

qNMR metabolomics for authenticity and winemaking processes discrimination

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Introduction

Nuclear Magnetic Resonance (NMR) is a spectroscopic technique that has been increasingly used for food analysis over the last 20 years. Its non-specific and non-selective properties make it a tool of choice for metabolomics, a recent science that studies all primary and secondary metabolites in a biological sample. NMR has many advantages including the ability to simultaneously analyze different families of molecules in a single analysis without prior preparation and from a small volume of material. Moreover, it is a quantitative technique since the signal area is proportional to the concentration of metabolites detected on the spectrum. This is known as quantitative NMR (qNMR).

Applied to wine, qNMR metabolomics makes it possible to observe the impact of different parameters on the chemical composition of wine such as grape variety, geographical origin or vintage (Valls Fonayet *et al.*, 2021). It is now known that proton NMR is a technique for distinguishing wines from different regions, grape varieties and vintages (López-Rituerto *et al.*, 2012; Godelmann *et al.*, 2013; Gougeon *et al.*, 2018). These results demonstrated that NMR is an efficient tool to guarantee the authenticity of wines. Fraud is a widespread problem in the wine industry. To overcome this problem, the creation of a database was carried out to collect a large number of authentic samples where each analysis is considered as an identity card. The collection leads to the elaboration of a profile for each region, grape variety or vintage. Thus, if there is a suspicion of fraud, the suspect sample is either directly compared to the authentic sample provided by the chateau or compared to a large set of data.

This collection has led to the discovery that NMR spectra distinguish wines not only within the same region but within the same appellation and from châteaux only a few kilometers away. This finding suggested that NMR analysis, rather than being able to distinguish environmental parameters, could be used to discriminate winemaking processes. We have studied several processes commonly used in oenology: winemaking temperature, filtration and the use of

enzymes. The aim of this work is to provide a better understanding of the interactions between oenological processes and wine by determining the metabolites responsible for differentiation through $^1\text{H-NMR}$ fingerprinting and chemometrics.

Material and methods

Sample preparation and analyses were done as described in our previous article (Gougeon *et al.*, 2019a). Briefly, wine samples were mixed with phosphate buffer and deuterated water solution (ratio 7:2:1). Trimethylsilylpropanoic acid sodium salt (TMSp) and calcium formate were used as frequency and quantification references, respectively. The pH was adjusted to 3.1 using a BTpH unit (Bruker BioSpin, Germany). The $^1\text{H-NMR}$ spectra were acquired on a 600 MHz spectrometer (Bruker BioSpin, Germany). Two specific sequences were used for the quantification of wine compounds. The ZGPR pulse program was used for water signal suppression. NOESYGPPS1D sequence was performed for the suppression of water and ethanol signals. Spectra were processed using Topspin 3.2 (Bruker Biospin, Germany) and analyzed using MestReNova 12 (Mestrelab Research, Spain). Spectra were submitted to phase and baseline corrections before data extraction. Finally, compounds were semi-automatically quantified using the plugin Simple Mixture Analysis (SMA) of MestReNova software.

Comparison between two wines were performed using an adapted z-score value approach allowing the calculation of a similarity score (*s* score) for each wine constituent (Gougeon *et al.*, 2019b). A-s-score higher than 2.0 (confidence interval of 95%) suggests significant difference between the two wines. SIMCA 16 software (Umetrics, Sweden) was used for unsupervised principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA). PCA was carried out in first stage to visualize the data. In a second stage, PLS-DA was completed to refine the discrimination between sets.

Results and discussion

$^1\text{H-NMR}$ Spectroscopy

A representative spectrum of wine after water and ethanol suppression was presented in Figure 1. Compounds were identified by using pure chemical standard, 2D-NMR experiments and literature data (Fotakis *et al.*, 2013). Today, nearly fifty wine constituents were identified and quantified including organic acids, alcohols, amino acids, phenolic compounds and sugars (Gougeon *et al.*, 2019a).

To perform an accurate quantification, $^1\text{H-NMR}$ spectra were exported into MestReNova 12

and treated using the plugin SMA (Gougeon *et al.*, 2018). Peak deconvolution was realized using global spectral deconvolution method (Cobas *et al.*, 2011). The precision of the method was validated by comparison with reference samples and by comparison with official OIV methods (Gougeon *et al.*, 2019b).

