



**ORIGINAL RESEARCH ARTICLE**

# Monitoring red wine maturation in oak barrels using <sup>1</sup>H NMR-based metabolomics

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Associate editor:  
David Jeffery



Received:  
14 March 2023

Accepted:  
11 April 2024

Published:  
6 May 2024

## ABSTRACT

Wine maturation in oak barrels is a well-known winemaking practice that, among other things, brings roundness and complexity to wine. Studying wine evolution during barrel maturation is crucial to ensure wine quality. Red wines produced in a wine growing estate of the Bordeaux region were monitored after one and twelve months of maturation in oak barrels in the estate's cellar. Wines were kept in oak casks from three different manufacturers. The evolution of the wine constituents during maturation was monitored by <sup>1</sup>H NMR-based metabolomics combined with multivariate statistical analysis. NMR spectra were submitted to targeted and untargeted approaches. The wine maturation effects were thereby monitored and the discriminant metabolites identified. The wines analysed after one month of maturation exhibit higher contents of amino acids, catechin and epicatechin, acetoin and choline than the wines analysed twelve months after maturation; for their part, the latter wines showed higher contents of acetic acid, ethyl lactate, arabinose and glucose. In addition, significant differences were observed between the wines depending on the barrel manufacturers.

**KEYWORDS:** Wine ageing, <sup>1</sup>H NMR, targeted analysis, fingerprinting



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## INTRODUCTION

Maturation of wine occurs between the end of the maceration and the bottling stage. Wine can be stored in different containers, including stainless-steel tanks, concrete tanks or oak barrels. Oak barrel maturing is very popular nowadays, because it positively influences the quality and aromas of the wines (Garde-Cerdán & Ancín-Azpilicueta, 2006). Many Bordeaux wines, especially the famous ones, are matured in oak barrels. The use of a barrel is not enough to produce a wine of high quality. It is also necessary to carry out this oenological method in a sustained way and with expertise. Wine ageing in an oak barrel promotes a balance between oxygen and the various constituents of wine, such as tannins, colours, aromas, proteins and enzymes. During this stage, wine is subject to many chemical changes due to reactions between the wine constituents, the micro-oxygenation through the staves and the compounds extracted from the oak barrel (Fernández de Simón *et al.*, 2014; Maioli *et al.*, 2022).

Recently, <sup>1</sup>H NMR-based metabolomics has emerged as a meaningful tool to ensure the traceability of alcoholic beverages, such as spirits (Teipel *et al.*, 2020) and wines (Le Mao *et al.*, 2023; Solovyev *et al.*, 2021; Valls Fonayet *et al.*, 2021). Based on quantitative analysis (targeted approach) or fingerprinting (untargeted approach), NMR metabolomics can be used to control geographical origin, real composition (including grape variety, for example) and vintage. More recently, <sup>1</sup>H NMR-based metabolomics has been used successfully to monitor wine quality during the winemaking process (Le Mao *et al.*, 2021; López-Rituerto *et al.*, 2022).

Red wine maturation in oak barrels induces numerous chemical transformations as a result of different mechanisms, including oxidative reactions and compounds released from oak (Alcaide-Hidalgo *et al.*, 2007; Ma *et al.*, 2022). In turn, this maturation process induces significant sensory changes in the final wine. Recently, Cassino *et al.* have demonstrated the interest of applying <sup>1</sup>H NMR when investigating wine evolution during bottle ageing (Cassino *et al.*, 2019). The aim of the present study was to investigate the evolution of wine during maturation in oak barrels via NMR-based metabolomics. Monitoring wine compound evolution during maturation could be useful for controlling overall wine quality. Red wine was conserved in new oak barrels provided by three different manufacturers, and analysed after one and twelve months conservation in the controlled environment of a cellar. The wine constituents were monitored via <sup>1</sup>H NMR spectrometry combined with multivariate statistical analysis.

## MATERIALS AND METHODS

### 1. Wine sample preparation

The red wine used for the experiment came from a Bordeaux winery in Pauillac (France), which is in the Medoc Controlled Denomination of Origin. It was produced from a blend of the three varieties Cabernet Sauvignon, Merlot and Cabernet Franc, all harvested in 2018. Following malolactic

fermentation, the wine was pressed, clarified using eggs and stabilised with SO<sub>2</sub>. Next, the wine was transferred to new 225 L French oak barrels from three different manufacturers (hereafter referred to as MA, MB and MC), which had undergone similar levels of toasting for a year; The MC barrels were of two different models (MC1 and MC2). During wine maturation, the usual operations linked to barrel management were performed in the cellar, including ullage and racking. Free SO<sub>2</sub> was maintained at approximately 30 mg/L. Wine samples were collected from six different barrels from each manufacturer after a month of maturation. This operation was repeated in the same barrels twelve months later.

For the NMR analysis, samples were prepared in the same way as in our previous work (Le Mao *et al.*, 2021). Briefly, wine samples underwent a first step of centrifugation for 3 min at maximum *g*force of 7 000 × *g* on a MiniSpin microcentrifuge from Eppendorf (Eppendorf, France). They were next prepared as follows: (7:2:1, v/v/v) wine, phosphate buffer (1 M, pH 2.6) and D<sub>2</sub>O containing 0.5 mM trimethylsilylpropanoic acid (TMSP) and 7 mM calcium formate (FCa). Finally, the pH was adjusted to 3.1 with HCl and NaOH solutions (1 M) by a small-scale semi-automatic system (BTpH, Bruker BioSpin, Germany). Finally, 600 µL of the wine solution was introduced into 5 mm tubes for NMR analysis.

