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Chemical composition, nutritional and *in vitro* functional properties of by-products from the Brazilian organic grape juice industry

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Abstract

Grape juice and wines (*Vitis* sp.) are the products that are most produced and consumed from different varieties of grapes. About 20% of pomace is generated from processing but there are very few products available with added grape pomace. Consequently, the aim of this study was to characterize flours made from grape skin (GSF) and grape pomace (GPF: seeds + skin) from organic *Vitis labrusca* L. cv. Bordeaux with respect to physicochemical composition, bioactive compounds content and antioxidant/reducing capacities. The by-products presented a high content of insoluble fibers (55.84 ± 0.63 and 51.02 ± 1.12 g/100 g, respectively); total lipids contents below 10 g/100 g; and >1.900 mg gallic acid equivalent (GAE)/100 g total phenolic content, in which total ortho-diphenols, flavonols, anthocyanins and water-soluble condensed tannins were the main chemical compounds. Regarding the in vitro functionality, which was measured by chemical methods, the GPF and GSF presented values of 1373.64 ± 72.94 and 361.48 ± 2.99 mg GAE/100 g iron reducing capacity; 1574.26 ± 8.94 and 1499.79 ± 31.39 mg ascorbic acid equivalent/100 g in the reduction of DPPH radical; and 2892.46 ± 61.69 and 426.34 ± 18.85 mg of quercetin equivalent/100 g of total reducing capacity, respectively. The results indicate that the GPF and GSF produced from the organic grape juice industry are promising materials for use by food companies.

Introduction

By-products from food companies need to be better used because, according to González-Centeno *et al.* (2013), these wastes are valuable raw materials, which are of growing interest due to the possibility of exploiting their natural bioactive compounds. Fruits and beverages, such as tea and red wine, are the main sources of phenolic compounds; they form a considerable part of dietary phytochemicals, which are related, in part, to a number of health benefits. Epidemiological evidence suggests that the consumption of phenolic compounds exerts protective effects against the occurrence of various diseases. However, several factors can affect the content of phenolic compounds in these foods such as variety, degree of ripeness, environmental conditions, processing and storage conditions (Kårlund *et al*., 2014).

Grapes (*Vitis* sp.) are one of the most cultivated fruit crops in the world, with more than 60 million tons produced annually. Winemaking is an important agricultural activity in several countries of southern Europe, such as Spain, Italy and France, and it produces huge amounts of grape pomace; this by-product mainly consists of skin, seeds and some stalks (Rondeau *et al*., 2013). According to Rondeau *et al.* (2013), in France, about 700,000 to one million ton of dry grape pomace are produced each year. Considering the growing demand for green materials and components, by-products such as grape pomace have obvious potential as a renewable raw material. Grapes are processed to make various products and this generates large amounts of semi-solid waste (grape pomace = a mixture of grape skin and seeds), with a high content of phenolic compounds, which is a valuable source of natural antioxidants (Ruberto *et al*., 2007; Hogan *et al*., 2010; Drosou *et al.*, 2015).

The importance of polyphenols, combined with the growing demand for nutraceutical compounds and antioxidants, makes the study of by-products from the food, nutraceutical, pharmaceutical and chemical industries of great importance (Balasundram *et al.*, 2006; Cheng *et al*., 2012; Sólyom *et al*., 2014; Jara-Palacios *et al*., 2015; Tournour *et al*., 2015). Although the composition of different grape pomaces is already known, the present study aimed to characterize the nutritional and *in vitro* functional properties of grape pomace, as well as determining some chemical markers of by-products from the organic Bordeaux grape (*Vitis labrusca* L.) juice industry.

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Materials and Methods

Chemicals

Ethanol, Folin-Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), gallic acid, sodium molibdate dihydrate, vanillin, quercetin, chlorogenic acid, (+)-catechin, and ascorbic acid were all obtained from Sigma-Aldrich (St. Louis, USA) and ultra-pure water was used in all experiments. All other reagents were of analytical grade.

Grape by-products

The dried organic Bordeaux grape (*Vitis labrusca* L.) by-products, namely grape skin flour (GSF) and grape pomace flour (GPF), which is mainly composed of partially defatted seeds and grape skin fragments, were kindly donated by the grape juice producer Uva'Só (producing region: Bento Gonçalves, Rio Grande do Sul, Brazil). The by-products were ground and sieved (28 mesh) to standardize the particle size. The materials were certified for organic agriculture by EcoCert Brazil, a third-party certification company in Brazil.

