Optimization of the extraction of phenolic compounds from olive pomace using response surface methodology[1]

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ABSTRACT

Extraction of olive oil gives rise to large quantities of pomace and liquid effluents, since on average only 21% of the weight of the olive corresponds to oil, the remaining 79% consists of water, bark, pulp and stone. With the intention to make available new forms of use of this residue, this research was proposed, with aimed to optimize the extraction of phenolic compounds from olive pomace resulting from oil extraction using methanolic extracts. The analysis of phenolic compounds (TPC) and the evaluation of the antioxidant activity (AA) were performed by spectrophotometry, and the individual phenols were carried out by LC-ESI-qTOF-MS. The data were evaluated by the application of the response surface methodology (RSM). The condition that promoted the highest TPC in an extract was using 40% methanol, 70 °C and 180 minutes (extract 7). The highest AA was in the extract obtained with 40% methanol, 45 °C and 180 minutes (extract 5). The highest individual phenol sum (IPS) was in the extract with 80% methanol, 45 ºC and 180 minutes (extract 6). Therefore, it is possible to conclude that the RSM was an interesting tool to measure the best conditions for extraction of phenolic compounds from olive pomace.

Keywords: residue; antioxidant activity; bioactive compounds.

INTRODUCTION

In European countries, where 95% of the world's olive oil production is concentrated (International Olive Council, 2018), the inherent residues of olive oil production are considered an environmental problem.

The amount and characteristics of the waste generated will depend on the extraction form of the olive oil. There are currently two distinct olive oil extraction methods: the traditional method, using hydraulic press, and the continuous extraction by centrifugation method, which the olive industry has adopted in recent decades (Bhatnagar *et al*., 2014).

In the hydraulic press extraction, a solid pomace and olive mill waste waters are generated. In the centrifugal extraction method, two distinct systems may be used: the three-phase system and the two-phase system. In the two-phase centrifugation system, only one pomace containing up to 80% moisture, including peel, pulp and stone, without the formation of olive mill waste waters is generated.

In the three-phase centrifugation system a solid pomace is formed, consisting of the pulp, rind and stone of the fruit, containing 25 to 50% moisture and 5 to 7% of olive oil. In addition, olive mill waste waters are formed in larger volume than in the traditional method, due to the addition of water in the three-phase centrifugation process. Generally speaking, the olive mill waste water produced in this system are mostly made up of water (83 to 94%), organic matter (4 to 16%) and mineral salts (0.4 to 2.5%) (Alú'datt *et al*., 2010).

The applications of olive pomace include their use as organic fertilizers and animal feed supplements (Innangi *et al*., 2017), fortification of food products, such as french

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fries (Bouaziz *et al*., 2010), pasta and bread (Simonato *et al*., 2019), refined edible oils (Sánchez de Medina *et al.*, 2012), and fermented milk (Aliakbarian *et al.*, 2015).

The olive pomace resulting from the oil extraction accounts with about 99.95% of the total phenolic content (TPC) of the olive fruit, with only less than 0.05% migrating to olive oil (Cecchi *et al.*, 2018).

The disposal of these residues may cause harmful effects on the environment due to its high organic content and phytotoxicity, due to recurrent high concentration of phenolic compounds, which ones have hard biological degradation, and antimicrobial effect, by affecting the processes of anaerobic digestion (Bhatnagar *et al*., 2014).

These residues can be substantially valued from the extraction of phenolic compounds that, given their wide range of bio-applications, can contribute to the recovery of this residue, with significant reduction of environmental impact. Therefore, the objective of this study was to optimize the extraction of phenolic compounds from olive pomace obtained in the two-phase extraction process using methanolic extracts.

MATERIAL AND METHODS

Sample and Chemicals

The olive pomace of two phase were supplied by a plant processing of olive oil, located at the city of Pinheiro Machado (31º29'59.2''S. 53º30'37.9''W) in Rio Grande do Sul, Brazil.

The samples were collected and subsequently frozen in an ultrafreezer at -80ºC. Afterwards, the samples were lyophilized, promoting the removal of 99% of water. All chemical products were of the highest analytic degree. Hydroxybenzoic Acid, Gallic Acid, Rutin, Catechin, Ferulic Acid, Caffeic Acid, Chlorogenic Acid, Vanillic Acid, Coumaric Acid, Syringic Acid, Tyrosol, Oleuropein, Hydroxytyrosol, were supplied by Sigma-Aldrich (St. Louis, USA); methanol and Folin Ciocalteu 2 N solution were obtained from Merck (Darmstadt, Germany). Water was purified by an Ultra Purification System (Mega Purity).

Experimental Design

The extraction parameters were optimized using Response Surface Methodology (RSM). A Central Composition Design (CCD) was used to identify the relationship between response functions and independent variables, as well as determine conditions that optimize the extraction process for total phenol content (TPC), antioxidant activity (AA) and individual phenol summatory (IPS) of olive pomace extracts. Concentration of methanol (X1), temperature (X2) and time (X3) were chosen for independent variables. The range and center point values of three independent variables presented in Table 1 and the choice of methanol as solvent were based on the results of preliminary experiments. Each variable to be optimized was encoded in three levels (-1, 0, +1). Eleven randomized experiments including three replicates as the central points were assigned based on CCD. The TPC, AA and IPS were selected as the responses (dependent variables) for the combination of the independent variables (Table 1). Three experiments of each condition were performed, and mean values were declared as measured responses. The predicted values of TPC, AA and IPS were obtained according to the recommended optimum conditions. The predicted and experimental values were compared in order to determine the validity of the model.

Preparation of the extract

Extraction by maceration was carried out in a water bath; where the lyophilized olive pomace sample (0.5 g) was mixed with 15 mL of aqueous methanol at defined concentrations (Table 1) and kept under agitation according to the time (Table 1) and temperature (Table 1) as determined in CCD. After extraction, the extracts were centrifuged at 7,000 × g for 15 min, and the supernatants were filtered through filter paper and transferred to a 20 mL volumetric flask, for the final volume to be adjusted with the respective concentrations of aqueous methanol.

