DETERMINATION OF THE POLYPHENOL OXIDASE ACTIVITY IN RELATIONSHIP TO TOTAL PHENOLIC AND ANTHOCYANIN CONTENT OF SOME ROMANIAN VINE VARIETIES (*VITIS VINIFERA* L.) FOR TABLE GRAPES

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Keywords: polyphenol oxidase, phenolic compounds, anthocyanins, table grapes varieties, *Vitis vinifera* L.

Abstract. The undesirable change in the grape colour, flavour and texture is associated with the enzymes polyphenol oxidase. Therefore, it is important to control their effect, as well as to establish their characteristics associated to the fruits. Grapes (skin, pulp and seeds) of ten Romanian vine varieties for table grapes (Splendid, Cetăţuia, Milcov, Transilvania, Someşan, Napoca, Gelu, Coarnă neagră selecţionată, Purpuriu and Radames) which are grown in the Ampelographic colection of University of Agricultural Sciences and Veterinary Medicine Iaşi, Romania, were analyzed for determination of polyphenol oxidase activities, total phenolics (flavonoids and non-flavonoids) and total monomeric anthocyanin content. Total phenolic content was higher in seeds, ranging from 4.36 to 5.35 g gallic acid equivalent/100 g fresh weight, of which flavonoids were between 65 and 88%. The highest polyphenol oxidase activity was determined in the grape extract of Radames variety (7.11 U/g/min), while total anthocyanin content was the most important in Napoca variety grape skins (343.86 mg cyanidin-3-glucoside equivalent/100 g fresh weight). There were found strong negative correlations between polyphenol oxidase activity (PA) and anthocyanin content (R^2=0.8854) as well as between PA and total phenolic content of grape skins (R^2=0.8586) and pulp (R^2=0.8831), emphasizing the destructive effect of the enzyme on these chemical classes of compounds.

INTRODUCTION

Polyphenol oxidase (PPO) also known as o-difenoloxidaza, catechol oxidase or tyrosinase (EC 1.10.3.1.), plays an important role in respiration, catalyzing the aerobic oxidation of polyphenols and their derivatives, to produce the corresponding quinones (Rocha and De Morais, 2005; Artenie *et al*., 2008). The purified enzyme had both cresolase and catecholase activities. Catecholase activity had a pH optimum in a range 3.5–4.5 and was characterized by a relatively high stability to heat. Cresolase activity presents a lag period which is modulated by different factors (enzyme concentration, substrate concentration, temperature or pH). The presence of o-diphenols (phenol molecules containing two hydroxyl substituents) cease the lag period, these acting as co-substrates (Valero *et al*., 1988). Belonging to the class of oxidoreductases, PPO is responsible for undesirable change in the grape colour (enzymatic darkening), flavour and texture. In damaged berries an unsavoury flavour and loss of the skin colour can be developed, which will interfere in the quality of the final product (De Pieri Troiani *et al*., 2003). Although peroxidase and polyphenol oxidase are considered to be involved in the oxidative mechanism of the grape juice, the PPO is the main responsible for that oxidation (Yokotsuka *et al*., 1991). On the other hand, the action of the PPO can be considered beneficial because it is responsible for the higher resistance against attacks performed by pathogens (once quinones formed are highly toxic), thereby allowing them to reduce the action of invading microorganisms (Alvarenga *et al*., 2011). Considering all these aspects PPO has attracted much attention to the researchers becoming a complex scientific and technological issue. Regarding the dynamic of this enzyme activity should be mentioned that all phenol oxidases have the maximum activity at the beginning of grape maturation followed by a decrease, in parallel to the increase in the content of phenolic substances and the accumulation of anthocyanins (Beceanu *et al*., 2011).

Polyphenols are secondary plant metabolites (Teissedre and Chervin, 2011), generally involved in the defense against ultraviolet radiation or aggression by pathogens in plants (Pandey and Rizvi, 2009). In food, polyphenols may contribute to the bitterness, astringency, colour, flavour, odour and oxidative stability (Vermerris and Nicholson, 2006). Recent years epidemiological studies strongly suggested that long term consumption of diets rich in plant polyphenols offered some protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Graf *et al*., 2005). The phenolic compounds occurring in grapes include flavonoids, in particular flavan-3-ols (catechins and procyanidins), anthocyanins and flavonols as well as nonflavonoid compounds such as hydroxycinnamic and hydroxybenzoic acids (Ribéreau-Gayon *et al*., 2006a).

