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Studying the evolution of anthocyanin-derived pigments in a typical red wine of Southern Italy to assess its resistance to aging

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ABSTRACT

In this study, the pigments of a young and 2 years old Primitivo red wines were investigated by using a column chromatography purification/fractionation step followed by HPLC-UV-ESI-MS^n. Compounds such as malvidin 3O-acetyl-4-vinyl-procyanidin, malvidin 3O-glucoside-di(epi)-catechin, and A-type peonidin and malvidin 3O-glucoside-(epi)catechin were identified in Primitivo for the first time; furthermore, this is a further evidence that malvidin 3O-glucoside-malvidin 3O-(*p*-coumaroyl)-glucoside and (malvidin 3O-glucoside)$_3$ can survive to the wine aging. Overall, the found anthocyanin-derived pigments were formed by malvidin, peonidin, and petunidin, which were mainly involved in the formation of pyranonathocyanins derivatives, accounting for 49% of the total content in the aged wine. The relative amounts of compounds, such as flavanol-anthocyanin adducts, which gave blue hues to the samples decreased whilst the reddish or violet ethyledene-bridge favanol-anthocyanin adducts increased in the aged wine. The predominance of pyranoanthocyanins, characterized by known higher stability compared to ethylidene-bridge favanol-anthocyanin and anthocyanin-flavanol compounds, and maybe their early formation (within two years) could be responsible for the reported rapid change of Primitivo color into orange hues, but also it would be able to slow down the natural loss of structure which wine is subject to.

1. Introduction

Primitivo is a red grape variety cultivated in Apulia region (Southern Italy), often resulting in a tannic, ruby-purple wine with spicy and red-fruit flavor. Primitivo is genetically identical to California Zinfandel (Bowers, Bandman, & Meredith, 1993) and Croatian Crljenak Kaštelanski and constitutes one parent of Croatian Plavac mali (Maletić et al. 2004).

Anthocyanins are the main compounds involved in the color of black and red grapes; in *Vitis vinifera* L. the monoglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin but also their acetyl-, *p*-coumaroyl-, and caffeoyl derivatives are present in quantities varying among varieties, climate, and viticulture conditions (Crupi et al., 2012; Coletta et al., 2013). At winemaking, they are partially extracted to the must becoming responsible for the color of young red wine (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006); then, during wine maturation and aging, their levels decrease with time, since they begin to react with other wine constituents leading to the formation of more stable pigments that are responsible for color changes as well as loss in astringency (Sun & Spranger, 2005).

Anthocyanins can condense with flavan-3-ols, as demonstrated in wines and model solutions, either directly (Salas et al., 2004; Hayasaka & Kennedy, 2003; Sun, Fernandes, & Spranger, 2010) or mediated by acetaldehyde (Atasanova, Fulckrand, Cheynier, & Moutounet, 2002; He et al., 2012) or other compounds (Pissarra et al., 2004). Moreover, they can give rise to cyclo-addition reaction at the carbon in position 4 and the hydroxyl group in position 5 with pyruvic acid (Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998; Romero & Bakker, 1999), acetaldehyde (Bakker

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& Timberlake, 1997), vinylphenol derivatives (Fulcrand, Dos Santos, Sarni-Manchado, Cheynier, & Favre-Bonvin, 1996; Hakånsson, Pardon, Hayasaka, De Sa, & Herderich, 2003; Hayasaka & Asenstorfer, 2002), and vinylflavanols (Mateus, Silva, Santos-Buelga, Rivas-Gonzalo, & De Freitas, 2002; Mateus et al., 2003), originating pyranoanthocyanins, which have been detected in red wine (Pati et al., 2006; Alcade-Eon, Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2006).

Because of the large number of anthocyanin-derived pigments and the low levels of some of them, especially in old red wines (Alcade-Eon et al., 2006), their analysis generally requires preliminary fractionation/purification steps (i.e. solid-phase extraction or column chromatography) prior to off-line chromatographic separation and identification (Gergely et al., 2000; Alcade-Eon, Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2007; Sun et al., 2010). Moreover, the attempt of overcoming the possible drawbacks (mainly time-consuming, hydrolysis phenomena, and irreversible absorption) of the aforementioned fractionation/purification methods by direct injection onto HPLC systems of raw red wine diluted samples without any pre-treatment has been limited by the difficulty of identifying and separating some minor pigments in aged wines from more concentrated compounds with which they coexist (Pati et al., 2006; De Villers, Vanhoenacker, Majek, & Sandra, 2004). This is particularly hard among the anthocyanins and their corresponding type A vitisins, which in the majority of the reversed phase LC methods elute very close together (Alcade-Eon, Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2004; Rentzsch, Schwartz, Winterhalter, Blanco-Vega, & Hermosin-Gutiérrez, 2010).

High performance liquid chromatography (HPLC) coupled to UV and mass spectrometry (MS) detections have provided decisive information for the identification of a large number of pigment families based on their spectroscopic features and fragmentation patterns (Alcade-Eon et al., 2007; Pati et al., 2006; Sun et al., 2010; He et al., 2012). However, in spite of the numerous wine polyphenols nowadays identified, the influence of a long aging period on the presence of oligomeric and polymeric pigments in Primitivo wine has been still few investigated.

Therefore, the goal of this study was to characterize the evolution of the anthocyanin-derived pigments present in a young and 2 years old Primitivo red wines by using a column chromatography purification/fractionation step followed by HPLC-UV-ESI-MS^n. Grouping this information could get new insight about the low color stability of this wine, a consistent problem still facing the wine makers, in order to better define the effective potential to age of Primitivo.

2. Materials and methods

2.1. Wine samples

2005 vintage grapes from a Primitivo 25 year old vineyard in Manduria territory (Taranto, Southern Italy), were picked at the technological maturity and processed according to the following winemaking procedure. About 300 kg of grapes (sugar content 19.3 °Brix, pH 3.35, total acidity 7.3 g L^{-1} of tartaric acid equivalents) previously destemmed, were crushed and collected in stainless steel 100-Kg-capacity tanks (three replicates). Before starting the fermentation, the crushed grapes were supplied with potassium metabisulphite (15 g 100 kg^{-1}), dry yeasts (15 g 100 kg^{-1}, *Saccharomyces cerevisiae*, Zymasil, AEB, Brescia, Italy), and yeast activator (25 g 100 kg^{-1}, Bioact Plus, Oliver Ogar, Montebello Vicentino, Italy). During the alcoholic fermentation (10 days long), two daily gentle punching down of the pulp cap into the fermenting juice were made. After running-off and light pressure of the marcs, the malolactic fermentation occurred spontaneously; afterwards, the wine was decanted and a second aliquot of potassium metabisulphite (15 g 100 kg^{-1}) was added. Finally, after another decantation performed in March, the wine was bottled in 1 L glass bottles and stored in the dark under controlled conditions of temperature (15–18 °C), for 2 years.

