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Quantitative Analysis of Proanthocyanidins (Tannins) From Cardinal Grape (*Vitis vinifera*) Skin and Seed by RP-HPLC

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ABSTRACT

BACKGROUND: Grape phenolics are structurally diverse, from simple molecules to oligomers and polymers usually designated "tannins or proanthocyanidins (PAs)" referring to their ability to interact with proteins. Those compounds have been attributed to a great number of biological activities beneficial for human health as they act as antioxidant, anti-inflammatory, antitumor, etc. **AIMS**: The objective of the current study was to quantify and to identify the PAs and determine the mean degree of polymerization (DPm) in seeds and skins of the grape cardinal variety cultivated in El-Tarf region, Algeria. **METHODS AND MATERIAL**: To determine PAs, Reverse Phase High-Performance Liquid Chromatography with Diode Array Detection *(RP-HPLC-DAD)* has been utilized. The DPm was determined after the reaction of thiolysis in the presence of toluene-α-thiol reagent. **RESULTS**: HPLC-DAD analysis of Cardinal skin and seed extract showed that epicatechin gallate (ECG) and epigallocatechin (EGC) were the major constitutive units of grape skin tannins and the mean degree of polymerization (DPm) was lower for seed PAs than for skin. **CONCLUSIONS**: This study showed the richness of skin and grape seeds in polyphenolic compounds (PAs). Therefore, these parts of grape can be used as a potential source of bioactive molecules to promote the health of populations in this region in Algeria.

KEYWORDS: Grape, Skin, Seed, Proanthocyanidins, RP-HPLC-DAD.

1. INTRODUCTION

Condensed tannins, also called proanthocyanidins (PAs), constitute the most abundant class of phenolics in grape berries [1]. Those compounds represent a class of phenolics that take the form of oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (-)-epicatechin. The grape (*Vitis vinifera*) especially seeds are a rich source of PAs. These grape seed PAs are mainly dimers, trimers, and highly polymerized oligomers of monomeric catechins. PAs act as powerful antioxidants with beneficial effects for human health including protection against free radical-mediated injury and cardiovascular disease and exhibit a strong antitumor and antimicrobial activity [2-4]. Furthermore, PAs contribute to the astringency and taste of many fruits and other plant products, such as fruit juices, tea and wine through interactions with salivary proteins [5]. The oligomeric and polymeric PAs may contribute significantly to grape tannin composition. To the best of our knowledge, no study involving PAs quantification and qualification has been published on grape grown in the region of North-East Algeria. Thus, the objective of the current study was to quantify and identify the grape skin and seed PAs from Cardinal grape, that is a purple-colored table grape cultivar, using RP-HPLC-DAD-UV-VIS method, and the DPm after thiolysis reaction using the toluene-α-thiol reagent. The PAs compositions of grape extracts (seed and skin) and the DPm from Cardinal cultivar were then compared and discussed.

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2. MATERIAL AND METHODS

2.1. Grape sample

Approximately, (2 kg) of grape Cardinal (red variety) was collected in late summer 2012 in the region of El-Tarf located in North-East of Algeria (36° 45' 00" N; 81° 10' 00" E). The experimental vineyard was raised in 1980. The distance of sowing was 3 ×1 m, with two rows support, and the training system was a "double-branched asymmetrical cordone". The sample was collected at commercial maturity with the Brix values of 17.65 °Brix. The sampling of grapes was done meticulously and berries were collected randomly from top, bottom, sun-exposed and unexposed clusters on each side of the vine. The sample was placed in clean, dry, plastic boxes and quickly transported and stored until analysis.

2.2. Sample treatment

Before extraction, skin and seed were manually separated from the whole grape berries and dried at oven temperature of 50°C until constant weight. The dried skin and seed were then crushed in a domestic mill for 2 min and then used for extractions.

2.3. Extraction and isolation of skin and seed PAs

According to Brossaud *et al.* [6], the extraction procedure was as follow: 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). Dried seed powder (0.1 g) was then extracted by maceration in 50 mL of a mixture of acetone/water (60:40) and 300 µL of methyl-4-hydrobenzoate (1g/L), with stirring for 70 min. Both extracts were centrifuged (10°C/10 min/10,000 rpm) and the supernatants were then filtered through glass microfiber filter GF / A 1.6 µm before drying under vacuum at 30°C and dissolved in 5 mL of methanol to yield a crude skin and seed PAs extracts, respectively. The extracts were chromatographed on Fractogel Toyopearl® HW-40(F) (300 mm × 10 mm i.d.) (Tosoh Corporation, Japan) to eliminate anthocyanins, flavonols, monomeric and dimeric flavanols with 30 mL of ethyl alcohol/water/TFA (11:9:0.001). The tannin fraction was eluted with acetone/water (6:4) (30 mL). A quantity of 300 µL of internal standard (50 mg of methyl 4-hydroxybenzoate in 100 mL of methanol) 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.

