

ANTIOXIDANT ACTIVITY OF TOKAJ ESSENCE

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ABSTRACT

The aim of the presented experiment was to measure the content of total polyphenols and antioxidant potential of Tokaj essence from the years 1999, 2006, 2007, 2009, 2013, 2015. Tokaj essence is produced by fermentation of concentrated must from botrytised grape berries. The highest content of polyphenols was determined in the essence of 1999 ($275.8 \pm 18.17 \text{ mg.L}^{-1}$) and the lowest in the sample of 2015 ($118.8 \pm 12.28 \text{ mg.L}^{-1}$). Antioxidant activity was determined by two methods DPPH and PRAC (permanganate reducing antioxidant capacity). Antioxidant DPPH method showed that the essence of year 1999 had significantly the highest activity ($63.4 \pm 0.81 \mu\text{mol.L}^{-1}$ Trolox) while essence of year 2015 ($47.5 \pm 1.58 \mu\text{mol.L}^{-1}$ Trolox) featured the lowest activity. The results of method PRAC, which determine the total reducing capacity of the essence, do not correlate with the results of DPPH and total phenolic content, because of higher content of saccharides, which gave false positive test results. The method PRAC is not suitable for measuring antioxidant activity of tokaj essence. The results showed that aging of wine increased the content of polyphenols and also its antioxidant activity. High content of polyphenols and the great antioxidant activity of Tokaj wine receive their beneficial effect to human health.

Keywords: antioxidant activity; botrytised berry; essence; total polyphenols

INTRODUCTION

Grape berry are generally used for production of wine and juice (Jang et al., 1996; Špakovská et al., 2012). Only in the Tokaj region the unique Tokaj essence are made from berries. Vine growing and wine production in Tokaj region are defined by the Act on Viticulture and Winemaking 349/2015 Statue, in § 30. Vineyard hunts are situated within the cadastral areas of the following villages: Černochovej, Veľká Bara, Malá Tŕňa, Veľká Tŕňa, Čerhov, Slovenské Nové Mesto, Viničky. The act allows growing of traditional varieties such as Furmint, Lipovina and Muškát žltý which ripen late, and turn into botrytised berries (Pospíšilová, 1981; Kakaš, 2005; Eftimová, 2008; Žadanský, 2009; Furdíková, & Malík 2009; Farkaš, 1998). Botrytised berries are only formed in dry, warm and long autumns when the berries have 19 – 20 °NM of sugar content and are infected with Botrytis cinerea (PERS.et Fries) so called Noble Rot.

Botrytis cinerea (PERS.et Fries.), perfect state Botryotinia fuckeliana (De Bary Whetzel.), is a saprophyte fungus covering infected berries with a grey coating of conidiophores (Eftimová, 2008). Fungus mycelium disrupts the skin of berries and consumes acids, which leads to fructose content increase and glucose content decrease. Hypertonic conditions for fungus development are being created, the fungus does not produce conidia, water evaporates and the berries shrunken and get dry (Kakaš, 2005; Eftimová, 2008; Furdíková &

Malík, 2009). The fungus, due to its oxidative enzyme, decomposes flavours such as linalool, geraniol and nerol into less volatile β -pinen, α -terpineol, furan oxides. The metabolism of fungus degrades carbohydrates, acids and crude, produces surplus of glycerol, gluconic acid and sotolon, which are typical of infected berries must. Glycerol increases naturally sugar free extract and sotolon gives sweet honey and nuts like aroma to botrytis wines (Furdíková & Malík, 2009).

Botrytised berries are used to make essence (nectar) and Tokaj wine selections 7,3,4,5. They are hand-picked from bunches of grapes, put into a tub with perforated base out of which Tokaj essence leaks, then. One puttonyos of botrytised berries (25 kg) provides as much as 1 to 1.5 litres of essence containing 25 to 60% of sugar. The essence ferments slowly and ripens for at least three years, out of these two in a wooden barrel (Kováč et al., 2005). Tokaj essence is used to enhance Tokaj wines and selection wines (Farkaš, 1998). Wine has beneficial effect of human health because of antioxidant activity (Špakovská et al., 2012).

