Journal of Food Composition and Analysis 92 (2020) 103547

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Research Article

Discrimination of five Greek red grape varieties according to the anthocyanin and proanthocyanidin profiles of their skins and seeds

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ARTICLE INFO

Keywords:
Anthocyanins
Proanthocyanidins
Chemical markers
Authentication
Greek varieties
Catechin
Epicatechin
Terroir
Grapes

ABSTRACT

The knowledge of grapes phenolic content is proven to be critical for the vinification process and the improvement of wine quality. This study was undertaken to determine the phenolic composition and to employ the phenolic profile as a varietal discrimination tool in five Greek red grape varieties. Ninety grape samples from two seasons (2017 and 2018) were analyzed after extraction with organic solvents. Their proanthocyanidin profile, expressed as percentages of flavan-3-ols, was determined in both skins and seeds by employing phloroglucinolysis followed by HPLC-UV and MS detection, and anthocyanin profile was identified only in the skin extracts by HPLC-UV. Significant differences were observed in proanthocyanidin and anthocyanin profiles of skin extracts between the samples of different varieties, but not in seeds. (-)-Epicatechin was the main subunit in Mandilaria, Kotsifali, Agiorgitiko and Xinomavro grapes while (-)-epigallocatechin gallate in Mavrotragano. Malvidin-3-*O*-glucoside was the predominant pigment in all grape samples analyzed with the exception of Kotsifali skin extracts, where peonidin-3-*O*-glucoside was the most abundant anthocyanin. In addition, Mavrotragano skin extracts were the richest in delphinidin and petunidin-3-*O*-glucosides, while Agiorgitiko and Xinomavro contained the highest amount of malvidin-3-*O*-glucoside. The results underline the significance of the skin phenolic composition as a tool for the discrimination of the Greek red grape varieties.

1. Introduction

Condensed tannins or proanthocyanidins, with a structure analogue to flavan-3-ol, are an important category of soluble polyphenols in grapes. This functional phenolic subclass consists of dimers and trimers up to oligomers and polymers with more than 40 subunits (Teixeira et al., 2013). Proanthocyanidins due to their bitter and astringent properties have a great importance to grape and wine oral sensory attributes (Chira et al., 2009; Kallithraka et al., 2014; Kyraleou et al., 2016; Quijada-Morín et al., 2012). Particularly, the molecular mass of tannins seems to differentiate bitterness and astringency, since tannins with larger molecular mass are perceived as more astringent (Harbertson et al., 2014) while smaller molecular mass tannins, such as (+)-catechin and (-)-epicatechin, are perceived as more bitter (Del Llaudy et al., 2008; McRae and Kennedy, 2011). Those two attributes of wines are associated with tannin concentration in grapes but are dictated by multiple factors during vinification, such as extraction during the maceration process (Del Llaudy et al., 2008; Gil et al., 2012), alterations in proanthocyanidin structure occurring during fermentation (McRae and Kennedy, 2011) and wine composition, for example ethanol, pH (Demiglio and Pickering, 2008; Fontoin et al., 2008) and anthocyanin content (Kallithraka et al., 2011).

Previous studies reported that it is possible to employ phenolic compounds of grapes, especially flavonols for taxonomical classification (Mattivi et al., 2006). Among phenolic compounds, anthocyanins are of great importance due to their role as sensory intensifiers (Costa et al., 2014; Kallithraka et al., 2014). Five glucosylated anthocyanins followed by their esterified derivatives with acetic or coumaric acid have been detected in the skins of red *Vitis vinifera* species, and the relative content of each anthocyanin has been used to evaluate red grape or wine profile (Costa et al., 2014; Kallithraka et al., 2005; Zhao et al., 2010). Among them, malvidin-3-*O*-glucoside is the chief anthocyanin in both grapes and wines of many European red vine varieties (Costa et al., 2014; Kallithraka et al., 2005). Anthocyanin profile and its evolution

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https://doi.org/10.1016/j.jfca.2020.103547
Received 13 December 2019; Received in revised form 23 May 2020; Accepted 27 May 2020
Available online 31 May 2020

highlight the local impact of a vineyard in the phenolic content of a variety (Costa et al., 2015) and provide indicative data on wine authentication, but also can be crucial to the direction of winemaking (Guidoni and Hunter, 2012).

The international varieties, for example Cabernet Sauvignon, Syrah, Merlot, were studied extensively for their phenolic and aromatic grape contents (Bordiga et al., 2011; Chira et al., 2011) which are important in their vinification procedures. In the last decade there is a great attention into the cultivation and the study of local varieties from different countries (Bordiga et al., 2011; Costa et al., 2014; Ćurko et al., 2014; Kallithraka et al., 2014; Kyraleou et al., 2015a,b; Petropoulos et al., 2017; Rinaldi et al., 2014).

Greek vineyard hosts numerous autochthonous varieties; many of them are part of its viticultural heritage. However, most of these varieties remain unexploited and lack complete characterization (Basalekou et al., 2019; Kallithraka et al., 2014, 2009). As a subsequence, their ability to improve wine quality properties is still limited. Nowadays, there is a keen interest on probing novel vinifications, to exploit the uniqueness of regional grape varieties (Costa et al., 2015, 2014). Lately, native Greek varieties have attracted international interest due their unique and distinct oenological characteristics and organoleptic properties, which have created a marketing advantage. The aim of this research was to discriminate five Greek red grape (*V. vinifera)* varieties based on the anthocyanin and proanthocyanidin profiles of their skins and seeds.

2. Materials and Methods

2.1. Chemicals

Methanol, ethanol, were of HPLC grade, chloroform, acetone, sodium metabisulfite, sodium carbonate, ethyl acetate, phloroglucinol, sodium hydroxide, L-(+)-tartaric acid, (+)-catechin, hydrochloric acid (37%), acetic acid, were purchased from Sigma Aldrich (Saint Louis, MO, USA). (-)-Epigallocatechin, (-)-epicatechin-3-O-gallate, (-)-epicatechin, were purchased from Extrasynthese (Genay Cedex, France). Anthocyanin mixture of standards containing 5 mmol L^{-1} each of delphinidin-3-*O*-monoglucoside (Dlp3-glu), cyanidin-3-*O*-monoglucoside (Cyn3-glu), petunidin-3-*O*-monoglucoside (Pt3-glu), peonidin-3-*O*-monoglucoside (Pn3-glu) and malvidin-3-*O*-monoglucoside (Mlv3-glu) was obtained from Polyphenol Laboratories (Sandnes, Norway).

