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Process Optimization and Stability of Waste Orange Peel Polyphenols in Extracts Obtained with Organosolv Thermal Treatment Using Glycerol-Based Solvents

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Abstract: This study was focused on the simultaneous organosolv treatment/extraction of waste orange peels (WOP) for the effective recovery of polyphenolic antioxidants. The treatments were performed with aqueous glycerol mixtures, which were acidified either with citric acid or hydrochloric acid (HCl). Process optimization was carried out using response surface methodology and comparative appraisal of the different processes tested, based on both the extraction efficiency factor (F_{EE}), severity factor (SF) or combined severity factor (CSF). Metabolite stability was also of major concern, and it was examined by deploying liquid chromatography-mass spectrometry. The results drawn suggested 90% (*w/w*) glycerol to be the highest-performing system, providing a yield in total polyphenols of 44.09 ± 5.46 mg GAE g^{-1} DM at 140 °C for 50 min, with a F_{EE} of 2.20 and an SF of 2.88. Acidification with 1% citric acid was proven less efficient and equally severe, whereas acidification with 1% HCl was less severe but also less efficient. The major disadvantage associated with the use of HCl was its detrimental impact on the polyphenolic composition of WOP since major metabolites, such as narirutin, hesperidin and didymin, did not survive the process. By contrast, the formation of lower molecular weight compounds was observed. With regard to antioxidant properties, the extract obtained with aqueous glycerol displayed significantly higher antiradical activity and reducing power, which was in line with its higher concentration in total polyphenols. It was concluded that organosolv treatment with aqueous glycerol under the conditions employed may boost polyphenol recovery from WOP, thus giving extracts with powerful antioxidant characteristics.

Keywords: antioxidants glycerol; organosolv treatment; polyphenols; waste orange peels

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1. Introduction

As the world's population is rapidly expanding, there is an increasing need for food production, which is accompanied by bioresource depletion, a generation of by-products and wastes, and, in several instances, severe ecosystem degradation. The residual biomass that is regularly produced by the agri-food sector may end up as waste dumped in landfills, resulting in environmental aggravation with serious associated health risks. However, it is now a well-consolidated knowledge that side streams originating from various agricultural activities and food processing, such as pruning, post-harvest handling, food production and consumption, represent an enormous pool of precious compounds. On this ground, circular economy strategies, based on innovative biorefinery concepts, have fostered the development of a range of value-added products destined for the food, pharmaceutical and cosmetics industries [1,2].

Amongst an assortment of economically important constituents that may be encountered in plant food processing residues (roots, leaves, peels, stems, seeds, etc.), polyphenols

have a prominent role as bioactive principles. This large family of secondary metabolites embraces several subclasses of substances, such as simple phenolic acids (e.g., gallic and protocatechuic acids), hydroxycinnamates (caffeic and ferulic acids, and their derivatives), and flavonoids (e.g., flavonols, flavones, flavanones and their glycosides). Numerous secondary metabolites have been proven to exert highly significant biological properties, including antioxidant, anti-inflammatory, antimicrobial, cardioprotective and chemoprotective effects [3,4]. As such, industrial interest has mainly focused on valorization technologies aiming at the exploitation of polyphenol-rich biomass [5,6].

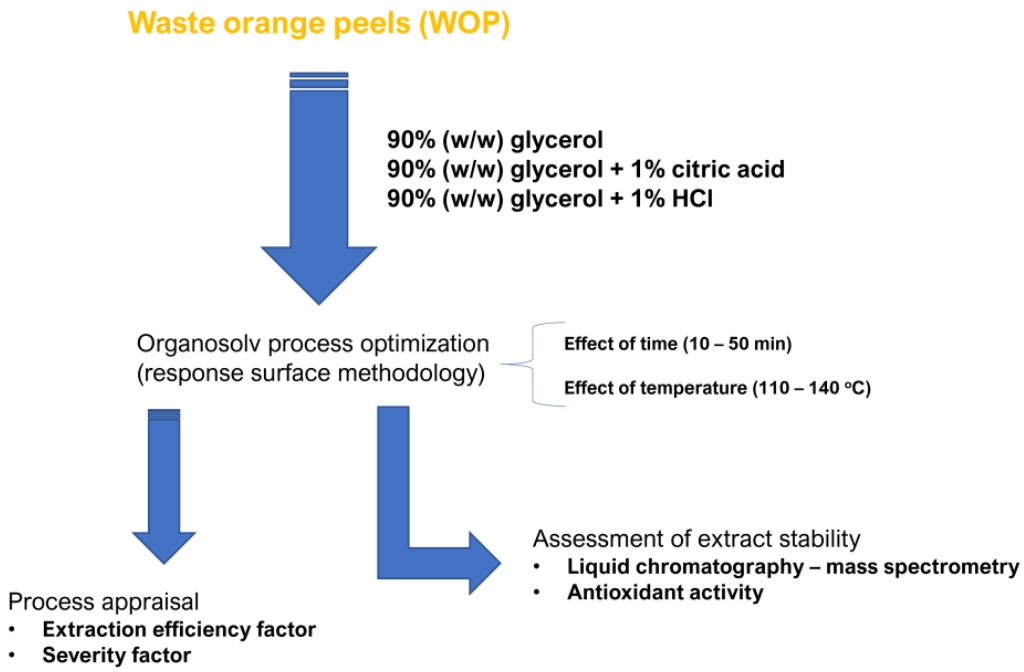
Citrus is, globally, the largest fruit crop, with oranges accounting for 60% of the total. The major processing deployed for oranges is the production of juice, where the yield is almost 50% on a fresh fruit weight basis. The remaining 50% is composed of residual peel, pulp, seeds, and discarded whole orange fruits [7]. The worldwide production of oranges in 2019 was 46 million metric tons, of which about 37% were further processed. As such, wastes generated from orange processing represent one of the largest sources of food processing waste. Therefore, it is imperative to establish feasible strategies for reuse/valorization of orange processing residues, with emphasis on high value-added products, including pectins, essential oil, polyphenols and a series of other commodities [8]. These compounds have been reported to have a wide range of industrial applications, mainly in the food, pharmaceutical and cosmetic sector. Furthermore, numerous studies have documented various pharmacological properties of citrus compounds, such as antimicrobial, antioxidant, anti-inflammatory, anti-cancer, etc., which have boosted their utilization as health supplements and nutraceuticals [9].

