Supplementary material

Seeking Optimal Extraction Method for Augmenting *Hibiscus sabdariffa* Bioactive Compounds and Antioxidant Activity

Athanasia Kourelatou [1], Theodoros Chatzimitakos [2], Vassilis Athanasiadis [2], Konstantina Kotsou [2], Ioannis Makrygiannis [2], Eleni Bozinou [2] and Stavros I. Lalas [2,*]

[1] Department of Chemical Engineering, University of Western Macedonia, 50100 Kozani, Greece; athanasiak83@hotmail.com
[2] Department of Food Science & Nutrition, University of Thessaly, Terma N. Temponera Str., 43100 Karditsa, Greece; tchatzimitakos@uth.gr (T.C.); vaathanasiadis@uth.gr (V.A.); kkotsou@agr.uth.gr (K.K.); ioanmakr1@uth.gr (I.M.); empozinou@uth.gr (E.B.)
* Correspondence: slalas@uth.gr; Tel.: +30-24410-64783

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.

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

Utilizing a Response Surface Methodology (RSM) approach, the total polyphenol content (TPC), and total anthocyanin content (TAC) was optimized, as well as the, antioxidant activity (evaluated through the DPPH free radical scavenging assay and the FRAP assay), total carotenoid content (TCC), and ascorbic acid content (AAC). This was achieved through adjustments to the extraction procedure involved 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 effects 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 1) 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 X_i and X_j represent the independent variables, and Y_k defines the predicted response variable. The model linear, quadratic, and interaction terms are represented by the intercept and regression coefficients, β_0, β_i, β_{ii}, and β_{ij}, respectively.

2.5. HPLC-Based Determination of the Pelargonin chloride Content and Other Polyphenolic Compounds

The analysis utilized a Shimadzu CBM-20A liquid chromatograph and a Shimadzu SPD-M20A diode array detector, both provided by Shimadzu Europa GmbH in Duisburg, Germany. Separation of compounds occurred on a Phenomenex Luna C18 (2) column from Phenomenex Inc. in Torrance, CA, USA, maintained at 40°C (100 Å, 5 μm, 4.6 × 250 mm). The mobile phase comprised 0.5% aqueous formic acid (A) and a mixture of 0.5% formic acid in acetonitrile/water (6:4) (B). The gradient program employed was as follows: starting at 0% B and increasing to 40% B, followed by a transition to 50% B over 10 minutes, further increasing to 70% B in the subsequent 10 minutes, and then maintaining this level for 10 minutes. The flow rate of the mobile phase was set at 1 mL/min. Retention time and

absorbance spectrum comparisons were made against those of pure chemical standards for compound identification. Quantification was accomplished using calibration curves ranging from 0 to 50 mg/L.

2.6. Analyses of Extracts

2.6.1. Determination of Total Polyphenol Content (TPC)

The determination of TPC was also conducted according to the technique established by Chatzimitakos et al. [1]. In a 1.5 mL Eppendorf tube, 100 µL of HS flower extracts were mixed with 100 µL of Folin–Ciocalteu reagent. After 2 min, 800 µL of sodium carbonate solution (5% *w*/*v*) was added, and the solutions were incubated for 20 min at 40 °C. The absorbance at 740 nm was measured with a Shimadzu spectrophotometer (UV-1700, Shimadzu Europa GmbH, Duisburg, Germany). Using a standard chemical, gallic acid generated a calibration curve (10–80 mg/L). The total polyphenol concentration (C_{TP}) was expressed as mg gallic acid equivalents (GAE) per L. The TPC was expressed as mg GAE per g of dry weight (dw) using the following Equation (S2):

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

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

2.6.2. Determination of Total Anthocyanin Content (TAC)

The TAC were determined using a previously published procedure [2]. In a 1.5-mL Eppendorf tube, 67 µL of extract was combined with 933 µL of hydrochloric acid solution (0.25 M in ethanol) and vortexed. After 10 min, the absorbance at 520 nm was measured using an ethanolic HCl solution as a blank. The total anthocyanin concentration (C_{TA}) was calculated as cyanidin-3-*O*-glucoside equivalents (CyE) [2], as shown in Equation (S3):

$$C_{TA}\ \text{(mg CyE/L)} = \frac{A \times MW \times F_D}{\varepsilon} \times 10^3 \quad \text{(S3)}$$

where A is the absorbance at 520 nm, MW is the cyanidin-3-*O*-glucoside molecular weight (449.2), F_D is the dilution factor, and ε=26,900. The TAC was then determined as follows in equation (S4):

$$\text{TAC (mg CyE/g dw)} = \frac{C_{TA} \times V}{w} \quad \text{(S4)}$$

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

2.6.3. Ferric-Reducing Antioxidant Power (FRAP) Assay

A previously described method [3] was employed. The 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 (C_{AA}, 50–500 µmol/L in 0.05 M HCl) using the following Equation (S5):

$$P_R\ \text{(µmol AAE/g dw)} = \frac{C_{AA} \times V}{w} \quad \text{(S5)}$$

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

2.6.4. DPPH Antiradical Activity Assay

The extracted polyphenols from the dried material were evaluated for their antiradical activity (AAR) using a slightly modified DPPH method, as previously established by Shehata et al [3]. In brief, 50 µL of the sample was mixed with a quantity of 1950 µL of a 100 µM DPPH solution in methanol, with the solution being kept at room temperature for 30 min in the dark right after. Following that, the absorbance was measured at 515 nm. Moreover, a blank sample was used instead of the sample, including DPPH solution and methanol, with the absorbance immediately being measured. To calculate the percentage of scavenging, Equation (S6) was employed:

$$\%\ \text{Scavenging} = \frac{A\text{control} - A\text{sample}}{A\text{control}} \times 100 \quad \text{(S6)}$$

An ascorbic acid calibration curve in Equation (S7) was used to evaluate antiradical activity (A_{AR}), which was expressed as μmol AAE per gram of dw:

$$A_{AR}\ (\mu\text{mol AAE/g dw}) = \frac{C_{AA} \times V}{w} \quad \text{(S7)}$$

