MONITORING WINEMAKING PROCESS USING TYROSINE INFLUENCE IN THE EXCITATION-EMISSION MATRICES OF WINE

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Abstract: Wine samples collected during the winemaking process have been analyzed employing a previously optimized UHPLC-FD method, determining their biogenic amines and amino acids profile. The results obtained have been submitted to a statistical analysis from which it was extracted that the most influential analyte was tyrosine. Thanks to its fluorescence, a method for its determination by excitation-emission matrices has been proposed. The accuracy of the method has been checked by means of Elliptical Joint Confidence Region test. The winemaking process has been monitored with this method, obtaining a faster and cheaper way to follow the process.

Keywords: tyrosine; wine; EEM; PARAFAC; chemometrics; chromatography

1. Introduction

Amino acids (AAs) are a subgroup within the organic acids that contribute to the organoleptic and nutritional properties (R. M. Callejón, Troncoso, & Morales, 2010; Robles, Fabjanowicz, Chmiel, & Płotka-Wasylka, 2019). In particular, AAs generate volatile compounds related to aroma (Petropoulos, Metafa, Kotseridis, Paraskevopoulos, & Kallithraka, 2018; Valdés et al., 2019). Moreover, they can be used to control the evolution of acidification during winemaking (Robles et al., 2019).

In wine, AAs can come directly from the raw material and are metabolized by yeasts, acting as a source of nitrogen (Arrieta & Prats-Moya, 2012; R. M. Callejón et al., 2010; Valdés et al., 2019). However, they can also appear as yeast waste after the fermentation or be generated due to enzymatic degradation of proteins (R. M. Callejón et al., 2010). Its concentration and profile depend on several factors (R. M. Callejón et al., 2010; Petropoulos et al., 2018; Valdés et al., 2019). Within them, tyrosine is a non-essential AA that reaches the human body through the hydroxylation of phenylalanine or through the intake of food containing it (PubChem, 2020; Schenck & Maeda, 2018). It forms part of many proteins, and is the precursor of some important neurotransmitters (Fernstrom & Fernstrom, 2007; PubChem, 2020). It also participates in the synthesis of melanin, catecholamines and some thyroid hormones (Slominski, Zmijewski, & Pawelek, 2012).

Apart from that, AAs are the precursors of biogenic amines (BAs), compounds with toxic activity that can be harmful for the human health (He et al., 2016; Meléndez, Sarabia, & Ortiz, 2016; Palomino-Vasco, Rodríguez-Cáceres, Mora-Diez, Pardo-Botello, & Acedo-Valenzuela, 2019; Papageorgiou et al., 2018). The concentration of BAs in wine could vary between a few and 50 mg L^{-1}, and although it is important to keep this concentration as low as possible, there are no laws regulating their maximum concentration in wine. Its profile also depends on several factors (Papageorgiou et al., 2018; Peña-Gallego, Hernández-Orte, Cacho, & Ferreira, 2012; Perestrelo, Bordiga, Locatelli, Silva, & Câmara, 2020). Hence, the joint determination of AAs and BAs is interesting to both researchers and

industry, since they can be used as a quality or safety index, or as an ageing or authenticity indicator (R. M. Callejón et al., 2010; He et al., 2016; Palomino-Vasco, Acedo-Valenzuela, Rodríguez-Cáceres, & Mora-Diez, 2019; Robles et al., 2019).

AAs can be determined directly by UV, but their absorption is in a very non-specific area where almost all compounds and solvents absorb. Only tyrosine, tryptophan and phenylalanine present chromophore groups that allow them to be determined by fluorescence. The determination and quantification of BAs in wine is also challenging due to the complexity of the matrix and the presence of several BAs in the same sample, which are normally present low concentrations. Moreover, BAs do not present adequate characteristics for their determination by spectrophotometric techniques due to their structure. Therefore, AAs and BAs should normally be pre-concentrated and/or derivatised or determined by MS. The most commonly used methods for their determination are separative techniques (R. M. Callejón et al., 2010; Ferré, González-Ruiz, Guillarme, & Rudaz, 2019; Önal, Tekkeli, & Önal, 2013; Palomino-Vasco, Acedo-Valenzuela, et al., 2019; Papageorgiou et al., 2018).

On another note, fluorescence spectroscopy is a non-invasive instrumental technique widely used in food matrices since it allows to obtain information about molecular structure and functions, and allows characterizing the foodstuffs. Other advantages are its sensitivity and selectivity, as well as being a quick and easy technique to use (Airado-Rodríguez, Durán-Merás, Galeano-Díaz, & Wold, 2011; Azcarate, Teglia, Karp, Camiña, & Goicoechea, 2017; Raquel M. Callejón et al., 2012; Carbonaro et al., 2019; Ríos-Reina et al., 2019). Within the different ways to employ fluorescence spectroscopy, excitation-emission matrices (EEMs) are one of the best ways to obtain a lot of information about the studied system, that can then be extracted using chemometrics. PARAFAC (PARAllel FACtor analysis) is the most used second-order algorithm for matrix decomposition, and it has been used in many food samples (Airado-Rodríguez et al., 2011; Azcarate et al., 2017; Raquel M. Callejón et al., 2012).

Several authors have employed the AAs and/or BAs profile of wines obtained by separative techniques in combination with chemometrics for wine differentiation or monitoring along time (R. M. Callejón et al., 2010; Jiménez Moreno, Torrea Goñ, & Ancín Azpilicueta, 2003; Ordóñez, Callejón, Troncoso, & García-Parrilla, 2017; Palomino-Vasco, Rodríguez-Cáceres, et al., 2019). However, we have no evidence of studies that determine the AAs and BAs profile during young wines winemaking. Therefore, this was the first objective of this research, employing a previously optimized chromatographic method. The statistical study of the obtained concentrations would give us information about the most influential analytes in the variance. Furthermore, and taking into account that some analytes are fluorescent, the second objective was the obtention of the EEMs of wine samples collected during winemaking. The application of chemometrics and the correlation between the chromatographic and the fluorescent results would result in the proposal of a simpler and more economical way of monitoring the winemaking which, to the best of our knowledge, has never been proposed.

2. Materials and Methods

2.1 Chemicals

The analytes determined in this study were putrescine (PUT), histamine (HIM), tyramine (TYM), glutamic acid (GLU), serine (SER), cadaverine (CAD), tryptamine (TRY), 2-phenylethylamine (PEA), lysine (LYS), arginine (ARG) and phenylalanine (PHE), purchased from Sigma-Aldrich (USA); agmatine (AGM), purchased from Alfa-Aesar (Germany); ethanolamine (ETA) and tyrosine (TYR), purchased from Merck (Germany); histidine (HIS), purchased from Fluka (Spain); and glycine (GLY) and tryptophan (TRP), purchased from Panreac (Spain). 1-octylamine (OCT; Fluka, Spain) was employed as internal standard in the chromatographic method. A stock solution of 10000 mg L^{-1} of each AA and of 5000 mg L^{-1} of each BA was prepared by solving the adequate amount of the

95 powder/liquid presentation in ultrapure water (Merck Millipore, USA). Solutions were stored at 4ºC in
96 darkness, and were daily used to prepare the working analyte solutions.

