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Optimization of the Extraction Parameters for the Isolation of Bioactive Compounds from Orange Peel Waste

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Abstract: More and more research is being focused on the production of value-adding products from waste materials. Food waste is not only a major global issue, but also an excellent source of bioactive compounds. In this study, the parameters that affect the extraction of the bioactive compounds (polyphenols, ascorbic acid, hesperidin, carotenoids) from orange peels, and their antioxidant properties, were optimized, using a response surface methodology (RSM) (examining the extraction temperature, time, and composition of the extraction solvent). In addition, the effect of two more techniques was examined [ultrasound (US) and pulsed electric field (PEF)], either separately or combined, so as to determine whether they can enhance the extraction of the compounds. From our results, it was apparent that orange peels are an excellent source of many bioactive compounds since the extracts contained hesperidin (16.26 mg/g dw), total polyphenols (34.71 mg GAE/g dw), ascorbic acid (1228.93 mg/100 g dw) and total carotenoids (52.98 μg CtE/g dw).

Keywords: orange peel extract; response surface methodology; ultrasound; pulsed electric field; polyphenols; carotenoids; ascorbic acid

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1. Introduction

Food waste is one of the biggest global issues [1]. Every year, 1.3 billion tons of food ends in waste, and it is estimated that in less than 10 years, food waste will reach two billion tons per year. Not only is food waste management unsustainable, both from an economic and an environmental point of view, but also, huge amounts of water were consumed for the irrigation of fields, so as to produce these foods. The sustainable management of food waste is mandatory, however, it is a major challenge.

Fruits are very popular around the globe and are widely consumed. One of the most widely consumed citrus fruits is the orange. Oranges are well known for their vitamin C content [2]. Vitamin C is responsible for the synthesis of collagen and elastin in the skin [3] while also, due to its strong antioxidant capacity, it protects the cells from oxidative stress and the skin from the ultraviolet rays of the sun [4]. What is less known is that orange peels are also rich in various nutrients, such as vitamin C, since just 6 g of peel provide 14% of the daily value of vitamin C intake, almost three times more than the inner-edible part of the fruit. In addition, the orange peel also contains substantial amounts of provitamin A—β-carotene. The two main forms of vitamin A in the human diet are vitamin A (retinol, retinyl esters) and provitamin A/carotenoids, such as β-carotene, which are converted into retinol. In general, many biological effects of carotenoids have been documented, such as the anti-inflammatory, antioxidant, immunomodulatory, antitumor, and mutagenesis inhibition effects [5]. Among the carotenoids, β-carotene is a powerful pigment that protects against cell damage caused by reactive oxygen species [6]. Moreover, citrus peels contain significant amounts of phenolic compounds [7]. Diets rich in polyphenols prevent a number of diseases that prevail in the modern world, such as obesity, diabetes, cancer, and heart

disease [8]. Last but not least, as reported by Park et al. [9] the citrus peel contains a high concentration of antioxidants, compared to the flesh of the fruit. Lately, more and more emphasis is being placed in the use of antioxidant compounds, since it is well grounded that they play a key role in avoiding many diseases. They assist in preventing oxidative damage that occur in lipids, proteins, and DNA. Owing to their importance, the consumption of foods rich in antioxidants is highly recommended. Moreover, many plant extracts are being prepared and evaluated, in terms of their antioxidant activity, so as to be used in antioxidant food supplements. As a consequence, the extraction of antioxidant compounds is a topic of increasing interest [2–4,6–9].

The majority of produced oranges are used for the flesh, which can be further processed into orange juice, jams, and other products, requiring farmers and industries to dispose of orange peels in landfills or find alternative ways to manage their waste. Due to their content in bioactive compounds, food and pharmaceutical industries utilize orange peels to prepare extracts [10–12]. Extracts recovered from orange peels have been used in foods and beverages, either as flavor enhancers, or to bestow health benefits, such as antioxidant, anti-inflammatory, and anticancer properties, or to produce cosmetics and fragrances. As such, there is an increased interest in the production of orange peel extracts. Extracts need to be prepared in a cost-efficient way, in the minimum possible amount of time and contain the maximum possible amount of bioactive compounds.

Up until now, few studies discuss the extraction of the bioactive compounds from orange peels [13–16]. However, either they focus on the extraction of one or two compounds (still leaving many bioactive compounds in the peels), or they use solvents (such as organic solvents or solvents that cannot be removed from the extract, such as deep eutectic solvents) and techniques (such as membrane separation, Soxhlet extraction, enzyme-assisted extraction, etc.) that are difficult to be employed for the large-scale extraction of the compounds from orange peels. The aim of this study was to examine and optimize the major parameters that affect the extraction of vitamin C, vitamin A, carotenoids, and polyphenols from orange peels. For the extraction of the abovementioned compounds, two techniques (in addition to the classical extraction), that can easily be used, as a pretreatment step to maximize the extraction yield, at an industrial scale were examined: ultrasound (US) and pulsed electric field (PEF). For the extraction of the compounds, water, ethanol and their mixtures were examined. Moreover, the extraction time and temperature were examined. A response surface methodology was employed, with all the abovementioned parameters, so as to maximize the extraction yield in the most efficient way. In addition, both the whole orange peel, as well as its parts (albedo and flavedo), were also examined, to have a better overview of their composition in the examined bioactive compounds.

2. Materials and Methods

2.1. Chemicals and Reagents

All solvents used were at least of HPLC grade and obtained from Carlo Erba (Val de Reuil, France). Gallic acid, anhydrous sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri-2-pyridinyl-1,3,5-triazine (TPTZ), and Folin–Ciocalteu reagent were obtained from Penta (Prague, Czech Republic). Chemical standards for the HPLC-based determination of polyphenols (i.e., hesperidin, caffeic acid, ferulic acid, narirutin, neochlorogenic acid, and chlorogenic acid), iron (III) chloride, hydrochloric acid, ascorbic acid, β-carotene, and trichloroacetic acid and were purchased from Sigma-Aldrich (Steinheim, Germany). Deionized water was used for all conducted experiments.

