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THE INFLUENCE OF TEMPERATURE AND TIME ON THE STABILITY OF THE ANTIOXIDANT ACTIVITY AND COLOUR PARAMETERS OF GRAPE MARC ETHANOLIC EXTRACT

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The interest to replace synthetic food colorants, preservatives and antioxidants in beverages with natural ones is leading researchers to explore the polyphenols of winery wastes. It is of great interest to see if these compounds could replace synthetic dyes in drinks. However, it is still necessary to study the stability of extracts containing grape phenolics during various technological treatments. This paper presents the study of the stability of the 50% ethanolic extract of marc resulting from the winemaking process. The extract was subjected to the following temperatures: -2°C for 12 hours; 4°C for 12 hours; 40°C for 15 minutes, 60°C for 15 minutes, 80°C for 15 minutes and 100°C for 2 minutes; after that the antioxidant activity and the colour parameters (CIELab) were measured. Three sets of extracts were kept for 2 weeks at -2°C, 4°C, and 25-30°C and afterwards the parameters mentioned above were measured once again. Furthermore, the total content of polyphenols and the content of tannins were determined using the Folin-Ciocalteu method. The results were expressed in mg gallic acid equivalents per litre and mg tannic acid equivalents per litre, respectively. The antioxidant activity was determined using the method based on the interaction with the ABTS radical, the results being expressed in % inhibition. The results have shown that the colour (CIELab parameters) and the antioxidant activity of the ethanolic extract of marc are relatively stable during thermal processes. High temperatures as well as prolonged storage at room temperature increased the values of antioxidant activity, chroma, and redness. However, they also produced the most significant effect of the overall colour of the extract, leading to the degradation of blue pigments and a shift towards orange hues.

Keywords: antioxidant activity, colour parameters, CIELab, grape marc extract, temperature

Introduction

The emerging interest to replace synthetic food colorants, preservatives and antioxidants in beverages with natural ones is leading researchers to explore natural sources of substances that exhibit such properties. Due to their intense colour and probable positive effects on health, the polyphenols of winery wastes could replace synthetic dyes in drinks. Many studies suggest that grape marc extracts could be used as tools to improve the colour of various foods (Negro *et al*., 2003; Spigno & DeFaveri, 2007; Hashim & Segupta, 1998). Pedroza *et al*. (2013) used mixtures of dehydrated waste grape skins and found them to be a useful tool for correcting colour loss before bottling. Furthermore, other classes of natural polyphenols could be used as antimicrobial agents and antioxidants. The results of Delgado Adamez *et al*. (2012) also suggest that the use of grape seed extract is a feasible alternative as antibacterial and antioxidant agents.

Given the fact that reducing the environmental impact of industrial waste is presently a major concern all over the world, wine making residues, such as marc and stalks, rich in polyphenols, can be a good source of natural colorants and additives (Spigno & De Faveri, 2007; Negro *et al.*, 2003). Grape marc is a residue of the winemaking industry with high potential for the industry of additives due to its high content of phenolics, which are one of the most active antioxidants compounds in plants. They act as both donors of hydrogen or electrons and stable radical intermediates (Lee *et al*., 2006).

Different synthetic antioxidants are used nowadays in food industry, which can sometimes pose problems for the human health. The use of substances such as butylated hydroxyanisole, butylated hydroxytoluene and tertiary-butylhydroquinone is discouraged due to their negative health effects (Lee *et al*., 2006), while the interest focuses on the use of natural antioxidants. However, more research is necessary to determine the stability of extracts containing grape phenolics during various technological treatments and to determine whether their antioxidant properties and their colour are not drastically modified by time and temperature. Thus, the objective of this study is to determine the influence of different temperature and time values and storage time and conditions on the antioxidant activity and colour parameters of ethanolic grape marc extract.

Materials and methods

Materials

The marc originating from red grape varieties was obtained from a Moldovan winery. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was obtained from Alfa Aesar (Thermo Fisher (Kandel) GmbH, Germany), and the Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany).

The marc used to obtain the extracts was dried at temperatures up to 65°C then chopped up to the state of powder and sieved. The extracts were obtained by extraction in 50% ethanol solution (1:10 ratio), continously stirring for 30 min at room temperature. Afterwards, the extract was subjected to the following

temperatures: -2°C for 12 hours; 4°C for 12 hours; 40°C for 15 minutes, 60°C for 15 minutes, 80°C for 15 minutes, and 100°C for 2 minutes; afterwards the antioxidant activity and the colour parameters (CIELab) were measured. Three sets of extracts were kept for 2 weeks at -2°C, 4°C, and 25-30°C and after that the parameters mentioned above were measured once again.