97 For the derivatization reaction, a boric acid/sodium borate buffer (0.6 M; pH 10.50) was weekly
98 prepared by diluting the adequate amount of boric acid (Merck, Germany) in ultrapure water (Merck
99 Millipore, USA), and adjusting the pH with NaOH (Panreac, Spain). A solution of 0.4 mg of o-
100 phthalaldehyde (OPA; Sigma-Aldrich, USA) in 10.0 mL of MeOH (Panreac, Spain) was also weekly
101 prepared. Then, the derivatization reagent was prepared by mixing in a 5.0 mL volumetric flask 1.6 mL
102 of the OPA solution and 1.2 mL of 2-mercaptoethanol (Sigma-Aldrich, USA). The flask was filled up
103 to the mark with the aforementioned boric acid/sodium borate buffer. Before its use, the derivatization
104 reagent was filtered (0.22 µm membrane nylon filters; Teknokroma, Spain).

105 Mobile phase employed for the chromatographic separation was composed with acetonitrile
106 UHPLC-grade (Sigma-Aldrich, USA), methanol UHPLC-grade (Panreac, Spain) and a TRIS buffer,
107 which was prepared by the dilution of the adequate amounts of Trizma® base (2-amino-2-
108 (hydroxymethyl)-1,3-propanediol; Sigma-Aldrich, USA) and tris(hydroxymethyl)aminomethane
109 hydrochloride (Acros, Spain) to obtain a concentration of 0.10 M and a pH of 8.30. Mobile phase was
110 filtered (0.22 µm membrane nylon filter; Teknokroma, Spain), and ultrasonicated before its use.

111 Synthetic wine employed was prepared by dissolving the adequate amount of L-(+)-tartaric acid
112 (Scharlau, Spain) to get a 3.0 g L^{-1} concentration, in a 13% (v/v) EtOH (Panreac, Spain) aqueous
113 solution. pH was adjusted to 3.50 employing NaOH (Panreac, Spain).

114 Polyvinylpolypirrolidone (PVPP; Sigma-Aldrich, USA) was employed to remove the
115 polyphenols of the wine samples.

116

117 ***2.2 Winemaking samples***

118 Wine samples were generously donated by the experimental winery of the University of
119 Extremadura during the winemaking process of a ‘Tempranillo’ grape young wine.

Samples were daily collected in the winery; and stored at -20ºC and in dark until analysis. Information about the winemaking process was given by the winemaker, who informed when the different fermentations began according to the typical analyses of the winery (i.e. density control or alcohol content).

2.3 Instrumentation and software

For the chromatographic analysis of the samples, an Agilent Model 1260 Infinity High Performance Liquid Chromatograph (Agilent Technologies Inc., USA) equipped with an online degasser, quaternary pump (G1311B), column oven compartment (G1316A), autosampler (G1329B), UV-VIS diode-array detector (G1315D) and fluorescence detector (G1321B) was employed. ChemStation software was used for data treatment and instrument control. The analytical separation was carried out in a Zorbax Eclipse XDB-C18 analytical column (100 x 4.6 mm; 1.8 µm; Agilent Technologies Inc., USA).

For the obtention of the fluorescence EEMs, a Cary Eclipse Varian spectrofluorometer (Agilent Technologies Inc., USA) connected by a GPIB488 card to a PC was employed. The instrument was equipped with two Czerny-Turner monochromators, a constant xenon light source and a photomultiplier tube as detector. Measurements were made in a 1.0 cm quartz cell. Cary Eclipse's own software was used for equipment control and data acquisition.

Central composite experimental design was carried out employing The Unscrambler v9.7 (CAMO Software, Japan). Statistical analyses were performed using XLSTAT software (Addinsoft, France). EEM_corr routine for MATLAB (Chiappini, Alcaraz, Goicoechea, & Olivieri, 2019) was freely downloaded from https://fbcb.web1.unl.edu.ar/laboratorios/ladaq/download/. Multivariate data analyses were done employing MatLab R2016B (The MathWorks Inc., USA) and the MVC2 routine (Olivieri, Wu, & Yu, 2009), available at www.iquir-conicet.gov.ar/descargas/mvc2.rar.

2.4 Chromatographic determination

The chromatographic determination of the analytes was carried out following an automatic UHPLC-FLD method previously proposed by our investigation group (Palomino-Vasco, Acedo-Valenzuela, et al., 2019). Briefly, it employed a fully automated derivatization reaction, making use of an injection program that mixes the reagents sequentially before injecting them into the column. Separation was performed at 50ºC, with a flow rate of 1.0 mL min^{-1}, and the fluorescent derivatives were monitored at 356/445 nm. The peak area/internal standard area ratio was used as analytical signal. Both the conditions of the automatic injection and the gradient used for the separation of the derivatives are summarized in Tables in the aforementioned article. Also, all the information about the establishment of the calibration curves as well as the analysis of real samples is extensively explained in it.

2.5 Fluorometric determination

The fluorescence EEMs of the analytes were obtained by increasing the excitation wavelength from 200 to 320 nm at 5 nm steps; and recording the emission spectra from 275 to 450 nm, every 1 nm. Excitation and emission slits were both set at 5 nm, and photomultiplier voltage was set at 600 V. All the measurements were made at room temperature.

For the establishment of the three-dimensional model, a central composite experimental design was employed, with a total of 16 samples. Also, four validation samples were incorporated. Twenty-six real wine samples were analysed.

Calibration and validation samples were prepared by the dissolution of the adequate aliquots of the stock analyte solutions with synthetic wine to a final volume of 10.0 mL. For the obtention of the EEMs of the real wine samples, a 10% (v/v) dilution in synthetic wine was employed. PVPP cleaning was made after the dilution and prior to the analysis. For this procedure, 0.5 g of PVPP were added to 10.0 mL of the diluted sample in a Falcon tube; and sonicated for 5 min to homogenate. Then, the tubes

were centrifuged for 2 min at 3000 rpm, to separate the phases. Cleaned wine was transferred to the quartz cell to its measurement.

3. Results and discussion

3.1 Chromatographic determination of the analytes during the winemaking process

All the samples collected during the winemaking process were chromatographed and compared, and only those corresponding to the most significant changes or corresponding to important milestones were analysed (i.e. start of the alcoholic or malolactic fermentation). Thus, the analytes concentrations of seven days of the process were obtained (Table 1). The samples were analysed employing the methodology aforementioned (section 2.4). Because the presence of matrix effect, it was decided to analyse the samples using the standard addition method in combination with the internal standard method, employing the peak area/internal standard area ratio as analytical signal. The quality parameters of this methodology, as well as the recovery values in real samples that demonstrate that the values obtained are accurate and real, can be found in the article where this method is optimised (Palomino-Vasco, Acedo-Valenzuela, et al., 2019).

