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Changes in the detailed pigment composition of red wine during maturity and ageing A comprehensive study

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Abstract

The qualitative and quantitative changes produced, during maturation and ageing, in the pigment composition of a red wine made from *Vitis vinifera* cv Tempranillo grapes have been studied. In order to determine the detailed composition of the main pigment families involved in the colour changes, a fractionation of the samples has been carried out. One-hundred and twenty-nine different compounds have been identified and their evolutions with wine age have also been established. The data obtained in the analyses of the fractions by high performance liquid chromatography–diode array detection coupled to mass spectrometry (HPLC–DAD–MS) have been used in pigment identification. In order to confirm the identity of some of these compounds, their syntheses have also been carried out. As far as we know, compounds originated by acylation of the monoglucosides of the anthocyanins with lactic acid as well as 3,7-diglucosides of anthocyanins have, among others, been reported here for the first time. The moments of appearance and disappearance of all the detected compounds have also been established as well as the changes in the levels of the different pigment families and subfamilies originated as a consequence of maturation and ageing of the wine in barrels or in bottles. As wine became older, the percentages of anthocyanins decreased slightly, whereas that of the anthocyanin-derived pigments increased and, above all, compounds providing the wine with orange hues (pyranoanthocyanins). In the last sample, they represented 70% of the anthocyanin-derived pigments. On the contrary, the percentage of compounds providing bluish hues to the wine, direct and acetaldehyde-mediated flavanol–anthocyanin condensation products, decreased. This change in the nature of the anthocyanin-derived pigment would explain the colour change observed in the samples, from purple towards orange hues.
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Keywords: Wine colour; Anthocyanins; Anthocyanin-derived pigments; Pyranoanthocyanins; Anthocyanin–flavanol condensation products; Ageing

1. Introduction

Anthocyanins are phenolic compounds which belong to the flavonoid family, responsible for the pigmentation of different parts of flowering plants (flowers, fruits, leaves, stems and roots and storage organs). They are the main compounds involved in the colour of black grapes and, during winemaking, they are extracted to the must. They are also responsible for the colour of young wine. In *Vitis vinifera* grapes, not only the monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin are present, but also their acetyl-, *p*-coumaroyl and caffeoyl derivatives.

Once extracted to the must and during maturation and ageing of the wine, their levels decrease with time, since they begin to react with other wine constituents. Anthocyanins can be involved in different kinds of reactions. First, anthocyanins can condense with flavanols, either directly [1–3] or by mediation of acetaldehyde [4–6] or other compounds [7] as demonstrated in model solutions and in wines. These reactions cause bathochromical shifts in the visible absorption maxima of the anthocyanins, the new pigments providing a bluish-red hue to the wine [3–5]. Other reactions causing anthocyanin transformation are those originating a new pyran ring in the anthocyanin structure by addition of different compounds, also present in wine, to the carbon in position 4 and the hydroxyl group in position 5 of the anthocyanin. Among these compounds, pyruvic acid [8,9], acetaldehyde [10,11], vinylphenol [12], vinylcatechol [13], vinylguaiacol [14], monomeric and dimeric procyanidins [4,5] and acetone

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[11,15] have been demonstrated to react with anthocyanin originating pyranoanthocyanins, which have been detected in red wines. The cycloaddition reaction causes a hypsochromical shift in the visible absorption maxima of the anthocyanins, thus producing a change in the wine colour towards orange hues [4,5,8–13,15]. More recently it has been shown that some of these pyranoanthocyanins can in turn react with other wine constituents, such as vinyl-flavanols originated in the cleavage of ethyl-linked flavanol oligomers and anthocyanin–ethyl-flavanol oligomers. The resulting compounds, named portisins [16], have their visible absorption maxima circa 575 nm and consequently, provide blue colour to the wine.

The formation of all these anthocyanin-derived pigments during ageing seems to cause the changes observed in the wine colour from the initial purple-red hue of young red wines to a brick-red hue, characteristic of aged wines. For a better understanding of these changes, therefore, not only the evolution of the levels of the anthocyanins has to be studied, but also those of the anthocyanin-derived pigments in order to establish which is the predominant pigment family at each moment of wine ageing. Some studies carried out in model solutions have determined the conditions that favour the formation of some of these derived pigments [9], others have established their stability regarding storage [17] whereas there are scarce studies of the evolutions of anthocyanin-derived pigments during winemaking, maturation and ageing [18].

The aim of the present study was to establish the changes undergone by the main pigment families (anthocyanins and anthocyanin-derived pigments) during maturation and ageing of a red wine made from *V. vinifera* cv Tempranillo grapes, as well as determine their moments of appearance and disappearance and the influence in their levels of wine maturation and ageing in oak barrels or in the bottle. Not only the main members of each pigment family have to be detected and quantified, since all the members contribute to the wine colour. As wine is a complex medium, the wine was fractionated in order to better detect and quantify most of the compounds responsible for wine colour.

2. Experimental

2.1. Samples

V. vinifera cv Tempranillo fresh grapes from the Spanish D.O. Rioja, were processed in the 2002 vintage by Bodegas RODA (Haro, La Rioja, Spain). The maceration and fermentation steps were carried out in wooden vats. Malolactic fermentation (MLF) took place after alcoholic fermentation and finished 1.5 months after harvest. Wine was transferred into new French oak barrels (medium toast) when it was 2.5 months old, where it was maintained for approximately 14 months. Then, the wine was bottled. Wine samples were collected at different stages of its maturation and ageing. Considering the day of harvest as the starting point of the winemaking process, samples were obtained at 4, 8, 13, 16 and 23 months. Thus, the first three samples corresponded to the maturation and first stages of the ageing in the barrel, the 16-month sample to the first days in the bottle and the last sample to the ageing in the bottle.

One milliliter of the samples was diluted to a final volume of 5 mL with acidified water (HCl, pH 0.5) and filtered through a 0.45 μm Millex® syringe driven Filter unit (Millipore Corporation, Bedford, MA, USA) prior to the HPLC–DAD–MS analysis.

2.2. Sample fractionation

Fifteen milliliters of each wine sample was fractionated with the method described by Alcalde-Eon et al. [19]. Eighty percent ethanol and 80% methanol have been used as elution solvents instead of 95% ethanol and 100% methanol, respectively, in order to avoid the slight shrinkage observed in the stationary phase when using the latter solvents. The obtained fractions underwent the same treatment as that reported by Alcalde-Eon et al. [19]. The freeze-dried fractions were weighed. From them, a solution of 10 mg of powder in 1 mL of acidic water (HCl, pH 0.5) was prepared, filtered through a 0.45 μm Millex® syringe driven Filter unit (Millipore Corporation) and then, analysed by HPLC–DAD–MS techniques.

2.3. HPLC–DAD–MS analysis

HPLC–DAD analysis was performed in a Hewlett-Packard 1100 series liquid chromatograph. An AQUA C18 reverse phase, 5 μm, 150 mm × 4.6 mm column (Phenomenex®, Torrance, CA, USA) thermostatted at 35 °C, was used.

The HPLC–DAD conditions had been previously employed with satisfactory results in our laboratory in the analysis of wine samples [19]. The solvents used were: (A) an aqueous solution (0.1%) of trifluoroacetic acid (TFA) and (B) 100% HPLC-grade acetonitrile, establishing the following gradient: isocratic 10% B for 5 min, from 10 to 15% B for 15 min, isocratic 15% B for 5 min, from 15 to 18% B for 5 min and from 18 to 35% B for 20 min, at a flow rate of 0.5 mL min^{-1}. Detection was carried out at 520 nm as the preferred wavelength. Spectra were recorded from 220 to 600 nm.

The mass analyses were performed using a Finnigan™ LCQ ion trap instrument (Thermoquest, San Jose, CA, USA) equipped with an electrospray ionisation (ESI) interface. The LC system was connected to the probe of the mass spectrometer via the UV cell outlet. Both the sheath gas and the auxiliary gas were a mixture of nitrogen and helium. The sheath gas flow was 1.2 L min^{-1} and the auxiliary gas flow, 6 L min^{-1}. The capillary voltage was 4 V and the capillary temperature 195 °C. Spectra were recorded in positive ion mode between 120 and 1500 *m/z*. The mass spectrometer was programmed to do a series of three consecutive scans: a full mass, an MS^2 scan of the most abundant ion in the full mass and an MS^3 of the most abundant ion in the MS^2. The normalised energy of collision was 45%.

2.4. Materials

Malvidin-3-glucoside was isolated from black grape skins in our laboratory using semi-preparative HPLC as described by Heredia et al. [20].

All solvents were HPLC quality and DL-lactic acid solution analytical grade.

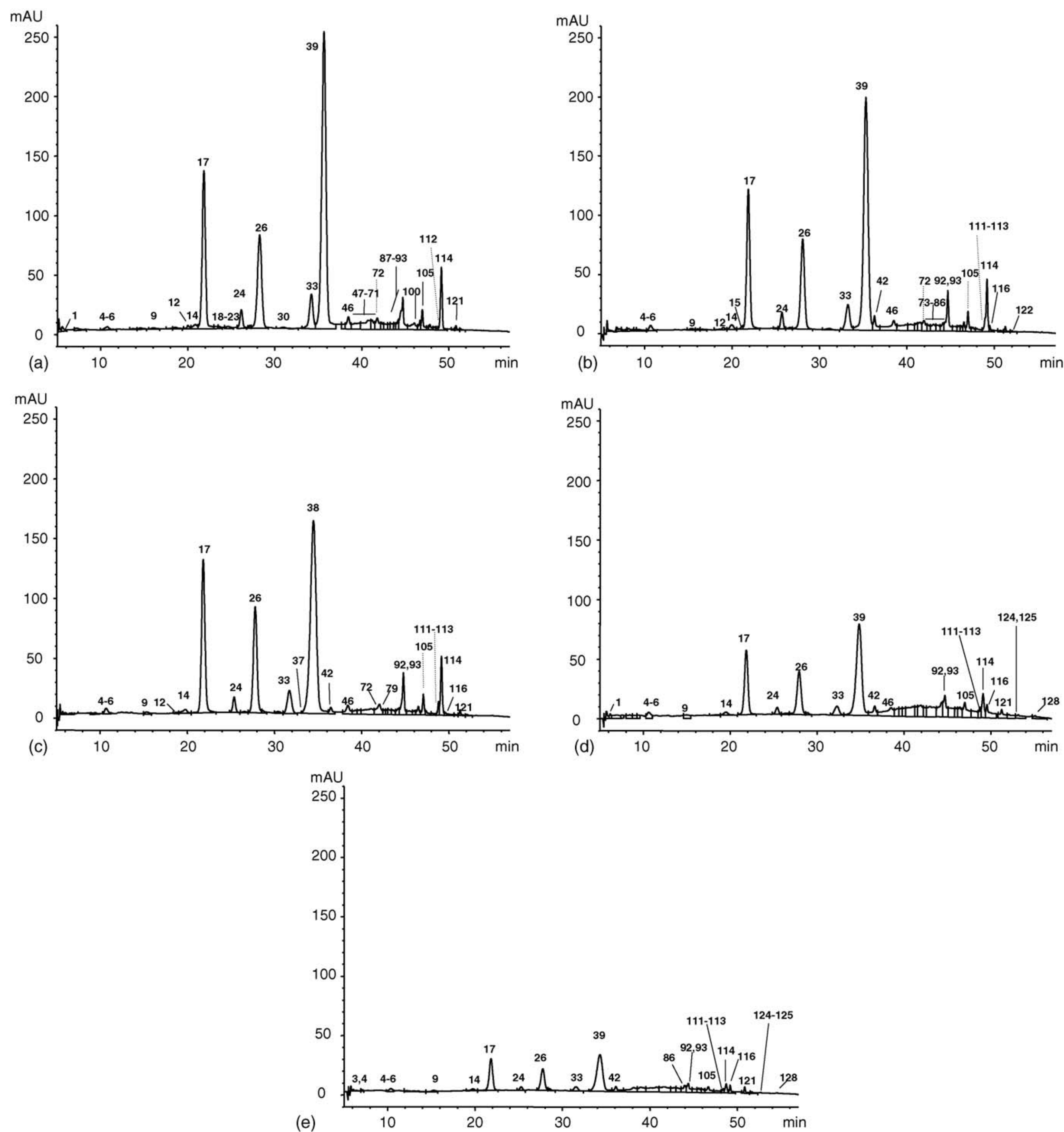

Fig. 1. Chromatograms recorded at 520 nm corresponding to (a) wine sample of 4 months, (b) wine sample of 8 months, (c) wine sample of 13 months, (d) wine sample of 16 months and (e) wine sample of 23 months. The compound numbers correspond to those in Tables 1–4.