Determination of total phenol content of olive pomace

The TPC was measured by a photometric Folin–Ciocalteu assay according to Swain & Hillis (1959) with a few adaptations. To 250 µL extract were added 4000 µL water and 250 µL Folin-Ciocalteu reagent (0.25 mol.L^{-1}) in a centrifuge tube and allowed to react for 3 minutes. Subsequently, 500 µL of sodium carbonate (1.0 mol.L^{-1}) was added. After 2 hours of reaction, the absorbance was measured in a spectrophotometer (Jenway 6705 UV/VIS) at 725 nm. Standard curve was defined by known concentrations of gallic acid, ranging between 0 and 200 mg.L^{-1} (R^2=0.9923), and results were expressed in milligrams of gallic acid equivalents (mg.kg^{-1} GAE).

Determination of the antioxidant activity

The antioxidant activity of the samples was assessed by standard antioxidant Trolox. Calibration curves of Trolox (concentrations 0-300 mg.L^{-1}) were made in FRAP (R^2 = 0.9954) post-column assays, and the results were expressed as Trolox equivalent antioxidant capacity (mg.kg^{-1} TEA). The analysis was conducted according to the method described by Silva (2013), with few modifications. To the extract was added 3000 µL of the FRAP reagent, and the reaction was conducted under heating at 37 ° C for 30 minutes. The reduction of the Fe^{3+}

to Fe^{2+} complex was obtained by reading the absorbance at 595 nm in a spectrophotometer (Jenway 6705 UV/VIS).

Identification of the Phenolic Compounds by LC-ESI-qTOF-MS

The same extracts analyzed for total phenolic content and antioxidant activity by spectrophotometer was used for identification of the phenolic compounds by LC-ESI-qTOF-MS. Samples were filtered through a 0.22 mM nylon membrane filter (Merck Millipore Corporation, Germany). After the samples were prepared, 10 µL was injected in a liquid chromatograph (UFLC, Shimadzu, Japan) coupled to a high-resolution mass spectrometer of the quadrupole type–flight time (Maxis Impact, Bruker Daltonics, Germany). A pre-column C18 (2.0 × 4 mm) and Luna C18 column (2.0 × 150 mm, 100 Å, 3 µm) (Phenomenex Torrance, USA) were used for the chromatographic separation using the mobile phases: water acidified with 0.1% formic acid (eluent A) and acetonitrile acidified with 0.1% formic acid (eluent B). For separation, a gradient was used: 0–2 min, 10% B; 2–15 min, 10–75% B; 15–18 min, 90% B; 18–21 min 90% B; 21–23 min, 10% B, 23–30 min, 10% B, 0.2 mL.min^{-1} flow and the column temperature was set at 40 °C. The mass spectrometer was operated in the ESI negative modes with spectra acquired over a mass range of m/z 50 to 1200, with capillary voltage at 3.5 kV, nebulization gas pressure (N_2) of 2 bar, drying gas at 8 L min^{-1}, source temperature 180 °C, RF collision of 150 Vpp; transfer 70 mS and pre-pulse storage of 5 mS. The equipment was calibrated with 10 mmol.L^{-1} sodium formate, covering the acquisition range of m/z 50 to 1200. Automatic MS/MS experiments were performed by adjusting the collision energy values as follows: m/z 100, 15 eV; m/z 500, 35 eV; m/z 1000, 50 e V, using nitrogen as the collision gas (Hoffmann *et al*., 2016). Data from MS and MS/MS were processed using Data analysis software 4.0 (Bruker Daltonics, Germany). Phenolic compounds were characterized by the UV/Vis spectrum (210–800 nm), and the exact mass and MSn fragmentation patterns were compared to the equipment library data and databases (Metlin, MassBank, Kegg Compound, ChemSpider) and compared with the isotopic standard. The quantification of phenolic compounds were performed by external calibration curve with eight concentrations (0.039; 0.078; 0.156; 0.312; 0.625; 1.250; 2.50 and 5 µg.mL^{-1}) with standards of each compound (Hydroxybenzoic acid (R^2 = 0.9988), Coumaric acid (R^2 = 0.9997), Vanillic acid (R^2 = 0.9999), Galic acid (R^2 = 0.9999), Caffeic acid (R^2 = 1.0000), Ferulic acid (R^2 = 0.9999), Syringic acid (R^2 = 0.9996), Chlorogenic acid (R^2 = 0.99969), Rutin (R^2 = 0.9998), Catechin (R^2 = 0.9989), Oleuropein (R^2 = 0.9996), Hydroxytyrosol (R^2 = 0.9962), Tyrosol (R^2 =0.9924)), and the results were expressed in mg.kg^{-1}.

Statistical Analysis

To evaluate the results for TPC, AA and IPS for experimental designs, it was used analysis of variance (ANOVA), which was carried out using the software Statistica 6.0 at level of 95% of confidence ($p < 0.05$). The experiments and analytical measurements were carried out in triplicate. The adequacy of the model was determined by evaluating the lack of fit, the coefficient of determination (R^2), and the F test value obtained from the ANOVA. The Tukey test was used for comparison of the means at 5% of significance. The relationship between the independent variables and the response variables was demonstrated by the 3D response surface plots.

RESULTS AND DISCUSSIONS

Optimization of phenolic compounds extraction

As shown in Table 2, concentrations of TPC ranged from 20886.2 to 23061.2 mg.kg^{-1} GAEof dried olive pomace, and all samples differed significantly by Tukey test ($p < 0.05$)

The highest concentration of phenolic compounds was obtained by the extract 7 (23061.2 mg.kg^{-1} GAE), in which the methanol concentration was 40% (level -1), the temperature of 70 ºC (level +1) and time of 180 minutes (level +1); followed by the extract 8, in which the concentration of 22809.4 mg.kg^{-1} GAE was obtained. The lowest yield was obtained by the extract 3 (20886.2 mg.kg^{-1} GAE), in which the methanol concentration was 40% (-1), the temperature of 70 ºC (+1) and the time of 60 minutes (-1). Similar results were reported in a study that evaluated the efficacy of ultrasound in the extraction of TPC from olive pomace, with 22020 mg.kg^{-1} GAE (Goldsmith *et al.*, 2018). The effects of the variables on the overall yield of the extraction were determined (Figure 1). For this, linear models with a 95% confidence interval were considered.