Antioxidant activity by reaction with ABTS radical

The antioxidant activity of the extracts was assessed by assay with the radical ABTS, which is based on the ability of antioxidants to reduce the radical and decrease its absorbance at 734 nm (Re *et al*., 1999).

ABTS is dissolved in water to 7 mM concentration. Afterwards, the ABTS radical cation is produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 hours before use. The oxidation of ABTS commences immediately, but the absorbance is not maximal and stable until after more than 6 hours. The radical is stable in this form for more than two days when stored in the dark at room temperature. In order to test the phenolic compounds, the ABTS radical is diluted to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. The sample solutions are diluted in such way that they would produce between 20%-80% inhibition of the blank absorbance, after the introduction of a 10 µL aliquot. After the addition of 1.0 mL of diluted ABTS radical solution to 10 µL of antioxidant compounds, the absorbance reading is taken at 30°C exactly 1 min after initial mixing and up to 6 min, using ethanol as a blank (Re *et al*., 1999). The results were expressed as % inhibition.

Total polyphenols by Folin-Ciocalteu assay

All phenolic compounds including tannins are oxidized by the Folin-Ciocalteu reagent. The blue coloration produced has a maximum absorption in the region of 750 nm, and is proportional to the total quantity of phenolic compounds originally present. The determination of the Folin-Ciocalteu index was performed by introducing the following into a test tube strictly in the mentioned order: 0.2 mL of sample, previously diluted; 6 mL of distilled water; 0.5 mL of Folin-Ciocalteu reagent. The mixture was vortexed, and after 1 min, 1.5 mL of aqueous sodium carbonate (20%) were added, the mixture was vortexed again and allowed to stay in the dark at room temperature for 120 min. Afterwards, the absorbance was determined at 750 nm through a path length of 1 cm against a blank prepared with distilled water in place of the sample. The results for total polyphenols are calculated from a calibration curve, using gallic acid (0-500 mg/L, R^2=0.9988) and tannic acid (0-500 mg/L, R^2=0.9991) as standards, and expressed in equivalents of gallic acid (mg GAE/L) and tannic acid (mg TAE/L), respectively (Singleton & Rossi, 1965).

Colour parameters (CIELab)

The CIELab parameters were determined using the Analytic Jena spectrophotometer (Germany). The calculations were made using the Specord programme provided by the same company. The transmittance of all extracts was

measured between 380 nm and 780 nm, every nm, in optical glass cuvette with the path length of 1 mm, using distilled water as reference. The illuminant was D65 and the observer was placed at 10°.

Statistical analysis

The accuracy was assessed using experimental methods of mathematical statistics, so the mean values and the standard deviations were calculated from 3 parallel experiments. ANOVA and post-hoc Tukey test were used to distinguish between means and evaluate the results. The considered significance level was $p \leq 0.05$. All calculations were made using IBM SPSS Statistics 23.

Results and discussion

The content of various classes of polyphenols, the antioxidant activity, and colour parameters of the initial extract are summarized in Table 1.

Table 1. Initial polyphenol content, antioxidant activity and colour parameters of grape marc extract (the results are expressed as means±standard deviations)

Indice	Values
Total polyphenols, mg GAE/L	3749±128
Total polyphenols, mg TAE/L	4398±140
Antioxidant activity, %inhibition	1393±37
Luminosity (L*)	65.6±0.1
Red/green component (a*)	30.00±0.18
Blue/yellow component (b*)	-7.14±0.09
Chroma (C*)	30.8±0.16
Hue (H*)	-4.12±0.08

Similar results for total polyphenols content of grape marc were obtained by other authors (Negro *et al*., 2003). Regarding the effect of drying conditions, Laurrari *et al*. (1997) have found that drying grape pomace at 60°C did not significantly affect the antioxidant activity and colour parameters of grape pomace and only temperatures of 100°C and 140°C had a significant impact on both the polyphenol content and the antioxidant activity. We can therefore assume that the drying conditions did not have a great impact on the polyphenol content and the antioxidant activity of the original grape skins.

The results from Figure 1 show the change in the antioxidant activity after different thermal treatments. Some of them had a statistically significant effect on this parameter, by either lowering it or increasing it. Generally, temperatures below 0°C had a negative effect on the antioxidant activity which decreased from 1393% inhibition to 1189% and 1065%, after 12 hours and two weeks of storage at this temperature, respectively. On the other hand, high temperatures did not exhibit a significant effect and, on the contrary, the highest temperatures, namely 80°C and 100°C, as well as prolonged storage at room temperature, increased the values of

antioxidant activity. This could be attributed to a probable loss of solvent through evaporation. On the other hand, the research of Kurzeja *et al*. (2012) has shown that High Temperature Short Time (HTST) decreased the number of radicals in the tested herbs used in the research, while the antioxidant activity increased, possibly because this parameter was enhanced just due to the effect of heat.