3. Results and discussion

3.1. Total pigment content

The total pigment content was calculated from the total area of the peaks obtained in the chromatograms of the non-fractionated samples recorded at 520 nm (Fig. 1) and expressed as malvidin-3-glucoside (Fig. 2).

As can be seen in Fig. 2, during the first year of maturation in oak barrels, the total pigment content hardly changed. Nevertheless, as shown in the chromatograms (Fig. 1), there was a change in the pigment profile. The most outstanding change was the decrease in the original grape anthocyanins, malvidin-3-glucoside undergoing the most intense change. During this part of the maturation in the barrel, new peaks also appeared in the chromatograms. Thus, it seems that the decrease in the origi-

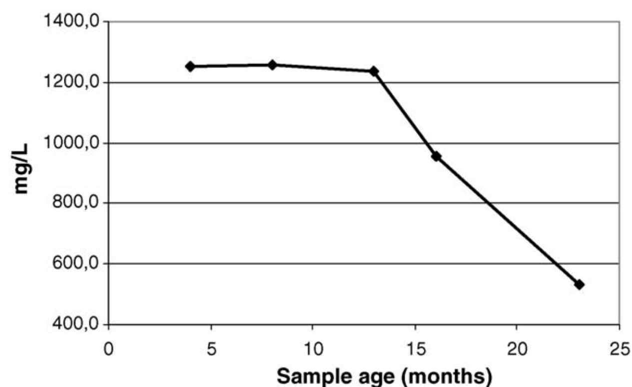

Fig. 2. Total pigment contents calculated from the total area of the chromatograms shown in Fig. 1 and expressed as mg/L of malvidin-3-glucoside.

nal grape anthocyanins was mostly due to the formation of new pigments, and above all, malvidin-3-glucoside derivatives. However, during the last months of ageing in the barrel and during ageing in the bottle the total pigment content decreased (Fig. 2), and as can be observed in the chromatograms (Fig. 1), not only the original anthocyanins decreased, but also some of the derived pigments. Thus, during this period, the pigment degradation rate was higher than that of formation.

3.2. *Qualitative and quantitative analyses of the main pigment families*

Four pigment families are considered in this study: anthocyanins, pyranoanthocyanins, direct flavanol–anthocyanin condensation products and acetaldehyde-mediated flavanol–anthocyanin condensation products. For each pigment family, the identification of all the members detected, the moment of their appearance/disappearance and the quantitative changes undergone during maturation and ageing in the barrel and bottle are reported here.

The identification of the compounds was carried out taking into account their retention times, UV–vis spectra and molecular and fragment ions supplied by the MS^n analysis. For some of the identified compounds, only retention time and *m*/*z* ratio of the molecular ion were available and the elution order and bibliographical data were useful in the identity assignment. Furthermore, some of these compounds have been synthesised in order to confirm the proposed identity.

The individual content of every detected compound was calculated from the sum of the areas obtained in the MS analysis in every fraction in which it appeared. It is worth pointing out that the concentration of the solution injected and the amount of freeze-dried material obtained from each fraction were taken into account in the calculation. The content of each pigment family in each sample was, therefore, calculated by summing the individual contents of all the members found.

3.2.1. *Anthocyanins*

The chromatographic and spectrum (UV–vis and MS) features of each detected compound belonging to this family are listed in Table 1. This family comprises monoglucosides of the anthocyanins, acetyl-, *p*-coumaroyl-, caffeoyl-derivatives of these monoglucosides, diglucosides and other acylated monoglucosides.

The retention times, UV–vis spectra, molecular and fragment ions of peaks **17**, **24**, **26**, **33** and **39** were in accordance with those of the 3-monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin, respectively. The UV–vis spectra of peaks **46**, **63**, **72**, **87** and **92** were similar to those of the monoglucosides but they eluted after them. Their molecular ions possessed 42 additional amu in relation to those of the monoglucosides of the anthocyanins. Thus, these compounds were identified as the acetyl derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin 3-glucosides, respectively. Peaks **93**, **103**, **105**, **112** and **114** were identified as the *trans*-*p*-coumaroyl derivatives of the monoglucosides of the anthocyanins taking into account their chromatographic and spectrum features, which were typical of this kind of acylated compounds. They showed higher retention times than those of the non-acylated and acetylated anthocyanins, a shoulder in the region of 309–313 nm in their UV–vis spectra, 146 additional amu in the *m*/*z* ratio of their molecular ions in relation to those of their corresponding monoglucosides and fragmentation patterns characteristic of acylated compounds. The fractionation of the wines has allowed the detection and identification of the *cis* isomers of all of these *p*-coumaroyl derivatives (peaks **84**, **96**, **97**, **107** and **108**). Although the *cis* isomers of peonidin and malvidin 3-*p*-coumaroylglucosides have already been detected in grapes [21] and wines [22,23], this is the first time, to our knowledge, that the *cis* isomers of delphinidin, cyanidin and petunidin 3-*p*-coumaroylglucosides have been detected in red wines.

Peak **100** showed a UV–vis spectrum similar to those of the *p*-coumaroyl derivatives of the monoglucosides but with a shoulder at 328 nm, instead of 313 nm, which was indicative of the acylation of the molecule with caffeic acid. The molecular ion of this compound yielded a signal in the mass spectrum at *m*/*z* 655 and, in the MS^2 analysis, it was fragmented into one single ion with *m*/*z* ratio at 331 (malvidin) by loss of 324 amu (162 amu corresponding to the glucose moiety and 162 to the caffeoyl moiety). Thus, compound **100** was identified as malvidin-3-(6″-caffeoylglucoside). Compounds **68**, **88** and **99** showed similar UV–vis spectra and the same fragmentation pattern as compound **100** originating, in the MS^2 analyses, fragment ions at *m*/*z*'s 303, 317 and 301, respectively. Their retention times and these data allowed the assignment of their identities: delphinidin (peak **68**), petunidin (peak **88**) and peonidin 3-(6″-caffeoylglucoside) (peak **99**). As far as we know, only the caffeoyl derivatives of peonidin and malvidin-3-glucosides (compounds **99** and **100**) have already been reported in grapes [21,24] and wines [22,23,25,26], the delphinidin and petunidin 3-caffeoylglucosides being reported here from the first time. The molecular ion of compound **94** had the same *m*/*z* ratio and yielded the same fragment ions in the MS^2 and MS^3 analyses as malvidin-3-(6″-caffeoylglucoside) and its retention time was lower than that of the latter. Taking into account that *p*-coumaric and caffeic acid are hydroxycinnamic acids and, therefore, may exist in both *cis* and *trans* conformations, and considering that the *cis* isomers of the *p*-coumaroyl derivatives elute before

Table 1
Chromatographic, UV–vis and MS spectrum data of the anthocyanins detected as well as their identities and samples in which they have been found

Peak	Rt	M^+ (m/z)	MS^2 frag.	MS^3 frag.	λ_{max} (nm)	Compound	Sample (months)				
							4	8	13	16	23
17	21.7	465	303	303	277, 342, 524	Dp-3-glc	*	*	*	*	*
24	26.1	449	287	287	279, 516	Cy-3-glc	*	*	*	*	*
26	28.1	479	317	317	277, 347, 525	Pt-3-glc	*	*	*	*	*
33	34.1	463	301	301	280, 517	Pn-3-glc	*	*	*	*	*
39	35.5	493	331	331	277, 348, 527	Mv-3-glc	*	*	*	*	*
46	38.3	507	303	303	276, 346, 527	Dp-3-acetylglc	*	*	*	*	*
63	41.0	491	287	287	280, 523	Cy-3-acetylglc	*	*	*	*	*
72	41.6	521	317	317	270, 529	Pt-3-acetylglc	*	*	*	*	*
87	43.6	505	301	301	280, 522	Pn-3-acetylglc	*	*	*	*	*
92	44.3	535	331	331	278, 350, 530	Mv-3-acetylglc	*	*	*	*	*
84	43.1	611	303	303	280, 301, 534	Dp-3-*p*-coumglc *cis*	*	*	*	*	*
93	44.3	611	303	303	282, 313, 531	Dp-3-*p*-coumglc *trans*	*	*	*	*	*
96	45.1	595	287	287	280, 301, 533	Cy-3-*p*-coumglc *cis*	*	*	*	*	*
103	46.3	595	287	287	284, 314, 524	Cy-3-*p*-coumglc *trans*	*	*	*	*	*
97	45.3	625	317	317	281, 301, 536	Pt-3-*p*-coumglc *cis*	*	*	*	*	*
105	46.6	625	317	317	282, 313, 532	Pt-3-*p*-coumglc *trans*	*	*	*	*	*
107	47.5	609	301	301	283, 300, 535	Pn-3-*p*-coumglc *cis*	*	*	*	*	*
112	48.6	609	301	301	283, 313, 526	Pn-3-*p*-coumglc *trans*	*	*	*	*	*
108	47.5	639	331	331	280, 301, 535	Mv-3-*p*-coumglc *cis*	*	*	*	*	*
114	48.7	639	331	331	282, 313, 532	Mv-3-*p*-coumglc *trans*	*	*	*	*	*
68	41.1	627	303	303	283, 331, 532	Dp-3-cafglc *trans*	*	n.d.	n.d.	n.d.	n.d.
88	43.6	641	317	317	283, 328, 531	Pt-3-cafglc *trans*	*	*	n.d.	n.d.	n.d.
99	45.6	625	301	301	283, 328, 525	Pn-3-cafglc *trans*	*	n.d.	n.d.	n.d.	n.d.
94	44.8	655	331	331		Mv-3-cafglc *cis*	*	*	n.d.	n.d.	n.d.
100	45.7	655	331	331	282, 328, 534	Mv-3-cafglc *trans*	*	*	*	*	*
10	16.7	627	303	303	279, 523	Dp-3,7-diglc	*	n.d.	n.d.	n.d.	n.d.
13	20.4	641	317	317	275, 521	Pt-3,5-diglc	*	n.d.	n.d.	n.d.	n.d.
23	24.6	641	317	317	275, 349, 522	Pt-3,7-diglc	*	*	*	*	*
28	28.7	625	301	301		Pn-3,7-diglc	*	*	n.d.	n.d.	n.d.
19	23.5	655	331	331	275, 524	Mv-3,5-diglc	*	*	n.d.	n.d.	n.d.
30	30.8	655	331	331	278, 350, 526	Mv-3,7-diglc	*	*	*	*	*
34	34.7	537				Dp-3-glc + L(+)lactic acid	*	*	*	*	*
44	37.5	551				Pt-3-glc + D(−)lactic acid	*	*	*	*	n.d.
52	39.2	551	317	317	278, 526	Pt-3-glc + L(+)lactic acid	*	*	*	*	*
57	40.5	535				Pn-3-glc + D(−)lactic acid	*	*	n.d.	n.d.	n.d.
73	41.7	535	301	301	281, 525	Pn-3-glc + L(+)lactic acid	*	*	*	*	*
61	40.8	565	331	331	278, 350, 530	Mv-3-glc + D(−)lactic acid	*	*	*	*	*
78	42.1	565	331	331	278, 348, 531	Mv-3-glc + L(+)lactic acid	*	*	*	*	*

Rt: retention time; M^+: molecular ion; frag.: fragments obtained in MS^2 or MS^3 analyses; Dp: delphinidin; Cy: cyanidin; Pt: petunidin; Pn: peonidin; Mv: malvidin; glc: glucose; acetylglc: (6″-acetylglucoside); *p*-coumglc: 6″-*p*-coumaroylglucoside; cafglc: 6″-caffeoylglucoside; diglc: diglucoside; (*) detected; n.d.: not detected.

the *trans* ones, compound **94** was identified as the *cis* isomer of malvidin-3-(6″-caffeoylglucoside), compound **100** being the *trans* isomer. To our knowledge this is the first time that compound **94** has been reported in red wines.

