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PROSPECTING FOR MACROMOLECULAR ANTIOXIDANTS OR NON-EXTRACTABLE POLYPHENOLS IN BAGASSE FROM WINERIES IN THE SÃO FRANCISCO RIVER VALLEY

[1]Antônio Maurício Sousa Lima, [1]Francisco Acácio de Sousa, [1]Joilna Alves da Silva, [2]Raimundo Wilane de Figueiredo, [3]Maria Auxiliadora Coêlho de Lima and *[1]Maria do Socorro Moura Rufino

[1]Master's program in Sociobiodiversity and Sustainable Technologies (MASTS), University for International Integration of the Afro-Brazilian Lusophony, Redenção-CE, Brazil; [2]Department of Food Engineering, Federal University of Ceará, Fortaleza-CE, Brazil; [3]Brazilian Agriculture Research Corporation (Embrapa Semiarid), Petrolina-PE, Brazil

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****Corresponding author:***
Maria do Socorro Moura Rufino

ABSTRACT

In this study, we evaluate industrial grape pomace from two wineries in the region known as São Francisco Valley, in Brazil, which were produced in two different cycles in 2015. The interest in this field of research arises from recent studies that have shown that macro-antioxidant (MACAN) substances have a positive effect on the body, especially because they favor the health of the gastrointestinal tract. Thus, in this research we analyze several varieties of grapes, such as Alicante Bouschet, Egiodola, Tempranillo, Touriga Nacional and Syrah. We quantify the contents of extractable polyphenols, antioxidant activity by the ABTS assay, and non-extractable polyphenols (MACAN). The varieties that showed the highest values regarding the total extractable polyphenols were Alicante Bouschet, 2nd cycle (seed) with 485.79 GAE/100g, and Egiodola 2nd cycle (skin) with 470.74 mg GAE/100g. In the quantification of antioxidant activity by the ABTS assay, the variety Egiodola 2nd cycle (seed) presented 21.06 μM Trolox/100g. The variety that showed the highest MACAN result was Tempranillo 1st cycle (seed) with 613.99 mg/100g. The MACAN compounds available in the analyzed grape pomace presented significant results, due to its high nutritional values that can be incorporated by the food industry in the formulation of new products.

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INTRODUCTION

Brazil is the third largest fruit producer in the world, only behind China and India. The non-exported production is consumed internally and absorbed by the food industry, and it is in this prosperous scenario of the primary sector that fruit production arises, with the production of grapes and their processing by the wine industry (KIST, 2018). Among the main national producing regions, the São Francisco Valley has been considered since the early 2000s as one of the most dynamic regions in Northeast Brazil. Grape and mango have gained prominence in production in the region, by conquering space and becoming consolidated in the commercial sector (SÁ *et al.,* 2015). The careful development of vitiviniculture transformed the lower-middle São Francisco valley region, reaching a degree of development and notoriety that makes it consolidated as a wine region of national and international importance. The main varieties used for the production of red wines in the São Francisco Valley are Syrah, Tempranillo, Touriga Nacional, Cabernet Sauvignon, Alicante Bouschet, Ruby Cabernet and Petit Verdot, with Syrah representing around 65% of red wines. A large volume of residue product called grape pomace arises from wine production, which is usually wasted by the industry. There is growing concern about the generation of such volume produced by the wine industry, estimating that, after processing, about 20% of the total weight of the grapes is discarded (GRUZ *et al.,* 2013). Studies also show that grape pomace is rich in biologically active compounds, such as low molecular weight polyphenols.

There are several methodologies for evaluating these compounds. However, the most common is solid-liquid extraction using mixtures of organic solvents with water. This mixture results in an extract associated with the analyzed food, which is called total extractable polyphenols – EPP. New studies have proven that there is a large number of compounds that remain during EPP extractions, which are called non-extractable polyphenols (NEPP), or macromolecular antioxidants (MACAN). A clear statement regarding these fractions portrays that non-extractable polyphenols are retained after aqueous-organic extraction (CAMACHO *et al.,* 2018). Studies have shown that plant foods contain high amounts of some types of high molecular weight antioxidants, the so-called MACAN. These can be divided mainly into two fractions: hydrolyzable polyphenols (HPP), which are compounds associated with polysaccharides or proteins, and non-extractable proanthocyanidins (NEPA), which are structures of high molecular weight. Macromolecular antioxidants cross the stomach and small intestine intact until they reach the colon, where they interact with the colonic microbiota in a fermentative process degrading the macromolecules, thus producing the antioxidant metabolites. These metabolites are absorbed through the colonic mucosa and reach the bloodstream about eight hours after ingestion, which distribute them to cells and tissues where they can have systemic effects. This indicates that macro-antioxidants can increase and prolong the antioxidant state and health effects (SAURA-CALIXTO, 2017). However, there are few data available on the content of MACAN in food (PÉREZ-JIMÉNEZ; TORRES, 2011). Likewise, data on MACAN dietary intake are also limited to some studies (ARRANZ; *et al.,* 2010; HERVERT-HERNÁNDEZ *et al.,* 2011). The lack of research on MACAN in tropical fruits, especially grapes produced in the Northeast region of Brazil, motivated the present study. We analyzed grape pomace of several grape varieties of grapes such as Alicante Bouschet, Egiodola, Tempranillo, Touriga Nacional, and Syrah, from wineries in São Francisco Valley, to quantify the total extractable polyphenols (EPP), the antioxidant activity by the ABTS assay, and the content of MACAN (including HPP and NEPA).

