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QUANTITATIVE ANALYSIS OF PROANTHOCYANIDINS (TANNINS) FROM GRAPE (*VITIS VINIFERA*) SEEDS BY REVERSE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Proanthocyanidins(PAs) are oligomeric and polymeric end products of the flavonoid biosynthetic pathway. They are present in the fruits, bark, leaves and seeds of many plants, where they provide protection against predation. At the same time they give flavor and astringency to beverages such as wine, fruit juices and teas, and are increasingly recognized as having a health promoting on human health. Seed extracts from five grape cultivars (*Vitis vinifera*) growing in El-Tarf (Algeria), were screened for their PAs composition and mDP (mean degree of polymerization). The study was realized by means of reversed-phase high-performance liquid chromatography coupled with photodiode array detector (RP-HPLC-DAD) analysis after thiolysis. The study revealed the presence of seven phenolic compounds belonging to the class of flavan-3-ol; Qualitative and quantitative differences among the cultivars were observed. The results confirm that grape seed of varieties studied are a potential source of PAs and can be used as easily accessible source of natural antioxidants.

1.Introduction

The polyphenolic compounds known as condensed tannins, or proanthocyanidins (PAs), are plant secondary metabolites synthesized via the flavonoid biosynthetic pathway. They occur in a wide range of plants and play an important role in defense against herbivores. 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 (Veluri *et al.*, 2006 ; Mink *et al.*, 2007; Doss *et al.*, 2009), PAs also contribute to the astringency and taste of many fruits and other plant products, such as fruit juices, tea and wine. It is well known that the concentration of polyphenolic compounds in grapes depends on the grape cultivar (Mattivi *et al.*, 2006) and other factors, such as ripening time, climate, soil and location of growth (Kennedy *et al.*, 2000). Several methods for the analysis of PAs have been proposed in the literature. Most of them are based on high performance liquid chromatography (HPLC) coupled with a photodiode array (PDA) detector. The aim of this study was to determine the PAs composition of grape seed extracts from

Vitis vinifera L. cv., Gros noir, Muscat noir, Cardinal, Muscat blanc and Victoria, all grown in the same geographical area and vintage. 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 mostly widespread in this region of Algeria. These grapes can be used fresh as well as for juice production. A RP-HPLC-DAD-UV-Vis method was used for the PAs analysis. The similarities and differences between the PAs compositions of grape seed extracts from different cultivars are discussed.

2. Material and methods

2.1.Plant material

Seeds from five grape cultivars, including Gros noir, Muscat noir, Cardinal, Muscat blanc and Victoria were examined. Samples collected at maturity, grown 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 (cultivars Gros noir and Cardinal), 1973 (cultivars Muscat noir and Muscat blanc); The distance of sowing was 3 ×1 m, with two rows support, and the training system was a "double-branched asymmetrical cordone" and 2011 (Victoria) with a distance of sowing of 3 x 3m, with two rows support, and the training system was a Pergola. Approximately 2kg of grape were collected for each cultivar in late summer 2012, from three different sites. All the samples were collected when the Brix values were in range 17 –21°Brix.

2.2. Sample preparation

Seeds from berries were manually separated from pulp, then, dried in oven at 50°C until constant mass, then, they were ground to a powder in a domestic mill, stored a -18°C until analysis.

2.3.Extraction of flavonoïds from grape seeds

Dried seed powder (0.1g) is subjected to extraction by maceration in 50 ml of a mixture of acetone/water (60:40) and 300µl of methyl-4-hydrobenzoate (1g/l), with stirring for 70 min, the extract was centrifuged (10°C/10 min/10 000 rpm). The supernatant was then filtered through glass microfiber filter GF / A 1.6µm. The solvent was removed to about dryness under reduced pressure by use of a rotary evaporator Buchi® R-111 at 30°C and redissolved in methyl alcohol to a volume of 5 ml to get a crude seed polyphenolic extract.

