

# PROANTHOCYANIDIN COMPOSITION AND EVOLUTION DURING GRAPE RIPENING AS AFFECTED BY VARIETY : NEBBIOLO AND BARBERA CV.

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## Abstract

**Aim :** Proanthocyanidin (PA) content, composition and evolution during berry development was determined in two cv., Nebbiolo and Barbera, quite different in their polyphenolic profile.

**Methods and results :** PAs were extracted separately from seeds and skins, before véraison until maturity. Intact extracts were fractionated according to PA degree of polymerization. Nebbiolo grapes presented a significantly higher PA content in both seeds and skins. PA depolymerization reactions as well as a weaker decrease in polymeric PAs occurred in Barbera skins. Oligomeric PAs prevailed over polymeric ones in Barbera seeds. At harvest, Nebbiolo skins presented a significantly higher percentage of oligomeric PAs, while monomeric and oligomeric flavanol percentages were significantly higher in Barbera seeds.

**Conclusion :** Barbera and Nebbiolo grapes differ also in their PA total amount and structure. PA content in grape skins and seeds, their distribution in monomeric, oligomeric and polymeric fractions, as well as their decrease rate during fruit ripening are different between the cv.

**Significance and impact of the study :** The fractionation results, obtained for Nebbiolo and Barbera grapes for the first time, highlight how grape variety affects the PA profile. Furthermore, this study is the basis for better understanding the astringency and bitter sensation in Barbera and Nebbiolo grapes and wines.

**Key words :** grapes, Nebbiolo cv., Barbera cv., proanthocyanidins, fractionation, ripening

## Résumé

**Objectif :** Détermination de la teneur en proanthocyanidines (PAs), leur composition et leur évolution pendant la maturation de deux variétés, Nebbiolo et Barbera, différentes pour leur profil polyphénolique.

**Méthodes et résultats :** Les PAs ont été extraites des pépins et de la pellicule, avant la véraison et jusqu'à la maturité. Les extraits intacts ont été fractionnés selon le degré de polymérisation des PAs. Les raisins Nebbiolo présentaient un niveau plus élevé de PAs, dans les pépins comme dans les pellicules ; à la vendange, les pellicules présentaient un pourcentage plus élevé de PAs oligomériques, tandis que les flavonoïdes monomériques et oligomériques étaient plus importants dans les pépins de raisin Barbera. Dans les pellicules des raisins Barbera, on a observé des réactions de dépolymérisation des PAs et une faible diminution de PAs polymériques.

**Conclusions :** Les raisins Barbera and Nebbiolo diffèrent aussi pour la teneur et la structure des PAs. Varient aussi les PAs des pépins et des pellicules, leur distribution en fractions monomériques, oligomériques et polymériques et leur diminution pendant la maturation.

**Signification et impact de l'étude :** Les résultats du fractionnement, obtenus pour la première fois pour les raisins Nebbiolo et Barbera, démontrent l'effet de la variété sur le profil des PAs. De plus, cette étude aide à mieux comprendre les sensations d'astringence et d'amertume qui caractérisent les raisins et les vins Barbera et Nebbiolo.

**Mots clés :** raisins, Nebbiolo cv., Barbera cv., proanthocyanidines, fractionnement, maturation

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## INTRODUCTION

Grape tannins represent a significant organoleptic component of wines and are an important contributor to wine quality.

The term tannin, in wine, describes proanthocyanidins (PAs) which are oligomers and polymers of flavan-3-ol units (Downey *et al.*, 2003). PAs are present mainly in skins (Souquet *et al.*, 1996) and seeds (Prieur *et al.*, 1994) of grape berries. Their accumulation occurs early in berry development and is completed when ripening starts (Bogs *et al.*, 2005). Flavan-3-ol monomers and PAs share the same biosynthetic pathway with anthocyanidins, which are red grape pigments, and with flavonols, which contribute to both bitter taste and color stability of red wine (Baranac *et al.*, 1997).

In grape berries, the phenolic and PA content depends on climatic and geographical factors, cultural practices, ripening stage, plant vigor and, consequently, on vine cultivar, clone and rootstock (Pérez Magariño and González-Sanjósé, 2002).

As anthocyanin, flavonol and hydrocinnamate profiles, PA composition could be used for the characterization and thus the classification of grape cultivars (Ferrandino *et al.*, 2012). Grape variety determines the polyphenolic profile in different cultivars. In fact, it is known that the patterns of some classes of flavonoids, such as anthocyanins, are under strict genetic control and that their distribution varies considerably among different grape cultivars (Mattivi *et al.*, 2006). Their profiles for each variety are relatively stable, while absolute concentrations can vary between different vintages.

Moreover, the diversity in wine grape PA composition leads to the employment of different oenological techniques (Kennedy and Jones, 2001; Mattivi *et al.*, 2003). As a consequence, the PA variation in grape skins and seeds, the rate of their decrease during fruit ripening and their extractability have to be considered when grapes at different maturity stages are used in winemaking (González-Manzano *et al.*, 2006). The mean degree of polymerization of PAs affects their relative bitterness and astringency. Monomers and small oligomers are more bitter than astringent, whereas the opposite is true for larger molecules (Bordiga *et al.*, 2011; Kallithraka *et al.*, 1997). Therefore, extracted during red winemaking, PAs are mostly responsible for the astringency, bitterness and structure of wines (Gawel, 1998; Haslam, 1974; Mc Rae and Kennedy, 2011), while they also play an important role in red wine aging due to their high reactivity (polymerization,

condensation with anthocyanins and oxidation reactions) (Ricardo-da-Silva *et al.*, 1991). These reactions with anthocyanins may be responsible for the loss of astringency during wine aging (Cheynier *et al.*, 1997; Remy *et al.*, 2000).