MATERIALS AND METHODS

Chemical reagents: The reagents Folin-Ciocalteu, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid, and ABTS (2,2-azino-bis 3-ethylbenzothiazoline-6-sulfonic), were acquired from Sigma-Aldrich. Other reagents used, such as potassium persulfate, sulfuric acid, hydrochloric acid, 70% acetone, 50% methyl alcohol, 99.8% ethyl alcohol and butanol, were purchased from Neon. In addition, we also used gallic acid from Dinâmica and FeCl3 from Êxodo Cientifica.

Plant Material: We used dry grape pomace samples made up of grape skin and seeds, from different production cycles of the 2015 harvest. The production cycles refer to grape phenology, in which each production cycle takes an average of 100 to 120 days to harvest. We studied twenty-two samples (skin and seeds) from five grape varieties: Alicante Bouschet, Egiodola, Tempranillo, Touriga Nacional, and Syrah. These varieties come from the wine processing industry of two wine companies located in the lower-middle São Francisco valley region, which we will call companies A and B. After identifying the samples, we transported them to the Tropical Fruit Laboratory, in the Food Engineering Department of the Federal University of Ceará (UFC), in Fortaleza-CE, where they were stored in a freezer at -20 ºC.

Preparation and analysis of polyphenol fractions: We extracted each sample by using organic solvents (methanol and acetone) through the method developed by Rufino *et al.* (2007), in which we used 0.5 grams per sample.

Total extractable polyphenols (EPP): This analysis followed the protocol developed by Montreau (1972) with adaptations by Rufino *et al.* (2007). We added an aliquot of 50 µL of the extract in test tubes and made up the final volume to 0.5 mL with distilled water. Then, we added 0.5 ml of Folin-Ciocalteau reagent, 1.0 ml of 20% Na_2CO_3, and 1.0 ml of distilled water. Finally, we homogenize the tubes and keep them at rest for 30 minutes. After that, we performed readings on a spectrophotometer at 700 nm and expressed the results in gallic acid equivalents mg/100g.

Antioxidant activity by ABTS•$^+$ free radical scavenging assay: The antioxidant activity was determined by the method developed by Miller *et al.* (1993), with adaptations by Rufino *et al*. (2007a). We added 10, 20, and 30 µL aliquots in test tubes for each sample in three repetitions. Then, added 3 ml of the ABTS stock solution and homogenates. The reading was performed after leaving the sample resting for 6 minutes, in a spectrophotometer at 734 nm, with results expressed in µM Trolox/100g.

Quantification of non-extractable polyphenols or MACAN: The method used was developed by Pérez-Jiménez *et al.* (2015). We weighed 0.5 gram of the sample and added aqueous organic solvents to obtain the extracts of HPP and NEPA, similarly to that performed in the EPP analysis (SAURA-CALIXTO, 2012). To perform the HPP analysis, we used the EPP analysis residues and added methanol 50% with pH 2.0, and also acetone 70%. Then, we heated samples in a water bath at 85ºC for one hour. After cooling, methanol and sulfuric acid were added to the residue (HARTZFELD *et al.,* 2002). Then the pH was adjusted to 5.5 and the samples were read on a spectrophotometer at 750 nm. The calibration curve had gallic acid as a standard. For the NEPA, we treated the EPP extraction residues with butanol/HCl/FeCl3 at 100° C for 1h (PÉREZ-JIMÉNEZ; SAURA-CALIXTO, 2015). Then, we performed the readings at two wavelengths, 450 and 555 nm. The results were compared with a type of proanthocyanidin (C. Siliqua), which is rich in high molecular weight proanthocyanidins. The sum of HPP and NEPA values are called macromolecular antioxidants (MACAN) and expressed in mg/100g

Statistical analysis: The data were submitted to analysis of variance and comparisons of means through the Tukey's test at the level of 5% with the aid of the R software (R DEVELOPMENT CORE TEAM, 2009). To facilitate the interpretation of the results, the varieties will be named by codes, as shown in Table 1.

