Optimizing Extract Preparation from Laurel (*Laurus nobilis* L.) Leaves Using a Pulsed Electric Field

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2.1. Chemicals and Reagents

Hydrochloric acid, methanol, *L*-ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), and all chemical standards for the HPLC determination of polyphenols were obtained from Sigma-Aldrich (Darmstadt, Germany). Ethanol, gallic acid, and the Folin-Ciocalteu reagent were bought from Panreac Co. (Barcelona, Spain). From Merck (Darmstadt, Germany), iron (III) chloride was purchased. Anhydrous sodium carbonate was purchased from Penta (Prague, Czech Republic). Deionized water was used for all conducted experiments.

2.3. Plant Extraction

The extraction procedure including PEF was based on a previous study [1]. Two custom stain-less steel chambers (Val-Electronic, Athens, Greece), a mode/arbitrary waveform generator (UPG100, ELV Elektronik AG, Leer, Germany), a digital oscilloscope (Rigol DS1052E, Beaverton, OR, USA), and a high-voltage power generator were used to perform the PEF processing of the samples. The optimal liquid-to-solid ratio and solvent concentration were initially investigated. For this purpose, laurel powder was properly weighed (Kern PLS 3100-2F, Kern & Sohn GmbH, Balingen, Germany) to achieve a liquid-to-solid ratio (10–50 mL/g) with 20 mL of extraction solvent (0–100 % *v/v* ethanol), as specified in Table S1. The dry sample initially underwent hydration by immersing in the solvent for 10 min. Following the completion of the PEF extraction process, the samples underwent a 10-min centrifugation at 10,000× *g* using a NEYA 16R Remi Elektrotechnik Ltd. (Palghar, India). Finally, the supernatants were collected and stored at –40 °C.

Table S1. The actual and coded levels of the independent variables were used to optimize the extraction process using the Screening design.

Independent Variables	Code Units	Coded Variable Level				
		1	2	3	4	5
Solvent concentration (C %, *v/v*)	X_1	0	25	50	75	100
Liquid-to-solid ratio (R, mL/g)	X_2	10	20	30	40	50

2.4. Optimization with Response Surface Methodology (RSM) and Experimental Design

The RSM technique was employed to achieve optimal efficiency in extracting bioactive compounds and evaluating antioxidant activity from laurel leaves extracts. Therefore, the main objective of the design was to effectively maximize the levels of these values. This was accomplished by optimizing the liquid-to-solid ratio (R, mL/g), solvent concentration (C %, *v/v*), extraction time (t, min), and PEF conditions, as discussed below. The optimization process was based on an experiment that utilized a Box-Behnken design with a main impact screening arrangement. The experiment consisted of 27 design points, including 3 center points. According to the experimental design, three levels of process variables were created. The overall model significance, as shown by the R^2 and *p* values, and the significance of the model coefficients, as represented by the equations, were assessed using analysis of variance (ANOVA) and summary-of-fit tests, with a minimum level of 95% confidence. In addition, the response variable was predicted as a function of the examined independent factors using a second-order polynomial model, as illustrated in Equation (S1):

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

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

To determine the greatest peak area and assess the effect of a substantial independent variable on the response, the RSM was applied. The development of three-dimensional surface response graphs was initiated to represent the model equation visually.

2.6. Polyphenol Determination

2.6.1. Total Polyphenol Content (TPC)

Total polyphenol content (TPC) was evaluated based on a previous study [2] and expressed as mg gallic acid equivalents (GAE) per g of dry weight (dw). Briefly, 200 μL of the sample was mixed with 200 μL of Folin-Ciocalteu reagent and after 2 min, 1600 μL of 5% w/v aqueous sodium carbonate solution was added. The mixture was incubated at 40 °C for 20 min and the absorbance was recorded at 740 nm in a Shimadzu UV-1700 PharmaSpec Spectrophotometer (Kyoto, Japan). The total polyphenol concentration (C_{TP}) was calculated from a gallic acid calibration curve. TPC was determined as mg gallic acid equivalents (GAE) per g of dry weight (dw), using the following Equation (S2):

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

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

2.6.2. HPLC Quantification of Polyphenolic Compounds

Individual polyphenolic compounds were identified and quantified from the laurel extracts using High-Performance Liquid Chromatography (HPLC), based on our prior research [2]. A Shimadzu CBM-20A liquid chromatograph and a Shimadzu SPD-M20A diode array detector (DAD) (both purchased by Shimadzu Europa GmbH, Duisburg, Germany) was employed for the analysis of laurel leaf extracts. The compounds were separated into a Phenomenex Luna C18(2) column from Phenomenex Inc. in Torrance, California, kept at 40 °C (100 Å, 5 μm, 4.6 mm × 250 mm). The mobile phase included 0.5% aqueous formic acid (A) and 0.5% formic acid in acetonitrile/water (3:2) (B). The gradient program required: initially from 0 to 40% B, then to 50% B in 10 min, to 70% B in another 10 min, and then constant for 10 min. The flow rate of the mobile phase was set at 1 mL/min. The compounds were identified by comparing the absorbance spectrum and retention time to those of pure standards and then quantified through calibration curves (0–50 μg/mL).

