Supplementary material

Enhancing antioxidant properties of *Prunus spinosa* fruit extracts via extraction optimization

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2.1. Chemicals

All solvents were purchased from Carlo Erba (Val de Reuil, France) and were of at least HPLC grade. Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) anhydrous sodium carbonate, 2,4,6-tri-2-pyridinyl-1,3,5-triazine (TPTZ), and Folin-Ciocalteu reagent were supplied by Penta (Prague, Czech Republic). Chemical standards for HPLC-based determination of polyphenols, such as procatechuic acid, catechin, cyanidin 3-*O*-glucoside, delphinidin 3,5-di-*O*-galactoside, rutin, quercetin 3-*O*-galactoside, delphinidin 3,5-di-*O*-glucoside, kaempferol 3-*O*-β-rutinoside, 3-*O*-caffeoylquinic acid (chlorogenic acid), kaempferol 3-glucoside, cyanidin 3-*O*-(6''-malonylglucoside), 5-*O*-caffeoylquinic acid (neochlorogenic acid), quercetin 3-β-D-glucoside, ferric (III) chloride, aluminum chloride, ascorbic acid, trichloroacetic acid, methanol, aqueous ethanol, sodium acetate, and hydrochloric acid were purchased from Sigma-Aldrich (Steinheim, Germany) and were all of HPLC grade. The deionized water used in the experiments was created using a deionizing column.

2.5.1. Determination of total polyphenol content (TPC)

The determination of TPC was also conducted according to the technique established by Athanasiadis et al. [1]. In a 1.5 mL Eppendorf tube, 100 μL of *P. spinosa* fruit 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 was employed to generate a calibration curve (10–80 mg/L). The TPC (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 (S1):

$$Y_{TP}\ (\text{mg GAE/g dw}) = \frac{C_{TP} \times V}{w} \quad \text{(S1)}$$

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

2.5.2. Determination of total flavonoid content (TFC)

A previously established technique [1] was followed, according to which a volume of 100 μL of a diluted sample (1:5) was mixed with 860 μL of aqueous ethanol (35% *v*/*v*) and 40 μL of a reagent that included 5% (*w*/*v*) aluminium chloride and 0.5 M sodium acetate. The mixture was left at ambient temperature for 30 min before the absorbance was measured at 415 nm. A rutin (quercetin 3-*O*-rutinoside) calibration curve (30–300 mg/L in methanol) was used to measure total flavonoid concentration (C_{TFn}). The TFC was expressed as mg rutin equivalents (RtE) per g dry weight (dw), using the following Equation (S2):

$$\text{TFC (mg RtE/g dw)} = \frac{C_{TFn} \times V}{w} \quad \text{(S2)}$$

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

2.5.3. Determination of total anthocyanins (TA)

The total pigments 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 pigment concentration (C_{TPm}) was calculated as cyanidin-3-*O*-glucoside equivalents (CyE) [2], as shown in equation (S3):

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

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 yield in total pigments (Y_{TPm}) was then determined as follows in equation (S4):

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

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

2.5.4. Ascorbic acid content

The ascorbic acid concentration was determined using a colorimetric assay established by Jagota et al. [3]. One hundred microliters of the extract was 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).

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

A previously employed assay [1] 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 antiradical activity (A_{AR}) was calculated employing Equation (S5):

$$A_{AR}\ (\mu\text{mol DPPH/g dw}) = \frac{\Delta A}{\varepsilon \times l \times C} \times Y_{TP} \quad (S5)$$

where $\Delta A = A_{515(i)} - A_{515(f)}$; ε (DPPH) = 11,126 × 10^{-6} μM^{-1} cm^{-1}; $C = C_{TP} \times 0.025$; Y_{TP} is the total polyphenol yield of the extract (mg/g), and l is the path length (1 cm).