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

2.6.5. Total Carotenoid Content (TCC) Determination

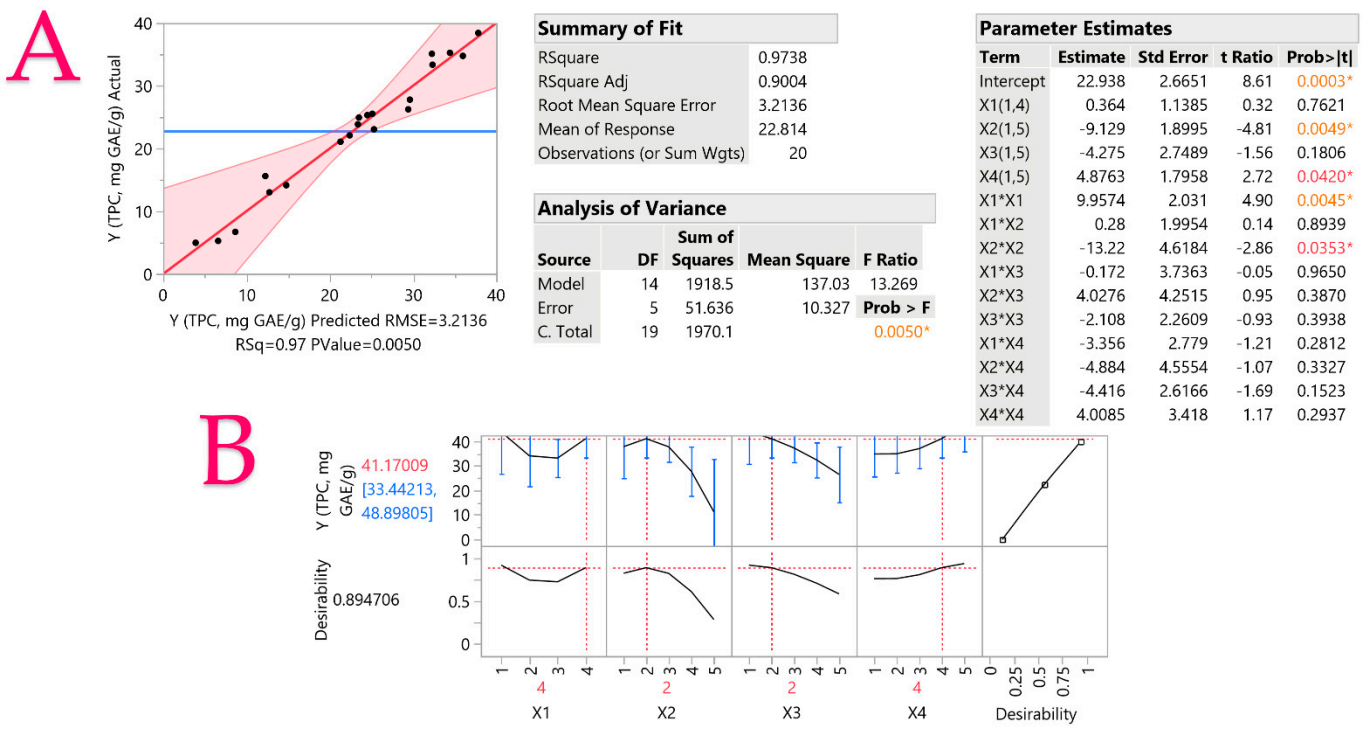
A slightly modified method introduced by Ayour et al. [4] was employed to determine the total carotenoid content (TCC) of the extracts. Briefly, a ten-fold dilution was used in the samples during their preparation, and therefore, the absorbance was recorded at 450 nm. The TCC was expressed as mg of β-carotene equivalents per gram of dried weight, using a calibration curve based on β-carotene.

2.6.6. Ascorbic Acid Content (AAC)

The ascorbic acid concentration was determined using a colorimetric assay established by Dani and Jagota [5]. One hundred microliters of the extract were added to 900 μL of 10% *w*/*v* trichloroacetic acid. After that, 500 μL of 10% (*v*/*v*) Folin–Ciocalteu reagent was added to the solution. The absorbance at 760 nm was measured after 10 min. A standard curve was created using ascorbic acid (10–80 mg/L).

2.7. Statistical Analysis

The design of the experiment, statistical analysis related to the response surface methodology, and distribution analysis were carried out utilizing JMP® Pro 16 software from SAS (Cary, NC, USA). Analyses were conducted in triplicate, and the extraction processes were executed three times. The results are presented as average values along with the corresponding standard deviations.



Summary of Fit

RSquare	0.9738
RSquare Adj	0.9004
Root Mean Square Error	3.2136
Mean of Response	22.814
Observations (or Sum Wgts)	20

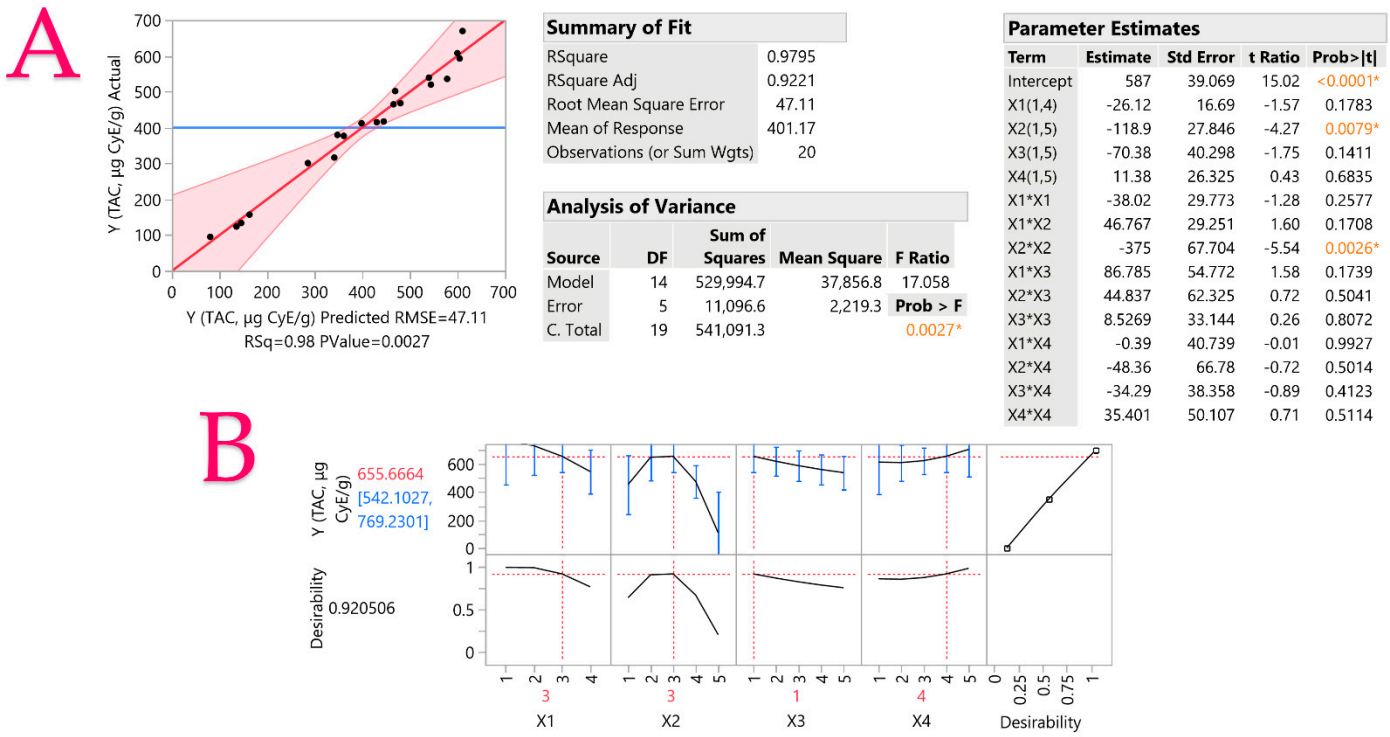
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	1918.5	137.03	13.269
Error	5	51.636	10.327	Prob > F
C. Total	19	1970.1		0.0050*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	22.938	2.6651	8.61	0.0003*
X1(1,4)	0.364	1.1385	0.32	0.7621
X2(1,5)	-9.129	1.8995	-4.81	0.0049*
X3(1,5)	-4.275	2.7489	-1.56	0.1806
X4(1,5)	4.8763	1.7958	2.72	0.0420*
X1*X1	9.9574	2.031	4.90	0.0045*
X1*X2	0.28	1.9954	0.14	0.8939
X2*X2	-13.22	4.6184	-2.86	0.0353*
X1*X3	-0.172	3.7363	-0.05	0.9650
X2*X3	4.0276	4.2515	0.95	0.3870
X3*X3	-2.108	2.2609	-0.93	0.3938
X1*X4	-3.356	2.779	-1.21	0.2812
X2*X4	-4.884	4.5554	-1.07	0.3327
X3*X4	-4.416	2.6166	-1.69	0.1523
X4*X4	4.0085	3.418	1.17	0.2937