Belonging to the class of phenolic compounds (flavonoid subclass) (Bąkowska-Barczak, 2005), anthocyanins (gr. *anthos* – flower, *kyanos* – blue) are generally accepted as the largest and most important group of water-soluble pigments in nature (Horbowicz *et al*., 2008). The anthocyanins are responsible for the red-blue shades of grapes, being located mainly in the skin and, more unusually, in the pulp („teinturier” grape) (Davies, 2004). Their flavylium cation

structure includes two benzene rings bonded by an unsaturated cationic oxygenated heterocycle, derived from the 2-phenyl-benzopyrylium nucleus (Ribéreau-Gayon *et al*., 2006b; Daayf and Lattanzio, 2008).

Frequently, negative correlations were found between PPO activity and total phenolic and anthocyanin content (Orak, 2007), therefore it is important to control enzyme effect, as well as to establish their characteristics in relation to the grapes. The purpose of this paper was to analyse the polyphenol oxidase activities, total phenolics (flavonoids and non-flavonoids) and total anthocyanin content of ten Romanian *Vitis vinifera* L. varieties for table grapes, and to establish relationships between referred factors.

MATERIALS AND METHODS

The research has been carried out on the grapes (skin, pulp and seeds) of ten *Vitis vinifera* L. indigenous table grape varieties (Gelu, Milcov, Cetățuia, Napoca, Someșan, Splendid, Transilvania, Coarnă neagră selecționată, Purpuriu and Radames), which are grown in the Ampelographic Collection of the University of Agricultural Sciences and Veterinary Medicine Iasi, Romania, at grape maturity of consumption. Grapevines genotype were 25 years old, grafted on Kober 5 BB (Berlandieri × Riparia). Planting distances were 2.2 m (between rows)/1.2 m (between plants), half-high training system, bilateral cordon, with pruning in fructification rings providing an average load of 40–45 buds/vine. Soil maintenance was "black field" and technological operations were specific to industrial vineyard ecosystem.

Upon harvest, whole grapes were immediately frozen at –80 °C. Frozen berries were separated (skin, pulp and seeds) (5 g each) and ground separately in a mortar using sieved inert sand as a grinding aid, and extracted in the dark by stirring with 50 mL of 0.1% HCl (v/v) in methanol overnight at room temperature. The samples were filtered and the solid residue washed with an additional 50 mL of 0.1% HCl (v/v) in methanol. Filtrates were combined resulting in a final ratio of 1:20 (g/mL). The resulting solutions were kept at –20 °C until analysis (Gambuti *et al*., 2009).

The Folin–Ciocâlteau method (Singleton and Rossi, 1965) was conducted for the colorimetric estimation of total polyphenols, measuring the absorbance at 750 nm (UV-vis Shimadzu 1700 Pharmaspec Spectrophotometer). A standard curve using different concentrations of gallic acid solutions (R^2=0.991) was done to report the results as grams gallic acid equivalent (GAE) per 100 g fresh weight (f.w.).

Flavonoids were precipitated by formaldehyde at pH<0.8. Five milliliters of a 32% HCl in distilled water (50/50 v/v) and 5 mL of formaldehyde (8 mg/L, in distilled water) were added to 10 mL of each extract. The mixture was vortexed, then left 24 h at room temperature. Its absorbance was measured in the same way as for the total phenolics. The flavonoid content as percentage of dry matter is (X-Y)%, where X is the total phenolic content and Y the non-flavonoid phenolic content as calculated (Tibiri *et al*., 2010).

Total monomeric anthocyanin determination was carried out through the pH differential method (Lee *et al*., 2005). The coloured oxonium form exists at pH 1.0, and the colorless hemiketal form predominates at pH 4.5. The difference in the absorbance at 520 nm is proportional to the pigment concentration. Although the main anthocyanin in grapes is malvidin-3-glucoside, results were calculated as cyanidin-3-glucoside equivalents (CE), based on molecular weight (449.2 g/mol) and molar extinction coefficient, in order to be able to compare data with other species.

