LUCRĂRI ŞTIINȚIFICE SERIA HORTICULTURĂ, 60 (1) / 2017, USAMV IAŞI

SEPARATION AND CHARACTERISATION OF THE MAIN PROANTHOCYANIDIN FRACTIONS OF GRAPE SEEDS

SEPARAREA ŞI CARACTERIZAREA PRINCIPALELOR FRACȚIUNI DE PROANTOCIANIDINE DIN SEMINȚELE DE STRUGURI

FILIMON V.R.[1], NECHITA Ancuța[1], DAMIAN Doina[1], PAŞA Rodica[1], FILIMON Roxana[1], BĂETU M.[1], MIHAI C.T.[2], NICULAUA M.[3]

e-mail: razvan_f80@yahoo.com

Abstract. *Grape seeds, as waste products of the winemaking industry, contain large amounts of monomers, oligomers and more highly polymerised proanthocyanidins (PA), being a good source of phytochemicals for the production of antioxidative dietary supplements. PA from defatted grape seeds were extracted by precipitation with diethyl ether from the crude alcoholic extract and fractionated into monomers (FI), oligomers (FII) and polymers (FIII) of flavan-3-ols by their separation on C18 Sep-Pak cartridges. FIII was the predominant class of proanthocyanidins (82.22%), while monomeric PA has only 5.71% of total. The ratio PA (by vanillin assay) / tannins (Bate-Smith assay) indicates the highest degree of polymerisation (DP) in FIII fraction (1.28). Thin layer chromatography (TLC) confirmed the presence of monomers in FI, the DP increasing significantly for the next two fractions. Oligomeric and polymeric PA showed the highest antioxidant activity (% scavenged DPPH), but the synergic antioxidant effect of PA classes was also observed.*

Key words: antioxidant activity, C18 cartridges, degree of polymerisation, grape seeds, tannins

Rezumat. *Semințele de struguri, ca deşeuri ale industriei de vinificație, conțin cantități mari de proantocianidine (PA) monomerice, oligomerice şi cu grad mare de polimerizare, fiind o sursă importantă de compuşi pentru producerea de suplimente alimentare cu rol antioxidant. PA din semințele de struguri delipidate, au fost extrase prin precipitare cu eter dietilic din extractul alcoolic crud şi fracționate în monomeri (FI), oligomeri (FII) şi polimeri (FIII) de unități flavan-3-ol prin separarea pe cartuşe de tip C18 Sep-Pak. FIII a constituit clasa predominantă de PA (82.22%), în timp ce formele monomerice au reprezentat doar 5.71% din totalul PA. Cromatografia în strat subțire (TLC) a confirmat prezența monomerilor în FI, gradul de polimerizare (GP) crescând semnificativ pentru fracțiunile FII şi FIII. Raportul catechine / taninuri a indicat cel mai ridicat GP pentru fracțiunea FIII (1.28). Formele oligomerice şi polimerice au prezentat cea importantă activitate antioxidantă, fiind remarcat efectul sinergic al claselor de PA.*

Cuvinte cheie: activitate antioxidantă, cartuşe C18, grad de polimerizare, semințe de struguri, taninuri

[1]Research - Development Station for Viticulture and Winemaking Iasi, Romania
[2]Biological Research Institute Iasi, Romania
[3]Research Center for Oenology of Romanian Academy - Iasi Branch, Romania

INTRODUCTION

Discovered by Masquelier J. in 1947, in pine bark and grape seeds, proanthocyanidins (PA) or condensed tannins are phenolic compounds consisting of chains of flavan-3-ol units of varying degree of polymerization (DP). PA can occur as polymers of up to 50 units without being susceptible to be cleaved by hydrolysis (Sun *et al*., 1998). In nature, PA serve in the defensive mechanisms of plants against pathogens and predators (Amil-Ruiz *et al*., 2011).

As waste products of the wine industry, grape seeds are concentrated sources of monomeric phenolic copounds such as (+)-catechin (0.16 mg/mL), (-)-epicatechin (0.66 mg/mL), and dimeric (0.329 mg/g dry weight (d.w.)), trimeric (0.384 mg/g d.w.) and polymeric (0.905 mg/g d.w.) PA (Khanal *et al*., 2009). In grape seeds, PA represents the major phenolic fraction being characterised by a lower degree of polymerization than those of berry skin (Iriti and Faoro, 2010).

Tannins are potent antioxidants (Reed, 1995). Specific studies have shown that the antioxidant power of PA is 20 times higher than vitamin E and 50 times higher than vitamin C (Shi *et al*., 2003). Extensive research suggested that PA help to protect the body from sun damage, improve flexibility in joints and arteries, improve blood circulation and have anti-carcinogenic activity (Nandakumar *et al*., 2008). However, all of these properties largely depend on their chemical structure and degree of polymerization (Sun *et al*, 1998).