Citrus flavonoids are a class of bioactive compounds, which occur largely in orange peels and may exhibit an array of activities, including anticancer, anti-inflammatory, antioxidant, and cardioprotective [10–12]. Owed to these properties, orange peel flavonoids have been extensively investigated, and a significant number of techniques for effective solid–liquid extraction have been proposed [13,14]. These techniques, which aim at optimizing polyphenol recovery, are solid–liquid extraction procedures implemented on the basis of the nature of the target compounds, solvent properties and their toxicity, cost, etc. In the Green Chemistry framework, the use of alternative solvents (non-petroleum-based liquids) is both an attractive and challenging concept for establishing eco-friendly processes for solid–liquid extraction [15,16].

In various solid-liquid extraction techniques, such as, i.e., ultrasound-assisted extraction, one of the major objectives is the decomposition of plant cell walls, which would buttress the release of intracellular metabolites (polyphenols) and their transfer into the liquid phase [17]. This, in turn, would increase mass transfer and, eventually, extraction yield. The disorganizing and/or partial degradation of plant cell wall biopolymers, such as hemicellulose and lignin, may very well be achieved through treatments involving thermal processing at elevated temperatures [18]. These technologies may be characterized as hydrothermal treatments when performed with water or aqueous solvent systems, or organosolv treatments, involving processing with organic solvents in combination with high temperature/high pressure [19]. The principal objective of such a procedure is biomass pretreatment to untangle cellulose–hemicellulose–lignin networks, to facilitate subsequent saccharification and sugar fermentation. However, partial polysaccharide decomposition would also assist polyphenol liberation from complex matrices, thus facilitating polyphenol extraction [20,21].

A recent investigation revealed that thermal treatments may be boosted by glycerol [22], and combinations of glycerol with HCl [23]. On such a ground, this examination aimed at deploying a simultaneous organosolv thermal treatment/extraction, targeting the recovery of antioxidant polyphenols from waste orange peels (WOP). The solvents used were glycerol and glycerol-based acidified aqueous mixtures to spot possible effects of the different solvents on the composition but also the stability of the extracts. Particular focus was on the changes in the polyphenolic profile, employing liquid chromatography–mass spectrometry. An overview of the experimental design is provided in Scheme 1. To the

best of the authors' knowledge, this is the first study on such a process implemented for polyphenol extraction from WOP.



Scheme 1. Overview of the experimental design of this study.

2. Materials and Methods

2.1. Chemicals

Hesperidin (hesperetin 7-*O*-rutinoside), hesperetin, ascorbic acid, sodium carbonate, 2,2-diphenylpicrylhydrazyl (DPPH), and gallic acid were from Sigma-Aldrich (Darmstadt, Germany). Iron(III) chloride hexahydrate and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Honeywell/Fluka (Steinheim, Germany). Ethanol was from Honeywell/Riedel-de Haen (Seelze, Germany). Folin–Ciocalteu reagent, citric acid and glycerol (99%) were from Merck (Darmstadt, Germany). Solvents of appropriate purity (HPLC grade) were used for chromatographic analyses.

2.2. Waste Orange Peels (WOP)

WOPs were collected shortly after the dejuicing of fresh oranges, from a catering facility (Chania, Greece), transferred to the laboratory within 30 min, and cut into pieces with a sharp cutter at an approximate size of 3 × 3 cm. WOP pieces were placed on aluminium trays to form layers with a thickness of no more than 1 cm and then dried in a laboratory oven (Binder BD56, Bohemia, NY, USA) for 7 h at 60 °C. The dried WOPs were then milled in a table domestic grinder and sieved to yield a powder with an average particle diameter of 0.850 mm. This material was stored in air-tight vessels at 4 °C.

2.3. Organosolv Treatment/Extraction Process

A volume of 10 mL of solvent was placed in a 25-mL Duran™ glass vial, and the vial was screw-capped. Heating at the desired temperature was accomplished by means of a temperature-controlled hot plate (Witeg, Wertheim, Germany), according to the experimental design (Table 1), and then 1 g of WOP was transferred into the vial. Treatment of the mixture was performed under continuous stirring at 400 rpm, for a predetermined resident time defined by the experimental design. The solvents used were glycerol (90% *w/w*, pH = 3.50), termed as GL, GL + 1% (*w/v*) citric acid (pH = 2.2), termed as GL + CA, and GL + 1% (*v/v*) HCl (pH = 0.35), termed as GL + HCl. Control extraction with distilled water was performed for 60 min at 70 °C, and with 60% (*v/v*) ethanol for 185 min at 70 °C, at the

same stirring speed. These conditions were selected based on the average values reported elsewhere [24]. After treatment, extracts were centrifuged at 10,000× *g* for 10 min, and the clear supernatant was used for all analyses.

Table 1. Process variables and their corresponding coded and actual levels used to set up the experimental design.

Process Variables	Codes	Coded Variable Level		
		−1	**0**	**1**
t (min)	X_1	10	30	50
T (°C)	X_2	110	125	140

2.4. Response Surface Methodology and Process Optimization

The organosolv process was optimized with respect to two key variables, temperature (*T*) and time (*t*), by deploying a central composite experimental design with 11 points, including three central points. Three coded levels (−1, 0 and 1) were set for each process (independent) variable, as dictated by the experimental design, and codification was performed as described elsewhere [25]. Coded and actual levels are presented in Table 1. The ranges used for each variable were chosen based on preliminary experiments and recent data [26]. The significance of individual model (equations) coefficients, as well as the overall significance of the models (R^2, *p*) were assessed by lack-of-fit and Analysis of variance (ANOVA) tests, at least at a 95% significance level.