Figure S1. Plot **A** displays the actual response versus the predicted response (Total polyphenol content – TPC, mg GAE/g) for the optimization of *H. sabdariffa* 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	0.9795
RSquare Adj	0.9221
Root Mean Square Error	47.11
Mean of Response	401.17
Observations (or Sum Wgts)	20

Analysis of Variance

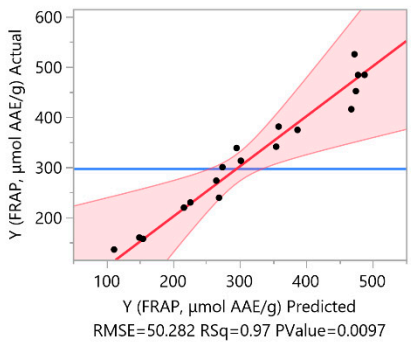
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	529,994.7	37,856.8	17.058
Error	5	11,096.6	2,219.3	Prob > F
C. Total	19	541,091.3		0.0027*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	587	39.069	15.02	<0.0001*
X1(1,4)	-26.12	16.69	-1.57	0.1783
X2(1,5)	-118.9	27.846	-4.27	0.0079*
X3(1,5)	-70.38	40.298	-1.75	0.1411
X4(1,5)	11.38	26.325	0.43	0.6835
X1*X1	-38.02	29.773	-1.28	0.2577
X1*X2	46.767	29.251	1.60	0.1708
X2*X2	-375	67.704	-5.54	0.0026*
X1*X3	86.785	54.772	1.58	0.1739
X2*X3	44.837	62.325	0.72	0.5041
X3*X3	8.5269	33.144	0.26	0.8072
X1*X4	-0.39	40.739	-0.01	0.9927
X2*X4	-48.36	66.78	-0.72	0.5014
X3*X4	-34.29	38.358	-0.89	0.4123
X4*X4	35.401	50.107	0.71	0.5114

Figure S2. Plot **A** displays the actual response versus the predicted response (Total anthocyanin content – TAC, µg CyE/g) for the optimization of *H. sabdariffa* 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.

A



Summary of Fit

RSquare	0.9652
RSquare Adj	0.8676
Root Mean Square Error	50.282
Mean of Response	296.43
Observations (or Sum Wgts)	20

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	350,182.3	25,013.0	9.8933
Error	5	12,641.4	2,528.3	Prob > F
C. Total	19	362,823.7		0.0097*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	268.05	41.699	6.43	0.0014*
X1(1,4)	-12.19	17.814	-0.68	0.5242
X2(1,5)	-102.1	29.721	-3.43	0.0185*
X3(1,5)	-117.1	43.011	-2.72	0.0416*
X4(1,5)	127.11	28.097	4.52	0.0063*
X1*X1	60.206	31.778	1.89	0.1167
X1*X2	26.746	31.221	0.86	0.4308
X2*X2	-242.5	72.263	-3.36	0.0202*
X1*X3	46.738	58.461	0.80	0.4603
X2*X3	128.87	66.522	1.94	0.1105
X3*X3	55.045	35.376	1.56	0.1804
X1*X4	-94.72	43.482	-2.18	0.0813
X2*X4	-163.2	71.276	-2.29	0.0706
X3*X4	-166.4	40.941	-4.06	0.0097*
X4*X4	179.49	53.481	3.36	0.0202*

B

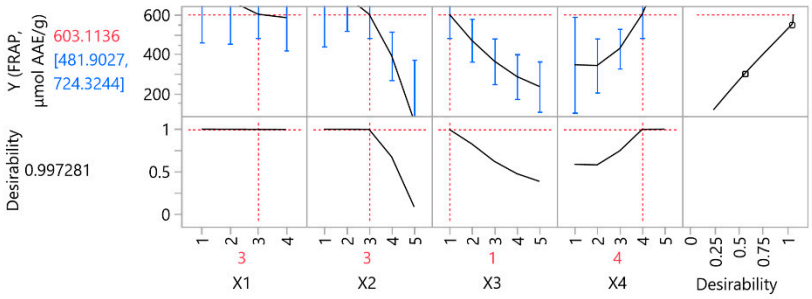
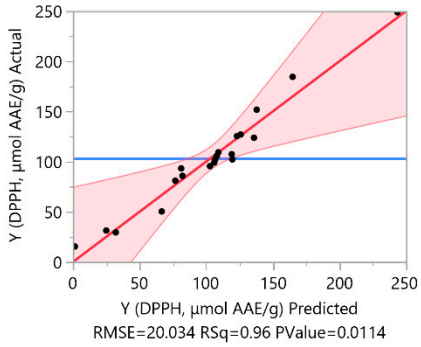


Figure S3. Plot **A** displays the actual response versus the predicted response (FRAP, µmol AAE/g) for the optimization of *H. sabdariffa* extracts using hydroethanolic solutions, different extraction techniques, and parameters, and plot **B** displays the desirability function. Asterisks and colored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

A



Summary of Fit

RSquare	0.9626
RSquare Adj	0.858
Root Mean Square Error	20.034
Mean of Response	103.37
Observations (or Sum Wgts)	20