There are a lot of publications on antioxidant activity of spontaneous and variety Tokaj wines, which provide e. g. cardioprotective, antiaging, cancer chemoprotective activity. (Staško et al., 2006; Pour Nikfardjam et al., 2003; 2006 a, b; Harmatha, 2009; Fikselová et al., 2010; Balová and Eftimová, 2015; Balová et al., 2016). The aim of our research was to measure the total content of

polyphenols and antioxidant potential of Tokaj essences which have not been analysed yet.

Scientific hypothesis

According to beneficial effect of tokaj essence on human health it is predicted that tokaj essence obtains high content of polyphenols with great antioxidant activity.

MATERIAL AND METHODOLOGY

Samples and chemicals

Our research included the samples of Tokaj essences of 1999, 2006, 2007, 2009, 2013, 2015 by TOKAJ & CO,

mixture of 300 μL DPPH (1 mg DPPH / 50 mL methanol) and 10 μL of Tokaj essence was measured after five minutes of incubation with the use of spectrophotometry comparison to methanol, at the wave length of 517 nm. TEAC method (Trolox equivalent antioxidant capacity) expressed the amount of known antioxidant Trolox, which is needed to reach reference sample like activity.

Determination of permanganate reducing antioxidant capacity

The method of determining total reduction ability PRAC



Figure 1 Creation of botrytised berries on Lipovina. Author: Eftimová, J.

Malá Trňa. Chemicals and standards were procured from Sigma-Aldrich (USA), the solvents were procured from Fisher (SR). The solutions were always freshly prepared and the samples of essences, until being used, were stored in dark glass containers at the temperature of 4 °C.

Spectrophotometric determination of total polyphenols content

The content was determined by a standard spectrophotometric method according to (Singleton et al., 1999), with the use of slightly modified Folin-Ciocalteu reagent (FC). 10 μL of sample was mixed with 200 μL of sodium carbonate (2%), 70 μL of distilled water and 20 μL of FC. The absorbance was measured after five minutes at the wave length of 750 nm with the use of spectrophotometric reader of microtiter plates Synergy 4 (BioTek, USA) and software Gen5™ (Reader Control and Data Analysis Software).

Determination of antioxidant activity

Antioxidant activity was determined with the use of radical DPPH (Brand-Williams et al., 1995) Reaction

(permanganate reducing antioxidant capacity) was employed. Reaction mixture consisting of 128 μL KMnO_4 solution (0.006 mol.L^{-1}) and 60 μL H_2SO_4 (1.2 mol.L^{-1}) was mixed with 10 μL of our sample. The absorbance of sample was measured with the use of spectrophotometry, at the wave length of 535 nm, after 5 minutes incubation. The results are expressed as the percentage potassium permanganate reduction.

$$PRAC [\%] = 100 - \left(\frac{\text{sample absorbance}}{\text{KMnO}_4 \text{ absorbance}} * 100 \right)$$

Statistical analysis

GraphPad Prisma 5 (GraphPad Software, Inc., USA) was used for statistical analyses. One-way ANOVA with Dunnett's post hoc test was used to evaluate the data. All samples was measured at least 5 times in all experiments. The results are expressed as the mean \pm S.E.M. Values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant.

RESULTS AND DISCUSSION

Table 1 Sugar and alcohol content in Tokaj essence.

	Sugar (g.L ⁻¹)	Alcohol (%)
1999	629.8	3.49
2006	615.4	4.18
2007	601.6	3.12
2009	413.48	1.5
2013	621.15	3.40
2015	562.13	0.0