2.4. Isolation of PAs

To separate PAs from crude seed extracts, 2 ml of these extracts were subjected to chromatography over Fractogel Toyopearl® HW-40(F) (300 mm × 10 mm i.d.) (Tosoh Corporation, Japon). Anthocyanins, flavonols, monomeric and dimeric flavanols were eliminated with 30 ml of ethyl alcohol/water/TFA (110:90:0:01) as the eluant at 1ml min^{-1} and PAs fraction was eluted with 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 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.

2.5. Characterization of PAs

Acetonic fraction was subjected to thiolysis reaction, this was performed as described previously (Cadot *et al.*, 2012) in duplicate, after addition of 120µl of toluene-α-thiol to 120µl to the fraction above and heating for 2 min at 90°C, this reaction allow the distinction between terminals units (released as flavan-3-ols) and extension units (released as the corresponding benzylthioether derivatives). Ration between

total unists and terminal units gives access to the mDP.

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

Identification and quantification of PAs was carried out using analytical reversed-phase HPLC according to the conditions adapted from those described by Brossaud et al. (1999), in a Waters Millenium HPLC–DAD system (Milford, MA) system with an auto-sampler and 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, washing and re-equilibration of the column. The column temperature was at 30°C, the flow-rate was set at 1 ml.min^{-1} and detection was monitored at 280nm. Peak identification was performed by comparison of retention times and UV –Vis spectra. Each sample of berries was extracted in duplicate and the acetonic fractions were analysed in duplicate too. Hence, the final result was the arithmetic average of four analyses.

2.6.Statistical analysis

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

3.Results anddiscussions

3.1. General

Condensed tannins or PAs are characterized by the properties to give combinations with 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 varieties, followed by Gros noir, which has a limited cultivation area. Victoria is an Italian variety, recently introduced in Algeria.

Analysis of polyphenols was carried out on the grape seeds, because this is part of berry that contains main class of polyphenols PAs (Guerrero *et al.*, 2009).

3.2. 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 mDP and the percentage of galloylation (%ECG) of PAs using toluene--α-thiol (benzyl mercaptan).

The PAs are listed in Table 1 (20 – 26). The total amount of PAs (TP, see Table 2), ranging from 565.70±113.56 mg/g (Victoria) to 1080.35±87.04 mg/g (Gros noir) which is not statistically different from the Muscat noir.

Table 1. Retention time of different PAs compounds in different table grape varieties

	Compound	Retention time (min)
	Proanthocyanidins	
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	Etalon interne (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

Table 2. Levels of TPAs, mDP, % CGE and % ECG of seed of different grape varieties analyzed

Variety	TP (mg/g of berries)	DPm	% ECG	% EGC
Muscat B	1935.85±60.88^{d}	5.68±0.02^{d}	26,25 ± 0.004^{d}	0.00
Gros Noir	1080.35±87.04^{c}	4.77±0.10^{c}	23,45±0.001^{c}	0.00
Cardinal	871.95±45.04^{b}	4.50±0.01^{b}	21.52±0.003^{a}	0.00
Muscat N	932.75±10.96^{b,c}	4.52±0.07^{b}	21,28±0.012^{a}	0.00
Victoria	565.70±113.56^{a}	4.14±0.14^{a}	22,00±0.001^{a,b}	0.00

TPAs: Total Proanthocyanidins
DPm: Mean of Polymerization Degree
ECG: Epicatechinegallat
EGC: Epigallocatechin
Results 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.

Based on works of Brossaud et al. (1999) on Cabernet franc berries grown on different sites of the vallée de la Loire (France) - 1995 vintage, the content of seed PAs (condensed tannins) oscillate between 3.363 and and 4.448 g / kg fresh weight. On other study, Lorrain et al. (2011) obtained on two French red grape varieties Cabernet sauvignon and Merlot, PAs seed contents ranging from 90.1 ± 4.0 and 92.2 ± 4.5 mg/g of dry weight, 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 (Cadot, 2010), harvest year (Sun *et al.*, 2001), the degree of maturation (Jordão *et al.*, 2001; Ó Marques *et al.*, 2005); these differences also highlight the impact of different soils, cultural practices, but also the harvest in metabolism way of tannins (Lorrain *et al.*, 2011). According to Mateus et al. (2001), low altitudes appear to be favorable for the synthesis of high concentrations of PAs in relation to weather conditions.