The polyphenol oxidase was assayed by spectrophotometry (420 nm), using catechol (Merck Romania) as a substrate according to Ermakov (1987), and reporting results as enzymatic units (U) per gram of f.w. and per minute (U/g/min). An enzymatic unit (U) represents the amount of enzyme that catalyzes the conversion of one micromole of catechol in one minute, at 25°C.

Relative humidity and total dry matter (OIV-MA-AS2-03A), total mineral content (OIV-MA-AS2-04), titratable acidity (OIV-MA-AS313-01) and evaluation by refractometry of the sugar concentration in grape (OIV-MA-AS2-02) were conducted according to the OIV (International Organisation of Vine and Wine) Compendium of international methods of analysis (2012).

Data have mentioned the standard deviation (± SD), and represent the average of three independent analyses. The method used to discriminate among the means was Fischer's least significant difference procedure at 95% confidence level. P values lower than 0.05 ($p \leq 0.05$) were considered to be significant. Simple regression analysis was performed to look for relationships between data.

RESULTS AND DISCUSSIONS

Determination of moisture and total dry matter is essential in vegetal tissue analysis, high proportion of humidity causing a poor stability of samples, favouring microbiological and enzymatic activity, and hydrolysis reactions (Maltini *et al*., 2003; Beceanu *et al*., 2011). Grapes at full maturity have a moisture content of about 70-85% (Fregoni, 1998; Keller, 2010) and a

total mineral content of about 0.22-0.54% (Creasy and Creasy, 2009; Beceanu, 2010). Moisture, total dry matter, minerals, sugar and acidity of Romanian varieties grape berries at technological maturity were shown in table 1. Sugar content varied between 171.99 g/L and 235.85 g/L, with a mean of 188.58±19.29 g/L, amid of a titratable acidity of 6.25±0.93 g/L tartaric acid (t.a.) Thus, the calculated sugar/acid ratio of grapes (“maturation index”) ranged from 23.17 at Purpuriu variety to 42.50 at Radames variety, with a mean of 30.87, values that were within the range presented by Nicolaescu and Cazac (2012), and much higher than the minimum value (20:1) required by the OIV (2008) for table grapes at maturity.

Table 1. Main chemical characteristics and technological parameters of the grapes at harvest

Variety	Moisture (%)	SD (±)	Total dry matter (%)	SD (±)	Minerals (%)	SD (±)	Sugars (g/L)	SD (±)	Acidity (g/L t.a.)	SD (±)
Purpuriu	83.48^{*}	1.02	16.52^{NS}	1.02	0.29^{NS}	0.09	183.05^{NS}	3.49	7.90^{***}	0.10
Splendid	83.67^{*}	0.87	16.33^{o}	0.87	0.37^{NS}	0.12	182.67^{NS}	3.03	5.87^{o}	0.42
Radames	79.19^{ooo}	2.01	20.81^{**}	2.01	0.50^{NS}	0.08	235.85^{***}	9.88	5.55^{ooo}	0.41
Cetăţuia	80.91^{NS}	1.45	19.09^{NS}	1.45	0.54^{NS}	0.14	180.41^{oo}	11.03	5.20^{ooo}	0.06
Coarnă neagră select.	83.91^{*}	0.98	16.09^{o}	0.98	0.38^{NS}	0.11	201.30^{**}	10.82	6.70^{**}	0.46
Transilvania	82.66^{NS}	1.11	17.34^{NS}	1.11	0.39^{NS}	0.09	187.65^{NS}	9.28	6.50^{*}	0.44
Someşan	84.84^{***}	2.01	15.16^{oo}	2.01	0.39^{NS}	0.07	175.08^{NS}	5.83	5.93^{o}	0.12
Napoca	81.19^{NS}	1.16	18.81^{NS}	1.16	0.40^{NS}	0.11	172.42^{ooo}	3.40	6.50^{*}	0.26
Gelu	80.94^{NS}	1.37	19.06^{NS}	1.37	0.43^{NS}	0.18	192.33^{NS}	11.44	4.94^{ooo}	0.08
Milcov	79.47^{oo}	2.11	20.53^{**}	2.11	0.49^{NS}	0.06	171.99^{ooo}	14.90	7.37^{***}	0.15
Mean	82.03	1.95	17.97	1.95	0.42	0.07	188.58	19.29	6.25	0.93
CV%	2.38	-	10.85	-	16.66	-	10.23	-	14.88	-

Note: Data expressed as mean values with standard deviation (n = 3). ^{NS}, *, **, *** - indicate non-significant and positive significant at $p \leq 0.05, 0.01, 0.001$, respectively; $^{o, oo, ooo}$ - negative significant at $p \leq 0.05, 0.01, 0.001$. CV% - coefficient of variation, ratio SD/mean (%).