Compounds **13** and **19** showed retention times, UV–vis spectra, molecular and fragment ions corresponding to petunidin and malvidin 3,5-diglucosides. On the contrary, compounds **10**, **23**, **28** and **30** also showed molecular ions at *m*/*z*'s corresponding to diglucosides (delphinidin (*m*/*z* 627), petunidin (*m*/*z* 641), peonidin (*m*/*z* 625) and malvidin (*m*/*z* 655), respectively) but their retention times, UV–vis spectra and fragmentation patterns did not correspond to those of the 3,5-diglucosides (Fig. 3). The retention times were higher than those of the 3,5-diglucosides. Their UV–vis spectra indicated that the hydroxyl group in position 5 of the anthocyanidins was not substituted, since the shoulder in the 440 nm region, typical of monoglucosides [27], was also present in these compounds (Fig. 3a). When the 3,5-diglucosides were fragmented in the MS^2 analyses two major ions appeared (Fig. 3b, fragmentation of compound **19**): the most abundant corresponding to the anthocyanidin by loss of 324 amu (two glucose moieties) and the other to the monoglucoside of the anthocyanidin. However, as can be seen in Fig. 3c, the fragmentation of compound **30** yielded the same ions but in different relative abundances. The ion corresponding to the monoglucoside of the anthocyanidins was present in a very low relative abundance (5%). This fragmentation pattern was also observed in compounds **10**, **23** and **28**. Furthermore, the presence in their MS^2 spectra of this ion indicated that the two existing glucose moieties should not be in the same position, since the cleavage of the glycosidic bond between two glucoside is not produced in the MS conditions usually employed [28]. The fact that the fragment ions corresponding to the monoglucosides of the anthocyanidins were in lower relative abundances than those obtained in the fragmentation of the 3,5-diglucosides (55%) could also be indicative

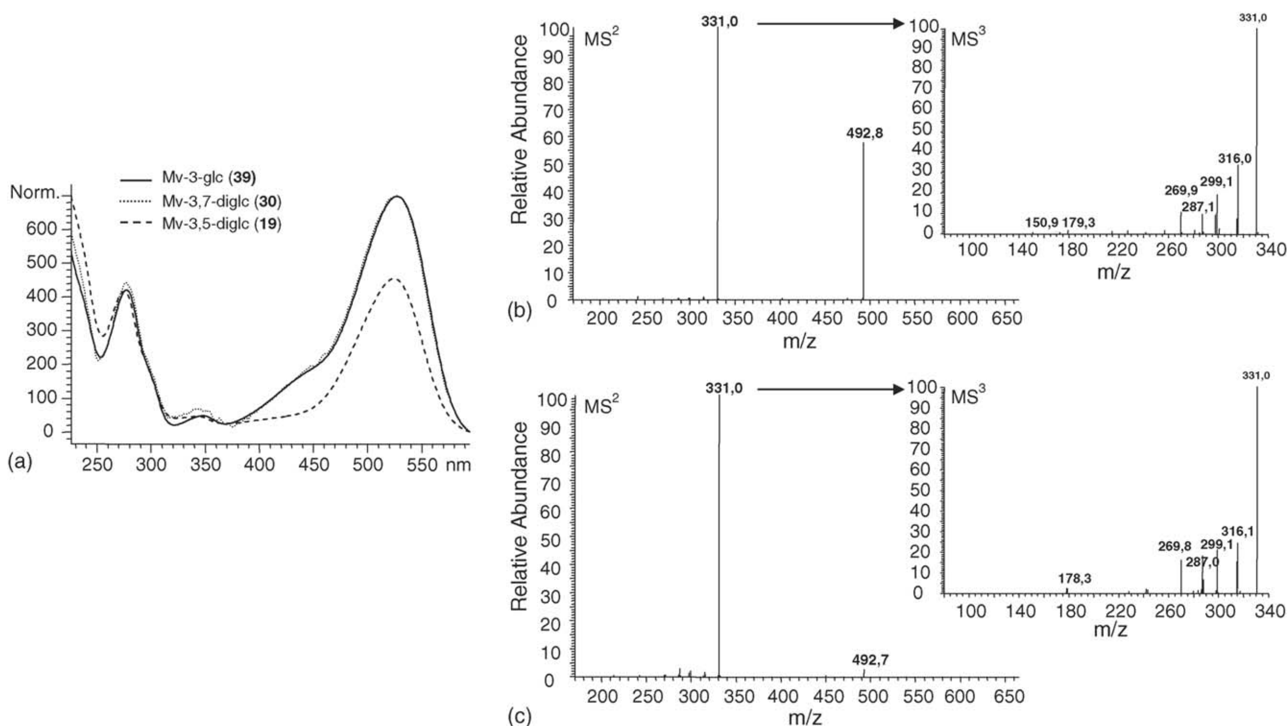

Fig. 3. UV–vis spectra (a) of malvidin 3-glucoside, 3,7-diglucoside and 3,5-diglucoside (compounds **39**, **30** and **19**, respectively) and MS^2 and MS^3 spectra of (b) malvidin-3,5-diglucoside (*m/z* 655) and (c) malvidin-3,7-diglucoside (*m/z* 655).

of the position of glucosilation since it has been demonstrated that the glycosidic linkage in position 7 of other flavonoid *O*-glycosides have high lability and are more easily cleaved than that in position 3 [29]. Thus, in the case of 3,7-diglucosides, it should be expected that the ions corresponding to the monoglucosides were all 3-monoglucosides, since the glycosidic bond in 7 is more easily cleaved, whereas in the case of 3,5-diglucosides, they might originate by cleavage of the glycosidic bond in position 3 or 5. This would explain the different fragmentation patterns in the case of 3,5-diglucosides and 3,7-diglucosides. Compounds **10**, **23**, **28** and **30** were, therefore, identified as delphinidin, petunidin, peonidin and malvidin 3,7-diglucosides, respectively. Although the Tempranillo variety belongs to the *V. vinifera* species and 3,5-diglucosides are known to be present in other *Vitis* species, but not in *V. vinifera*, the fractionation of the wine and the high sensitivity of HPLC–DAD–MS techniques has allowed their detection. Baldi et al. [24] also detected 3,5-diglucosides in extracts of skins from *V. vinifera* grapes by applying HPLC/DAD and HPLC/MS techniques after purification of the extract. Nevertheless, this is the first time that the presence of 3,7-diglucosides of the anthocyanins have been reported in red wines, although 3,7-diglucosides have been detected in other natural sources [30].

The UV–vis spectrum of compound **78** was practically identical to that of malvidin-3-glucoside, but 4 nm bathochromically shifted in the visible maximum (Fig. 4a). This compound eluted after the monoglucosides of the anthocyanins, which could indicate the acylation of the molecule. The absence of any additional shoulder in the 309–330 nm range was indicative of the aliphatic nature of the acyl group. The molecular ion yielded a signal in the MS spectrum at *m/z* 565 and, when it was fragmented in the MS^2 analysis, it originated one single fragment ion at *m/z* 331 by the loss of 234 amu (Fig. 4b). The fragmentation of this fragment ion in the MS^3 analysis revealed that the anthocyanidin of compound **78** was malvidin (see MS^3 spectrum in Fig. 4b and compare it to MS^3 spectra of compounds **19** and **30** in Fig. 3b and c). Taking into account that it has been reported that in the fragmentation of the acyl derivatives the cleavage of the linkage between the sugar and the acid is not usually observed [28], the loss of 234 amu would correspond to a glucose moiety (162 amu) and an acyl residue of 72 amu. During malolactic fermentation of the wines, the malic acid is transformed into lactic acid (molecular weight 90 g/mol) and this acid might react with the monoglucosides of the anthocyanins in an esterification reaction between the carboxyl group of the acid and the hydroxyl group in position 6″ of the sugar, originating compound **78**. Two isomers of lactic acid can be found in wines, L(+) and D(−) lactic acid, in average concentrations of 3.10 g/L for the L(+) isomer and 0.15 g/L for the other [31]. Thus, esterification of the anthocyanins might occur with both compounds. Compound **61** possessed the same UV–vis spectrum (Fig. 4a), molecular ion and fragmentation pattern as compound **78** (Fig. 4b and c). Its retention time was lower than that of the latter and it was present in smaller amounts. Compound **78** was, therefore, identified as the product resulting from the esterification of malvidin-3-glucoside with L(+) lactic acid and compound **61** as malvidin-3-glucose acylated with the D(−) isomer. In order to confirm their identities and mechanism of formation, a commercial racemic mixture of L(+) and D(−) lactic acid was added in excess to an acidic solution of malvidin-3-glucoside and the resulting solution was maintained

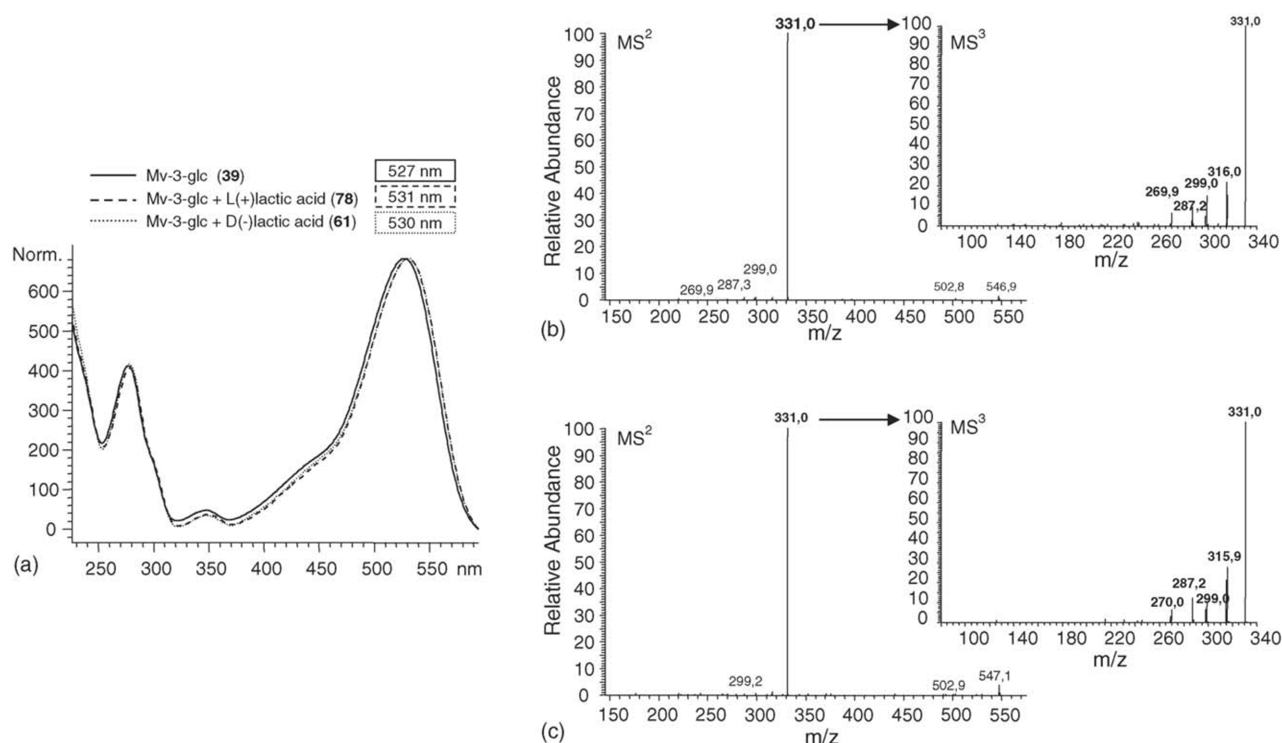

Fig. 4. UV–vis spectra (a) of malvidin 3-glucoside, malvidin-3-glucoside acylated with L(+)lactic acid and with D(−)lactic acid (compounds **39**, **78** and **61**, respectively) and MS^2 and MS^3 spectra of (b) compound **78** (*m*/*z* 565) and (c) compound **61** (*m*/*z* 565).