2.7. Antioxidant Capacity of the Extracts

2.7.1. Ferric-Reducing Antioxidant Power (FRAP) assay

The ferric-reducing antioxidant power (FRAP) was calculated as μmol of ascorbic acid equivalents (AAE) per gram of dw based on a previous established methodology by Shehata et al. [3]. In a 1.5-mL Eppendorf tube, 50 μL of properly diluted sample was mixed with 50 μL of FeCl₃ solution (4 mM in 0.05 M HCl). The mixture was incubated for 30 min at 37 °C, with 900 μL of TPTZ solution (1 mM in 0.05 M HCl) being immediately added right after, and the absorbance was measured after 5 min at 620 nm. The ferric-reducing power (P_R) was calculated using an ascorbic acid calibration curve (C_{AA}) in 0.05 M HCl with ranging values (50–500 μM). The P_R was calculated as μmol of ascorbic acid equivalents (AAE) per g of dw, using Equation (S3):

$$P_{\text{R}} \text{ (μmol AAE/g dw)} = \frac{C_{\text{AA}} \times V}{w} \tag{S3}$$

where V is represented (in L) as the entire volume of the extraction medium and w (in g) represents the dried weight of the material.

2.7.2. DPPH• Antiradical Activity Assay

The antiradical activity for DPPH• (expressed as μmol AAE per gram of dw) was evaluated based on a previous procedure [3]. The extracted polyphenols from the dried material were evaluated for their antiradical activity (A_{AR}) 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. 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 (S4) was employed:

$$\% \text{ Scavenging} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \tag{S4}$$

An ascorbic acid calibration curve in Equation (S5) was used to evaluate antiradical activity (A_{AR}), which was expressed as μmol AAE per g of dw:

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

2.8. Statistical Analysis

The statistical analysis related to Box-Behnken design for the response surface methodology and distribution analysis, which were applicable through JMP® Pro 16 software (SAS, Cary, NC, USA). The quantitative analysis was performed in triplicate, and the extraction procedures were repeated at least twice for each batch of laurel leaf extract. The results are represented in the form of means and standard deviations. Kinetics analysis, bivariate analysis, Pareto plot analysis, principal component analysis (PCA), multivariate correlation analysis (MCA), and partial least squares (PLS) analysis were conducted through JMP® Pro 16 software.
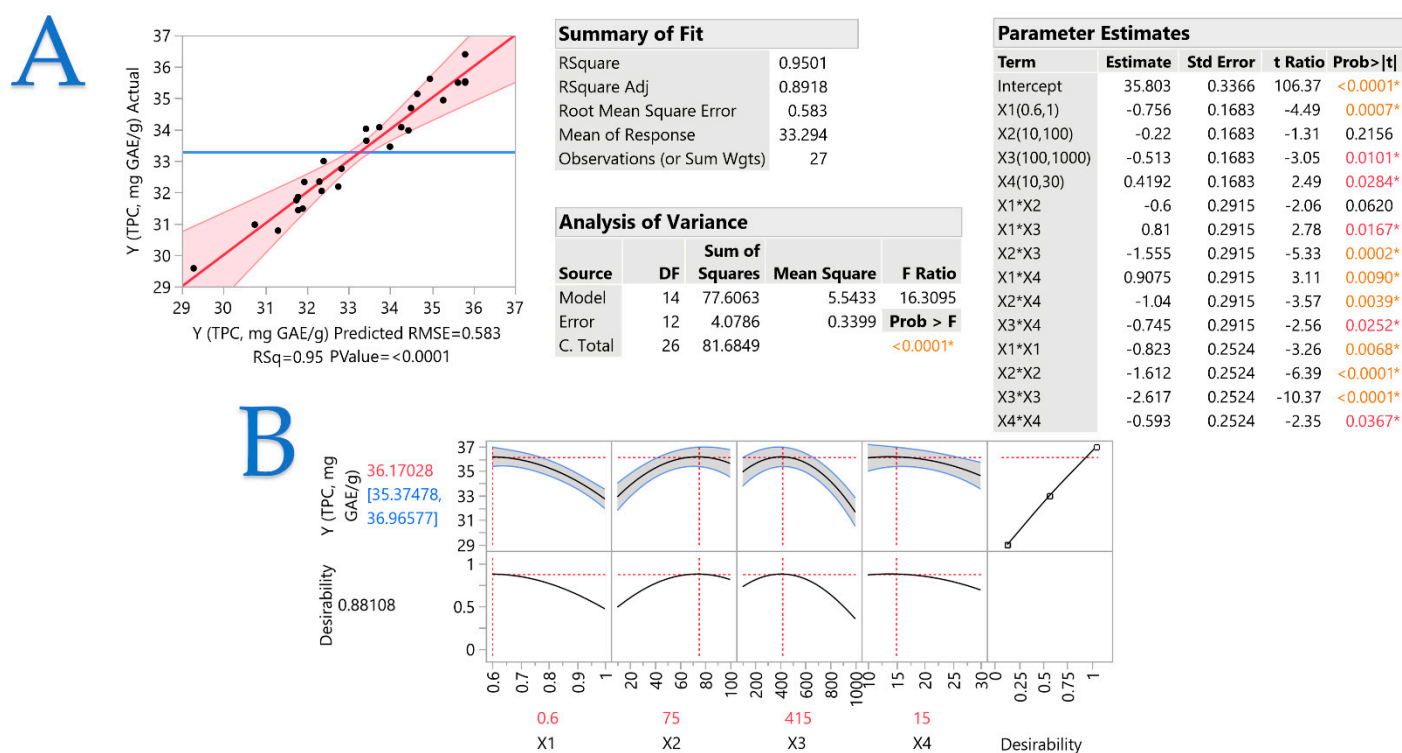
Figure S1. Plots **A** and **B** display the actual response versus the predicted response (Total polyphenol content – TPC, mg GAE/g) for the optimization of *L. nobilis* leaf extracts carried out with hydroethanolic solution, different extraction PEF parameters, and the desirability function. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model.