2.5.6. Ferric-reducing antioxidant power (FRAP) assay

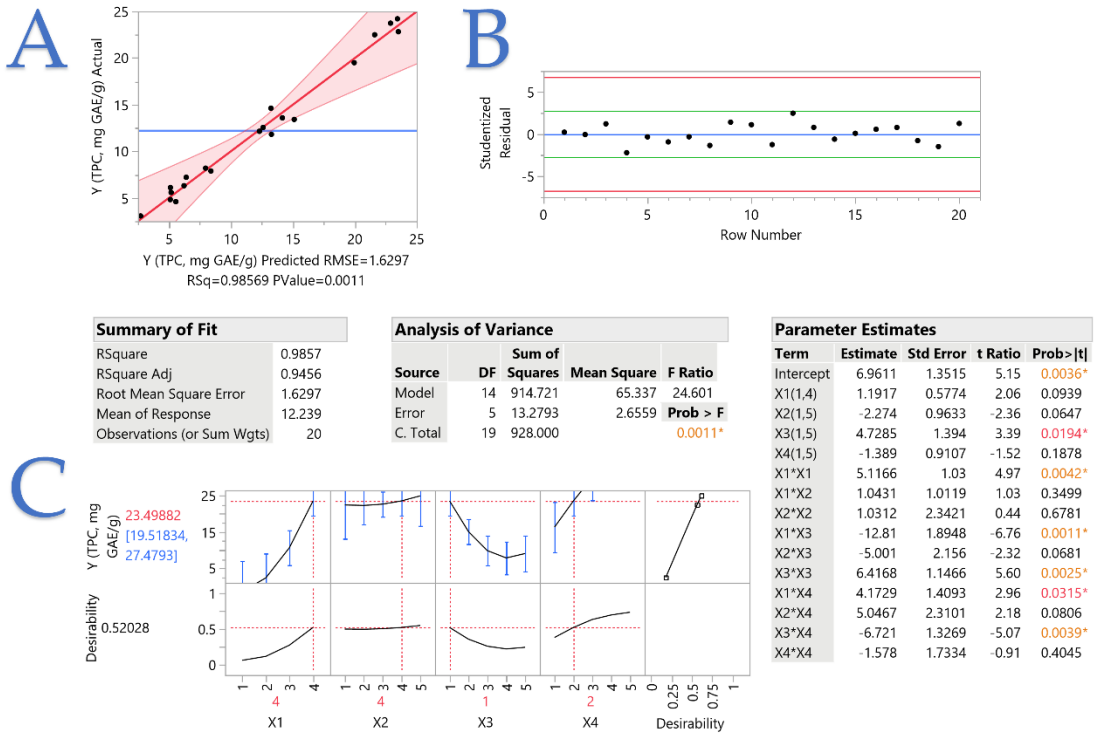
A previously described method [1] was employed. The amount of 0.05 mL ferric (III) chloride solution (4 mM in 0.05 M HCl) was well mixed with the diluted sample extract (0.05 mL, 1:50) and then incubated in a water bath at 37 °C for 30 min. After that, 90 μL of TPTZ solution (1 mM in 0.05 M HCl) was 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 (S6):

$$P_R\ (\mu\text{mol AAE/g dw}) = \frac{C_{AA} \times V}{w} \quad (S6)$$

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

2.5.7. Color analysis

A colorimeter (Lovibond CAM-System 500, The Tintometer Ltd, Amesbury, UK) was used to determine the color of the extracts. In particular, each Eppendorf tube with the extract sample was placed in a colorimeter for CIELAB color determination. The lightness psychometric index, L^*, and two color coordinates, a^* and b^*, were defined.