at 35 °C. The reaction took place very fast and two new peaks appeared. These new compounds possessed the same retention times and spectrum features as compounds **61** and **78**, confirming the identities proposed for them. These compounds have not been described previously in wines. This is the first time that the existences of acylated anthocyanins not initially present in grapes are reported originating from the grape anthocyanins. Compounds **34**, **44**, **52**, **57** and **73** showed the same fractionation patterns as those acylated with lactic acid, and the MS^2 and MS^3 analyses revealed the anthocyanin involved in these acylation reactions. Only one of the two possible isomers was detected for delphinidin-3-glucoside and it was assumed to be the L(+) one (compound **34**). Nevertheless, both isomers were detected in the cases of petunidin and peonidin 3-glucosides, the less abundant of them, D(−), always eluting before the other. Thus, compounds **44** and **52** corresponded to petunidin-3-glucosides acylated with D(−) and L(+) lactic acid, respectively, and compounds **57** and **73** to peonidin-3-glucoside derivatives.

Although the total anthocyanin content (considering monoglucosides, diglucosides and all the acyl-derivatives) decreased during all the period studied in this work, the rate was different as time passed (Fig. 5a). The decrease rate was higher during the first months of maturation in the barrel than in the last ones and this rate increased again during ageing in the bottle. Since the monoglucosides of the anthocyanins are the most abundant subfamily in red wines within the family, it seems reasonable that the changes undergone by the monoglucosides as wine became older were practically identical to those of the whole family. Again, the most important decrease took place at the beginning of the ageing in the barrel, the levels remaining practically stable in the last 3 months of this ageing. The decrease observed during the 7 months of ageing in the bottle was lower than that observed in the first months of ageing in the barrel and lower than that observed for all the family.

The evolution of the levels of the acetyl and *p*-coumaroyl derivatives was very similar (Fig. 5b). Ageing in the barrel affected both kinds of compounds in the same way. However, *p*-coumaroyl derivatives seemed to be more resistant towards the conditions undergone during ageing in the bottle, since during ageing in the barrel the acetyl derivatives were present in the wine in higher amounts than the *p*-coumaroyl ones and during ageing in the bottle this proportion inverted. A slight increase in the levels of the *p*-coumaroyl derivatives was observed in the sample of 13 months. Taking into account the possibility of acylation of the glucosides of the anthocyanins with acids during wine maturation, as demonstrated with acid lactic derivatives, *p*-coumaric acid present in wine might also react with anthocyanins originating acylated compounds with the same features as those originally present in grapes. Thus, the decrease of the *p*-coumaroyl derivatives coming from grapes should partially be compensated by the formation of new *p*-coumaroyl derivatives and this could also explain the lower decrease rate in the final stages of the *p*-coumaroyl derivatives than the acetyl ones. Among the *p*-coumaroyl derivatives, the *cis* isomers disappeared faster than the *trans* ones. In the first sample (4 months old), almost 20% of the *p*-coumaroyl derivatives were *cis* isomers, representing only 10% in the last sample.

The *trans* isomers of the caffeoyl derivatives of delphinidin, petunidin, peonidin and malvidin 3-glucosides as well as the *cis* isomer of malvidin-3-caffeoylglucoside were present in the first

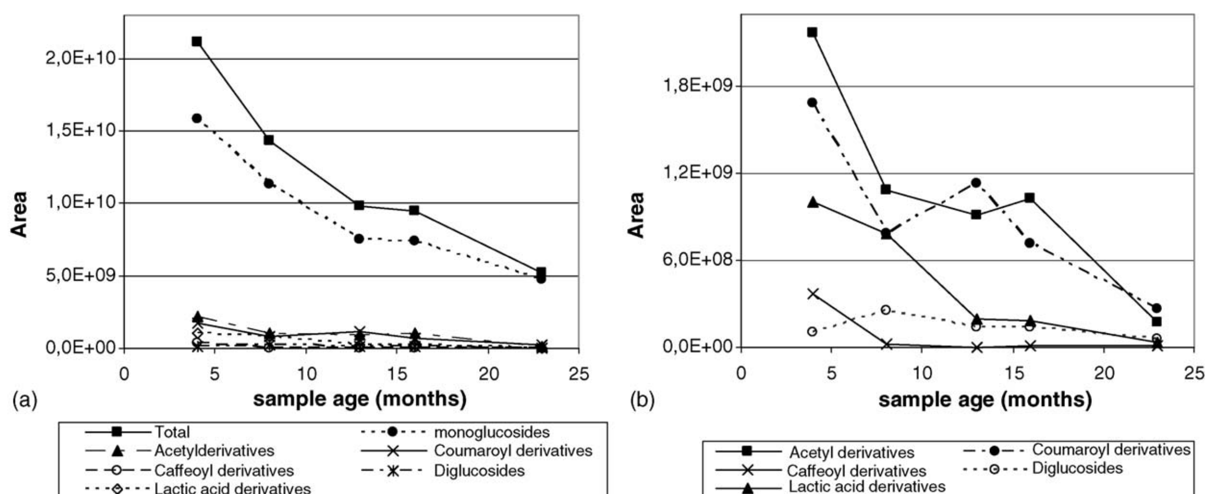

Fig. 5. (a) Changes produced in the levels of anthocyanins during maturation and ageing. (b) Detail of the changes produced in the acyl derivatives and diglucosides of the anthocyanins.

sample, but as the wine became older, only the *trans* malvidin-3-caffeoylglucoside was detected (Table 1). As expected, the highest levels of caffeoyl derivatives were found in the first sample, at 4 months from harvest. Four months later, only 7% of the initial compounds remained in the wine. From this point onwards the decrease in their levels continued, but at a slower rate (Fig. 5b).

The 3,5-diglucosides found in this wine, were only detected in the first two samples, and their levels, very low when compared to monoglucosides, also decreased as the wine became older, both kinds of pigments undergoing similar changes.

As stated previously, this is the first time that 3,7-diglucosides of the anthocyanins are reported in red wines. Nevertheless, their origin remains uncertain because their evolution with time does not fit with that expected for anthocyanins originally present in grapes. At 4 months from the harvest, the levels of these 3,7-diglucosides seemed to be slightly higher than those of the 3,5-diglucosides and instead of decreasing with time, the highest levels were reached when the wine was 8–13 months old. If these compounds came from grapes, their levels should decrease, or at least remain stable with time, but not increase. Further studies in fresh Tempranillo grapes have still to be made in order to elucidate their nature and origin.

The evolution of the anthocyanins acylated with lactic acid was similar to those of the other acylated anthocyanins: the highest levels were found at the first months of ageing in the barrel and from then onwards they decreased, rapidly in a first stage and more slowly during the end of ageing in the barrel and during ageing in the bottle. As lactic acid is originated during malolactic fermentation, the highest amount of lactic acid was reached at 1.5 month from the day of harvest (end of MLF). That could explain why the levels of lactic acid derivatives remained practically stable from the fourth to the eighth month: during this period there should still be lactic acid available which would react with the anthocyanins not involved in other reactions. This is the difference in the evolution between these compounds and the other acyl compounds: the latter are already present in grapes and their highest levels are dependent on the initial extraction of the anthocyanins from the grapes to the must whereas the levels of the former ones depend on the amount of lactic acid produced by bacteria, on the availability of the anthocyanins and on the reactivity of both compounds.

3.2.2. *Pyranoanthocyanins*

Pyranoanthocyanins originated in the cycloaddition of pyruvic acid, acetaldehyde, acetone, 4-vinylcatechol, 4-vinylphenol, 4-vinylguaiacol and vinylcatechin with anthocyanins (monoglucosides and their acyl derivatives) were detected in this wine, showing different moments of appearance (Table 2) and different evolutions with wine age.

The retention times, UV–vis spectra, molecular ions and fragmentation pattern of compounds **15**, **25**, **27**, **36**, **42**, **60**, **89** and **91** were in agreement with those of pyranoanthocyanins originated in the cycloaddition of pyruvic acid and different anthocyanins (A-type vitisins or vinylformic adducts). Compounds **15**, **25**, **27**, **36** and **42** were identified as vinylformic adducts of delphinidin, cyanidin, petunidin, peonidin and malvidin 3-glucosides, and compounds **60**, **89** and **91** as vinylformic adducts of petunidin, peonidin and malvidin 3-(6″-*p*-coumaroylglucosides) taking into account bibliographical data [8,10] and studies carried out in our laboratory [19,23,32]. Although the levels of the acetyl and *p*-coumaroyl derivatives of the anthocyanins were similar (Fig. 5b), no A-type vitisins originated from acetylated anthocyanins were detected in this wine. Romero and Bakker [33] observed, in model solutions containing a grape skin extract with malvidin-3-glucoside, malvidin-3-acetylglucoside and malvidin-3-*p*coumaroylglucoside as main pigments and pyruvic acid in different concentrations and maintained at different temperatures, that 3-acetylvitisin A, originated by interaction of pyruvic acid with malvidin-3-acetylglucoside, was not detected or was formed in very low concentrations in the assays carried out at temperatures higher than 15 °C, whereas vitisin A and its *p*-coumaroyl derivative were formed, at slow rates, in these conditions. Thus, in the wine analysed, the absence of acetylated A-type vitisins might be related to the temperature at which the wine was maintained during its maturation and ageing.

