Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.47420

Optimization of antioxidant extraction and characterization of oil obtained by pressing cold from *Vitis labrusca* seeds

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Abstract

This study aimed to optimize the extraction solvents for Bordo grape (*Vitis labrusca)* seeds by response surface methodology regarding to the antioxidant activity (AA) and *trans*-resveratrol content. Fatty acids (FA) and AA of the oil obtained by pressing cold method were also determined. The extraction optimization was determined by the statistical simplex-centroid mixing scheme, enabling the analysis of solvents effects (water, ethanol and acetone) and their mixtures on the responses. AA was performed by DPPH, ABTS and FRAP methods, and by total phenolic compounds and flavonoids. FA were determined by GC and *trans*-resveratrol by HPLC. The extracts containing ternary fraction of solvents showed greater AA, increasing about 20 times compared to pure solvent. The composition that showed the best response ranged between 45-48% of water, 14-20% of ethanol and 35-38% of acetone for both grape seeds, with polarity ranged from 0.705 to 0.706. The oil from seeds showed high concentrations of PUFA, particularly linoleic acid. The optimized extraction method improved the use of this residue as a potential antioxidant source for food industry.

Keywords: industrial by-products; solvent extraction; mixture modeling; phenolic compounds; *Trans*-resveratrol; fatty acids.

Practical Application: Optimized extract has high antioxidant activity and can be used as a preservative at food industry.

1 Introduction

Grapes are highlighted as a worldwide consumed fruit and also present a high quantity of phenolic compounds *in natura* as in its derivatives (Spigno et al., 2007). Such phenolic compounds have antioxidant potential and anti-inflammatory, antimicrobial and anticarcinogenic action (Teixeira et al., 2014).

The grape industry generates a large number of residues as peel, seeds and pulp, which also contain antioxidants (Santos et al., 2011). This has aroused the interest of the industry itself in recycling, in order to reduce costs, environmental impacts, and add value to the product (Spigno & De Faveri, 2007). Seeds of vinification were the residues that started the exploitation of the phenolic compounds of this class, followed by bagasse (Melo, 2010). By this way, considering the diversity of polyphenols sources from plant matrices and their physicochemical structures, as well as its properties (such as antioxidant activity), it is hard to find a universal extraction method that can be applied in all samples (Alara et al., 2018). Aspects such as time, temperature, particle size and type of extraction are crucial because they alter the yield of the extracted antioxidant compounds (Alcântara et al., 2019). In addition, the extractor solvent polarity also affects the antioxidants extracted (Bosso et al., 2016). In this regard, the extraction optimization for each sample plays a key role because it also enhances the global economic process (Mukherjee et al., 2014). In this manner, the development of grape seeds extraction method can add commercial and industrial values for grape culture.

By this way, this study seeks to optimize the extraction process by statistical simplex-centroid mixing scheme, assessing the obtained extracts, as well as, the oil extracted from seeds by cold pressing in terms of antioxidant activity, resveratrol content and fatty acids.

2 Materials and methods

2.1 Reagents

Folin-Ciocalteau reagent, gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-1,3,5-triazine (TPTZ), 2,2-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), standard nonadecanoic acid methyl ester (19:0) and *trans*-resveratrol internal standard were from Sigma-Aldrich. All other reagents were of analytical grade.

2.2 Samples

Unfermented Bordo seeds were obtained from an industry in the city of Toledo, Paraná State, Brazil, before fermentation process. Fermented seeds were obtained at a winery located in Quatro Pontes municipality, Paraná State, Brazil, after fermentation process. The seeds were separated from the peels and peduncles, washed, sun-dried, sieved, packed in vacuum, and stored at room temperature.

Received 06 Oct., 2020
Accepted 18 Nov., 2020
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2.3 Experimental design and grape seed extracts preparation

In order to study the influence of different solvents on the extraction of antioxidants, an experimental simplex centroid design was planned, using the response surface methodology for modeling mixtures in Statistica software, version 10.0 (StatSoft, 2011), with a 0.05 significance level. Table 1 presents the coded levels, where the independent variable is the proportion of solvents (water, ethanol, and acetone), and dependent variables is the antioxidant activity obtained by several methods.

Seeds were ground into powder using a knife mill (Solab - PS 30), in mesh 20 sieves. About 10 g of the samples were weighted, added to 100 mL of the extraction solvent, and kept 4 hours in an orbital shaker at 250 rpm, 45 °C, and protected from light. The extracts were filtered and placed in a rotary evaporator at 45 °C under reduced pressure. Then, they were freeze-dried for 5 days and stored in a dark room at -18 °C.

2.4 Oil extraction using a hydraulic press

To extract the oil a stainless steel template, composed of a chamber for seed storage, a piston, and a hydraulic press (EMIC, 2000 kN), was used. The load applied by the downward piston press was received by the other piston that crushed the material, extracting the oil collected through a door. A 50 stainless steel mesh was used at the bottom of the template to obtain an oil free of impurities. It was used 100 grams of dried grape seed (fermented and unfermented), at 85 tonnes of pressure and 300 seconds as extraction time.

2.5 Physicochemical analyzes

Moisture (012/IV), ash (018/IV), lipids (032/IV), and proteins contents (037/IV) were performed according to the methodology described by Instituto Adolfo Lutz (2008). Total nitrogen was converted into protein by the specific factor for conversion of 6.25.

2.6 Antioxidant activity

Capture of free radicals by DPPH method

The methodology employed was described by Bondet et al. (1997), with modifications. Methanolic solutions (40 µL) of grape seeds (oil and extracts 2.5 mg mL^{-1}) were pipetted and 3.0 mL of methanolic DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (0.0842 mmol L^{-1}) were added. After 30 minutes in the dark, the absorbance was read in a UV-VIS spectrophotometer (PG Instruments Ltda, Model T 80+) at 517 nm. The blank used was methanol and Trolox (0-2500 µmol L^{-1}) was used for calibration curve. Results were expressed in µmol Trolox equivalent (TE) g^{-1} of extract or oil (R^2 = 0.9997).