Table 1. Identification of grape varieties: skin and seed

Name of the variety (skin and seed)	Code: Company / Variety
Alicante Bouschet 1st cycle	(A/A)
Egiodola 1st cycle	(A/B)
Tempranillo 1st cycle	(A/C)
Touriga Nacional 1st cycle	(A/D)
Syrah 2nd cycle	(A/E)
Egiodola 2nd cycle	(A/F)
Tempranillo 2nd cycle	(A/G)
Touriga Nacional 2nd cycle	(A/H)
Alicante Bouschet 2nd cycle	(A/I)
Syrah 1st cycle	(B/J)
Tempranillo 1st cycle	(B/K)

RESULTS

Determination of Extractable Polyphenols – EPP: In Table 2, the results found ranged from 237.37 to 485.79 mg GAE/100g, where the latter value belongs to the Alicante Bouschet variety, 2nd cycle (seed) from company A. Other varieties that had similar results were Egiodola 2nd cycle (skin), Tempranillo 1st cycle (seed), and Egiodola 2nd cycle (seed). The coefficient of variance was 7.74% for the seed samples and 4.84% for the skin samples.

Determination of Antioxidant Activity – ABTS: In Table 3, the variety that had better results than the others were Egiodola 2nd cycle (seed) with 21.06 µM Trolox/100g, followed by Tempranillo 2nd cycle (seed) with 20.06 µM Trolox/100g, both from company A. These results converge with the previous table, which may indicate that the higher the content of polyphenols, the greater the antioxidant activity by the method. The variation coefficient was 21.33% for the seed samples and 25.78 for the skin samples.

Determination of Macromolecular antioxidants – MACAN: Table 4 shows the values in relation to the MACAN content, which consists of the sum of hydrolyzable polyphenols (HPP) and non-extractable proanthocyanidins (NEPA). The results are expressed in mg/100g of dry matter. For the determination of HPP, the values varied from 119.54 to 314.62 mg/100g, with emphasis on Tempranillo 1st cycle (seed), from company B. The coefficient of variance was 4.86% for the seed samples and 4.34% for the skin samples. As for the NEPA content, the Tempranillo 1st cycle (seed), from company A, presented 379.53 mg/100g of dry matter. The percentage of the coefficient of variance was 4.35% for the seed samples, and 1.64% for the skin samples. As for the total content of MACAN, the Tempranillo 1st cycle (seed), from company B, presented 613.99 mg/100g, thus standing out among the others.

DISCUSSION

Total Extractable Polyphenols: The results presented in Table 2 confirm the study presented by Braga (2016), in which the extract of Pinot Noir was evaluated and the result was 768.56 mg GAE/g. Another study, carried out by Silva (2010), evaluating the Syrah variety, found 360.1 mg GAE/g. This shows that the variation of the results found in this research, in

Table 2. Total extractable polyphenols in extracts of grape pomace varieties

Company / Variety	Skin	Seed
(A/A)	291.59 ^{ef}	363.38 ^{bc}
(A/I)	249.99 ^{gh}	485.79 ^{a}
(A/B)	371.87 ^{bc}	345.86 ^{c}
(A/F)	470.74 ^{a}	468.20 ^{a}
(A/E)	249.99 ^{gh}	257.00 ^{d}
(A/C)	281.13 ^{fg}	470.09 ^{a}
(A/G)	356.77 ^{cd}	467.72 ^{a}
(A/D)	253.30 ^{gh}	342.29 ^{c}
(A/H)	237.37 ^{h}	423.96 ^{ab}
(B/J)	328.58 ^{de}	253.09 ^{d}
(B/K)	400.02 ^{b}	366.63 ^{bc}

Table 3. Total Antioxidant Activity by the ABTS method in extracts from industrial grape pomace

Company / Variety	Skin µM Trolox/100g	Seed µM Trolox/100g
(A/A)	9.33 ^{bc}	18.19 ^{ab}
(A/I)	9.12 ^{bc}	16.88 ^{ab}
(A/B)	7.54 ^{bc}	12.76 ^{b}
(A/F)	15.37 ^{a}	21.06 ^{a}
(A/E)	11.02 ^{ab}	13.34 ^{ab}
(A/C)	8.16 ^{bc}	17.37 ^{ab}
(A/G)	10.70 ^{abc}	20.06 ^{a}
(A/D)	9.88 ^{abc}	13.35 ^{ab}
(A/H)	8.40 ^{bc}	11.68 ^{b}
(B/J)	13.07 ^{a}	11.69 ^{b}
(B/K)	11.67 ^{ab}	10.74 ^{c}

