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ANTHOCYANIN CONTENT AND COMPOSITION OF FRESH AND DRY POMACE FROM *VITIS VINIFERA* L. WINE CULTIVARS

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INTRODUCTION

Grapes are one of the main crops in the world, with an annual production of 60 million tons. About 80% of total production is used for winemaking, leaving about 15-20% of their weight as pomace (Oreopoulou and Russ, 2007). Phenolic substances of grapes, including anthocyanins, are concentrated in the skin and seeds, more precisely, that part which remains as pomace after grape processing. Annually, worldwide are produced 5 to 9 million tons of grape pomace (Louli et al., 2004). In Europe, only about 10,000 tons of grape skins are processed annually, resulting about 50 tonnes of anthocyanin dye, these numbers being in an upward trend (Davies, 2004).

Anthocyanins (gr. *anthos* - flower and *kyanos* - blue) are natural pigments, belonging to flavonoid class, responsible for red-blue-violet colour of grapes, being the most important group of water-soluble plant pigments visible to the human eye (Mazza and Miniati, 1993; Jing and Giusti, 2014). Localized usually in dark grape skins, anthocyanins are extracted only partially (30-40%) through winemaking processes, so, pomace resulting from the production of red wines contains significant amounts of these phenolic compounds (Câmpeanu *et al.*, 1989). In the case of its drying, the percentage of anthocyanin dye extracted is lower, influenced by the physico-chemical processes of organic compounds degradation (Rein, 2005).

According to Davies (2004), commercial anthocyanin extracts are predominantly prepared from *Vitis* species berry skin or pomace from the wine industry, which is cheaply and available in large amounts. In grapes, around 20 different anthocyanins have been reported, but the common commercial recipes contain principally only relatively simple 3-glucosides of cyanidin, delphinidin and malvidin. These compounds have limited colour stability with regard to pH and are therefore limited in their food applications (Bąkowska-Barczak, 2005).

Grape anthocyanins represents approximately 38% of total phenolic compounds (Ţârdea, 2007), their total content varying between 30-1250 mg · 100 g fresh skins, depending on the grape cultivar, the area of growing and technology applied (Davies, 2004; Kallithraka et al., 2009). Anthocyanin composition of species is determined primarily by the genetic factor, the relative content of each anthocyanin being constant for a specific variety (Mazza and Miniati, 1993). More precisely, the percentage with which an anthocyanin is participating at the profile showed small variations from one year to another (Pomar et al., 2005). Regardless of these factors, malvidin-3-glucoside remained the main anthocyanin identified in *Vitis vinifera* L. Glycosidic moieties of grape anthocyanins are usually represented by glucose, with the formation of 3-O-monoglycoside anthocyanins at C_3 position (Ford et al., 1998).