Table 1. Experimental design to the extraction of antioxidants compounds of Bordô grape seeds with their encoded levels.

Extracts	Water (W)	Ethanol (E)	Acetone (A)
1	1[a]	0	0
2	0	1	0
3	0	0	1
4	1/2	1/2	0
5	1/2	0	1/2
6	0	1/2	1/2
7	1/3	1/3	1/3

[a]Volumetric proportion: 1= 100%; 1/2= 50%; 1/3= 33.33%.

Capture of free radicals by ABTS$^{\bullet+}$ method

The antioxidant activity by ABTS (2,2-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) method followed the methodology described by Re et al. (1999). First, the $ABTS^{\bullet+}$ radical from a mixture of ABTS at 7 mmol L^{-1} with 140 mmol L^{-1} of potassium persulfate, was maintained in the dark at room temperature for 16 hours. After the incubation period, the solution was diluted with ethanol (HPLC grade) until obtaining an absorbance of 0.70 (± 0.01). In the absence of light, a sample of 30 µL was pipetted (extracts and oil 2.5 mg mL^{-1}) and 3.0 mL of $ABTS^{\bullet+}$ radical solution was added. After 6 minutes, the absorbance was read at 734 nm in a UV-VIS spectrophotometer. Trolox (0-2000 µmol L^{-1}) was used for calibration curve and the results were expressed in µmol Trolox equivalent (TE) g^{-1} of extract or sample (R^2 = 0.9995).

Power reduction of Fe (III) by FRAP method

The reduction power were determined by the FRAP method and evaluated according to the methodology (adapted) described by Benzie & Strain (1996). Solutions were prepared with 3 mL of FRAP reagent, preheated at 37 °C, 300 µL of distilled water, and 100 µL of sample. The resulting solution was homogenized and incubated in a water bath at 37 °C for 60 minutes. Absorbance was read at 593 nm in a spectrophotometer and results were expressed in µmol of ferrous sulfate equivalent per gram of extract or oil (µmol EQ$FeSO_4 \cdot 7H_2O$ g^{-1}), by the calibration curve (0-2000 µmol L^{-1}) of standard $FeSO_4 \cdot 7H_2O$ (R^2 = 0.9990).

Evaluation of total phenolic compounds by Folin-Ciocalteau (FC) method

The method described by Singleton & Rossi (1965) to determine the concentration of total polyphenols were employed. The methanolic solutions from the seeds (extracts and oil 2.5 mg mL^{-1}) were prepared, 250 µL were pipetted, 250 µL of the Folin-Ciocalteu reagent (diluted at 1:1 in distilled water) was added, 500 µL of the saturated solution of Na_2CO_3 and 4.0 mL of distilled water). After 25 minutes in the dark and at room temperature, it was centrifuged for 10 minutes at 3000 rpm and the absorbance read at 725 nm. The results were determined with gallic acid (0-200 mg L^{-1}) as standard and expressed in mg of gallic acid equivalent (GAE) g^{-1} of extract or oil (R^2 = 0.9997).

Flavonoid content (FLA)

The procedure adopted to determine the flavonoids content was adapted from Woisky & Salatino (1998). The methanolic solutions from grape seeds were prepared (extract and oil 2.5 mg L^{-1}), then 500 µL were transferred to test tubes, 250 µL of aluminum chloride 5% (m/v in methanol) and 4.25 mL of

methanol were added. After 30 minutes in the dark and at room temperature, the absorbance was read at 425 nm. Quercetin was used as standard (0-100 mg L^{-1}) and results was expressed as mg quercetin equivalents (EQ) g^{-1} of extract or oil (R^2 = 0.9976).

2.7 Trans-resveratrol

The methodology for separation and quantification of *trans*-resveratrol was adapted from Souto et al. (2001). About 18 mg of sample were diluted in 1 mL of methanol and injected into an HPLC (Dionex UltiMate 3000) system with a UV-VIS detector, at 50 °C. With isocratic elution, C18 column (250 mm × 4,6 mm) with a particle diameter of 5 µm (Agilent Eclipse XDB), the mobile phase consisted of acetonitrile and water (25%:75%), 3.0 of pH (adjusted with orthophosphoric acid), and an injection volume of 20.0 µL. The injection flow rate was 1.5 mL min^{-1}, with the analyte detected at 306 nm after a 6-minute run. *Trans*-resveratrol in methanol (0.1-10.0 mg L^{-1}) was used as standard and results were expressed in mg L^{-1} of *trans*-resveratrol (R=0.9944).

2.8 Fatty acids

The procedure of Hartman & Lago (1973), with adaptations from Maia & Rodriguez-Amaya (1993), was used to determine the fatty acids content in the seeds oils. The methylation reaction was carried out with sodium hydroxide solution in methanol, followed by esterification with acid catalysis (ammonium chloride, methanol, and sulfuric acid). The separation of the fatty acids methyl esters (FAME) was carried out by a Perkin Elmer automatic gas chromatograph, Clarus 680 model, coupled with a flame ionization detector (GC-FID) and a capillary column of fused silica Select Fame CP 7420 (100 m long, 0.25 mm inner diameter, and 0.25 µm of coating film). The gases flows were 1.1 mL min^{-1} for the carrier gas (H_2), 40 mL min^{-1} to H_2, and 400 mL min^{-1} to the synthetic air flame. The injector and the detector remained at 240 °C and 275 °C, respectively. The FAME separation column used had a programmed temperature of 80 °C for 1 minute, followed by heating on a temperature ramp of 15 °C min^{-1} until 180 °C. Then one heated to 220 °C with a 3 °C min^{-1} ramp, remaining for 2 minutes, and finally a 5 °C min^{-1} ramp until 250 °C for 5 minutes. The samples injections were performed with a volume of 2 µL using a 5 µL syringe (Split 1:100). Given the determined peak areas, FA could be identified by comparing with retention times of standard FAME, comparing with individual patterns and added patterns. The FAME quantification was performed in relation to the internal standard nonadecanoic acid methyl ester (19:0) (1.026 mg mL^{-1} in n-heptane). The results were expressed in mg of FA per gram of total lipids (Equation 1) (Visentainer & Franco, 2012).

$$C_x = \left(A_x \cdot M_{19:0} \cdot F_{TC}\right) / \left(A_{19:0} \cdot M_A \cdot F_{CEA}\right) \quad (1)$$

where: C_X = concentration of the fatty acid x in mg g^{-1} of total lipids; A_X = area of methyl esters corresponding to the fatty acid x; $A_{19:0}$ = internal standard area; $M_{19:0}$ = internal standard mass added to the sample (mg); M_A = mass of total lipids (g); F_{TC} = theoretical correction factor; F_{CEA} = conversion factor from FAME to FA.