As it can be seen in Table 1, most analytes undergo hardly any changes during the winemaking process. The analytes that varied the most were TYR (whose concentration was the highest in general and, moreover, increased with the passing of the days) and ARG (whose concentration in the must was high, but decreased greatly after the start of alcoholic fermentation, remaining constant the rest of the days). Although studies found in the literature do not reach a clear consensus on the behaviour of the evolution of each AA during winemaking, some authors have found similar results regarding the increase in TYR concentration throughout the days of fermentation (Izquierdo Cañas, García Romero, Gómez Alonso, Fernández González, & Palop Herreros, 2008; Lorenzo et al., 2017). These studies propose that this increase may be caused by the presence of alcoholic fermentation lees, as well as by yeast autolysis, which generates several protein degradation products, including AAs. Furthermore, it is

also suggested that the prophylaxis of acid-lactic bacteria with carboxylase activity during malolactic fermentation may influence the increase in TYR concentration. On the other hand, in the case of the ARG, other authors do seem to agree, and several have reported a significant decrease in its concentration along time (Izquierdo Cañas et al., 2008). Thus, the values obtained during this study seem to be in accordance with those found in the literature.

It can also be noted that on 10th day (start of malolactic fermentation) there was a slight increase in the concentration of TRP, as well as another slight increase on 25th day. However, in general, except in the case of TYR and ARG, the initial and final concentrations of all analytes were very similar. On the other hand, LYS was detected on all the samples, but in concentrations below the LOQ, and HIM, TYM, TRY and PEA were not detected in any samples. This can be an indication of the good quality of the wine, since the content of BAs is very low and, in addition, neither HIM or TYM, which are the two most harmful BAs for the human beings, were present.

3.2 Statistical analysis of the chromatographic data

Since there was no great variability in the data obtained except for TYR and ARG, it was decided to study it statistically to check whether these were indeed the most influential analytes during the winemaking process, which could simplify its monitorization.

Firstly, a Principal Component Analysis (PCA) was carried out. Covariance matrix was employed because data was dimensionally homogenous and presented similar mean values. This type of PCA assigns more weight to those variables with greater variance. For those concentrations found below the LOD or LOQ, the missing value was replaced by the corresponding LOD/2 or LOQ/2.

It was obtained that two principal components explained 93.0% of the variance. The loadings graph is showed in Figure 1A. PC1 explained 82.0% of variance, and was principally constituted by the positive contribution of TYR (82.1%) and the negative contribution of ARG (16.4%). On the other hand, PC2 explained 11.0% of variance, and was principally constituted by the positive contribution of

ARG (81.8%), TYR (16.4%) and TRP (1.4%). Taking this information into account, it can be said that the analyte that have the most weight in the data variance and, therefore, have the greatest influence on the winemaking process is TYR, followed far behind by ARG and TRP.

Figure 1B shows the scores graph obtained for the different samples. Considering the information obtained by the winemaker when the samples were collected, some stages of the winemaking process can be seen into the graph. Thus, the initial must (day 0) is the most different sample (highest concentration of ARG); the 3rd day started the alcoholic fermentation (drastic reduction of ARG concentration); the 10th day started the malolactic fermentation; and then, the samples post-malolactic fermentation presented an increasing TYR concentration.

It can therefore be said that one way of monitoring the winemaking process would be to control the concentration of TYR in the samples, as its concentration raised during the winemaking process, and it is the most influential analyte as shown in the statistical analysis, since it explains the 82.1% variance within the Principal Component 1, which accounts for 82.0% of the explanation of the total variance.

In order to contrast the information obtained through PCA, it was decided to carry out a cluster analysis, both non-hierarchical and hierarchical. In the case of the non-hierarchical analysis, the k-means methodology was selected. Although it was not possible to separate the samples into different groups, the class profile obtained made it clear that the most influential analyte was TYR (Figure 1C). On the other hand, Ward's method was selected as the hierarchical methodology. In this case, three different classes were obtained (Figure 1D). The first class included the initial samples (days 0 and 3), which corresponded with the must and the alcoholic fermentation start. The second class consisted exclusively of the 10th day, which corresponds to the start of malolactic fermentation. Finally, the last class is made up of the remaining samples, which are those collected after the start of malolactic fermentation. The class profile obtained with this method (Figure 1E) presented the same information as the previous one, TYR being the most influential analyte. However, in this case, ARG presented

some importance in class 1 (where it was produced the drastic reduction of the ARG concentration), and TRP was important in class 2 (the slight increase in its concentration that was aforementioned in section 3.1).

3.3 Fluorometric approach to the problem

Bearing in mind the information obtained of the statistical analysis, and taking into account that TYR presents fluorescence, a new approach to the monitoring of the winemaking process was proposed. For that reason, the following experiences were made for determining the best conditions for the fluorometric measurements.

Firstly, and employing conventional mode, EEMs of the fluorometric analytes were registered between 250 and 500 nm (each 1 nm), exciting between 200 and 320 nm (each 5 nm), with a voltage of 600 V and the slits opened 5 nm. It was checked that TYR, TRP and PHE, and their respective biogenic amines (TYM, TRY and PEA), were fluorescent (aqueous medium, no pH adjustment). The most fluorescent analytes were TYR and TYM, followed by TRP and TRY. PHE and PEA presented a lot lower florescence. It was also checked that each biogenic amine presented the same $\lambda_{ex}/\lambda_{em}$ as its precursor amino acid, although each pair of analytes presented the maximum fluorescence at different $\lambda_{ex}/\lambda_{em.}$ So, TYM and TYR presented the maximum fluorescence at 276/356 nm; TRY and TRP presented it at 274/301 nm; and PHE and PEA presented it at 257/280 nm. Taking into account that TRY, TYM and PEA were not detected in any wine sample, and that the concentrations of PHE ranged between 0.1 – 0.2 mg L^{-1} and its low fluorescence, only TYR and TRP were finally selected as analytes for the fluorometric method.

Synthetic wine was registered in these conditions to check that no fluorescence (except light dispersion) was observed. No fluorescence of wine sample was observed due the internal filter effect. The same was observed when front-face mode was used, and therefore, dilution of wine samples was necessary to observe fluorescence.

Wine dilutions were made with MilliQ water and synthetic wine to search for differences, and it was decided to use synthetic wine to help buffer the samples. Also, tests were made to find the best dilution, trying to maintain the greater quantity of wine and obtaining the less internal filter, finally choosing 10% dilution. After dilution, it was studied how the cleaning of the wine with PVPP affected, and it was found that PVPP eliminated both polyphenols and part of the internal filter from the sample.