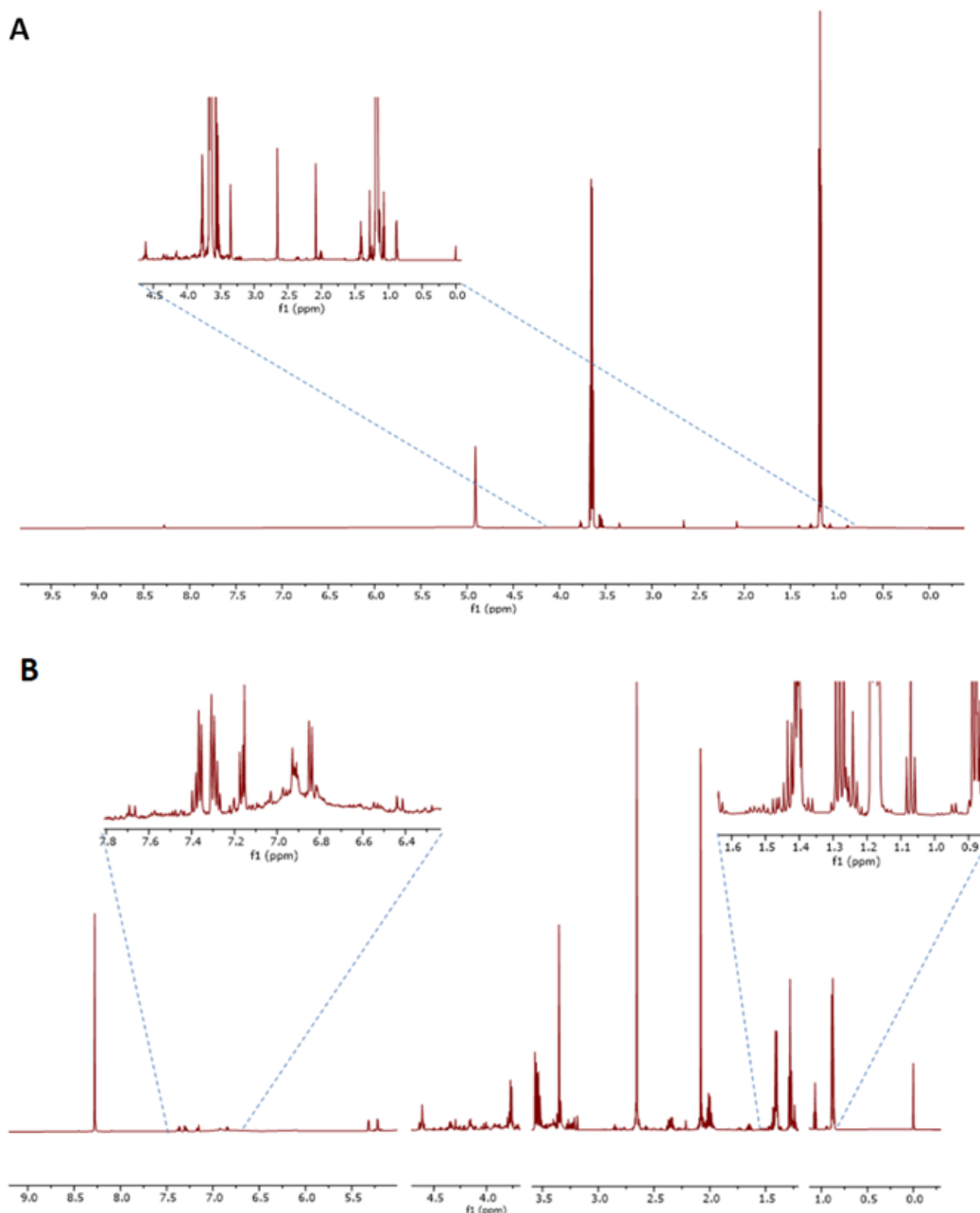


Figure 1. Representative ¹H-NMR spectra of a wine sample after signal suppression (A: water suppression using ZGPR sequence; B: water and ethanol signals suppression using NOESYGPPS1D sequence).