2.9 Statistical analysis

All analyses were performed in triplicate and the results expressed as mean ± standard deviation. The results containing two averages were submitted to test-T (5% of probability), and the results with three or more averages were submitted to the analysis of variance (ANOVA), followed by a comparison of the averages by Tukey test (5% of probability) performed by StatSoft (2011).

3 Results and discussion

3.1 Physicochemical analyzes

Table 2 shows the physicochemical composition values of fermented and unfermented seeds of Bordô grape.

By comparing the experimental data obtained for the seeds, it is possible to verify that the averages were alike, featuring a physicochemical composition very similar for both. The differences reported between the values obtained in the physicochemical compositions analyzes can be attached to different forms of treatment, of cultivars, agroclimatic factors, and the winery management (Cheng et al., 2012).

Santos et al. (2011) reported in their study similar moisture values of four grape seeds varieties (from 8.87 up to 10.50%). Regarding proteins, these same authors obtained smaller values (from 6.5 up to 7.7%) analyzing those samples. Santos et al. (2011) also observed lower protein content in Izabel, Niagara, Benitaka and Brazil grape seeds than that observed in this study. The protein content indicates a potential use of these residues, once they are differentiated in a diet, able to be inserted into restricted feed systems, and also aid in maintaining weight loss (Mohd Adzim Khalili et al., 2009).

According to Luque-Rodríguez et al. (2005), the highest concentration of lipid on the grapes is found in seeds and may vary from 10 to 16% depending on the cultivar analyzed. It was confirmed for both samples of present study.

3.2 Antioxidant activity

According to the results (Table 3), the extracts containing ternary fraction of solvents (water, ethanol and acetone) showed best results for DPPH essays (1296.00 ± 256.62 and 1704.00 ± 80.13 µmol TE g^{-1} sample) and ABTS (1684.80 ± 127.27 and 2835.77 ± 116.71 µmol TE g^{-1} sample), followed by the binary compositions, highlighting the fraction of water/acetone (DPPH: 1184.88 ± 42.86 and ABTS: 1452.13 ± 14.04 µmol TE g^{-1} sample), and water/ethanol (DPPH: 1453.33 ± 58.84 and ABTS: 1980.13 ± 61.49 µmol TE g^{-1} sample) for fermented and unfermented seeds, respectively. The lowest antioxidant activity

Table 2. Physicochemical composition of Bordô grape seeds.

	Fermented (%)	Unfermented (%)
Moisture	10.16 ± 0.19^{B}	12.52 ± 0.20^{A}
Ash	1.76 ± 0.05^{A}	1.67 ± 0.02^{B}
Protein	8.60 ± 0.27^{A}	8.26 ± 0.27^{A}
Lipid	13.46 ± 0.18^{A}	13.73 ± 0.15^{A}

Different letters in the same line represent significant differences (P<0.05) by test-T.

Table 3. Antioxidant activity of fermented and unfermented Bordô grape seed extracts.

Extracts	DPPH	ABTS	FRAP	Phenolic compounds	Flavonoids	trans – resveratrol
	μmol TE g^{-1} extract	μmol TE g^{-1} extract	μmol $EFeSO_4$ g^{-1} extract	mg EAG g^{-1} extract	mg EQ g^{-1} extract	(mg L^{-1})
Fermented						
W (1)	104.44 ± 11.49^{C}	62.26 ± 0.00^{E}	599.6 ± 4.24^{D}	18.35 ± 1.25^{D}	0.36 ± 0.03^{C}	ND
E (1)	269.77 ± 71.75^{C}	160.26 ± 21.68^{E}	180.93 ± 40.07^{F}	24.86 ± 1.51^{CD}	1.22 ± 0.09^{BC}	0.21 ± 0.03^{C}
A (1)	62.66 ± 5.33^{C}	117.82 ± 16.67^{E}	51.60 ± 3.30^{G}	14.19 ± 0.71^{D}	0.58 ± 0.05^{C}	0.04 ± 0.00^{C}
W/E (1/2:1/2)	952.00 ± 32.00^{B}	1140.13 ± 75.08^{C}	4192.53 ± 29.70^{B}	190.71 ± 3.03^{B}	8.42 ± 0.38^{A}	1.39 ± 0.07^{AB}
W/A (1/2:1/2)	1184.88 ± 42.86^{AB}	1452.13 ± 14.04^{B}	4557.11 ± 78.12^{A}	263.07 ± 8.61^{A}	8.80 ± 0.30^{A}	1.18 ± 0.12^{B}
A/E (1/2:1/2)	342.00 ± 14.00^{C}	370.71 ± 33.89^{D}	349.27 ± 32.26^{E}	42.86 ± 1.89^{C}	1.84 ± 0.15^{B}	0.24 ± 0.07^{C}
A/E/W (1/3:1/3:1/3)	1296.00 ± 256.62^{A}	1684.80 ± 127.27^{A}	4061.09 ± 59.09^{C}	244.44 ± 17.99^{A}	8.12 ± 0.86^{A}	2.01 ± 0.70^{A}
Unfermented						
W (1)	247.11 ± 34.21^{EF}	324.93 ± 23.24^{E}	791.89 ± 48.57^{C}	31.64 ± 2.13^{D}	1.14 ± 0.19^{D}	0.22 ± 0.05^{D}
E (1)	394.22 ± 71.02^{E}	578.71± 2.77^{E}	500.82 ± 45.82C^{D}	44.76 ± 3.17^{CD}	1.82 ± 0.38^{CD}	1.99 ± 0.19^{C}
A (1)	121.33 ± 39.62^{F}	321.37 ± 79.88^{E}	276.04 ± 50.53^{D}	21.91 ± 2.73^{D}	1.18 ± 0.10^{D}	2.78 ± 0.27^{C}
W/E (1/2:1/2)	1060.44 ± 49.55^{C}	1385.46 ± 128.33^{C}	4424.36 ± 111.91^{B}	221.16 ± 4.48^{B}	10.38 ± 0.51^{B}	8.94 ± 1.12^{AB}
W/A (1/2:1/2)	1453.33 ± 58.84^{B}	1980.13 ± 61.49^{B}	5372.66 ± 215.72^{A}	292.56 ± 9.86^{A}	11.75 ± 0.41^{A}	8.28 ± 0.24^{B}
A/E (1/2:1/2)	705.33 ± 25.75^{D}	918.31 ± 76.19^{D}	789.33 ± 32.51^{C}	64.19 ± 5.48^{C}	2.29 ± 0.28^{C}	2.59 ± 0.16^{C}
A/E/W (1/3:1/3:1/3)	1704.00 ± 80.13^{A}	2835.77 ± 116.71^{A}	4512.66 ± 155.06^{B}	311.66 ± 19.72^{A}	12.36 ± 0.57^{A}	9.84 ± 0.37^{A}

