# *Influence of pH and ionic strength on the color parameters and antioxidant properties of an ethanolic red grape marc extract*

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# Influence of pH and ionic strength on the colour parameters and antioxidant properties of an ethanolic red grape marc extract

(*Abbreviated running title:* ***Study of some medium factors influencing grape marc extract properties***)

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## Highlights

- Gallic, protocatechuic, ferulic, chlorogenic and salicylic acids were identified
- $CaCl_2$ decreased antioxidant activity, but enhanced colour intensity
- Different pH values had slight influence on the antioxidant activity

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**Keywords:** grape marc extract, antioxidant activity, colour parameters, polyphenols, pH, CIELab

***Abstract*:** The aim of this paper was to investigate the influences of pH and several salts on the antioxidant activity and colour of an ethanolic grape marc extract. Furthermore, the phenolic content of the extract was analysed using HPLC and spectrophotometric methods, while the total antioxidant activity was assessed by reaction with ABTS radical. Gallic acid, procyanidin B1, polydatin, catechin, epicatechin, hyperoside, ferulic, chlorogenic, and salicylic acids were among the main identified polyphenols. Different pH values had slight influence on the antioxidant activity; the highest value being determined for the pH 3.7. The redness, blueness, chroma and hue were significantly enhanced at pH 3.7 and 2.6. The chromaticity decreased at pH=5.5 and pH=7.4, so the extract should be used with care in products with such media. The presence of salts did not significantly affect the antioxidant activity, except the higher concentration of $CaCl_2$, which decreased antioxidant activity, but enhanced colour intensity.

***Practical application:*** The data presented in this paper could be used for the development of a new food dye with antioxidant properties of natural origin. The optimal medium conditions, i.e. pH and ionic strength for the use of an ethanolic red grape marc extract, have been identified. The information could be used in product development and product formulation, especially when functional foodstuffs are envisaged. Consequently, this paper would be of significant interest for food chemists, food technologists, food manufacturers and especially manufacturers of food dyes and all those using natural substances in their production process.

## 1. Introduction

The "clean label" is a growing global trend and involves aspects, which range from sustainability of food production to use of non-synthetic ingredients (Global Food Forums, 2017). The use of natural pigments is part of the latter and recent publications report that the global market will grow by 6.22% revenue until 2019 (Cortez et al., 2017). Furthermore, due to their structure and properties, these substances could play a double technological role in foods, i.e. act as both colourants and antioxidants. Many of these pigments/antioxidants can be sourced from the by-products of the food industry, which, at present, are not utilized at full potential. For example, in the process of winemaking, around 25% of the grape weight results in waste, most of which is afterwards composted and reintroduced in the vineyards (Dwyer et al., 2014). Studies suggest that, depending on the winemaking technique, around 70% of phenolics remain in that waste after processing (Ratnasooriya & Rupasinghe, 2012). These phenolics present also a source of valuable bioactive compounds, which may be used in different pharmaceutical, nutraceutical, and food formulations. Traditionally, natural antioxidant extracts are intended for medical use, however, because of many uncertainties related to their bioavailability and metabolism, their application in food systems is more promising (Astley, 2003) where they can be used as antioxidants, colour compounds, and antimicrobial agents(Oliveira et al., 2013). If implemented globally, such trends could impact different aspects of the entire food chain namely contribute to a more sustainable agriculture, increase the availability of some important nutrients in diets and reduce the risk of certain degenerative diseases while improving the sensory properties of foodstuffs.

Nevertheless, the pigments found in grape skins, such as the anthocyanins, degrade rapidly and form colourless or brown compounds(Ngo & Zhao, 2009), which is why it is important to consider the optimal technological conditions and other ingredients in foods which may influence their antioxidant activity and colour. The discovery of acylated anthocyanins and their stability has opened new pathways for food producers since these pigments present lower susceptibility to temperature, light and pH change. Caffeic, *p*-coumaric, ferulic, and sinapic acids, as well asa range of aliphatic acids like acetic, malic, malonic, oxalic, and succinic acid are some of the most important acylating agents(Bakowska-Barcak, 2005). Even though recent studies suggest that during alcoholic fermentation, the grape anthocyanins stabilise via interaction with other compounds, i.e. acetic acid originating from yeast metabolism(Campos, 2009), a study on the stability of the antioxidant activity and colour of these newly formed compounds is necessary. Moreover, several physicochemical parameters could affect phenolics, in particular, the pH and the complexation with other compounds present in the food matrix (Cortez et al., 2017).