2.2. Grape Samples

The grape samples were collected from 45 vineyards of 9 different regions of Greece, the island of Santorini, the island of Paros, the island of Creta, Naoussa, Amynteo, Goumenissa, Thessaloniki (North Greece), Nemea and Rapsani (Central Greece) for two consecutive seasons (2017 and 2018). Mavrotragano (Variety Number of Vitis International Variety Catalogue, VIVC, 40210) grapes were collected from vineyards of the island of Santorini and Thessaloniki. Mandilaria (VIVC 7300) grapes from the islands of Santorini and Paros. Kotsifali (VIVC 6446) grapes from the island of Creta. Agiorgitiko (VIVC 102) grapes from Nemea. Xinomavro (VIVC 13284) grapes from Goumenissa, Naoussa, Amynteo and Rapsani (Supplementary Table 1). These varieties are cultivated in an area of 70,634.2 acres (30319.4 Agiorgitiko; 19668 Xinomavro; 11657.6 Kotsifali; 8577.5 Mandilaria; 411.7 Mavrotragano) (Source: Ministry of Rural Development and Food, Hellenic Republic, 2020) and they are important Greek red grape varieties producing 'Protected Designation of Origin' (P.D.O.) or 'Protected Geographical Indication' (PGI) red wines.

Samples of 500 berries were collected at commercial harvest. Analytical grape parameters as total soluble solids (°Brix) by refractometry and titratable acidity (g L^{-1} tartaric acid) were also determined in a sample of 200 berries (Supplementary Table 1). An amount of 100 berries was weighed and the skins and seeds were manually removed. The skins and seeds were freeze-dried separately and were ground to obtain powder. Skins were analysed for anthocyanins and proanthocyanidins and seeds were analysed for proanthocyanidins with the following procedures.

2.3. Analysis of anthocyanins

Anthocyanins were extracted from 1 g of dried skin powder for three different time duration steps (4, 18 and 24 h) with 20 mL, 10 mL and 10 mL acidified methanol (1 mL L^{-1} of 0.012 mol HCl L^{-1}). After centrifugation, the supernatants were combined and analyzed. The content of anthocyanins was determined by HPLC. The equipment used consisted of a Jasco AS-1555 Intelligent Sampler, a Jasco PU 2089 Plus Quaternary Gradient Pump, coupled to a UV-vis detector (Jasco MD-910 Multiwavelength Detector) set at 520 nm and a Jasco LC-Net II/ADC (Jasco Corporation, Tokyo, Japan). A Restek Pinnacle II C18 (Restek Corporation, Bellefonte, PA, USA) (250 mm x 4.6 mm, 4 μm) column was employed. Eluent A was 100 mL L^{-1} aqueous formic acid and eluent B was methanol at a flow rate 1 mL min^{-1}. The elution was as follows: 90% A for 1 min, then from 90 to 50% A in 22 min, from 50 to 5% A in 10 min and finally isocratic for a further 2 min. Identification was based on comparing retention times and UV spectra of the peaks detected with those of original compounds or on previous observations (Kyraleou et al., 2015a,b; Kallithraka et al., 2005). The following compounds were identified: Dlp3-glu, Cyn3-glu, Pt3-glu, Pn3-glu, Mlv3-glu, as well as acetyl (Ac): petunidin-3-*O*-(6-acetyl)glucoside (Pt3-Ac-glu), peonidin-3-*O*-(6-acetyl)glucoside (Pn3-Ac-glu), malvidin-3-*O*-(6-acetyl)glucoside (Mlv3-Acglu) and coumaroyl (Cm) glucosides: delphinidin-3-*O*-(6-p-coumaroyl)glucoside (Dlp3-Cm-glu), cyanidin-3-*O*-(6-p-coumaroyl)glucoside (Cyn3-Cm-glu), petounidin-3-*O*-(6-p-coumaroyl)glucoside (Pt3-Cm-glu), malvidin-3-*O*-(6-p-coumaroyl)glucoside (Mlv3-Cm-glu). The anthocyanin content was expressed as mg g^{-1} skin fresh weight of Mlv3-glu equivalents. The profile of individual anthocyanins was expressed as the percentage of each anthocyanin per total anthocyanin content. The percentages of cyanidin based (A1: Cyn3-glu, Pn3-glu) and delphinidin based (A2: Dlp3-glu, Pt3-glu, Mlv3-glu) anthocyanins (Castellarin et al., 2006; Castellarin and Di Gaspero, 2007) and the percentages of methoxylated (Pn3-glu, Pt3-glu and Mlv3-glu) and non-methoxylated anthocyanins (NonOMe), as Dlp3-glu, Cyn3-glu and non methoxylated anthocyanins were calculated according to monoglucoside anthocyanins. All analyses were performed in triplicate.

2.4. Analysis of proanthocyanidins

Two different solvents were used in skin or seed powder to extract tannins, according to previously published method (Chira et al., 2009; Kyraleou et al., 2015a,b). An amount of 3 g of the powder (skins or seeds) was first extracted with 25 mL of 80% acetone in water for 3 h and then with 25 mL of 60% methanol in water for 2.5 h. The supernatants were combined and evaporated under reduced pressure at 30 °C to remove organic solvents. The residue was lyophilized, was weighted and re-dissolved in 5% of ethanol and then the lipophilic material was removed by chloroform. The aqueous fractions was collected and lyophilized to obtain dry powder. The final tannin extracts were weighted and dissolved in methanol (5 g L^{-1}). Acid-catalyzed depolymerization occurred in the presence of phloroglucinol (50 g L^{-1} phloroglucinol, 10 g L^{-1} ascorbic acid, 0.1 N HCl, in methanol) for 20 min at 50 °C. The reaction was terminated by addition of 1 mL aqueous sodium acetate (40 mM). Reaction products were analyzed on an LC-MS 2010A instrument coupled to a single quadrupole mass spectrometer equipped with an electrospray ion source (Shimadzu, Kyoto, Japan). The mass spectrometer was operated in positive-ion mode. The source's temperature was set at 70 °C, the capillary voltage at 3.5 kV and the cone voltage at −30 eV. The absorbance was recorded at 280 nm and mass spectra were recorded in the range of 50–1500 amu. Separation was