*Vitis vinifera* L. cv. Nebbiolo and Barbera are two cultivars widely planted in northern Italy and particularly in Barolo DOCG (*Denominazione di origine controllata e garantita*) and Barbaresco DOCG wines are produced with Nebbiolo grapes and represent two of the most famous wine regions in the world, both situated in Piedmont. These wines are characterized by a light color and a high level of acids and tannins. In a sense, Nebbiolo can be compared to Pinot Noir, which also produces light-colored, aromatic wines with high acidity. However, Nebbiolo wines tend to be more tannic in their youth, the best examples often requiring decades to reach a balance between the aromatic complexity and the vibrant acids and tannins. They are long-lived wines that improve with age. On the other hand, the Barbera grape variety is widely planted in Piedmont and in other Italian regions as well as overseas. Barbera wines tend to be much more colored, fruitier, riper and less tannic than Nebbiolo wines. The full characterization of skin and seed PAs of those two important Piedmont cultivars is then fundamental to better understand their composition and evolution during fruit ripening and to choose the best time for harvest and modulate their extraction into wine.

Previous studies have focused more on anthocyanin profile characterization and other physico-chemical differences between these two cultivars (Di Stefano *et al.*, 2002). Recently, anthocyanin, flavonol and hydrocinnamate profiles have been studied at maturity and their importance from a taxonomical point of view for the classification of a great number of Italian *V. vinifera* cultivars, including Nebbiolo and Barbera, was investigated (Ferrandino and Guidoni, 2010; Ferrandino *et al.*, 2012; Mattivi *et al.*, 2006).

While little information is available about Nebbiolo and Barbera PA content and evolution during berry development (Di Stefano *et al.*, 2002), no study involving PA fractionation is mentioned.

The aim of this study is the evaluation of PA potential and composition as affected by grapevine cultivar at both maturity and different stages of berry development. Potential differences between the two cultivars are investigated too.

## MATERIALS AND METHODS

### 1. Sampling and extraction of phenolic compounds from solid parts of the grape

Nebbiolo and Barbera grape berries grown during the 2011 harvest season were collected in Cisterna d'Asti, Piedmont (Italy), every fifteenth day during berry development, from July 15<sup>th</sup> (before véraison) until September 28<sup>th</sup> (commercial maturity). The second sampling date corresponded to the beginning of véraison. All samples consisted of randomly collected berries gathered with their pedicles, from different sides of the bunches, both shaded and sunlit, within the same vineyard block for each cultivar.

Three replicates of 25 berries were chosen each time from a pool of 300 – 400 berries for each cultivar and then weighed.

The seeds and skins were separated from the berry mesocarp, and the intact tissues (i.e., whole seed or skin) were extracted separately.

Preliminary tests have shown that 50 mL of acetone/water (60:40) were enough to extract the totality of phenolic compounds from grape samples of both cultivars (data not reported).

Briefly, the berry samples were processed as follows: isolated skins and seeds were homogenized (Ariete Magic Choppy 1824) separately with 30 mL of acetone/water (60:40). The extraction was carried out at room temperature for 30 min, then the suspension was centrifuged (x 15 min) and the pellet washed twice with the same solvent. The final volume of extracts was 50 mL for each sample. The oxidation of phenolic compounds was limited by saturation with nitrogen during the preparation of both skin and seed extracts. In particular, the solvents were saturated with nitrogen and the same gas was used to saturate the headspace during both the homogenization and extraction processes.

Afterwards, acetone was removed under reduced pressure, then the residue was dissolved in a tartaric buffer pH 3.2 (L<sup>-1</sup> tartaric acid, 22 mL NaOH 1 N, 12 % v/v ethanol and 100 mg L<sup>-1</sup> Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) and brought up to 30 mL of total volume.

### 2. Spectrophotometric determinations

Among the proposed methods to study flavanols, spectrophotometric measurement after acidic hydrolysis or reactivity towards vanillin represent cheap and easy-to-use measurement approaches. Moreover, the chemistry behind those analytical methods provides different information and the two

results cannot be equivalent. Acidic catalysis carried out during the analysis of total PAs evaluates oligomeric and polymeric structures. The evaluation of flavanols by the vanillin assay method (V) takes into account both monomeric flavan-3-ols as well as oligomeric and polymeric structures accessible to the electrophilic addition of vanillin (6 and 8 free positions). Even if « the higher the amount in oligomeric and polymeric flavanols, the greater the response in acidic hydrolysis and reactivity to vanillin », the indexes provide different information. The former reaction hydrolyzes flavanols into their monomeric components and measures them. The vanillin assay takes into account the property of flavanols to react with aldehyde. This property is strictly related to the spatial conformation and sterical hindrance of PAs. Thus, on the one hand, the two spectrophotometric methods provide complementary but different information, on the other hand, the V/PAs ratio of intact extracts and of each fraction also provides some information about PA structure during berry development (Di Stefano *et al.*, 2002).