2.5. Extraction Efficiency Factor

This factor relates the total polyphenol extraction yield to the extraction temperature and time as follows [24]:

$$\mathrm{EE} = \frac{\mathrm{Y_{TP}}}{t \times T}\ \mathrm{mg\ g^{-1}\ min^{-1}\ {}^{\circ}C^{-1}} \quad (1)$$

where EE is the extraction efficiency, Y_{TP} is the yield in total polyphenols expressed as mg gallic acid equivalents (GAE) per g dry mass, *t* is the extraction time given in min and *T* is the extraction temperature, expressed in °C. The extraction efficiency factor (F_{EE}) is defined as:

$$F_{EE} = -log(\mathrm{EE}) \quad (2)$$

2.6. Severity Factor (SF)

This factor describes the severity of the organosolv treatment, as a function of resident time, *t*, and temperature, *T* [27], and it may be used to compare the different extraction conditions [28]:

$$R_0 = t \times e^{\left(\frac{T-100}{14.75}\right)} \quad (3)$$

$$\mathrm{SF} = logR_0 \quad (4)$$

R_0 and the value 100 °C represent the severity of the reference temperature, respectively, while the value 14.75 is an empirical parameter related to temperature and activation energy. For the HCl-catalyzed treatment (GL + HCl), the effect of pH was taken into consideration by using the combined severity factor (CSF) [29]:

$$\mathrm{CSF} = logR_0 - \mathrm{pH} \quad (5)$$

2.7. Total Polyphenol and Antioxidant Activity Determination

Total polyphenol determination was carried out with the Folin–Ciocalteu methodology, adopting a previously published protocol [30]. In short, 0.05 mL of Folin–Ciocalteu reagent was mixed with 0.02 mL of the sample and 0.78 mL of deionized water. After exactly 1 min, 0.15 mL of sodium carbonate (20%) was added, and the mixture was allowed to react for

60 min. The absorbance was obtained at 750 nm and the results were given as mg total polyphenols per g dry mass (DM), using gallic acid as the calibration standard.

For the ferric-reducing power (P_R), 0.05 mL of $FeCl_3$ (4 mM in 0.05 M HCl) and 0.05 mL of the sample were incubated for 30 min in a water bath at 37 °C. Then, 0.9 mL of TPTZ (1 mM in 0.05 M HCl) was added, and absorbance readings were recorded at 620 nm after exactly 5 min. The P_R was determined as µmol ascorbic acid equivalents (AAE) per gram of DM, using an ascorbic acid calibration curve (0.02–0.2 mM) [30].

For the antiradical activity (A_{AR}), 0.025 mL of the sample was mixed with 0.975 mL of DPPH (100 µM in methanol) and the absorbance at 515 nm was obtained immediately after mixing ($A_{515(i)}$), and after 30 min ($A_{515(f)}$). AAR was then determined as follows:

$$A_{AR}\left(\mu\text{mol DPPH g}^{-1}\text{ dw}\right) = \frac{C_{DPPH}}{C_{TP}} \times \left(1 - \frac{A_{515(f)}}{A_{515(i)}}\right) \times Y_{TP} \quad (6)$$

where C_{DPPH} is the initial molar concentration of DPPH (µmol L^{-1}), C_{TP} is the total polyphenol concentration of the extract expressed as mg GAE per liter, and Y_{TP} is the extraction yield in mg GAE g^{-1} DM. A_{AR} was expressed as µmol DPPH per g DM [30].

2.8. Liquid Chromatography–Diode Array–Mass Spectrometry (LC–DAD–MS) Analyses

All chromatographic analyses were performed by deploying a previously reported methodology [31]. Briefly, a Finnigan AQA mass spectrometer (San Jose, CA, USA), a P4000 pump, and a UV6000LP diode array detector were used. Chromatographic separations were carried out at 40 °C, with a Fortis RP-18 column, 150 mm × 2.1 mm, 3 µm, with a 10-µL injection loop. Electrospray ionization (ESI), in positive ion mode, was used to acquire mass spectra. Chromatographic setup, regarding the elution program and acquisition of mass spectra, has been given in detail elsewhere [31].

2.9. Statistical Processing

The organosolv treatments were repeated at least twice, and all analytical determination were in triplicate. The values given are means ± standard deviation. JMP™ Pro 13 software (SAS, Cary, NC, USA) was used to set up the design of the experiment and to carry out statistical treatment for the response surface methodology and distribution analyses. Linear regressions were done with SigmaPlot™ 12.5 (Systat Software Inc., San Jose, CA, USA).

3. Results and Discussion

3.1. Organosolv Process Modeling

The process was designed to assess the simultaneous influence of two critical variables (t and T) and to detect possible synergistic effects between them. The assessment of the models established, and the response surface suitability was done considering the lack-of-fit and ANOVA tests (Figures A1–A3, inset tables) and the proximity of the predicted and measured values (Table 2). The mathematical models (second-degree polynomial equations), given by including only the significant terms, are shown in Table 3, along with the R^2 and *p*-values. For all three models, R^2 was equal to or higher than 0.96, and the *p*-values for lack-of-fit (confidence interval of 95%) were highly significant. Thus, it can be supported that the models had a very satisfactory adjustment to the experimental data and that the process variables tested accounted for at least 96% of the variability of Y_{TP}. Therefore, only about 4% of the variability may be attributed to other factors. The three-dimensional diagrams deriving from the models (Figure 1) provide the visualized effect of the process variables on the response (Y_{TP}), but also depict the differences between the three solvents used.

Table 2. Measured and predicted response values for each design point considered for the deployment of response surface methodology.

