



Multivariate analysis of the polyphenol composition of Tempranillo and Graciano red wines

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ABSTRACT

Vitis vinifera L. cv Graciano is often used as a blending partner of Tempranillo based wines because it is considered to contribute significantly to the quality. The aim of this study is to discriminate between Tempranillo and Graciano monovarietal wines, and those made by the incorporation of 20% of Graciano variety in two different stages (at the end of malolactic fermentation and mixing the two grape varieties in the pre-fermentative maceration stage) of the winemaking process of the Tempranillo variety. To achieve this, supervised and unsupervised pattern recognition tools were applied to the data obtained in the study of the detailed polyphenolic composition, colour and other oenological parameters (143 variables). Patterns related to stages in the winemaking and ageing process, different wines and vintages can be observed using principal component analyses. Furthermore, linear discriminant analysis has been applied in order to characterise the wine samples. From the 143 variables, flavan-3-ols have exerted a profound influence on wine differentiation.

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1. Introduction

Vitis vinifera L. cv Graciano represents only a small proportion of the total grapes grown in Spain. In the Rioja region this variety is commonly used as a blending partner of Tempranillo-based wines to which it adds brightness, aroma, tannins and acidity. Although less usual some wineries also produce varietal Graciano wines. Differences in the phenolic profiles between Graciano and Tempranillo have been studied [1–3] and it has been shown that flavanols from grape skins of Graciano are more effective copigments of anthocyanins than those of Tempranillo, and the opposite occurs for seed flavanols, where Tempranillo was more effective [2]. On the other hand, it has been proposed that copigmentation could assist the extraction of polyphenolics from grapes [4] and that it could also act as a first stage in the formation of new pigments that determine the colour of aged red wines [5]. Taking into consideration the aforementioned, it could be reasonably expected that if the contact of the fermenting must with the skins and seeds of both varieties was facilitated, the final wine could result in higher contents of some polyphenolics, which would affect not only the sensorial properties but also its ageing potential.

Nowadays the use of techniques such as HPLC-DAD coupled to ESI/MSⁿ allows the determination of a large quantity of phenolic

compounds in red wines [6]. The huge amount of data that can be obtained makes the use of chemometry a necessary approach to help selecting relevant information [7–9]. Chemometric tools have been used in a wide range of foods [10,11] including wine [12]. Specifically, the phenolic composition, colour and oenological parameters of wine have been studied using different procedures for classificatory purposes and for recognising grape cultivars [13–20] and also for studying the relationship between the colour and phenolic composition of red wines obtained under different winemaking conditions [3,21,22].

The aim of this study is to discriminate between Tempranillo and Graciano varietal wines, and those made by both blending these wines at the end of malolactic fermentation and mixing the two grape varieties in the pre-fermentative maceration stage. To achieve this, supervised and unsupervised pattern recognition tools were applied to the data obtained in the study of the detailed polyphenolic composition, colour and other oenological parameters (143 variables). Also, the variables that can explain most of the variance observed in the dataset have been investigated.

2. Experimental

2.1. Samples

The wines used in this study were obtained from red grapes of *V. vinifera* L. of the Tempranillo and Graciano varieties, harvested in 2005 and 2006 and processed by Bodegas Roda (Haro, La Rioja,

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Table 1
Stages of sampling of wines T, G, M and W during winemaking and ageing.

Stages	Time
1	End of alcoholic fermentation
2	Middle of post-fermentative maceration
3	Beginning of malolactic fermentation
4	Middle of malolactic fermentation
5 ^a	End of malolactic fermentation
6	Before 1st rack
7	Before 2nd rack
8	Before 3rd rack
9	Clarification and bottling
10	Ageing in bottle during 5 months
11	Ageing in bottle during 9 months
12	Ageing in bottle during 12 months

^a In the case of W wines samples, were collected from stage 05.

Spain). In the studied samples notations T and G were used to design Tempranillo and Graciano varietal wines respectively; W was the wine obtained by the blend of these wines (proportion 80:20) at the end of malolactic fermentation, and M the wine resulting of the co-vinification of the two grape varieties mixed in proportion 80:20 in the pre-fermentative maceration stage. Samples of each wine were collected in triplicate during winemaking and ageing (Table 1). The total number of wine samples collected was 264, 132 per vintage. Analysis of each sample was also performed in triplicate and the mean value obtained was used in the statistical treatment.