Physicochemical characterization

Moisture, ash, proteins, and total lipids were determined according to the AOAC official procedures (methods 925-09, 923-03, 920-87, 920-85, respectively). The moisture content was measured by gravimetry after oven drying at 105°C for 7 h, and the ash content was calculated after total combustion at 550°C for 6 h. Total protein was determined using the Kjeldhal methodology, and a factor of 6.25 was adopted to express the total protein content. Total lipid content was quantified by extracting the samples with hexane for 6 h using a Soxhlet apparatus. Total dietary fiber content, including soluble and insoluble fibers, was determined after digestion and washing the residue with hot water and ethanol (Horwitz and Latimer, 2005).

Extraction and analysis of bioactive compounds

In order to quantify the bioactive compounds, the Bordeaux grape pomace (10 g) was extracted three times with 40 mL of a HCl/propanone/water solution (0.1:70:29.9 v/v) using a ultrasonic device for 45 min and the content (120 mL) was then concentrated to 50 mL using a rotatory evaporator following the procedures described by Deng *et al.* (2011).

The total phenolic content (TPC) was assessed using the modified Folin–Ciocalteu assay (Singleton *et al*., 1999) adapted for microplates (Granato *et al.*, 2014a). Firstly, 25 µL of properly diluted samples were mixed with 25 µL of Folin–Ciocalteu reagent and 200 µL of ultrapure water. Then, 25 µL of a saturated sodium carbonate (10.6 g/100 mL) solution was added to the mixture after 5 min reaction and the absorbance was measured at λ = 725 nm after 1 h using a microplate reader (Epoch, BioteK, USA). The baseline was recorded by the absorbance of all the reagents in their proper proportions to replace the corresponding rate for the sample by ultrapure water. Gallic acid was used as a standard to generate the analytical curve (linearity: 0-180 mg/L; R^2=0.977) and the TPC of the extracts was expressed as mg of gallic acid equivalent/100 g of flour.

Total ortho-diphenols content was estimated by the colorimetric method that uses sodium molybdate (Durán *et al*., 1991). Briefly, a 50 µL aliquot of grape pomace extract was mixed with 200 µL of a 5 g/100 mL sodium molybdate dihydrate ethanolic solution (EtOH:H_2O, 1:1 v/v) and left to react for 25 min. The absorbance was recorded at = 370 λ m against a blank (ultrapure water) using a microplate spectrophotometer (Epoch, BioteK, USA). Chlorogenic acid was used as a standard to generate the analytical curve (linearity: 0-160 mg/L; R^2=0.981) and results were expressed as mg of chlorogenic acid equivalent/100 g of flour.

The content of water-soluble condensed tannins was estimated using a modified colorimetric method conducted in acidic medium (H_2SO_4) containing vanillin (Horszwald and Andlauer, 2011). For this purpose, 25 µL of grape pomace extract was diluted in methanol and 150 µL of a 4 g/100 mL vanillin solution and 75 µL of a 32 mL/100 mL H_2SO_4 solution were added in 96-well microplates. After 15 min at room temperature the absorbance was read at λ = 500 nm against a blank (methanol). Water replaced the sample as the blank. (+)-Catechin was used as a standard to generate the analytical curve (linearity: 0-240 mg/L; R^2=0.985) and the results were expressed as mg of (+)-catechin equivalent/100 g of flour.

The total content of flavonols was estimated using the colorimetric method outlined by Yermakov et al. (1987) with modifications. In a microplate containing 96 wells, 80 µL of diluted extract or water (blank) and 80 µL of a 2% $AlCl_3.6H_2O$ ethanolic solution were mixed and left to react for 5 min. Then, 120 µL of a 0.61 mol/L CH_3COONa solution was added in each well and the absorbance was read at λ = 440 nm after 2.5 h using a microplate reader (Epoch, BioteK, USA).The baseline was recorded by the absorbance of all the reagents in their proper proportions to replace the corresponding rate for the sample by ultrapure water. Quercetin was used as a standard to generate the analytical curve (linearity: 0-70 mg/L;

R^2=0.998) and the results were expressed as mg of quercetin equivalent/100 g of flour.