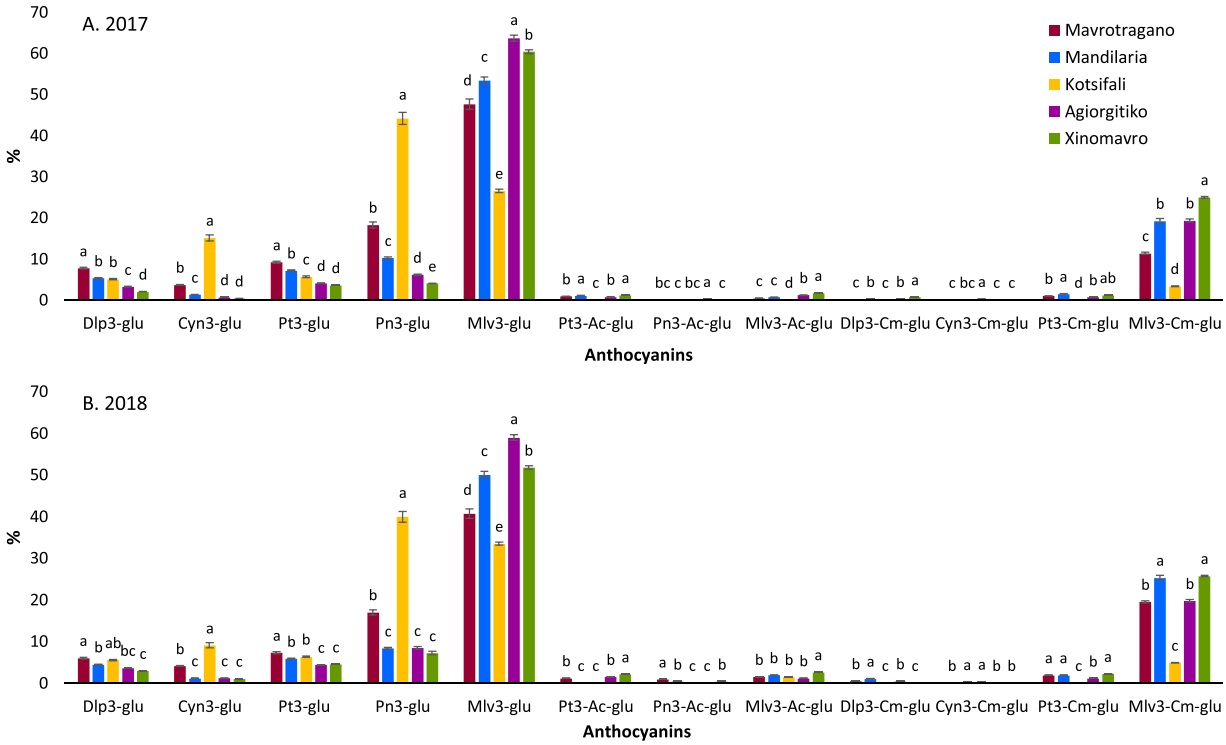

Fig. 1. Profile of individual anthocyanins, concentration of individual anthocyanin per total anthocyanin concentration 100x, in skins from the varieties Mavrotragano, Mandilaria, Kotsifali, Agiorgitiko and Xinomavro for A. 2017 and B. 2018 seasons. Bars indicate ± S.E. of the mean value. Significant differences among varieties are indicated by different letters (Tukey's test, $p < 0.05$)[1].

performed on an XTerra RR C18 (100 × 4.6 mm, 3.5 µm) reversed-phase column (Waters, Milford, MA, USA) at a flow rate of 0.5 mL min^{-1}, using a 20 µL injection volume and the following elution program: eluent A from 80% to 40% in 20 min, which was kept isocratic for further 10 min and then from 40% to 80% in 2 min. Eluent A was 0.1% aqueous acetic acid and eluent B methanol. All analyses were performed in triplicate which enable the qualification of all the individual polymer subunits, calculation of mean polymerization degree (mDP), and the percentage of (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC) and (-)-epicatechin gallate (ECG) according to previously published methods (Kennedy and Jones, 2001; Kyraleou et al., 2017).

2.5. Statistical analysis

Data statistical analysis was submitted to a variance analysis (ANOVA), on Statistica V.7 Software (StatsoftInC., Tulsa, OK, USA). Comparison of mean values were performed using Tukey's HSD test when samples were significantly different after ANOVA ($p < 0.05$). Principal Component Analysis (PCA) was employed to examine any possible grouping of samples and was conducted in the R statistical environment, using the factormineR package (Lê et al., 2008). Data was visualized using the factorextra and ggplot2 packages (Ginestet, 2011).

[1] Dlp3-glu: delphinidin-3-*O*-monoglucoside; Cyn3-glu: cyanidin-3-*O*-monoglucoside; Pt3-glu: petunidin-3-*O*-monoglucoside; Pn3-glu: peonidin-3-*O*-monoglucoside; Mlv3-glu: malvidin-3-*O*-monoglucoside. Pt3-Ac-glu: petunidin-3-*O*-(6-acetyl)glucoside; Pn3-Ac-glu: peonidin-3-*O*-(6-acetyl)glucoside; Mlv3-Ac-glu: malvidin-3-*O*-(6-acetyl)glucoside; Dlp3-Cm-glu: delphinidin-3-*O*-(*6*-p-coumaroyl) glucoside Cyn3-Cm-glu: cyanidin-3-*O*-(*6*-p coumaroyl) glucoside; Pt3-Cm-glu: petounidin-3-*O*-(*6*-p-coumaroyl) glucoside;Mlv3-Cm-glu: malvidin-3-*O*-(*6*-p-coumaroyl) glucoside.

3. Results and discussion

3.1. Anthocyanin composition

As it can be observed in Fig. 1, the anthocyanin profiles of the studied varieties differed significantly. This heterogeneity is mainly due to the effect of the different variety and less to the season effect. The predominant anthocyanin of Agiorgitiko, Xinomavro, Mandilaria and Mavrotragano grapes was Mlv3-glu, as it has been previously reported for a great number of varieties including native Greek varieties (García-Beneytez et al., 2002; Han et al., 2015; Petropoulos et al., 2017; Theodorou et al., 2019), while Kotsifali grapes were the richest in Pn3-glu (followed by Mlv3-glu) for both seasons (Fig. 1). According to a previous study, Pn3-glu has been determined as the prevalent pigment in skins of the Spanish variety Garnacha Tintorera (García-Beneytez et al., 2002), in a few Italian varieties as Galliopo, Moscato Rosa, Nebbiolo etc (Mattivi et al., 2006) and at similar contents with Mlv3-glu in the Portuguese variety Alvarilhão (Costa et al., 2014). The colour of a young red wine is primarily attributed to its monomeric anthocyanin content. During ageing the covalent associations of anthocyanins with other anthocyanins or flavonoid compounds are responsible for the presence of condensation-derived adducts and consequently for the colour change (both intensity and hue) and stabilization. It has been shown that the role of the individual anthocyanins to association interactions is important and thus the different anthocyanin profiles observed for the studied varieties are expected to affect the wine colour parameters (González-Manzano et al., 2008; He et al., 2010).