2.2. Sample and Extract Preparation

Fresh oranges of the Merlin (Washington navel) cultivar (*Citrus sinensis* L.), collected at the maturity stage, were either purchased or donated from selected orchards in the Argos area (Greece) in December 2021. In order to ensure that the oranges were at the maturity stage, the physical and chemical parameters were taken into account. The color of the fruit limb/twig was green, the fruit rind (epicarp) color was orange and the weight of the fruits

were in the range of 150–200 g. Furthermore, the total soluble solids (TSSs) (°Brix) were between 10 and 12, the titratable acidity (TA) (expressed as % citric acid) was >0.8 and finally the TSS/TA ratio was >12.

The oranges were washed with tap water and dried with a paper towel. The orange peels were removed manually, cut into smaller pieces (~2 × 2 cm), and placed in a Biobase BK-FD10P freeze-dryer (Shandong, China) for 24 h, in order to remove water. The freeze-dried peels were pulverized and placed in sieves, in order to be separated according to size. For the preparation of the extracts, the powdered orange peels with an average particle diameter of 470 μm were used (the powder from the smaller average particle diameter was not used, since its amount was substantially smaller, compared to the used particles, and we opted for the extraction of compounds from the major fraction of the powder).

For the preparation of the extracts, 1 g of pulverized orange peels was placed in 25-mL Duran bottles, along with 20 mL of the tested solvent, and extraction was carried out, according to the conditions described in Section 2.3. For the extraction of the compounds, a standard extraction procedure (ST) was followed, by stirring the mixture at a fixed temperature for a set amount of time (details for each extract are given in Section 2.3). In addition, some samples were subjected to ultrasound treatment (US) or pulsed electric field (PEF) pretreatment, or both (PEF + US) before the ST. The samples were moisturized with the respective solvent for 30 min before the ST. In the case of the US pretreatment, the moisturization lasted 10 min and the US treatment, 20 min. Similarly, the moisturization before the PEF was completed after 10 min and the PEF pretreatment was completed in 20 min. For the extracts prepared by both pretreatment methods, the moisturization was carried out in 10 min, and after 20 min of the PEF and 20 min of the US, the ST was carried out. Once the extraction was completed, the mixture was centrifuged at 3396× *g* for 10 min and the supernatant was retracted, transferred to amber-glass vials, and stored in the freezer.

For the PEF treatment of the samples, a high voltage power generator (Leybold, LD Didactic GmbH, Huerth, Germany), a digital oscilloscope (Rigol DS1052E, Rigol Technologies, Inc, Beaverton, OR, USA), a function/arbitrary waveform generator (UPG100, ELV Elektronik AG, Leer, Germany), and two custom-made stainless-steel chambers (Val-Electronic, Athens, Greece) were employed [17]. The pulse duration was 10 μs, the period was 1 ms (frequency: 1000 Hz) and the electric field density was set at 1.0 kV cm^{-1}. The US treatment of the samples was carried out in a Elmasonic P (Elma Schmidbauer GmbH, Singen, Germany), operated at 37 kHz and the temperature was maintained at 30 °C.

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

Maximizing the extraction yield for the tests for hesperidin (HSP) concentration, the total polyphenol content (TPC), antioxidants (FRAP and DPPH), ascorbic acid (AA), and total carotenoid content (TCC) was the goal of the experimental design. Thus, testing for the hesperidin concentration, total polyphenols, antioxidants, ascorbic acid, and total carotenoids were the design's response. The extraction technique, the solvent (EtOH) concentration (*C*, % *v/v*), the extraction time (*t*, min), and the extraction temperature (*T*, °C) were optimized to achieve this. An experiment with a main effects screening design and 20 design points served as the foundation for the optimization. As per the experimental design, the process variables were established in five levels. Table 1 lists the coded and actual levels. The analysis of variance (ANOVA) and the summary-of-fit tests were used to evaluate the overall model significance (R^2, *p*), as well as the significance of the model (equations) coefficients, at a minimum level of 95%.

Additionally, the response variable was predicted using a second-order polynomial model presented in the following Equation (1), as a function of the investigated independent factors:

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

where, Y_k is the predicted response variable; X_i and X_j are the independent variables; β_0, β_i, β_{ii}, and β_{ij} are the intercept, regression coefficients of the linear, quadratic, and interaction terms of the model, respectively. The RSM was also used to calculate the greatest peak area and to examine the impact of a significant independent variable on the response. To display the model equation visually, the 3D surface response graphs were built.

Table 1. Independent variables and their related actual and coded levels utilized to optimize the process.

Independent Variables	Code Units	Coded Variable Level				
		1	**2**	**3**	**4**	**5**
Technique	X_1	ST	US+ST	PEF+ST	PEF+US+ST	–
C (%, *v*/*v*)	X_2	0	25	50	75	100
t (min)	X_3	15	30	60	120	180
T (°C)	X_4	20	35	50	65	80

2.4. Total Polyphenol Content (TPC) Determination

The Folin–Ciocalteu assay was used to determine the total polyphenol content (TPC) of the extracts, in accordance with a previously reported procedure [18]. In brief, 100 µL of the orange peel extracts were mixed with an equal volume of the Folin–Ciocalteu reagent in an Eppendorf tube. Two min later, 800 µL of Na_2CO_3 solution (5% *w*/*v*) was added and the solutions were heated at 40 °C for 20 min. Finally, using a Shimadzu spectrophotometer (UV-1700, Shimadzu Europa GmbH, Duisburg, Germany), the absorbance at 740 nm was measured. A calibration curve was also prepared, using gallic acid as a standard compound. 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 (2):

$$Y_{TP}\ (\text{mg GAE/g dw}) = \frac{C_{TP} \times V}{w} \quad (2)$$

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

2.5. Ferric Reducing Antioxidant Power (FRAP) Assay

For the evaluation of the FRAP, a previously described method was employed [19]. In an Eppendorf tube, 0.05 mL of $FeCl_3$ solution (4 mM in 0.05 M HCl) was mixed with the sample extracts and the solutions were incubated for 30 min at 37 °C. Next, 0.90 mL of TPTZ solution (1 mM in 0.05 M HCl) was added, and after 5 min, the absorbance at 620 nm was measured. A calibration curve was prepared using ascorbic acid as a standard compound. P_R was determined as µmoL ascorbic acid equivalents (AAE) per g of dw, using the following Equation (3):

$$P_R\ (\mu\text{moL AAE/g dw}) = \frac{C_{P_R} \times V}{w} \quad (3)$$

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

2.6. DPPH Radical Scavenging Activity

A previously described procedure was used to assess the DPPH radical scavenging activity [18]. In brief, 25 µL of the prepared extract was added to 975 µL of DPPH solution (100 µM). Following a thorough mixing, the absorbance of the solution was measured at