Mean ± standard deviation. Volumetric proportion: 1= 100%; 1/2= 50%; 1/3= 33.33%. W = water; E = ethanol; A = acetone. Different letters in the same column represent significant differences between averages for different fractions of solvents for each seed variety (P<0.05) by Tukey test. ND = not detected.

results were obtained for pure solvents. This same behavior was described by DiCiaula et al. (2014) over the effects of solvents on the total polyphenols content, and antioxidant capacity of the crude extracts of *Schinus terebinthifolius Raddi (Anacardiaceae*) peel. Regarding the performance of solvents binary mixtures, Cheng et al. (2012), studying wine extracted with different solvents (water, methanol, ethanol, and acetone), also reported that the water/acetone extracts reflected in higher antioxidant activity when compared to the ethanol/water extracts.

The analysis of the best extracting solvents to FRAP methodology presented the largest average in the binary fraction of acetone and water for fermented and unfermented seed, respectively (4557.11 ± 78.12 and 5372.66 ± 215.72 μmol $EQFeSO_4$ g^{-1}). Rockenbach et al. (2008) also report this behavior in a study to determine the influence of solvent (acetone and ethanol - 50% and 70%) in the total polyphenol content of grape bagasse extracts (*Vitis vinifera*) in Tannat and Ancelota cultivars.

The content of phenolic compounds and flavonoids showed better extraction in binary and ternary mixtures than in pure solvents, showing similar behavior to the DPPH and ABTS, with lower antioxidants extraction capacity in a pure fraction. The extract of fermented grape seed, for example, obtained with pure water and acetone had its content of phenolic compounds increased, respectively, from 1.8 and 1.4% to about 26% with combination of both solvents.

According to the antioxidant activities obtained for fermented grape seeds, the surface model analysis delineated in DPPH, ABTS, FRAP, total phenolic compounds and flavonoids were a special Cubic (P <0.001), the most significant and best fitted to predict the mixture behavior, with correlation coefficient (R) values of 0.9711; 0.9964; 0.9999; 0.9968, and 0.9930, respectively.

Equations 2-6 represent the models with optimal values evaluation for the antioxidant essays.

$$DPPH = 104.44*W + 269.77*E + 62.66*A + 3059.55*W*E + 4405.33*W*A + 703.11*E*A + 6556.00*W*A*E \quad (2)$$

$$ABTS = 62.27*W + 160.27*E + 117.82*A + 4115.47*W*E + 5448.35*W*A + 926.67*E*A + 10954.94*W*A*E \quad (3)$$

$$FRAP = 599.60*W + 180.93*E + 51.60*A + 15209.07*W*E + 16926.04*W*A + 932.00*E*A + 2958.87*W*A*E \quad (4)$$

$$FC = 18.35*W + 24.85*E + 14.19*A + 676.41*W*E + 987.18*W*A + 93.33*E*A + 812.48*W*A*E \quad (5)$$

$$FLA = 0.36*W + 1.22*E + 0.58*A + 30.52*W*E + 33.31*W*A + 3.74*E*A - 2.86*W*A*E \quad (6)$$

where: *W = water, E = ethanol,*A = acetone regarding volume fraction, FC = total phenolic compounds, and FLA = flavonoids content.

Figure 1a and 1b represents the results of the response surfaces from DPPH and ABTS methods, respectively, also showing optimal working conditions for the fermented seeds due to the mixture of solvents employed in the extraction. The reddish region in the graphics shows that the composition of the solvents with higher antioxidant activity for both methods is the ternary proportion. Expressing the maximum value for DPPH (1350.45 μmol TE g^{-1} extract) in the composition 0.43:0.23:0.34 water/ethanol/acetone (v/v/v) with desirability of 0.843. As for the ABTS test, the maximum (1748.43 μmol TE g^{-1} extract) in the composition

