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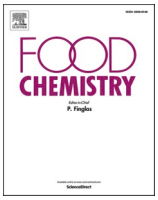

# Grape skin anthocyanin extraction from red varieties during simulated maceration: Influence of grape seeds and pigments adsorption on their surface

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ABSTRACT

The impact of seeds on anthocyanin extraction from skins was assessed on four Italian red winegrape varieties presenting different anthocyanin profile. Grape skins were macerated alone or in presence of seeds for ten days in model solutions. Aglianico, Nebbiolo, Primitivo, and Sangiovese cultivars showed differences in the anthocyanin extraction rate, content, and profile. The presence of seeds did not significantly affect the anthocyanin content and forms extracted from skins and kept into solution, but it generally led to an increase in the polymerization rate. For the first time, anthocyanins adsorbed on seed surface have been quantified after maceration. The amount of anthocyanins retained by seeds was less than 4 mg/kg berries and it seems variety-dependent, with a possible role of seeds number and weight. Individual anthocyanin forms were adsorbed mainly according to their abundance in the solution, but cinnamoyl-glucoside anthocyanin forms showed a higher affinity with seed surface.

## 1. Introduction

The color of red wines is one of the main aspects that affect consumer judgment, and it depends primarily on the anthocyanin content. Anthocyanins are characterized by a flavonoid ring-based structure and the color of the molecule is associated with the fully conjugated 10 electron A–C ring π-system. The five main anthocyanidins (delphinidin, cyanidin, peonidin, petunidin, and malvidin) differ according to the substitution on the B-ring and are present in grapes (*Vitis vinifera* L.) as glucosylated forms (anthocyanins). Further differentiation is due to the six-hydroxyl acylation of glucose (Waterhouse, 2002). Anthocyanins are subjected to a strongly pH-dependent structural transformation that results in color variations (Dangles & Fenger, 2018; Tang et al., 2019).

Grape phenolics are extracted through a concentration-driven diffusion mechanism from berry tissues into the grape must during maceration and alcoholic fermentation. Among them, anthocyanins are located only in red grape skins (except for red-fleshed *tenturier* varieties) and are mainly extracted during the first days of maceration as influenced by several factors. In particular grape ripening, maceration temperature, alcohol content, and contact area have a decisive role (Setford, Jeffery, Grbin, & Muhlack, 2017). The typical anthocyanin extraction kinetic shows a concentration decrease after an initial fast increase, as the polymerization, oxidation, and precipitation rates increase (Cheynier, Souquet, Kontek, & Moutounet, 1994; Tindal, Jeffery, & Muhlack, 2021). Furthermore, anthocyanin content depletion is also due to the resorption by grape (Medina-Plaza et al., 2020) and yeast (Morata, Gómez-Cordovés, Colomo, & Suárez, 2005) cell wall materials.

Nevertheless, monomeric anthocyanins represent only a part of the molecules responsible for the color of the wine as they are involved in non-covalent or covalent interactions with other phenolic compounds resulting in more stable pigments, which determine the final color (He et al., 2012; Unterkofler, Muhlack, & Jeffery, 2020). In the first case, copigmentation is the anthocyanin non-covalent association among themselves or with other phenolic species, leading to the stabilization in their colored forms (Boulton, 2001). Among reaction products of anthocyanins, pyranoanthocyanins result from the addition of

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acetaldehyde, pyruvic acid, and other yeast fermentative metabolites (Marquez, Serratosa, & Merida, 2013), while other polymeric pigments are formed by the condensation between anthocyanins and tannins (He et al., 2012). Moreover, a recent study reported the formation of anthocyanin-anthocyanin adducts as a very important contributor to the wine color (Kumar, Tian, & Harrison, 2022). Thus, the evolution and the stabilization of the wine color are mainly associated with the formation of polymeric pigments, which depends primarily on the initial concentration of anthocyanins and other phenolics, particularly tannins (Ristic, Bindon, Francis, Herderick, & Iland, 2010). The important role of tannins in color stabilization is confirmed by the fact that the addition of exogenous tannin formulations during maceration enhances the polymeric pigment formation, the color intensity, and the preservation of anthocyanin forms with different extents according to the grape variety used (Paissoni, Río Segade, Carrero-Carralero, Montanini, Giacosa, & Rolle, 2020).

The wine tannin content is directly linked with the presence of these compounds in grape skins and seeds, which represent their main sources (Rousserie, Rabot, & Geny-Denis, 2019). Some studies reported that the presence of seeds during fermentation resulted in a more stable wine color (Kovac, Alonso, & Revilla, 1995; Sparrow, Dambergs, Bindon, Smith, & Close, 2015). In contrast, other authors found that the concentration of polymeric pigments during simulated maceration did not increase if model wine solutions were added with a supplementary quantity of seeds or tannins, while a significant increase was reported when skins or anthocyanins were added (Kumar et al., 2022; Sparrow et al., 2015). These observations suggested that quantitative and qualitative differences in the initial phenolic composition influence the polymeric pigment formation and that the polymerization mechanism and the role of seeds are still not completely understood.

The wine phenolic profile is affected by many factors such as vintage, soil, terroir, ripeness grade, and extraction techniques, but the most important factor is the grape variety (Sartor, Caliari, Malinovski, Toaldo, & Bordignon-Luiz, 2017). Italian red grape varieties are known to be very different from each other, showing differences in phenolic content and profile (Mattivi, Prast, Nicolini, & Valenti, 2003), and physico-mechanical characteristics, resulting in highly distinguishable final products. In fact, these features, highlighted in grape, are differentiating also the respective monovarietal wines (Giacosa et al., 2021).

'Aglianico', 'Nebbiolo', 'Primitivo', and 'Sangiovese' are well-known Italian varieties used to produce high-quality red wines. Concerning anthocyanins, 'Nebbiolo' is the only grape variety showing a disubstituted derivative prevalence (>55% with about 45% of peonidin-3-*O*-glucoside). In contrast, 'Primitivo' and 'Aglianico' have a high ratio of malvidin-3-*O*-glucoside derivatives (>50%). 'Sangiovese' has a relatively high share of cyanidin-3-*O*-glucoside (>18%) and peonidin-3-*O*-glucoside (>15%), whereas low or absent acylation rate (Mattivi, Guzzon, Vrhovsek, Stefanini, & Velasco, 2006). Given the diversity of Italian grape cultivars, variety-specific studies are needed to deepen the knowledge of how phenolics are extracted in each variety to better manage their winemaking strategy. The purpose of this work was to investigate the extraction kinetics of anthocyanin compounds of these four important Italian red wine grape cultivars in terms of qualitative and quantitative differences in anthocyanin extraction. To better understand how grape seeds affect the skins maceration process, skins were macerated alone and in presence of seeds during standardized maceration in model solution with increasing alcohol content, with the latter approach used to limit the influence of yeast fermentative variables. Seed management techniques (such as seed removal) are common practices in winemaking aimed to limit the excessive extraction of undesired tannins during maceration (Bautista-Ortín, Busse-Valverde, López-Roca, Gil-Muñoz, & Gómez-Plaza, 2014): in this sense, this study can provide useful information for managing the maceration process by exploring how the presence or absence of seeds can affect the extraction and stabilisation of colour pigments. Furthermore, the role of seed surface in the adsorption of anthocyanins extracted from skins was investigated for the first time to our knowledge, leading to a quali-quantitative assessment of this phenomenon.

