BIOGENIC AMINES PROFILE IN RED WINES REGARDING AGING AND STORAGE CONDITIONS

Abstract: The determination and quantification of eight biogenic amines in red wines without sample clean-up has been carried out by an ultra-high performance chromatographic method with fluorescent detection based on the *o*-phthaldialdehyde derivatization reaction. In these conditions, several monovarietal '*Tempranillo*' wines (young, oak and aged ones) have been analyzed and the total concentrations of biogenic amines ranged between 22.2 and 73.4 mg L^{-1}, which is in concordance with other Spanish red wines. No significant differences of total biogenic amines content between young and oak wines have been observed, but total biogenic amines content in aged wine is significant different. A study of the influence of the storage conditions through time has been carried out with three types of wine (young, oak and aged wine). Principal Components Analysis (without and with Varimax rotation) and hierarchical and non-hierarchical cluster analysis grouped the samples by wine aging. On the other hand, Discriminant Analysis allowed to classify the observations of each wine by storage time and conditions (storage temperature and bottle closing).

Keywords: biogenic amines; UHPLC-FD; wine; Principal Components Analysis; cluster analysis; Discriminant Analysis; storage conditions

1. Introduction

Amines are nitrogenous bases which are usually synthetized in food by decarboxylation of amino acids (Lange and Wittmann, 2002). When this process is performed by the microbial action of living organisms they are designated as biogenic amines (BAs). Their synthesis in wines is affected by several factors, as the availability of free amino acids of the grapes, the presence of microorganisms with amino acids decarboxylases, or the favorable conditions of such microorganisms for the growth and production of their enzymes (Beneduce et al., 2010; Nouadje et al., 1997; Shalaby et al., 1996).

The presence of BAs in foodstuffs has traditionally been used as an indicator of undesired microbial activity, and relatively high amounts of certain BAs have been correlated with deterioration of foods and/or their defective manufacture. The BAs content in wine depends on the processes involved in the vinification, the amino acidic composition of the must, the presence of yeasts and lactic acid bacteria, grape variety and region (García-Villar et al., 2006; Lange and Wittmann, 2002; Nouadje et al., 1997).

Besides being a quality index, some BAs can cause health problems, including palpitations, hypo- and hyper-tensive crisis, headache and nausea among others. Its effects can be particularly significant when are consumed in high quantities or when the ability to metabolize them is reduced by the ingestion of monoaminoxidase inhibitor drugs or alcohol, so its determination in alcoholic beverages such as wine is essential (Kirschbaum et al., 1999; Lange and Wittmann, 2002; Romero et al., 2000; Yamamoto et al., 1980). Among BAs in food, histamine (HIM) is the most potentially hazardous one, presenting psychoactive and cutaneous effects, as well as affections in the blood vessels and gastrointestinal problems. Tyramine (TYM) is another important BA, as it increases blood pressure and can cause tachycardia, and also has synergistic effects with HIM; and phenylethylamine (PEA) is a potent migraine inductor. On the other hand, putrescine (PUT) or cadaverine (CAD) are not considered to be toxic but have been described as being able to enhance HIM toxicity, as well as produce negative effects on sensory quality, giving putrefaction and rotting flesh flavor to foodstuffs (Arlorio et al.,

1998; Kirschbaum et al., 1999; Lange and Wittmann, 2002; Moreno-Arribas and Polo, 2008; Redruello et al., 2013; Sentellas et al., 2016).

This toxicity has led to general agreement that they should not be allowed to accumulate in food (Arlorio et al., 1998; Beneduce et al., 2010). Although there is no legislation dealing with BAs content in wines, some countries recommend limit HIM concentration to a few milligrams per liter (2 - 10 mg L^{-1}) (Busto et al., 1994; García-Villar et al., 2006; Peña-Gallego et al., 2012).

Regarding wine consumption in Spain, Spanish people prefer red wines, mainly produced from '*Tempranillo*' grapes, and which have been aged in oak barrels ("www.degustacastillayleon.es," 2019). In Spanish red wines, and according to the literature, HIM concentrations varied between not detected (n.d.) and 19.6 mg L^{-1}, so those limits were exceeded. PUT, TYM, ethanolamine (ETA) and CAD were also reported in high concentrations (from not detected to 99.9; 59.7; 27.8, and 14.1 mg L^{-1}, respectively). The BAs in the lowest concentrations were agmatine (AGM; n.d. - 6.7 mg L^{-1}), PEA (n.d. – 5.15 mg L^{-1}) and tryptamine (TRY; n.d. – 4.7 mg L^{-1}) (Peña-Gallego *et al*., 2012).

This BAs content can be modified during the storage of the wine. Several studies show the evolution of the BAs profile over time during barrel aging (Jiménez Moreno et al., 2003). However, the consumer may be concerned about the evolution of the BAs once the wine is in their hands and the bottle is opened. As far as we have found, there is only one study analysing the evolution of BAs in opened wine bottles (Ordóñez et al., 2017), in which they studied the evolution of the BAs profile in white wine and red wine of two qualities, for 10 days, under different storage conditions.

BAs determination in fermented food is complicated because its low concentrations and the complexity of the samples, being, usually, necessary some clean-up and/or preconcentration steps. BAs are determined by several techniques, being HPLC using C18 columns the most commonly employed. The chromatographic methods developed for its determination have been reviewed by Hernández-Cassou & Saurina in 2011 (Hernández-Cassou and Saurina, 2011), Ordóñez *et al*. in 2016 (Ordóñez et al., 2016) and Papageorgiou *et al.* in 2018 (Papageorgiou et al., 2018). In these methods, a pre- or post-

column derivatization step is necessary because these compounds do not have adequate chromatographic or absorption/fluorescence properties. The most used derivatization reagents are *o*-phthaldialdehyde (OPA), fluorenylmethylchloroformate and dansyl chloride (Busto et al., 1994; Hernández-Cassou and Saurina, 2011; Ordóñez et al., 2016).

Ultra-High Performance Liquid Chromatography (UHPLC) is becoming very important lately. This technique employs columns with a smaller particle size (minor than 2 μm), as well as greater pressures (up to 600 bar), obtaining better separation capacity, and faster and more sensible results. However, Ordóñez *et al*. (Ordóñez et al., 2016) and Papageorgiou *et al*. (Papageorgiou et al., 2018) have reviewed the recent trends in the determination of biogenic amines in foodstuffs, finding only four methods employing UHPLC in wines. Two of them (Iijima *et al*., 2013; Latorre-Moratalla *et al*., 2009) employed ion pair chromatography, derivatizing with OPA post-column, and with fluorescence detection (FD), at 40°C. The other two methods (Jia et al., 2012; Lee et al., 2015) performed a precolumn derivatization with dansyl chloride and a solid phase extraction (SPE) of the sample, detecting the derivatives with MS/MS. The times of analysis ranged between 8 and 21 minutes, obtaining in all cases good analytical figures of merit.

Sample treatment is usually done to eliminate possible interferences and to concentrate the analytes. Solvent extraction is the most commonly used technique, being solid phase (micro) extraction the most used. Liquid-liquid extraction and its different variants (liquid-liquid microextraction; dispersive liquid-liquid microextraction; etc.) as well as ionic liquids are also employed. On the other hand, it is also common to add different substances to help clean the sample: polyvinylpyrrolidone (elimination of phenolic compounds and related substances) and acids or organic solvents for protein precipitation (Ordóñez et al., 2016; Papageorgiou et al., 2018).

