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Identification of Proanthocyanidins Compounds in Skins of Some Table Grape *Vitis Vinifera* Varieties from Algeria Grown in Mediterranean Climate by High-Performance Liquid Chromatography

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ABSTRACT

Introduction: Proanthocyanidins (PAs) are some of the most abundant polyphenolic substances in the plant kingdom. PAs are an integral part of the human diet, found in high concentrations in fruits such as apple, pear, grape and in chocolate, wine, and tea.
Materials and Methods: A range of five colored and white table grape (*Vitis vinifera*) varieties from El-Tarf region (North-East of Algeria) were assayed for their skin content of PAs and Mean of Polymerization Degree (DPm). The study was realized by means of reversed-phase high performance liquid chromatography coupled with photodiode array detector (RP-HPLC-DAD) analysis after thiolysis.
Results: The results show the presence of seven compounds which are identified as belonging to the class of flavan-3-ol. The two navy-blue colored grape cultivars Gros Noir and Muscat noir showed the highest PAs content (average content 1721.90 and 1739.45 mg per g fresh berry weight, respectively) and the highest percentage of epigallocatechin (EGC) (18.58 and 21.83%, respectively).
Conclusion: The presence of such amounts of PAs makes these varieties a potential source of PAs compounds and can be used as easily accessible source of natural antioxidants for use in replacement of synthetic antioxidants.

KEYWORDS: *Vitis Vinefera*; Grape skins; Proanthocyanidins; DPm; RP-HPLC.

ABBREVIATIONS: PAs: Proanthocyanidins; DPm: Mean of Polymerisation Degree; EGC: Epi-Gallo Catechin; ECG: Epicatechin Gallate; RP-HPLC-DAD: Reversed Phase-High Performance Liquid Chromatography with Photo Diode Array Detector; TFA: Trifluoroacetic Acid.

INTRODUCTION

PAs, also called condensed tannins, are oligomers and polymers of monomeric flavans linked through the specific single (B linkages) and double (A linkages) bonds. These secondary plant metabolites have substantial antioxidant activity. They are prevalent in some foods and dietary supplements including several types of berries, red grapes and their wines, and seeds, chocolate, cinnamon, pycnogenol, and *Ginkgo biloba*.[1] Their presence in food affects food quality parameters such as astringency, bitterness, sourness, sweetness, salivary viscosity, aroma, and color formation. In the grapes, flavanols are either present in the form of monomers or in the form of polymers forming condensed tannins or PAs in the hypodermic layers of the skin and the soft parenchyma of seeds between the cuticle and the hard seed coat.[2-4] PAs are a class of the most studied polyphenolic phytochemicals, due to the relative importance of their antioxidant activity and other biological activities. They are an integral part of the human nutrition,

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and in recent years, several studies have generated analytical data on the PAs profile, as well as their effects on the human health.[5-7] Several chromatographic approaches had been reported for the analysis of PAs and are also proposed in the literature. Reversed-phase high-performance liquid chromatography (RP-HPLC) uses various detection techniques, and is currently the most common method employed.[8-10] To the best of our knowledge, no research has been yet reported on the determination of PAs in the grapes grown in the vines in this particular region of Algeria. The purpose of this study was to investigate the content of PAs and the DPm in skin extracts of five table grape varieties from *Vitis vinifera* grown in El-Tarf region (North-East of Algeria). Two grape cultivars, Muscat blanc and Victoria, have a yellow-green colored grape berry. Gros Noir and Muscat noir are navy-blue colored grape cultivars and Cardinal is purple-colored grape cultivar. These entire table grape varieties studied are most widespread in this region of Algeria. A RP-HPLC-DAD-UV/VIS method was used for this analysis. The similarities and differences between the tannins` compositions in the grape skin extracts from different cultivars are discussed.

