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Mathematical Modeling and Optimization of the Microwave Assisted Extraction of the Natural Polyphenols and Flavonoids from the Raw Solid Waste of the Orange Juice Industry

Konstantinos Petrotos[1], Ioannis Giavasis[2], Konstantinos Gerasopoulos[1], Chrysanthi Mitsagga[2], Chryssoula Papaioannou[1] and Paschalis Gkoutsidis[1]

1. Department of Agrotechnology, School of Agricultural Sciences, University of Thessaly, Geopolis Campus, Periferiaki Odos Larisas Trikalon, Larisa 41500, Greece

2. Department of Food Science and Nutrition, School of Agricultural Sciences, University of Thessaly, Karditsa Campus, End of N. Temponera Street, Karditsa 43100, Greece

Abstract: Orange pomace is the solid waste of the orange juice industry which accounts for approximately 50% of the quantity of the fruits processed into juice and is a good raw material for production of high added value products with diverse uses. Orange pomace is rich in polyphenols and flavonoids which can substitute the potentially hazardous or less desirable chemical antioxidants/antimicrobials used in agro-food and cosmetics industry. In this work, an eco-friendly aqueous microwave assisted extraction of orange pomace was investigated and optimized in order to produce aqueous bioactive antioxidant/antimicrobial extracts. A three factorial Response Surface Optimization methodology with centered Box & Behnken experimental design was used to obtain optimum values of total polyphenols and total flavonoids and build predictive models for their optimal extraction conditions. The three optimization factors in terms of applied process parameters were (a) water/solid ratio, (b) extraction temperature and (c) extraction time. The effectiveness and statistical soundness of the two corresponding models regarding optimal total polyphenols and flavonoids were verified by Analysis of Variance (ANOVA).

Key words: Orange pomace, microwave assisted extraction, response surface optimization, Box & Behnken, polyphenols, flavonoids.

1. Introduction

Orange fruit belongs to the species *Citrus sinensis* and in the family *Rutacae* and it originates from China. It is designated as sweet orange in order to be discriminated from the bitter orange which is known as *Citrus aurantium* [1] and it is cultivated in tropical and subtropical regions. In 2017, the global production of fresh orange fruits was 73 million MT, from which Brazil has the largest share of 24%, followed by China and India. According to Lohrasbi et al. [2], 33% of the global orange fruits production is industrially processed and 15 million MT of solid orange fruit waste, known as raw orange pomace is produced, which roughly represents the 50% of the total orange fruits which are processed [3]. The orange pomace consists of peels, seeds, pulp, and segment residue [4].

In an investigation carried out by Sharma et al. [5] it was found out that the orange peel could be a valuable raw material for production of a variety of high added value products as it contains substantial quantities of flavonoids, carotenoids, edible fibers, polyphenols, essential oils and ascorbic acid as well as sugars that could be a good substrate of various

Corresponding author: Konstantinos Petrotos, PhD, professor, research field: food process engineering.

fermentations. Putnik et al. [6] reviewed these alternative valorization options for orange pomace which include production of antioxidants, edible fibres, pectin, essential oil, enzymes, bioethanol, ellagic acid, biogas and use of the pomace as soil improvement material or animal feed. These options can be used separately or in combination in order to achieve the reuse and exploitation of the solid waste of the orange juice industry in a circular economy. Regarding the average composition of orange pomace, Gotmare and Gade [7] reported that sun-dried orange pomace consists of (*w*/*w*) 9.2% ± 0.01% moisture, 14.17% ± 0.36% crude fiber, 12.43 ± 0.20 protein, 7.8 ± 0.01 ash, and 52.90 ± 0.43 carbohydrates. The phenolic content of orange pomace includes polyphenols belonging mainly in the class of flavonoids and in particular flavones, flavonols, flavonons, isoflavons, anthocynidines and flavanols. The main flavonoids of orange fruit are hesperidin, narirutin, eriocitrin, naringin, neohesperidin, neoeriocitrin, and among them hesperidin and narirutin are the most dominant [6, 8]. Also, a series of short chain bioactive phenolic acids including caffeic, chlorogenic, ferulic, cinapic and p-cumaricare are reported as constituents of the phenolic profile of orange pomace [9].

The extraction of high added value phenolics from orange pomace is one of the first steps of any scheme of bio-refinery targeting a total discharge of this solid waste. Thus, a variety of novel and innovative methods have been used for the effective extraction of orange pomace phenols. Luengo et al. [10] successfully used pulsed electric field technology (PEF) in order to increase the yield of the extracted orange pomace phenolics. In this study, a 159% increase of the total polyphenols extraction yield and 2-3 fold increase of the main orange pomace flavonoids (hesperidin and naringin) were observed at 1-7 kV/cm and tPEF = 60 μs, 20 pulses, f = 1 Hz and an improvement of yield was observed in parallel to the increase of the applied voltage. Ma et al. [11] and Khan et al. [12] commented on the advantages of using ultrasound assisted extraction at 25 kHz/150 W/30 °C/15 min which led to high extraction yield in short time, using low extraction temperature and thus having a low carbon foot print [13]. Furthermore, Londono-Londono et al. [14] studied the aqueous ultrasound assisted extraction of the orange pomace and reported an extraction yield for total polyphenols equal to 19.595 ± 2.114 mg GAE·kg^{-1} of dry matter at 60 kHz, 30 min, 40 °C and solid to liquid ratio 1/10. In addition, Dahmoune et al. [15] & Mhiri et al. [16] optimized microwave assisted extraction of orange pomace at short extraction time 122-180 s, microwave power 200-500 W, and extraction temperature 120-135 °C with solid to liquid ratio 1/25 to 1/30, which led to polyphenols extraction yield of 12.20-15.74 mg GAE·kg^{-1} of dry orange pomace. Min et al. [17] & Kim et al. [18] presented the advantages of using pressurized fluid extraction for extracting phenolics from orange extracts including the ability of this method to extract the highest quantity of methoxylated flavones. The proposed extraction conditions were T = 200 °C, P = 1.4 MPa, t = 60 min. Casquete et al. [19, 20] studied the extraction of orange pomace phenolics with high pressure and pointed out that the optimum operational conditions were 300 MPa, 10 min or alternatively 500 MPa, 3 min. They also found out that the application of high pressure led to the increased antimicrobial effectiveness of the obtained extracts against Gram positive and Gram negative bacteria like *Acinetobacter* and *Listeria*. Ariel et al. [21] & Ergüt et al. [22] investigated the supercritical carbon dioxide extraction of orange pomace at various combinations of pressure and temperatures. The results of these studies suggest that lower (25 to 35 MPa) than higher (200 MPa) operating pressures are more favorable for the total antioxidant capacity of the obtained extracts (apparently due to a pro-oxidative effect of high pressure), although the yield of total polyphenols appeared to be higher at higher pressures. The concentration of total polyphenols extracted by this

technology reported to range from 18 to 27.8 mg GAE·kg^{-1} of dry orange pomace. The temperature used was 40-60 °C and the use of ethanol co-solvent was found to be beneficial in achieving higher yields.

The target of the present work is to investigate the microwave assisted extraction of raw orange pomace and obtain the optimum conditions in terms of extraction temperature, extraction time and water to raw orange pomace ratio in order to get maximum yields of polyphenols and/or flavonoids. This information can then be used in order to produce bioactive aqueous orange pomace extracts of high added value in an economically feasible way, which would also lead to the improvement of the carbon footprint of the orange juice production industry. The optimized aqueous orange pomace extracts can be used either as natural antioxidants or as natural antimicrobials (extracts with high polyphenols and/or flavonoids content) in food/nutraceutical, cosmetic and agro-protection applications.

2. Materials and Methods

2.1 Orange Pomace

The orange pomace was kindly supplied by the Greek orange juice producer, ALBERTA S.A. which is established in Argos Peloponnese-Greece. The orange variety from which the obtained pomace was coming was the well-known orange fruit variety "NAVEL". The obtained pomace was passed through a commercial meat mincer (model CANDY COMET supplied by D. Tomporis Co, 92 Cyprus str, Larisa, Greece) with a 3 mm hole diameter sieve in order to become comminuted and then it was kept in properly sealed vacuum plastic bags (2 kg per each bag) at -25 °C until used for extraction. Air of heat drying was not applied to the orange pomace, in order to avoid oxidative degradation of the bioactive compounds.