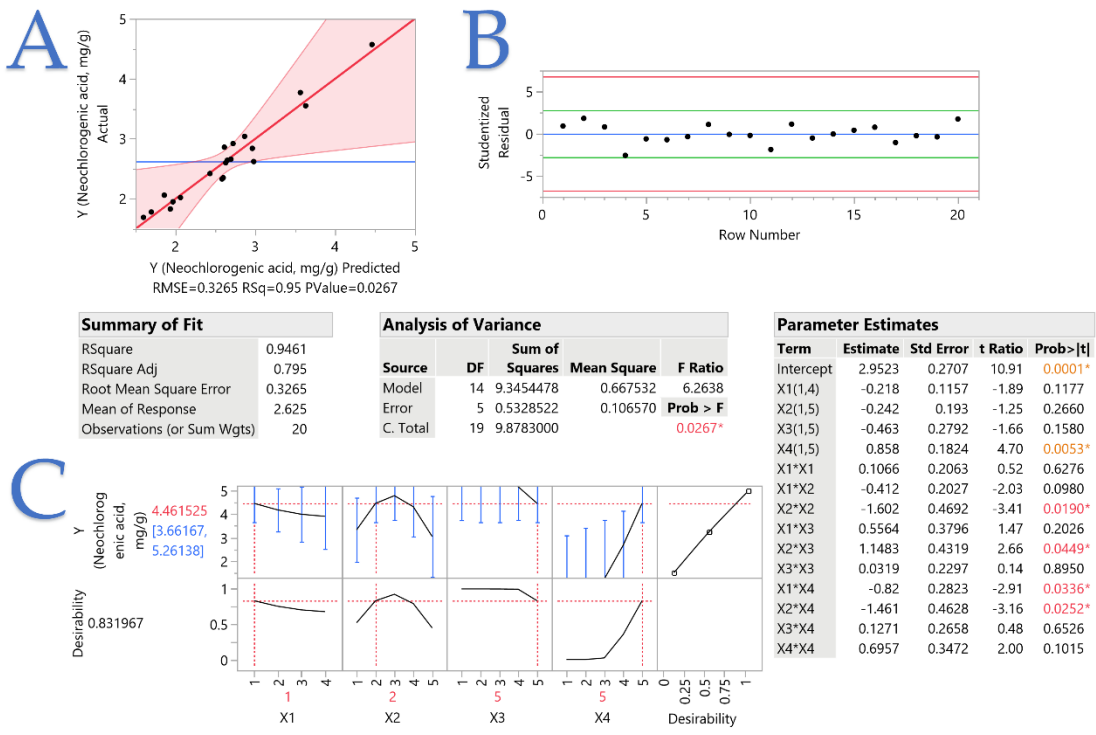


Summary of Fit	
RSquare	0.9857
RSquare Adj	0.9456
Root Mean Square Error	1.6297
Mean of Response	12.239
Observations (or Sum Wgts)	20

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	914.721	65.337	24.601
Error	5	13.2793	2.6559	Prob > F
C. Total	19	928.000		0.0011*

Parameter Estimates				
Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	6.9611	1.3515	5.15	0.0036*
X1(1,4)	1.1917	0.5774	2.06	0.0939
X2(1,5)	-2.274	0.9633	-2.36	0.0647
X3(1,5)	4.7285	1.394	3.39	0.0194*
X4(1,5)	-1.389	0.9107	-1.52	0.1878
X1*X1	5.1166	1.03	4.97	0.0042*
X1*X2	1.0431	1.0119	1.03	0.3499
X2*X2	1.0312	2.3421	0.44	0.6781
X1*X3	-12.81	1.8948	-6.76	0.0011*
X2*X3	-5.001	2.156	-2.32	0.0681
X3*X3	6.4168	1.1466	5.60	0.0025*
X1*X4	4.1729	1.4093	2.96	0.0315*
X2*X4	5.0467	2.3101	2.18	0.0806
X3*X4	-6.721	1.3269	-5.07	0.0039*
X4*X4	-1.578	1.7334	-0.91	0.4045

Figure S1. Plots A, B, and C display the actual response versus the predicted response (Total polyphenol content – TPC, mg GAE/g) for the optimization of *Prunus spinosa* extracts carried out with hydroethanolic solutions and different extraction methods, as well as the studentized residuals and the desirability function, respectively. 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	0.9461
RSquare Adj	0.795
Root Mean Square Error	0.3265
Mean of Response	2.625
Observations (or Sum Wgts)	20

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	9.3454478	0.667532	6.2638
Error	5	0.5328522	0.106570	Prob > F
C. Total	19	9.8783000		0.0267*