2.2. Wine polyphenol analysis

For the anthocyanins and flavonols analysis 1 mL of wine samples were diluted (1:1) with 0.1N HCl (Panreac® Barcelona, España), filtered through 0.45 µm Millex® HV syringe driven filter units and injected into the chromatographic system. For the flavan-3-ols and phenolic acids analysis 2 mL of each wine sample diluted (1:1) with 0.1N HCl were eluted through Oasis® MCX 3 cm³ (Waters Corporation Mildford, MA, USA) cartridges previously conditioned with 2 mL methanol and 2 mL water, with the objective of eliminating the red pigments [23]. After washing with 4 mL of ultrapure water, flavan-3-ols and the phenolic acids were eluted with 8 mL methanol, whereas anthocyanins and the flavonols were retained in the cartridges. A small volume of water was added to the eluate and concentrated under vacuum at lower than 30 °C until complete elimination of methanol. The volume of the aqueous residue was adjusted to 0.5 mL with ultrapure water (Direct Q3, Millipore-Waters, Bedford, MA), filtered (0.45 µm) and analysed by HPLC-DAD-MS. All analyses were performed in triplicate.

2.3. HPLC-DAD-MS analysis

The HPLC-DAD analysis was performed in a Hewlett-Packard 1100 series liquid chromatograph. The LC system was connected to the probe of the mass spectrometer via the UV cell outlet. The mass analyses were performed using a Finnigan™ LCQ ion trap detector (Thermoquest, San Jose, CA, USA) equipped with an API source, using an electrospray ionisation (ESI) interface. The HPLC-DAD-MS analysis conditions of red pigments and flavonols were carried out in accordance with García-Marino et al. [3] selecting an additional wavelength at 360 nm to achieve the analysis of flavonols. Analyses of flavan-3-ols and phenolic acids were carried out as described by García-Marino et al. [24] selecting an additional wavelength at 330 nm to achieve the analysis of phenolic acids.

2.4. Quantification

For the quantitative analysis, calibration curves were obtained using standards of anthocyanins (delphinidin, cyanidin, petuni-

Table 2
Pigments analysed.

Pigments	
1	Direct condensation product between malvidin-3-O-glucoside and galliccatechin
2	Delphinidin-3,5-diglucoside
3	Direct condensation product between petunidin-3-O-glucoside and catechin
4	Petunidin-3,5-diglucoside
5	Delphinidin-3,7-diglucoside
6	Vitisin A of delphinidin-3-O-glucoside
7	Direct condensation product between peonidin-3-O-glucoside and catechin
8	Direct condensation product between malvidin-3-O-glucoside and catechin
9	Delphinidin-3-O-glucoside
10	Petunidin-3,7-diglucoside
11	Cyanidin-3-O-glucoside
12	Vitisin A of petunidin-3-O-glucoside
13	Petunidin-3-O-glucoside
14	Malvidin-3,7-diglucoside
15	Vitisin A of peonidin-3-O-glucoside
16	Peonidin-3-O-glucoside
17	Vitisin A of malvidin-3-O-glucoside
18	Malvidin-3-O-glucoside
19	Delphinidin-3-O-(6'-acetyl)-glucoside
20	Malvidin-3-O-hexose
21	Vitisin B of malvidin-3-O-glucoside
22	Malvidin-3-O-pentoside
23	Malvidin-3-O-glucoside-8-ethyl-catechin
24	Petunidin-3-O-(6'-acetyl)glucoside
25	Malvidin-3-O-glucoside-8-ethyl-galliccatechin
26	Malvidin-3-O-glucoside-8-ethyl-epicatechin
27	Cyanidin-3-O-hexoside
28	Malvidin-3-O-glucoside-8-ethyl-metoxycatechin
29	Malvidin-3-O-(6'-p-coumaroyl)glucoside-8-ethyl-catechin
30	Peonidin-3-O-(6'-acetyl)glucoside
31	Vitisin A of malvidin-3-O-(6'-p-coumaroyl)glucoside
32	Malvidin-3-O-(6'-acetyl)glucoside
33	Delphinidin-3-O-(6'-p-coumaroyl)glucoside
34	Petunidin-3-O-(6'-p-coumaroyl)glucoside (cis)
35	Malvidin-3-O-(6'-p-cafeoyl)glucoside
36	Cyanidin-3-O-(6'-p-coumaroyl)glucoside
37	Petunidin-3-O-(6'-p-coumaroyl)glucoside (trans)
38	Malvidin-3-O-(6'-p-coumaroyl)glucoside (cis)
39	Peonidin-3-O-glucoside-4-vinylcatechol
40	Peonidin-3-O-(6'-p-coumaroyl)glucoside (trans)
41	Malvidin-3-O-(6'-p-coumaroyl)glucoside (trans)
42	Malvidin-3-O-glucoside-4-vinylphenol adduct
43	Malvidin-3-O-glucoside-4-vinylguaicol adduct
44	Malvidin-3-O-(6'-acetyl)glucoside-4-vinylphenol adduct
45	Malvidin-3-O-(6'-p-coumaroyl)glucoside-4-vinylphenol
46	Malvidin-3-O-(6'-p-coumaroyl)glucoside-4-vinylguaicol
47	Total anthocyanidin-monoglucosides
48	Total anthocyanidin-diglucosides
49	Total acetyl anthocyanins
50	Total caffeoyl anthocyanins
51	Total p-coumaroyl anthocyanins
52	Total vitisins
53	Total vinyl anthocyanin adducts
54	Total pyranoanthocyanidins
55	Acetaldehyde-mediated flavanol-anthocynidin condensation products
56	Direct flavanol-anthocyanin condensation products
57	Total condensation products
58	Total pigments
59	Total anthocyanins
60	Total acylated anthocyanins
61	Total derived pigments
62	A-type vitisins
63	B-type vitisins
64	Anthocyanin-4-vinylphenol adducts
65	Anthocyanin-4-vinylcatechol adducts
66	Anthocyanin-4-vinylguaicol adducts