Red wine was analyzed at the end of fermentations and after 2 years.

2.2. Chemicals

Trifluoroacetic acid, ethanol RPE, and HPLC grade water were purchased from J.T. Baker (Deventer - Holland). LC-MS grade solvent acetonitrile was purchased from Riedel-de Haën (Steinheim - Germany). Delphinidin, cyanidin, petunidin, peonidin, and malvidin 3-O-glucoside chloride was purchased from Extrasynthese (Genay – France).

2.3. Wine fractionation

For fractionation of the aged wine, 10 mL of sample was placed onto a 230 × 15 mm i.d. Toyopearl HW-40(S) gel column (Tosoh, Japan) which was then passed with 150 mL distilled water to wash out phenolic acids. The elution solvent was ethanol/0.1% aqueous trifluoroacetic acid (60:40, v/v) by which all the pigments retained in the column were eluted and collected in 10 fractions of 5 mL. Flow rate was regulated at 0.2 mL min^{-1} using a peristaltic pump. Finally, since there was no interest in correlating the fractions composition to sensorial characteristic such as in other works (Weber, Greve, Durner, Fischer, & Winterhalter, 2013; Wollmann & Hofmann, 2013), for reducing the analysis time the fractions 1 and 2 (A), 3–6 (B), 7–10 (C) were grouped together, respectively, on the basis of their UV–Vis maxima wavelengths (λ_{max}) registered in the range 190–700 nm and of their chromatographic behavior.

The young wine was analyzed without fractionation as reported by Pati et al. (2006).

2.4. HPLC-UV-ESI-MS analysis

As previously described (Pati et al., 2006), separation and identification of anthocyanin-derived pigments were carried out by means of an HPLC-UV-ESI-MS^n system consisting of a 600-MS multi solvent delivery pump (Waters, Milford, MA, USA), an UV–Vis detector (model G1315B DAD system, 1100 series Agilent Technologies, Palo Alto, CA, U.S.A.) and an LCQ ion trap mass spectrometer (Thermo Electron Co., San Jose, CA, USA).

Briefly, after filtration through 0.45 μm pore size regenerated cellulose filters (VWR International, USA) wine diluted samples (1:2, v/v with water) and/or the separated fractions were injected onto a reversed stationary phase column, Luna C18 (150 × 2 mm i.d., particle size 3 μm, Phenomenex, USA) protected by a C18 Guard Cartridge (4.0 × 2.0 mm i.d., Phenomenex). HPLC separation was accomplished using a binary mobile phase composed of (solvent A) water/acetonitrile (95:5, v/v) and (solvent B) water/acetonitrile (10:90, v/v), both containing 0.1% (v/v) trifluoroacetic acid. The following gradient was adopted: isocratic 2% B for 2 min; from 2 to 10% B for 6 min; from 10 to 13% B for 22 min; from 13 to 20% B for 20 min; from 20 to 30% B for 25 min; followed by washing and re-equilibrating the column. The column temperature was not controlled and the flow was maintained at 0.2 mL min^{-1}. UV–Vis detection wavelength was set at 520 nm. HPLC-ESI-MS data were acquired under positive ion mode using Xcalibur (Thermoquest) software. In the preliminary stage, an optimization of the ESI interface and ion optics parameters was accomplished in order to maximize the signal of malvidin 3-O-glucoside ion (*m/z* 493), chosen as the reference compound. To this aim, a 40-ppm solution

of malvidin 3-O-glucoside in solvent A was infused (at a flow rate of 20 μL/min) in the ESI interface using the syringe pump incorporated into the mass spectrometer. The optimized electrospray/ion optics parameters were as follows: spray voltage, 4.5 kV; sheath gas (nitrogen), 0.9 L min^{-1}; capillary voltage, 35.0 V; capillary temperature, 200 °C; tube lens offset voltage, 15.0 V.

Typically, three runs were performed during HPLC-ESI-MS analysis of each sample. First, an MS full-scan acquisition (*m/z* range 50–2000) was performed to obtain preliminary information on the predominant *m/z* ratios observed during the elution. Subsequently, MS^2 spectra were acquired by fragmenting some precursor ions selected in the MS spectra. Tentative compound identification was achieved by combining different information: UV absorption, capacity factors (k'), and mass spectra (MS and MS^2) which were compared with those from pure standards, when available, and interpreted with the help of structural models already hypothesized in the literature (Alcade-Eon et al., 2004; Alcade-Eon et al., 2007; Pati et al., 2006). Semi-quantitation was performed using extracted ion chromatograms (EIC): for each compound, the EIC at the corresponding molecular ion was obtained and the relevant peak was integrated; subsequently, peak areas were summed with respect to the type of pigment to calculate the percentage content of the different classes determined in the wines.

3. Results and discussion

3.1. HPLC-UV-MS analysis of raw wine

Fig. 1 shows the chromatogram of a young and 2 years old Primitivo raw wine samples diluted (1:2, v/v) with water. Unlike the young Primitivo wine (Fig. 1a), it was difficult to achieve a total identification of the compounds present in the older wine (Fig. 1b). This drawback is very frequent in ESI-MS analysis of minor constituents in complex matrix (like aged wine) and is probably caused by competitive ionization between the low concentrated analytes of interest and the matrix. Moreover, as it usually happens for aged wines (Wang, Race, & Shrikhande, 2003; Alcade-Eon et al., 2004; Blanco-Vega, Gómez-Alonso, & Hermosín-Gutiérrez, 2014), the "hump" under the chromatogram indicated the presence of a lot of not-well resolved peaks corresponding to co-eluting species. Only the identity of 7 pigments could be tentatively assigned by means of their k' and molecular ions $[M]^+$ in their mass spectra (Table 1). Therefore, a fractionation of this wine was needed to obtain a more complete analysis of the pigments present, by eliminating those compounds responsible of the "hump" in the chromatogram of the raw aged wine (Fig. 1b).

