Supplementary material

Exploring Varied (Green) Extraction Methods to Optimize Galia Melon Antioxidant Potential

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2.1. Chemicals and Reagents

All solvents were at least of HPLC grade and purchased from Carlo Erba (Valde Reuil, France). Chemical standards of polyphenolic compounds, such as 3-hydroxytyrosol, hesperidin, catechin, rutin, pelargonin chloride, luteolin-7-glucoside, cyanidin-3-*O*-glucoside and chlorogenic acid, were acquired from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid, ascorbic acid, trichloroacetic acid, ferric (III) chloride, aluminum chloride, and sodium acetate were also obtained from Sigma-Aldrich (Steinheim, Germany). Gallic acid, anhydrous sodium carbonate, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,4,6-tri-2-pyridinyl-1,3,5-triazine (TPTZ) were obtained by Penta (Prague, Czech Republic). For all experiments, deionized water was used.

2.3. Response Surface Methodology (RSM) Optimization of Extraction and Experiment Design

Utilizing the Response Surface Methodology (RSM) technique, the extraction of total polyphenol content (TPC), polyphenolic compounds, and antioxidant activity was measured using the FRAP, DPPH method aimed for optimal yield. The design's objective was to enhance the content of Galia melon peel in TPC, polyphenolic compounds, and antioxidant activity. This was achieved through adjustments to the extraction procedure involving parameters such as solvent concentration (ethanol, EtOH) represented as *C*, % *v*/*v*, extraction duration denoted as *t*, min, and extraction temperature indicated as *T*, °C. An experiment employing a main effect screening design with twenty design points formed the basis for optimization. Process variables were set at five levels, as outlined in Table 1, indicating both coded and actual levels. Analysis of variance (ANOVA) and summary-of-fit tests were employed to establish overall model significance (R^2, *p*-value) and the significance of model coefficients (equations). Additionally, a second-order polynomial model (Equation S1) was utilized to forecast the dependent variable based on the analyzed independent factors:

$$Y_k = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad \text{(S1)}$$

where Y_k is the predicted response variable; X_i and X_j are the independent variables; β_0, β_i, β_{ii}, and β_{ij} are the intercept, regression coefficients of the linear, quadratic, and interaction terms of the model, respectively.

2.4. Analyses of Extracts and HPLC-Based Analysis of the various Polyphenolic compounds

2.4.1. Total Polyphenol Content (TPC) Determination

Following a previously established methodology [1], the total polyphenol content (TPC) of the extracts was determined using the Folin-Ciocalteu assay. In brief, a 1.5 mL Eppendorf tube was filled with 100 μL of Galia melon peel extracts and 100 μL of Folin-Ciocalteu reagent. The solution was heated at 40 °C for 20 min before 800 μL of Na_2CO_3 solution (5% *w*/*v*) was added. Ultimately, a Shimadzu spectrophotometer (UV-1700, Shimadzu Europa GmbH, Duisburg, Germany) was used to record the absorbance at 740 nm. A calibration curve was further prepared using gallic acid as a standard compound. The total polyphenol concentration (C_{TP}) was expressed as mg gallic acid equivalents (GAE) per L. The extraction yield in total polyphenols (Y_{TP}) was expressed as mg GAE per g of dry weight (dw), using the following Equation (S2):

$$Y_{TP}\ (\text{mg GAE/g dw}) = \frac{C_{TP} \times V}{w} \quad \text{(S2)}$$

where V is the volume of the extraction medium (in L) and w is the dry weight of the sample (in g).

2.4.2. Ferric reducing antioxidant power (FRAP) assay

A previously described method [2] was employed. Amount of 50 μL ferric (III) chloride solution (4 mM in 0.05 M HCl) was well mixed with the diluted sample extract (50 μL, 1:50) and then incubated in a water bath at 37 °C for 30 min. After that, 900 μL of TPTZ solution (1 mM in 0.05 M HCl) was added, and the absorbance at 620 nm was measured after exactly 5 min. Ferric reducing antioxidant power (P_R) was determined as μmol ascorbic acid equivalents (AAE) per g of dw, using an ascorbic acid calibration curve (50–500 μmol/L in 0.05 M HCl) using the following Equation (S3):

$$P_R\ (\mu\text{mol AAE/g dw}) = \frac{C_{AA} \times V}{w} \quad \text{(S3)}$$

where V is the volume of the extraction medium (in L) and w is the dry weight of the sample (in g).