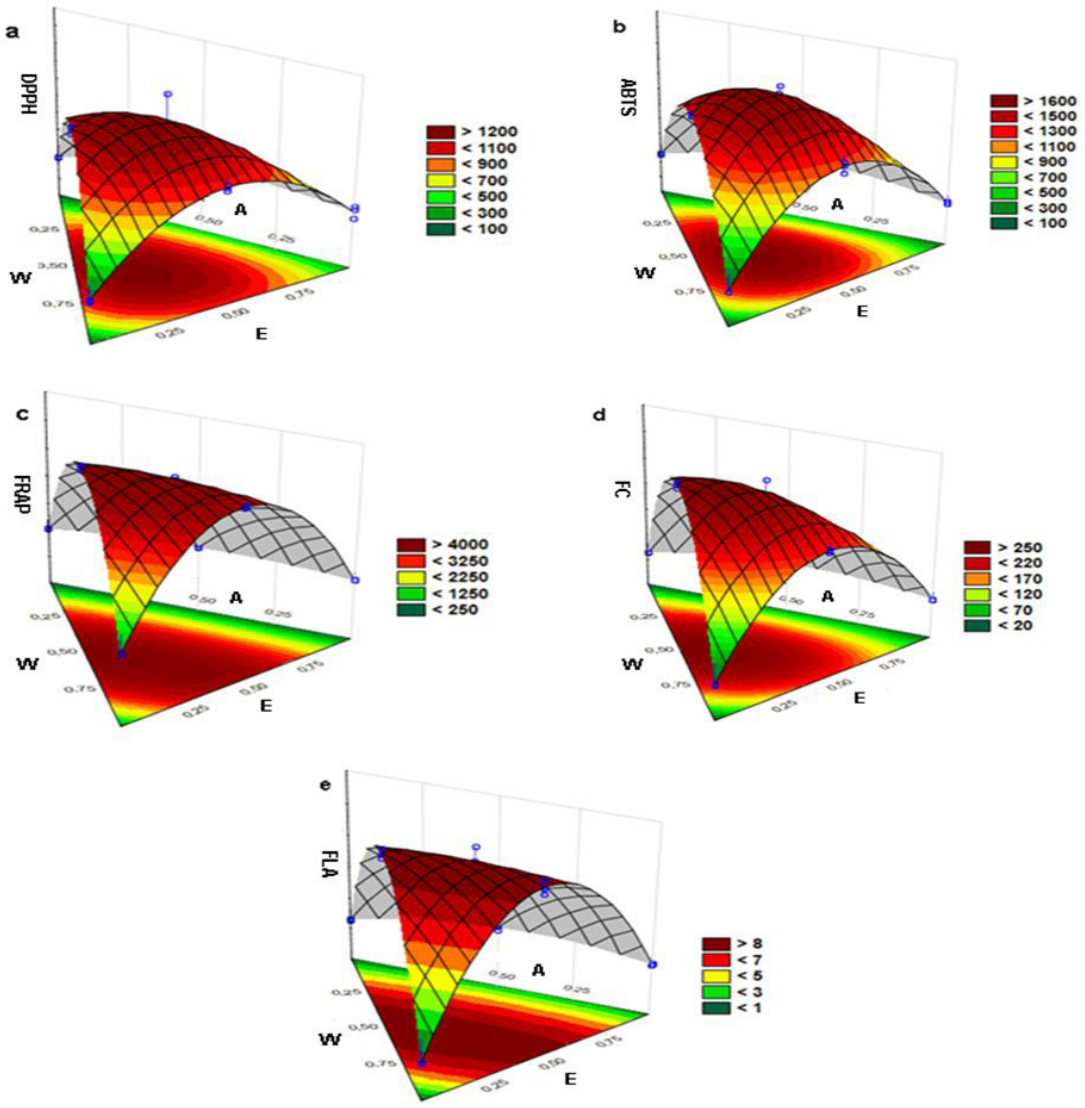

Figure 1. Response surface of the cubic special model for antioxidant potential in the (a) DPPH assay (μmol TE g^{-1} extract); (b) ABTS (μmol TE g^{-1} extract); (c) FRAP (μmol EQ$FeSO_4$ g^{-1} extract); (d) FC = phenolic compounds (mg EAG g^{-1} extract); and (e) FLA = flavonoids (mg EQ g^{-1} extract) for the extraction of fermented Bordo seed according to the solvents (W = water; A = acetone; E = ethanol).

was 0.42:0.24:0.34 water/ethanol/acetone (v/v/v), and 0.984 of desirability.

Figure 1c shows the response surface of the fermented seed for the FRAP method, showing best results with the ternary composition, with maximum value (4580.65 μmol EQ$FeSO_4$ g^{-1} extract) in the composition 0.50:0.10:0.40 water/ethanol/acetone (v/v/v), and desirability of 0.985. The water acts enhancing the antioxidant compounds in this method, once the responses with this solvent are greater than in the fractions without it. Water combined with other organic solvents contributes to forming a moderately polar medium favoring the extraction of polyphenols since mediums with extreme polarities are not characterized as good extractor (Liu et al., 2000).

Figure 1d and 1e show the data obtained for the response surface for flavonoids and phenolic compounds, respectively, according to the proportion of the solvents. The ternary mixture of solvents in the composition 0.47:0.12:0.41 water/ethanol/acetone (v/v/v) showed better response (269.15 mg EAG g^{-1} extract) for the phenolic compounds test with 0.999 of desirability. This same fraction of solvents in the composition 0.48:0.11:0.41 water/ethanol/acetone (v/v/v), favors the maximum extraction of flavonoids (8.82 mg EQ g^{-1} extract), with 0.966 of desirability. In both tests, it is perceived that its extraction is favored by the increase of water and acetone.

For unfermented grape seeds, the same procedure was performed to provide the response surface for the tests. The correlation presented to the special cubic model was the best fit ($p<0.001$ and R: 0.9939; 0.9924; 0.9977; 0.9950, and 0.9959) to determine DPPH, ABTS, FRAP, phenolic compounds and flavonoids, respectively.