MATERIALS AND METHODS

Plant Materials, Experimental Design and Climatic Conditions

Skins from five table grape cultivars, including Gros Noir, Muscat Noir, Cardinal, Muscat Blanc and Victoria were examined. Experiments were carried out during the 2012 season in a commercial vineyard, located at El-Tarf, East-Northern Algeria (36° 45' 00" N; 81° 10' 00" E) where the climate is of the Mediterranean type, with hot and dry summers and mild rainy winters. Soils horizons present a silty sandy and silty sandy clay texture with the following average characteristics: clay 2 to 6%; silt 44.9 to 59.1%; sand 38.9 to 49.4%; organic matter 2.33±0.75 to 4.22±0.18; pH (H_2O) 7.57±0.13 to 7.83±0.64. Approximately 2 kg of grapes was collected from each cultivar in late summer 2012, from three different sites. All the samples were collected when the Brix values were in the range 17-21°Brix.

Sample Preparation

Skins from berries were manually separated from the pulp and dried in an oven at 50 °C until a constant mass was reached. They were then grounded to powder in a domestic mill and, stored at -18 °C until analysis.

According to Brossaud et al[11], the extraction procedure was as follows: dried skin powder (2 g) was successively extracted twice with 80 ml of methyl alcohol:water:TFA (80:20:0.05) and afterward twice with 50 ml of a mixture acetone:water (60:40) (25 °C/15 min/250 rpm). The extract was centrifuged (10 °C/10 /10,000 rpm), the supernatant was then filtered through glass microfiber filter GF/A 1.6 µm, before drying under vacuum at 30 °C and then dissolved in 5 ml of methanol to yield a crude skin flavanols extract.

Isolation of Proanthocyanidins (PAs)

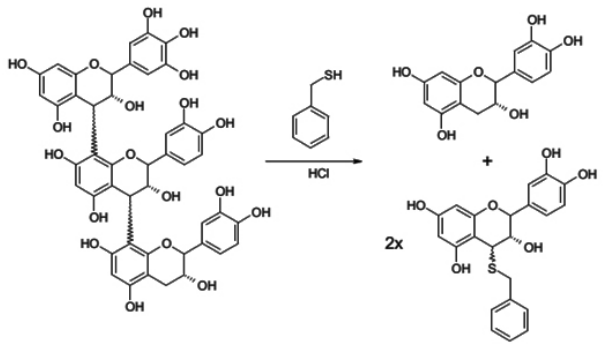
To separate PAs from crude skin extracts, 2 ml of these extracts were subjected to chromatography over Fractogel Toyopearl® HW-40(F) (300 mm × 10 mm i.d.) (Tosoh Corporation, Japan). Anthocyanins, flavonols, monomeric and dimeric flavanols were eliminated using 30 ml of ethyl alcohol:water:TFA (110:90:0:01) as the eluant at 1ml min^{-1}, and PAs fraction was eluted using 30 ml of acetone:water (60:40) at 1ml min^{-1}. To this fraction, 300 µl of internal standard (50 mg of methyl 4-hydroxybenzoate in 100 ml of MeOH) was added. The acetonic fraction was dried using a rotary evaporator Bucchi® under vacuum at 30 °C and then dissolved in 5 ml methanol for the thiolysis reaction.

Characterization of Proanthocyanidins (PAs)

Acetonic fraction was subjected to thiolysis reaction which was performed as described previously[12] in duplicate, after the addition of 120 µl of toluene-α-thiol to 120 µl to the fraction above and heating for 2 min at 90 °C, this reaction allows the distinction between terminals units (released as flavan-3-ols) and extension units (released as the corresponding benzylthioether derivatives, 2x) (Figure 1). Reaction between total units and terminal units gives us the access to the mean degree of polymerization (DPm).

The thiolysis reaction medium (20 µl) filtrated through a membrane filter with an aperture size of 0.45 µm. This was analyzed by RP-HPLC.