In Mavrotragano skins the most abundant anthocyanins were Mlv3-glu > Pn3-glu > Mlv3-Cm-glu. Unlike the results obtained for a great number of varieties (Kallithraka et al., 2009; Mattivi et al., 2006), Pn3-glu was the second most abundant pigment in 2017 while in 2018 its content was similar to that of Mlv3-Cm-glu. In addition, Dlp3-glu and Pt3-glu were found in Mavrotragano at highest percentages (7.7% and 9.2%, for 2017 and 6.0 and 7.2% for 2018, respectively), followed by Mandilaria and Kotsifali during both harvest seasons. Cyn3-glu was

fairly represented in Mavrotragano grapes with a contribution of 3.6% (in 2017) and 4.1% (in 2018). Concerning Mandilaria grapes, Mlv3-glu had the highest contribution to total anthocyanins (average 51.6% during both seasons). In addition, the percentage contribution of Mlv3-Cm-glu was also highest in Mandilaria grapes during both harvest seasons (19.2% in 2017 and 25.3% in 2018), in accordance with the results of a previous published study concerning Greek native grape varieties (Kallithraka et al., 2009). The lowest contribution of Mlv3-glu (26.6% in 2017 and 33.4% in 2018) and the highest of Pn3-glu (44.1% and 39.9% for 2017 and 2018) to total anthocyanins respectively, were observed at Kotsifali grapes. Kotsifali was also a variety rich in Cyn3-glu (15% in 2017 and 9.1% in 2018) when compared to the other varieties examined. Another interesting observation concerning this variety was that acetylated anthocyanins were not detected in skin extracts in 2017, while in 2018 only a minor amount of Mlv3-Ac-glu was detected. Coumaroylated derivatives were also underrepresented in Kotsifali, with Mlv3-Cm-glu having the lowest contribution during both harvest seasons (3.3% and 4.9% respectively) (Fig. 1). However, although Cyn3-Cm-glu was absent from all the varieties during 2017 season, it was only detected in Kotsifali berries. The results obtained in this study are in agreement with the data reported by previous study concerning colour parameters of Kotsifali wines (Basalekou et al., 2016). In general, the wines of this variety are characterized by low colour intensity and high hue (hº) values respectively and this might be mainly due to both the absence of esterified anthocyanins and the low content of Mlv3-glu.

Similarities concerning the profiles of monomeric anthocyanins between Agiorgitiko and Xinomavro skin extracts were observed with Mlv3-glu having the highest contribution to total anthocyanins during 2017 (63% and 60.5%, respectively) and 2018 (59% and 52% respectively) in accordance with Theodorou et al. (2019), while Dlp3-glu, Cyn3-glu, Pt3-glu and Pn3-glu showed the lowest contribution in comparison with the other varieties (Fig. 1). However, Xinomavro was richer in coumaroylated anthocyanin derivatives and poorer in anthocyanin mono-glucosides than Agiorgitiko. Moreover, Pn3-Ac-glu was determined only in Agiorgitiko during 2017 season.

To better describe the variations in the anthocyanin patterns among the five varieties, the identified pigments were separated into two groups (esterified and non-esterified) based on their similar chemical characteristics. An important factor that affects wine colour intensity is the contribution of non-esterified (Cyn3-glu, Dlp3-glu, Pt3-glu, Pn3-glu, Mlv3-glu) and esterified anthocyanins (acetylated and coumaroylated). Non-esterified anthocyanins, are more sensitive to oxidation reactions in berries and wines (He et al., 2010), while acetylated and coumaroylated represent a more stable form of anthocyanins (Rinaldi et al., 2015) with higher resistance to oxidative effects caused by exposure to solar radiation and to high temperatures (Downey et al., 2004). In this study, the concentration of non-esterified anthocyanins was higher than that of the esterified for all the varieties studied and for both experimental seasons (Fig. 2). The higher percentages of non-esterified anthocyanins were observed, during 2017 and 2018 seasons, in Kotsifali grapes (95.3% and 93.5% respectively), while the lowest in Xinomavro (70.5% and 67.2% respectively) in accordance with previous results (Theodorou et al., 2019). Higher percentages of non-esterified anthocyanins were observed in several red varieties, due to the high concentrations of Cyn3-glu or Pn3-glu, especially in those that were used for the production of white wines, such as Gewürztraminer (Mattivi et al., 2006). A previous study reported that although Pn3-glu and Cyn3-glu were detected in grapes, they were absent or found in much lower concentrations in the corresponding wines where Mlv3-glu was the main anthocyanin (García-Beneytez et al., 2002). In Agiorgitiko grapes the levels of non-esterified and esterified anthocyanins were 77% and 23%, for both seasons (Fig. 2). Anthocyanin profile of Mavrotragano and Mandilaria was mostly affected by the harvest season. In Mavrotragano skins non-esterified anthocyanins were 86% (in 2017) and 74% (in 2018), while for Mandilaria the calculated percentages were 77% (in 2017) and 69% (in 2018) respectively. These differences mainly concern Mlv3-glu and Mlv3-Cm-glu, since harvest season mainly affected the percentage contribution of these two compounds (in 2018 % Mlv3-glu decreased while % Mlv3-Cm-glu increased, for both varieties).

Total non-esterified (TNonEstA) and esterified (TEstA) anthocyanins were expressed as mg per g skin fresh weight (f.w.) (mg Mlv3-glu g^{-1} of skin f.w.) and as mg per g of berry (mg Mlv3-glu g^{-1} berry) (Table 1). In addition the values of the individual anthocyanins are provided (Supplementary Table 2). TNonEstA ranged from 1.67 to 10.85 mg Mlv3-glu g^{-1} of skins f.w. according to variety and they were found in lower concentrations in 2018 compared to 2017 season. The skins of Xinomavro contained the lowest concentration, while Mavrotragano and Mandilaria skins were characterized by the highest concentrations in 2017 and 2018 seasons, respectively (Table 1). The lowest content in berries (mg Mlv3-glu g^{-1} berry) was observed in Xinomavro, while harvest season did not affect significantly the content in Agiorgitiko and Xinomavro (Table 1). TEstA were in lower concentrations to all the varieties compared to TNonEstA. The highest content was observed in Mandilaria and the lowest in Kotsifali for both seasons (Table 1).

According to the number and the pattern of the substitution of hydroxyl and methoxy groups on the B-ring, the anthocyanin colour parameters and especially their hº values differ. Cyanidin based anthocyanin (A1) pigments (Cyn3-glu, Pn3-glu) exhibit a red colour whereas delphinidin based (A2) anthocyanins (Dlp3-glu, Pt3-glu, Mlv3-glu) are purple to blue (Castellarin et al., 2006; Castellarin and Di Gaspero, 2007). Moreover, methoxylated (OMe) as Pn3-glu, Pt3-glu, Mlv3-glu, compounds are more stable than non-methoxylated anthocyanins (NonOMe), as Dlp3-glu, Cyn3-glu (Castellarin and Di Gaspero, 2007). It was observed that the percentage of A2 was higher than that of A1 for Mavrotragano, Mandilaria, Agiorgitiko and Xinomavro varieties, while the opposite was observed for Kotsifali grapes (Fig. 2). In the skin extracts of Kotsifali grapes %A2 derivatives was lower than the %A1 by 10.9% and 4.4% for 2017 and 2018, respectively. In addition, %A2 was highest in Xinomavro (96.6%) and Agiorgitiko (95.3%) in 2017 season, followed by Mandilaria (91.5%). In 2018 season the above three varieties contained similar amounts of %A2, (92% to 94%). In addition, Mavrotragano and Mandilaria skin extracts contained the lowest percentages of OMe pigments during both harvest seasons (86% and 83% in average, respectively) (Fig. 2). Moreover, Agiorgitiko and Xinomavro contained the lowest percentages of NonOMe anthocyanins during both harvest seasons and high values of %OMe (Fig. 2). These values are similar to those reported for Pinot Gris and Pinot Noir by Castellarin and Di Gaspero (2007).