2.4.3. Radical scavenging activity (A_{AR}, DPPH assay)

A previously employed assay [3] of DPPH scavenging was followed. A volume of 25 μL of diluted sample extract (1:5) was mixed with 975 μL of DPPH solution (100 μmol/L in methanol), and the absorbance at 515 nm was measured immediately after mixing ($A_{515(i)}$) and exactly 30 min later ($A_{515(f)}$). The capacity to scavenge the DPPH radical was expressed as:

$$\text{Inhibition (\%)} = \frac{A_{515(i)} - A_{515(f)}}{A_{515(i)}} \times 100 \quad \text{(S4)}$$

The antiradical activity (A_{AR}) was expressed as μmol ascorbic acid equivalents (AAE), using an ascorbic acid calibration curve (C_{AA}, 50–1.000 μM) and the following Equation (S5):

$$A_{AR}\ (\mu\text{mol AAE/g dw}) = \frac{C_{AA} \times V}{w} \quad \text{(S5)}$$

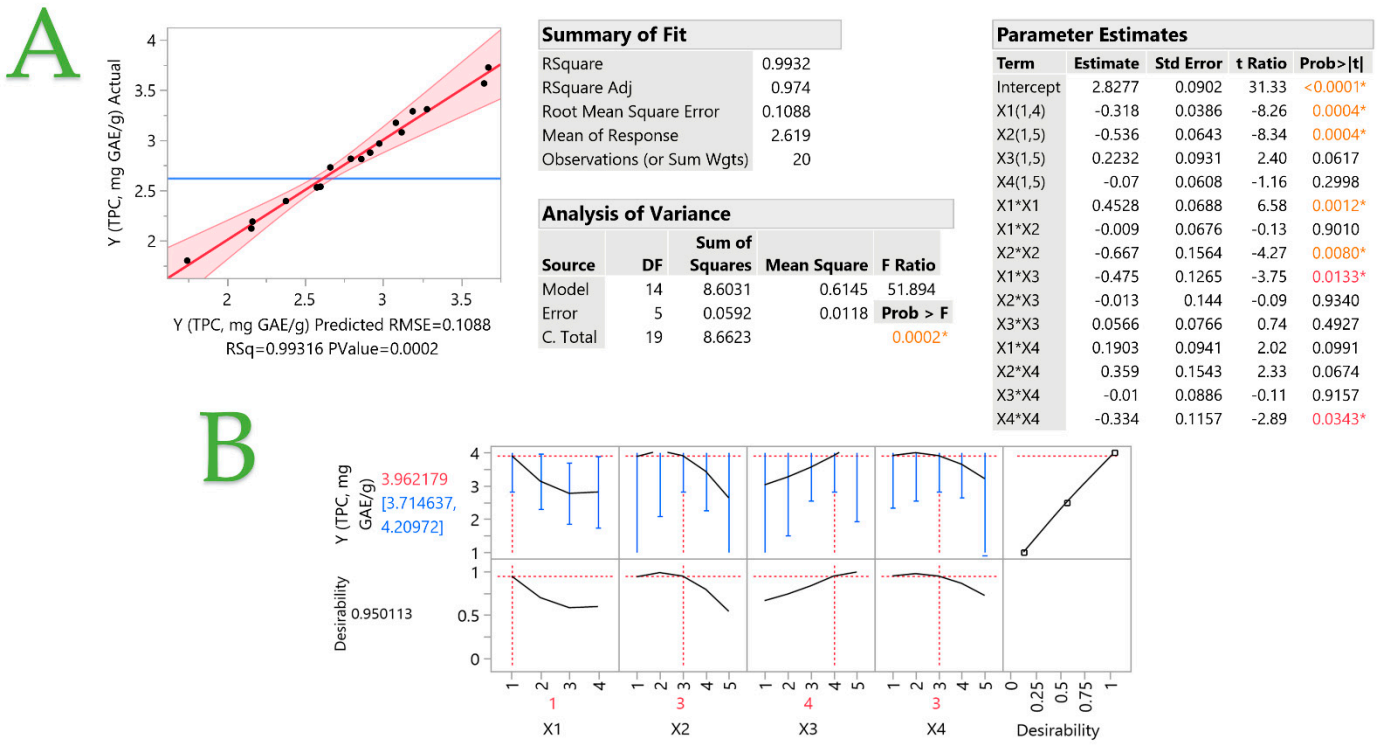
where V is the volume of the extraction medium (in L) and w is the dry weight of the sample (in g).

