Food Chemistry xxx (2018) xxx-xxx

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com

Affinity and selectivity of plant proteins for red wine components relevant to color and aroma traits

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ARTICLE INFO

Keywords:
Red wine
Fining
Plant proteins
Wine aroma
Wine color
Anthocyanidins

ABSTRACT

The effects of fining with various plant proteins were assessed on Aglianico red wine, using both the young wine and wine aged for twelve and twenty four months, and including wine unfined or fined with gelatin as controls. Color traits and fining efficiency were considered, along with the content of various types of phenolics and of aroma-related compounds of either varietal or fermentative origin. All agents had comparable fining efficiency, although with distinct kinetics, and had similar effects on wine color. Individual plant proteins and enzymatic hydrolyzates differed in their ability to interact with some anthocyanins, with specific proanthocyanidins complexes, and with some aroma components of fermentative origin. Changes in varietal aroma components upon fining were very limited or absent. Effects of all the fining agents tested in this study on the anthocyanidin components were most noticeable in young red wine, and decreased markedly with increasing wine ageing.

1. Introduction

Flavonoids are important components of grapes, essential to wine quality. They are responsible for the color and astringency of red wines as well as for the yellow hue of oxidized white wines, and are also involved in the development of haze and precipitates, along with other technological problems, such as clogging of filtration membranes and adsorption on surfaces (Smith, Mcrae, & Bindon, 2015). The reactions of anthocyanins and proanthocyanidins play a major role in changes taking place during wine ageing, that include conversion of grape anthocyanins to other pigments responsible for changes in color intensity and tonality, and for decreased astringency (Cáceres & Harbertson, 2014).

The main purpose of using a protein-based fining agent is to soften red wines, and is reportedly related to the efficiency of proteins in complexing the phenolic compounds in the wine and in their removal through precipitation (Gonzalez-Neves, Favre, & Gil, 2014; Sarni-Manchado, Deleris, Avallone, Cheynier, & Moutounet, 1999). Similar studies have been carried out on the relevance of the interaction among phenolics and aroma components (Dufour & Bayonove, 1999; Vincenzi, Panighel, Gazzola, Flamini, & Curioni, 2015) and on the potential impact of fining agents of various nature on aroma compounds of varietal origin (Lubbers, Charpentier, & Feuillat, 1996; Moio, Ugliano, Gambuti, Genovese, & Piombino, 2004; Nasi, Ferranti, Amato & Chianese, 2008; Volley, Lamer, Dubois, & Feuillat, 1990).

Commonly used protein fining agents include gelatin, casein, egg albumin, and proteins of plant origin able to replace the animal ones. In response to winemakers' interest in replacing animal-derived fining agents with plant-based products, plant proteins were investigated as possible wine fining agents (Lefebvre et al., 2000). Following studies included a variety of plant-derived proteins in both intact and partially hydrolyzed form, (Gambuti, Rinaldi, & Moio, 2012; Gazzola, Vincenzi, Marangon, Pasini, & Curioni, 2017; Marchal, Marchal-Delahaut, Lallement, & Jeandet, 2002; Maury, Sarni-Manchado, Lefebvre, Cheynier, & Moutounet, 2003; Noriega-Dominguez, Duran, Virseda, & Marín-Arroyo, 2010; Simonato, Mainente, Selvatico, Violoni, & Pasini, 2013; Tschiersch, Nikfardjam, Schmidt, & Schwack, 2010). Fining efficiency of all the tested plant proteins and of enzymatically prepared protein hydrolysates was related to their capability to precipitate condensed species, which was found – in most cases - to relate to the pro-

Abbreviations: LC-MS, liquid chromatography-mass spectrometry; ESI, electrospray ionization; TFA, trifluoroacetic acid; TIC, total ion current; OPCs, oligomeric proanthocyanidins complexes.

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https://doi.org/10.1016/j.foodchem.2018.02.085
Received 21 April 2017; Received in revised form 15 February 2018; Accepted 15 February 2018
Available online xxx
0308-8146/

tein surface hydrophobicity (Granato, Nasi, Ferranti, Iametti, & Bonomi, 2014; Granato et al., 2010; Le Bourvellec & Renard, 2013). Molecular features of the target compounds (size, hydrophobicity, degree of polymerization and formation of adducts) and their concentration were major factors in the effectiveness of the various proteins. All these factors relate to the characteristics of the original grapes and to the winemaking conditions (Castillo-Sanchez et al., 2008; Gonzalez-Neves et al., 2014; Karamanidou, Kallithraka, & Hatzidimitrio, 2011; Le Bourvellec & Renard, 2013; Smith et al., 2015). Other recent studies also reported on the impact of combined treatments (e.g., separate and sequential use of bentonite and proteins) on some relevant traits in young red wines (Ben Aziz, Mouls, Fulcrand, Douieb, & Hajjaj, 2017), or on the influence of other wine components – such as sugars and polyols – on the fining process (Maury, Sarni-Manchado, Poinsaut, Cheynier, & Moutounet, 2016).

Here we evaluated the effect of plant-derived proteins on the same *Aglianico* red wine either right after winemaking or after aging for twelve or twenty-four months. High-resolution separative techniques and mass-spectrometry based approaches were combined to compare fining-related changes in the qualitative and quantitative anthocyanidin profiles and – in the case of young wine – in the profile of volatiles. This experimental plan allowed the effects of the interaction between fining proteins and some of the relevant compounds in both young and aged wine to be evaluated, with the final goal of addressing the nature and role of the possible molecular determinants of specific interactions between the fining agents and molecules relevant to sensory traits.

In fining trials were included also two preparations obtained from partial enzymatic hydrolysis of pea proteins, in order to assess whether the modification of their size and of the number/accessibility of hydrophobic sites on their surface (Bonomi, Mora, Pagani, & Iametti, 2004; Granato et al., 2010; Nakai & Li-Chan, 1988), could increase their fining efficiency while minimizing possible negative effects on wine quality, as suggested by previous studies on partially hydrolyzed cereal proteins (Marchal et al., 2002; Maury et al., 2003; Tschiersch et al., 2010).

2. Materials and methods

2.1. Experimental wines

The young red wine used in this study (*Aglianico* del Taburno) was produced in 2012 by Cantine Tora (located in Torrecuso, Benevento, a DOC area in the Campania Region), from *Aglianico* grapes grown in local vineyards. The same wine was used for all the tests carried out after twelve and twenty-four months of aging. Wine had the following chemical characteristics, as assessed by standard OIV methods (International Organisation of Vine and Wine, 2005): alcohol content, 15.10% (v/v); titratable acidity, 6.71 g/L (as tartaric acid); volatile acidity, 0.29 g/L (as acetic acid); pH 3.31; free sulfur dioxide, 18 mg/L; total sulfur dioxide, 30 mg/L.

