Elsevier Editorial System(tm) for Food Chemistry

Manuscript Draft

Manuscript Number: FOODCHEM-D-16-03799R2

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Article Type: Research Article (max 7,500 words)

Keywords: Hyperspectral imaging; grape; winemaking byproducts; extractable anthocyanins; copigments.

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Aqueous supernatants were analyzed by HPLC-DAD and extractable anthocyanin contents were obtained. Principal component analyses and analyses of variance were carried out with the aim of studying trends related to the extractable anthocyanin contents. Significant differences were found among grapes with different levels of predicted extractable anthocyanin contents. Moreover, no significant differences were found on the extractable anthocyanin contents using different copigment concentrations in grape skin macerations.

Evaluation of the influence of white grape seed extracts as copigment sources on the anthocyanin extraction from grape skins previously classified by near infrared hyperspectral tools.

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ABSTRACT

Hyperspectral imaging has been used to classify red grapes (*Vitis vinifera* L.) according to their predicted extractable total anthocyanin content (i.e. extractable total anthocyanin content determined by a hyperspectral method). Low, medium and high levels of predicted extractable total anthocyanin content were established. Then, grape skins were split into three parts and each part was macerated into a different model wine solution for a three-day period. Wine model solutions were made up with different concentration of copigments coming from white grape seeds.

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Keywords

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1. Introduction

The majority of flavonoids in red wine come from grape solid parts. These compounds are transferred to wine at the maceration stage. Wine flavonoids are all polyphenolic compounds, having multiple aromatic rings presenting hydroxyl groups (Waterhouse, 2002). Flavonoids have well-known health benefits (Rice-Evans, Miller, & Paganga, 1997).

Flavanols is the most abundant class of flavonoids in red wine and more than 50 per cent of the aforesaid family of compounds come from grape seeds. Anthocyanins come from grape skins and transfer to red wine their characteristic color. This color is based on the fully conjugated flavylium chromophore (Waterhouse, 2002). The color of red wine is an important quality parameter, it is usually the first characteristic perceived by consumers, who tends to prefer wines with a deep color and hue (García-Marino, Escudero-Gilete, Heredia, Escribano-Bailón, & Rivas-Gonzalo, 2013). These features make winemakers, who are continuously looking for high quality wines, give a lot of importance to the color of their wines. Wine color depends largely on anthocyanin content, nonetheless other factors such as, pH, SO_2 content or copigmentation can modify it (Boulton, 2001; Heredia, Francia-Aricha, Rivas-Gonzalo, Vicario, & Santos-Buelga, 1998; Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006).

Wine anthocyanin content depends mainly on the amount of anthocyanins released from grape skin to wine, i.e. the extractable anthocyanin content. Therefore, it is really important to control the amount of anthocyanins that may be extracted from grapes to wine. It is well known that extractability of anthocyanins from skins depends significantly on grape ripeness. Riper grapes have higher cell wall degradation hence they have higher extraction degree (Hernández-Hierro , Quijada-Morín, Martínez-Lapuente, Guadalupe, Ayestarán, Rivas-Gonzalo, et al., 2014; Ribéreau-Gayon,

Dubourdieu, Doneche, Lonvaud, Glories, Maujean, et al., 2006). As a consequence, it is possible to use grape ripeness to control wine anthocyanin content, even though there is heterogeneity of extractable anthocyanin content within the same ripeness stage (Nogales-Bueno, Baca-Bocanegra, Rodríguez-Pulido, Heredia, & Hernández-Hierro, 2015). For example, the soluble solid content of grape must or grape ripening stage affects wine anthocyanin content (Canals, Llaudy, Valls, Canals, & Zamora, 2005; Fournand, Vicens, Sidhoum, Souquet, Moutounet, & Cheynier, 2006; Hernández-Hierro, Quijada-Morín, Rivas-Gonzalo, Rivas-Gonzalo, & Escribano-Bailón, 2012; Torchio, Cagnasso, Gerbi, & Rolle, 2010; Zouid, Siret, Jourjon, Mehinagic, & Rolle, 2013). Furthermore, other methodologies can be used to increase the amount of anthocyanins in wine, e.g., thermic treatments, carbonic maceration, pectolytic enzymes, yeast selection, etc. (Sacchi, Bisson, & Adams, 2005).