CIEL*a*b* parameters are becoming increasingly popular among food scientists and processors for the description and standardisation of foodstuffs' colour. Although difficult at first glance, this colour space is in fact easy to interpret with little training and could save a great amount of expensive sensory work performed in industrial environments for simple colour match, if the data becomes available.

The objective of this study was to research the influence of pH and various ions on the antioxidant activity and colour of an ethanolic grape marc extract. This paper also offers some answers on the interactions and effectiveness of antioxidants/colourants in different food media.

## 2. Materials and Methods

### 2.1 *Materials*

The red grape marc was sourced from a Moldovan winery. D (-)-quinic acid (98%), sinapic acid (98%), methyl 4-hydroxy-3-methoxycinnamate (99%), ABTS(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) were obtained from Alfa Aesar (Germany), Folin-Ciocalteu reagent was provided by Merck (Germany), (+)-catechin(98%), morin hydrate, ellagic acid (≥95%), benzoic acid, quercetin, caffeic acid, (+)-rutintrihydrate, syringic acid, ferulic acid, gallic acid (98%), protocatechuic acid, gentisic acid, parahydroxybenzoic acid, salicylic acid (99.9%), para-coumaric acid were purchased from Sigma (Germany, Japan, China). Procyanidin B1, procyanidin B2, polydatin, hyperoside were purchased from Extrasynthese (France). Trans-resveratrol was purchased from TCI Europe (Belgium). Quercetin (>95%) was obtained from Sigma-Aldrich. All the spectrophotometric measurements were made using Analytic Jena Specord 200 Plus spectrophotometer (Germany).

### *2.2 Extraction*

The marc was dried at the temperature up to 65°C, chopped up to a powdery state, and sieved. The initial samples were obtained by extraction in ethanol 50% (v/v) at the ratio 1g marc: 10 mL solvent, under stirring for 30 min at room temperature(Cristea et al., 2015). The extraction parameters have been optimised during the earlier stages of the research (unpublished data). These extracts were stored in the dark, at the temperature (t) of 4°C, and then used in the experiments involving pH modification and the addition of salts. Moreover, their composition was determined.

### *2.3 Studies on the ionic strength*

Three different salts widely used in food production, i.e. NaCl, $CaCl_2$, $KNO_3$ were added at different concentrations (0.001 M, 0.01 M, and 0.1 M). The extracts were then stored at t = 4±1°C for 12 hours, after which the antioxidant activity and the colour parameters (CIEL*a*b*) were measured. The parameter $(A-A_0)/A_0$ was calculated and expressed as a percentage, in order to assess the hyperchromic shift, where A=absorption after salt addition, $A_0$=absorbtion of the extract in the absence of the salts, both at λ=520 nm (Gonzalez-Manzano et al., 2009; Malaj et al., 2013).

### *2.4 Studies on pH*

The extracts were brought to the following values of pH: 2.6; 3.7; 5.5; 7.4, and 8.0 using adequate buffers, i.e. buffer pH=3.7 (glycocoll and sodium chloride), buffer pH=5.5 (sodium citrate), pH=7.4 (PBS – phosphate-buffered saline and sodium dihydrogenphophate), but also NaOH (0.1 M) and HCl (0.1 M), then stored at t = 4±1° C for 12 hours. Control samples were prepared by diluting the extracts with the same volumes of ethanol 50% (v/v) as the ones of the buffers used for pH adjustment. Afterwards, the antioxidant activity and the colour parameters (CIELab) were determined.

### *2.5 Antioxidant activity by reaction with ABTS radical*

The antioxidant activity of the extracts was measured using the assay with ABTS radical. ABTS was dissolved in distilled water to 7 mM concentration, after which the ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark for 12-16 hours before use. Before analysis, the ABTS radical solution was diluted and equilibrated to an absorbance of 0.70 (±0.02) at 734 nm. 2.0 mL of diluted ABTS radical solution were added to 20 μL of sample then the absorbance was measured after 1 to 6 minutes after the initial mixing, using ethanol as a blank (Re et al., 1999). The results were expressed as mmol trolox equivalent (TE) /L, from a calibration curve (0-2000 μmol/L; $R^2$=0.9974).

### *2.6 Total polyphenols by Folin-Ciocalteu method*

The determination of total polyphenols was performed by introducing the following into a test tube: 0.2 mL of the sample, previously diluted; 6 mL of distilled water; 0.5 mL of Folin-Ciocalteau 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. The absorbance was measured at 750 nm through a path length of 1 cm against a blank prepared with distilled water in place of the sample(Singleton & Rossi, 1965). The results of total polyphenols were calculated from a calibration curve of gallic acid (0-500 mg/L, $R^2$=0.9988), and expressed in mg equivalents of gallic acid (mg GAE).