Parameter Estimates				
Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	2.9523	0.2707	10.91	0.0001*
X1(1,4)	-0.218	0.1157	-1.89	0.1177
X2(1,5)	-0.242	0.193	-1.25	0.2660
X3(1,5)	-0.463	0.2792	-1.66	0.1580
X4(1,5)	0.858	0.1824	4.70	0.0053*
X1*X1	0.1066	0.2063	0.52	0.6276
X1*X2	-0.412	0.2027	-2.03	0.0980
X2*X2	-1.602	0.4692	-3.41	0.0190*
X1*X3	0.5564	0.3796	1.47	0.2026
X2*X3	1.1483	0.4319	2.66	0.0449*
X3*X3	0.0319	0.2297	0.14	0.8950
X1*X4	-0.82	0.2823	-2.91	0.0336*
X2*X4	-1.461	0.4628	-3.16	0.0252*
X3*X4	0.1271	0.2658	0.48	0.6526
X4*X4	0.6957	0.3472	2.00	0.1015

Figure S2. Plots A, B, and C display the actual response versus the predicted response (Neochlorogenic acid, mg/g) for the optimization of *Prunus spinosa* extracts carried out with hydroethanolic solutions and different extraction methods, as well as the studentized residuals and the desirability function, respectively. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model.

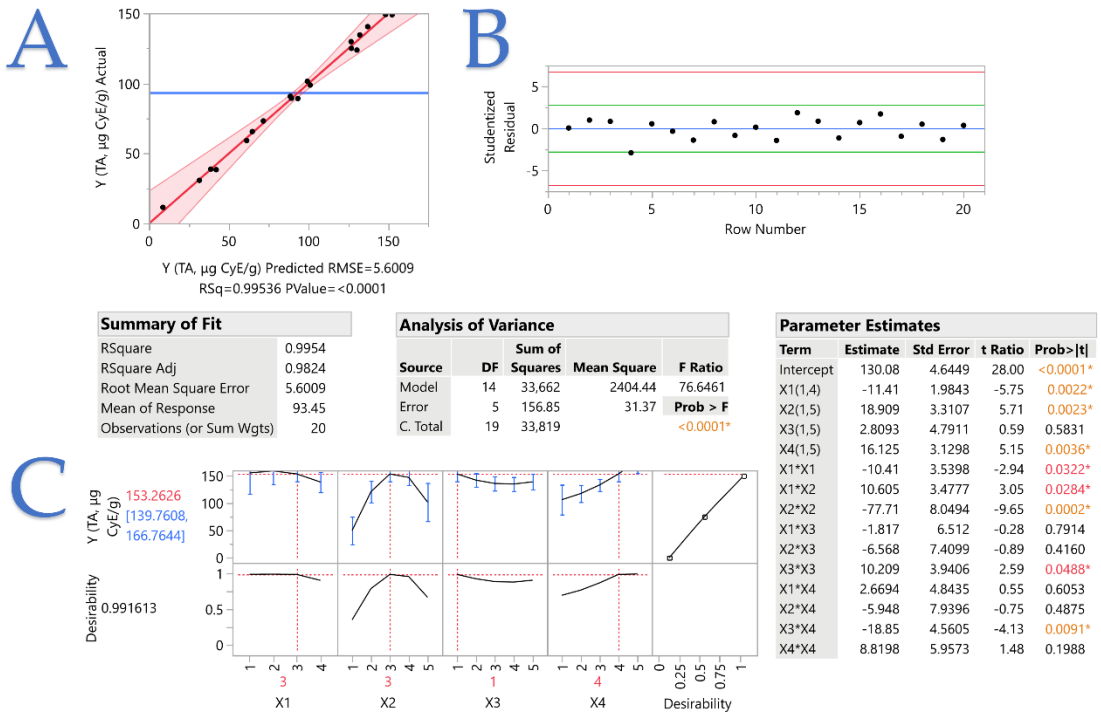


Figure S3. Plots A, B, and C display the actual response versus the predicted response (Total anthocyanins – TA, µg CyE/g) for the optimization of *Prunus spinosa* extracts carried out with hydroethanolic solutions and different extraction methods, as well as the studentized residuals and the desirability function, respectively. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model.