515 nm ($A_{515(i)}$), as well as after 30 min of incubation, in the absence of light ($A_{515(f)}$). The antiradical activity (A_{AR}) was calculated using Equation (4):

$$A_{AR}\ (\mu\text{moL DPPH/g dw}) = \frac{\Delta A}{\varepsilon \times l \times C} \times Y_{TP} \quad (4)$$

where, $\Delta A = A_{515(i)} - A_{515(f)}$; ε (DPPH) = 11,126 $\times$ 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).

2.7. Ascorbic Acid (AA) Content

The ascorbic acid (AA) content was determined using a modified colorimetric assay [20]. A 100 µL sample aliquot was added to 900 µL trichloroacetic acid (10% *w/v*) and to the resulting solution, 500 µL of 10% (*v/v*) Folin–Ciocalteu reagent was added. Ten min later, the absorbance was read at 760 nm. A standard curve was prepared using ascorbic acid.

2.8. Total Carotenoid (TCC) and Vitamin A Content

The total carotenoid content (TCC) was determined using a colorimetric assay developed by Biswas et al. [21]. An aliquot of 100 µL of the sample was mixed with 900 µL of ethanol and the solution was vigorously shaken for 30 s. The absorbance was read at 449 nm. The TCC was determined using a calibration curve, using β-carotene as a standard compound. Vitamin A was determined using a conversion factor suggested by the US Department of Agriculture (USDA) [1 International Unit (IU) of Vitamin A = 0.60 µg of β-carotene].

2.9. HPLC-Based Determination of the Hespridin Content and Other Phenolic Compounds

The content of the extracts in the HSP and other phenolic compounds (i.e., caffeic acid, ferulic acid, narirutin, neochlorogenic acid, and chlorogenic acid) was determined using an SPD-M20A diode array detector, after the high-performance liquid chromatography (HPLC)-based separation of the compounds with a CBM-20A liquid chromatograph (Shimadzu Europa GmbH, Duisburg, Germany). The stationary phase [Phenomenex Luna C18(2) column (100 Å, 5 µm, 4.6 $\times$ 250 mm; Phenomenex, Inc., Torrance, CA, USA] was placed in a furnace and the temperature was maintained at a constant at 40 °C. Aqueous formic acid (0.5% *v/v*) (A) and acetonitrile/water (6:4 *v/v*) containing formic acid (0.5% *v/v*) (B) were used as a mobile phase and the flow rate was set at 1 mL/min. The following gradient elution program was employed for the elution of the compounds: 5% B to 40% B in 40 min, then to 50% B in 10 min, and finally to 70% B in 10 min, and kept constant at 70% B for 10 min. The total run time was 70 min. The target compounds were identified by comparing the retention times and the absorbance spectra to that of the pure chemical standards. For the quantification of the compounds, the calibration curves (0–500 µg/mL) were prepared and used.

2.10. Statistical Analysis

The experimental design, statistical analysis related to the response surface methodology, and the distribution analysis were all created using the JMP® Pro 16 (SAS, Cary, NC, USA) software. All extraction procedures described above were carried out three times and for each extract, the analyses were carried out in triplicates. The results were expressed as mean values of all measurements (3 $\times$ 3 = 9 total measurements for each extract) and the standard deviation was also calculated from the nine samples.

3. Results and Discussion

3.1. Extraction Optimization

The extraction of the bioactive compounds, recently, has become a hot topic of research. Transforming orange peel waste into value-added products, is of high importance, given their wide consumption. Ascorbic acid, carotenoids, and polyphenols are among the most abundant compounds in orange peels, which also have a wide applicability in the food

industry [7]. In order to extract the maximum possible quantity of the aforementioned compounds from orange peels, we examined the main parameters that affect the extraction of the bioactive compounds. The parameters examined were the composition of the solvent (water, ethanol, and their 25, 50, and 75% (*v*/*v*) mixtures), the extraction time (ranging between 15 and 180 min), and the extraction temperature (studied in the range 20–80 °C). The solid-to-liquid ratio is also known to be an important parameter in the extraction processes. Based on our preliminary experiments, we found that the optimum solid-to-liquid ratio was 20 mL of solvent per 1 g of orange peel. A lower amount of solvent resulted in an inadequate extraction of the compounds, whereas an increased amount did not yield any better results. Thus, this solid-to-liquid ratio was used for the preparation of all of the extracts. In addition to examining the extraction parameters, the use of the US as a pretreatment step was also examined, since the US is commonly used in the preparation of extracts. Moreover, another up-and-coming technique that can maximize the extraction yield was also examined (i.e., PEF). These two techniques have a low energy consumption and can easily be used to prepare extracts on a bigger scale. The two techniques were examined individually, prior to the ST, as well as used one after the other, to examine their combinatorial effect (the PEF was carried out first and followed by the US).