2.2 Description of the Microwave Extractor and of the Extraction Methodology

The extraction of the orange pomace samples was contacted by using the Lab Scale microwave extractor NEOS-GR/MILESTONE TECHNOLOGIES which is established in the premises of PELLAS NATURE Co. (Edessa, Greece) and it is illustrated in Fig. 1.

The extraction trials of the orange pomace samples were conducted following the procedure described below. The frozen orange pomace samples (2 kg bags)

Fig. 1 The setup of the lab scale microwave extractor NEOS-GR/Milestone Technologies.

were first thawed at ambient temperature and 100 g of each sample was then collected and used as the extraction sample. The 100 g orange pomace sample was mixed with distilled water in the PYREX glass beaker (of either 2 L or 5 L capacity, depending on the quantity of the total mixture). The quantity of the water used in each trial was according to the water/solid ratio suggested by the experimental plan (shown below). The loaded beaker was then adjusted on the TEFLON base in the extractor and the desired values of the extraction temperature and time were set via the electronic panel of the extractor, according to the experimental plan, and the lab scale extractor was set in automatic operation. The progress of the extraction was monitored by using a camera and agitation was applied by using the relevant facility of the extractor. After the end of each extraction trial the extract was collected, filtered in plain filter paper and the filtrate was filled in plastic bottles and coded accordingly in order to distinguish different samples. The collected samples were kept frozen at -25 °C in the Laboratory of Food and Biosystems Engineering (University of Thessaly) for a short period until the selected bioactivity parameters were analyzed.

2.3 Total Polyphenols Determination Method

For the determination of the total polyphenols as GAE (gallic acid equivalents) of the obtained orange extracts, a slightly modified version of the method of Singleton et al. [23] and Waterhouse [24] was used. According to this method, initially a gallic acid solution was prepared by dissolving 0.5 g gallic acid in 10 mL pure ethanol and the solution was then transferred in a 100 mL volumetric flask and the rest of the volume was filled by distilled water (preparation of a gallic acid stock solution of 5,000 ppm). In addition, in a 1 L glass beaker, 200 g of anhydrous sodium carbonates were dissolved in 800 mL distilled water and the solution was boiled until the salt was fully dissolved. The solution was then cooled and kept at 24 h in dark, which resulted in the formation of crystals of anhydrous sodium carbonate, which were removed by filtration the next day. The clear filtrate was finally dissolved in a total volume of 1 L by adding the remaining distilled water in a 1 L volumetric flask. Consequently, a set of standards of gallic acid was prepared by diluting 0 mL, 1 mL, 2 mL, 3 mL, 5 mL, 10 mL, and 20 mL of the gallic acid stock solution in six volumetric flasks of 100 mL each and filled with distilled water up to 100 mL volume in order to prepare standard solutions of 0, 50, 100, 150, 250, 500 and 1,000 ppm gallic acid. From each standard solution a quantity of 20 μL was mixed with 1.58 μL distilled water and 100 μL Folin Ciocalteu reagent in a glass tube and within 8 min a quantity of 100 μL sodium carbonate solution was added and the tubes were incubated for 2 h at 20 °C, after which their absorbance was measured with a UV-Vis photometer (model EVOLUTIONTM 201, Thermo-Scientific Co., Shanghai, China) against the blind solution (0 ppm gallic acid concentration). The standard curve depicting gallic acid concentration vs. absorbance was constructed using the Microsoft Excel software and its R^2 value was 0.9982. Calculation of the total polyphenols of extracts of orange pomace was carried out following the same procedure and using the following equation of the standard curve:

$$\text{Total polyphenol concentration of extract in ppm of GAE} = \text{absorbance at 765 nm}/0.001 \quad (1)$$

Before each respective measurement the relevant orange pomace extract was diluted with water to 1:30 dilution ratio, in order to obtain a readily measurable concentration of polyphenols in the extract. Each measurement of total polyphenols was carried out in triplicate and the result was the average of the three obtained values.

2.4 Total Flavonoids Determination Method

The total flavonoids content expressed as mg of $QE \cdot L^{-1}$ of the obtained orange pomace extracts was determined by using the colorimetric method of $AlCl_3$, as described by Chandra et al. [25]. The method is

based on the principle that $AlCl_3$ reacts with the hydroxyls of the flavonoids and produces a colored complex which has maximum absorbance at 420 nm. The total flavonoids content was expressed as quercetin equivalents (QE) per L of extract. The determination method for the total flavonoids was carried out as below: 1.0 mL of the orange pomace extract or standard solution (used for the construction of the calibration curve) was added in a glass test tube to which 3 mL methanol, 200 μL of aqueous solution of 10% *w*/*v* $AlCl_3$, 200 μL 1 M potassium acetate solution and 5.6 mL distilled water were added. The tube was then agitated by vortex and incubated for 30 min at ambient temperature for the completion of the chemical reaction. The absorbance of each sample was measured at 420 nm against a blind solution which contained all the reagents except for the pomegranate pomace extract, which was replaced by distilled water.

For the construction of the calibration curve, a quercetin stock solution of 1,000 ppm was prepared as well as a series of standard solutions of 50, 100, 200, 500 and 1,000 ppm by serial dilutions of the stock. The absorbance of standard solutions was measured and plotted against their concentration and the linear equation obtained by Excel was used for the determination of the concentration of the total flavonoids of the orange pomace extracts. The R^2 value of the obtained linear correlation was 0.9834.

Calculation of the total flavonoids of extracts of orange pomace was carried out following the same procedure and using the following equation of the standard curve:

$$\text{Total flavonoids concentration mg QE}\cdot\text{L}^{-1}\text{ of extract} = \text{absorbance at 420 nm } 0.0055 \quad (2)$$

Each measurement of total flavonoids was carried out in triplicate and the result was the average of the three obtained values.

2.5 Chemicals Used for Antioxidant Tests

All the chemicals used for the above mentioned antioxidant tests were selected from the standard catalog of Sigma Aldrich company and they were supplied by the Greek representative Life Sciences Chemilab, 33 Amarantou str, Thessaloniki 56431, Greece.

2.6 Modeling and Optimization Methodology

The methodology used for modeling and optimization had the following aims:

- Modeling and optimization of total polyphenol content in the orange pomace extract;
- Modeling and optimization of total flavonoids content in the orange pomace extract;
- Simultaneous optimization of total polyphenols and total flavonoids content in the orange pomace extract.

A central composite Box & Behnken experimental design was used to pre-select the experimental points along with response surface methodology (RSM) to obtain the 3rd order mathematical models for total polyphenols and total flavonoids of the orange extracts and predict the optimum values. Three factors were used as optimization factors and in particular: (a) the ratio of extraction water to orange pomace in a range from 5 to 30, (b) the extraction temperature in a range of 40 °C to 80 °C and (c) the extraction time in a range of 30 min to 90 min and three optimization responses: (a) total polyphenol content, (b) total flavonoids content, (c) total polyphenols and total flavonoids simultaneously. The Design Expert 7.0.0 statistical software was used to perform modeling and optimization and construct the predictive mathematical models. Third order (cubic) polynomial mathematical models were adopted along with the appropriate regression technique and response transformations which were proved effective in order to obtain reliable models. The reliability of the obtained cubic models was validated by Statistical analysis (ANOVA) and in all cases the statistical significance of the derived cubic models, as well as the desirable no-significance of lack of fit, have been proved.