Therefore, in this paper, 8 biogenic amines have been determined and quantified employing an UHPLC-FD in Spanish '*Tempranillo*' red wine, after derivatization with OPA, without sample clean-

94 up, besides filtration. Significant differences in the total biogenic amine content of wine of different
95 aging have been looked for. Also, a study of the influence of storage conditions in the BAs profile had
96 been carried out, increasing the storage time to one month and using three types of red wine of different
97 aging (young, oak and aged wine). Principal Components Analysis and Discriminant Analysis were
98 performed to group and classify the samples.
99
100 **2. Materials and Methods**
101 ***2.1 Chemicals***
102 Eight BAs were determined in this study: PUT, HIM, TYM (Sigma), AGM (Alfa Aesar), CAD,
103 TRY, PEA (Aldrich) and ETA (Merck). Octylamine (OCT; Fluka) was used as internal standard (IS).
104 A stock solution of 500 mg L^{-1} of each BA was prepared (stored refrigerated and in darkness) by
105 solving adequate amounts of the presentation in 0.1 M HCl (Panreac) and was used to daily prepare
106 working analyte solutions. Also, a 0.4 mg mL^{-1} stock solution of OPA (Aldrich) was weekly prepared
107 in methanol UHPLC-grade (Panreac).
108 Boric acid (Merck) and NaOH (Panreac) were used to weekly prepare a boric acid/sodium
109 borate buffer (pH 10.50; 0.4 M). 2-mercaptoethanol (2-ME; Sigma-Aldrich) was used in the
110 derivatization reaction to improve the sensitivity and stability of the derivatives (Ordóñez et al., 2016;
111 Peña-Gallego et al., 2012).
112 Mobile phase was acetonitrile UHPLC-grade (Sigma-Aldrich) and Trizma® base buffer (2-
113 amino-2-(hydroxymethyl)-1,3-propanediol; Sigma-Aldrich), prepared in a concentration of 0.08 M and
114 adjusted the pH at 8.30 with diluted HCl (Panreac). Before its use, mobile phase was filtered (0.22 µm
115 membrane nylon filter; Teknokroma), and ultrasonicated.
116 The red wines analyzed were obtained from local markets and were kept refrigerated and in
117 darkness, except for the samples employed in the storage conditions study at room temperature. A total
118 amount of thirteen wine brands were taken for this study.

2.2 Instrumentation and software

An Agilent Model 1260 Infinity High Performance Liquid Chromatograph (Agilent Technologies) was employed to carry out the experiences. It was equipped with an online degasser, quaternary pump (G1311B), column oven compartment (G1316A), autosampler (G1329B), UV-VIS diode-array detector (G1315D) and fluorescence detector (G1321B). Data treatment and instrument control were performed using the ChemStation software. Previous experiences were performed on a Zorbax Eclipse XDB-C18 analytical column (50 x 4.6 mm; 1.8 µm; Agilent Technologies), but finally a Zorbax Eclipse XDB-C18 analytical column (100 x 4.6 mm; 1.8 µm; Agilent Technologies) was employed for the analytical separation.

pH measurements were performed using a Crison Micro pH 501 meter (Barcelona, Spain), equipped with combined glass/saturated calomel electrode, which was daily calibrated.

ACOC software (programmed in MATLAB code) (Galeano Diaz *et al*., 2007) was used to build the calibration curves and calculate analytical figures of merit. Student t tests of mean comparation were performed using IBM SPSS Statistics v.19 (SPSS Inc., Chicago, USA). Principal Components Analysis and Discriminant Analysis were performed using XLSTAT software (Addinsoft, Paris, France).

2.3 Derivatization reaction

The derivatization reaction was slightly modified from the proposed in the official method for determination of BAs (OIV, 2009). Briefly, in a glass tube, 0.4 mL OPA, 0.6 mL methanol, 1.0 mL of boric acid/sodium borate buffer and 0.3 mL of 2-ME were mixed in a vortex. Then, 0.1 mL of IS (5.0 mg L^{-1}) was added, and:

a) <u>For calibration curve:</u> 0.1 mL aliquots of mixed BAs solutions (concentration between 0.019 and 1.0 mg L^{-1}) were added. The solutions were vortexed.

b) For real samples:

- *Determination of ETA and PUT:* wine sample was diluted in ultrapure water (1:1). Then, 0.05 mL of HCl 0.1 M were placed and vortexed.
- *Determination of the other BAs:* 0.05 mL of wine (without dilution) and 0.05 mL of each BAs addition standard (0.05 – 0.30 mg L^{-1} of each BA) were placed and vortexed.

2.4 Calibration curve

Calibration curve was stablished by the internal standard method. To build it, standard solutions were prepared as aforementioned (section 2.3), by addition of aliquots of mixed BAs solutions in variable concentration (0.019 – 1.0 mg L^{-1}). PTFE membrane filters (0.22 µm) were used to filter them before transferred to topaz vials and be immediately injected into chromatographic system. The chromatograms obtained under the optimized conditions were processed using the ChemStation package.

2.5 UHPLC-FD method

The derivatives were eluted at 1.0 mL min^{-1}, using a mobile phase composed by Trizma® base buffer (eluent A) and ACN (eluent B). A gradient mode was established, consisting in a first 0 – 2 min step from 20% to 30% B; 2 – 7 min step from 30% to 40% B; 7 – 10 min step from 40% to 100% B; and a final step 10 – 13 min down to initial conditions (20% B). The column was reequilibrated during 2 min.

5 µL of derivatized sample were injected, and the eluate was fluorimetrically monitored (356/445 nm), using the peak area/IS area ratio as analytical signal.

2.6 Analysis of wine

To determine ETA and PUT a dilution of wine was necessary (1:1, wine:water). The derivatization was carried out as mentioned in paragraph 2.3, by internal standard method, and injecting the samples by triplicate. The concentration of each analyte was determined substituting the analytical signal (BA area/IS area ratio) into the calibration curve.

The other BAs could not be determined by the internal standard method, because of the existence of matrix effect, so they were determined by standard addition method combined with IS. Thus, the concentration of each analyte was determined by extrapolation of the curves stablished between areas ratio (BA area/IS area) versus concentration of BAs added. In this case, no dilution of the sample was necessary. The procedures described previously (sections 2.3 and 2.5) were followed. All the solutions were prepared by duplicate.

For its analysis, compounds were identified by comparing retention times with their standards, and by spiking different solutions. The analytical signal employed was the peak area/IS area ratio obtained measuring the fluorescence at 445 nm (exciting at 356 nm).

At the end of the working day, the column was cleaned by the injection of 100 µL of MeOH and going through the column first ultrapure water for 40 minutes and then ACN for 40 minutes.

2.7 Influence of the storage conditions in the evolution of BAs profile in opened bottles

The influence of the storage conditions in the BAs profile of opened bottles was performed comparing four different conditions: room temperature versus refrigerated, and stopped with cork versus maintained in argon atmosphere. Samples were analyzed as aforementioned (section 2.6). All aliquots were tempered before analysis.

