Phenolic Compounds Extraction in Enzymatic Macerations of Grape Skins Identified as Low Level Extractable Total Anthocyanin Content

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ABSTRACT: Anthocyanins in wine principally depends on grape skin extractable anthocyanin content, i.e., the amount of anthocyanins present in grape skin that are released to wine during the maceration stage. This amount of extractable anthocyanins is closely linked to the cell wall degradation of skin cells. Indeed, among other methodologies, the maceration in presence of different enzymes can be used to increase cell wall degradation, and therefore, the amount of anthocyanins extracted from grape skins to wine.

Vitis vinifera L. cv. Tempranillo and Syrah red grapes have been identified as samples with low anthocyanin extraction potential by near infrared hyperspectral imaging. Grape skins have been macerated in presence of cellulase, glucosidase and pectinase. Then, colour of the supernatants and phenolic compounds extracted from grape skins (total phenols, total flavanols and total and individual anthocyanins) have been determined.

Cellulase and glucosidase have shown a positive effect in the extraction of phenolic compounds from these grapes. Macerations carried out in the presence of cellulase have produced supernatants with a more intense colour (lower lightness and higher chroma values), and a higher extraction of flavanols and anthocyanins than the respective control essays. However, pectinase treatments have produced the opposite effect, which could be partially explained by an eventual interaction between the cell wall polysaccharides liberated by pectinase and the phenolic compounds extracted.

Synergy effects do not appear between cellulase and glucosidase. Moreover, the negative effect of the addition of pectinase might be due to the interactions between the cell wall material liberated by pectinase and the phenolic compounds extracted.

Keywords: Wine; grape skins; phenolic compounds; enzymes; extraction.

Practical Application: In the present study, grape samples with a low anthocyanin extraction potential have been identified and these samples have been macerated in presence of different enzymes. The applied enzymes were three of the most common enzymes that are applied in the wine industry. Individual enzymes and mixtures have been applied to Syrah and Tempranillo grape skin samples and the results have been compared to control macerations. Knowledge in this topic will help the production of quality wines.

Introduction

Enzymes play an important role in the winemaking process. They usually come from grape itself, the indigenous microbiota on the grape and the microorganisms present during winemaking. Since the action of these enzymes is difficult to control in some cases and is not enough in others, manufacturers traditionally have developed better adapted enzymatic preparations with the aims of improving the juice extraction, must clarification, aroma freeing and colour extraction and stabilization (Ribéreau-Gayon et al., 2006).

Regarding to the colour extraction and stabilization, it mainly depends on the quantity and type of phenolic compounds extracted from grape skins to wine. Anthocyanins are the compounds directly responsible of the colour of red wines. However, other phenolic compounds, such as flavanols or phenolic acids, can modulate and stabilize wine colour (Boulton, 2001). Similarly, phenolic compounds are also responsible for other wine attributes related to wine quality (astringency, sourness, bitterness, etc.) or to their health properties (antioxidant activity, free radical scavenging capacity, etc.) (Jackson, 2000; Martín Bueno et al., 2012). In consequence, the effect that different enzymes or enzymatic preparation have on the phenolic compounds extraction from grape skin is being widely studied.

Romero-Cascales, Fernández-Fernández, Ros-García, López-Roca, and Gómez-Plaza (2008) characterized and quantified the activities of six different enzymatic preparations. They studied the phenolic composition and wine colour in post-fermentative and 12-month Monastrell wines. They obtained a general increase in the total phenol content and higher colour intensity after 12 month of storage. In the case of glucosidase activity, which can degrade anthocyanins by breaking down the linkage of the glucose to the anthocyanidin (side effect in enzymatic

preparations), these authors found a positive significant difference in the extraction of total phenols and tannins using the suppliers dosage of enzymatic preparations. However, they did not obtain any difference in colour parameters even taking into account that the aforesaid side effect may cause anthocyanin's degradation. Parley (1997) and Río Segade et al. (2015) also found differences in colour and phenolic content respectively using different enzymatic preparations. Benucci et al. (2017) evaluated the effectiveness of enzymes applied, individually and in enzyme mixes, on the extraction of anthocyanins, oligomeric flavanols and polymeric flavanols from withered grape skins during simulated maceration. They found that the effectiveness of individual enzymes and mixes of enzymes in improving the extractability of phenolic compounds were variety dependent. Finally, Castro-López, Gómez-Plaza, Ortega-Regules, Lozada, and Bautista-Ortín (2016), Samoticha, Wojdyło, Chmielewska, Politowicz, and Szumny (2017) and A. Bautista-Ortín, Ben Abdallah, Castro-López, Jiménez-Martínez, and Gómez-Plaza (2016) studied the proanthocyanidin extraction in presence of maceration enzymes.

In all these studies, enzymatic macerations were carried out taking into account grape samples collected at veraison or in the harvest, i.e. grape samples in the same stage of maturity. However, it is well known that there is heterogeneity in the content of polyphenols and extractable polyphenols within grape samples with the same maturity degree (Kontoudakis et al., 2011; Martinez-Sandoval et al., 2016; Nogales-Bueno, Baca-Bocanegra, Rodríguez-Pulido, Heredia, & Hernández-Hierro, 2015; Zouid, Siret, Jourjon, Mehinagic, & Rolle, 2013). In consequence, it is not possible to know if the abovementioned enzymatic macerations would affect similarly to grape samples with different contents of extractable polyphenols (e.g. high

extractable content or low extractable content). Actually, it would be of interest to know if the presence of enzymes during the maceration stage also increases the amount of polyphenols extracted from grape samples of low extractable polyphenol contents.