2.2. Wine fining trials

Fining agents for experimental activities included commercially available protein extracts from soybean and pea, lentil flour, and wheat gluten proteins (all from Prodotti Gianni, Milan, Italy). These extracts had a protein content ranging from 82 in the case of legume proteins to 91% in the case of gluten, all expressed on a dry weight basis. Water content was in the 6–8% range (w/w). The study also included two experimental preparations obtained from enzymatic hydrolysis of pea proteins (PH1 and PH2), that were prepared by treating pea protein isolates with two different food grade enzymes. PH1 was prepared by using a trypsin-like protease (Amano N, PRZ1250448N, Amano Enzyme Europe Limited, Chipping Norton, UK), whereas a chymotrypsin-like enzyme (Europa Protease 2, Europa Bioproducts Ltd, Wicken, UK) was used to prepare PH2. In both cases, the hydrolysis was carried out at 37 °C for 2 h, and arrested by lowering the pH to 2.5 with HCl. Insoluble materials were recovered by centrifugation (10 min at 3000×*g*), washed with water, and lyophilized. MS characterization of PH1 and PH2 confirmed hydrolysis with both enzymes, with disappearance of large-sized proteins and a similar size distribution of the hydrolytic products. Peptide size was centered around 20000 Da in both PH1 and PH2, but different polypeptides were present in PH1 and PH2 as a consequence of the different specificity of the enzymes.

Fining tests were carried out by adding protein fining agents in plastic-stoppered 100-mL cylinders, that were completely filled with about 120 mL of wine to minimize air exposure and ensuing oxidation. Plant-derived proteins were used at the maximum dosage commonly employed for animal proteins (20 g/hl for gelatin and egg proteins), as done in previous studies (Granato et al., 2014). An untreated sample was used as a negative control, and a cold-water soluble gelatin (PULVICLAR S, Enartis, Italy) was included in this study for comparative purposes. Gelatin was used at the same concentrations (20 g/hl) used for plant proteins.

2.3. Clarification kinetics and spectrophotometric measurements

Turbidity was measured in Nephelometric Turbidity Units (NTU) using a turbidimeter (LP 2000, Hanna Instruments). Optical density, turbidity, and the volume of lees were measured in duplicate 1, 4, 10, 20, 32, 48, 58 and 168 h after the addition of fining agents. Kinetics were studied at room temperature (20 °C ± 2 °C). Absorbance at 420, 520, and 620 nm was determined in an UV–VIS spectrophotometer (mod. 1601, Shimadzu). All analyses were carried out in triplicate.

2.4. Structural characterization of phenolic compounds after wine fining

Phenolic compounds were isolated by loading 5 mL of wine on a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) previously conditioned by sequential washing with 5 mL of methanol and 5 mL of water. The cartridge was washed three times with 10 mL of water and the samples eluted with 70% aqueous ethanol containing 0.1% TFA. Samples were stored at −18 °C until used. LC/MS analysis was carried out by means of a LC/MS single quadrupole instrument (HP1100-MSD, Agilent Technologies, Santa Clara, CA, USA) by using a C18 column (Vydac, Hesperia CA, USA; 2.1 * 250 mm). The eluant was 0.1% (v/v) TFA in HPLC-grade water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). Oligomeric proanthocyanidins complexes (OPCs) were separated at a constant flow-rate of 0.2 mL/min, with a linear gradient of solvent B in the following proportions (v/v): 4 min, 0% B; 4–14 min, 0–18% B; 14–22 min, 18–28% B, 22–24 min, 28% B; 24–26 min, 28–60% B; 26–27 min; 60–80% B; and 27–30 min, 80–100% B. The total run time was 30 min. Detection was carried out at 280 and 520 nm. For LC/ESI-MS analysis, proanthocyanidins were characterized according to the conditions used for wine model solutions (Granato et al., 2010). To increase sensitivity of ESI-MS measurements, the samples were assayed twice, scanning in the positive ion mode from *m/z* 100 to 1000, and from *m/z* 1000 to 2000, at a scan rate of 4.90 s per scan and 0.1 s inter-scan delay. The source temperature was 180 °C. The capillary voltage was 3.6 kV and the cone voltage was maintained either at 40 or 25 V, according to different experiments. N_2 was used as both drying and spraying gas. Calibration curves were prepared by using (+)-catechin in the 50–250 mg/L concentration range, as reported in previous studies (Granato et al., 2014).

2.5. Determination of volatile compounds in wines after fining

Analysis of volatile compounds (including either varietal molecules, such as terpenes, and volatile phenols or non-varietal compounds, such as acids, esters, aldehydes, lactones, etc.) was performed by solid phase micro-extraction (SPME) and gas chromatography-mass spectrometry (GC/MS, Nasi, et al., 2008). SPME holders and fibers were from Supelco (Aldrich, Bornem, Belgio). SPME fibers were immersed in the headspace of the samples (120 mL) of wine until equilibrium was reached. The internal standard 1-octanol (0.500 mg/L) was added to the wine before the extraction. Thermal desorption of the analytes from the fiber inside the GC injection port was carried out in the split mode (1/10) at a desorption temperature of 250 °C during 1 min. For GC/MS analysis, all samples were analyzed with an HP 7890 gas chromatograph coupled to a 5975C quadrupole HP mass spectrometer. The gas chromatograph was equipped with an HP-5 capillary column (30 m × 0.32 mm ID), with He as the carrier gas. For analysis of aroma compounds, the GC oven temperature was increased from 40 °C (held for 7 min) to 180 °C, at 5 °C/min. The mass spectrometer operated in electron mode (EI, 70 eV) and scans covered the 45–350 *m/z* range. In other cases, a SIM method was used (for terpene compounds *m/z* 93, 12, 136). The identification of odorous components was done by referring to the National Institute of Standards and Technology (NIST) library and by comparison with suitable standards, as reported elsewhere (Nasi et al., 2008). Quantitative determinations were obtained by means of calibration curves in the concentration ranges typical of wines for each compound. Seven concentration levels and five replicates per level were used – in the range of verified linearity – for calibration purposes.

2.6. Statistical analysis

Analysis of variance (ANOVA) was performed by utilizing Statgraphics XV version 15.1.02 (StatPoint Inc., Warrenton, VA, USA). Samples were used as factor. When the factor effect was found to be significant ($p \leq 0.05$), significant differences among the respective means were determined using Fisher's Least Significant Difference (LSD) test.

3. Results and discussion

3.1. Fining efficiency of plant proteins

Fig. 1 shows the time course of wine clarification for the various proteins. All of the plant proteins tested here are insoluble in wine and they have to be eliminated by decanting or filtering after fining. Indeed, turbidity of wines increased after addition of plant proteins but slowly stabilized to values close to that of wine treated with gelatin. After 7 days, all treated wines had turbidity ranging between 5.7 and 22.9 NTU.

