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Evolution of red wine anthocyanins during malolactic fermentation, postfermentative treatments and ageing with lees

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Abstract

A comparative study was conducted on nine batches of wine, from the same initial wine, subjected to malolactic fermentation and ageing in barrels, under different technological conditions: Malolactic fermentation in barrel or in tank, with or without wine clarification, ageing with or without lees and stirring or no stirring of the lees. Samples were taken of the initial wine, of the wine at the end of malolactic fermentation, of the wines after clarifying treatments, and after 3, 6, 9, 12 and 14 months of ageing in the barrel, making a total of 48 wines. As a result of the anthocyanin analysis of all the wines studied, a total of 21 different anthocyanin compounds were detected, which can be classified into four groups: simple glucosides, acetyl glucosides, cinnamoyl glucosides and pyroanthocyanins. During MLF, it was shown that the effect of the container used seems to be more important than the metabolic activity of the bacteria responsible for the process. From application of the LSD test, significant differences were found in the concentrations of all the anthocyanin compounds identified due to ageing time and significant differences were also revealed for most anthocyanin compounds in relation to the manufacturing method, especially the presence or absence of lees.

Keywords: Industrial red winemaking; Phenolic compounds; Lactic acid bacteria; Oak barrel; Stainless-steel tank; Lees storage; Technological procedures; Statistical treatments

1. Introduction

Besides alcoholic fermentation, red winemaking also requires malolactic fermentation (MLF) and ageing of wine in barrels and/or in bottles. The main purpose of MLF is to reduce wine acidity, transforming the malic acid, a dicarboxylic acid, into lactic acid, a monocarboxylic acid. Moreover, during this process volatile compounds are also formed that enrich the wine's aromatic quality (Henick-Kling, 1995; Moreno-Arribas & Polo, 2005; Pozo-Bayón et al., 2005). After MLF has finished, the wine is submitted to different treatments to clarify it and stabilize it, and is stored in oak barrels for ageing for a variable period of time, ranging from a few months to over a year. In recent years, in an attempt to obtain more complex quality wines from an organoleptic perspective, with their own distinguishing personality, new production technologies are being introduced in the wineries, including ageing of wine in barrels to which the lees from the malolactic and alcoholic fermentations has been added. Another production method consists in carrying out the malolactic fermentation in the oak barrels in which the ageing takes place, after which the wine is aged further with its own lees. For ageing with lees, the wine is regularly stirred, with greater or lesser frequency, to facilitate the transfer of compounds from the lees to the wine.

Traditional red wine production technology, in which malolactic fermentation is carried out in tanks and ageing in barrels, has been widely studied. However, less research has been done on malolactic fermentation in barrels, in which the control of the process is minor and, therefore, entails more risks. There are also few studies on the

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changes occurring during ageing in barrel with lees. Most research in this area has focussed on the changes in non-anthocyanin phenolic compounds (Hernández, Estrella, Carlavilla, Martín-Álvarez, & Moreno-Arribas 2006), some nitrogen compounds (Alcaide-Hidalgo, Moreno-Arribas, Martín-Álvarez, & Polo, 2007), and polysaccharides (Escot, Feuillat, Dulau, & Charpentier, 2001), and has been conducted in wines to which yeast walls, or autolyzed yeasts obtained in model solutions, have been added (Guilloux-Benatier & Chassagne, 2003; Salmon, Vuchot, Doco, & Moutounet, 2003). To our knowledge, no studies have focussed on the influence of this production method on changes in anthocyanins during industrial wine manufacture.

Grape anthocyanin pigments are mainly located in the skin, being largely responsible for the colour of red wine. The main anthocyanins identified in *Vitis vinifera* spp. grapes and wines are the 3-*O*-glucosides, 3-*O*-acetyl glucosides and 3-*O*-*p*-coumaroyl glucosides and delphinidin, cyanidin, petunidin, peonidin and malvidin, as well as the 3-*O*-caffeoyl glucosides of malvidin and peonidin (Atanasova, Fulcrand, Cheynier, & Moutounet, 2002; Monagas, Gómez-Cordovés, & Bartolomé, 2005; Monagas, Núñez, Bartolomé, & Gómez-Cordovés, 2003). The anthocyanin profile and concentration of wines is largely dependent on grape variety, but also on the technology applied in their winemaking, and the reactions that take place during maturation and ageing in wood.

Given the importance of phenolic compounds to the final quality, and therefore, to the consumer acceptance of red wines, and because there are not nearly enough data in the literature about the changes that anthocyanins undergo during MLF and ageing of wine on lees, in the present work we report the changes in these compounds during 14 months of ageing in oak barrels as a result of different ways of performing these technological processes.

2. Materials and methods

2.1. Manufacture of the wines

A red wine was industrially elaborated and submitted to different experiments in a winery. A description of the samples is shown in Table 1 and these were manufactured as follows: the wine used was a quality red wine from the AOC (Appellation of Origin Controlled) Navarra (Spain), from *Vitis vinifera* L., c.v Tempranillo grapes, manufactured in 10,000 l stainless-steel tanks. This wine was called the initial wine (Wi). After alcoholic fermentation, part of this wine (approximately 3000 l) underwent malolactic fermentation (MLF) in oak barrels (technological variants A, B and C), whereas in the rest of the wine (equivalent to approx. 7000 l) MLF was carried out in stainless-steel tanks. After the malolactic fermentation in stainless steel tanks had finished, part of the wine was transferred to oak barrels, without removing the lees (variants D, E and F), while the rest was given the following postfermentation treatments before being transferred to the corresponding barrels: racking (variant G), racking and clarification with albumin and bentonite (variant H), and racking, clarification, cold stabilization and filtration (variant I). The barrels with wine of variants B and E, were treated with weekly *batônnage*, while *batônnage* was monthly in barrels C and F. *Batônnage* was not carried out in barrels A and D. Malolactic fermentation was carried out by inoculation of a commercial lactic acid bacteria, *Oenococcus oeni* (ITV 04 A1), provided by Oenofrance (Rueil-Malmaison, France).