Figure 1. Fractionation of a Trimer by Thiolyse



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Identification and quantification of PAs was carried out using analytical RP-HPLC according to the conditions adapted from those described by Brossaud et al,[11] in a Waters Millenium HPLC–DAD system (Milford, MA, USA) system with an autosampler and a quaternary pump coupled to a diode array detector. A 250×4.6 mm (internal diameter), 5 µm, reversed-phase Lichrospher 18 RP100, column (Merck, Darmstadt, Germany) was used and the elution solvents were: A, water/acetic acid (97.5:2.5) and B, acetonitrile:water:acetic acid (80:17.5: 2.5); isocratic elution with 100% A for 5 min, followed by linear gradients from 100% to 90% in 30 min; from 90% to 80% in 30 min, from 80% to 0% in 5 min, from 0% to 100% in 5 min (Table 1), washing and re-equilibration of the column. The column temperature was at 30 °C, the flow-rate was set at 1 ml/min and detection was monitored at 280 nm. Peak identification was performed by comparing the retention time and UV/VIS spectra. Each sample of berries was extracted in duplicate and their acetonic fractions were analyzed by the same method. Hence, the final result was the arithmetic average of the four analyses.

Statistical Analysis

Results are expressed as mean±standard deviation (SD). Statistical analysis was carried out using the STATISTICA software version 5.0 (Copyright© StatSoft, France). Differences between the mean results were first analyzed using the ANOVA test and the least significant differences (Fisher's LSD) were calculated following significant *F* test ($p \leq 0.05$).

RESULTS AND DISCUSSION

General

Condensed tannins or PAs are characterized by the properties to give combinations with the proteins and other polymers such as polysaccharides. The tannins are characterized by a sensation of astringency (dry mouth).

The cultivars of *Vitis vinifera* selected for this study are to date widely cultivated in this area. Muscat noir, Cardinal and Muscat blanc are the main Algerian variety, followed by Gros noir, which has a limited cultivation area. Victoria is an Italian variety, recently introduced in Algeria.

Proanthocyanidins (PAs) and Derivatives

Depolymerization in the presence of acid and nucleophile followed by HPLC analysis is a useful tool for quantification and characterization of PAs. This method allows determining the nature and concentration of terminal and extension units and consequently calculating the mean DP (DPm) and the percentage of galloylation (%ECG) of PAs using toluene--α-thiol (benzyl mercaptan).

The PAs identified in this study are listed in Table 2 (20-26). The total amount of PAs (Table 3), vary significantly ($p<0.05$) between 447.40 mg/g (Victoria) to 1739.45±107.27 mg/g (Muscat noir).

Based on works of Brossaud et al[11] on Cabernet franc berries grown on different sites of the *vallée de la Loire* (France)-1995 vintage, the PAs contents (condensed tannins) oscillate between 1.239 and 1.759 g/kg fresh weight, between 3,363 and 4.448 g/kg fresh weight for skins and seeds, respectively.

The levels of grape PAs vary considerably, depending on the variety, environmental conditions, especially water supply and sunlight exposure, berry size and number of seeds,[13] harvest year,[14] the degree of maturation.[9,15] These differences not only highlight the impact of different types of soils, cultural practices, but also the harvest in metabolism way of tannins.[16] According to Mateus et al[17] low altitudes appear to be favorable for the synthesis of high concentrations of PAs in relation to weather conditions which coincide with high values recorded in this study for all varieties cultivated at very low altitude.

Muscat noir variety showed the highest flavanol content (1739.45 mg/g of berries). These findings are consistent with previous reports related to grape varieties grown around the world[18,19] and confirm that grape extracts are a rich source of PAs, usually oligomers and polymers of polyhydroxy flavan-

Table 1. Linear Gradient Used for the Separation of Flavan-3-ols

Temps	% A	%B
0	95	5
3.4	88.5	11.5
5	80	20
23	50	50
25	40	60
28	5	95
32	5	95
35	95	5
38	95	5

Table 2. Retention Time of Different PAs Compounds in Different Table Grape Varieties

	PAs Compounds	Retention time (min)
20	Catéchine (C)	8.100±0.079
21	Epicatéchine (EC)	9.112±0.075
22	Epicatéchine-3-O-Gallate (ECG)	11.609±0.129
EI	Internal Standard (EI)	15.006±0.127
23	Epigallocatechine-SH (EGC-SH)	18. 263±0.129
24	Catechine-SH (C-SH)	20. 822±0.136
25	Epicatechine-SH (EC-SH)	21.563±0.134
26	Epicatéchine-3-O-Gallate-SH (ECG-SH)	23.725±0.128