3. Results

3.1 Modeling and Optimization of the Total

Polyphenol Content of the Orange Pomace Extracts

The results of the total polyphenol content of the orange extracts are presented in Table 1. In particular, three (triplicate) microwave extraction experiments were carried out for each one of the seventeen (17) sets of Box & Behnken Design experimental conditions (51 experiments in total) and the total polyphenol content of each one of the obtained three extracts was determined (the average values of them are listed in Table 1). Using the Design Expert software Version 7.0.0 the data presented in Table 1 were analyzed by Surface Response Methodology (RSM) and the mathematical modeling yielded the following total orange polyphenols model equation:

$$(\text{Orange pomace extract total polyphenols } \left(\frac{\text{mg}}{\text{L}}\right))^2 = + 65514.29417 + 570.53600 \times \text{Ratio} - 2204.52654 \times \text{Temperature} \neg 1282.98279 \times \text{Time} \neg 23.19865 \times \text{Ratio} \times \text{Temperature} - 4.43019 \times \text{Ratio} \times \text{Time} + 42.20999 \times \text{Temperature} \times \text{Time} + 22.73185 \times \text{Ratio}^2 + 18.52827 \times \text{Temperature}^2 + 1.36228 \times \text{Time}^2 \neg 0.39466 \times \text{Ratio}^2 \times \text{Temperature} + 0.31129 \times \text{Ratio} \times \text{Temperature}^2 \neg 0.35712 \times \text{Temperature}^2 \times \text{Time} \quad (3)$$

In Table 2, the statistical analysis by ANOVA is presented. The ANOVA conclusion is that the model is significant while its lack of fit is not significant, which implies that the developed model is a good tool for prediction of total orange polyphenols in the extracts with regards to the three experimental factors that were investigated.

Furthermore, according to ANOVA analysis, the R^2 value = 0.9821 of the above equation is very high and very close to the adjusted R^2 = 0.9284, which means there is very good prediction by the developed model equation. The goodness of model prediction is also supported by the data presented in Fig. 2, which depicts the predicted vs. actual total polyphenols value,

Table 1 Total polyphenols, total flavonoids concentration of raw orange pomace extracts.

A/A	Water to solid ratio	Temperature (°C)	Extraction time (min)	Concentration of total polyphenols in the extract expressed as gallic acid equivalents ($mg \cdot L^{-1}$)	Concentration of total flavonoids in the extract expressed as quercetin equivalents ($mg \cdot L^{-1}$)
1	5.00	80	60.00	55.8103 ± 8.2884	10.100 ± 0.232
2	17.50	60	60.00	59.9923 ± 11.7475	4.100 ± 0.143
3	5.00	60	90.00	84.9413 ± 8.7639	8.700 ± 0.254
4	5.00	60	30.00	29.9603 ± 7.8163	7.000 ± 0.126
5	30.00	60	90.00	62.4443 ± 14.3117	9.6000 ± 0.246
6	17.50	40	90.00	26.1799 ± 5.8525	8.9000 ± 0.139
7	17.50	60	60.00	55.6207 ± 4.1698	6.3636 ± 0.098
8	17.50	80	90.00	64.7517 ± 5.2868	9.800 ± 0.293
9	17.50	80	30.00	102.6790 ± 2.4602	9.000 ± 0.131
10	30.00	80	60.00	80.0380 ± 21.0222	9.000 ± 0.165
11	30.00	60	30.00	65.0167 ± 25.7963	8.000 ± 0.143
12	5.00	40	60.00	38.3607 ± 5.2154	5.000 ± 0,076
13	30.00	40	60.00	66.4820 ± 10.7232	6.900 ± 0.111
14	17.50	40	30.00	74.0787 ± 17.0264	2.000 ± 0.014
15	17.50	60	60.00	55.3780 ± 4.2610	4.900 ± 0.037
16	17.50	60	60.00	43.2640 ± 9.5671	5.600 ± 0.102
17	17.50	60	60.00	57.2523 ± 13.5735	3.800 ± 0.058

Table 2 ANOVA statistical analysis of the predictive model for the total polyphenols concentration of orange pomace extracts.

Response	Total polyphenols of orange pomace				
Transform	Power	Lambda: 2	Constant: 0		
Stepwise regression with alpha to enter = 0.100, alpha to exit = 0.100					
ANOVA for response surface reduced cubic model analysis of variance table (partial sum of squares—type III)					
Source	Sum of squares	df	Mean square	*F* value	*p*-value Prob > *F*
Model	9.438E + 007	12	7.865E + 006	18.28	0.0063
A	47.73	1	47.73	1.110E - 004	0.9921
B-	1.833E + 007	1	1.833E + 007	42.61	0.0028
AB	29,408.62	1	29,408.62	0.068	0.8066
AC	1.104E + 007	1	1.104E + 007	25.67	0.0071
BC	5.990E + 005	1	5.990E + 005	1.39	0.3033
A^2	92,332.20	1	92,332.20	0.21	0.6672
B^2	4.375E + 006	1	4.375E + 006	10.17	0.0332
C^2	6.329E + 006	1	6.329E + 006	14.71	0.0185
A^2B	3.042E + 006	1	3.042E + 006	7.07	0.0564
AB^2	4.845E + 006	1	4.845E + 006	11.26	0.0284
B^2C	3.673E + 007	1	3.673E + 007	85.39	0.0008
Pure error	1.721E + 006	4	4.301E + 005		
Cor total	9.610E + 007	16			
Std. dev.	655.85		R-squared	0.9821	
Mean	3,968.78		Adj R-squared	0.9284	
C.V.%	16.53		Pred R-squared	N/A	
Press	N/A		Adeq precision	17.188	
Model: is significant.			Lack of fit: is not significant.		

where all the predicted points are very close to the actual central linear plot. In addition, in the Figs. 3-5 the plots illustrate the paired interactions of the factors A = water/solid ratio, B = extraction temperature, C = extraction time on the total polyphenols extraction yield. Furthermore, by using the developed RSM cubic model and the facility of Expert Design 7.0.0 statistical package, the maximum value of 111.58 mg/L of orange total polyphenols was achieved under the following conditions: water to orange pomace ratio = 29.88, extraction temperature: 80 °C, extraction time: 30.05 min.

3.2 Modeling and Optimization of the Total Flavonoids Content of the Orange Pomace Extracts

The results of the total flavonoids content of the orange extracts are presented in Table 1. In particular, three (triplicate) respective microwave extraction experiments were carried out for each one of the seventeen (17) sets of Box & Behnken Design experimental conditions (51 experiments in total) and the total flavonoids contents of each one of the three extracts were determined (the average of them is listed in Table 1). Using the total orange flavonoids concentrations presented in Table 1 and applying mathematical modeling by RSM methodology by Design Expert Statistical software, a reliable mathematical model was constructed to simulate the response (total orange flavonoids concentration) vs. the three experimental factors: (a) water to solid ratio, (b) extraction temperature (°C) and (c) extraction time (min). The model equation (Eq. (3)) has the following form:

$$\left(\text{Orange pomace total flavonoids } \left(\frac{\text{mg}}{\text{L}}\right)\right)^2 = +55.36534 - 1.93887 \times \text{Ratio} - 1.45575 \times \text{Temperature} - 0.95429 \times \text{Time} - 0.043620 \times \text{Ratio} \times \text{Temperature} - 0.025071 \times \text{Temperature} \times \text{Time} + 0.13962 \times \text{Ratio}^2 + 0.041665 \times \text{Temperature}^2 + 0.025526 \times \text{Time}^2 \quad (4)$$

