Vineyard in One Tree Hill near Adelaide, Southern Australia.

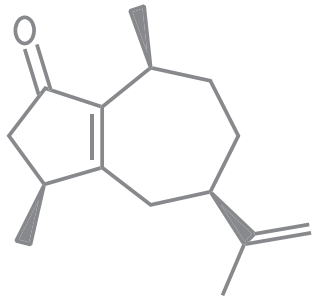
Searching for the pepper in Shiraz

Rotundone is a key flavor compound with a distinct black pepper note. Rotundone occurs naturally in pepper, but also in wines such as Australian Shiraz and others from around the world, for example in the Austrian Green Veltliner. Australian wine scientists are searching for clues as to how the peppery compound is formed in the hope that this knowledge would enable them to optimize both growth conditions for the Shiraz grape and the ensuing wine-making process. Finding answers requires an efficient analysis method. Researchers turned to Membrane Assisted Solvent Extraction (MASE) combined with heart-cut GC/MS for more efficient determination of rotundone in wine and grapes.

When it comes to wine, tasting beats reading reviews. But a little reading could offer a good starting point when you set out to differentiate noble drops from decent everyday wines or even inferior ones. When mingling with real or would-be connoisseurs, the following vocabulary samples could help you fit in: A wine scientist is called an oenologist; wine expert in a classy restaurant: Sommelier; and while merry wine drinkers enjoy wine tasting, highbrow tasting is referred to as a degustation. But even experts can find themselves at wit's end when sensory impressions do not solve the puzzles they face and hard facts are required to ensure best possible product quality and product acceptance.

When faced with a wine with its hundreds of volatile compounds that influence the flavor, the technique of choice for the analyst is gas chromatography with mass selective detection (GC/MS). This is the tool used by Tracey E. Siebert and Sheridan R. Barter from the Australian Wine Research Institute in Glen Osmond (Adelaide), Australia. The scientists conduct basic research; they are not trying to outperform sommeliers.

Siebert's and Barter's goal is to find out how wine flavor compounds are formed in order to find ways to optimize growth conditions for best taste and bouquet. The Australian scientists had set their sights on rotundone, a bicyclic sesquiterpene containing a conjugated ketone group. With its characteristic black pepper note, rotundone is considered a key flavor compound in Australian Shiraz wine. Shiraz is the number one grape grown in Australia. It is highly valued due to its high yield and resistance to cold weather, and of course due to the



Structural formula of (-)-Rotundone

GC/MS-System comparable to the one used by Siebert and Barter for automated MASE and 2D-GC/MS Rotundone determination

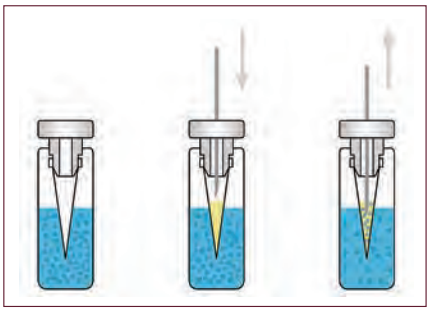
excellent tasting wine it produces. Shiraz vines were originally cultivated in France (Syrah). Today the wine is not only popular in Australia, but also widely grown in South Africa, Latin America, the United States, and Canada.

Wine under the influence

Many factors influence quality and character of the final wine; it is generally accepted that controlling the grape maturation process is key. Knowledge of specific factors that influence, for example, how much rotundone is formed in the vine and how growth conditions could be optimized could help improve quality, taste, flavor, and ultimately product value. Such knowledge might enable wine producers to provide more uniform year-to-year wine quality and taste. But Sieber and Barter are convinced that such insights can only be gained based on access to efficient and sensitive analysis methods.

The scientists presented their work in May 2013 in Palm Springs, California, USA during the 37th International Symposium for Capillary Chromatography (ISCC) [1]. Previously used analysis methods were labor intensive and time consuming. Large sample volumes had to be handled and extensive sample preparation was required for matrix elimination and analyte concentration since Rotundone is present only at very low concentration levels.

According to literature: "The flavor threshold of Rotundone is at 16 ng/L in red wine and 8 ng/L in water. A limit of quantitation (LoQ) of < 8 ng/L would be necessary for juices, grapes, mash and wine. These are complex aqueous samples that contain sugars, ethanol, anthocyanins or polyphenols." [2].