Table 1
Technological procedures for the manufacture of wines

	Technological procedures
Wi (initial wine)	
WMLFb (wine that performed MLF in oak barrel)	A (ageing on lees) B (ageing on lees, with weekly "batônnage") C (ageing on lees, with monthly "batônnage")
WMLFs (wine that performed MLF in stainless-steel tank)	D (ageing on lees) E (ageing on lees, with weekly "batônnage") F (ageing on lees, with monthly "batônnage") G (without lees, with racking) H (without lees, with racking and clarification) I (without lees with racking, clarification and cold stabilization)

For storage of the wines during MLF and wine ageing, 225 l new barrels of French oak (*Quercus sessilis*) were used. During ageing, four barrels of each technological batch were considered and the wines from the barrels were mixed and homogenized before analysis. Wine samples were taken before (initial wine, Wi) and after MLF (WMLFs and WMLFb) and during the three months of the experiment until 14 months of ageing in the new oak barrels. In total 48 wine samples were analyzed by HPLC-PAD-MS (Table 1). At each sampling time, wine samples were collected, centrifuged for 15 min at 5000*g* and immediately refrigerated until analysis. Each analytical assay was performed at least in duplicate.

2.2. Anthocyanin analysis

2.2.1. Liquid chromatography (LC)

A Waters (Milford, MA) HPLC equipped with a 600-MS controller, a 717 plus autosampler, and a 996 photodiode-array detector was used. A gradient of solvent A (water/formic acid, 90:10, v/v) and solvent B (water/methanol/formic acid, 45:45:10, v/v/v) was applied to a reverse-phase Waters Nova-pack C_{18} column (150 mm × 3.9 mm, 4 μm) as follows: 15–80% B linear (0.8 ml/min) from 0 to 30 min, 80% B isocratic (0.8 ml/min) from 30 to 43 min, followed by washing (100% methanol) and re-equilibration of the column from 43 to 75 min. Detection was performed

by scanning from 260 to 600 nm. Quantification was carried out by area measurement at 530 nm and expressed as malvidin-3-glucoside (Extrasynthese, Genay, France) by a standard calibration curve; coefficients of variation were 2.0%. Quantification of delphinidin-3-(6″-*p*-coumaroyl-glucoside) and malvidin-3-glucoside pyruvate was carried out jointly by more exact valuation. One hundred microlitres of wine, previously filtered through a 0.45 μm membrane were injected onto the column. Injections were carried out in duplicate.

2.2.2. Liquid chromatography/electrospray mass spectrometry (LC-/ESI-MS)

A Hewlett-Packard series 1100 (Palo Alto, CA) chromatography system equipped with a diode array detector (DAD) and a quadrupole mass spectometer (Hewlett-Packard series 1100 MSD) with a electrospray interface was used. Chromatographic and separation conditions were the same as those reported above. Nitrogen was used as the nebulizing and drying gas. ESI conditions were as follows: drying gas (N_2) flow and temperature, 10 l/min and 350 °C; nebulizer pressure, 380 Pa (55 psi), and capillary voltage, 4000 V. The ESI was operated in positive mode, scanning from *m*/*z* 100 to *m*/*z* 1500, using the following fragmentator voltage gradient: 100 V from 0 to 17 min, and 120 V from 17 to 55 min. Analyses were conducted in duplicate.

2.3. Statistical analysis

The statistical methods used for the data analysis were: cluster analysis (Ward's method from standardized variables) to discover natural groupings of the wine samples, two-way analysis of variance (ANOVA) to test the effect of two factors studied (technological factor and ageing time) and least significant differences (LSD) test for the comparisons of means. STATISTICA programme for Windows, version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com). This programme was run on a personal computer.

3. Results and discussion

3.1. Identification of anthocyanins and anthocyanin-derived pigments

As a representative example, the anthocyanidin LC chromatograms corresponding to the wine that performed MLF in stainless steel are illustrated in Fig. 1. The anthocyanins can be classified into three groups according to their acylation: anthocyanidin-3-glucosides, 3-(6″-acetyl-glucosides), 3-(6″-*p*-coumaroylglucosides) and 3-(6″-*p*-caffeoylglucosides). A fourth group would be formed by several more complex anthocyanin-derived pigments (Table 2).

The 3-glucosides (peaks 1, 2, 3, 4 and 5), the 3-(6″-acetylglucosides) (peaks 6, 9, 11, 12 and 14), the 3-(6″-*p*-coumaroylglucosides) (peaks 13, 17, 18 and 19) and the 3-(6″-*p*-caffeoylglucosides) (peaks 15 and 16) were identified in all

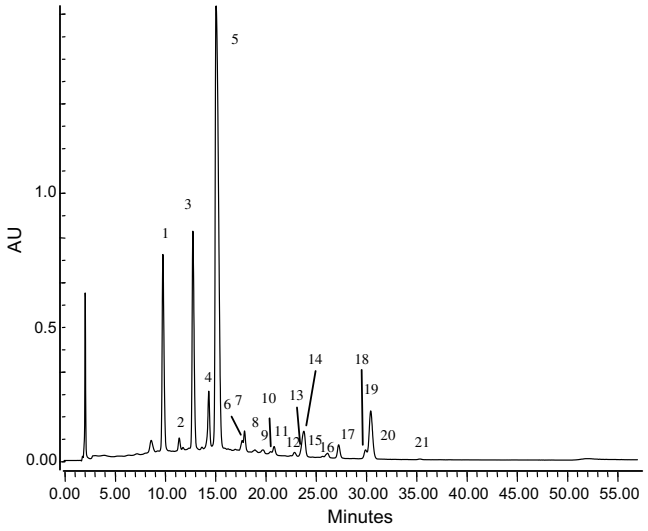

Fig. 1. HPLC chromatographic profile of the anthocyanins determined in the initial wine (before MLF). The retention times of compounds not detected in this wine are also indicated in the chromatogram. For peak identification, see Table 2.