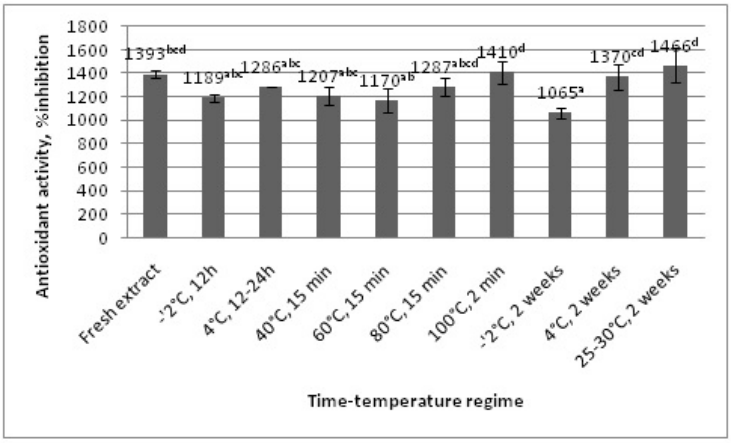

Figure 1. Influence of temperature on the antioxidant activity of grape marc extract (the results are expressed as means±standard deviations; different letters designate significantly different results)

Some other authors also suggested that during thermal treatments new antioxidant compounds may be generated (Jeong *et al*., 2004). The power of certain antioxidants is associated with their reducing power and thus associated with the presence of reductones (Jayaprakasha *et al*., 2001).

The research on the sterilization of spice herbs undertaken by Kurzeja *et al*. (2012) has shown that high temperature influences both antioxidant activity and colour parameters. Table 2 summarizes the obtained values for L*, a*, b*, and H*.

The values of luminosity were comprised between 62 and 68, the highest value being observed in the extracts subjected to 60°C for 15 min, -2°C for two weeks, and 25-30°C for two weeks. This value is higher by approximately 3 units than the value determined in the fresh extract; therefore, prolonged exposure to very low temperatures and room temperatures could lead to some loss of pigment. Some authors even suggest a linear correlation between the anthocyanin content and all CIELab parameters. Furthermore, high values of L* in grape extracts were associated with low total anthocyanins (Liang *et al*., 2011). On the other hand, only a slight increase was observed in the extract kept in the dark, at 4°C, which suggests that these storage conditions would be better in terms of preservation of pigment quality.

The statistical analysis has shown that only the results obtained for the extract subjected to 100°C for 2 min are significantly different from the others in terms of

luminosity and redness. However, the value of a* has increased, which means that there was a colour shift towards more red tones, whereas Laurrari *et al*. (1997) found a loss of red colour in grape pomace peels subjected to 140°C. Given the decrease in luminosity, there is more evidence that there was a loss of solvent during the process.

Table 2. The change of colour parameters during various thermal treatments (the results are expressed as means±standard deviations; different letters designate significantly different results)

Temperature-time regime	L*	a*	b*	H*
Fresh extract	65.60±0.12^{a}	30.00±0.19^{a}	-7.14±0.09^{a}	-4.12±0.07^{a}
-2°C, 12h	67.85±0.58^{a}	28.91±0.16^{a}	-6.80±0.95^{a}	-4.23±0.69^{a}
4°C, 12-24h	65.58±0.12^{a}	30.03±0.17^{a}	-7.15±0.09^{a}	-4.12±0.08^{b}
40°C, 15 min	67.76±0.40^{a}	29.32±0.20^{a}	-7.10±0.24^{a}	-4.05±0.18^{b}
60°C, 15 min	68.50±0.16^{a}	28.28±0.02^{a}	-6.10±0.18^{a}	-4.57±0.14^{b}
80°C, 15 min	66.73±1.53^{a}	29.58±0.97^{a}	-4.02±0.35^{b}	-7.35±0.86^{a}
100°C, 2 min	62.52±2.33^{b}	33.27±2.45^{b}	-3.87±0.54^{b}	-8.66±1.15^{a}
-2°C, 2 weeks	68.35±0.20^{a}	28.24±0.34^{a}	-6.50±0.20^{a}	-4.27±0.09^{b}
4°C, 2 weeks	66.52±0.15^{a}	29.61±0.11^{a}	-6.22±0.01^{a}	-4.12±0.08^{b}
25-30°C,2 weeks	68.41±0.13^{a}	27.77±0.14^{a}	0.79±0.05^{c}	35.30±2.47^{c}

The results in columns a* and b* show the evolution of the red/green component and blue/yellow component, respectively. Both parameters are relatively stable and only the shift of the blue/yellow component towards positive values in the extracts subjected to 100°C for two minutes, -2°C for two weeks and, especially, in the extract kept at room temperature and exposed to light, suggests the degradation of blue pigments and the evolution towards yellow tones. This could be a sign of contribution of other pigments, which usually involves pyroanthocyanin formation resulting in red-orange hues (Torchio *et al*., 2011).