### *2.7 Total flavonoids by Folin-Ciocalteu*

The total flavonoid content was determined using formaldehyde precipitation in strong acidic medium, following the method described by Spranger et al. (2008). 2.5 mL of the extract were placed in a brown-coloured vial. Afterwards, 1.25 mL of HCl diluted with distilled water (50:50 by volume) and 1.25 mL of formaldehyde were added in the same vial. The mixture was left to stand for 24 hours at t=4°C. After 24 hours, the mixture was filtered and the non flavonoid polyphenol content was determined by the method described in section 2.6(Filimon et al., 2017). The total flavonoid content was calculated by the difference between the total content of polyphenols previously determined and the polyphenol content remained after the flavonoids precipitation with formaldehyde.

### *2.8 Total polyphenols by Abs 280*

The total polyphenolic content was also determined by measuring the absorbance at 280 nm and expressed as mg equivalent of gallic acid (mg GAE) by construction of a calibration curve(0-50 mg/L, $R^2$=0.9958)(Patras et al., 2017), following the method described by Ribereau-Gayon et al., 2006.

### *2.9 The content of anthocyanins by difference of pH*

The contents of total and monomeric anthocyanins were determined by reading the absorbance at 520 nm and 700 nm, 20 minutes after the addition of 4 mL of pH=1.0 (sodium acetate) and respectively, pH=4.5 (sodium citrate) buffer solutions to 1 mL of appropriate diluted sample(Giusti& Wrolstad, 2001 with modifications). The results were calculated with the following formulae and expressed as malvidin-3-glucoside equivalents (mg ME)/L extract:

Total anthocyanins, mg ME/L = ($A_T$ x MW x d x 1000)/(ε x 1)

Monomeric anthocyanins, mg ME/L = ($A_M$ x MW x d x 1000)/(ε x 1)

$A_T = (Abs_{520} - Abs_{700})_{pH\ 1.0}$

$A_M = (Abs_{520} - Abs_{700})_{pH\ 1.0} - (Abs_{520} - Abs_{700})_{pH\ 4.5}$

MW – molecular weight of malvidin-3-glucoside (493.4 g/mol)

d - dilution factor

ε - molar absorptivity of malvidin-3-glucoside (ε =37700)

1 – pathlength (1 cm)

### *2.10 Total cinnamic acids derivatives*

The content of total cinnamic acids derivatives was determined by acidifying 0.25 mL of extract with 0.25 mL acidified ethanol (0.1% in 95% ethanol) and 4.55 mL HCl (2%). After 20 min, the absorbance was read at 320 nm and the results were expressed as caffeic acid equivalents (CAE) based on a calibration curve (0-50 mg/L, $R^2$=0.9994) with standard of caffeic acid(Sant'Anna et al., 2012; Demir et al., 2014).

### *2.11 Total flavonols*

The content of total flavonols was determined by acidifying 0.25 mL of extract with 0.25 mL acidified ethanol (0.1% in 95% ethanol) and 4.55 mL HCl (2%). After 20 min, the absorbance was read at 360 nm and the results were expressed as quercetin equivalents (QE) based on a calibration curve (0-50 mg/L, $R^2$=0.9967) with standard of quercetin(Sant'Anna et al., 2012; Demir et al., 2014).

### *2.12 Colour parameters (CIEL*a*b*)*

The CIEL*a*b* parameters were determined using the Analytic Jena Specord 200 Plus (Germany) spectrophotometer, as mentioned previously. The calculations were made using the software WinASPECT PLUS provided by the same company. The transmittance of all the samples 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 at $10^{o}$. The results present three colorimetric coordinates, i.e. luminosity (L*), red/green component (a*), blue/yellow component (b*) and two derived magnitudes, i.e. chromaticity (C*), hue (H*). The overall colour difference (ΔE*) between the control and each extract with modified medium, by either addition of salt or pH change, was calculated, using the formula $\boldsymbol{\Delta E^*} = \sqrt{(\boldsymbol{\Delta L^*})^2 + (\boldsymbol{\Delta a^*})^2 + (\boldsymbol{\Delta b^*})^2}$, where ΔL* - difference of luminosity between the control and the sample with modified medium, Δa*- difference of red/green components between the control and the sample with modified medium, Δb* - difference of blue/yellow component between the control and the sample with modified medium(OIV, 2013).