Equations 7-11 represent the model obtained for each method for unfermented grape seeds.

$$DPPH = 247.11*W + 394.22*E + 121.33*A + 2959.11*W*E + 5076.44*W*A + 1790.21*E*A + 9666.68*W*A*E \quad (7)$$

$$ABTS = 324.93*W + 578.71*E + 321.38*A + 3734.78*W*E + 6627.91*W*A + 1873.07*E*A + 28834.13*W*A*E \quad (8)$$

$$FRAP = 791.89*W + 500.82*E + 276.16*A + 15112.00*W*E + 19354.58*W*A + 1604.71*E*A - 491.67*W*A*E \quad (9)$$

$$FC = 31.64*W + 44.76*E + 21.91*A + 731.82*W*E + 1063.12*W*A + 123.41*E*A + 1775.00*W*A*E \quad (10)$$

$$FLA = 1.14*W + 1.81*E + 1.18*A + 35.58*W*E + 42.34*W*A + 3.18*E*A + 53.25*W*A*E \quad (11)$$

where: *W = water, E = ethanol,*A = acetone regarding volume fraction, FC = total phenolic compounds, and FLA = flavonoids content.

Figure 2a and 2b shows the response surfaces generated for the DPPH and ABTS assay, respectively for the unfermented seeds, as a function of the solvents, where the best response was the ternary composition of the solvents for both. The maximum value obtained for DPPH (1741.46 μmol TE g^{-1} extract) was in the composition 0.40:0.25:0.35, water/ethanol/acetone (v/v/v) with 0.973 of desirability and for ABTS (2869.78 μmol TE g^{-1} extract) in the composition 0.38:0.28:0.34 water/ethanol/acetone (v/v/v), with 0.969 of desirability.

The response for the FRAP antioxidant activity is favored with the reduction of the ethanol (solvent) in the fraction. As shown in Figure 2c, the best response was in the binary mixture of the solvents water and acetone, with the maximum

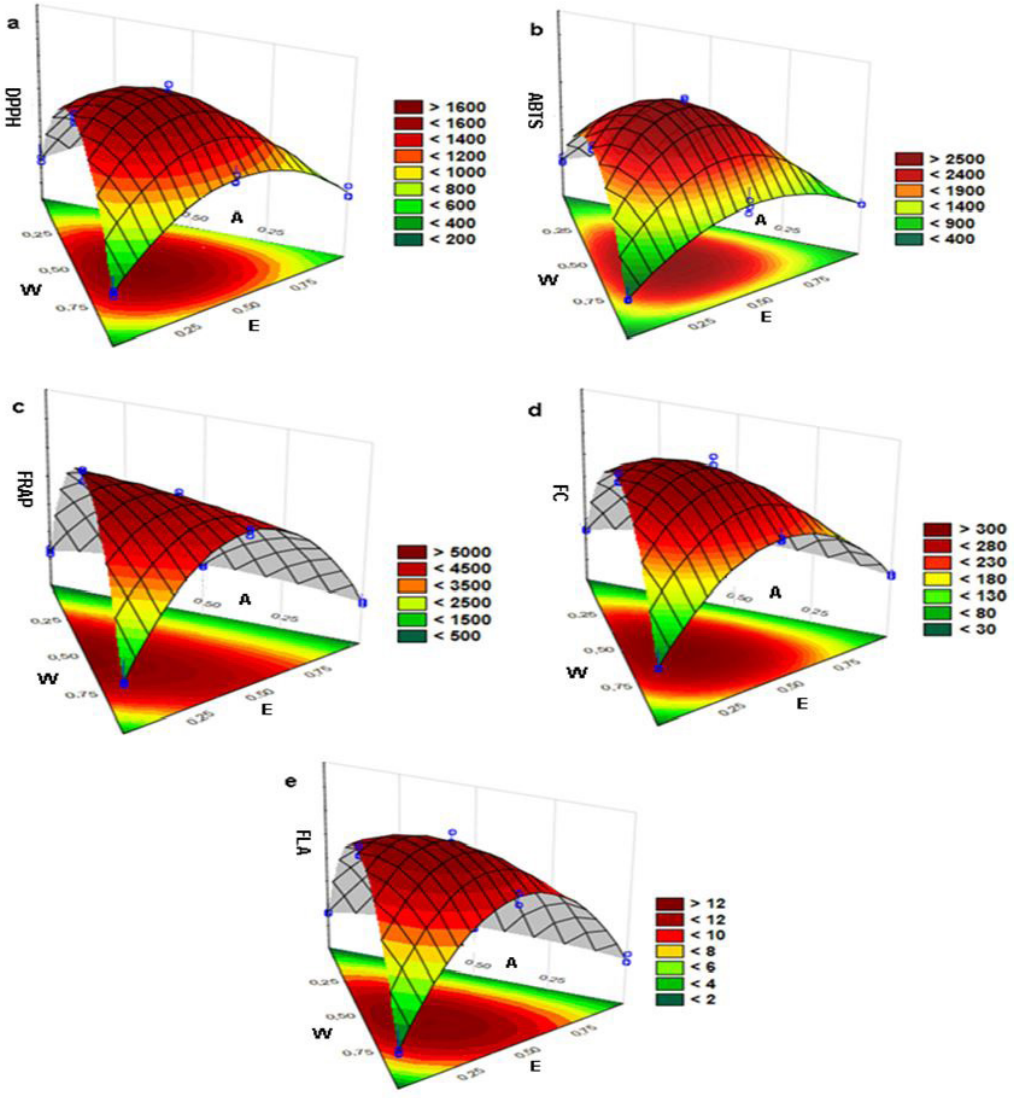

Figure 2. Response surface of the cubic special model for antioxidant potential in the (a) DPPH assay; (b) ABTS; (c) FRAP; (d) FC = phenolic compounds; and (e) FLA = flavonoids for the extraction of unfermented Bordo seed as a function of the solvents (W = water; A = acetone; E = ethanol).

value obtained (5375.88 μmol EQ$FeSO_4$ g^{-1} extract) in the composition 0.52:0.48 (v/v), and 0.971 of desirability.

The response surfaces for the total phenolic compounds and flavonoids (Figure 2d and 2e), as a function of solvent proportion, show the best results in ternary fractions: (328.75 mg EAG g^{-1} extract) 0.45:0.21:0.34 water/ethanol/acetone (v/v/v) of composition and 0,993 of desirability for the phenolic compounds. As for flavonoids, (13.02 mg EQ g^{-1} extract) the best composition of 0.46:0.27:0.27 water/ethanol/acetone (v/v/v), with desirability of 1.000.