In the present study, grape samples have been classified according to their content of extractable polyphenols by means of near infrared hyperspectral imaging. Then, samples with a low anthocyanin extraction potential have been selected and these samples have been macerated in presence of different enzymes. The applied enzymes were cellulase, glucosidase and pectinase, three of the most common enzymes that make up the commercial enzymatic preparations that are applied in the wine industry. Individual enzymes and mixtures have been applied to Syrah and Tempranillo grape skin samples and the results have been compared to control macerations. Total phenols, flavanols, total and individual anthocyanins and colour have been evaluated in the different model wine extractions. To the best of our knowledge, the effect that different enzymes have in the extraction of phenolic compounds has not been evaluated yet in grapes with a low anthocyanin extraction potential.

Materials and Methods

In order to facilitate the understanding of the entire procedure carried out in this study, a schematic representation of this process is shown in Fig. 1.

Sample selection by near infrared hyperspectral imaging

Sample collection.

Two hundred *V. vinifera* L. cv. Tempranillo and Syrah red grape samples, one hundred per variety, were collected from two vineyards located in the Condado de Huelva Designation of

Origin D.O. (Andalusia, Spain). Samples were collected on two different dates, when Syrah and Tempranillo vineyards were respectively harvested (August 11 and 07, 2014). Due to the heterogeneity of the grape within a grape cluster (Ribéreau-Gayon et al., 2006), single grapes were collected from the top, middle and bottom of the cluster and from the sunlight and shade side in order to obtain a representative sample set. The samples were refrigerated and immediately transported to the laboratory.

Near infrared data acquisition and predicted extractable anthocyanin content determination.

Near infrared hyperspectral images were acquired for each individual grape. Hyperspectral imaging device is described in detail elsewhere in Nogales-Bueno, Hernández-Hierro, Rodríguez-Pulido, and Heredia (2014).

Afterward, hyperspectral images were calibrated (a two point calibration) and segmented and the average absorbance spectrum was calculated for each grape sample. Then, the partial least square (PLS) prediction model developed for Nogales-Bueno et al. (2015) was applied to grape spectra in order to predict the extractable anthocyanin content for each sample. This methodology has already been applied with good results for predicting the extractable anthocyanin contents in different studies (Baca-Bocanegra, Nogales-Bueno, Heredia, & Hernández-Hierro, 2018; Nogales-Bueno, Baca-Bocanegra, Jara-Palacios, Hernández-Hierro, & Heredia, 2017). Previously to the extractable anthocyanin content prediction, it was evaluated if these spectral samples were within the spectral space where the calibration model can be applied. For this purpose, a Principal Component Analysis (PCA) was applied to the spectral matrix and samples were ordered according to their distance from the centre of the spectral

space. This distance was measured following the advice of the software developer (Infrasoft International LLC, 2000). According to this advice, Mahalanobis distance (H) was calculated for each sample and samples with a H distance from the spectral space greater than 3 were removed from the data set (Garrido-Varo, Garcia-Olmo, & Fearn, 2019; Infrasoft International LLC, 2000).

Sample selection.

Grape samples were sorted according to their predicted extractable anthocyanin content. In this way, samples with a low capacity for the extraction of anthocyanins were identified following the procedure described elsewhere in Nogales-Bueno, Baca-Bocanegra, Jara-Palacios, et al. (2017) and they were used in the subsequent analysis.

Enzymatic treatments

Grape skins, from grapes identified as samples with a low anthocyanin extraction potential, as described in section 2.1., were removed from the whole grapes, divided into two parts and weighed. For each sample, a half skin was macerated in presence of enzyme (enzymatic maceration) while the other half was macerated without enzyme (control maceration). Enzymatic macerations were carried out in presence of three of the most common enzymes that make up the commercial enzymatic preparations: cellulase (C), glucosidase (G) and pectinase (P) (Fluka Biochemika, references 22178, 49291 and 17389 respectively). Enzymes were applied individually and in mixtures of enzymes (CG, CP and GP). The activities for C, G and P are respectively 0.8, 0.8 and 1.0 U mg^{-1} at their respective optimal conditions of pH and temperature. Although these conditions are different from the pH and temperature typical in the vinification processes, the macerations were carried out under vinification conditions (pH

3.6-4.0 and 20-30 ºC) in order to verify the technological applicability of these commercial enzymes.

Control and enzymatic macerations were carried out in model wine hydroalcoholic solution for a maceration period of 72 h. This model wine consisted in a 12.5% ethanol solution with 4 g L^{-1} tartaric acid and pH adjusted at 3.6 with NaOH 0.5 M. After this time, grape skin, i.e. substrate, was removed from the essay. The concentration of enzyme in the enzymatic macerations was 15 mg L^{-1}. For ease of comparison, in macerations carried out with a mix of enzymes, the combined concentration also was 15 mg L^{-1} (7.5 mg L^{-1} per each enzyme). For all samples, it was kept constant the ratio of skin weight and model wine solution (1:20 w:v (g mL^{-1})). Supernatants were used in the subsequent analysis.

Supernatant analyses

Colour.

The whole visible spectra (380–770 nm) of the supernatants was measured at constant intervals (Δλ = 2 nm) with an Agilent 8453 UV–Vis spectrophotometer (Palo Alto, USA), using 10 mm path length plastic cells and distilled water as white reference. The CIELAB colour parameters (L^*, a^*, b^*, C^*_{ab} and h_{ab}) were calculated for from transmittance spectra by using the original software CromaLab® (Heredia, Álvarez, González-Miret, & Ramírez, 2004), following the recommendations of the Commission Internationale de l'Eclairage (CIE, 2004): 10° Standard Observer and D65 Standard Illuminant as references.

Total phenols.

The total phenol concentration of the supernatants were determined using the Folin–Ciocalteu spectrophotometric method (Singleton & Rossi, 1965). Results were presented as mg of gallic

192 acid equivalents per gram of grape skin. Total phenol analysis was carried out in duplicate on an
193 Agilent 8454 UV–visible spectrophotometer (Palo Alto, USA), which is equipped with a diode
194 array detector (DAD).