The content of total monomeric anthocyanins was determined by UV-Vis spectrophotometry using the differential pH method as described by Lee *et al.* (2005) with minor modifications. Buffer solutions of potassium chloride at pH 1.0 (0.025 mol/L) and sodium acetate at pH 4.5 (0.40 mol/L) were used in the experiments. An amount of 0.40 mL of diluted grape pomace extract was mixed with 3.60 mL of each buffer solution in separate tubes and the mixtures were read at λ = 520 and λ = 700 nm against a blank (ultrapure water). The absorbance (A) was calculated by: $A= (A_{520} – A_{700})$ pH1 - $(A_{520} – A_{700})$ pH4.5. Then, the total content of monomeric anthocyanins (MA) was estimated by: MA = [(A x MW x DF x 1000)]/Ma, where MW: molecular weight (493.20), DF: dilution factor, and Ma: molar absorptivity of malvidin-3-glucoside (28.000). The results were expressed as mg of malvidin-3-glucoside equivalent/100 g of flour.

Reducing capacity and antioxidant activity

The iron reducing capacity (IRC) of the extracts was estimated using the Prussian Blue method (Price and Butler, 1977) with the modifications proposed by Margraf *et al*. (2015). Briefly, an aliquot of 100 µL of a 0.50 mmol/L ferric chloride hexahydrate ($FeCl_3.6H_2O$) solution diluted in HCl 0.01 mol/L was added to 100 µL of properly diluted sample or water (blank) (80-100 times in distilled water) and left to react for 2 min. Then, 100 µL of a 0.50 mmol/L $K_3[Fe(CN)_6]$ solution was added to each well. The absorbance was measured at λ = 725 nm after 25 min reaction using a microplate reader (Epoch, BioteK, USA). Gallic acid was used as standard for the analytical curve (linearity: 0-45 mg/L; R^2=0.979) and the IRC was expressed as mg of gallic acid equivalent/100 g of flour.

The total reducing capacity (TRC) was quantified using the Folin-Ciocalteu (FC) method, in which FC phenol reagent is diluted in isobutanol and the reaction occurs in alkaline medium (Berker *et al*., 2013). This assay was used to measure the reducing potential of both water-soluble and lipophilic antioxidants in the grape pomace extracts. Briefly, isobutanol was used to dilute the FC reagent at a proportion of 1:2 (v/v) and then 50 µL of extract previously diluted in propanone (or pure propanone - blank) was mixed with 75 µL of the FC-isobutanol reagent. Then, 875 µL of a 0.10 mol/L NaOH solution and 1.50 mL of ultrapure water were added to the tube, which was vortexed for 10 s. After 20 min reaction, 250 µL of the reaction mixture was placed in microplates and the absorbance was read at λ = 665 nm against a blank (ultrapure water) using a microplate reader (Epoch, BioteK, USA). Quercetin was used as standard for the analytical curve (linearity: 0-360 mg/L; R^2=0.992) and the TRC was expressed as mg of quercetin equivalent/100 g of flour.

The antioxidant activity of both the grape skin and grape pomace extracts in relation to the DPPH radical was quantified by using a protocol described by Brand-Williams *et al.* (1995) with modifications for microplates (Granato *et al*., 2015a). The assay was conducted in a buffered system at pH 6.0 using 50 mmol/L sodium phosphate and pure ethanol as a solvent of the DPPH radical in a 1:1 (v/v) proportion (Zheng *et al*., 2015). An aliquot of 40 µL of grape pomace extract (diluted with the buffered ethanol solution) was mixed with 260 µL of a 0.10 mmol/L DPPH radical solution in a 96-well microplate. The mixture was allowed to react for 30 minutes at 25°C in the dark and the absorbance was measured at λ = 525 nm against a blank (buffered ethanol solution). An analytical curve was prepared with ascorbic acid (linearity: 0–15 mg/L; R^2=0.995) and results were expressed as mg ascorbic acid equivalent/100 g.

Statistical analysis

The results were presented as means ± SD (n=3) and the differences between extracts were compared using Student's-t test for independent samples, taking $p<0.05$ (Nunes *et al.*, 2015). Graphs were constructed to facilitate the interpretation of the experimental data (Granato *et al.*, 2014b). Action v.2.9 and Microsoft Excel 2010 were used for the statistical analysis.

Results and Discussion

The physicochemical data are shown in Table 1. It can be observed that the grape pomace flour presented higher values ($p<0.05$) of total fiber, moisture and ash content than the grape skin flour. On the other hand, there was no difference in the protein and lipid contents. Tseng and Zhao (2013) studied the composition of red wine grape pomace (*Vitis vinifera* L. cv. Pinot Noir) and found values of 5.63 ± 0.10 g/100 g; 5.07 ± 0.05 g/100 g; 10.32 ± 0.22 g/100 g; 11.09 ± 0.33 g/100 g; and 61.32 ± 1.69 g/100 g for moisture, ash, proteins, lipid and dietary fiber respectively. Some of these values were similar to the results obtained in the present study.