Different qualitative (thin layer chromatography) and quantitative (normal phase HPLC, chromatographic columns and resins) techniques were proposed to separate PA according to their DP, but having as main shortcomings that they are very difficult to use for routine analysis. Taking into account the different physico-chemical and health promoting properties of PA classes, their separation and specific use is of great interest in food, pharmaceutical and cosmetic fields.

In present work, was used an improved simplified method for the separation and quantification of PA from grape seeds, based on their DP, using preconditioned neutral C18-max Sep-Pak cartridges, silica-based bonded phase, with strong hydrophobicity. C18 cartridges are characterised by lower cost, higher accuracy and faster protocol, similar to reversed-phase HPLC columns.

MATERIAL AND METHOD

Grape seeds of autochthonous wine cultivar Fetească neagră were separated from skins, dried and grinded. Grinded seeds were defatted with hexane 99% (24 h), phenolic compounds being extracted with 96% ethanol (72 h) and concentrated by vacuum evaporation (Heidolph rotavapor) at 35 °C. PA were subsequently extracted by precipitation with diethyl ether 99.5%, and dried at 30 °C. Dried brute precipitate of PA was then fractionated in compliance with their DP into monomers (FI), oligomers (FII) and polymers (FIII) of flavan-3-ols by separation on C18-max Sep-Pak cartridges, 500 mg sorbent / 6 mL volume (Waters Corporation, USA). Aqueous solution of PA (100 mg) was passed through the C18 cartridge, previously activated with ethanol 96% and distilled water. Elution was carried out with 10 mL of distilled water to eliminate phenolic acids and eventually sugars or proteins. After the cartridge was dried, elutions were carried out first with 25 mL of ethyl acetate to elute catechins and oligomeric PA, resulting the fraction (F) I + II, and then with 20 mL of ethanol to elute the polymeric PA (FIII). For the separation of monomers from oligomeric PA, FI+II was evaporated to dryness, dissolved in distilled water, and then redeposited onto the same preconditioned cartridge. Separation was realised by sequential elution with 20 mL of diethyl ether (FI) and then with 20 mL of ethanol (FII). Each PA fraction was evaporated in glass capsules and analysed gravimetrically (Shimadzu ATX 220).

PA purity was calculated as the sum of the three fractions. For each fraction was determined the total catechin content by vanillin-HCl assay according to Caceres-Mella A. *et al.* (2013) and tannin concentration (Procyanidolic Index) using the methodology proposed by Bate-Smith E. (1981).

Commercial TLC silica gel 60 F254, 20×20 cm aluminium sheets (Merck, Germany) were used to control the DP of PA in each fraction obtained from C18 Sep-Pak cartridges. The chromatography was carried out using an ascending elution with toluene/acetone/acetic acid (3:3:1, v/v/v), according to the method proposed by Sun B. *et al.* (1998). 10% (w/v) vanillin in concentrated HCl was used for peak detection.

Antioxidant activity of PA fractions (1 mg/mL) was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Stock solution: 4.0 mg DPPH in 100 mL 96% ethanol. Sample (25 μL) / DPPH (975 μL) solution was mixed, measured at λ 517 nm (T_0), incubated at 37°C for 30 min and measured at λ 517 nm (T_{30}), using a UV-mini spectrophotometer (Shimadzu, Japan) and 10 mm path length Helma® glass cells. Inhibition (%) = [(Absorbance T_0 − Absorbance T_{30})/Absorbance T_0] × 100.

RESULTS AND DISCUSSIONS

After PA precipitation from the crude alcoholic extracts, grape seeds PA (100 mg) were separated into fractions on C18 Sep-Pak cartridges. After gravimetric determinations, the monomeric PA (FI) represents only 5.20 mg/100 mg d.w., oligomeric PA (FII) 11.00 mg/100 mg d.w. and polymeric PA (FIII) 74.90 mg/100 mg d.w. (tab. 1). Total amount of 91.15 mg, resulting from the cumulative of fractions, indicates the degree of purity of the PA brut precipitate.

Table 1

Total proanthocyanidin content of individual fractions by gravimetry

Fraction (F)	Type of PA	PA (mg/100 mg)	% of total PA
I	monomers	5.20	5.71
II	oligomers	11.05	12.07
III	polymers	74.90	82.22
Total	-	91.15	100.00

These results are slightly higher than those presented by US Food and Drug Administration for grape seed extracts, respectively up to 78.00% for polymeric flavan-3-ols and 5.50% for monomeric flavan-3-ols (FDA, 2003), but more closely to those reported by Monagas *et al.* (2003), which showed that the polymeric fraction in grape seeds represents 75 to 81% of total flavan-3-ols content.