195 *Total flavanols.*

196 The total flavanol concentration of the supernatants were determined following a modification
197 of the method described by Vivas, Glories, Lagune, Saucier, and Augustin (1994). Twenty
198 microlitres of supernatant was mixed with 180 µL of methanol and 1 mL of
199 p-dimethylaminocinnamaldehyde (DMACA) reagent. After 10 min of reaction, the absorbance
200 of these solutions was recorded in duplicate at 640 nm. This measure was carried out on an
201 Agilent 8454 UV–visible spectrophotometer (Palo Alto, USA), which is equipped with a diode
202 array detector (DAD). A calibration curve of (+)-catechin (Sigma–Aldrich, St. Louis, USA) was
203 used for quantification purposes and all measurements were within the linear range of this
204 calibration curve. After the spectrophotometric analysis, the results were presented as mg of
205 catechin equivalents per gram of grape skin.

206 *Anthocyanins.*

207 The anthocyanic profile of the supernatants was determined by means of chromatographic
208 analysis. After a 1:2 dilution with 0.1 HCl, the supernatant was filtered through 0.45 µm pore
209 size filters and then it was directly injected into the chromatographic system. Chromatographic
210 analyses were performed in duplicate. The chromatographic method followed was a
211 modification (Hernández-Hierro, Nogales-Bueno, Rodríguez-Pulido, & Heredia, 2013) of the
212 method described by García-Marino, Hernández-Hierro, Rivas-Gonzalo, and Escribano-Bailón

(2010). Up to 15 anthocyanins were identified. Finally, the results were presented as mg of malvidin-3-*O*-glucoside equivalents per gram of grape skin.

Data analysis

Hyperspectral data was processed with MATLAB R2012b (The Mathworks, Natik, MA, USA, 2012). Near infrared spectra processing and prediction of extractable anthocyanin contents in grape skins were performed on Win ISI® (v1.50) (Infrasoft International, LLC, Port. Matilda, PA, USA). Moreover, statistical analyses, such as PCA (based on the correlation matrix) or multivariate analysis of variance (MANOVA) were implemented on Statistica v.8.0 (StatSoft Inc., OK, USA, 2007).

Results and Discussion

Selected samples

By the application of the procedure described in Nogales-Bueno et al. (2015), it was obtained that 50 grape spectra do not meet the $H < 3$ criterion and they were eliminated from the spectral matrix and then they were not used in further steps. As several studies have previously reported (Cozzolino et al., 2004; Janik, Cozzolino, Dambergs, Cynkar, & Gishen, 2007; Nicolaï et al., 2007), this result confirms that the applicability of prior developed models is reduced within different seasons. However, as authors declared in their previous study, it would be beneficial to have models with a higher applicability by taking into account more seasons in a further model, which could use the obtained outliers on its development.

Thus, the prediction model was applied to the remaining 150 samples and the predicted extractable anthocyanin content was obtained for these samples. A total of 18 grapes were identified as samples with a low anthocyanin extraction potential. Among these samples, there were only 3 Tempranillo samples. This result is in concordance with the result obtained in the previous study, where the extractable anthocyanin content was higher for Tempranillo than for Syrah grapes (Nogales-Bueno et al., 2015).

Skins of selected samples were macerated in wine model solutions with and without enzyme as described in section 2.2. Three skin samples were individually macerated for each enzymatic treatment (C, G, P, CG, CP and GP).

Data analysis

Colour and phenolic composition.

After a three-day period, supernatants from enzymatic and control maceration were analysed and the mean and standard deviation of the colorimetric parameters and phenolic contents were calculated for each enzyme.

Table 1 shows the mean and standard deviation of the colorimetric parameters. The comparison between control and enzymatic macerations shows that supernatants obtained in presence of enzyme have a lower L^* values, with the exception of pectinase (P) maceration, and a higher C^*_{ab} values, with the exception of glucosidase-pectinase (GP) maceration. Both results could indicate a higher extraction of phenolic compounds by the enzymatic macerations, with the exception of these two enzymatic preparations.

The extractable phenolic contents of grape skins, in presence or absence of enzyme, are shown in the Table 2. The main phenolic families are presented in the table. By comparison, enzymatic

maceration produces higher extractions of total phenols, flavanols and anthocyanins, especially for C, G, CG and CP treatments. However, the presence of pectinase in P and GP enzymatic macerations does not show a similar effect. Other enzyme formulations, which contain some of these enzymes, have been applied to different matrixes in order to improve the extraction of phenolic compounds. Glucosidase activity shown an increase of the phenolic content in post-fermentative and 12-month Monastrell wines (Romero-Cascales et al., 2008). Moreover, cellulase activity have improved the phenolic extraction in grape, apple and mulberry leaves (Kim et al., 2005; Parley, 1997; Qadir et al., 2019; Río Segade et al., 2015).

Next, due to the importance that anthocyanins have in the wine quality, individual anthocyanins were determined. Up to 15 anthocyanins were identified. They were grouped in basis of their basic structure as coumaroyls anthocyanins (Cyanidin-3-*O*-(6'-*p*-coumaroyl)-glucoside, Petunidin-3-*O*-(6'-*p*-coumaroyl)-glucoside (*trans*), Malvidin-3-*O*-(6'-*p*-coumaroyl)-glucoside (*cis*), Peonidin-3-*O*-(6'-*p*-coumaroyl)-glucoside (*trans*), Malvidin-3-*O*-(6'-*p*-coumaroyl)-glucoside (*trans*)), acetyls anthocyanins (Delphinidin-3-*O*-(6'-acetyl)-glucoside, Cyanidin-3-*O*-(6'-acetyl)-glucoside, Petunidin-3-*O*-(6'-acetyl)-glucoside, Peonidin-3-*O*-(6'-acetyl)-glucoside, Malvidin-3-*O*-(6'-acetyl)-glucoside) and non-acylated anthocyanins or anthocyanin monoglucosides (Delphinidin 3-*O*-glucoside, Cyanidin 3-*O*-glucoside, Petunidin 3-*O*-glucoside, Peonidin 3-*O*-glucoside, Malvidin 3-*O*-glucoside).