2.4. Characterization of polymeric PAs

The characterization of condensed tannins by depolymerization is frequently employed. 120 µL of each fraction was placed in a glass ampoule with an equal volume of reagent toluene-α-thiol. After sealing, the mixture was shaken and heated at 90°C for 2 min, then quickly cooled with cold water, to stop the reaction. This technique allows the distinction between the terminal units released in the form of flavan-3-ols, the intermediate and upper units released in the form of benzyl thioethers [6]. The thiolysis reaction medium (20 µL) filtrated through a membrane filter (0.45 µm) was then analyzed directly by RP-HPLC under the following conditions: flow rate 1 mL/min at 30°C, solvent A, water/acetic acid (97.5:2.5); solvent B, acetonitrile /water/acetic acid (80:17.5: 2.5), elution with linear-gradient (Table 1) followed by washing the re-equilibration, detection UV 280 nm. Each part of grape (skin and seeds) was extracted in duplicate and the acetonic fractions were analyzed in duplicate too. Hence, the final result was the arithmetic average of four analyses. The compounds identified in the seeds and Cardinal grape skin are listed in Table 2 (20 – 26) and Figure 1 shows the chromatographic profiles of identified tannins eluted according to their retention time.

Table 1: Linear gradient used for the separation of flavan-3-ols

Time (min)	% 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

2.5. Statistical analysis

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

3. RESULTS

The total amount of PAs (Table 3), ranging from 537.25±35.28 mg/g to 1332.90±95.88 mg/g of berries for skin and seed, respectively. As expected, the seed PAs content is higher than in the skin, which is in accordance with the previous studies [6-8].

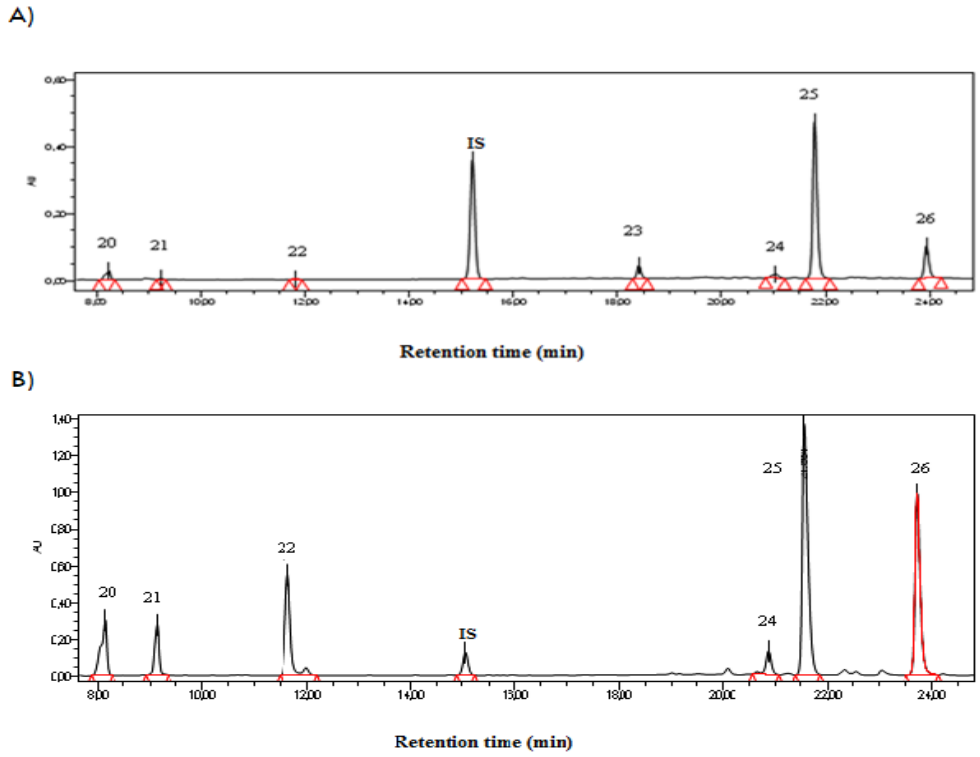

Figure 1: Proanthocyanidin chromatographic pattern of Cardinal skin (A) and seed (B) extract recorded at 280 nm. For key to substances, refer to Table 2

As shown in Table 3, the values obtained for the DPm; 16.33±1.19 for skin and 4.59±0.01 for seed respectively, show that seed tannins are in oligomeric and monomeric forms (DPm varies from 2 to 12 - 15) and skin PAs are in the polymeric form [7].