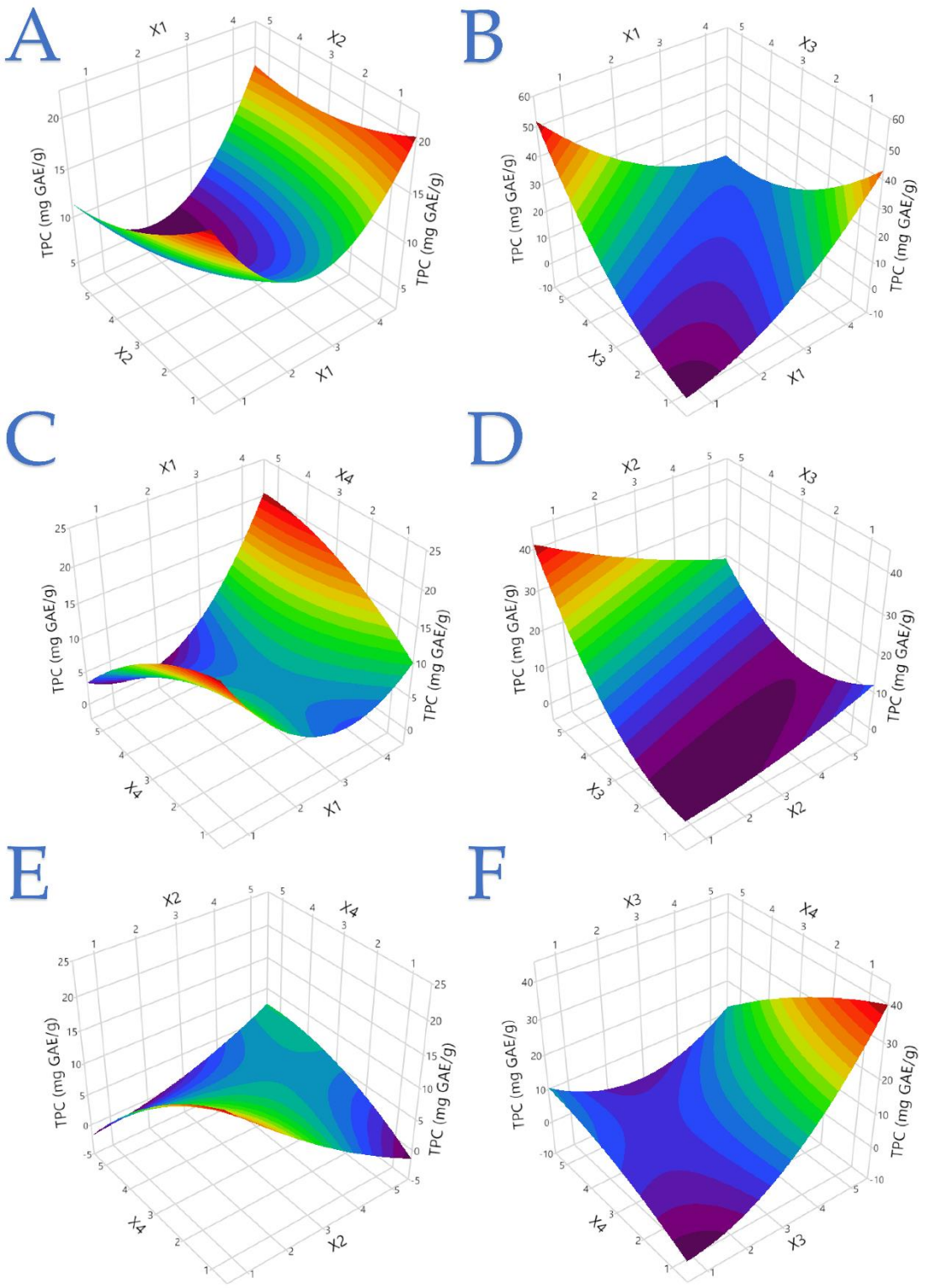


Figure S4. The optimal extraction of *Prunus spinosa* extracts using different extraction methods and hydroethanolic solutions is shown in 3D graphs that show the impact of the process variables considered in the response (Total polyphenol content – TPC, mg GAE/g). Plot (A), covariation of X_1 and X_2; 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.

