ATR-MIR spectroscopy and multivariate analysis in alcoholic fermentation monitoring and lactic acid bacteria spoilage detection

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Wine production processes still rely on post-production evaluation and off-site laboratory analyses to ensure the quality of the final product. Here we propose an *at-line* methodology that combines a portable ATR-MIR spectrometer and multivariate analysis to control the alcoholic fermentation process and to detect wine fermentation problems. In total, 36 microvinifications were conducted, 14 in *normal fermentation conditions* (NFC) and 22 *intentionally contaminated fermentations* (ICF) with different lactic acid bacteria (LAB) concentrations. ATR-MIR measurements were collected during alcoholic and malolactic fermentations and relative density, pH, and L-malic acid were analyzed by traditional methods. Partial Least Squares Regression could suitably predict density and pH in fermenting samples (root mean squared errors of prediction of 0.0014 $g \cdot mL^{-1}$ and 0.06 respectively). With regard to ICF, LAB contamination was detected by multivariate discriminant analysis when the difference in L-malic acid concentration between NFC and ICF was in the order of 0.7-0.8 $g \cdot L^{-1}$, before the end of malolactic fermentation. This methodology shows great potential as a fast and simple *at-line* analysis tool for detecting fermentation problems at an early stage.

Keywords: Process monitoring, alcoholic fermentation, wine, malolactic fermentation contamination, ATR-MIR, Process Analytical Technologies

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1. Introduction

The production of wine is based on alcoholic fermentation, which consists in the biochemical transformation of sugar into ethanol by yeasts. There are many factors that have an influence over the complexity and quality of the final product such as the grape quality and variety, yeast strain or cellar practices used (Suárez-Lepe & Morata, 2012). However, even with the best raw materials and starting under the optimal conditions, problems during alcoholic fermentation can occur, in which yeast or other microorganisms synthetize undesirable compounds that negatively affect the quality of the wine. Stuck and sluggish fermentations along with contamination-related processes are the most common problems that can appear during alcoholic fermentation (Hernández, León, & Urtubia, 2016). Nutrient deficiencies, sudden temperature changes or the imposition of undesired and non-inoculated yeast are the main causes of stuck and sluggish fermentations. Spoilage processes are due to the growth of unwanted microorganisms in the must, such as acetic acid or lactic acid bacteria (LAB), which are part of the normal microbiota found on the surface of leaves and grapes but can also be found in the environment of wineries (Portillo, Franquès, Araque, Reguant, & Bordons, 2016). Although the "*piqûre acétique*" is the most widely known spoilage, the "*piqûre lactique*" can also pose very important problems in some wines.

LAB are responsible for the biochemical transformation of L-malic acid into L-lactic acid releasing carbon dioxide. This process, called malolactic fermentation, is promoted in red wines to decrease their acidity since, from an organoleptic point of view, a lower acidity is more compatible with the high tannicity of these wines (Cappello, Zapparoli, Logrieco, & Bartowsky, 2017). However, in white wines, this second fermentation is usually undesired because it increases pH and reduces their typical freshness, leading to wines with worse organoleptic quality (Cozzolino, Mccarthy, & Bartowsky, 2012).

In the winemaking industry, a control of the alcoholic fermentation process is required in order to avoid problems that result in low quality wines and consequently, in economic losses. In the cellar, the process is mostly controlled by determining temperature, density and pH, which are usually

measured twice a day, together with a visual and aroma evaluation of the fermenting grape must. These parameters are related to sugars, acids and other minor compounds that ultimately impact substantially the colour and/or aroma of the wine (Bisson, 1999). These parameters are sufficient to control the process when the fermentation progresses well. However, these control measures sometimes fail to timely detect problems when they could still be solvable by applying corrective measures to the must. This is why the implementation of novel process control strategies to obtain real-time information during alcoholic fermentation has a growing interest in the oenological field (Cozzolino, 2016).