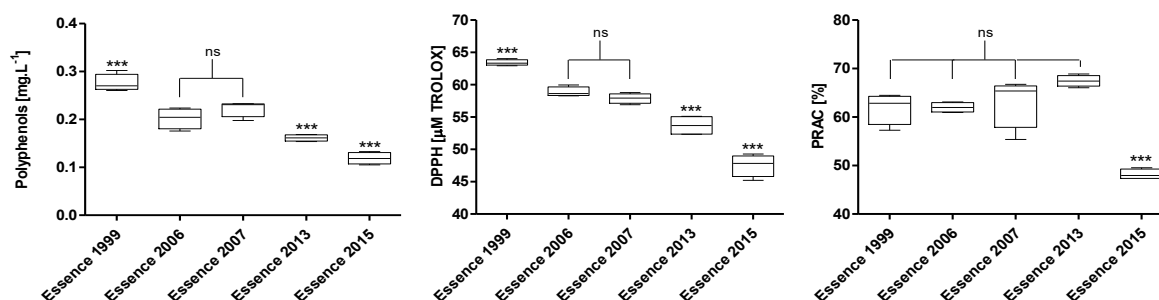


Figure 1 Concentration of total polyphenols in Tokaj essence samples expressed in mg.L⁻¹. Note: ***statistically significant, $p < 0.001$, “ns” refers to statistically not significant values. Samples of Essence 1999 and Essence 2007 show statistical significance $p < 0.01$. Samples of Essence 2006 and Essence 2013 along with Essence 2013 and Essence 2015 show statistical significance $p < 0.05$. Antioxidant activity measured by DPPH of Tokaj essence samples converted to μM Trolox. Total reduction ability (PRAC) of Tokaj essences samples expressed as percentage of potassium permanganate reduction. ***statistically significant $p < 0.001$, “ns” statistically not significant values.

The basis for Tokaj essence production is botrytised berries. The conditions for botrytised berries formation were only met in: 1999, 2006, 2007, 2013, and 2015. The essences of 1999, 2006, 2007, 2013, and 2015 featured the average of 562.13 to 629.8 g.L⁻¹ of sugar. The essence of 2009 only had 413.48 g.L⁻¹ of sugar, which is under the required limit of 450 g.L⁻¹. That is why, the company uses it to produce Tokaj essence selection wine. Significant changes in sugar formation in the berries happen in the period of transformation berries to botrytised berries (Kakaš, 2005). The skin of infected berries splits, evaporation of water increases and carbohydrates get concentrated (Kakaš, 2005; Eftimová, 2008; Furdíková & Malík, 2009). Alcohol content in the essences was from 0 to 4.18 volume % (Table 1). No alcohol is presented in fresh Tokaj essence, as it was determined in essence of 2015. However during maturation the content of alcohol increases. The concentration of alcohol depends on concentration of sugar, in case of essence of 2009 were detected both – low sugar content 413.48 g.L⁻¹ and low alcohol content 1.5%.

Total polyphenols

Significantly highest concentration of total polyphenols (275.8 ±18.17 mg.L⁻¹, $p < 0.001$) was detected and measured in the oldest essence sample of 1999. The essences of 2007 (223.1 ±16.84 mg.L⁻¹) and 2006 (202.2 ±21.55 mg.L⁻¹) also featured a high concentration of total polyphenols. The essences of 2006 and 2007 did not show significant mutual differences, but significantly differed from other essences. The results may be contributed to the age of essences, which is about the same in case of years 2006 and 2007. The lowest content was detected in the youngest essence sample of 2015 (118.8 ±12.28 mg.L⁻¹, $p < 0.001$) (Figure 1).

Polyphenolic substances (anthocyanins, flavonols, flavanols, tannins, sinapinic and hydroxybenzoate acids and their derivatives) of grapes, wines and also of Tokaj essence feature antioxidant activity and many health benefits (Caciget al., 2006). According to Lianga et al. (2012) grape contains at least 4% of polyphenols, including flavonoids up to 3.5% (quercetin-3-glucuronid, isoquercetin, hyperosid, kaempferol-3-glucoside), stilbens (resveratrol, 3,4',5-trihydroxy-trans-stilben), condensed tannins (catechins) and phenolic acids (Košťalová et al., 2012; Špakovská et al., 2012; Jakubcová et al., 2015) consider grapes to be the source of mainly catechin, epicatechin and gallic acid. Resveratrol is mainly found in the berries skin. Its content depends on vine variety and the year of picking (Pour Nikfardjam et al., 2003; 2006 b). Protective substances phytoalexins (resveratrol) are formed under the skin of *Botrytis cinerea* infected berry (PERS.et Fries.), however it is changed to dimers and higher oligomers (Pour Nikfardjam et al., 2006a).