Table 4. Macro-antioxidant content in extracts from industrial grape pomace

Company / Variety	Skin			Seed		
	HPP	NEPA	MACAN	HPP	NEPA	MACAN
(A/A)	236.97 ^{a}	362.50 ^{a}	599.47	125.95 ^{fg}	293.40 ^{cd}	419.35
(A/I)	251.64 ^{a}	308.91 ^{b}	560.55	192.47 ^{cd}	254.97 ^{de}	447.44
(A/B)	187.38 ^{bc}	360.54 ^{a}	547.92	160.47 ^{e}	317.74 ^{bc}	478.21
(A/F)	201.78 ^{b}	352.24 ^{a}	554.02	237.41 ^{b}	353.22 ^{ab}	590.63
(A/E)	214.92 ^{b}	327.68 ^{a}	542.60	230.60 ^{b}	299.28 ^{c}	529.88
(A/C)	185.35 ^{bc}	359.73 ^{a}	545.08	119.54 ^{g}	379.53 ^{a}	499.07
(A/G)	200.81 ^{b}	272.89 ^{d}	473.70	193.33 ^{bc}	326.39 ^{bc}	519.72
(A/D)	154.55 ^{d}	309.98 ^{b}	464.53	168.37 ^{de}	348.50 ^{ab}	516.87
(A/H)	159.91 ^{cd}	293.04 ^{c}	452.95	150.14 ^{ef}	332.09 ^{bc}	482.23
(B/J)	254.92 ^{a}	265.58 ^{d}	520.50	288.31 ^{a}	228.04 ^{e}	516.35
(B/K)	254.82 ^{a}	262.72 ^{d}	517.54	314.62 ^{a}	299.37 ^{c}	613.99

relation to the cycle and/or company, may have occurred due to several factors, such as: climate; storage of pomace; wine making process, among others. However, it can be said that the pomace of both varieties is rich in phenolic compounds.

Total antioxidant activity: In the research carried out by Melo *et al.* (2011), they evaluated Isabel grape pomace, in which they found values ranging from 50.0 to 120.0 µM Trolox/100g in the aqueous and ethanol extracts. Another study by Rockebach *et al.* (2011) in red grape pomace of the Cabernet Sauvignon, Merlot, Bordeaux, and Isabel varieties, obtained values ranging from 193.36 to 485.42 µM Trolox/100g. The results reaffirm that grapes are an excellent source of bioactive compounds, beneficial to health. As for pomace, even though it is currently discarded by the wineries and only partially contains bioactive compounds, the functional potential that it still has is notorious and can be u sed in the formulation of new food products (MELO *et al.,* 2011).

Macro-antioxidants*:* The results presented in Table 4 are far superior to those performed by Pérez-Jiménez *et al.* (2014), who carried out a survey on the content of MACAN in various foods in the Mediterranean and found 146 mg/100g of dry matter in red grape. Due to the lack of further research evaluating the content of MACAN in tropical fruits, mainly in grape pomace, we used other reference studies, such as those by Pérez-Jiménez *et al.* (2013). They evaluated red fruits such as açaí, apple, apple pomace and blackberry pomace, and the results found were, respectively: 1.240 mg/100g of dry matter; 37-43 mg/100g of fresh matter; 18-23 mg/100g of fresh matter and 1.685 mg/100g of fresh matter for hydrolyzed polyphenol content. Another study by Rufino *et al.* (2010), where they evaluated the content of hydrolyzable tannins in tropical fruits such as cashew and acerola, found values of 12.1 and 3.9 $g.kg^{-1}$ of dry matter. In this same research, the content of non-extractable polyphenols from cashew was 52.0 $g.kg^{-1}$ of dry matter. This research demonstrates that tropical fruits contain high levels of extractable and non-extractable polyphenols, corroborating with Saura-Calixto (2017) who reports on the important role of these compounds in preventing and combating gastrointestinal and cardiovascular diseases. Thus, it is estimated that the content of MACAN is associated with the content of polyphenolic compounds found primarily in the skin and seeds of grapes (MONRAD *et al.,* 2010).

Conclusion: Varieties of grape pomace from the lower-middle São Francisco valley region have high levels of polyphenols, antioxidant and macro-antioxidant activity. Therefore, they can be used in the formulation of new products, as they are rich in dietary fiber.

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