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Optimization of Ultrasound-Assisted Extraction of Free Phenolic Compounds and *in vitro* Biological Activity from peach fruit Using Response Surface Methodology

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Abstract: In this study, the ultrasonic extraction (UAE) of free phenolic compounds and relative biological activities of the Bulgarian peach variety 'Filina' was optimized using chemometric techniques (response surface methodology). A Box–Behnken design was used to reveal the variation of hydro module, temperature, duration, and extractant on the total phenolic content, total flavonoid content, antioxidant potential, and inhibitory activity on each yield. The results revealed that optimal conditions included a hydro module of 20, a duration of 39.328 minutes, a temperature of 70 °C, and an extractant of 96.638% to retrieve the highest level of bioactive compounds. The calculated parameters were discovered to be following the projected values.

Keywords: green methods; environmental-friendly; sustainability; phytochemicals; mathematical evaluation

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1. Introduction

Prunus fruits are constantly studied for their beneficial properties as well as their highly preferred taste. Fruits in general are not consumed in quantities that are suggested by the WHO [1]. Efforts have been made to promote fruit consumption. The genus *Prunus* is probably spread and known worldwide which explains the interest in creating new varieties as well as intraspecific hybrids [2]. *Prunus persica* fruits provide a wide range of local and introduced varieties that ripen from June to October. Local varieties are often understudied in terms of their bioactivity. The “Filina” peach variety is a native Bulgarian cultivar that has not been promoted enough although it has shown its potential. The variety is a result of the combination of "July Lady" and "Maycrest". Authors have documented its chemical composition as well as its antioxidant properties [3] and enzyme inhibition abilities [4]. The majority of important and significant polyphenol compounds i.e. flavonoids, and phenolic acids can be found in peach fruits.

Phenolic compounds are often associated with enhanced health. The biological advantages of fruit phenolic compounds are widely studied and the results continue to prove the fruits’ abilities to positively alter conditions like increased insulin levels, cholesterol levels, cancer cell growth, inflammation, among others [5]. Phenolic compounds

are beneficial not only to the food industry but also to fields like cosmetology and pharmacology. Each research design followed a different extraction approach as there is no unified extraction method that has shown maximum efficiency. Polarity variation of the solvent has shown that differences exist in the properties of the resulting extracts [6,7]. The extraction of phenolic compounds is dependent on the duration, temperature, solvent/sample ratio, specificity of the plant matrix, among others. Ultrasonic-assisted extraction is often documented as highly potent for plant-based matrices [8].

Planning, examining, and foreseeing extraction conditions have all been performed with the use of response surface methodology (RSM). RSM is a reliable and promising tool in terms of bioactive compounds extraction [9]. It can help create the most favorable, cost-effective, and sustainable conditions (short extraction, low solvent, consumption, minimum environmental impact) [10,11].

This study attempted to optimize the extraction duration, temperature, and solvent ration to positively alter the number of phenolic compounds and their respected biological activities. Currently, no studies, on the optimization of antioxidant-rich extracts from "Filina" fruits using mathematical approaches, exist. The Box-Behnken design was applied to predict the model and to optimize the extraction conditions (temperature, time, liquid/solvent ratio) based on total phenolic and flavonoid contents, as well as antioxidant, and enzymatic activities. This work can act as a core for other researchers to assess and quantify the biological activity of *Prunus persica* L.

2. Materials and Methods

2.1 Fruit samples

The whole fruit of the Bulgarian peach variety "Filina" was harvested in 2022 undamaged, at eating ripeness in the Fruit Growing Institute, Plovdiv, BG (lat. 42.10384828045957 and long. 24.72164848814686). No bactericides were applied to plants during testing. Fruits on the trees were considered ripe when the growth of the fruit had stopped and the fruit began to soften, exhibited a red color (representative for the variety), and was easily detached. The fruits were cut into peace with a ceramic knife and quickly frozen and then freeze-dried with a vacuum freeze dryer (BK-FD12S, Biobase, Shandong, China). The dried samples were ground to powder and kept before extraction in a dry and cool place.

2.2. Ultrasound-assisted Extraction of Free Phenolic Compounds

The extracts with respect to the levels of independent variables in Table 1 were obtained in an ultrasonic bath operated at a frequency of 35 kHz with a maximum input power of 240 W (UST 5.7150 Siel, Gabrovo, Bulgaria). After that the extracts were centrifugated at 6000 1/min for 40 min, filtered throughout a syringe filter (0.45μm), and vaccum-evaporatred to dryness (IKA RV10 digital, IKA HB 10 digital water bath -IKA®-Werke GmbH & Co., Germany). The residue was dissolved in 5 ml ethanol and kept in a freezer until further analyses.

2.3. Determination of the phenolic contents (TPC)

The TPC was analyzed following the method of Kujala et al. [12] with some modifications. Each extract (0.1 mL) was mixed with 0.5 mL Folin–Ciocalteu reagent and 0.4 mL 7.5 % Na_2CO_3. The mixture was vortexed and left for 5 min at 50°C. After incubation, the absorbance was measured at 765 nm. The TPC is expressed as mg gallic acid equivalents (GAEs) per gram dry weight (g dw).

2.4. Determination of Total Flavonoid Content (TFC)

The total flavonoid content was evaluated according to the method described by Kivrak et al. [13] An aliquot of 0.5 mL of the sample was added to 0.1 mL of 10 % $Al(NO_3)_3$, 0.1 mL of 1 M CH_3COOK, and 3.8 mL of ethanol. After incubation at room temperature for 40 min, the absorbance was measured at 415 nm. Quercetin (QE) was used as a standard and the results are expressed as μg quercetin equivalents (QE)/g dw.

2.5. Evaluation of Antioxidant Activities

2.5.1. DPPH• Radical Scavenging Assay

The ability of the extracts to donate an electron and scavenge 2,2-diphenil-1-picrylhydrazyl (DPPH) radicals was determined by the slightly modified method of Brand-Williams et al. [14] as described by Mihaylova et al. [15]. A freshly prepared 4×10^{-4} M solution of DPPH was mixed with the samples in a ratio of 2:0.5 (*v*/*v*). The light absorption was measured at 517 nm after a 30 min incubation. The DPPH radical scavenging activity is presented as a function of the concentration of Trolox—Trolox equivalent antioxidant capacity (TEAC) and is defined as the concentration of Trolox with equivalent antioxidant activity expressed as μM TE/g dw.

2.5.2. Ferric-Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was carried out according to the procedure of Benzie and Strain [16] with slight modification. The FRAP reagent was prepared fresh daily and was warmed to 37°C prior to use. One hundred and fifty microliters of samples were allowed to react with 2850 μL of the FRAP reagent for 4 min at 37°C, and the absorbance was recorded at 593 nm. The absorbance was recorded at 593 nm and the results are expressed as μM TE/g dw.

2.5.3. Cupric Ion-Reducing Antioxidant Capacity (CUPRAC) Assay

The CUPRAC assay was carried out according to the procedure of Apak et al. [17]. One milliliter of $CuCl_2$ solution (1.0×10^{-2} M) was mixed with 1 mL of neocuproine methanolic solution (7.5×10^{-3} M), 1 mL of CH_3COONH_4 buffer solution (pH 7.0), and 0.1 mL of samples followed by the addition of 1 mL of water (total volume = 4.1 mL) and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as a standard and the results are expressed as μM TE/g dw.