Table 2
Chromatographic, UV–vis and MS spectrum data of the pyranoanthocyanins detected as well as their identities and samples in which they have been found

Peak	Rt	M^+	MS^2 frag.	MS^3 frag.	λ_{max} (nm)	Compound	Sample (months)				
							4	8	13	16	23
15	21.0	533	371	371	297, 368, 507	A-type vitisin of Dp-3-glc	n.d.	*	*	*	*
25	27.0	517				A-type vitisin of Cy-3-glc	nd	n.d.	nd	*	n.d.
27	28.7	547	385	385	299, 371, 508	A-type vitisin of Pt-3-glc	n.d.	*	*	*	*
36	35.0	531	369	369	503	A-type vitisin of Pn-3-glc	n.d.	n.d.	n.d.	*	*
42	36.0	561	399	399	299, 372, 510	Vitisin A	n.d.	*	*	*	*
60	40.7	693				A-type vitisin of Pt-3-*p*-coumglc	n.d.	n.d.	*	*	n.d.
89	43.8	677	369	369	284, 508	A-type vitisin of Pn-3-*p*-coumglc	n.d.	*	*	*	*
91	44.1	707	399	399	271, 514	A-type vitisin of Mv-3-*p*-coumglc	n.d.	*	*	*	*
22	24.4	489	327	327		B-type vitisin of Dp-3-glc	n.d.	*	*	*	*
32	33.5	503	341	341	492	B-type vitisin of Pt-3-glc	n.d.	*	*	*	*
47	38.5	487	325	325		B-type vitisin of Pn-3-glc	*	*	*	*	n.d.
55	39.5	517	355	355	294, 358, 490	Vitisin B	*	*	*	*	*
71	41.4	529	325	325		B-type vitisin of Pn-3-acetylglc	*	*	n.d.	n.d.	n.d.
80	42.4	559	355	355	298, 361, 494	B-type vitisin of Mv-3-acetylglc	*	*	*	*	n.d.
67	41.1	501	339	339	475	Acetone derivative of Pn-3-glc	n.d.	*	*	*	n.d.
77	42.1	531	369	369	480	Acetone derivative of Mv-3-glc	n.d.	*	*	*	*
98	45.5	581	419	419	264, 412, 503	Dp-3-glc 4-vinylphenol adduct	*	*	*	*	*
109	47.5	565				Cy-3-glc 4-vinylphenol adduct	n.d.	*	*	*	*
111	48.3	595	433	433	264, 413, 502	Pt-3-glc 4-vinylphenol adduct	*	*	*	*	*
119	50.5	579	417	417	278, 406, 500	Pn-3-glc 4-vinylphenol adduct	*	*	*	*	*
121	51.0	609	447	447	263, 412, 504	Mv-3-glc 4-vinylphenol adduct	*	*	*	*	*
125	53.2	651	447	447	298, 416, 505	Mv-3-acetylglc 4-vinylphenol adduct	*	*	*	*	*
117	49.9	727	419	419		Dp-3-*p*-coumglc 4-vinylphenol adduct	nd	n.d.	nd	*	*
123	52.4	741	433	433	314, 504	Pt-3-*p*-coumglc 4-vinylphenol adduct	n.d.	n.d.	n.d.	*	*
127	54.6	725	417	417	314, 501	Pn-3-*p*-coumglc 4-vinylphenol adduct	n.d.	n.d.	n.d.	*	*
128	55.2	755	447	447	264, 313, 416, 505	Mv-3-*p*-coumglc 4-vinylphenol adduct	n.d.	*	*	*	*
86	43.5	597	435	435	509	Dp-3-glc 4-vinylcatechol adduct	*	*	*	*	*
104	46.5	611	449	449	510	Pt-3-glc 4-vinylcatechol adduct	*	*	*	*	*
113	48.6	595	433	433	506	Pn-3-glc 4-vinylcatechol adduct	*	*	*	*	*
116	49.2	625	463	463	510	Mv-3-glc 4-vinylcatechol adduct	*	*	*	*	*
120	50.9	667	463	463	513	Mv-3-acetylglc 4-vinylcatechol adduct	n.d.	n.d.	*	*	*
110	47.6	743	435	435		Dp-3-*p*-coumglc 4-vinylcatechol adduct	*	*	*	*	*
118	50.5	757	449	449		Pt-3-*p*-coumglc 4-vinylcatechol adduct	n.d.	*	*	*	*
124	53.2	771	463	463	312, 511	Mv-3-*p*-coumglc 4-vinylcatechol adduct	*	*	*	*	*
122	52.0	639	477	477	511	Mv-3-glc 4-vinylguaiacol adduct	*	*	*	*	*
126	54.0	681	477	477	514	Mv-3-acetylglc 4-vinylguaiacol adduct	n.d.	*	*	*	*
129	55.7	785	477	477	514	Mv-3-*p*-coumglc 4-vinylguaiacol adduct	n.d.	*	*	*	*
115	49.0	805				Mv-3-glc 4-vinyl*epi*-catechin adduct	*	*	*	*	*

Rt: retention time; M^+: molecular ion; frag.: fragments obtained in MS^2 or MS^3 analyses; Dp: delphinidin; Cy: cyanidin; Pt: petunidin; Pn: peonidin; Mv: malvidin; glc: glucose; acetylglc: 6″-acetylglucoside; *p*-coumglc: 6″-*p*-coumaroylglucoside; (*) detected; n.d.: not detected.

The chromatographic and spectrum features of compound **55** were consistent with those reported for the pyranoanthocyanin originated in the cycloaddition of acetaldehyde with malvidin-3-glucoside [10,23,25,32] also named vitisin B by Bakker and Timberlake [10]. Compounds **22**, **32**, **47**, **71** and **80** were identified as B-type vitisins of delphinidin, petunidin, peonidin 3-glucosides and peonidin and malvidin 3-acetylglucosides, respectively. Unlike in the case of A-type vitisins, products resulting from the reaction between acetylated anthocyanins and acetaldehyde have been detected but not those originated from *p*-coumaroyl derivatives. In model solutions containing malvidin 3-glucoside, 3-acetylglucoside, 3-*p*-coumaroylglucoside, pyruvic acid and acetaldehyde [34], it has been shown that the decrease in the levels of the anthocyanins was not only due to the formation of A-type vitisins, but also to the interactions between anthocyanins and acetaldehyde. The decrease was reported to be higher in the case of malvidin-3-acetylglucoside than in the monoglucoside and *p*-coumaroylglucoside. Furthermore, no acetylvitisin A was observed to be formed. Thus, the decrease in the levels of the acetylated anthocyanin might be attributed to the formation of anthocyanin-derived compounds in whose formation acetaldehyde is involved, and among them, B-type vitisins. This should partially explain why in the analysed wine A-type vitisins of acetylated anthocyanins have not been detected and why B-type vitisins of acetylated anthocyanins have been found.

The UV–vis spectrum of compound **77** was similar to that of compound **55** (vitisin B), indicating similar structure, but with the visible maximum at lower wavelength (λ_{max} of vitisin B: 490 nm; compound **77**: 480 nm). The retention time was higher than that of compound **55**, which indicated that in the molecule there should be an additional residue that decreased polarity. The molecular ion corresponding to this compound possessed the same *m*/*z* ratio (531) as that of the A-type vitisin

of peonidin-3-glucoside (compound **36**). The fragmentation of this molecular ion in the MS^2 analysis was the same for both compounds and only the loss of a glucose moiety was observed. Nevertheless, the MS^3 analysis was very useful in the identification of this compound, since the aglycone was fragmented and the losses observed were different in both cases. Those observed for compound **77** were the same as those produced in the fragmentation of the malvidin aglycone, whereas the aglycone of compound **36** was fragmented as the peonidin aglycone. Thus, compound **77** should originate from malvidin-3-glucoside. The molecular ion showed 38 additional amu. All these data allowed the identification of compound **77** as the pyranoanthocyanin originated in the reaction between malvidin-3-glucoside and acetone (vinylmethyl adduct). The possibility of formation of this kind of compounds has already been demonstrated [11,15] and their existence has been reported in wines [14,23,26]. Similarly, compound **67** was identified as the pyranoanthocyanin originated in the reaction between peonidin-3-glucoside and acetone. Although Hayasaka and Asenstorfer [14] detected a signal in the MS spectra of fractions obtained from red wines at the *m*/*z* corresponding to compound **67**, no identity was proposed for it. In order to confirm their identity, synthesis of these compounds was carried out in our laboratory employing similar conditions to those described by Lu and Foo [15] using an extract of grape anthocyanins instead of isolated compounds. The reaction took place very fast and the original anthocyanins were converted into pyranoanthocyanins. Those originated from peonidin and malvidin 3-glucosides possessed the same retention times, UV–vis spectra, molecular ions and were fragmented in the MS^2 and MS^3 analyses as compounds **67** and **77**, respectively, thus confirming their identities.

The fractionation of the samples has allowed the obtaining of the UV–vis spectra of peaks that were barely detectable in the whole wine because they were either in low amounts or co-eluted with more abundant compounds which hid their spectra. This is the case of some of the 4-vinylphenol, 4-vinylcatechol and 4-vinylguaiacol derivatives whose UV–vis and MS spectrum features were easily determined in the fractions, which were very useful in the assignment of their identities, since some of the compounds had similar retention times and molecular ions with the same *m*/*z* ratio.

The UV–vis spectra of compounds **98**, **109**, **111**, **119**, **121**, **125**, **117**, **123**, **127** and **128** were characteristic of the pyranoanthocyanins originated in the cycloaddition between 4-vinylphenol and different anthocyanins. The retention times, *m*/*z* ratios of the molecular and fragment ions allowed their identification. Compounds **98**, **109**, **111**, **119** and **121** were the 4-vinylphenol derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin 3-glucosides, respectively. The loss of 204 amu observed in the fragmentation of compound **125**, and those of 308 amu observed in compounds **117**, **123**, **127** and **128** indicated that these compounds derived from acylated anthocyanins. Their identity is shown in Table 2. Compound **121** was first described by Fulcrand et al. [12] and the presence in wines of these pyranoanthocyanins has been widely reported [14,19,23,26,32].

Pyranoanthocyanins originated in the cycloaddition of 4-vinylcatechol to different anthocyanins were also found in the samples. Compounds **86**, **104**, **113** and **116** were identified as delphinidin, petunidin, peonidin and malvidin 3-glucosides 4-vinylcatechol adducts, taking into account their chromatographic and spectrum features (see Table 2) and by comparing them to those obtained in other wine analyses carried out in our laboratory [19,23]. Similarly, compounds **120**, **110**, **118** and **124** were identified as malvidin-3-acetylglucoside, delphinidin, petunidin and malvidin-3-*p*-coumaroylglucosides 4-vinylcatechol adducts. The presence of most of these vinylcatechol adducts has already been reported in wines [13,14,19,23,26].

With regard to the vinylguaiacol adducts, only malvidin derivatives were detected. Compounds **122**, **126** and **129** corresponded to malvidin-3-glucoside, malvidin-3-acetylglucoside and malvidin-3-*p*-coumaroylglucoside 4-vinylguaiacol adducts. The existence of these compounds has already been reported [14,19,23].

Another pyranoanthocyanin detected in all the samples was that originated in the cycloaddition reaction between the monoglucoside of malvidin and vinyl*epi*-catechin (compound **115**). The identification of this compound was carried out considering its retention time, which was similar to that of the vinyl*epi*-catechin adduct of malvidin-3-glucoside when was analysed in the same conditions [19,23] and the *m*/*z* ratio of its molecular ion, since its UV–vis spectrum and MS^n fragmentations were not available as it co-eluted with more abundant compounds. This kind of compounds was first detected in model solutions containing malvidin-3-glucoside, catechin and acetaldehyde [4] and its presence in wines has also been reported [14,35,36].

The formation of all these sub-families of pyranoanthocyanins did not take place simultaneously. In the 4 months old wine sample neither the A-type vitisins nor those originated in the reaction with acetone were detected, the rest of the pyranoanthocyanins being present in the wine from the first months of ageing in the barrel.