Table 3

Flavonols, flavan-3-ols, phenolic acids, chromatic parameters and other variables analysed.

Flavonols		Flavan-3-ols		Phenolic acids		Colorimetric parameters		Other variables	
1	Myricetin-3-O-galactoside	1	Procyanidin tetramer	1	Gallic acid	1	L^*	1	pH
2	Myricetin-3-O-glucoside	2	Procyanidin pentamer	2	Protocatechuic acid	2	a^*	2	Mean degree of polymerisation (mDP)
3	Myricetin aglycone	3	(+)-gallocatechin	3	Vanillic acid	3	b^*	3	%Copigmentation
4	Quercetin-3-O-galactoside	4	Procyanidin tetramer	4	Syringic acid	4	C^*_{ab}		
5	Quercetin-3-O-glucoside	5	Procyanidin dimer (B1)	5	trans-Caftaric acid	5	h_{ab}		
6	Quercetin glucuronide	6	Procyanidin dimer (B3)	6	cis-Cutaric acid	6	s^*_{uv}		
7	Quercetin-7-O-neohesperidoside-3-O-rutinoside	7	Prodelphinidin dimer	7	trans-Cutaric acid				
8	Quercetin aglycone	8	(–)-Epigallocatechin	8	trans-Fertaric acid				
9	Kaempferol-3-O-galactoside	9	Procyanidin trimer	9	p-Coumaroyl hexose (1)				
10	Kaempferol-3-O-glucoside	10	Procyanidin trimer	10	trans-Caffeic acid				
11	Kaempferol aglycone	11	(+)-Catechin	11	p-Coumaroyl hexose (2)				
12	Isorhamnetin aglycone	12	Procyanidin tetramer	12	p-Coumaric acid				
13	Total flavonols	13	Procyanidin trimer	13	Total hydroxybenzoic acids				
14	Total myricetin derivatives	14	Procyanidin dimer (B4)	14	Total hydroxycinnamic acids				
15	Total quercetin derivatives	15	Procyanidin dimer (B6)	15	Total phenolic acids				
16	Total kaempferol derivatives	16	Procyanidin dimer (B2)						
17	Total isorhamnetin derivatives	17	(–)-Epicatechin						
18	Total myricetin glycosides	18	Procyanidin trimer						
19	Total myricetin aglycons	19	Galloyl dimer						
20	Total quercetin glycosides	20	Procyanidin tetramer						
21	Total quercetin aglycons	21	Total flavan-3-ols						
22	Total kaempferol glycosides	22	Total procyanidins						
23	Total kaempferol aglycons	23	Total prodelphinidins						
24	Total isorhamnetin aglycons	24	Total procyanidin monomers						
		25	Total procyanidin dimers						
		26	Total procyanidin trimers						
		27	Total procyanidin tetramers						
		28	Total procyanidin pentamers						
		29	Total galloylated flavan-3-ols						

din, peonidin and malvidin 3-O-glucosides), flavonols (myricetin, quercetin and kaempferol), flavanols (catechin, gallocatechin, epicatechin gallate, dimer B₂ and trimer epicatechin-4,8-epicatechin-4,8-catechin) and phenolic acids (3,4-dihydroxybenzoic acid and 4-hydroxycinnamic acid). Anthocyanins were purchased from Polyphenols Labs. (Sandnes, Norway). Myricetin, kaempferol, gallocatechin and epicatechin gallate were purchased from Extrasynthèse (Genay, France). Quercetin, catechin, 3,4-dihydroxybenzoic acid and 4-hydroxycinnamic acid were purchased from Sigma (Steinheim, Germany). Procyanidin dimer and trimer were obtained in our laboratory in accordance with Escribano-Bailón et al. [25].