### *2.13 HPLCanalysis of polyphenols*

The polyphenol composition was analysed using the Agilent 1100 Series HPLC. The gradient was optimised using trifluoroacetic acid (TFA) as an eluent acidification of 1% $CH_3OH$ (A channel) and 50% $CH_3OH$ (B channel) acidified to 2.15 pH with TFA. The column system was composed of a pre-column SecurityGuard ULTRA Cartridges HPLC C18 for 4.6 mm ID couplet to Kinetex 5 µm C18 100 Å 250×4.6 mm columns manufactured by Phenomenex at 35$^{o}$C. The injection volume was 20 µL and the run time 90 min. The phases were A: $H_2O$: $CH_3OH$ (99:1) and B: $H_2O$: $CH_3OH$ (50:50), with a flow of 1.5 mL/min. The detection was carried out at 256 nm, 280 nm, 324 nm, and 365 nm. The gradient of elution was 100% (A): for 10 min; 82% (A): 18% (B) for the next 10 min; 70% (A): 30% (B) for 10 min; 65% (A): 35% (B) for 6 min; 40% (A): 60% (B) for 15 min; 20% (A): 80% (B) for 5 min; 100% (B) for 15 min. and 100% (A) for 10 min. The content of specific polyphenols was determined by comparison of retention times and peaks of the red grape marc chromatogram with the ones from the chromatogram of a synthetic mixture containing the following standards: (+)-catechin, morin hydrate, ellagic acid, benzoic acid, quercetin, caffeic acid, (+)-rutintrihydrate, syringic acid, ferulic acid, gallic acid, protocatechuic acid, gentisic acid, parahydroxybenzoic acid, salicylic acid, para-coumaric acid, D (-)-quinic acid, sinapic acid, methyl 4-hydroxy-3-methoxycinnamate, procyanidin B1, procyanidin B2, polydatin, hyperoside, and trans-resveratrol.

### *2.14 Statistical analysis*

The mean values and the standard deviations were calculated from 3 parallel experiments using three extraction procedures for polyphenol composition and antioxidant activity analysis, and the same extract to study the influence of different treatments. One-way 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.

## 3. Results and Discussion

### *3.1 The phenolic content and the antioxidant activity of the original grape marc extract*

The phenolic composition and the antioxidant activity of the initial extract were analysed and the results are presented in Table 1. These data demonstrate that the used red grape marc is an important source of antioxidants and could be of interest to food processors and consumers. In addition, the composition of extracts can be used to explain the changes in colour and antioxidant activity observed after pH change and the addition of salts.

**Table 1. Composition of polyphenols and antioxidant activity of the grape marc extract used for experiments (the results are presented as means±standard deviations of three experiments)**

The total concentrations of polyphenols measured by the two methods had comparable values, although the content of polyphenols obtained by Folin-Ciocalteu method was higher. It is documented that there are many interfering substances when the total polyphenol content is determined by the Folin-Ciocalteu method. Any substance with reducing properties such as reducing sugars, ascorbic acid, some proteins interact with the Folin-Ciocalteu reagent (Box, 1983). In this way, this reagent determines not only the content of polyphenols, but the reducing potential of a solution (Singleton & Rossi, 1965) and therefore it is considered suitable for the determination of the total antioxidant activity.

Other authors have obtained similar results, even though the content of different phenolics is greatly affected by several factors such as, grape variety, extraction method, type and volume of solvent and others. Negro et al. (2003) have obtained the values 4.19 g/100 g for polyphenols, 3.94 g/100 g for flavonoids and 0.98 g/100 g for anthocyanins, all the results being expressed as g/100 g dry marc. Their results for polyphenols and flavonoids are similar to those obtained in the present research, if the values are compared in the same units.

Sant'Anna et al. (2012) obtained the maximum total polyphenol extraction from wine marc at the solid-to-liquid ratio of 1 g dried marc to 50 mL of 50% ethanol. Moreover, the yields of extraction ranged from 11 to 22 mg GAE/g(Sant'Anna et al., 2012).

