	6.49	12.64
dimethyl trisulfide	trisulfide	0.9944	0.01	0.003	0.12-24.04	6.06	10.93
methional	thioaldehyde	0.9803	80.54	24.18	10.84-2167.63	13.80	13.86
2-mercaptoethanol	thioalcohol	0.9949	34.50	10.36	2.94-587.07	7.38	12.89
2-methylthio-1-ethanol	thioalcohol	0.9968	15.79	4.74	1.86-372.57	9.63	13.29
3-methylthio-1-propanol (methionol)	thioalcohol	0.9955	17.11	5.14	19.7-3941.91	9.98	11.34
4-methylthio-1-butanol	thioalcohol	0.9968	0.02	0.01	0.04-8.84	11.03	13.72
benzothiazole	thiazole	0.9949	0.54	0.16	0.16-32.86	8.88	18.36

Even though the method is straightforward, we would like to draw the attention to the following:

- Utmost care must be taken when handling the samples (for example during sample transfer), as the most volatile substances will evaporate if treated carelessly, thus leading to inaccurate and non-reproducible results.
- Working with the standard solutions and the internal standard solutions require highly skilled operators. Simply doing the wet chemistry part (for example making up the standards for the calibration) required separate micro-syringes for each pure substance, working constantly under a fumehood, sealing the solutions and sampling them through the septum of the various vials, *etc*.
- Besides the volatile nature of the compounds, they are also toxic and prone to cross-contamination (since S shows affinity for a range of chemicals commonly present in a lab).
- The glassware used in the method is treated with a special washing solution which, in addition to cleaning, deactivates the surface of the glass to stop any of the S compounds being retained on the surface and contaminating the samples.

d) CONCLUSIONS

The main issues with the determination of the RSC derive from the compounds' high volatility and affinity for various chemicals commonly present in the work environment. Using a combination of low tech and high tech equipment, a large variety of RSC can be measured. The simple, straightforward determination of H_2S can be done with the use of the commercially available tubes, taking into account a series of experimental conditions to ensure accuracy and repeatability. The same type of issues due to volatility and contamination can be encountered in the measurement of an array of RSC by GC-MS/MS if adequate care is not taken. Nevertheless, the developed method has shown to be linear, sensitive, and reproducible in the conditions tested.

This method can be applied further to a variety of projects that cover fundamental knowledge (such as the elucidation of pathways related to microorganisms and their metabolites in wine) which will translate into applied knowledge. Some examples of practical applications could be the influence on the production of RSC of grape variety, choice of yeast and additional nutrients (their nature and quantity), impact of Brett and LAB spoilage, impact of aging, oxidation and microoxygenation, the use of Cu^{2+} during winemaking, *etc*.

6. ACCUMULATED OUTPUTS

a) TECHNOLOGY DEVELOPED, PRODUCTS AND PATENTS

The measurement of H_2S using the tubes is easy to implement and doesn't require any high-tech setup. It can easily be transferred to the industry for the producers to measure H_2S *in situ* if necessary, while taking into account the restrictions on the measuring conditions to ensure accuracy.

At the other end of the technology spectrum, the RSC method requires state-of-the-art equipment. The method will be added to the portfolio of analyses to be performed on commercial and experimental wines. It will become an essential tool for future projects (some of them suggested in the Conclusions section). The method will be set up using CAF equipment, therefore once the method is up and running, industry could have direct access to this analysis.

b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

The H_2S measuring method can be demonstrated to ensure adequate transfer and implementation. The H_2S and RSC methods will be communicated via a popular science article.

c) HUMAN RESOURCES DEVELOPMENT/TRAINING

Student Name and Surname	Student Nationality	Degree (e.g. MSc Agric, MComm)	Level of studies in final year of project	Graduation date	Total cost to industry throughout the project
Honours students					
Masters Students					
PhD students					
S Vannevel	Belgium	MSc	PhD upgraded	Dec 2018	

Postdocs					
Support Personnel (not a requirement for HORTGRO Science)					
W Kuhn	SA	n/a	n/a	n/a	
L Mokwena	SA	n/a	n/a	n/a	
A Schulze	SA	n/a	n/a	n/a	

PERSONS PARTICIPATING IN THE PROJECT (Excluding students)

Initials & Surname	Highest Qualification	Degree/ Diploma registered for	Race (1)	Gender (2)	Institution & Department	Position (3)	Cost to Project R
AS Buica	PhD	n/a	W	F	DVO	PL	
M Stander	PhD	n/a	W	F	CAF	Coll	
L Mokwena	MSc	n/a	B	M	CAF	Co	
W Kuhn	Hons	n/a	W	F	CAF	Co	
A Schulze	MSc	n/a	W	F	IGWS	Co	

(1)Race B = African, Coloured or Indian
W = White

(2)Gender F = Female
M = Male

(3)Position Co = Co-worker (other researcher at your institution)
Coll = Collaborator (participating researcher that does not receive funding for this project from industry)
PF = Post-doctoral fellow
PL = Project leader
RA = Research assistant
TA = Technical assistant/ technician

d) PUBLICATIONS (POPULAR, PRESS RELEASES, SEMI-SCIENTIFIC, SCIENTIFIC)
n/a

e) PRESENTATIONS/PAPERS DELIVERED
n/a

7. BUDGET

TOTAL COST SUMMARY OF THE PROJECT

YEAR	CFPA	DFTS	Deciduous	SATI	Winetech	THRIP	OTHER	TOTAL
2016					500 000			500 000
2017					0			0

EVALUATION BY INDUSTRY

This section is for office use only

Project number	
Project name	
Name of Sub-Committee*	

Comments on project

Committee's recommendation (Review panel in the case of PHI)

- Accepted. ☐
- Accepted provisionally if the sub-committee's comments are also addressed. Resubmit this final report by_____ ☐
- Unacceptable. Must resubmit final report. ☐

Chairperson_____ Date_____

*SUB-COMMITTEES

Winetech
Viticulture: Cultivation; Soil Science; Plant Biotechnology; Plant Protection; Plant Improvement;
Oenology: Vinification Technology; Bottling, Packaging and Distribution; Environmental Impact; Brandy and Distilling; Microbiology

Deciduous Fruit
Technical Advisory Committees: Post-Harvest; Crop Production; Crop Protection; Technology Transfer
Peer Work Groups: Post-Harvest; Horticulture; Soil Science; Breeding and Evaluation; Pathology; Entomology