### 2. NMR spectroscopic analysis of wines

<sup>1</sup>H NMR experiments were performed on an Avance III NMR spectrometer (Bruker, France) operating at 600.27 MHz and equipped with a 5 mm TXI probe with Z-gradient coils. All measurements were performed at 293 K, using Topspin 4.0.8 software (Bruker, France). Three magnetic pulse sequences were used: zg30 to determine the resonance frequency of the water signal; zgpr and noesygpps1d (1D-NOESY) for the suppression of the water; and ethanol signals with 8 and 32 scans (ns) respectively. Data acquisition parameters were set as follows: free induction decay (FID) was collected in a time domain (TD) of 64K data points and with a spectral width (SW) of 16 ppm, an acquisition time (AQ) of 3.40 s and a relaxation time (RD) of 5 s per scan. The 90° pulse calibration was automatically calibrated for each sample. The FID was multiplied by an exponential function corresponding to a line broadening factor of 0.3 Hz before Fourier transformation. Manual phase and baseline correction was applied to the resulting spectrum, which was then manually phased and zero aligned using the TMSP signal.

In the targeted analyses, 32 components (Table S1) were quantified using the Simple Mixture Analysis (SMA) plugin of the MestReNova 12.0 software (Mestrelab Research, Spain). The untargeted approach was performed with NMRProcFlow 1.4 online. 1D-NOESY spectra were exported into NMRProcFlow for alignment, baseline correction and intelligent bucketing binning with variable size bin (De Meyer *et al.*, 2008). The spectra were first calibrated to 0.00 ppm using TMSP, and then underwent local baseline correction by selecting the region upstream and downstream of the water and ethanol signals.

The signals were then aligned and cut into 536 bins (Table S2). Regions affected by solvent suppression were excluded from the binning. For the analysis, the data were divided into three parts comprising the aromatic region (10.0 – 5.5 ppm), the carbohydrate region (5.5 – 3.7 ppm), and the aliphatic region (3.6 – 0.5 ppm).

### 3. Statistical analysis

All the data collected from the targeted and untargeted analyses were independently exported as an Excel XML file in SIMCA 16.0 software (Sartorius, Germany) to perform multivariate statistical analysis (MSA). Scaling to unit variance (uv) and mean centring were applied. Principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA) were used to visualise the distribution of the metabolic variance and to sharpen the separation between observations groups respectively. The reliability of the models was verified by analysis of variance testing of cross-validated predictive residuals (CV-ANOVA). Validity and degree of overfit were checked by a permutation test. The accuracy of the models was estimated based on area under the receiver operating characteristic curves (AUC-ROC curves) and misclassification tables. The potential discriminant metabolites were identified using the variable importance of projection (VIP) and the loadings scaled as the correlation coefficient ( $p(\text{corr})$ ) values.

For the selected compounds, the statistical significance based on the analysis of variance (ANOVA) or t-test were calculated using GraphPad Prism 9.3.0 software (GraphPad Software, USA).

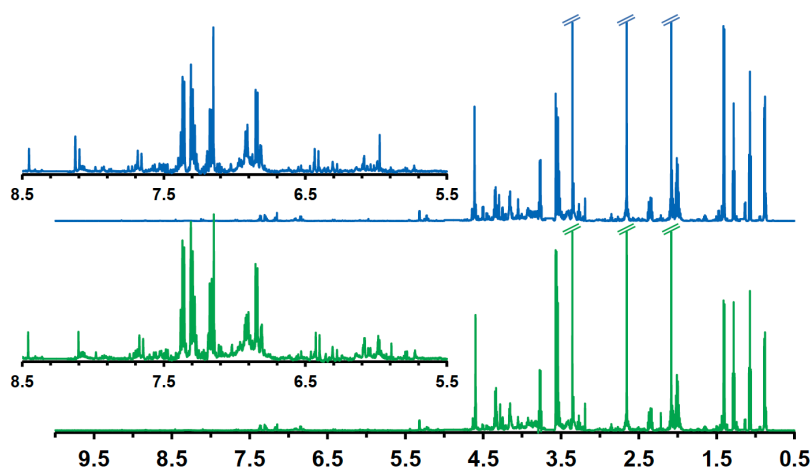
## RESULTS AND DISCUSSION

### 1. Effect of wine maturation in oak barrels

#### 1.1. $^1\text{H}$ NMR Fingerprinting

In this first trial, the effect of wine maturation in oak barrels was monitored using a combination of  $^1\text{H}$  NMR-based metabolomics and MSA. The wines matured in the different oak barrels of each manufacturer were analysed after one and twelve months of maturation ( $n = 24$  each).

To observe the effect of the maturation process on the overall  $^1\text{H}$  NMR spectrum, a metabolomics approach based on  $^1\text{H}$  NMR fingerprinting was applied to the wine spectra. Representative  $^1\text{H}$  NMR spectra corresponding to one and twelve months of maturation are shown in Figure 1. In the untargeted analysis,  $^1\text{H}$  NMR spectra were processed using the software NMRProcFlow (Jacob *et al.*, 2017). A total of 536 bins were obtained with the full range of 10.0 - 0.5 ppm (Table S2).



**FIGURE 1.** Typical  $^1\text{H}$  NMR spectra (1D-NOESY) of wine samples after one (green) and twelve months (blue) of maturation in oak barrels.