The hue is influenced by the prolonged time of exposure to light and room temperature with the evolution of colour towards yellow hues and loss of the blue ones. The same evolution is confirmed by the increase in b* value. The evolution of the b* parameter is strictly dependent on the temperature and the time of exposure: the higher the temperature, the higher the shift towards yellow. Other authors also found that high temperatures (>100°C) increase the hue angle and the difference in colour of red grape pomace peels (Laurrari *et al.*, 1997).

Figure 2 depicts the change of chroma during various temperature-time regimes. Chroma characterizes the quality of colour. Generally, the colour quality is not influenced by high or very low, freezing temperatures and remains relatively stable. The highest value was observed in the extract subjected to the temperature of 100°C for 2 minutes. Even though the standard deviation is higher than in other

cases, the increase in colour quality could be explained again by the water loss through evaporation.

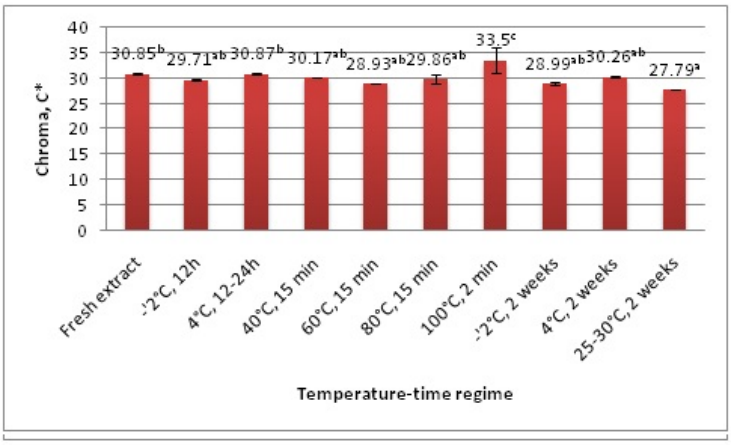

Figure 2. Chroma of each extract (the results are expressed as means±standard deviations; different letters designate significantly different results)

With regard to CIELab parameters, the darkening and the shift towards red and yellow hues were observed by other authors (Kurzeja *et al*., 2012). The authors have also found a decrease of the L* value in comparison with the unsterilized samples and have related this effect with the loss of water which occurred during sterilization.

Table 3. Overall colour difference between the fresh extract and the extracts subjected to various thermal treatments

Time-temperature regime	ΔE between the fresh extract and extract subjected to respective regime
-2°C, 12h	2.53
4°C, 12-24h	0.03
40°C, 15 min	2.26
60°C, 15 min	3.53
80°C, 15 min	3.35
100°C, 2 min	5.56
-2°C, 2 weeks	3.33
4°C, 2 weeks	1.36
25-30°C, 2 weeks	8.71

Table 3 shows the overall difference in colour between the freshly prepared extract and those subjected to various thermal regimes. Overall, the colour was stable and

it did not change. It is generally accepted that wine tasters can distinguish the colour of two wines through the glass when ΔEab^* is higher than 5 units and wine can be used as a model in this case since it is an ethanolic grape extract. Furthermore, the differences that can be distinguished by the human eye also depend on the colour intensity (Kontoudakis *et al*., 2011). Other authors report that the perceptibility thresholds of CIELab colorimetric differences are ΔE^*=0.8-1 (Gonnet, 2001) and ΔE^* =3 (Martinez *et al*., 2011). Therefore, changes perceptible by the human eye occurred in the extract subjected to 100°C for two minutes and the one kept for two weeks at room temperature.

Conclusion

The results have shown that the colour (CIELab parameters) and the antioxidant activity of the ethanolic extract of marc are relatively stable during thermal processes. High temperatures, i.e. 80ºC and 100ºC, as well as prolonged storage (two weeks) at room temperature, increased the values of antioxidant activity, chroma and redness. On the other hand, they led to the degradation of blue pigments and a shift towards orange hues. Moreover, the same temperatures, as well as the prolonged exposure to room temperature and light, also produced the most significant effect on the overall colour of the extract.

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