Lentil flour, PH1 and soy proteins were – in this order – the most efficient fining agents in the young red wine, giving final NTU values of 5.7 ± 0.2, 9.9 ± 0.3, and 10.31 ± 0.7, respectively, all lower than the final NTU value in the control un-fined wine (15.5 ± 0.2), at difference with other plant-based fining agents. The hydrolyzed pea proteins (PH1 and PH2) had a slower fining rate than other matrices, probably because of the longer time needed for flocculate formation from smaller polypeptides, but at the end of the treatment their fining efficiency was better than that of the corresponding intact proteins (final NTU values: 9.9 ± 0.3 for PH1; 17.4 ± 1.2 for PH2; 18.5 ± 0.8 for intact pea proteins) and comparable to that of gelatin (final NTU, 14.4 ± 1.0). At contrast, gluten gave a marked decrease in turbidity in the first 24 h but, at

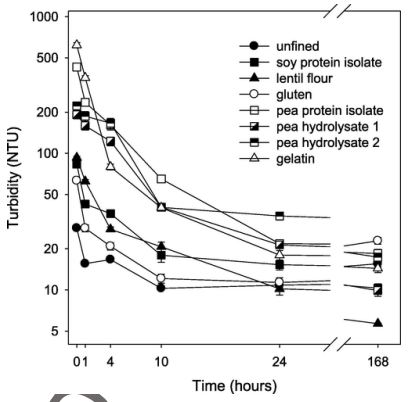

Fig. 1. Time course of *Aglianico* red wine clarification. All treatments were carried out at 0.2 g protein/L.

the end of the process (i.e., after seven days), gluten gave the highest final NTU observed in this study (22.90 ± 2.1).

It has to be noted that the results reported here were obtained by using similar amounts of the various fining agents, thus allowing a straightforward comparison of their relative efficiencies and of the time course of the fining process. Further studies will be needed to assess whether the fining efficiency or the time course of fining may change when varying the amounts of individual fining agents.

3.2. Interaction of plant proteins with proanthocyanidins

The removal of proanthocyanidins and of their derivatives by various plant proteins with respect to controls was evaluated through a quantitative LC-ESI MS analysis, that gave the results summarized in Fig. 2. Lentil and soy proteins – closely followed by PH1 – showed the highest ability to interact with proanthocyanidins in red wine, with a decrease in residual proanthocyanidin ranging from 40% (for monomeric compounds) to 70% (for proanthocyanidin trimers).

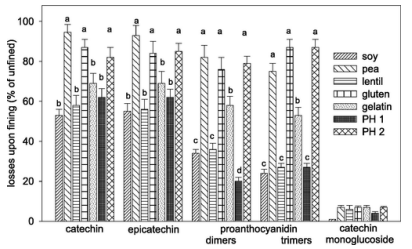

Fig. 2. Percentage loss of individual low molecular weight proanthocyanidins in fined *Aglianico* red wine, as assessed by LC-ESI MS analysis. Results are expressed in comparison to unfined wine. Values in the same group with identical superscripts are not significantly different (LSD; $p \leq 0.05$).

A different efficiency in the removal of monomeric and dimeric proanthocyanidins was observed for soy and gluten proteins in different wines. Gluten proteins had the lowest impact on proanthocyanidins in red wine, although they interacted extensively with proanthocyanidins in white wine (Granato et al., 2014). Whether this originates from limited competition among various protein-adsorbed species (in white wine) rather than from some co-operative binding (in red wine) remains to be assessed. The nature and amount of surface hydrophobic regions in the various proteins also may come into play (Granato et al., 2010), as soy proteins showed a behavior opposite to that of gluten proteins when comparing red and white wines.

A different affinity for proanthocyanidins was observed for pea proteins and their hydrolysis products. Pea proteins were the least effective removing agent, giving a 3–5% decrease in catechin- epicatechin and 15–22% decreases in OPC dimers and trimers. In contrast, PH1 showed a much higher ability to interact with OPCs than the native proteins, giving a decrease ranging from 30% (for monomeric species) to more than 70% (for dimers and trimers). Also, PH1 was noticeably more effective than PH2. These differences are likely related to the different molecular size, hydrophobicity, and conformation of pea proteins and of their hydrolytic fragments. In particular, hydrolysis with chimotrypsin-like enzymes (acting on hydrophobic regions, and used for preparation of PH2) gave products with a lower fining ability than that observed for PH1 (prepared by using a trypsin-like enzyme, and thus retaining in intact form its hydrophobic sequences).

These observations confirm that formation of insoluble (and, therefore, removable) protein-OPC complexes is not governed simply by hydrophobic forces, as the formation of insoluble complexes – and the ensuing clarifying effects – requires multiple and simultaneous types of interactions, each of them with distinct thermodynamic and kinetics features (Granato et al., 2010). Thermodynamic aspects relate to protein/protein and protein/phenolics affinity, and are intertwined with the kinetics of individual steps in the formation of insoluble protein aggregates incorporating the target species. In this frame, one possible explanation of the better behavior of hydrolyzed pea proteins with respect to the native ones was that the large native proteins could impair accessibility to phenolics binding sites, that are expected to be hydrophobic in nature (Granato et al., 2010), and are likely buried inside the native proteins or at the protein–protein interface in intact protein aggregates (Iametti, Scaglioni, Mazzini, Vecchio, & Bonomi, 1998). Selective hydrolysis with enzymes that do not act on hydrophobic residues (as done for PH1) could improve the accessibility of hydrophobic binding sites, and favor cross-interactions among proteins and the eventual precipitation of insoluble complexes.

3.3. Effects of plant proteins on anthocyanins and chromatic characteristics

Many factors responsible for the color of red wine could be affected by fining treatments, with particular reference to anthocyanins and to additional components that cause both a violet shift in color (bathochromic effect) and an increase in color intensity (hyperchromic effect) (Zhang, He, Zhou, Liu, & Duan, 2015). Changes in the red wine color related to the fining treatments were analyzed by both color measurements and UV/Vis spectrophotometry at 420, 520, and 620 nm (to include the blue component of young red wines). Although all fining agents decreased the color intensity at the end of the fining process, the tonality remained stable, as indicated by changes in color intensity index and tonality during wine treatments (Figs. 1S and 2S). Absorbance changes at specific wavelengths were expected to provide information about the influence of fining proteins on color. In general, all treatments slightly lowered the intensity of the three color components. The effect on the yellow and red components was slightly more pronounced than that on the blue one (Fig. 2S), that reportedly relates to the association between pigments and co-pigments, and involves other substances, mainly derivatives of the flavonol and flavone subgroups (Boulton, 2001). However, none of the observed variations in tonality was statistically significant.

The anthocyanin pattern in the various samples was outlined by carrying out a thorough anthocyanin profiling by means of a combined liquid chromatography/electrospray ionization mass spectrometry (LC-ESI MS) approach. Forty-four anthocyanin derivatives were identified and quantified, as listed in Table 1.