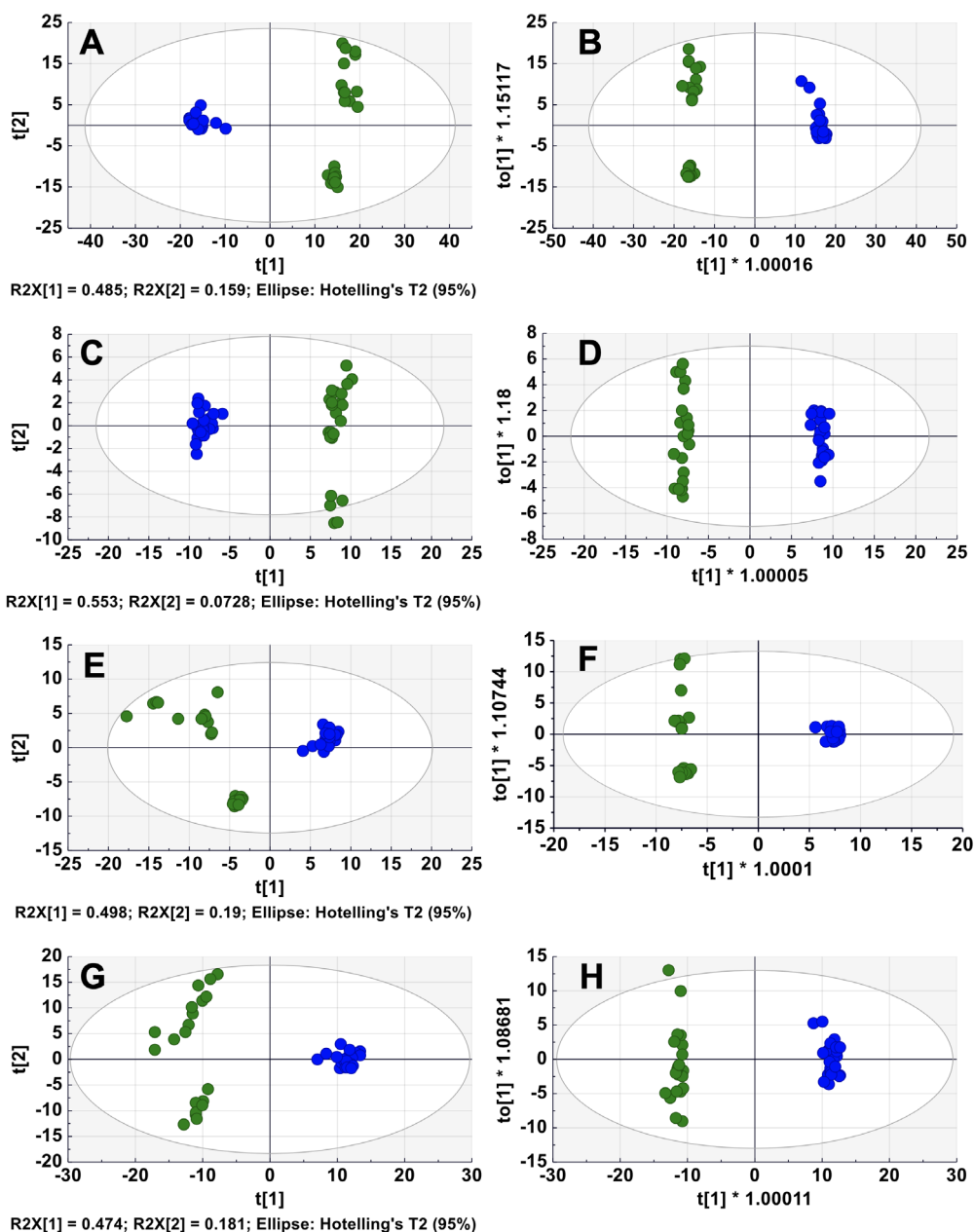
Unsupervised PCA followed by supervised OPLS-DA were applied to observe classification trends based on fingerprinting data (Wheelock & Wheelock, 2013; Worley & Powers, 2016). MSA was applied to the entire peak lists derived from the full spectrum binning (full spectrum, 10.0 – 0.5 ppm). In addition, to better observe the influence of the different families of compounds, the data were divided into three parts: the aromatic region (10.0 – 5.5 ppm), containing mainly phenolic compound signals; the carbohydrate region (5.5 – 3.7 ppm), containing mainly sugar signals; and the aliphatic region (3.6 – 0.5 ppm), containing the signals of organic acids and alcohols. Depending on their side chain, signals of amino acids were distributed across the three regions.

The PCA score plot ( $R^2X = 0.857$ ;  $Q^2 = 0.729$ ) applied to the data matrix obtained from  $^1\text{H}$  NMR full spectrum is shown in Figure 2A. A clear differentiation between the wines matured one and twelve months in oak barrels can be seen along the first principal component (PC1). The two first principal components explain 48.5 % and 15.9 % of the total variance respectively. The effect of the different barrels can be seen to be less pronounced after twelve months of maturation. Further classification of the wines is represented in Figure 2B in the form of a OPLS-DA score plot based on the full spectrum data. The performance and validation parameters for the OPLS-DA model are summarised in Table 1. A highly satisfactory OPLS-DA model was obtained to discriminate

the two stages of wine maturation in oak barrels. The model quality evaluation is generally based on the  $R^2Y$  and  $Q^2$  values (Li *et al.*, 2017). In metabolomics,  $Q^2 > 0.5$  is considered to be good and  $Q^2 > 0.9$  excellent. In addition, the difference between  $R^2Y$  and  $Q^2 > 0.3$  suggests irrelevant or outlying data. The values obtained based on full spectrum data indicate the high quality of the OPLS-DA model ( $R^2Y$  and  $Q^2$  of 0.995 and 0.993 respectively). The model was validated by the CV-ANOVA p-value ( $< 0.05$ ) and permutation test, for which  $R^2Y$  and  $Q^2$  values intercept in Y-axis are expected to be  $< 0.4$  and negative respectively (Yuan *et al.*, 2020). As shown in

Table 1,  $R^2Y$ - and  $Q^2$ -intercept values (100 permutations) meet these criteria. In addition, the correct classification rate (CCR) obtained from the misclassification table and the area under the curve (AUC) obtained from the receiver operating characteristic (ROC) plot (100 % and 1.0 respectively) confirm the highly predictive performance of the model.

The OPLS-DA loadings plot for the discrimination between one and twelve months maturation in oak barrels indicates both positive and negative contributions of the different regions of the NMR spectrum (Figure S1).