## 2. Materials and methods

### 2.1. Grape material and grape must analysis

*Vitis vinifera* L. cultivars Aglianico, Nebbiolo, Primitivo, and Sangiovese were selected, among the main Italian red wine grape varieties, for this experiment. Grapes from the ampelographic collection of Grinzane Cavour (Piemonte region, northwestern Italy) were harvested at ripeness (about 21–24 °Brix soluble solids content) in 2019 vintage. Ten kilograms of berries of each variety have been collected and transported to the laboratory. Grapes were manually destemmed and two replicates of 100 g of berries from each variety were randomly sampled and crushed to perform compositional analyses on the grape juice. Before analysis, the grape must was centrifugated (Heittich 32R, Tuttlingen, Germany) at 4000 × *g* at 20 °C for 15 min. The determination of total soluble solids was performed by refractometry, using a calibrated refractometer (Atago CO. LTD, Tokyo, Japan) and expressed as degrees Brix. Total acidity was evaluated by titration following the official method OIV-MA-AS313-01 (OIV, 2016); pH was analyzed by potentiometry using an InoLab 730 calibrated pHmeter (WTW, Weilheim, Germany), according to the OIV-MA-AS313-15 method (OIV, 2016).

### 2.2. Grape sample preparation: Density sorting

All the remaining berries were sorted according to their density by flotation in different saline solutions following the methods reported by Fournand, Vicens, Sidhoum, Souquet, Moutounet, & Cheynier (2006) and Rolle, Torchio, Giacosa, Río Segade, Cagnasso, & Gerbi (2012). This operation was carried out to obtain a homogeneous set of berries to be used for anthocyanin extraction. The density flotation was performed using saline solutions containing from 130 to 190 g of NaCl/L (corresponding to densities between 1087 and 1125 kg/m$^3$). For each cultivar, the most represented class was chosen for this experiment, which corresponded to 1101 kg/m$^3$ for 'Nebbiolo' and 1092 kg/m$^3$ for 'Aglianico', 'Primitivo', and 'Sangiovese'. The grape juice characterization was determined also for density sorted berries, following the same procedure reported in Section 2.1.

### 2.3. Grape skin anthocyanin potential: Total extraction

To evaluate the potential anthocyanin content of the four grape varieties, berry skins from sorted berries were macerated in a highly extracting solution prepared according to Río Segade et al. (2014). The buffer solution was prepared with 5 g of tartaric acid/L, 12% (v/v) ethanol, 2 g of sodium metabisulphite/L, and then buffered at pH 3.2 using 1 mol/L NaOH. For each cultivar, three replicates of 10 berries each were weighted and used for the total extraction. Skins were manually separated from the pulp, then quickly inserted in 40 mL of the extracting solution, and subsequently frozen. Samples were then thawed to reach room temperature, homogenized with an immersion blender (Ultra-Turrax T25, IKA, Staufen, Germany), and centrifuged at 4000 × g at 20 °C for 5 min. The supernatant was taken, diluted to 50 mL of total volume using the same buffer solution, and the resulting solution was used for anthocyanin analysis.

### 2.4. Simulated macerations in model solutions of grape skins and seeds

For each cultivar, two treatments were established corresponding to different extraction protocols: skins were macerated alone and in presence of seeds ("skins + seeds"), and three replicates were prepared for each combination. Density sorted berries were used in this extraction trial. Simulated macerations were performed in 100 mL of a model solution prepared with 5 g of tartaric acid/L, 100 mg of sodium

metabisulphite/L, and buffered at pH 3.40 using a 1 mol/L NaOH solution. For each grape cultivar, six sets of 80 g of berries were randomly selected among the density sorted berries previously obtained. Berries belonging to three sets were weighed and peeled. Skins and seeds were cleaned from the pulp residues and then inserted into the same glass container inside the model solution. The other three sets of berries were weighted, peeled without pulp and, in this case, only the grape skins were obtained and used for the extraction. Simulated macerations took place for ten days at 27 °C temperature. To monitor each maceration, samples (3 mL) were taken at 48, 72, 96, 144, and 168 h. Following the procedure described by Paissoni et al. (2020), at each sampling point, the liquid taken was replaced with absolute ethanol (min. 99.8% v/v) to simulate the alcohol content increase typical of a fermentation process. This addition resulted in about 3% v/v ethanol in each sampling point, leading to approx. 15% v/v ethanol content in the sample after 168 h to the end of the maceration (240 h).

### 2.5. Chemical analysis of extracts

During the maceration in model solution with increasing ethanol content, the anthocyanin concentration in the extract was monitored at each sampling point by spectrophotometric analyses, which were performed using a UV-1800 spectrophotometer (Shimazdu Corporation, Kyoto, Japan). The total anthocyanin index (TAI) was determined following the method proposed by Di Stefano & Cravero (1991) and described by Petrozziello et al. (2018). TAI was determined by reading the absorbance at 540–536 nm after diluting the sample in an ethanol: water:37% hydrochloric acid (70:30:1, v/v) solution. TAI values were expressed as mg of malvidin-3-*O*-glucoside chloride equivalents/kg of berries. TAI was also determined at the end of the maceration (240 h) and on the total extraction solutions obtained from the skins.

The anthocyanin profile and single form contents of all extracts were analyzed by HPLC following the method reported by Río Segade et al. (2014). Each skin extract was diluted 1:1 using an HCl solution at pH 0.5 and filtered with a 0.45 μm PTFE membrane filter (Paissoni et al., 2020). Afterwards, 50 μL were injected in the HPLC system equipped with a LiChroCART analytical column (25 cm × 0.4 cm) (Merck, Darmstadt, Germany). The mobile phase consisted of A = formic acid:water (10:90, v/v) and B = formic acid:methanol:water (10:50:40, v/v), and the analysis was performed in gradient mode starting from 28% of solvent B, increasing up to 45% of B in 15 min, 70% in 20 min, and 90% in 10 min. Individual anthocyanins were detected at 520 nm. Using an external standard calibration, the results were expressed as mg of malvidin-3-glucoside chloride/kg of berries. The percentage of the di- and tri-substituted forms was calculated by dividing the sum of the concentrations of the respective forms by the total concentrations of all the detected forms. Acquisition and processing were performed using the Agilent ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The extracts were then stored at −20 °C for further analysis.

Monomeric pigments (%MON), small polymeric pigments (%SPP), and large polymeric pigments (%LPP) were then analyzed according to the method proposed by Harbertson, Picciotto, & Adams (2003). This last method is based on the absorbance measurement at 520 nm of the solutions resulting from the use of bovine serum albumin to precipitate polymeric forms and the decolorization of monomeric forms with sulfur dioxide.

### 2.6. Extraction of anthocyanins adsorbed on seed surface

After simulated maceration of grape skins and seeds together in the same extracting media, the seeds adsorbed red pigments on their surface. To extract anthocyanins adsorbed on seed surface, a further extraction was performed on seeds. The extraction was carried out using the methanol:formic acid:water 50:1.5:48.5 (v/v) solvent, according to Gao, Girard, Mazza, & Reynolds (1997). For each replicate, approximately 1 g of seeds (previously macerated together with grape skins) were macerated in 20 mL of the extracting solution for 7 days at room temperature. At the end of the maceration, the liquid was taken, diluted to 25 mL of total volume, and the resulting solution was used to determine the anthocyanins adsorbed on the seed surface following the protocol described in section 2.5.