Design Point	Independent Variables		Response (Y_{TP}, mg GAE g^{-1} DM)					
	t (min) (X_1)	*T* (°C) (X_2)	GL		GL + HCl		GL + CA	
			Measured	Predicted	Measured	Predicted	Measured	Predicted
1	10 (−1)	110 (−1)	11.63	12.31	13.04	11.84	16.67	17.21
2	10 (−1)	140 (1)	20.02	21.74	27.48	27.84	22.61	21.81
3	50 (1)	110 (−1)	16.85	17.02	28.65	28.46	25.61	25.69
4	50 (1)	140 (1)	42.86	44.09	23.02	24.33	36.92	35.66
5	10 (−1)	125 (0)	15.05	12.58	20.38	21.18	19.28	19.54
6	50 (1)	125 (0)	27.51	26.11	28.86	27.73	29.53	30.70
7	30 (0)	110 (−1)	16.28	15.43	23.07	24.42	20.39	19.77
8	30 (0)	140 (1)	36.65	33.68	32.04	30.35	25.04	27.05
9	30 (0)	125 (0)	19.68	20.10	28.31	28.72	25.12	23.44
10	30 (0)	125 (0)	18.40	20.10	28.35	28.72	22.99	23.44
11	30 (0)	125 (0)	18.39	20.10	29.18	28.72	23.76	23.44

Table 3. Mathematical models of extraction optimization, generated after deploying response surface methodology.

Solvent	2nd Order Polynomial Equations	R^2	*p*
GL	$20.10 + 6.76X_1 + 9.12X_2 + 4.41X_1X_2 + 4.45{X_2}^2$	0.97	0.0009
GL + HCl	$28.72 + 3.28X_1 + 2.97X_2 - 5.03\ X_1X_2 - 4.27{X_1}^2$	0.96	0.0012
GL + CA	$23.44 + 5.58X_1 + 3.64X_2$	0.96	0.0015

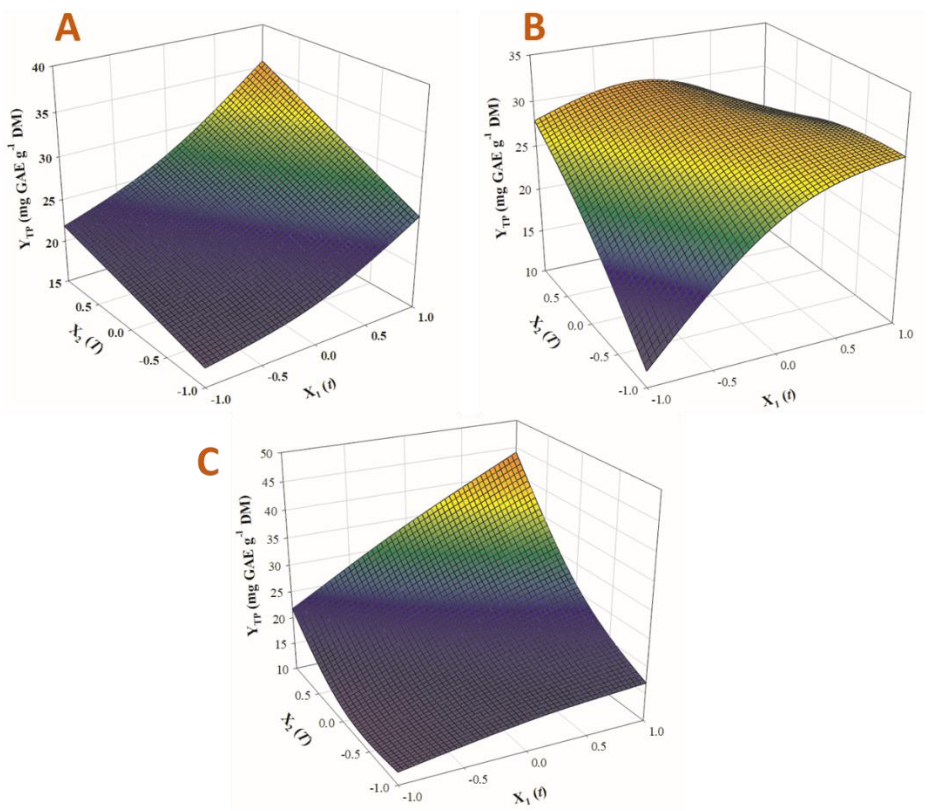


Figure 1. Three-dimensional graphs depicting the response (Y_{TP}) as a function of process variables. (**A**), extraction with GL + CA; (**B**), extraction with GL + HCl; (**C**), extraction with GL.

For the process with GL, both *t* (X_1) and *T* (X_2) were significant, and they had a positive effect on the response. The same held true for their cross term (X_1X_2), but also the quadratic term of *T* (X_2^2) (Table 3). Likewise, for the process with GL + CA, both *t* (X_1) and *T* (X_2) were significant, but no significant cross or quadratic terms were seen. The process with GL + HCl exhibited a differentiated pattern, in that the processing time, beyond a certain limit, had a negative impact on the response. This was manifested by both the negative cross term X_1X_2 and the quadratic term of X_1^2. For each process, the maximum predicted response was calculated using the desirability function (Figures A1–A3), and presented in Table 4, along with the predicted optimum *t* and *T* values. Both processes with GL and GL + CA required 50 min at 140 °C to attain maximum Y_{TP}, whereas the process with GL + HCl was significantly less demanding in resident time. However, under optimum conditions, the process with GL + HCl afforded a maximum Y_{TP} of 30.54 mg GAE g^{-1} DM, but the processes with GL + CA and GL were 16.8% and 35.5% more efficient, respectively.

Table 4. Maximum predicted responses under optimized extraction conditions, and the corresponding F_{EE} and *SF* (or *CSF*) values.

Solvent	Maximum Predicted Response (mg GAE g^{-1} DM)	Optimal Conditions		Indices	
		***t* (min)**	***T* (°C)**	F_{EE}	***SF* or *CSF***
GL	44.09 ± 5.46	50	140	2.20	2.88
GL + HCl	30.54 ± 2.63	26	140	2.08	2.24 *
GL + CA	35.66 ± 3.44	50	140	2.29	2.88
Water	13.24 ± 0.93	60	70	2.50	0.89
60% ethanol	14.21 ± 0.99	185	70	2.96	1.38

* Combined severity factor (CSF).