Finally, the instrumental conditions were adjusted and measurements were made in the range of 275 - 450 nm (every 1 nm), with the sample being excited every 5 nm between 200 and 320 nm. No changes in voltage or slits opening were necessary.

3.4 Establishment of the fluorometric calibration and validation with the chromatographic data

Once the conditions of the fluorometric measurements were optimized, a central composite experimental design was proposed to establish the calibration data test. TRP concentrations ranged between 0.16 – 1.00 mg L^{-1}, and TYR concentrations ranged between 0.19 – 2.00 mg L^{-1} (taking into account the concentrations found by the chromatographic method and the dilution of the samples). Three more samples were added that presented only one analyte or any of them. Also, four validation samples were prepared with analytes concentrations different from those employed in the calibration data set. On the other hand, the wine samples analysed by UHPLC-FLD were also fluorometrically measured and, taking as nominal concentrations the concentrations obtained by the chromatographic method, acted as a second validation set.

After obtaining the EEMs, the *EEM_corr* routine (Chiappini et al., 2019) was used to eliminate Rayleigh dispersion. PARAFAC was then applied to determine the number of components and to predict the concentrations of the samples. Both the test samples and the previously analysed wine samples were employed as validation files, obtaining that three components explained the system, by both the CORCONDIA and the model fit criteria. For the determination of the number of components, all modes were restricted to non-negative, since neither concentrations nor spectra can take negative

values. The three-dimensional spectra of the components obtained are represented in Figure 2. The first one (C1) corresponds to TRP, while the third one (C3) corresponds to TYR. The second one (C2) must be some unknown component in the wine.

Then, the concentrations of the validation samples were predicted and the results were compared with the nominal values. Also, eleven replicates with the analyte's concentration values of one of the test samples were prepared and predicted, to obtain the values of repeatability. In the optimized conditions, a relative standard deviation (RSD) of 0.87% and 0.75% was obtained for TYR and TRP measurements, respectively.

The prediction of the test samples was good for both analytes. However, in the wine validation set, the prediction was only good for TYR values, which was checked employing the Elliptical Joint Confidence Region test or EJCR (Figure 3) (Mandel & Linnig, 1957), taking into account the applied dilution. Nominal values or chromatographic concentrations, as appropriate, as well as the percentage recovery are shown in the Table 2. Predicted values for TRP in wine samples were outside the calibration range and the predictions were not good, so no data is presented. The reason why the prediction of TRP in real samples is not good, although it is in test samples, may be that TRP has lower fluorescence intensity than TYR. This fact, together with its low concentrations in the wine samples (as can be seen in the values obtained by chromatography in Table 1), may have caused very low signals for its correct quantification. Furthermore, the emission wavelength of TRP is inside the UV zone (i.e.: 301 nm), while TYR emission wavelength (i.e.: 356 nm) is closer to the visible zone, so its signal may have been affected by more constituents of the wine matrix, which could have influenced the signal or generated more internal filtering.

3.5 Monitoring of the winemaking process by EEMs

Although the prediction of TRP in real wine samples is not good, the monitoring of the winemaking process could be carried out by quantifying TYR concentration, since it generated good

results in accordance with the chromatographically obtained values and, moreover, it is the most influential analyte according to the statistical study, as previously mentioned (section 3.2).

It was therefore decided to measure fluorometrically all the samples collected during the winemaking process. The results are showed in Figure 4. The concentration of TYR undergoes a progressive increase from the initial must until approximately the start of malolactic fermentation (day 10^{th}), almost doubled at this point. In the following days, TYR concentration variated between 5.21 - 7.68 mg L^{-1}, presenting ups and downs with respect to the value assumed by the polynomial of grade three which approximately explains its behaviour (red line in Figure 4). These variations can be explained by two reasons. First, wine is a live system, in which the yeasts themselves are responsible for homogenizing the sample inside the vat, since during the malolactic fermentation the winery staff do not touch the wine. For this reason, the sample is not completely homogenized, and there may be more variations than expected. Secondly, it must be taken into account that there are two opposite processes that take place at the same time and that affect the concentration of TYR: on the one hand, the yeasts consume TYR and part of it is transformed into TYM; on the other hand, the proteolysis and autolysis of the yeasts results in more TYR.

Regarding the slight increase of TYR concentration in the last day, other authors (Lorenzo et al., 2017) have studied how different parameters of winemaking (i.e.: temperature and alcoholic degree) affect the concentrations of different AAs and BAs. In all the conditions they tested, the TYR concentration after the malolactic fermentation was higher than that found after the alcoholic fermentation, and they concluded that the final concentrations of the analytes depended on these conditions. In our case, the rebound is not as pronounced, but the differences in the type of grapes, temperature, alcoholic degree, yeast strains, etc. must be taken into account. In any case, this rebound on the last day could be used as an indicator that fermentation is over and it is time to transfer the wine to the bottles.

4. Conclusions

The determination and quantification of amino acids and biogenic amines in wine samples collected during the winemaking process have been carried out by UHPLC-FLD. Then, a statistical analysis of the data was carried out (PCA and Cluster Analysis), from which it was concluded that the most influential analyte of data variance was TYR.

Thanks to the fluorescence of TYR, a method has been developed for its determination and quantification by means of EEMs. An attempt has been made to determine TRP (a less influential but also fluorescent analyte) together, but the quantification did not obtain good results. However, the results for TYR concentration have been validated with the chromatographic methodology by means of the ellipse test. The monitoring of TYR concentration throughout the winemaking process allowed to determine the start and the end of malolactic fermentation. Therefore, EEMs could be used as a much faster and cheaper method for monitoring the winemaking process through TYR concentration monitoring.