### 2.7. Statistical analysis

Statistical analyses were performed using R statistic software (R Foundation for Statistical Computing, Vienna, Austria). The normality and homoscedasticity ANOVA assumptions were evaluated using Shapiro–Wilk's and Levene's tests, respectively. For each variable, a one-way analysis of variance (ANOVA) with the Bonferroni LSD posthoc test was used to evaluate significant differences among treatments. Differences were considered statistically significant at $p < 0.05$. ANOVA with Welch's correction and Games-Howell Test was performed when ANOVA assumptions were violated. Statistical correlations were evaluated as Pearson coefficient (r).

## 3. Results and discussion

### 3.1. Grape characterization and potential anthocyanin content

Grape must composition of original (unsorted) and density sorted samples is reported in Table 1. As expected, density sorting minimized the differences within a sample. The total soluble solids degree of unsorted samples ranged between 21.0 and 22.2 °Brix for all varieties, except for 'Nebbiolo', which resulted in a higher value, for which a higher density class was chosen as the most representative for this variety. Titratable acidity and pH values showed bigger differences among cultivars, even in sorted berries, confirming their variety-related nature.

Sorted berries were used in the anthocyanin extraction experiments.

**Table 1**
Grape juice characterization of the four Italian red wine grape varieties used in the experiment.

| Grape cultivar | Sample type | Density class | °Brix | pH | Titratable acidity |
|---|---|---|---|---|---|
| | | kg/m$^3$ | ° | – | g/L as tartaric acid |
| **Aglianico** | Original sample | – | 21.0 ± 0.1 | 2.93 ± 0.01 | 11.3 ± 0.2 |
| | Density-sorted sample used for phenolic extraction | 1092 | 21.8 ± 0.1 | 2.95 ± 0.01 | 11.3 ± 0.1 |
| **Nebbiolo** | Original sample | – | 24.2 ± 0.1 | 3.14 ± 0.03 | 7.4 ± 0.3 |
| | Density-sorted sample used for phenolic extraction | 1101 | 23.4 ± 0.1 | 3.19 ± 0.01 | 6.8 ± 0.1 |
| **Primitivo** | Original sample | – | 21.8 ± 0.1 | 3.27 ± 0.01 | 6.5 ± 0.1 |
| | Density-sorted sample used for phenolic extraction | 1092 | 22.2 ± 0.1 | 3.33 ± 0.01 | 6.5 ± 0.3 |
| **Sangiovese** | Original sample | – | 22.2 ± 0.1 | 3.48 ± 0.01 | 5.1 ± 0.1 |
| | Density-sorted sample used for phenolic extraction | 1092 | 22.1 ± 0.1 | 3.45 ± 0.01 | 5.4 ± 0.1 |

Except for density class, data are expressed as mean values ± standard deviation ($n = 2$).

The grape skins potential anthocyanin content determined by HPLC (as sum of detected monomeric anthocyanins; SDA) and the concentration of individual forms for the four varieties are reported in Table 2. A significant correspondence between SDA values and the spectrophotometric determinations (as TAI which comprises all red-colored pigments) was found ($r = 0.9929$, $p < 0.001$). 'Aglianico' showed the highest SDA value, confirmed by the highest TAI value. Following, 'Primitivo' and 'Sangiovese' showed no significant differences neither in SDA nor in TAI values. 'Nebbiolo' showed the lowest SDA and TAI values. The anthocyanin profiles obtained were generally in line with those reported by other authors (Mattivi et al., 2006). 'Aglianico' and 'Primitivo' showed the highest content of malvidin-3-*O*-glucoside, which represented the 57.3% and 49.6%, respectively, of total anthocyanins, while 'Nebbiolo' resulted in a high percentage of di-substituted forms showing the highest amount of peonidin-3-*O*-glucoside. Similarly, 'Sangiovese' was characterized by a high concentration of di-substituted forms, particularly evidenced by the highest amount of cyanidin-3-*O*-glucoside. This value represents 24.7% of the detected anthocyanins, which is higher compared to what was previously reported (Mattivi et al., 2006). The share of acetyl- and cinnamoyl-glucosides is very high for 'Primitivo' (27.0%), then followed by 'Aglianico' (20.8%) and 'Nebbiolo' (11.8%), while 'Sangiovese' did not evidence these anthocyanin forms (Table 2). The positioning of these varieties according to anthocyanin content is coherent with the situation generally found in the wines produced from these varieties (Giacosa et al., 2021), but their ratio changes due to the nature of anthocyanins, their easiness of extraction, and losses by degradation reactions in winemaking (e.g. in Nebbiolo and Sangiovese with a high prevalence of di-substituted forms).

### 3.2. Anthocyanin extraction during simulated maceration

For each grape cultivar considered, two extraction experiments using model solutions, simulating wine maceration conditions with ethanol increase through time, were compared: grape skins extraction alone ("skins") or in presence of grape seeds ("skins + seeds"). Their anthocyanin extraction kinetics are available in Fig. 1. The maximum anthocyanin extraction from skins was reached at 48 h sampling point for all varieties, consistently to what was already reported in literature (Paissoni et al., 2020). Afterwards, the anthocyanin loss led to a slow decrease in TAI values, following a concentration pattern also coherent with previously-built models for wine maceration (Tindal et al., 2021). At the end of the simulated skin maceration (240 h), 'Aglianico' showed the highest TAI value, which corresponded to 521.6 ± 40.9 mg of malvidin-3-*O*-glucoside chloride equivalents/kg of berries for skins maceration (Table 2), followed by 'Primitivo' (355.5 ± 20.9 mg/kg) and 'Sangiovese' (225.4 ± 8.1 mg/kg), while 'Nebbiolo' had the lower anthocyanin concentration (220.6 ± 6.0 mg/kg).

The comparison with the potential anthocyanin content available in grape skins evidences that, after 240 h of wine-like maceration, 62% of the total anthocyanin (TAI) content was found in 'Aglianico' skins extracts; while 56% and 54% of the total content were observed in 'Primitivo' and 'Nebbiolo' skins extracts, respectively. 'Sangiovese' skins showed the lowest extraction rate (35%). These differences can be correlated with a different extent of the anthocyanin resorption on skin cell wall materials (Medina-Plaza et al., 2020). Therefore, the anthocyanin content at the end of the maceration was strongly affected by the variety and depended on the total anthocyanins (Table 2) and their extractability. These differences, however, can be also influenced by the tannin composition, which is another important variety-related factor. As reported by other authors (Sparrow et al., 2015), the presence of grape skin and seed tannins can modulate the preservation of skin anthocyanins by increasing the formation of more stable pigments.

Regarding the influence of seeds in the macerating media, within each variety, no significant differences were found in the anthocyanin extraction kinetics (Fig. 1) among treatments (grape skins *vs* skins + seeds), although only one exception has been found. On the second day of maceration, 'Sangiovese' skins resulted in a higher anthocyanin concentration with respect to the combined maceration of skins and seeds (+12.4%; $p < 0.05$); nevertheless, no significant differences were found in the following sampling points. Therefore, it is possible to assert that the presence of seeds did not significantly impact the anthocyanin extraction from grape skins or they preservation during maceration. Our results agreed with the published literature; indeed, Bautista-Ortín et al. (2014) reported that seeds removal operation during maceration did not significantly affect the anthocyanin concentration in wines.