Table 3 shows the mean and standard deviation for the individual anthocyanins and for the main anthocyanic families: monoglucosides, acetyls and coumaroyls. If concentrations for control and enzymatic macerations are compared, it can be appreciated that C and, to a lesser extent, G treatments produce a higher extraction of anthocyanins for the major part of the

277 compound, whereas P treatment produce the opposed effect. Regarding to the mixtures of
278 enzymes, the CG treatment do not produce an effect similar to the expected if the individual
279 enzyme effects are taken into account. Moreover, in the case of the CP and GP treatments,
280 pectinase seems to counteract the positive effects of cellulase and glucosidase in the individual
281 treatments.

282 *Statistical analysis.*

283 In order to check if the differences between control and enzymatic macerations obtained with
284 the use of the different enzymes and mixtures of enzymes are significant differences, a
285 MANOVA was carried out using colorimetric parameters and phenolic contents as dependent
286 variables and different enzymatic treatments (C, G, P, CG, CP, GP) and the type of maceration
287 (control or enzymatic macerations) as independent variables or factors (i.e., data in Tables 1, 2
288 and 3). As result of this statistical analysis, no significant differences could be found. Differences
289 appreciated in the data sets are probably eclipsed by the high standard deviations of the data.
290 These high standard deviations are due to the fact that three different grapes skins were
291 individually macerated for each enzymatic treatment (a half for each maceration type). As
292 stated previously, there is a high heterogeneity in the content of extractable polyphenols
293 among different grape samples with a similar stage of maturity, even in each extractability
294 level.

295 In a further effort to obtain a general overview extracted from the experimental data, a PCA
296 was performed. This technique is a pattern recognition tool that allows looking for trends in an
297 unsupervised way among the different factors taken into account. PCA was performed using

colorimetric parameters and phenolic contents as dependent variables. The different enzymatic treatments and the type of maceration were evaluated as factors.

The scores of the first two principal components after PCA performed on supernatant colour and phenolic parameters of the grape skins are shown in Fig. 2a. Samples are plotted in the space defined by the first and second principal components, which described 67.61% (PC1) and 13.31% (PC2) of the data variability. Apparently, there are no trends in this graph. The samples processed in control or enzymatic macerations or with different enzymatic treatments seem to be overlaid. Nevertheless, if only samples treated with cellulase and glucosidase are shown (Fig. 2b), a trend can be inferred. Samples macerated in presence of their respective enzyme appear on the left of samples macerated without the enzyme. This trend is surely linked to the differences observed in the experimental data (Tables 1, 2 and 3). Moreover, this trend is mainly observed in the PC1 direction, thus, this principal component surely collects the major part of the variability of the data linked to the different extraction rate observed.

The influence that each variable have in PC1 and PC2 are shown in the Fig. 2c. It can be seen that the colorimetric variable L^* is which have an effect more intense (positive) on PC1, whereas a number of variables have a negative effect on this principal component. If graph is zoomed in on this region (Fig. 2d), more details can be observed. Chroma, total and monoglucoside anthocyanins, and different individual anthocyanins show a negative influence on the PC1. Notably, most of anthocyanin monoglucosides and their sum (M_{Total}) and the three cyaniding derivatives appear in this region. Therefore, samples with a low value of L^*, high value of C^*_{ab} and a high extraction of these anthocyanins show a negative PC1 score, they appear on the left region of the Fig. 2a graph, and *vice versa*.

320 *Discussion*

321 Among the different enzymes tested, glucosidase and, to a larger extent, cellulase have a
322 positive influence on the extraction of phenolic compounds from grape skins previously
323 classified as samples with a low capacity for the extraction of anthocyanins. In this study, an
324 increase of approximately 120% in the extraction of phenolic compounds has been achieved in
325 the macerations in presence of cellulase. A result larger than those obtained in wine (110%)
326 (Romero-Cascales et al., 2008) and in grape skin (Benucci et al., 2017) were the total
327 anthocyanins and proanthocyanins were increased in a 10% in presence of cellulase activity.
328 This slight increase can be due to the fact that grapes used in the present study were previously
329 identified as grapes with low extraction potential. Therefore, the enzyme had a larger room for
330 improvement.

331 Samples extracted in presence of these enzymes show lower L^* and higher C^*_{ab} values than
332 control extractions. These enzymes should have weakened the skin cell wall. The relationship
333 between cell wall degradation and phenolic extractability has been previously studied
334 (Hernández-Hierro et al., 2014; Nogales-Bueno, Baca-Bocanegra, Rooney, et al., 2017; Quijada-
335 Morín, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailón, 2015). A number of previous
336 studies also obtain a degradation of the cell wall and an increase of phenolic compounds
337 extraction with the application of cellulase (Apolinar-Valiente, Romero-Cascales, Gómez-Plaza,
338 & Ros-García, 2016; A. Bautista-Ortín et al., 2016; Benucci et al., 2017; Castro-López et al.,
339 2016) and glucosidase (Romero-Cascales et al., 2008) enzymes to grape samples. However,
340 mixtures of cellulase and glucosidase enzymes have not shown a synergy effect. It has been

found that synergetic effects are not always obtained in the enzymatic macerations of grape skins (Benucci et al., 2017).

Another interesting result of this study is that pectinase does not show a positive influence on the phenolic extraction from these samples. This enzyme seems to produce the opposite effect. It is known that the degradation of the cell wall produces the liberation of different polysaccharides and these polysaccharides present a high affinity for the proanthocyanidins and may reduce the proanthocyanidin content in the solution (A. B. Bautista-Ortín, Cano-Lechuga, Ruiz-García, & Gómez-Plaza, 2014; A. Bautista-Ortín et al., 2016; Bindon, Smith, & Kennedy, 2010; Zhu, 2017).