3.2. Efficiency and Severity of the Process

The processes deployed were appraised by both F_{EE} and SF (or CSF) to obtain a picture regarding both the efficiency and severity, on a quantitative basis. It can be seen in Table 4 that the F_{EE} determined for the GL + HCl process was 2.08, while the corresponding values for the processes with GL and GL + CA were 2.20 and 2.29. According to the categorization proposed by Morsli et al. [24], the process with GL + HCl may be characterized as being of moderate efficiency, but the processes with both GL and GL + CA were of low efficiency. Similarly, the SF for GL and GL + CA were identical (2.88), but the CSF for the GL + HCl treatment was 2.24. This indicated that the addition of HCl might contribute to lower process severity.

The yield in total polyphenols (Y_{TP}) achieved with GL under optimized conditions was 44.09 ± 5.46 mg GAE g^{-1} DM. This level was 30.1% higher than that attained with GL + HCl and 19% higher than that with HCl + CA. Compared to control extractions performed with water and 60% aqueous ethanol, the yield was 70% and 67.8% higher, respectively (Table 4). Yet, a critical issue pertaining to yield is the comparison with data reported in the literature. More specifically, the yields in total polyphenols were usually from 7 to more than 26 mg GAE g^{-1} DM, attained with various extraction techniques, such as microwave-assisted extraction [32,33], ultrasound-assisted extraction [34], cyclodextrin-aided extraction [35], etc. There is only one study reporting a yield of 75.77 mg GAE g^{-1} DM, obtained with deep eutectic solvent extraction [36]. On this ground, it could be argued that GL extraction under the optimized conditions established in this study could be a very effective means of producing WOP extracts enriched in antioxidant polyphenols. However, this claim merits profound investigation.

The differences observed apparently lay in the different compositions of the solvents tested. Considering the highly acidic pH of the GL + HCl, it might be hypothesized that this feature enabled optimal polyphenol extraction at a significantly shorter processing time. More rapid recovery of total polyphenols could be ascribed to faster and/or more effective decomposition/deconstruction of lignin–hemicellulose–cellulose complexes, which in turn, could enable faster entrainment of intracellular metabolites (polyphenols) into the liquid

(solvent). Such a phenomenon could be fostered by proton-catalyzed cleavage of some bonds of lignocellulosic materials, which might be significant for lignin/hemicellulose disintegration since this mechanism is involved in separating lignin from biomass. For example, it has been documented that organosolv treatments with deep eutectic solvents composed of a carboxylic acid exhibited higher performance, owed to the active carboxylic acid protons, which could facilitate proton-catalyzed reactions that result in the cleavage of bonds, such as glycosidic bonds, ether bonds and lignin-carbohydrate links [37]. Furthermore, acidified aqueous glycerol (90%, *w/w*, 1.2% HCl, *w/w*) has been reported to provide better glycan digestibility for rice husks. This finding implied that this solvent was effective in untangling lignin–hemicellulose–cellulose complexes [23].

In general, the use of various inorganic and organic acids has been shown to enhance the organosolv treatment of biomass and the extraction efficiency of polyphenols. Glycerol-based organosolv treatment with oxalic acid was shown to promote efficient disintegration of the cellulose–hemicellulose–lignin complex in sugarcane residues [38], and a similar outcome was also found for sulfuric acid-assisted glycerol pretreatment [39] and formic acid-catalyzed glycerol-based organosolv pretreatment [40] of sugarcane bagasse. Effective disintegration of flavedo and albedo tissues of orange peels was also performed with dilute aqueous solutions of glycolic acid [41]. Moreover, organosolv pretreatment using a combination of ethanol and sulfuric acid was successfully implemented for the extraction of polyphenols from spent coffee waste [42], while the addition of HCl in hydroethanolic solution exerted a positive effect on polyphenol extraction from red grape pomace [43]. On the other hand, negative effects of tartaric acid in combination with glycerol have also been demonstrated, with regard to polyphenol and pigment extraction from red grape pomace [44].

3.3. Effect on Metabolite Profile and Stability

Each of the optimally produced extracts with GL, GL + CA and GL + HCl, along with the control extracts obtained with water and 60% ethanol, was subjected to LC–DAD–MS analysis to depict the profile of major polyphenolic metabolites. Extracts produced with water, aqueous ethanol and GL displayed virtually the same pattern, irrespective of the relative abundance of the principal constituents (Figure 2). Based on the analytical data given in Table 5, peak 1 was tentatively assigned to 6,8-di-*C*-glycosylapigenin (vicenin-2), as previously proposed [45,46]. More particularly, this compound gave a molecular ion at m/z = 595, which was further confirmed by its Na^+ adduct at m/z = 617. Likewise, peak 2, tentatively identified as narirutin, yielded a molecular ion at m/z = 581, a Na^+ adduct at m/z = 603 and the aglycone fragment at m/z = 273. Peak 3 was assigned to hesperidin, based on its molecular ion at m/z = 611, its Na^+ adduct at m/z = 633 and the aglycone fragment at m/z = 303. Didymin (peak 4) was tentatively identified by the molecular ion at m/z =595, the Na^+ adduct at m/z = 617, and the aglycone (isosakuranetin) at m/z = 287. Peak 5 was tentatively ascribed to sinensetin, based on the molecular ion at m/z = 373 and its Na^+ adduct at m/z = 395. Finally, peaks 6 and 7 were tentatively identified as nobiletin and demethylnobiletin, respectively, based on their corresponding molecular ions at m/z = 403 and 389 [31,46].

On the other hand, the extract obtained with GL + HCl showed a highly diversified profile, where narirutin, hesperidin and didymin were not detected. By contrast, four new major peaks appeared and were assigned as A, B, C and D (Figure 2). Peaks A and B had identical UV-vis spectra, exhibiting λ_{max} at 274 nm, and they also yielded the same molecular ion (m/z = 213) and major fragment (m/z = 173). Likewise, peaks C and D had identical UV-vis spectra, exhibiting λ_{max} at 280 nm and molecular ions corresponding to m/z = 201, which was further confirmed by the Na^+ adduct at m/z = 223. A fragment ion at m/z = 195 was also detected.