3. Results and discussion

3.1 Optimization of chromatographic conditions and evaluation of method performance

193 Some previous experimental studies were performed and finally a Zorbax Eclipse XDB-C18
194 analytical column (4.6 x 100 mm; 1.8 µm) was employed, using a mobile phase constituted by
195 Trizma® base (eluent A) and ACN (eluent B), in a gradient elution and at 50°C (column temperature).
196 The chosen gradient was as follows: 2 minutes from initial conditions (20% B) to 30% B; a lineal
197 gradient step 2 – 7 min up to 40% B, a lineal gradient step 7 – 10 min up to 100% B, and finally, a
198 lineal gradient step 10 – 13 min down to initial conditions for 2 min. In these conditions, adequate
199 resolution between all peaks was obtained, as can be seen in Figure 1. This figure also shows the
200 chromatogram obtained of a real wine sample.
201 Calibration curves were established between 0.019 and 1.0 mg L^{-1} of BAs to assess the
202 linearity, injecting three times each standard under the optimum conditions. Fluorescence emission was
203 measured at 445 nm, exciting at 356 nm. Calibration curves were built employing the internal standard
204 method. Also, percentage of recovery was calculated, and the mean value obtained was 104 ± 15%.
205 These values, together with the figures of merit are listed in Table 1.
206
207 ***3.2 Analysis of BAs in wine samples***
208 Calibration curves equations obtained by internal standard method were compared with those
209 obtained by standard addition method combined with IS, observing a matrix effect for all BAs except
210 ETA and PUT. For these two BAs, only a dilution was employed and were determined by internal
211 standard method. The other BAs were found in lower concentrations and no dilution was necessary.
212 Taking into account their matrix effect, standard addition method combined with IS was selected as
213 determination method.
214 At the end of each working day, the column was cleaned injecting 100 µL of MeOH, with a
215 cleaning program consisting on 40 minutes of ultrapure water and 40 minutes of ACN (0.5 mL min^{-1}).

In these conditions, more than 250 injections of samples can be injected into the column without loss of efficiency.

3.3 BAs content in just opened bottles

Several red monovarietal ('*Tempranillo*') young, oak and aged wines were analyzed by the developed method. In Table 2 characteristics of the analyzed wines are shown, and Table 3 shows the concentrations of the BAs found. Total concentration ranged between 22.2 and 73.4 mg L^{-1}, which is in concordance with the values found in the literature, being between 2.49 and 150.64 mg L^{-1} (Peña-Gallego *et al*., 2012).

In the literature, ETA is only determined in two of fifteen methods found, with concentrations between n.d. and 27.8 mg L^{-1}. However, the developed method permitted to determine ETA and it was found in all the analyzed samples in high concentrations (13.0 – 27.7 mg L^{-1}). PUT also appeared in all samples with a great variability (5.88 and 42.6 mg L^{-1}) as it occurs in other Spanish wines (Peña-Gallego *et al*., 2012).

HIM, AGM, TYM and CAD were found in lower concentrations (n.d.-10.3; 4.3; 4.1, and 2.5 mg L^{-1}, respectively). Other Spanish red wines present higher concentrations for all these BAs, especially for TYM which can reach 59.7 mg L^{-1} (Peña-Gallego *et al*., 2012). The presence of HIM and TYM in lower concentrations in analyzed wines may present an advantage, since these BAs present a synergistic effect, and are the most dangerous for health.

Finally, TRY was not detected in any of the analyzed wines, and PEA was only detected in one of them. Wines of other regions of Spain present concentrations of these BAs that range between n.d.-4.7 and 5.15 mg L^{-1}, respectively (Peña-Gallego *et al*., 2012).

No significant differences have been observed in the total BAs concentration between young and oak red wines. Differences between young and/or oak wines with aged ones could not be calculated because only an aged wine was analyzed.

3.4 Influence of the storage conditions in the BAs profile

A study was carried out to check whether there were changes in the profile of BAs according to storage conditions in order, if so, to be able to decide which are the optimal conditions for the storage of wine once the bottles are opened. For this purpose, evolution of BAs profile was monitored for one month in three types of red wine: young (Y6), oak (O6) and aged (A1). Samples were kept under four different conditions: room temperature (25ºC) or refrigerated (4ºC) and stopped with cork or maintained in Ar atmosphere.

For the creation of the Ar atmosphere it was necessary to first remove the air with a vacuum pump, and then, to introduce the Ar in the head space. Ar was used instead of N_2 to create an inert atmosphere because it is denser than air, which implies that less quantity is needed to remove it. Also, it was more difficult to be displaced again by the air.

During the studied period of time, few differences have occurred (Table 4). In the young red wine, all BAs (except of TRY) were found. The main BAs present were ETA and PUT, which concentrations slightly decreased along time. Concentrations of AGM and TYM, however, presented the opposite tendency. Total concentrations ranged between 39.8 and 44.0 mg L^{-1}, decreasing a little along time, and showing concentrations more similar to those obtained for the oak red.

In the oak red wine, the most important change was the apparition of TYM, which concentration rose up to 6.7 – 7.2 mg L^{-1} in the seventh day (causing a higher total concentration of BAs content on day 7) to then decrease to 2.70 – 3.0 mg L^{-1} in the 30^{th} day. CAD also appeared in the sample after the 30 days of storage, in concentrations between 1.1 and 1.9 mg L^{-1}. The other BAs which appeared in the sample (PUT, ETA and HIM) suffered minor changes. PUT followed a tendency to decrease along time, while ETA remained almost constant. Lastly, HIM, decreased slightly from day 0 to day 7, and then remained constant. AGM, TRY and PEA were not detected in any of the analyzed

samples. Total concentrations ranged between 34.7 and 45.3 mg L^{-1}, being in general lower than in the young red.

Finally, aged red presented all BAs except for TRY and PEA. The most important change was the apparition of AGM in the seventh day, which continued to increase along time. TYM presented a decrease in the seventh day, to return to its initial concentrations on day 30^{th}. Others BAs remained almost constant over time, as the total concentrations, which varied between 20.4 and 25.6 mg L^{-1}.

It is interesting to note that, according to this study, total concentrations of BAs presented a tendency to decrease as the aging time of the wine increases. Student t-test of mean comparison has been carried out to check if there are significant differences between the means. Assuming that the variances are different (as Levene test was not passed), young wine and oak wine do not present significant differences (as observed in the previous test in section 3.3). On the other hand, aged wine does differ significantly from young and oak wines.

3.5 Statistical analysis of the data

Taking into account the previous results, it was decided to study the data statistically, in order to check whether it was possible to classify them as a function of the type of wine and the different storage conditions. Firstly, a Principal Component Analysis (PCA) was carried out to check whether it was possible to group the different samples according to the type of wine (young, oak or aged) based on the BAs concentration. Missing data (undetected BAs concentrations) have been replaced by the value LOD/2. By means of the cross-validation method, it was obtained that two principal components explain 91.9% of the variance. Taking into account the loadings, and as is shown in Figure 2A, the first principal component (PC1; 60.7% explained variance) is constituted by the positive contribution of PEA, ETA, TYM, HIM and PUT; while CAD and AGM have a negative effect. The positive effect of the second principal component (PC2; 31.2% explained variance) is formed by the positive contribution of CAD, AGM, PEA and ETA, and the negative effect of TYM, HIM and PUT.