Antioxidant activity

DPPH assay

The highest antioxidant capacity determined by DPPH method was in the essence sample of 1999 (63.4 ±0.81 $\mu\text{mol.L}^{-1}$, $p < 0.001$), and the lowest in the essence sample of 2015 (47.5 ±1.58 $\mu\text{mol.L}^{-1}$, $p < 0.001$). The average antioxidant activity of Tokaj essence samples is 56.3 ±1.28 $\mu\text{mol.L}^{-1}$. The samples showed statistically significant differences $p < 0.001$, except the essences of 2006 and 2007, due to their small age difference (Figure 1).

The measurements of Tokaj essences showed that the level of free radicals uptake determined with the use of DPPH method and polyphenols concentration correlate positively ($R^2 = 0.9471$). The highest level of polyphenols and antioxidant capacity were measured in the essence

sample of 1999. The lowest level of polyphenols as well as antioxidant capacity was measured in the essence sample of 2015. The results show that antioxidant activity can be attributed to polyphenols present in the essences (Figure 2). Pharmacological force of vine berries can be attributed to polyphenols, mainly flavonoids (quercetin, kaempferol, rutin) and stilbenoids (Arnous and Mayer, 2008; Chéze et al., 2001) which is in correlation with our observations. Author Chlebo et al. (2011) claims that resveratrol and its derivatives are substances to be responsible for medicinal properties of Tokaj wines and essences.

Correlations between PRAC assay, DPPH assay and total content of polyphenols

Obtained results show that the level of total reduction ability does not correlate with antioxidant activity measured by DPPH method ($R^2 = 0.3837$), nor they correlate with concentration of total polyphenols ($R^2 = 0.2696$) (Figure 3).

Positive correlations were observed by Keylor et al. (2015) between total polyphenolics content and anthocyanins content in water extract of *Cornus mas* L., which indicates important contribution of polyphenols to result of PRAC assay. Popović et al. (2012) reported that

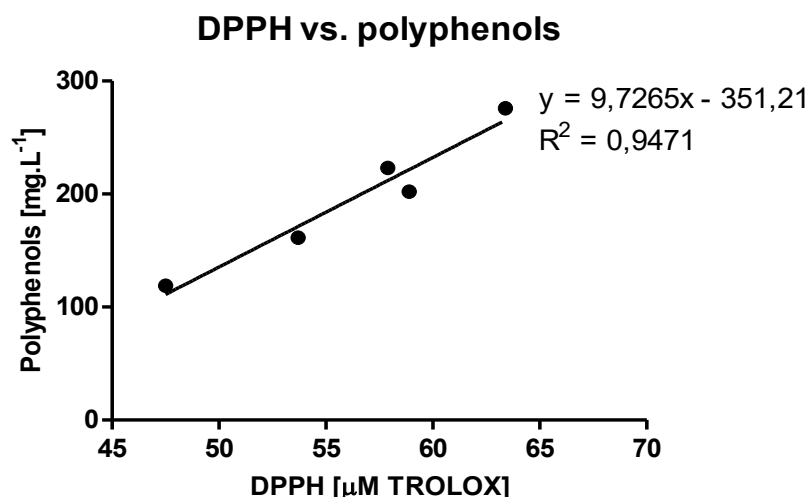


Table 2 Correlation of antioxidant activity determined with the use of DPPH method and concentration of total polyphenols.