These peaks presumably represent artifacts arising from the decomposition of other extract constituents; however, it was not clear whether the disappearance of narirutin, hesperidin and didymin was associated with the formation of A, B, C and D. Nevertheless,

it was evident that their formation was attributed to the presence of HCl since none of these compounds was detected in the extracts produced with the other solvents tested.

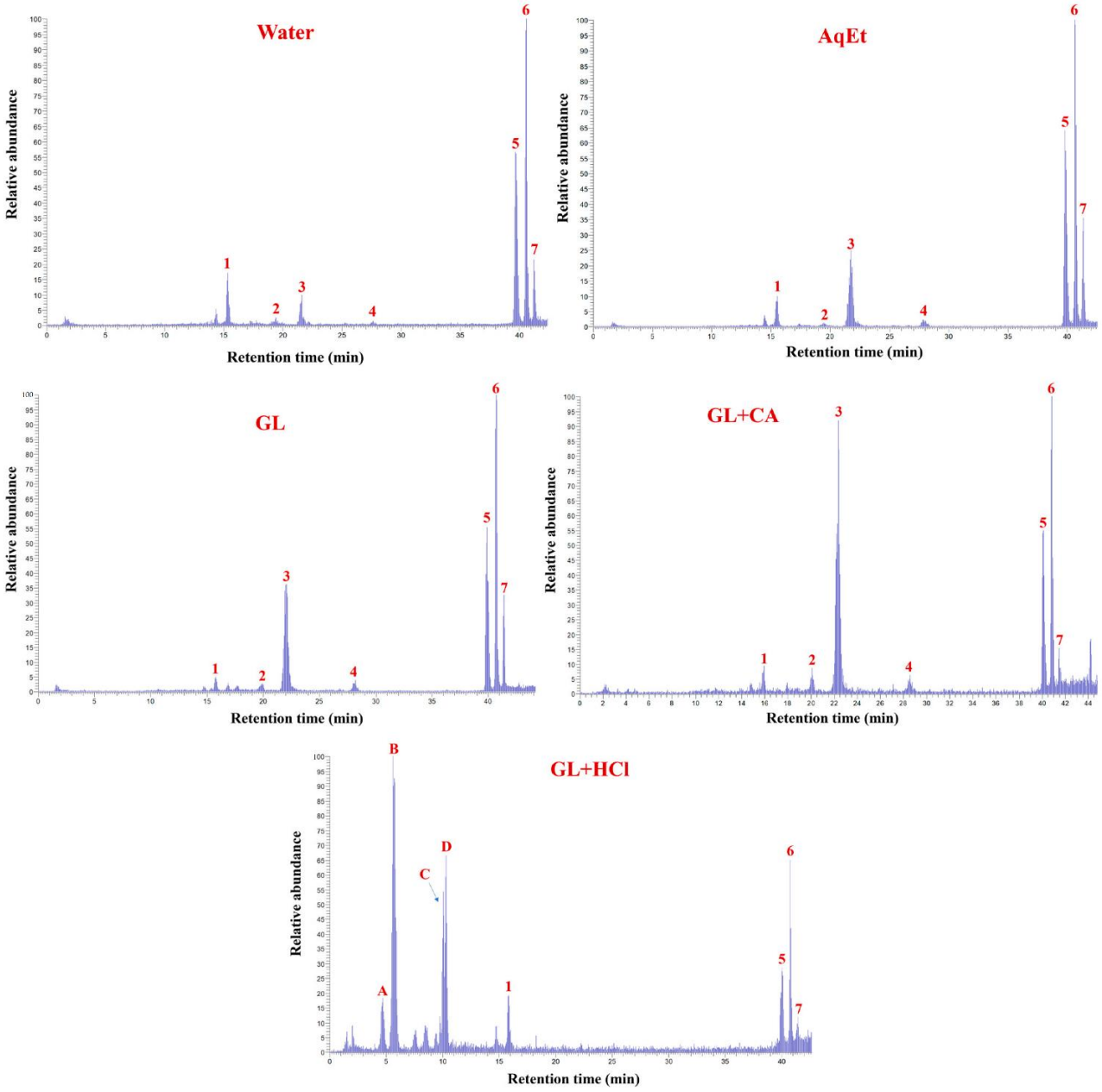


Figure 2. Selected ion chromatograms of WOP extracts produced with the solvents tested. AqEt corresponds to the extract produced with 60% (*v*/*v*) ethanol. Peak assignment: 1, apigenin 6,8-di-C-hexoside (vicenin-2); 2, narirutin; 3, hesperidin; 4, didymin; 5, sinensetin; 6, nobiletin; 7, demethylnobiletin; A, B, C and D, unknown degradation products.

3.4. Process Impact on Antioxidant Properties

The antioxidant activity of the extracts produced under optimized conditions was evaluated by measuring both the A_{AR} and P_R. The extract obtained with GL was found to be the most active based on both A_{AR} and P_R (Figure 3), and this supremacy was highly significant ($p < 0.05$). This outcome was in line with the richness of GL extracts in total polyphenols, as shown in Table 4. The extract produced with GL + HCl exhibited 17.3% higher A_{AR} and 16.7% higher P_R, respectively, when compared with GL + CA, although the extract with GL + CA was 14.4% richer in total polyphenol.

Table 5. UV-vis characteristics and mass spectroscopy information of the major polyphenolic compounds detected in the extracts analyzed.