2.4.4. HPLC-Based Analysis of the various Polyphenolic compounds

The content of the extracts in phenolic compounds (i.e., gallic acid, neochlorogenic acid, catechin, chlorogenic acid, epicatechin, kaempferol) was determined using SPD-M20A diode array detector, after the high-performance liquid chromatography (HPLC)-based separation of the compounds with a CBM-20A liquid chromatograph (Shimadzu Europa GmbH, Duisburg, Germany) [4]. The stationary phase [Phenomenex LunaC18(2) column (100 Å, 5 μm, 4.6 × 250 mm; Phenomenex, Inc., Torrance, CA, USA] was placed in a furnace and the temperature was maintained at a constant at 40 ◦C. Aqueous formic acid (0.5% v/v) (A) and acetonitrile/water (6:4 v/v) containing formic acid (0.5% v/v)(B) were used as a mobile phase and the flow rate was set at 1 mL/min. The following gradient elution program was employed for the elution of the compounds: 5% B to 40% Bin 40 min, then to 50% B in 10 min, and finally to 70% B in 10 min, and kept constant at 70% B for 10 min. The total run time was 70 min. The target compounds were identified by comparing the retention times and the absorbance spectra to that of the pure chemical standards. For the quantification of the compounds, the calibration curves (0–500 μg/mL) were prepared and used.

2.5. Statistical Analysis

The design of the experiment, statistical analysis related to the response surface methodology (RSM), distribution analysis, multivariate correlation analysis (MCA), partial least squares (PLS) analysis, and distribution analysis were carried out utilizing JMP® Pro 16 software from SAS, located in Cary, NC, USA. Quantitative analysis was conducted in triplicate, and the extraction processes were executed at least twice. The results are presented as medians along with their corresponding standard deviations.



Summary of Fit

RSquare	0.9932
RSquare Adj	0.974
Root Mean Square Error	0.1088
Mean of Response	2.619
Observations (or Sum Wgts)	20