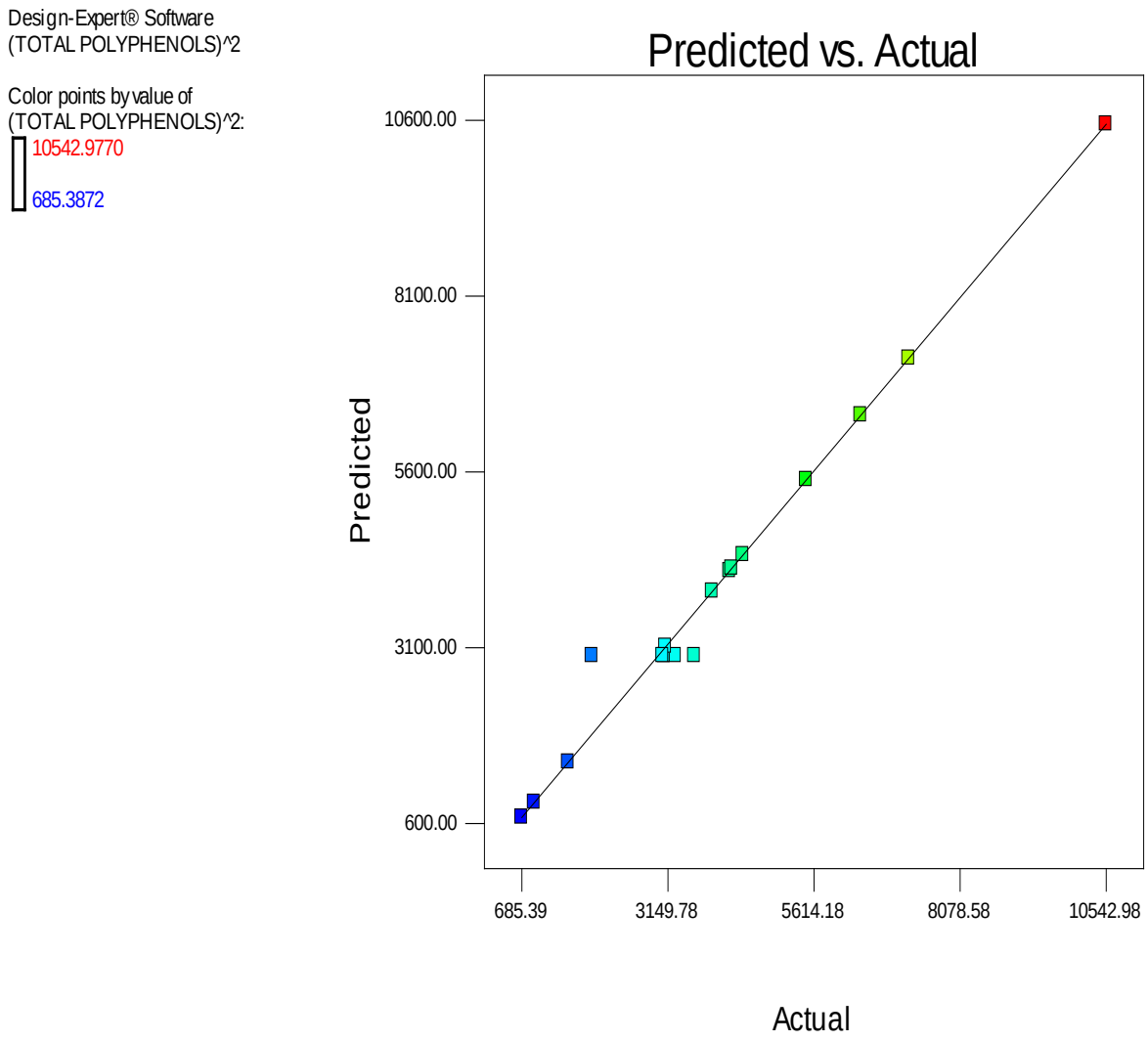

Fig. 2 Correlation of predicted vs. actual values of total orange polyphenols.

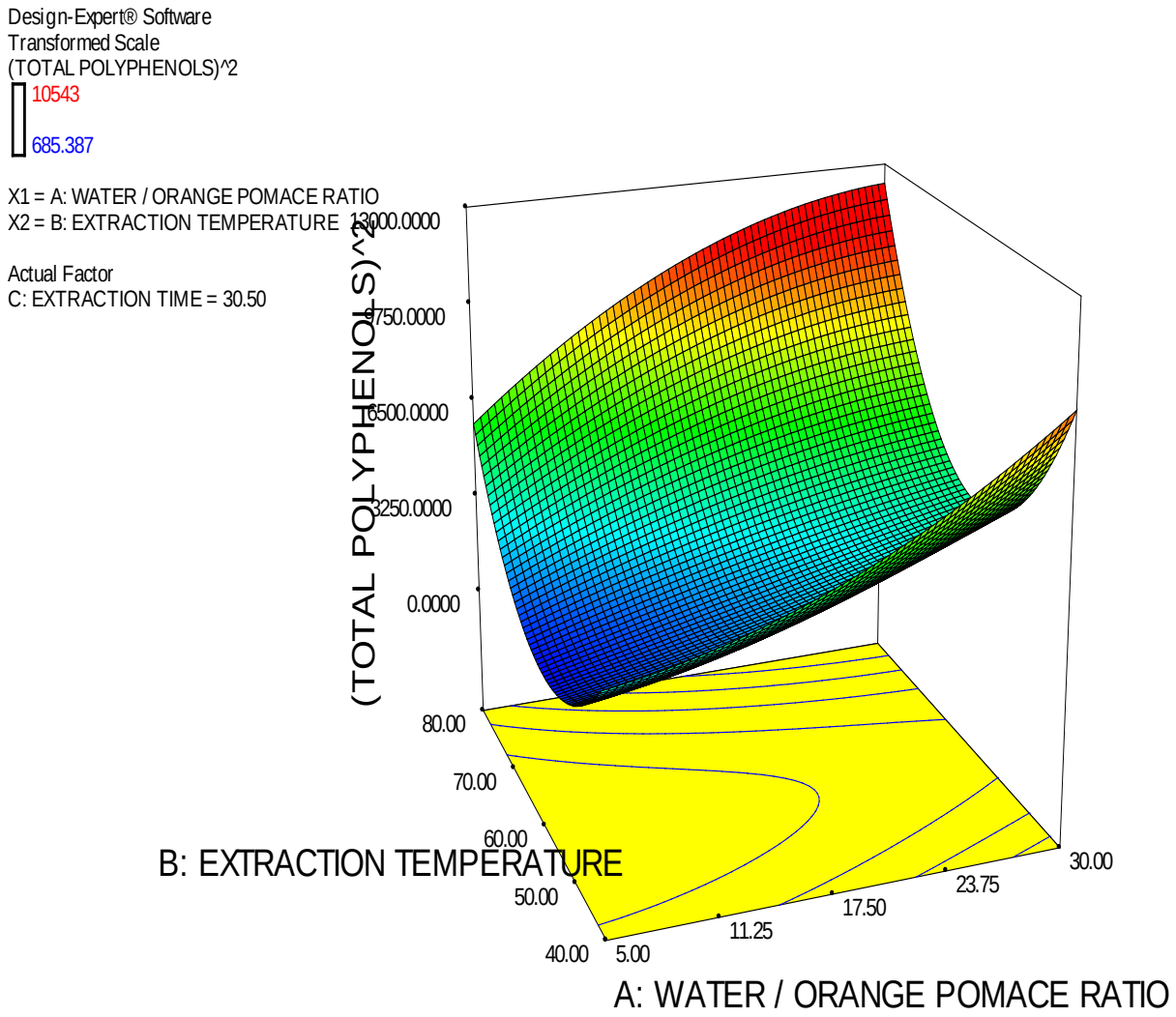

Fig. 3 Response surface plot of total orange polyphenols: (A × B interaction). A: water/solid ratio × B: extraction temperature (°C).