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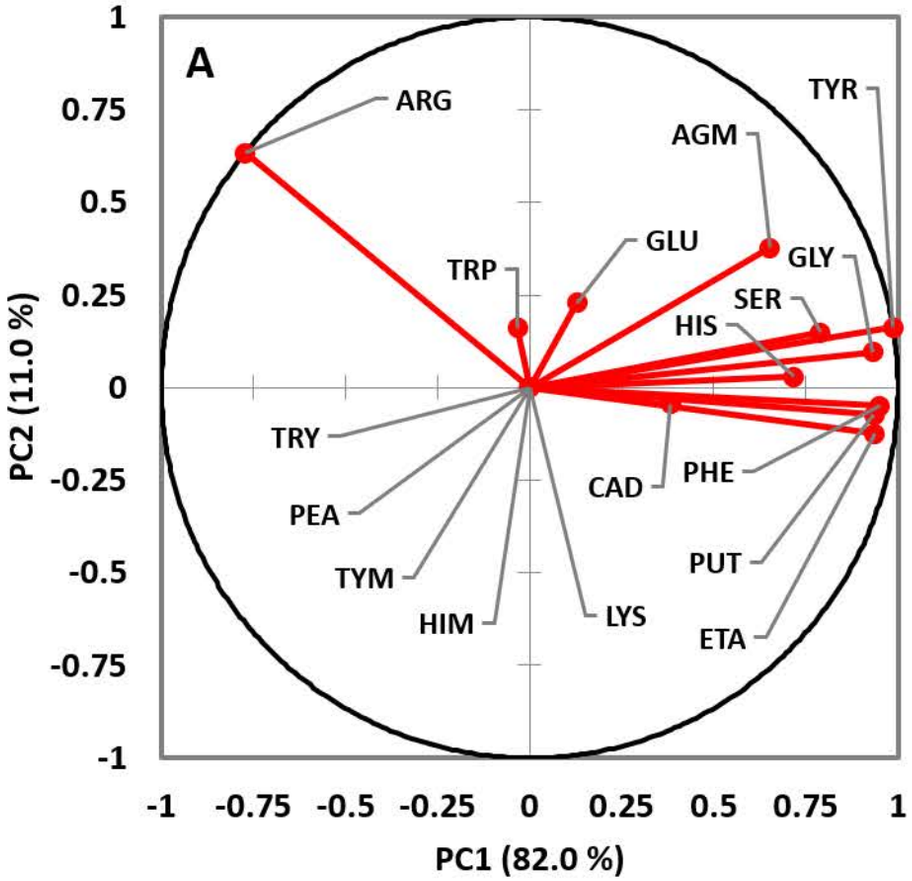
FIGURE CAPTIONS

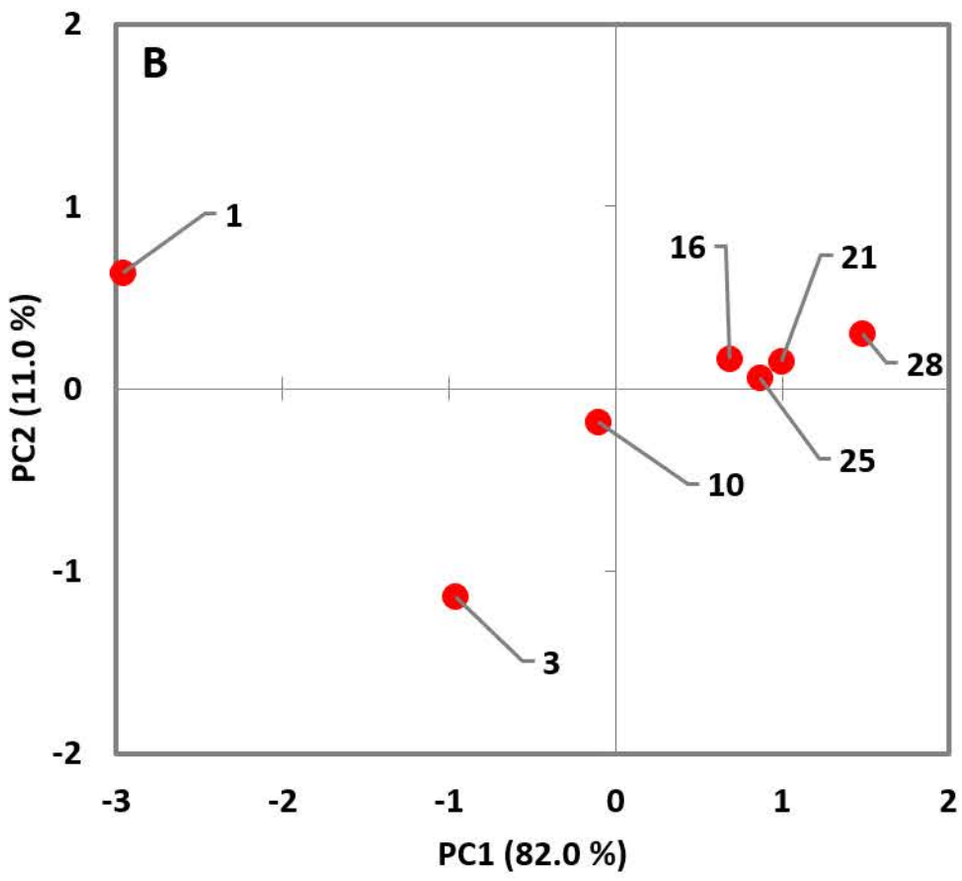
Figure 1. Statistical analysis of the concentrations of biogenic amines and amino acids obtained by UHPLC-FD. Representation of the loadings graph (A) and the scores graph (B) obtained after Principal Component Analysis. The numbers identifying the samples correspond to the days on which the samples were collected. Class profile obtained by k-means method (C); and dendrogram (D) and class profile (E) obtained by Ward´s method, obtained after Cluster Analysis.

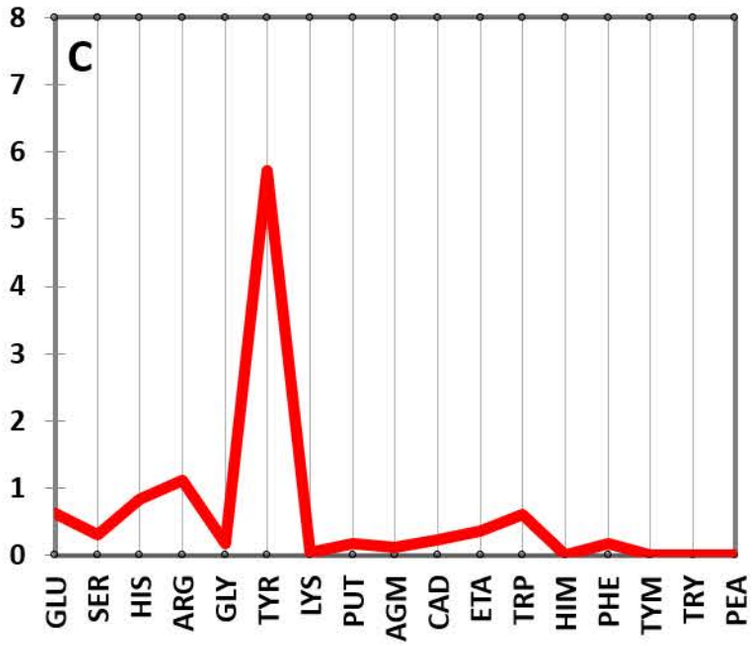
Figure 2. PARAFAC components obtained, represented as three-dimensional spectra. Component 1 (C1) corresponds to TRP and component 3 (C3) corresponds to TYR. Component 2 (C2) corresponds to an unknown component of the wine samples.