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	51,709.5	3,693.53	9.2027
Error	5	2,006.8	401.35	Prob > F
C. Total	19	53,716.2		0.0114*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	140.76	16.614	8.47	0.0004*
X1(1,4)	-6.518	7.0977	-0.92	0.4006
X2(1,5)	-42.39	11.842	-3.58	0.0159*
X3(1,5)	-46.34	17.137	-2.70	0.0426*
X4(1,5)	33.302	11.195	2.97	0.0310*
X1*X1	-31.92	12.661	-2.52	0.0531
X1*X2	-32.7	12.439	-2.63	0.0466*
X2*X2	-72.22	28.792	-2.51	0.0539
X1*X3	-0.654	23.292	-0.03	0.9787
X2*X3	80.39	26.504	3.03	0.0290*
X3*X3	13.032	14.095	0.92	0.3976
X1*X4	-41.72	17.324	-2.41	0.0610
X2*X4	-71.31	28.399	-2.51	0.0538
X3*X4	-30.27	16.312	-1.86	0.1226
X4*X4	13.428	21.308	0.63	0.5563

B

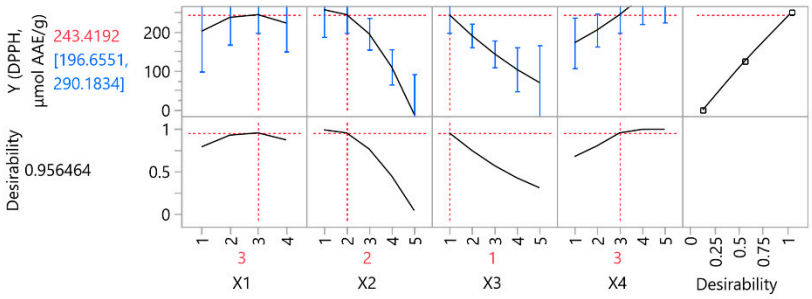
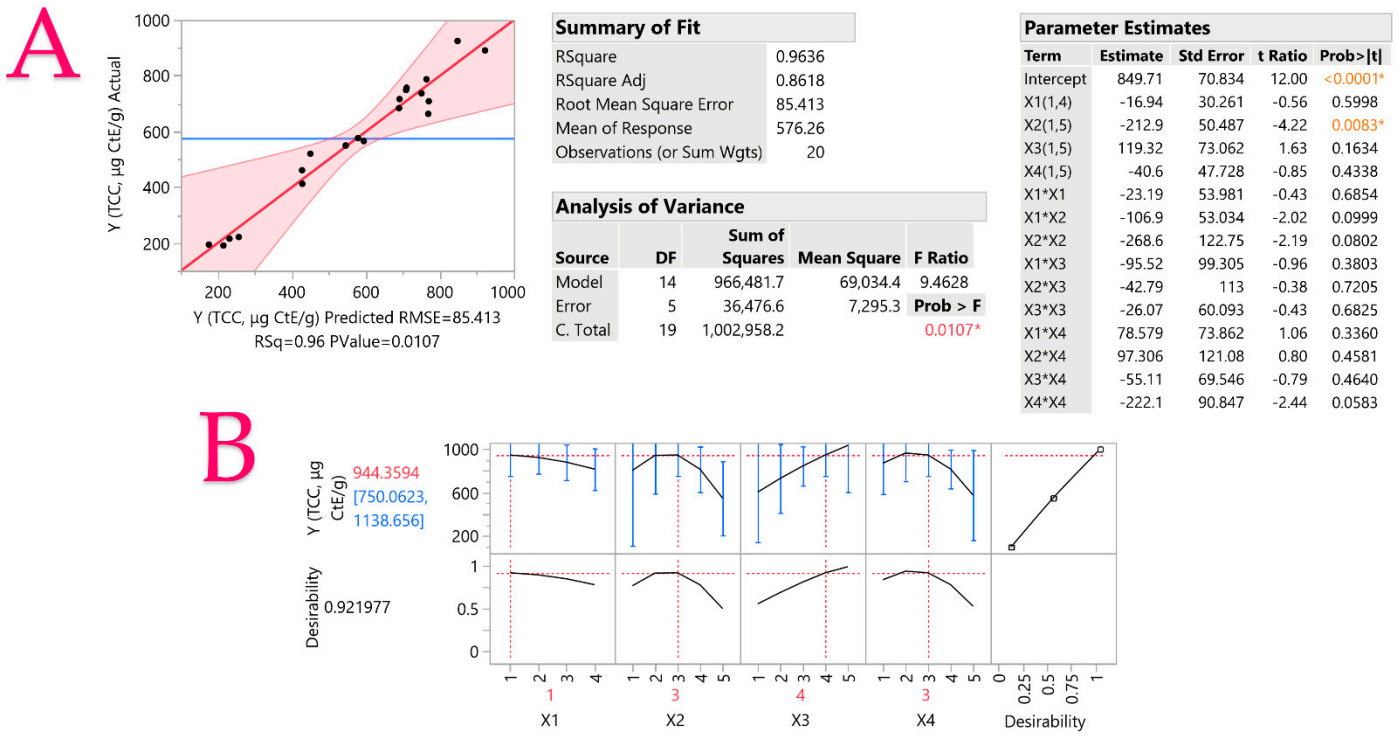


Figure S4. Plot **A** displays the actual response versus the predicted response (DPPH, µmol AAE/g) for the optimization of *H. sabdariffa* extracts using hydroethanolic solutions, different extraction techniques, and parameters, and plot **B** displays the desirability function. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model.



Summary of Fit

RSquare	0.9636
RSquare Adj	0.8618
Root Mean Square Error	85.413
Mean of Response	576.26
Observations (or Sum Wgts)	20