2.6. Enzyme-Inhibitory Activities

2.6.1. α-Glucosidase Inhibitory Assay (Alfa-Gl)

The reaction mixture contained 10 μL of extract (a minimum of five extract concentrations were tested in order to calculate the IC_{50}) and 30 μL of α-glucosidase (0.1 U/mL, G5003-100UN, Sigma-Aldrich, Merck, Darmstadt, Germany). It was incubated for 15 min at 37 °C in a microplate reader (SPECTROstar Nano Microplate Reader, BMG LABTECH, Ortenberg, Germany). Afterwards, 25 μL of 1 mM 4-nitrophenyl-α-D-glucopyranoside (N 1377, Sigma-Aldrich, Merck, Darmstadt, Germany) was added. The reaction mixture was then shaken and incubated at 37 °C for 10 min. The reaction was terminated by adding 60 μL of 0.2 M sodium carbonate solution. Blanks were prepared by adding the extract after the termination of the reaction. The absorbance at 405 nm was measured using a microplate reader. Enzyme without inhibitor was used as a negative control. The α-glucosidase inhibition percentage of blank corrected data was assessed using the following formula (1):

$$\% \text{ Inhibition} = 100 - (A_{405} \text{ Blank corrected sample}/A_{405} \text{ Blank corrected control}) \times 100 \quad (1)$$

The results are expressed as concentration of extract (IC50) in mg/mL that inhibited 50% of α-glucosidase.

2.6.2. Acetylcholineesterase Inhibitory Assay (AChE)

The experimental conditions of the *in vitro* AChE-inhibitory assay were based on the method described by Lobbens et al. [18] with slight modifications. The acetylcholinesterase inhibitory assay was carried out in a 96-well microplate. Each well contained 30 μL AChE (final concentration of 0.05 U/mL, C3389-500U, Sigma Aldrich, Merck, Darmstadt, Germany), 125 μL 1.5 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, D 218200, Sigma Aldrich, Merck, Darmstadt, Germany) dissolved in phosphate-buffered saline (PBS) pH 7.5, 45 μL PBS pH 7.5, and 25 μL test solution or 25 μL negative control (water). A blank sample was prepared by adding buffer instead of enzyme. The microplate was shaken for 10 s and left at 30°C for 5 min. Subsequently, 30 μL of 7.5 mM acetylthiocholine (ATCI,

01480, Sigma Aldrich, Merck, Darmstadt, Germany) dissolved in water was added to each well and the absorbance was measured at 412 nm every 30 s for 1 min. The blank corrected data were plotted against time and the reaction rate (the slope of the plot) was calculated. Finally, the inhibition was calculated by comparing the reaction rate in the test solution compared to the negative control. The experiment was performed in triplicate.

The inhibition was expressed as a percentage as follows (2):

$$\%\text{inhibition} = 100 - (\text{Slope sample}/\text{Slope negative control}) \times 100 \quad (2)$$

The results are expressed as concentration of extract (IC_{50}) in mg/mL that inhibited 50% of acetylcholinesterase.

2.7. Experimental Design and Statistical Analysis

In present study Response surface methodology (RSM) was used. Using the Box-Behnken design (BBD), the influence of four independent variables - HM (ratio) (X_1), Duration (X_2), Temperature (X_3), and Extractant (X_4) - on different physicochemical parameters of peaches was investigated. The coded and actual levels of independent variables used in the RSM design are listed in Table 1. The dependent variables in this study are TPC, TFC, FRAP, CUPRAC, DPPH, ABTS, α-glucosidase, and AChE. They are denoted from Y1 to Y8, respectively. A second-order polynomial equation was used to express these dependent variables as a function of the independent variables as follows:

$$Y_j = \beta_0 + \sum_{i=0}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i>0}^{k} \beta_{ij} X_i X_j + E \quad (3)$$

where Y_j (j=1, 2, … , 8) represents the responses to be modeled; β_0 is the constant coefficient; β_i is the coefficient of linear effect; β_{ij} is the coefficient of interaction effect; β_{ii} is the coefficient of squared effect; k is the number of variables; and X_i and X_j define the independent variables (Hydro module (HM) (ratio) (X_1), Duration (X_2), Temperature (X_3), and Extractant (X_4)). The statistical significance of the coefficients was verified by means of the Student's t-test (α = 0.05), goodness-of-fit was established as the determination coefficient (R^2), and the model consistency by the Fisher F test (α= 0.05).

The experimental design and statistical analysis were performed using Design-Expert software (Version 13.0.5.0, State-Ease Inc., Minneapolis, MN, USA). The complete experimental design consisted of 29 experimental runs, taken in random order. Center Points per Block were established at 5 to be able to estimate the pure error sum of squares.

Table 1. Coded and decoded levels of independent variables used in the RSM design.

Independent variables	Symbols	Levels		
		-1	0	+1
Hydro module (ratio)	X_1	5	10	20
Duration	X_2	20	30	40
Temperature	X_3	50	60	70
Extractant	X_4	80%	90%	99.9%

3. Results and discussion

RMS is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes [19]. RMS is preferred because it minimizes the number of experiments for a specific number of factors and their levels [20]. In general, RMS has two main types of designs - the Box-Behnken design (BBD) and the central-composite designs (CCD). The BBDs differ from the CCDs in that they use fewer runs and only three levels, compared to the five in CCD. For this reason, the BBD was preferred in the present study. Table 2 summarizes the obtained models on this study.

Table 2. Coded dependent variables used in the RSM design.

Dependent variables	Symbols	Model

TPC	Y_1	$Y_1 = 90.11+9.26X_1-4.93X_2+6.95X_3$
TFC	Y_2	$Y_2 = 15.34+3.17X_1+2.49X_2-4.49X_4-6.59X_1X_4+4.14X_2X_3+7.36X_2X_4+8.5X_3X_4$
FRAP	Y_3	$Y_3 = 288.82+23.13X_3+18.85X_4-72.77X_1X_2+42.96X_2X_3+20.81X_3X_4-19.04X_1^2 - 19.38X_2^2+35.21X_4^2$
CUPRAC	Y_4	There is no fit model.
DPPH	Y_5	$Y5 = 99.82-5.98X_1+3.02X_2-5.1X_1X_3+13.68X_2X_3-15.66X_2X_4+11.25X_3X_4-12.63X_2^2-6.48X_4^2$
ABTS	Y_6	$Y_6 = 238.93-28.45X_2+35.71X_3+123.95X_1X_3-58.88X_1X_4+85.91X_2X_4+53.69X_1^2-53.25X_2^2 - 33.74X_4^2$
α-Glucosidase	Y_7	$Y_7 = 0.04-0.02X_4+0.016X_1X_4+0.37X_1^2+0.057X_2^2-0.013X_3^2$
AChE	Y_8	$Y_8 = 0.24-0.064X_1+0.08X_2+0.05X_3-0.12X_4+0.07X_1X_2+0.05X_1X_3+0.19X_2X_3-0.054X_2X_4+0.26X_1^2+0.25X_2^2-0,082X_3^2-0,076X_4^2$

The software product Design-Expert defines the dependence of TPC on the input variables as linear (Table 3) with statistically insignificant coefficients in front of the added members. Consequently, only three factors influence the TPC value, namely Hydro module (ratio), Duration, and Temperature (Table 4).