#### 2.1 Total proanthocyanidin (PA) determination

Total PAs were determined by spectrophotometry according to Di Stefano (Di Stefano *et al.*, 1989; Di Stefano and Cravero, 1991) using a Beckman D.U. 640 spectrophotometer. Briefly, 0.2 mL of each intact extract was added to 12.3 mL ethanol and 12.5 mL 37 % HCl containing 300 mg L<sup>-1</sup> FeSO<sub>4</sub>\*7H<sub>2</sub>O. The absorbance at 532 nm was measured before and after hydrolysis (50 min in a thermostatic bath at ), and the concentration of PAs was determined according to a calibration curve obtained with cyanidin-3-glucoside. The results are expressed as mg per kg of fresh weight.

#### 2.2 Flavan-3-ol (V) determination by the vanillin assay (V)

The analysis was carried out according to Di Stefano *et al.* (1989) and Di Stefano and Cravero (1991). In particular, 0.5 mL of each intact extract previously diluted with methanol was added to 3 mL vanillin (4 % in methanol) and then acidified with 1.5 mL 37 % HCl. The electrophilic reaction of vanillin with flavanols and PAs was followed by measuring the absorbance at 500 nm after 15 min reaction at room temperature. A calibration curve was obtained by known amount of (+) catechin. The results are expressed as mg per kg of fresh weight.

### 3. Fractionation of phenolic compounds

The fractionation of Nebbiolo and Barbera grape PAs according to their degree of polymerization was performed as reported by Sun and coworkers *et al.* (1995; 1996) with minor modification. Briefly, 1 mL of each intact extract (skins or seeds) was fractionated on a 500 mg C<sub>18</sub> Sep-Pak (Waters) (previously conditioned with 2 mL methanol and 4 mL deionised water) into three fractions by different organic solvents. Monomeric flavan-3-ols were eluted with 25 mL of diethyl ether, oligomeric PAs with 25 mL of ethyl acetate, and polymeric PAs with methanol, collected directly in a 10 mL flask.

The first two fractions were taken to dryness, then dissolved with methanol and transferred in 10 mL flasks. Sample fractionation and analyses were carried out in triplicate.

Since monomeric flavan-3-ols are not transformed in anthocyanins and thus not measured by the acidic hydrolysis, their content in the diethyl fraction was evaluated using the vanillin assay; oligomeric and polymeric flavanols in the ethyl acetate and methanol fractions were characterized by PAs and V indexes in order to assess both their total content in flavanols as well as their reactivity towards vanillin. Combined values of V and PAs were used to gain information about flavanol structure. The results are expressed as mg per kg of fresh weight.

### 4. Statistics

All data are expressed as the arithmetic mean  $\pm$  standard deviation (SD) of three replicates. At harvest time, the percentages of oligomeric and polymeric PAs of skin and seed flavanols were

calculated, and the differences between the cv. were tested for significance by applying the t student test.

## RESULTS AND DISCUSSION

### 1. Skin intact extracts

Barbera grape skins were significantly less rich in PAs and flavan-3-ols measured by the vanillin assay than Nebbiolo grape skins (Figure 1a).

PA and V measurements showed that PA content in skin was high prior to véraison, then decreased during ripening, as previously reported for other cultivars (Downey *et al.*, 2003) as well as for Nebbiolo and Barbera (Di Stefano *et al.*, 2002). This decrease may be due to the deviation of the intermediate metabolites (cyanidin and delphinidin) towards the synthesis of anthocyanins as they share the same biosynthetic pathway (Baranac *et al.*, 1997) or to little-known phenomena involving PA transformation and oxidation.

From a varietal point of view, the decrease was stronger and probably started before the first sampling in Nebbiolo grapes in comparison to Barbera (Figure 1a).

The decrease of V value in both varieties at véraison suggests that during this period PAs also modify their structure. A more rapid decrease was noticed for Nebbiolo skins (Figure 1a). Moreover, PA structure, as revealed by the V/PAs ratio, appeared quite different for the two grape cultivars: PAs in berry skin identified Barbera and Nebbiolo with respect to both their content and structure (Table 1).

Moreover, great differences between V and PAs indexes can be observed in both Barbera and

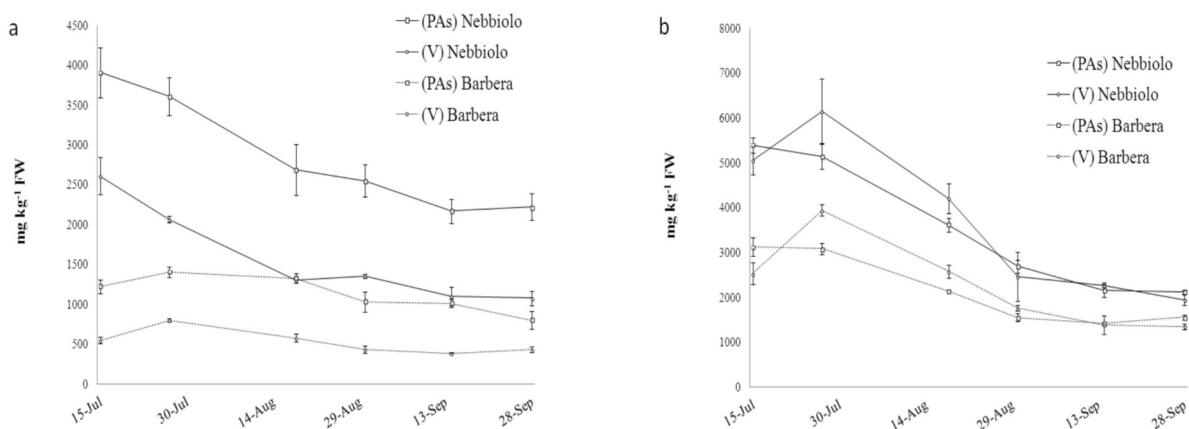


Figure 1. PA and V evolution in Barbera and Nebbiolo intact skin (a) and seed (b) extracts during grape ripening. Data reported as mg kg<sup>-1</sup> of fresh weight, mean values  $\pm$  SD (n = 3).