A response surface methodology (RSM) was employed to assess the impact of each extraction factor and to optimize the compound extraction process. The responses were the FRAP and DPPH values, the TPC and HSP content, as well as the content in AA and the TCC. In addition to the HSP content that was determined with HPLC-DAD, other polyphenolic compounds were also identified and examined (i.e., caffeic acid, ferulic acid, narirutin, neochlorogenic acid, and chlorogenic acid). The suitability of the response surface and model fitting was assessed using ANOVA and summary-of-fit tests, taking into account how well the measured and predicted values corresponded. The experimental conditions for the preparation of the extracts, as well as the measured responses, can be seen in Table 2. Furthermore, in Table S1, the concentrations of all of the examined polyphenols are presented. In Table 3, the statistical parameters, second-order polynomial equations (models), and coefficients derived for each model are presented. It can be seen that the coefficients were >0.97, suggesting a good fit for the developed models. The maximum predicted values (Table 4) for the hesperidin concentration, the total polyphenols, antioxidants (FRAP and DPPH), ascorbic acid, and total carotenoids, as well as the optimal levels for each of the four variables taken into consideration were calculated using the desirability function (Figures S1–S6, respectively). Furthermore, 3D response graphs for each examined response can be seen in Figures S7–S12. Finally, a representative chromatogram of the identified polyphenols in the extracts can be seen in Figure S13.

A principal component analysis was also carried out, so as to have a better overview of the results, by reducing the dimensionality of the multivariate data. As can be seen in Figure 1, the two principal components were selected (Eigenvalues > 1) that could explain 81.5% of the variance, which was determined to be a statistically significant parameter ($p < 0.0001$). Component 1 explained 49.1% of the variability and showed a positive correlation with DPPH, FRAP, polyphenols, and hesperidin and a negative correlation with ascorbic acid and carotenoids. Component 2 can explain 32.4% of the variance and has a positive correlation with all of the examined variables. As can be seen from the PCA plots in Figure 1, the loading direction of DPPH, FRAP, and the polyphenols are the same, whereas the loading direction of hesperidin is different. Owing to this, it can be assumed that the polyphenols with the antioxidant parameters are correlated (>0.7), but not very well with hesperidin (<0.4). Moreover, ascorbic acid and the carotenoids showed the best correlation (0.94), which was determined to be a statistically significant parameter ($p < 0.0001$). Finally, the objective of the hierarchical cluster analysis was to construct a dendrogram with the optimal extraction of the orange peels that were deemed to be the most comparable, by the various extraction techniques, and the hydroethanolic solutions used in the study being placed on branches that are close to each other (constellation plot). Therefore, according to Figure 2, design point 10 (X_1:2, X_2:5, X_3:5, X_4:5) was clustered separately, which provides

the convincing evidence that it is superior to all other extracts for the extraction of both ascorbic acid and the total carotenoids. Additionally, design point 14 (X_1:3, X_2:4, X_3:5, X_4:4) was also clustered separately, which may be considered convincing evidence that it is better than all other extracts in terms of hesperidin, as well as the total polyphenols and antioxidants.

Table 2. Coded values of the four independent variables investigated and the responses of the dependent variable (hesperidin, total polyphenols, FRAP, DPPH, ascorbic acid, and total carotenoids); Hesperidin (HSP) is expressed in mg/g dw, the total polyphenol content (TPC) is expressed in mg GAE/g dw, FRAP is expressed in μmoL AAE/g dw, DPPH is expressed in μmoL DPPH/g dw, ascorbic acid is expressed in mg/100 g dw and the total carotenoid content (TCC) is expressed in μg CtE/g dw.

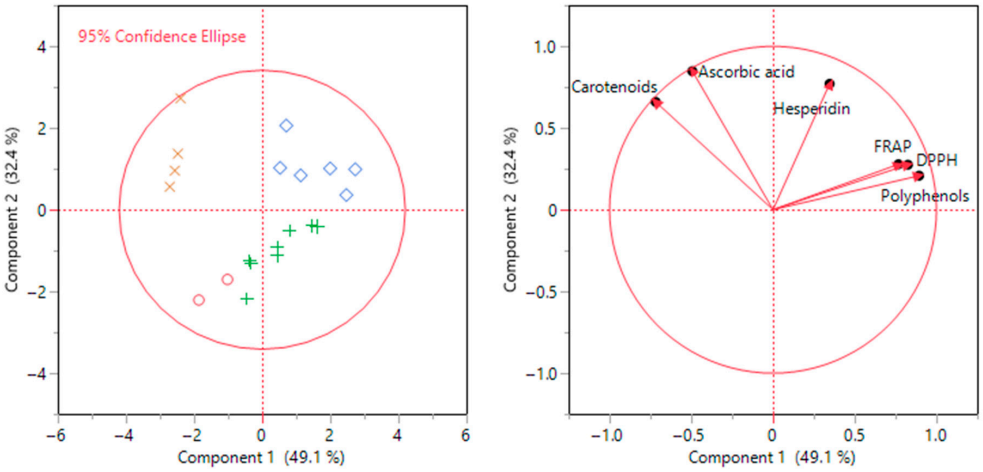
Design Point	Independent Variables				Responses					
	X_1	X_2	X_3	X_4	HSP	TPC	FRAP	DPPH	AA	TCC
1	1	1	4	3	7.04	15.99	37.66	39.01	52.83	0.58
2	1	2	5	1	3.41	13.27	13.08	42.24	97.81	3.90
3	2	3	3	1	5.04	22.11	65.34	60.77	176.86	6.59
4	1	4	3	2	13.67	26.61	53.31	70.17	347.55	11.88
5	1	5	1	4	4.74	17.14	16.78	48.70	820.61	42.90
6	4	1	5	2	0.90	22.53	49.45	41.12	11.91	4.39
7	2	2	4	2	3.75	21.22	53.05	49.02	209.96	6.11
8	1	3	2	5	6.30	27.70	78.07	49.73	252.39	6.97
9	2	4	1	3	10.19	24.14	65.21	69.52	605.33	18.08
10	2	5	5	5	11.64	14.76	67.63	48.05	1228.93	52.24
11	2	1	2	4	3.41	20.08	83.10	39.69	191.15	9.69
12	3	2	2	3	5.40	28.12	94.43	59.98	253.44	5.63
13	4	3	4	4	13.04	30.53	100.93	62.26	128.05	0.60
14	3	4	5	4	16.26	24.75	87.73	58.20	702.75	20.23
15	3	5	4	1	6.55	19.11	55.81	35.88	1029.24	44.90
16	3	1	3	5	2.80	19.44	92.07	58.31	198.71	5.00
17	4	2	1	5	3.46	27.23	110.67	63.76	289.65	6.32
18	3	3	1	2	11.84	29.62	102.88	81.23	383.20	2.16
19	4	4	2	1	10.22	30.72	99.25	68.68	501.89	9.16
20	4	5	3	3	6.67	19.38	57.73	26.58	870.26	43.44

Table 3. The hydroethanolic solution extraction with the various techniques of orange peel was optimized using the mathematical models developed using the response surface methodology; There were only significant terms in the models.