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Table 3. Levels of Total PAs, DPm, %CGE and %ECG of Skin of Different Grape Varieties Analyzed

	Skins			
Varieties	**Total PAs (mg/g of berries)**	**DPm**	**% ECG**	**% EGC**
Muscat blanc	511.35±7.14[a]	28.55±0.56[e]	4,23±0.0002[b]	11,63±0.003[b]
Cardinal	574.95±5.16[a]	14.34±0.14[b]	7,21±0.0023[d]	12,89±0.001[c]
Gros noir	1721.90±87.96[b]	11.04±0.14[a]	5,7±0.0023[c]	18,58±0.001[d]
Muscat noir	1739.45±107.27[c]	18.54±0.15[d]	3,19±0.0012[a]	21,83±0.003[e]
Victoria	447.40±31.25[a]	15.03±0.49[b,c]	6,96±0.0046[d]	9,93±0.001[a]

Total PAs: Total Proanthocyanidins
DPm: Mean of Polymerization Degree
ECG: Epicatechine gallat
EGC: Epigallocatechin
Results are expressed as mg per g of berries. Values with the same letter in each column do not differ significantly ($p<0.05$). The results are classified in ascending order; a<b<c<d<e.

3-ols such a (+)-catechin and (−)-epicatechin, in the form of gallate esters or glycosides.

The content of total monomeric and oligomeric flavanols was estimated by thiolysis assay, the monomeric form of PAs represented by the flavan-3-ols epicatechin and catechin monomer were detected in skin extracts. This could not be quantified (peak between thresholds detection and quantification) (Figures 2 and 3) either due to the fact that catechin is present in a small proportion (less than 10%)[14] or an inter-conversion between catechin and EGC during the maturation of grapes, that were analyzed. According to Liang et al[10] the contents of (+) -catechin and epicatechin are significantly lower than those of the other two monomers (ECG and EGC).

In the present study, DPm, % ECG and % EGC recorded values vary significantly from one variety to another ($p<0.05$) from 11.04±0.14 (Gros noir) to 28.55±0.56 (Muscat blanc); from 3.19±0.0012 (Muscat noir) to 7.21±0.0023% (Cardinal) and from 9.93±0.001 (Victoria) to 21.83±0.003% (Muscat noir), respectively.

Figure 2. PAs Chromatographic Pattern of Gros noir (A), Cardinal (B) and Muscat noir (C) Skin Extract Recorded at 280 nm. For List of Substances, See Table 2