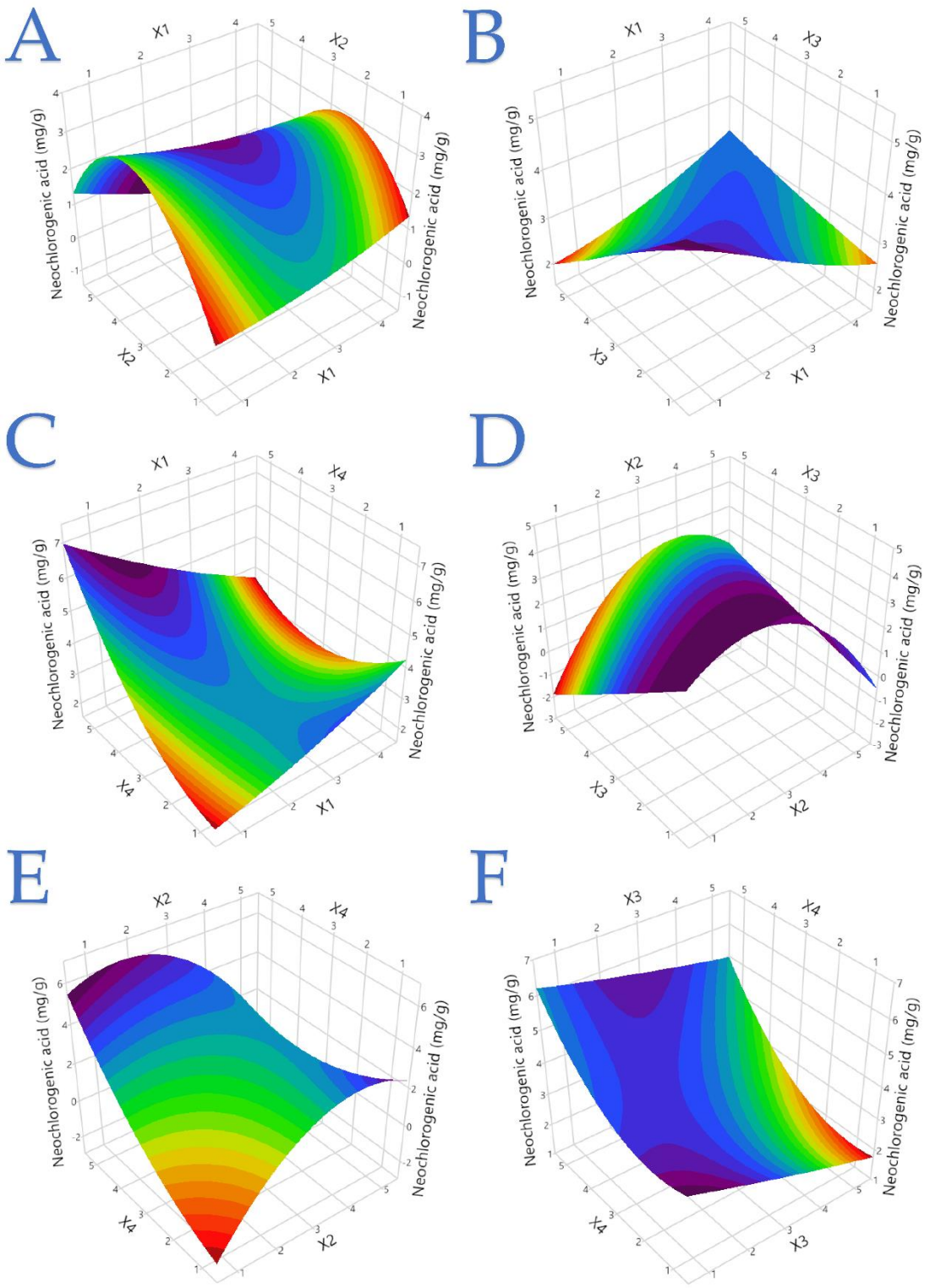


Figure S5. The optimal extraction of *Prunus spinosa* extracts using different extraction methods and hydroethanolic solutions is shown in 3D graphs that show the impact of the process variables considered in the response (Neochlorogenic acid, mg/g). Plot (A), covariation of X_1 and X_2; plot (B), covariation 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.