**FIGURE 2.** MSA of  $^1H$  NMR fingerprinting data after one and twelve months of maturation in oak barrels.

A and B: PCA and OPLS-DA score plots from full spectrum data (10.0 - 0.5 ppm) respectively; C and D: PCA and OPLS-DA score plots from aromatic region data (10.0 - 5.5 ppm) respectively; E and F: PCA and OPLS-DA score plots from carbohydrate region data (5.5 - 3.7 ppm); G and H: PCA and OPLS-DA score plots from aliphatic region data (3.6 - 0.5 ppm). Green = after one month of maturation; blue = after twelve months of maturation.

**TABLE 1.** Performance and validation parameters of OPLS-DA models for classification of wine maturation in oak barrels from targeted and untargeted NMR data.

Model parameters	Targeted data	Untargeted data			
		Full spectrum	Aromatic	Carbohydrate	Aliphatic
No of variables	32	536	128	124	284
R <sup>2</sup> X	0.616	0.627	0.608	0.791	0.557
R <sup>2</sup> Y	0.985	0.995	0.996	0.996	0.995
Q <sup>2</sup>	0.964	0.993	0.992	0.991	0.993
NC	1 + 2 + 0	1 + 1 + 0	1 + 1 + 0	1 + 2 + 0	1 + 1 + 0
Permutation tests (R <sup>2</sup> ; Q <sup>2</sup> )	0.340; - 0.553	0.179; - 0.324	0.340; - 0.367	0.187; - 0.404	0.106; - 0.342
CV-ANOVA	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
AUC	1.0	1.0	1.0	1.0	1.0
Fisher's probability	3.1 × 10 <sup>-14</sup>	3.1 × 10 <sup>-14</sup>	3.1 × 10 <sup>-14</sup>	3.1 × 10 <sup>-14</sup>	3.1 × 10 <sup>-14</sup>
CCR (%)	100 %	100 %	100 %	100 %	100 %

NC = No. of components; AUC = area under the ROC curve; CCR = correct classification rate.

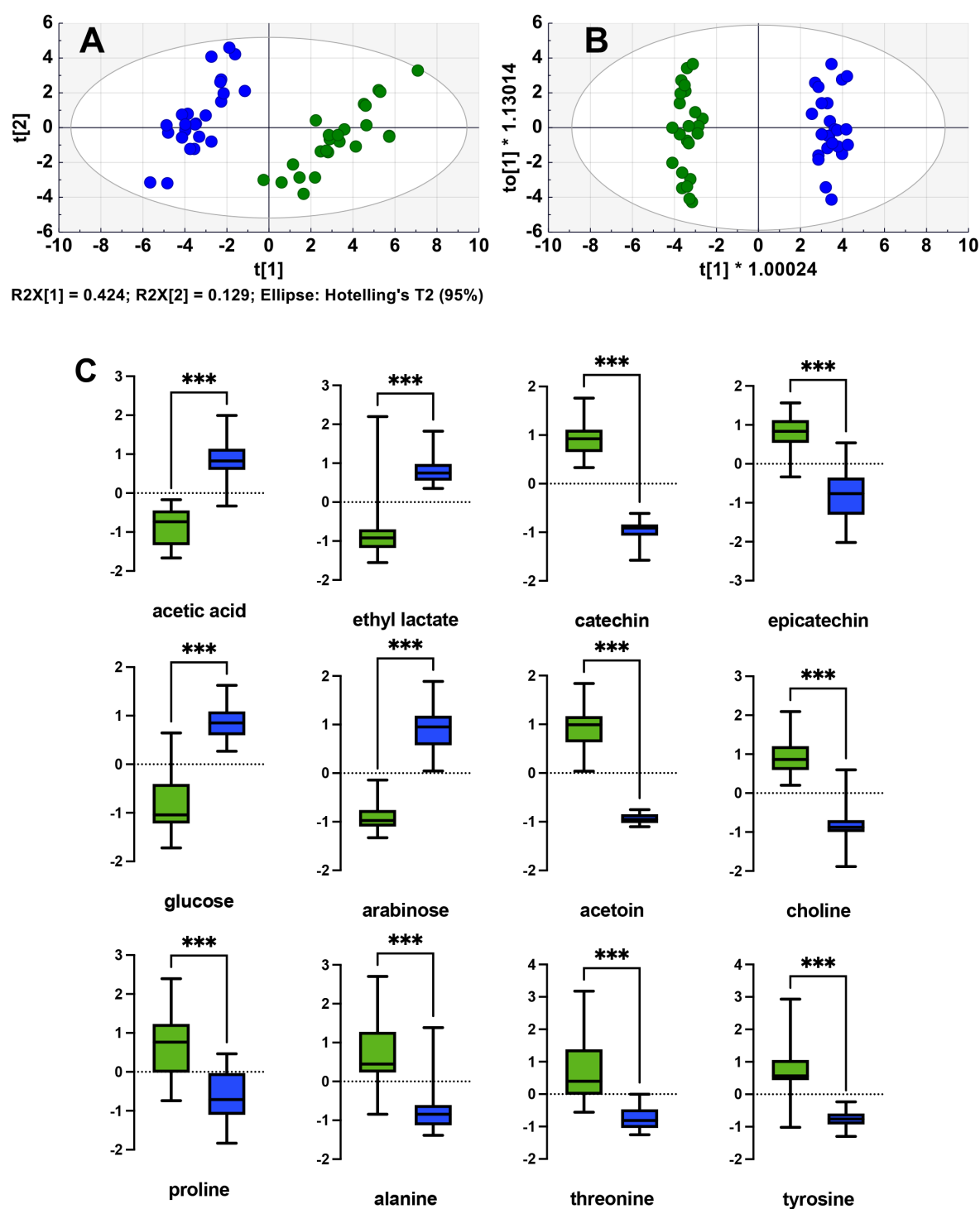
To further identify the NMR signals involved in the classification, PCA and OPLS-DA were applied on the three different regions selected above. The score plots are shown in Figure 2: PCA and OPLS-DA models for the aromatic region (Figures 2C and 2D respectively), carbohydrate region (Figures 2E and 2F respectively) and aliphatic region (Figures 2G and 2H respectively). In the PCA score plots, a clear and significant separation appears between the samples of one and twelve months of ageing along PC1 for the three regions ( $R^2X > 0.7$  and  $Q^2 > 0.6$  for each region). The first PC explains 55.3, 49.8 and 47.4 % for the aromatic, carbohydrate and aliphatic regions respectively. The wine samples analysed after twelve months of maturation seem more homogeneous than those analysed after one month. Like in the full spectrum analysis, the OPLS-DA models exhibited two well-differentiated clusters when classifying one and twelve months of maturation. The results of model performances and validation tests for the different <sup>1</sup>H NMR regions are summarised in Table 1. All the models showed  $R^2Y$  and  $Q^2$  values  $> 0.99$ , and a CV-ANOVA p-value  $< 0.05$ . As with the full spectrum data, the permutation tests (100 permutations) indicated the absence of overfitting. The CCR and AUC values (100 % and 1.0 for each model) confirm the excellent performance of the different models.