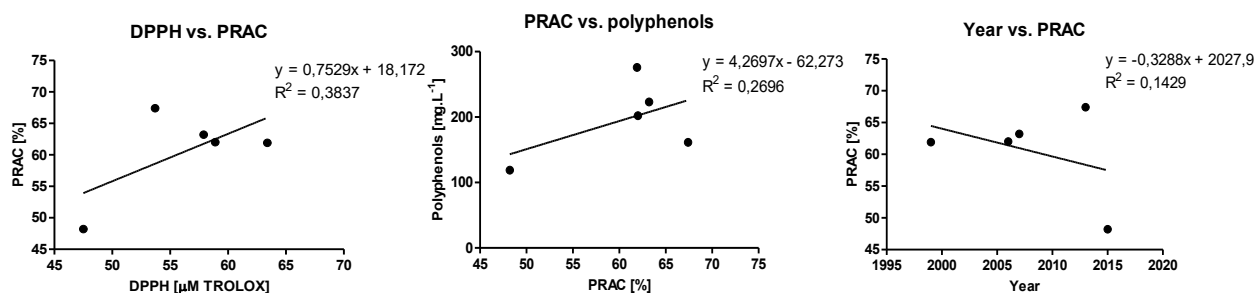


Figure 3 Correlation between antioxidant activities as determined with the use of DPPH and PRAC methods. Correlation of antioxidant activity as defined with the use of PRAC method and concentration of total polyphenols. Correlation of antioxidant activity (PRAC method) and essence age.

PRAC assay

Determined by PRAC method, essences samples of 1999, 2006 and 2007 were found to have approximately similar and statistically not significant rate of potassium permanganate reduction ability, the average being $60.6 \pm 3.83\%$ (Figure 1). The highest reduction ability ($67.4 \pm 2.58\%$) was measured in the essence sample of 2013, while the lowest one was measured in the essence sample of 2015, $48.2 \pm 1.05\%$, which was statistically significant in relation to other essences ($p < 0.001$). The results, which are not in correlation with results of DPPH assay, will be discussed in part 3.3.

permanganate chemiluminescence detection is suitable to assess the antioxidant status of fruit juices, teas and other beverages. However no author tested this assay for Tokaj essence. Observed discrepancy in correlation with total polyphenols content and DPPH assay was caused by presence of sugar high levels and alcohol in the essences. The method is a disadvantageous one since besides antioxidant substances which react (polyphenols), there are also other substances (sugar) with a reduction potential present, which give false positive results. That is why the method is not suitable one to test the samples of Tokaj essences.

Relative values of antioxidant activity

Due to difficulty of absolute value comparison of Tokaj essences antioxidant activity and to make the data clearer, the obtained results were expressed as relative values. The sample with highest absolute absorbance was given the highest relative value of 1. Other values were then calculated with respect to a given sample. Relative values along with deviations are summarized in Table 2.

polyphenols was detected in the oldest sample of 1999 ($275.8 \pm 18.17 \text{ mg.L}^{-1}$) (Figure 4).

Similar results were observed in case of result of DPPH assay. Figure 4 shows the correlation of essence age and antioxidant activity measured by DPPH method, reaching a high value of $R^2 = 0.9181$. Correlation results make it clear that the oldest essences will usually feature the highest antioxidant activity, the opposite being true for

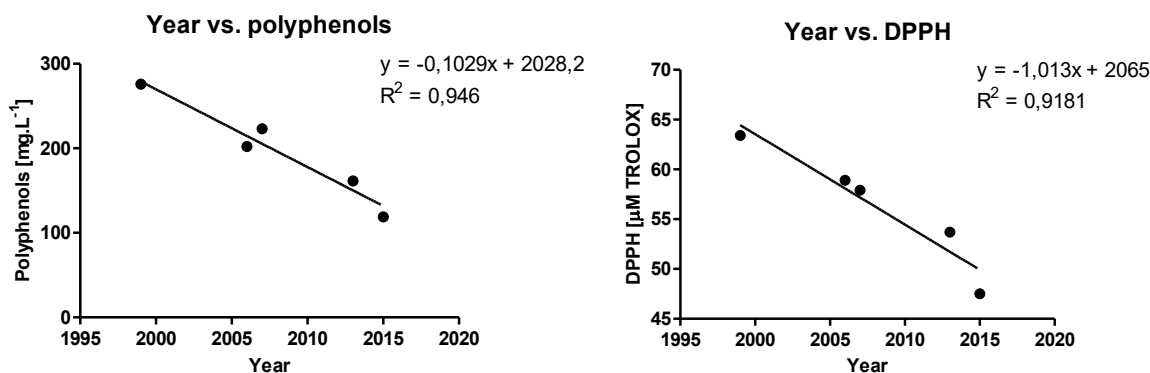


Figure 4 Correlation of sample age and concentration of polyphenols and antioxidant activity determined by DPPH assay.