### 3.3. Anthocyanin profile of extracts from simulated macerations

At the end of the maceration, the anthocyanin content of extracts was determined by HPLC and reported in Table 2. Anthocyanin profiles were generally in line with those obtained after potential anthocyanin extraction from grape skins (Table 2). Regarding the impact of seeds on the anthocyanin extraction in model solution, all the intra-varietal comparisons among treatments were not significant, with only one exception. The petunidin-3-*O*-glucoside concentration in 'Nebbiolo' skin extract has been found lower compared to the simultaneous maceration of skins and seeds (−6.7%, $p < 0.01$). However, the presence of seeds did not significantly affect either the content of total anthocyanins (TAI) or that of the total individual forms (Table 2). Thus, anthocyanins are extracted and preserved from skins regardless of the presence of seeds during maceration for all the varieties studied.

Anthocyanin single forms (SDA) extracted from skins and detected by HPLC showed a similar tendency with respect to the spectrophotometric (TAI) index that involves all the coloured pigments, but higher values were found for the second one in all varieties (from +0.6% to +4.0%), except for 'Aglianico' which showed an unexpected result (−4.3%). However, in the latter case, the differences between the results of the two determinations (TAI and SDA) were not significant (*t-test* $p > 0.05$) due to the higher variability between the replicates analyzed. The SDA extraction percentage, calculated within each cultivar as comparison with the potential skin extraction, was lower for 'Sangiovese' (34%), whereas 'Nebbiolo' average value (53%) was slightly above 'Primitivo' (52%), followed by 'Aglianico' (66%).

Regarding anthocyanin profile, expressed as percentage, some differences are present between the corresponding to total extraction from grape skins and simulated macerations in model solutions (Fig. 2). Malvidin-3-*O*-glucoside increased its share (28.4–64.6% share depending on the variety and simulated extraction type; +11.6% average share increase) in model wine macerations, confirming its pronounced preservation among free forms. Instead, cyanidin-3-*O*-glucoside marked high reductions in share for total extractions when compared to maceration in model solutions (−4.6% on average), followed by delphidin-3-*O*-glucoside (−3.1%) but not by peonidin-3-*O*-glucoside (−0.2%), the latter also considered as quite sensible to losses during winemaking (González-Neves, Gil, & Barreiro, 2008). The minimum amount of sulfur dioxide (100 mg/L as $Na_2S_2O_5$) present in the model media could have had a role in this behavior, also given that delphidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside are both more susceptible to oxidation as they are *o*-diphenyl anthocyanins (Cheynier, Souquet, Kontek & Moutounet, 1994). However, the anthocyanin forms with the most important decrease in their share from total extraction to model solutions were the cinnamoyl-glucosides (−4.7%). Given that these forms are considered quite resistant to losses by oxidation, in this case the reason could be their weak extraction in model conditions, or possible depolymerization phenomena (García-Beneytez, Revilla, & Cabello, 2002). Nevertheless, for all forms combination phenomena could have impacted their presence as free forms at the end of the simulated maceration process.

**Table 2**
Anthocyanin profile and content of four Italian red wine grape varietes determined by HPLC on berry skin potential extraction, and on simulated 240-hour extractions in presence of grape skins or grape skins + seeds.

| Grape cultivar | Extraction type | Delphinidin-3-*O*-glucoside | Cyanidin-3-*O*-glucoside | Petunidin-3-*O*-glucoside | Peonidin-3-*O*-glucoside | Malvidin-3-*O*-glucoside | Sum of acetyl-glucosides | Sum of cinnamoyl-glucosides | Di-substituted free forms | Tri-substituted free forms | SDA (HPLC) | TAI (spectr.) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | mg/kg berries | | | | | | | % | | mg/kg berries | mg/kg berries |
| **Potential content in grape skins** | | | | | | | | | | | | |
| **Aglianico** | **Grape skins potential** | 68.9 ± 14.1 b | 4.8 ± 0.8 c | 73.3 ± 12.3 b | 32.0 ± 2.5 c | 469.3 ± 14.4 a | 29.1 ± 1.2 a | 141.3 ± 3.8 a | 4.5 ± 0.3 d | 74.6 ± 1.4 a | 818.8 ± 39.3 a | 847.3 ± 58.9 a |
| **Nebbiolo** | **Grape skins potential** | 21.2 ± 1.5 c | 52.8 ± 9.2 b | 16.3 ± 1.0 d | 175.0 ± 26.8 a | 86.7 ± 0.6 d | 14.3 ± 0.6 c | 32.9 ± 4.0 b | 59.3 ± 2.0 a | 29.9 ± 2.1 d | 399.3 ± 31.6c | 408.4 ± 29.3 c |
| **Primitivo** | **Grape skins potential** | 35.0 ± 0.1 c | 12.1 ± 0.1 c | 42.7 ± 0.5 c | 51.6 ± 1.8 c | 299.3 ± 6.2 b | 19.5 ± 0.1 b | 143.5 ± 1.5 a | 10.5 ± 0.1 c | 62.5 ± 0.1 b | 603.8 ± 10.0b | 631.3 ± 2.9 b |
| **Sangiovese** | **Grape skins potential** | 98.4 ± 5.4 a | 153.7 ± 6.5 a | 88.7 ± 4.8 a | 102.2 ± 2.4 b | 179.7 ± 7.9 c | nd | nd | 41.1 ± 0.8 b | 58.9 ± 0.8 c | 622.8 ± 23.8b | 640.2 ± 32.8 b |
| **Sign.** | | *** | ***# | *** | *** | *** | ***# | *** | ***# | *** | *** | *** |
| **Extractable content after 240 h in model solution** | | | | | | | | | | | | |
| **Aglianico** | **Skins** | 32.1 ± 5.3 | 2.5 ± 0.4 | 43.9 ± 5.8 | 20.0 ± 1.6 | 348.9 ± 26.5 | 21.7 ± 1.7 | 70.6 ± 6.8 | 4.2 ± 0.1 | 78.7 ± 0.3 | 539.7 ± 46.9 | 521.6 ± 40.9 |
| | **Skins + Seeds** | 33.7 ± 5.0 | 2.5 ± 0.5 | 44.3 ± 5.1 | 19.1 ± 2.4 | 345.8 ± 21.0 | 22.2 ± 1.6 | 74.4 ± 7.3 | 4.0 ± 0.2 | 78.2 ± 0.5 | 542.0 ± 42.7 | 539.2 ± 38.2 |
| | **Sign.** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **Nebbiolo** | **Skins** | 9.1 ± 0.4 | 18.3 ± 0.6 | 10.4 ± 0.1b | 91.4 ± 6.8 | 65.1 ± 0.9 | 8.1 ± 0.5 | 10.8 ± 0.3 | 51.4 ± 1.6 | 39.7 ± 1.3 | 213.2 ± 6.2 | 220.6 ± 6.0 |
| | **Skins + Seeds** | 10.1 ± 0.2 | 21.6 ± 1.5 | 11.1 ± 0.1 a | 97.2 ± 2.7 | 63.1 ± 2.5 | 8.6 ± 0.2 | 10.5 ± 0.7 | 53.4 ± 1.6 | 37.9 ± 1.2 | 222.2 ± 1.0 | 208.8 ± 22.8 |
| | **Sign.** | ns | ns# | ** | ns | ns# | ns | ns | ns | ns | ns | ns |
| **Primitivo** | **Skins** | 10.6 ± 0.9 | 4.2 ± 0.7 | 19.1 ± 1.1 | 26.1 ± 3.9 | 189.0 ± 2.4 | 12.7 ± 0.7 | 53.6 ± 1.0 | 9.6 ± 1.2 | 69.3 ± 0.5 | 315.3 ± 6.5 | 355.5 ± 20.9 |
| | **Skins + Seeds** | 10.8 ± 1.1 | 4.2 ± 0.7 | 19.2 ± 1.5 | 27.2 ± 3.3 | 190.9 ± 15.0 | 12.8 ± 0.8 | 54.1 ± 6.6 | 9.9 ± 1.0 | 69.2 ± 0.7 | 319.2 ± 26.3 | 331.7 ± 28.6 |
| | **Sign.** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **Sangiovese** | **Skins** | 18.6 ± 2.9 | 23.9 ± 3.8 | 27.3 ± 2.6 | 34.7 ± 2.3 | 105.5 ± 1.7 | nd | nd | 27.8 ± 1.1 | 72.2 ± 1.1 | 210.0 ± 13.2 | 225.4 ± 8.1 |
| | **Skins + Seeds** | 20.1 ± 3.7 | 25.3 ± 3.3 | 29.3 ± 4.8 | 37.6 ± 3.8 | 114,0 ± 17.5 | nd | nd | 27.8 ± 2.3 | 72.2 ± 2.3 | 227.2 ± 31.4 | 244.4 ± 27.5 |
| | **Sign.** | ns | ns | ns | ns | ns# | ns | ns | ns | ns | ns | ns |