In previous works carried out in our laboratory, hyperspectral imaging has been used to determine the extractable total anthocyanin content in grapes for Syrah and Tempranillo varieties (Nogales-Bueno, et al., 2015). These hyperspectral methods are not as accurate as traditional methods, however, they can screen parameters of interest without sample destruction and reagent consumption (Nogales-Bueno, Hernández-Hierro, Rodríguez-Pulido, & Heredia, 2014; Sun, 2010).

Besides anthocyanin content, other parameters such as pH, SO_2 content and copigment content also have a great impact on wine color. However, these parameters are not flexible, they are usually fixed by other technologic or sensorial aspects. Only copigment content can be modified by winemakers in order to improve wine color, if the astringency is controlled. Wine copigmentation is currently well-known (Boulton, 2001), a large number of copigmentation studies have been developed in the recent years (Bimpilas, Panagopoulou, Tsimogiannis, & Oreopoulou, 2016; García-Marino, et

al., 2013; González-Manzano, Dueñas, Rivas-Gonzalo, Escribano-Bailón, & Santos-Buelga, 2009; Gordillo, Rodríguez-Pulido, Escudero-Gilete, González-Miret, & Heredia, 2012; Gordillo, Rodríguez-Pulido, González-Miret, Quijada-Morín, Rivas-Gonzalo, García-Estévez, et al., 2015; Hermosín-Gutiérrez, Lorenzo, & Espinosa, 2005). In these studies it has been confirmed that the addition of different copigments improves the red wine color stabilization. Among these copigments, flavanols (i.e., catechin, epicatechin, etc.) present a good potential for copigmentation (Gordillo, Rodríguez-Pulido, Escudero-Gilete, et al., 2012). Thus, different winemaking byproducts, such as grape seeds which are rich in flavanols, could have a good potential for copigmentation. However, the addition of copigments with the aim of improving or stabilizing wine color can also have negative effects. For example, the copigment source could absorb pigment from the wine and modify its color. Moreover, the copigment could hamper the extraction equilibrium of anthocyanins compounds handicapping wine color.

In this study, hyperspectral imaging is used to select grape skins with different anthocyanin extractability levels. Following this, the amount of anthocyanins extracted from these grape skins is evaluated using chemical extractions. The extractions are carried out in presence of different levels of copigments coming from white grape seeds. The main aim of this study is to evaluate the influence of white grape seed extracts as copigment sources on the anthocyanin extraction from grape skin. To our knowledge, this is the first time that the aforementioned aims have been jointly faced.

2. Materials and methods

2.1. Samples

Samples were collected from two vineyards located in the Condado de Huelva Designation of Origin D.O. (Andalusia, Spain). *V. vinifera* L. cv. Syrah and

Tempranillo red grape samples were collected when the vineyards were harvested (August 12 and 27, 2013 respectively). Both varieties are typically grown in Spain for producing quality red wines and being a resistant cultivar to warm climatic conditions (Gordillo, Rodríguez-Pulido, Mateus, Escudero-Gilete, González-Miret, Heredia, et al., 2012).

One hundred single berries were collected for each variety from the top, middle and bottom of the cluster and in the sunlight and shade side of this. Afterwards, samples were refrigerated and they were immediately carried to the laboratory, tempered and subjected to the hyperspectral analysis.

White grape seed were collected from *V. vinifera* L. cv Zalema, a white cultivar autochthonous to the South of Spain where it represents over 90% of the overall production (Hernanz, Gallo, Recamales, Meléndez-Martínez, González-Miret, & Heredia, 2009). Seed were obtained from winemaking Zalema byproducts which had been previously characterized by Jara-Palacios, Gordillo, González-Miret, Hernanz, Escudero-Gilete, and Heredia (2014).