Table 3. ANOVA for Quadratic model for Response 1: TPC.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2739.79	14	195.70	4.33	0.0049	significant
A-hydromodule (ratio)	1141.70	1	1141.70	25.24	0.0002	
B-Duration	340.86	1	340.86	7.54	0.0158	
C-Temperature	443.07	1	443.07	9.80	0.0074	
D-Extractant	196.55	1	196.55	4.35	0.0559	
AB	49.35	1	49.35	1.09	0.3139	
AC	20.11	1	20.11	0.4446	0.5158	
AD	58.34	1	58.34	1.29	0.2751	
BC	19.05	1	19.05	0.4211	0.5269	
BD	5.30	1	5.30	0.1171	0.7373	
CD	74.47	1	74.47	1.65	0.2203	
A^2	18.72	1	18.72	0.4138	0.5304	
B^2	143.38	1	143.38	3.17	0.0967	
C^2	48.84	1	48.84	1.08	0.3164	
D^2	131.04	1	131.04	2.90	0.1108	

Other temperature dependent studies on the topic of phenolic compounds are available in literature [21]. Duration is also reported as critical to phenolic content availability and possible damage and degradation [22].

Table 4. ANOVA for Linear model Response 1: TPC.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2114.34	4	528.58	10.08	< 0.0001	significant
A-hydromodule (ratio)	1095.66	1	1095.66	20.89	0.0001	
B-Duration	291.47	1	291.47	5.56	0.0269	
C-Temperature	579.36	1	579.36	11.05	0.0028	
D-Extractant	147.96	1	147.96	2.82	0.1060	

The Extractant's effect was not statistically significant. However, many papers reveal that different extracts of the same plant result in different levels of phenolic compounds [23]. The F-value of the model itself is 10.08 and the p-value is < 0.0001, indicating that the model is significant. The coefficient of determination is $R^2 = 0.63$. Fig. 1 reveals some of the most important model informational graphs. The Perturbation plot (Fig. 1a) represents the factors with the most significant influence. The hydro module's and the duration's graphs have the steepest slope. Since the extraction of bioactive molecules is evaluated as

a first and most important step in functional ingredients to foods, pharmaceuticals, cosmetology, among others, researchers have pointed out that an increase in the hydro module improves the diffusion rate in a solid–liquid extraction [24]. Fig. 1b presents the actual versus predicted values.

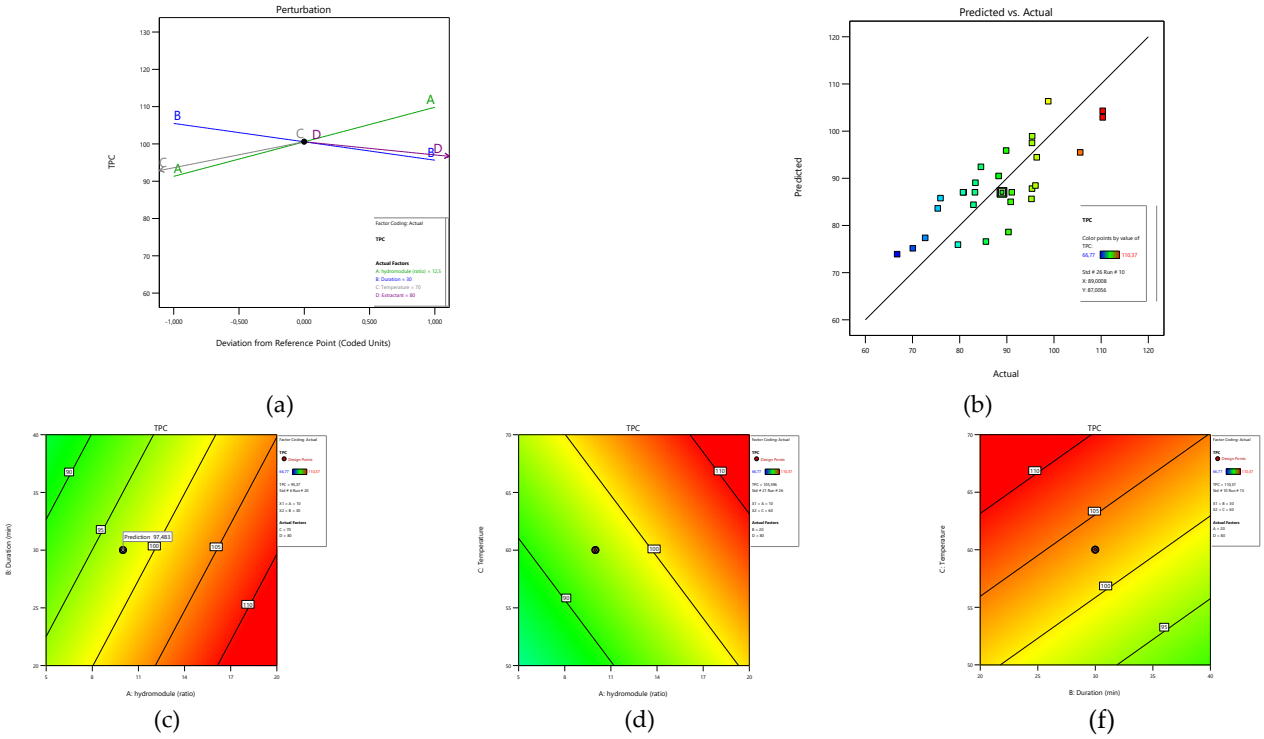


Figure 1. Perturbation and response surface plots: TPC

The response surface plots (Fig. 1c-f) visualize the variation of the values of two independent variables within the experimental domain while holding the other two constants. Fig. 1c reveals that the maximum TPC value can be achieved by keeping the temperature and the extractant at 70 °C and 80%, respectively. Considering the effect of hydro module and temperature on the TPC (max. 105.596 mgGAE/100g fw), Fig. 1d predicts an optimal duration of 20 minutes and an 80% extractant (110.37 mgGAE/100g fw).

A two-factor interaction terms have been added to further describe the dependence of TFC on the input variables. The results within the framework of the model appeared not statistically significant and are not visualized. An attempt was also made to define the model as linear. The results are presented in Table. 5.

Table 5. ANOVA for Linear model for Response 2: TFC.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	277.13	4	69.28	1.60	0.2074	not significant
A-hydromodule (ratio)	126.49	1	126.49	2.92	0.1006	
B-Duration	36.09	1	36.09	0.8322	0.3707	
C-Temperature	4.93	1	4.93	0.1136	0.7390	
D-Extractant	109.65	1	109.65	2.53	0.1249	
Residual	1040.92	24	43.37			
Lack of Fit	1021.34	20	51.07	10.43	0.0174	significant
Pure Error	19.58	4	4.89			
Cor Total	1318.05	28				

It turned out that this model was also not suitable because the model's F-value of 1.60 meant that the model was not robust to noise. There was a 20.74% chance that such a large F-value is due to noise. Finally, the 2FI model was chosen, and the results are presented in the Table 6.

Table 6. ANOVA for 2FI model for Response 2: TFC.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1085.61	8	135.70	11.68	< 0.0001	significant
A-hydromodule (ratio)	128.26	1	128.26	11.04	0.0034	
B-Duration	65.29	1	65.29	5.62	0.0279	
D-Extractant	212.31	1	212.31	18.27	0.0004	
AB	52.76	1	52.76	4.54	0.0457	
AD	186.50	1	186.50	16.05	0.0007	
BC	68.50	1	68.50	5.89	0.0248	
BD	216.94	1	216.94	18.67	0.0003	
CD	288.59	1	288.59	24.83	< 0.0001	
Residual	232.44	20	11.62			
Lack of Fit	212.86	16	13.30	2.72	0.1721	not significant
Pure Error	19.58	4	4.89			
Cor Total	1318.05	28				

It shows that the F-value is 11.68, which means that the model is significant. There is only a 0.01% chance that the F-value is due to noise. The coefficient of determination is R^2 = 0.82. The temperature has no effect on the extractions of TFC (Fig. 2). Other authors also support this finding in their work stating that the flavonoid and total phenolic contents were not influenced by temperature, time, and milling treatment [25].