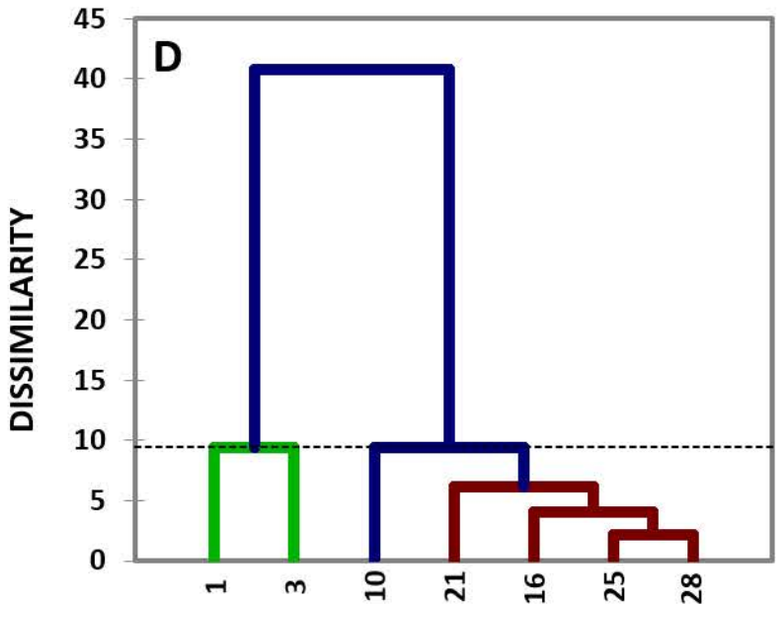
Figure 3. Elliptical joint confidence regions (EJCR) test for TRP in test samples (red line), TYR in test samples (green line) and TYR in wine samples previously analysed by UHPLC-FLD (blue line). Ideal point (1, 0) represented by a black point.

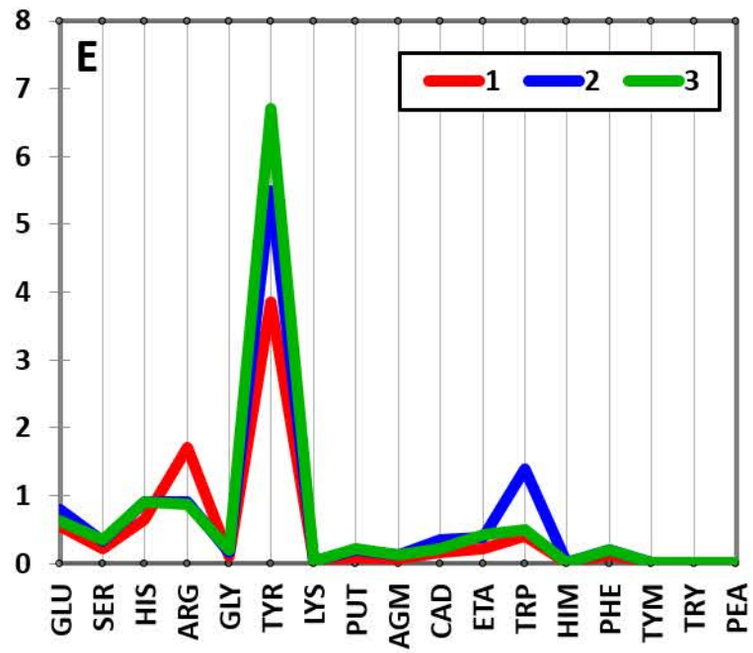
Figure 4. Variation in TYR concentration (expressed in units of mg L^{-1}) over the days of the winemaking process (black points). In red, the polynomial function of grade 3 that approximately explains the concentration trend.

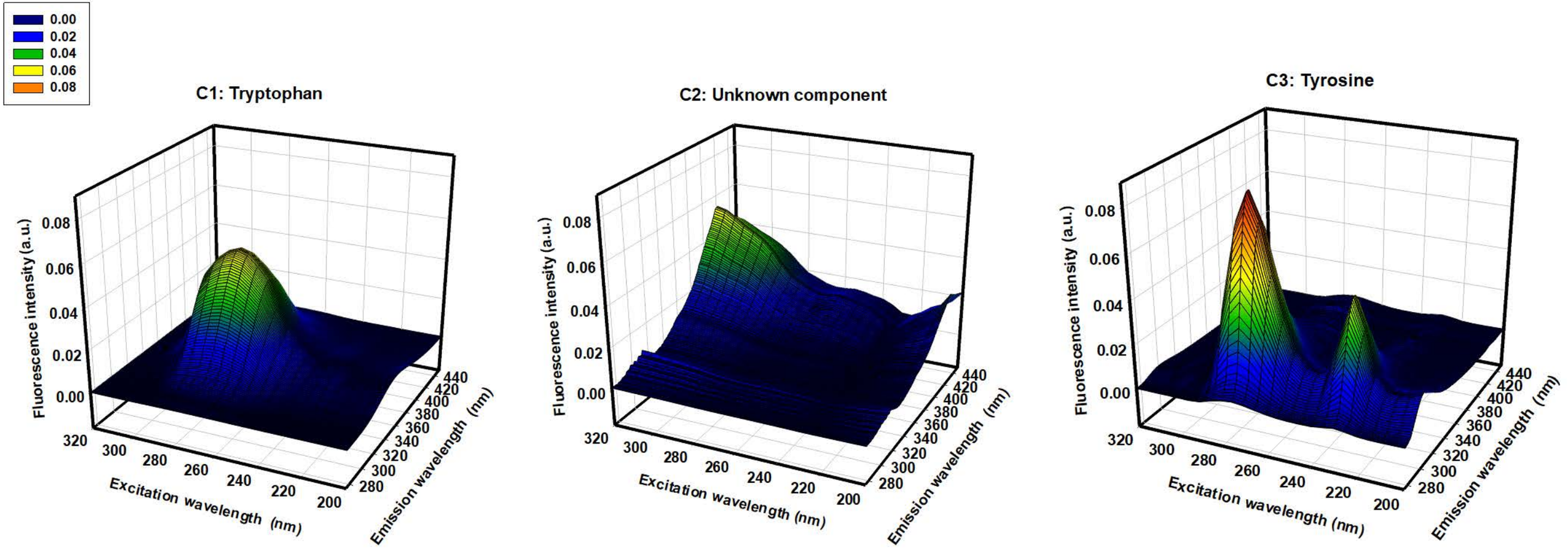

A
ARG
TYR
AGM
GLU
GLY
TRP
SER
HIS
TRY
CAD
PHE
PEA
TYM
PUT
HIM
LYS
ETA
PC2 (11.0 %)
PC1 (82.0 %)
1
0.75
0.5
0.25
0
-0.25
-0.5
-0.75
-1
-1 -0.75 -0.5 -0.25 0 0.25 0.5 0.75 1