Table 2
Anthocyanin compounds identified by LC/ESI-MS in the analyzed wines

Peak No.	Compounds[a]	Tr (min)	m/z (M+)
Simple glucosides			
1	Delphinidin-3-glucoside	9.8	465
2	Cyanidin-3-glucoside	11.2	449
3	Petunidin-3-glucoside	12.5	479
4	Peonidin-3-glucoside	14.0	463
5	Malvidin-3-glucoside	14.8	493
Acetyl glucosides			
6	Delphinidin-3-(6″-acetylglucoside)	17.2	507
7	Malvidin-3-glucoside pyruvate (Vitisin A)	17.5	561
9	Cyanidin-3-(6″-acetylglucoside)	19.2	491
11	Petunidin-3-(6″-acetylglucoside)	20.2	521
12	Peonidin-3-(6″-acetylglucoside)	22.2	505
14	Malvidin-3-(6″-acetylglucoside)	23.0	535
Cinnamoyl glucosides			
13	Delphinidin-3-(6″-*p*-coumaroylglucoside)	22.8	611
15	Peonidin-(6″-caffeoylglucoside)	24.8	625
16	Malvidin-3-(6″-caffeoylglucoside)	25.3	655
17	Petunidin-3-(6″-*p*-coumaroylglucoside)	26.3	625
18	Peonidin-3-(6″-*p*-coumaroylglucoside)	30.1	609
19	Malvidin-3-(6″-*p*-coumaroylglucoside)	30.5	639
Pyroanthocyanins			
8	Malvidin-3-(6″-acetylglucoside) pyruvate	18.5	603
10	Malvidin-3-glucoside-ethyl-catechin	19.8	809
20	Malvidin-3-glucoside-4-vinyl-epicatechin	31.8	805
21	Malvidin-3-(6″-acetylglucoside)-4-vinylphenol	35.2	651

[a] The numbers of compounds correspond to those of the peak chromatogram (Fig. 1).

Table 3
Concentrations of anthocyanins (mg/l) in the wines before and after MLF

	Before MLF (Wi)	After MLF	
		WMLFb	WMLFs
Simple glucosides			
Delphinidin-3-glucoside	59.8	58.5	81.6
Cyanidin-3-glucoside	2.82	2.87	4.51
Petunidin-3-glucoside	64.7	64.2	92.3
Peonidin-3-glucoside	15.8	15.5	22.7
Malvidin-3-glucoside	209	209	308
Acetyl glucosides			
Delphinidin-3-(6″-acetylglucoside) + Malvidin-3-glucoside pyruvate	4.74	5.12	9.14
Cyanidin-3-(6″-acetylglucoside)	1.88	1.63	2.13
Petunidin-3-(6″-acetylglucoside)	2.28	1.75	2.75
Peonidin-3-(6″-acetylglucoside)	1.70	1.46	2.49
Malvidin-3-(6″-acetylglucoside)	7.07	6.84	10.2
Cinnamoyl glucosides			
Delphinidin-3-(6″-*p*-coumaroylglucoside)	6.48	6.27	9.30
Peonidin-(6″-caffeoylglucoside)	0.27	0.25	0.33
Malvidin-3-(6″-caffeoylglucoside)	1.81	1.67	2.52
Petunidin-3-(6″-*p*-coumaroylglucoside)	5.42	5.29	7.82
Peonidin-3-(6″-*p*-coumaroylglucoside)	1.62	1.07	1.78
Malvidin-3-(6″-*p*-coumaroylglucoside)	21.5	15.7	29.6
Pyroanthocyanins			
Malvidin-3-(6″-acetylglucoside) pyruvate	1.20	1.19	1.69
Malvidin-3-glucoside-ethyl-catechin	0.45	0.34	0.48
Malvidin-3-glucoside-4-vinyl-epicatechin	0.11	0.11	0.14
Malvidin-3-(6″-acetylglucoside)-4-vinylphenol	0.51	0.52	0.60

Wi: Initial wine; WMLFb: wine that performed MLF in oak barrel; WMLFs: wine that performed MLF in stainless-steel tank.

the wines analyzed. Cyanidin-3-(6″-*p*-coumaroylglucoside) was absent although other authors have reported its presence in grape skins and wines from the Tempranillo variety (Revilla, Pérez-Magariño, González-SanJosé, & Beltrand, 1999; Monagas et al., 2003). The presence of peonidin-3-(6″-caffeoyl-glucoside) (15), a compound reported in few studies (Mazza, Fukumoto, Delaquis, Girard, & Ewert, 1999; Monagas et al., 2003), was also found in the wines.

Several different series of anthocyanin-derived pigments were also detected by LC/ESI-MS. A first family of pigments (peaks 7 and 8) eluted soon after malvidin-3-glucoside and had a polarity similar to that of the anthocyanidin-3-glucosides and the anthocyanidin-3-acetyl-glucosides (Table 2). Peak 7 corresponded to the product resulting from the C-4/C-5 cycloaddition of pyruvic acid and malvidin-3-glucoside (malvidin-3- glucoside pyruvate or vitisin A) (Bakker & Timberlake, 1997; Fulcrand, Benabdeljail, Rigaud, Cheynier, & Moutounet, 1998). Adducts of malvidin-3-(6″-acetylglucoside) (8) with pyruvic acid were also detected in the wines.