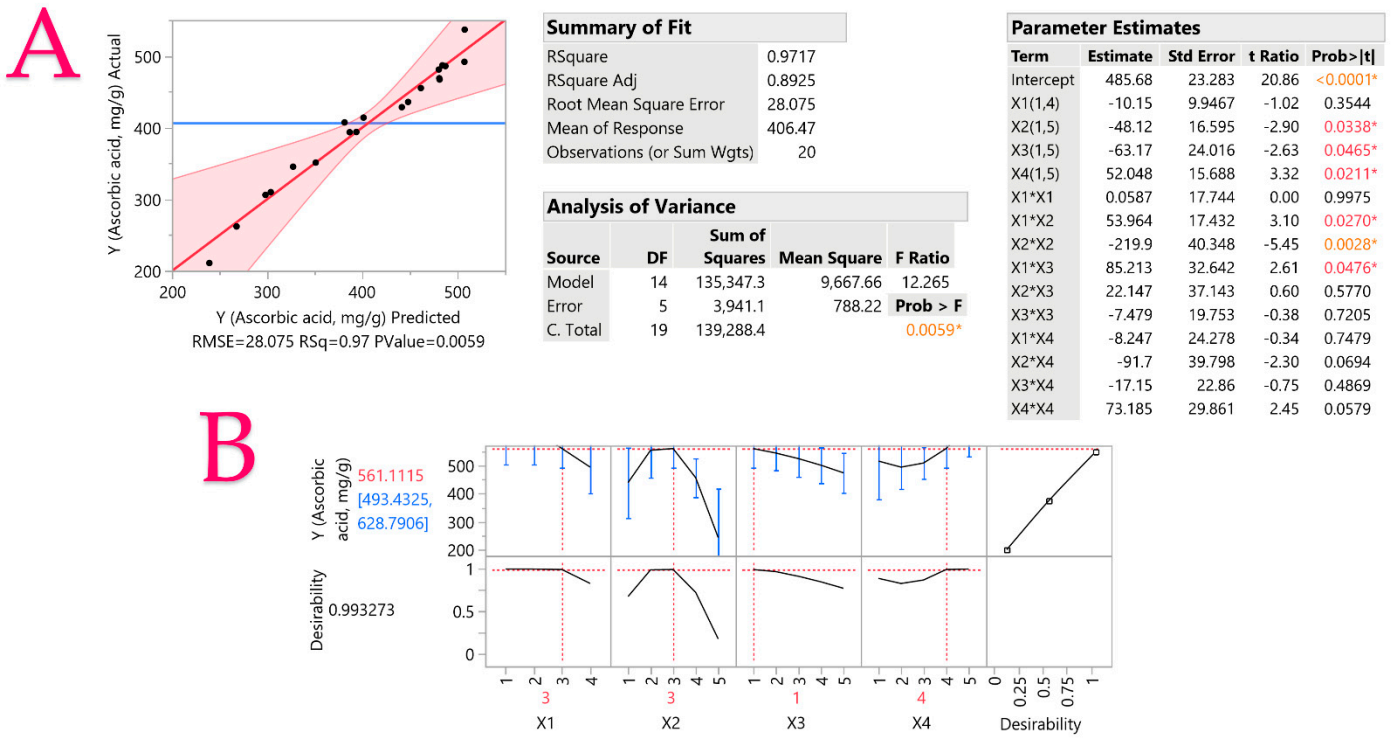
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	966,481.7	69,034.4	9.4628
Error	5	36,476.6	7,295.3	Prob > F
C. Total	19	1,002,958.2		0.0107*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	849.71	70.834	12.00	<0.0001*
X1(1,4)	-16.94	30.261	-0.56	0.5998
X2(1,5)	-212.9	50.487	-4.22	0.0083*
X3(1,5)	119.32	73.062	1.63	0.1634
X4(1,5)	-40.6	47.728	-0.85	0.4338
X1*X1	-23.19	53.981	-0.43	0.6854
X1*X2	-106.9	53.034	-2.02	0.0999
X2*X2	-268.6	122.75	-2.19	0.0802
X1*X3	-95.52	99.305	-0.96	0.3803
X2*X3	-42.79	113	-0.38	0.7205
X3*X3	-26.07	60.093	-0.43	0.6825
X1*X4	78.579	73.862	1.06	0.3360
X2*X4	97.306	121.08	0.80	0.4581
X3*X4	-55.11	69.546	-0.79	0.4640
X4*X4	-222.1	90.847	-2.44	0.0583

Figure S5. Plot **A** displays the actual response versus the predicted response (Total carotenoid content – TCC, µg CtE/g) for the optimization of *H. sabdariffa* 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	0.9717
RSquare Adj	0.8925
Root Mean Square Error	28.075
Mean of Response	406.47
Observations (or Sum Wgts)	20

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	135,347.3	9,667.66	12.265
Error	5	3,941.1	788.22	Prob > F
C. Total	19	139,288.4		0.0059*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	485.68	23.283	20.86	<0.0001*
X1(1,4)	-10.15	9.9467	-1.02	0.3544
X2(1,5)	-48.12	16.595	-2.90	0.0338*
X3(1,5)	-63.17	24.016	-2.63	0.0465*
X4(1,5)	52.048	15.688	3.32	0.0211*
X1*X1	0.0587	17.744	0.00	0.9975
X1*X2	53.964	17.432	3.10	0.0270*
X2*X2	-219.9	40.348	-5.45	0.0028*
X1*X3	85.213	32.642	2.61	0.0476*
X2*X3	22.147	37.143	0.60	0.5770
X3*X3	-7.479	19.753	-0.38	0.7205
X1*X4	-8.247	24.278	-0.34	0.7479
X2*X4	-91.7	39.798	-2.30	0.0694
X3*X4	-17.15	22.86	-0.75	0.4869
X4*X4	73.185	29.861	2.45	0.0579

Figure S6. Plot **A** displays the actual response versus the predicted response (Ascorbic acid content, mg/100 g) for the optimization of *H. sabdariffa* 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.