**Table 1. V/PAs ratio in intact skin and seed extracts of Barbera and Nebbiolo grapes during ripening.**

	15-juil	30-juil	14-Aug	29-Aug	13-Sept	28-sept
<b>Intact skin extracts</b>						
V/PAs Nebbiolo	0.67	0.57	0.49	0.53	0.51	0.48
V/PAs Barbera	0.45	0.57	0.44	0.42	0.38	0.54
<b>Intact seed extracts</b>						
V/PAs Nebbiolo	0.94	1.20	1.16	0.92	1.06	0.92
V/PAs Barbera	0.81	1.28	1.20	1.13	0.98	0.86

Nebbiolo intact skin extracts, particularly at the end of maturation. This suggests that large flavanols which compose oligomeric and polymeric PAs are not reactive towards vanillin due to sterical hindrance or the involvement of nucleophilic sites in linkages between flavanols: polymeric and complex structures are abundant in grape skins.

## 2. Seed intact extracts

As for skin extracts, Nebbiolo seed intact extracts were characterized by higher PA and V content in comparison to Barbera extracts but the trend during grape ripening was similar for both cultivars (Figure 1b). In Barbera, PAs reached a maximum value at the first sampling (3119.55 mg kg<sup>-1</sup>), while flavan-3-ols by the vanillin assay (V), as for the skins, reached a maximum value at the second sampling. Similar behavior can be noticed for Nebbiolo seed extracts.

During this trial, V values increased until the second sampling then decreased, while PAs continuously decreased for both varieties: this suggests that (i) the accumulation of flavans determined by the vanillin assay continues for a longer period than that of polymeric structures or (ii) PAs continuously change towards more complex, probably less extractable structures.

Degradation processes are the main reason for PA decrease (Figure 1b). In particular, since it is highly unlikely that the decrease of seed flavanols is due to metabolic reactions, their decrease during ripening could be due to oxidation phenomena and subsequent formation of covalent bonds between the product quinones and polysaccharidic or proteic structures of seeds, making them more difficult to extract during ripening with organic solvents (Kennedy *et al.*, 2000b; Pourcel *et al.*, 2005). Decreased PA extractability after véraison has been previously reported (Kennedy and Waterhouse, 2000a; Mateus *et al.*, 2001).

The V/PAs ratio in seed extracts was different considering the two grape cultivars (Table 1). Despite this, it is noteworthy that V and PAs indexes were similar in seeds at least at maturity, in both Nebbiolo and Barbera intact extracts, which was not the case for skins. This result highlights that almost the whole flavanols which compose PAs are reactive towards vanillin: this suggests a modest degree of polymerization or more linear structures in seeds.

## 3. Fractionation

The fractions obtained were characterized for their total PA content as well as their V content (as determined by the vanillin assay). In order to evaluate the recovery of the fractionation method employed, the amount of PAs and V of intact extract was compared to the sum of PAs and V determined in diethyl ether, ethyl acetate and methanol fractions (Table 2).

A good correspondence was observed between the PAs and V indexes of skin and seed intact extracts and the sum of the same indexes measured in the fractions (Table 2): the fractionation method is not responsible for degradation processes or flavanol losses. The method was therefore employed for a first evaluation of the flavanol composition of grape skins and seeds and its evolution during ripening.

## 4. Barbera skin extract fractionation

In the monomeric fraction of Barbera skins, flavan-3-ol monomers (V) increased during ripening, showing the highest value (57.17 mg kg<sup>-1</sup>) at the last sampling (Figure 2a). Similar results were noticed by Fujita *et al.* (2007) for Cabernet-Sauvignon but not for Sagrantino grape skins (Di Stefano *et al.*, 2008), where the V value in the diethyl ether fraction remained unchanged during grape ripening.