Peak 10 was assigned to a dimer of malvidin-3-glucoside and catechin linked by an ethyl bridge (acetadehyde-mediated condensation), first reported in model solutions (Bakker, Picinelli, & Bridle, 1993) and then detected in wines (Atanasova et al., 2002; Mateus, Silva, Vercauteren, & De Freitas, 2002; Monagas et al., 2003; Revilla et al., 1999; Vivar-Quintana, Santos-Buelga, Francia-Aricha, & Rivas-Gonzalo, 2002). It was the only representative compound of the family of the oligomeric condensed anthocyanin-derived pigments detected in the wines analyzed.

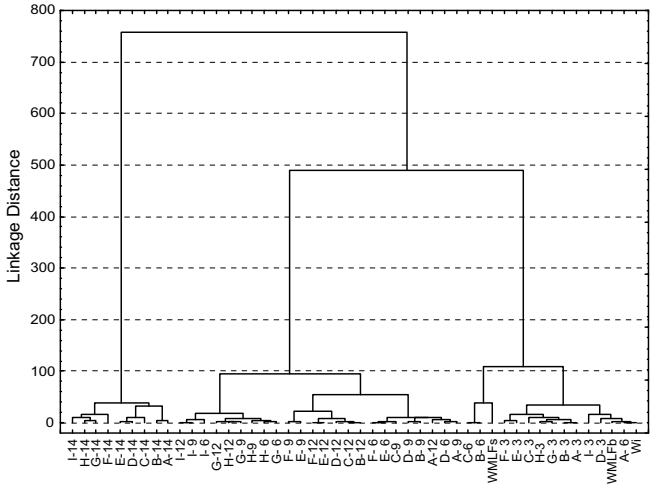

Fig. 2. Dendrogram of the wines from their concentration of anthocyanins (mg/l). The wine samples are labelled according to the levels of the technological procedure (A–I) and ageing time (3–14 months) factors.

Additional anthocyanin-derived pigments exhibiting low polarity (peaks 20 and 21) were identified in the studied wines. Peak 20, presenting an $[M]^+$ of *m/z* 805, was

Table 4
Factors effect, mean, standard deviation (SD) and range of concentration of anthocyanins (mg/l) of wines, and levels of the technological procedure (A–I) and ageing time (3–14 months) factors, with the higher and lower values obtained from LSD test

	Factors effect		Mean	SD	Range		Technol. procedure		Ageing time (months)	
	Technol. Procedure	Ageing Time			Min. value	Max. value	Higher values	Lower values	Higher values	Lower values
Simple glucosides										
Delphinidin-3-glucoside	*	*	60.0	11.8	36.2	86.3	A, B, C	G, I	3, 14	12
Cyanidin-3-glucoside	*	*	2.88	0.31	2.33	3.42	A, B, C	I	3	6, 9, 12, 14
Petunidin-3-glucoside	*	*	56.6	11.8	35.4	85.4	A, B, C	G, I	3	12
Peonidin-3-glucoside	*	*	12.6	2.98	7.65	18.6	A, B, C	G, I	3	12
Malvidin-3-glucoside	*	*	169	33.6	112	232	A, B, C	G, I	3	12
Acetyl glucosides										
Delphinidin-3-(6″-acetylglucoside) + Malvidin-3-glucoside pyruvate	*	*	5.25	0.84	3.84	7.50	B, C	F, I	14	12
Cyanidin-3-(6″-acetylglucoside)	*	*	1.54	0.49	0.74	2.78	A, B	H, I	3, 14	12
Petunidin-3-(6″-acetylglucoside)	*	*	2.75	1.84	1.11	7.66	A, B	G, I	14	6, 12
Peonidin-3-(6″- acetylglucoside)	n.s.	*	1.85	2.04	0.42	7.02	A, B, C	I	14	9, 12
Malvidin-3-(6″- acetylglucoside)	*	*	4.80	2.69	0.34	9.63	A, B, C	I	3, 6	14
Cinnamoyl glucosides										
Delphinidin-3-(6″-*p*-coumaroylglucoside)	*	*	4.58	2.19	0.74	8.83	A, B, C	G,H,I	3, 6	14
Peonidin-(6″-caffeoylglucoside)	n.s.	*	1.08	1.78	0.12	5.55			14	3, 6, 9, 12
Malvidin-3-(6″-caffeoylglucoside)	*	*	1.62	0.68	0.65	3.38	A, B, C, E	G, I	3, 14	9, 12
Petunidin-3-(6″-*p*-coumaroylglucoside)	n.s.	*	6.64	4.75	2.32	19.6			14	3, 6, 9, 12
Peonidin-3-(6″-*p*-coumaroylglucoside)	n.s.	*	1.38	0.56	0.37	2.95			3	12
Malvidin-3-(6″-*p*-coumaroylglucoside)	*	*	12.5	7.52	0.35	25.0	A, B, C	G, H, I	3, 6	14
Pyroanthocyanins										
Malvidin-3-(6″-acetylglucoside) pyruvate	n.s.	*	1.07	0.28	0.56	1.88			14	12
Malvidin-3-glucoside-ethyl-catechin	n.s.	*	0.62	0.47	0.17	1.88			14	3, 6, 9, 12
Malvidin-3-glucoside-4-vinyl-epicatechin	n.s.	*	1.46	1.30	0.11	3.97			14	3, 6
Malvidin-3-(6″-acetylglucoside)-4-vinylphenol	*	*	0.60	0.22	0.11	1.04	A, B, C	G, H, I	12	3, 14

* Significant effect of the factor ($P < 0.05$); n.s.: non significant effect of the factor ($P > 0.05$); LSD: least significant difference.

identified as a vinyl-epicatechin-derived pigment of malvidin-3-glucoside. Peak 21, with a molecular mass (*m/z* 651) of malvidin-3-(6″-acetylglucoside), corresponds to the 4-vinylphenol adduct of malvidin-3-(6″-acetylglucoside). The presence of pigments resulting from the anthocyanin-vinylphenol condensation reaction has also been reported during the ageing with yeast of sparkling wines (Pozo-Bayón, Monagas, Polo, & Gómez-Cordovés, 2004).