The total content of the different groups of phenolic compounds studied was calculated from the sum of the individual concentrations obtained for each individual compound, expressed in mg L^{−1} of wine.

2.5. Mean degree of polymerisation (mDP)

The mean degree of polymerisation of wine flavanols was calculated in accordance with the method described by González-Manzano et al. [26].

2.6. Copigmentation

The contribution of the copigmentation phenomenon to the absorbance at 520 nm of the wines (% copigmentation) was determined in accordance with the method proposed by Boulton [4] using a Hewlett Packard UV–vis HP-3853 spectrophotometer.

2.7. Analysis of the chromatic parameters

A Hewlett Packard UV–vis HP3853 was used for scanning between 380 and 770 nm at 2 nm intervals with a 2 mm path-length quartz cell, and CIE 1964 10° standard observer and the CIE D₆₅ illuminant observer, as references to calculate the tristimulus values recommended by the “Comission Internationale de l’Éclairage” [27]. The CIELAB and CIELUV colour spaces were used and parameters measured included: lightness (L^*), hue (h_{ab}), red-green coordinate (a^* , $-a^*$) and yellow-blue coordinate (b^* , $-b^*$), Chroma (C^*_{ab}) and saturation (s^*_{uv}). Calculations were made using the CromaLab® software [28].

2.8. Chemometric analysis

Chemometric tools could be divided into quantitative and qualitative methods. The latter could be subdivided into supervised and unsupervised pattern recognition tools. Unsupervised methods are applied as a preliminary stage prior to supervise modelling, such as classification, so as to observe trends in the data indicating relationships between samples and between variables. Supervised pattern recognition methods usually indicate whether samples fall into pre-defined groups, how well, and what causes this separation. The human capability of visually recognising regularities in data is still unsurpassed by these computer methods [8,9].

The SPSS 13.0 for Windows software package (SPSS, Inc., Chicago, IL) was used for data processing. The unsupervised pattern recognition method used for data analysis was principal components analysis (PCA), which was applied from the correlation matrix of the original variables. The supervised pattern recognition method was linear discriminant analysis (LDA). Stepwise feature selection was employed to select the most significant variables for