Muscat blanc variety showed the highest flavanol content (1935.85±60.88 mg/g of berries). These findings are consistent with previous reports relating to grape varieties grown around the world (Negro *et al.*, 2003; Rockenbach *et al.*, 2011) and confirm that

grape seed extracts are a rich source of PAs, usually oligomers and polymers of polyhydroxy flavan-3-ols such a (+)-catechin and (−)-epicatechin, in the form of gallate esters or glycosides.

Results of Cadot et al. (2006) on Grape seeds from *Vitis vinifera* L. cv Cabernet franc suggest that evolution of the tannins in the seeds is related to evolution of cell wall, but more investigations should be done, in particular, about the composition and structure of the grape seed cell wall and the evolution of the cellular structures, the oxidation of phenolic compounds and their implication in the changes in cells walls, and the impact of cellular death on the extractability of PAs.

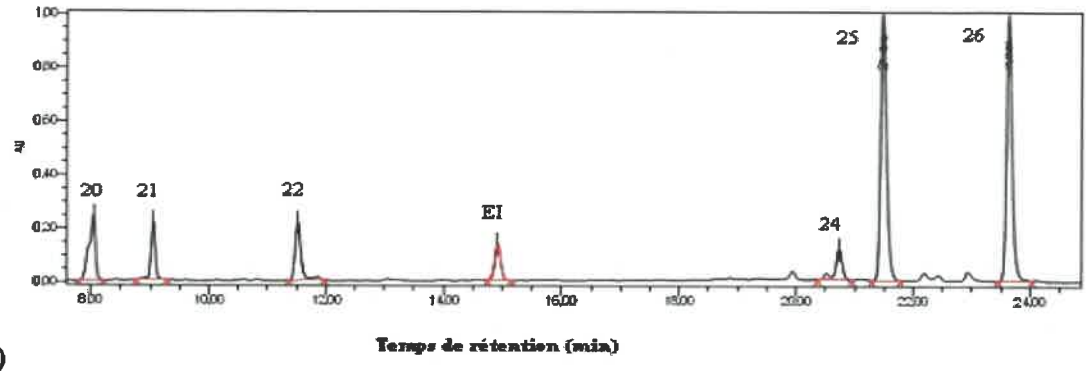
The content of total monomeric and oligomeric flavanols was estimated by thiolysis assay, the monomeric form of PAs represented by the flavan-3-ols epicatechin and catechin monomer were detected in seed extracts, but could not be quantified (peak between thresholds detection and quantification) (Fig. 1 and 2), this is probably due to the fact that catechin is present in a small proportion (less than 10%) (Sun *et al.*, 2001), or to an inter-conversion between catechin and EGC during the maturation of grapes analyzed. According to Liang et al. (2012), the contents of (+) - catechin and epicatechin are significantly lower than those of the other two monomers (ECG and EGC).

mDP and % ECG values recorded vary significantly from one variety to another ($p < 0.05$) from 4.14 ± 0.14 (Victoria) to 5.68 ± 0.02 (Muscat blanc) and 21.28 ± 0.012 (Muscat noir) - which does not differ significantly from Victoria - to 26.25 ± 0.004% (Muscat blanc), respectively. The work of Obreque-Slier et al. (2010) on grape of two varieties (Carmenere and Cabernet Sauvignon) from Chile, show mDP of 2.0 ± 0.2 and 1.8 ± 0.2, while the ECG varies between 20.6 ± 5.5 and 18.7 ± 5.5 (seeds) for the two varieties respectively.