The anthocyanin derivatives identified in this study included adducts with pyruvic acid and acetaldehyde, and derivatives formed by copigmentation. Copigmentation involves complexation phenomena, generally at low bond energy (hydrogen bonds and hydrophobic interactions), either between the various forms of anthocyanins or between anthocyanins and other usually colorless – phenolic compounds, that results in changes in intensity of specific color traits. Among the copigmentation-relevant species detected in this study and reportedly relevant to wine color (Zhang et al., 2015), the vinyl-phenol derivatives could have resulted from the decarboxylation of *p*-coumaric acid by yeast decarboxylases and by the consequent reaction with malvidin, either as a monoglucoside or as acylated monoglucosides (*p*-coumarylglucoside). Further rearrangements could involve carbon 4 of the anthocyanin and the oxygen on carbon 5, leading to the formation of a new oxygen heterocyclic species, which is colorless, but recovers an unsaturated structure (and, therefore, its color) upon oxidation.

Another group of pigments identified in *Aglianico* young wine resulted from the addition of pyruvic acid onto anthocyanins. Compared with other pigments in wine, these co-pigments were present in small quantities (Table 1), and have been reported to be relatively stable, as changes in their concentration occur very slowly during ageing (Bakker & Timberlake, 1997).

By comparing anthocyanin-related compounds in fined and control wines, it is evident that all protein fining agents were found to be more or less able to interact with anthocyanins, although the effects of interaction were dissimilar for individual compounds. Indeed, the residual amount of some compounds was higher in the treated wine than in wine undergoing spontaneous settling. This was observed for delphinidin, peonidin, and malvidin-3O-glucoside upon treatment with gluten and pea proteins, and for several co-pigmented anthocyanins in wine fined with lentil proteins. A possible explanation could be related to the capability of these fining agents to interact with pigments preventing them from become bound to the solids that were removed by the racking step after the spontaneous settling of reference wine.

The effects of the various fining agents on individual anthocyanins may be exemplified by taking into account changes involving some of the most abundant and most relevant species among those listed in Table 1. Taking malvidin-3-O-glucoside as an example, the data in Table 1 make it evident that the efficiency of removal decreased in the order: PH1 > SI = LE > gelatin > PH2, whereas GL and PI were ineffective. However, only soybean isolates, PH1 and gelatin were effective in removing relevant (and comparatively abundant) malvidin-related species, such as (*epi*)cat-mv-3-O-glu, mv-3-O-glu-4-vinylguaiacol, and mv-3-O-glu-8-ethyl-(*epi*)cat. Lentil proteins were ineffective in removing these complex malvidin-related species, in spite of lentil proteins having the highest fining efficacy in terms of lowering NTU values after prolonged fining (see Fig. 1 and related comments).

3.4. Effects of fining agents on volatile composition

Our analysis of the impact of the use of plant proteins as fining agents in red wines was completed by characterizing the volatile compounds profile in the fined products by LC/MS (Nasi et al., 2008). As reported in the Supplementary Table 1S, *Aglianico* has a complex varietal aroma composition. Terpenes such as α-pinene, β-pinene,

Table 1
Mean values of concentration (mg/l) and standard deviations (n = 2) for anthocyanic phenolics belonging to different chemical families (monomeric anthocyanins, anthocyanin-flavan-3-ol adducts mediated by acetaldehyde, pyranoanthocyanins and hydroxyphenyl-pyranoanthocyanins) as identified by HPLC-MS in young *Aglianico* wine either unfined or fined with various plant proteins (SI, soy protein isolate; LE, lentil flour; GL, gluten; PI, pea protein isolate; PH1, pea protein hydrolysate1; PH2, pea protein hydrolysate2; GE, gelatin). RT, retention time; n.d.: not detected.