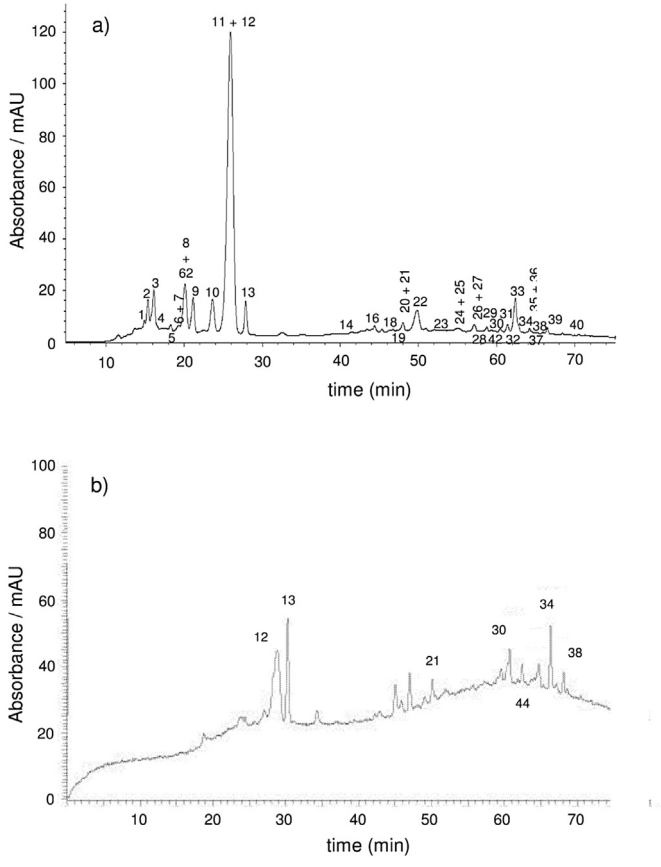

Fig. 1. HPLC-UV-Vis chromatogram of the raw a) young and b) 2 years old red wine (Primitivo cv.) recorded at 520 nm.

In the elution of the wine through the Toyopearl HW-S gel column with ethanol/0.1% aqueous trifluoroacetic acid (60:40, v/v), different colored bands were formed. As previously stated, the eluates were collected in 10 fractions, which were further grouped in 3 fractions according to their UV–Vis spectra and chromatographic behavior in order to simplify the analysis. Indeed, preliminary HPLC-UV-Vis analyses of all the eluates were carried out in order to determine the chemical nature of the major components of each; the eluates 1 and 2, 3–6, and 7–10, respectively, showed similar chromatograms, with peaks that had the same retention times and UV–Vis spectra in all cases, only differing in the proportions in which they were present in the different eluates. Thus, "fraction A" was formed by the combination of eluates 1 and 2 and was mainly characterized by anthocyanidins monoglucosides and different types of pyranoanthocyanins, whilst "fraction B" and "fraction C" contained prevalently vinyl-linked anthocyanin-flavonol and ethyl-linked anthocyanin-flavonol, were obtained grouping together the eluates 3–6 and 7–10, respectively (Table 1, Fig. 2).

3.2. HPLC-UV-MS analysis of young wine and aged wine fractions

Fig. 3 shows the UV–Vis chromatograms at 520 nm of fractions A-C of the 2 years old Primitivo wine, in which more than 50 compounds were identified. Their k' and ions from MS and MS/MS analyses were reported in Table 1. Peaks 8, 10, and 12 were identified as the monoglucosides of petunidin, peonidin, and malvidin, respectively, as reported elsewhere (Pati et al., 2006); likewise, peaks 22, 24, and 27 were attributed to malvidin 3O-acetylglucoside, malvidin 3O-caffeoylglucoside, and mavidin 3O-*p*-coumaroylglucoside. As shown by the fragmentation pattern equivalent to compound 27, another malvidin 3O-*p*-coumaroylglucoside was also observed into the fraction B (peak 33, Fig. 3b), thus, on the basis of their elution order, it was assumed that those molecules (peaks 27 and 33) were *cis*- and *trans*-isomers (Crupi et al., 2012). It is worth pointing that the afore mentioned compounds were found in both wines, whilst other acyl anthocyanins, such as peonidin 3O-acetylglucoside (peak 20) and cyanidin, petunidin, and peonidin 3O-(*p*-coumaroyl)-glucoside (peaks 25, 26, and 32), as well as delphinidin and cyanidin glucosides (peaks 3 and 5) were only detected in the young wine (Fig. 1a, Table 1).

Five compounds corresponding to carboxy-pyranoanthocyanins derived from the reaction between glucosides anthocyanidins and pyruvic acid (A-type vitisins, Fig. 2b) were also identified in both wines. In particular, peaks 9, 11, and 13 were attributed to petunidin, peonidin, and malvidin 3O-glucoside pyruvic derivatives because their molecular ions in the MS analysis (*m/z* 547, 531, and 561, respectively) were fragmented into aglycones (*m/z* 385, 369, and 399, respectively) by losing a dehydrated-glucose moiety

Table 1
Chromatographic and mass spectral data of the compounds identified into Primitivo young wine and 2 year old wine fractions.