With regards to specific phenolics, gallic acid, procyanidin B1, catechin, epicatechin, ferulic acid methyl ester, hyperoside, polydatin, ferulic, chlorogenic and salicylic acids were the main compounds found in the grape marc extract. Tournmour et al., (2015) analysed grape pomace from Portuguese cultivars. The obtained values for antioxidant activity (ORAC) and total polyphenols were comprised between 906 and 2337 μmolTE/ g and respectively, 142.4 ± 1.1 mg GAE/g of dry pomace, which are higher than the ones obtained in this study. The results of HPLC analysis revealed the presence of gallic acid, caffeic acid, (+) catechin, syringic acid, and (-)catechin, the latter two being the major identified compounds (Tournmour et al., 2015). Different results may be explained by the fact that the marc was obtained from different grape varieties and has resulted from different winemaking techniques(Apolinar-Valiente et al., 2015). Ramirez-Lopez and DeWitt (2014) have analysed commercial dried grape pomace by high-performance liquid chromatography electrospray ionization mass spectrometry and have determined a total of 16 phenolic compounds among which epicatechingallate, catechin hydrate, quercetin, caffeic, ferulic, gallic and protocatechuic acids, i.e. components or derivatives also identified in the present study.

### *3.2 The effect of pH on the antioxidant activity and colour*

Figure 1 presents the change of the antioxidant activity after pH modification of the red grape marc extract.

**Figure 1. The dependence of the antioxidant activity on the grape marc extract's pH (errors bars present the standard deviation of three determinations, different letters designate statistically different results)**

Different values of pH had little influence on the antioxidant activity of the ethanolic grape marc extract. The highest value was determined for the pH=3.7 although not significantly different from the control which had the initial pH=4.4. The analysis between pairs of treatment revealed a statistically significant difference between the values found at pH=3.7 and pH=2.6; pH=3.7 and pH=5.5.

Altukaya et al. (2016) studied the influence of pH on the antioxidant activities of lettuce extract with quercetin, green tea extract, and grape seed extract. The authors found enhanced scavenging effect with increasing pH values and have explained this effect by the increase of the electron-donating ability upon deprotonation and stabilization in alkaline solutions (Altukaya et al., 2016).Saeedeh et al. (2007) have evaluated the antioxidant activity of drumstick leaves, mint leaves and carrot tuber extracts as well as its stability at different pH values, i.e. 4 and 9. The antioxidant activity of mint and carrot extracts was found to be higher at pH 9 than at pH 4, while the one of drumstick extract remained the same under both pH conditions (Saeedeh et al., 2007).

The antioxidant activity has been correlated with the number of hydroxyl groups and their hydrogen donating abilities (Lemanska et al., 2001; Jabbari & Gharib, 2012; Chen et al., 2014). Additional OH groups in ortho position increase the scavenging activity of polyphenols, especially at pH values superior to 4(Altukaya et al., 2016). Thus, the structure of each phenolic compound must be taken into account when explaining the change of the antioxidant activity of the extract.

**Table 2. CIEL*a*b* parameters' dependence on the pH of the grape marc extracts (results are expressed as means±standard deviation, different letters designate significantly different results between pairs of test and control for each value of pH)**

The highest decrease in luminosity was observed for the pH values of 2.6, 3.7, and 8.8. Furthermore, the redness, the blueness, the chroma and the hue angle were significantly enhanced at acidic pHs namely 2.6 and 3.7, values found in products such as lemon juice, vinegar, various syrups, and fruit juice, ketchup, fermented dairy products, pickled vegetables, various preserves, respectively. These changes are explained by the fact that the flavylium cation is stabilised by the excess of $H^+$. On the other hand, the red/green (a*) and blue/yellow (b*) parameters were shifted towards green and yellow values, respectively, at pH≥7.4, because of the degradation of the red and blue pigments. A significant decrease of chroma which translates as colour quality was observed for pH=5.5 and pH=7.4. The results obtained for pH≥5.5 suggest that grape marc extract should be used with great care in foods with such media. Ready-to-eat meals, bread, nectars, soups and sauces, cheese, and tea are among the products with a pH>5.5. All these modifications caused significant changes in the overall colour, although of different nature, in both acidic and alkaline media. In strong acidic medium, the colour has been enhanced, while in alkaline medium it changed from red-blue to green-yellow. According to Gonnet (2001) and Martinez et al. (2011), these colour changes will be perceived by the human eye.

### *3.3 The influence of the ionic strength on the antioxidant activity and CIEL*a*b* parameters*

The antioxidant activity was not affected significantly by the presence of added salts. Only the higher concentration of $CaCl_2$ decreased its value from 29.59 mmolTE/L to 17.30 mmol TE/L, modification which was found to be statistically significant (fig. 2). The decrease of the antioxidant activity (fig. 2) caused by the addiction of 0.01 M of calcium chloride was also close to significance threshold. Subsequent verification using Scheffé and Holm-Bonferoni multiple comparisons showed that the value is significantly different when the treatments are only compared to the control. Therefore, $CaCl_2$ could have a negative impact on the antioxidant activity of the grape marc ethanolic extract when concentrations equal or higher than 0.01 M are added.