3.2. Evolution of anthocyanins during MLF in different containers

Table 3 shows the concentrations (mg/l) of the different anthocyanins identified in the wines before (initial wine, Wi) and after MLF (WMLFs and WMLFb). As observed in other red wines from the Tempranillo cultivar (Monagas et al., 2005; Revilla, López, & Ryan, 2005), simple glucosides were the most abundant groups of the anthocyanins in the different wines studied. The cinnamoyl glucosides (including both *p*-coumaryl and caffeoyl anthocyanins) and the acetylated glucosides, represented the second and third group, respectively, while the anthocyanin-derived pigments were found in low concentrations.

As can be noted, the wine that performed MLF in stainless steel had higher concentrations of most anthocyanins than had the wine that performed this process in barrel. These results are especially evident for all the simple glucosides studied, the acetyl glucosides, delphinidin-3-(6″-acetylglucoside) plus malvidin-3-glucoside pyruvate, and the cinnamoyl glucosides, particularly petunidin-3-(6″-acetylglucoside), delphinidin-3-(6″-*p*-coumaroylglucoside) and malvidin-3-(6″-*p*-coumaroylglucoside). In contrast, no differences were observed in the complex anthocyanidin-derived compounds of the wines during MLF. On the other hand, the oak barrel wine presented very similar concentrations of most of the analyzed anthocyanins to those obtained in the initial wine (Table 3). These data suggest that the effect of the container used for MLF seems to be more important than differences due to lactic acid bacteria metabolic activity during this process.

Some cinnamoyl glucosides, especially malvidin-3-(6″-*p*-coumaroylglucoside) and peonidin-3-(6″-*p*-coumaroylglucoside), were found at higher concentrations in the wine before (Wi) than after MLF in barrel (MLFb). The concentrations of anthocyanins likely decrease because of their hydrolysis by lactic acid bacteria. These observations

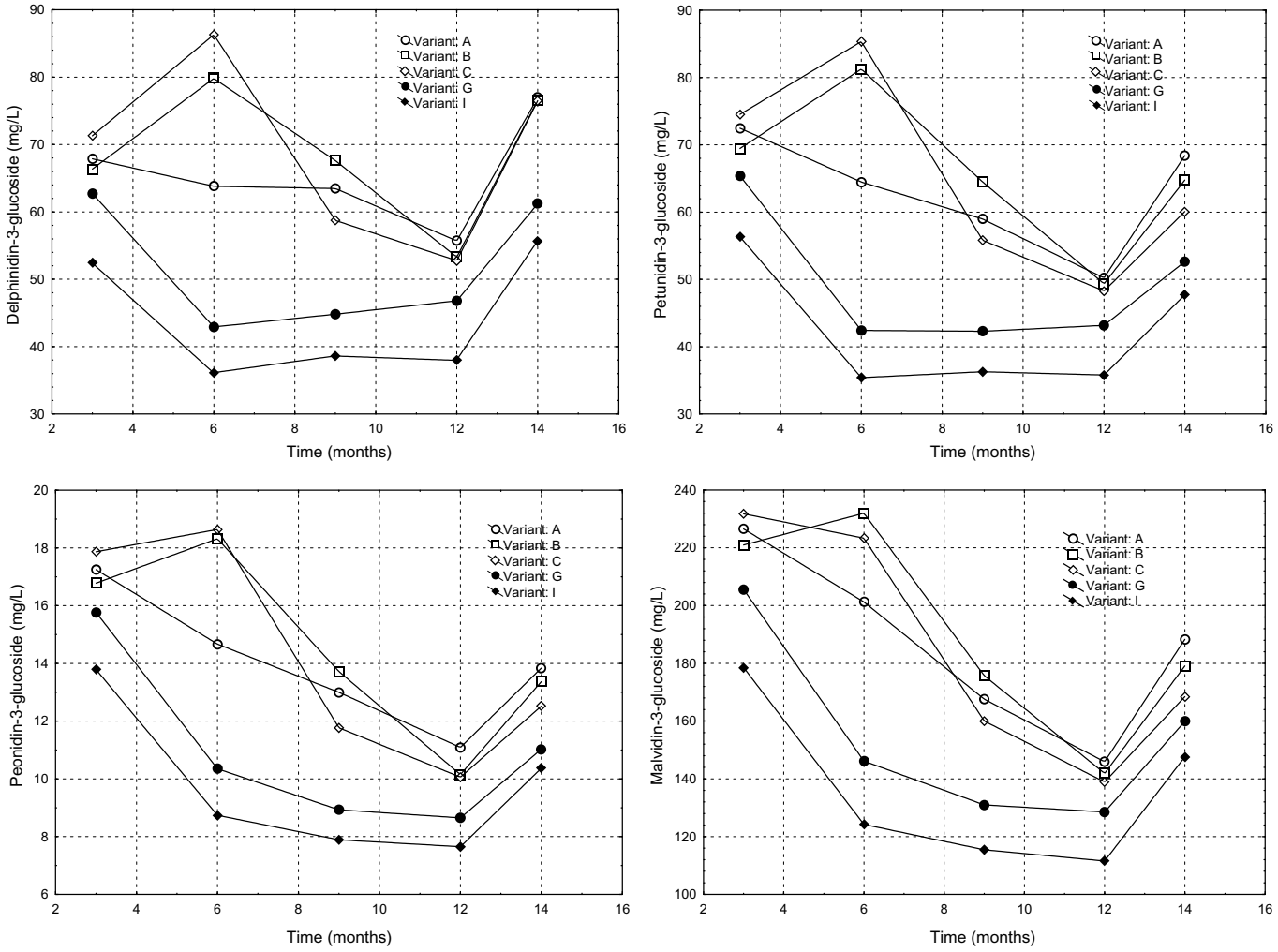

Fig. 3. Evolution of simple glucosides significantly affected by the technological variants.

support, at least partially, previous results that indicated that the hydrolysis of these cinnamoyl anthocyanins by lactic acid bacteria enzymes during MLF could be a source of free *p*-coumaric and caffeic acid in the present wines (Hernández et al., 2006).