Diagnostics of the model are done by plots of the normal distribution of residuals, residuals versus predicted values, and predicted versus actual values presented in Fig. 2. Based on them, it can be said that the residuals are normally distributed and there are no extreme values among them.

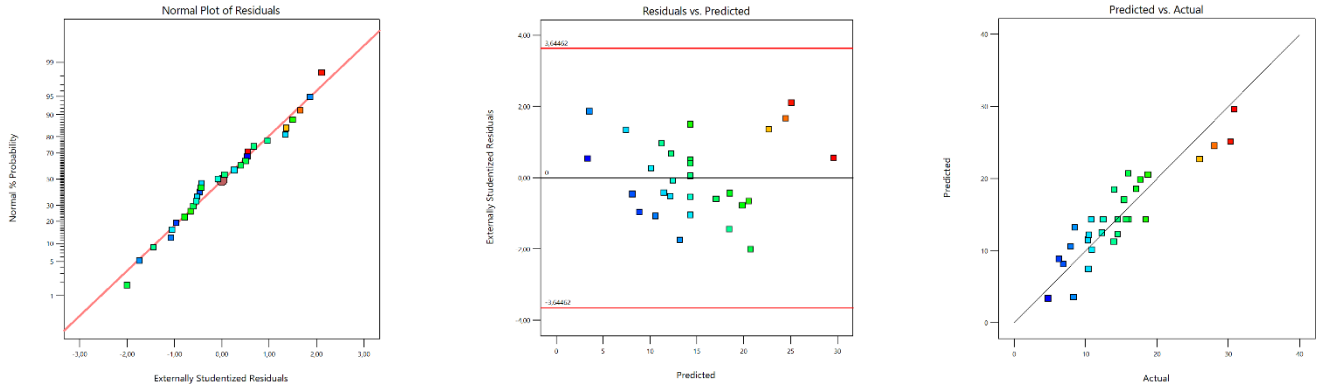
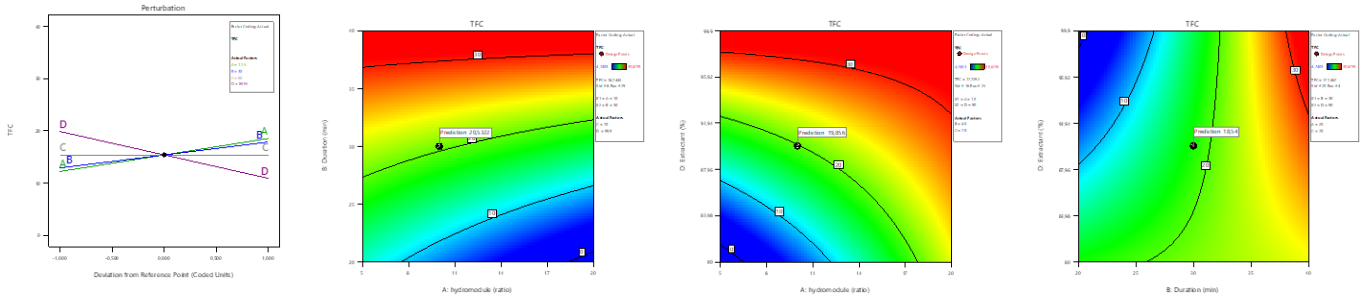


Figure 2. Diagnostics of the TFC model.

Fig. 3 presents the perturbation and contour plots. It is clear that the extractant factor has the most significant influence.



(a) (b) (c) (d)

Figure 3. Perturbation and response surface plots: TFC.

Fig. 3b shows the influence of the factors hydro module and duration while the temperature and the extractant are constants, 70 °C, and 99.9%, respectively. A design point with its actual and predicted value is also presented. Fig. 3c shows the influence of the factors hydro module and extractant while the temperature and the duration are constants, 70 °C and 40, respectively. Under these conditions, the maximum TFC value is 17.729 μg QE/g dw. Fig. 3d shows the influence of the factors duration and extractant while the temperature and the hydro module are constants, 70 °C and 20, respectively.

The model describing the dependence of FRAP on the independent variables is quadratic and the results are presented in Table. 7. The F-value of the model is 23.64, which indicates a significance. There is only a 0.01 chance that an F-value this large could occur due to noise.

Table 7. ANOVA for Reduced Quadratic model Response 3: FRAP.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	59815.01	8	7476.88	23.64	< 0.0001	significant
C-Temperature	6420.18	1	6420.18	20.30	0.0002	
D-Extractant	4262.40	1	4262.40	13.48	0.0015	
AB	25850.75	1	25850.75	81.73	< 0.0001	
BC	7383.79	1	7383.79	23.35	0.0001	
CD	1732.05	1	1732.05	5.48	0.0298	
A^2	1926.74	1	1926.74	6.09	0.0227	
B^2	2525.69	1	2525.69	7.99	0.0104	
D^2	8337.57	1	8337.57	26.36	< 0.0001	
Residual	6325.58	20	316.28			
Lack of Fit	5361.37	16	335.09	1.39	0.4089	not significant
Pure Error	964.21	4	241.05			
Cor Total	66140.60	28				

Based on the diagnostics of the model done by plots, it can be said that the residuals are normally distributed and there are no extreme values among them (Fig. 4).