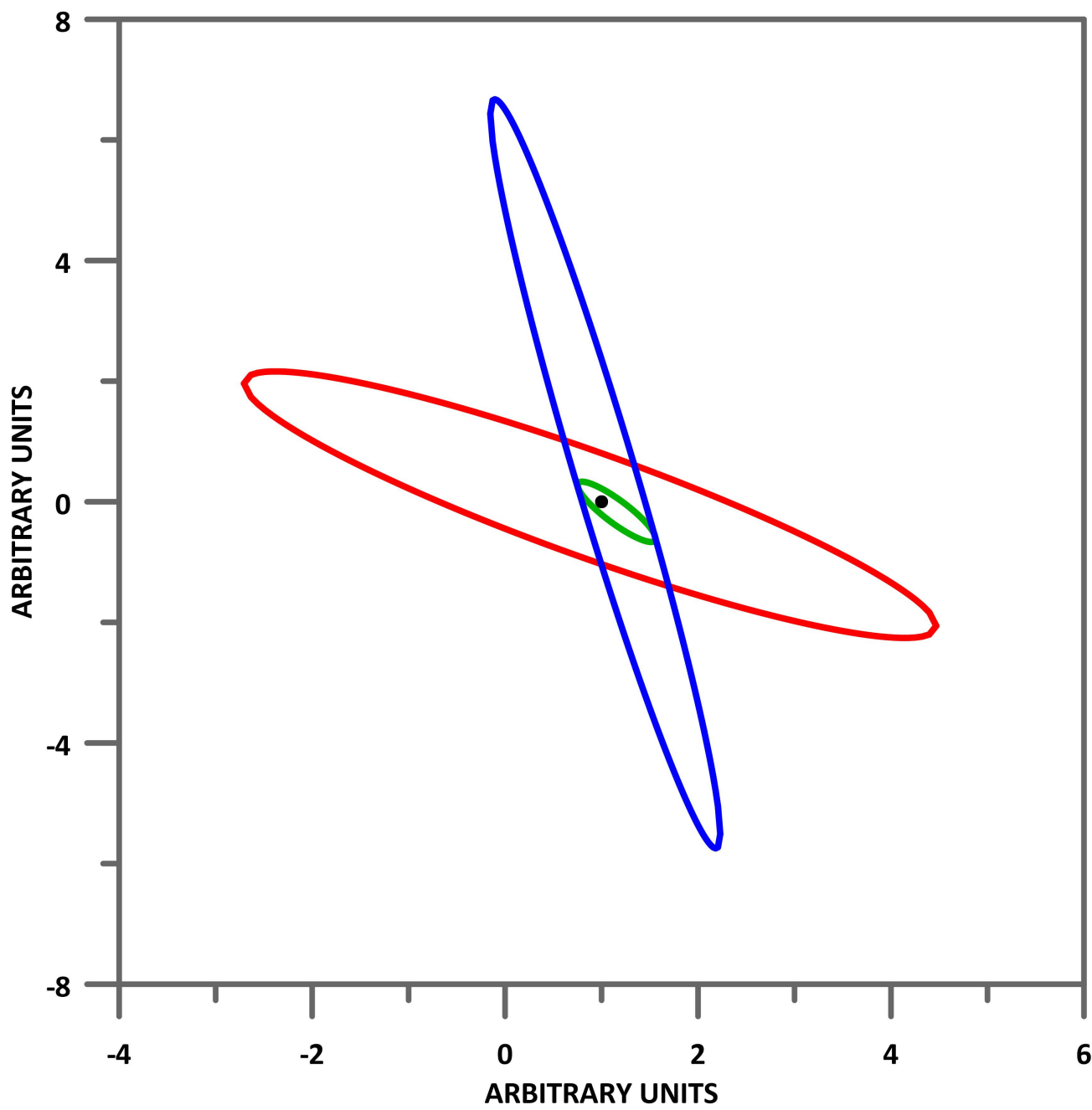

B
1
16
21
28
10
25
3
PC2 (11.0 %)
PC1 (82.0 %)
2
1
0
-1
-2
-3 -2 -1 0 1 2


C
GLU SER HIS ARG GLY TYR LYS PUT AGM CAD ETA TRP HIM PHE TYM TRY PEA
8
7
6
5
4
3
2
1
0


D
DISSIMILARITY
SAMPLING DAY
1 3 10 21 16 25 28
45
40
35
30
25
20
15
10
5
0


E
1
2
3
GLU SER HIS ARG GLY TYR LYS PUT AGM CAD ETA TRP HIM PHE TYM TRY PEA
8
7
6
5
4
3
2
1
0


0.00
0.02
0.04
0.06
0.08
C1: Tryptophan
C2: Unknown component
C3: Tyrosine
Fluorescence intensity (a.u.)
0.00
0.02
0.04
0.06
0.08
Excitation wavelength (nm)
320
300
280
260
240
220
200
Emission wavelength (nm)
280
300
320
340
360
380
400
420
440