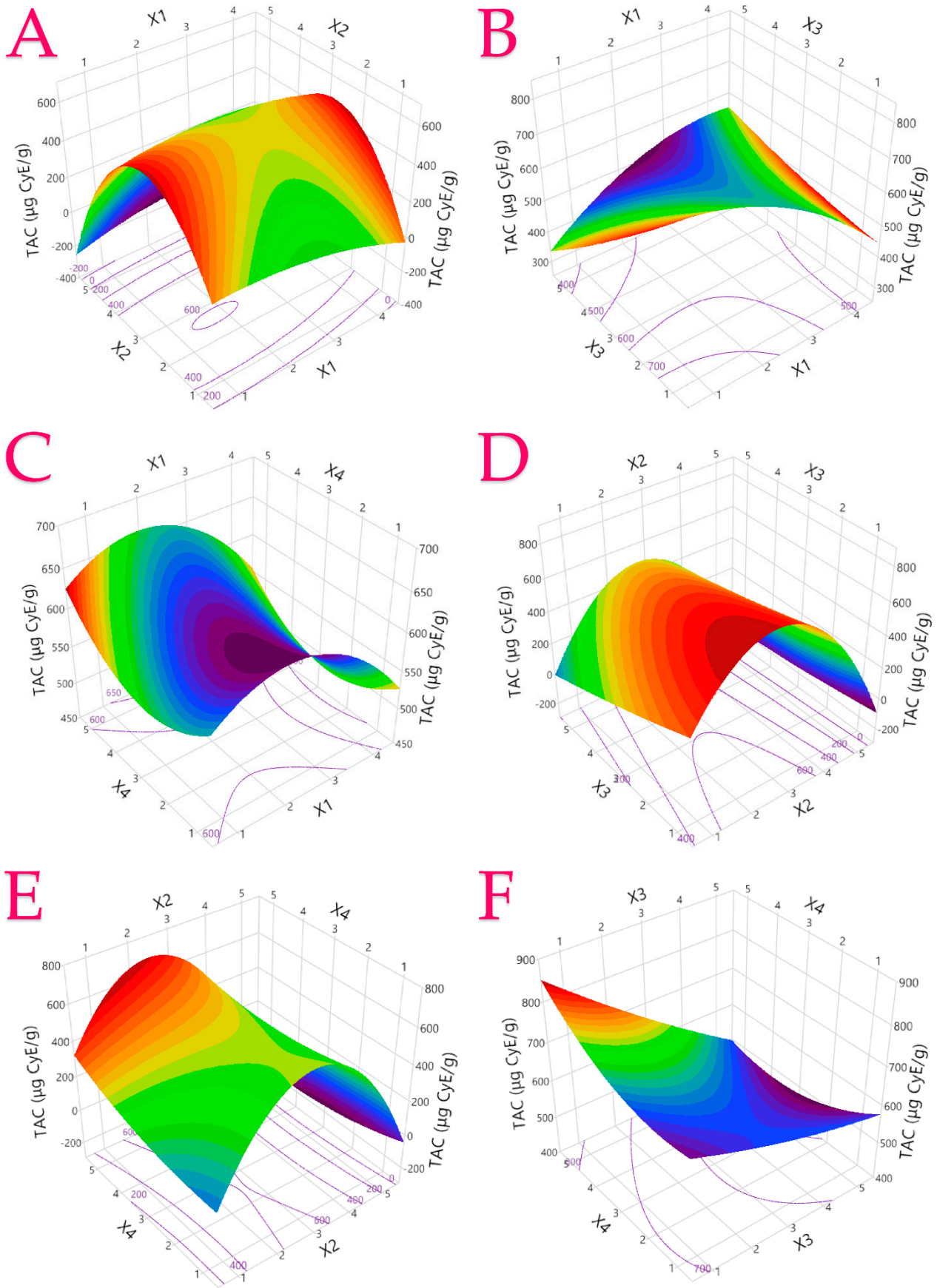


Figure S7. The optimal extraction of *H. sabdariffa* extracts is shown in 3D graphs that show the impact of the process variables considered in the response (Total anthocyanin content – TAC, µg CyE/g). Plot (**A**), covariation of X_1 and X_2; plot (**B**), covariation of X_1 and X_3; plot (**C**), covariation 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.

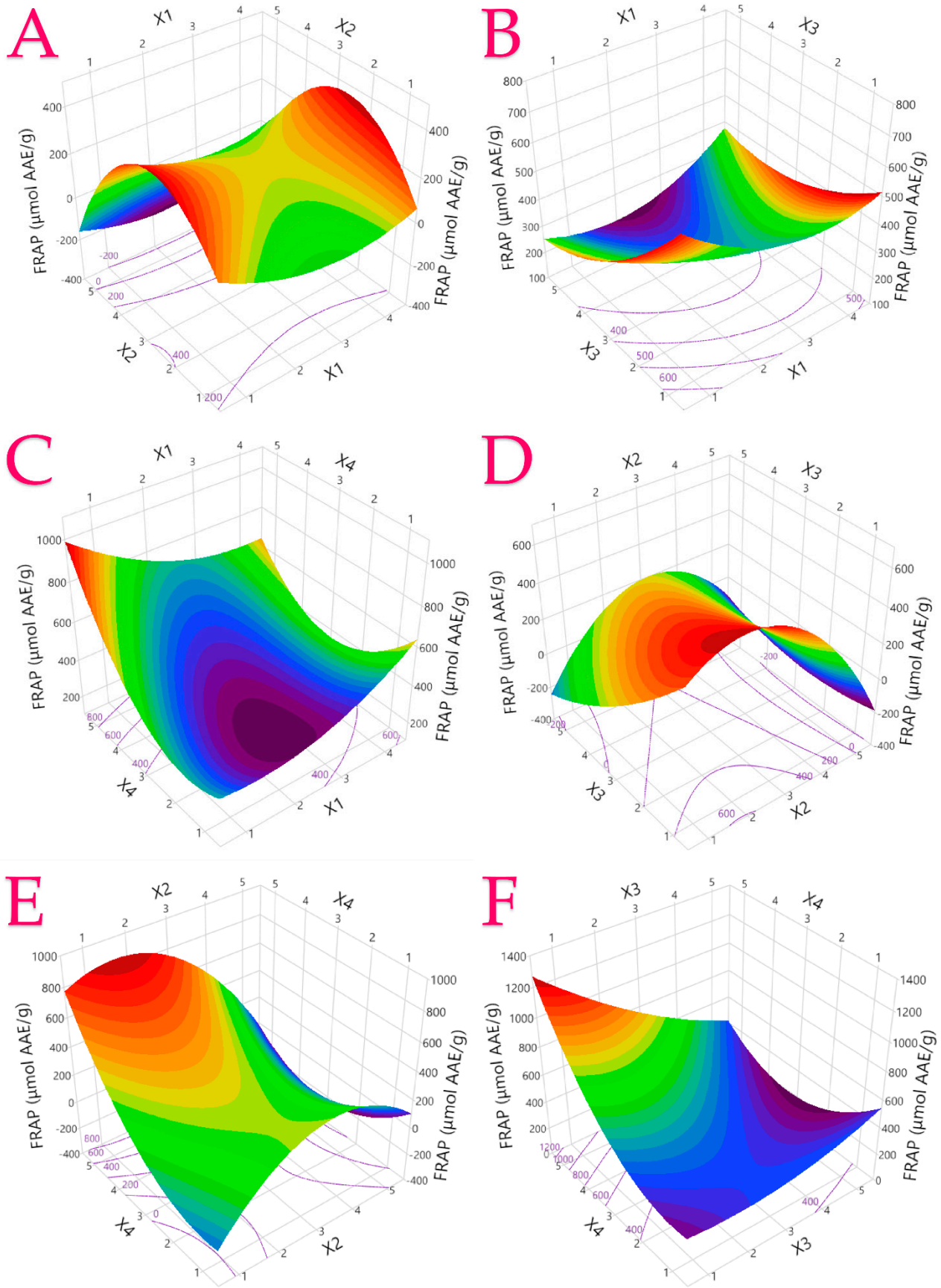


Figure S8. The optimal extraction of *H. sabdariffa* extracts is shown in 3D graphs that show the impact of the process variables considered in the response (FRAP, µmol AAE/g). Plot (**A**), covariation of X_1 and X_2; plot (**B**), covariation of X_1 and X_3; plot (**C**), covariation of X_1 and X_4; 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.

