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Organosolv Treatment/Polyphenol Extraction from Olive Leaves (*Olea europaea* L.) Using Glycerol and Glycerol-Based Deep Eutectic Solvents: Effect on Metabolite Stability

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Abstract: Olive leaves (OLL) are an agri-food waste that may be regarded as a bioresource rich in bioactive polyphenolic metabolites. In this examination, simultaneous organosolv treatment/extraction of OLL polyphenols at elevated temperatures (>110 °C) has been optimized using glycerol, but also two glycerol-based deep eutectic solvents (DES). The assessment of the processes was based on the severity factor and the extraction efficiency factor. In any case, the treatment/extraction with a DES composed of glycerol and citric acid (GL-CA) was found to be the less severe and the most effective in recovering polyphenols from OLL, giving a yield of 69.35 mg gallic acid equivalents per g dry mass. On the other hand, liquid chromatography-mass spectrometry investigation revealed that extraction with either DES used provided extracts with differentiated polyphenolic profile than that obtained when water or 60% (v/v) aqueous ethanol was used as solvents. On the ground of these analysis, evidence emerged regarding hydrolysis of flavone glucosides when the treatment was performed with an alkaline DES composed of glycerol and sodium citrate. The extracts produced also exhibited diversified antioxidant activity, a fact putatively attributed to the different polyphenolic profiles. It was concluded that organosolv treatment/extraction of OLL for polyphenol recovery opens new endeavors in the valorization of this particular waste, but metabolite stability is an issue that merits profounder study.

Keywords: antioxidants; deep eutectic solvents; extraction; glycerol; olive leaves; polyphenols

1. Introduction

According to recent estimations, about 140 billion tons of biomass are generated globally per annum from the agricultural sector [1]. A considerable part of this biomass is characterized as waste, comprised mainly of leaves, seeds, peels, roots, stalks, small branches, straw residues, etc. These agri-food side streams may pose severe environmental concerns, if not properly managed and disposed, yet they are also recognized as bioresources to produce bioenergy, feed, fertilizers, platform chemicals and high value-added commodities. The orientation of the global economy towards sustainable routes has thus enabled the implementation of alternative strategies, aimed at minimizing the volume of non-renewable materials used today, and valorize waste biomass in a biorefinery concept [2,3].

One of the most prominent families of compounds abundant in various agro-industrial residues are polyphenols. Polyphenols are secondary plant metabolites and embrace a number of subfamilies, such as simple phenols, flavonoids, tannins, etc. [4]. A plethora of polyphenolic substances has been demonstrated to possess biologically important activities, including mainly antioxidant, anti-inflammatory and antimicrobial effects, but also



Citation: Houasni, A.; Grigorakis, S.; Kellil, A.; Makris, D.P. Organosolv Treatment/Polyphenol Extraction from Olive Leaves (*Olea europaea* L.) Using Glycerol and Glycerol-Based Deep Eutectic Solvents: Effect on Metabolite Stability. *Biomass* **2022**, *2*, 46–61. https://doi.org/ 10.3390/biomass2010004

Academic Editor: Chandrashekhar P. Joshi

Received: 3 February 2022 Accepted: 2 March 2022 Published: 7 March 2022

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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). long-term health-related properties, i.e., protection against cancer and cardiovascular disorders [5]. As such, a great effort has focused on their effective recovery from polyphenol-rich agri-food waste biomass, and their exploitation as bioactive food, pharmaceutical and cosmetic ingredients [6,7].

An environmental challenge tightly associated with the countries of the Mediterranean basin is the handling of by-products and wastes unavoidably generated from the olive oil industry. These rejected materials can have a negative impact on the environment, and therefore their proper management is of undisputed significance. One of the major olive industry by-products are the olive leaves, originating from pruning practices and olive harvesting, and it has been estimated that from 1 hectare of olive trees, as much as 2500 kg may be retrieved. It has also been reported that for every liter of olive oil produced, 6.23 kg of pruning residues (branches and leaves) may be generated [8]. Other studies estimated that out of 1500 kg pruning wastes, about 375 kg of olive leaves may be retrieved [9], and channeled into a biorefinery process for cellulose, bioethanol and fuel production [10], but also antioxidants, lignin and bioethanol production [11]. Olives leaves are a unique plant tissue, in that they contain a high load of bioactive polyphenolic compounds, the major representative being the secoiridoid derivative oleuropein.