Table 1: Independent variable and coded levels used in Central Composition Design

Independent variable	Units	Coded Levels		
		-1	0	+1
Methanol concentration (X1)	%	40	60	80
Temperature (X2)	ºC	45	57.5	70
Time (X3)	minutes	60	120	180

Table 2: Coded and real (in parenthesis) values for the concentration response of total phenol content (TPC), antioxidant activity (AA) and individual phenol summatory (IPS)

Extract	Concentration (%)	Temperature (ºC)	Time (min)	Response value					
				TPC ($mg.kg^{-1}$ GAE)		AA ($mg.kg^{-1}$ TEA)		IPS ($mg.kg^{-1}$)	
				Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	-1(40)	-1(45)	-1(60)	21224.4^{i}±0.8	21051.6	18772.4^{d}±17.2	19170.9	1227.4^{d}±4.0	1282.7
2	+1(80)	-1(45)	-1(60)	21231.5^{h}±0.3	21158.6	18124.6^{e}±25.8	14550.6	1343.6^{c}±5.0	1282.7
3	-1(40)	+1(70)	-1(60)	20886.2^{k}±0.1	20918.2	19443.9^{b}±17.2	16246.5	1063.3^{h}±3.0	1036.5
4	+1(80)	+1(70)	-1(60)	21088.9^{j}±1.3	20811.2	19253.8^{c}±17.1	20028.9	1012.5^{i}±1.7	1036.5
5	-1(40)	-1(45)	+1(180)	21652.4^{d}±0.1	21662.0	20405.5^{a}±34.4	17208.0	1377.7^{b}±3.7	1434.7
6	+1(80)	-1(45)	+1(180)	22024.3^{c}±1.1	21769.0	2711.8^{k}± 25.7	3487.0	1481.3^{a}±3.5	1434.7
7	-1(40)	+1(70)	+1(180)	23061.2^{a}±0.8	22865.9	13884.9^{f}±17.3	14283.5	888.6^{i}±1.3	884.5
8	+1(80)	+1(70)	+1(180)	22809.4^{b}±0.2	22758.9	12539.5^{g}±8.6	8965.4	856.8^{k}±1.3	884.5
9	0(60)	0(57.5)	0(120)	21330.0^{e}±1.3	21624.5	10510.8^{i}±8.7	14242.6	1141.7^{f}±1.0	1159.6
10	0(60)	0(57.5)	0(120)	21263.9^{g}±1.7	21624.5	10939.0^{h}±8.7	14242.6	1106.4^{g}±4.2	1159.6
11	0(60)	0(57.5)	0(120)	21296.9^{f}±1.5	21624.5	10082.5^{j}±8.5	14242.6	1176.9^{e}±2.0	1159.6

*± = Standard Deviation.

**^{a,b,c,d,e,f,g,h,i,j,k}= Values with different lowercase letters in a column are significantly different ($p < 0.05$).

The effects considered significant can be observed from the p value, where all values smaller than 0.05 are significant. The p value is the probability of observing a statistical test value greater than or equal to that found. This value is used to evaluate the significance of the coefficients, so that the smaller the p value, the greater the significance of the coefficient of variation.

The time variable presented positive and significant effect, when the extraction time was increased from level -1 (60 min) to level +1 (180 min), there was an increase in the effect of 1279.1. This can be justified by the increase in the contact time between the solvent and the sample, which leads to a greater penetration of the solvent and, consequently, favors the extraction of the phenolic compounds. According to Yingngam *et al*. (2015), reduced extraction times do not allow efficient penetration of the solvent into the extract, preventing the extraction of the compounds of interest. Some studies corroborate these findings, since an increase in TPC content was observed due to an increase in extraction time (Sun *et al*., 2011; Dent *et al*., 2013; Chen *et al*., 2018).

The interaction between temperature and extraction time promoted a positive and significant effect on the extraction of phenolic compounds. With the increase of the extraction time and the heating of the sample, the integrity of the cell wall weakened, promoting greater extraction of these compounds, due to the increased solubility with the solvent (Liu *et al*., 2013). The temperature had a positive and significant effect, when this variable was elevated to -1 (45 ºC), at +1 (70 ºC) there was an increase in the effect of 428.28 (Figure 1). This is justified because the high temperature favors the mass transfer process, leading to the reduction of the viscosity of the solvent and facilitating its penetration, besides favoring the degradation of the matrix and the cellular structure, which makes the cells more permeable (Tabaraki *et al*., 2012; Liu *et al*., 2013). In addition, there is a weakening of the interactions between phenolic compounds and proteins, and between phenolic compounds and polysaccharides; therefore, increasing the rate of diffusion. Yuan *et al*. (2018) observed similar results in the optimization of phenolic compounds extraction by maceration from Oregon hazelnut residues in the United States.

The interaction between the methanol concentration and the temperature showed a significant negative effect. This behavior can be related to the reduction of the dielectric constant of the aqueous solution mixture with increasing temperature, which reduces the polarity of the solvent, resulting in a lower extraction of phenolic compounds (Chiang *et al*., 2017). The solvent concentration variable as well as its interaction with time were not significant. Equation 1 presents the first order

coded model, which describes the TPC as a function of the independent variables (concentration, temperature and time).

The model was validated by analysis of variance (Table 3), in which a correlation coefficient of 0.94 was obtained, indicating that the model was significant.

TCP = 21624.46 + 214.14 temperature + 639.54 time - 53.51 concentration tmperature + 334.34 temperature time (1)

The $F_{calculated}$ values of 583.19 and $F_{tabulated}$ of 4.46 (Table 1 in supplementary material), demonstrate that the model was predictive, thus allowing the construction of the response surfaces shown in Figures 2 (a), (b) and (c).(b)

In Figure 2 (a), it is possible to observe that with the increase of temperature and time variables to higher levels there is a higher concentration of TPC, which is evident by the intensification of the dark color presented in the graph. Figure 2 (b) shows that the region with the highest TPC is at the highest levels of extraction times, however, the methanol concentration had no influence on the TPC, and in Figure 2 (c) it is possible to observe that the darkest and highest TPC region is concentrated at the highest temperature and concentration levels.

Determination of the AA by FRAP Assay

According to Table 2 all samples differed significantly by Tukey test ($p < 0.05$), and the highest AA by the FRAP method was obtained in extract 5 (20405.5 mg.kg^{-1} TEA), in which the methanol concentration was 40% (-1), the temperature of 45 ºC (-1) and time of 180 minutes (+1). The lowest AA was observed in extract 6 (2711.8 mg.kg^{-1} TEA), where the methanol concentration was 80% (+1), temperature of 45 °C (-1) and time of 180 minutes (+1).