	RT, min	m/z	Unfined	Fined wine						
				SI	LE	GL	PI	PH1	PH2	GE
de-3-O-glu	19.8	465	5.47 ± 0.09	2.12 ± 0.08	2.29 ± 0.03	5.74 ± 0.05	6.61 ± 0.03	2.37 ± 0.02	4.92 ± 0.08	4.70 ± 0.01
cy-3-O-glu	20.5	449	0.40 ± 0.04	0.19 ± 0.00	0.20 ± 0.03	0.44 ± 0.01	0.37 ± 0.01	0.23 ± 0.01	0.24 ± 0.02	0.34 ± 0.04
pe-3-O-glu	21.90	463	4.69 ± 0.22	0.31 ± 0.15	3.01 ± 0.00	4.82 ± 0.19	5.41 ± 0.03	2.81 ± 0.05	3.67 ± 0.13	4.04 ± 0.09
mv-3-O-glu	21.90	493	77.08 ± 1.09	41.03 ± 0.35	41.91 ± 0.03	72.68 ± 0.15	87.18 ± 0.55	36.68 ± 0.16	67.08 ± 0.25	60.87 ± 0.26
pt-3-O-glu	20.90	479	11.06 ± 0.45	5.59 ± 0.48	5.48 ± 0.28	11.39 ± 0.22	12.85 ± 0.05	5.44 ± 0.09	9.18 ± 0.00	10.15 ± 0.68
Monoglucosides residual, % of unfined wine				**50**	**54**	**96**	**114**	**48**	**87**	**81**
pt-3-O-(6-O p-coumaryl)-glu	26.90	625	0.91 ± 0.08	0.51 ± 0.03	0.98 ± 0.01	0.73 ± 0.04	0.62 ± 0.06	0.53 ± 0.02	0.53 ± 0.05	0.59 ± 0.04
de-3-O-(6-O p-coumaryl)-glu	25.70	611	0.43 ± 0.11	0.17 ± 0.03	0.63 ± 0.01	0.56 ± 0.08	0.43 ± 0.07	0.23 ± 0.00	0.23 ± 0.12	0.28 ± 0.04
cy-3-O-(6-O p-coumaryl)-glu	26.70	595	0.35 ± 0.08	n.d.	0.31 ± 0.01	0.27 ± 0.00	0.12 ± 0.01	0.10 ± 0.01	0.10 ± 0.00	0.12 ± 0.00
pe-3-O-(6-O p-coumaryl)-glu	28.11	609	1.69 ± 0.38	1.13 ± 0.25	1.68 ± 0.40	1.49 ± 0.05	1.16 ± 0.10	1.02 ± 0.16	1.02 ± 0.06	1.13 ± 0.08
mv-3-O-(6-O p-coumaryl)-glu	28.16	639	11.03 ± 1.25	6.91 ± 0.11	12.34 ± 0.70	10.99 ± 0.80	7.87 ± 0.05	6.50 ± 0.31	6.50 ± 0.45	6.57 ± 0.22
p-cumaroyl glucosides residual, % of unfined wine				**60**	**58**	**97**	**111**	**60**	**71**	**87**
pt-3-O-(6-O p-caffeoyl)-glu	28.16	641	1.06 ± 0.10	0.67 ± 0.00	1.14 ± 0.35	0.98 ± 0.21	0.81 ± 0.00	0.73 ± 0.40	0.73 ± 0.09	0.59 ± 0.13
pe-3-O-(6-O p-caffeoyl)-glu	27.90	625	0.17 ± 0.09	0.11 ± 0.05	0.17 ± 0.01	0.14 ± 0.00	0.11 ± 0.13	0.08 ± 0.07	0.08 ± 0.05	0.07 ± 0.00
mv-3-O-(6-O p caffeoyl)-glu	26.40	655	0.18 ± 0.03	0.15 ± 0.02	0.25 ± 0.01	0.24 ± 0.01	0.17 ± 0.03	0.13 ± 0.01	0.13 ± 0.02	0.16 ± 0.00
cy-3-O-(6-O p-caffeoyl)-glu	28.00	611	0.10 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	n.d.	0.10 ± 0.00	0.16 ± 0.02	0.16 ± 0.03	0.14 ± 0.01
p-caffeoyl glucosides residual, % of unfined wine				**69**	**73**	**90**	**111**	**64**	**78**	**84**
mv-4-vinylphenol	20.44	447	0.54 ± 0.22	0.16 ± 0.09	0.41 ± 0.01	0.24 ± 0.13	0.18 ± 0.06	0.13 ± 0.00	0.13 ± 0.04	0.19 ± 0.00
mv-3-O-glu-4-vinylphenol	27.28	609	0.32 ± 0.05	0.10 ± 0.00	0.27 ± 0.02	0.21 ± 0.01	0.07 ± 0.00	0.10 ± 0.01	0.10 ± 0.01	0.14 ± 0.00
pe-3-O-glu-4-vinylguaiacol	29.01	609	0.32 ± 0.00	0.22 ± 0.08	0.31 ± 0.00	0.26 ± 0.01	0.19 ± 0.03	0.20 ± 0.00	0.20 ± 0.00	0.21 ± 0.01
mv-3-O-glu-4-vinylguaiacol	28.16	639	11.10 ± 0.98	4.47 ± 0.94	12.15 ± 0.46	10.90 ± 1.09	7.87 ± 0.10	6.42 ± 0.55	6.42 ± 0.55	6.63 ± 0.23
mv-3-O-glu pyruvic acid (vitisin A)	25.89	561	0.24 ± 0.09	0.15 ± 0.01	0.10 ± 0.05	0.24 ± 0.05	0.22 ± 0.00	0.14 ± 0.06	0.14 ± 0.00	0.24 ± 0.10
pe-3-O-glu pyruvic acid	25.16	531	2.61 ± 0.17	1.52 ± 0.52	2.25 ± 0.12	2.02 ± 0.24	2.05 ± 0.21	1.40 ± 0.13	1.40 ± 0.14	1.41 ± 0.41
de-3-O-glu pyruvic acid	19.90	533	1.86 ± 0.05	1.29 ± 0.10	2.06 ± 0.44	1.72 ± 0.12	1.44 ± 0.22	1.22 ± 0.15	1.22 ± 0.01	1.41 ± 0.14
mv-3-O-coumaroyl- glu pyruvic acid	25.50	707	0.11 ± 0.01	0.16 ± 0.01	1.08 ± 0.12	0.95 ± 0.11	0.83 ± 0.09	0.22 ± 0.09	0.22 ± 0.00	0.19 ± 0.00
(*epi*)cat-mv-3-O-glu	19.80	781	2.24 ± 0.52	0.75 ± 0.09	2.75 ± 0.10	2.66 ± 0.12	2.28 ± 0.21	0.65 ± 0.01	0.65 ± 0.15	0.83 ± 0.29
(*epi*)cat-pe-3-O-glu	19.80	751	0.25 ± 0.08	0.04 ± 0.01	0.26 ± 0.03	0.23 ± 0.00	0.20 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
(*epi*)cat-mv-3-O-couglu	25.02	927	0.27 ± 0.05	0.06 ± 0.01	0.31 ± 0.01	0.28 ± 0.00	0.27 ± 0.01	0.05 ± 0.00	0.05 ± 0.02	0.05 ± 0.00
di(*epi*)cat-mv-3-O-glu	20.82	1069	0.15 ± 0.02	0.01 ± 0.01	0.14 ± 0.01	0.14 ± 0.00	0.12 ± 0.05	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.01
mv-3-O-glu-8-ethyl-(*epi*)cat	23.97	809	4.92 ± 0.52	2.01 ± 0.11	5.93 ± 0.21	4.73 ± 0.35	4.14 ± 0.25	2.03 ± 0.44	2.03 ± 0.12	1.99 ± 0.45
mv-3-O-glu-8-ethyl-(*epi*)cat	24.58	809	8.27 ± 0.78	3.57 ± 0.23	8.90 ± 0.41	6.56 ± 0.35	6.14 ± 0.32	3.19 ± 0.35	3.19 ± 0.12	1.17 ± 0.09
mv-3-O-glu-8-ethyl-(*epi*)cat	25.03	809	2.82 ± 0.21	1.02 ± 0.04	3.26 ± 0.19	3.06 ± 0.15	1.48 ± 0.08	1.02 ± 0.12	1.02 ± 0.35	3.02 ± 0.17
mv-3-O-glu-o-ethyl(epigallo)gallocat	23.34	821	0.55 ± 0.01	0.10 ± 0.01	0.60 ± 0.05	0.46 ± 0.12	0.56 ± 0.22	0.13 ± 0.01	0.13 ± 0.05	0.10 ± 0.01
mv-3-O-glu-o-ethyl(epigallo)gallocat	25.00	821	0.37 ± 0.05	0.03 ± 0.01	0.31 ± 0.01	0.29 ± 0.01	0.32 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.05 ± 0.01
pe-3-O-coumaroyl-glu-8-ethyl-(*epi*)cat	27.45	925	0.25 ± 0.06	0.04 ± 0.00	0.24 ± 0.01	0.17 ± 0.04	0.16 ± 0.03	0.08 ± 0.00	0.08 ± 0.03	0.07 ± 0.01
mv-3-O-coumaroyl-glu-8-ethyl-(*epi*)cat	27.45	955	2.63 ± 0.25	0.88 ± 0.30	2.26 ± 0.23	2.21 ± 0.16	1.80 ± 0.32	0.87 ± 0.15	0.87 ± 0.10	0.97 ± 0.26
mv-3-O-glu-4-vinyl(*epi*)cat	23.24	805	1.45 ± 0.35	0.71 ± 0.25	1.36 ± 0.15	1.17 ± 0.12	1.20 ± 0.15	0.71 ± 0.09	0.71 ± 0.05	0.68 ± 0.13
mv-3-O-glu-4-vinyl(*epi*)cat	25.00	805	0.87 ± 0.20	0.45 ± 0.05	0.88 ± 0.18	0.80 ± 0.15	0.78 ± 0.09	0.44 ± 0.02	0.44 ± 0.12	0.42 ± 0.19
mv-3-O-glu-4-vinyl-di(*epi*)cat	21.14	1093	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	n.d.	0.01 ± 0.00	n.d.	n.d.	n.d.
mv-3-O-glu-4-vinyl-di(*epi*)cat	24.31	1093	0.09 ± 0.01	0.02 ± 0.01	0.09 ± 0.00	0.08 ± 0.01	0.09 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.01
mv-3-O-cou-glu-4-vinyl-(*epi*)cat	28.60	951	0.77 ± 0.10	0.31 ± 0.01	0.76 ± 0.00	0.61 ± 0.01	0.40 ± 0.02	0.30 ± 0.01	0.30 ± 0.02	0.34 ± 0.09
mv-3-O-cou-glu-4-vinyl-(*epi*)cat	29.30	951	0.19 ± 0.09	0.11 ± 0.01	0.26 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.10 ± 0.00	0.10 ± 0.09	0.16 ± 0.02
mv-3-O-ac-glu-4-vinyl-(*epi*)cat	25.00	847	0.11 ± 0.01	0.05 ± 0.00	0.11 ± 0.01	0.13 ± 0.01	0.11 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	0.03 ± 0.00
mv-3-O-ac-glu-4-vinyl-(*epi*)cat	26.29	847	0.41 ± 0.14	0.12 ± 0.01	0.37 ± 0.09	0.33 ± 0.01	0.30 ± 0.20	0.12 ± 0.11	0.12 ± 0.15	0.18 ± 0.01
mv-3-O-(6-O-ac)-glu	25.43	535	9.25 ± 0.90	6.11 ± 0.10	9.43 ± 0.45	8.51 ± 0.50	6.83 ± 0.20	5.31 ± 0.12	5.31 ± 0.15	6.15 ± 0.29
mv-3-O-glu acetaldehyde	23.15	517	1.91 ± 0.21	1.97 ± 0.20	1.93 ± 0.04	1.54 ± 0.12	1.36 ± 0.10	1.08 ± 0.10	1.08 ± 0.12	1.83 ± 0.09
Total anthocyanin content			**171.41**	**85.66**	**129.95**	**161.80**	**166.01**	**83.43**	**121.67**	**118.51**