Wine authenticity

In order to guarantee the authenticity of a wine, two approaches can be used. The first approach is to directly compare suspect sample with the sample from the authentic bottle. If it is a fraud, the two spectra will be different either in composition (differences detected at the level of the molecules observed) or in content (concentrations of the different constituents of the wine). To allow the comparison of the data and to take into account the wine aging in bottle, a statistical tool called *s* score has been developed (Gougeon *et al.*, 2019b). This technique was validated by comparison of usual wine analysis performed using OIV official methods and qNMR metabolomics. Compared to classical methods, NMR could reduce the volumes of wine required and the duration of analysis as a first approach of authenticity control of wines. To illustrate the process, the *s* score comparison of an authentic and a fake wine to the reference wine provided by the winery were presented in the Figure 2. Wines presenting more than four *s* scores higher than two could be considered significantly different.

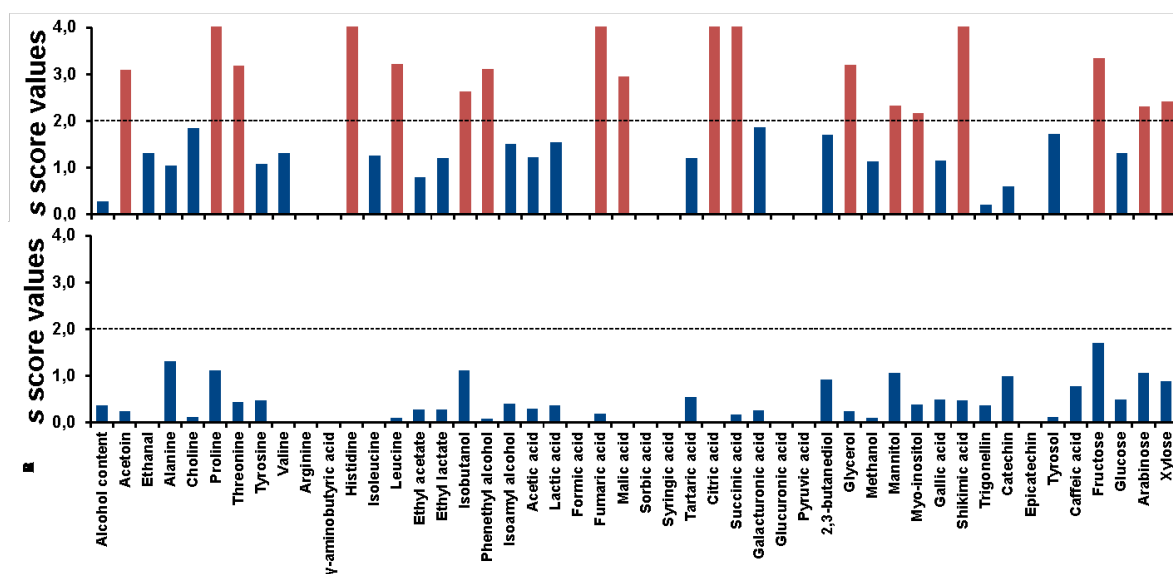


Figure 2. Comparison of *s* score values of a fake wine (A) and an authentic one (B) to the reference wine provided by the winery.

The alternative, when an authentic sample cannot be obtained, is to compare the suspect sample with a database. Indeed, we know that the composition of wine varies according to grape variety, vintage, geographic origin and viticultural practices. Thus, the collection of a large number of samples leads to the elaboration of a profile for each appellation, region or grape variety. When an unknown sample is confronted with the database, we are able to determine its origin through statistical analysis (Gougeon *et al.*, 2019a). To illustrate the ability of qNMR metabolomics to discriminate wines, analytical data of wines from different vintages

of three close wineries of Bordeaux were compared by partial least squares discriminant analysis (PLS-DA). As shown in Figure 3, the three sets of wines are clearly discriminate over a near ten-year period.