Data are expressed as mean values ± standard deviation of three replicates ($n = 3$). Individual anthocyanin forms, SDA (sum of detected anthocyanins), and TAI (total anthocyanin index detected through spectrophotometric analysis) are expressed as mg of malvidin-3-*O*-glucoside chloride equivalents/kg of berries. nd: not detected. Sign.: *, **, ***, and "ns" indicate significant differences at $p < 0.05$, 0.01, 0.001, and not significant, respectively, among values within the same column (comparison of skins potential anthocyanins among varieties, or between extractable contents inside each variety) according to ANOVA or Welch's ANOVA (#). Values followed by different letters within a column are significantly different ($p < 0.05$, according to Bonferroni LSD or Games-Howell post-hoc tests for ANOVA and Welch's ANOVA, respectively).

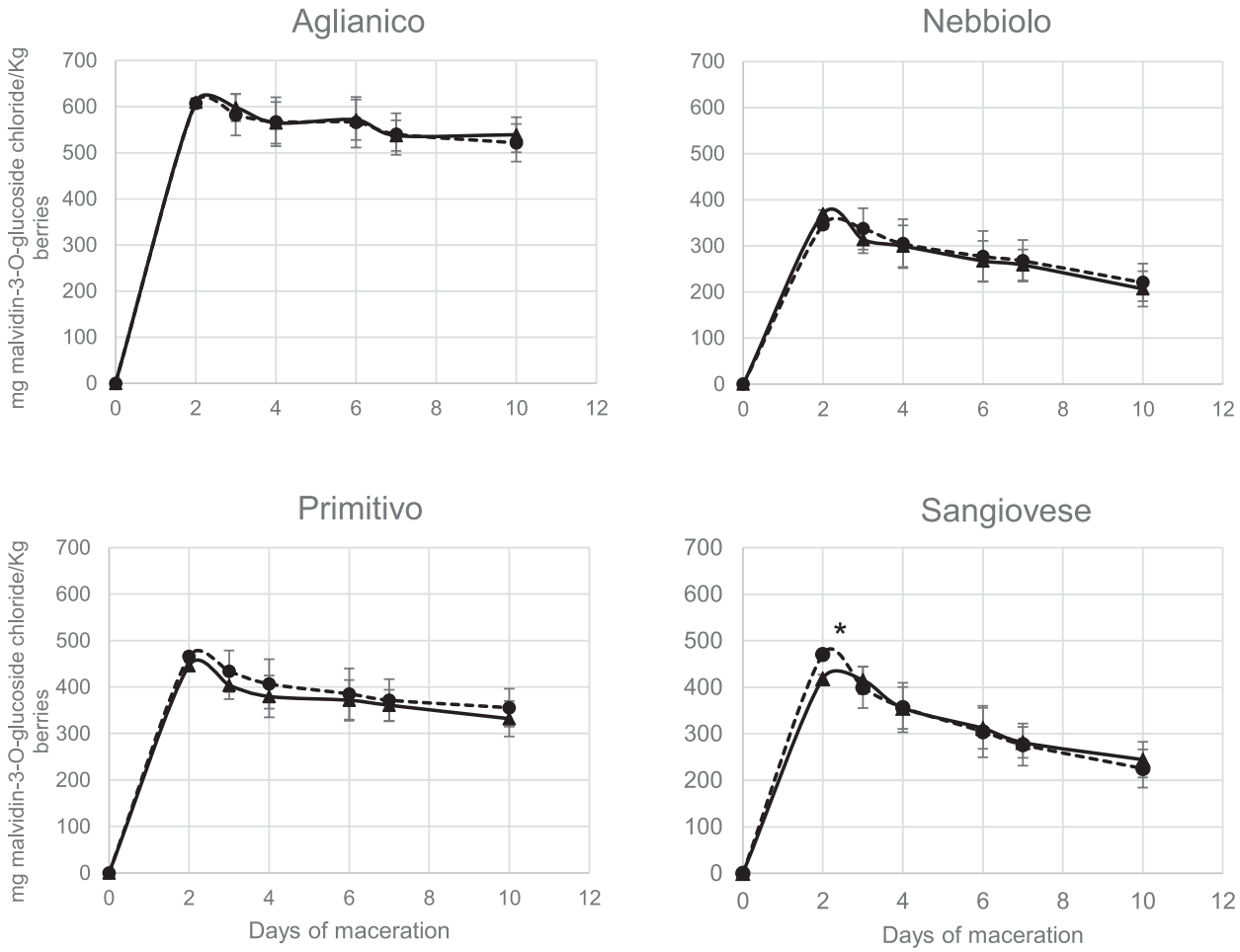


**Fig. 1.** Anthocyanin extraction kinetics from skins (- -●- - circles, dashed line) and skins and seeds (—▲— triangles, continuous line) of four Italian red grapevine varieties during ten days of simulated maceration in model solution. Data are expressed as mg of malvidin-3-*O*-glucoside chloride equivalents/kg of berries. At each sampling point, data are reported as mean values and bars show standard deviation ($n = 3$). Sign.: * indicate significant differences at $p < 0.05$ (according to *t-test*) between skins and skins + seeds extraction.