A-type vitisins reached detectable levels at 8 months (Fig. 6a and Table 2). They then increased until ageing in the bottle, from where they began to decrease. The highest amount was reached at the end of ageing in the barrel. As expected, the vinylformic adduct of malvidin-3-glucoside (vitisin A) was the most abundant member of this sub-family. It seems that during the maturation and ageing steps occurring in barrels, A-type vitisins were formed and, after bottling, the reaction was not favoured and the levels decreased. Asenstorfer et al. [18] demonstrated that the formation of vitisin A is controlled by the existence in wine of a suitable oxidant rather than by the levels of malvidin-3-glucoside or pyruvic acid, since the formation of this pyranoanthocyanin requires an oxidation reaction step. They also stated that oxygen is not the oxidant species that promotes vitisin A production, but it can be converted into reactive oxygen species, a conversion that is promoted by some wine components, such as ellagitannins [37]. The normal barrel handling operations favour the contact of the wine with air and thus, with oxygen. Furthermore, during maturation and ageing of the wines in oak barrels, ellagitannins

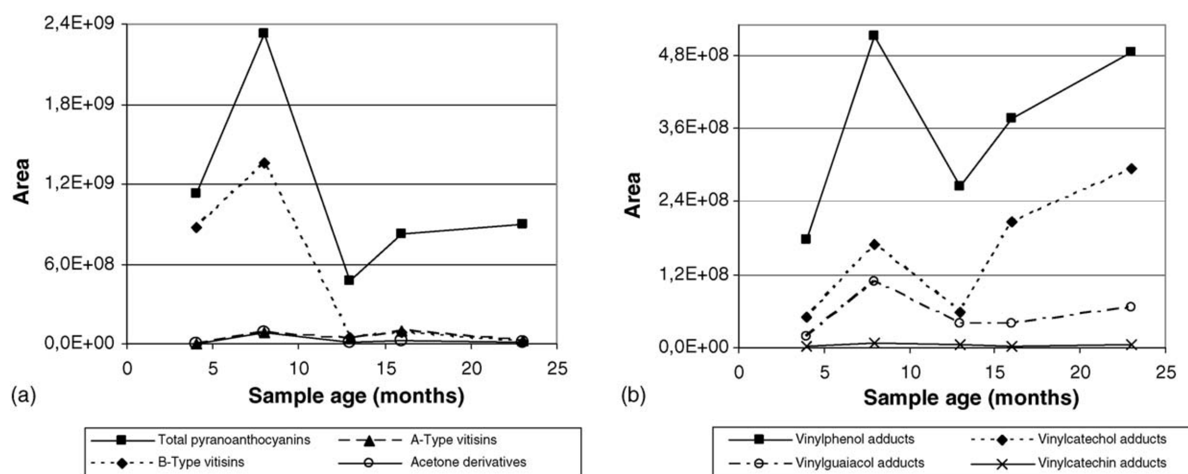

Fig. 6. Changes produced during wine maturation and ageing in (a) the total pyranoanthocyanin, A-type vitisin, B-type vitisin and acetone derivative contents and in (b) the vinylphenol, vinylcatechol, vinylguaiacol and vinylcatechin adduct contents.

are extracted from wood into the wine [38] and there, they can favour the oxidation reactions. Thus, the synthesis of A-type vitisins can be promoted during ageing in the barrel, provided there are anthocyanins and pyruvic acid available. Nevertheless, during ageing in the bottle, the formation of oxidant species is not favoured, since there is no air contact and no more extraction from oak wood of compounds that could promote their formation. This would explain the decrease in the levels of A-type vitisins observed during wine ageing in the bottle.

Unlike A-type vitisins, B-type ones were already detected, in high amounts, in the youngest sample and the highest level was reached when the wine was 8 months old. From this moment onwards, they rapidly decreased attaining much lower levels than those found in the first sample. In the last 3 months of ageing in the barrel they seemed to stabilise and slightly decreased during ageing in the bottle. As previously mentioned, the acetaldehyde reacted better with acetylated anthocyanins than with *p*-coumaroylated ones, since the B-type vitisins of the latter were not detected in any of the samples.

Compounds resulting from the reaction between anthocyanins and acetone did not appear until the 8th month (Fig. 6a and Table 2). From here, their behaviour was very similar to that of B-type ones. The 8 month-old sample presented the highest levels, decreasing very fast until the 16th month, stabilising during the last months of ageing in the barrel and slightly decreasing during ageing in the bottle.

It is worth pointing out that the real contribution to wine colour of B-type vitisins and acetone derivatives will not be established until the determination of their molar absorptivity, since they both showed higher MS areas (Fig. 6a) than those that would be expected from their absorbances observed in the chromatograms recorded at 520 nm. It has to be determined if the high MS areas correspond to high concentrations in wine and if the areas obtained in the chromatogram at their visible maxima (490 nm for B-type vitisins and 480 nm for acetone derivatives) would be much higher than those obtained at 520 nm.

Vinylphenol, vinylcatechol and vinylguaiacol derivatives have been detected from the first sample, and their evolutions in time were similar, but not identical. Two formation periods can be observed (Fig. 6b): one from the 4th to the 13th month and the other from the 13th month to the end point of the study. The vinylphenol derivatives were always the most abundant. The vinylphenol derivatives of the five monoglucosides have been detected as well as that of malvidin-3-acetylglucoside and those of petunidin, peonidin and malvidin 3-*p*-coumaroylglucosides. As observed in B-type vitisins, the highest levels of these compounds were attained when the sample was 8 months old and then a decrease in both kinds of compounds was produced. Nevertheless, the decrease was not as strong as occurs in B-type vitisins and was followed by an increase during the 3 last months of ageing in the barrel and by a slight increase or a stabilisation during ageing in the bottle. It has been demonstrated that two different mechanisms can be involved in the formation of these vinylphenol derivatives. Fulcrand et al. [12] stated that they are formed in the reaction of the anthocyanin with the vinylphenol originated in the wine by decarboxylation of the *p*-coumaric acid by yeasts. In order to confirm this mechanism, they carried out the synthesis of the malvidin-3-glucoside 4-vinylphenol adduct by adding 4-vinylphenol to the anthocyanin solution. The reaction took place very fast and the target compound was obtained. More recently, Schwarz et al. [39] have demonstrated that these compounds can also be originated in the reaction between the anthocyanin and the intact hydroxycinnamic acid. However, this reaction seemed to require more time to be completed. This synthesis has also been carried out in our laboratory confirming the slow formation rate. Taking into account all these data, the evolution of vinylphenol derivatives can be explained as follows: during the first months of ageing in the barrel there is vinylphenol available, as a consequence of yeast activity, and this compound can react with free anthocyanins yielding high levels of pyranoanthocyanins. Once all the vinylphenol has reacted, the levels slowly begin to fall. Simultaneously, the existing free *p*-coumaric acid and that continuously released from tartaric esters [40] might be slowly reacting with anthocyanins. In the last months of ageing in the barrel, the formation rate is probably higher than the disappearance one, causing a global increase of their levels. During

ageing in the bottle, in addition to the pyranoanthocyanin formation, the degradation of the previously formed compounds should begin. This would explain the slower increase or the stabilisation trend observed in this period. In relation to the vinylcatechol adducts, the behaviour is similar to that described for the vinylphenol ones, but with slight differences (Fig. 6b). The formation of pyranoanthocyanins from vinylcatechol (from the beginning to the eighth month) is slower than in the case of vinylphenol and might be due either to a lower amount of available vinylcatechol, since it seems that it cannot be formed by yeast by enzymatic decarboxylation of caffeic acid [41], or to a lower reactivity of this compound in relation to vinylphenol. Furthermore, the rate of the reaction that takes place directly from caffeic acid is higher, obtaining, at the end of the ageing in the barrel and during ageing in the bottle, higher levels than at 8 months, the point at which vinylphenol derivatives had reached their highest levels. This difference might be due to the higher contents in wines of caffeic acid than *p*-coumaric acid [31] and to a higher reactivity of the anthocyanins towards caffeic acid than towards *p*-coumaric acid, as stated by Schwarz et al. [39].

Vinylguaiacol derivatives showed similar behaviour (Fig. 6b) to vinylcatechol ones in the period corresponding to the formation from vinylguaiacol. However, it seems that formation directly from ferulic acid was less important than in the former case and, after the decrease observed in the 13-month sample, the levels remained stable and then increased slightly during the last months of ageing in the barrel and during ageing in the bottle, respectively, not reaching the levels of the eighth month. It is worth pointing out that among these three hydroxycinnamic acids, namely, *p*-coumaric, caffeic and ferulic acids, the ferulic acid possesses the lowest content in wines [31] and that would explain the slight formation of 4-vinylguaiacol adducts directly from the acid when compared with 4-vinylphenol and 4-vinylcatechol adducts.

Only the vinyl*epi*-catechin derivative of malvidin-3-glucoside was detected in this wine. It was not very important in quantitative terms, and its evolution with wine age (Fig. 6b) was dependent on that of the acetaldehyde-mediated flavanol–anthocyanin and flavanol–flavanol condensation products, since this compound is formed from ethyl-catechin units released from these condensation products. In fact, in this wine, when its evolution was compared to that of acetaldehyde-mediated anthocyanin-(*epi*)catechin condensation products, it has been seen that the moments of formation of this pyranoanthocyanin corresponded to the decrease in the levels of anthocyanin–ethyl-(*epi*)catechin dimers.

3.2.3. Direct anthocyanin–flavanol condensation products

Thirty-four different compounds belonging to this family were detected (Table 3). Compounds **4** and **14** were the most abundant. Their retention times, *m*/*z* ratio of the molecular ions and fragmentation patterns were in agreement with those of direct condensation products between malvidin-3-glucoside and gallocatechin (compound **4**, *m*/*z* 797) and malvidin-3-glucoside and catechin (compound **14**, *m*/*z* 781) when analysed in the same conditions [19,23]. The presence of compound **14** in wines as well as its chromatographic and spectrum features has been widely reported [2,3,22,26,32], whereas there are less bibliographical data concerning the direct condensation product between malvidin-3-glucoside and gallocatechin [23]. Compounds **18** and **29** possessed molecular ions with the same *m*/*z* ratios as compounds **4** and **14**, respectively, and were fragmented identically to the latter ones. Their retention times were higher in relation to those of the latter, and were assigned to direct condensation products between malvidin-3-glucoside and *epi*-gallocatechin (compound **18**) and *epi*-catechin (compound **29**). To our knowledge, this is the first time that these compounds have been detected in wine. The possibility of being direct condensation products in which (gallo)catechin is linked to malvidin-3-glucoside through different positions in the anthocyanin molecule (C_6 instead of C_8) might also be considered, although it is more unlikely. Compounds **1**, **2**, **3** and **5** showed the same fragmentation pattern as compound **4**. The *m*/*z* ratio of their molecular ions and the fragments originated in the MS^2 and MS^3 analyses indicated that they were, respectively, direct condensation products between gallocatechin and delphinidin, cyanidin, petunidin and peonidin 3-glucosides. Compound **21** was identified as the direct condensation product between gallocatechin and malvidin-3-acetylglucoside and compounds **31**, **37**, **38**, **48** and **45** as direct condensation products between gallocatechin and delphinidin, cyanidin, petunidin, peonidin and malvidin 3-*p*-coumaroylglucoside, respectively, taking into account their retention times, *m*/*z* of their molecular ions, and, when available, fragmentation patterns. Compound **66** showed the same molecular ion as compound **45** and it was proposed to be the resulting compound from the direct reaction between malvidin-3-*p*-coumaroylglucoside and *epi*-gallocatechin. As far as we know, all these gallocatechin derivatives have not been described in wine previously. The fragmentation patterns of compounds **6**, **8**, **9** and **12** indicated that, in the direct condensation reaction, catechin was involved and the molecular and fragment ions revealed the identity of the anthocyanins with which catechin had reacted, being, respectively, delphinidin, cyanindin, petunidin and peonidin 3-glucosides. Compounds **7**, **11**, **16** and **20** were identified as the *epi*-isomers of compounds **6**, **8**, **9** and **12**, respectively. The direct condensation products between (*epi*)catechin and acylated anthocyanins were also identified using the same criteria employed in the identification of the other direct condensation compounds. Thus, compound **41** was identified as that containing malvidin-3-acetylglucoside and catechin, compounds **51**, **54**, **64**, **75** and **85** as those formed by delphinidin, cyanidin, petunidin, peonidin and malvidin 3-*p*-coumaroylglucosides and catechin and compounds **58**, **70**, **81**, **90** and **102** as the *epi*-isomers of the former (see Table 3 for more details).