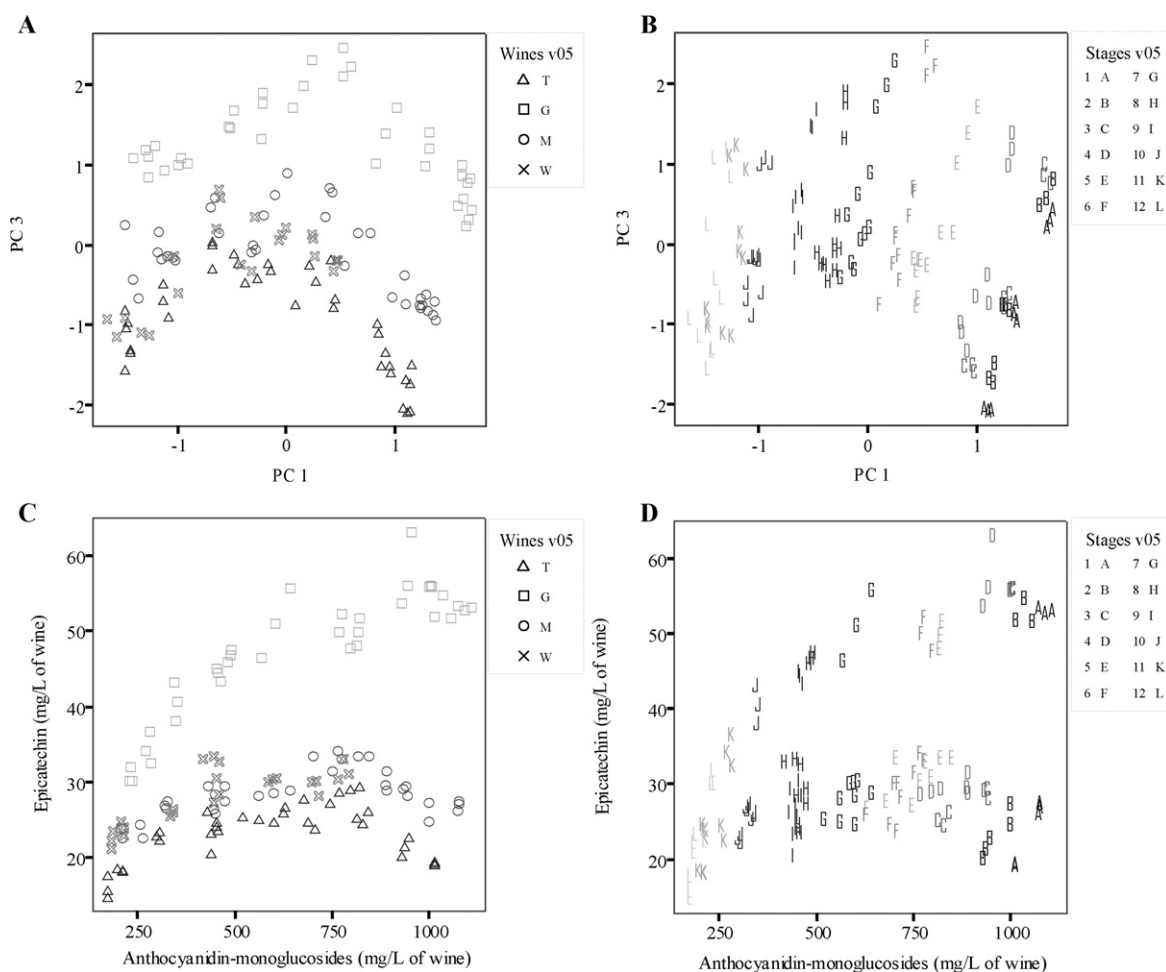


Fig. 1. Representation of the wine samples (2005 vintage) on the plane defined by the first and third principal components, PC 1 (41.6%) and PC 3 (9.4%) (A and B) and representation of the wine samples on the plane defined by epicatechin and anthocyanidin-monoglucosides (C and D). Differences between wines (A and C). Differences between stages in the winemaking process (B and D).

the discrimination between classes using *F*-statistic to test the significance of the change in Wilks' Lambda by adding or removing a variable. The prediction ability was estimated considering the percentage of samples adequately classified by the rules developed with the training set using a leave-one-out cross-validation procedure. The total variables used were 143, corresponding to detailed phenolic composition, colour parameters, mDP, % copigmentation and pH. They are summarised in Table 2 (pigments) and Table 3 (other variables).

3. Results and discussion

3.1. Principal component analysis

Principal component analysis was used as an unsupervised pattern recognition method with the dataset from the 2005 vintage. Fig. 1A and B shows the projection of the wine samples on the plane defined by the first and third principal components. The first principal component (PC 1) describes 41.6% of the variability in the data and the third (PC 3) 9.4%. Although the second component (PC 2) accounts for 14.6% of the variability in the data, it is not represented since no easy to explain tendency was found for it. This might be due to this component is influenced by factors for which no related information was available. PC 3 allows distinction between T (placed below) and G (placed above), whereas M and W wines are located between them and they are hardly distinguishable (Fig. 1A). PC1 allows the visualisation of differences between stages of the

winemaking process (Fig. 1B). From the obtained loadings, the variables most related with PC 3 and PC 1 were the contents of epicatechin and anthocyanidin-monoglucosides, respectively. Fig. 1C and D shows the plots of wine samples considering only these variables. In this plot, T and G can also be distinguished whereas M and W wines are located between them in a similar way to that shown in Fig. 1A and B, which indicates the high relation between the aforementioned variables and PC 1 and PC 3, respectively.

Similar results were obtained with the dataset from the 2006 vintage, in this case, the projection of the wine samples on the plane defined by the first and second principal components PC 1 (38.3%) and PC 2 (19.9%) (Fig. 2A and B) shows a similar pattern to the 2005 vintage plot, as stated above the variable most related with PC 1 was the content of anthocyanidin-monoglucosides which is related to stages of the winemaking process (Fig. 2B). PC 2 allows distinction between wines (Fig. 2A) and the variable most related to this PC was the content in the flavonol quercetin. The plots using the anthocyanidin-monoglucosides and quercetin contents are also presented (Fig. 2C and D); these plots show a similar distribution pattern to those in Fig. 2A and B and they are also similar to the plots obtained for the 2005 vintage. It is noticeable that in 2006 the differences between wines are related to PC 2, thus a greater amount of data variability than in vintage 2005 was related to the type of wine.

In order to increase the variability, the samples from both vintages, 2005 and 2006, were used in a new PCA analysis. The plot of