Responses	Second-Order Polynomial Equations (Models)	R^2	*P*	Equation
HSP	$Y = 20.47 - 11.9X_1 + 10.27X_2 - 13.89X_3 + 3.74X_4 + 1.75{X_1}^2 - 1.14{X_2}^2 + 1.22{X_3}^2 - 0.85{X_4}^2 + 0.03X_1X_2 + 0.36X_1X_3 + 0.24X_1X_4 + 0.564X_2X_3 - 1.4X_2X_4 + 1.65X_3X_4$	0.9852	0.0012	(5)
TPC	$Y = -13.41 + 0.38X_1 + 18.34X_2 - 3.47X_3 + 11.75X_4 + 1.61{X_1}^2 - 2.46{X_2}^2 + 0.07{X_3}^2 - 1.11{X_4}^2 - 1.1X_1X_2 - 0.09X_1X_3 - 1.21X_1X_4 + 0.69X_2X_3 - 1.11X_2X_4 + 0.39X_3X_4$	0.9754	0.0043	(6)
FRAP	$Y = 68.42 + 10.26X_1 + 30.44X_2 - 40.97X_3 + 6.7X_4 + 1.56{X_1}^2 - 6.36{X_2}^2 + 1.27{X_3}^2 + 0.06{X_4}^2 - 0.92X_1X_2 + 0.49X_1X_3 - 2.19X_1X_4 + 5.1X_2X_3 - 3.74X_2X_4 + 4.39X_3X_4$	0.9702	0.0067	(7)
DPPH	$Y = 8.83 + 9.54X_1 + 53.25X_2 - 20.13X_3 - 3.59X_4 + 0.88{X_1}^2 - 4.7{X_2}^2 + 2.11{X_3}^2 - 0.41{X_4}^2 - 4.27X_1X_2 - 1.79X_1X_3 + 0.9X_1X_4 - 1.27X_2X_3 - 3.69X_2X_4 + 4.77X_3X_4$	0.9846	0.0014	(8)
AA	$Y = -1623.99 + 821.1X_1 - 92.47X_2 + 129.02X_3 + 315.89X_4 - 92.52{X_1}^2 + 58.51{X_2}^2 + 18.41{X_3}^2 - 15.37{X_4}^2 - 11.36X_1X_2 - 61.88X_1X_3 - 39.62X_1X_4 + 2.66X_2X_3 - 5.03X_2X_4 - 21.3X_3X_4$	0.9894	0.0005	(9)
TCC	$Y = -12.52 + 23.55X_1 - 29.84X_2 + 11.85X_3 + 1.84X_4 - 3.6{X_1}^2 + 5.22{X_2}^2 - 0.84{X_3}^2 + 0.14{X_4}^2 - 0.12X_1X_2 - 0.94X_1X_3 - 0.46X_1X_4 + 0.74X_2X_3 + 2.11X_2X_4 - 2.25X_3X_4$	0.9671	0.0085	(10)

Table 4. Maximum predicted responses and the ideal extraction conditions for the hesperidin concentration, total polyphenols, antioxidants, ascorbic acid, and total carotenoids using various extraction techniques and hydroethanolic solutions. The desirability functions were used to establish the predictions (see Figures S1–S6).

Responses	Maximum Predicted Response	Optimal Conditions			
		Technique (X_1)	C (%) (X_2)	t (min) (X_3)	T (°C) (X_4)
HSP	16.26 ± 2.05 mg/g dw	PEF+ST (3)	75 (4)	180 (5)	65 (4)
TPC	34.71 ± 3.86 mg GAE/g dw	PEF+US+ST (4)	50 (3)	30 (2)	35 (2)
FRAP	110.67 ± 22.72 µmoL AAE/g dw	PEF+US+ST (4)	25 (2)	15 (1)	80 (5)
DPPH	81.23 ± 6.29 µmoL DPPH/g dw	PEF+ST (3)	50 (3)	15 (1)	35 (2)
AA	1228.93 ± 173.33 mg/100 g dw	US+ST (2)	100 (5)	180 (5)	80 (5)
TCC	52.98 ± 13.6 µg CtE/g dw	PEF+ST (3)	100 (5)	120 (4)	65 (4)



Eigenvalues

Number	Eigenvalue	Percent	Cum Percent	ChiSquare	DF	Prob>ChiSq
1	2.9467	49.112	49.112	102.871	14.002	<0.0001*
2	1.9444	32.407	81.519	76.017	12.636	<0.0001*
3	0.5963	9.938	91.457	41.265	9.754	<0.0001*
4	0.3011	5.019	96.476	29.125	5.979	<0.0001*
5	0.1993	3.321	99.797	22.142	2.931	<0.0001*
6	0.0122	0.203	100.000	.	.	.