Extracts 5 and 6 showed a large difference in AA, although only differing in methanol concentration, probably because the set of parameters used promoted lower extraction of compounds in extract 6.

The effects of the variables on AA by the FRAP method (Figure 3) were determined, where linear models with a 95% confidence interval were considered.According to Figure 3, the time was the variable that presented the most expressive effect. When time was elevated from level -1 (60 min) to +1 (180 min), it was promoted a reduction effect of AA of 6513.3, probably because in longer extraction periods there was a greater degradation of compounds with antioxidant activity.

Solvent concentration showed a negative and significant effect on the AA response, when it was increased from level -1 (40%) to level +1 (80%), there was a reduction of AA in 4969.3. The interaction between solvent concentration and time presented a negative and significant effect. Similar result also was reported in previous study (Aybastier *et al*., 2013). The interaction between solvent concentration and temperature exerted a positive and significant effect against the AA response by the FRAP method. The temperature variable presented $p > 0.05$, and consequently did not present statistical significance. Similarly, the interaction between temperature and time had no significant effect on AA response by the FRAP method.

Equation 2 presents the coded model, which describes the AA by the FRAP method, as a function of the independent variables (concentration, temperature and time).

AA = 14242.6-2484.6 concentration + 638.5 time - 3256.6 time + 2100.7 concentration temperature - 2275.15 concentration + 638.5 time (2)

The model was validated by analysis of variance, in which a correlation coefficient of 0.84 was obtained,

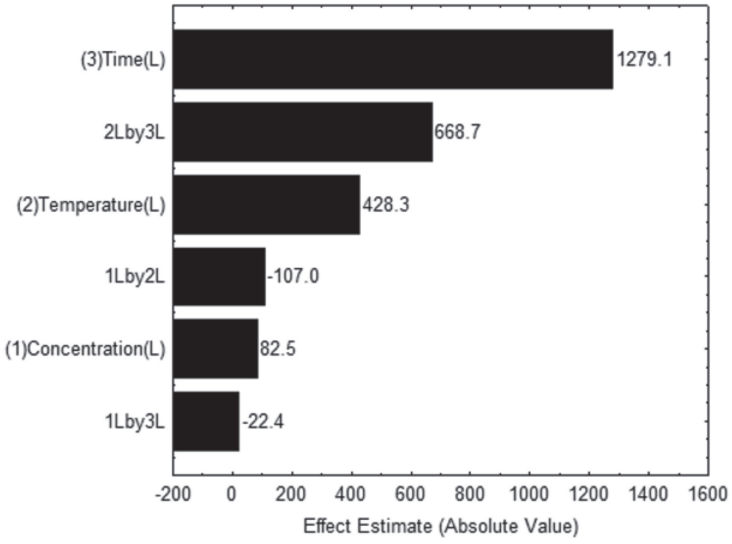

Figure 1: Estimated effects of concentration (1), temperature (2) and time (3) parameters on TPC response.

indicating that the model was significant. The $F_{calculated}$ values of 206.8 and $F_{tabulated}$ of 4.46 demonstrated that the model was predictive, thus allowing the construction of the response surfaces, shown in Figures 4 (a), (b) and (c).

According to Figure 4 (a), the darker region of the graph (higher AA) is concentrated at lower levels of the time variable; however, temperature had no influence on AA. In Figure 4 (b) the darkest region is concentrated at the lowest levels of solvent concentration and time, and in Figure 4 (c) it can be seen that the darkest region is concentrated at the lowest level of concentration and temperature.

Identification of the Phenolic Compounds by LC-ESI-qTOF-MS

According to table 3, the sum of the compounds ranged from 856.8 to 1481.3 $mg.kg^{-1}$. In ascending order of average concentration were found: catechin (0.68 $mg.kg^{-1}$), ferulic

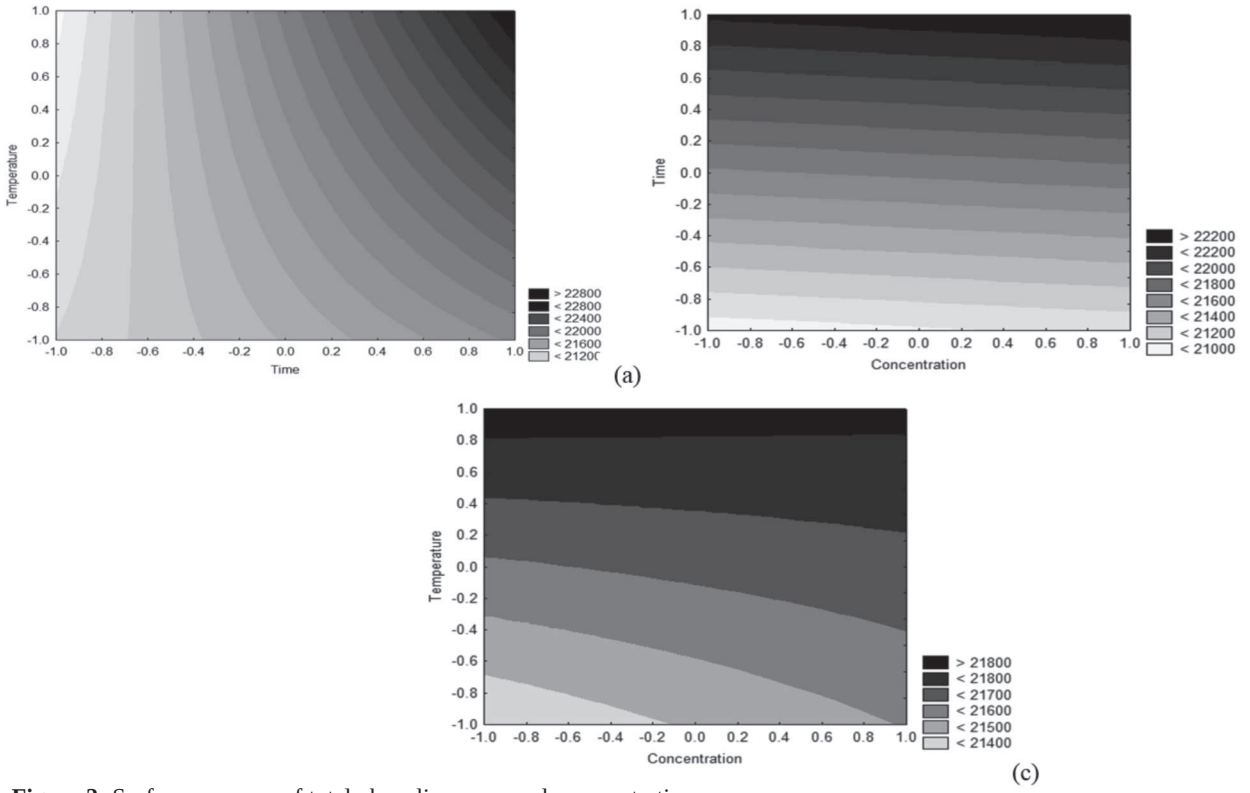

Figure 2: Surface response of total phenolic compounds concentration.