Table 1 (*Continued*)

	RT, min	m/z	Unfined	Fined wine					
				SI	LE	GL	PI	PH1	PH2	GE
Residual anthocyanin content, % of initial				**49.97**	**75.81**	**94.39**	**96.85**	**48.67**	**70.98**	**69.14**

limonene, 3-carene, and geraniol were detectable in *Aglianico* wine, along with β-damascenone, a norisoprenoid compound associated to rosewood nuances (Karamanidou et al., 2011). Varietal and fermentative aroma-relevant molecules detected in *Aglianico* wine and their individual sensory attributes and odor thresholds are listed in Table 1S. All the varietal components in the untreated wine were retained upon fining with all the proteins tested in this study.

An example of the TIC tracings of untreated wine compared to one fined with lentil proteins, the most effective fining agent (at least in terms of residual turbidity of the treated wine, see Fig. 1 and related comments), is presented as Supplementary material (Fig. 3S). Peaks with the largest areas in the TIC chromatograms corresponded to fermentation-derived compounds such as various esters, fatty acids, and alcohols. Quantitative data for the compounds most affected by fining with the various fining agents are presented in Figs. 3 (for ethyl esters) and 4 (for phenylethyl alcohol and its acetate ester, and for isoamyl acetate and benzaldehyde).

In general, gelatin was the fining agent causing the largest decrease in the three ethyl esters considered in Fig. 3. As for the components considered in Fig. 4, benzaldehyde and phenylethyl acetate were the components most affected by the fining process, that had marginal effects on the levels of phenyethyl alcohol and of isoamyl acetate. The decrease in benzaldehyde upon fining ranged from 75 to 55%, with gelatin being most effective in its removal. Benzaldehyde is related to bitter almond notes, that are often considered a non-desirable trait. Upon fining, the levels of phenyl ethyl acetate dropped to 10% of what found in unfined wine, almost regardless of the specific fining agent being tested. Fining had much less pronounced effects on the levels of phenyl ethyl alcohol, if not for gelatin lowering its content by about 50%. Both phenyl ethyl alcohol and its acetate ester contribute to floral notes. In consideration of their relative abundance (100 mg/L for the alcohol and 0.3 mg/L for the acetate), it is expected that the fining

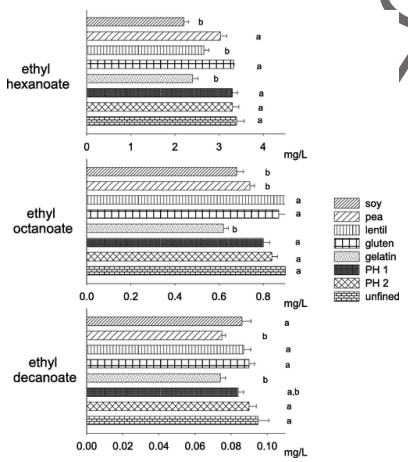

Fig. 3. Percentage loss of ethyl esters in *Aglianico* wine after various fining treatments, as obtained by means of static headspace-GC/MS analysis. Values in the same panel with identical superscripts are not significantly different (LSD; $p \leq 0.05$).

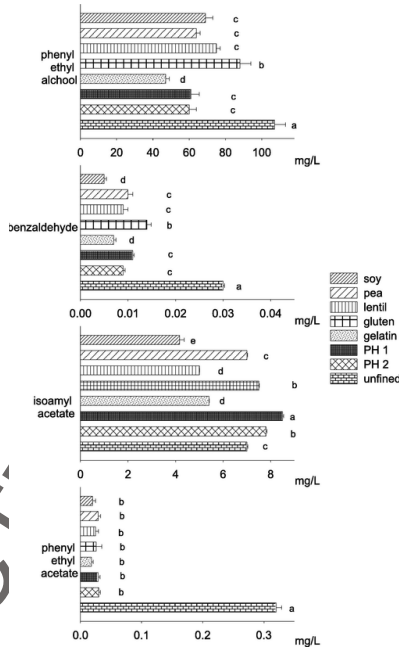

Fig. 4. Percentage loss of various aroma compounds in *Aglianico* wine after various fining treatments, obtained by means of static headspace-GC/MS analysis. Values in the same panel with identical superscripts are not significantly different (LSD; $p \leq 0.05$).

process may have a limited impact on the aroma traits related to these specific components.

On the basis of the chemical and biochemical characteristics of the proteins used here (and of the chemical and physical properties of individual aroma components) it is not possible at the moment to provide a straightforward rationale for some aroma-relevant molecules more being affected by fining or less than others. Again, as pointed out before in our comments on the fining efficacy, it may be possible that using different amounts of individual fining agents – rather than the identical amount used in this study for comparative purposes – will affect the aromatic profile of the product. These aspects, including the kinetics of the various interactions discussed so far and their dependence on the amount of the various fining agents, will be the subject of further studies.

3.5. Interaction of plant proteins with anthocyanins in aged red wines

The same plant proteins tested as fining agents on young *Aglianico* wine were used – at the same concentration – to treat the same wine after twelve and twenty-four months of ageing. The approach used to understand the positive or negative effects of fining on wine quality was focused on profiling the products of reactions involving antho-

cyanins derivatives, and allowed to evaluate the presence, abundance, and sensitivity to fining of acetaldehyde-mediated condensation products and of pyranoanthocyanins. All these species are reportedly important for improvement and stabilization of wine color, as they have shown to be resistant to pH variations, to SO_2 bleaching, and to further anthocyanin oxidation (Escribano-Bailon, Alvarez-Garcia, Rivas-Gonzalo, Heredia, & Santos-Buelga, 2001).