Pairwise Correlations

Variable	by Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob
Carotenoids	Ascorbic acid	0.9422	20	0.8570	0.9773	<0.0001*
FRAP	Polyphenols	0.7486	20	0.4578	0.8947	0.0001*
DPPH	Polyphenols	0.7057	20	0.3827	0.8750	0.0005*
DPPH	FRAP	0.5571	20	0.1521	0.8019	0.0107*
DPPH	Hesperidin	0.4834	20	0.0520	0.7627	0.0308*
Polyphenols	Hesperidin	0.3924	20	−0.0607	0.7114	0.0870
Ascorbic acid	Hesperidin	0.3867	20	−0.0673	0.7081	0.0921
FRAP	Hesperidin	0.2956	20	−0.1691	0.6527	0.2058
Carotenoids	Hesperidin	0.1557	20	−0.3080	0.5597	0.5120
Ascorbic acid	FRAP	−0.0833	20	−0.5071	0.3729	0.7269
Ascorbic acid	DPPH	−0.1635	20	−0.5651	0.3008	0.4910
Ascorbic acid	Polyphenols	−0.2455	20	−0.6206	0.2210	0.2967
Carotenoids	FRAP	−0.3085	20	−0.6608	0.1552	0.1856
Carotenoids	DPPH	−0.4175	20	−0.7259	0.0307	0.0670
Carotenoids	Polyphenols	−0.4541	20	−0.7466	−0.0145	0.0443*

Figure 1. Orange peel extraction plots utilizing the principal component analysis (PCA), using various extraction techniques and hydroethanolic solutions. PCA 1 and PCA 2′s axis scores were shown. Each of the six distinct bays, each of which has a different line allocated to it, corresponds to one of the six variables used in the PCA. Physicochemical properties include the concentration of hesperidin, total polyphenols, antioxidants (FRAP and DPPH), ascorbic acid, and total carotenoids. Pairwise correlation analysis was used to estimate the physicochemical properties. Asterisks and colored values indicate the statistically significant values.

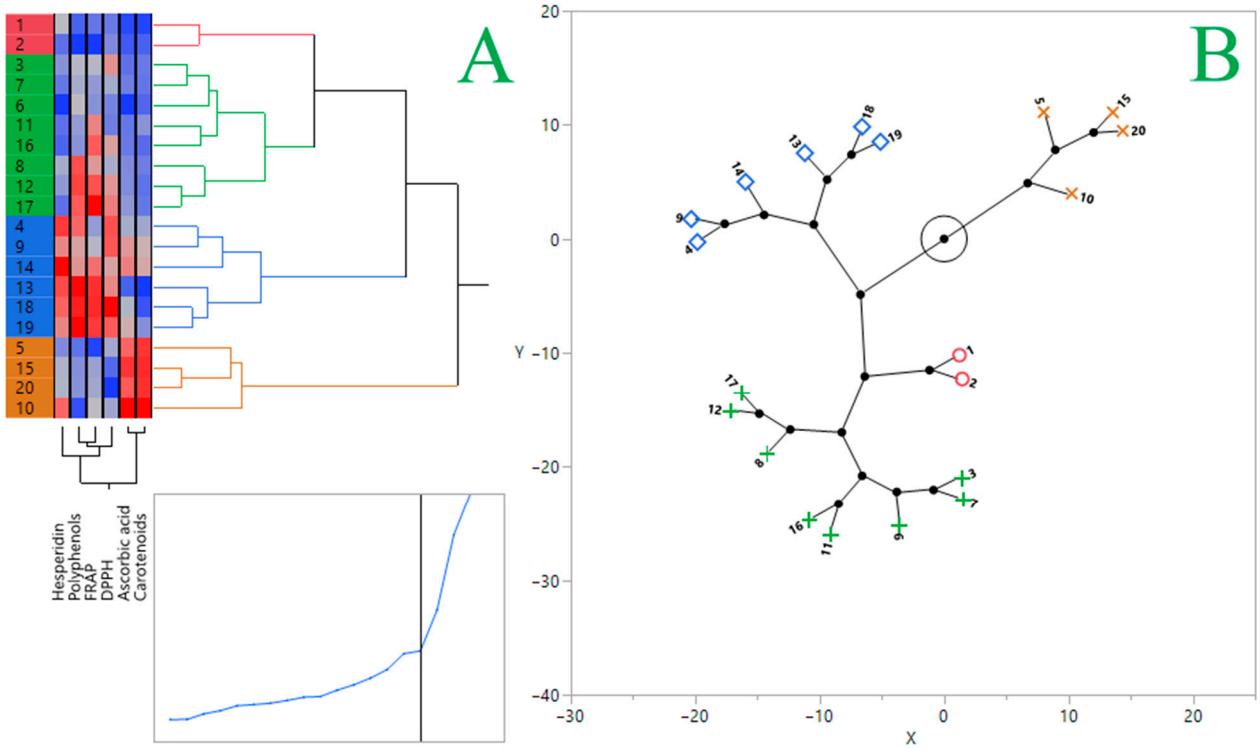


Figure 2. Hierarchical cluster analysis for the optimal extraction of the orange peels, using various extraction techniques and hydroethanolic solutions. Plot (**A**), hierarchical clustering as a dendrogram, and plot (**B**), constellation plot.

3.2. Antioxidant Properties of the Extracts

With regard to the FRAP values of the extracts, the extraction technique (X_1), and the $X_2 \times X_2$ interaction were found to be statistically significant parameters ($p < 0.05$). As can be seen in Figure S3, the optimum extraction technique to maximize FRAP was the combination of both the PEF and US, prior to ST. Moreover, the ethanol concentration in the extraction solvent was found to have a great effect on the FRAP values, with increasing the concentration of ethanol resulting in the lower FRAP values. On the contrary, the extraction time and temperature had a lower impact on the FRAP values of the extracts. The FRAP values of the extracts ranged from 13.08 to 110.67 μmoL AAE/g dw, suggesting that the examined parameters, if finely tuned, can cause a 10-fold increase in the FRAP values. As regards the DPPH values of the extracts, the interactions $X_1 \times X_2$, $X_2 \times X_2$, $X_3 \times X_3$, $X_2 \times X_4$, and $X_3 \times X_4$, were found to be statistically significant parameters ($p < 0.05$). From Figure S4 it can be seen that the optimum extraction technique is the use of PEF, prior to ST, while the optimum extraction solvent is the 50% (*v*/*v*) ethanol: water mixture. Contrarily to the abovementioned results, it was evident that the higher the extraction time and temperature, the lower the DPPH values of the extracts. The DPPH scavenging activity of the extracts ranged between 26.58 and 81.23 μmoL DPPH/g dw.