Linear functions of desirability were employed to define the composition of extraction solvents for fermented and unfermented seeds, maximizing the antioxidant activity. For fermented seeds, the values 57.33, 824.66, and 1592.00 μmol EQTrolox g^{-1} were used for DPPH test; 62.26, 918.53, and 1774.8 μmol E.Trolox g^{-1} for ABTS test; 49.26, 2347.63, and 4646.00 μmol E$FeSO_4$ g^{-1} for FRAP test; 13.69, 141.42, and 269.15 mg EAG g^{-1} for total phenolics compounds; 0.34, 4.73 and 9.12 mg EQ g^{-1} for flavonoids, with desirability of 0, 0.5, and 1 for all tests corresponding to these values.

For unfermented seeds, the values 76.00, 931.33 and 1786.67 μmol E.Trolox g^{-1} were used for DPPH test; 255.60, 1603.47, and 2951.33 μmol E.Trolox g^{-1} for ABTS test; 173.26, 2849.63, and 5526.00 μmol EFeSO4 g^{-1} for FRAP test; 19.72, 175.24, and 330.77 for total phenolic compounds, and 1.00, 7.00, and 13.00 mg EQ g^{-1} for flavonoids, corresponding to desirability values of 0, 0.5, and 1 for all tests.

The predicted values found by desirability (Table 4), were similar to the results obtained experimentally for both fermented and unfermented seeds. According to DiCiaula et al. (2014), the choice of the extraction method has a considerable effect on the quality of the extract, but the nature of the solvent used for extraction provides the most obvious influence in the qualitative composition of the extract.

Considering the polarity of the solvents involved in the extractions with water (1.000), ethanol (0.654), and acetone (0.355) (Reichardt & Welton, 2010), it is clear their influence on the extraction methods. Knowing the fractions with the best responses to the extraction of antioxidant compounds, one can calculate the volumetric proportion employed in each seed, according to Snyder (1968). The composition obtained as the best response for the unfermented seeds (45% water, 20% ethanol, and 35% acetone) showed 0.705 of polarity, while for the fermented seeds (48% water, 14% ethanol, and 38% acetone) showed 0.706, very close numbers.

In a big picture, the use of solvents alone for extraction with high polarities, as water, or low polarities, such as acetone, did not show good results in the extraction of antioxidant compounds for grape seeds. Although the binary combination of solvents has increased the extractable compounds, it has not been fully effective in most of these extractions. However, one can see that the solvents in the ternary fraction, which showed moderate polarity, presented a better universal extraction capacity (Liu et al., 2000).

The two types of seeds, although representing residues with different treatments, presented composition and polarity of the solvent extraction mixture very close. Moreover, the results of antioxidant activity were similar. However, one can see that after the desirability analysis for the best extraction fraction of solvents, the unfermented grape seed excelled at all submitted tests.

3.3 Trans-resveratrol

The *trans*-resveratrol content found in the two seeds showed statistical differences ($P < 0.05$) (Table 3). The highest indexes of the polyphenol were in the unfermented grape seed extracts, highlighting the ternary fraction of the extraction solvents water/ethanol/acetone (9.84 ± 0.37 mg L^{-1} of *trans*-resveratrol), followed by the binary fractions water/ethanol (8.94 ± 1.12 mg L^{-1} of *trans*-resveratrol), and water/acetone (8.28 ± 0.24 mg L^{-1} of *trans*-resveratrol). The composition of the solvents, in particular, the ternary mixture water, ethanol and acetone favored the extraction of *trans*-resveratrol polyphenol. According to Casas et al. (2010), *trans*-resveratrol content found in these samples ranged similarly to those found in white wine (0.01 up to 8 mg L^{-1}).

3.4 Seeds oil

The volume of oil obtained by cold pressing had a yield of 13.0% for both seeds. This value was very close to the obtained by the Soxhlet method which ranged from 13.46 to 13.73% for fermented and unfermented seeds, respectively. This indicates that the oil extraction method may not be a decisive factor in relation to the yield of lipid content in the seeds. However, according to (Ribeiro et al., 2019), the yield and physicochemical quality can be affected by the oil extraction method. Table 5 shows that most of the results had no significant difference ($P < 0.05$) between the concentrations of fatty acids in the analyzed seeds.

It were identified 12 fatty acids (FA) in both seeds studied, where linoleic acid (18:2n-6) was the major, followed by oleic acid (18:1n-9), and palmitic acid (16:0). Similar results were found in other studies of grape seeds (Cao & Ito, 2003;

Table 4. Predicted *versus* obtained values for antioxidant activity.

Fermented seed		
Assay	**Predicted values**	**Obtained values**
DPPH	1195.38[a]	1144.00 ± 124.19
ABTS	1684.81	1138.00 ± 137.76
FRAP	4585.22	3449.33 ± 98.99
FC	264.55	179.97 ± 8.65
FLA	8.10	8.79 ± 1.13
Unfermented seed		
DPPH	1800.24[b]	1340.00 ± 97.07
ABTS	2630.95	1486.88 ± 127.25
FRAP	5120.55	4512.66 ± 175.24
FC	341.57	220.31 ± 3.88
FLA	13.53	10.89 ± 1.01

[a]water:etanol:acetone - 0.48:0.14:0.38 (v/v); [b]water:etanol:acetone - 0.45:0.20:0.35 (v/v). Where DPPH (μmol TE g^{-1} extract), ABTS (μmol TE g^{-1} extract), FRAP (μmol E$FeSO_4$ g^{-1} extract), FC = phenolic compounds (mg EAG g^{-1} extract) and FLA = flavonoids (mg EQ g^{-1} extract).

Table 5. Fatty acids and antioxidant activity of grape seeds oil.