Conclusion

In this study, different enzymes have been tested in order to know if they can modify the amount of phenolic compounds extracted from grape skins previously identified as samples with a low anthocyanin extraction potential. The chosen enzymes have been cellulase, glucosidase and pectinase, three enzymes typically present in commercial enzymatic preparations. Their individual and combined effects have been tested.

Cellulase has shown a positive effect in the extraction of phenolic compounds from these low-anthocyanic-extraction grapes. Macerations carried out in the presence of this enzyme have produced supernatants with lower L^* and higher C^*_{ab} values than control macerations. Moreover this enzyme also produced a higher extraction of proanthocyanidins and anthocyanins than the respective control essays. To a lesser extent, glucosidase produced a

similar effect than cellulase, whereas their enzymatic mixture produced more inconsistent results.

On the contrary, supernatants obtained from pectinase treatments have less colour (more L^* and less C^*_{ab} values) and extractable phenols than those obtained from controls. This effect could be explained by an interaction between the cell wall material liberated by pectinase and the phenolic compounds extracted.

Further studies should be developed in order to clarify several aspects. Firstly, the non-appearance of a synergy effect between cellulase and glucosidase. Secondly, how to reduce the interactions between the cell wall material liberated by pectinase and the phenolic compounds extracted in grapes with low potential for the phenolic extraction. And thirdly, understanding why the pectinase, an enzyme widely apply in oenology, produced this negative effect in grapes with low potential for the extraction anthocyanic compounds.

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Author Contributions (*required*)

Julio Nogales-Bueno developed the statistical analyses and drafted the manuscript. Berta Baca-Bocanegra designed and executed the experimental part. Francisco José Heredia supervised

the study, results and manuscript. José Miguel Hernández-Hierro planned the study and interpreted the results.

Nomenclature or Appendix (*optional*)

a^* and b^*, CIELAB colorimetric coordinates; C, cellulase; C^*_{ab}, CIELAB chroma; CG, enzymatic mixture of cellulase and glucosidase; CP, enzymatic mixture of cellulase and pectinase; DMACA, p-dimethylaminocinnamaldehyde; G, glucosidase; GP, enzymatic mixture of glucosidase and pectinase; H, Mahalanobis distance; h_{ab}, CIELAB hue; L^*, CIELAB lightness; MANOVA, multivariate analysis of variance; P, pectinase; PC, principal component; PCA, principal component analysis.

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Tables

Table 1. Mean and standard deviation of the colorimetric parameters determined for control and enzymatic macerations. Control macerations were carried out without presence of enzyme.

		Control macerations						Enzymatic macerations					
		$^{\alpha}$C	$^{\beta}$G	$^{\chi}$P	$^{\delta}$CG	$^{\varepsilon}$CP	$^{\phi}$GP	$^{\alpha}$C	$^{\beta}$G	$^{\chi}$P	$^{\delta}$CG	$^{\varepsilon}$CP	$^{\phi}$GP
Colorimetric parameters	L^*	48.46 ± 25.69	57.54 ± 9.43	72.98 ± 28.89	78.69 ± 15.64	70.70 ± 15.81	56.49 ± 6.52	42.01 ± 31.30	51.56 ± 2.74	74.59 ± 26.23	75.50 ± 13.82	67.58 ± 19.38	54.88 ± 7.59
	a^*	40.66 ± 23.81	39.10 ± 15.88	21.88 ± 28.91	21.11 ± 17.69	24.99 ± 10.34	41.16 ± 1.26	40.54 ± 20.85	42.04 ± 12.86	20.78 ± 26.14	22.62 ± 13.39	25.87 ± 11.66	37.34 ± 7.07
	b^*	12.18 ± 4.25	11.17 ± 1.38	11.67 ± 1.90	8.32 ± 0.93	8.78 ± 3.36	9.64 ± 0.78	17.22 ± 7.16	10.84 ± 2.36	13.05 ± 3.07	7.10 ± 3.36	10.79 ± 4.00	10.22 ± 2.23
	C^*_{ab}	42.63 ± 23.71	41.01 ± 14.57	27.26 ± 25.44	23.59 ± 15.86	27.08 ± 8.41	42.28 ± 1.32	44.08 ± 21.94	43.44 ± 12.93	27.86 ± 20.78	24.81 ± 10.51	28.97 ± 8.48	38.71 ± 7.40
	h_{ab}	19.99 ± 9.24	18.51 ± 10.74	48.12 ± 31.12	30.66 ± 22.78	22.66 ± 15.69	13.17 ± 0.91	24.15 ± 3.47	14.90 ± 2.67	50.00 ± 34.71	25.15 ± 25.95	26.54 ± 19.62	15.24 ± 0.59

$^{\alpha}$C: cellulase; $^{\beta}$G: glucosidase; $^{\chi}$P: pectinase; $^{\delta}$CG: cellulase and glucosidase; $^{\varepsilon}$CP: cellulase and pectinase; $^{\phi}$GP: glucosidase and pectinase.

Table 2. Mean and standard deviation of the phenolic extractable contents in grape skins for the main phenolic families determined for control and enzymatic macerations. Control macerations were carried out without presence of enzyme.