Both the grape skin flour and grape pomace flour presented high levels of extractable phenolic antioxidants (Makris *et al*., 2007). Fontana *et al.* (2013) emphasize that grape pomace is a potential source of phytochemicals which can be recovered as functional compounds for industrial use and can be

Table 1. Physicochemical characterization of organic grape pomace flour and grape skin flour

Composition	GPF*	GSF**	t-value	p-value
Moisture (g/100 g)	8.59 ± 0.43	2.39 ± 0.57	14.982	<0.001
Ash (g/100 g)	5.37 ± 0.16	3.85 ± 0.08	15.191	<0.001
Proteins (g/100 g)***	11.44 ± 0.06	9.83 ± 1.36	2.057	0.109
Lipids (g/100 g)	7.69 ± 0.95	8.54 ± 0.30	-1.488	0.211
Insoluble fiber (g/100 g)	55.84±0.63	51.02 ± 1.12	-7.516	<0.001
Soluble fiber (g/100 g)	8.04 ± 0.77	3.79 ± 0.46	-9.450	<0.001
Total fiber (g/100 g)	63.88 ± 1.05	54.81 ± 0.77	14.296	<0.001

*GPF: grape pomace flour; **GSF: grape skin flour; ***Factor: N*6.25

Table 2. Bioactive compounds in organic grape pomace flour and grape skin flour

	GPF*	GSF**	t-value	p-value
Ortho-diphenols[a]	852.68 ± 46.62	467.88 ± 8.76	14.038	<0.001
Total phenolic content[b]	2179.68 ± 35.15	1976.98 ± 57.36	5.219	0.006
Flavonols[c]	698.03 ± 16.18	95.35 ± 3.99	72.345	<0.001
Total condensed tannins[d]	2933.86 ± 100.75	587.94 ± 16.34	40.069	<0.001
Monomeric anthocyanins[e]	102.82 ± 13.86	26.05 ± 1.68	9.523	0.001

*GPF: Grape pomace flour; **GSF: Grape skin flour; a Expressed as mg of chlorogenic acid equivalent/100 g; b Expressed as mg of gallic acid equivalent/100 g; c Expressed as mg of quercetin equivalent/100 g; d Expressed as mg of (+)-catechin equivalent/100 g; e Expressed as mg of malvidin-3-glucoside equivalent/100 g

potentially used as a raw material to develop new food formulations (Table 2). The results found for total phenolic content were 2179.68 ± 35.15 and 1976.98 ± 57.36 mg GAE/100 g for both pomace and grape skin flours, respectively. These values were higher than found by Ky and Teissedre (2015) in a study of aqueous and ethanol extracts of the pomace and skin of different varieties of grapes; the values in that study were 1282.20 and 1956.60 mg GAE/100 g for the Grenache variety in aqueous and ethanol extract, respectively. The amounts found in the present study were also higher than those found for the pulps of pineapple, monbin, guava, papaya, mango, passion fruit, sapodilla and tamarind, and are higher than by-products of guava, soursop, papaya, mango, passion fruit and sapodilla, found by Silva *et al.* (2014) in a broad study using tropical fruits from Brazil.

Montealegre *et al.* (2006) point out that anthocyanins, catechins and other flavanols, phenolic acids and stilbenes are the major phenolic constituents found in grape pomace. Souza *et al*. (2014) obtained values of 1918.00 ± 3.1 mg/100 g for total phenolic content in relation to freeze-dried extract of Bordeaux grape by-products. Iora *et al*. (2015) found values for total phenolic compounds varying between 3014.55 ± 9.09 mg/100 g and 5101.82 ± 119.03 mg/100 g, in Merlot, Tanat and Cabernet Sauvignon grape pomace produced in Brazil. Rockenbach *et al.* (2011a) verified that there was a greater concentration of phenolic compounds in the seeds (2128 to 16,518 mg of catechin equivalent/100 g) than in the skins (660 to 1839 mg catechin equivalent/100 g) of conventional grape pomace from varietals (*V. vinifera* and *V. labrusca*) widely produced in Brazil. Bordeaux and Isabel grape pomace presented a TPC of 633.10 ± 2.40 mg/100 g and 326.20 ± 0.68 mg/100 g, respectively. It is worth noting that there is a varietal effect on the chemical composition, especially on the profile and content of phenolic compounds, of grape pomaces (de la Cerda-Carrasco *et al*., 2015).