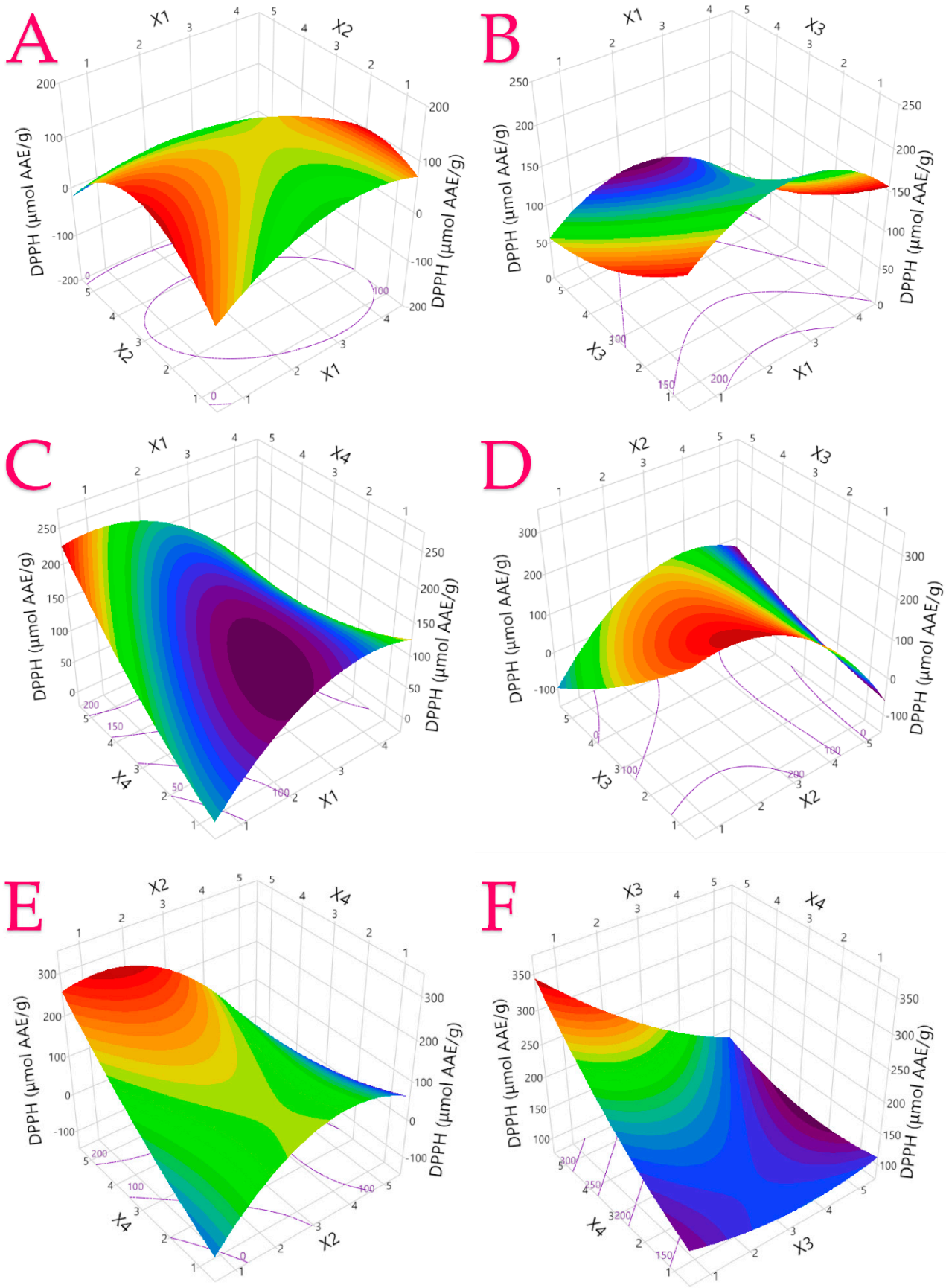


Figure S9. The optimal extraction of *H. sabdariffa* extracts is shown in 3D graphs that show the impact of the process variables considered in the response (DPPH, µmol AAE/g). Plot (**A**), covariation of X_1 and X_2; plot (**B**), covariation of X_1 and X_3; plot (**C**), covariation of X_1 and X_4; 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.

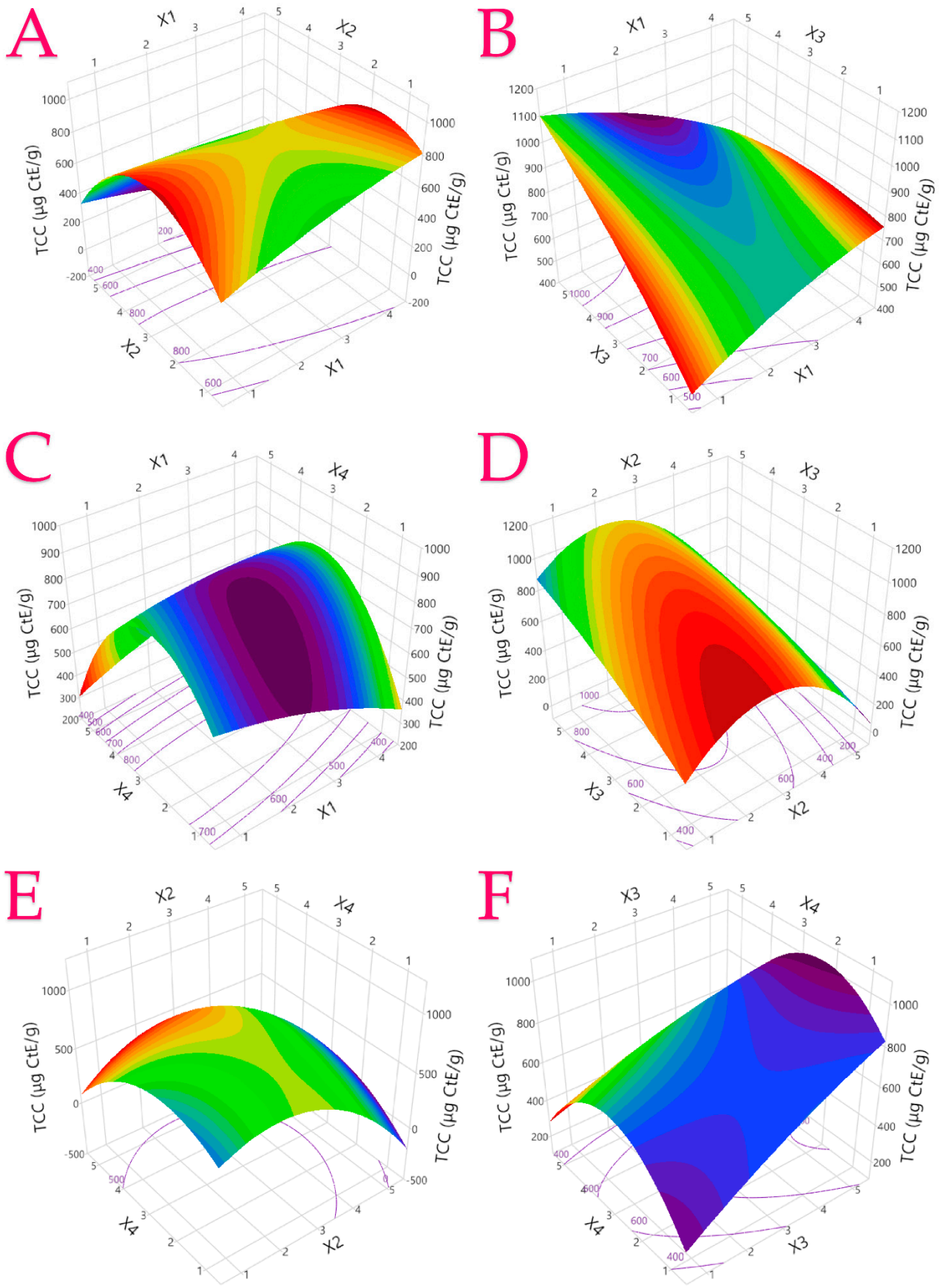


Figure S10. The optimal extraction of *H. sabdariffa* extracts is shown in 3D graphs that show the impact of the process variables considered in the response (Total carotenoid content – TCC, µg CtE/g). Plot (**A**), covariation of X_1 and X_2; plot (**B**), covariation of X_1 and X_3; plot (**C**), covariation 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.