The extraction processes developed to extract polyphenolic compounds from plant material rely on solid-liquid techniques, deployed according to the nature of the compounds to-be-extracted, the cost, properties, and toxicity of suitable solvents, but also the unit operations required, the purity of the product, etc. In the framework of Green Chemistry, the use of alternative solvents (i.e., deep eutectic solvents—DES) and technologies (i.e., ultrasound irradiation, microwave heating) are gaining acceptance for establishing benign protocols for solid-liquid extraction, and on such a basis a high number of extraction procedures have been proposed for polyphenol recovery from olive leaves [12]. One of the major objectives for applying techniques such as ultrasonication, is the disruption of plant cell walls, in order to facilitate release of intracellular metabolites and their entrainment into the liquid phase [13]. This has been shown to increase mass transfer and, consequently, extraction yield.

On the other hand, biomass treatment aimed at disorganizing and/or partly degrading biopolymers such as lignin and hemicellulose, which are principal cell wall constituent, may be very efficiently performed through thermal processing at elevated temperatures [14]. The key scope of such processes is the pretreatment of biomass to untangle and/or partly depolymerize biopolymer networks of cellulose-hemicellulose-lignin and maximize subsequent production of fermentable sugars, whereas the retrieval of polyphenols is an issue largely disregarded. Several of these technologies are characterized as hydrothermal treatments, but also organosolv treatments, encompassing processing with organic solvents through combination with high temperature/high pressure conditions [15].

Recent examinations demonstrated that these thermal treatments may be fostered by the use of DES [16], glycerol [17], and combinations of glycerol with HCl [18]. On this basis, the study presented herein had as objective the implementation of a simultaneous thermal treatment/extraction technique to recover bioactive polyphenols from olive leaves, through a response surface experimental design. The solvents used were glycerol and glycerol-based DES, to identify possible solvent effects on extract composition and stability. Particular emphasis was given to the investigation of polyphenol conversions, due to elevated temperatures employed, using liquid chromatography—mass spectrometry. To the best of the authors' knowledge, this is the first report of such a process applied to polyphenol extraction from olive leaves.

2. Materials and Methods

2.1. Chemicals

Hydroxytyrosol, ammonium acetate, sodium carbonate, ascorbic acid, luteolin 7-Oglucoside, oleuropein, and 2,2-diphenylpicrylhydrazyl (DPPH) were from Sigma-Aldrich (Darmstadt, Germany). 2,4,6-Tripyridyl-s-triazine (TPTZ) and iron (III) chloride hexahydrate were from Honeywell/Fluka (Steinheim, Germany). β-Cyclodextrin was from Wacker Chemie AG (Burghausen, Germany). Methanol and ethanol were from Honeywell/Riedelde Haen (Seelze, Germany). Citric acid, Folin-Ciacalteu reagent, sodium citrate tribasic dihydrate (>99%) and glycerol (99%) were from Merck (Darmstadt, Germany). L-Lactic acid was from Fisher Scientific (Loughborough, UK). All chromatographic analyses were performed with solvents of appropriate purity (HPLC grade).

2.2. Deep Eutectic Solvent (DES) Synthesis

For DES synthesis, glycerol was used as the hydrogen bond donor (HBD), and citric acid and trisodium citrate as hydrogen bond acceptors (HBAs). The DES composed of glycerol/citric acid, termed as GL-CA, had HBD/HBA molar ratio of 4:1 [19] and the DES composed of glycerol/trisodium citrate, termed as GL-SC, a respective ratio of 15:1 [20]. To synthesize the DES, accurately weighted mass of HBD and HBA were mixed at predetermined molar proportions, under continuous stirring at 500 rpm, and heated at 70 °C until perfectly transparent liquids were formed. The usual time to achieve this was approximately 60 min, depending on HBD/HBA combination. The DES produced were kept in glass screw-cap vials, at room temperature, and stability (appearance of crystals) was ascertained by periodic visual inspection over several weeks.