The Process Analytical Technologies (PAT) approach follows this trend. PAT is a system for designing, analysing and controlling a manufacturing process, through timely measurements of critical quality attributes of raw and *in-process* materials and processes in order to ensure final product quality. The hypothesis behind PAT is that quality must be controlled through process control and not only by evaluating postproduction information (Simon, Pataki, Marosi, Meemken, Hungerbühler, et al., 2015). This is specially advantageous when applied over expensive or complex samples such as pharmaceuticals or food products (Lourenço, Lopes, Almeida, Sarraguça, & Pinheiro, 2012; Van Den Berg, Lyndgaard, Sørensen, & Engelsen, 2013). For this reason, the winemaking industry is a sector where PAT could be widely applied.

In the last decades, the use of spectroscopy to determine oenological parameters has increased considerably. Spectroscopic methods are fast, clean and provide large amounts of information with minimum sample preparation. Near and Mid Infrared Spectroscopy (FT-NIR and FT-MIR) have been widely used to monitor wine fermentations because information can be obtained *on-time* all along the process (Buratti, Ballabio, Giovanelli, Zuluanga Dominguez, Moleset al., 2011; Urtubia, Pérez-Correa, Meurens, & Agosin, 2004). Several authors have reported good prediction of sugars (glucose and fructose), ethanol, volatile acids, phenolic compounds or volumic mass in must, fermenting must and wine samples (Cozzolino, 2016; Di Egidio, Sinelli, Giovanelli, Moles, & Casiraghi, 2010; dos Santos, Páscoa, & Lopes, 2017). In some cases, the prediction of chemical

parameters has allowed detecting some problems such as sluggish fermentations (Urtubia, Pérez-Correa, Pizarro, & Agosin, 2008). Among these studies, those using MIR spectroscopy with attenuated total reflectance (ATR-MIR) stand out because this technique only requires one drop of sample and provides well resolved water peaks (Teixeira dos Santos, Páscoa, & Lopes, 2017; Shah, Cynkar, Smith, & Cozzolino, 2010). All these advantages, together with the fact that modern MIR spectrometers can also be portable, make this technique a very suitable tool in a cellar not only to monitor different fermentation parameters but also to detect fermentation problems as we demonstrated in a previous study (Cavaglia, Giussani, Mestres, Puxeu, Busto, et al., 2019).

The present research aims to evaluate the application of a portable ATR-MIR spectrometer and multivariate analysis techniques to control the progress of alcoholic fermentations and to detect problems at an early stage. Density and pH were evaluated using regression models, whereas discriminant models were used to detect wine fermentation deviations due to LAB contamination.

2. Materials and methods

2.1. Grape must and microorganisms

Concentrated white must was provided by *Mostos Españoles* S.A., (Ciudad Real, Spain) and it was stored at -20 ºC until its use. Its defrosting was done at 5 ºC and it was then diluted with MilliQ water to adjust the sugar concentration to 200 ± 10 g/L. The diluted must was supplemented with 0.30 g/L of ENOVIT® (SPINDAL S.A.R.L. Gretz Armainvilliers, France) and 0.30 g/L of Actimaxbio* (Agrovin, Ciudad Real, Spain) in order to ensure a sufficient final concentration of yeast assimilable nitrogen.

The commercial dry *Saccharomyces cerevisiae* yeast strain used was “E491” (Vitilevure Albaflor, YSEO, Danstar Ferment A.G., Denmark). Regarding to lactic acid bacteria, a commercial freeze-dried blend of *Oenococcus oeni* and *Lactobacillus plantarum* “Co-inoculant Bacteria 3.2” (Anchor Oenology, South Africa) was used. Rehydration of the microorganisms was done following the suppliers’ instructions.

2.2. Microvinifications

Three small-scale alcoholic fermentation or microvinification batches were carried out as follows. For each sample, 350 mL of diluted must were added into 500 mL Erlenmeyer flasks and they were inoculated with 0.105 g of active dry yeast rehydrated in 2 mL of MiliQ water for 30 minutes at 25 ºC, reaching a final concentration of $3 \cdot 10^6$ CFU·mL^{-1}. To prepare the simulated contaminated samples, LAB co-inoculations were done taking into account the producer instructions (1 g = $1 \cdot 10^{11}$ CFU·mL^{-1}) to reach different final concentrations ranging between $1 \cdot 10^6$ and $1 \cdot 10^7$ CFU·mL^{-1}. All microvinifications were kept under a constant temperature of 18 ºC until the end of alcoholic and malolactic fermentations. Alcoholic fermentation was considered finished when density was under 0.995 g·L^{-1} whereas malolactic fermentation ended when L-malic acid concentration was < LOD (0.06 g·L^{-1}).