Peak	k'	Compound[a]	$[M]^+$ (*m/z*)	MS/MS	Sample	
					Young	Old
Fraction A						
Free anthocyanins						
3	5.83	Delphinidin 3O-glucoside[b]	465	303	*	n.d.
5	6.71	Cyanidin 3O-glucoside[b]	449	287	*	n.d.
8	7.50	Petunidin 3O-glucoside[b]	479	317	*	*
10	9.04	Peonidin 3O-glucoside[b]	463	301	*	*
12	9.90	Malvidin 3O-glucoside[b]	493	331	*	*
20	19.04	Peonidin 3O-acetylglucoside[c]	505	301	*	n.d.
22	19.50	Malvidin 3O-acetylglucoside[c]	535	331	*	*
24	21.78	Malvidin 3O-caffeoylglucoside[c,i]	655	331	*	*
25	21.90	Cyanidin 3O-(*p*-coumaroyl)-glucoside[c,i]	595	287	*	n.d.
26	22.76	Petunidin 3O-(*p*-coumaroyl)-glucoside[c,i]	625	317	*	n.d.
27	22.83	Malvidin 3O-*cis*-(*p*-coumaroyl)-glucoside[d,i]	639	331	*	*
32	24.63	Peonidin 3O-(*p*-coumaroyl)-glucoside[c,i]	609	301	*	n.d.
A-type vitisins						
9	7.67	Petunidin 3O-glucoside pyruvic derivative[e]	547	385	*	*
11	9.78	Peonidin 3O-glucoside pyruvic derivative[e]	531	369	*	*
13	10.57	Malvidin 3O-glucoside pyruvic derivative[e]	561	399	*	*
19	18.53	Peonidin 3O-(*p*-coumaroyl)-glucoside pyruvic derivative[e,i]	677	369	*	*
21	19.06	Malvidin 3O-(*p*-coumaroyl)-glucoside pyruvic derivative[e,i]	707	399	*	*
Hydroxyphenyl pyranoanthocyanins						
41	24.03	Petunidin 3O-glucoside-4-vinyl-phenol[e]	595	433	n.d.	*
43	24.61	Peonidin 3O-glucoside-4-vinyl-guaiacol[e]	609	447	n.d.	*
44	24.65	Malvidin 3O-glucoside-4-vinyl-guaiacol[e]	639	477	n.d.	*
45	24.71	Peonidin 3O-glucoside-4-vinyl-catechol[e]	595	433	n.d.	*
36	25.88	Malvidin 3O-glucoside-4-vinyl-catechol[e]	625	463	*	*
37	26.11	Peonidin 3O-glucoside-4-vinyl-phenol[e]	579	417	*	*
39	26.71	Malvidin 3O-glucoside-4-vinyl-phenol[e]	609	447	*	*
Flavanol pyranoanthocyanins						
23	21.04	Malvidin 3O-acetylglucoside-4-vinyl-(epi)catechin[c]	847	643,491	*	*
30	23.79	Malvidin 3O-glucoside-4-vinyl-(epi)catechin[c]	805	643,491	*	*
34	25.19	Malvidin 3O-glucoside-4-vinyl-(epi)catechin[c]	805	643,491	*	*
8,8-Ethylidene Flavanol-Anthocyanin adducts						
14	15.63	Malvidin 3O-glucoside-8-ethyl-(epi)catechin[c,e]	809	647,519,357	*	*
16	17.08	Malvidin 3O-glucoside-8-ethyl-(epi)catechin[c,e]	809	647,519,357	*	*
18	17.94	Malvidin 3O-glucoside-8-ethyl-(epi)catechin[c,e]	809	647,519,357	*	*
29	23.50	Peonidin 3O-(*p*-coumaroyl)-glucoside-8-ethyl-(epi)catechin[c,e,i]	925	635,617,327	*	*
31	24.21	Malvidin 3O-(*p*-coumaroyl)-glucoside-8-ethyl-(epi)catechin[c,e,i]	955	665,647,357	*	*
40	27.95	Malvidin 3O-(*p*-coumaroyl)-glucoside-8-ethyl-(epi)catechin[c,e,i]	955	665,647,357	*	*
Flavanol-anthocyanin adducts						
1	5.29	(epi)catechin-peonidin 3O-glucoside[c]	751	589, 463, 437	*	n.d.
2	5.50	(epi)catechin-malvidin 3O-glucoside[c]	781	619, 493, 467	*	n.d.
	5.88	di(epi)catechin-malvidin 3O-glucoside[c]	1069	907, 781, 619	*	n.d.
6	7.21	(epi)catechin-malvidin 3O-glucoside[c]	781	619, 493, 467	*	n.d.
7	7.25	di(epi)catechin-malvidin 3O-glucoside[c]	1069	907, 781, 619	*	n.d.
Anthocyanin dimers and trimers						
62	7.5	$(\text{Malvidin glucoside})_3$[h]	1477	1315, 1153, 822	*	*
42	24.08	Malvidin 3O-glucoside malvidin 3O-(*p*-coumaroyl)-glucoside[e]	1131	969,823,661	*	*
Fraction B						
Anthocyanin-tannin derivative						
46	8.09	Malvidin 3O-glucoside-(epi)catechin[g]	783	631,621,469	n.d.	*
47	9.39	Peonidin 3O-glucoside-(epi)catechin[g]	753	601,591,439	n.d.	*
Flavanol pyranoanthocyanins						
15	17.00	Malvidin 3O-glucoside-4-vinyl-di(epi)catechin[c]	1093	931,803,641	*	*
17	17.75	Malvidin 3O-glucoside-4-vinyl-di(epi)catechin[c]	1093	931,803,641	*	*
48	18.53	Malvidin 3O-acetylglucoside-4-vinyl-di(epi)catechin[f]	1135	931,845	n.d.	*
49	23.50	Petunidin 3O-glucoside-4-vinyl-(epi)catechin	791	629,477	n.d.	*
50	25.34	Peonidin 3O-glucoside-4-vinyl-(epi)catechin	775	613,461	n.d.	*
35	25.82	Malvidin 3O-(*p*-coumaroyl)-glucoside-4-vinyl-catechin[c,i]	951	643,491	*	*
38	26.50	Malvidin 3O-(*p*-coumaroyl)-glucoside-4-vinyl-(epi)catechin[c,i]	951	643,491	*	*
Free Anthocyanins						
27	22.76	Malvidin 3O-*cis*-(*p*-coumaroyl)-glucoside[d,i]	639	331	*	*
33	25.00	Malvidin 3O-*trans*-(*p*-coumaroyl)-glucoside[d,i]	639	331	*	*
Fraction C						
Anthocyanin-Flavanol adducts						
51	10.80	Malvidin 3O-glucoside-di(epi)catechin[f]	1071	919,909,781	n.d.	*
8,8-Ethylidene Flavanol-Anthocyanin adducts						
53	14.96	Malvidin 3O-glucoside-8-ethyl-(epi)catechin[c,e]	809	647,519,357	n.d.	*
54	16.41	Malvidin 3O-glucoside-8-ethyl-(epi)catechin[c,e]	809	647,519,357	n.d.	*
55	17.27	Malvidin 3O-glucoside-8-ethyl-(epi)catechin[c,e]	809	647,519,357	n.d.	*
59	24.49	Malvidin 3O-(*p*-coumaroyl)-glucoside 8-ethyl-(epi)catechin[c,e,i]	955	665,357	n.d.	*
60	27.14	Malvidin 3O-(*p*-coumaroyl)-glucoside 8-ethyl-(epi)catechin[c,e,i]	955	665,357	n.d.	*
61	27.45	Malvidin 3O-(*p*-coumaroyl)-glucoside 8-ethyl-(epi)catechin[c,e,i]	955	665,357	n.d.	*

Table 1 (*continued*)

Peak	k'	Compound[a]	$[M]^+$ (*m/z*)	MS/MS	Sample	
					Young	Old
Flavanol-pyranoanthocyanins						
52	14.02	Malvidin 3O-glucoside-4-vinyl-tri(epi)catechin[c]	1381	1219,803	n.d.	*
56	21.03	Malvidin 3O-(caffeoyl)-glucoside 4-vinyl-di(epi)catechin[c,i]	1255	965,931,641	n.d.	*
28	23.08	Malvidin 3O-(*p*-coumaroyl)-glucoside 4-vinyl-di(epi)catechin[c,i]	1239	931,641	*	*
57	23.51	Malvidin 3O-(*p*-coumaroyl)-glucoside 4-vinyl-di(epi)catechin[c,i]	1239	931,641	n.d.	*
58	23.83	Malvidin 3O-(*p*-coumaroyl)-glucoside 4-vinyl-di(epi)catechin[c,i]	1239	931,641	n.d.	*

k': capacity factor values; M^+: molecular ions; MS/MS: product ions; *: detected; n.d.: not detected.
[a] Detailed structures are reported as Supplementary Material (Figure S1 – S5).
[b] Identified by comparison with standard compounds.
[c] Identified by comparison with literature data (Pati et al., 2006; Asenstorfer et al., 2001).
[d] Identified by comparison with literature data (Crupi et al., 2012).
[e] Identified by comparison with literature data (Alcade-Eon et al., 2004; Heier et al., 2002).
[f] Identified by comparison with literature data (Blanco-Vega et al., 2014).
[g] Identified by comparison with literature data (Duenās et al., 2006).
[h] Identified by comparison with literature data (Oliveira, da Silva, Parola, Mateus, Brás, João Ramos, & de Freitas, 2013).
[i] The characteristic absorption maximum around 320 nm in the UV–Vis spectra confirmed the presence of coumaroyl or caffeoyl moieties in the molecules.