3.3. Evolution of anthocyanins during the ageing of wines

In an attempt to obtain a preliminary view of the main causes for the variation in phenolic compounds, cluster analysis was carried out on the quantified anthocyanin data of all the wines studied. Fig. 2 shows the dendrogram obtained. The squared Euclidean distance was taken as a measure of proximity between two samples, and Ward's method was used as the linkage rule. The variables were previously standardized. As can be observed in this figure, there are two large groups of wines, one comprised of all the variants studied at 14 months of ageing, and a second larger group, comprised of wines of all the variants up to 12 months ageing. Two subgroups can also be identified in this second group. One is formed by the initial wine, the wines after MLF, and the wines of 3 and 6 months ageing in barrel with weekly and monthly "batônnage", and the second includes the rest of the wines at 6 months, together with the wines at 9 and 12 months, revealing the influence of ageing time in barrel and on lees on anthyocyanin composition of both MLF type wines. Fig. 2 also shows how, within each subgroup, samples of the variants G, H and I (especially at 6, 9, 12 and at 14 months) form a group in which the I variants appear to be separate from the rest, showing the influence of racking and overall clarification and cold treatments.

To test the effects of the technological variant and ageing time factors on the anthocyanin composition of the wines, two-way ANOVA was carried out to analyse the first order effects (the interaction and the within-error terms were pooled). The results obtained are shown in Table 4, together with the mean, standard deviation, minimum and maximum values of the anthocyanin concentrations in these wines. During ageing of the wines, the simple glucosides and malvidins were also the predominant anthocyanins, as is shown for most *Vitis vinifera* wines. It can be observed how there are indeed significant differences in the concentrations of all anthocyanin compounds identified in

relation to ageing time and also significant differences in most of the anthocyanin compounds analyzed in relation to the mode of manufacture (3-glucosides and 3-6″-acetylglucosides) from all anthocyanidins and, delphinidin-3-(6″-acetylglucoside) + malvidin-3-glucosidepyruvate, delphinidin-3-(6″- *p*-coumaroylglucoside), malvidin-3-(6″-caffeoylglucoside), malvidin-3-(6″-*p*-coumaroylglucoside) and malvidin-3-(6″-acetylglucoside)-vinylphenol). Table 4 summarises the main significant differences in concentrations of anthocyanins according to the levels of the technological procedure (A–I variants) and ageing time (3 to 14 months) factors; the higher and lower values obtained from the LSD test. To compare the means, are included in the table. As an example, Figs. 3–5 show the long-term evolution over 14 months of ageing for 11 of these compounds, affected by the technological variants (delphinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, cyanidin-3-(6″-acetylglucoside), petunidin-3-(6″-acetylglucoside),malvidin-3-(6″-acetylglucoside), Delphinidin-3-(6″-*p*-coumaroylglucoside), malvidin-3-(6″-caffeoylglucoside), malvidin-3-(6″-*p*-coumaroylglucoside) and malvidin-3-(6″-acetyl glucoside)-vinylphenol.

All the different anthocyanin-3-glucosides identified in the wines were influenced by both the time of ageing and the technological procedures investigated (Table 4). Their concentrations progressively decreased during the first 12 months of ageing, and later an important increase was observed at 14 months. As an example, Fig. 3 shows the evolution of delphinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside. The wines of variants A, B and C, those which performed MLF in barrel and were aged in the presence of lees, had the highest values of these compounds, while the wines of variants G and especially I, those submitted to racking, clarification, filtration and cold stabilization, presented the lowest values with evolutions different from the A–C variants during the 12 first months (Table 4 and Fig. 3). This suggests that the concentrations of these compounds also seem to be influenced by the post-fermentation technological treatment, especially the racking and the cold stabilization, as indicated previously.

Acetyl glucosides, except peonidin-3-(6″-acetyl glucoside), are affected by both the technological variant and the ageing time factors (Table 4). Fig. 4 shows the evolution of cyanidin-3-(6′-acetylglucoside), petunidin-3-(6′-acetylglucoside) and malvidin-3-(6″-acetylglucoside). All of them were maintained more (petunidin derivative) or less stable or slightly decreased during the first 12-months of the ageing period, but later, at 14 months, the contents of cyanidin and petunidin-3-(6′-acetylglucosides) (Fig. 4) increased while malvidin-3-(6″-acetylglucoside) significantly decreased. With respect to the influence of the technological procedure, the results also show that the concentrations of these compounds also seem to be significantly affected by the treatments used in the manufacture of the wines, since the I-wines presented the lowest values of these anthocyanins.