The number of samples of each batch, the initial must parameter values (which are slightly different to simulate the natural maturity variability in grapes) and codification used are specified in Table 1. The normal fermentation conditions were coded as NFC and the intentionally contaminated fermentations as ICF. ICF samples were divided into 5 groups: ICF1, ICF2, ICF3, ICF4 and ICF5, according to the concentrations of LAB inoculated. The aim of using different concentrations of LAB was to promote the transformation of L-malic acid into L-lactic acid at different points of the alcoholic fermentation.

2.3. ATR-MIR analysis

The samples were collected at least once a day to follow both alcoholic and malolactic fermentations until both were finished. The sampled volume was 1.5 mL, which was centrifuged at 10000 rpm for 10 minutes to avoid the scattering effect in the spectroscopic measurements due to the presence of microorganisms. The pellet was discarded while the supernatant was kept in 1.5 mL eppendorfs for further analysis. Right after sample collection, spectra were obtained using a portable 4100 ExoScan FTIR instrument (Agilent, California, USA), equipped with an

interchangeable spherical ATR sampling interface, consisting on a diamond crystal window. A drop of sample was placed onto the crystal using a Pasteur pipette and the spectra were acquired right afterwards. Each sample was analysed in triplicate and an air background was recorded between samples. Each sample was measured applying our previously optimized methodology (Cavaglia, Giussani, Mestres, Puxeu, Busto, et al., 2019). After each measurement, the crystal was thoroughly cleaned with deionized water and cotton wipes. Spectra were collected in absorbance mode from 4000 to 650 cm^{-1}. The resolution and number of scans that provided the best results were 8 cm^{-1} and 32, respectively. Measurements were made at 63 ± 1 ºC, as this was the stabilization temperature of the crystal. Spectra were examined using the Microlab PC software (Agilent, California, USA), and saved as *.spc* files.

2.4. Standard sample analysis

As it is done in a cellar, density and pH were determined along the alcoholic fermentation to ensure the normal progress of this process. Density was measured using an electronic portable densimeter (Densito2Go, Mettler Toledo, United States) and pH was measured with a portable pH-meter with a 201 T portable electrode (7+ series portable pH-meter, XS Instruments, Italy). The remaining volume of the supernatant was used for L-malic acid analysis using an Y15 Analyser (Biosystems, Barcelona, Spain) in order to follow the malolactic fermentation. Measurements were performed in parallel to ATR-MIR analysis of the samples.

2.5. Multivariate analysis

For each sample, the average of the three recorded spectra was used in all the models described below.

The collected data had a three-dimensional structure, with *I* samples, *J* wavenumbers and *K* sampling times. This 3-way array was rearranged in different ways (Figure 1), depending on the aim of the study.

First, a global approach was developed using all the spectra collected throughout the fermentation process for all the experiments, to explore the main information contained in the data and to correlate the spectra with the fermentation parameters. A time-wise unfolding of the 3-way array was performed to obtain a matrix with dimensions (*IK* x *J*), in which rows were the spectra recorded for *I* samples at *K* sampling times and columns were the *J* spectroscopic wavenumbers.

The individual examination of each sampling time was also considered for the discrimination between NFC and ICF samples for each experimental batch following a local approach. For each experimental batch, different *K* matrices (one for each time sampled) with dimensions (*I* x *J*) were thus independently investigated.

Principal Component Analysis (PCA) was applied to visualize the variability among data both through alcoholic and malolactic fermentations and to detect potential outliers, while Partial Least Squares Regression (PLSR) models were developed to predict fermentation parameters.