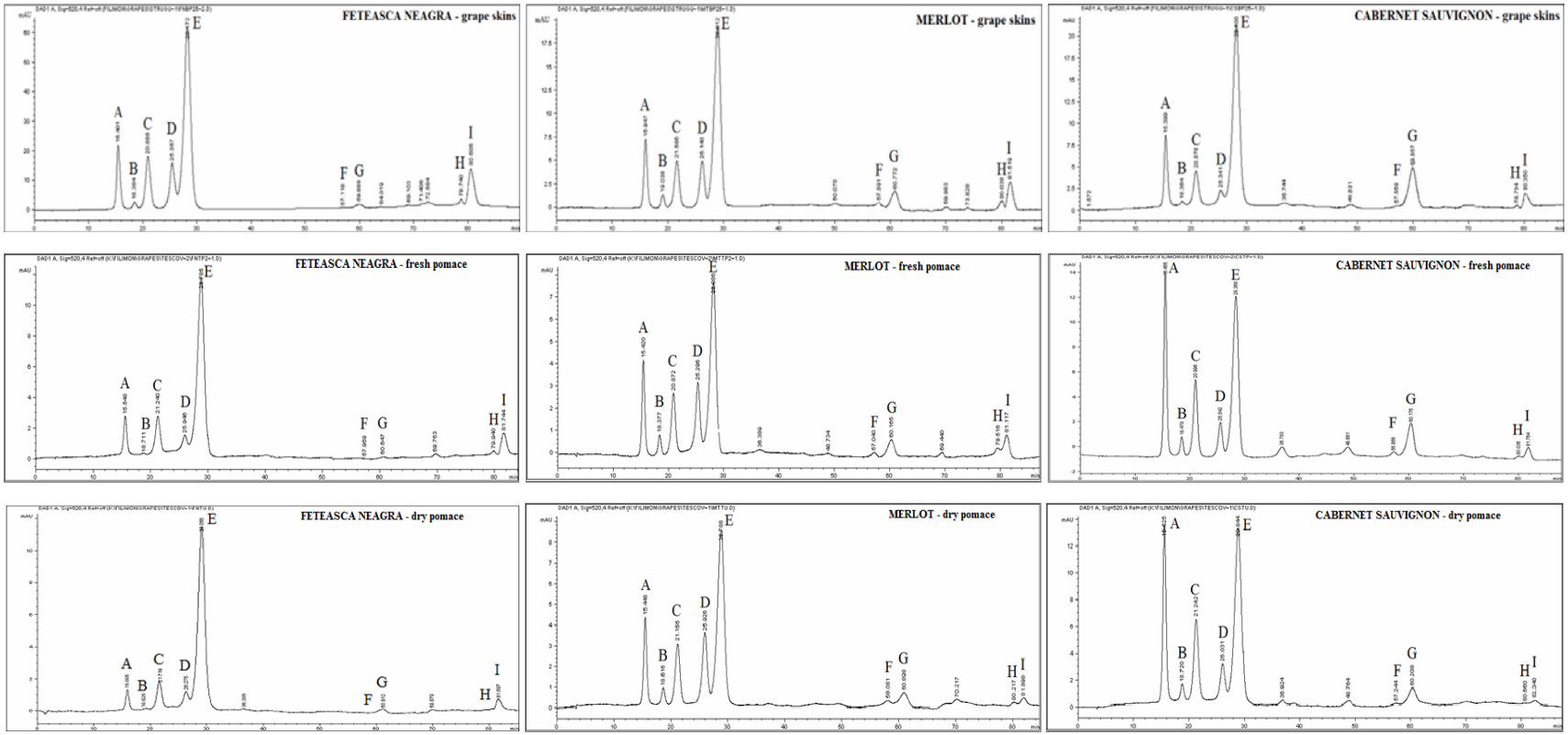
According to Tamborra et al. (2003), the exceptional colouring properties of grape anthocyanins are largely due to the existence of acylated anthocyanins, which can reach up to 50% of total anthocyanins. Anthocyanin acylation improves the colour and stability of the pigment even at a higher pH of solution (Bąkowska-Barczak, 2005). Acylated pigments are more stable at pH variation than unacylated ones (Horbowicz, 2008).

Currently anthocyanins are one of the most reliable alternatives to replace synthetic dyes limited to consumption (Kähkönen et al., 2003; Fanali et al., 2011). Knowing the type and quantities of pigments available in grapes and their pomace, the main representatives and the relationship between them, is possible to appreciate their functional and technological value and also the structure and stability of the colour obtained.

MATERIAL AND METHODS

For conducting the determinations were selected three *Vitis vinifera* L. cultivars (cv.) of black grapes for red wines, cultivated on large areas in Romania: Cabernet Sauvignon (CS), Merlot (Mt) and old Romanian cv. Feteasca neagră (FN), all growing in the Ampelographic collection of the Faculty of Horticulture, University of Agricultural Sciences and Veterinary Medicine Iaşi, Romania.

Wine technology applied was classic, with grape crushing and declustering, followed by pressing (winepress), the remaining pomace being recovered. A part of the grape pomace was dried in cool and dark place at room temperature (20±2 °C).