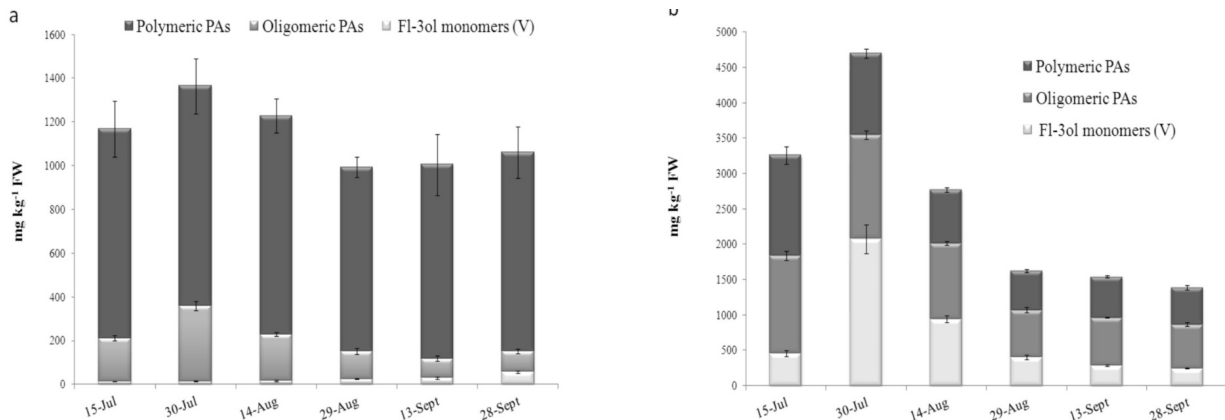
Both oligomeric (ethyl acetate fraction) and polymeric structures (methanol fraction) were characterized by the highest PA value before

**Table 2. Proanthocyanidin (PA) and flavanol (V) content of intact extracts and of the sum of fractions in Barbera (a) and Nebbiolo (b) skins and seeds during ripening. Data reported as mg kg<sup>-1</sup> of fresh weight, mean values ± SD.**

a	Skins				Seeds			
	PA	Sum of fractions PA	Flavanols (V)	Sum of fractions (V)	PA	Sum of fractions PA	Flavanols (V)	Sum of fractions (V)
15-Jul	1226.62 ± 86.11	1194.94 ± 135.11	549.96 ± 42.14	578.65 ± 30.93	3119.55 ± 205.6	2986.73 ± 144.19	2527.75 ± 240.78	2136.88 ± 56.8
30-Jul	1404.84 ± 64.33	1393.98 ± 119.71	801.34 ± 18.05	779.9 ± 27.34	3081.02 ± 130.73	2901.12 ± 17.87	3938.46 ± 125.08	3828.61 ± 68.71
14-Aug	1318.68 ± 61.08	1273.62 ± 63.18	580.03 ± 46.73	567.64 ± 56.43	2144.97 ± 4.58	1959.91 ± 23.08	2580.82 ± 143.78	2155.14 ± 21.87
29-Aug	1032.08 ± 125.32	995.02 ± 54.36	435.64 ± 43.95	370.69 ± 75.64	1552.20 ± 89.31	1375.63 ± 94.61	1720.11 ± 63.78	1378.14 ± 96.14
13-Sept	1013.45 ± 48.03	1099.4 ± 70.11	384.41 ± 12.71	408.04 ± 58.42	1415.28 ± 34.28	1364.54 ± 19.6	1387.45 ± 207.70	1091.56 ± 45.96
28-Sept	799.81 ± 111.05	881.5 ± 68.89	430.59 ± 33.80	517.76 ± 83.51	1488.34 ± 52.68	1317.24 ± 99.54	1311.71 ± 62.80	1129.33 ± 115.7

b	Skins				Seeds			
	PA	Sum of fractions PA	Flavanols (V)	Sum of fractions (V)	PA	Sum of fractions PA	Flavanols (V)	Sum of fractions (V)
15-Jul	3908.65 ± 312.68	3822.13 ± 117.60	2609.52 ± 234.01	2416.25 ± 84.93	5389.39 ± 176.60	5254.32 ± 149.54	5052.62 ± 310.87	4802.57 ± 38.99
30-Jul	3607.9 ± 238.42	3581.6 ± 51.85	2031.43 ± 38.55	1896.07 ± 36.82	5132.48 ± 279.58	5150.84 ± 382.59	6148.22 ± 721.09	6017.04 ± 181.1
14-Aug	2692.51 ± 318.40	2587.3 ± 43.56	1306.93 ± 43.02	1238.34 ± 37.01	3611.96 ± 150.18	3719.23 ± 234.29	4204.93 ± 335.54	4114.97 ± 139.28
29-Aug	2547.63 ± 203.09	2496.68 ± 95.71	1351.94 ± 27.44	1284.36 ± 13.24	2688.3 ± 145.14	2574.42 ± 176.69	2462.15 ± 553.10	2281.39 ± 45.34
13-Sept	2171.29 ± 151.72	2023.9 ± 117.26	1099.72 ± 114.99	992.77 ± 8.78	2147.31 ± 137.57	2083.16 ± 141.58	2270.43 ± 46.43	1973.42 ± 196.26
28-Sept	2219.51 ± 167.41	2154.29 ± 113.41	1075.64 ± 92.18	1018.03 ± 166.9	2113.27 ± 44.84	2015.01 ± 201.16	1938.21 ± 119.66	1699.69 ± 129.01



**Figure 2. Flavan-3-ol monomer and oligomeric and polymeric PA content and evolution in Barbera skins (a) and seeds (b) during ripening. Data reported as mg kg<sup>-1</sup> of fresh weight, mean values ± SD (n = 3)**

véraison, which then decreased slightly or remained constant during the last period of grape ripening (Figure 2a): the accumulation of oligomeric and polymeric flavanols occurred during pre-véraison, then their content decreased. Similar results were reported for Sagrantino grapes (Di Stefano *et al.*, 2008).

PAs changed their structure during grape ripening. In particular, more complex structures which are less

reactive to the vanillin addition were present. The main result was a lower V/PAs ratio in the skin methanolic fraction (Table 3).