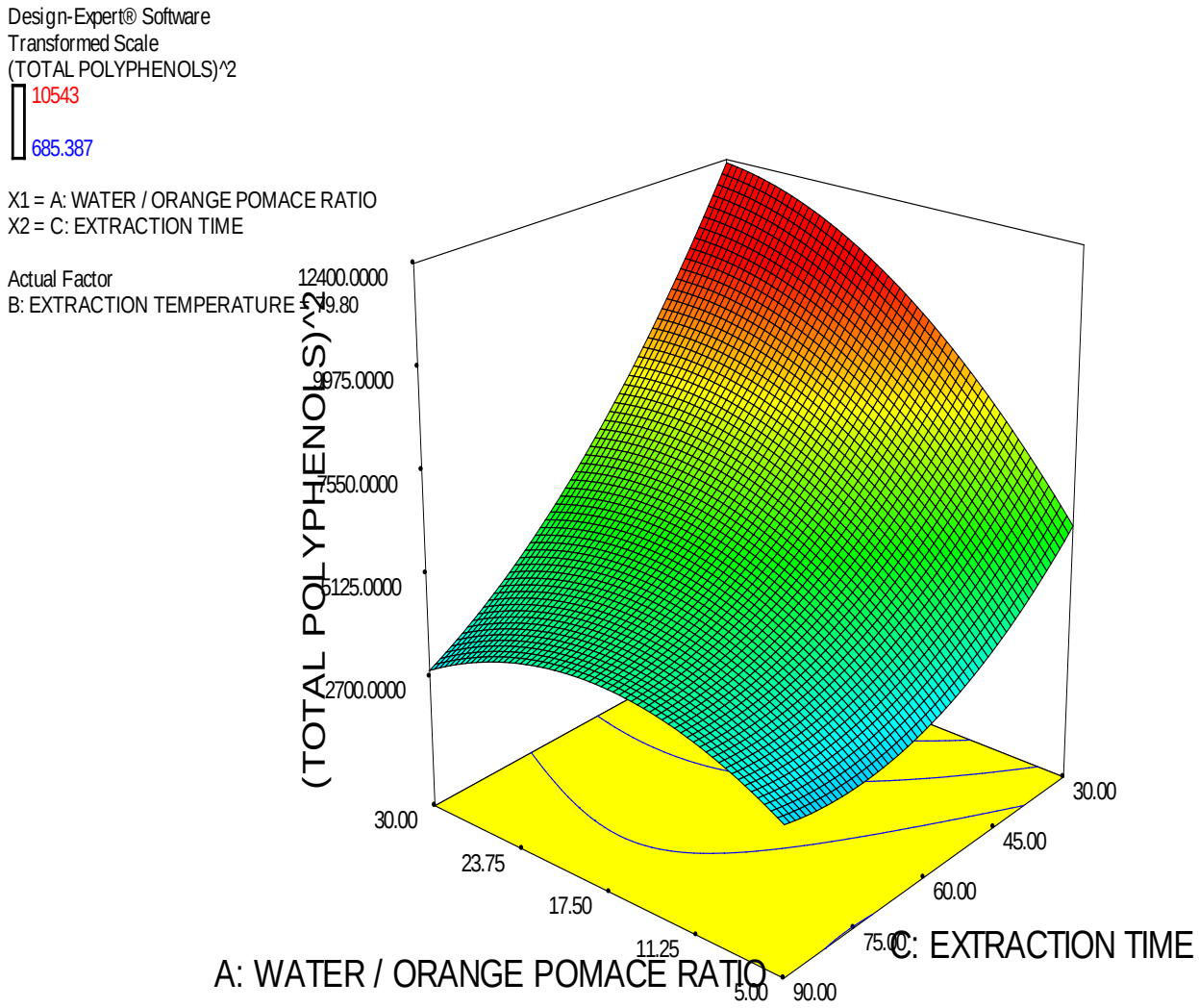

Fig. 4 Response surface plot of total orange polyphenols: (A × C interaction). A: water/solid ratio × C: extraction time (min).

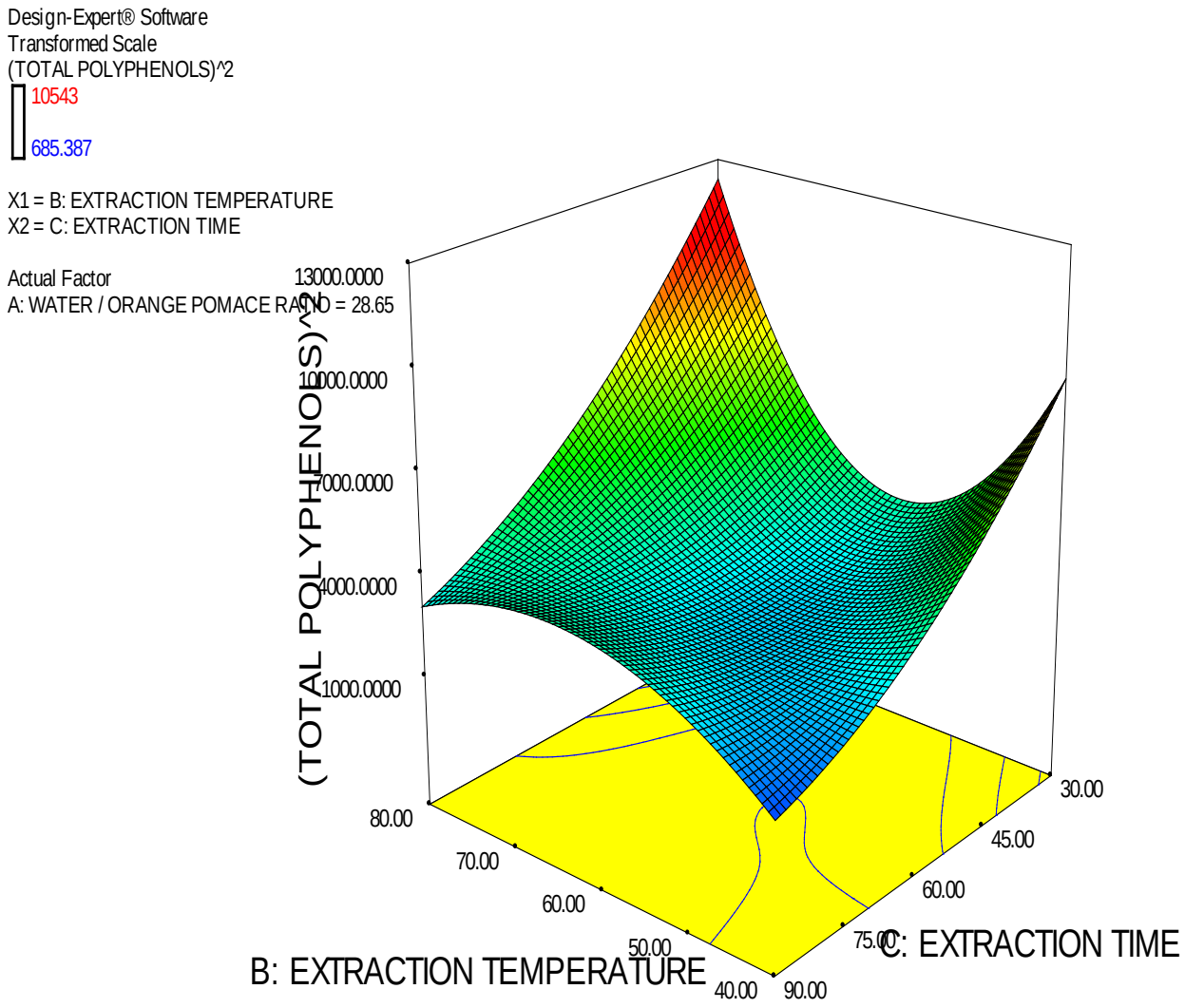

Fig. 5 Response surface plot of total orange polyphenols: (B × C interaction). B: Extraction Temperature (°C) × C: time (min).

In Table 3, the statistical analysis by ANOVA is presented. The ANOVA conclusion is that the model is significant while its lack of fit is not significant, which implies that it is a good tool for prediction of total orange flavonoids in the extracts vs. the three experimental factors involved. The R^2 value = 0.9528 is very high and very close to the adjusted R^2 = 0.9055 which means there is very good prediction by the developed model equation. The goodness of model prediction is additionally supported by the data presented in Fig. 6 where the predicted vs. actual values of total orange flavonoids are correlated and all the individual predicted points come very close to the actual central linear plot. In addition, in Figs. 7-8 the plots illustrate the paired interactions of the factors A = water/solid ratio, B = extraction temperature, C = extraction time regarding the total flavonoids extraction yield. Furthermore, using the developed RSM cubic model and the facility of Expert Design 7.0.0 statistical package, the maximum value of orange total flavonoids, which corresponds to the maximum value of 10.88 mg/L of total flavonoids, was achieved under the following conditions:

Water to solid orange pomace ratio = 5.42;

Extraction temperature: 79.62 °C;

Extraction time: 89.27 min.

Table 3 ANOVA statistical analysis of the predictive model for the total flavonoids concentration of orange pomace extracts.