		Control macerations						Enzymatic macerations					
		$^{\alpha}$C	$^{\beta}$G	$^{\chi}$P	$^{\delta}$CG	$^{\varepsilon}$CP	$^{\phi}$GP	$^{\alpha}$C	$^{\beta}$G	$^{\chi}$P	$^{\delta}$CG	$^{\varepsilon}$CP	$^{\phi}$GP
Phenolic contents	$^{\gamma}$Total phenols	7.54 ± 4.65	5.64 ± 1.29	6.18 ± 1.51	3.52 ± 2.09	5.11 ± 0.47	5.11 ± 0.41	8.94 ± 5.82	5.64 ± 0.70	6.06 ± 1.80	3.59 ± 0.83	5.32 ± 0.32	5.16 ± 1.19
	$^{\eta}$Total flavanols	0.85 ± 0.58	0.67 ± 0.09	0.95 ± 0.26	0.43 ± 0.25	0.74 ± 0.35	0.69 ± 0.13	0.92 ± 0.62	0.74 ± 0.12	0.91 ± 0.32	0.48 ± 0.07	0.85 ± 0.24	0.54 ± 0.28
	$^{\tau}$Total anthocyanins	1.71 ± 1.20	1.34 ± 0.79	1.01 ± 1.36	0.70 ± 0.47	0.92 ± 0.50	1.54 ± 0.05	2.29 ± 1.60	1.61 ± 0.77	0.70 ± 0.85	0.79 ± 0.42	0.93 ± 0.59	1.30 ± 0.38

$^{\alpha}$C: cellulase; $^{\beta}$G: glucosidase; $^{\chi}$P: pectinase; $^{\delta}$CG: cellulase and glucosidase; $^{\varepsilon}$CP: cellulase and pectinase; $^{\phi}$GP: glucosidase and pectinase; $^{\gamma}$Total phenols: expressed as mg of gallic acid equivalents per gram of grape skin; $^{\eta}$Total flavanols: expressed as mg of catechin equivalents per gram of grape skin; $^{\tau}$Total anthocyanins: expressed as mg of malvidin-3-*O*-glucoside equivalents per gram of grape skin.

Table 3. Anthocyanic profile of grape skins for control and enzymatic macerations. Mean and standard deviation are provided. Control macerations were carried out without presence of enzyme.

		Control macerations						Enzymatic macerations					
		$^{\alpha}$C	$^{\beta}$G	$^{\chi}$P	$^{\delta}$CG	$^{\varepsilon}$CP	$^{\phi}$GP	$^{\alpha}$C	$^{\beta}$G	$^{\chi}$P	$^{\delta}$CG	$^{\varepsilon}$CP	$^{\phi}$GP
$^{\psi}$Anthocyanic profile	$^{\gamma}$DfG	0.10 ± 0.08	0.06 ± 0.04	0.03 ± 0.04	0.03 ± 0.02	0.04 ± 0.02	0.05 ± 0.04	0.16 ± 0.13	0.06 ± 0.04	0.02 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.05 ± 0.05
	$^{\eta}$CyG	0.03 ± 0.03	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.04 ± 0.03	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01
	$^{\iota}$PtG	0.13 ± 0.09	0.08 ± 0.06	0.05 ± 0.06	0.04 ± 0.03	0.05 ± 0.02	0.08 ± 0.06	0.20 ± 0.16	0.09 ± 0.05	0.04 ± 0.03	0.05 ± 0.03	0.05 ± 0.03	0.07 ± 0.05
	$^{\varphi}$PnG	0.11 ± 0.09	0.07 ± 0.04	0.04 ± 0.03	0.07 ± 0.05	0.08 ± 0.04	0.07 ± 0.03	0.14 ± 0.10	0.06 ± 0.03	0.04 ± 0.03	0.07 ± 0.04	0.08 ± 0.04	0.07 ± 0.03
	$^{\kappa}$MvG	0.73 ± 0.53	0.58 ± 0.32	0.41 ± 0.60	0.28 ± 0.23	0.35 ± 0.28	0.61 ± 0.60	0.99 ± 0.73	0.72 ± 0.28	0.29 ± 0.41	0.31 ± 0.20	0.36 ± 0.32	0.53 ± 0.16
	$^{\lambda}$DfA	0.02 ± 0.02	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.02
	$^{\mu}$CyA	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	$^{\nu}$PtA	0.03 ± 0.02	0.03 ± 0.02	0.02 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.04 ± 0.03	0.03 ± 0.02	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02
	^{o}PnA	0.04 ± 0.02	0.03 ± 0.02	0.06 ± 0.07	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.07	0.05 ± 0.03	0.04 ± 0.02	0.04 ± 0.06	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
	$^{\pi}$MvA	0.25 ± 0.24	0.28 ± 0.22	0.22 ± 0.34	0.14 ± 0.07	0.22 ± 0.17	0.40 ± 0.34	0.30 ± 0.32	0.36 ± 0.27	0.12 ± 0.18	0.17 ± 0.08	0.21 ± 0.19	0.32 ± 0.07
	$^{\theta}$CyC	0.03 ± 0.04	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.04 ± 0.04	0.02 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	$^{\rho}$PtC	0.04 ± 0.03	0.03 ± 0.01	0.03 ± 0.04	0.01 ± 0.01	0.02 ± 0.01	0.06 ± 0.04	0.05 ± 0.03	0.05 ± 0.01	0.02 ± 0.02	0.01 ± 0.01	0.02 ± 0.02	0.04 ± 0.01

	$^{\sigma}MvC_{cis}$	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	$^{\tau}PnC$	0.04 ± 0.04	0.02 ± 0.01	0.02 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.02	0.05 ± 0.03	0.02 ± 0.00	0.02 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
	$^{\upsilon}MvC_{trans}$	0.14 ± 0.12	0.07 ± 0.03	0.07 ± 0.11	0.02 ± 0.02	0.02 ± 0.01	0.09 ± 0.11	0.17 ± 0.14	0.09 ± 0.03	0.04 ± 0.06	0.02 ± 0.01	0.03 ± 0.02	0.05 ± 0.01
	$^{\varpi}M_{total}$	1.09 ± 0.80	0.81 ± 0.47	0.54 ± 0.72	0.43 ± 0.34	0.54 ± 0.29	0.82 ± 0.07	1.52 ± 1.10	0.95 ± 0.41	0.40 ± 0.49	0.48 ± 0.29	0.55 ± 0.35	0.73 ± 0.26
	$^{\omega}A_{total}$	0.35 ± 0.30	0.37 ± 0.27	0.32 ± 0.46	0.21 ± 0.10	0.30 ± 0.17	0.52 ± 0.04	0.43 ± 0.41	0.46 ± 0.32	0.20 ± 0.26	0.25 ± 0.11	0.30 ± 0.20	0.43 ± 0.12
	$^{\xi}C_{total}$	0.28 ± 0.23	0.15 ± 0.06	0.14 ± 0.18	0.06 ± 0.03	0.08 ± 0.03	0.21 ± 0.05	0.33 ± 0.25	0.20 ± 0.04	0.09 ± 0.10	0.06 ± 0.02	0.08 ± 0.04	0.14 ± 0.01