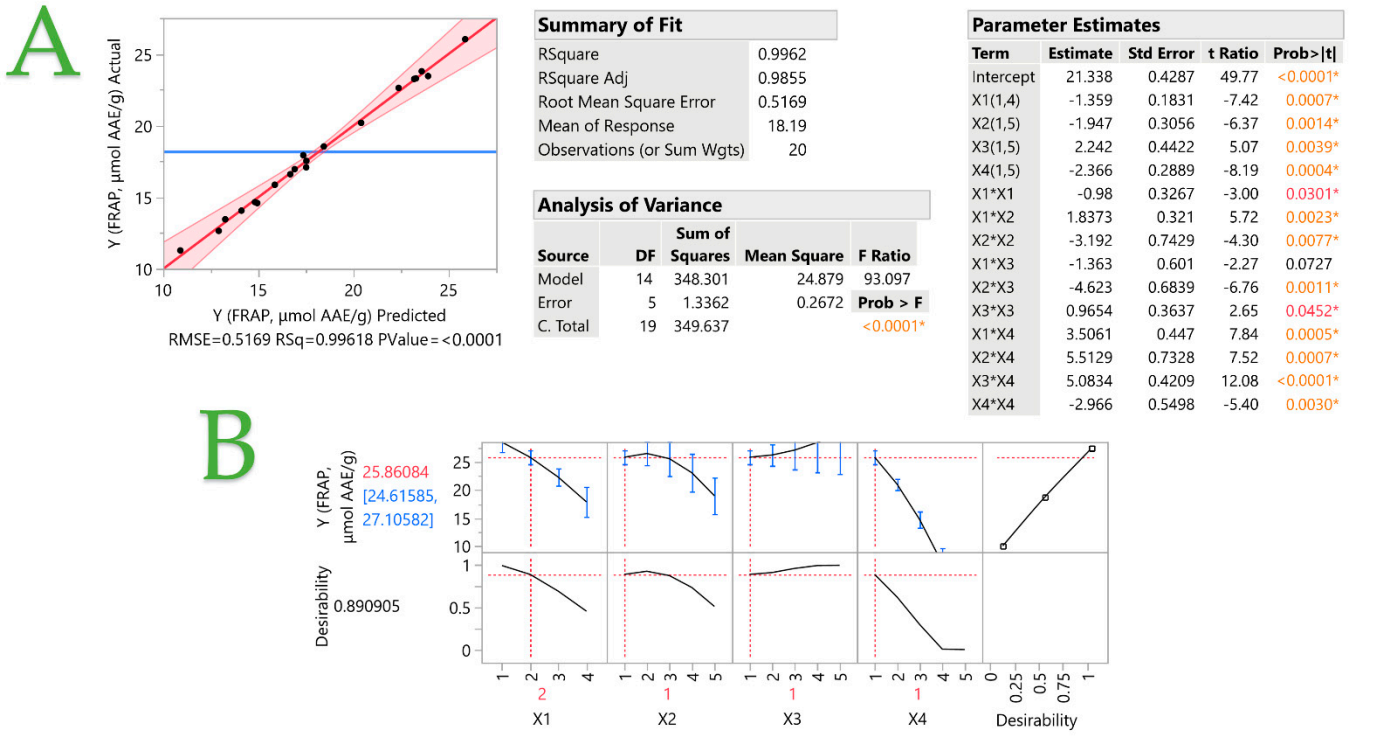
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	8.6031	0.6145	51.894
Error	5	0.0592	0.0118	Prob > F
C. Total	19	8.6623		0.0002*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	2.8277	0.0902	31.33	<0.0001*
X1(1,4)	-0.318	0.0386	-8.26	0.0004*
X2(1,5)	-0.536	0.0643	-8.34	0.0004*
X3(1,5)	0.2232	0.0931	2.40	0.0617
X4(1,5)	-0.07	0.0608	-1.16	0.2998
X1*X1	0.4528	0.0688	6.58	0.0012*
X1*X2	-0.009	0.0676	-0.13	0.9010
X2*X2	-0.667	0.1564	-4.27	0.0080*
X1*X3	-0.475	0.1265	-3.75	0.0133*
X2*X3	-0.013	0.144	-0.09	0.9340
X3*X3	0.0566	0.0766	0.74	0.4927
X1*X4	0.1903	0.0941	2.02	0.0991
X2*X4	0.359	0.1543	2.33	0.0674
X3*X4	-0.01	0.0886	-0.11	0.9157
X4*X4	-0.334	0.1157	-2.89	0.0343*

Figure S1. Plot **A** displays the actual response versus the predicted response (Total polyphenol content – TPC, mg gallic acid equivalents (GAE)/g) for the optimization of Galia melon peel extracts using hydroethanolic solutions, different extraction techniques, and parameters, and plot **B** displays the desirability function. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model.



Summary of Fit

RSquare	0.9962
RSquare Adj	0.9855
Root Mean Square Error	0.5169
Mean of Response	18.19
Observations (or Sum Wgts)	20

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	348.301	24.879	93.097
Error	5	1.3362	0.2672	Prob > F
C. Total	19	349.637		<0.0001*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	21.338	0.4287	49.77	<0.0001*
X1(1,4)	-1.359	0.1831	-7.42	0.0007*
X2(1,5)	-1.947	0.3056	-6.37	0.0014*
X3(1,5)	2.242	0.4422	5.07	0.0039*
X4(1,5)	-2.366	0.2889	-8.19	0.0004*
X1*X1	-0.98	0.3267	-3.00	0.0301*
X1*X2	1.8373	0.321	5.72	0.0023*
X2*X2	-3.192	0.7429	-4.30	0.0077*
X1*X3	-1.363	0.601	-2.27	0.0727
X2*X3	-4.623	0.6839	-6.76	0.0011*
X3*X3	0.9654	0.3637	2.65	0.0452*
X1*X4	3.5061	0.447	7.84	0.0005*
X2*X4	5.5129	0.7328	7.52	0.0007*
X3*X4	5.0834	0.4209	12.08	<0.0001*
X4*X4	-2.966	0.5498	-5.40	0.0030*

Figure S2. Plot **A** displays the actual response versus the predicted response (FRAP, µmol ascorbic acid equivalents (AAE)/g) for the optimization of Galia melon peel extracts using hydroethanolic solutions, different extraction techniques, and parameters, and plot **B** displays the desirability function. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model.