Lower values for vanillin assay are explained through the fact that vanillin reacts only with free flavan-3-ol fraction or with the terminal units of PA (Price *et. al*, 1978). The total content of tannins (Procyanidolic Index or Bate-Smith assay) varied between 8.51 and 33.17 g CE/L (tab. 2). Close values for FIII and brut precipitate (Bp), which include all the three fractions, confirmed the high percentage of polymeric PA forms in the initially obtained precipitate.

Table 2

Total PA (as catechins) of individual fractions

Fraction (F)	OD 500 nm (Vanillin assay)	OD 550 nm (Bate-Smith assay)	PA (catechins) (Vanillin assay) (g CE/L)	PA (tannins) (Bate-Smith assay) (g CE/L)	Cat/Tan (OD)
I	1.068	0.440	6.91	8.51	2.43
II	1.810	0.844	11.69	16.31	2.14
III	2.191	1.716	14.14	33.17	1.28
Bp	2.121	1.792	13.69	34.64	1.18

Note: Bp – initial brute precipitate (includes all three PA fractions); OD – optical density; CE – catechin echivalent; Cat/Tan – catechins/tannins ratio.

The ratio between the optical density of vanillin-coloured combinations and anthocyanidins formed by heating in acidic medium was used as the indicator of tannin DP. This ratio should be lower as the DP is higher (Goldstein and Swain, 1963). Thus, Cat/Tan ratio was the lowest in FIII and brute precipitate (Bp), confirming the presence of PA with higher DP in these samples (see tab. 2).

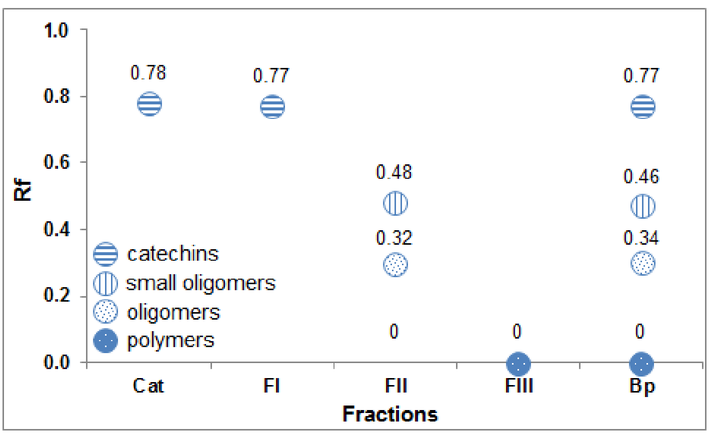

Fig. 1 TLC of grape seed PA fractions isolated on C18 Sep-Pak cartridges
Note: Cat - catechins; FI, FII, FIII - PA fractions; Bp - brut precipitate.

PA composition of each fraction was verified by TLC, using pure catechin solutions as standard. Individual compounds appeared as spots separated vertically (fig. 1). For each spot was calculated the retention factor (Rf), as ratio between the distance migrated over the total distance covered by the solvent (18 cm). The Rf values were compared with catechin standard and data reported by Sun *et al*. (1998). The migration of compounds on plates was according to their DP, confirming the presence of catechin units (monomers) in FI (similar migration to pure catechins) and of low molecular weight PA (oligomers) in FII.

FIII did not contain monomers or oligomers of PA, being composed by highly polymerised PA (no migration). TLC technique give only a qualitative answer on PA separation, further investigations (like HPLC technique) being necessary.

Grape seed extracts are known as powerful antioxidants that protect the body from premature aging, disease and decay (Shi *et al*., 2003).

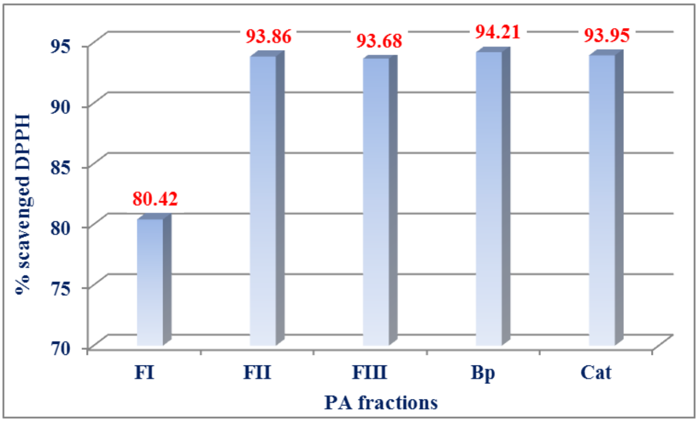

Fig. 2 Antioxidant activity of grape seed PA fractions isolated on C18 cartridges
Note: Bp - brut precipitate; Cat - catechin

The antioxidant activity of PA fractions (1 mg/mL) was high, FII being the most reactive, followed very closely by the polymeric fraction (FIII) (fig. 2). The highest antioxidant activity (94.21%) was that of the crude PA precipitate (Bp), highlighting the synergistic effect of PA classes. The results are consistent with those presented by Yilmaz and Toledo (2004), oligomeric and polymeric PA accounting for most of the superior antioxidant capacity of grape seeds.