551 $^{\alpha}$C: cellulase; $^{\beta}$G: glucosidase; $^{\chi}$P: pectinase; $^{\delta}$CG: cellulase and glucosidase; $^{\varepsilon}$CP: cellulase and pectinase; $^{\phi}$GP: glucosidase and pectinase; $^{\gamma}$DfG: Delphinidin 3-
552 *O*-glucoside; $^{\eta}$CyG: Cyanidin 3-*O*-glucoside; $^{\iota}$PtG: Petunidin 3-*O*-glucoside; $^{\varphi}$PnG: Peonidin 3-*O*-glucoside; $^{\kappa}$MvG: Malvidin 3-*O*-glucoside; $^{\lambda}$DfA: Delphinidin-
553 3-*O*-(6′-acetyl)-glucoside; $^{\mu}$CyA: Cyanidin-3-*O*-(6′-acetyl)-glucoside; $^{\nu}$PtA: Petunidin-3-*O*-(6′-acetyl)glucoside; ^{o}PnA: Peonidin-3-*O*-(6′-acetyl)glucoside; $^{\pi}$MvA:
554 Malvidin-3-*O*-(6′-acetyl)glucoside; $^{\theta}$CyC: Cyanidin-3-*O*-(6′-*p*-coumaroyl)glucoside; $^{\rho}$PtC: Petunidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*trans*); $^{\sigma}MvC_{cis}$: Malvidin-
555 3-*O*-(6′-*p*-coumaroyl)glucoside (*cis*); $^{\tau}$PnC: Peonidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*trans*); $^{\upsilon}MvC_{trans}$: Malvidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*trans*); $^{\varpi}M_{total}$:
556 total monoglucosides; $^{\omega}A_{total}$: total acetyls; $^{\xi}C_{total}$: total coumaroyls; $^{\Psi}$Anthocyanic profile: all variables are expressed as mg of malvidin-3-*O*-glucoside
557 equivalents per gram of grape skin.