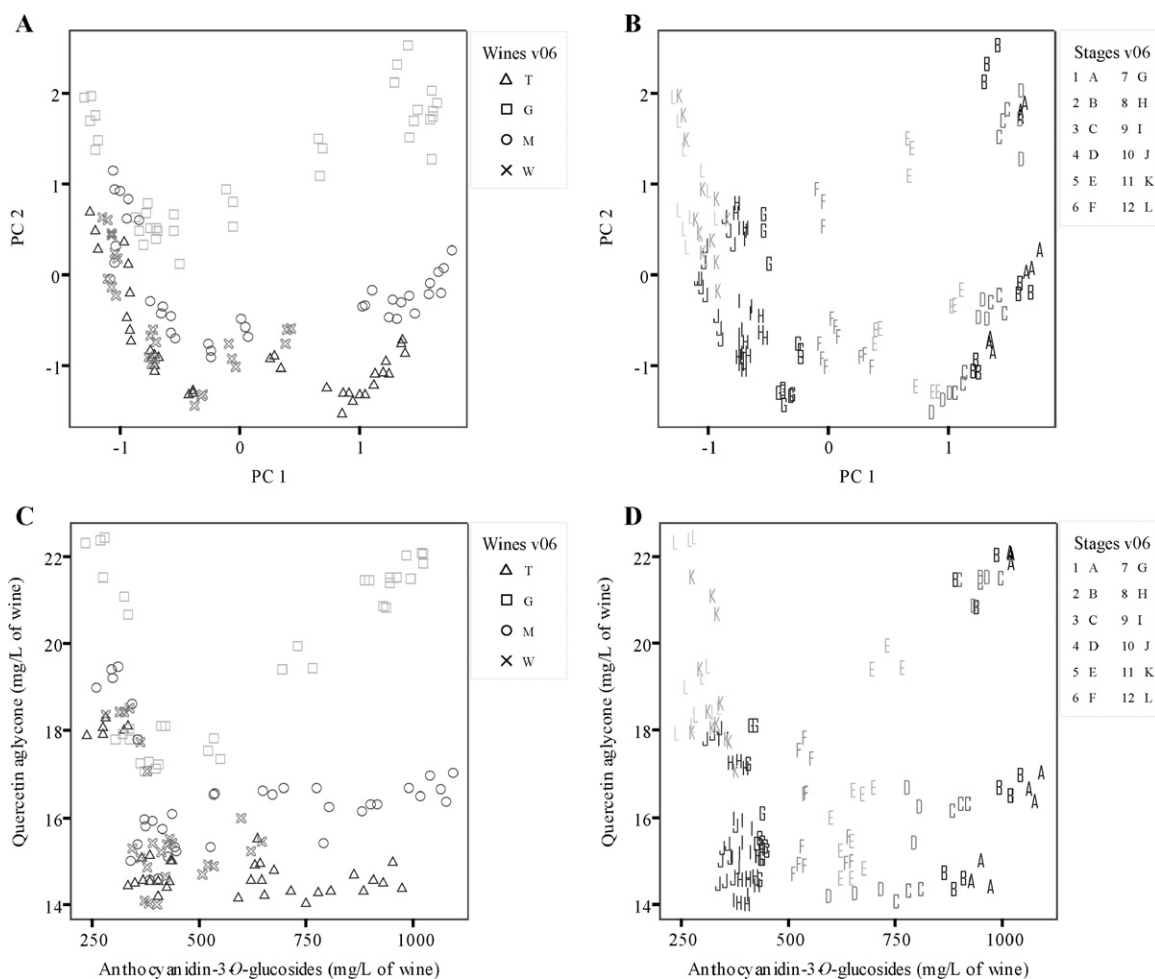


Fig. 2. Representation of the wine samples (2006 vintage) on the plane defined by the first and third principal components, PC 1 (38.3%) and PC 2 (19.9%) (A and B) and representation of the wine samples on the plane defined by quercetin aglycone and anthocyanidin-monoglucosides (C and D). Differences between wines (A and C). Differences between stages in the winemaking process (B and D).

the wine samples in the space defined by PC 1 (31.3%), PC 2 (18.6%) and PC 3 (11.7%) shows differences between the wines and stages in the winemaking process (Fig. 3A and B). However, in this case, another and more intense pattern was also observed related to the vintage that allowed a clear distinction between the wines of 2005 and 2006 vintages. Fig. 3C emphasises this pattern showing the plane defined by PC 1 and PC 2. In general, the principal components PC 1, PC 2 and PC 3 stand for the differences between stages of the winemaking process, vintages and types of wines, respectively. From the obtained loadings the variables most related to these PCs were the content of malvidin-3-O-glucoside followed by total anthocyanidin-monoglucosides' contents, for PC1, which also made a great contribution in the models where the 2005 and 2006 vintages were considered separately. For PC 3 the most related variable was the total content of pyranoanthocyanidins, and for PC 2 the total content of anthocyanidin-diglucosides. Anthocyanidin-diglucosides are minor anthocyanins in *V. vinifera* although in recent years they have been extensively found in grapes and wine from this species [3,6,29,30]. The fact that in our study their contents allow the distinction between vintages seems to indicate that their variations in grapes (and consequently in wines) are related to weather conditions.