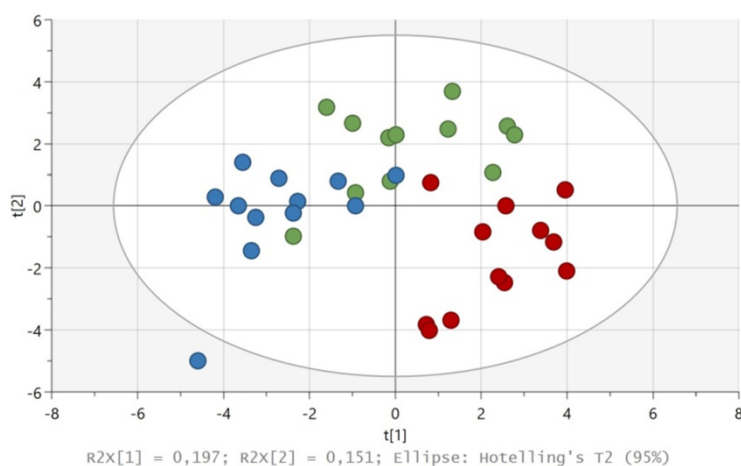


Figure 3. PLS-DA score plot based on qNMR analyses of wines from three close wineries of Bordeaux (winery A: blue circle; winery B: red circle; winery C: green circle) over a decade.

Winemaking processes discrimination

Three processes commonly used in oenology were studied: thermovinification, filtration and enzyme addition. The results are presented in Figure 4.

Thermovinification is a process initially used to eliminate the laccases produced by *Botrytis cinerea*, responsible for organoleptic defects. In this study two sets of wines processed using thermovinification or conventional temperature were analyzed by qNMR metabolomics. The PLS-DA score plot (Figure 4A) show that thermo-vinified samples are significantly different from samples vinified at conventional temperatures. The loading plot indicates that the molecules involved in the discrimination are: galacturonic, lactic and succinic acids and xylose for thermovinified wines; and fructose, proline, choline and acetic acid for samples vinified at conventional temperature. These results prove that thermovinification does not only have an impact on phenolic compounds and the color of the wine, but also on other compounds such as organic acids, sugars and amino acids.

Filtration is a post-fermentation process that contributes to the clarification and conservation of wines. Filtered and unfiltered wines were analyzed by qNMR metabolomics. The PLS-DA score plot (Figure 4B) shows that NMR analyses discriminate filtered samples from unfiltered samples. The molecules most impacted by filtration are succinic and lactic acids, isobutanol, glycerol and acetoin. These data suggest that filtration process not only impacts large molecules but also low molecular weight ones.