### *3.4. Impact of seeds on the formation of polymeric pigments*

The anthocyanin polymerization rate of extracts was also evaluated. Polymeric pigments in seed and skin simultaneous maceration were compared to those obtained in skin alone-extracts (Fig. 3). No increase in polymerization rate (with respect to skins alone) was observed when Sangiovese skins and seeds were macerated together. In the maceration of skins together with seeds, %MON were found significantly lower for 'Nebbiolo' (−4.0%), 'Aglianico' (−2.7%), and 'Primitivo' (−2.5%). % MON reduction led to %LPP significant increase for 'Nebbiolo' (+3.1%) and 'Aglianico' (+2.0%), while 'Primitivo', despite its significant reduction of %MON in skins + seeds sample, showed a slight increasing tendency in both %LPP and %SPP but without significant differences. Therefore, the presence of seeds during maceration generally led to a decrease of the monomeric pigments fraction and thus an increase in the polymerization rate. Our results agreed with those of Sparrow et al. (2015) who found that non-bleachable pigments increased where 'Pinot noir' seeds were added to skin simulated maceration. Lastly, we may hypothesize that 'Sangiovese' resulted in a non-significant increase due to the lower amount of tannins extractable from seeds compared to the other varieties (Mattivi et al., 2003).

Kumar et al. (2022) studied the depletion of 'Pinot noir' monomeric anthocyanins in model wine at different anthocyanin and seed tannin concentrations. They highlighted that the formation of polymeric pigments increased during aging and found that model wine added of seed tannins resulted in a faster depletion of monomeric anthocyanins, which led to an increased polymeric pigments content. In literature, it is well reported that high initial tannin and anthocyanin concentrations resulted in a high polymerization rate (Ristic et al., 2010). Tannins play a fundamental role in the formation of polymeric pigments (Tindal et al., 2021). Given that seeds are an important source for tannins (Rousserie et al., 2019), tannins extracted from seeds may have enhanced the formation of polymeric pigments, increasing both %LPP and %SPP. Moreover, it must be noted that the polymeric pigment formation may be higher in real winemaking conditions as fermenting yeast metabolites are involved in reactions between tannins and anthocyanins (He et al., 2012; Marquez, Serratosa, & Merida, 2013).

Anthocyanin-tannin and anthocyanin-anthocyanin adduct formations preserve the wine color since the anthocyanin chromophore is protected from the nucleophilic attack on C4, minimizing the bisulfite bleaching and hydration reaction (He et al., 2012). Polymeric pigments formation during the maceration in presence of seeds can lead to more color stability, confirming what was previously found about seeds and color stabilization. Several authors agreed on the positive role of seed presence during maceration, which may enhance the color stability and increase the color intensity (Bautista-Ortín et al., 2014; Kovac et al., 1995). It should be noted that the presence of seeds during maceration, and the subsequent interaction between anthocyanins and tannins, can modulate also the sensory profile of the wine with a relevant impact on the mouthfeel properties and astringency (Rinaldi et al. 2015).

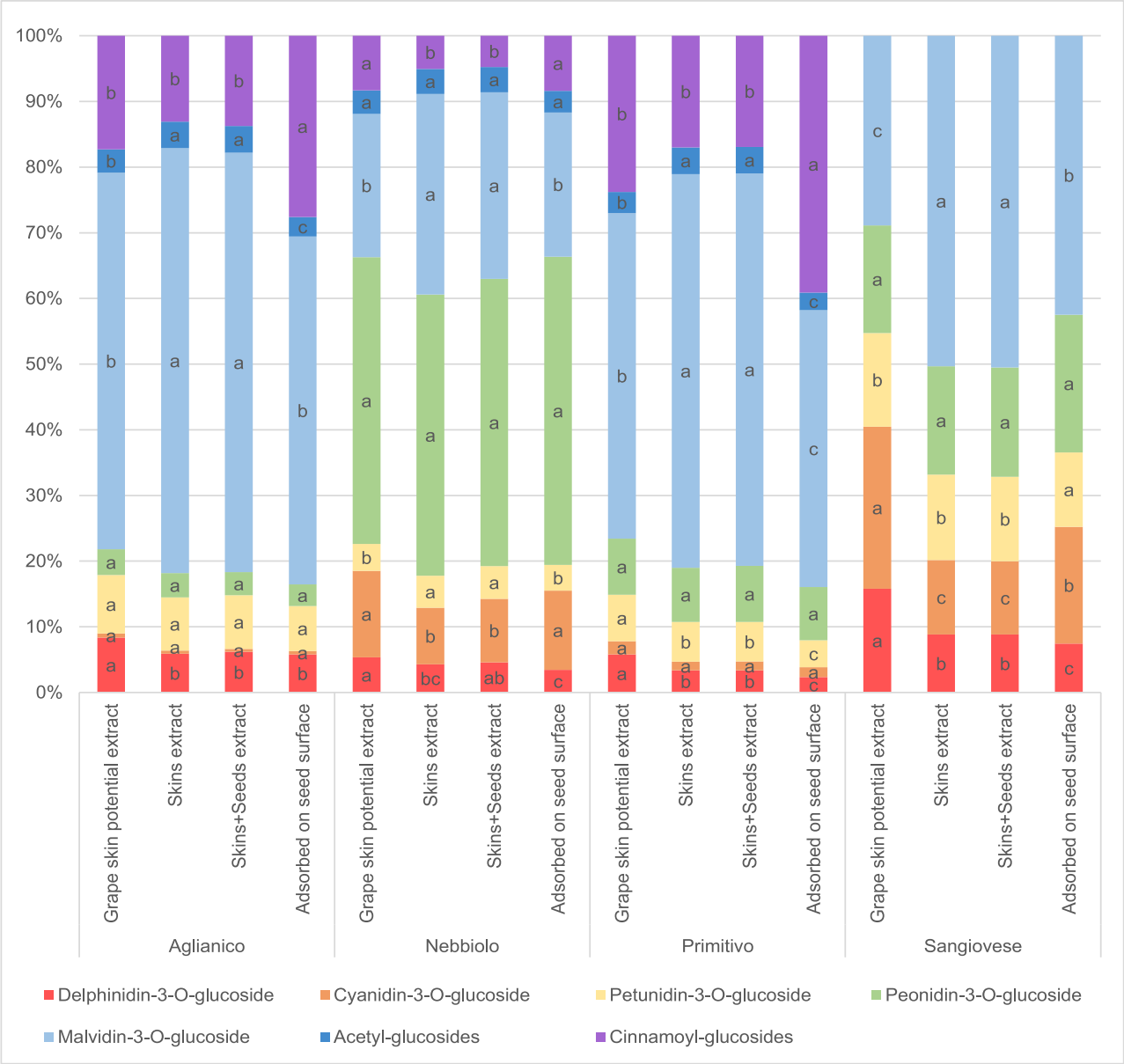


**Fig. 2.** Relative (percentage) profile of anthocyanins obtained in grape skins potential extracts, skins extracts, skins and seeds extracts, and of those adsorbed on seed surface after model wine maceration with skins of four Italian grape varieties. Different letters within each grape cultivar and parameter indicate significant differences ($p < 0.05$, one-way ANOVA with Bonferroni LSD post-hoc test).

### 3.5. *Quantification of anthocyanins adsorbed on seed surface*

During the combined maceration of seeds and skins in model solution, seeds retained anthocyanins on their surface. To investigate this behavior, at the end of the maceration, seeds have been subjected to further extraction in a stronger media. Anthocyanins retained by seeds have been obtained and the anthocyanin profile was determined by HPLC (Table 3). As a hypothesis, the amount of adsorbed coloring matter should be dependent primarily on the number of seeds contained per kg of berries, which is a varietal character related to the berry weight and ripening (Ristic & Iland, 2005). Moreover, also seed weight (as an indicator of their size) could be involved in anthocyanins adsorption since it determines the seed surface that enters in contact with the liquid.