In the three PCA analyses performed, the variations in the contents of anthocyanidin-monoglucosides play a decisive role for distinguishing between different stages of the winemaking. The changes in the amount of these compounds during the winemaking

process can be related to their involvement in the formation of new pigments (including pyranoanthocyanidins) or to their degradation by oxidative or enzymatic processes [31–33].

Polyphenols whose variations allow the distinction between the different wines were not the same in all of the PCA analyses performed (i.e. epicatechin in 2005, quercetin in 2006 and pyranoanthocyanidins in 2005 + 2006). This could be attributed to the different phenolic composition of the grape in each vintage. Differences between the polyphenolic profiles of a given cultivar reflects to a great extent its genetic potential but environmental stimuli also play critical roles in polyphenols' biosynthesis and this effect is reflected in the polyphenolic profiles [18].

3.2. Linear discriminant analysis

Linear discriminant analysis was performed as a supervised pattern recognition method with the dataset from the 2005 vintage so as to allocate the wine samples to their wine group. Thirty-one variables were retained that allowed 100% of the samples were correctly classified in both the internal and leave-one-out cross-validation procedures. The most represented family was flavan-3-ols with eleven variables that correspond to this family of phenolic compounds. A new LDA model was developed using only these eleven variables in order to use a single family of compounds as classifiers. Using this approach 100% of samples were correctly classified in internal validation and 96.2% in the leave-one-out