A: Dp-3-gl; B: Cy-3-gl; C: Pt-3-gl; D: Po-3-gl; E: Mv-3-gl; F: Po-3-ac-gl; G: Mv-3-ac-gl; H: Po-3-cm-gl; I: Mv-3-cm-gl.

Fig. 1. HPLC–DAD chromatograms of purified extracts of *V. vinifera* L. fresh grape skins and fresh and dried pomace (λ 520 nm)

After three weeks, the samples were sufficiently dry for storage (average moisture content 7.5%). The drying yield of the fresh pomace was about 20%, from 1 kg of fresh grape pomace yielding approximately 200 g of dry pomace, depending on the variety. Dry pomace was grinded (<0.2 mm).

Fresh (crushed in a mortar) and dried pomace (grinded) was extracted in the dark by stirring with 0.1% HCl (v/v) in methanol overnight at room temperature (20±2 °C). Were used 10 g of fresh grape skins in 150 mL 0.1% HCl (v/v) in methanol, 10 g of fresh grape pomace in 150 mL 0.1% HCl (v/v) in methanol and 10 g of dried grape pomace in 200 mL 0.1% HCl (v/v) in methanol. The samples were filtered on a Buchner funnel (Whatman no. 1) and the solid residue washed with the same amount initially used of 0.1% HCl (v/v) in methanol. Filtrates were combined and dried in vacuum using a Büchi R-200 rotary evaporator at 32 °C.

The remaining solid was dissolved in 0.01% HCl (v/v) deionized water and successively purified through a 0.5 g sorbent weight Grace Pure® C-18 SPE 500 mg (6 mL), as reported by Wrolstad (2001). Anthocyanins and other polyphenolics were adsorbed onto the solid-phase, while sugars, acids and other water-soluble compounds were removed. Less polar polyphenolics were subsequently eluted with ethyl acetate. Anthocyanins were eluted with methanol containing 0.01% HCl (v/v) and evaporated in vacuum at 32 °C. The remaining solid was dissolved in 0.01% HCl (v/v) aqueous solution in order to have a known concentration solution (1mg/mL). The solutions were stored at −20 °C until used.

High-performance liquid chromatography (HPLC) - diode array detection (DAD) - electrospray ionization (ESI) - mass spectrometry (MS) were performed using an Agilent 1100 Series system. Chromatographic separation was carried out on a 250×4.6 mm, 5 µm SS Wakosil C18 RP column, thermostatted at 32 °C. The mobile phase: 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) at a flow rate of 1 mL/min. The following gradients were used: 0 min - 10% B; 10 min - 15% B; 35 min - 18% B; 50 min - 20% B; 75 min - 25% B. Absorbance spectra were recorded every 2 s, between 250 and 600 nm, with a bandwidth of 4 nm and chromatograms were acquired at 520, 440, 310 and 280 nm. ESI-MS parameters were: capillary voltage: 4000 V, drying gas temperature: 350 °C, gas flow (N_2):10 L/min, nebulizer pressure: 50 psi. The instrument was operated in positive ion mode, scanning from m/z 50 to 2000 at a scan rate of 2 sec/cycle.

Individual and total quantities of anthocyanins were expressed as cyanidin-3-glucoside equivalent (CE) per 100 g of plant material. The calibration curve was produced by the integration of absorption peaks generated from the analysis of a dilution series of cyanidin-3-glucoside (≥96% Kuromanin chloride, Extrasynthese, France).

RESULTS AND DISCUSSIONS

Based on chromatograms recorded at λ 520 nm and the mass spectra specific to each individualised compound, in grapes and pomace were identified nine anthocyanins. The order of anthocyanin elution from the chromatographic column was as follows: 3-glucoside (3-gl) forms of delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Po) and malvidin (Mv), followed by the 3-acetyl-glucoside (3-ac-gl) and 3-coumaroyl-glucoside (3-cm-gl) forms of Po and Mv, glucose being the only sugar moiety identified in anthocyanin composition of *V. vinifera* L. grape extracts. Using ESI-MS system were identified two other acylated anthocyanins: Dp-3-ac-gl and Mv-3-caffeoyl-gl, as traces, being often below the limit of identification and quantification of the analytical system used. According to Ribereau-Gayon et al. (2006) only monoglucoside anthocyanins and acylated monoglucoside anthocyanins have been reported in *Vitis vinifera* L. grapes, acylation being made with p-coumaric, caffeic and acetic acids.