Figure S2 — Plot A

Summary of Fit

RSquare	0.9591
RSquare Adj	0.9113
Root Mean Square Error	9.3201
Mean of Response	518.18
Observations (or Sum Wgts)	27

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	24,427	1744.82	20.0865
Error	12	1042.4	86.87	Prob > F
C. Total	26	25,470		<0.0001*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	515.1	5.381	95.73	<0.0001*
X1(0.6,1)	-15.33	2.6905	-5.70	<0.0001*
X2(10,100)	15.577	2.6905	5.79	<0.0001*
X3(100,1000)	-2.13	2.6905	-0.79	0.4439
X4(10,30)	10.403	2.6905	3.87	0.0022*
X1*X2	21.373	4.6601	4.59	0.0006*
X1*X3	-22.64	4.6601	-4.86	0.0004*
X2*X3	19.857	4.6601	4.26	0.0011*
X1*X4	-40.65	4.6601	-8.72	<0.0001*
X2*X4	-7.015	4.6601	-1.51	0.1581
X3*X4	-16.14	4.6601	-3.46	0.0047*
X1*X1	3.8929	4.0357	0.96	0.3538
X2*X2	17.224	4.0357	4.27	0.0011*
X3*X3	-0.006	4.0357	-0.00	0.9989
X4*X4	-14.19	4.0357	-3.52	0.0043*

RMSE=9.3201 RSq=0.96 PValue=<0.0001

Y (FRAP, µmol AAE/g): 579.4291 [563.9482, 594.9101]; Desirability 0.838747
X1 = 0.6; X2 = 23; X3 = 550; X4 = 27

Figure S2. Plots **A** and **B** display the actual response versus the predicted response (FRAP, µmol AAE/g) for the optimization of *L. nobilis* leaf extracts carried out with hydroethanolic solution, different extraction PEF parameters, and 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

Summary of Fit

RSquare	0.9561
RSquare Adj	0.9049
Root Mean Square Error	3.0617
Mean of Response	209.22
Observations (or Sum Wgts)	27

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	2449.0	174.932	18.6614
Error	12	112.49	9.374	Prob > F
C. Total	26	2561.5		<0.0001*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	208.95	1.7677	118.21	<0.0001*
X1(0.6,1)	-10.99	0.8838	-12.44	<0.0001*
X2(10,100)	0.0425	0.8838	0.05	0.9624
X3(100,1000)	-1.335	0.8838	-1.51	0.1568
X4(10,30)	2.7683	0.8838	3.13	0.0087*
X1*X2	-7.853	1.5309	-5.13	0.0002*
X1*X3	0.5725	1.5309	0.37	0.7149
X2*X3	2.18	1.5309	1.42	0.1799
X1*X4	-7.877	1.5309	-5.15	0.0002*
X2*X4	-0.335	1.5309	-0.22	0.8305
X3*X4	-2.682	1.5309	-1.75	0.1052
X1*X1	-1.83	1.3258	-1.38	0.1926
X2*X2	5.8046	1.3258	4.38	0.0009*
X3*X3	-1.747	1.3258	-1.32	0.2123
X4*X4	-1.619	1.3258	-1.22	0.2454

RMSE=3.0617 RSq=0.96 PValue=<0.0001

Y (DPPH, µmol AAE/g): 233.6843 [228.5968, 238.7717]; Desirability 0.953639
X1 = 0.6; X2 = 90; X3 = 470; X4 = 26