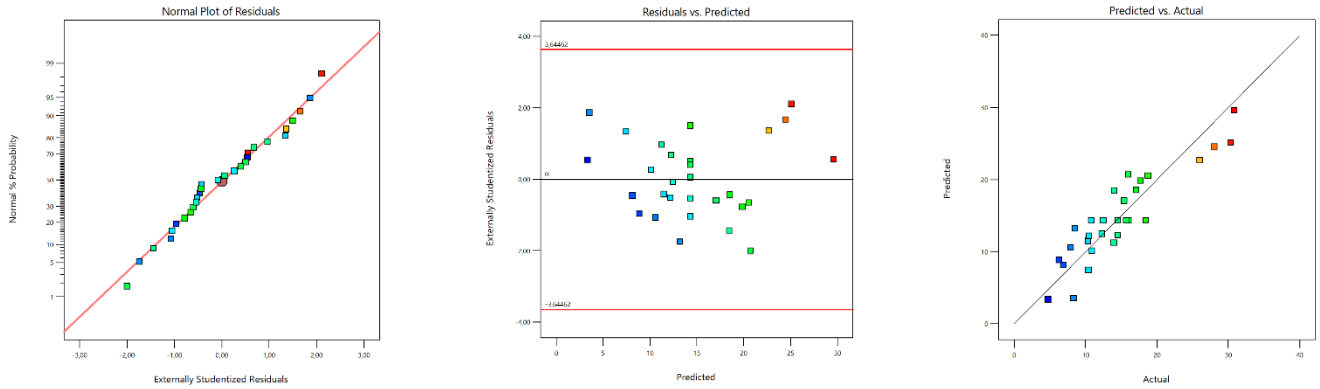


Figure 4. Diagnostics of the FRAP model. 253

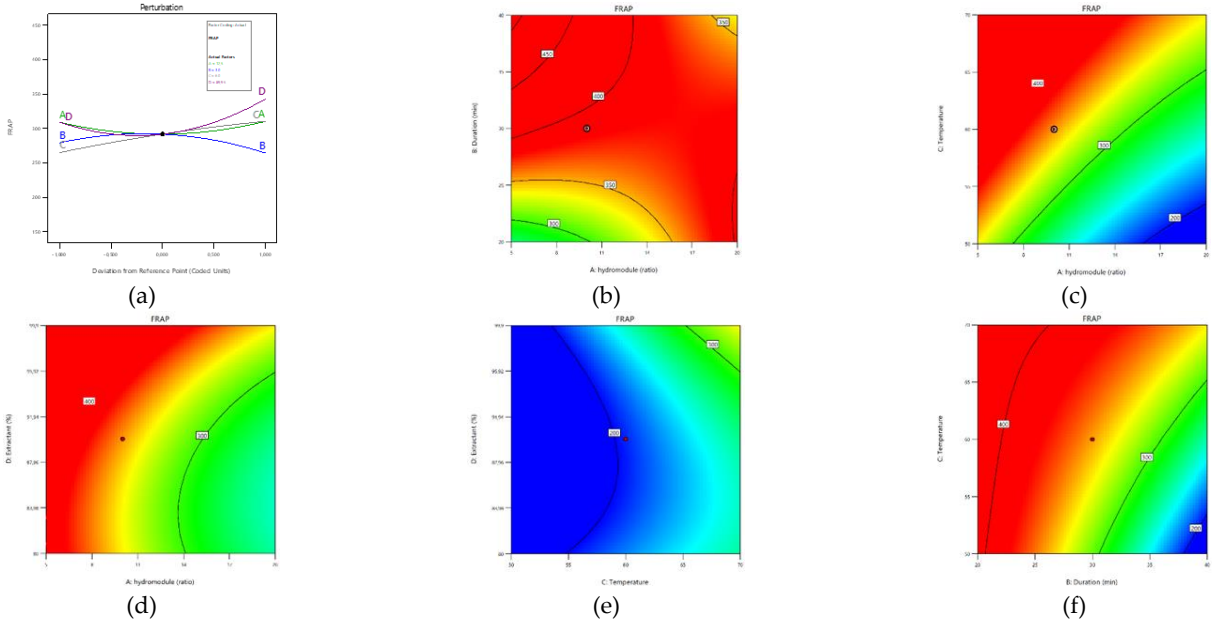


Figure 5. Perturbation and response surface plots: FRAP 254

Following the perturbation and contour plots in Fig. 5 the maximum FRAP values could be obtained with a hydro module 10 for a duration time of 30 min. Temperature is revealed as the least significant. Fig. 5d shows the effect of hydro module and extractant on the FRAP while keeping duration and temperature at values of 40 and 70 °C, respectively. Under these conditions, the maximum FRAP could be obtained with a hydro module 10 and an extractant of 90. 255 256 257 258 259 260

A suitable model has not been established to describe the dependence of the CUPRAC on the independent variables. A linear, 2FI and a quadratic model were tried, but all three were found to be insignificant. 261 262 263

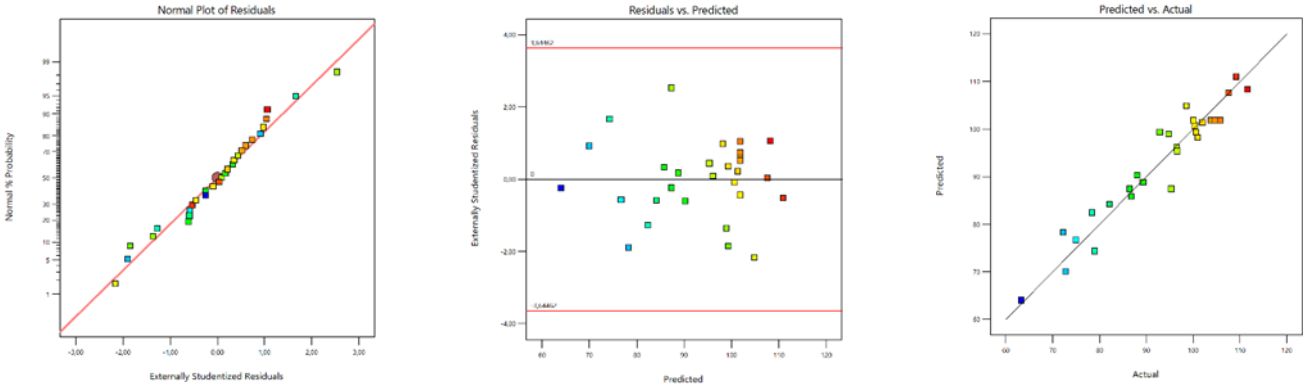
The model describing the dependence of DPPH on the independent variables is quadratic (Table 8). 264 265

Table 8. ANOVA for Reduced Quadratic model Response 3: DPPH. 266

Model	**4112.52**	**8**	**514.06**	**32.20**	**< 0.0001**	**significant**
A-hydromodule (ratio)	455.80	1	455.80	28.55	< 0.0001	
B-Duration	109.75	1	109.75	6.87	0.0163	
AC	127.13	1	127.13	7.96	0.0105	
BC	748.28	1	748.28	46.87	< 0.0001	
BD	980.55	1	980.55	61.42	< 0.0001	

CD	506.13	1	506.13	31.70	< 0.0001	
B^2	1099.99	1	1099.99	68.90	< 0.0001	
D^2	289.55	1	289.55	18.14	0.0004	
Residual	319.30	20	15.97			
Lack of Fit	300.81	16	18.80	2.07	0.321	not significant
Pure Error	18.50	4	4.62			
Cor Total	4431.82	28				

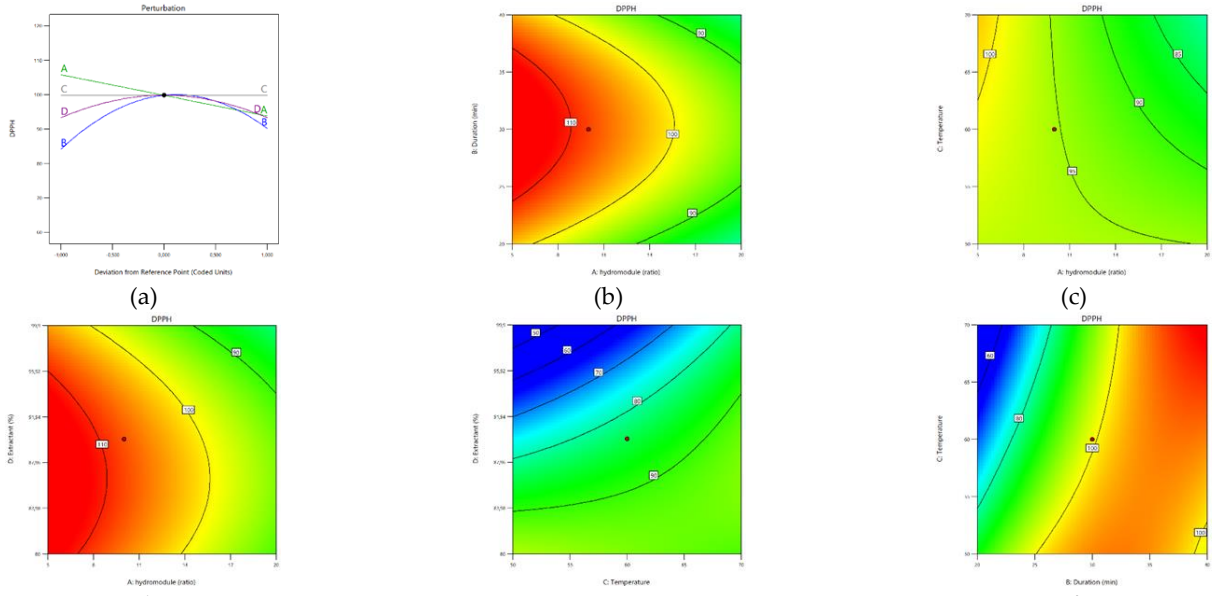
Diagnostics of the model are done by plots of the normal distribution of residuals, residuals versus predicted values, and predicted versus actual values presented in Fig. 6. Based on them, it can be said that the residuals are normally distributed and there are no extreme values among them.