According to Mazza and Miniati (1993) and Horbowicz *et al*. (2008), anthocyanin content (AC) of grapes varies greatly depending on many factors (e.g. genetic factor, light, temperature, technology etc.), ranging between 30 and 900 mg cyanidin-3-glucoside equivalent (CE)/100 g f.w. At Romanian *V. vinifera* L. varieties for table grapes studied, AC had a mean value of 260.31 mg CE/100 g f.w., with a high standard deviation (±) of 70.97 mg CE/100 g f.w. (table 2).

Table 2. Anthocyanin content (AC) and total phenolic content (TPC) of grapes (skins, pulp and seeds)

Variety	AC (mg CE/ 100 g f.w.)	SD (±)	Skin TPC (g GAE/ 100 g f.w.)	SD (±)	Pulp TPC (g GAE/ 100 g f.w.)	SD (±)	Seed TPC (g GAE/ 100 g f.w.)	SD (±)
Purpuriu	248.48^{ooo}	0.21	1.66^{NS}	0.09	0.33^{NS}	0.02	4.38^{NS}	0.45
Splendid	218.87^{ooo}	1.54	1.70^{NS}	0.11	0.33^{NS}	0.01	4.45^{NS}	0.12
Radames	103.70^{ooo}	1.00	1.56^{NS}	0.08	0.32^{NS}	0.05	4.06^{NS}	0.30
Cetăţuia	299.23^{***}	2.29	1.77^{NS}	0.08	0.34^{NS}	0.01	4.58^{NS}	0.40
Coarnă neagră selecţionată	293.16^{***}	2.16	1.81^{NS}	0.14	0.34^{NS}	0.02	5.07^{NS}	1.08
Transilvania	274.68^{***}	1.02	1.75^{NS}	0.24	0.34^{NS}	0.02	5.15^{NS}	1.21
Someşan	318.92^{***}	0.95	1.80^{NS}	0.07	0.34^{NS}	0.04	4.80^{NS}	0.68
Napoca	343.86^{***}	1.74	1.80^{NS}	0.11	0.34^{NS}	0.08	4.88^{NS}	1.14
Gelu	198.33^{ooo}	1.33	1.67^{NS}	0.08	0.33^{NS}	0.02	4.49^{NS}	0.98
Milcov	303.84^{***}	2.18	1.75^{NS}	0.06	0.34^{NS}	0.03	4.59^{NS}	1.27
Mean	260.31	70.97	1.73	0.08	0.34	0.01	4.65	0.33
CV%	27.27	-	4.60	-	2.11	-	7.16	-

Note: Data expressed as mean values with standard deviation (n = 3). ^{NS}, *, **, *** - indicate non-significant and positive significant at $p \leq 0.05, 0.01, 0.001$, respectively; $^{o, oo, ooo}$ - negative significant at $p \leq 0.05, 0.01, 0.001$. CV% - coefficient of variation, ratio SD/mean (%).

Among the three main parts of the grape berry, the most important total phenolic content was recorded in seeds (mean 4.65±0.33 g GAE/100 g f.w.), followed by skins (mean 1.73±0.08 g GAE/100 g f.w.) and pulp (mean 0.34±0.01 g GAE/100 g f.w.).

Statistical analysis indicate very significant differences to the mean for AC values ($p \leq 0.001$), while for TPC values of skins, pulp and seeds the influence of genetic factor (variety) was nonsignificant ($p > 0.05$).

The phenylalanine metabolism products include the chemical groups of flavonoids and non-flavonoids. Major flavonoid groups in grapes are tannins (e.g. flavanol oligomers and polymers), flavonols and anthocyanins (Brossaud *et al*., 1999). The non-flavonoids form a smaller class (stilbenes, hydroxycinnamic and hydroxybenzoic acids) and are often associated with the flavonoids (Rentzsch *et al*., 2009). Within the berry, the non-flavonoids compounds are primarily found in the pulp (Creasy and Creasy, 2009), while flavonoids are found mainly in skin and seeds (Ribéreau-Gayon *et al*., 2006a; Cotea *et al*., 2009).