$Abs_{440}/Abs_{\lambda max}$ ratio offers information about anthocyanin glycosylation (Longo et al., 2007). Values calculated for each anthocyanin ranged from 22 to 38%, indicated the substitution in the C_3 position of the flavylium ring. In addition, $Abs_{310}/Abs_{\lambda max}$ ratio confirmed that *V. vinifera* L. berry anthocyanins were acylated with aromatic acids in the case of last four compounds identified: 78-81% for acetylated anthocyanins and 61-65% for coumaroylated ones.

Chromatograms of fresh and dried pomace extracts presented the same structure of anthocyanin profile as grapes, with no total degradation of any compound (Fig. 1).

Mv-3-gl had the largest participation rate at the total area corresponding to anthocyanins (>50% in fresh grape skin extracts). At the opposite, Cy-3-gl, the most widespread anthocyanin in nature, occupied the lowest proportion of total chromatographic area (1-3% for fresh grape extracts).

Evolution of the percentage of area occupied by each anthocyanin throughout grapes processing and subsequently with pomace drying, was specific for each cultivar, being observed a general decreasing trend, mainly for esterified anthocyanins (acetylated and coumaroylated).

In Table 1 are presented the chromatographic and spectral characteristics of the identified anthocyanins (in grape skin, fresh and dry pomace) and the percentage of area from total anthocyanin profile of each individual compound.

Individual quantities of the main anthocyanins identified are shown in Table 2. In CS grape skins, Mv-3-gl showed the most important concentration, with over 870 mg CE · 100 g^{-1} fresh weight (f.w.), with a total anthocyanin amount of 1569.88 mg CE · 100 g^{-1} f.w.

Table 1. Chromatographic and spectrometric characteristics of anthocyanins identified in *Vitis vinifera* L. grapes and the corresponding area percentages

Compound	Retention time (~min)	[M] ±*m/z*	Fragments, *m/z*	λ_{max} UV/vis (nm)	Area, 520 nm (%)									Identification
					Feteasca neagra			Cabernet Sauvignon			Merlot			
					FS	FP	DP	FS	FP	DP	FS	FP	DP	
1	15.94	465	303	278/524	10.06	8.13	4.03	11.38	22.73	22.54	10.47	13.44	11.88	Delphinidin-3-O-glucoside
2	19.03	449	287	280/518	1.47	0.68	0.47	1.03	2.81	2.17	1.96	2.70	2.40	Cyanidin-3-O-glucoside
3	21.58	479	317	280/528	11.88	11.18	7.70	8.92	13.88	15.42	10.27	11.89	12.12	Petunidin-3-O-glucoside
4	26.14	463	301	280/518	10.88	2.93	4.32	3.95	6.25	7.20	9.71	14.06	15.37	Peonidin-3-O-glucoside
5	28.91	493	331	278/526	**53.22**	**67.78**	**74.58**	**55.44**	**39.95**	**46.87**	**51.89**	**46.69**	**51.35**	Malvidin-3-O-glucoside
6	57.89	505	301, 463	284/518	0.27	0.38	1.18	0.92	1.07	0.43	1.03	0.80	1.04	Peonidin-3-O-acetyl-glucoside
7	60.72	535	331, 493	278/528	1.13	0.63	2.09	11.93	9.98	4.29	5.16	5.05	3.84	Malvidin-3-O-acetyl-glucoside
8	80.04	609	301, 463	282/522	0.73	0.69	0.66	1.17	0.22	0.35	1.98	1.15	0.63	Peonidin-3-O- coumaroyl-glucoside
9	81.52	639	331, 493	282/528	10.36	7.60	4.96	5.26	3.11	0.71	7.53	4.22	1.37	Malvidin-3-O- coumaroyl-glucoside

Note: FS - fresh skins; FP - fresh pomace; DP - dry pomace.