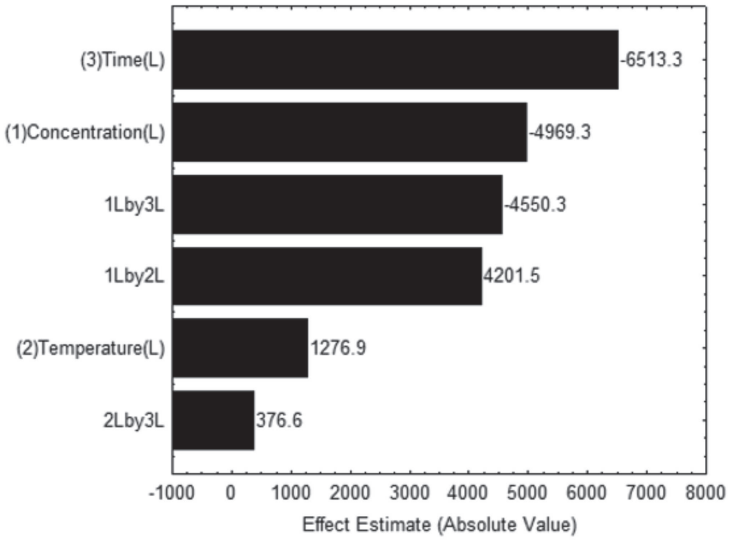

Figure 3: Estimated effects of concentration (1), temperature (2) and time (3) parameters on AA response.

acid (1.75 mg.kg^{-1}), hydroxybenzoic acid (2.73 mg.kg^{-1}), gallic acid (4.07 mg.kg^{-1}), rutin (4.52 mg.kg^{-1}) coumaric acid (4.55 mg.kg^{-1}), oleuropein (6.16 mg.kg^{-1}), chlorogenic acid (6.24 mg.kg^{-1}), vanillic acid (10.37 mg.kg^{-1}), caffeic acid (18.38 mg.kg^{-1}), syringic acid (132.5 mg.kg^{-1}), hydroxytyrosol (136.7 mg.kg^{-1}) and tyrosol (833.7 mg.kg^{-1}). In general, phenolic alcohols were present in higher concentration (930.4 mg.kg^{-1}), followed by phenolic acids (173.8 mg.kg^{-1}), secoiridoids (6.16 mg.kg^{-1}) and flavonoids (5.11 mg.kg^{-1}).

Albahari *et al*. (2018) optimized extraction of olive pomace phenolic compounds by applying ultrasound, and

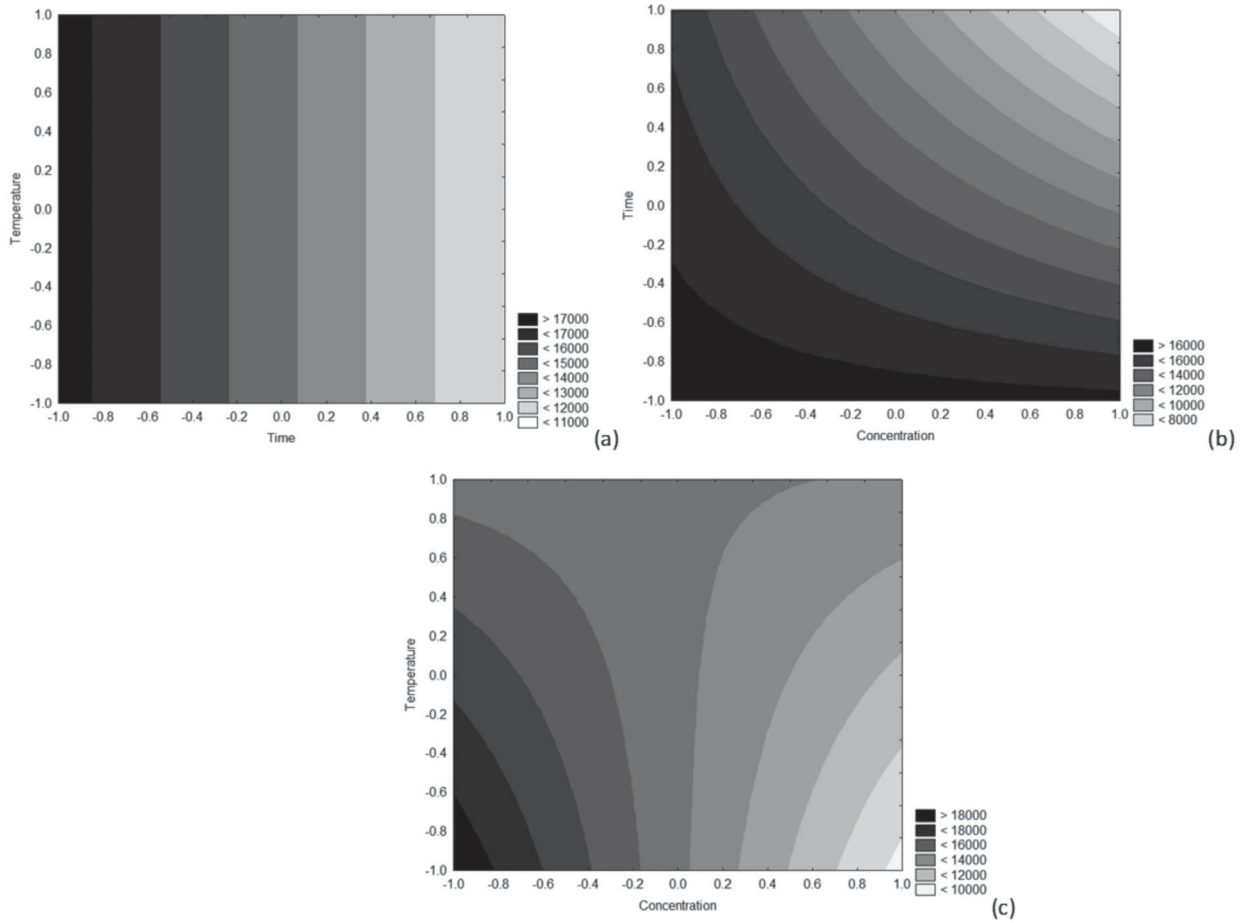

Figure 4: Surface response of antioxidant activity in extracts.