The principal component analysis (PCA) was applied to anthocyanin data from both seasons in order to obtain possible categorization of grape varieties. Fig. 3 shows the projection of the variables and the varieties onto the first two principal components (PC). The first PC explains 69.6% of the total variance and it opposes % OMe, % coumaroylated (Cm) and % esterified anthocyanins (%Est) to % NonOMe and % non-esterified (NonEst). The second principle component explains 13.3% of the total variance and opposes total non-esterified anthocyanins (TNonEstA, mg Mlv3-glu g^{-1} skin f.w.), %Pt3-glu and % Dlp3-glu to %Pn3-glu, %Cyn3-glu and %A1. The PCA enables the discrimination of two varieties (and to a lesser extent another one) based on their anthocyanin content (Fig. 3). Mavrotragano variety is mainly situated on the right and upper part of the diagram and it is categorization is based on its high contents of %Pt3-glu and %Dlp3-glu. Kotsifali which is situated on the right and lower part of the diagram is the second variety which can be discriminated by PCA. The high contribution of %Pn3-glu and %Cyn3-glu to total anthocyanins as well as the presence of A1 pigments are the main parameters responsible for the discrimination of this variety. Xinomavro is situated in the center of the left part of the diagram. It is well separated from Mavrotragano and Kotsifali due to its high content of esterified (both coumaroylated and acetylated) and OMe anthocyanins. However, there is an overlapping

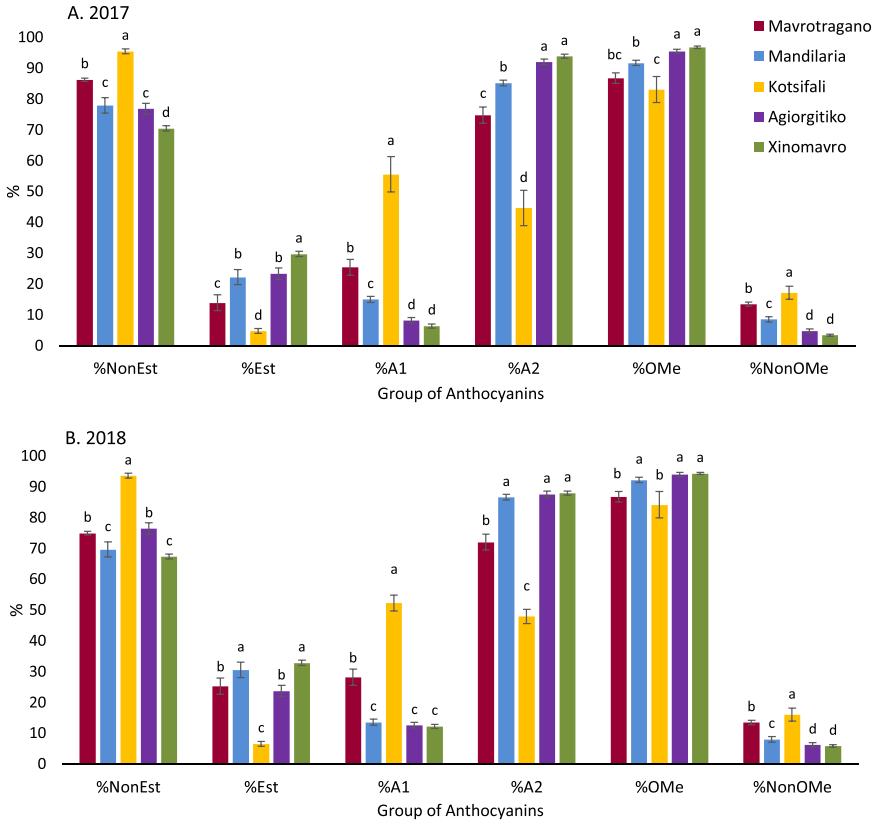

Fig. 2. Pattern of the different anthocyanin groups in skins from the varieties Mavrotragano, Mandilaria, Kotsifali, Agiorgitiko and Xinomavro for A. 2017 and B. 2018 seasons. Bars indicate ± S.E. of the mean value. Significant differences among varieties are indicated by different letters (Tukey's test, $p < 0.05$)[2].

Table 1
Total non-esterified and esterified anthocyanins of skins from the varieties Mavrotragano, Mandilaria, Kotsifali, Agiorgitiko and Xinomavro for 2017 and 2018 seasons.

Season	Variety	Total Non-Esterified Anthocyanins		Total Esterified Anthocyanins	
		mg Mlv g^{-1} fresh weight of skins	mg Mlv g^{-1} berry	mg Mlv g^{-1} fresh weight of skins	mg Mlv g^{-1} berry
2017	**Mavrotragano**	10.85 ± 0.33^{a}	0.601 ± 0.01^{a}	1.71 ± 0.13^{b}	0.096 ± 0.00^{c}
	Mandilaria	8.54 ± 0.16^{b}	0.556 ± 0.01^{b}	2.49 ± 0.11^{a}	0.164 ± 0.00^{a}
	Kotsifali	6.98 ± 0.21^{c}	0.461 ± 0.01^{c}	0.26 ± 0.01^{d}	0.017 ± 0.00^{d}
	Agiorgitiko	5.52 ± 0.08^{d}	0.510 ± 0.01^{b}	1.58 ± 0.03^{b}	0.147 ± 0.00^{b}
	Xinomavro	2.64 ± 0.02^{e}	0.221 ± 0.01^{d}	1.11 ± 0.01^{c}	0.093 ± 0.00^{c}
2018	**Mavrotragano**	3.14 ± 0.91^{c}	0.524 ± 0.01^{b}	1.06 ± 0.05^{c}	0.176 ± 0.00^{b}
	Mandilaria	6.13 ± 0.15^{a}	0.627 ± 0.01^{a}	2.68 ± 0.08^{a}	0.275 ± 0.00^{a}
	Kotsifali	2.73 ± 0.21^{c}	0.282 ± 0.01^{c}	0.19 ± 0.01^{d}	0.020 ± 0.00^{d}
	Agiorgitiko	4.96 ± 0.09^{b}	0.534 ± 0.05^{b}	1.53 ± 0.02^{b}	0.165 ± 0.02^{b}
	Xinomavro	1.67 ± 0.04^{d}	0.202 ± 0.01^{d}	0.81 ± 0.11^{c}	0.098 ± 0.01^{c}

space between Xinomavro, Mandilaria and Agiorgitiko which does not enable the complete discrimination of these varieties. Finally, no discrimination exists among Mandilaria and Agiorgitiko varieties due to similarities in their anthocyanin composition.[3]