Table 2 reports the effect of fining on the anthocyanin profile in *Aglianico* red wines treated after twelve and twenty-four months of ageing, and makes it possible a comparison among changes related to both wine aging and to fining wines of different age. Both one- and two-year aged wine showed a decrease in total anthocyanin content, along with the age-related formation of co-pigmented products. The observed decrease in total anthocyanin content of aged wines with respect to the young one is well explained by a combination of reactions with various other compounds in the wine, as well as by breakdown reactions. This is exemplified in Table 2, among others, by the ageing-related increase of caffeoyl and vinylguaiacol derivatives of peonidin, with a concomitant decrease in the levels of the original monoglucoside. Table 2S in the accompanying Supplementary materials offers a comprehensive vision of the most relevant data presented in Tables 1 and 2.

There are some aspects of the interactions among fining proteins and anthocyanins that are highlighted when comparing the effects of the various fining agents on wines of different age. By analyzing the data presented in Table 1 and 2 (and the comprehensive comparison in Table 2S) it is evident that the anthocyanin derivatives progressively formed upon aging are removed more efficaciously by the fining process than the corresponding non-modified glucosides. Whether this behavior relates to a decrease in their polarity or to ageing-related changes in the complex wine matrix remains to be assessed.

Another point arising from the comparison among the effects of fining wines of different age is that even the fining agents that proved most effective in anthocyanin removal from young Aglianico wine (namely, soybean, PH1, and gelatin) were quite ineffective when used on one-year aged wine. Conversely, all fining agents removed more than 85% of total anthocyanins from two-year aged wine. This suggests that both the chemical nature of the involved compounds and their concentration play a role in the formation of insoluble protein aggregates incorporating the target anthocyanins, and – ultimately – in their removal.

In an attempt to relate our analytical data to wine color traits, spectrophotometric measurements were used to carry out a comparative scrutiny of fined and unfined wine of the same age. As observed for young wine, fining of aged wines resulted in a decreased color intensity, but the tonality remained unaffected, as indicated by the ratio between absorbance parameters at various wavelengths (data not shown). In other words, fining-dependent modifications in the anthocyanin profiles of aged wine discussed above were not accompanied by statistically significant fining-dependent changes in wine tonality, regardless of the type of fining agent used.

4. Conclusions

A first conclusion that may be drawn from this work is that proteins of plant origin are at least as effective – at the same addition rate – as animal-derived proteins in fining of red wine, even when winemaking involves grapes with complex varietal aroma composition. Varietal aroma compounds in young Aglianico wine were not affected by the fining treatment. The impact of fining with plant proteins on the aroma components of fermentative origin in young wine was much less marked than that of gelatin, used here as a reference animal protein. Also, the effects of fining on fermentative aroma components was much lower in this study than what previously reported for white wine (Granato et al., 2014). This should alert researcher in this area as for the feasibility of extending or generalizing observations made on model systems to real ones.

Also noteworthy is the observation that fining with plant proteins did not alter the color traits of the treated wine, at least in terms of tonality, despite the ability of some of the plant proteins studied here (most notably, soybean, lupin and one of the pea protein hydrolysate) to selectively remove specific anthocyanins components. Whether the observed selectivity has an impact on organoleptic traits other than color was beyond the scope of this study, and remains to be assessed, possibly under actual winemaking conditions. This type of studies will also allow to evaluate the combination of treatments and agents that could be best suited for improving quality traits - or for removing undesired components – in a given wine.

This study also demonstrates that simple biotechnological interventions (such as limited proteolysis of some of the plant proteins used here) may have a positive impact on their behavior as fining agents. In this frame, this study points out that some of the proteolyzed pea proteins used in this study have a ligand specificity quite different – from both a qualitative and quantitative standpoint – from that observed in the corresponding native proteins. The different fining efficacy and specificity reported here for hydrolyzates prepared with different enzymes underscores the necessity of careful selection of the appropriate hydrolysis conditions, and suggests the possibility of exploiting some features of plant protein hydrolysates in further studies aimed at developing a possible targeted action on wine-relevant chemical components.

Future studies based on the molecular analyses reported here will necessarily involve an appropriate evaluation of the sensory impact of the fining process on young red wine, and could be completed by a thorough analysis of the chemical and sensory changes occurring upon ageing of the red wine fined at an early stage. From the applicative standpoint, further studies also may wish to address how the time course and efficiency of fining (and of the related molecular changes, as explored in this study) may relate to the amount of individual fining agents and of their derivatives. These studies may be also helpful in improving our current understanding of the nature and relevance of the various intermolecular interactions that are relevant to the fining process as a whole, also when different types of grapes and different winemaking procedures are involved.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.02.085.

Table 2
Mean values of concentration (mg/l) and standard deviations (n = 2) of anthocyanic phenolic compounds identified by HPLC-MS in Aglianico wine of the given vintage either unfined or fined with various plant proteins (SI, soy protein isolate; LE, lentil flour; GL, gluten; PI, pea protein isolate; PH1, pea protein hydrolysate 1; PH2, pea protein hydrolysate 2; GE, gelatin; n.d., not detected).