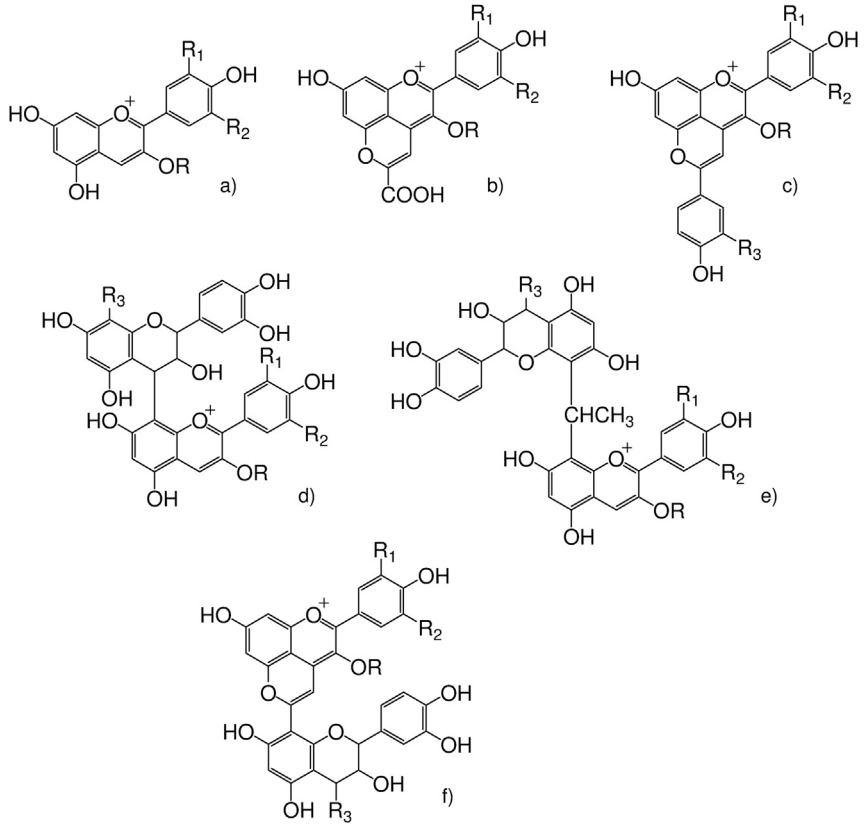

Fig. 2. Structures of the main anthocyanins and anthocyanin-derived red wine pigments found in Primitivo: a) grape native anthocyanins; b) A-type vitisins; c) hydroxyphenyl-pyranoanthocyanins or pinotins (R_3 = H, phenol-pyranoanthocyanins; R_3 = OH, catechol-pyranoanthocyanins; R_3 = OCH_3, guaiacol-pyranoanthocyanins); d) (4 → 8)-anthocyanin-flavanol adducts (R_3 = H or (epi)catechin); e) 8,8-ethylidene-flavanol-anthocyanin adducts (R_3 = H or (epi)catechin); f) 10-flavanol-pyranoanthocyanins (R_3 = H or (epi)catechin). For all structures: R = glucose, *p*-coumaroyl, or caffeoyl glucose; R_1 = H and R_2 = OH, OCH_3, R_1 = OH and R_2 = OH, OCH_3, or R_1 and R_2 = OCH_3.

(Table 1). While compounds 19 and 21 can be proposed as peonidin and malvidin 3O-*p*-coumaroyl-glucoside pyruvic derivatives, since they had molecular ions (*m/z* 677 and 707) 146 u heavier respect to peaks 11 and 13 (Pati et al., 2006; Alcade-Eon et al., 2004; Heier, Blaas, Droβ, & Wittkowski, 2002). Acetylated A-type vitisin was absent in according with what reported in model solutions (Romero & Bakker, 1999) and in wine (Alcade-Eon et al., 2006).

Similarly to other red wines (Hayasaka & Asenstorfer, 2002), seven hydroxyphenyl-pyranoanthocyanins (pinotins) originated from the cycloaddition between anthocyanidins glucosides and vinylphenol, vinylcatechol, or vinylguaiacol were revealed (Fig. 2c). By matching the information from their MS intense signals (*m/z* 595, 579, and 609) and MS/MS product ions (*m/z* 433, 417, and 447), together with their elution order to those present in the literature (Alcade-Eon et al., 2004), peaks 41, 37, and 39 were assigned to petunidin, peonidin, and malvidin 3O-glucoside-4-vinyl-phenol, respectively. Then, because of the appearance in their mass spectra of molecular ions and aglycones of 16 and 30 u heavier than compounds 37 and 39 (maybe due to an additional hydroxyl or methoxyl group in the vinylphenol structure) and considering the

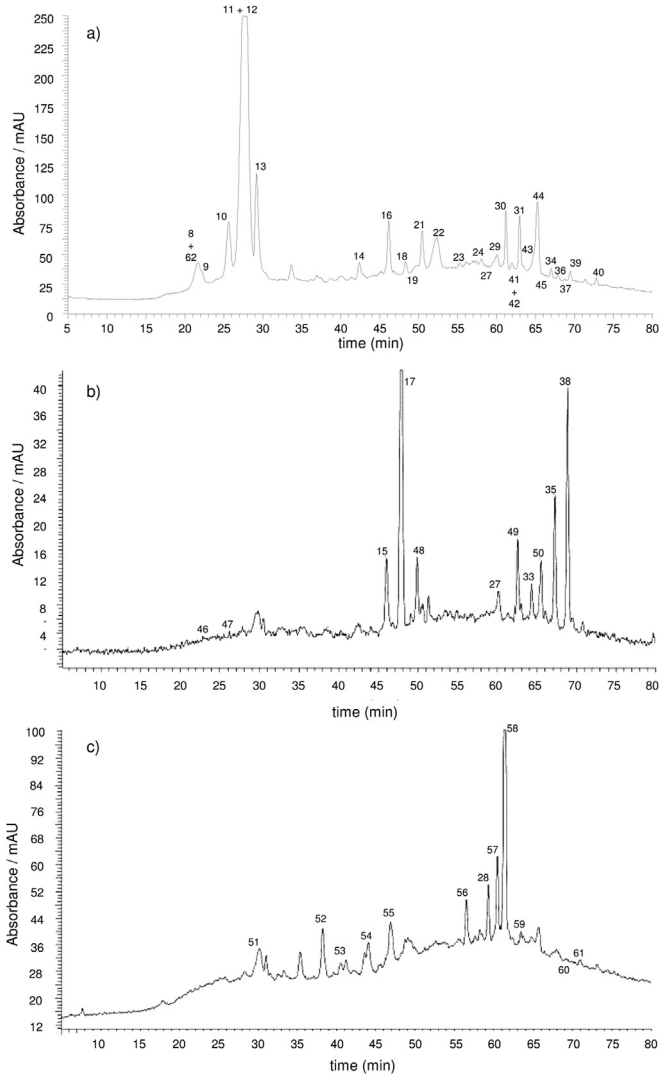

Fig. 3. HPLC-UV-Vis chromatogram of a) fraction A, b) fraction B, and c) fraction C recorded at 520 nm.