As an example of the changes in the cynnamoyl (coumaroyl and caffeoyl) anthocyanins, Fig. 5 shows the evolution of delphinidin-3-(6″-*p*-coumaroylglucoside), malvidin-3-(6″-caffeoylglucoside) and malvidin-3-(6″-*p*-coumaroylglucoside). As reflected in these figures, the evolutions of these compounds were different depending on their structure, according to acylation with *p*-coumaric or

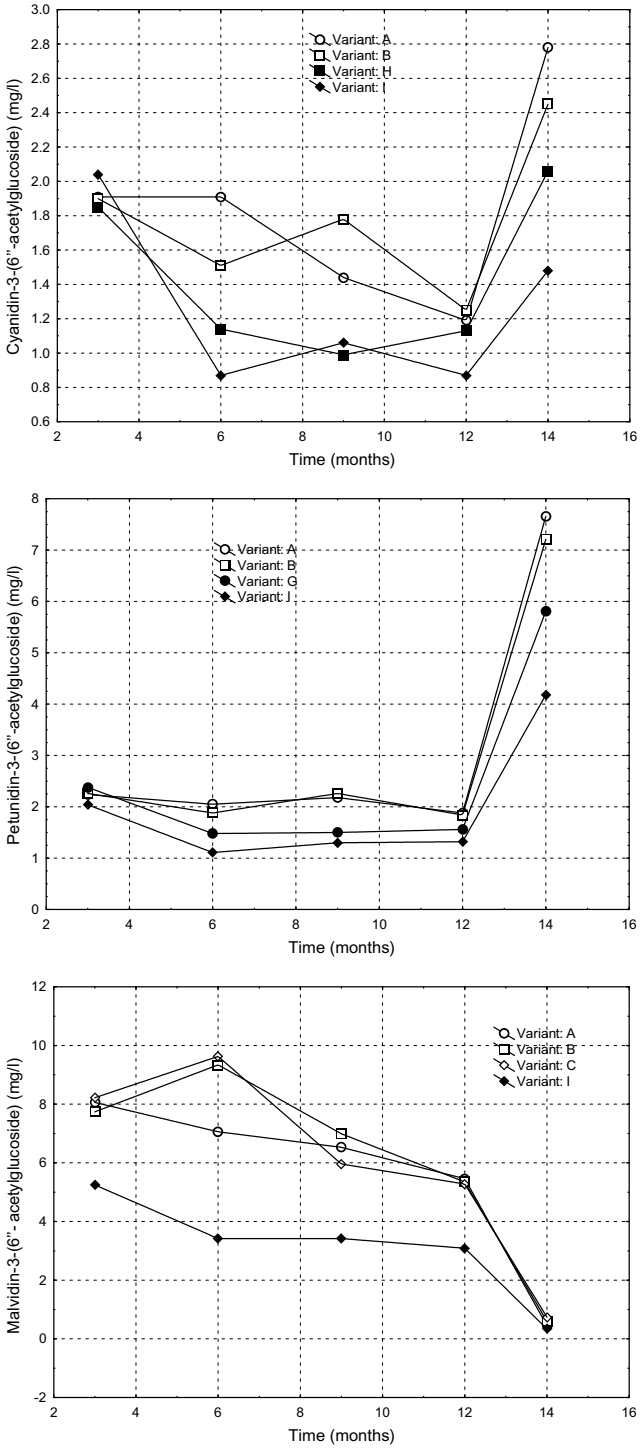

Fig. 4. Evolution of acetyl glucosides significantly affected by the technological variants.

acetic acids. In the *p*-coumaroyl-glucosides (of delphinidin and malvidin) an important decrease was produced between 12 and 14 months of ageing (as an example see Fig. 5), while, in the caffeoyl-glucosides, an opposite trend was observed in the same period of ageing. This trend is similar to the rest of the acylated anthocyanins. However, in both groups of anthocyanins, the influence of the technological variable was the same (Table 4). The wines of variants A, B and C, i.e. MLF performed in barrel and aged in the presence of lees had the highest values of delphinidin-3-(6″-*p*-coumaroylglucoside), malvidin-3-(6″-caffeoylglucoside) and malvidin-3-(6″-*p*-coumaroylglucoside), while wines of the variants G, H and I, submitted to racking, clarification, filtration and cold stabilization, presented the lowest values of these compounds.

During ageing of the wines, most of the pyranoanthocyanins, except malvidin-3-(6″-acetylglucoside)-vinylphenol, were only significantly influenced by the time of ageing but not by the technological procedure (Table 4). The evolution of malvidin-3-(6″-acetylglucoside)-vinylphenol is shown in Fig. 5, where a tendency was observed to increase up to 12 months, which was most pronounced in variants G, H and I, and to decrease at 14 months of ageing. According to the results, obtained, it should be pointed out that a prolonged time of ageing on lees (14 months for the studied wines) did not result in the generation of new pigments. Anthocyanin-pyruvic adducts remained stable or experienced a slight decrease between 3 and 12 months, followed by an increase at 14 months. By contrast, both malvidin3-(6″-acetylglucoside)-vinyl-(epi)catechin and malvidin-3-(6″-acetylglucoside)-vinylphenol showed similar evolutions but different from the latter pyranoanthocyanins, since an increase was detected between 3 and 12 months, followed by a decrease at 14 months of ageing in barrel. The vinylphenol derivative was influenced by the technological variant, in particular the presence or absence of lees during ageing. In fact, the A–F wines (i.e. aged on lees) presented higher values of the content of this compound than the G–I wines (i.e. aged without lees) (Table 4). The formation of malvidin 3-glucoside-4-vinylphenol, as postulated by Schwarz, Tobias, Wabnitz, and Winterhalter (2003), requires only the presence of malvidin and