**Figure 2. Change of the antioxidant activity for different salts and different concentrations added to the grape marc extract (error bars present the standard deviations of three replicates, different letters designate statistically different results)**

Several studies show that flavonoids also act as metal chelators, thus the interaction between metal ions and flavonoids may change their antioxidant activity (Jabbari & Gharib, 2012). Jabbari & Gharib (2012) have determined that metal chelation with cerium (IV) enhances the radical scavenging activity of flavonoids. These results were not confirmed for the ions studied in the present experiment.

The most significant effect on all colour parameters was exerted by calcium chloride (table 3). Moreover, it was observed that the higher the concentration of the added salt, the higher the effect on the colour of the extract. The addition of this salt caused mainly a decrease of luminosity and an increase of redness, which resulted in enhanced chromaticity and important overall colour differences. Considering the fact that certain salts could modify the pH of a solution, the latter was measured after the addition of calcium chloride (table 3). The results show a gradual decrease of pH, with a difference of 1.2 between the control and the extract containing 0.1 M $CaCl_2$. Nonetheless, the same concentration of salt reduced the pH of distilled water from 7.8±0.2 to 7.5±0.2 when 0.1 M of calcium chloride was added, while the other two tested concentrations, i.e. 0.001 M and 0.01 had an insignificant effect on water pH. This difference in pH could be attributed to the formation of partially dissociated complexes between calcium and anions of carboxylic and weak acids (Joseph, 1946). Consequently, the enhancement in colour would be attributed to the stabilization of the flavylium ion in acidic medium, however the overall colour difference in this case is greater than in the case of pH modification using buffer solutions (table 2). The enhancement of colour through the addition of metals and divalent ions was documented by other authors(Cortez et al., 2017). This phenomenon could also be explained by the processes of polymerization and complexation between anthocyanins and metal ions(Negro et al., 2003). The significant increase of colour quality and intensity is interesting and could be used in the creation of new food dyes of natural origin. Furthermore, it would be interesting to investigate if such colourant could act as a calcium delivery system, if used in calcium-enriched dairy products such as yoghurts. Thus, detailed kinetic and nutritional studies are recommended.

**Table 3. Change of CIEL*a*b* parameters for different salts and different concentrations added to the grape marc extract (standard deviations are based on three replicates, different letters designate significantly different results)**

Ngo & Zhao (2009) have studied the stabilization of anthocyanins on thermally processed red d'Anjou pears through complexation with Sn in the presence of hydrochloric acid, formaldehyde, and tannic acid. The treatment resulted in red pigments and although their nature is unknown, all four reagents were required to provide the stabilization. Polymerization was considered by the authors as the main responsible reaction. Furthermore, the authors observed both bathochromic and hyperchromic shifts when Sn was added alone (Ngo & Zhao, 2009). Other metals that have been studied are tin, copper, aluminium, magnesium, and potassium in the quest to obtain a natural blue food dye from anthocyanins(Yoshida et al., 2009; Cortez et al., 2017). Only, a slight influence on the blueness of the extract was observed in this study.

The parameter $(A-A_0)/A_0$ was also calculated for the extracts treated with different salts (fig. 3). A drastic hyperchromic shift was observed when $CaCl_2$ was added to the extract, the intensity of which increased with the greater concentration of the salt. Given $\lambda$=520 nm, the anthocyanins are the main molecules involved. The analysis of the results has shown that the addition of calcium salts in a concentration of 0.01 M can significantly improve the colour of the extract without affecting its radical-scavenging ability.

**Figure 3. Change of ($A$-$A_0$)/$A_0$ entity in the grape marc extracts with different salts added in different concentrations (error bars present the standard deviations of three replicates, different letters designate statistically different results)**

The capacity of anthocyanins to form metal complexes is related to the ortho-dihydroxyl arrangement on the B ring. While the glucosides of cyanidin, delphinidin and petunidin can form such complexes, the ones of malvidin, pelargonidin and peonidin cannot, therefore it is unlikely that pigment-metal complexes play a significant role in the colour of grape marc extracts,some authors suggest(Boulton, 2001). Molecular stacking is necessary and special conditions are required to achieve stable colour formation. Previous studies indicate that the chirality of stacking is influenced by the glucosyl residues. Moreover, glycosyl residues are indispensable in metal-anthocyanin complex formation(Cheynier et al., 2012).

A research on the composition and structure of the anthocyanins present in the extract, as well as a study on the interactions between the respective anthocyanins, the weak acids present in the extract and calcium ions are recommended.