elution order which respect the decreasing polarity, the peaks 43, 44 and the peaks 45, 36 were identified as pyranoanthocyanins formed by reaction of peonidin and malvidin 3O-glucoside with vinylguaiacol and vinylcatechol, respectively (Table 1). It is worth noting that the compounds 36, 37, and 39 were found into both wines, while the other pinotins were only present into the older sample (Figs. 1a and 3a).

Some well resolved chromatographic peaks referring to isobaric ions with similar MS/MS spectra were achieved for species with $[M]^+$ at *m/z* 805 and 809 (Figs. 1a and 3a, Table 1), which are vinyl-linked flavanol anthocyanin (Fig. 2f) and ethyliden-bridged flavanol anthocyanin (Fig. 2e) pigments, respectively. In particular, the MS/MS fragmentation of the two molecular ions at *m/z* 805 gave rise to the base peak corresponding to the aglycon ion (loss of 162 u) which was consecutively fragmented to *m/z* 491 (loss of 152 u) arising from a RDA cleavage, indicative for the presence of catechin or epicatechin unit in the compound. Then, peaks 30 and 34 could be attributed to malvidin 3O-glucoside-4-vinyl-(epi)catechin isomers due to the stereoisomery of catechin/epicatechin attached to the C10 position. On the basis of its fragmentation pattern, in which the dominant fragment was originated by the loss of a dehydrated-acetylglucoside $[M-204]^+$ from the $[M]^+$ at *m/z* 847 (Table 1), also compound 23 was imputed to a vinyl-linked anthocyanin flavanol pigment, namely malvidin 3O-acetylglucoside-4-vinyl-(epi)catechin. Fraction B revealed the presence of other vinyl-linked flavanol anthocyanins, generating from the reaction between malvidin coumaroyl-glucosides and vinylcatechin and vinylepicatechin (peaks 35 and 38), also observed into Primitivo young wine (Fig. 1a). Indeed, the molecular ion at *m/z* 951, and fragments at *m/z* 643 and 491, respectively, originating from the consecutive loss of a coumaroyl-glucose moiety $[M-308]^+$ and 152 u by retro Diels-Alder (RDA) fission of an heterocyclic ring of a catechin or epicatechin unit, were detected. Similar observation about the MS fragmentation patterns allowed to attribute the peaks 49 and 50, with molecular ions at *m/z* 791 and 775, to petunidin 3O-glucoside-4-vinyl(epi)catechin and peonidin 3O-glucoside-4-vinyl(epi)catechin (Table 1); these compounds, only revealed into the older sample, have been never reported in Primitivo wine. Showing the same $[M]^+$ at *m/z* 1093 and the same fragmentation with the ions at *m/z* 931 and 803 due to the loss of 162 u (dehydrated-glucose moiety) and 290 u by the elimination of an (epi)catechin molecule, peaks 15 and 17 can be imputed to two isomers of malvidin 3O-glucoside-4-vinyl-procyanidin; analogously, in the MS/MS spectrum of peak 48, the elimination of 162 and 290 u from its molecular ion at *m/z* 1135, which is 42 u higher than 1093, allowed to name it malvidin 3O-acetylglucoside-4-vinyl-procyanidin. It is very interesting that the latter compound was identified in Primitivo for the first time, unlike the other two procyanidin-anthocyanins isomers were previously found into a year old wine (Pati et al., 2006).

Also the highest peaks in the chromatogram of fraction C (peaks 28, 52, 56–58, Fig. 3c) can be attributed to vinyl-linked pigments, namely to three isomers of the trimer malvidin 3O-(*p*-coumaroyl)-glucoside-4-vinyl-di(epi)catechin and to the trimer malvidin 3O-(caffeoyl)-glucoside-4-vinyl-di(epi)catechin and the tetramer malvidin 3O-glucoside-4-vinyl-tri(epi)catechin, as confirmed by their molecular ion at *m/z* 1239, 1255, and 1381, respectively, and by the presence of dominant fragments corresponding to the aglycon ions (loss of 308, 324, and 162 u, respectively) together with the common loss of terminal flavanol moieties (at 290 and 578 u) in their fragmentation patterns (Pati et al., 2006; Asenstorfer, Hayasaka, & Jones, 2001).

The MS/MS pattern related to the three molecular ions at *m/z* 809 (peaks 14, 16, 18, Figs. 1a and 3a) showed three main fragment ions at *m/z* 647 (loss of a dehydrated-glucose residue), 519 (loss of an (epi)catechin unit), and 357 (consecutive loss of dehydrated glucose and (epi)catechin) (Table 1); these data confirmed the identification of the pigments as three isomers of malvidin 3O-glucoside-8-ethyl-(epi)catechin (Pati et al., 2006). By matching their MS and MS/MS patterns with literature, peaks 29, 31, and 40 were identified as peonidin 3O-(*p*-coumaroyl)-glucoside-8-ethyl-(epi)catechin and two malvidin 3O-(*p*-coumaroyl)-glucoside-8-ethyl-(epi)catechin isomers, respectively (Figs. 1a and 3a, Table 1) (Pati et al., 2006; Alcade-Eon et al., 2004). Four others stereoisomers of malvidin 3O-glucoside-8-ethyl-(epi)catechin (peaks 53 and 54) and malvidin 3O-(*p*-coumaroyl)-glucoside-8-ethyl-(epi)catechin (peaks 60 and 61), having different k' respect to those into faction A and young wine, were only detected into the fraction C of the 2 years old Primitivo (Fig. 3c, Table 1). Furthermore, a direct adduct between malvidin 3O-glucoside and flavanol dimer was found (peak 51) for the first time into an aged Primitivo; indeed, the mass of its molecular ion (*m/z* 1071) together with the appearance of the heterocycle-ring-fission fragment ion at *m/z* 290 in the MS^2 spectrum allow to hypothesize that adduct linkage involved the C4 position of the anthocyanin unit and the C8 position of the flavanol unit (Table 1, Fig. 3c) (Blanco-Vega et al., 2014).