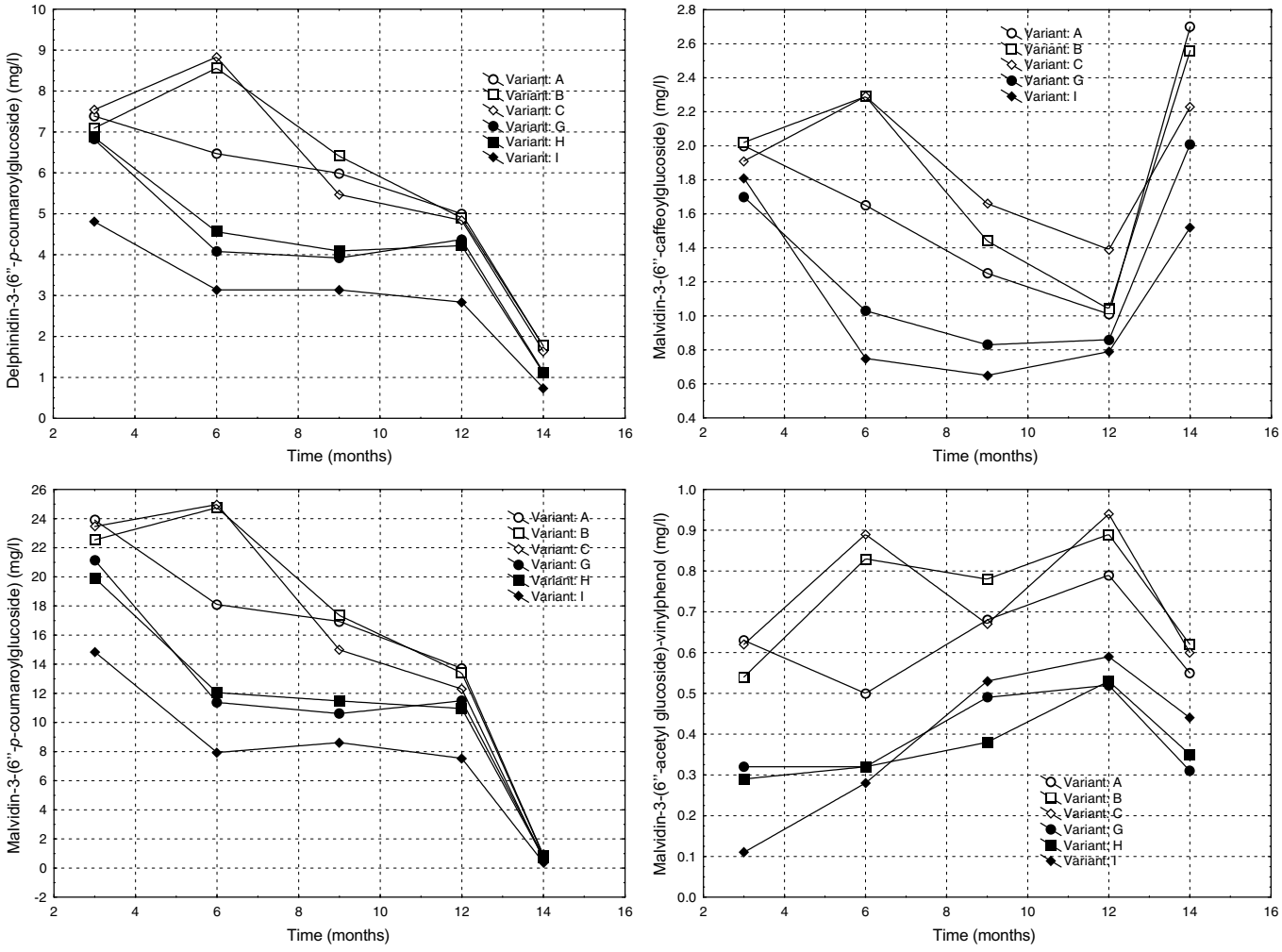

Fig. 5. Evolution of delphinidin-3-(6″-*p*-coumaroylglucoside), malvidin-3-(6″-caffeoylglucoside), malvidin-3-(6″-*p*-coumaroylglucoside) and malvidin-3-(6″-acetylglucoside)-4-vinylphenol significantly affected by the technological variants.

p-coumaric acid, the latter known to be present in the studied wines (Hernández et al., 2006). However, according to the results obtained in the present work, the formation of anthocyanin-vinylphenol adducts also seems to be favoured by yeast (as previously suggested, Pozo-Bayón et al., 2004) and lactic acid bacteria from lees. Moreover, the rate of formation of anthocyanin–vinylphenol adducts does not depend on the presence of oxygen, since no differences were encountered between wines due to *batonnâge* (Table 4), in agreement with previous results (Schwarz et al., 2003).

4. Conclusions

In relation to MLF, it has been demonstrated that most anthocyanin compounds identified in the wines studied (with the exception of pyroanthocyanins) were affected differently, depending on the type of container (oak barrel or stainless-steel tank) used for the process. Different evolution patterns were found between the different types of anthocyanins during wine ageing in barrels. The concentrations of most of the compounds identified, except for cyanidin-3-glucoside, malvidin-3-(6″-acetylglucoside), delphinidin-3-(6″-*p*-coumaroylglucoside) and malvidin-3-(6″-*p*-coumaroylglucoside), diminished during the first 12 months ageing in barrel, possibly due to typical oxidation and condensation reactions occurring in barrels. In wines aged in the presence of lees (variants A–F), another possible reason for these compounds being reduced during this period, could correspond to their adsorption to yeast walls, previously described during alcoholic fermentation during red wine manufacture (Morata et al., 2003), during the biological ageing of some Sherry-wines (Barón, Mayén, Mérida, & Medina, 1997; Cortés, Moreno, Zea, Moyano, & Medina, 1998; Fabios, López-Toledano, Mayen, Mérida, & Medina, 2000), and during the ageing of sparking wines on lees (Pozo-Bayón et al., 2004). Later on, the increase occurring at 14 months of ageing in barrels is noteworthy, especially of acetyl glucosides, cinnamoyl glucosides and pyranoanthocyanins that can be related to depolymerisation processes following condensation and copigmentation reactions of phenolic compounds that take place during oxidative ageing of wine in barrels, in which the low molecular weight and monomeric free forms of anthocyanins are involved (Boulton, 2001; Fernández de Simón, Hernández, Cadahía, Dueñas, & Estrella, 2003). Finally, from the statistical treatments applied, it can be concluded that the anthocyanin content of wines largely depends on the technological procedures used in the wineries, since wines with different phenolic characteristics were obtained over the 14 months of ageing, in relation to storage, or not, with lees, and the post-fermentation procedures studied here.

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