Figure 2B shows the scores graph obtained according to the principal components aforementioned, where there are three well-differentiated groups, corresponding to the three types of wine analyzed. Young wine appears in the upper right quadrant, dominated by the contribution of PEA and ETA, that corresponds to the higher values of these two BAs in young wine. Oak wine is grouped in the lower right quadrant, which is influenced by the positive contribution of PC1 and the negative of PC2. This translates into above-average concentrations for TYM, PUT and HIM. Finally, aged wine appears on the left side of the graph, due to their lower than the average concentration in the BAs content.

Although the interpretation of the PCA was simple, Varimax rotation was also applied in an attempt to simplify it further. Rotated PC1 was formed by the positive contribution of all the BAs except CAD, AGM and PEA. The contribution of almost all BAs was reduced compared to the non-rotated loadings, obtaining a rotated PC1 based principally on the contribution of PUT (58.2 %) and HIM (30.5 %). With respect to rotated PC2, it had changed and was formed by the positive contribution of all BAs. Rotated PC2 was basically conditioned by ETA (94.4 %), while the rest of BAs had practically no effect. The loadings plot after Varimax rotation is shown in Figure 2C.

After rotation, the scores slightly modified their position, with young wines in the upper part of the graph (positive value of PC2), oaks in the lower right quadrant (positive value of PC1) and aged wines in the lower left quadrant (lower concentrations of the mean in all the BAs, especially ETA).

Thus, discriminant parameters can be found for each type of wine. ETA discriminates between young (high concentration) and aged wines (low concentration), while high concentrations of PUT and HIM differentiate oak wines.

In order to reaffirm the classification obtained by PCA, non-hierarchical and hierarchical cluster analyses have been also carried out.

In the case of the non-hierarchical analysis, the k-means method has been employed, which allows the observations to be classified into previously established and independent groups, searching

315 for the minimum residual variance within each group. In this way, three groups were established
316 (young, oak and aged wine), obtaining a 100% correct classification.

317 On the other hand, for the hierarchical cluster analysis, Ward's method was chosen, an
318 agglomerative method that starts from as many groups as observations, and that joins them looking for
319 the minimum residual variance. In this way, homogeneous groups with similar sizes are obtained. After
320 the analysis, three clusters were obtained, corresponding to the three types of wine. Figure 3 shows the
321 dendrogram obtained. As it can be seen, in the case of oak and aged wine, the smaller clusters grouped
322 the observations according to the day of storage. In addition, it can be observed that young and aged
323 wine are more similar to each other than to oak wine, which could be related to the fact that young and
324 aged wine are more related to ETA, and oak wine with PUT and HIM (as discussed previously).

325 Also, the class profile obtained in both cluster analyses revealed that the greatest differences
326 between the three types of wine were found in the concentrations of ETA, PUT and HIM, which was in
327 accordance with the results obtained in PCA with Varimax rotation.

328 In order to analyse the existence of differences within each type of wine according to storage
329 conditions, three Discriminant Analyses (DA) were performed for each wine (type of bottle closing,
330 storage temperature and day of sampling). The results obtained with the three types of wine were
331 similar, and allowed observations to be grouped according to storage conditions.

332 Regarding the classification according to the type of bottle closing used, a single factor
333 composed by the positive contribution of AGM, CAD, HIM, TYM and PEA; and the negative
334 contribution of PUT and ETA, allowed to correctly differentiate the samples stored with cork stopper
335 and those maintained in Ar atmosphere. Samples stored with cork stopper obtain scores located in the
336 left half of the graph (Figure 4A), which corresponds to a greater contribution of those BAs that have a
337 negative influence on the classification factor (PUT and ETA). Wines stored in Ar atmosphere
338 produced higher concentrations of HIM and TYM, two of the most dangerous BAs, so cork stopped

wines presented a better BAs profile. In addition, the observations made to the just opened wines were correctly assigned to the cork stopper group (original bottle closing).

Similar results were obtained in the classification according to storage temperature. Again, a single factor was enough to discriminate the observations, in this case composed by the positive contribution of all ABs except CAD and HIM. Thus, samples with higher concentrations of these BAs (25ºC) appear in the negative zone of the classification factor (Figure 4B). Thus, refrigeration conditions are better to maintain the wines. All observations were correctly classified, and those corresponding to the just opened wines were assigned to the 4ºC group, because before their analysis, bottles were stored in refrigeration.

Finally, the classification of the observations according to the day of analysis also required a single discriminant factor, consisting of the positive effect of ETA, PUT, HIM and TYM; and the negative of CAD, AGM and PEA. The concentrations of CAD, AGM and PEA increase with storage time, which allows the separation of the observations according to these variables, obtaining the groups of observations further to the left of the graph (Figure 4C) the more time passes (due to a greater negative contribution). Again, the observations were correctly assigned to all types of wine.

These results are in agreement with what was obtained by Ordóñez *et al*. (Ordóñez et al., 2017). However, their study only allowed the classification of the samples according to the type of wine and the storage time, not being able to differentiate the samples according to the temperature or type of bottle closing and concluding that the different storage conditions did not produce important changes. In our study, our results show a greater power to discriminate, since small differences in the biogenic amine profile of wines allows their classification based on the type of wine and on the different storage conditions: temperature, bottle closing and storage time. In addition, a more in-depth statistical study has been carried out, which has made it possible to reaffirm the information obtained on the BAs profile of three types of wine. This way, the optimal conditions for the opened wine bottles storage can be selected.

4. Conclusions

In this work, several monovarietal '*Tempranillo*' red wines have been analyzed employing an UHPLC method with fluorescent detection, after OPA derivatization and without sample clean-up (besides filtration).

The total concentration of BAs in the analyzed wines is in concordance with other Spanish red wines. Also, no significant differences between young and oak total BAs content has been found, but there are significant differences between young/oak and aged wines. On the other hand, a study of the evolution of the BAs profile through time with different storage conditions have been carried out in three types of red wine (young, oak and aged wine), to select the optimal conditions for opened bottles storage. A decreasing tendency in the total concentration of BAs as the aging time of the wine increases has been observed. Statistical analyses have been performed to group the samples according to their aging time employing PCA (without and with Varimax rotation) and cluster analysis (hierarchical and non-hierarchical); and according to the storage conditions using DA. In each case, a single classification factor was employed.

Our results suggest that cork stopper and refrigeration are the best conditions to maintain the wines and prevent the increase of HIM and TYM concentrations, the two more hazardous BAs. However, as the production of BAs is linked to microbial activity, further studies, as well as microbiological analysis, are necessary to determine the factors that affect the evolution of BAs profile and set conclusions.