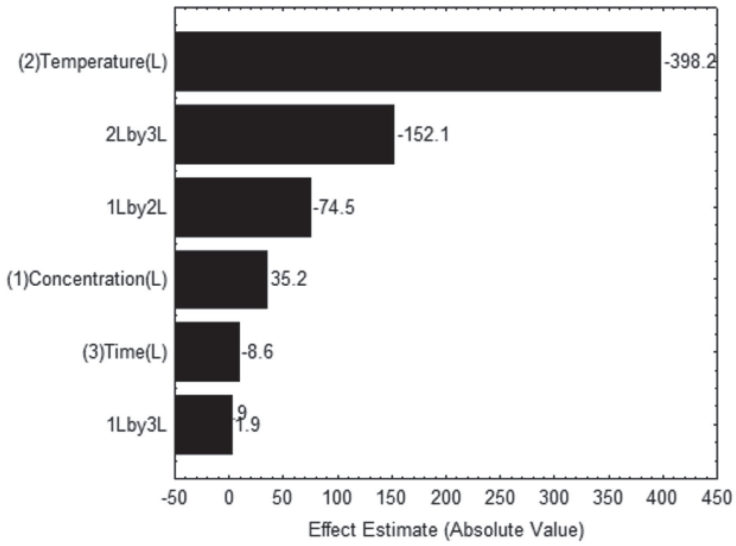

Figure 5: Estimated effects of concentration (1), temperature (2) and time (3) parameters on IPS response.

Table 3: Quantification of individual phenols by LC-ESI-qTOF-MS

	Extracts										
Phenols (mg.kg^{-1})	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Hydroxybenzoic Acid	2.6±0.06	3.7±0.34	2.8±0.01	2.2±0.02	2.9±0.03	4.6±0.07	3.6±0.08	3.7±0.09	1.1±0.01	1.7±0.02	1.4±0.08
Coumaric Acid	6.9±0.15	6.3±0.00	5.5±0.03	5.5±0.00	5.9±0.23	5.3±0.00	4.2±0.04	3.5±0.01	2.1±0.00	2.7±0.15	2.3±0.01
Vanillic Acid	10.1±1.43	11.6±2.37	12.8±1.29	12.3±2.81	11.6±0.25	9.4±0.11	14.5±1.08	10.1±0.72	6.8±0.30	7.9±0.04	7.1±1.01
Gallic Acid	4.2±0.51	4.4±0.1.1	4.9±0.18	4.4±0.03	5.4±0.13	4.3±0.70	3.1±0.35	3.1±0.13	3.1±0.11	3.7±0.05	3.6±0.40
Caffeic Acid	20.4±0.02	22.4±0.01	20.6±0.12	23.0±0.18	18.1±0.13	21.1±0.20	21.4±0.02	20.3±0.12	9.2±0.10	13.9±0.25	11.8±0.01
Ferulic Acid	1.9±0.01	1.7±0.06	1.7±0.01	1.7±0.00	1.9±0.04	1.8±0.17	1.7±0.02	1.9±0.15	1.7±0.02	1.6±0.00	1.7±0.02
Syringic Acid	157.5±0.14	150.8±1.16	158.7±2.23	148.8±1.67	161.4±3.95	153.2±3.77	141.8±0.05	135.2±3.57	83.3±2.01	88.5±1.16	78.1±1.26
Chlorogenic Acid	6.5±0.01	6.7±0.00	6.6±0.01	6.8±0.01	6.7±0.00	6.8±0.03	6.1±0.04	6.0±0.11	5.2±0.02	5.7±0.04	5.6±0.01
Rutin	5.2±0.09	6.3±0.17	4.3±0.17	5.6±0.43	4.8±0.29	5.8±0.34	3.7±0.16	5.1±0.17	2.0±0.06	3.1±0.16	2.8±0.32
Catechin	0.69±0.05	0.60±0.18	0.88±0.05	0.74±0.07	0.83±0.05	0.69±0.29	0.57±0.13	0.57±0.13	0.62±0.19	0.65±0.01	0.66±0.05
Oleuropein	6.9±0.03	6.8±0.12	5.8±0.07	5.9±0.03	6.8±0.09	6.5±0.01	5.9±0.21	7.3±0.10	4.9±0.00	5.4±0.08	5.5±0.01
Hydroxytyrosol	161.2±0.42	139.5±0.12	187.6±0.13	142.9±6.38	198.7±0.31	154.9±1.07	165.4±0.31	102.3±0.19	83.8±0.24	86.9±0.10	80.6±0.63
Tyrosol	852.4±0.03	993.9±0.12	663.1±0.07	664.6±0.03	963.5±0.09	1115.4±0.01	530.5±0.21	576.3±0.10	937.1±0.00	892.0±0.08	982.2±0.01
Total	1227.4	1343.6	1063.3	1012.5	1377.7	1481.3	888.6	856.8	1141.7	1106.4	1176.9

they observed the concentration of 1117 mg.kg^{-1} of tyrosol, similar to that found in the present study for trial 6 (1115.4 mg.kg^{-1}), which was obtained by 80% methanol, 45 ºC and 180 minutes.

The extract 5 presented the highest concentration of hydroxytyrosol (198.7 mg.kg^{-1}), wich was obtained with 40% methanol, 45 ºC and 180 minutes. Similar result was reported by Chanioti & Tzia (2018), whose optimized the extraction of phenolic compounds from olive pomace by assisted ultrasound (230 mg.kg^{-1}).