Assay	Fermented (mg g^{-1} oil)	Unfermented (mg g^{-1} oil)
Fatty acids		
14:0	0.65 ± 0.04^{A}	0.49 ± 0.00^{B}
15:0	0.39 ± 0.02^{A}	0.35 ± 0.01^{A}
16:0	72.35 ± 3.49^{A}	66.05 ± 0.35^{B}
16:1	1.16 ± 0.07^{A}	0.77 ± 0.01^{B}
17:0	0.68 ± 0.02^{A}	0.65 ± 0.00^{A}
18:0	33.26 ± 0.73^{A}	32.94 ± 0.15^{A}
18:1n9c	184.62 ± 7.03^{A}	175.13 ± 0.29^{A}
18:2n6t	6.59 ± 0.23^{A}	5.98 ± 0.24^{B}
18:2n6c	744.94 ± 37.86^{A}	713.90 ± 2.77^{A}
20:0	1.68 ± 0.04^{A}	1.73 ± 0.00^{A}
18:3n3	3.96 ± 0.23^{A}	3.54± 0.03^{B}
20:1	0.35 ± 0.01^{A}	0.36 ± 0.03^{A}
∑ SFA	109.04 ± 4.36^{A}	102.24 ± 0.52^{A}
∑ MUFA	186.13 ± 7.11^{A}	176.27 ± 0.31^{A}
∑ PUFA	755.50 ± 38.33^{A}	723.43 ± 3.90^{A}
IA	0.08 ± 0.00 ^{A}	0.08 ± 0.00^{A}
IT	0.22 ± 0.00 ^{A}	0.22 ± 0.00 ^{A}
HH	12.88 ± 0.06 ^{A}	13.50 ± 0.08 ^{B}
Antioxidant activity		
DPPH (µmol TE g^{-1} sample)	20.00 ± 5.65^{A}	15.33 ± 2.31^{A}
ABTS (µmol TE g^{-1} sample)	13.60 ± 4.6^{A}	14.26 ± 0.00^{A}
FRAP (µmol $EFeSO_4$ g^{-1} sample)	17.93 ± 0.94^{A}	10.60 ± 1.88^{B}
Phenolic compounds (mg EAG g^{-1} sample)	ND	ND
Flavonoids (mg EQ g^{-1} sample)	0.05 ± 0.00^{B}	0.17 ± 0.00^{A}

Mean ± standard deviation. Different letters in the same line represent significant differences (P<0.05) by T-test. Comparison was done between different treatments of seed (fermented and unfermented). PUFA = poliunsaturated fatty acids; MUFA = monounsaturated fatty acids; SFA = saturated fatty acids; ND = not detected; IA = atherogenicity index = (12:0 + 4 x 14:0 + 16:0)/[ΣMUFA + Σ(n-6) + Σ(n-3)]; IT = thrombogenicity index = (14:0 + 16:0 + 18:0)/[0,5 x ΣMUFA + 0,5 x Σ(n-6) + 3 x Σ(n-3) + Σ(n-3)/Σ(n-6)] (Bono et al., 2012); HH = ratio between hypocholesterolemic and hypercholesterolemic (HH) fatty acids = [(Σ 18:1 cis-9, 18:2 n-6, 20:4n-6, 18:3 n-3)/(Σ 14:0 e 16:0)] (Santos-Silva et al., 2002).

Rockenbach et al., 2010; Santos et al., 2011). The other FA showed low concentrations for both seeds. An organism can biosynthesize saturated and unsaturated fatty acids of the omega 9 family. However, it does not produce linoleic acid (omega 6), a necessary component to be inserted in a diet (Aguiar et al., 2011). The nutritional quality of the lipid fraction of the Bordô seeds samples (Table 5) was similar since the index of atherogenicity (IA) and thrombogenicity (IT) were identical for both. These indexes indicate the potential for stimulating platelet aggregation, i.e., the lower the IA and IT values, more antiatherogenic FA are present in the lipid fraction, and greater the prevention of the onset of coronary diseases (Tonial et al., 2010; Turan et al., 2007). In this manner, the obtained results for Bordô seed oils suggest they are good sources of lipids for the human organism, according to Shinagawa et al. (2015). The ratio of hypocholesterolemic and hypercholesterolemic (HH) FA was also close in both samples (unfermented 13.50; fermented 12.88). This relationship is linked to the incidence of cardiovascular diseases since it considers the functional activity of FA in the metabolism of plasma cholesterol transport lipoproteins (quantity and type). Thereby, higher values of HH correspond to products with a desirable composition of FA (Tonial et al., 2010).

Regarding the antioxidant activity, the oils from both samples showed lower values than the extracts, while phenolic compounds and *trans*-resveratrol were not found in oils. According to (Mahanna et al., 2019), after quantify the levels of *trans*-resveratrol in grape seed-oil, only a fraction of this polyphenol remained after the oil extraction, probably due its polarity.

4 Conclusion

The experimental design obtained by the special cubic model predicted that the best results of the antioxidant activity were obtained for the ternary mixture of the solvents (water, ethanol, and acetone), increasing about 20 times this bioactivity for both seeds when compared to pure solvents. The quantification of *trans*-resveratrol demonstrated this polyphenol is also present in most of the ternary composition of the extractor solvent. It was possible to observe the close relationship between the polarities of the extractor solvents involved in response of the antioxidant activity. The composition that showed the best response ranged between 45-48% of water, 14-20% of ethanol and 35-38% of acetone for both grape seeds, with polarity ranged from 0.705 to 0.706. By the same way, oils of both seeds presented a similar fatty acid profile, being this vinification by-product characterized by high content of polyunsaturated fatty acids, predominating linoleic acid.

Therefore, the use of this optimized mixture of solvents can be applied in grape seeds antioxidant extraction and results at an extract with stronger bioactivity. In addition, the developed extraction method preserve bioactive compounds from grape seeds, adding value on this by-product that can be availed on food industry as a preservative.

Acknowledgements

The authors acknowledge to Unipar, Campus Toledo, for borrowing the universal press to obtain the oil and Gilmar Camargo for donating the oil extraction template.

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