As regards anthocyanin-tannin derivatives, two protonated molecules $[M+H]^+$ at *m/z* 783 (peak 46) and 753 (peak 47) were

detected (Table 1), whose fragmentation patterns, characterized by the major ions at *m/z* 631 and 601, respectively, due to the neutral loss of 152 u by RDA decomposition of the flavanol unit and less abundant $[M+H-162]^+$ product ions corresponding to the loss of a glucose, were similar to that obtained by Duenãs, Fulcrand, and Cheynier (2006) for a malvidin 3O-glucoside-catechin adduct (A-T) with an A-type linkage (Fig. 4). These data, together with the lack of fragments at *m/z* 343 and 313 or at *m/z* 331 and 301, respectively, typical of a flavene form (Sánchez-Ilárduya et al. 2014), as well as the absence of absorption in the visible region, corroborated the hypothesis that the peaks 46 and 47 were the colorless A-type peonidin 3O-glucoside-(epi)catechin and malvidin 3O-glucoside-(epi)catechin, respectively. These bycyclic compounds, which indirectly influence the wine color, were previously identified into model solutions but also into Roja aged red wines (Duenãs et al., 2006; Sánchez-Ilárduya et al. 2014). On the contrary, tannin-anthocyanin derivatives (peaks 1, 2, 4, 6, and 7) having molecular ions and fragmentation patterns typical of (epi)catechin-petunidin or malvidin 3O-glucoside adducts (Pati et al., 2006), were only revealed into young Primitivo (Fig. 1a, Table 1).

Finally, it is really interesting that the anthocyanin dimer malvidin 3O-glucoside- malvidin 3O-(*p*-coumaroyl)-glucoside (peak 42) and trimer (malvidin 3O-glucoside)$_3$ (peak 62) showing a molecular ion at *m/z* 1131 and 1477, respectively, and fragmentation patterns consistent with what reported in literature (Vidal, Meudec, Cheynier, Skouroumounis, & Hayasaka, 2004; Alcade-Eon et al., 2007; Oliveira, da Silva, Parola, Mateus, Brás, João Ramos, & de Freitas, 2013), were detected into both wines because this is the first evidence that such compounds survive to the wine aging. Indeed, their presence in red wine is maybe due to their extraction from grape skin during fermentation rather than obtained from the degradation of larger instable oligomers, which could be rapidly formed in the young wine as intermediates. This statement is confirmed by the fact that the synthesis of dimers and trimers was not observed in wine model solutions containing malvidin 3O-glucoside (Oliveira, da Silva, Parola, Mateus, Brás, João Ramos, & de Freitas, 2013).

3.3. Color contribution of each pigment class

The aging capacity of a wine is influenced by a number of factors, including the amount of tannin, acid, grape variety, and alcohol present in the wine, as well as the storage conditions of the wine; however, knowing the type and evolution of anthocyanin derived pigments can be of crucial importance for assessing the resistance to aging of a red wine (Ribéreau-Gayon et al., 2006). Therefore the percentage content of the seven different classes of pigments considered in this work were compared between young and 2 years old Primitivo wines (Fig. 5), in order to establish which is the predominant pigment family at the two moments of wine aging. As expected, a strong decrease of monomeric anthocyanins was revealed in the aged wine respect to young Primitivo; in particular

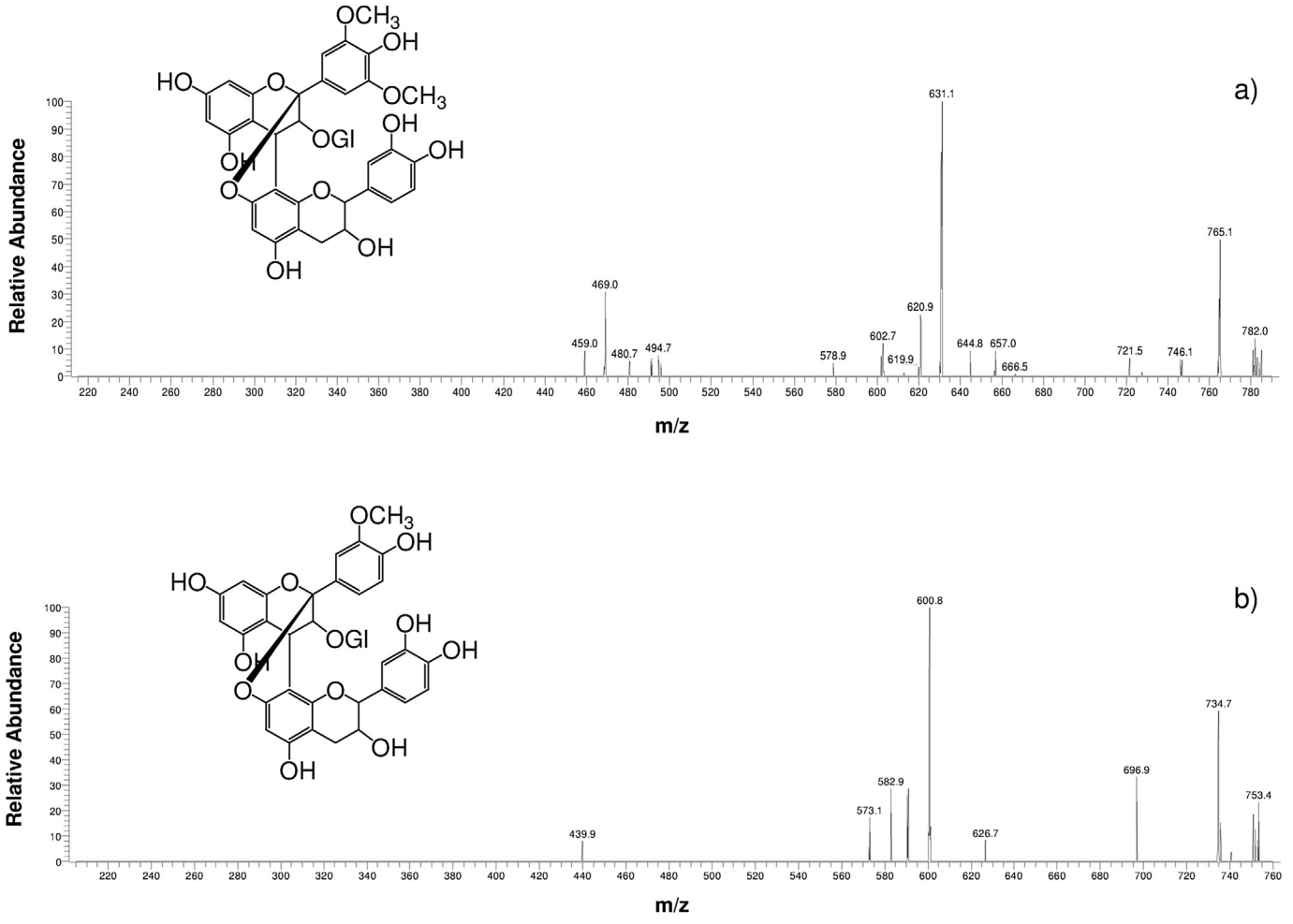

Fig. 4. MS/MS spectra of the A-type (epi)catechin-malvidin 3O-glucoside (a), and (epi)-catechin-peonidin 3O-glucoside (b).