**5. Barbera seed extract fractionation**

A clear difference in flavan-3-ol monomers (V) and oligomeric and polymeric PAs was noticed in seed extract fractionation in comparison to skin extracts. In particular, a higher amount of V and polymeric PAs was measured in seeds (Figure 2b). An increase

**Table 3. V/PAs ratio of oligomeric and polymeric fractions in Barbera skins and seeds during grape ripening.**

	15-juil	30-juil	14-Aug	29-Aug	13-sept	28-sept
<b>Barbera skins</b>						
V/PAs (oligomers)	0.52	0.61	0.63	0.50	1.07	0.87
V/PAs (polymers)	0.48	0.55	0.41	0.32	0.32	0.34
<b>Barbera seeds</b>						
V/PAs (oligomers)	0.53	0.64	0.67	0.82	0.72	0.70
V/PAs (polymers)	0.66	0.67	0.65	0.78	0.55	0.67

of flavan-3-ol monomers in seeds prior to véraison followed by a decrease towards the harvest stage was highlighted, similar to what was observed for other cultivars (Di Stefano *et al.*, 2002 and 2008; Fujita *et al.*, 2007; Kennedy *et al.*, 2000b). This trend suggests that monomeric flavan-3-ols are also involved in degradation processes, mainly oxidation reactions. Their involvement in polymerization reactions for the synthesis of PAs seems unlikely, according to the characterization of oligomeric and polymeric fractions.

Actually, the polymeric PAs and V underwent a progressive decrease from the first to the fourth sampling, then it remained almost constant until ripening (Figure 2b). Similar results were reported in literature (Di Stefano *et al.*, 2008).

The total amount of flavano-3-ol monomers and PAs reached its maximum close to the véraison stage, as for Cabernet-Sauvignon seeds (Fujita *et al.*, 2007).

In Barbera seeds, the amount of oligomeric PAs was higher than that of polymeric PAs during the entire ripening period (Figure 2b), except the first sampling, and significantly higher at harvest (Figure 4). Similar results were obtained for Carménère seeds (Obreque-Slier *et al.*, 2010) during ripening but not at harvest when no significant differences between oligomers and polymers were noticed.

This result is in contrast with some previous works where polymeric PAs are the greatest fraction in red wines (Sun *et al.*, 1996), as well as in pulp, seeds and skins of some varieties (Monagas *et al.*, 2003). A possible explanation for this result could be the spectrophotometric method employed to measure flavanols in the oligomeric and polymeric fractions. Usually, in the cited works, the vanillin assay is used to determine flavanols in fractions, while in this work the acidic hydrolysis has been employed. As reported above, a clear difference in the obtained results can be

noticed, especially in extracts where a huge amount of oligomeric and polymeric flavanols is present: the hindrance or the involvement of nucleophilic sites in polymers could be responsible for low response in vanillin assay, which does not occur in acidic hydrolysis.

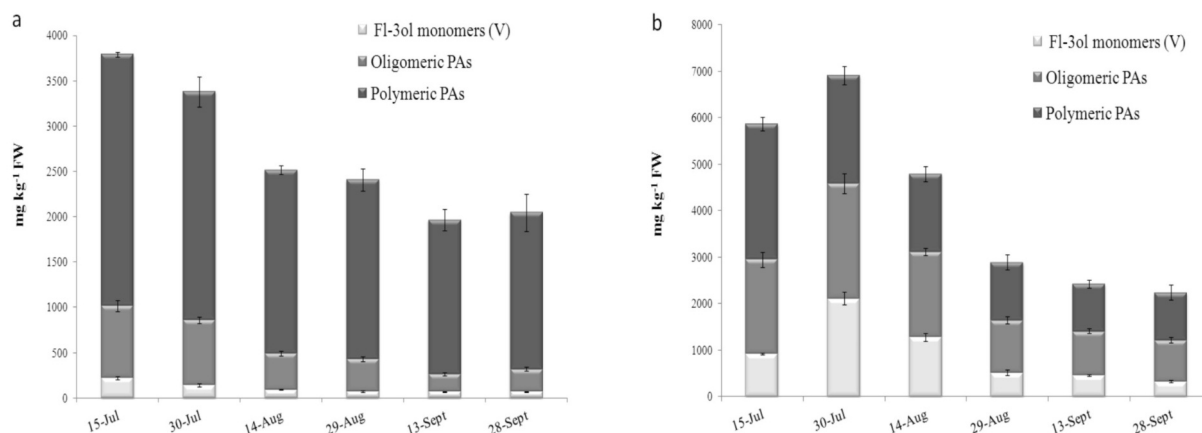
## 6. Nebbiolo skin extract fractionation

The PAs determined in the methanol fraction (polymeric PAs) were the most abundant, as previously reported for Barbera skin extract. Unlike the observation in Barbera cv., flavan-3-ol monomers (V) decreased during ripening in Nebbiolo skin (Figure 3a). The evolution of monomeric fraction in grape skins seems to be a varietal character: different trend was observed for Sagrantino cv. (Di Stefano *et al.*, 2008), where the content of monomeric flavan-3-ols was constant.

Both oligomeric and polymeric fractions were characterized by a decrease in PAs during grape ripening. The highest PA content was measured at the first sampling date. It can be assumed that already in the first sampling oxidation reactions were active. An increase in V/PAs ratio was measured during ripening in the oligomeric fraction, thus suggesting a modification in the molecular structure (Table 4).