2.2. Sample selection by hyperspectral image

Hyperspectral imaging was used to develop a hyperspectral method for the screening of the extractable total anthocyanin content in grape skins as described elsewhere by (Nogales-Bueno, et al., 2015).

Grapes were ordered according their predicted extractable total anthocyanin content (PETAC). Then three groups were created for each variety: low, medium and high levels of PETAC. Finally two samples were selected from each group obtaining a total of 12 samples (i.e. 2 varieties × 3 groups × 2 samples)

2.3. Model wine macerations

136 Firstly, a stock model wine solution was made up of 4 g L^{-1} tartaric acid and 12.5%
137 ethanol, adjusted at pH 3.6 with NaOH 0.5 M. Then, 20 g of white grape seeds were
138 macerated in the model wine solution for three days as described elsewhere in Jara-
139 Palacios, et al. (2014). The concentration of total phenols in this stock solution was 2.6
140 g L^{-1} expressed as gallic acid equivalents. It was determined using the Folin–Ciocalteu
141 method (Singleton & Rossi, 1965). Fig. 1 summarizes the qualitative phenolic
142 composition for this stock solution based in data previously reported elsewhere by
143 (Jara-Palacios, et al., 2014) . Two model wine solutions were made up from the stock
144 solution with total phenols concentrations of 0.1 and 0.2 g L^{-1} expressed as gallic acid
145 equivalents (hereinafter solutions B and C respectively). Additionally, another model
146 wine solution without copigments (hereinafter solution A) was also used in the study as
147 control.
148 With the aim of studying the influence that copigments from grape seeds have in grape
149 skin anthocyanin extraction, the following methodology was carried out: grape skins
150 were split into three parts and each part was immersed in a different model wine
151 solution (A, B or C). The ratio of skin weight and model wine solution was kept
152 constant for all samples (1:20 w:v (g mL^{-1})). Macerations went on for a three-day
153 period. Then, these supernatants were used in the subsequent chromatographic analysis.
154 Fig. 2 shows the whole process.

155 2.4. Chromatographic analysis

156 The aqueous supernatants obtained from A, B and C extractions were diluted 1:2 with
157 0.1 M HCl, filtered through 0.45 μm pore-size filters and directly injected into the
158 chromatographic system to determine the anthocyanins. Anthocyanins chromatographic
159 analysis was carried out following a modification of García-Marino et al. (2010) as
160 described elsewhere in Hernández-Hierro et al. (2013). As result, extractable

anthocyanin contents (EAC) were obtained. EAC were expressed as mg of malvidin-3-*O*-glucoside equivalents per gram of grape skin. All analyses were performed in duplicate.

2.5. Statistical analysis

All statistical analyses were performed using Statistica v.8.0 software (StatSoft Inc., OK, USA, 2007). A PCA was applied to EAC data (individual anthocyanins and families described in section 2.4) in order to look for different trends into the samples and mainly between different copigment concentrations. Univariate analyses of variance (Tukey *post hoc* test) were applied to look for differences in the EAC (dependent variables) among two independents variables or factors: copigment concentrations of the model wine solutions, A, B or C, used in the anthocyanin extraction and levels of PETAC. The statistically significant level was considered at $\alpha = 0.05$.

3. Results and discussion

3.1. Sample selection by hyperspectral image and anthocyanins analysis

As result of sample selection three levels were created for PETAC: low, medium and high. Then, two samples were selected for each group and variety. Table 1 shows the thresholds which determine the different levels for both varieties.