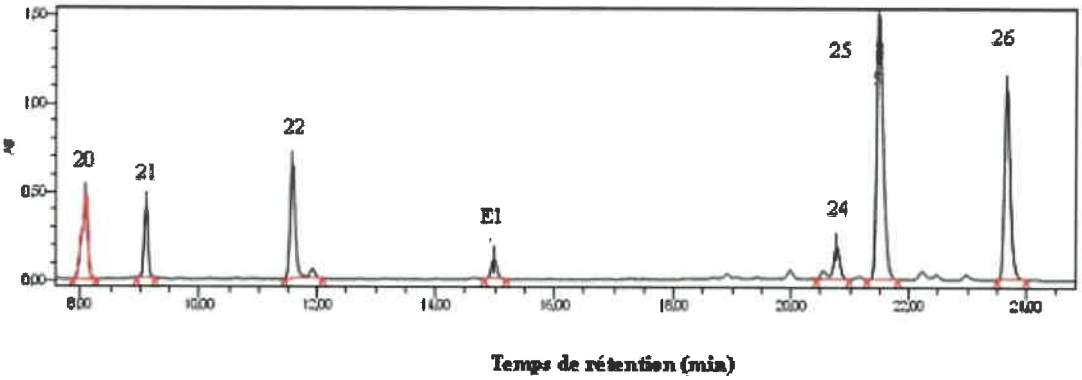
Muscat blanc and Cardinal exhibit a high mDP when comparing with others varieties, which explains their astringency character. According to Cadot et al. (2006), the homogeneous polymerization during the PAs synthesis between fruit set and ripening increases astringency as they increase in size, while the combination with anthocyanins decreases the reactivity, and therefore the astringency of the compounds formed. Regardless of the vintage, the mDP and the percentage of galloylation of seed tannins appear to be appropriate tools to discriminate Muscat blanc and Cardinal variety from others.

The values obtained for the mDP, show that seed tannins are in oligomeric and monomeric forms (mDP varies from 2 to 12-15) (Jordão and Correia, 2012).

A)



B)



C)

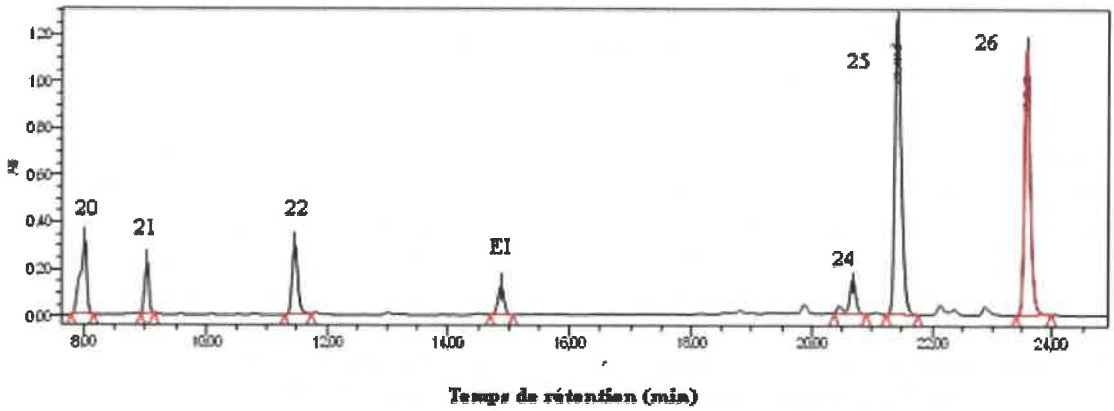


Figure 1. Typical PAs HPLC trace of a seed grape extract monitored at 280nm of Gros noir **(A)**, Cardinal **(B)** and Muscat noir **(C)**

A)