Table 2 Relative values of Tokaj essences antioxidant activities determined by DPPH and PRAC methods.

Samples	DPPH	PRAC
Essence 1999	1 ±0.013	0.92 ±0.074
Essence 2006	0.92 ±0.017	0.92 ±0.023
Essence 2007	0.91 ±0.017	0.94 ±0.133
Essence 2013	0.84 ±0.028	1.00 ±0.038
Essence 2015	0.75 ±0.025	0.71 ±0.016

When determining the relative value of antioxidant activity by DPPH radical, the highest relative activity (1) was found in the essence sample of 1999, while its relative value determined by PRAC method was 0.92 ± 0.074 . The highest relative value determined by PRAC method was found in the essence of 2013, however, its relative value determined by DPPH method was 0.84 ± 0.028 . The lowest relative activity was observed in the essence of 2015 (0.75 ± 0.025 by DPPH method, 0.71 ± 0.016 by PRAC method). Discrepancy in relative antioxidant values of both methods confirm our suggestion, that PRAC method is not suitable for detection of Tokaj essence and other samples with high content of sugar.

Aging of Tokaj essences

Ripening of wine in the tuff cellars is a part of winemaking technological process (slow oxidation in wooden barrels), which significantly impacts character, taste and quality of wine (Farkaš, 1998; Francis et al., 2010; Kakaš, 2005; Eftimová, 2008; Žadanský, 2009; Špakovská, 2012). Correlation dependence of the essence age on total polyphenols concentration shows their significant relationship ($R^2 = 0.946$). The youngest essence of 2015 features the lowest polyphenols concentration ($118.8 \pm 12.28 \text{ mg.L}^{-1}$), however, it grows with the essence ageing. That is why the highest concentration of

young essences.

In contrast with total polyphenols concentration and antioxidant activity determined by DPPH method, there was no correlation observed ($R^2 = 0.1429$) between the essences age and total reduction ability (Figure 3). However, it is expectable, since PRAC assay gives false positive results.

During aging of wine, the content of reducing sugars and biogenic amines is changed and depend on the age of wine (Vasko, 2001). Biogenic amines (such as histamine, tyramine and putrescine) can develop during wine ageing and also botrytization (Souflerros et al., 2007). The aging of white and red wines are connected with polyphenols. Acetaldehyde, produced during oxidative aging, enhance the polymerization of anthocyanins (presented in red wines) and flavonoid tannins (Hajos et al., 2000). Tartaric acid is oxidized to glyoxylic acid, which binds catechins and other phenolic compounds to long polymers (Somers, 1986). Must from botrytised berries recovery decreases, more tartaric acid than malic acid is being degraded while sugar content and quality of wine increase (Kakaš, 2005). Grapes infected by Botrytis cinerea produced phytoalexins resveratrol, which is polymerized by fungus to its derivatives such as δ – viniferin (resveratrol trans-dehydrodimer) (Fulcrand et al., 1997; Harmatha and Dinan, 2003). Botrytis cinerea forms enzymes stilbenoxidase and laccases, which play same role in phenolic metabolism

(Bavaresco et al., 2016). Enzymes laccases serve as a catalysers of oxide reduction conversion of phenol substances which contribute to golden and brown colour of Tokaj wines.

CONCLUSION

The results obtained from Tokaj essences analysis (1999, 2006, 2007, 2013, 2015) are original ones and confirm the fact that essences are a source of polyphenols and have antioxidant activity. They can be used in prophylaxis of diseases triggered by oxidation stress. Since Tokaj essences are used to make Tokaj spontaneous selection wines, they contribute to previously known theoretical knowledge of the antioxidant potential of Tokaj wines. In a correlation comparison of the two methods of antioxidant activity and total polyphenols determination, we found out that the level of total reduction ability does not correlate with antioxidant activity, nor it correlates with the total amount of polyphenols in the essences samples. The experiment has proven that PRAC method is not a suitable one to measure antioxidant activity of Tokaj essences with high content of sugars and low alcohol content.

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Acknowledgments:

This work was supported by the Slovak Research and Development Agency [APVV-15-0377].

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