After chromatographic analysis (2.4. section), up to 15 anthocyanins were identified and EAC were obtained. Taking into account their basic structure, anthocyanins were also grouped as 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), 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*)), non-

186 acylated anthocyanins (Delphinidin 3-*O*-glucoside, Cyanidin 3-*O*-glucoside, Petunidin
187 3-*O*-glucoside, Peonidin 3-*O*-glucoside, Malvidin 3-*O*-glucoside) and acylated
188 anthocyanins as sum of acetyls and coumaroyls anthocyanins. The sum of them was
189 also expressed as total anthocyanins.
190 A univariate analysis of variance, a statistical method used to analyze the differences
191 among group means and their associated procedures, was performed to check the
192 goodness of the hyperspectral calibration model used for sample selection. Levels of
193 PETAC, shown in Table 1, were used as independent variable whereas the EAC were
194 used as dependent variables or factors. With the aim of comparing the levels of PETAC
195 with those predicted by the hyperspectral method only samples without any external
196 factor should be considered. Then, only samples macerated in model wine solution A
197 were taken into account for this analysis. Results are shown in Table 2. Significant
198 differences ($p < 0.05$) were found for almost all dependent variables among samples
199 depending on the level of PETAC, although two groups were usually found instead of
200 the proposed three groups. An important fact to be highlighted is that the hyperspectral
201 model tested was developed for the prediction of extractable total anthocyanin content,
202 however, significant differences were found even among individual compounds. Three
203 groups were found only for total non-acylated anthocyanins, whereas no significant
204 differences were found for total acetyls anthocyanins. Non-acylated anthocyanins region
205 has a better-defined chromatographic profile than total acetyls region. This explains the
206 different variances.
207 These results confirm the potential that hyperspectral imaging has for the identification
208 of different levels of PETAC, and particularly for sorting grapes into low and high
209 anthocyanin cession groups. Therefore, hyperspectral imaging, a reproducible, fast,
210 reliable, non-contact, and non-destructive analytical technique, can be used to identify

grapes with low anthocyanin cession. In this way, wineries might use hyperspectral imaging for implementation of corrective measures which allow improving anthocyanin extraction in these samples. Moreover, hyperspectral imaging can be used to identify grapes with high anthocyanin cession in order to produce high quality wines.

3.2. Influence of white grape seed extracts as copigment sources on the anthocyanin extraction.

With the aim of studying the influence that copigments coming from white grape seeds have on grape skin EAC, a PCA was carried out. PCA is an unsupervised pattern recognition technique that allows looking for trends among the different factors taken into account. PCA was carried out using EAC as dependent variables and several factors were evaluated. Regarding the results, more than 90% of the data variability is described for the first 3 principal components and PC1 and PC2 describe 61.23% and 22.51% respectively. PCA shows some trends among different varieties and levels of PETAC. PC2 allows a variety separation whereas a PC1-PC2 combination allows an extractability separation as is shown in Fig. 3. However, no trends were found among EAC from samples extracted in model wine solution with different concentrations of copigments A, B and C.

Moreover, a univariate analysis of variance was carried out using EAC as dependent variables and copigment concentrations of the model wine solutions, A, B or C, used in the anthocyanin extraction as independent variable or factor. Results are shown in Table 3. No significant differences were found among the three different copigment concentrations present into the solutions A, B and C. Therefore, there is no evidence that the presence of different levels of copigments coming from white grape seeds could hamper the extraction equilibrium of anthocyanins compounds handicapping wine color. The important implication of these findings is that a winemaking byproduct (i.e.,

white grape seeds) can be used as a copigment source without modifying (reducing or increasing) the amount of anthocyanins extracted from grape skins.

4. Conclusion

The procedure previously reported using near infrared hyperspectral imaging presents a good potential for selecting grapes according to their PETAC. This tool may allow identifying grapes with low anthocyanin cession and implementing a number of corrective treatments in order to improve anthocyanin extraction in these samples.

In addition, evidence is provided to show that copigments coming from white grape seeds do not reduce the amount of anthocyanins extracted from grape skins during the maceration stage. Therefore, these copigments can improve or stabilize wine color without hampering the extraction equilibrium of anthocyanins compounds. Nonetheless, further studies would be necessary in order to test the effect that copigments coming from different copigment sources, such as oak wood, white grape skin, grape pomace, etc., have on the anthocyanin extraction.

Abbreviations

PETAC, predicted extractable total anthocyanin content; EAC, extractable anthocyanin contents; PCA, principal component analysis; PC, principal component.