'Nebbiolo' had the highest number and weight of seeds/kg of berries and showed the highest amount of retained anthocyanins expressed as mg of malvidin-3-*O*-glucoside chloride equivalents/kg of berries. Consistently, 'Primitivo', which showed both the lowest weight and number of seeds/kg of berries, resulted in the lowest retained anthocyanin concentration (1.54 ± 0.74 mg/kg of berries). However, 'Sangiovese' and 'Aglianico' had a similar seed weight with respect to 'Nebbiolo', but the anthocyanin adsorption on seeds was 2.04 ± 0.34 and 1.93 ± 0.19 mg/kg, respectively. Significant differences are observable also considering the expression as amount of adsorbed anthocyanins per weight of seeds (μg/g of seeds). 'Nebbiolo' seeds showed the highest retained amount (103.3 ± 15.0 μg/g of seeds), followed by 'Primitivo', which showed the second-highest adsorption rate (69.3 ± 1.0 μg/g of seeds) despite resulting in the lowest total retained amount per kg of berries, due to the lowest seeds number and weight with respect to the other varieties. Therefore, seeds had a different anthocyanin adsorption capacity, and this characteristic appeared to be variety-dependent also considering that grapevine seeds have a different composition, structure, and shape among varieties (Bordiga, Travaglia, Locatelli, Coïsson, & Arlorio, 2011; Cervantes, Martín-Gómez, Espinosa-Roldán, Muñoz-Organero, Tocino, Cabello-Sáenz de Santamaría, 2021;

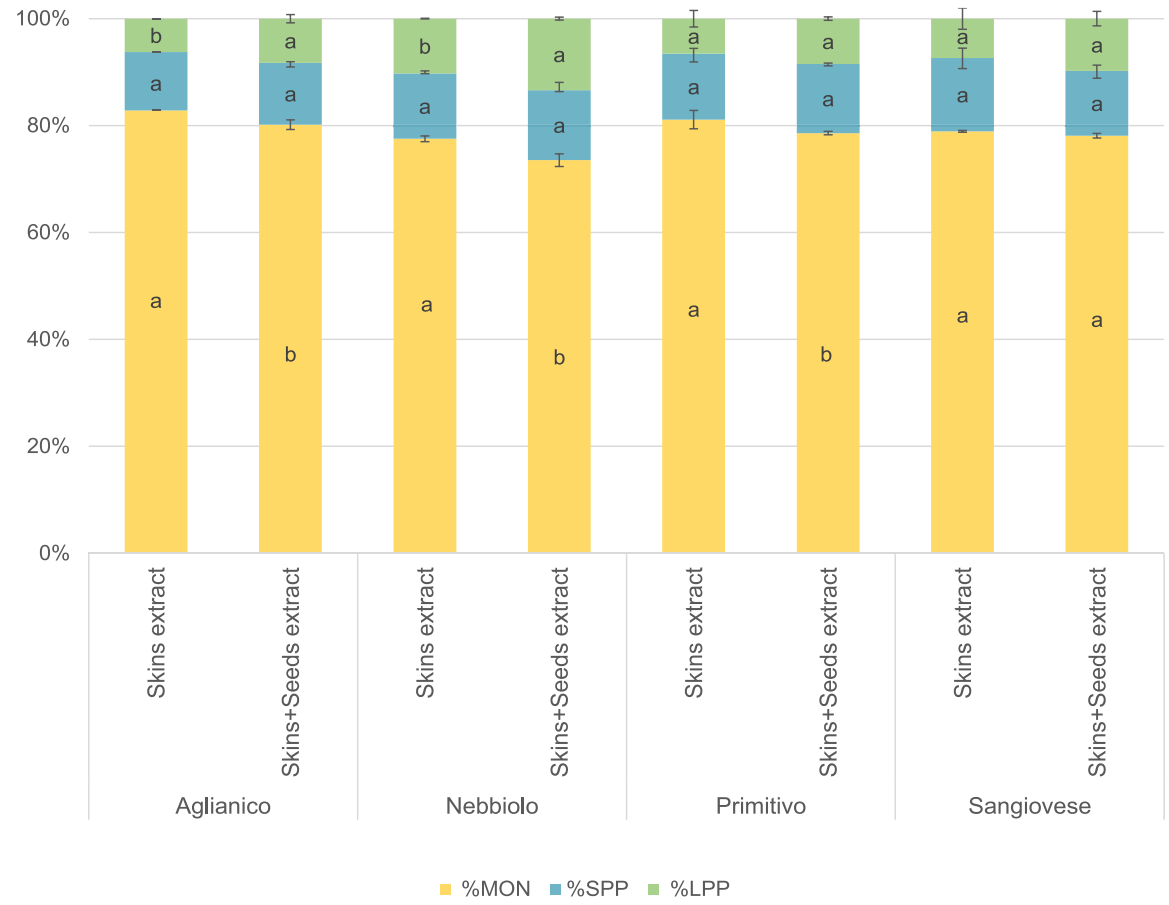


**Fig. 3.** Percentages of large polymeric pigments (%LPP), small polymeric pigments (%SPP), and monomeric pigments (%MON) detected in berry skin and skin together with seed extracts obtained. Data are reported as mean values and bars show standard deviation ($n = 3$). Different letters within each grape cultivar and parameter indicate significant differences ($p < 0.05$, one-way ANOVA with Bonferroni LSD post-hoc test).

**Table 3**
Anthocyanins adsorbed by grape seeds during simulated maceration of seeds and skins in model solution.

| Grape cultivar | No. seeds/ kg berries | g seeds/ kg berries | Delphinidin-3-*O*-glucoside | Cyanidin-3-*O*-glucoside | Petunidin-3-*O*-glucoside | Peonidin-3-*O*-glucoside | Malvidin-3-*O*-glucoside | Sum of acetyl-glucosides | Sum of cinnamoyl-glucosides | SDA (HPLC) from seed adsorption | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | µg/g seeds in the extracting solution | | | | | | | µg/g seeds in the extracting solution | mg/kg berries on the wine-like extraction |
| **Aglianico** | 989 ± 23 b | 39.52 ± 0.54 a | 2.80 ± 0.33 b | 0.26 ± 0.02 c | 3.31 ± 0.26 b | 1.59 ± 0.15 c | 25.67 ± 0.48 | 1.44 ± 0.07 b | 13.73 ± 5.43 b | 48.8 ± 5.1 c | 1.93 ± 0.19 b |
| **Nebbiolo** | 1175 ± 31 a | 38.43 ± 1.26 a | 3.53 ± 0.33 ab | 12.43 ± 1.62 a | 4.04 ± 0.54 b | 48.46 ± 6.83 a | 22.69 ± 3.62 | 3.38 ± 0.54 a | 8.74 ± 1.90 b | 103.3 ± 15.0 a | 3.97 ± 0.57 a |
| **Primitivo** | 496 ± 47 c | 22.26 ± 10.93 b | 1.63 ± 0.22 c | 1.03 ± 0.24 c | 2.85 ± 0.40 b | 5.60 ± 1.11 bc | 29.24 ± 3.61 | 1.85 ± 0.15 b | 27.10 ± 5.39 a | 69.3 ± 1.0 b | 1.54 ± 0.74 c |
| **Sangiovese** | 935 ± 35 b | 36.11 ± 1.03 a | 4.23 ± 1.06 a | 10.04 ± 1.90 b | 6.44 ± 1.44 a | 11.83 ± 2.26 b | 24.00 ± 4.66 | nd | nd | 56.5 ± 11.0 bc | 2.04 ± 0.34 b |
| **Sign.** | *** | * | **# | *** | ** | **# | ns | ***# | *** | *** | ** |