Many studies have shown that the barrel acts as an active vessel for wine constituents (del Alamo-Sanza & Nevares, 2018; Jordão & Cosme, 2022; Li & Duan, 2019). Wines aged in barrels develop more complex aromas and acquire roundness. Nevertheless, it is the know-how of the winegrower that will ultimately define the quality of the wine. In this study, <sup>1</sup>H NMR fingerprinting combined with MSA

was carried out to investigate wine maturation in oak barrels. These results show that maturation has a significant impact on all families of compounds as observed via <sup>1</sup>H NMR-based metabolomics. Interestingly, a higher degree of dispersion was observed in the orthogonal components for the wine samples analysed at one month than those analysed at twelve months of maturation.

## 1.2. Marker identification of wine maturation

The results presented above show that maturation has a significant impact on all families of compounds as observed via <sup>1</sup>H NMR-based metabolomics. The complexity of the <sup>1</sup>H NMR spectrum and numerous spectral features extracted from MSA make direct profiling from untargeted analysis difficult. To identify the maturation markers, a targeted analysis of the main compounds was carried out using a method developed in the laboratory (Le Mao *et al.*, 2021). Briefly, compounds were identified by a combination of 2D NMR experiments and pure standards. This method makes it possible to identify different wine metabolites including organic acids, amino acids, alcohols, sugars, polyphenols, sugars and other organic compounds (Table S1). Compounds were quantified by global spectral deconvolution using our own spectral database implemented in the simple mixture analysis (SMA) plugin of MestReNova 12.0 software. In this study, thirty-two compounds were assayed from the targeted data. The fingerprinting data was processed via MSA. The PCA and OPLSDA score plots are shown in Figures 3A and 3B respectively. The PCA model ( $R^2X = 0.647$ ;  $Q^2 = 0.547$ ) exhibits a clear differentiation between the two classes of wines along PC1. The two first principal components explain 42.4 and 12.9 % of the variance respectively.



**FIGURE 3.** MSA of  $^1\text{H}$  NMR targeted data after one and twelve months of maturation in oak barrels.

A and B: PCA and OPLS-DA score plots respectively; C: boxplots from mean-centred values of significantly discriminant metabolites. Statistical significance was checked by unpaired *t* test or Mann-Whitney's test ( $***p < 0.001$ ). Green = one month of maturation; blue = twelve months of maturation.

The OPLS-DA model exhibits two clusters to discriminate one and twelve months of maturation.  $R^2Y$  and  $Q^2$  values close to 1 (0.985 and 0.964 respectively), and the CV-ANOVA p-value ( $< 0.05$ ) indicate the goodness of the model (Table 1). The permutation test (100 permutations) indicates the absence of over fit. In addition, the CCR and AUC values from the ROC curves (100 % and 1.0 respectively) confirm the excellent performance of the model.

The main discriminant metabolites in the two classes of wine were identified based on a combination of variable importance in projection (VIP) and  $p(\text{corr})$  values (Le Mao *et al.*, 2021). Only metabolites that showed VIP and  $p(\text{corr})$  values of  $> 1.0$  and  $> 0.5$  respectively were considered potential markers and confirmed by the t-test ( $p < 0.05$ ). Following this methodology, a total of twelve discriminant markers were identified (Figure 3C): five amino acids (tyrosine, threonine, alanine, proline and leucine), one organic acid (acetic acid), two sugars (glucose and arabinose), two phenolic compounds (catechin and epicatechin), one ester (ethyl lactate) and choline. Wines analysed after one month of maturation exhibited higher contents of amino acids, catechin and epicatechin, acetoin and choline, whereas wines after twelve months had higher contents of acetic acid, ethyl lactate, arabinose and glucose.

The results indicate that it is possible to monitor wine maturation using  $^1\text{H}$  NMR-based metabolomics. Both targeted and untargeted approaches discriminate the two classes of wines. The untargeted approach is more informative, but is more difficult to analyse. The discriminant metabolites identified in this study are globally in agreement with those previously highlighted during the wine ageing in bottle (Cassino *et al.*, 2019). Increasing of acetic acid amounts could be connected to the presence of acetic acid bacteria or to ethanol oxidation into acetaldehyde and later into acetic acid, due to the oxygenation process in oak barrels. Similarly, increasing ester levels during ageing, such as ethyl lactate, could be the result of the reaction of ethanol with organic acids (Shinohara & Shimizu, 1981). Decreasing of catechin and epicatechin levels during wine maturation was expected. These monomeric flavanols are subjected to various polymerization and condensation reactions during wine ageing (Pérez-Magariño & González-San José, 2004).