Acknowledgements

The Spanish MINECO is thanked for J. Nogales-Bueno FPI grant (BES-2012-060192) and project AGL2014-58486-C2. Universidad de Sevilla is thanked for M.J. Jara-Palacios postdoctoral grant and also for B. Baca-Bocanegra predoctoral grant (VPPI-Universidad de Sevilla). The authors also thank the technical staff of Biology Service [Servicios Generales de Investigación (SGI), Universidad de Sevilla].

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Figure captions

Fig. 1. Percentages of phenolic compounds, coming from white grape seeds, into the stock model wine solution. (a) Phenols. (b) Flavonols. (c) Flavanols. (d) Phenolic acids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Fig. 2. Schematic representation of the entire process. Hyperspectral screening of the predicted extractable total anthocyanin content (PETAC), model wines elaboration, macerations and chromatographic analyses of the extractable anthocyanin contents (EAC).

Fig. 3. Score plot of extractable anthocyanin contents (EAC) in the space defined by PC1 and PC2. (a) Codified as Syrah and Tempranillo grapes. (b) Codified as Low, Medium and High levels of predicted extractable total anthocyanin content (PETAC). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 1. Thresholds for different levels of predicted extractable anthocyanin content (mg g^{-1} of skin grape, expressed as malvidin-3-*O*-glucoside equivalents).

Cultivar	PETAC[α]	Minimum	Maximum
Syrah	Low	0.42	1.20
	Medium	1.20	2.38
	High	2.55	2.82
Tempranillo	Low	0.23	1.19
	Medium	1.55	2.40
	High	2.41	3.43

[α]PETAC: Predicted extractable total anthocyanin content;

Table 2. Extractable anthocyanin contents (mg g^{-1} of skin grape, expressed as malvidin-3-*O*-glucoside equivalents) for different levels of predicted extractable total anthocyanin content. Means ± standard errors of means (n = 24). For each anthocyanin, different letters in the same row indicate statistical differences (Tukey test, α=0.05).

	PETAC[α]		
EAC[β]	Low	Medium	High
Delphinidin-3-*O*-glucoside	0.040 ± 0.004[a]	0.088 ± 0.009[ab]	0.15 ± 0.03[b]
Cyanidin-3-*O*-glucoside	0.008 ± 0.001[a]	0.017 ± 0.003[a]	0.031 ± 0.003[b]
Petunidin-3-*O*-glucoside	0.070 ± 0.007[a]	0.11 ± 0.01[a]	0.17 ± 0.02[b]
Peonidin-3-*O*-glucoside	0.06 ± 0.01[a]	0.080 ± 0.007[ab]	0.13 ± 0.02[b]
Malvidin-3-*O*-glucoside	0.37 ± 0.04[b]	0.55 ± 0.04[a]	0.68 ± 0.04[a]
Delphinidin-3-*O*-(6′-acetyl)-glucoside	0.006 ± 0.001[a]	0.010 ± 0.002[ab]	0.012 ± 0.002[b]
Cyanidin-3-*O*-(6′-acetyl)-glucoside	0.005 ± 0.001[a]	0.008 ± 0.001[ab]	0.011 ± 0.002[b]
Petunidin-3-*O*-(6′-acetyl)glucoside	0.014 ± 0.004[a]	0.017 ± 0.004[a]	0.024 ± 0.005[a]
Peonidin-3-*O*-(6′-acetyl)glucoside	0.0050 ± 0.0005[a]	0.019 ± 0.005[ab]	0.04 ± 0.01[b]
Malvidin-3-*O*-(6′-acetyl)glucoside	0.14 ± 0.04[a]	0.18 ± 0.05[a]	0.24 ± 0.06[a]
Cyanidin-3-*O*-(6′-*p*-coumaroyl)glucoside	0.032 ± 0.002[a]	0.038 ± 0.006[a]	0.045 ± 0.007[a]
Petunidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*trans*)	0.014 ± 0.001[a]	0.012 ± 0.003[a]	0.030 ± 0.003[b]
Malvidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*cis*)	0.0067 ± 0.0007[a]	0.0046 ± 0.0004[ab]	0.009 ± 0.001[b]
Peonidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*trans*)	0.022 ± 0.003[a]	0.018 ± 0.004[a]	0.047 ± 0.006[b]
Malvidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*trans*)	0.066 ± 0.009[a]	0.07 ± 0.01[a]	0.16 ± 0.03[b]
Total non-acylated	0.55 ± 0.06[a]	0.85 ± 0.05[b]	1.16 ± 0.04[c]