Response	Total flavonoids of orange pomace				
Transform	Power	Lambda: 2	Constant: 0		
Stepwise regression with alpha to enter = 0.100, alpha to exit = 0.100					
ANOVA for response surface reduced cubic modelanalysis of variance table (partial sum of squares—type III)					
Source	Sum of squares	df	Mean square	*F* value	*p*-value Prob > *F*
Model	15,377.51	8	1,922.19	20.17	0.0002
A	136.70	1	136.70	1.43	0.2653
B-	5,213.74	1	5,213.74	54.72	< 0.0001
C	2,631.75	1	2,631.75	27.62	0.0008
AB	475.68	1	475.68	4.99	0.0559
BC	905.11	1	905.11	9.50	0.0151
A^2	2,003.94	1	2,003.94	21.03	0.0018
B^2	1,169.49	1	1,169.49	12.27	0.0080
C^2	2,222.23	1	2,222.23	23.32	0.0013
Residual	762.26	8	95.28		
Lack of fit	303.03	4	75.76	0.66	0.6515
Pure error	459.23	4	114.81		
Cor total	16,139.77	16			
Std. dev.	9.76		*R*-squared	0.9528	
Mean	54.34		Adj R-squared	0.9055	
C.V.%	17.96		Pred R-squared	0.7552	
Press	3,950.56		Adeq precision	12.651	
Model: is significant.			Lack of fit: is not significant.		

3.3 Simultaneous Optimization of Total Polyphenols Content and Total Flavonoids Content of Orange Extracts

In addition, the simultaneous maximization of the total polyphenol content and total flavonoids content of the orange pomace extracts was carried out. The methodology to obtain the mutual polyphenol and flavonoids optimum was based on the facility of Design Expert software to calculate the values of the extraction parameters which lead to common convergence of the total polyphenol content and total flavonoids content to their independent optima.

By this methodology the following set of optimum extraction conditions was obtained:

Water to solid orange pomace ratio = 29.88;

Extraction temperature: 80 °C;

Extraction time: 30.05 min.

At the above mentioned optimum extraction conditions for simultaneous optimization of total polyphenols and total flavonoids in the obtained orange pomace extracts the optimum values found to be: 111.58 $mg \cdot L^{-1}$ for total polyphenol content (100% of the independent polyphenols optimum) and 10.10 $mg \cdot L^{-1}$ for total flavonoids (92.87% of the independent flavonoids optimum) respectively.

The recommended optimum extraction conditions and the predicted optimum values of response (antioxidant values) independently, or in combination of two are summarized in Table 4.

3.4 Validation of the Mathematical Models Developed to Predict the Total Polyphenols Content, Total Flavonoids Content of Orange Pomace Extracts

For the validation of the precision of the developed mathematical models, five respective sets of values of experimental conditions have been selected. The selected sets of experimental values were evenly

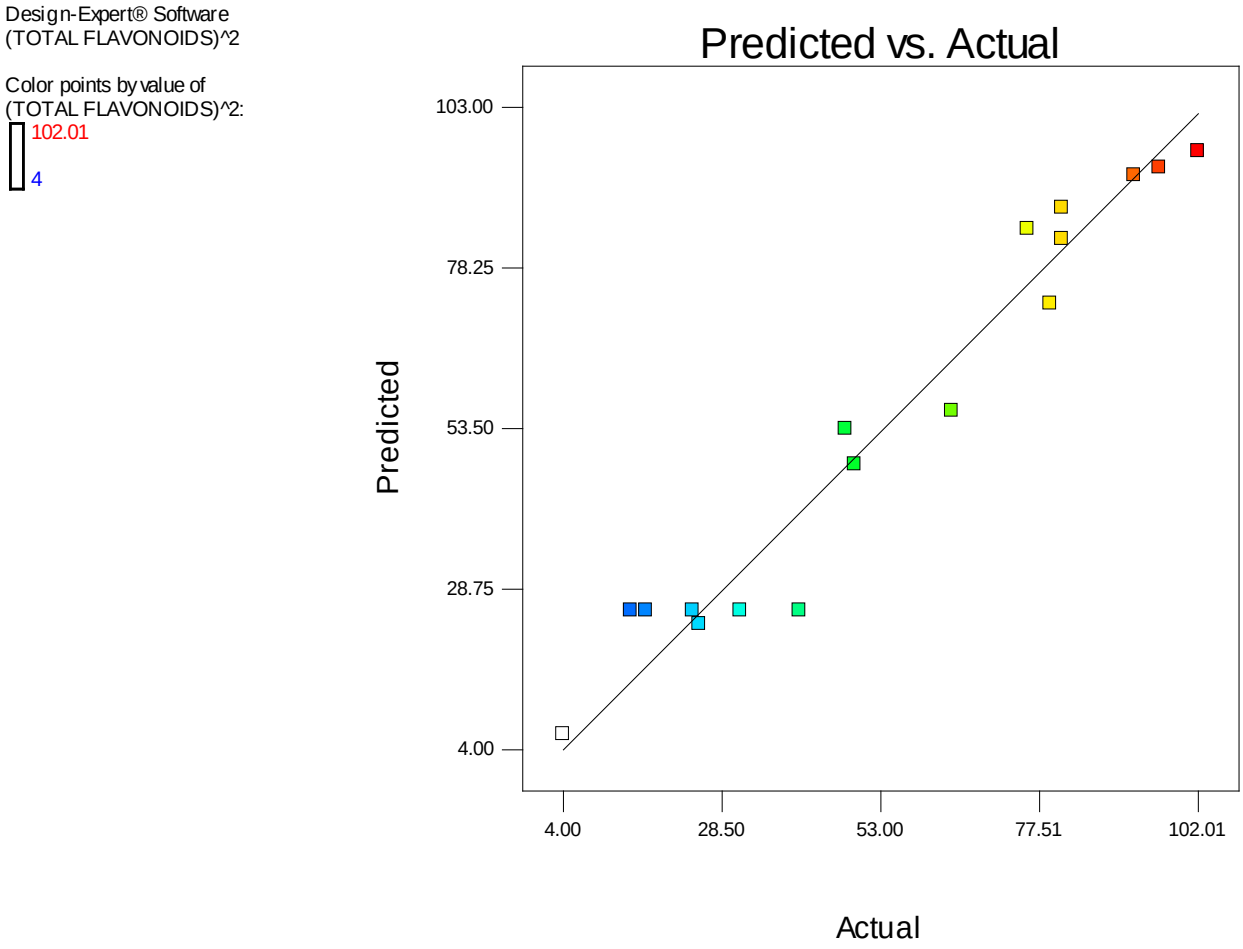

Fig. 6 Correlation of predicted vs. actual values of total orange flavonoids.