Table 2. Individual quantities of the main anthocyanins identified in *Vitis vinifera* L. grape and pomace extracts (mg CE·100 g^{-1})

Variety / Anthocyanin	delphinidin -3-gl	cyanidin -3-gl	petunidin -3-gl	peonidin -3-gl	malvidin -3-gl	peonidin -3-ac-gl	malvidin -3-ac-gl	peonidin -3-cm-gl	malvidin -3-cm-gl	Σ ant. ac. + Σ ant. cm.	Σ ant. ac. /Σ ant. cm.	Σ ant. gl. /Σ ant.-COOR	Total anthocyanins
Fresh grape skins													
Feteasca neagra	83.62	12.22	98.75	90.45	**442.45**	2.23	9.43	6.11	86.16	103.93	0.13	7.00	**831.43**
Merlot	101.32	19.00	99.35	93.92	**502.19**	10.00	49.93	19.18	72.90	152.01	0.65	5.37	**967.78**
Cabernet Sauvignon	178.64	16.12	140.05	62.08	**870.30**	14.41	187.35	18.30	82.65	302.71	2.00	4.19	**1569.88**
Fresh pomace													
Feteasca neagra	28.05	2.33	38.60	10.10	**233.92**	1.31	2.18	2.39	26.22	32.10	0.12	9.75	**345.10**
Merlot	58.55	11.75	51.79	61.27	**203.43**	3.47	22.02	4.99	18.41	48.89	1.09	7.91	**435.66**
Cabernet Sauvignon	157.69	15.99	99.80	43.33	**277.10**	7.41	69.23	1.55	21.56	99.75	3.32	5.95	**693.66**
Dry pomace													
Feteasca neagra	19.74	2.30	37.69	21.15	**364.91**	5.76	10.22	3.23	24.27	43.48	0.58	10.25	**489.28**
Merlot	93.65	18.92	95.57	121.15	**404.84**	8.19	30.30	5.00	10.78	54.27	2.44	13.53	**788.40**
Cabernet Sauvignon	349.84	33.75	239.32	111.74	**727.37**	6.70	66.53	5.47	11.08	89.78	4.42	16.28	**1551.80**

Note: 3-gl: 3-glucoside; 3-ac-gl: 3-acetyl-glucoside; 3-cm-gl: 3-coumaroyl-glucoside; Σ ant. ac.: total amount of acetylated anthocyanins; Σ ant. cm.: total amount of coumaroylated anthocyanins; Σ ant. gl.: total amount of glycosylated anthocyanins; Σ ant.-COOR: total amount of esterified anthocyanins.

The lowest total anthocyanin content was found in fresh grape skins of autochthonous cv. FN, 831.43 mg CE · 100 g^{-1} f.w. Dp-3-gl varied in the range of 83.62 to 178.64 mg CE · 100 g^{-1} f.w., being along with Pt-3-gl and Po-3-gl the main secondary anthocyanins of grape extracts. Among acylated anthocyanins, Mv-3-ac-gl was best represented. In fresh grape skin extracts, the sum (Σ) of acetyl and coumaroyl anthocyanins varied between 103.93 and 302.71 mg CE · 100 g^{-1} f.w., while the ratio between Σ of glucoside anthocyanins / Σ of esterified anthocyanins was higher at FN cv. (7.00), due to lower concentrations of acylated anthocyanins (103.93 mg CE · 100 g-1 f.w.).