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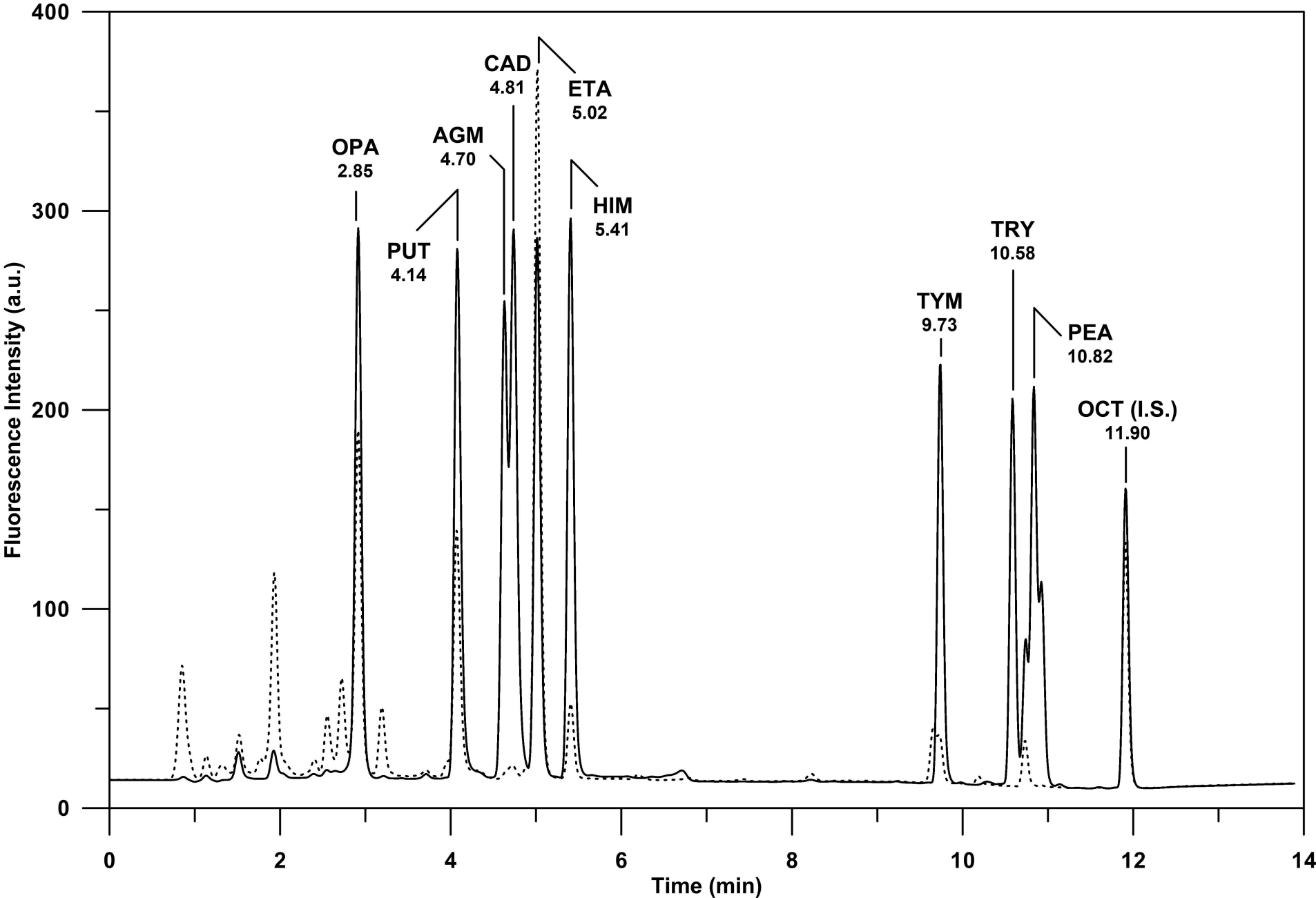
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ETA
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10.58
PEA
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OCT (I.S.)
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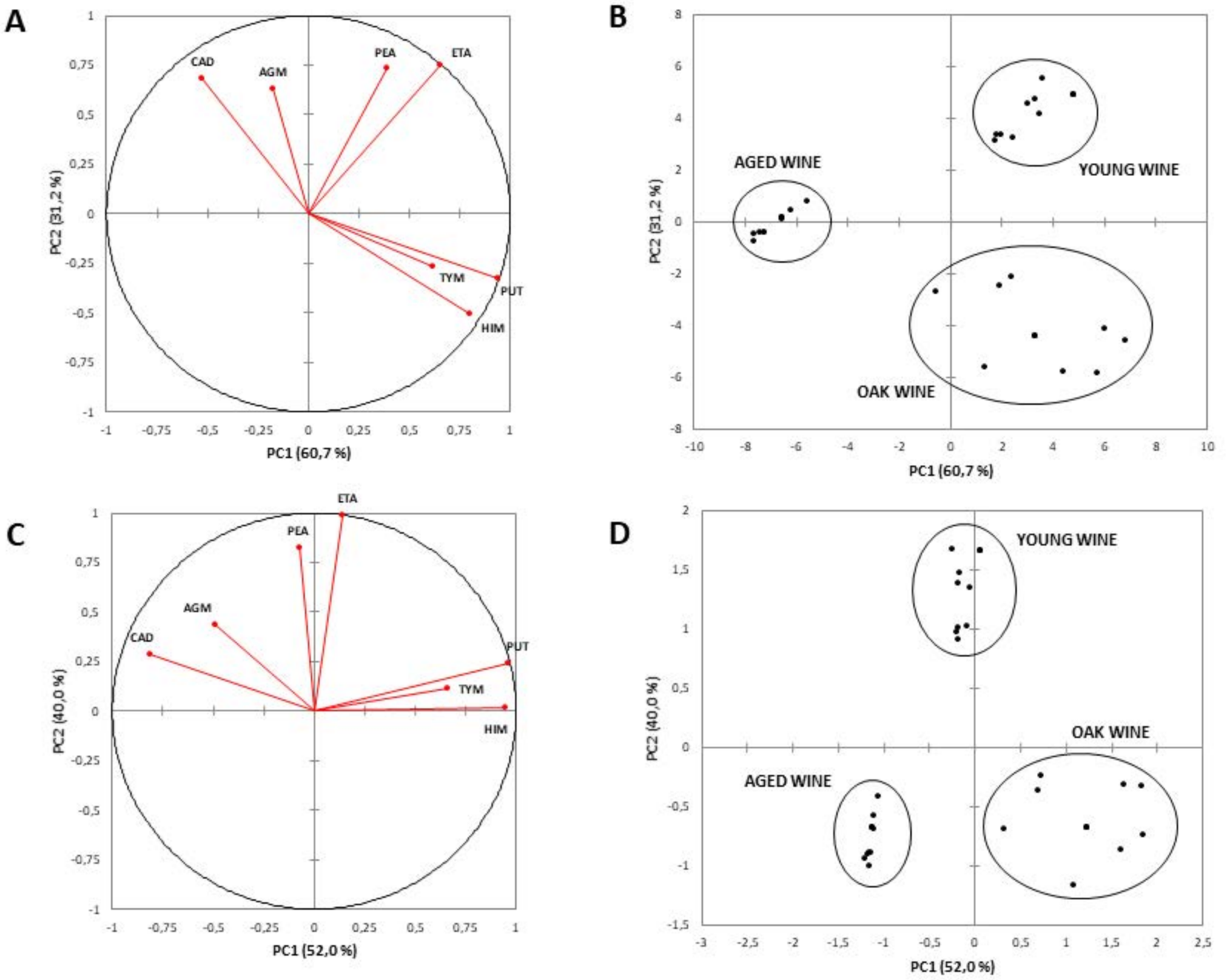
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PC2 (31,2 %)
PC1 (60,7 %)
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AGED WINE