8
4
0
-4
-8
ARBITRARY UNITS
-4
-2
0
2
4
6
ARBITRARY UNITS

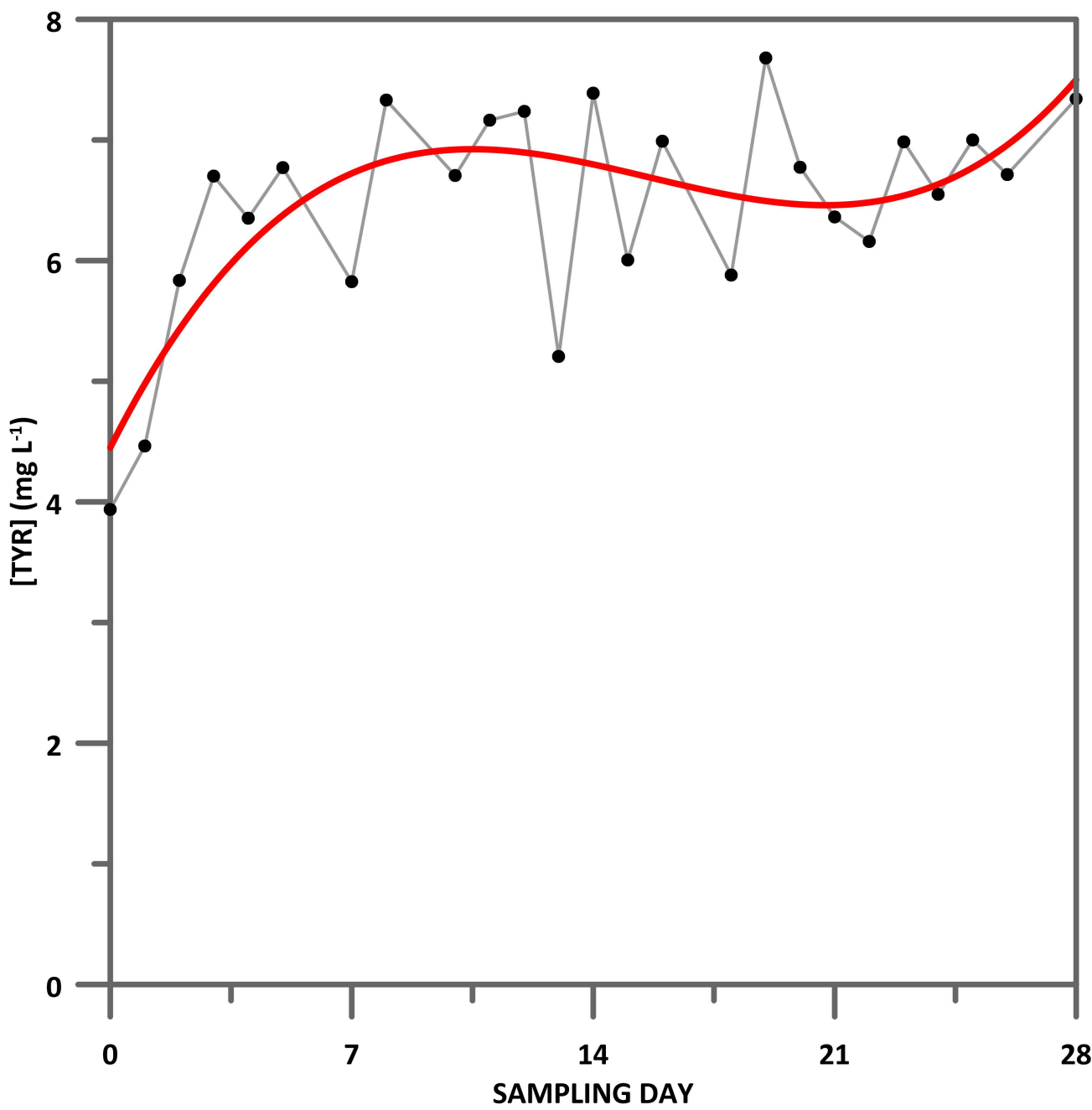

8
6
4
2
0
[TYR] (mg L-1)
0
7
14
21
28
SAMPLING DAY

Table 1. *Analytes concentrations (standard deviation) chromatographically obtained for seven different days of winemaking sampling. LOD and LOQ stand for Limit of Detection (calculated by Long-Winfordner method) and Limit of Quantification (calculated as 3.3 times LOD), respectively, and are referred to those obtained in the validation of the UHPLC-FD method* (Palomino-Vasco, Acedo-Valenzuela, Rodríguez-Cáceres, & Mora-Diez, 2019). *All the concentrations are expressed in mg* L^{-1}.

DAY	GLU	SER	HIS	ARG	GLY	TYR	LYS	PUT	AGM	CAD	ETA	TRP	HIM	PHE	TYM	TRY	PEA
0	0.6 (0.2)	0.20 (0.06)	0.6 (0.1)	2.9 (0.3)	0.08 (0.01)	3.3 (0.3)	<LOQ	0.05 (0.01)	0.08 (0.02)	0.16 (0.03)	0.14 (0.02)	0.6 (0.1)	<LOD	0.10 (0.04)	<LOD	<LOD	<LOD
3	0.5 (0.1)	0.23 (0.08)	0.7 (0.1)	0.5 (0.1)	0.12 (0.01)	4.4 (0.4)	<LOQ	0.14 (0.03)	0.06 (0.02)	0.18 (0.03)	0.31 (0.04)	<LOQ	<LOD	0.15 (0.07)	<LOD	<LOD	<LOD
10	0.8 (0.1)	0.32 (0.08)	0.9 (0.2)	0.9 (0.2)	0.17 (0.04)	5.5 (0.7)	<LOQ	0.19 (0.03)	0.13 (0.04)	0.34 (0.04)	0.39 (0.06)	1.4 (0.2)	<LOD	0.2 (0.1)	<LOD	<LOD	<LOD
16	0.7 (0.1)	0.26 (0.08)	1.0 (0.2)	1.0 (0.2)	0.17 (0.02)	6.4 (0.8)	<LOQ	0.24 (0.04)	0.10 (0.05)	0.23 (0.04)	0.39 (0.06)	0.5 (0.1)	<LOD	0.2 (0.1)	<LOD	<LOD	<LOD
21	0.5 (0.2)	0.4 (0.1)	0.8 (0.2)	0.9 (0.2)	0.22 (0.05)	6.7 (0.5)	<LOQ	0.22 (0.04)	0.10 (0.03)	0.16 (0.03)	0.48 (0.04)	<LOQ	<LOD	0.2 (0.1)	<LOD	<LOD	<LOD
25	0.7 (0.3)	0.4 (0.1)	1.0 (0.2)	0.8 (0.2)	0.21 (0.03)	6.5 (0.8)	<LOQ	0.20 (0.02)	0.16 (0.03)	0.26 (0.03)	0.45 (0.04)	0.8 (0.1)	<LOD	0.2 (0.1)	<LOD	<LOD	<LOD
28	0.6 (0.1)	0.34 (0.07)	0.8 (0.2)	0.8 (0.2)	0.19 (0.05)	7.2 (0.9)	<LOQ	0.20 (0.03)	0.15 (0.03)	0.25 (0.03)	0.40 (0.05)	0.51 (0.07)	<LOD	0.2 (0.1)	<LOD	<LOD	<LOD
LOD	0.21	0.08	0.10	0.03	0.3	0.05	0.02	0.01	0.03	0.01	0.01	0.12	0.03	0.10	0.03	0.01	0.02
LOQ	0.68	0.26	0.32	0.09	0.10	0.18	0.07	0.03	0.10	0.05	0.02	0.41	0.08	0.35	0.09	0.03	0.08

Table 2. *Nominal and predicted concentrations of TYR (SD) and TRP (SD), expressed in mg L^{-1}, as well as percentage of recovery, in test samples and in wine samples collected during the winemaking process.*

	TYR			**TRP**		
Test sample	**Nominal concentration**	**Predicted concentration**	**Recovery (%)**	**Nominal concentration**	**Predicted concentration**	**Recovery (%)**
1	0.75	0.66 (0.02)	88.6	0.75	0.50 (0.05)	66.3
2	1.90	1.99 (0.03)	105.0	0.20	0.21 (0.07)	103.5
3	0.25	0.15 (0.03)	60.7	1.00	1.3 (0.1)	124.7
4	1.50	1.61 (0.03)	107.4	0.40	0.30 (0.06)	75.1
	MEAN RECOVERY (%)		**90.4**	**MEAN RECOVERY (%)**		**92.4**
Day of sampling	**UHPLC-FLD concentration**	**Predicted concentration**	**Recovery (%)**			
0	3.3 (0.3)	3.7 (0.3)	112.1			
3	4.4 (0.4)	6.5 (0.2)	147.7			
10	5.5 (0.7)	6.5 (0.2)	118.2			
16	6.4 (0.8)	6.8 (0.2)	106.3			
21	6.7 (0.5)	6.1 (0.2)	91.0			
25	6.5 (0.8)	6.8 (0.2)	104.6			
28	7.2 (0.9)	7.1 (0.2)	98.6			
	MEAN RECOVERY (%)		**111.2**			