CONCLUSIONS

1. Defatted grape seeds of wine cultivar Fetească neagră contained large amounts of proanthocyanidins, mainly in the polymeric form (>82%).

2. Oligomeric and polymeric PA showed the highest antioxidant activity, but the synergic antioxidant effect of PA classes was observed.

3. The use of C18 type cartridges to separate the PA fractions based on their DP is an efficient, accurate, fast and relatively inexpensive method for tannins preliminary analysis.

4. Considering the new possibilities of transforming these non-hydrolysable tannins into water-soluble compounds by controlled oxidation, grape seeds became a good source of phytochemicals, providing excellent protection against oxidative stress.

Acknowledgements: *This paper was published under the frame of MEN-UEFISCDI, PN-II-PT-PCCA-2013-4-0333, contract no. 183/2014.*

REFERENCES

1. **Amil-Ruiz F., Blanco-Portales R., Munoz-Blanco J., Caballero J.L., 2011** - The strawberry plant defense mechanism: a molecular review. Plant Cell Physiol., 52 (11), p. 1873–903.
2. **Bate-Smith E., 1981** - *Astringent tannins of the leaves of Geranium species*. Phytochemistry, 20, p. 211–216.
3. **Caceres-Mella A., Pena-Neira A., Narvaez-Bastias J., Jara-Campos C., Lopez-Solis R., Canals J.M., 2013** - *Comparison of analytical methods for measuring proanthocyanidins in wines and their relationship with perceived astringency*. Int. J. Food Sci. Tech., 48, p. 2588–2594.
4. **Da Silva R.J.M., Rigaud J., Cheynier V., Cheminat A., Moutounet M., 1991** - *Procyanidin dimers and trimers from grape seeds*. Phytochemistry, 4, p. 1259-1264.
5. **FDA, 2003** - *Grape seed extract (GSE) notification*. US Food and Drug Administration, Silver Spring, Maryland, USA. Available online at: https://www.fda.gov/downloads /Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm267216.pdf.
6. **Goldstein J.L., Swain T., 1963** - *Methods for determining the degree of polymerization of flavans.* Nature, 198, p. 587-588.
7. **Iriti M., Faoro F., 2010** - *Bioactive chemicals and health benefits of grapevine products.* In Bioactive foods in promoting health: Fruits and Vegetables (Watson R.R. and Preedy V.R. eds.). Chapter 38, p. 581–620. Elsevier, Academic Press, London, UK.
8. **Khanal R. C., Howard L. R., Prior R. L., 2009** - *Procyanidin content of grape seed and pomace, and total anthocyanin content of grape pomace as affected by extrusion processing*. J. Food Sci., 74(6), p. H174-H182.
9. **Monagas M., Gómez-Cordovés C., Bartolomé B., Laureano O., Da Silva R.J.M., 2003** - *Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from Vitis vinifera L. Cv. Graciano, Tempranillo, and Cabernet Sauvignon.* J. Agric. Food Chem., 51, p. 6475-6481.
10. **Nandakumar V., Singh T., Katiyar S.K., 2008** - *Multi-targeted prevention and therapy of cancer by proanthocyanidins.* Cancer Lett., 269(2), p. 378–387.
11. **Price M.L., Van Scoyoc S. , Butler L. G., 1978** - *A critical evaluation of the vanillin reaction as an assay for tannin in Sorghum grain*. J. Agric. Food Chem., 26 (5), p. 1214–1218.
12. **Reed J.D., 1995** - *Nutritional toxicology of tannins and related polyphenols in forage legumes.* J. Animal Sci., 73, p. 1516–1528.
13. **Saito M., Hosoyama H., Ariga T., Kataoka S., Yamaji N., 1998** - *Antiulcer activity of grape seed extract and procyanidins*. J. Agric. Food Chem., 46 (4), p. 1460 –1464.
14. **Shi J., Yu J., Pohorly J.E., Kakuda Y., 2003** - *Polyphenolics in grape seeds-biochemistry and functionality*. J. Med Food. Winter, 6(4), p. 291-299.
15. **Sun B., Conceição L., Da Silva R.J.M., Spranger I., 1998** - *Separation of grape and wine proanthocyanidins according to their degree of polymerization*. J. Agric. Food Chem., 46, p. 1390-1396.
16. **Yilmaz Y., Toledo R.T., 2004** - *Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic acid*. J. Agric. Food Chem., 52 (2), p. 255-260.