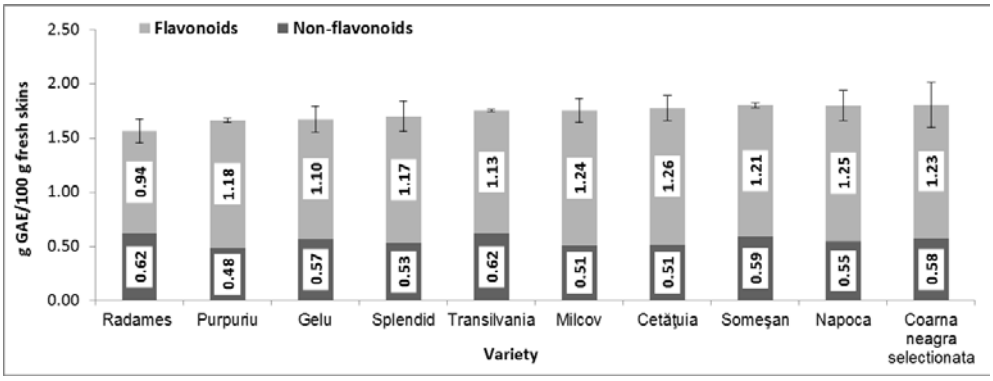

Fig. 1. Flavonoid and non-flavonoid content in grape skins of analysed varieties

In table grape samples of Romanian varieties, flavonoids were the most important class of phenolic compounds in skins (0.94–1.26 g GAE/100 g f.w.) (Fig. 1) and seeds (3.00–3.94 g GAE/100 g f.w.) (Fig. 2), while in pulp flavonoids were exceeded categorically by non-flavonoid phenolic compounds (Fig. 3).

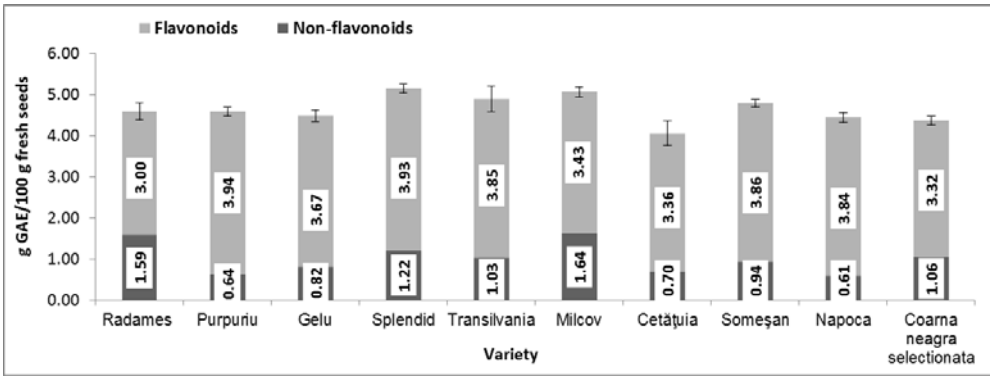

Fig. 2. Flavonoid and non-flavonoid content in grape seeds of analysed varieties

Percentage of flavonoids from total phenolic ranged in skins from 64.57% (Transilvania variety) to 71.05% (Cetăţuia variety), with a mean of 67.71±3.45%, in grape seeds from 65.28% (Milcov variety) to 86.35% (Splendid variety), with a mean of 78.13±7.08%, and in grape pulp from 9.35% (Gelu variety) to 27.27% (Purpuriu variety), with a mean value of 22.29±5.94%.

Ribéreau-Gayon *et al*. (2006a) mention that ripe grapes contain an orthophenol oxygen oxidoreductase, also known as cresolase or catechol oxidase, with an extremely variable activity depending on the grape variety and degree of ripeness. When physical disruption of cell occurs (due to mechanical damage or rot infections), phenolics are brought in contact with oxygen and polyphenol oxidases convert phenolics to quinones (Hernandez *et al*., 2009). This reaction is dominant if the grape pulp is rich in hydroxycinnamic acids (Keller, 2010).