	age	unfined	SI	LE	GL	PI	PH1	PH2	GE
cy-3-O-glu	1y	0.24 ± 0.12	0.67 ± 0.01	0.23 ± 0.02	0.44 ± 0.11	0.63 ± 0.01	0.25 ± 0.06	0.81 ± 0.09	0.14 ± 0.02
	2y	*0.30 ± 0.01*	*0.16 ± 0.01*	*n.d.*	*n.d.*	*n.d.*	*0.15 ± 0.00*	*0.17 ± 0.04*	*n.d.*
pe-3-O-glu	1y	2.36 ± 0.36	2.34 ± 0.30	1.49 ± 0.21	1.93 ± 0.19	3.41 ± 0.25	2.07 ± 0.26	2.76 ± 0.14	1.58 ± 0.20
	2y	*0.65 ± 0.03*	*0.70 ± 0.01*	*0.34 ± 0.01*	*n.d.*	*n.d.*	*0.50 ± 0.05*	*0.44 ± 0.03*	*0.29 ± 0.01*
mv-3-O-glu	1y	10.65 ± 0.40	8.61 ± 0.20	7.81 ± 0.22	7.22 ± 0.22	9.71 ± 0.39	8.99 ± 0.52	7.15 ± 0.25	8.46 ± 0.19
	2y	*9.64 ± 0.51*	*2.46 ± 0.21*	*2.61 ± 0.29*	*2.30 ± 0.41*	*2.53 ± 0.20*	*2.29 ± 0.19*	*2.29 ± 0.15*	*2.15 ± 0.20*
pt-3-O-glu	1y	0.82 ± 0.12	0.78 ± 0.15	0.62 ± 0.01	0.83 ± 0.21	1.02 ± 0.26	0.72 ± 0.05	1.04 ± 0.09	0.69 ± 0.09
	2y	*0.84 ± 0.01*	*0.20 ± 0.03*	*0.19 ± 0.01*	*n.d.*	*0.18 ± 0.03*	*0.30 ± 0.02*	*0.20 ± 0.04*	*n.d.*
de-3-O-glu	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.27 ± 0.01*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
pe-3-O-(6-O p-coumaryl)-glu	1y	0.70 ± 0.10	0.46 ± 0.04	0.44 ± 0.16	0.49 ± 0.08	0.52 ± 0.10	0.54 ± 0.09	0.39 ± 0.02	0.49 ± 0.12
	2y	*0.16 ± 0.02*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
mv-3-O-(6-O p-coumaryl)-glu	1y	2.40 ± 0.26	1.43 ± 0.19	1.58 ± 0.07	1.64 ± 0.12	1.93 ± 0.20	1.87 ± 0.12	1.20 ± 0.18	1.65 ± 0.04
	2y	*0.52 ± 0.07*	*0.17 ± 0.01*	*0.16 ± 0.01*	*0.16 ± 0.01*	*0.23 ± 0.00*	*n.d.*	*n.d.*	*0.17 ± 0.00*
de-3-O-(6-O p-coumaryl)-glu	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.16 ± 0.02*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
cy-3-O-(6-O p-coumaryl)-glu	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.17 ± 0.01*	*n.d.*	*n.d.*	*n.d.*	*0.17 ± 0.0*	*0.14 ± 0.01*	*0.12 ± 0.0*	*n.d.*
pe-3-O-(6-O p-caffeoyl)-glu	1y	1.79 ± 0.16	0.92 ± 0.15	0.17 ± 0.07	1.16 ± 0.02	1.36 ± 0.21	1.35 ± 0.12	0.81 ± 0.01	1.41 ± 0.05
	2y	*2.27 ± 0.27*	*0.70 ± 0.11*	*1.15 ± 0.11*	*1.12 ± 0.19*	*n.d.*	*0.96 ± 0.07*	*0.92 ± 0.09*	*n.d.*
mv-3-O-(6-O p-caffeoyl)-glu	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.14 ± 0.01*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
pe-3-O-glu-4-vinylguaiacol	1y	0.97 ± 0.10	0.55 ± 0.09	0.68 ± 0.16	0.73 ± 0.12	0.72 ± 0.07	0.68 ± 0.17	0.49 ± 0.06	0.68 ± 0.19
	2y	*2.08 ± 0.24*	*0.78 ± 0.15*	*1.08 ± 0.24*	*1.32 ± 0.14*	*0.15 ± 0.01*	*1.29 ± 0.14*	*1.11 ± 0.12*	*n.d.*
mv-3-O-glu-4-vinylguaiacol	1y	0.14 ± 0.12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.12 ± 0.12
	2y	*0.29 ± 0.03*	*0.11 ± 0.01*	*n.d.*	*0.13 ± 0.0*	*0.20 ± 0.02*	*n.d.*	*n.d.*	*1.67 ± 0.19*
pe-3-O-glu pyruvate	1y	0.26 ± 0.03	0.16 ± 0.00	0.16 ± 0.02	0.19 ± 0.01	0.22 ± 0.01	0.21 ± 0.03	0.14 ± 0.00	0.20 ± 0.02
	2y	*1.79 ± 0.20*	*0.17 ± 0.01*	*2.29 ± 0.23*	*0.25 ± 0.01*	*0.32 ± 0.03*	*0.24 ± 0.01*	*0.23 ± 0.01*	*0.21 ± 0.02*
de-3-O-glu pyruvate	1y	0.34 ± 0.05	n.d.	n.d.	0.23 ± 0.03	n.d.	n.d.	n.d.	n.d.
	2y	*1.49 ± 0.19*	*0.25 ± 0.01*	*0.30 ± 0.02*	*0.24 ± 0.02*	*0.43 ± 0.03*	*0.25 ± 0.01*	*0.27 ± 0.02*	*0.23 ± 0.02*
mv-3-O-cou-glu pyruvate	1y	0.47 ± 0.07	0.26 ± 0.01	0.22 ± 0.02	0.27 ± 0.03	0.45 ± 0.04	0.50 ± 0.00	0.21 ± 0.01	0.28 ± 0.02
	2y	*0.20 ± 0.01*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
mv-3-O-(6-O-ac)-glu	1y	2.75 ± 0.46	1.62 ± 0.14	1.86 ± 0.17	1.81 ± 0.13	2.18 ± 0.32	2.32 ± 0.22	1.56 ± 0.19	2.01 ± 0.14
	2y	*1.17 ± 0.14*	*0.44 ± 0.05*	*0.42 ± 0.02*	*0.48 ± 0.04*	*0.54 ± 0.09*	*0.05 ± 0.02*	*0.45 ± 0.04*	*0.43 ± 0.01*
mv-3-O-glu acetaldehyde	1y	0.70 ± 0.08	0.42 ± 0.09	0.55 ± 0.02	0.55 ± 0.12	0.50 ± 0.04	0.48 ± 0.01	0.38 ± 0.03	0.47 ± 0.04
	2y	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
(*epi*)cat-mv-3-O-glu	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.70 ± 0.03*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
di(*epi*)cat-mv-3-O-glu	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.01 ± 0.00*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
mv-3-O-glu-8-ethyl-(*epi*)cat	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.18 ± 0.00*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
mv-3-O-glu-8-ethyl-(*epi*)cat	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.36 ± 0.01*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
mv-3-O-glu-8-ethyl-(*epi*)cat	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.06 ± 0.02*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
mv-3-O-glu-o-ethyl(epigallo)gallocat	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.16 ± 0.01*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
mv-3-O-coumaroyl-glu-8-ethyl-(*epi*)cat	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.14 ± 0.01*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
mv-3-O-glu-4-vinyl(*epi*)cat	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 2 (*Continued*)

	age	unfined	SI	CE	GL	PI	PH1	PH2	GE
	2y	*0.45 ± 0.07*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
mv-3-O-glu-4-vinyl(*epi*)cat	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.18 ± 0.01*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
mv-3-O-couglu-4-vinyl-(*epi*)cat	1y	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2y	*0.19 ± 0.01*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Total anthocyanin content	1y	**26.45**	**19.84**	**17.80**	**18.52**	**23.43**	**21.30**	**18.04**	**19.26**
	2y	***16.14***	***2.20***	***2.20***	***2.20***	***1.77***	***1.99***	***2.06***	***2.54***
Residual anthocyanin content after fining, % of initial	1y		**73.9**	**67.3**	**70.0**	**88.6**	**80.5**	**68.2**	**72.8**
	2y		***13.6***	***13.6***	***13.6***	***10.9***	***12.3***	***12.7***	***15.7***

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