Total acetyls	0.16 ± 0.05[a]	0.24 ± 0.06[a]	0.32 ± 0.08[a]
Total coumaroyls	0.14 ± 0.01[a]	0.14 ± 0.03[a]	0.29 ± 0.04[b]
Total acylated	0.31 ± 0.06[a]	0.37 ± 0.04[ab]	0.62 ± 0.12[b]
Total	0.85 ± 0.13[a]	1.22 ± 0.07[a]	1.78 ± 0.13[b]

[α]PETAC: Predicted extractable total anthocyanin content; [β]EAC: Extractable anthocyanin contents.

Table 3. Extractable anthocyanin contents (mg g^{-1} of skin grape, expressed as malvidin-3-*O*-glucoside equivalents) for different levels of copigment concentrations of the model wine solutions, A, B or C, used in the anthocyanin extraction. Means ± standard errors of means (n = 72), and means followed by the same letter within same row are not significantly different ($P < 0.05$).

	Copigment concentration		
EAC[α]	A[β]	B[γ]	C[δ]
Delphinidin-3-*O*-glucoside	0.09 ± 0.01[a]	0.10 ± 0.01[a]	0.11 ± 0.02[a]
Cyanidin-3-*O*-glucoside	0.018 ± 0.002[a]	0.017 ± 0.002[a]	0.019 ± 0.003[a]
Petunidin-3-*O*-glucoside	0.12 ± 0.01[a]	0.13 ± 0.01[a]	0.14 ± 0.02[a]
Peonidin-3-*O*-glucoside	0.09 ± 0.01[a]	0.084 ± 0.007[a]	0.09 ± 0.01[a]
Malvidin-3-*O*-glucoside	0.54 ± 0.03[a]	0.57 ± 0.04[a]	0.60 ± 0.05[a]
Delphinidin-3-*O*-(6′-acetyl)-glucoside	0.009 ± 0.001[a]	0.011 ± 0.001[a]	0.011 ± 0.001[a]
Cyanidin-3-*O*-(6′-acetyl)-glucoside	0.0079 ± 0.0009[a]	0.008 ± 0.001[a]	0.008 ± 0.001[a]
Petunidin-3-*O*-(6′-acetyl)glucoside	0.018 ± 0.003[a]	0.021 ± 0.003[a]	0.021 ± 0.003[a]
Peonidin-3-*O*-(6′-acetyl)glucoside	0.020 ± 0.005[a]	0.022 ± 0.004[a]	0.016 ± 0.003[a]
Malvidin-3-*O*-(6′-acetyl)glucoside	0.19 ± 0.03[a]	0.20 ± 0.03[a]	0.20 ± 0.03[a]
Cyanidin-3-*O*-(6′-*p*-coumaroyl)glucoside	0.038 ± 0.003[a]	0.040 ± 0.003[a]	0.038 ± 0.003[a]
Petunidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*trans*)	0.019 ± 0.002[a]	0.022 ± 0.002[a]	0.023 ± 0.003[a]
Malvidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*cis*)	0.0068 ± 0.0006[a]	0.0079 ± 0.0007[a]	0.0077 ± 0.0008[a]
Peonidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*trans*)	0.029 ± 0.004[a]	0.027 ± 0.003[a]	0.0.29 ± 0.003[a]
Malvidin-3-*O*-(6′-*p*-coumaroyl)glucoside (*trans*)	0.10 ± 0.01[a]	0.11 ± 0.02[a]	0.11 ± 0.01[a]