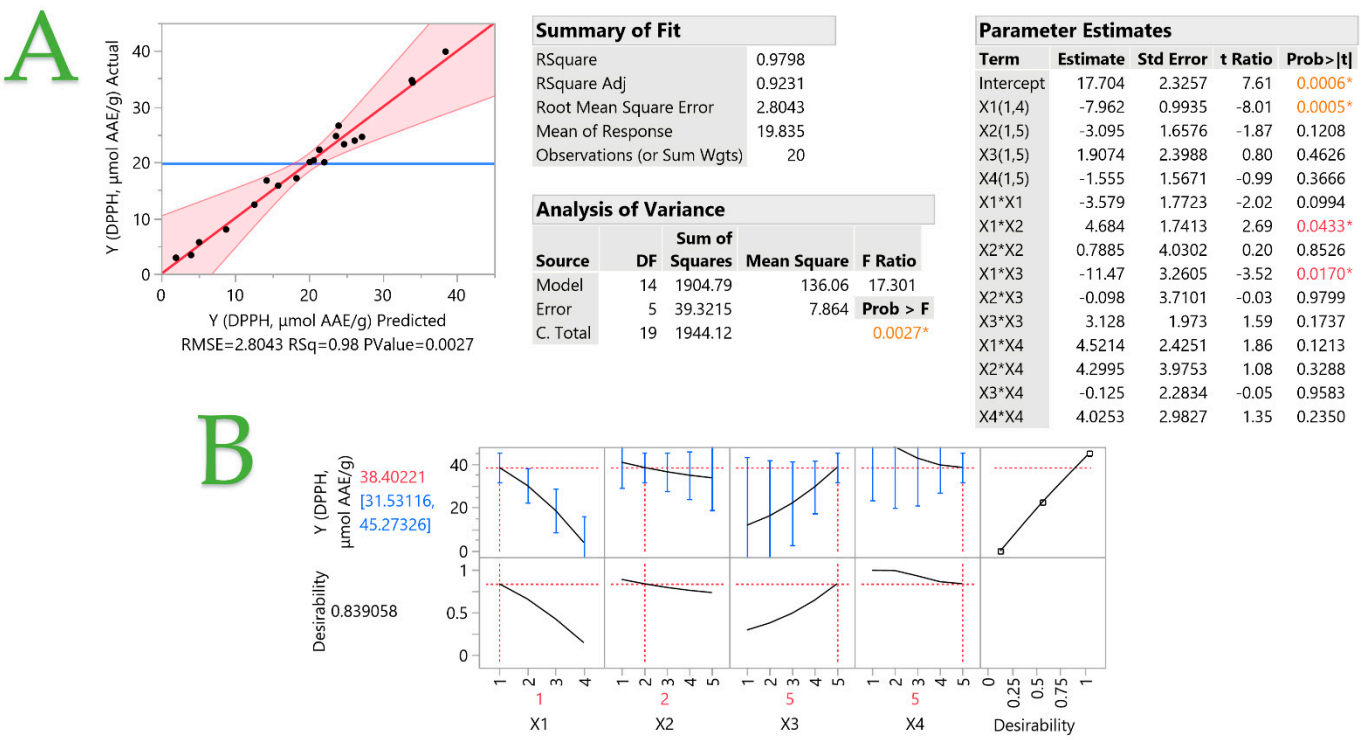


Figure S3. Plot **A** displays the actual response versus the predicted response (DPPH, µmol ascorbic acid equivalents (AAE)/g) for the optimization of Galia melon peel extracts using hydroethanolic solutions, different extraction techniques, and parameters, and plot **B** displays the desirability function. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model.