Based on our results, it was evident that the extracts with the highest antioxidant activity can be prepared in a short time (i.e., 15 min), albeit at a different temperature. For the maximum activity in the DPPH assay, a temperature of 35 °C is suggested, since, at higher extraction temperatures, the DPPH scavenging activity decreases, suggesting that the extracted compounds are temperature sensitive, and probably degraded to phenolic acids [22]. A similar decrease in the DPPH activity of the orange peel extracts, with the increasing extraction temperature, was also recorded by Haya et al. [23]. The decrease in the DPPH activity, in relation to the extraction temperature, follows the same trend as the TPC (Figures S2 and S4), suggesting that the observed effect may be due to the extracted polyphenols, to a high extent. As regards the maximum activity to the FRAP assay, the

higher the extraction temperature, the higher the FRAP activity, with the optimum extract prepared at 80 °C. This suggests that the compounds responsible for the observed effect, are more thermally stable, compared to the previous case. As can be seen in Figures S3 and S5, the AA content and the FRAP activity has the same trend, as regards the temperature, suggesting that the FRAP activity, may be attributed, to a higher extent, in the AA content of the extracts.

3.3. TPC of the Extracts

As regards the TPC of the extracts, the technique (X_1) was the only statistically significant parameter, besides the interactions $X_1 \times X_1$, $X_1 \times X_2$, $X_2 \times X_2$, $X_1 \times X_4$, $X_2 \times X_4$, and $X_4 \times X_4$. From Figure S2, it can be seen that the TPC is maximized when the PEF and US are used before the ST. Moreover, 50% (*v*/*v*) ethanol in water is the optimum extraction solvent. The TPC was not found to vary significantly with the extraction time, while the temperature of the extraction was found to be significant, with the increasing temperatures achieving a lower TPC. This can be due to the degradation of the polyphenolic compounds, as discussed above. The TPC of the extracts ranged between 13.27 and 34.71 mg GAE/g dw. In previous studies, it was demonstrated that the use of the US or PEF can increase the TPC of the prepared extracts [14,24]. However, to the best of our knowledge, up until now, the two techniques have not been used in combination, so as to examine their combinatorial effect. Based on our findings, the use of both techniques can maximize the TPC of the extracts. In the study of Ntourtoglou et al. [25], it was also reported that the use of the PEF prior to the US can increase the TPC of grape stem extracts by up to 35%.

3.4. Hesperidin Content of the Extracts

Regarding the hesperidin (HSP) content of the extracts, the extraction technique (X_1), the ethanol concentration (X_2), and the extraction time (X_3) were found to be statistically significant parameters ($p < 0.05$), as well as the interactions $X_1 \times X_1$, $X_2 \times X_2$, $X_3 \times X_3$, $X_2 \times X_4$, $X_3 \times X_4$, and $X_4 \times X_4$. From Figure S1, it can be seen that more HSP is extracted, as the concentration of ethanol in the extraction solvent increases up to 75%, while a marginally lower quantity is extracted with pure ethanol. The results are similar to or better than previous studies [26–29]. In these studies, focusing on the extraction of HSP from citrus fruit peels, it was evident that the optimum concentration of ethanol or methanol in water, was approximately 70% (*v*/*v*). This can be justified by the lower polarity of the solvent, compared to plain water, which is more suitable for HSP (the log octanol/water partition coefficient is ~0.3, according to the European Chemicals Agency) and the fact that HSP is nearly insoluble in water. Furthermore, both methanol and ethanol were examined for their potential to extract HSP, and in many cases, methanol was found to be more suitable. However, methanol was not examined in our case, since the methanolic extracts cannot be used directly in the food industry, due to the toxicity of methanol. Thus, further steps are needed to remove methanol, rendering the whole method more time- and energy-consuming. Therefore, we examined only ethanol, which can be used directly for the preparation of alcoholic beverages. As regards the extraction temperature, the HSP content of the extracts increased as the temperature increased. This is in contrast with the TPC of the extracts. This can be justified by the high thermal stability that HSP exhibits, as well as the fact that as the temperature increases, the solubility of HSP increases, resulting in a higher content [29].

3.5. AA Content and the TCC of the Extracts

Regarding the AA content of the extracts, the composition of the solvent (X_2) was found to be a statistically significant parameter ($p < 0.05$), as well as the interactions $X_1 \times X_1$, and $X_2 \times X_2$. As can be seen in Figure S5, the use of the US only, prior to the ST, was found to be the most beneficial technique to maximize the extraction yield. In addition, the maximum content in AA was achieved in the extracts that were prepared with pure ethanol. Finally, the extraction time and temperature had no significant effect

in the extraction yield, suggesting that the extracts with an increased content in AA can be prepared fast, with a low energy consumption. In the study of Escobedo et al. [16], it was reported that the highest amount of AA that could be extracted was 98.2 mg/100 g. Similarly, Elkhatim et al. [30] reported that 100 g of the whole peel of oranges contained 110.4 mg of ascorbic acid. Based on these, it can be inferred that the PEF and ultrasound system greatly enhanced the amount of vitamin C.

As regards the TCC of the extracts, the composition of the solvent (X_2), and the interaction $X_2 \times X_2$ were found to be statistically significant parameters ($p < 0.05$). The maximum TCC can be achieved by employing the PEF prior to the ST and pure ethanol. Similarly, in the case of AA, the temperature and time of the extraction did not significantly affect the TCC of the extracts. Although there are reports suggesting the use of non-polar solvents for the extraction of carotenoids, such as hexane [31], it is known that the main carotenoid in orange peels is violaxanthin, which can readily be extracted by more polar solvents, due to the polar functional groups of the molecule [32,33].

3.6. Albedo and Flavedo Extracts

In order to have a better overview of the part of the peel that contains most of the compounds, the albedo (inner/white part of the peel) and flavedo (the outer/orange part of the peel) of the peels were also examined. They were subjected to extraction with the optimum parameters for each examined variable, as described in Table 4. The results can be seen in Table 5 (in the case of the polyphenols, the extraction was carried out, according to the optimum conditions for the TPC). As can be seen, the flavedo extract contains all the examined compounds (aside from narirutin) at a much higher concentration, compared to the albedo part.