To summarise, all the monoglucosides of the anthocyanins, their *trans*-*p*-coumaroyl derivatives and malvidin-3-acetylglucoside were found to be involved in direct condensation reactions, not only with catechin and gallocatechin, but some of them with their *epi*-isomers as well. The compounds containing (*epi*)catechin were more abundant than those formed from (*epi*)gallocatechin and, among them, those originated from the *epi*-isomers were less abundant. The direct condensation reactions with acyl derivatives seem to be slower than with monoglucosides, since the former ones did not appear until

Table 3
Chromatographic, UV–vis and MS spectrum data of the direct condensation products detected as well as their identities and samples in which they have been found

Peak	Rt	M^+	MS^2 frag.	MS^3 frag.	λ_{max} (nm)	Compound	Sample (months)				
							4	8	13	16	23
1	5.7	769	607	439	531	Dp-3-glc-GC	*	*	*	*	*
2	7.1	753	591	453	282, 524	Cy-3-glc-GC	*	*	*	*	*
3	7.2	783	621	453	279, 532	Pt-3-glc-GC	*	*	*	*	*
5	10.8	767	605	437		Pn-3-glc-GC	*	*	*	*	*
4	10.6	797	635	467	281, 531	Mv-3-glc-GC	*	*	*	*	*
18	22.3	797	635	467		Mv-3-glc-EGC	*	*	*	*	*
21	24.4	839				Mv-3-acetylglc-GC	n.d.	*	*	*	n.d.
31	30.9	915	607	439		Dp-3-*p*-coumglc-GC	n.d.	*	*	*	n.d.
37	35.1	899				Cy-3-*p*-coumglc-GC	n.d.	*	*	n.d.	n.d.
38	35.4	929				Pt-3-*p*-coumglc-GC	n.d.	*	*	*	n.d.
48	38.5	913	605	437		Pn-3-*p*-coumglc-GC	n.d.	*	*	*	*
45	38.3	943	635	467		Mv-3-*p*-coumglc-GC	n.d.	*	*	*	*
66	41.1	943				Mv-3-*p*-coumglc-EGC	n.d.	*	*	*	*
6	10.8	753	591	439	282, 534	Dp-3-glc-C	*	*	*	*	*
7	14.8	753	591	439		Dp-3-glc-EC	*	*	*	*	*
8	14.9	737	575	423	286, 526	Cy-3-glc-C	*	*	*	*	*
11	18.0	737	575	423		Cy-3-glc-EC	*	*	*	*	*
9	16.2	767	605	453	279, 532	Pt-3-glc-C	*	*	*	*	*
16	21.6	767	605	453		Pt-3-glc-EC	*	*	*	*	*
12	20.3	751	589	437	283, 524	Pn-3-glc-C	*	*	*	*	*
20	24.3	751	589	437		Pn-3-glc-EC	*	*	*	*	*
14	21.0	781	619	467	280, 532	Mv-3-glc-C	*	*	*	*	*
29	29.9	781	619	467	279, 533	Mv-3-glc-EC	*	*	*	*	*
41	35.9	823	619	467		Mv-3-acetylglc-C	n.d.	*	*	*	*
51	39.0	899	591	439		Dp-3-*p*-coumglc-C	n.d.	*	*	*	*
58	40.5	899				Dp-3-*p*-coumglc-EC	n.d.	*	n.d.	n.d.	n.d.
54	39.5	883				Cy-3-*p*-coumglc-C	n.d.	*	*	*	n.d.
70	41.4	883				Cy-3-*p*-coumglc-EC	n.d.	*	n.d.	n.d.	n.d.
64	41.0	913	605	453		Pt-3-*p*-coumglc-C	n.d.	*	*	*	*
81	42.6	913				Pt-3-*p*-coumglc-EC	n.d.	*	n.d.	n.d.	n.d.
75	41.8	897	589	437		Pn-3-*p*-coumglc-C	n.d.	*	*	*	*
90	43.8	897				Pn-3-*p*-coumglc-EC	n.d.	*	n.d.	n.d.	n.d.
85	43.4	927	619	467	290, 538	Mv-3-*p*-coumglc-C	*	*	*	*	*
102	46.0	927				Mv-3-*p*-coumglc-EC	n.d.	*	n.d.	n.d.	n.d.

Rt: retention time; M^+: molecular ion; frag.: fragments obtained in MS^2 or MS^3 analyses; Dp: delphinidin; Cy: cyanidin; Pt: petunidin; Pn: peonidin; Mv: malvidin; Glc: glucose; acetylglc: 6″-acetylglucoside; *p*-coumglc: 6″-*p*-coumaroylglucoside; GC: gallocatechin; EGC: *epi*-gallocatechin; C: catechin; EC: *epi*-catechin; (*) detected; n.d.: not detected.

the eighth month and the latter ones were already present in the first analysed sample (see Table 3). Among acyl derivatives, *p*-coumaroyl ones seemed to react better, as the direct condensation products involving them were formed in higher amounts. Moreover, only the malvidin-3-acetylglucoside participated in the direct condensation reactions, whereas all the *trans*-*p*-coumaroyl derivatives reacted with the flavanols. The evolutions of (*epi*)catechin and (*epi*)gallocatechin derivatives were similar (Fig. 7). The most important difference was found in the ageing in the bottle period. The (*epi*)gallocatechin derivatives disappeared more slowly in this period than (*epi*)catechin ones. Furthermore, as the wine became older, the percentage of (*epi*)gallocatechin derivatives over the total direct condensation products increased, whereas that of (*epi*)catechin decreased, which might indicate higher resistance of direct (*epi*)gallocatechin–anthocyanin condensation products in relation to (*epi*)catechin ones. The fragmentation patterns observed for all these direct flavanol–anthocyanin condensation products indicated that they were F–A+ dimers, in which the flavanol

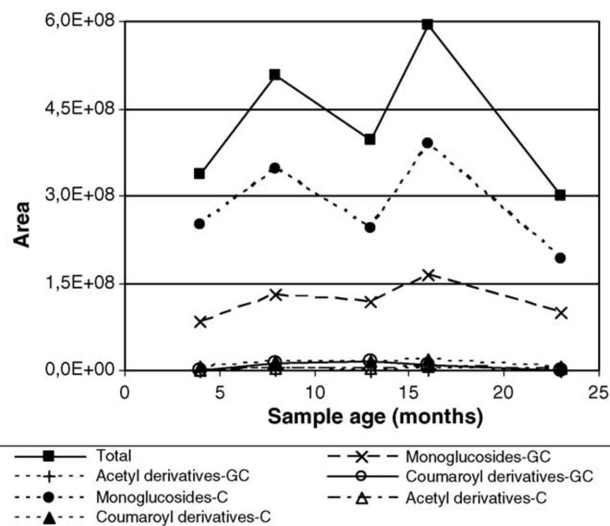

Fig. 7. Changes produced in the direct condensation products during maturation and ageing. GC: (*epi*)gallocatechin, C: (*epi*)catechin.

is the top unit of the dimer and the anthocyanin the base one [19,23]. The formation of these compounds requires the flavanol to be a carbonium ion and the anthocyanin to be in its hydrated hemiketal form [2]. This carbonium ion is originated in the cleavage of tannin interflavanic bonds from the top units of the flavanol oligomers and polymers. Taking into account the results obtained in model solutions [42] it seems reasonable that the breakdown of these procyanidins should also occur in wines, leading to a decrease in the mean polymerisation degree in time and a release of the carbonium ions to the wine. At wine pH, a high percentage (80%) of the anthocyanins are in their hydrated form [43] and, thus, they could act as nucleophiles and attack position 4 of the carbonium ion, originating the F–A+ compounds. These compounds have been synthesised in model solutions employing a protocol adapted from the synthesis of procyanidins and thus confirming the mechanism of formation [3]. During maturation and ageing in the barrel, as stated previously, oak wood continuously releases ellagitannins [38] and it has been shown that these compounds speed the condensation of procyanidins [37]. Taking into account that anthocyanins can compete with flavanols in the nucleophilic attack on the carbonium ion [42] and that F–A+ compounds have been synthesised similarly to procyanidin dimers [3], ellagitannins should also be expected to favour the direct condensation between flavanols and anthocyanins by the mechanism previously stated. Furthermore, ellagitannins, along with the available oxygen can create, in barrels, oxidative conditions that favour the cleavage of the interflavanic bond of proanthocyanidins [44] and, thus, release more carbonium ions. These hypotheses might explain the increase observed in the levels of these compounds during maturation and ageing in oak barrel, when ellagitannins are continuously extracted from the wood, and the subsequent decrease during ageing in the bottle, where, despite the existence of anthocyanins in hemiketal form, lesser amounts of carbonium ions are released and the condensation reaction is less favoured, since no more ellagitannins can be extracted.

3.2.4. Acetaldehyde-mediated anthocyanin–flavanol condensation products

The chromatographic and spectrum features of the compounds found in wine belonging to this family are shown in Table 4.

The UV–vis spectrum of compound **79** was characteristic of acetaldehyde-mediated anthocyanin–flavanol condensation products. Its retention time, *m*/*z* ratio of the molecular ion (809) and the fragmentation pattern indicated that it was one of the two possible diasteroisomers [4,19,23,45] originated in the acetaldehyde-mediated condensation between catechin and malvidin-3-glucoside. Compound **65** had the same spectrum features as compound **79** and eluted before it. This compound was the other diasteroisomer resulting from the reaction of malvidin-3-glucoside with ethyl-catechin. Compound **83** also showed the same molecular ion with the same fragmentation pattern, and taking into account the results of other studies carried out in our laboratory [4,19,23,45] it was identified as malvidin-3-glucoside-ethyl-*epi*-catechin. The molecular ions of compounds **101** and **106** possessed, respectively, 42 and 146 additional amu when compared to those of compounds **65**, **79** and **83**, and have been identified, also taking into account their fragmentation patterns, as malvidin-3-acetyl and malvidin-3-*p*-coumaroyl ethyl-catechin dimers, respectively.

The chromatographic and spectrum data of compounds **40**, **43**, **53**, **56**, **76** and **82** allowed their identification. For the assignment of the catechin isomer (*epi*- or not) involved in the reaction, the same criteria employed for the identification of compounds **65**, **79** and **83** were used. Their identities are shown in Table 4.

Compounds **59**, **69** and **74** possessed molecular ions with the same *m*/*z* ratio (825) and their fragmentation patterns and fragment ions originated in the MS^2 and MS^3 analyses were those described for malvidin-3-glucoside-ethyl-(*epi*)gallocatechin dimers [19]. Taking into account their elution order, amounts in each sample and by comparing them to those of malvidin-3-glucoside-ethyl-(*epi*)catechin dimers, they were identified as shown in Table 4. Similarly, compounds **35**, **49**, **50** and **62** were, respectively, identified as delphinidin, cyanidin, petunidin and peonidin 3-glucosides ethyl-gallocatechin dimers, and compound **95** as malvidin-3-acetylglucoside-ethyl-gallocatechin dimer.