YOUNG WINE
OAK WINE
PC2 (31,2 %)
PC1 (60,7 %)
C
ETA
PEA
AGM
CAD
PUT
TYM
HIM
PC2 (40,0 %)
PC1 (52,0 %)
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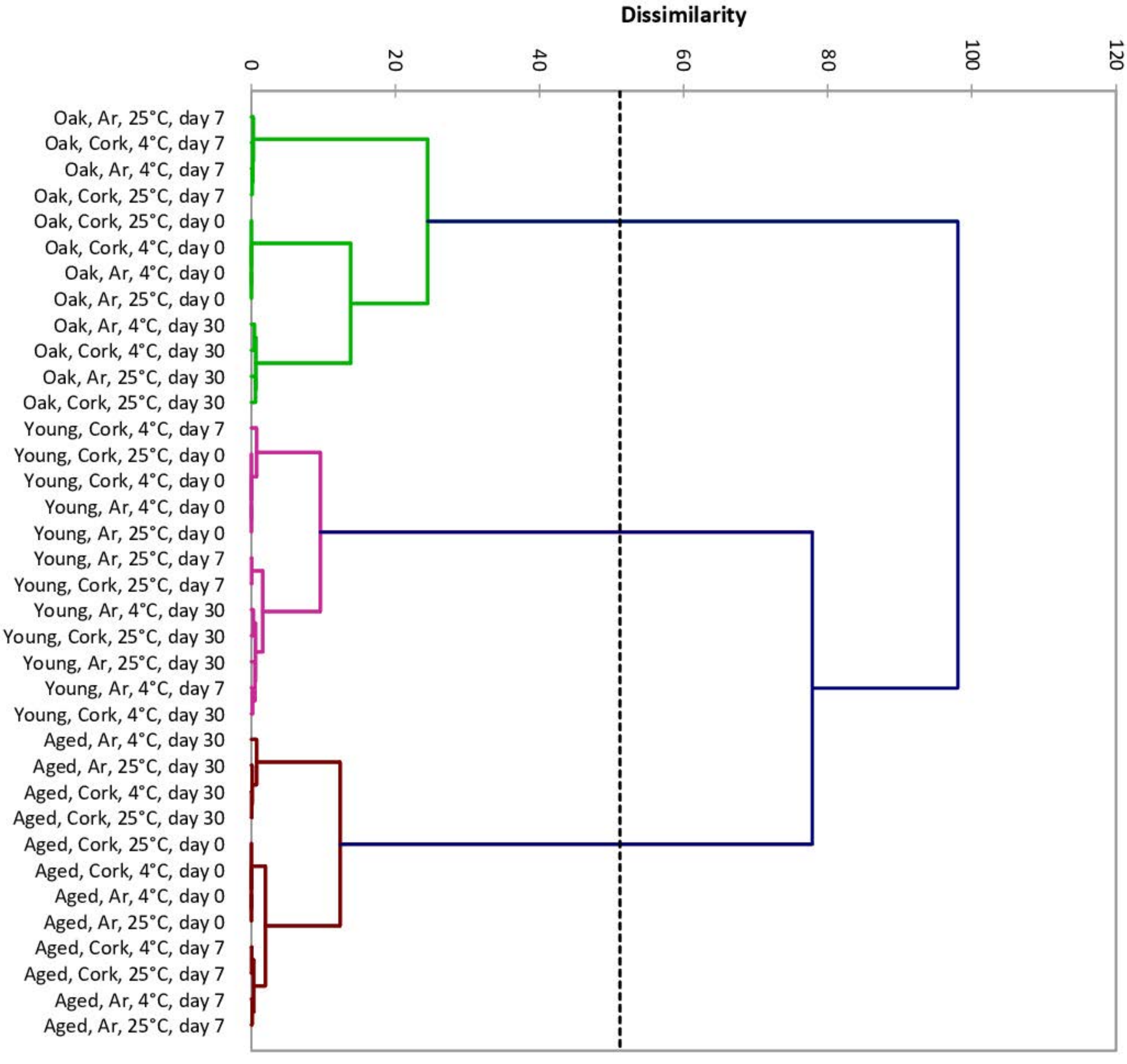
YOUNG WINE
OAK WINE
AGED WINE
PC2 (40,0 %)
PC1 (52,0 %)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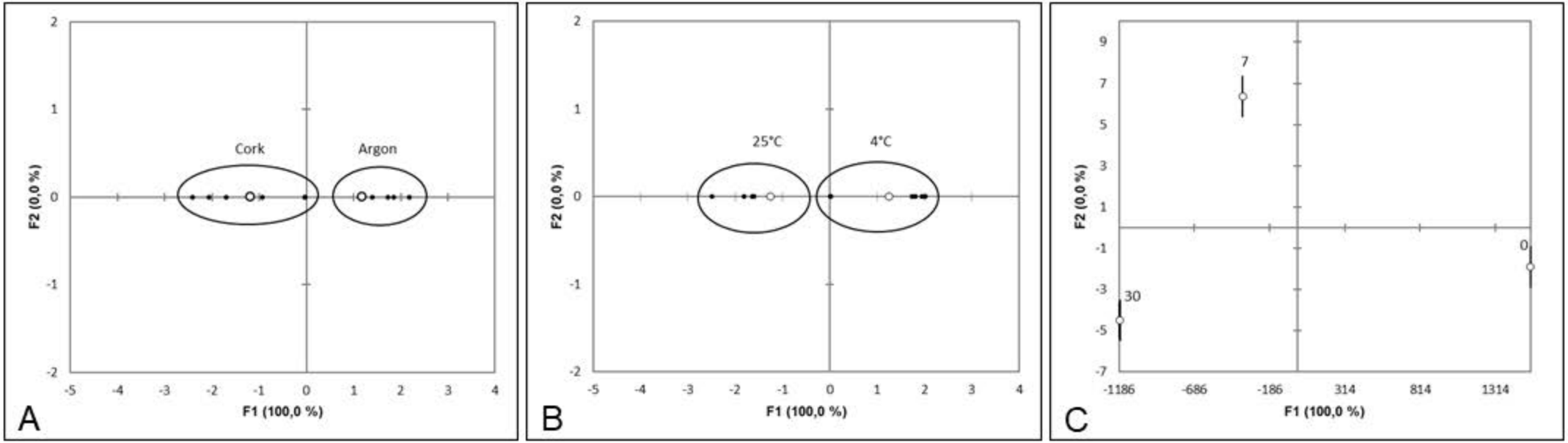
A
Cork
Argon
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F1 (100,0 %)
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F2 (0,0 %)
F1 (100,0 %)