4. DISCUSSION

Regarding PAs contents, as published on two red grape varieties (Cabernet Sauvignon and Merlot), Lorrain *et al.* [9] recorded values varying from 90.1 ± 4.0 to 92.2 ± 4.5 in seed and from 57.4 ± 0.4 to 63.8 ± 0.1 mg/g on dry weight in skin. According to Brossaud *et al.* [6] on Cabernet Franc berries cultivated at different sites in the Loire Valley (France) - vintage 1995, the contents of PAs (condensed tannins) oscillated between 1.239 and 1.759 g / kg of the fresh weight and between 3.363 and 4.448 g / kg of the fresh weight in skins and seeds, respectively. These results are different from those found in our study. Those differences depend on the grape variety, environmental conditions, in particular water supply and sunlight exposure, berry size and number of seeds [10], variety and year of harvest [11], degree of maturation [12,13]. These differences also may highlight the impact of the different terroirs, the cultural practices, but also the grape harvest on

Table 2: Retention time of different identified proanthocyanidins in Cardinal skin and seed

	Proanthocyanidins	Retention time (min)
20	Catechin (C)	8.100 ± 0.079
21	Epicatechin (EC)	9.112 ± 0.075
22	Epicatechin-3-O-Gallate (ECG)	11.609 ± 0.129
EI	Internal standard (IS)	15.006 ± 0.127
23	Epigallocatechin-SH (EGC-SH)	18. 263 ± 0.129
24	Catechin-SH (C-SH)	20. 822 ± 0.136
25	Epicatechin-SH (EC-SH)	21.563 ± 0.134
26	Epicatechin-3-O-Gallate-SH (ECG-SH)	23.725 ± 0.128

Each value in the table is the mean ± standard deviation (n = 4).

Table 3: Proanthocyanidins, DPm, ECG, and EGC of Cardinal skin and seed

	Skin	Seed
Proanthocyanidins (mg/g of berries)	537.25±35.28	1332.90±95.88
DPm	16.33±1.19	4.59±0.01
ECG %	6.08 ± 0.0001	23.11 ± 0.004
EGC %	17.14±0.006	0.00 ±0.00

The results are expressed as mean ± standard deviation (n = 4); DPm: mean degree of polymerization; ECG: epicatechin gallate; EGC: epigallocatechin.

the metabolism path of the tannins [9]. According to Mateus *et al.* [14], the low altitudes appear to be favorable for the synthesis of important PAs concentrations in relation to climatic conditions, which coincide with high values recorded in this study for cardinal cultivated at very low altitude. Values of DPm were recorded on two varieties from Chili (Cabernet Sauvignon and Carménère) oscillated from 6.4±1.1 to 10.0±3.7 in skin and from 1.8±0.2 to 2.0±0.2 in seed, respectively for the two varieties [15]. The acid catalysis method used for fractionation of tannins may be, particularly, causing the differences recorded. According to Cadot *et al.* [16], homogenous polymerization of PAs during synthesis between fruit set and veraison increases astringency as their size increases, while combination with anthocyanins decreases the reactivity, and hence the astringency, of the compounds formed; So the astringency of cardinal variety directly depend on the DPm of its PAs. Table 3 results visibly show that skin PAs differ from those of seed by a lower percentage of galloylation (% ECG), high DPm and the presence of the prodelphinidin (EGC), as it was also observed by other authors for other varieties [1, 15,17].

5. CONCLUSION

The results of the present study show differences between total PAs, monomer PAs (ECG and EGC) and DPm of the two grape compartments (skin and seeds); but it remains that seeds and skin of Cardinal grape variety cultivated in this region of Algeria, are important sources of PAs known for their high antioxidant activity. This suggests their use as raw material for the extraction of these bioactive molecules, and their use, for example as specific additives in the food industry to replace chemical additives or as food supplements.

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