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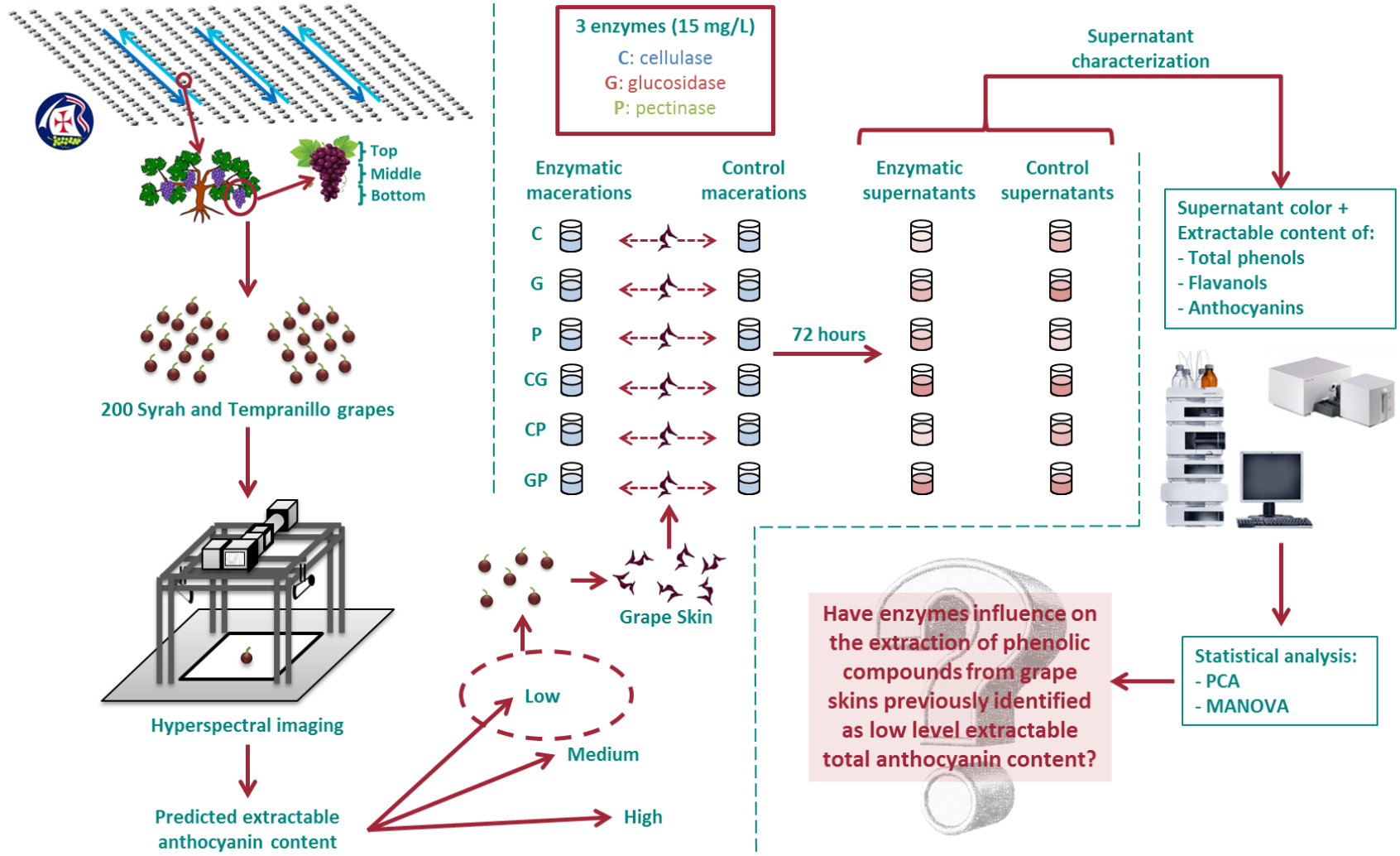
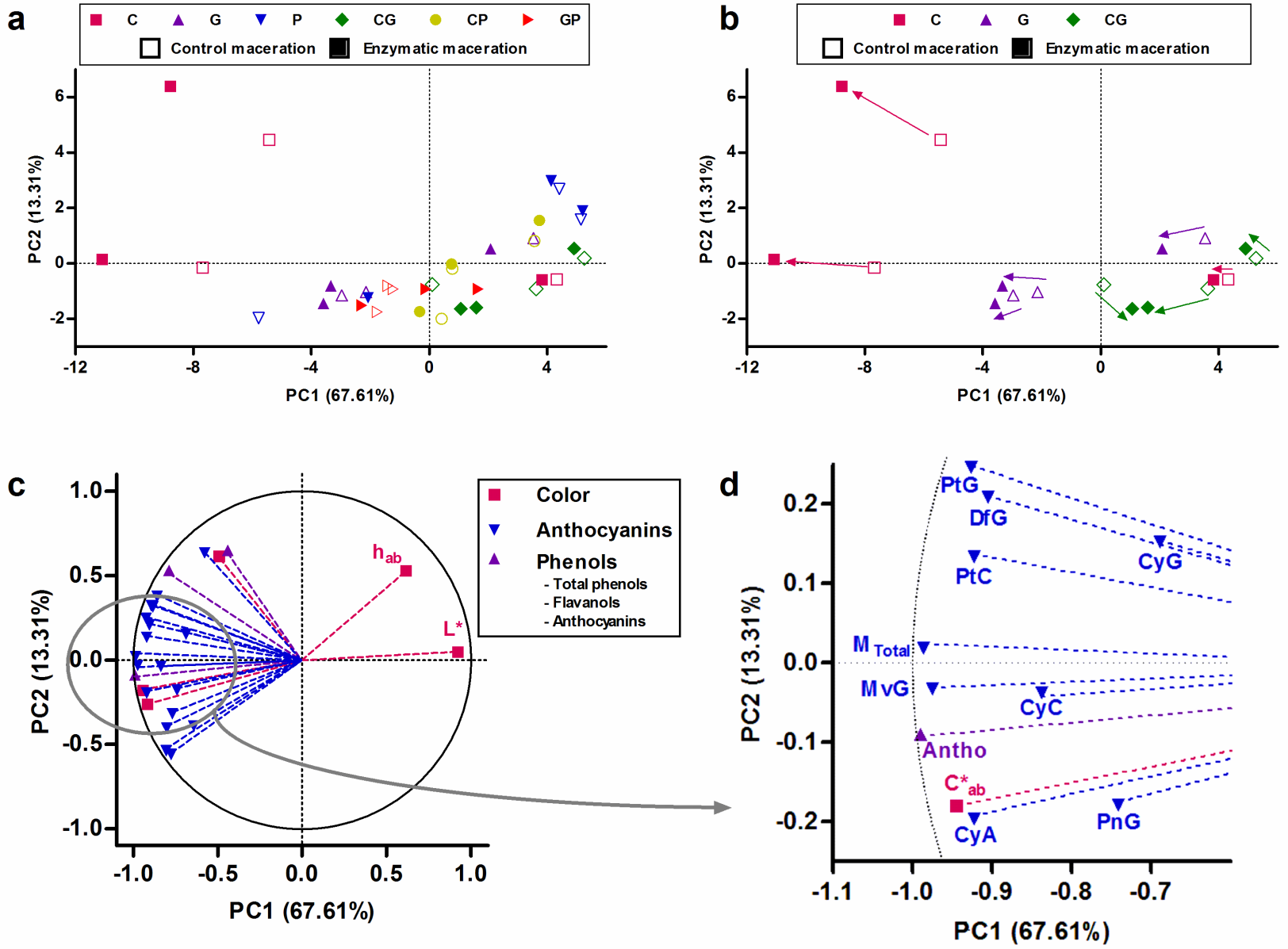
559 **Figures** (graphs, charts, line drawings, photographs)



560

561 **Fig. 1**: Schematic representation of the entire process. Sample collection, prediction of extractable anthocyanin content by near
562 infrared hyperspectral imaging, selection of samples with a low anthocyanin extraction potential, enzymatic and control maceration
563 of grape skins and supernatant and statistical analyses.
564


a
C
G
P
CG
CP
GP
Control maceration
Enzymatic maceration
PC2 (13.31%)
PC1 (67.61%)
b
C
G
CG
Control maceration
Enzymatic maceration
PC2 (13.31%)
PC1 (67.61%)
c
Color
Anthocyanins
Phenols
- Total phenols
- Flavanols
- Anthocyanins
h ab
L*
PC2 (13.31%)
PC1 (67.61%)
d
PtG
DfG
CyG
PtC
M Total
MvG
CyC
Antho
C*ab
CyA
PnG
PC2 (13.31%)
PC1 (67.61%)

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566 **Fig. 2**: (a) Score plot of the first two principal components after PCA performed on supernatant colour and phenolic parameters of
567 the grape skins for all the enzymatic treatments. Non-filled markers correspond to control macerations whereas filled markers
568 correspond to enzymatic macerations. (b) Score plot represented only for C, G and CG enzymatic treatments. Arrows indicate the
569 trends produced by the presence of the enzyme in the enzymatic macerations. (c) Projection of the colorimetric and phenolic
570 variables on the principal component plane. (d) Detail of this projection where variables with a higher influence on PC1 have been
571 identified. Abbreviations in this figure: please, see Tables 1 and 3 foot notes.