Schematic diagram of Membrane Assisted Solvent Extraction (MASE): The semi-permeable membrane insert is immersed into the sample (left). Extraction solvent is added into the MASE insert and analytes diffuse through the membrane into the extraction solvent while particulate matter is kept in the sample (center).
The extract is aspirated by the syringe for introduction to the GC/MS (right).

Critical factor for success: The extraction

The critical factor that helps ensure analytical success or failure is the extraction step, according to Siebert and Barter.

Searching for a suitable technique, the scientists came across automated Membrane Assisted Extraction (MASE). MASE was developed in cooperation between the Environmental Research Institute Leipzig-Halle and GERSTEL.

This type of liquid – liquid extraction is based on using a semi-permeable membrane as a phase boundary, which keeps particulate matter and other matrix components out of the extraction solvent. Further sample clean-up steps such as filtration and centrifugation are no longer needed. MASE extracts – even of heavily matrix laden samples are clean and can be injected directly to the GC/MS or LC/MS system for analysis. Additionally, MASE enables extraction using sample-solvent mixtures that do not normally result in phase separation. As an example, HPLC compatible polar solvents can be used to extract aqueous samples in which the solvents are normally miscible. MASE extends the analytical possibilities considerably [3,4]

Experimental

The grape juice/extract or wine sample was added to a 20 mL autosampler vial, the MASE membrane bag placed inside the vial, and the vial capped. Sample preparation and introduction were performed automatically by the GERSTEL MultiPurpose Sampler (MPS). MASE was performed by adding 750 µL of hexane into the MASE membrane bag. The sample was extracted for 60 min at 35 °C. A 100 µL aliquot of the resulting extract was injected (Large Volume Injection - LVI) into a GERSTEL Cooled Injection System (CIS 4) inlet mounted in an Agilent GC 7890.

Sample preparation:

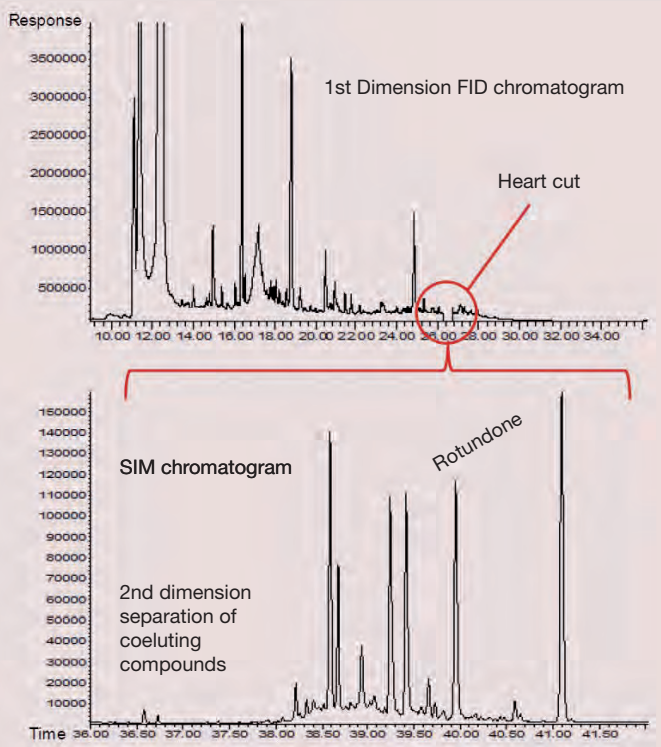
Grapes: A 100 g sample of berries was gently blended and centrifuged and the juice supernatant collected. The solid residue was further extracted by adding 60 mL of an ethanol/water mixture (50:50) and internal standard (IS) to the solids and shaking for 24 hours before centrifuging and collecting the supernatant. The supernatant and the juice initially collected were combined and topped up with water to a volume of 200 mL. Of the final volume, a 15 mL aliquot was taken and used for analysis.

Wine: A 15 mL sample of wine was taken and used directly for analysis.