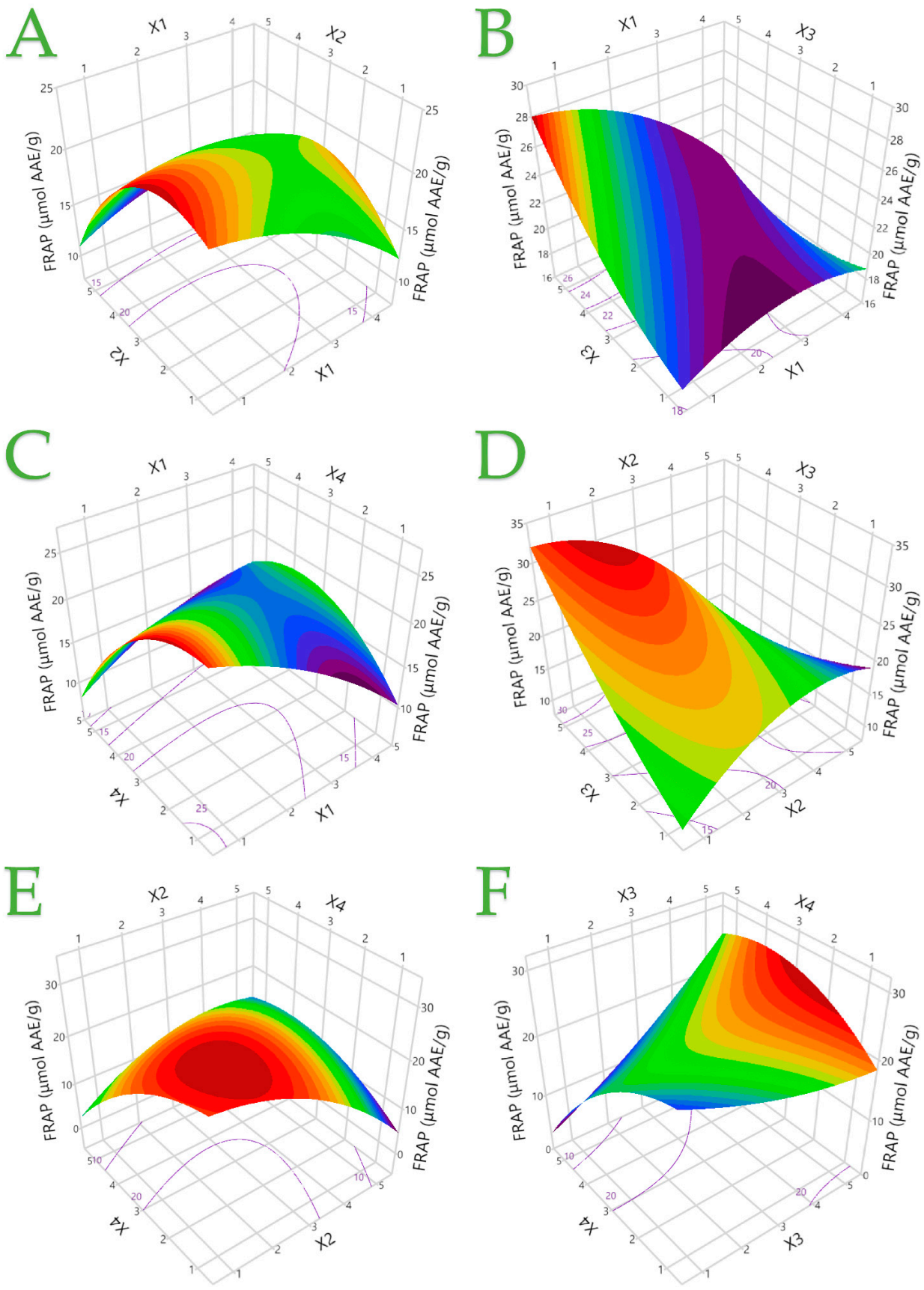


Figure S4. The optimal extraction of Galia melon peel extracts is shown in 3D graphs that show the impact of the process variables considered in the response (FRAP, µmol ascorbic acid equivalents (AAE)/g). Plot (**A**), covariation of X_1 and X_2; plot (**B**), covariation of X_1 and X_3; plot (**C**), covariation of X_1 and X_4; plot (**D**), covariation of X_2 and X_3; plot (**E**), covariation of X_2 and X_4; plot (**F**), covariation of X_3 and X_4.