Souza *et al*. (2014) obtained values of 538.00 ± 0.40 mg/100 g for total anthocyanins in freeze-dried extract of Bordeaux grape by-products. Values for total monomeric anthocyanins ranging between 1246.85 ± 64.27 mg/100 g and 2092.93 ± 71.57 mg/100 were found by Iora *et al.* (2015) in Merlot, Tanat and Cabernet Sauvignon grape pomace produced in southern Brazil. Rockenbach *et al.*

(2011b) obtained values of 112.20 ± 0.50 mg/100 g and 18.40 ± 0.06 mg/100 g for total monomeric anthocyanins in pomace from Bordeaux and Isabel grapes, respectively. The flavonol content showed statistical significance ($p<0.001$) between the samples, with values of 698.03 ± 16.18 and 95.35 ± 3.99 mg of quercetin equivalent/100 g for the pomace and skin respectively. Flavonols, such as quercetin, rutin, myricetin, kaempferol, and their glucosides, are highly present in grape berries and juices (Granato *et al*., 2015b), and some content from juice is also retained in the pomaces (skin and seeds).

In the present study, the values for ortho-diphenols in the pomace (852.68 ± 46.62 mg chlorogenic acid/100 g) were also higher ($p<0.001$) than for the grape skin flour (467.88 ± 8.76 mg chlorogenic acid/100 g. The content of water-soluble condensed tannins in the extracts showed significant differences ($p<0.001$), with values of 2933.86 ± 100.75 and 587.94 ± 16.34 mg of (+)-catechin equivalent/100 g for the grape pomace and skin flours, respectively. These values are lower than those obtained by Abarghuei *et al.* (2015) in *Vitis vinifera* grape pomace (total tannins: 4970 mg/100 g). Condensed tannins, which are a mixture of monomers and dimers of procyanidins, can be extracted from grape pomace using organic solvents in acidic medium. Bindon *et al.* (2010) suggest that a part of proanthocyanidin oligomers is presumably linked via covalent attachment to polysaccharides in the cell wall material. This may explain the high tannin content in the residual pomace.

According to Samavardhana *et al.* (2015), analytical results for bioactive composition obtained by different research groups are affected by the method used to extract the bioactive compounds (i.e., polarity of solvents, time and temperature of extraction) and by the process used to obtain the wine or juice. The effect of the type of extraction method was also observed by Otero-Pareja *et al*. (2015), where higher values for total phenolic content were obtained for a hydroalcoholic mixture than the values obtained using pure ethanol or water. These authors also verified that there are effects related to temperature and pressure depending on the extraction method: the temperature can increase the extraction or generate the degradation of thermolabile compounds, and the use of higher pressure increases the contact between solvent and analyte. According to Ky and Teissedre (2015), ethanol facilitates the dissolution of grape pomace tissue, thus releasing a larger amount of polyphenols.

Ramirez-Lopez and DeWitt (2014) found that in obtaining phenolic compounds through the use of organic solvents (50% acetone-water, 70% methanol-water, petroleum ether, and 0.01% mixture water-pectinase) a concentration of 70% methanol-water was more effective in recovering anthocyanin monoglucosides, while using 50% methanol-water was more effective in recovering total flavonoids, flavonols, phenolic acids and stilbenes. They also found that organic solvents were responsible for higher values among the solvents that were used. Ky and Teissedre (2015) observed that extraction with 70% ethanol-water was most efficient in obtaining flavan-3-ols and anthocyanins in grape pomace.