## 4. Conclusions

This study has brought further proof that grape marc extracts contain high amounts of polyphenols from different classes, which results in a fairly high antioxidant activity. The main identified phenolics were gallic acid, polydatin, procyanidin B1, ferulic acid methyl ester, catechin, epicatechin, hyperoside, ferulic, chlorogenic, and salicylic acids.

The results of this research have shown that the presence of several ions does not significantly affect the antioxidant activity. The most noticeable effect was exhibited by high concentration of $CaCl_2$, which decreased antioxidant activity from 29.59 mmol TE/L to 17.30 mmol TE/L. Furthermore, calcium salts have also shown the most significant effect on all colour parameters, by visibly enhancing the colour of grape marc extract. This phenomenon was explained by the complexation between anthocyanins and metal ions or the decrease of pH. The increase of colour intensity is potentially interesting for the food industry since it could be exploited for the formulation of new food dyes.

Different pH values had little influence on the antioxidant activity of the ethanolic grape marc extract. The highest value was determined for the pH=3.7, although, not significantly different from the control sample. The redness, blueness, the chroma and the hue angle were also significantly enhanced at this value of pH, as well as at pH=2.6. The colour of the extract was however affected in a negative way by pH values higher than 5.5, which is why the extract should be used with care in products with such media.

## Conflict of interest

The authors have declared no conflict of interest.

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**Table 1. Composition of polyphenols and antioxidant activity of the grape marc extract used for experiments (the results are presented as means±standard deviations of three experiments)**

| Polyphenols and antioxidant activity | Value (per 1 L of extract and 100 g grape marc) |
|---|---|
| Total polyphenols (Folin-Ciocalteu) | 3749±128 mg GAE |
| Total polyphenols (Abs280) | 2791±70 mg GAE |
| Total flavonoids | 3699±70 mg GAE |
| Total anthocyanins | 138±2 mg ME |
| Monomeric anthocyanins | 116±2 mg ME |
| Cinnamic acids derivatives | 446±21 mg CAE |
| Flavonols | 358±15 mg QE |
| **Individual polyphenols** | |
| Gallic acid | 7.40±0.60 mg |
| Protocatechuic acid | 0.48±1.60 mg |
| p-hydroxybenzoic acid | Traces |
| Procyanidin B1 | 3.90±0.30 mg |
| m-hydroxybenzoic acid | 0.14±0.01 mg |
| Catechin | 29.10±13.20 mg |
| Vanillic acid | 1.20±0.80 mg |
| Procyanidin B2 | 0.30±0.10 mg |
| Epicatechin | 5.10±0.00 mg |
| Ferulic acid | 4.00±2.00 mg |
| Sinapic acid | 0.48±0.16 mg |
| Trans-resveratrol | Traces |
| Hyperoside | 4.50±0.00 mg |
| Cis-resveratrol | 0.12±0.00 mg |
| Ferulic acid methyl ester | 24.40±8.50 mg |
| Quercetin | 0.30±0.20 mg |
| Caffeic acid | Traces |
| Chlorogenic acid | 2.80±0.00 mg |
| Polydatine | 7.20±0.00 mg |
| Salicylic acid | 70.00±0.00 mg |
| **Antioxidant activity** | 29.59±0.00 mmolTE |

**Table 2.CIEL*a*b* parameters' dependence of pH (results are expressed as means±standard deviation, different letters designate significantly different results between pairs of test and control for each value of pH)**