(a) (b) (c)

Figure 6. Diagnostics of the DPPH model.

Fig. 7 presents the perturbation and contour plots. The perturbation plot (Fig. 7a) showed that the extractant and duration have the most significant influence, while the impact of temperature had no effect. Fig. 7b revealed the effect of hydro module and duration on the DPPH antioxidant activity of peach fruits while keeping temperature and extractant at values of 70 °C and 99.9, respectively.



(a) (b) (c)

(d) (e) (f)

Figure 7. Perturbation and response surface plots: DPPH

The DPPH assay is frequently used since its method is published back in 1995. Researchers are still relying on it while evaluating the antioxidant capacity of plant matrices [26]. Under the abovementioned conditions, the maximum DPPH values (112,1608 μM TE/g dw) could be obtained with a hydro module of 10 for a duration time of 30 min. Fig. 7c shows the effect of hydro module and temperature on the DPPH scavenging ability while keeping duration and extractant at values of 20 and 99.9, respectively. Under these conditions, the maximum DPPH values could be obtained with a hydro module of 10 and a temperature of 60 °C. Fig. 7d shows the effect of hydro module and extractant on the DPPH while keeping duration and temperature at values of 40 and 70°C, respectively. Under these conditions, the maximum DPPH parameters could be obtained with a hydro module of 10 and an extractant of 90. Fig. 7e shows the effect of temperature and extractant on the DPPH while keeping hydro module and duration at values of 20 and 40, respectively. Under these conditions, the maximum DPPH could be obtained at 60 °C and an extractant of 90. Fig. 7f shows the effect of duration and temperature on the DPPH while keeping hydro module and extractant at values of 5 and 80, respectively. Under these conditions, the maximum DPPH (83,39945 μM TE/g dw) could be obtained with a duration of 30 and a temperature of 60 °C. Other authors also present predicted values of antioxidant activity based on conditions like temperature, duration, hydro module, stating that such results can aid in presenting the extract of choice as a functional ingredient [27]. It has to be noted that some extracts exhibit a slower reaction with the DPPH radical resulting in a less than the actual antioxidant capacity [28]. Thus, it is of importance to provide expected values under different conditions since most researchers are aiming at standardization of methods and reliability of results in different laboratories.

A quadratic model described the dependence of ABTS on the independent variables and the results are presented in a Table 9.

Table 9. ANOVA for Reduced Quadratic model Response 3: ABTS.

Model	**1.713E+05**	**8**	**21413.80**	**12.41**	**< 0.0001**	**Significant**
B-Duration	9710.72	1	9710.72	5.63	0.0278	
C-Temperature	13444.53	1	13444.53	7.79	0.0113	
AC	66007.99	1	66007.99	38.26	< 0.0001	
AD	16949.28	1	16949.28	9.82	0.0052	
BD	29524.68	1	29524.68	17.11	0.0005	
A^2	15323.59	1	15323.59	8.88	0.0074	
B^2	19075.15	1	19075.15	11.06	0.0034	
D^2	7659.68	1	7659.68	4.44	0.0479	
Residual	34504.33	20	1725.22			
Lack of Fit	30827.64	16	1926.73	2.10	0.2479	not significant
Pure Error	3676.69	4	919.17			
Cor Total	2.058E+05	28				

Fig. 8 presents the perturbation and contour plots where it can be seen that the hydro module, the duration, and the extractant have a quadratic influence, while the temperature has a linear impact. Fig. 8b shows the effect of hydro module and duration on the ABTS values while keeping temperature and extractant at 70 °C and 99.9, respectively. Under these conditions, the maximum ABTS (216,5005 μM TE/g dw) could be obtained with a hydro module of 10 and a duration time of 30 min. Other authors state that the UAE extraction of plant matrices reveals dose-dependent ABTS values [29].

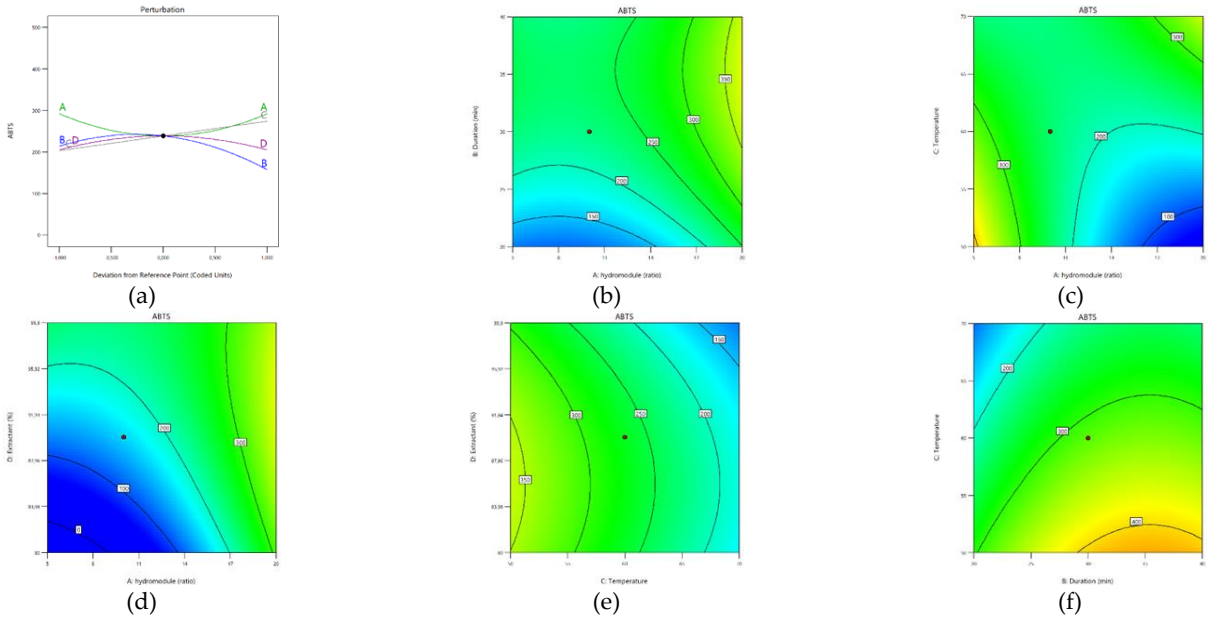


Figure 8. Perturbation and contour plots: ABTS

Fig. 8c shows the effect of hydro module and temperature on the ABTS while keeping duration and extractant at values of 40 and 99.9, respectively. Under these conditions, the maximum ABTS could be obtained with a hydro module of 10 and a temperature of 60 °C. Fig. 8d shows the effect of hydro module and extractant on the ABTS while keeping duration and temperature at values of 40 and 70°C, respectively. Under these conditions, the maximum ABTS could be obtained with a hydro module of 10 and an extractant of 90. Fig. 8e shows the effect of temperature and extractant on the ABTS while keeping hydro module and duration at values of 5 and 20, respectively. Under these conditions, the maximum ABTS could be obtained at 60 °C and an extractant of 90. Fig. 8f shows the effect of duration and temperature on the ABTS while keeping hydro module and extractant at values of 5 and 99.9, respectively. Under these conditions, the maximum ABTS (461,6783 μM TE/g dw) could be obtained with a duration of 30 and a temperature of 60 °C.