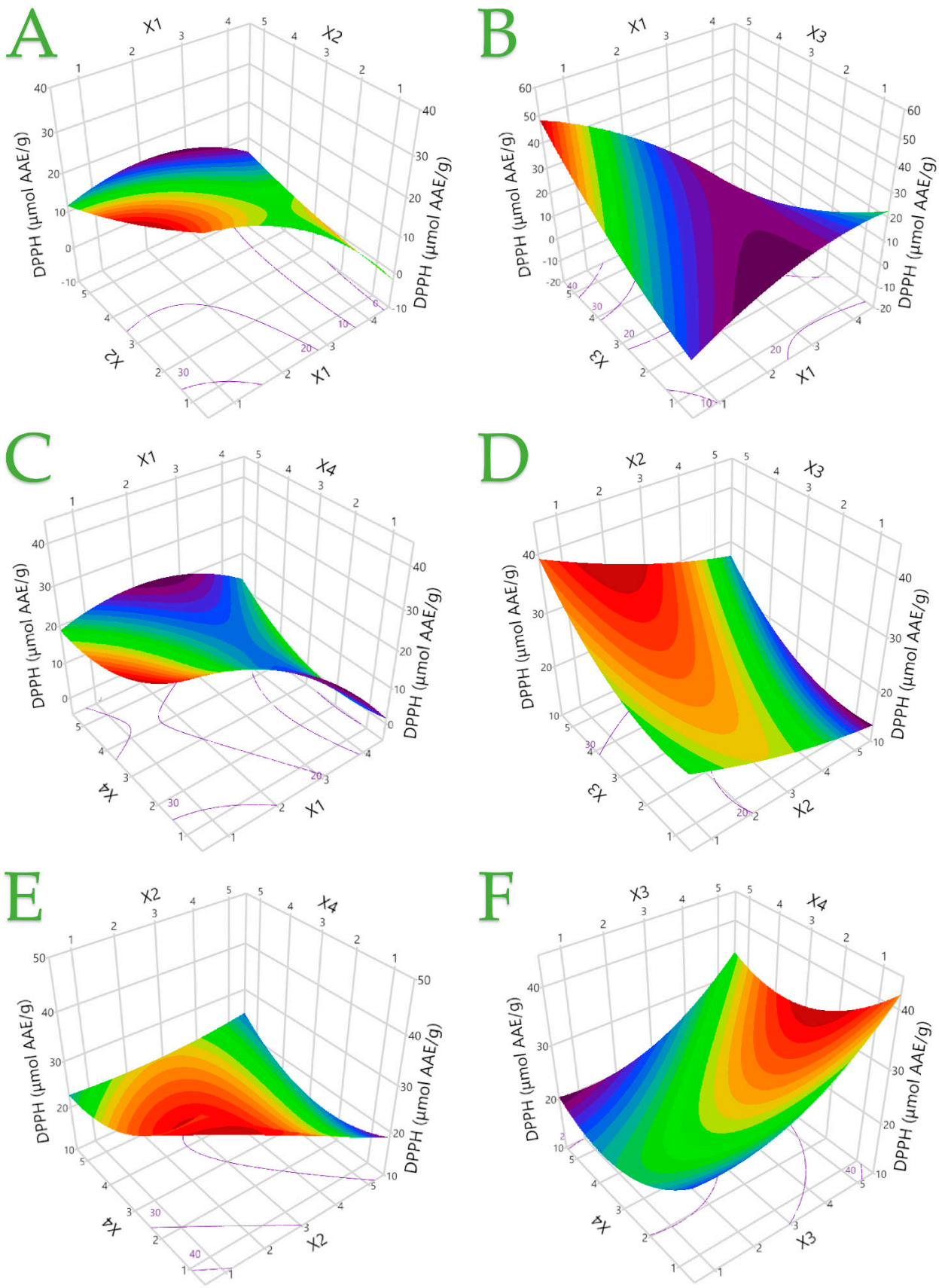


Figure S5. The optimal extraction of Galia melon peel extracts is shown in 3D graphs that show the impact of the process variables considered in the response (DPPH, µmol ascorbic acid equivalents (AAE)/g). Plot (**A**), covariation of X_1 and X_2; plot (**B**), covariation of X_1 and X_3; plot (**C**), covariation of X_1 and X_4; plot (**D**), covariation of X_2 and X_3; plot (**E**), covariation of X_2 and X_4; plot (**F**), covariation of X_3 and X_4.

References

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3. Chatzimitakos, T.; Athanasiadis, V.; Makrygiannis, I.; Kotsou, K.; Palaiogiannis, D.; Bozinou, E.; Lalas, S.I. Nutritional Quality and Antioxidant Properties of Brown and Black Lentil Sprouts. *Horticulturae* **2023**, *9*, 668, doi:10.3390/horticulturae9060668.
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