2.3. Olive Leaves (OLL)

The plant material was collected and handled following a previously described procedure [21]. Briefly, OLL were hand-picked by a small olive tree plantation (*Olea europaea* cv. Koroneiki) located within the premises of M.A.I.Ch. Collection was accomplished by a few vicinal trees and from different sides of each tree (sun-exposed, shaded), to obtain a representative gross sample. The material was air-dried for 2 weeks in a dark and dry chamber, at room temperature (25 ± 2 °C), and then ground and sieved to provide a powder with 0.350-mm average particle diameter. This material was kept in tightly sealed vessels, at 4 °C.

2.4. Thermal Treatment/Extraction

In a 25-mL DuranTM glass vial, 10 mL of solvent was placed, and the vial was screw capped and heated at the desired temperature, in accordance with the experimental design (Table 1). Once the solvent acquired the temperature set, exact mass of 1 g of OLL was introduced into the vial, and the mixture was treated for predetermined resident time, as dictated by the experimental design, under continuous stirring at 400 rpm, on a temperature-controlled hot plate (Witeg, Wertheim, Germany). The solvents used were glycerol (90% *w*/*w*, pH = 3.50), GL-CA (90% *w*/*w*, pH = 1.40) and GL-SC (90% *w*/*w*, pH = 7.85). Control extraction with distilled water was performed for 60 min at 70 $^{\circ}$ C, and with 60% (v/v) ethanol for 185 min at 70 °C, under the same stirring speed [22]. Furthermore, an additional control extraction was also carried out with a recently developed optimized process, employing as extraction medium a DES composed of L-lactic acid/ammonium acetate (7:1) [21]. This DES was used as 54% water mixture (w/w) and contained 0.7% (w/v) β -cyclodextrin. The conditions employed for the extraction with this DES were a liquid-to-solid ratio of 100 mL g^{-1} , stirring speed of 300 rpm, resident time 150 min and extraction temperature 80 °C. All extracts were centrifuged at $10,000 \times g$ for 10 min prior to analyses.

Table 1. Process variables and their corresponding coded and actual levels.

Process Variables	Codes	Coded Variable Level		
		-1	0	1
<i>t</i> (min)	X ₁	10	30	50
<i>T</i> (°C)	X ₂	110	125	140

2.5. Experimental Design and Response Surface Methodology

Optimization of the thermal treatment/extraction process with regard to two major variables, time (*t*) and temperature (*T*), was accomplished by implementing response surface methodology. The design of experiment selected was a central composite with three central points and, in total, 11 design points. The process (independent) variables (*t*, *T*) were set in 3 levels, -1, 0 and 1, in compliance with the experimental design, and codified as described elsewhere [23]. The actual and coded levels are given in Table 1. The choice of the ranges for both variables was based on preliminary experiments. The overall significance of the models (\mathbb{R}^2 , *p*) and the significance of each coefficient of the models (equations) was evaluated by lack-of-fit and ANOVA tests, at a minimum level of 95%.

2.6. Extraction Efficiency Factor

This newly established factor [22] provides an account of total polyphenol extraction yield (\mathbf{Y}_{TP}), given as mg gallic acid equivalents per g dry mass (DM), as related to extraction time (*t*, min) and temperature (*T*, °C). The expression of extraction efficiency (EE) is as follows:

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

Based on this, the extraction efficiency factor (F_{EE}) is defined as:

$$F_{\rm EE} = -log(\rm EE) \tag{2}$$

2.7. Severity Factor (SF)

This factor, established by Overend and Chornet in 1987 [24], provides a measure of the severity of biomass hydrothermal and organosolv processing conditions, as a result of specific combinations of temperature and time, and it was used in this study to compare the different extraction conditions [25]:

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

$$SF = log R_0 \tag{4}$$

where R_0 is the severity and the value 100 °C represents the reference temperature. The value 14.75 is an empirical parameter related to temperature and activation energy, assuming first order kinetics.