A quadratic model explained the dependence of α-glucosidase on the independent variables (Table 10).

Table 10. ANOVA for Reduced Quadratic model Response 3: Alfa-Gl.

Model	**0.0378**	**5**	**0.0076**	**66.55**	**< 0.0001**	**significant**
D-Extractant	0.0041	1	0.0041	36.47	< 0.0001	
AD	0.0010	1	0.0010	9.15	0.0060	
A^2	0.0071	1	0.0071	62.30	< 0.0001	
B^2	0.0221	1	0.0221	194.63	< 0.0001	
C^2	0.0011	1	0.0011	9.57	0.0051	
Residual	0.0026	23	0.0001			
Lack of Fit	0.0023	19	0.0001	1.80	0.3018	not significant
Pure Error	0.0003	4	0.0001			
Cor Total	0.0404	28				

Figure 9 presents the perturbation and contour plots. The hydro module, duration, and extractant have a quadratic influence, while the temperature - a linear (Fig. 9a). Fig. 9b shows the effect of the hydro module and duration on the α-glucosidase while keeping temperature and extractant at 50 °C and 80, respectively.

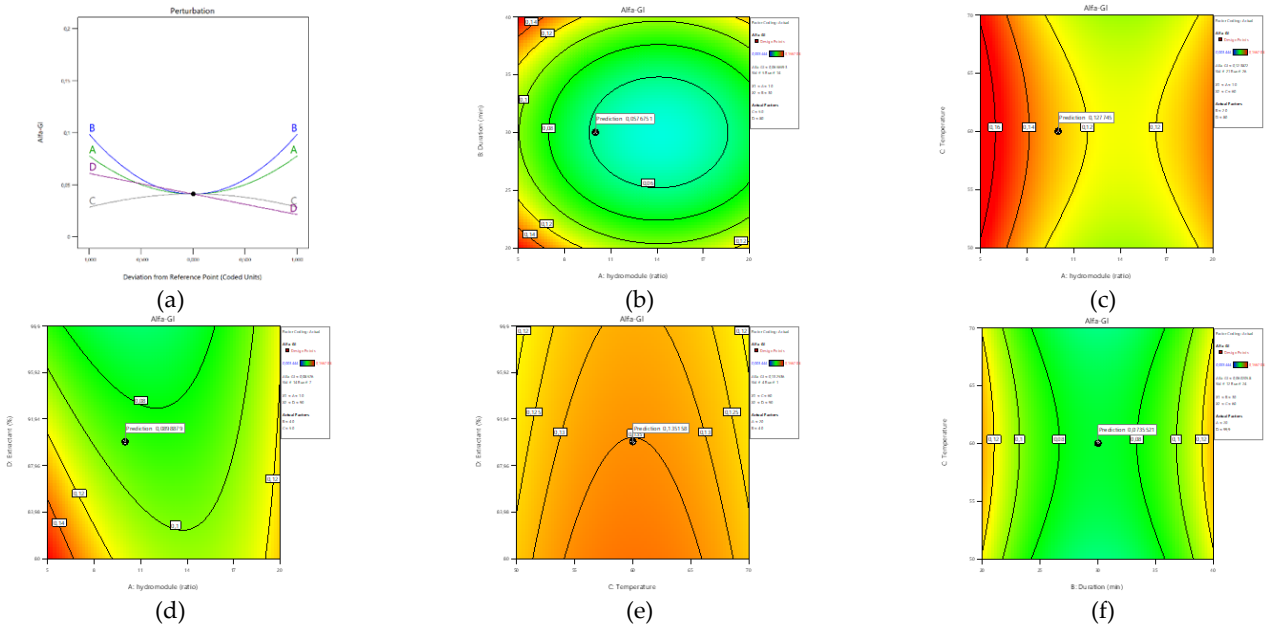


Figure 9. Perturbation and response surface plots: α-glucosidase inhibition

Under these conditions, the maximum α-glucosidase inhibition (IC_{50} 0,08 mg/mL) could be obtained with a hydro module of 10 for a duration time of 30 min. Fig. 9c shows the effect of hydro module and temperature on the α-glucosidase inhibition potential while keeping duration and extractant at values of 20 and 80, respectively. Under these conditions optimum results could be obtained with a hydro module of 10 and a temperature of 60 °C. Fig. 9d shows the effect of hydro module and extractant on the α-glucosidase inhibition while keeping duration and temperature at values of 40 and 50°C, respectively. In this view, the optimal conditions are a hydro module of 10 and an extractant of 90. Fig. 9e shows the effect of temperature and extractant on the α-glucosidase activity while keeping hydro module and duration at values of 20 and 40, respectively. Under these conditions, maximal values could be obtained at 60 °C and an extractant of 90. Fig. 9f shows the effect of duration and temperature on the α-glucosidase inhibition potential while keeping hydro module and extractant at values of 20 and 99.9, respectively. Under these conditions, the optimal effect (IC_{50} 0,13 mg/mL) could be achieved with a duration of 30 and a temperature of 60 °C. Other authors [30] stated that solid/solvent ratio and extraction time were key process parameters in the optimization of extraction conditions of antioxidant and α-glucosidase inhibitory of weed fruits.

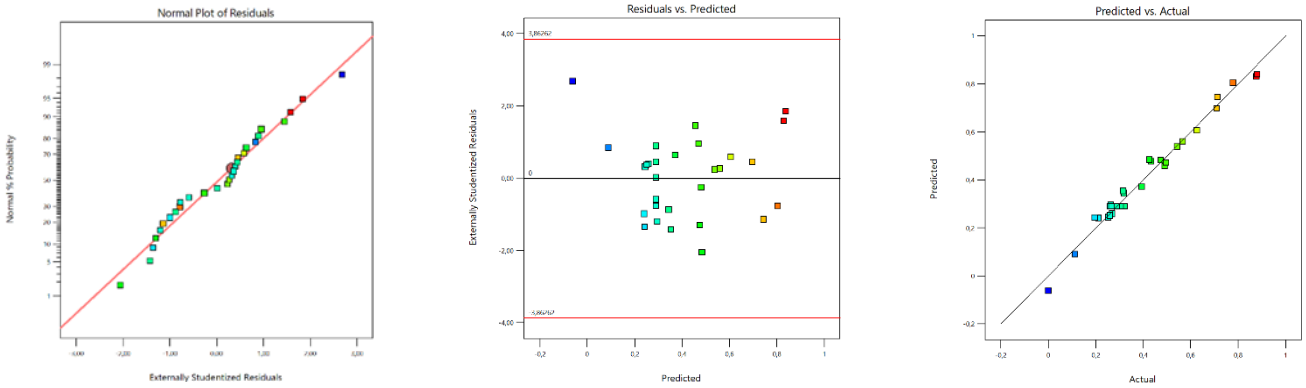
A quadratic model revealed the dependence of acetylcholinesterase on the independent variables (Table 11). In this case, A, B, C, D, AB, AC, BC, BD, A2, B^2, C^2 and D2 are significant model terms.