Table 1. *Validation parameters for the chromatographic determination of BAs by the developed UHPLC-FD method, using the internal standard calibration.* $\lambda_{ex}/\lambda_{em}$ *= 356/445 nm.*

	PUT	**AGM**	**CAD**	**ETA**	**HIM**	**TYM**	**TRY**	**PEA**
t_R (min)	4.14	4.70	4.81	5.02	5.41	9.73	10.58	10.82
Linear range (mg L^{-1})	0.074 – 0.75	0.054 – 1.00	0.071 – 0.75	0.036 – 0.30	0.058 – 1.00	0.019 – 0.50	0.038 – 0.50	0.021 – 0.45
Regression equation	y = 3.73 (0.07) x + 0.06 (0.03)	y = 2.22 (0.02) x + 0.009 (0.012)	y = 3.99 (0.07) x + 0.02 (0.03)	y = 8.8 (0.2) x + 0.05 (0.03)	y = 2.61 (0.03) x + 0.02 (0.02)	y = 3.40 (0.02) x + 0.007 (0.006)	y = 3.05 (0.04) x + 0.005 (0.012)	y = 4.53 (0.04) x – 0.007 (0.009)
R^2	0.9961	0.9990	0.9965	0.9942	0.9988	0.9995	0.9981	0.9991
Linearity (%)	98.27	99.11	98.35	97.88	99.04	99.41	98.78	99.16
LOD (µg L^{-1})	22.4	16.3	21.4	10.8	17.6	5.6	11.6	6.3
LOQ (µg L^{-1})	73.9	53.8	70.6	35.6	58.1	18.5	38.3	20.8
Analytical sensitivity (γ^{-1}) (µg L^{-1})	16.8	11.7	16.1	8.4	12.6	3.7	7.7	5.2
RSD intraday (%) (low point)	5.24 (0.15 mg L^{-1})	2.70 (0.25 mg L^{-1})	4.57 (0.15 mg L^{-1})	4.24 (0.05 mg L^{-1})	3.20 (0.25 mg L^{-1})	4.22 (0.15 mg L^{-1})	4.40 (0.15 mg L^{-1})	6.05 (0.05 mg L^{-1})
RSD intraday (%) (high point)	2.37 (0.75 mg L^{-1})	1.62 (1.0 mg L^{-1})	1.44 (0.75 mg L^{-1})	1.45 (0.30 mg L^{-1})	1.58 (1.0 mg L^{-1})	1.90 (0.50 mg L^{-1})	1.49 (0.50 mg L^{-1})	1.69 (0.45 mg L^{-1})
RSD interday (%) (low point)	5.52 (0.15 mg L^{-1})	3.64 (0.25 mg L^{-1})	4.65 (0.15 mg L^{-1})	2.62 (0.05 mg L^{-1})	2.62 (0.25 mg L^{-1})	2.22 (0.15 mg L^{-1})	3.70 (0.15 mg L^{-1})	5.23 (0.05 mg L^{-1})
RSD interday (%) (high point)	4.55 (0.75 mg L^{-1})	3.02 (1.0 mg L^{-1})	5.57 (0.75 mg L^{-1})	3.28 (0.30 mg L^{-1})	3.28 (1.0 mg L^{-1})	2.51 (0.50 mg L^{-1})	3.66 (0.50 mg L^{-1})	4.25 (0.45 mg L^{-1})
Recovery (%)	126.7	96.0	101.0	78.9	109.7	121.3	100.4	95.9

Table 2. *Characteristics of the monovarietal ('Tempranillo' grape) analysed wines.*

TYPE	WINE	PROCEDENCE	OENOLOGICAL REGION
Young wine	Y1	Puebla Sancho Pérez (Badajoz)	Matanegra (Zafra – Río Bodión)
	Y2	Los Santos de Maimona (Badajoz)	Matanegra (Zafra – Río Bodión)
	Y3	Cañamero (Cáceres)	Cañamero
	Y4	Valdefuentes (Cáceres)	Montánchez
	Y5	Mérida (Badajoz)	Tierra de Barros (Tierra de Mérida – Vegas Bajas)
	Y6	Puebla Sancho Pérez (Badajoz)	Matanegra (Zafra – Río Bodión)
Oak wine	O1	Fuente del Maestre (Badajoz)	Matanegra (Zafra – Río Bodión)
	O2	Bienvenida (Badajoz)	Matanegra (Tentudía)
	O3	Cañamero (Cáceres)	Cañamero
	O4	Valdefuentes (Cáceres)	Montánchez
	O5	Almendralejo (Badajoz)	Tierra de Barros
	O6	Fuente del Maestre (Badajoz)	Matanegra (Zafra – Río Bodión)
Aged wine	A1	Fuente del Maestre (Badajoz)	Matanegra (Zafra – Río Bodión)

Table 3. *Concentrations of BAs found in the analysed wines (mg L^{-1} ± SD). n.d. = not detected.*

WINE	PUT	AGM	CAD	ETA	HIM	TYM	TRY	PEA	TOTAL
Y1	14.1 ± 0.9	2.5 ± 0.1	1.7 ± 0.2	22 ± 1	6.0 ± 0.2	1.4 ± 0.2	n.d.	n.d.	47.7
Y2	13.7 ± 0.5	n.d.	0.59 ± 0.07	22 ± 3	2.4 ± 0.2	0.7 ± 0.1	n.d.	n.d.	39.4
Y3	14.7 ± 0.9	3.0 ± 0.2	2.4 ± 0.2	20 ± 2	n.d.	2.52 ± 0.09	n.d.	n.d.	42.6
Y4	27.4 ± 0.6	4.3 ± 0.5	2.5 ± 0.3	26 ± 1	7.3 ± 0.5	3.0 ± 0.3	n.d.	n.d.	70.5
Y5	42.6 ± 0.9	n.d.	n.d.	16 ± 1	10.3 ± 0.2	3.3 ± 0.2	n.d.	n.d.	72.2
Y6	12.1 ± 0.4	0.37 ± 0.08	1.5 ± 0.1	23 ± 1	4.7 ± 0.1	2.2 ± 0.1	n.d.	0.19 ± 0.06	44.1
O1	9.8 ± 0.4	0.97 ± 0.09	0.99 ± 0.08	16 ± 1	5.2 ± 0.2	2.4 ± 0.1	n.d.	n.d.	35.4
O2	6.9 ± 0.3	n.d.	n.d.	18.0 ± 0.3	1.3 ± 0.2	1.4 ± 0.2	n.d.	n.d.	27.6
O3	23 ± 3	2.8 ± 0.2	2.1 ± 0.2	19 ± 2	8.3 ± 0.2	4.1 ± 0.3	n.d.	n.d.	59.3
O4	34.1 ± 0.6	2.5 ± 0.2	1.9 ± 0.3	27.7 ± 0.6	5.3 ± 0.4	1.9 ± 0.4	n.d.	n.d.	73.4
O5	14.0 ± 0.6	n.d.	n.d.	22.3 ± 0.3	1.1 ± 0.3	n.d.	n.d.	n.d.	37.4
O6	14.2 ± 0.7	n.d.	n.d.	14.5 ± 0.1	9.1 ± 0.4	n.d.	n.d.	n.d.	37.8
A1	5.88 ± 0.04	n.d.	1.5 ± 0.2	13.0 ± 0.4	1.3 ± 0.1	0.5 ± 0.2	n.d.	n.d.	22.2
YOUNG AVERAGE	20.8	1.7	1.5	21.5	5.1	2.2	n.d.	n.d.	52.8
OAK AVERAGE	17.0	1.0	0.8	19.6	5.1	1.6	n.d.	n.d.	45.1