Figure S3. Plots **A** and **B** display the actual response versus the predicted response (DPPH, µmol AAE/g) for the optimization of *L. nobilis* leaf extracts carried out with hydroethanolic solution, different extraction PEF parameters, and the desirability function. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model.
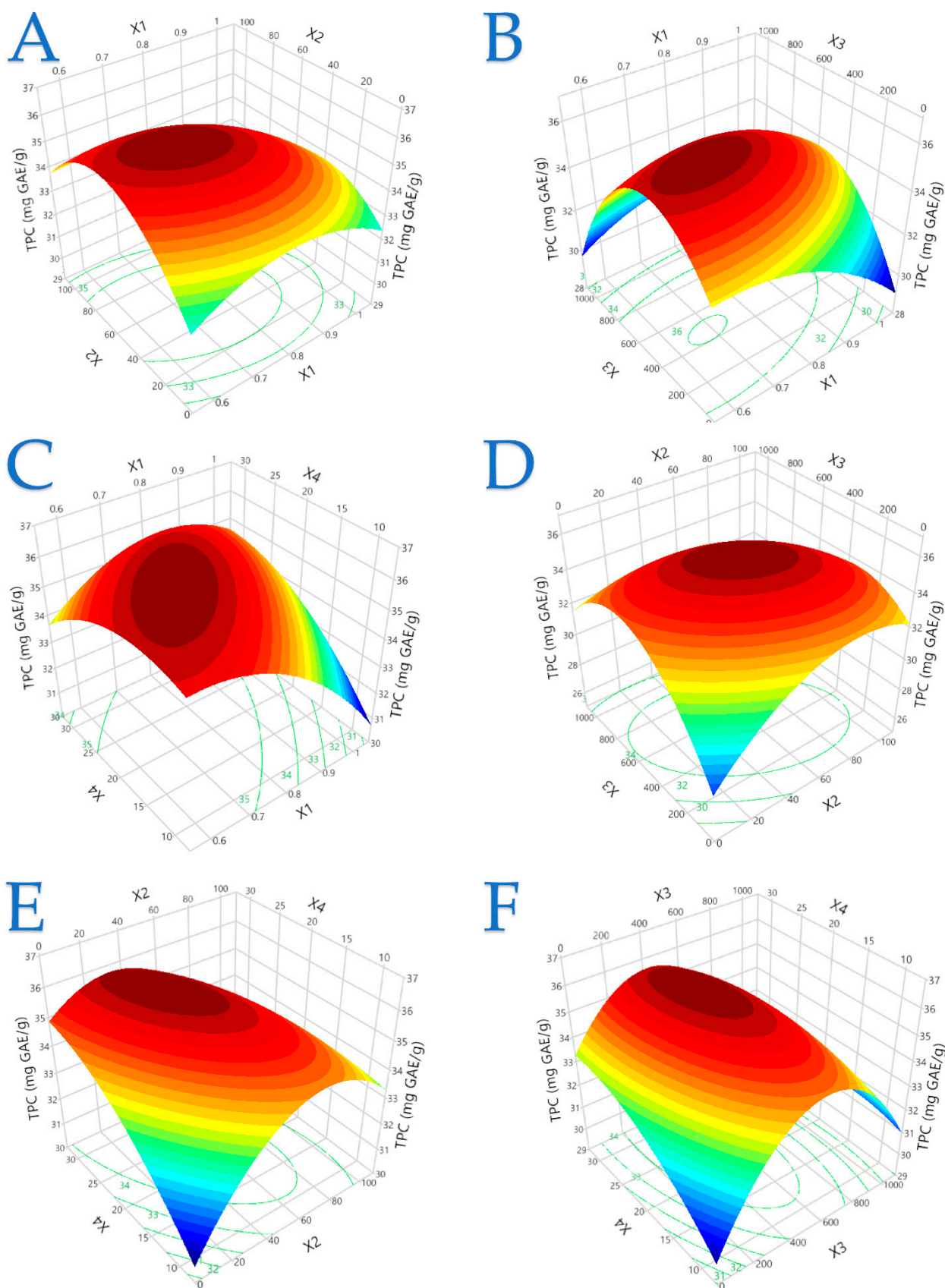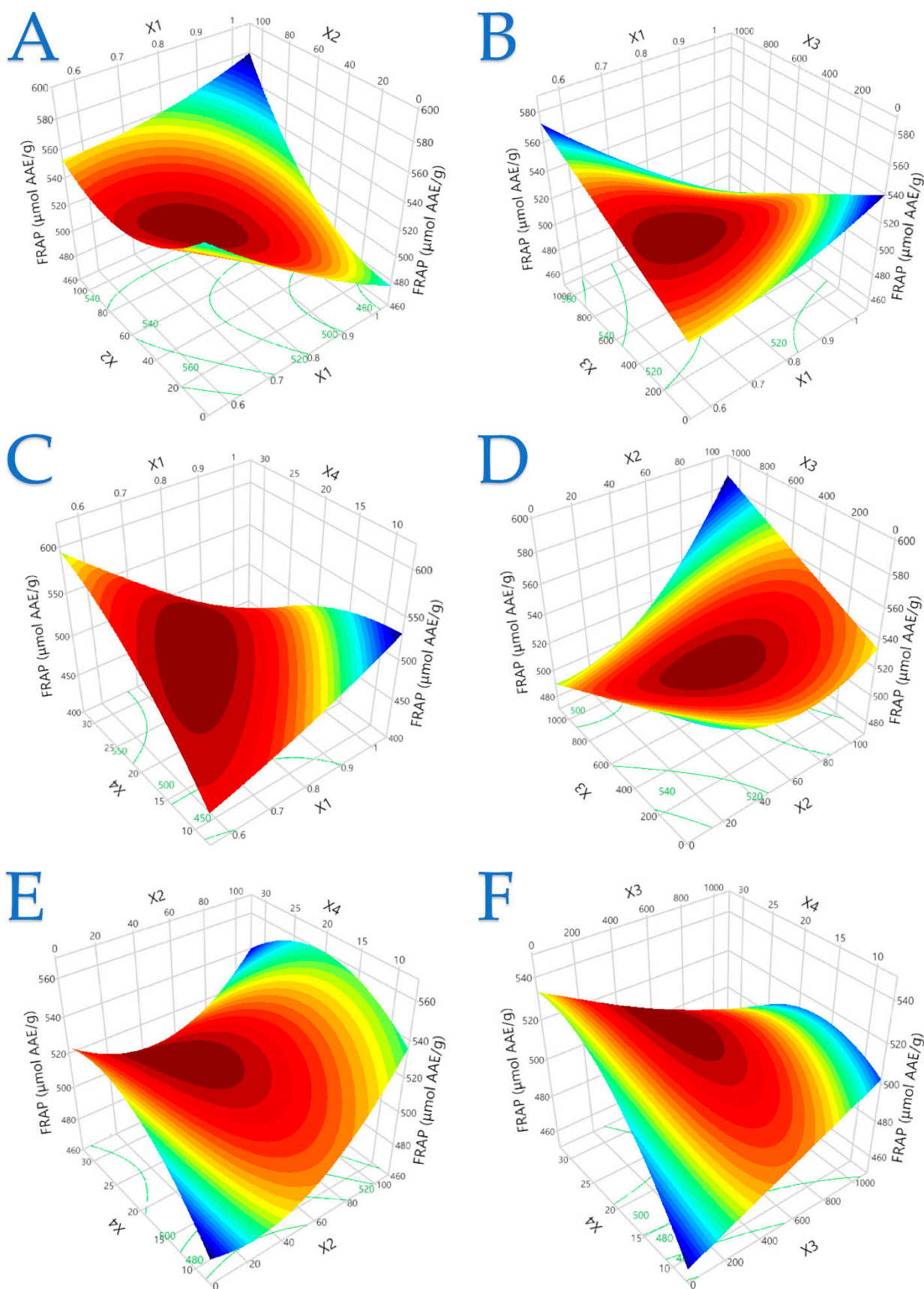
Figure S4. The optimal extraction of *L. nobilis* leaf extracts is shown in 3D graphs that show the impact of the process variables considered in the response (Total polyphenol content – TPC, mg GAE/g). Plot (**A**), covariation of X_1 and X_2; plot (**B**), covariation of X_1 and X_3; plot (**C**), 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 *L. nobilis* leaf 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.
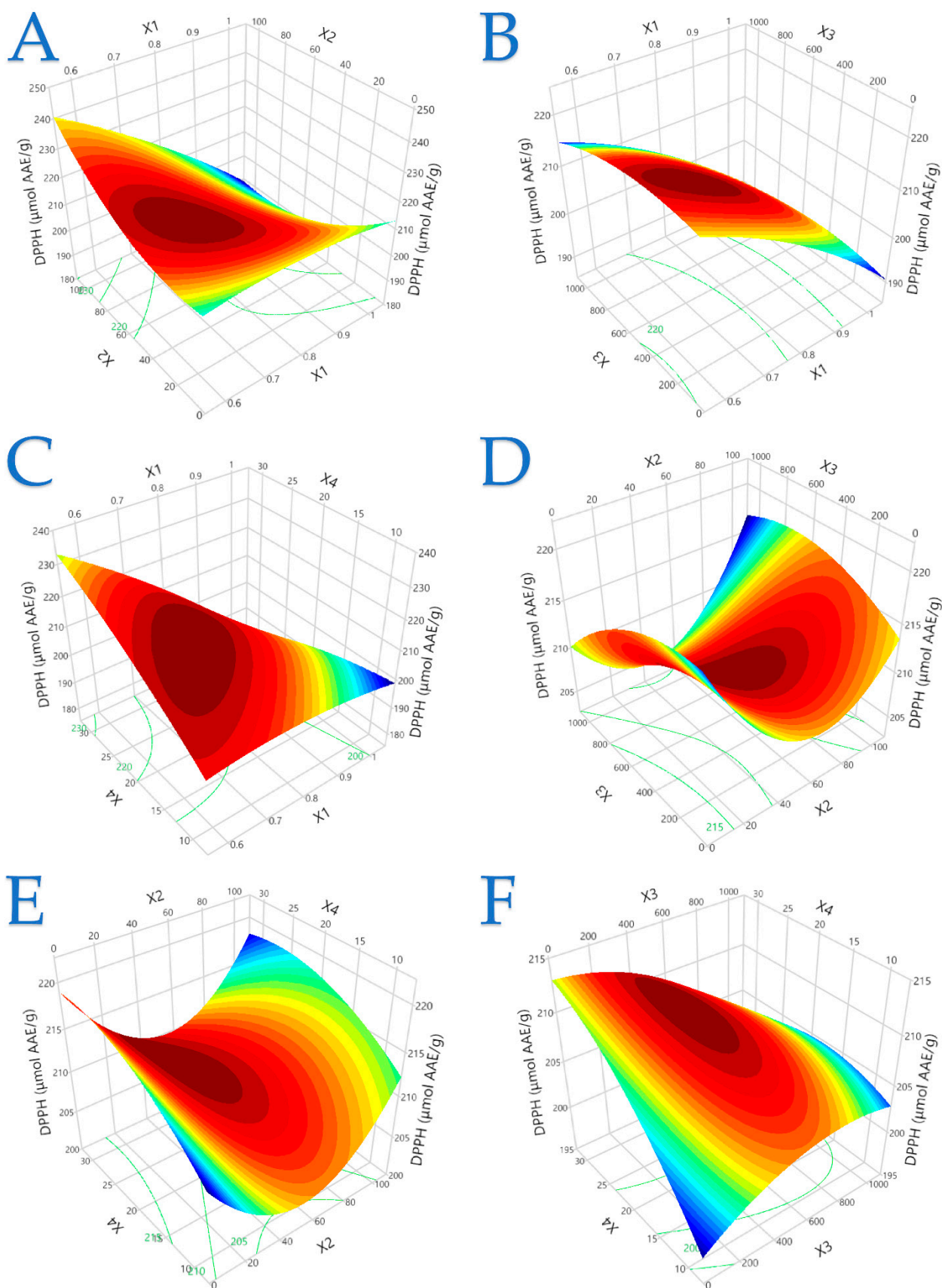
Figure S6. The optimal extraction of *L. nobilis* leaf extracts is shown in 3D graphs that show the impact of the process variables considered in the response (DPPH, μmol AAE/g). Plot (**A**), covariation of X_1 and X_2; plot (**B**), covariation of X_1 and X_3; plot (**C**), covariation of X_1 and X_4; 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.
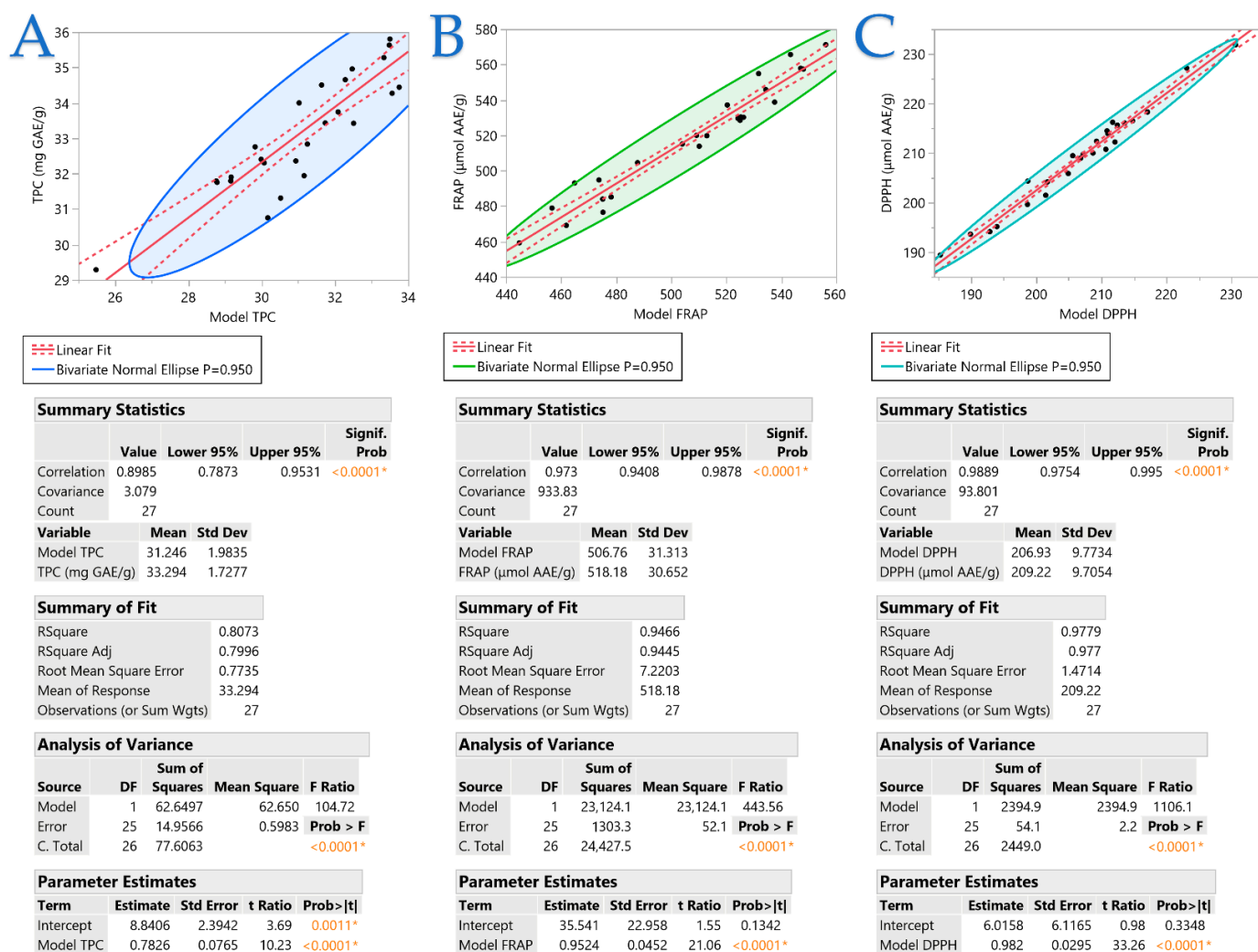
A (TPC plot: TPC (mg GAE/g) vs Model TPC)