2.8. Determinations

Total polyphenol analysis was carried out adopting a previously described methodology [26]. Results were reported as mg total polyphenols per g dry mass (DM), using gallic acid as the reference standard. Antiradical activity (A_{AR}) and ferric-reducing power (P_R) of the extracts were estimated by employing well-established assays, as described elsewhere [26]. Results were expressed as µmol DPPH per g DM and µmol ascorbic acid equivalents (AAE) per g DM, respectively.

2.9. Liquid Chromatography—Diode Array—Mass Spectrometry (LC—DAD—MS)

For the chromatographic determinations, a published methodology was used [21]. In short, a Finnigan (San Jose, CA, USA) P4000 pump, a UV6000LP diode array detector and a Finnigan AQA mass spectrometer were used. Separations were accomplished on a Fortis RP-18 column, 150 mm \times 2.1 mm, 3 µm, with a 10-µL injection loop, at 40 °C. Mass spectra acquisition was carried out with electrospray ionization (ESI) in positive ion mode. All settings concerning the elution program, mass spectra acquisition and quantitation have been given in detail elsewhere [21].

2.10. Statistical Treatments and Analyses

The design of experiment and all statistics related to response surface methodology, as well as distribution analyses, were accomplished with JMPTM Pro 13 (SAS, Cary, NC, USA). Linear regressions were carried out with SigmaPlotTM 12.5 (Systat Software Inc., San Jose, CA, USA). All processes were repeated at least twice and all quantitative determinations in triplicate. The values were given as means \pm standard deviation.

3. Results and Discussion

3.1. Process Modelling

The simultaneous organosolv treatment and extraction process deployed had as objective the examination of the performance of specific solvents on the extraction yield in total polyphenols, at elevated temperatures. In Table 2 can be seen the measured and predicted values of Y_{TP} for all three solvents tested. For all three models derived, the total R² were equal or higher than 0.95, and the *p* value for lack-of-fit (based a confidence interval of 95%) was highly significant (Figures S1–S3). Thus, it could be supported that the equations showed excellent adjustment to the experimental data. According to the models (mathematical equations) presented in Table 3, the independent variables considered (*t*, *T*) gave diverging outcome, depending on the solvent used.

Table 2. Measured and predicted values of the response for all design points considered for the optimization of the extraction of OLL polyphenols, with the three solvents tested.

Design Point	Independent Variables		Response (Y _{TP} , mg GAE g^{-1} dw)						
	t (min) (X ₁)	<i>T</i> (°C) (X ₂)	GL		GL-CA		GL-SC		
			Measured	Predicted	Measured	Predicted	Measured	Predicted	
1	10 (-1)	110 (-1)	61.10	61.24	70.29	69.35	54.82	53.42	
2	10(-1)	140 (1)	65.94	66.07	51.34	51.71	57.02	57.25	
3	50 (1)	110(-1)	64.57	64.81	53.14	52.96	64.42	64.25	
4	50(1)	140 (1)	60.53	60.76	60.00	61.12	71.61	73.07	
5	10(-1)	125 (0)	63.40	63.13	57.97	58.55	54.01	55.18	
6	50 (1)	125 (0)	62.73	62.26	56.02	55.06	69.80	68.51	
7	30 (0)	110(-1)	68.28	67.91	62.12	63.25	60.04	61.57	
8	30 (0)	140 (1)	68.67	68.30	60.05	58.51	69.59	67.90	
9	30 (0)	125 (0)	67.05	67.58	58.67	58.90	64.25	64.58	
10	30 (0)	125 (0)	67.11	67.58	58.48	58.90	66.38	64.58	
11	30 (0)	125 (0)	68.08	67.58	59.18	58.90	63.00	64.58	

Table 3. Models derived from the optimization of the polyphenol extraction from OLL with the three solvents tested and their statistical significance.