Finally, Partial Least Squares Discriminant Analysis (PLS-DA) was used to detect LAB spoilage. PLS-DA is similar to PLSR, but in this method the vector **y** contains dummy variables (0 or 1) for the classes you want to discriminate (here, NFC and ICF). The method seeks the optimal number of latent variables (LVs) that maximize the covariance (and thus the discrimination) between the infrared spectra and the classes. A discrimination threshold (between 0 and 1) is calculated taking into account the probability of classification error of the samples into the classes (Pérez, Ferré, & Boqué, 2008).

To proceed with the study of the spectra, different pre-processing strategies were tested including first and second derivatives (to emphasise small peaks), Savitzky-Golay smoothing (to reduce noise) and Standard Normal Variate (SNV) (to reduce the variability between samples due to scatter). This step is crucial because the outcome of a multivariate model has a strong dependence on the pre-processing applied. According to the data matrix used in the calculation, different pre-processing combinations were tried and compared. Only those giving the best results are shown.

After spectral pre-processing, data were mean-centered. The theoretical basis of these treatments can be found elsewhere (Rinnan, Van Den Berg, & Engelsen, 2009).

In addition, to optimize the regression models and further reduce their complexity, a variable selection strategy based on the Selectivity Ratio algorithm was considered. It is based on the idea of progressively excluding variables in the **X** data block and evaluate the effectiveness of the **Y** prediction until the combination of **X** variables is optimized (Rajalahti, Arneberg, Berve, Myhr, Ulvik, et al., 2009).

Regression models were validated considering three different validation strategies and the best model was selected by evaluating the best compromise between the higher percentage of explained variance in **Y** and the minimum RMSECV/RMSEP (Root Mean Square Error of Cross-Validation/Prediction). In the first validation strategy, an internal cross-validation (CV) was performed, where groups of samples (accounting for 5% of the total number of samples) were left out each time and used for prediction. The procedure was iterated 20 times and the average RMSECV was considered. In the second strategy, data were split into random halves and each half was used as calibration set in one model and as validation test set in the other. Thus, a random vector of zeros and ones was built, where zeros were considered calibration samples and ones were validation samples. The data split procedure was repeated 10 times to reduce the dependence of data splits in the performance of the models and the average RMSEP error was evaluated. Finally, the third strategy consisted on applying the Kennard-Stone sample selection algorithm which divides the data into calibration and test sets taking into account the distribution of the samples in the principal components space. This algorithm selects the samples for the calibration set providing uniform coverage over the **X** data, including samples at the limits of the measurements ranges (Kennard & Stone, 1969). This methodology tends to be overoptimistic, and for this reason the number of samples to be included in the calibration test was optimized, assuring a RMSEP comparable to the ones obtained by the other strategies.

In the case of the PLS-DA models, different internal CV strategies were tested, depending on the number of samples available in each case. A leave-one-out CV was used when the number of samples ≤ 6, while a leave-two-out CV was used when the number of samples ≥ 6.

All multivariate data analyses were performed using the PLS Toolbox v8.7 (Eigenvector Research Inc., Eaglerock, USA) with MATLAB R2015b (The MathWorks, Natick, USA).

3. **Results and discussion**

3.1. ATR-MIR spectra

The evolution of the ATR-MIR spectra during alcoholic fermentation is shown in Figure 2. The region from 850 to 649 cm^{-1} was excluded as it did not contain useful information, resulting in low quality models. As previously reported (Cozzolino & Curtin, 2012; Wu, Xu, Long, Zhang, Wang, et al., 2015), the regions that show most of the variability during wine alcoholic fermentation in the mid-infrared region are mainly found between 950 to 1500 cm^{-1}, where CH_2, C-C-H, H-C-O bonds and C-C, C-O stretching vibrations absorb, and between 3000 to 3700 cm^{-1}, where O-H stretching absorbs.