Table 11. ANOVA for Reduced Quadratic model Response 3: AChE.

Model	**1.36**	**13**	**0.1045**	**58.51**	**< 0.0001**	**significant**
A-hydromodule (ratio)	0.0498	1	0.0498	27.90	< 0.0001	
B-Duration	0.0711	1	0.0711	39.83	< 0.0001	
C-Temperature	0.0270	1	0.0270	15.09	0.0015	
D-Extractant	0.1656	1	0.1656	92.73	< 0.0001	
AB	0.0203	1	0.0203	11.39	0.0042	
AC	0.0116	1	0.0116	6.50	0.0223	
BC	0.1457	1	0.1457	81.55	< 0.0001	
BD	0.0117	1	0.0117	6.56	0.0217	
CD	0.0072	1	0.0072	4.05	0.0624	
A^2	0.3292	1	0.3292	184.29	< 0.0001	

B^2	0.4151	1	0.4151	232.37	< 0.0001	
C^2	0.0432	1	0.0432	24.18	0.0002	
D^2	0.0378	1	0.0378	21.17	0.0003	
Residual	0.0268	15	0.0018			
Lack of Fit	0.0240	11	0.0022	3.08	0.1446	not significant
Pure Error	0.0028	4	0.0007			
Cor Total	1.39	28				

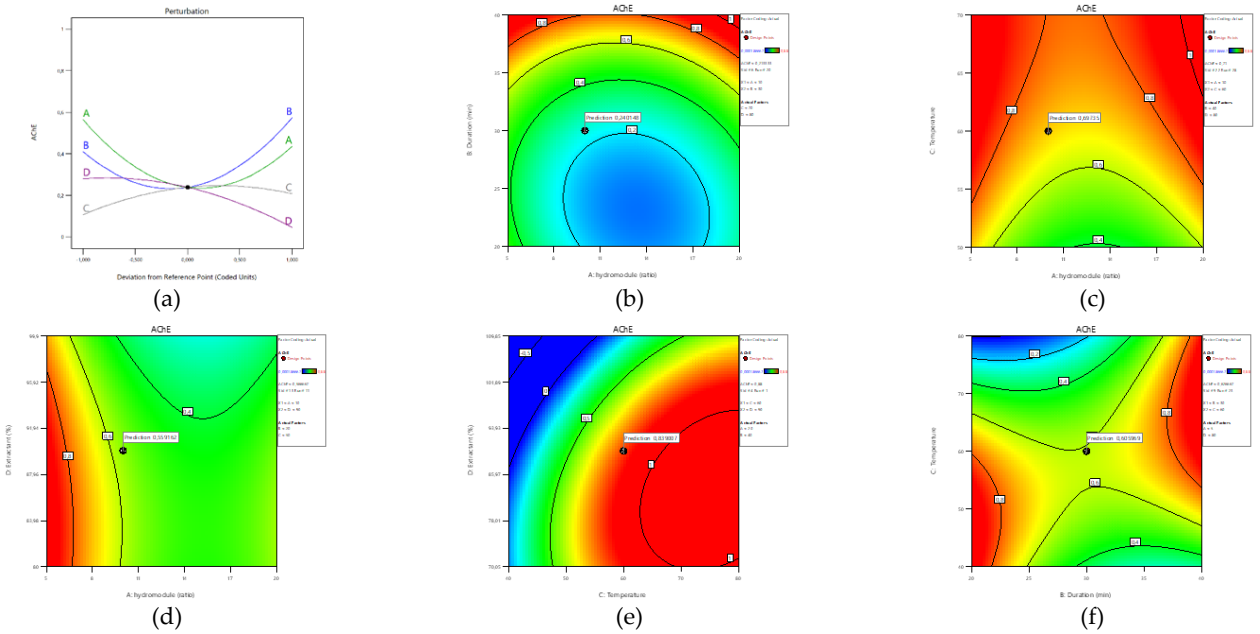
Diagnostics of the model are done by plots of the normal distribution of residuals, residuals versus predicted values, and predicted versus actual values presented in Fig. 10. Based on them, it can be said that the residuals are normally distributed and there are no extreme values among them.



(a) (b) (c)

Figure 10. Diagnostics of the AChE model

Fig. 11 presents the perturbation and contour plots with quadratic influence of the factors. The influence of factors hydro module and duration is the most significant (Fig. 11a). Fig. 11b shows the effect of hydro module and duration on the Ache inhibition potential while keeping temperature and extractant at values of 70 °C and 80, respectively. Under these conditions, the maximum AChE (IC_{20} 0,27 mg/mL) could be obtained with a hydro module of 10 for a duration time of 30 min.



(a) (b) (c)

(d) (e) (f)

Figure 11. Perturbation and response surface plots: AChE inhibition

Fig. 11c shows the effect of hydro module and temperature on the Ache while keeping duration and extractant at values of 40 and 80, respectively. Under these conditions, the maximum Ache could be obtained with a hydro module of 10 and a temperature of 60 °C. Fig. 11d shows the effect of hydro module and extractant on the Ache while keeping duration and temperature at values of 20 and 50°C, respectively. Under these conditions, the maximum Ache could be obtained with a hydro module of 10 and an extractant of 90. Fig. 11e shows the effect of temperature and extractant on the Ache while keeping hydro module and duration at values of 20 and 40, respectively. Under these conditions, the maximum Ache could be obtained at 60 °C and an extractant of 90. Fig. 11f shows the effect of duration and temperature on the Ache while keeping hydro module and extractant at values of 5 and 80, respectively. Under these conditions, the maximum Ache could be obtained with a duration of 30 and a temperature of 60 °C. Other authors have reported the optimal conditions for UAE in terms of high AChE inhibitory activity to be the following: methanol concentration of 85.06%, ultrasonic time of 39.1 min, and material-to-liquid ratio of 1.06:10 (g/mL) [31].

5. Conclusions

The effect of UAE extraction conditions of *Prunus persica* L. from the "Filina" cultivar on the polyphenolic antioxidants were optimized using a Box-Behnken experimental design with four variables and three levels. Using the response surface method, the optimal extraction conditions for the extraction of bioactive compounds were found to be: hydro modulus of 20, duration of 39.328 minutes, temperature of 70 °C, and extractant of 96.638%. In addition, empirical relationships between input variables and responses have been established. For five of the responses, namely FRAP, DPPH, ABTS, α-glucosidase and AChE - the dependence is second order. The model for TPC is linear, while for TFC, the model is linear with interactions. The only non-reportable model is CUPRAC.

In conclusion, it can be said that *Prunus persica* L. can be used as a basis for the extraction of bioactive compounds and antioxidants to be put into functional foods and/or drug candidates. This work can act as a core for other researchers to assess and quantify the biological activity of *Prunus persica* L. However, the purification of bioactive compounds and *in vivo* evaluation should be further investigated.

Supplementary Materials: Not applicable.

Author Contributions: Conceptualization, D.M.; M.T. and I.D.; methodology, D.M.; M.T. and I.D.; software, M.T.; validation, D.M., M.T.; formal analysis, D.M and I.D.; investigation, D.M.; resources, D.M.; data curation, D.M.; writing—original draft preparation, D.M.; M.T.; A.P. and I.D.; writing—review and editing, D.M.; M.T.; A.P.; A.L. and I.D.; visualization, M.T; supervision, D.M. and A.L.; project administration, D.M.; funding acquisition, D.M. All authors have read and agreed to the published version of the manuscript.

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