Table 4. *Evolution of the BAs profile in different red wines through storage time in different storage conditions. Concentrations are expressed in mg L^{-1} (SD). n.d. = not detected.*

		DAY	PUT	AGM	CAD	ETA	HIM	TYM	TRY	PEA	TOTAL
YOUNG RED	Argon 4°C	0	12.1 (0.4)	0.37 (0.08)	1.5 (0.1)	22.9 (0.9)	4.7 (0.1)	2.2 (0.1)	n.d.	0.19 (0.06)	44.0
		7	11.1 (0.2)	1.0 (0.2)	1.8 (0.2)	21.6 (0.6)	4.9 (0.3)	2.2 (0.2)	n.d.	0.39 (0.09)	43.0
		30	9.8 (0.4)	0.84 (0.09)	1.5 (0.2)	20.3 (0.3)	4.9 (0.3)	2.5 (0.2)	n.d.	0.5 (0.2)	40.3
	Argon 25°C	0	12.1 (0.4)	0.37 (0.08)	1.5 (0.1)	22.9 (0.9)	4.7 (0.1)	2.2 (0.1)	n.d.	0.19 (0.06)	44.0
		7	11 (1)	0.8 (0.1)	2.0 (0.1)	22 (1)	4.3 (0.2)	2.1 (0.1)	n.d.	0.3 (0.1)	42.5
		30	10.3 (0.3)	1.0 (0.1)	2.1 (0.4)	19.8 (0.7)	4.5 (0.2)	2.1 (0.2)	n.d.	0.5 (0.3)	40.3
	Cork 4°C	0	12.1 (0.4)	0.37 (0.08)	1.5 (0.1)	22.9 (0.9)	4.7 (0.1)	2.2 (0.1)	n.d.	0.19 (0.06)	44.0
		7	11.2 (0.5)	0.5 (0.1)	1.3 (0.2)	22.8 (0.7)	3.7 (0.2)	1.7 (0.2)	n.d.	0.3 (0.1)	41.2
		30	11.2 (0.6)	1.0 (0.2)	1.7 (0.1)	20.2 (0.4)	4.1 (0.2)	2.0 (0.2)	n.d.	0.4 (0.1)	40.6
	Cork 25°C	0	12.1 (0.4)	0.37 (0.08)	1.5 (0.1)	22.9 (0.9)	4.7 (0.1)	2.2 (0.1)	n.d.	0.19 (0.06)	44.0
		7	10.8 (0.6)	0.7 (0.1)	1.9 (0.2)	21.7 (0.8)	4.3 (0.2)	1.99 (0.09)	n.d.	0.3 (0.1)	41.7
		30	10 (1)	0.8 (0.3)	1.7 (0.4)	20.1 (0.6)	4.6 (0.5)	2.2 (0.4)	n.d.	0.4 (0.3)	39.8
OAK RED	Argon 4°C	0	14.2 (0.7)	n.d.	n.d.	14.5 (0.1)	9.1 (0.4)	n.d.	n.d.	n.d.	37.8
		7	16.7 (0.9)	n.d.	n.d.	15.9 (0.9)	7.7 (0.2)	6.9 (0.2)	n.d.	n.d.	47.2
		30	9.4 (0.3)	n.d.	1.5 (0.2)	14.0 (0.7)	7.1 (0.2)	2.7 (0.2)	n.d.	n.d.	34.7
	Argon 25°C	0	14.2 (0.7)	n.d.	n.d.	14.5 (0.1)	9.1 (0.4)	n.d.	n.d.	n.d.	37.8
		7	15.3 (0.6)	n.d.	n.d.	13.6 (0.9)	6.9 (0.3)	7.2 (0.2)	n.d.	n.d.	43.0
		30	12.9 (0.5)	n.d.	1.5 (0.1)	12.3 (0.3)	7.9 (0.2)	3.0 (0.1)	n.d.	n.d.	37.6
	Cork 4°C	0	14.2 (0.7)	n.d.	n.d.	14.5 (0.1)	9.1 (0.4)	n.d.	n.d.	n.d.	37.8
		7	16.5 (0.8)	n.d.	n.d.	15.7 (0.4)	6.4 (0.3)	6.7 (0.2)	n.d.	n.d.	45.3
		30	11.8 (0.4)	n.d.	1.1 (0.1)	15.4 (0.6)	7.3 (0.1)	2.70 (0.08)	n.d.	n.d.	38.3
	Cork 25°C	0	14.2 (0.7)	n.d.	n.d.	14.5 (0.1)	9.1 (0.4)	n.d.	n.d.	n.d.	37.8
		7	16.1 (0.7)	n.d.	n.d.	14.3 (0.5)	8.1 (0.3)	6.7 (0.2)	n.d.	n.d.	45.2
		30	12.1 (0.4)	n.d.	1.9 (0.2)	15.8 (0.3)	7.3 (0.2)	3.0 (0.1)	n.d.	n.d.	40.1

AGED RED	**Argon 4°C**	0	5.88 (0.04)	n.d.	1.5 (0.2)	13.0 (0.4)	1.3 (0.1)	0.5 (0.2)	n.d.	n.d.	22.2
		7	5.3 (0.2)	0.33 (0.07)	1.76 (0.03)	12.1 (0.5)	1.43 (0.05)	0.39 (0.03)	n.d.	n.d.	21.3
		30	5.1 (0.1)	0.89 (0.06)	1.59 (0.09)	11.7 (0.5)	1.60 (0.08)	0.6 (0.1)	n.d.	n.d.	21.5
	Argon 25°C	0	5.88 (0.04)	n.d.	1.5 (0.2)	13.0 (0.4)	1.3 (0.1)	0.5 (0.2)	n.d.	n.d.	22.2
		7	5.4 (0.2)	0.2 (0.1)	1.6 (0.2)	11.9 (0.3)	1.1 (0.1)	0.2 (0.1)	n.d.	n.d.	20.4
		30	6.3 (0.5)	1.04 (0.09)	2.0 (0.2)	14.0 (0.6)	1.8 (0.1)	0.5 (0.2)	n.d.	n.d.	25.6
	Cork 4°C	0	5.88 (0.04)	n.d.	1.5 (0.2)	13.0 (0.4)	1.3 (0.1)	0.5 (0.2)	n.d.	n.d.	22.2
		7	5.7 (0.2)	0.4 (0.1)	2.0 (0.1)	12.1 (0.4)	1.28 (0.07)	0.24 (0.07)	n.d.	n.d.	21.7
		30	5.7 (0.3)	1.1 (0.1)	2.2 (0.2)	13.4 (0.4)	2.0 (0.1)	0.61 (0.07)	n.d.	n.d.	25.0
	Cork 25°C	0	5.88 (0.04)	n.d.	1.5 (0.2)	13.0 (0.4)	1.3 (0.1)	0.5 (0.2)	n.d.	n.d.	22.2
		7	5.6 (0.1)	0.49 (0.07)	1.83 (0.09)	12.1 (0.3)	1.37 (0.06)	0.33 (0.05)	n.d.	n.d.	21.7
		30	5.8 (0.2)	1.1 (0.1)	2.14 (0.09)	12.9 (0.2)	1.7 (0.1)	0.6 (0.1)	n.d.	n.d.	24.2