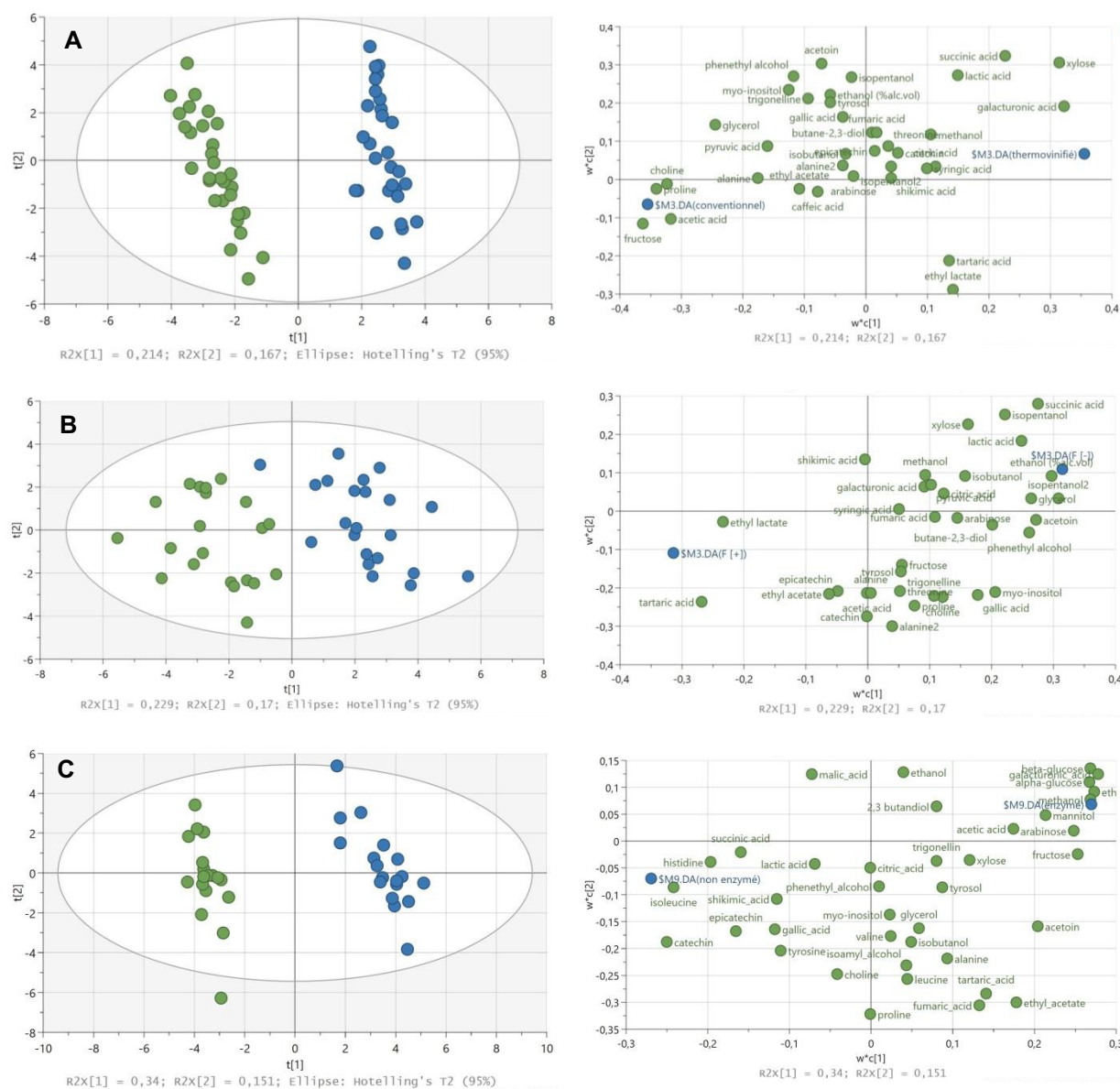


Figure 4. PLS-DA score and loadings plots based on qNMR analyses of wines from three winemaking processes (A: thermovinification; B: filtration; C: enzyme addition).

The use of enzyme is a very widely used process for the clarification and stabilization during winemaking. Wines with or without addition of enzymes were analyzed by qNMR metabolomics. Analytical results also show that this process has an impact on the wine metabolome since the untreated samples are significantly different from those that have undergone enzyme addition (Figure 4C). The PLS-DA loadings show that the molecules involved are: glucose, galacturonic acid, methanol, ethyl lactate, mannitol, arabinose and fructose for treated samples and catechin, histidine and isoleucine for untreated samples. This is the first time that molecules impacted by the use of enzymes are highlighted. Indeed, the differences are generally found by sensory analysis but never by classical chemical analysis.

Conclusion

The results obtained illustrate the capacity of qNMR combined with chemometrics to discriminate wines. Applied to winemaking processes, qNMR metabolomics allows discriminating not only physical processes such as filtration, but also chemical processes such as thermovinification, and enzymatic treatment. In addition, the metabolites involved in the discrimination of these winemaking processes are highlighted. To conclude, ¹H-NMR metabolomics is a fast technique that could be used as a tool to help professionals decide on technical itineraries.

Acknowledgments

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Abstract

qNMR Metabolomic applied to wine offers many possibilities. The first application that is increasingly being studied is the authentication of wines through environmental factors such as geographical origin, grape variety or vintage (Gougeon et al., 2019). Another less common approach is from a qualitative point of view by studying the various oenological practices used that are an integral part of the elaboration of a wine. We wondered whether quantitative NMR could be used to dissociate the physical or chemical processes commonly used in oenology. The objective of this work was to provide a better understanding of the interactions between oenological processes and wine by determining the metabolites responsible for differentiation through ¹H-NMR fingerprinting and chemometrics.

About 40 molecules were quantified on wine samples that have undergone several physical and chemical processes. Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA) and S-score were performed for the analytical discrimination of winemaking processes.

The results obtained show that qNMR combined with chemometrics allows to dissociate not only physical processes such as filtration, but also chemical processes such as maceration temperature, enzymatic treatment and fining. In addition, the metabolites involved in the discrimination of these winemaking processes could also be determined.

The ¹H-NMR metabolomics is a fast technique that could be used as a tool to help professionals decide on technical itineraries.

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