The evaluation of the PAs in the polymeric fraction during ripening showed a decrease in both PAs and V values. The decrease of such values was stronger in Nebbiolo skin extracts in comparison to Barbera ones, thus suggesting that this behavior depends on the grape variety.

Nebbiolo skins presented a higher oligomeric PA content, as a percentage of total skin flavanols, in comparison to Barbera skins during ripening, and a significantly higher content at harvest (Figure 5).



**Figure 3.** Flavan-3-ol monomer and oligomeric and polymeric PA content and evolution in Nebbiolo skins (a) and seeds (b) during ripening. Data reported as mg kg<sup>-1</sup> of fresh weight, mean values  $\pm$  SD (n = 3).

**Table 4.** V/PAs ratio of oligomeric and polymeric fractions in Nebbiolo skins and seeds during grape ripening.

	15-juil	30-juil	14-Aug	29-Aug	13-sept	28-sept
<b>Nebbiolo skins</b>						
V/PAs (oligomers)	0.63	0.55	0.48	0.56	0.79	0.74
V/PAs (polymers)	0.59	0.53	0.45	0.48	0.45	0.44
<b>Nebbiolo seeds</b>						
V/PAs (oligomers)	0.81	0.81	0.88	0.82	0.81	0.75
V/PAs (polymers)	0.75	0.80	0.71	0.62	0.66	0.62

As for the oligomeric fraction, a decrease in the V/PAs ratio was observed during ripening for the polymeric structures: an increase in the molecular complexity seems to occur. From a varietal characterization point of view, the polymeric PAs showed minor molecular complexity in Nebbiolo skin extracts (V/PAs: 0.44) in comparison to Barbera ones (V/PAs: 0.33) at harvest (Tables 3, 4).

### 7. Nebbiolo seed extract fractionation

Similarly to Barbera seed extract fractionation, clear differences were noticed in the amount of monomeric, oligomeric and polymeric fractions in comparison to skin extract. Also for Nebbiolo seed extracts, the amount in monomeric and oligomeric fractions was high. Similar results are reported in two different studies for Cabernet-Sauvignon seeds (Fujita *et al.*, 2007; Obreque-Slier *et al.*, 2010), while opposite data were shown for Graciano, Tempranillo and Cabernet-Sauvignon cv. (Monagas *et al.*, 2003), where polymeric PAs prevailed both in seeds and skins. In the mentioned studies the same method for

the extraction and determination (vanillin assay) of phenolic compounds was used. Thus, environmental parameters could also influence the relative content of monomeric, oligomeric and polymeric flavanols, especially in seeds (Downey *et al.*, 2003).

As observed previously for Barbera and already reported for other varieties (Kennedy *et al.*, 2000b), an increase in flavan-3-ol monomers (V value in the diethyl ether fraction) was observed prior to véraison, then the content in such flavanols progressively decreased during ripening.

In the oligomeric fraction, both oligomeric PAs and V reached a maximum level at the second sampling, thus close to véraison, then their value decreased during ripening (Figure 4b). At the same time, V/PAs ratio remained quite constant (Table 4), thus suggesting that the molecular complexity for the oligomeric fraction did not vary.

The polymeric fraction was characterized by a decrease in both PA and V values during ripening:

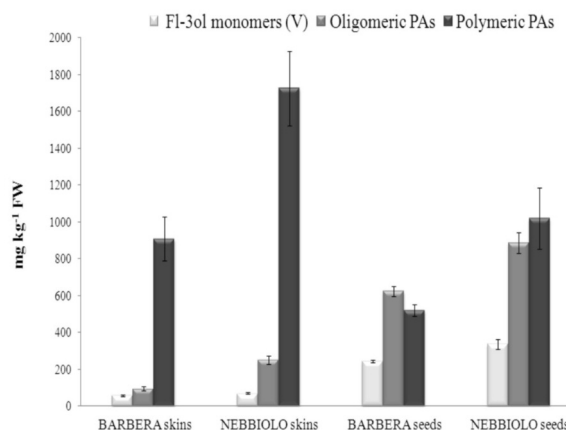
the accumulation of these compounds stopped early probably before the first sampling. As reported for the Barbera cv., transformation reactions of flavanols could be the main reason for the decline of such compounds in grape seeds. Moreover, these reactions contribute to a decrease in flavanol extractability (Kennedy *et al.*, 2000b).

The V/PAs ratio at harvest was 0.62 in the polymeric fraction and 0.75 in the oligomeric fraction (Table 4). Polymeric PAs are characterized by a higher molecular complexity than the oligomeric ones.

In this study, the data were obtained under the same environmental and analytical conditions. Thus, from a varietal point of view, Barbera seed oligomeric PAs were more abundant than the polymeric PAs, as mentioned before, both during ripening and at the harvest date, while no significant differences were noticed for Nebbiolo seeds (Figures 2b, 3b, 4). Besides important PA quantitative differences between the two cv. at harvest (Figure 4), significant qualitative differences were noticed when the percentages (%) of monomers, oligomers and polymers were calculated in skins and seeds. Nebbiolo skins were characterized by a significantly more important % of oligomeric PAs ( $P < 0.05$ ) in comparison to Barbera skins, while Barbera seeds contained a significantly higher % ( $P < 0.01$ ) of flavan-3-ol monomers and oligomeric PAs than Nebbiolo skins at harvest (Figure 5).