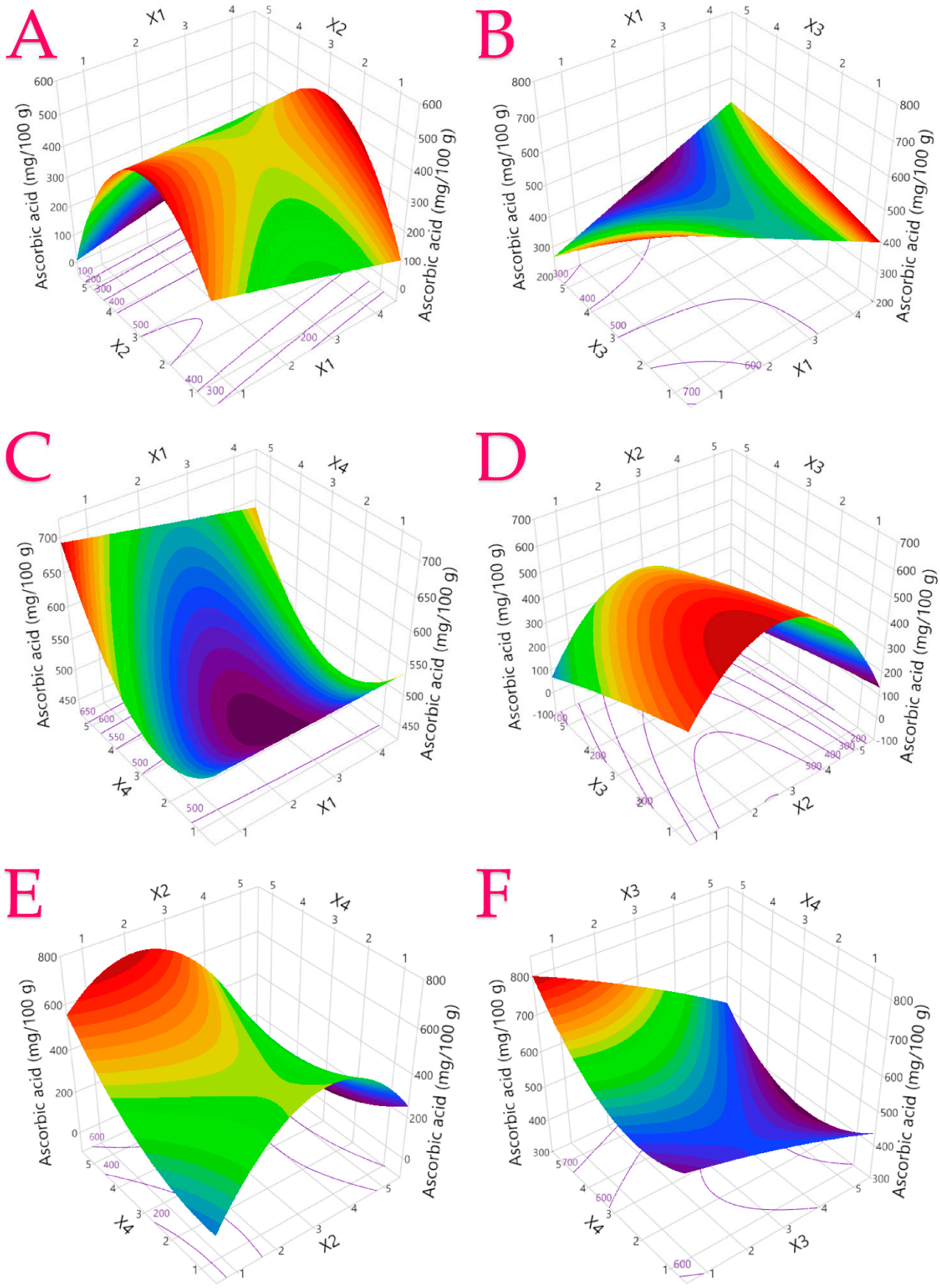


Figure S11. The optimal extraction of *H. sabdariffa* extracts is shown in 3D graphs that show the impact of the process variables considered in the response (Ascorbic acid content, mg/100 g). Plot (**A**), covariation of X_1 and X_2; plot (**B**), covariation of X_1 and X_3; plot (**C**), covariation 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

1. Chatzimitakos, T.; Athanasiadis, V.; Kotsou, K.; Bozinou, E.; Lalas, S.I. Response Surface Optimization for the Enhancement of the Extraction of Bioactive Compounds from *Citrus Limon* Peel. *Antioxidants* **2023**, *12*, 1605, doi:10.3390/antiox12081605.
2. Bozinou, E.; Lakka, A.; Poulianiti, K.; Lalas, S.; Makris, D.P. Cyclodextrins as High-Performance Green Co-Solvents in the Aqueous Extraction of Polyphenols and Anthocyanin Pigments from Solid Onion Waste. *Eur. Food Res. Technol.* **2021**, *247*, 2831–2845, doi:10.1007/s00217-021-03839-2.
3. Shehata, E.; Grigorakis, S.; Loupassaki, S.; Makris, D.P. Extraction Optimisation Using Water/Glycerol for the Efficient Recovery of Polyphenolic Antioxidants from Two Artemisia Species. *Sep. Purif. Technol.* **2015**, *149*, 462–469, doi: 10.1016/j.seppur.2015.06.017.
4. Ayour, J.; Alahyane, A.; Harrak, H.; Neffa, M.; Taourirte, M.; Benichou, M. Assessment of Nutritional, Technological, and Commercial Apricot Quality Criteria of the Moroccan Cultivar "Maoui" Compared to Introduced Spanish Cultivars "Canino" and "Delpatriarca" towards Suitable Valorization. *J. Food Qual.* **2021**, *2021*, 6679128, doi:10.1155/2021/6679128.
5. Dani, H.M.; Jagota, S.K. A New Calorimetric Technique for the Estimation of Vitamin C Using Folin Phenol Reagent. *Anal. Biochem.* **1982**, *127*, 178–182.