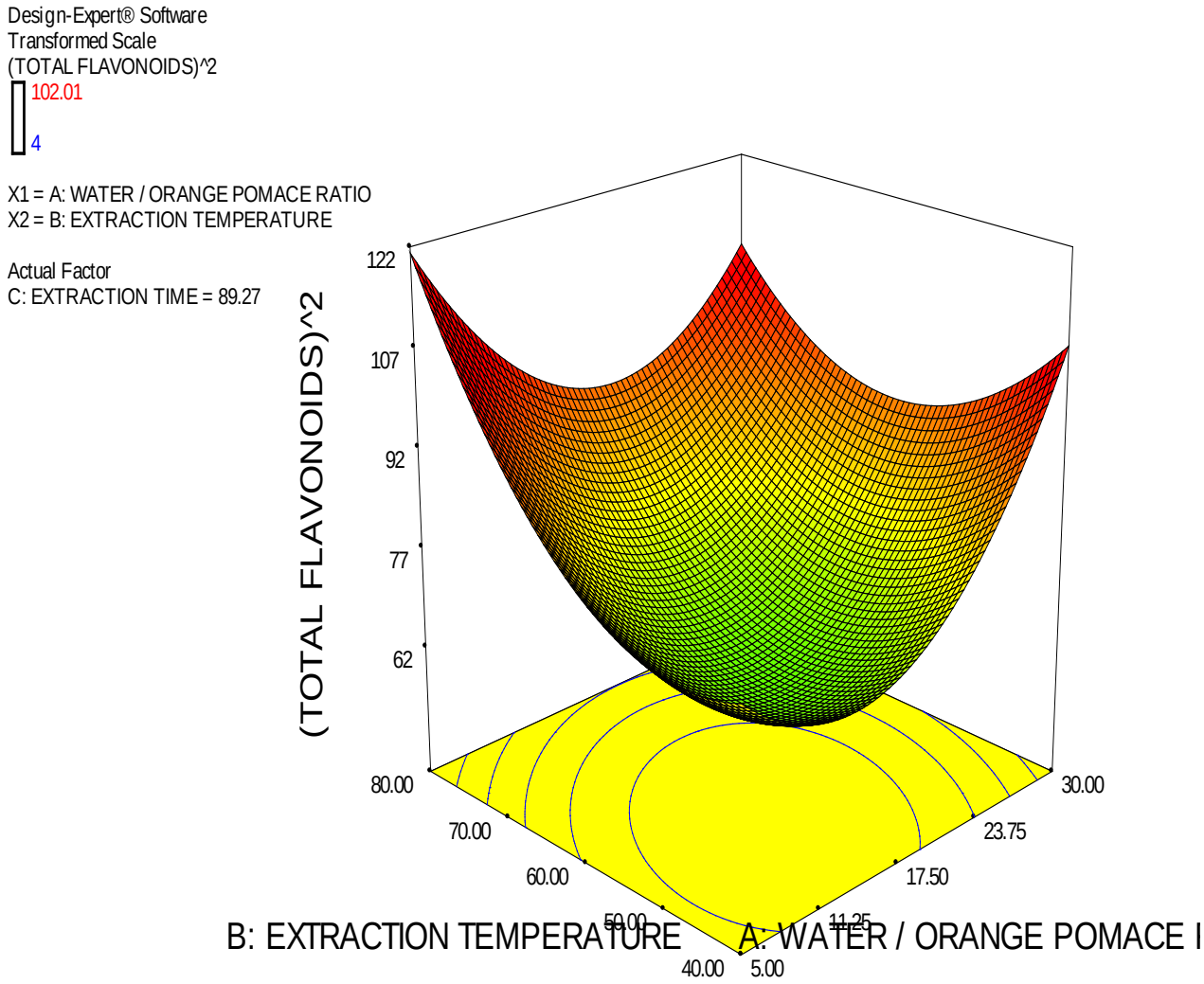

Fig. 7 Response surface plot of total orange flavonoids: (A × B interaction). A: water/solid ratio × B: extraction temperature (°C).

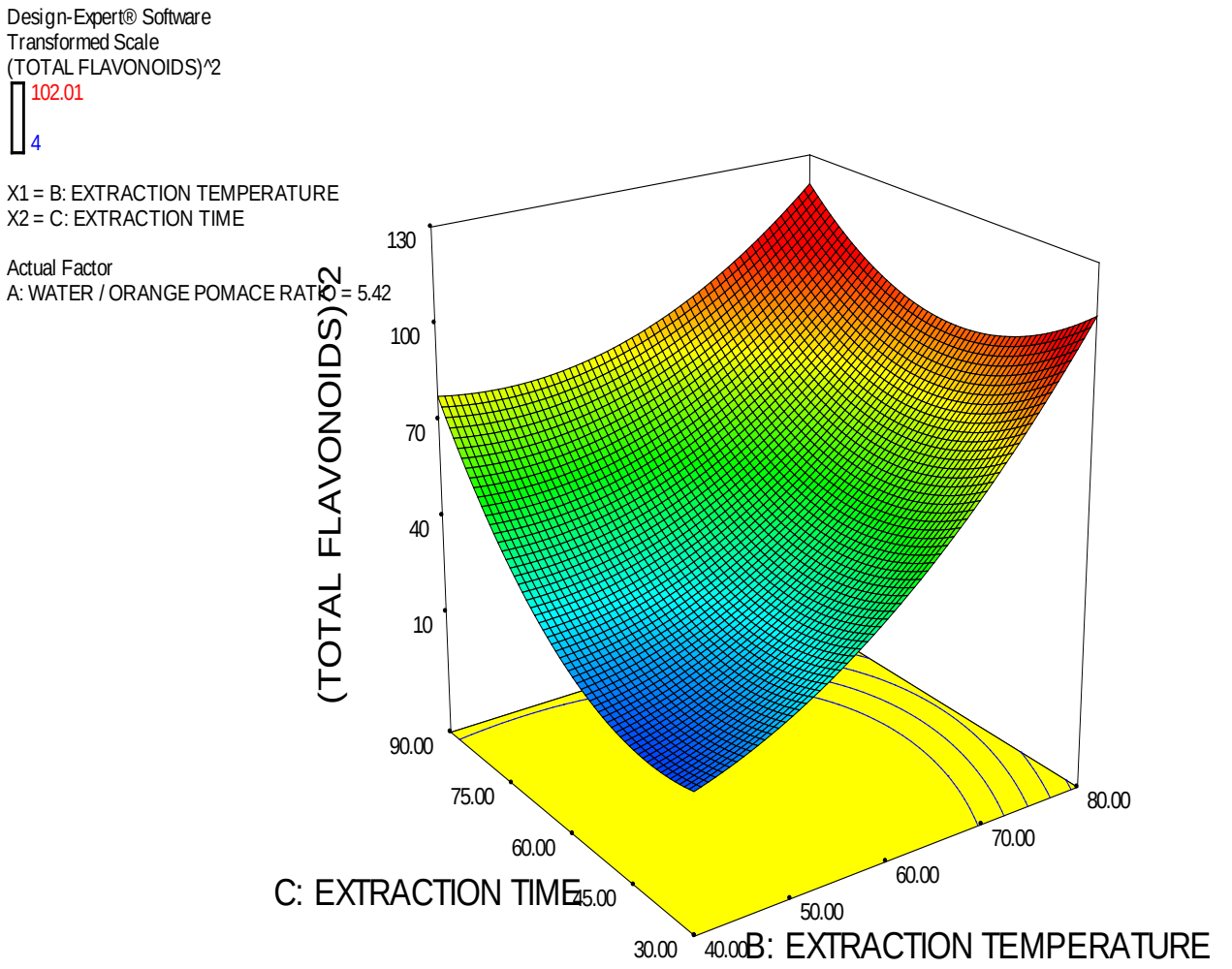

Fig. 8 Response surface plot of total orange flavonoids: (B × C interaction). B: extraction temperature (°C) × C: extraction time (min).

Table 4 Optimum values of antioxidant parameters vs. extraction parameters.

	Optimized extraction conditions			Optimized antioxidant parameters	
Optimization target	Water/orange pomace ratio	Temperature (°C)	Time (min)	Maximum total polyphenols content ($mg \cdot L^{-1}$)	Maximum total flavonoids content ($mg \cdot L^{-1}$)
Total polyphenols	29.88	80	30.05	111.58	
Total flavonoids	5.42	79.62	89.27		10.88
Total polyphenols + total flavonoids	29.88	80	30.05	111.58	10.10

spread within the experimental grid of points and independent to those previously used to develop the models. Consequently, for each one of them, microwave assisted extraction was carried out and the total polyphenols and flavonoids concentrations of the obtained orange pomace extracts were measured, while the developed models were used to predict the values at the same conditions.

The experimentally obtained values as well as the predicted values of the two above mentioned antioxidant parameters are listed in Table 5 along with the calculated % errors of prediction.

Table 5 Validation data for the developed mathematical models.

a/a	Water/pomace ratio	Extraction temperature (°C)	Extraction time (min)	PM (1)	PP (2)	Error (%)	FM (3)	FP (4)	Error (%)
1	10	50	45	42.15	40.29	4.41	3.32	3.44	3.65
2	10	70	45	56.85	58.84	3.50	6.88	7.17	4.24
3	10	70	75	71.32	68.50	3.96	8.13	7.88	3.11
4	25	50	45	54.24	55.14	1.65	4.68	4.83	3.24
5	25	70	45	68.85	70.58	2.51	7.15	7.06	1.25
	%Mean error of the prediction					3.21			3.10

(1) PM: measured values of total polyphenols concentration of orange pomace extracts.
(2) PP: predicted values of total polyphenols concentration of orange pomace extracts (Eq. (2)).
(3) FM: measured values of total flavonoids concentration of orange pomace extracts.
(4) FP: predicted values of total flavonoids concentration of orange pomace extracts (Eq. (3)).
% Mean error = absolute value of (predicted value-measured value) × 100/measured value.

Table 6 Results of the statistical *t*-test analysis applied between predicted and measured values of the total polyphenols and total flavonoids concentrations of orange pomace extracts.

a/a		*T*-value	*P*-value	Statistical significance (2-tailed) significance level = 0.05
1	Pair 1 PM-PP	-0.012256	0. 99081	*p*-value = 0. 99081 > 0.05. No significant difference between predicted and measured total polyphenols concentrations.
2	Pair 2 FM-FP	0.461347	0. 66852	*p*-value = 0. 66852 > 0.05. No significant difference between predicted and measured total flavonoids concentrations.