Table 5. Composition of the extracts from the albedo and flavedo parts of the orange peels, prepared using the optimum conditions for each parameter.

Examined Parameter	Albedo	Flavedo
HSP (mg/g dw)	9.2 ± 0.7	12.8 ± 0.9
TPC (mg GAE/g dw)	9.2 ± 0.3	14.9 ± 0.5
FRAP (μmol AAE/g dw)	9.5 ± 0.6	29.7 ± 0.3
DPPH (μmol DPPH/g dw)	34 ± 3	122 ± 2
AA (mg/100 g dw)	448 ± 2	1381 ± 5
TCC (μg CtE/g dw)	36 ± 3	48.7 ± 0.4
Caffeic acid (mg/g dw)	0.05 ± 0.01	0.14 ± 0.02
Ferulic acid (mg/g dw)	0.05 ± 0.01	0.14 ± 0.03
Narirutin (mg/g dw)	1.9 ± 0.1	0.30 ± 0.02
Neochlorogenic acid (mg/g dw)	0.05 ± 0.01	0.39 ± 0.02
Chlorogenic acid (mg/g dw)	0.16 ± 0.01	0.95 ± 0.05

4. Conclusions

In this study, the parameters that affect the extraction of the bioactive compounds from orange peels were optimized using a response surface methodology approach. Aside from the main extraction parameters, two additional pretreatment techniques were examined (PEF and US). It was evident that for the examined compounds in the extract, different extraction conditions are needed, so as to achieve the maximum recovery. Moreover, the addition of the pretreatment techniques was a major benefit for the proposed extractions, since they significantly increased the amount of the bioactive compounds. Therefore, these techniques are highly recommended as a pretreatment step, since they are environmentally friendly, have a low energy consumption, are fast, and can easily be used for the large-scale preparation of extracts. Overall, our results can serve as a benchmark for future studies, aiming to further optimize the extraction of the bioactive compounds, as well as they can be used for the preparation of the extracts, so as to result in a more sustainable waste orange peel management system. The advantage of using the proposed optimized

extraction parameters is the maximization of the extraction yield of the target compound(s), in the most efficient way, resulting in extracts that can more easily be used in the food and beverage industries. The practical applications of the proposed procedure include the production of orange peels extracts that can be used in the food and beverage industries, for cosmetics production, for pharmaceutical preparation, as well as natural pigments to replace synthetic pigments. A limitation of this study is the examination of one variety of oranges. Further studies should focus on other varieties of oranges, as well as to consider further optimization of the parameters that affect the PEF and the US treatment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su142113926/s1, Figure S1: Plots of the actual vs. predicted response (Hesperidin, mg/g) (plot A) and the desirability function with the extrapolation control (plot B) were created to optimize the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. Statistics pertaining to the assessment of the resulting model are provided in the inset tables. Asterisks and colored values indicate the statistically significant values; Figure S2: Plots of the actual vs. predicted response (Polyphenols, mg GAE/g) (plot A) and the desirability function with the extrapolation control (plot B) were created to optimize the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. Statistics pertaining to the assessment of the resulting model are provided in the inset tables. Asterisks and colored values indicate the statistically significant values; Figure S3: Plots of the actual vs. predicted response (FRAP, μmoL AAE/g) (plot A) and the desirability function with the extrapolation control (plot B) were created to optimize the extraction of the orange peel using various extraction techniques and hydroethanolic solutions. Statistics pertaining to the assessment of the resulting model are provided in the inset tables. Asterisks and colored values indicate the statistically significant values; Figure S4: Plots of the actual vs. predicted response (DPPH, μmoL DPPH/g) (plot A) and the desirability function with the extrapolation control (plot B) were created to optimize the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. Statistics pertaining to the assessment of the resulting model are provided in the inset tables. Asterisks and colored values indicate the statistically significant values; Figure S5: Plots of the actual vs. predicted response (ascorbic acid, mg/100 g) (plot A) and the desirability function with the extrapolation control (plot B) were created to optimize the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. Statistics pertaining to the assessment of the resulting model are provided in the inset tables. Asterisks and colored values indicate the statistically significant values; Figure S6: Plots of the actual vs. predicted response (Carotenoids, μg CtE/g) (plot A) and the desirability function with the extrapolation control (plot B) were created to optimize the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. Statistics pertaining to the assessment of the resulting model are provided in the inset tables. Asterisks and colored values indicate the statistically significant values; Figure S7: 3D graphs depicting the effect of the process variables considered in the response (Hesperidin, mg/g), to optimize the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. 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: 3D graphs depicting the effect of the process variables considered in the response (Polyphenols, mg GAE/g), to optimize the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. 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: 3D graphs depicting the effect of the process variables considered in the response (FRAP, μmoL AAE/g), to optimize the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. 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: 3D graphs depicting the effect of the process variables considered in the response (DPPH, μmoL DPPH/g), to optimize the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. 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: 3D graphs depicting the effect of the process variables considered in the response (ascorbic acid, mg/100 g), to optimize

the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. 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 S12: 3D graphs depicting the effect of the process variables considered in the response (Carotenoids, µg CtE/g), to optimize the extraction of the orange peel, using various extraction techniques and hydroethanolic solutions. 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 S13: Representative chromatogram of an orange peel extract, at 320 nm, depicting the identified polyphenols; Table S1: Coded values of the four independent variables investigated and the actual concentration of the phenolic compounds; Results are expressed in mg/g dw; ND: not detected.

Author Contributions: Conceptualization, V.A., T.C. and S.I.L.; methodology, V.A. and T.C.; software, V.A.; validation, V.A.; formal analysis, V.A. and T.C.; investigation, K.K., D.P. and E.B.; resources, S.I.L.; data curation, K.K., D.P. and E.B.; writing—original draft preparation, K.K.; writing—review and editing, V.A., T.C., K.K., D.P., E.B. and S.I.L.; visualization, V.A.; supervision, S.I.L.; project administration, V.A. and T.C. All authors have read and agreed to the published version of the manuscript.

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