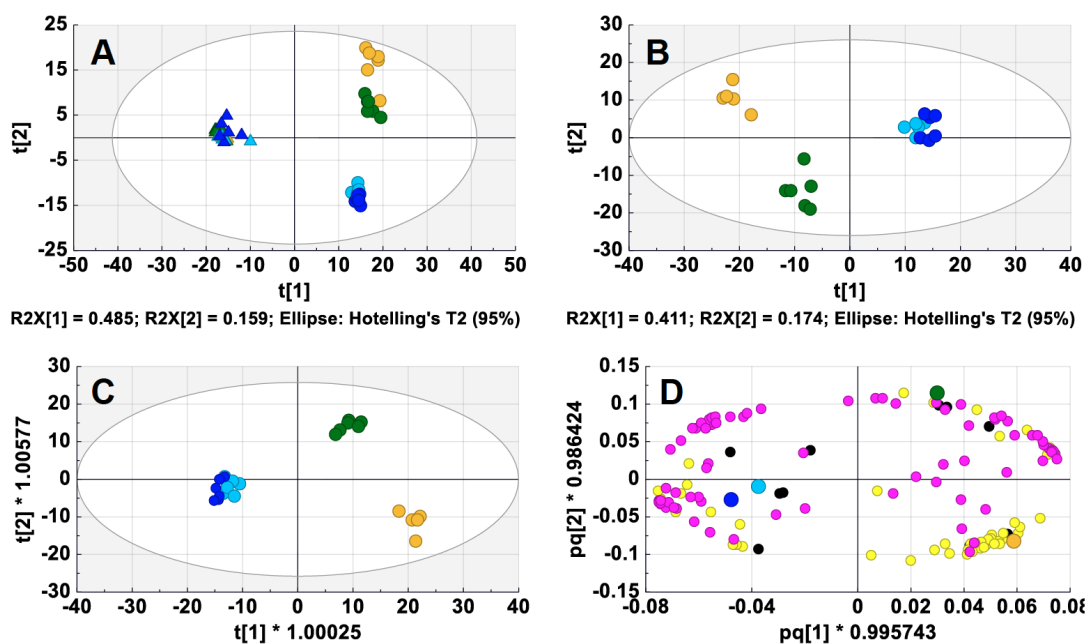
Even if few studies have been conducted on residual sugars in red wines, they are known to be a source of energy for microorganisms and to undergo different reactions. The evolution of monosaccharide content during wine maturation could be due to different phenomena, such as wood hemicellulose hydrolysis and polyphenol degradation on the one hand, and microorganism consumption or Maillard reactions with amino acids on the other (del Alamo *et al.*, 2000; Zhang *et al.*, 2018). In the present study, an increase in two monosaccharides (glucose and arabinose) during wine maturation was observed. An increase in arabinose content has been associated with hemicellulose degradation of the barrel (del Alamo *et al.*, 2000), and in the same study, the observed increase in hexoses may have been linked to an increase in pentoses. An increase in acetoin levels in red

wine during wine ageing in the bottle has previously been described (Han *et al.*, 2019). Acetoin formation has been associated with acetaldehyde levels and diacetyl reduction during wine ageing. In the present study, a decrease in acetoin was observed during barrel maturation. This trend is consistent with the decrease in acetoin content during wine ageing in the bottle reported by Cassino *et al.* (Cassino *et al.*, 2019). The formation and evolution of acetoin in the presence of microorganisms is well known. Further studies will be necessary to clarify its evolution during wine maturation. Even if choline is considered an essential nutrient for humans that is mainly found in food (Zeisel *et al.*, 1991), little is known about choline in wine. In line with the results of Cassino *et al.* (2019) we observed a decrease in choline levels during wine ageing (Cassino *et al.*, 2019). Amino acid content and its evolution during wine ageing have been closely correlated with production methods (Alcaide-Hidalgo *et al.*, 2007). In our case, the decrease in amino acids was probably due to different factors, such as processes linked to the filtering and racking of the wine before and during maturation in the oak barrels. In addition, the decrease in amino acids may have been due to an interaction with tannins or to the Strecker reaction with aldehydes (Pripis-Nicolau *et al.*, 2000).

## 2. Effect of oak barrel origin

As mentioned previously, MSA from NMR fingerprinting shows higher sample heterogeneity after one month of maturation than after 12 months. One of the possible reasons for this is the quality of the barrels: barrel properties can act on both the oxygenation process and the release of oak compounds. To strengthen this hypothesis, the MSA was repeated taking into account the origin of the barrel given that they came from three different manufacturers (MA, MB, and MC) with two different qualities for the manufacturer MC (MC1 and MC2).

The PCA score plot of all the wine samples from the NMR fingerprinting taking into account the barrel origin (i.e., the different manufacturers) is shown in Figure 4A. It is clear from the plot that the observed heterogeneity after one month of maturation in the barrels is linked to the manufacturer, whereas the wine samples are more homogeneous after twelve months of maturation regardless of barrel origin. To confirm this trend, the samples taken at one month ( $n = 24$ ) and after twelve months ( $n = 24$ ) of maturation were analysed separately. Regarding wine samples analysed after one month of maturation, the PCA score plot from NMR fingerprinting ( $R^2X = 0.647$ ;  $Q^2 = 0.547$ ) shows a clear discrimination between the three manufacturers (Figure 4B). The first two principal components explain 41.1 % and 17.4 % of the total variance. Wine samples from manufacturers MA and MB ( $n = 6$  each) are discriminated from those of MC ( $n = 12$ ) along PC1. A clear classification between MA and MB wine samples can be observed along PC2, whereas no evident separation can be seen between the two barrel qualities of the third manufacturer (MC1 and MC2  $n = 6$  each). By contrast, no clear classification can be seen on the PCA score plot based on barrel origin for wine samples analysed after twelve months of maturation (Figure S2A).