The concentration of hydroxytyrosol and tyrosol observed in the present study for olive pomace was higher than the content reported for Blanquette olive oil (11 and 1 mg.kg^{-1}, respectively) and Rougette (5 and 11 mg.kg^{-1}, respectively) found by Yakhlef *et al*. (2018).

In the literature, the main compounds described in olive pomace are oleuropein, hydroxytyrosol, tyrosol, luteolin, apigenin, vanillic acid, caffeic acid and rutin (Servili *et al*., 1999; Alu'datt *et al*., 2010; Sánchez de Medina *et al*., 2012; Chanioti & Tzia, 2018; Albahari *et al*., 2018; Seçmeler *et al*., 2018; Nunes *et al*., 2018). In the present study, different molecules than those previously described were found (galangin, kaempferol and chrisin), which may be related to the optimization of the extraction conditions, which results in a more efficient removal of the phenolic compounds from olive pomace.

Differences in the extraction variables did not qualitatively modify the phenol profile, but it was observed quantitatively differences, since all the compounds were identified in the 11 extracts; however, at different concentrations (Table 3).

As shown in Table 2, the highest IPS was obtained in the trial 6 (1481.3 mg.kg^{-1}) (Table 2), which was obtained by 80% methanol, 45 ºC and 180 minutes, and all samples differed significantly by Tukey test ($p < 0.05$).

It was possible to observe by the analysis of effects of the variables (Figure 5) that when the temperature variable was elevated from level -1 to +1 there was a reduction in the effect of 398.2 against the analyzed response. Similarly, the interaction between temperature and time promoted a significant and negative effect, which indicates that when these variables were elevated from -1 to +1 there was a reduction in the effect of 152.1.

In contrast, the variables concentration, interaction between concentration and temperature, interaction between concentration and time, in addition to the variable time analyzed alone, did not exert a significant effect on the IPS response, since they presented $p > 0.05$ (Figure 5).

Equation 3 presents the coded model, which describes the IPS as a function of the independent variables (concentration, temperature and time).

$$IPS = 1159.6 - 199.1\ temperature - 76.0\ temperature\ time \quad (3)$$

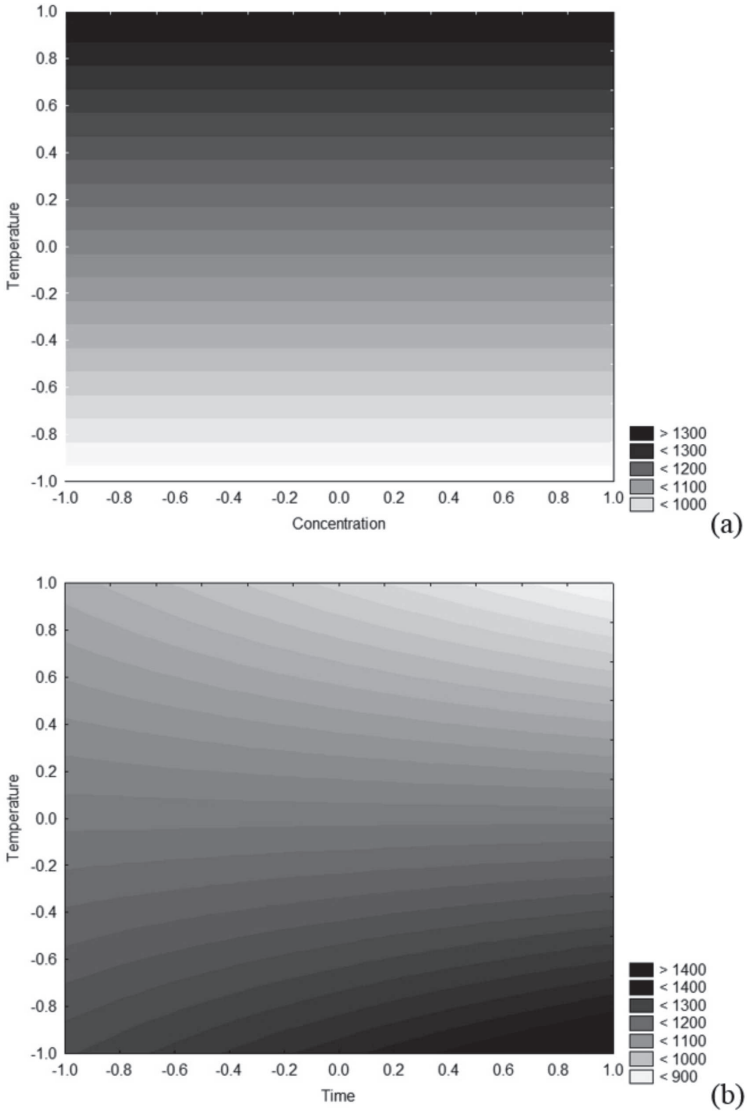

Figure 6: Surface response of individual phenol summatory.

The model was validated by analysis of variance (Table 3), in which a correlation coefficient of 0.98 was obtained, indicating that the model was significant. The $F_{calculated}$ values of 28.43 and $F_{tabulated}$ of 4.46 (Table 1 in supplementary material), demonstrate that the model was predictive, thus allowing the construction of the response surfaces shown in Figures 6 (a) and (b).

In Figure 6 (a), it is observed that the darkest region (highest IPS) is concentrated at the highest temperature level, regardless of the concentration level. Figure 6 (b) shows that the region with the highest IPS is located at the highest extraction time level and lowest temperature level.

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CONCLUSIONS

Through surface response methodology it was possible to observe that the conditions that promoted the highest TPC were obtained by using 40% methanol, 70 °C and 180 minutes. The highest AA was found in the extract obtained with 40% methanol, 45 °C and 180 minutes. The extract that showed the highest IPS was the one obtained using 80% methanol, 45 ºC and 180 minutes.

The response surface methodology proved to be a great alternative for reducing the number of tests, allowing the optimization of the phenol extraction process with reduced number of experiments, promoting reduction on cost and analysis time.

REFERENCES

Alu'datt MH (2010) Optimization, characterization and quantification of phenolic compounds in olive cake. Food Chemistry, 123:117-122.

Albahari P, Jug M, Radiæ K, Jurmanoviæ S, Brnèiæ M, Brnèiæ SR & Èepo DV (2018) Characterization of olive pomace extract obtained by cyclodextrin-enhanced pulsed ultrasound assisted extraction. LWT-Food Science and Technology, 92:22-31.