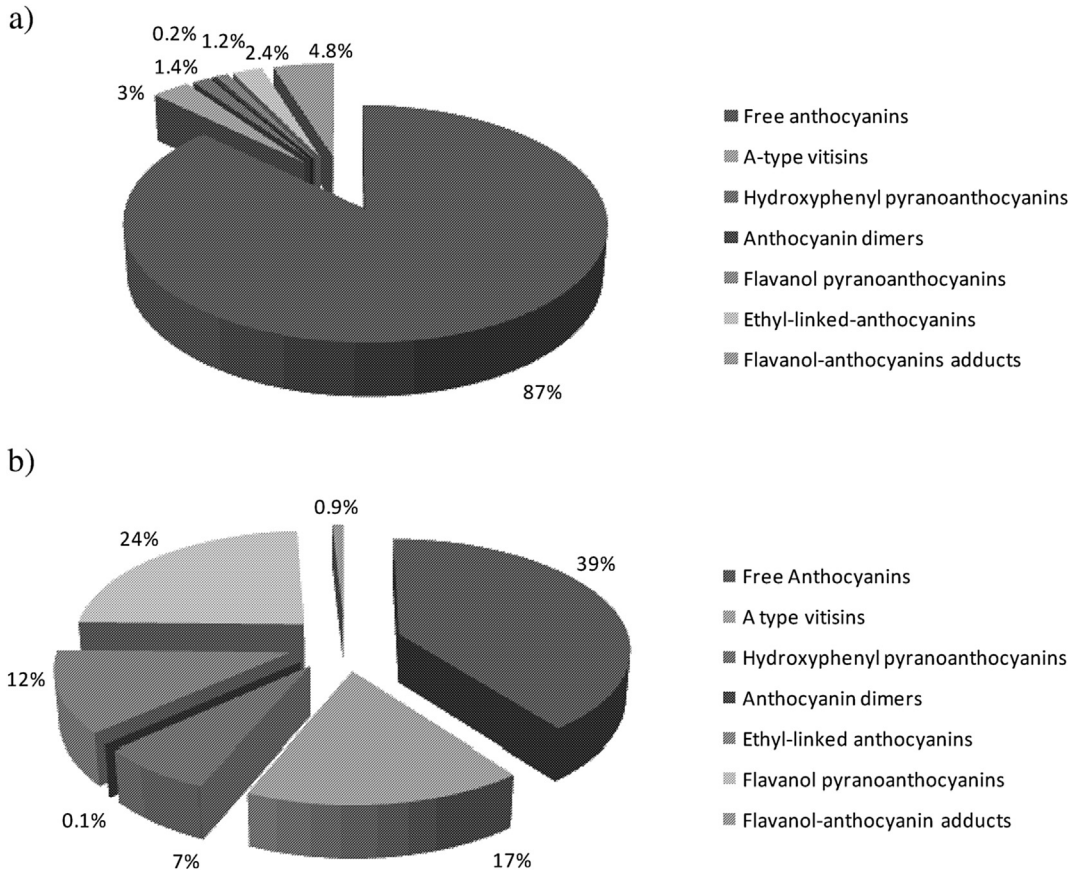

Fig. 5. Distribution (percentage) of anthocyanin-derived red pigments in Primitivo: a) young wine; b) two years old wine.

the absence of both free and derivative forms of delphinidin and cyanidin (Table 1), which were probably degraded during the maturation but not oxidized to form hydroxyphenyl or catechin adducts, as revealed in other aged wines (Alcade-Eon et al., 2006; Wang et al., 2003). Also a relative high percentage (39%) of the grape native pigments, especially malvidin compounds, was still found in the older wine (Fig. 5b, Table 1).

Interestingly, all the found anthocyanin-derived pigments were formed by malvidin, peonidin, and petunidin which are known to be the most stable anthocyanins (He et al., 2012) and are mainly involved in the formation of pyranonathocyanins derivatives, known to have their absorbance maxima within the range of 500–515 nm and accounting for 49% of the total content in the aged wine (Fig. 5b). Furthermore, it can be seen that the relative amounts of compounds, such as flavanol-anthocyanin adducts, which gave blue hues to the samples decreased (from 4.8 to 0.9%) as wine became older, and the reddish or violet ethyledene-bridge favanol-anthocyanin adducts were greatly affected by disappearance during aging, passing from a relative ratio of almost 1:2 up to 1:4 respect to vinyl-linked anthocyanins in the older wine (Fig. 5). Then, overall, it is the predominance of pyranoanthocyanins, characterized by higher stability compared to ethyledene-bridge favanol-anthocyanin and anthocyanin-flavanol compounds (Alcade-Eon et al., 2006; Boido, Alcade Eon, Carrau, Dellacassa, & Rivas-Gonzalo, 2006), and maybe their early formation (within two years) that would be responsible for the more rapid change of Primitivo color into orange hues compared to other international wines (Blanco-Vega et al., 2014), but also it would be able to slow down the natural loss of structure which wine is subject to (Ribéreau-Gayon et al., 2006).

4. Conclusion

In this work, the percentage content of seven different classes of pigments were compared between young and 2 years old Primitivo wines. More than fifty anthocyanin-derived pigments were detected whose structures have been elucidated through a comparison with the literature data and interpretation of MS and MS/MS spectra. Compounds such as malvidin 3O-acetyl-4-vinyl-procyanidin, malvidin 3O-glucoside-di(epi)-catechin, and A-type peonidin and malvidin 3O-glucoside-(epi)catechin were identified in Primitivo for the first time; then, malvidin-3O-glucoside-malvidin-3O-*p*-coumaroylglucoside dimer and (malvidin-3O-glucoside)$_3$ trimer anthocyanins were detected into both wines showing a further evidence that such compounds survive to the wine aging. The pyranoanthocyanin pigments were the most abundant in the aged wine, accounting for 49% of the total pigment content. This, and maybe their early formation (within two years), can be responsible for the often reported rapid change of Primitivo color into orange hues compared to other international wines, but also it would be able to slow down the natural loss of wine structure as pyranoanthocyanin are the most stable known pigments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://

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