3.2. Alcoholic fermentation

All the spectra arranged in a time-wise unfolded matrix (Figure 1) were used to build a global PCA model. The evolution of each batch during alcoholic fermentation was best described when applying the following pre-processing combination: Savitzky-Golay second order polynomial smoothing through 7 points, SNV and mean-centering. The first 2 principal components accounted for the 99.31% of the data variability (97.39% for PC1 and 1.92% for PC2). As it can be noticed in figure 3, when comparing the evolution in time of the PC1 scores with the evolution of the density curve with the values registered during the fermentation process, both plots show a similar trend. The loadings plot of PC1 shows that the most important region to follow the progress of alcoholic

fermentation is between 950-1700 cm^{-1} (data not shown), which was not surprising as this region mainly corresponds to sugars and ethanol absorptions (Cozzolino, Cynkar, Shah & Smith, 2011). Moreover, the PCA model built using the spectra shows small differences between batches. A hypothesis is that this behaviour could be related to small changes in the initial sample density, since all samples come from the dilution of the same must in the same experimental conditions. In other words, the spectra recorded by the portable instrument allowed to distinguish between experiments, confirming the capability of the spectroscopic technique coupled with chemometrics to spot small differences between fermentation processes.

3.3. Prediction of chemical parameters

As mentioned above, PC1 scores and density showed a similar trend when depicted against time. From this important result arose the idea of using the spectroscopic data to predict density by means of PLSR. All the available NFC experiments were used in this regression model (final data matrix dimensions 566 samples x 850 variables).

By applying the Selectivity Ratio algorithm, the spectroscopic regions selected were 967 to 1175 cm^{-1} and 1483 to 1771 cm^{-1}. The validation errors for the density models using the different CV strategies are shown in Table 2. For the first model, a subset of 28 samples was used. The number of LVs to be considered was optimized taking into account the higher percentage of explained variance of **Y** data and the lower RMSECV/ RMSEP values. For the subsequent models, only one LV was used. The Kennard-Stone algorithm showed that only 29 calibration samples were necessary to build a model with an RMSEP value comparable to the ones obtained by the other validation methods.

Similar results have been reported using NIR spectroscopy. Fernandez-Novales et al. obtained an RMSECV of 0.0065 $g \cdot mL^{-1}$ for the prediction of density in wine fermenting samples (Fernández-Novales, López, González-Caballero, Ramírez, & Sánchez, 2011). In our study, we showed for the first time that the spectroscopic information obtained with a portable ATR-MIR spectrometer with

PLSR can be used to predict density in must and fermenting samples, obtaining very satisfactory results considering the lower optical robustness of the instrument compared to benchtop devices. pH is another chemical parameter that is usually determined to control alcoholic fermentation. In this study, PLSR was applied to predict pH following the same methodology as for density (in this case, the data matrix dimensions were 427 samples x 850 variables). The Selectivity Ratio algorithm selected regions all along the spectroscopic range, suggesting that pH prediction requires information from the full spectrum. A combination of Savitzky-Golay second order polynomial smoothing through 15 points, SNV and mean-centering pre-processing gave the best results. For all models, 5 LVs were needed to achieve good predictions. In the first model built with all the samples, a subset of 22 samples was used for internal validation. The validation based on the Kennard-Stone selection method needed 43 calibration samples to obtain errors comparable to those of the other validation methods; therefore, 384 validation samples were used to test the model. Results from the different validation strategies for the pH models are summarised in Table 2. Swanepoel et al. obtained an standard error of prediction (SEP) of 0.05 pH units for grape and must samples using FT-MIR in the transmission mode (Swanepoel, du Toit, & Nieuwoudt, 2007). Using ATR-MIR, Shah et al. obtained a standard error of cross-validation (SECV) of 0.07 for the pH of grape juice samples (Shah, Cynkar, Smith, & Cozzolino, 2010). Our results show that the portable spectrometer used in this study can perform a fast and simple control of the progress of alcoholic fermentation with an acceptable error when combined with a chemometric strategy to manage the recorded spectra. Additionally, the fact that similar validation errors were obtained using different validation strategies shows the robustness of the models.