Aliakbarian B, Casale M, Paini M, Casazza AA, Lanteri S & Perego P (2015) Production of a novel fermented milk fortified with natural antioxidants and its analysis by NIR spectroscopy. LWT-Food Science and Technology, 62:376-383.

Aybastýer Ö, Iþýk E, Þahin S & Demir C (2013) Optimization of ultrasonic-assisted extraction of antioxidant compounds from blackberry leaves using response surface methodology. Industrial crops and products, 44:558-565.

Bhatnagar A, Kaczala F, Hogland W, Marques M, Paraskeva C, Papadakis V & Sillanpää M (2014) Valorization of solid waste products from olive oil industry as potential adsorbents for water pollution control – A review. Environmental Science and Pollution Research, 2:268-298.

Bouaziz M, Feki I, Ayadi M, Jemai H & Sayadi S (2010) Stability of refined olive oil and olive pomace oil added by phenolic compounds from olive leaves. European Journal of Lipid Science and Technology, 112:894-905.

Cecchi L, Migliorini M, Zanoni B, Breschi C & Mulinacci N (2018) An effective HPLC based approach for the evaluation of the content of total phenolic compounds transferred from olives to virgin olive oil during the olive milling process. Journal of the Science of Food and Agriculture, 98:3636-3643.

Chanioti S & Tzia C (2018) Extraction of phenolic compounds from olive pomace by using natural deep eutectic solvents and innovative extraction techniques. Innovative Food Science & Emerging Technologies, 48:228-239.

Chen S, Zeng Z, Hu N, Bai B, Wang H & Suo Y (2018) Simultaneous optimization of the ultrasound-assisted extraction for phenolic compounds content and antioxidant activity of *Lycium ruthenicum* Murr. fruit using response surface methodology. Food Chemistry, 242:01-08.

Chiang PS, Lee DJ, Whiteley CG & Huang CY (2017) Antioxidant phenolic compounds from *Pinus morrisconicola* using compressional-puffing pretreatment and water–ethanol extraction: Optimization of extraction parameters. Journal of the Taiwan Institute of Chemical Engineers, 70:07-14.

Dent M, Dragovic-uzelac V, Penic M, Brncic M, Bosiljkov T & Levaj B (2013) The effect of extraction solvents, temperature and time on the composition and mass fraction of polyphenols in Dalmatian wild sage (*Salvia officinalis* L.) extracts. Food Technology and Biotechnology, 51:84-91.

Goldsmith CD, Vuong QV, Stathopoulos CE, Roach PD & Scarlett CJ (2018) Ultrasound increases the aqueous extraction of phenolic compounds with high antioxidant activity from olive pomace. LWT-Food Science and Technology, 89:284-290.

Hoffmann JF, Zandoná GP, Santos PS, Dallmann CM, Madruga FB, Rombaldi CV & Chaves FC (2016) Stability of bioactive compounds in butiá (Butia odorata) fruit pulp and nectar. Food Chemistry, 237:638-644.

Innangi M, Niro E, D'ascoli R, Danise T, Proietti P, Nasini L, Regni L, Castaldi S & Fioretto A (2017) Effects of olive pomace amendment on soil enzyme activities. Applied Soil Ecology, 119:242-249.

International Olive Council (2018) Statistics. Available at: http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures. Accessed on: September 10th, 2019.

Liu Z, Dang J, Wang Q, Yu M, Jiang L, Mei L & Tao Y (2013) Optimization of polysaccharides from *Lycium ruthenicum* fruit using RSM and its anti-oxidant activity. International Journal of Biological Macromolecules, 61:127-134.

Nunes MA, Costa AS, Bessada S, Santos J, Puga H, Alves RC & Oliveira MBP (2018) Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid-and water-soluble components. Science of the total environment, 644:229-236.

Sánchez de Medina V, Priego-Capote F, Luque de Castro MD (2012) Characterization of refined edible oils enriched with phenolic extracts from olive leaves and pomace. Journal of agricultural and food chemistry, 60:5866-5873.

Seçmeler Ö, Üstündað ÖG, Fernández-Bolaños J & Rodríguez-Gutiérrez G (2018) Effect of subcritical water and steam explosion pretreatments on the recovery of sterols, phenols and oil from olive pomace. Food chemistry, 265:298-307.

Servili M, Baldioli M, Selvaggini R, Macchioni A & Montedoro G (1999) Phenolic compounds of olive fruit: one-and two-dimensional nuclear magnetic resonance characterization of nüzhenide and its distribution in the constitutive parts of fruit. Journal of Agricultural and Food Chemistry, 47:12-18.

Simonato B, Trevisan S, Tolve R, Favati F & Pasini G (2019) Pasta fortification with olive pomace: Effects on the technological characteristics and nutritional properties. LWT-Food Science and Technology, 114:108368.

Sun Y, Wengin X, Wengin Z & Qiuhui H (2011) Optimizing the extraction of phenolic antioxidants from kudingcha made from *Ilex kudingcha* C.J. Tseng by using response surface methodology. Separation and Purification Technology, 78:311-320.

Swain T & Hillis WE (1959) The phenolic constituents of *Prunus domestica* L.- The quantitative analysis of phenolic constituents. Journal of the Science of Food and Agriculture, 10:63-68.

Tabaraki R, Heidarizadi E & Benvidi A (2012) Optimization of ultrasonic-assisted extraction of pomegranate (*Punica granatum* L.) peel antioxidants by response surface methodology. Separation and Purification Technology, 98:16-23.

Yakhlef W, Arhab R, Romero C, Brenes M, Castro A & Medina E (2018) Phenolic composition and antimicrobial activity of Algerian olive products and by-products. LWT- Food Science and Technology, 93:323-328.

Yingngam B, Supaka N & Rungseevijitprapa W (2015) Optimization of process parameters for phenolics extraction of *Cratoxylum formosum* ssp. formosum leaves by response surface methodology. Journal of Food Science and Technology, 52:129-140.

Yuan B, Lu M, Eskridge KM, Isom LD & Hanna MA (2018) Extraction, identification, and quantification of antioxidant phenolics from hazelnut (*Corylus avellana* L.) shells. Food Chemistry, 244:07-15.