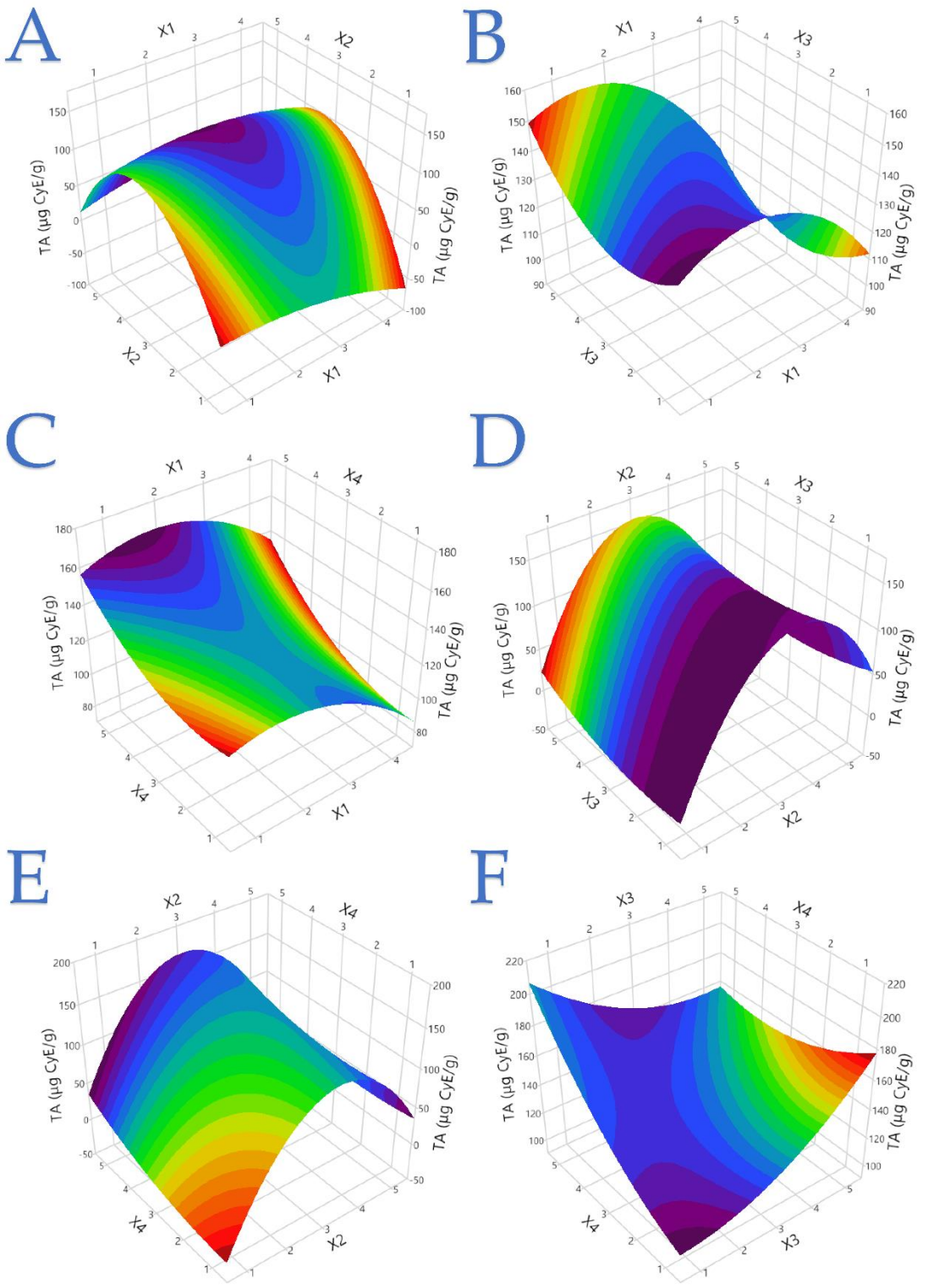


Figure S6. The optimal extraction of *Prunus spinosa* extracts using different extraction methods and hydroethanolic solutions is shown in 3D graphs that show the impact of the process variables considered in the response (Total anthocyanins – TA, µg CyE/g). Plot (A), covariation of X_1 and X_2; plot (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.