All these identified compounds were found in the first sample but, as wine became older, most of them attained non-detectable levels, the malvidin derivatives being the only detected compounds (see Table 4). Thus, the highest levels of these compounds were reached in the first months of ageing in the barrel. The evolutions of (*epi*)gallocatechin and (*epi*)catechin derivatives were similar (Fig. 8). After the fourth month, the levels significantly decreased and remained practically constant, with slight fluctuations. This behaviour is in accordance with their reactivity [45]. They are rapidly formed, which explains why the highest levels were reached in the first sample, but they are also rapidly broken down releasing ethyl-(*epi*)catechin or ethyl-(*epi*)gallocatechin units which can, in turn, react again with anthocyanins or other dimers, giving rise to more condensed products or even polymers. In turn, those condensed products can also be broken down and release ethyl-flavanol units

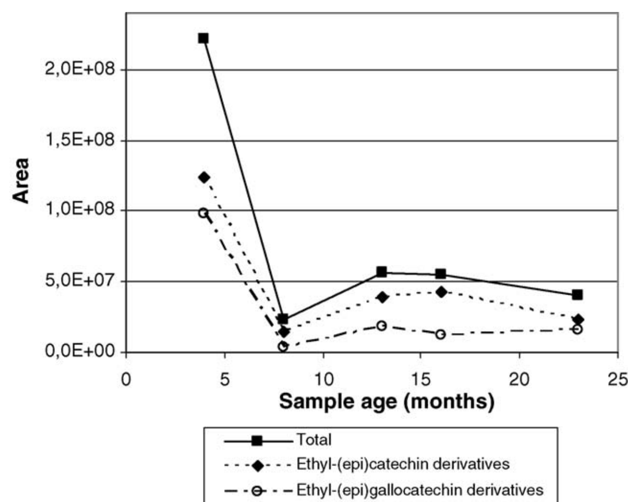

Fig. 8. Changes produced in the acetaldehyde-mediated condensation products during maturation and ageing.

Table 4
Chromatographic, UV–vis and MS spectrum data of the acetaldehyde-mediated condensation products detected as well as their identities and samples in which they have been found

Peak	Rt	M^+	MS^2 frag.	MS^3 frag.	λ_{max} (nm)	Compound	Sample (months)				
							4	8	13	16	23
40	35.8	781	329	329		Dp-3-glc-ethyl-C	*	n.d.	n.d.	*	n.d.
43	36.7	781				Dp-3-glc-ethyl-EC	*	n.d.	n.d.	*	n.d.
53	39.5	765				Cy-3-glc-ethyl-C	*	n.d.	n.d.	n.d.	n.d.
56	39.6	795	343	343		Pt-3-glc-ethyl-C	*	n.d.	n.d.	*	*
76	42.0	779	327	327		Pn-3-glc-ethyl-C	*	*	n.d.	*	*
82	43.1	779				Pn-3-glc-ethyl-EC	*	n.d.	n.d.	*	*
65	41.1	809	357	357		Mv-3-glc-ethyl-C	*	*	*	*	*
79	42.2	809	357	357	282, 539	Mv-3-glc-ethyl-C	*	*	*	*	*
83	43.1	809	357	357	276, 537	Mv-3-glc-ethyl-C	*	*	*	*	*
101	45.8	851	357	357		Mv-3-acetylglc-ethyl-C	*	*	n.d.	n.d.	n.d.
106	47.4	955	357	357		Mv-3-*p*-coumglc-ethyl-C	*	*	*	*	n.d.
35	34.7	797	329	329		Dp-3-glc-ethyl-GC	*	n.d.	n.d.	n.d.	*
49	38.6	781				Cy-3-glc-ethyl-GC	*	n.d.	n.d.	n.d.	n.d.
50	38.7	811	343	343		Pt-3-glc-ethyl-GC	*	n.d.	n.d.	*	*
62	40.9	795				Pn-3-glc-ethyl-GC	*	n.d.	n.d.	n.d.	n.d.
59	40.6	825	357	357		Mv-3-glc-ethyl-GC	*	n.d.	*	*	*
69	41.1	825	357	357	539	Mv-3-glc-ethyl-GC	*	*	*	*	*
74	41.8	825	357	357		Mv-3-glc-ethyl-GC	*	n.d.	*	*	*
95	45.0	867				Mv-3-acetylglc-ethyl-GC	*	n.d.	n.d.	n.d.	n.d.

Rt: retention time; M^+: molecular ion; frag.: fragments obtained in MS^2 or MS^3 analyses; Dp: delphinidin; Cy: cyanidin; Pt: petunidin; Pn: peonidin; Mv: malvidin; Glc: glucose; acetylglc: 6″-acetylglucoside; *p*-coumglc: 6″-*p*-coumaroylglucoside; GC: gallocatechin; EGC: *epi*-gallocatechin; C: catechin; EC: *epi*-catechin; (*) detected; n.d.: not detected.

which can originate new anthocyanin–ethyl-flavanol dimers that can be detected, thus explaining the fluctuation observed during the last months of ageing in the barrel and bottle (Fig. 8).

3.3. Colour contribution of each pigment family

Fig. 9, shows the relative amounts of each pigment family in the samples, calculated as a percentage of their MS areas over the total area. For calculating the total area not only the areas of all the identified compounds were considered but also those of unknown compounds which were detected in the samples and could contribute to wine colour. Eighty to 90% of the total area was due, in all the samples, to the anthocyanin family. However, the contribution to the wine colour was not so high, since at wine pH, approximately only 15% of the anthocyanins are in the flavylium coloured form [43]. On the contrary, anthocyanin-derived pigments are almost all in coloured forms at wine pH. Thus, although the percentages corresponding to anthocyanin-derived pigments were lower than that of anthocyanins, their contributions to wine colour might be as important as that of anthocyanins. In Fig. 10, only the relative percentages of the anthocyanin-derived pigments are considered in order to explain their contribution to wine colour. The direct flavanol–anthocyanin condensation products have their visible maxima at wavelengths slightly higher than the anthocyanins from which they are formed (see Tables 1 and 3). Thus, they will give a slightly bluish hue to the wine. The visible maxima of the acetaldehyde-mediated flavanol–anthocyanin condensation products (circa 540 nm) are bathochromically shifted in relation to those of anthocyanins and direct condensation products and, therefore, they will give the wine a more bluish hue. All the pyranoanthocyanins have their visible absorbance maxima at lower wavelengths than anthocyanins, thus providing an orange hue to the wine. However, there are important differences in λ_{max} value according to the nature of the pyranoanthocyanin. A-type vitisins, vinylcatechol and vinylguaiacol adducts of malvidin-3-glucoside have their absorbance maxima within the range of 510–515 nm (see Table 2), giving the wine a reddish-orange hue. Vinylphenol adducts would give the wine an orange hue, since their maxima are close to 500–503 nm. B-type vitisins and acetone derivatives have their visible maxima close to 490 nm in the first case, and close to 480 nm in the second. The colour provided by these two compounds is also orange, but with a brownish hue. The knowledge of the colour provided by each kind of pigment, along with their relative amounts and correspondence with their concentrations in wine, is necessary for the assessment of the contribution of each pigment family to the final colour of the wine. In the case of B-type vitisins, as previously stated, it has still to be demonstrated whether their high MS areas really correspond to high concentrations in order to establish if their high relative amounts in the first samples are real.

Taking into account all these considerations and looking at Fig. 10, it can be seen that the relative amounts of compounds which give orange hues to the samples increase as wine becomes older and that those of compounds that give blue hues decrease. The colour provided by the anthocyanins at wine pH would be modified towards bluish or orange hues in accordance with the proportion of each type of compounds and this would explain the change in the colour, from purple-bluish hues to orange ones, undergone by wine as it becomes older.

Fig. 9. Relative contents of the main pigment families and changes observed during maturation and ageing of the wine.

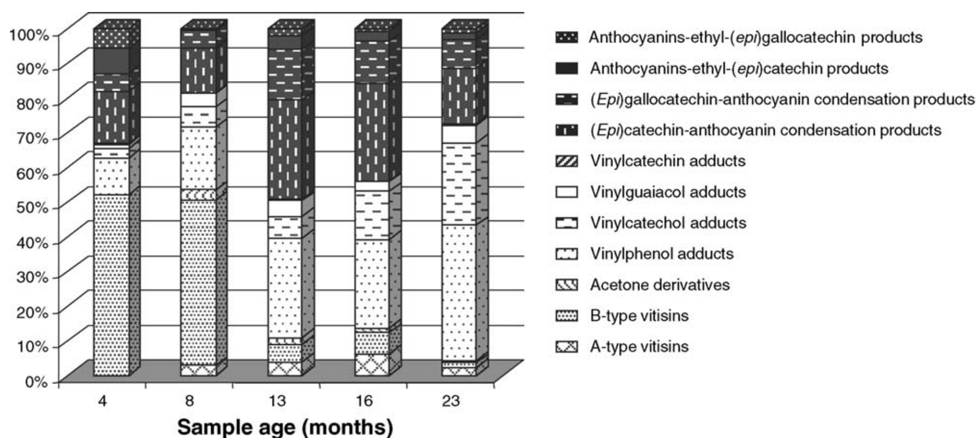

Fig. 10. Relative contents of the main anthocyanin-derived pigments, considering only the derived pigments in each sample.

4. Conclusions

The changes occurring in the pigment composition of a wine during its maturation and ageing have been studied here. The fractionation of the samples has allowed the detection and identification of 129 different compounds, belonging to four pigment families: anthocyanins, pyranoanthocyanins, direct flavanol–anthocyanin condensation products and acetaldehyde-mediated flavanol–anthocyanin condensation products. Some of these compounds have been described here for the first time. The fractionation has also allowed the monitoring of the quantitative changes undergone by the detected compounds during wine maturation and ageing in oak barrels and during ageing in the bottle. From these data, the evolutions of the levels of each pigment family have been established. Thus, it has been shown that the levels of the anthocyanins decrease with ageing, probably due, at first, to the formation of new anthocyanin-derived pigments, and, during ageing in the bottle, to a degradation of all the pigments present in wines. During the first months of ageing in the barrel, B-type vitisins, vinylphenol, vinylcatechol and vinylguaiacol adducts, direct and acetaldehyde-mediated condensation products are formed, whereas A-type vitisins and acetone derivatives are formed later. The levels of A-type vitisins and direct condensation products increased during the ageing in the barrel, but decreased in the ageing in the bottle. The levels of acetone derivatives and B-type vitisins reached the highest values at the first months of maturation in the barrel and then decreased, remaining more or less stable during ageing. Vinylphenol, vinylcatechol and vinylguaiacol adducts showed two periods of formation: one, during maturation in the barrel and the other during ageing in the barrel and bottle, the mechanisms of reaction being different in both periods. Vinylphenol and vinylguaiacol adducts reached the highest levels during the first period of synthesis, whereas vinylcatechol adducts were originated in higher amounts from caffeic acid. Thus, as wine became older, the percentages of orange compounds increased, partially explaining the changes in colour produced during wine ageing. It has been shown that the levels of A-type vitisins, direct condensation products and anthocyanins are the most affected by the transfer of the wine from barrel to bottle during its ageing. Ellagitannins released by oak barrel might favour the synthesis of these two anthocyanin-derived pigments and might protect anthocyanins against degradation. On the contrary, the changes produced in the rest of the anthocyanin-derived pigments seemed to be more influenced by the concentrations of the products taking part in their formation reaction than by the environment in which it took place, either in the barrel or in the bottle.

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