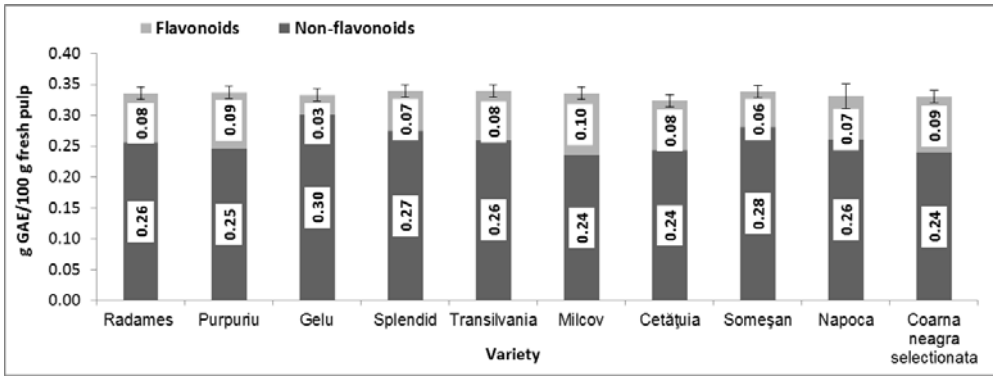

Fig. 3. Flavonoid and non-flavonoid content in grape pulp of analysed varieties

Polyphenol oxidase activity in *Vitis vinifera* L. Romanian varieties berries had a maximum of 7.11 U/g/min (Radames resistant variety), with a mean value of 5.30±0.99 U/g/min (Fig. 4).

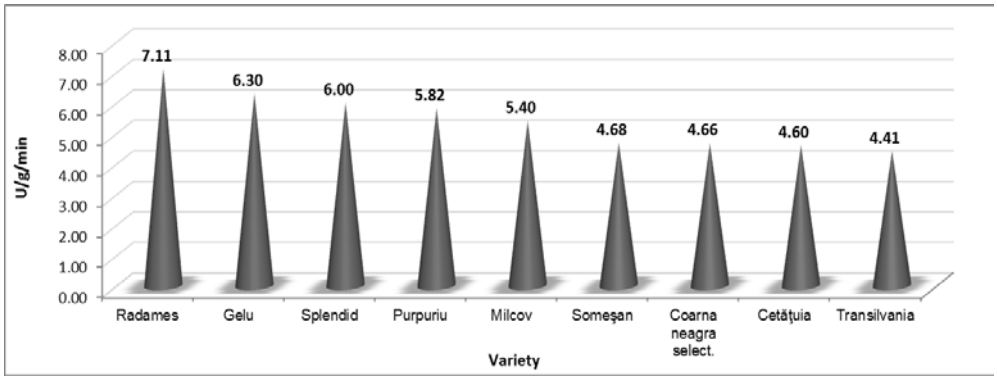

Fig. 4. Polyphenol oxidase activity of Romanian varieties grape berry

Should be noted the positive correlation between the anthocyanin and total phenolic content of grape skins (R^2=0.9291) (Fig. 5). This fact highlights that anthocyanins are part of the

phenolic compounds class, belonging to the flavonoid subclass, as suggested by the coefficient of determination (R^2=0.8471) of the anthocyanin – flavonoid relationship (Fig. 6).

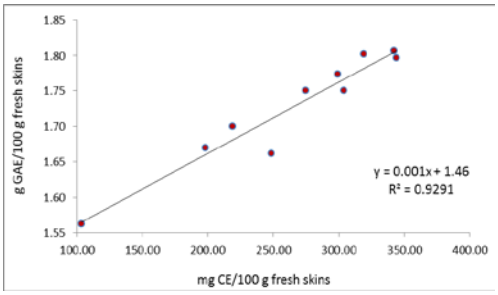

Fig. 5. Correlation of anthocyanin content and total phenolic content of grape skins

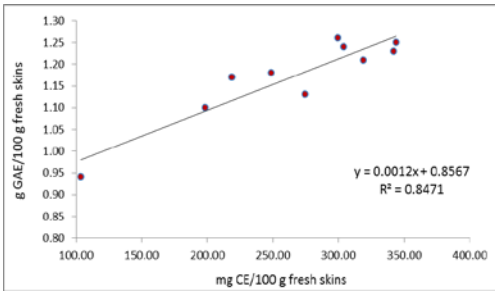

Fig. 6. Correlation of anthocyanin content and flavonoid content of grape skins

The main goal of this study was to identify correlations (positive or negative) between polyphenol oxidase activity and the concentrations of various classes of phenolic compounds in grape berry morphological parts. In this respect, in table 3 are presented the correlation coefficients of relationships identified between the experimental parameters.