Data are expressed as mean values ± standard deviation of three replicates ($n = 3$). Individual anthocyanin forms and SDA (sum of detected anthocyanins) are expressed as µg of malvidin-3-*O*-glucoside chloride equivalents/g of seeds. nd: not detected. Sign.: *, **, ***, and "ns" indicate significant differences at $p < 0.05$, 0.01, 0.001, and not significant, respectively, among values within the same column according to ANOVA or Welch's ANOVA (#). Values followed by different letters within a column are significantly different ($p < 0.05$, according to Bonferroni LSD or Games-Howell post-hoc tests for ANOVA and Welch's ANOVA, respectively).

Mattivi, Vrhovsek, Masuero, & Trainotti, 2009).

In general, in the model wine extractions the anthocyanin amount retained by seed surface represented a small percentage compared to the total extractable amount from skins; thus, the impact of anthocyanin adsorption on seeds can be considered negligible. To confirm this, as previously indicated for all the varieties studied, the anthocyanin content at the end of the simultaneous maceration (skins + seeds) was not significantly lower compared to the maceration without seeds (Fig. 1, Table 2).

It is worth noting that 'Nebbiolo', whose skin extract showed the highest concentration of peonidin-3-*O*-glucoside (Table 2) resulted in the highest peonidin concentration adsorbed by seeds (48.46 ± 6.83 μg/g of seeds, $p < 0.01$; Table 3). Likewise, 'Sangiovese', which is characterized by a high percentage of di-substituted forms, resulted in a high amount of cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside adsorbed by seeds (10.04 ± 1.90 and 11.83 ± 2.26 μg/g of seeds, respectively). Therefore, seeds adsorbed different anthocyanin forms mainly depending on their abundance in the solution. Nonetheless, malvidin-3-*O*-glucoside adsorbed content (μg/g of seeds) showed no significant difference among varieties regardless of several differences detected on grape skin potential content (Table 2).

The profiles of anthocyanins adsorbed on seed surface, expressed as percentage, were compared to those obtained at the end of simulated macerations in the model solutions (Fig. 2). For the latter, no differences were found in the percentages of the individual anthocyanin forms between the maceration of skins alone and the combination of seeds and skins. In contrast, some differences have been found among individual forms retained by seeds: cinnamoyl-glucosides were always retained in a higher percentage compared to the share during skins + seeds simulated maceration, on the other hand, acetyl-glucosides and malvidin-3-*O*-glucoside were less adsorbed (by percentage) on seed surface in all varieties.

In light of this data, we may hypothesize that individual anthocyanin forms have a different inclination to be adsorbed on seed surface, as also emerged in prior studies on different substrates. Padayachee et al. (2012) demonstrated that acylated anthocyanins from purple carrot juice were generally more adsorbed on plant cell wall composites than non-acylated as they have a higher molecular weight and a greater affinity with cell wall materials. Furthermore, the seed structure may have a role in determining the quantity and the form of anthocyanins retained. It is known that seed cell walls are mainly composed by cellulose, hemicellulose, lignin, and pectins (Rousserie et al., 2019), and these plant cell wall materials (cellulose and cellulose-pectin composites) are reported to adsorb anthocyanins through ionic and hydrophobic interactions (Padayachee et al., 2012). In literature, it is widely proven that grape skins are involved in anthocyanin adsorption due to interactions between cell walls and phenolic substances (Medina-Plaza et al., 2020). Therefore, the seed surface adsorption phenomenon may be based on the same mechanism. Inoue, Kobayashi, Hoshino, Hisamoto, Watanabe-Saito, & Okuda (2019) analyzed the adsorption properties of anthocyanins by insoluble cell wall materials extracted from seeds in model solutions. Authors found that seed cell wall materials can adsorb up to 4% of the grape skin anthocyanin content. Moreover, they found that the adsorption occurred to different extents according to the variety. However, the seed-derived cell wall material adsorption capacity was up to 6-fold lower than that from skins, suggesting that seeds had a minor role in causing the wine anthocyanin adsorption. To the best of our knowledge, no studies have been conducted to assess the mechanism through which anthocyanins are retained by seed surface.

It should be noted that the anthocyanins extraction and adsorption may result differently during a regular fermentation since i) treatments necessary to manage the solid parts may affect the adsorption mechanisms; ii) the influence of yeasts and enzyme activities could play a role in the modification of cell wall materials; iii) during the sample preparation, the seeds surface was cleaned from the pulp; iv) density sorted berries were used in the phenolic extraction to limit the variability of the original samples. Therefore, in the future, the influence of all these aspects should be assessed.

## 4. Conclusion

The role of grape seeds presence during maceration in anthocyanin extraction and preservation was assessed in this study. Differences in the total anthocyanin content and anthocyanin profile among varieties were confirmed. The presence of seeds had no impact on the anthocyanin extraction from grape skins during simulated maceration; indeed, the kinetics and the total anthocyanin content after 240 h did not show significant differences when compared to skin-alone maceration. Moreover, seeds did not affect the anthocyanin profiles of skin extracts. The presence of seeds during the simulated maceration led to an increase in the polymeric pigment fraction over time, confirming that seeds contribution enhanced the color stability. These results highlight that seeds had a key role in wine colour stabilization and their removal in real maceration condition may reduce the share of polymeric pigments.

For the first time to our knowledge, the anthocyanins adsorbed by the seed surface during the maceration in presence of skins were obtained and quantified. The anthocyanin concentration retained by seeds was generally very low, and impacted for less than 4 mg/kg of berries. 'Nebbiolo' showed the highest seed number and seed weight per kg of grapes: indeed, it resulted in the highest anthocyanin concentration adsorbed during the maceration. However, the existence of a direct correlation among these factors was not confirmed on all varieties. In light of these data, it is possible to hypothesize that the total amount of coloring matter retained by seeds depends on varietal characteristics such as the number, weight, size, and surface structure of seeds. Individual anthocyanins were retained by seeds mainly according to their abundance in the extract. We may also hypothesize that cinnamoyl-glucoside anthocyanin forms have a higher affinity with seed surface. Furthermore, the seed surface adsorption phenomenon may be based on anthocyanins-seed cell walls interaction, similar to what happens with skin cell wall materials.

## CRediT authorship contribution statement

**Simone Giacosa:** Conceptualization, Investigation, Formal analysis, Writing – review & editing. **Lorenzo Ferrero:** Writing – original draft, Formal analysis, Visualization. **Maria Alessandra Paissoni:** Investigation, Data curation, Formal analysis, Writing – review & editing. **Susana Río Segade:** Investigation, Writing – review & editing. **Vincenzo Gerbi:** Funding acquisition, Resources, Writing – review & editing. **Luca Rolle:** Conceptualization, Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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