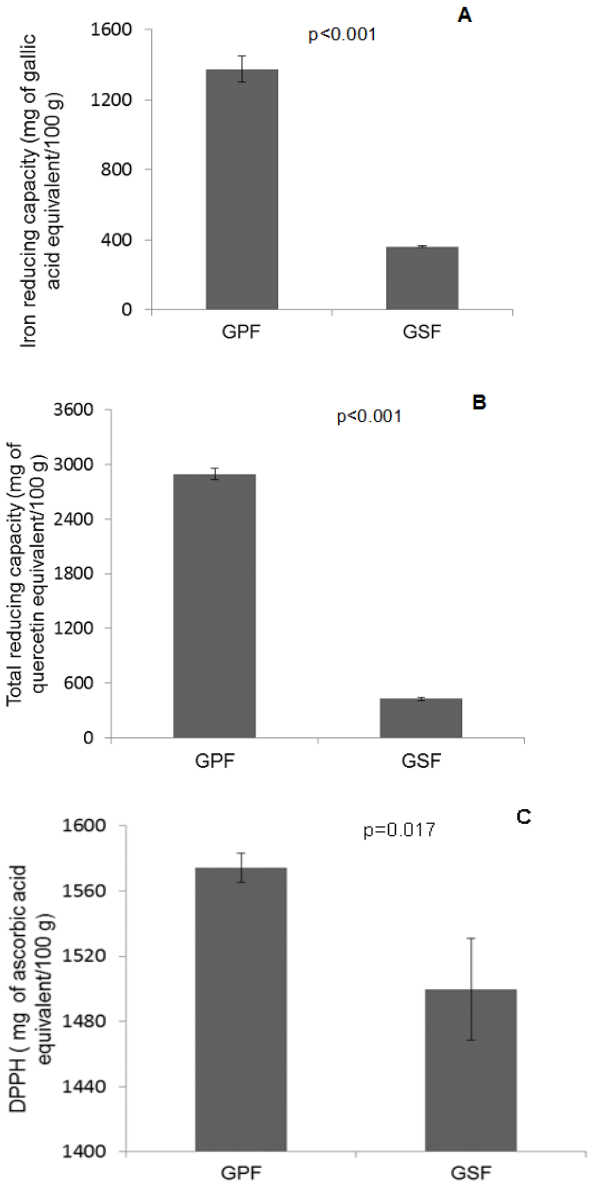

Figure 1. *In vitro* functionality of organic Bordeaux grape pomace flour (GPF) and grape skin flour (GSF). (A) Iron reducing capacity (mg GAE/100 g), (B) total reducing capacity (mg QE/100 g), (C) free-radical scavenging activity toward DPPH (mg AAE/100 g)

The *in vitro* antioxidant activity and reducing capacity of organic Bordeaux grape pomace were measured by evaluating the iron reducing capacity

(IRC), total reducing capacity (TRC) and scavenging of DPPH radical (Figure 1). The IRC mean values were 1373.64 ± 72.94 and 361.48 ± 2.99 mg of gallic acid equivalent/100 g for the grape pomace and grape skin flours, respectively. The results for TRC were 2892.46 ± 61.69 and 426.34 ± 18.85 mg of quercetin equivalent/100 g for the grape pomace and grape skin flours respectively. In the evaluation of antioxidant capacity by the DPPH method, the samples of pomace and grape skin obtained 1574.26 ± 8.94 and 1499.79 ± 31.39 mg of ascorbic acid equivalent/100 g, respectively. These values corroborate the results found by Rockenbach *et al.* (2011b) indicating a high antioxidant activity of grape skin flour and grape pomace flour in relation to the DPPH radical.

It is possible to observe that the grape pomace flour presented higher ($p<0.001$) antioxidant activity and reducing capacity compared to the grape skin flour. This effect was related to the higher content of phenolic compounds present in the grape pomace flour. Plaza *et al*. (2014) point out that the antioxidant capability of chemical compounds or food extracts is dependent on many specific chemical structural characteristics, in which the number and/or position of hydroxyl groups is one of the main factors. Another aspect that also impacts on the quantitative antioxidant level of an extract/chemical compound, is the type of assay used to measure such bioactivity (concentration of reagents, time of reaction, solvent used).

Tseng and Zhao (2013) obtained values for DPPH radical scavenging activity of 37.46 ± 1.86 mg of ascorbic acid equivalent/g for wine grape pomace (*Vitis vinifera* L. cv. Pinot Noir); the pomace showed values higher than the skin. Studies by Rockenbach *et al.* (2011a) and Rockenbach *et al.* (2011b) found values of 188.02 ± 2.50 μmol of Trolox equivalent/g and 3640.00 ± 63.00 μmol of Trolox equivalent/100 g for the antioxidant capacity of Isabel grape skin and pomace respectively.

Conclusion

The results from the physicochemical characterization indicated that the grape pomace flour and grape skin flour from the organic grape juice industry presented a high content of insoluble fibers, and high levels of o-diphenols, water-soluble condensed tannins, flavonols, monomeric anthocyanins, as well as considerable free-radical scavenging activity in relation to DPPH and reducing capacity. Considering the large amount of by-products generated by the organic grape juice/wine industry in Brazil, grape skin flour and grape pomace flour represent promising materials for food companies in terms of product development and/or enrichment of different food/beverage formulations.

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