**FIGURE 4.** MSA of  $^1\text{H}$  NMR fingerprinting data in the four different oak barrel models.

A: PCA score plot from data after one (circles) and twelve months (triangles) of maturation; B: PCA score plot from data after one month of maturation; C: OPLS-DA score plots from data after one month of maturation, and D: OPLS-DA score and loading plots from data after one month of maturation. Orange = manufacturer MA; green = manufacturer MB; light blue and dark blue = MC1 and MC2 qualities of manufacturer MC respectively; black circles = loadings from aromatic region signals (10.0 – 5.5 ppm); yellow circles = loadings from carbohydrate region signals (5.5 – 3.7 ppm); purple circles = loadings from aliphatic region signals (3.6 – 0.5 ppm).

**TABLE 2.** Performance and validation parameters of OPLS-DA models for classification of wines after one month of maturation in barrels from different manufacturers.

Model parameters	Targeted data	Untargeted data
No of variables	32	536
$R^2X$	0.368	0.726
$R^2Y$	0.926	0.886
$Q^2$	0.883	0.716
NC <sup>a</sup>	1 + 1 + 0	1 + 1 + 0
Permutation tests ( $R^2$ ; $Q^2$ )	0.488; - 0.587	0.518; - 0.501 (MA)
		0.474; - 0.521 (MB)
		0.484; - 0.409 (MC1)
		0.510; - 0.412 (MC2)
CV-ANOVA	< 0.05	< 0.05
AUC <sup>b</sup>	1.0	0.877 (MA)
		0.976 (MB)
		0.998 (MC1)
		0.843 (MC2)
Fisher's probability	$3.7 \times 10^{-7}$	$3.1 \times 10^{-14}$
CCR (%) <sup>c</sup>	100 %	83 % (MA)
		100 % (MB)
		100 % (MC1)
		83 % (MC2)

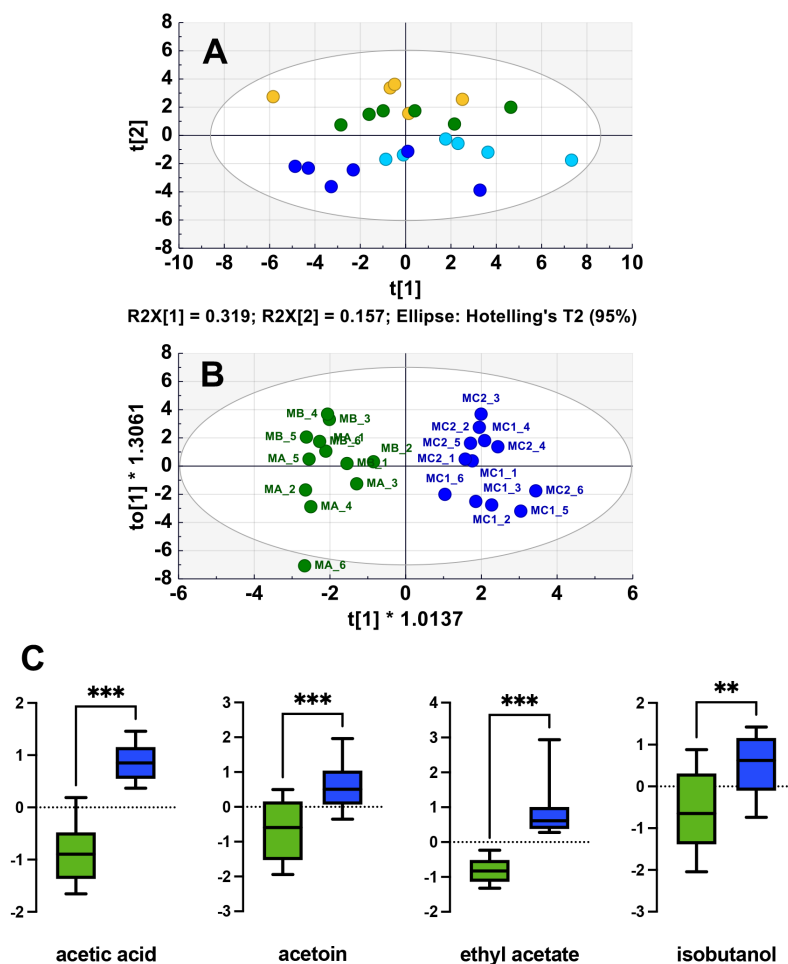
NC = No. of components; AUC = area under the ROC curve; CCR = correct classification rate.



Based on the profile obtained via  $^1\text{H}$  NMR metabolomics, the wines seem to have undergone different maturation kinetics depending on barrel quality, yet they tend towards a fairly similar chemical profile. Similarly to the PCA models from NMR fingerprinting, the PCA model based on NMR targeted data from wine samples collected after twelve months of maturation did not show any discrimination (Figure S2B). Figure 5A shows the PCA score plot of NMR targeted data from wines after one month in the barrels ( $R^2X = 0.476$ ;  $Q^2 = 0.200$ ). While this model is less efficient than the one built from untargeted NMR data, a clear separation between MC wine samples and the other wine samples (MA and MB) can be observed. The first two principal components explain 31.9 and 15.7% of the total variance. This result is consistent with the non-targeted analysis data: the wines kept in the MC barrels undergo a process significantly different from those matured into the MA and MB barrels.

Supervised OPLS-DA following a validation protocol was performed on untargeted data extracted from the full NMR spectrum of wine samples after one month of maturation.