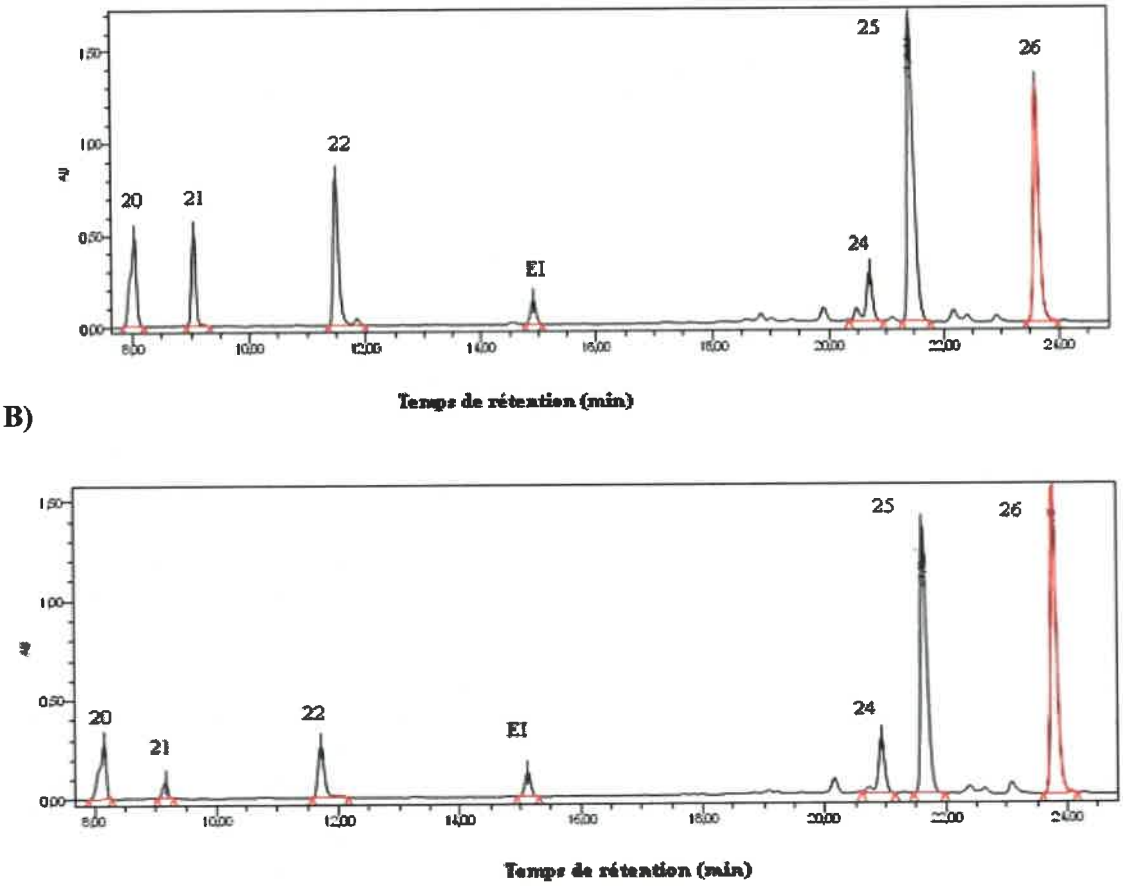


Figure 2. Typical PAs HPLC trace of a seed grape extract monitored at 280nmof Victoria **(A)** and Muscat blanc **(B)**

4. Conclusions

Results obtained show that the PAs composition and its various subunits differ significantly from one variety to another. This could be due to differences in the astringency of the studied varieties. Given the heterogeneity of these observations, the debate remains open about the possible changes affecting the tannins during the maturation phase. Today it is known with the contribution of new biochemical analysis techniques and knowledge on the expression of genes involved in the production of these compounds as active biosynthesis of tannin of the skin and seeds (the pulp also) takes place in the green stage, when the formation of the berry. During ripening, there is no accumulation of tannins, wherease the synthesis of anthocyanins takes place, the biosynthetic pathway is partially common to that of tannins. This study show also, that the mDP and the percentage of galloylation of seed tannins can be used as a significant tools to discriminate Muscat blanc and Cardinal variety from others.

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