Peak	Rt (min)	UV-Vis	$[M+H]^+$ (*m*/*z*)	Other Ions (*m*/*z*)	Tentative Identity
A	4.70	274	213	173	Unknown
B	5.64	274	213	173	Unknown
C	10.08	280	201	223 (Na^+ adduct), 195	Unknown
D	10.31	280	201	223 (Na^+ adduct), 195	Unknown
1	15.87	270, 340	595	617 (Na^+ adduct)	Apigenin 6,8-di-C-hexoside (vicenin-2)
2	19.92	286, 328	581	603 (Na^+ adduct), 273	Narirutin
3	22.02	280, 332	611	633 (Na^+ adduct), 303	Hesperidin
4	28.15	280, 330	595	617 (Na^+ adduct), 287	Didymin
5	39.86	248, 264, 334	373	395 (Na^+ adduct)	Sinensetin
6	40.69	254, 340	403	-	Nobiletin
7	41.35	270, 330	389	-	Demethylnobiletin

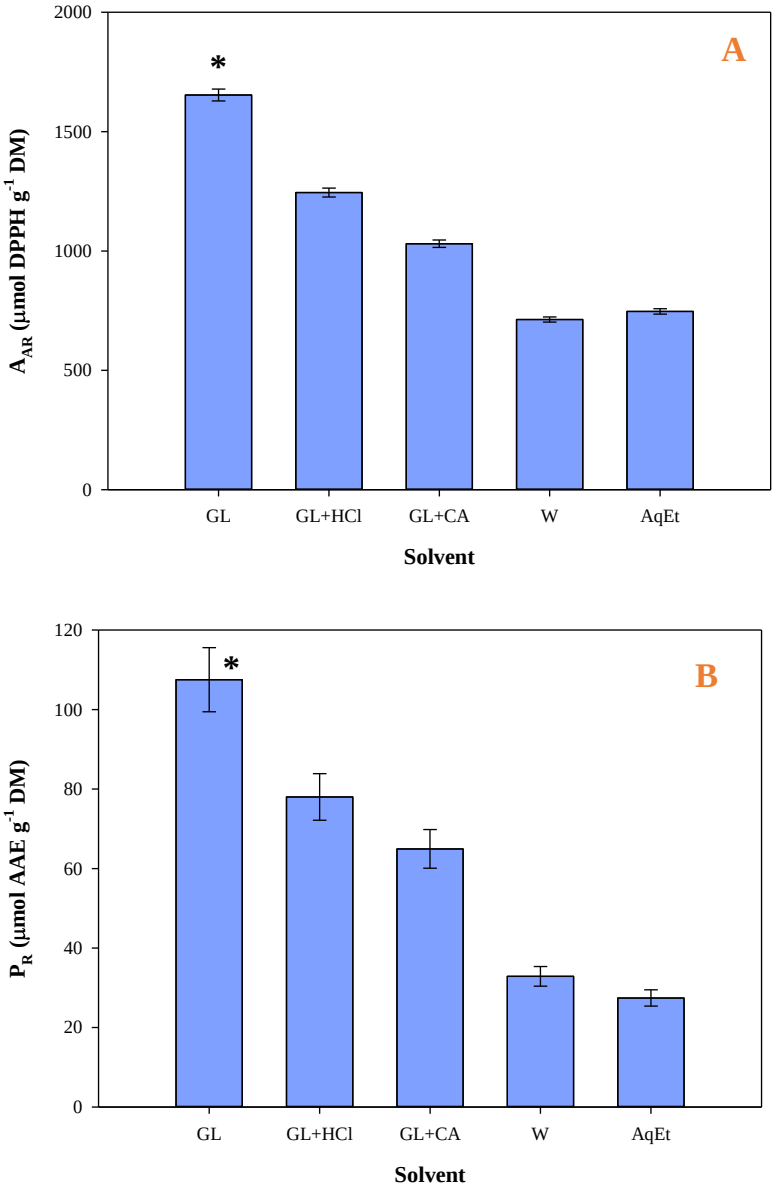


Figure 3. Antiradical activity (**A**) and ferric-reducing power (**B**) of the extracts obtained with the solvents tested. Asterisk (*) denotes statistically higher value ($p < 0.05$).

Such a discrepancy could be attributed to the polyphenolic profile, which, in the case of GL + HCl, was fundamentally different from that of GL + CA. Therefore, it could be argued that the compounds formed during extraction with GL + HCl might provide higher antioxidant activity. Yet, due to a lack of evidence concerning the nature of peaks A, B, C and D, which occurred only in the GL + HCl extracts, no further claims can be sustained about this issue. However, it was evident that alterations in the polyphenolic composition of the extracts could impact to some extent, their antioxidant properties.

4. Conclusions

This study explored the possibility of performing a glycerol-based organosolv treatment to achieve enhanced recovery of polyphenolic antioxidants from waste orange peels. The results drawn indicated that, under the conditions employed, water/glycerol mixtures at elevated temperatures may provide extracts with very satisfactory total polyphenol yields and improved antioxidant characteristics. Acidification of the extraction medium, either with citric acid or HCl, had a rather negative impact on process efficiency and severity. Furthermore, the addition of HCl was shown to provoke drastic changes in the polyphenolic profile, which suggested that the effect of HCl on orange peel polyphenols could be detrimental. The addition of HCl provoked the complete disappearance of narirutin, hesperidin and didymin, while it gave rise to four other unknown substances. Based on the evidence that emerged out of this examination, it could be substantiated that glycerol-based organosolv treatment is a very promising path toward the valorization of waste orange peels and the production of value-added products, using a green, food-grade and low-cost extraction medium. On the other hand, further optimization might be possible by testing conditions that would enable higher efficiency and lower severity. Such a study is currently in progress.

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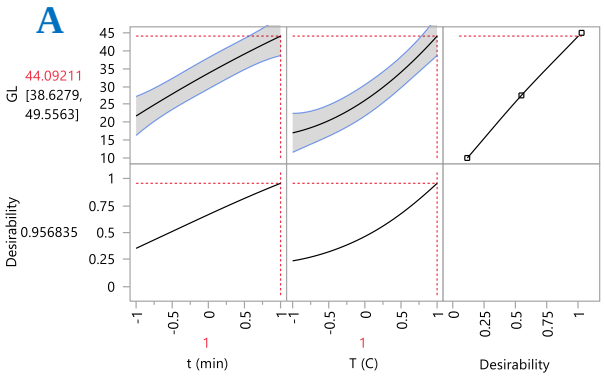
Appendix A



Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	20.099474	1.223855	16.42	<.0001 *
t (min)	6.765	0.973971	6.95	0.0010 *
T (C)	9.125	0.973971	9.37	0.0002 *
t (min)*T (C)	4.41	1.192866	3.70	0.0140 *
t (min)*t (min)	-0.758684	1.49891	-0.51	0.6343
T (C)*T (C)	4.4513158	1.49891	2.97	0.0312 *