(1) PM: measured values of total polyphenols concentration of orange pomace extracts.
(2) PP: predicted values of total polyphenols concentration of orange pomace extracts (Eq. (2)).
(3) FM: measured values of total flavonoids concentration of orange pomace extracts.
(4) FP: predicted values of total flavonoids concentration of orange pomace extracts (Eq. (3)).

From the calculation of % mean error of the prediction for the two antioxidant parameters of orange pomace extracts obtained by microwave assisted extraction, it is concluded that the errors of prediction of 3.21% for total polyphenols and 3.10% for total flavonoids are rather low, which confirms the prediction effectiveness of the produced mathematical models.

In addition, *t*-test was performed between the measured and the predicted values of the antioxidants composition of the orange extracts and the results, which are presented in Table 6, suggesting that there is no statistically significant difference between them.

4. Discussion

According to the results of the present study the maximum amount of the polyphenols extracted by microwave assisted extraction is 111.58 $mg \cdot L^{-1}$ of orange extract. Furthermore, as all the measured extracts are 1:30 diluted and the used orange pomace amount is 100 g the total volume of the extract is 3 L and this equivalently means that the quantity of the total extracted polyphenols was: 3 L × 111.58 $mg \cdot L^{-1}$ × 10 = 3,347.4 mg of GAE·kg^{-1} of fresh orange pomace. In the same way, the quantity of the total flavonoids extracted was: 3 L × 10.88 $mg \cdot L^{-1}$ × 10 = 326.4 mg of QE·kg^{-1} of fresh orange pomace. Considering that the total solids content of the orange pomace was analytically determined to be 18% *w*/*w* and in order to allow the direct comparison with the literature values that are expressed on dry basis, the above mentioned optimum values of total polyphenols and total flavonoids were expressed on dry basis as 18,607.8 mg of GAE·kg^{-1} of fresh orange pomace and 1,813.3 mg of QE·kg^{-1} of dried orange pomace, respectively.

According to Putnik et al. [6] the total polyphenols concentration of orange dry peel extracts falls in

between 9,100-49,200 mg GAE·kg^{-1} of dry peel while the concentration of flavonoids falls in the range of 2,000-30,000 mg QE·kg^{-1} of dry peel. Also, Dahmoune et al. [15] and Mhiri et al. [16] reported extraction yields of total polyphenols for orange dry peel extracts in the range of 12,200-15,740 mg of GAE·kg^{-1}, using water/ethyl alcohol solvents. In addition, Londono-Londono et al. [14] cited that aqueous ultrasound assisted extraction of the orange pomace yielded total polyphenols of 19,595 ± 2,114 mg GAE·kg^{-1} of dry orange.

Furthermore, Ariel et al. [21] & Ergüt et al. [22] by supercritical carbon dioxide extraction of orange pomace found out that the yield of total polyphenols extracted by this technology yielded from 18,000 to 27,800 mg GAE·kg^{-1} of dry orange pomace, depending on the experimental conditions. Also, according to Barbosa et al. [27], substantial differences are observed among various researchers, with regard to the reported values of total polyphenols and the antioxidant activity of solid orange waste extracts. This can be attributed to the variations of the raw materials (different cultivars and solid tissue composition) and to the use of different solvents and extraction systems and conditions. In addition, Anagnostopoulou et al. [28], reported an extraction yield of total polyphenols from orange waste in the range 36.3-2,540 mg GAE·kg^{-1} of dry matter (DM), while Casquete et al. [19] obtained 2,840 mg GAE·kg^{-1} DM. Finally, Escobedo-Avellaneda et al. [29] achieved a total polyphenols yield of 5,886-6,799 mg GAE·kg^{-1} DM, after extraction of orange peels only. It can be concluded from the above that, in general, the yield of the extracted total polyphenols from the orange juice industry solid waste lies in the wide range of 36.3-49,200 mg GAE·kg^{-1} while the yield of total flavonoids was reported to be in the range of 2,000-30,000 mg QE·kg^{-1} of dry solid waste.

Comparing the literature values with the findings of the present work, it is concluded that our values for total polyphenols are somewhere in the middle of the reported range, while the values of total flavonoids were slightly less than the lower limit of the literature. This can be attributed, firstly to the quality of the raw orange pomace used in the present work, which is substantially different than the dry finely comminuted orange peel used in most cases by other researchers, and secondly, to the mechanical removal of the orange essential oil before the smashing of the fruits, which was applied by the producer that supplied us with orange pomace in this study. This process removes a significant part of the natural antioxidants found in the orange peel (in the form of essential oil) and reduces the concentration of the phenolics in the solid orange waste. In addition, the orange cultivar and the extractor dynamics can be considered as parameters that could cause this difference of optimal antioxidants composition of the extracts.

5. Conclusions

In the present work, for the first time, the aqueous eco-green microwave assisted extraction of phenolics from bioactive raw orange pomace was investigated and optimized. The essential difference of the present study in comparison with previous studies is that raw, humid raw pomace was used as extraction material instead of the dried orange pomace or dry peel in powder form, which has been used in previous studies. This can improve the economics and logistics of potential industrial production of orange extracts because it omits the drying and milling steps involved in the case that dry orange pomace or peel is used as raw material for the extraction. According to the results of the present study, natural antioxidant extracts can be produced by microwave extraction from raw orange pomace at condition optimized by Response Surface Methodology with Box & Behnken experimental design. The optimum extraction yield for total polyphenols was found to be 3,347.4 mg of GAE·kg^{-1} of fresh orange pomace while for total flavonoids the optimum extraction yield value was found to be 326.4 mg of QE·kg^{-1} of fresh orange

pomace. The same values expressed on dry basis were found to be 18,607.8 mg of GAE·kg^{-1} and 1,813.33 mg of QE·kg^{-1} for total polyphenols and total flavonoids, respectively.

The corresponding optimum values of the extraction parameters to achieve the above mentioned maximum polyphenol and flavonoids extraction yields were found to be: (a) extraction temperature = 80 °C, water/solid ratio = 1:29.88 and extraction time = 30.5 min for total polyphenols and (b) extraction temperature = 79.62 °C, water/solid ratio = 1:5.42 and extraction time = 89.27 min for total flavonoids.

It is also worth noticing that the set of extraction parameters corresponding to extraction temperature = 80 °C, water/solid ratio = 1:29.88 and extraction time = 30.05 min could be an effective compromise in order to get simultaneously optimized the total polyphenols, the total of the orange pomace extract.

In addition, the best fitting RSM mathematical models which were developed to predict the extraction yields of total polyphenols, total flavonoids of the orange pomace extracts were of cubic form, with appropriately transformed (squared) optimization responses. Yet another important observation, according to the data listed in Table 4, is that in order to achieve the optimum (maximum) yield of polyphenols an operation at high temperature and short time with high water to solid ratio is required, while the optimum flavonoids yield is obtained again at high temperature and long time but by using lower water/pomace ratio.

Another important conclusion is the large variance of the extraction yield of the total phenolics and total flavonoids of the orange pomace extracts, taking into account the available literature. This can be attributed to the varying composition and pre-extraction processing of the raw material as well as to the respective extraction technology. This, in turn, establishes the need for accurate and effective pilot-scale optimization process—before the industrial-scale extraction of orange pomace—using the specific raw materials intended to be used in the industrial scale operation. Equally important for process optimization is the novel statistical methodology described in the present work, which employs higher degree (cubic) RSM models and appropriately transformed optimization responses, in contrast to previous works where typically second order models have been used, compromising the precision of the obtained optimized extraction parameters.

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Author Contributions

(a) Konstantinos Petrotos, project administration, methodology, writing—original draft preparation.

(b) Ioannis Giavasis, supervision and paper correction.

(c) Konstantinos Gerasopoulos, investigation.

(d) Chrysanthi Mitsagga, investigation.

(e) Chryssoula Papaioannou, validation.

(f) Paschalis Gkoutsidis, investigation.

Conflicts of Interest

The authors declare no conflict of interest.

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