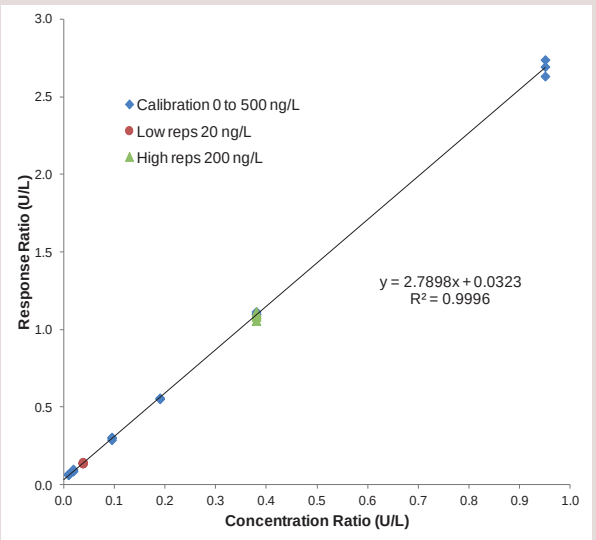
The separation took place on a multidimensional heart-cut GC system, based on an Agilent Capillary Flow Technology (CFT) Deans' Switch configured with two separation columns. A CryoTrap System (CTS, GERSTEL) was connected between the first and second dimension columns for analyte focusing prior to the analytical separation. An FID monitor detector was used on Column 1 and an Agilent 5975C MSD (Agilent Technologies) was used in Selected Ion Monitoring (SIM) mode for the analytical separation on Column 2. Stable isotope dilution was performed using d5-Rotundon as the internal standard.

According to Siebert and Barter, the described method combining MASE and LVI minimizes sample preparation workload significantly. Problems with interferences were compensated using internal standards. By heart-cutting the key part of the chromatogram for separation onto a second column, a cleanly separated and clearly defined rotundone peak is obtained. The scientists reported the analysis method to be accurate, precise, rugged, and sensitive leading to limits of quantitation in the low ppt range and high sample throughput. The statistical data speak volumes: "In the Shiraz grapes (0 to 2000 ng/L) as well as in wine (0 to 500 ng/L), the limits of quantitation (LOQs) were 5 ng/L. The linear correlation (R^2) was >0,999 and the reproducibility expressed as standard deviation was below 3 % (n=6) both for low and high concentration values – a very good result", the wine scientists reported. They went on to say that "the described method will allow more detailed research on the formation of rotundone in the grape. This includes the maturation processes involved as well as whether yeast plays a role by influencing the extraction process. Such knowledge will make it more likely that we will be able to describe how rotundone is synthesized in the grape and ultimately control the rotundone concentration in the wine." Further investigations are planned and a scientific publication of the research results is scheduled to appear in the near future.

Main FID-Chromatogramm and heart cut MSD chromatogram showing a clearly defined rotundone peak. The method developed by Siebert and Barter was used to determine rotundone in Shiraz grapes and in wine following MASE analyte extraction.



Rotundone in red wine: calibration and repeatability



Instrument method parameters

GERSTEL MPS XL, CIS and CTS	
LVI:	100 µL at 0.58 µL/sec
He carrier gas flow:	100 mL/min
CIS:	Glass wool packed liner
CIS:	20 °C, solvent vent, 20 psi (0.12 min); splitless, 47.6 psi, 12 °C/sec to 240 °C (2.5 min); split, 12 °C/sec to 275 °C (2 min)
Column 1:	VF-35ms (30 m x 0.25 mm x 0.25 µm), medium polarity phase; He 47.6 psi
Column 2:	VF-200ms (30 m x 0.25 mm x 0.25 µm), unique selectivity with dipole-dipole interactions He 37.7 psi
Column 3:	Deactivated fused silica (0.70 m x 0.1 mm)
GC Oven:	80°C (1 min), 5°C/min to 210°C, cool 15°C/min to 130°C (2 min), 10°C/min to 280°C (10 min)
Heart-cut:	26.25 to 26.75 min
CTS:	-20°C (34 min), 20°C/sec to 300°C (1 min)
MSD SIM:	m/z 147, 161, 163, 203, 208, 218, 223

Literature

[1] Siebert, T. E. and Barter, S. R.: Determination of the potent flavour compound rotundone in grapes and wine using MDGC-MS and membrane assisted solvent extraction, Posterpräsentation, 37th International Symposium for Capillary Chromatography (2013) Palm Springs, USA.

[2] Wood, C.; Siebert, T. E.; Parker, M. et al.: From Wine to Pepper: Rotundone, an Obscure Sesquiterpene, Is a Potent Spicy Aroma Compound, J. Agric. Food Chem. 56 (2008) 3738-3744

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