Table S1. Design points under investigation and the actual concentration of polyphenolic compounds, represented in mg/g dw.

Polyphenolic compounds (mg/g)	Design points																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Gallic acid	0.17	N.D. *	N.D.	N.D.	N.D.	0.05	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Procatechuic acid	0.10	0.04	0.03	0.02	0.02	0.04	0.03	0.03	0.02	0.01	0.07	0.03	0.03	0.02	0.01	0.08	0.03	0.03	0.03	0.01
Neochlorogenic acid	3.04	3.77	2.92	2.62	1.83	3.55	2.66	2.86	2.42	1.95	2.35	4.57	2.84	2.64	1.78	1.69	2.33	2.60	2.02	2.06
Catechin	0.63	0.70	0.14	0.28	0.26	0.42	0.38	0.19	0.29	0.27	0.50	0.64	0.15	0.37	0.24	0.41	0.36	0.21	0.27	0.26
Cyanidin 3-*O*-glucoside	0.15	0.79	1.00	0.60	0.58	0.27	0.21	1.04	0.98	0.71	N.D.	1.49	0.97	0.93	0.55	N.D.	0.19	0.97	0.76	0.56
Delphinidin 3,5-di-*O*-galactoside	0.19	0.62	0.54	0.30	0.26	0.29	0.20	0.63	0.52	0.30	N.D.	0.97	0.51	0.41	0.26	N.D.	0.23	0.61	0.40	0.28
Chlorogenic acid	0.30	0.27	0.20	0.15	0.14	0.39	0.17	0.19	0.18	0.14	0.17	0.40	0.19	0.20	0.14	0.15	0.15	0.18	0.16	0.16
Delphinidin 3,5-di-*O*-glucoside	0.07	0.29	0.49	0.28	0.23	0.12	0.12	0.57	0.71	0.23	N.D.	0.46	0.41	0.49	0.22	N.D.	0.09	0.60	0.58	0.21
Cyanidin 3-*O*-(6′′-malonylglucoside)	0.32	0.93	0.88	0.34	0.30	0.42	0.46	0.97	0.70	0.36	0.11	1.25	1.00	0.55	0.31	0.04	0.38	1.07	0.51	0.33
Rutin	0.13	0.19	0.19	0.15	0.13	0.15	0.15	0.20	0.21	0.15	0.09	0.20	0.19	0.22	0.15	0.09	0.13	0.21	0.17	0.16
Quercetin 3-*O*-galactoside	0.06	0.08	0.07	0.05	0.04	0.06	0.07	0.08	0.08	0.05	0.05	0.08	0.07	0.09	0.05	0.03	0.05	0.08	0.06	0.06
Quercetin 3-β-D-glucoside	0.05	0.08	0.08	0.07	0.06	0.05	0.07	0.09	0.09	0.07	0.04	0.08	0.08	0.09	0.07	0.03	0.06	0.09	0.07	0.07
Kaempferol 3-*O*-β-rutinoside	0.10	0.13	0.13	0.12	0.14	0.08	0.11	0.14	0.15	0.16	0.08	0.13	0.13	0.16	0.13	0.05	0.10	0.15	0.14	0.16
Kaempferol 3-glucoside	0.06	0.07	0.07	0.06	0.06	0.05	0.06	0.08	0.08	0.07	0.05	0.07	0.07	0.08	0.06	0.03	0.05	0.08	0.06	0.06
SUM	5.35	7.96	6.75	5.04	4.04	5.95	4.69	7.06	6.41	4.47	3.50	10.38	6.64	6.26	3.97	2.61	4.15	6.87	5.22	4.39

* N.D.: not detected.

References

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3. Jagota, S.K.; Dani, H.M. A New Colorimetric Technique for the Estimation of Vitamin C Using Folin Phenol Reagent. *Anal. Biochem.* **1982**, *127*, 178–182, doi:10.1016/0003-2697(82)90162-2.