Total non-acylated	0.85 ± 0.06[a]	0.91 ± 0.07[a]	0.9 ± 0.1[a]
Total acetyls	0.24 ± 0.04[a]	0.26 ± 0.04[a]	0.26 ± 0.04[a]
Total coumaroyls	0.19 ± 0.02[a]	0.21 ± 0.02[a]	0.21 ± 0.02[a]
Total acylated	0.43 ± 0.05[a]	0.47 ± 0.06[a]	0.46 ± 0.05[a]
Total	1.3 ± 0.1[a]	1.4 ± 0.1[a]	1.4 ± 0.1[a]

[α]EAC: Extractable Anthocyanin Contents; [β]A: Model wine solution without copigments; [γ]B: Model wine solution with a total phenols concentration of 0.1 g L^{-1} expressed as gallic acid equivalents; [δ]C: Model wine solution with a total phenols concentration of 0.2 g L^{-1} expressed as gallic acid equivalents.

Figure 1

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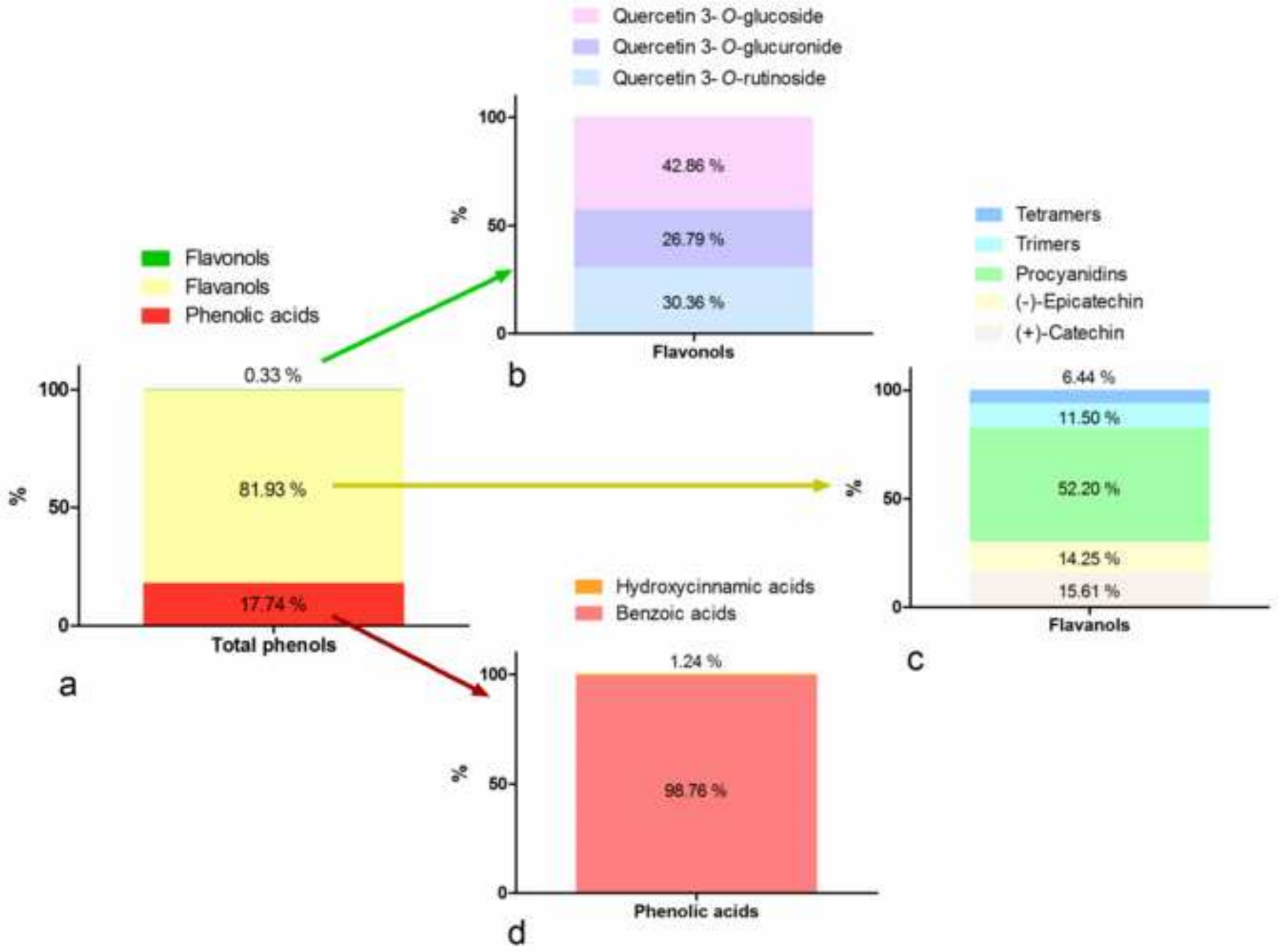