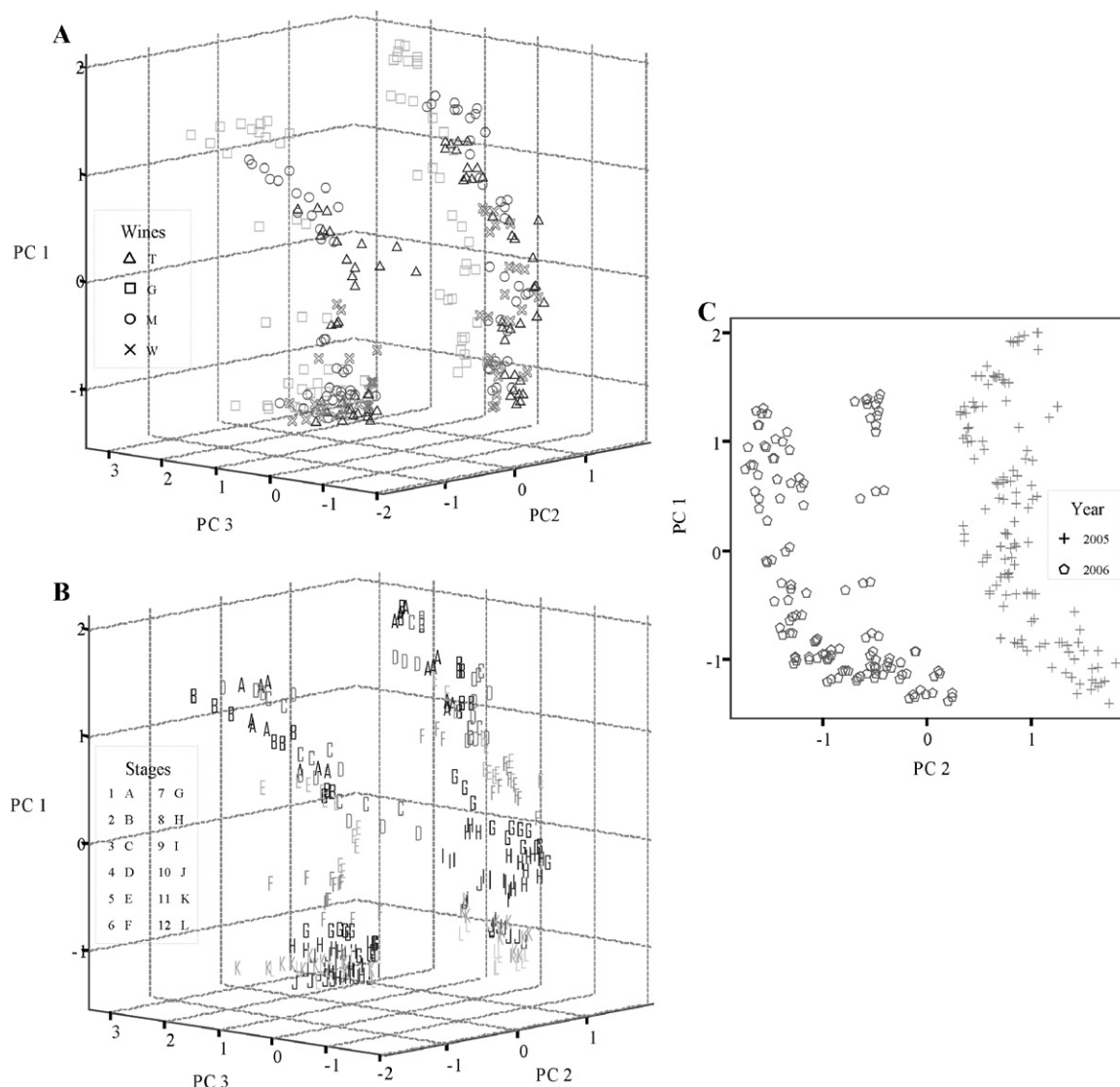


Fig. 3. Representation of the wine samples in the space defined by the first, second and third principal components, PC 1 (31.3%), PC 2 (18.6%) and PC 3 (11.7%) (A and B) and in the plane defined by PC 1 and PC 2 (C) (2005 and 2006 vintages together). (A) Differences between wines. (B) Differences between stages in the winemaking process. (C) Differences between vintages.

cross-validation procedure. Using the variables corresponding to other phenolic families, the percentage of samples correctly classified was lower in all cases. The same procedure was applied to the dataset from the 2006 vintage. Forty-one variables were retained, which allowed 100% of samples were correctly classified in both the internal and leave-one-out cross-validation procedures. The most represented family was also flavan-3-ols with twelve variables that were used to develop a new LDA model, in which 91.7% of samples were correctly classified in the internal validation and 85.6% in the leave-one-out cross-validation procedure. Similarly, when variables of the rest of the families were used the percentage of samples correctly classified was lower in all cases. In the case of the 2005 and 2006 vintages together, fifty-four variables were retained, and 100% of samples were correctly classified in the internal validation and 98.5% in the leave-one-out cross-validation procedure (Fig. 4). The most represented family was again flavan-3-ols with sixteen variables (Table 3, variables 1, 2, 5, 7–10, 12, 14–17, 19, 20, 23 and 24). An LDA model was developed using only these variables: 82.4% of samples were correctly classified in the internal validation and 79.0% in the leave-one-out cross-validation procedure. Again, worse classification results were obtained using variables of other phenolic families.

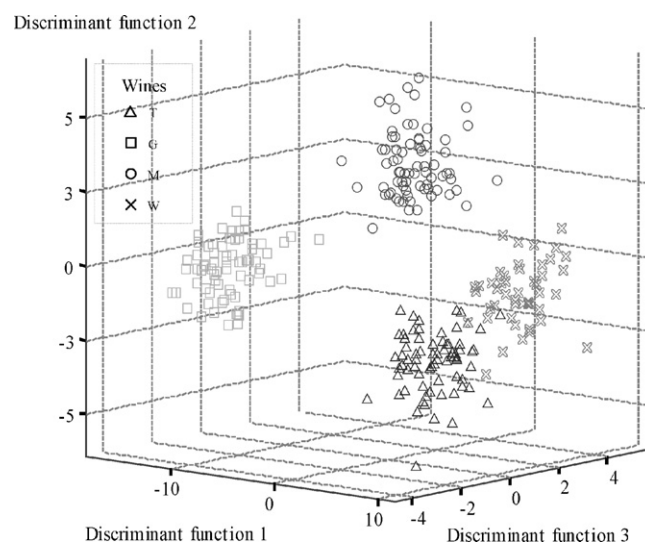


Fig. 4. Representation of the wine samples in the space defined by the first, second and third discriminant functions (2005 and 2006 vintages together).

It appears that flavan-3-ols are predominant factors regarding differentiation between T, G, M and W wines. Flavan-3-ol composition is dependent on the ripeness stage and growth conditions, but it is also greatly affected by genetics [18,34]. This group of flavonoids was also utilised in order to classify wines according to their geographical origin [17], cultivars [20] or both [18]. In the present study the generated models have permitted discrimination not only between different cultivars (T and G wines), but also between different oenological practices (M and W wines). Therefore, the different procedures used for the winemaking of M and W wines (mixing grapes or blending wines) would imply important differences related to the qualitative and/or quantitative phenolic profile, which are mainly reflected in the flavanols composition. Although other families of compounds or more easily obtained parameters, such as colour parameters, have been separately evaluated, in all cases the results obtained were not as good as using flavan-3-ol compounds.

4. Conclusions

Based on the results obtained it can be concluded that patterns related to stages of the winemaking process, type of wines and vintages can be differentiated using principal component analyses. Moreover, the variables most related to the principal components obtained in these analyses have been identified, which provide a chemical explanation of the observed tendencies.

Furthermore, linear discriminant analysis has been applied in order to classify the samples of the different studied wines (T, G, M and W). The generated models have permitted discrimination not only between different cultivars (T and G wines) wines, but also between different oenological practices (M and W wines). From the 143 considered variables, flavan-3-ols have proved to be the most profoundly influential for wine differentiation.

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