3.2. Proanthocyanidin composition

The importance of tannins in the sensory properties of red wine is well documented, particularly with respect to astringency and bitterness. It is known that the individual subunit composition of PAs is an important parameter that affects astringency, with (-)-epicatechin (EC) being more astringent than (+)-catechin (C) (Kyraleou et al., 2016; Quijada-Morín et al., 2012). According to previous studies, the intensity of astringency is positively correlated with the presence of galloylated subunits (%ECG) in the tannin structure due to the enhanced reaction with proteins (Rinaldi et al., 2014; Vidal et al., 2003); however, this

[2] Dlp3-glu: delphinidin-3-*O*-monoglucoside; Cyn3-glu: cyanidin-3-*O*-monoglucoside; Pt3-glu: petunidin-3-*O*-monoglucoside; Pn3-glu: peonidin-3-*O*-monoglucoside; Mlv3-glu: malvidin-3-*O*-monoglucoside; NonEst: Cyn3-glu, Dlp3-glu, Pt3-glu, Pn3-glu, Mlv3-glu; Ac: acetylated anthocyanins; Cm: coumaroylated anthocyanins; Est: Ac and Cm; A1: Cyn3-glu and Pn3-glu; A2: Dlp3-glu, Pt3-glu and Mlv3-glu; OMe: Pn3-glu, Pt3-glu and Mlv3-glu; NonOMe: Dlp3-glu and Cyn3-glu.; TNonEstA: total non-esterified anthocyanins in mg per g skin fresh weight

[3] Non-esterified anthocyanins: delphinidin-3-*O*-monoglucoside; cyanidin-3-*O*-monoglucoside; petunidin-3-*O*-monoglucoside; peonidin-3-*O*-monoglucoside; malvidin-3-*O*-monoglucoside. Esterified anthocyanins: acetylated and coumaroylated glucosides. Values are expressed as the means ± standard error. Significant differences among the varieties are indicated by different letters (Tukey's test, $p < 0.05$).

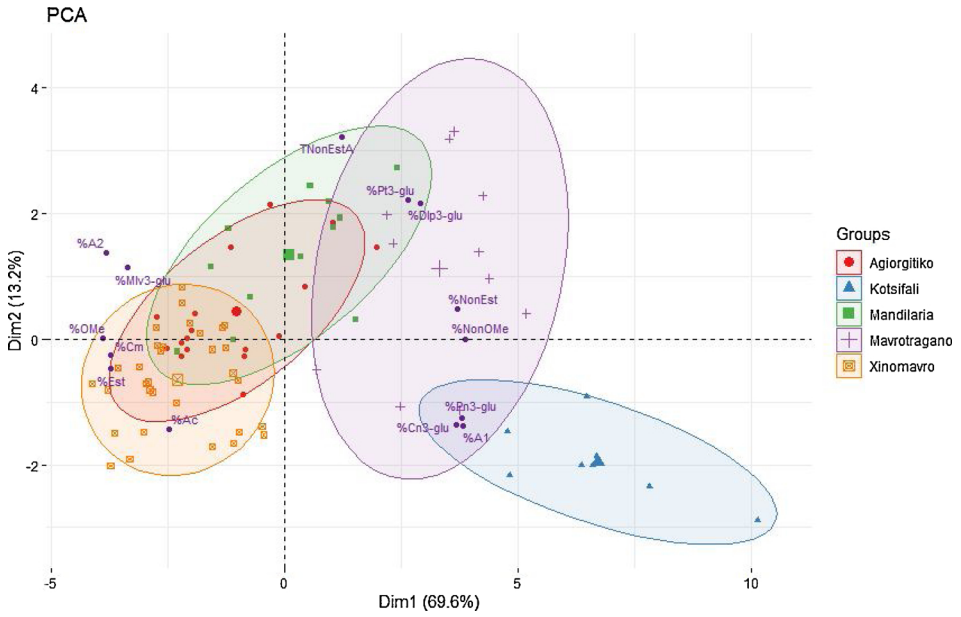

Fig. 3. PCA plot based on the anthocyanin profile and groups according to the variety[2].

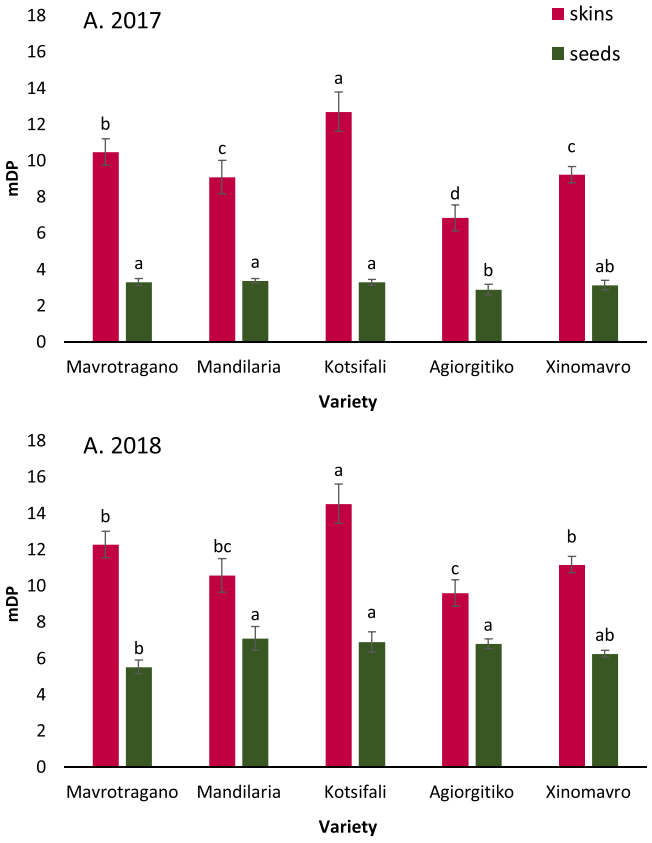

Fig. 4. Mean polymerization degree (mDP) of skins and seeds proanthocyanidins from the varieties Mavrotragano, Mandilaria, Kotsifali, Agiorgitiko and Xinomavro for A. 2017 and B 2018 seasons. Bars indicate ± S.E. of the mean value. Significant differences among varieties are indicated by different letters (Tukey's test, $p < 0.05$)[4].

relationship was not confirmed in all studies (Kyraleou et al., 2016). Chira et al. (2012) reported that %ECG of seed tannins was negatively correlated with astringency while in skins the opposite was observed. Concerning the presence of EGC, most researchers agree that EGC subunits in skins are negatively correlated with the perceived intensity of astringency (Chira et al., 2012; Quijada-Morín et al., 2012; Rinaldi et al., 2014), probably due to the increase in B ring hydroxylation.