Figure 2

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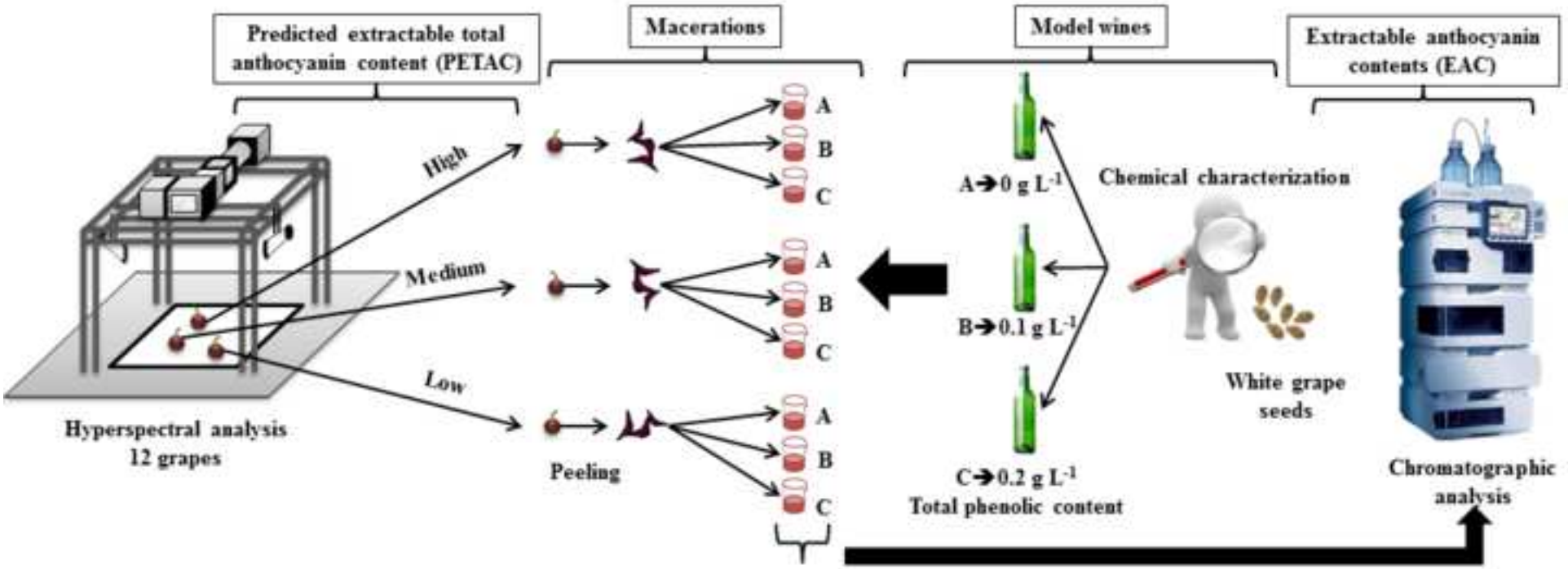

Figure 3

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