Legend:
- Linear Fit
- Bivariate Normal Ellipse P=0.950

Summary Statistics

	Value	Lower 95%	Upper 95%	Signif. Prob
Correlation	0.8985	0.7873	0.9531	<0.0001*
Covariance	3.079			
Count	27			

Variable	Mean	Std Dev
Model TPC	31.246	1.9835
TPC (mg GAE/g)	33.294	1.7277

Summary of Fit

RSquare	0.8073
RSquare Adj	0.7996
Root Mean Square Error	0.7735
Mean of Response	33.294
Observations (or Sum Wgts)	27

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	62.6497	62.650	104.72
Error	25	14.9566	0.5983	Prob > F
C. Total	26	77.6063		<0.0001*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	8.8406	2.3942	3.69	0.0011*
Model TPC	0.7826	0.0765	10.23	<0.0001*

B (FRAP plot: FRAP (µmol AAE/g) vs Model FRAP)

Legend:
- Linear Fit
- Bivariate Normal Ellipse P=0.950

Summary Statistics

	Value	Lower 95%	Upper 95%	Signif. Prob
Correlation	0.973	0.9408	0.9878	<0.0001*
Covariance	933.83			
Count	27			

Variable	Mean	Std Dev
Model FRAP	506.76	31.313
FRAP (µmol AAE/g)	518.18	30.652

Summary of Fit

RSquare	0.9466
RSquare Adj	0.9445
Root Mean Square Error	7.2203
Mean of Response	518.18
Observations (or Sum Wgts)	27

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	23,124.1	23,124.1	443.56
Error	25	1303.3	52.1	Prob > F
C. Total	26	24,427.5		<0.0001*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	35.541	22.958	1.55	0.1342
Model FRAP	0.9524	0.0452	21.06	<0.0001*

C (DPPH plot: DPPH (µmol AAE/g) vs Model DPPH)

Legend:
- Linear Fit
- Bivariate Normal Ellipse P=0.950

Summary Statistics

	Value	Lower 95%	Upper 95%	Signif. Prob
Correlation	0.9889	0.9754	0.995	<0.0001*
Covariance	93.801			
Count	27			

Variable	Mean	Std Dev
Model DPPH	206.93	9.7734
DPPH (µmol AAE/g)	209.22	9.7054

Summary of Fit

RSquare	0.9779
RSquare Adj	0.977
Root Mean Square Error	1.4714
Mean of Response	209.22
Observations (or Sum Wgts)	27

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	2394.9	2394.9	1106.1
Error	25	54.1	2.2	Prob > F
C. Total	26	2449.0		<0.0001*

Parameter Estimates

| Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	6.0158	6.1165	0.98	0.3348
Model DPPH	0.982	0.0295	33.26	<0.0001*