The resulting OPLS-DA score plot confirms the discrimination between wine samples produced in barrels from the three different manufacturers (Figure 4C). The model performances are summarised in Table 2.  $R^2Y$ ,  $Q^2$  ( $> 0.71$ ) and the CV-ANOVA p-value ( $< 0.05$ ) indicate the goodness of fit of the model. The permutation test (100 permutations) indicates the absence of overfitting. The CCR and AUC values from the ROC curves ( $> 83\%$  and  $> 0.84$  respectively) confirm the good performance of the model. Wine samples kept in MA and MB barrels are discriminated from those kept in MC barrels along the first component. Meanwhile, the difference between wines kept in MA and MB barrels can be observed in the first orthogonal component. Wines can be classified as two main groups, the wine samples kept in MC barrels being significantly different from the others. The variables involved in the discrimination between the wine samples can be visualised in the OPLS-DA loading plot (VIP  $> 1$ ) (Figure 4D). Loadings have been classified according to the three previously mentioned spectral regions: the aromatic, carbohydrate and aliphatic regions.



**FIGURE 5.** MSA of  $^1\text{H}$  NMR wine targeted data after one month maturation in the four different oak barrel models.

A: PCA score plot (orange, green, light blue and dark blue circles = MA, MB, MC1 and MC2 manufacturers respectively); B: OPLS-DA score plot (green circles = MA and MB; blue circles = MC1 and MC2); C: Boxplots of significantly discriminant metabolites (green = MA and MB; blue = MC1 and MC2). Statistical significance was checked by unpaired t test or Mann-Whitney's test (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

The discriminating loadings from NMR signals of the aliphatic region are distributed on either side of the vertical axis. This spectral region specific to alcohol and organic acid signals was strongly impacted by storage in oak barrels for one month. Loadings from the signals of the carbohydrate spectral region were found to be specific to wine samples kept in MA and MB barrels. This spectral region is characteristic of monosaccharides. The higher content in monosaccharides may be due to different oak quality depending on the different producer (del Alamo *et al.*, 2000). Finally, some loadings from the aromatic region were observed to be distributed on either side, indicating that the release of phenolic compounds is linked to the quality of the barrel of each manufacturer.

To identify the specific markers, the data from the targeted analysis of the NMR spectra were used. As the PCA model based on targeted analysis correctly discriminated MC wines after one month of ageing from the others (Figure 5A), it was possible to classify the wine samples in two groups. The first set contains the MA and MB samples ( $n = 6$  each), and the second contains all the MC wines ( $n = 12$ ). The targeted NMR data shows that OPLS-DA modelling (Table 2) gave a satisfactory  $R^2Y, Q^2$  ( $> 0.96$ ) and CV-ANOVA  $p$  value ( $< 0.05$ ). Figure 5B shows the OPLS-DA score plot, which compares the two sets of wines (MA and MB versus MC). A clear discrimination between the two groups was observed. As can be seen in Table 2, permutation tests (100 permutations) indicate the absence of overfitting. The CCR and AUC values from the ROC curves (100 % and 1.0 respectively) confirm the efficiency of the model. As noted above, the main discriminant metabolites between the two classes were identified using VIP and  $p(\text{corr})$  values ( $> 1.0$  and  $> 0.5$  respectively) and verified by carrying out a  $t$ -test ( $p < 0.05$ ); four discriminant markers were thereby identified (Figure 5C): acetic acid, acetoin, ethyl acetate and isobutanol. The wines stored in MC barrels contained significantly higher contents of these four compounds after one month of maturation. As previously mentioned, the evolution of these first three compounds is directly related to the process of oxidation. The increase in isobutanol content is more difficult to explain, since this compound is mainly associated with the fermentation process. Nevertheless, an additional amount may have come from the metabolic activity of microorganisms (yeasts and bacteria). Its synthesis is favoured by the presence of oxygen (Jackson, 2020). All these data clearly indicate a difference in oxygen permeability of the two groups of wines. MC barrels, regardless of their quality, have a greater permeability to oxygen at least during the first month. This difference can come from different factors such as oak origin and anatomy or barrel manufacture (del Alamo-Sanza & Nevares, 2018).

## CONCLUSION

In this study, both targeted and non-targeted  $^1\text{H}$  NMR analyses were carried out to monitor wine maturation in oak barrels. The results show a clear distinction between samples taken after one and twelve months in barrels. The results of the non-targeted analysis show that phenolic, carbohydrate

and aliphatic regions were impacted. In the targeted analyses, twelve molecules were identified whose levels varied significantly during barrel maturation. During ageing, amino acids, catechin, epicatechin and acetoin decreased, while acetic acid, ethyl lactate and sugars (arabinose and glucose) increased. NMR-based metabolomics proved to be a useful tool for the overall analysis of wine maturation in oak barrels. Despite being a preliminary study, a significant difference between the manufacturers was observed in terms of ageing kinetics after one month of maturation. This difference could be due to the level of permeability to oxygen of the respective manufacturer's barrels. Finally, the  $^1\text{H}$  NMR profiles were more homogeneous after twelve months of maturation. It would be interesting to compare these profiles to those obtained by other techniques to complete this work. This preliminary study shows that NMR makes it possible to follow the evolution of wines in barrels. In order to thoroughly evaluate the real potential of using NMR for studying wine quality, future work will need to focus on complementing this approach with a more complete analysis that integrates the dosage of other wine compounds, such as phenolics and volatiles.

## ACKNOWLEDGEMENTS

This work was supported by *Association Nationale de la Recherche et de la Technologie* (Inès Le Mao was the recipient of a CIFRE PhD fellowship from ANRT and Baron Philippe de Rothschild S.A.). We would also like to thank Château Mouton Rothschild and Fondation de Bordeaux (donors: Baron Philippe de Rothschild SA, Chateau Cheval Blanc, Château Lafite Rothschild, Le Domaine Clarence Dillon, Château Petrus) for their financial support. The work was supported by the Bordeaux Metabolome Facility and ANR (MetaboHUB ANR-11-INBS-0010 and WAPNMR ANR-21-CE21-0014 projects).

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