The mean degree of polymerization (mDP) and the subunit composition of PAs and of skin and seed tannins of the five varieties studied are shown in Figs. 4 and 5 respectively. According to our knowledge, this is the first time that the tannin structural characteristics of Mandilaria, Kotsifali and Mavrotragano grapes have been reported. It was also calculated the weight of tannin extracts from skins and seeds as mg per 1 g of f.w. of seed or skin (Supplementary Table 3). Seeds were richer in tannin extract than skins, with the exception of Mavrotragano in 2017 season. Moreover, Mandilaria was the richest variety in seed tannin extract while the weight of all tannin extracts was greatly affected by the harvest season.

The mDP values of skin PAs in 2017 season varied from 6.8 to 12.7 (Fig. 4A) while in seeds the respective values lower (2.8 to 3.4) in accordance with other studies (Ćurko et al., 2014; Kyraleou et al., 2017). In 2018 season, the mDP values of both seeds and skins were higher than the respective values of 2017 season (Fig. 4B). Although these results were similar to those reported for the Greek varieties Agiorgitiko (Petropoulos et al., 2017) and Xinomavro (Kyraleou et al., 2015a,b), they were lower compared to several were international varieties as for example, Merlot (Chira et al., 2009), Cabernet Sauvignon (Bordiga et al., 2011) and Syrah (Kyraleou et al., 2017).

According to the results, significant differences were observed among the mDP values of skin PAs between the different varieties. During both experimentation seasons, Kotsifali skin tannins were the most polymerized followed by those of Mavrotragano. Agiorgitiko seemed to have the shortest skin tannins. In 2017 season the mDP values differ significantly among Mandilaria and Agiorgitiko while in 2018 season no significant differences were observed (Fig. 4). Moreover, mDP values of Xinomavro skin tannins were higher than those of Agiorgitiko in agreement with previous studies (Kyraleou et al., 2015a,b; Petropoulos et al., 2017).

In a previous study (Kyraleou et al., 2015a,b) the mDP values of Xinomavro seeds and skins were measured after separation of PAs in oligomeric and polymeric fractions. In this study, the mDP values are referred to the total PAs of skins or seeds, since no fractionation was carried out to separate them according to their size. In general, according to the previous study (Kyraleou et al., 2015a,b) polymeric PAs represented an average of 76% of the extracted seed proanthocyanidins,

[4] mDP: mean degree of polymerization

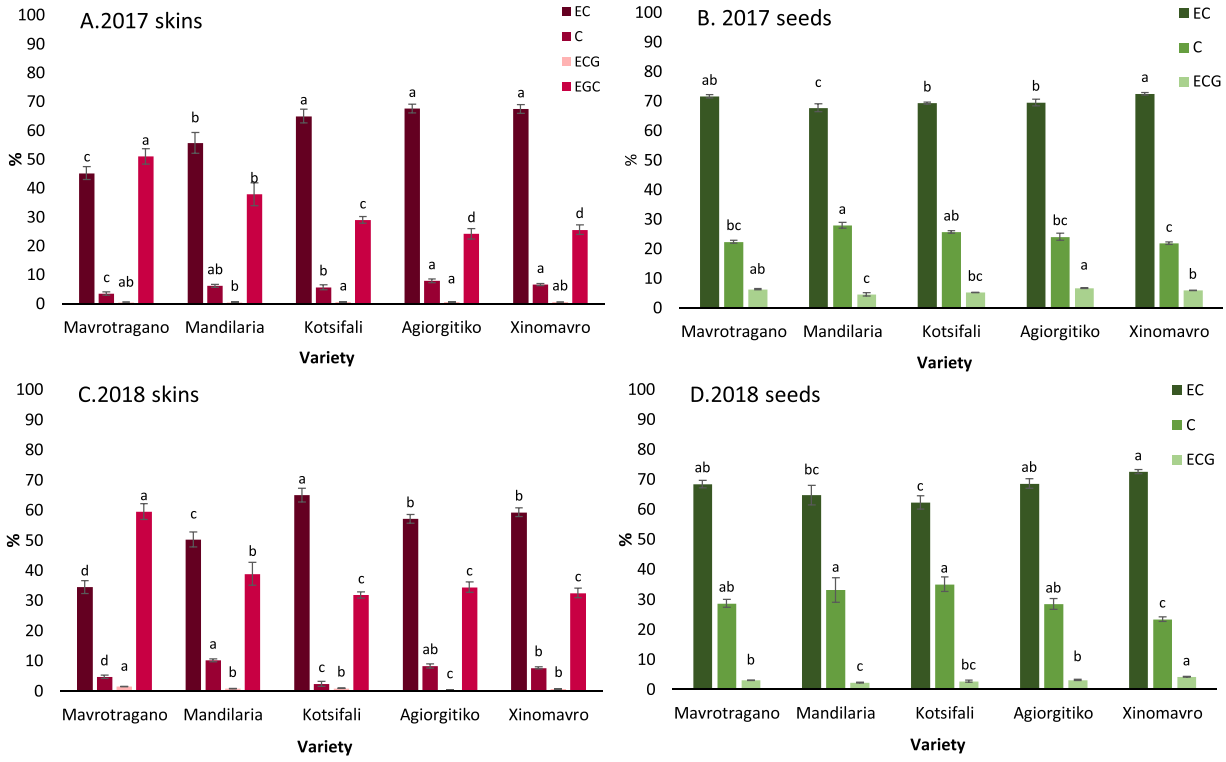

Fig. 5. Proanthocyanidin composition (in percentage of subunits %) of skins for A. 2017 and C.2018 seasons and seeds for B. 2017 and D. 2018 seasons, from the varieties Mavrotragano, Mandilaria, Kotsifali, Agiorgitiko and Xinomavro. Bars indicate ± S.E. of the mean value. Significant differences among varieties for each subunit are indicated by different letters (Tukey's test, $p < 0.05$)[5].

whereas in skins this value was higher, with an average of 96%. Similar results have also been reported for several other varieties (Kyraleou et al., 2017; Monagas et al., 2003). Since total PAs are mainly composed by polymeric complexes, a comparison of the results obtained without fractionation with those of the polymeric fraction might be possible.