Figure S7. Bivariate analysis of TPC (A), FRAP (B), and DPPH (C) assays by each model estimate; Line of Fit and confidence limits (curves) for the expected values also presented; Asterisks and colored values indicate statistically significant values, while inset tables include statistics on the evaluation of the resulting bivariate platform model.
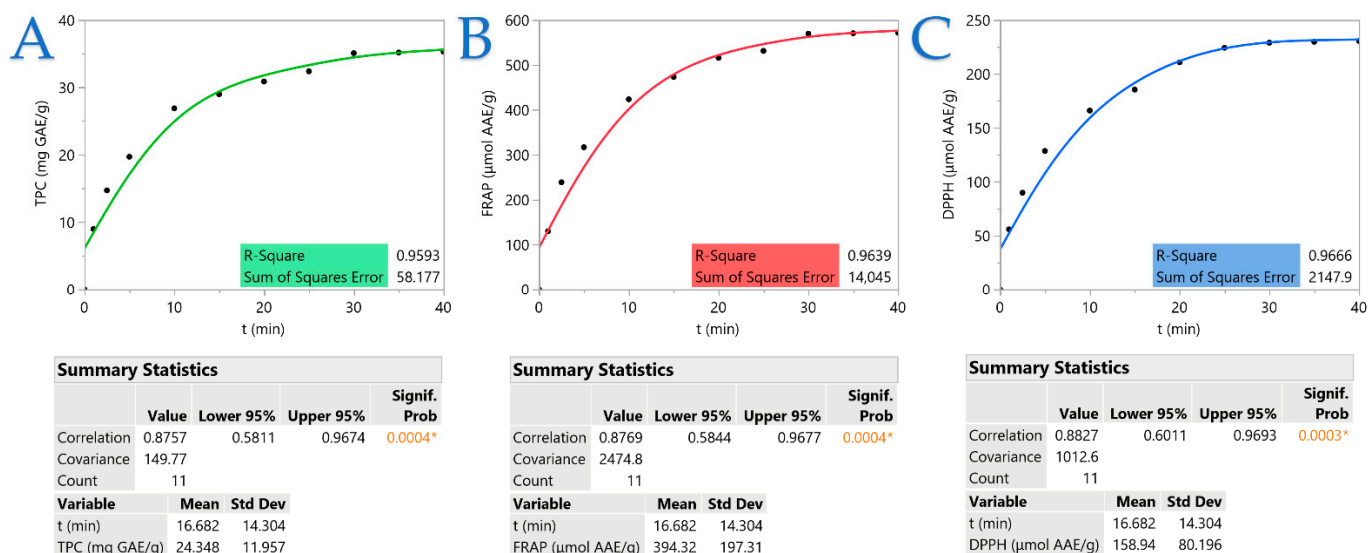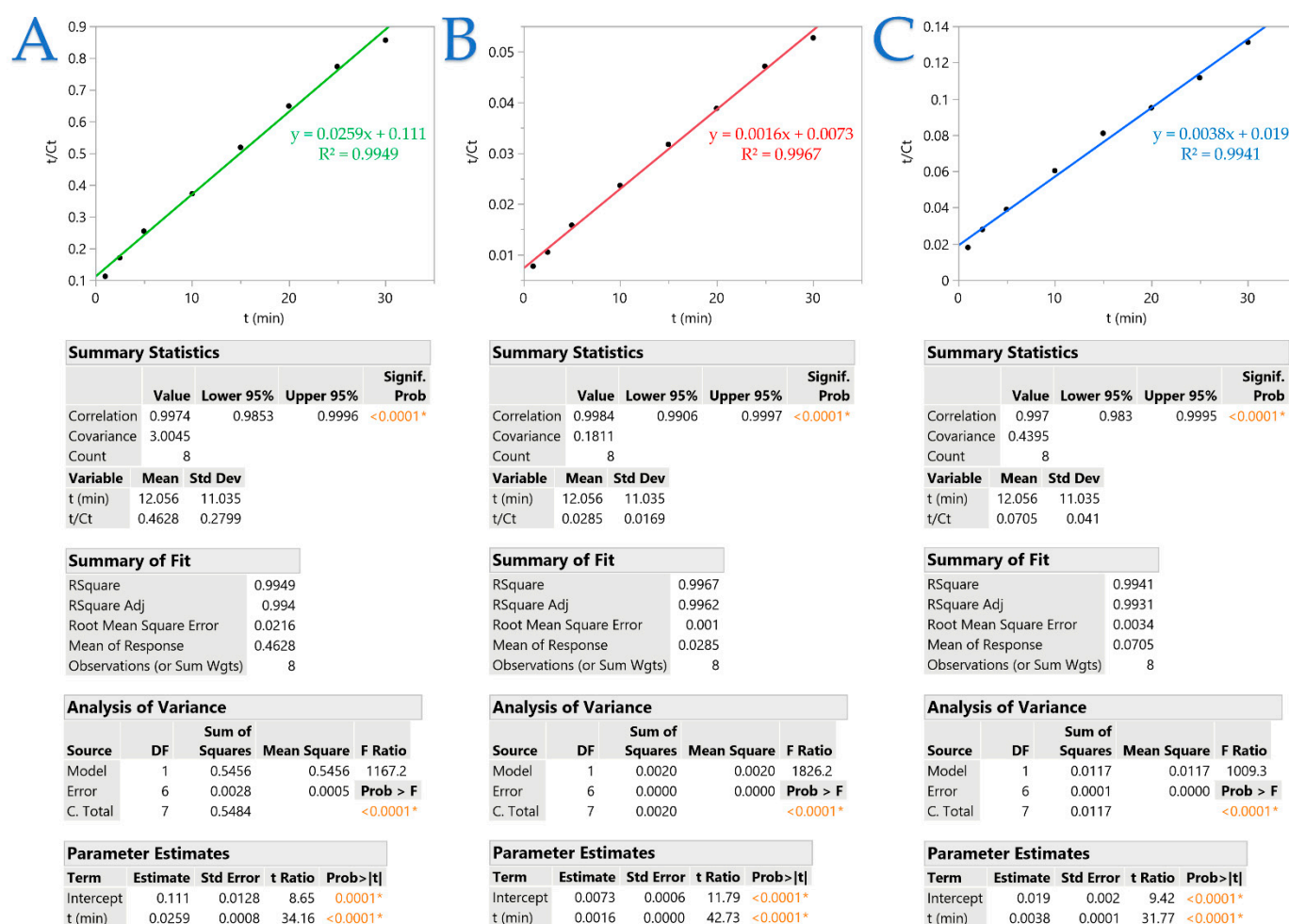
Figure S8. Time course of TPC (**A**), FRAP (**B**), and DPPH (**C**) assays during extraction from laurel leaves using PEF, under the optimal extraction PEF conditions (X_1:0.6, X_2:55, X_3:355); Asterisks and colored values indicate statistically significant values, while inset tables include statistics on the evaluation of the resulting bivariate platform model.
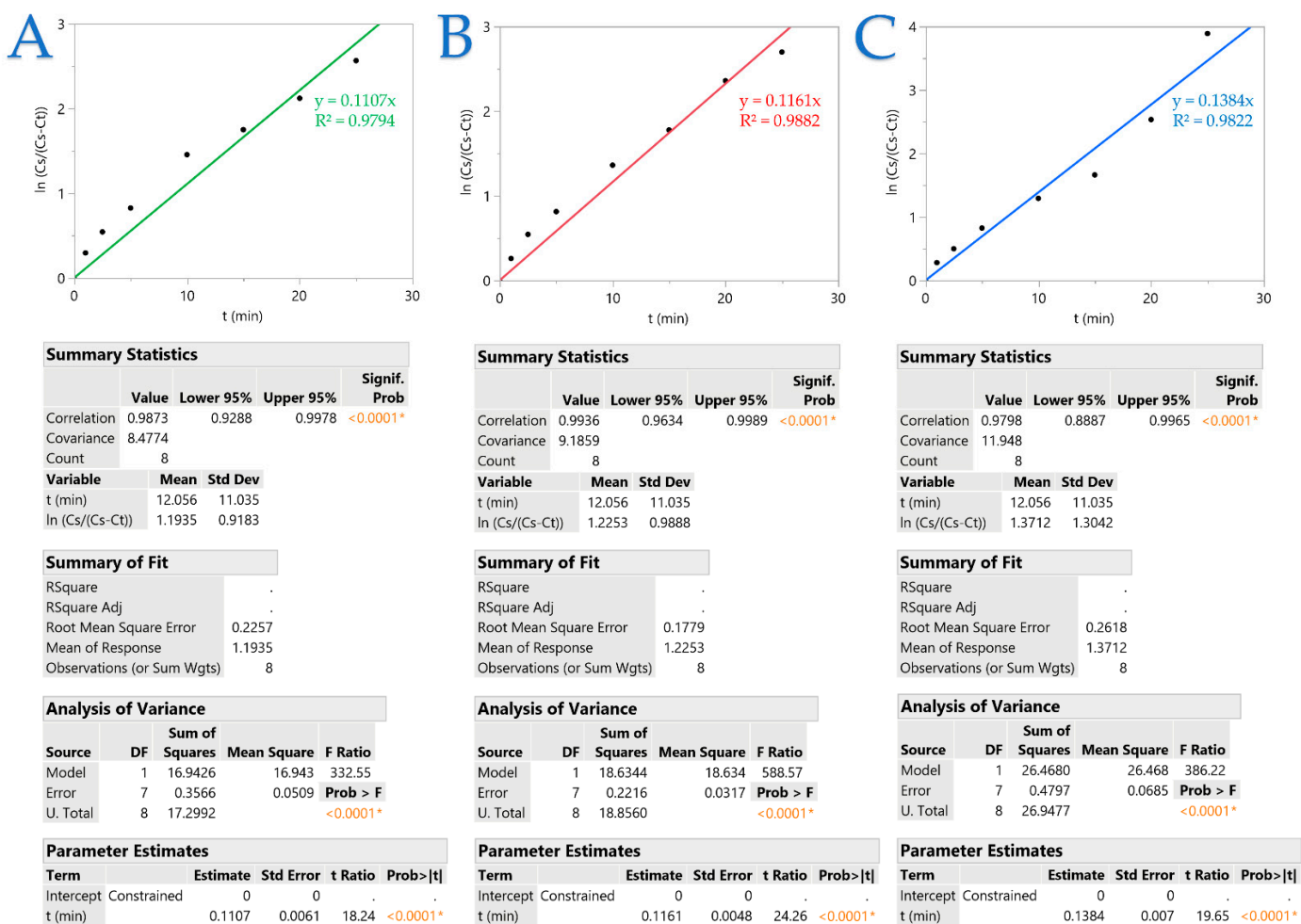
Figure S9. First-order kinetic models of extraction of TPC (**A**), FRAP (**B**), and DPPH (**C**) assays from laurel leaves using PEF, under the optimal extraction PEF conditions (X_1:0.6, X_2:55, X_3:355); Asterisks and colored values indicate statistically significant values, while inset tables include statistics on the evaluation of the resulting bivariate platform model.

Figure S10. Second-order kinetic models of extraction of TPC (**A**), FRAP (**B**), and DPPH (**C**) assays from laurel leaves using PEF, under the optimal extraction PEF conditions (X_1:0.6, X_2:55, X_3:355); Asterisks and colored values indicate statistically significant values, while inset tables include statistics on the evaluation of the resulting bivariate platform model.

References

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