Lack Of Fit

Source	DF	Sum of Squares	Mean Square	F Ratio
Lack Of Fit	3	27.357712	9.11924	16.5674
Pure Error	2	1.100867	0.55043	Prob > F
Total Error	5	28.458579		0.0575
				Max RSq
				0.9988

Figure A1. *Cont.*

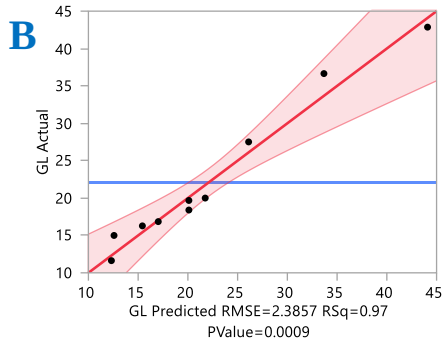
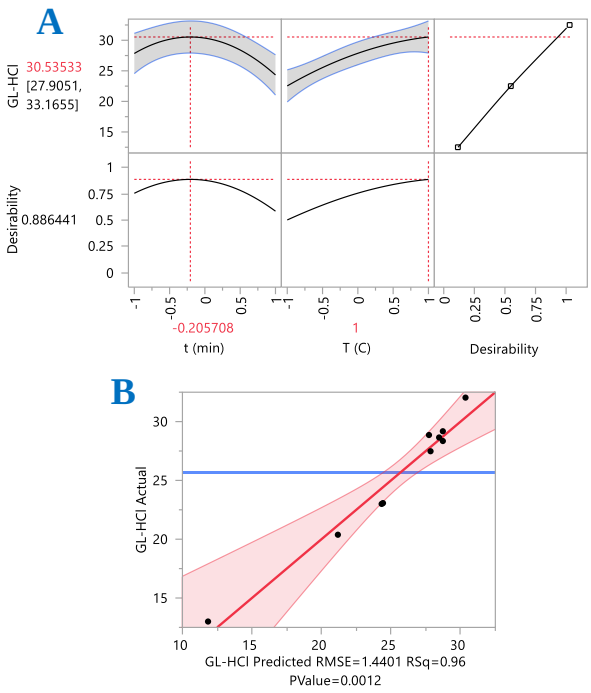


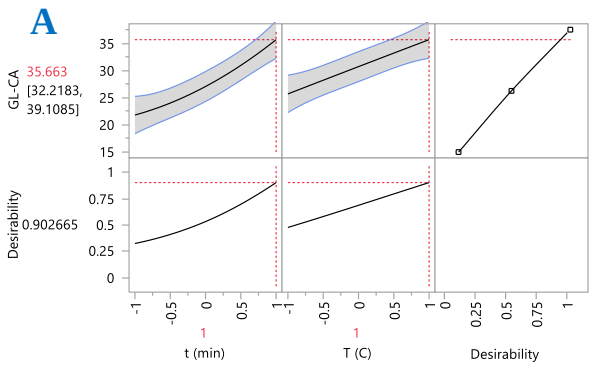
Figure A1. Desirability function (graph **A**), and plot of predicted vs. actual values of the response (Y_{TP}) (plot **B**), for the optimization of the extraction of WOP polyphenols performed with glycerol. Inset tables provide statistics associated with the assessment of the model derived. Values with color and asterisk are statistically significant.



Parameter Estimates				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Intercept	28.724737	0.73874	38.88	<.0001 *
t (min)	3.275	0.587906	5.57	0.0026 *
T (C)	2.9666667	0.587906	5.05	0.0039 *
t (min)*T (C)	-5.0325	0.720035	-6.99	0.0009 *
t (min)*t (min)	-4.271842	0.904768	-4.72	0.0052 *
T (C)*T (C)	-1.336842	0.904768	-1.48	0.1996

Lack Of Fit				
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Lack Of Fit	3	9.886536	3.29551	13.6611
Pure Error	2	0.482467	0.24123	**Prob > F**
Total Error	5	10.369003		0.0690
				Max RSq
				0.9983

Figure A2. Desirability function (graph **A**), and plot of predicted vs. actual values of the response (Y_{TP}) (plot **B**), for the optimization of the extraction of WOP polyphenols performed with GL + HCl. Inset tables provide statistics associated with the assessment of the model derived. Values with color and asterisk are statistically significant.



Parameter Estimates				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Intercept	23.438947	0.77162	30.38	<.0001 *
t (min)	5.5833333	0.614073	9.09	0.0003 *
T (C)	3.6433333	0.614073	5.93	0.0019 *
t (min)*T (C)	1.3425	0.752082	1.79	0.1343
t (min)*t (min)	1.6826316	0.945038	1.78	0.1351
T (C)*T (C)	-0.027368	0.945038	-0.03	0.9780

Lack Of Fit				
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Lack Of Fit	3	9.255691	3.08523	2.9999
Pure Error	2	2.056867	1.02843	**Prob > F**
Total Error	5	11.312557		0.2599
				Max RSq
				0.9930

Figure A3. *Cont.*

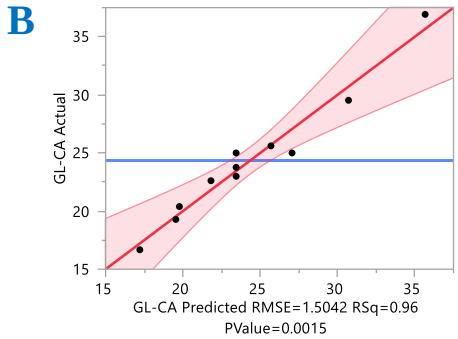


Figure A3. Desirability function (graph **A**), and plot of predicted vs. actual values of the response (Y_{TP}) (plot **B**), for the optimization of the extraction of WOP polyphenols performed with GL + CA. Inset tables provide statistics associated with the assessment of the model derived. Values with color and asterisk are statistically significant.

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