Regarding seed PAs, no significant differences were observed among the mDP values of Mavrotragano, Mandilaria, Kotsifali and Xinomavro, while Agiorgitiko was characterized by the shortest PAs in 2017 season (Fig. 4). However, a different pattern was observed in 2018 season, where the lowest mDP value was determined in Mavrotragano seeds.

The mDP values of seed, skin or wine tannins are characterized by high heterogeneity and are mostly affected by maturity stage and harvest season (Ćurko et al., 2014; Kyraleou et al., 2016; Petropoulos et al., 2017). Therefore, this value could hardly be considered as an index, which could characterize and discriminate the different grape varieties. However, it is important to underline that both the average size and the composition of skin and seed tannins exert a significant influence on wine tannin structural characteristics, which affect their organoleptic properties.

In general, the average mDP of wine PAs is lower than that of the corresponding calculated values of grape seeds or skins (Basalekou et al., 2019; Cosme et al., 2009). This is mainly due to the predominance of the degradation reactions of the higher molecular weight proanthocyanidins over the polymerization reactions, which take place simultaneously. Moreover, shorter tannins are more easily extracted than the larger ones which are retained in the cells (Downey and Hanlin, 2010; Kyraleou et al., 2016). It is also well documented that the maturity stage of grapes and several winemaking parameters influence the extraction of grape PAs into the corresponding wine (e.g. fermentation temperature, skin maceration, cold maceration, addition of enzymes and oenological tannins).

However, unlike mDP values, the subunit composition of skin PAs is strongly related with that determined in the corresponding wines (Petropoulos et al., 2017; Quijada-Morín et al., 2012) mainly due to the presence of prodelphinidin subunits (ECG). These findings are in agreement Hanlin et al. (2011), who reported that although the size of wine's PAs was comparable to that of the seeds (mDP 4 to 17), their composition resembled more with that of skin PAs. Grape variety therefore, might have a strong influence on wine PAs composition since it greatly affects the proportion of skin or seed PAs extracted in the wine.

Given the strong influence of grape PAs on the content and composition of wine tannins, the determination of the subunit composition of skin and seed PAs was of great interest in this study. Concerning skin extracts, EC was the major subunit determined in Mandilaria, Kotsifali, Agiorgitiko and Xinomavro extracts, with a percentage contribution to total PAs higher than 50% during both seasons, while in Mavrotragano the dominant subunit was EGC (Fig. 5). Moreover, the skins of Kotsifali, Agiorgtiko and Xinomavro varieties were the richest in the sum of EC and C during both seasons, compared to the other two varieties (Fig. 5). EGC had the highest contribution in Mavrotragano skin PAs for both seasons; however in 2018 the percentage contribution of this compound was higher than in 2017 season. The results are generally in agreement with the findings of previous studies. Monagas et al. (2003) and Bordiga et al. (2011) reported that EC was the predominant subunit of skin PAs while other researchers found C as the main subunit in grape skins in some varieties (Hanlin et al., 2010).

As far as the presence of subunits either at terminal or extension positions on the tannin polymer is concerned, C was detected only in terminal positions (data not shown) in Agiorgitiko grape skins, while in the other four varieties, it was determined as both terminal and extension subunit. Moreover, ECG in skins was detected only as extension subunit (data not shown) and at trace levels (less than 0.5%), in all five varieties in 2017 season, while in 2018 season its contents were higher

[5] C: (+)-catechin; EC: (-)-epicatechin; ECG: (-)-epicatechin gallate; EGC: (-) – epigallocatechin.

(Fig. 5).

Regarding seeds, significant amounts of EC were present in every variety, while EGC was entirely absent in agreement with the findings of previous studies (Hanlin et al., 2011; Kyraleou et al., 2017; Rinaldi et al., 2014). The pattern of PAs subunits in seeds (Fig. 5) did not vary significantly among the varieties, unlike skins. According to the results obtained, the percentages of EC, C, and ECG in 2017 season ranged from 67% to 73%, 21% to 28% and 4% to 7%, respectively. While in 2018 season the respective percentages were 62-72%, 23-34% and 2-4% respectively (Fig. 5). In 2018 season, the values of C were higher compared to those in 2017 season, while the opposite was observed for ECG. Xinomavro seeds contained the highest percentage of EC during both seasons, while the lowest was determined in Mandilaria seeds in 2017 season and Kotsifali in 2018 season. Most previous studies involving different varieties reported EC as the predominant subunit in seed extracts of Merlot (Chira et al., 2011; Rinaldi et al., 2015), Carmenere, Marzemino, and Syrah (Mattivi et al., 2009), Xinomavro (Kyraleou et al., 2015a,b) and Cabernet Sauvignon (Chira et al., 2011; Rinaldi et al., 2015).

All the subunits of seeds' PAs were detected in both terminal and extension positions. The higher proportions of EC and ECG were determined in extension (> 50%) and terminal positions (> 3%), respectively. Concerning C, it was determined at similar contents at both, terminal and extension positions in Mavrotragano, Mandilaria and Kotsifali seeds. In contrast, in the seeds of Agiorgitiko and Xinomavro grapes, C was participating less as extension than the terminal subunit.

4. Conclusions

The purpose of the current study was to discriminate five Greek red grape varieties according to their phenolic composition. One of the most significant findings of this study is the variety dependence of skin anthocyanins and proanthocyanidins. Mavrotragano discrimination was based on the higher percentage contribution of Pt3-glu and Dlp3-glu in skin total anthocyanins and EGC subunits in skin proanthocyanidins. The main characteristics of Kotsifali grapes were the higher percentage contributions of Pn3-glu, Cyn3-glu and 2OH pigments to total anthocyanins in combination with the most polymerized skin PAs. Anthocyanin composition between Mandilaria and Agiorgitiko varieties was quite similar, however the lower mDP values of skin PAs of Agiorgitiko might be a significant discrimination tool. Xinomavro was characterized by higher contents of Est and OMe anthocyanins which resulted in its partly differentiation from Mandilaria and Agiorgitiko. In contrast, fewer differences were observed among the studied parameters of seed proanthocyanins suggesting that possibly seed PA structural characteristics are most affected by harvest season rather than by the variety.

CRediT authorship contribution statement

Maria Kyraleou: Conceptualization, Project administration, Writing - original draft, Writing - review & editing. **Stamatina Kallithraka:** Conceptualization, Supervision, Resources, Funding acquisition, Writing - review & editing. **Eugenia Gkanidi:** . **Stefanos Koundouras:** Resources. **David T. Mannion:** Formal analysis. **Kieran N. Kilcawley:** 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.

Acknowledgements

First author was supported by State Scholarships Foundation of Greece (IKY). The authors would like also to express their gratitude to the Greek wineries for providing grape samples for this study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2020.103547.

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