3.4. Malolactic fermentation deviation

The spectra recorded during the experiments in which LAB co-inoculations were performed (ICF) showed only minor changes with respect to the ones recorded in NFC due to the small concentration changes involved in the malolactic fermentation process. The main information in both NFC and

292 ICF spectra is ascribable, in fact, to the alcoholic fermentation evolution (sugars and ethanol
293 signals).
294 To focus the attention on the malolactic fermentation process, each batch was individually studied
295 to avoid the variability among batches. In addition, the PCA models were built using the
296 spectroscopic region between 1320 and 1109 cm^{-1}, which is related to organic acid molecules
297 involved in the malolactic fermentation as previously reported (Grassi, Vigentini, Sinelli, Foschino,
298 & Casiraghi, 2012; Picque, Lefier, Grappin, & Corrieu, 1993).
299 Three models were calculated, one for each batch experiment. The best results were obtained with a
300 combination of 1st derivative Savitzky-Golay second order polynomial smoothing through 15
301 points, SNV and mean-centering as pre-processing methods~~. In this case, the 1st derivative~~
302 ~~emphasised the slight changes in small peaks.~~ All models explained more than 98% of the
303 variability using 3 PCs.
304 The scores for two PCs against time are depicted for each batch in figure 4. Samples are labelled
305 according to the LAB co-inoculated concentrations. It can be observed that the evolution of
306 malolactic fermentation takes different directions in the PCA space with respect to time and it is
307 even possible to distinguish among the different LAB concentrations in the second and third
308 batches. The models allowed to observe the different trends between ICF and NFC samples before
309 the end of malolactic fermentations, and in some cases, before the end of alcoholic fermentation
310 (batch 3). A deep investigation of these plots allowed to qualitatively determine at which sampling
311 time the trajectories of ICF samples started to deviate from NFC. In batches 1 and 2, trajectories
312 showed different trends 100 hours after the beginning of alcoholic fermentation, whereas in batch 3,
313 it was possible to qualitative see the different trajectories after 50 hours.
314
315 *3.5. Discrimination between NFC and ICF*
316 Starting from the qualitative results previously shown (section 3.4), PLS-DA models for each batch
317 were built at individual sampling times (local models) to determine at which sampling time the

trajectories of ICF samples started to deviate from NFC. In other words, to determine as soon as possible when the deviation from the NFC occurred because of LAB spoilage. For each PLS-DA model at each sampling time (Figure 1) the **y** vector was built by assigning 1s to ICF samples and 0s to NFC samples.

The first sampling time to find a discrimination threshold between the two groups with a 100% correct classification was defined as the deviation time. The deviation time was confirmed with a local model of the consecutive sampling time when 100% correct classification was achieved. For all models, only one LV was needed for a successful discrimination of the classes.

Samples deviated from NFC in the first batch 213 hours after the beginning of the fermentation. In the second batch, ICF1 deviated after 187 hours whereas ICF2 and ICF3 deviated after 145 hours and 138 hours, respectively**.** In the third batch the difference of ICF4 and ICF5 from NFC was detected after 56 and 58 hours, respectively.

At those deviation times, malolactic fermentation was around 50%-60%, which means that it is possible to differentiate the spectra before the end of malolactic fermentation, allowing to make corrective measures in wineries. Manley et al. considered the possibility of using FT-NIR to detect if malolactic fermentation has started, is in progress or has been completed in white wine, where L-lactic acid values were between 0-0.3 $g \cdot L^{-1}$, 0.3-2 $g \cdot L^{-1}$ and above 3 $g \cdot L^{-1}$, respectively. They reported good classification of each class, with >95% of recognition rates (Manley, van Zyl, & Wolf, 2001). In our study, for all PLS-DA models, the difference in L-malic acid concentration between NFC and ICF samples ranged from 0.7 to 0.8 $g \cdot L^{-1}$. Despite the fact that this decrease in L-malic acid concentrations result in a slight increase in pH, this is the first time that an ATR-MIR device is used to detect deviations from NFC before the end of malolactic fermentation.

4. Conclusions

It has been demonstrated that a portable ATR-MIR spectrometer with multivariate analysis is a valuable analytical tool to rapidly control the progress of alcoholic fermentation in white wine.

Here, the ability of this portable device has been proved to effectively predict density and pH in fermenting must samples. The methodology presented shows great potential as a fast and simple *at-line* analysis tool for the detection of fermentation problems, as is possible to use this instrument to rapidly assess a LAB spoilage during alcoholic fermentation. Upon this findings, further research will be developed based on PAT strategies to give the winemaker the possibility to correct the process and to obtain good quality wines.

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