## CONCLUSIONS

Flavanols are accumulated in Nebbiolo and Barbera skins and seeds prior to véraison. Then, as reported for many varieties, their total content decreases due to the deviation of the intermediate metabolites towards

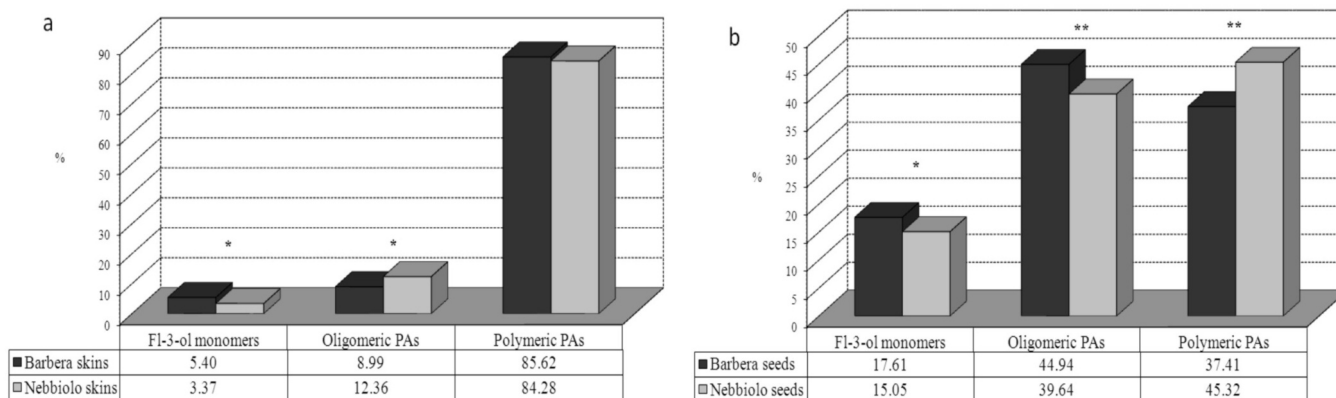


**Figure 4. Content of flavan-3-ol monomers and oligomeric and polymeric PAs in Barbera and Nebbiolo skins and seeds at harvest. Data reported as mg kg<sup>-1</sup> of fresh weight, mean values  $\pm$  SD (n = 3).**

the synthesis of anthocyanins or to little-known transformation and oxidation phenomena.

The amount of total flavanols in grape seeds and skins depends on the variety. In particular, Nebbiolo grapes accumulate higher flavanol content in both seeds and skins than Barbera. Moreover, differences between acidic hydrolysis and vanillin assay values suggest that flavanol composition and complexity in skin and seed extracts represent a varietal characteristic: further evaluation of the flavanol composition is needed.

The fractionation method used in this study has allowed an evaluation of the flavanol composition of grape skins and seeds and their evolution during ripening, revealing further differences between the cultivars.



**Figure 5. Percentages of flavan-3-ol monomers and oligomeric and polymeric PAs in Barbera and Nebbiolo skins (a) and seeds (b) at harvest. Data reported as mean values. \* $P < 0.05$ , \*\* $P < 0.001$ .**

During grape ripening, flavan-3-ol monomers increase in Barbera skins, indicating a modest depolymerization reaction, while the opposite happens in Nebbiolo grapes. An increase of monomers was also noticed in Cabernet-Sauvignon skins, while a different trend was observed for Sagrantino cv.: the content of monomeric flavan-3-ols was constant during ripening.

Nebbiolo skins present higher content in oligomeric PAs as a percentage in comparison to Barbera skins both during ripening and at harvest. Moreover, the decrease in polymeric PAs, weaker in Barbera skins in comparison to Nebbiolo, depends on the grape variety.

A rapid and consistent decrease of flavan-3-ol monomers for both Barbera and Nebbiolo seeds during grape ripening suggests that they are probably more involved in oxidation reactions.

Polymeric PAs prevailed in skins, while monomeric and oligomeric structures prevailed in seeds of both cv. The spectrophotometric method used for the evaluation of PA content is also the reason for this result. However, the abundance of monomeric, oligomeric and polymeric flavanols in seeds, as others studies on Cabernet-Sauvignon demonstrate, has not been clearly illustrated. In addition to analytic method, environmental parameters seem to influence the relative content of each fraction in seeds.

In our study, the data collected under the same environmental and analytical conditions highlight that Barbera seed oligomeric PAs are more abundant than the polymeric ones during ripening and at harvest, while no significant differences are noticed for Nebbiolo seeds. According to literature, similar results to Barbera were obtained for Carmènère seeds during ripening. Finally, Barbera seeds had also a significantly higher percentage of flavan-3-ol monomers and oligomeric PAs than Nebbiolo ones at harvest.

Differences evidenced in this study regarding the total amount of flavanols and their distribution in monomeric, oligomeric and polymeric fractions during grape ripening and at harvest could be the basis to better understand the astringency and bitter sensation in Barbera and Nebbiolo grapes and wines. Moreover, a good knowledge of skin and seed PA composition is important during the winemaking process because the extraction of PAs is not only dependent on the maceration time but also influenced by the characteristics of the grapes.

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