Table 3. Correlation of the experimental parameters

Parameter	PPO	AC	Skin TPC	Skin NFC	Skin FC	Pulp TPC	Pulp NFC	Pulp FC	Seed TPC	Seed NFC	Seed FC
PPO	1										
AC	**-0.9180**	1									
Skin TPC	**-0.9266**	**0.9639**	1								
Skin NFC	0.0502	-0.2783	-0.1195	1							
Skin FC	**-0.7772**	**0.9204**	0.8711	-0.5917	1						
Pulp TPC	**-0.9397**	**0.8611**	**0.9020**	-0.0068	0.7358	1					
Pulp NFC	**0.8132**	-0.8174	-0.7567	0.3980	-0.8113	-0.7015	1				
Pulp FC	**-0.8833**	**0.8698**	0.8280	-0.3343	0.8377	0.8029	-0.9880	1			
Seed TPC	**-0.8713**	0.7948	**0.8401**	0.2337	0.5666	0.8770	-0.6483	0.7319	1		
Seed NFC	-0.3872	0.5598	0.4509	-0.0263	0.3791	0.3592	-0.4568	0.4598	0.5740	1	
Seed FC	-0.4561	0.1824	0.3521	0.2695	0.1526	0.4936	-0.1485	0.2310	0.3769	-0.5422	1

Note: AC – anthocyanin content; TPC – total phenolic content; NFC – non-flavonoid content; FC – flavonoid content.

Presence of polyphenol oxidase in a high concentration had a negative influence on the anthocyanin content of grapes ($r = –0.9180$; $p<0.001$), a greater enzyme activity corresponding to lower AC values. According to Kader *et al*. (1997), phenolases such as polyphenol oxidase are common anthocyanin degradation enzymes, but the destruction of anthocyanins is more efficient when other phenolic compounds (e.g. catechol, caftaric acid, chlorogenic acid) are present. Also, it is generally known that inactivation of enzymes improves anthocyanin stability in fruits (Garcia-Palazon *et al*., 2004).

Along with an increase in PPO activity, grape skins total phenolics followed the same trend as for anthocyanins ($r = –0.9266$; $p<0.001$), flavonoid fraction being more affected ($r = –0.7772$; $p<0.05$) than non-flavonoid one. A low level of total phenolic content was strongly correlated to a higher enzyme activity in pulp ($r = –0.9397$; $p<0.001$) and slightly lower in seeds ($r = –0.8713$; $p<0.05$).

It was observed that only in grape berry pulp non-flavonoid content was positively correlated to an intense PPO activity, while in skins and seeds were not set a relationship between these parameters. Thus, in the pulp an intense PPO activity was achieved on the background of a high content of phenolic acids (non-flavonoids). In parallel, it was noticed that varieties with a high content of phenolic compounds in the skin have also a high content of phenolic compounds in pulp and seeds.

CONCLUSIONS

Anthocyanin content of grape skins was highly correlated to the total phenolic content, and moreover with the flavonoid content of skins, confirming their appurtenance to this subclass of phenolic compounds. The flavonoid content was higher in grape skins and seeds, while non-flavonoids were predominant in grape pulp.

An intense polyphenol oxidase activity in grapes was negatively correlated to a lower total anthocyanin and total phenolic content of skins, pulp and seeds. Only in pulp non-flavonoid content was positively correlated to an intense polyphenol oxidase activity due to the fact that in pulp predominate phenolic acids that can be used by enzyme as substrate. Considering the possible destructive action of polyphenol oxidase on anthocyanins in grapes, further studies are necessary in order to evaluate the influence of an intense enzyme activity on the chromatic parameters of berry skins.

Experimental data obtained can be of interest to researchers in viticulture, vine breeding and pharmaceutical industry, and can serve as basis of comparison for future studies.

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