After pressing the grapes, the percentage of total anthocyanins remaining in the pomace (comparing to grapes raw material) was: 41.51% at FN, 45.02% at Mt and 44.02% at CS. These variations can be attributed to the winemaking technologies, more precisely, to the inability of obtaining a constant yield of pressing in the experimental research system. Also, it was observed a selective extraction of anthocyanins with grape processing. Thus, if in the fresh pomace of FN, Mv-3-gl was found in a percentage of 52.87% in comparison with grapes, at Mt and CS the percentage of Mv-3-gl was much lower (40.51%, and 31.84% respectively). For all secondary anthocyanins the extraction trend was inversed in comparison with the major anthocyanin, meaning that fresh pomace of CS retained the most important quantities of each representative, followed by Mt and FN. Although at the other cultivars Cy-3-gl decreased after grape processing, at CS cv. the concentration of Cy-3-gl found in the fresh pomace was very similar to grapes (16 mg CE · 100 g^{-1} f.w.). This is probably due to their higher stability in concentrated solutions through self-association and copigmentation reactions (Whiting, 1995; Rein, 2005), or due to the presence in higher concentration of acylated anthocyanins in CS grapes (almost double than in Mt grapes), monoglucoside forms being better protected.

Drying the pomace resulted in a concentration of anthocyanins up to 2.6 times for Mv-3-gl at CS (727.37 mg CE·100 g^{-1} dry pomace). Although these amounts may be considered important if it is considered the drying yield of fresh samples (1 kg of fresh pomace yielded 200 g of dry pomace) by mathematical calculation can be observed that the values will be reduced by up to 5 times when results will be reported to the initial fresh plant material.

On the background of lower initial concentrations in grape extracts, was observed a decrease in the quantities corresponding to 3-gl forms of Dp, Cy and Pt, with pomace drying, suggesting a low stability of these compounds under the influence of physico-chemical degradation processes.

Among acylated anthocyanins, acetyl-glucoside and coumaroyl-glucoside forms of Mv were most strongly represented, reaching up in the case of acetylation to 187.35 mg CE · 100 g^{-1} f.w. at CS grapes (Table 2). In dried pomace extracts, acylated anthocyanins were present in low concentrations, being subsequently degraded during grape processing and pomace drying at all analysed cultivars. Acylated anthocyanins are considered protective compounds that provide stability to the extracts in which they are present. In addition to its own resistance to the degrading factors, acylated anthocyanins are compounds "sacrificing" themselves, suffering the most important degradation and transformations during extracts storage, thus protecting glucoside anthocyanins, more susceptible without acylation.

CONCLUSIONS

Anthocyanins profile of *Vitis vinifera* L. analysed cultivars determined by means of LC/MS technique, was unique and included five monoglucoside and four acylated forms of anthocyanins, with malvidin-3-glucoside as the major representative. Other acylated forms were also present in the profile as traces. After grape processing, fresh pomace retained a significant percentage of anthocyanins, the degree of solubility in the grape must, being a peculiarity of each anthocyanin. Drying the pomace resulted in a selective degradation of anthocyanins. Fresh and dried pomace of Cabernet Sauvignon had the most important total anthocyanin content, at this variety acylated forms being best represented.

Significant quantities of anthocyanins registered justifies the extraction of these phenolic compounds from the considered material and demonstrates that grape pomace, both fresh and dried, is an accessible and sustainable source of vegetal pigment, with a huge economic and nutraceutical potential still insufficiently exploited.

ABSTRACT

By-products resulting from grape processing currently represent an environmental issue, their recovery and reintroduction in the food industry is one of the main goals of sustainable agriculture. Anthocyanin content and composition of grapes and their fresh and dry pomace (after processing) of three *Vitis vinifera* L. wine cultivars growing in Iasi vineyard, NE of Romania, was evaluated in order to identify new sustainable sources of vegetal pigments. HPLC-DAD/ESI-MS anthocyanin profile was unique and included five monoglucosyl forms and four acylated forms of anthocyanins, with malvidin-3-glucoside as main representative. Other acylated forms were also present in the profile as traces. Grape processing and pomace drying resulted in a decrease of total pigment content, mainly for esterified anthocyanins (acetylated and coumaroylated). After grape pressing, fresh pomace

of Merlot grapes retained the highest percentage of anthocyanins comparing with raw material (45%). Important quantities of anthocyanins justifies the extraction of these phenolic compounds from the considered material and demonstrate that grape pomace, both in fresh and dry state, is a valuable and accessible source of pigment.

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