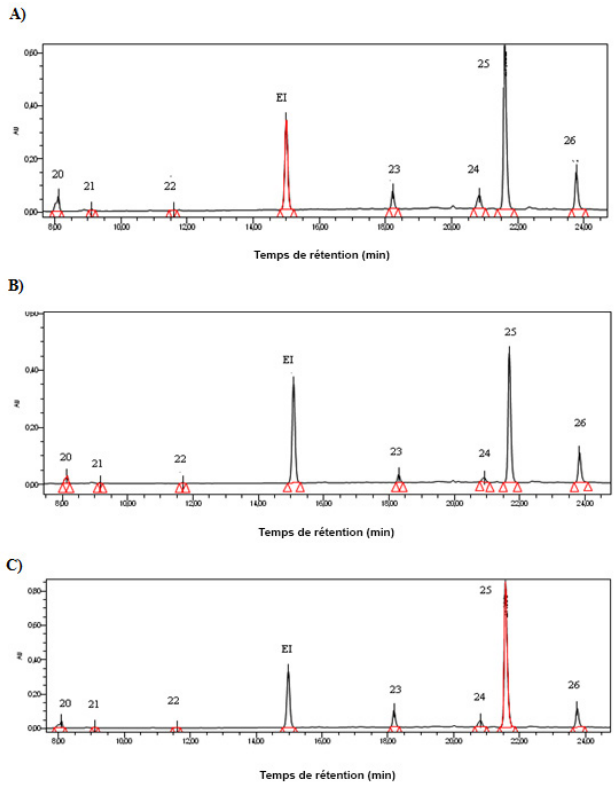

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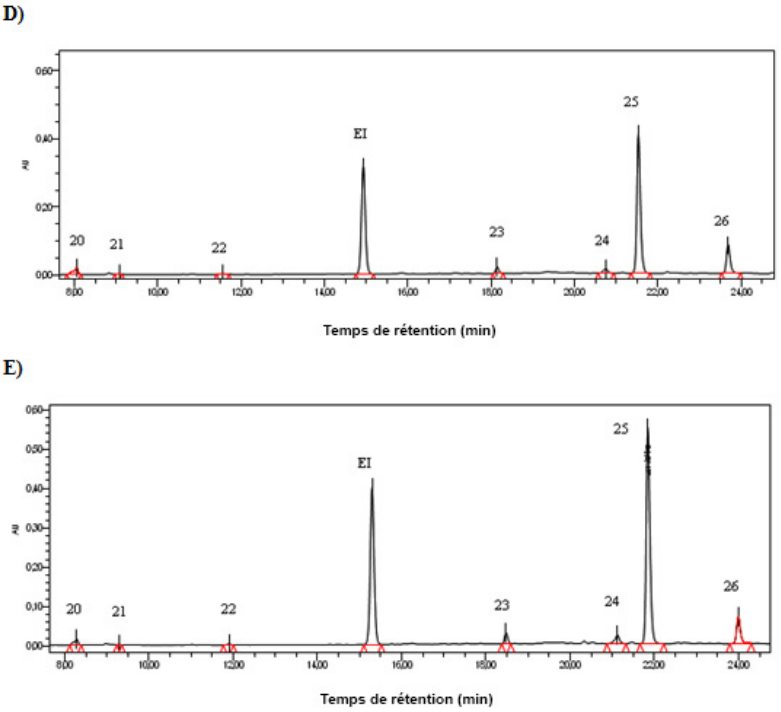
Figure 3: PAs Chromatographic Pattern of Victoria **(D)** and Muscat Blanc **(E)** Skin Extract Recorded at 280 nm. For List of Substances, See Table 2.



The work of Obreque-Slier et al[20] on grape of two varieties (Carmenere and Cabernet Sauvignon) from Chile, show DPm of 10.0±3.7 and 6.4±1.1, while the ECG varies between 12.2±0.3 et 7.3±2.9 for the two skin extract varieties, respectively.

The present results are different from those of some previous reports, on several varieties cultivated in various countries, in which (+)-catechin was always the predominant flavan-3-ol monomer, followed by (-)-epicatechin and (-)-epigallocatechin. On the other hand, the EGC and ECG monomers have not been detected in grape skin.[21] These differences suggest that the proportion of flavan-3-ol monomers varies considerably between the different grape varieties with perhaps a certain regional influence and possibly of viticultural management. Moreover, the methods of the acid catalysis used for the fractionation of the tannins are different.

Differences among the grape varieties studied, climatic factors, year and viticultural practices may be partly responsible for the distinct differences in values and trends of DPm compared to other published results.[10] The values obtained for the DPm, also show that skin PAs are in polymeric forms (DPm> 12-15).[22]

Finally, the results obtained show that the proanthocyanidin composition and its various subunits differ significantly from one variety to another. This could be the reason for the differences in the astringency of the grapes studied.

Muscat blanc exhibits a high DPm when compared with others varieties, which explains its astringency character. According to Cadot et al,[4] the homogeneous polymerization during the PAs synthesis between fruit and *véraison* (veraison) stage increases astringency as they increase in size, while the combination with anthocyanins decreases the reactivity, and therefore the astringency of the compounds formed.

CONCLUSION

Statistically significant difference in the contents of PAs compounds between the studied cultivars was noticed. From these findings, it may be concluded that the amounts and distribution of PAs compounds in grape skins depend directly on the cultivar, ripening time and fertilization, as the other factors, such as climate and location of growth were the same for all the cultivars studied by us.

The skin extracts of the grape varieties had high PAs contents which known to have a high antioxidant activity. This confirms that grape skin is a potential source for the extraction of tannins and suggests that although the differences in the PAs content between the varieties analyzed was significant, the levels of PAs are such that extracting from these grape skins might be economically viable for use in industry to make specific food additives or dietary supplements.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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