| CIEL*a*b* parameters | L* | a* | b* | C* | H* | ΔE* |
|---|---|---|---|---|---|---|
| **Control 2.6** | 86.9±0.8$^{a}$ | 11.5±0.4$^{a}$ | -2.7±0.2$^{a}$ | 11.8±0.3$^{a}$ | -4.12±0.51$^{a}$ | |
| **pH=2.6** | 72.1±2.2$^{b}$ | 48.1±3.1$^{b}$ | -5.3±0.1$^{b}$ | 48.4±3.1$^{b}$ | -9.07±0.50$^{b}$ | 39.56±3.04 |
| **Control 3.7** | 83.5±0.1$^{a}$ | 14.9±0.1$^{a}$ | -4.7±0.1$^{a}$ | 15.6±0.1$^{a}$ | -3.05±0.01$^{a}$ | |
| **pH=3.7** | 81.4±4.5$^{a}$ | 22.2±3.2$^{b}$ | -3.6±0.7$^{b}$ | 22.4±3.2$^{b}$ | -6.19±0.42$^{b}$ | 8.25±5.42 |
| **Control 5.5** | 74.4±0.8$^{a}$ | 22.4±0.6$^{a}$ | -5.5±0.3$^{a}$ | 23.0±0.6$^{a}$ | -4.00±0.09$^{a}$ | |
| **pH=5.5** | 79.6±0.1$^{b}$ | 11.7±0.1$^{b}$ | -1.0±0.1$^{b}$ | 11.7±0.1$^{b}$ | -11.80±1.60$^{b}$ | 12.72±0.88 |
| **Control 7.4** | 74.4±0.8$^{a}$ | 22.4±0.6$^{a}$ | -5.5±0.3$^{a}$ | 23.0±0.6$^{a}$ | -4.00±0.09$^{a}$ | |
| **pH=7.4** | 77.2±0.1$^{a}$ | -0.7±0.2$^{b}$ | 9.3±0.3$^{b}$ | 9.3±0.3$^{b}$ | -0.46±1.02$^{b}$ | 27.58±0.81 |
| **Control 8.8** | 83.5±0.1$^{a}$ | 14.9±0.1$^{a}$ | -4.7±0.1$^{a}$ | 15.6±0.1$^{a}$ | -3.05±0.01$^{a}$ | |
| **pH=8.8** | 81.3±0.2$^{a}$ | -3.2±0.1$^{b}$ | 14.7±0.5$^{b}$ | 15.1±0.4$^{a}$ | -0.16±0.26$^{b}$ | 26.62±0.41 |

**Table 3. Change of CIEL*a*b* parameters for different salts and different concentrations (standard deviations are based on three replicates, different letters designate significantly different results)**

| Salt and concentration | L* | a* | b* | H* | C* | ΔE* |
|---|---|---|---|---|---|---|
| **Control** | 65.60±0.12$^{cd}$ | 30.00±0.19$^{ab}$ | -7.14±0.09$^{cd}$ | -4.12±0.08$^{b}$ | 30.84±0.16$^{ab}$ | - |
| **NaCl 0.001 M** | 65.76±0.14$^{cd}$ | 31.61±0.33$^{ab}$ | -7.66±0.18$^{bcd}$ | -4.05±0.85$^{b}$ | 32.52±0.35$^{ab}$ | 1.70±0.17 |
| **NaCl 0.01 M** | 65.79±0.52$^{cd}$ | 31.95±0.37$^{ab}$ | -7.81±0.21$^{b}$ | -4.01±0.07$^{b}$ | 32.89±0.40$^{ab}$ | 2.07±0.45 |
| **NaCl 0.1 M** | 65.88±0.10$^{c}$ | 35.99±0.28$^{b}$ | -9.18±0.10$^{ab}$ | -3.83±0.01$^{b}$ | 37.14±0.30$^{b}$ | 6.33±0.09 |
| **$KNO_3$ 0.001 M** | 68.96±0.68$^{e}$ | 27.89±0.81$^{a}$ | -5.75±0.50$^{a}$ | -4.80±0.31$^{ab}$ | 28.48±0.88$^{a}$ | 4.20±0.93 |
| **$KNO_3$ 0.01 M** | 68.52±0.15$^{de}$ | 29.54±0.52$^{ab}$ | -6.29±0.22$^{cd}$ | -4.62±0.09$^{ab}$ | 30.21±0.55$^{a}$ | 3.08±0.36 |
| **$KNO_3$ 0.1 M** | 67.27±0.20$^{de}$ | 32.44±0.47$^{b}$ | -7.23±0.10$^{cd}$ | -4.41±0.01$^{ab}$ | 33.24±0.48$^{ab}$ | 2.96±0.29 |
| **$CaCl_2$ 0.001 M*** | 59.82±2.68$^{b}$ | 45.55±6.70$^{c}$ | -9.80±0.88$^{a}$ | -4.56±0.29$^{ab}$ | 46.60±6.74$^{c}$ | 16.80±7.03 |
| **$CaCl_2$ 0.01 M**** | 51.59±0.72$^{a}$ | 64.65±1.15$^{c}$ | -9.40±0.42$^{a}$ | -6.84±0.42$^{a}$ | 65.33±1.08$^{d}$ | 37.44±1.79 |
| **$CaCl_2$ 0.1 M***** | 47.46±0.94$^{a}$ | 69.00±0.19$^{c}$ | -6.25±1.07$^{d}$ | -11.24±2.10$^{a}$ | 69.29±0.16$^{d}$ | 43.02±1.28 |

*pH=4.1±0.1 after the addition of the salt; **pH=3.7±0.1 after the addition of salt; ***pH=3.2±0.1 after the addition of salt

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