Solvent	2nd Order Polynomial Equations	R ²	p
GL	$\begin{array}{r} 67.58-2.22{X_1}{X_2}-4.89{X_1}^2\\ 58.90-1.74{X_1}-2.37{X_2}+6.45{X_1}{X_2}-2.09{X_1}^2+1.98{X_2}^2\\ 64.58+6.66{X_1}+3.16{X_2}\end{array}$	0.98	0.0002
GL-CA		0.97	0.0008
GL-SC		0.95	0.0029

The effect the variables exerted in each case was visualized in the form of 3-dimentional diagrams (Figure 1), which depicted the differences between the three solvents. For the process performed with GL, the effect of neither individual variable was statistically significant, but the process was influenced by the combined effect of *t* and *T* (cross term X_1X_2), as well as the quadratic effect of *t* (X_1^2) (Figure S1). This finding evidenced that the performance of the treatment with GL was negatively affected by *t*, beyond a certain point. Likewise, the equation derived for the treatment with GL-CA suggested that both individual variables had negative impact on the treatment performance, but their joint effect (cross term X_1X_2), as well as the quadratic effect of *T* (X_2^2) were positive (Table 3). By contrast, treatment



with GL-SC was positively affected by both *t* and *T*, yet no cross or quadratic terms were statistically significant.

Figure 1. 3D-plots illustrating the effect of the simultaneous variation of process variables (t, T) on the response (Y_{TP}). (**A**), extraction with GL; (**B**), extraction with GL-CA; (**C**), extraction with GL-SC.

Based on the desirability function (Figures S1–S3), maximum Y_{TP} and optimized variable values could be predicted (Table 4). The maximum Y_{TP} displayed no significant difference and varied from 68.66 (GL) to 73.07 (GL-SC) mg GAE g⁻¹ DM. On the contrary, notable variations were observed for the optimum *t*, as the process with GL-SC required 5-fold longer period to achieve maximum Y_{TP} , compared to GL-CA. Similarly, the optimum *t* for the treatment with GL was 2.5-fold longer compared to GL-CA. With respect to *T*, the process with GL-CA was the least demanding, the optimum value being 110 °C, as opposed to processes with GL and GL-SC, which both required 140 °C to attain maximum Y_{TP} . Recent findings suggested that, on average, the resident optimum time for polyphenol extraction with a glycerol-based DES was 210 min, at an average *T* of 68 °C [22]. However, optimum *t* of the process with GL-CA was 10 min, at 110 °C. This result strongly indicated that at elevated *T*, the resident *t* might be significantly shortened.

Solvent	Maximum Predicted Response (mg GAE g ⁻¹ DM)	Optimal Conditions		Indices	
		<i>t</i> (min)	<i>T</i> (°C)	F _{EE}	SF
GL	68.66 ± 1.02	25	140	1.71	2.58
GL-CA	69.35 ± 2.78	10	110	1.20	1.29
GL-SC	73.07 ± 4.39	50	140	1.98	2.88

Table 4. Maximum predicted values for the response (Y_{TP}), optimal predicted values for the process variables (*t*, *T*) and values of F_{EE} and *SF* determined for each process.

3.2. Process Severity and Efficiency

To put process evaluation on a quantitative basis, first the severity factor (*SF*) was determined. This factor has been used to describe sugar recovery and lignin reduction when treating various herbaceous feedstocks, such as corn stover, hardwood chips etc. [25], and it may reflect how the combined effect of *t* and *T* could contribute towards disorganizing lignin-hemicellulose complexes in plant cell walls. Since such a phenomenon could foster plant cell disintegration and liberation of intracellular metabolites (polyphenols) into the liquid phase (solvent), then for an organosolv process, such as the one used in this study, SF could be used to provide an account of the severity of the process. SF was determined used the optimum *t* and *T* values required to attain maximum Y_{TP} and it can be seen in Table 4 that the process with GL-CA was the least severe exhibiting an SF of 1.29, whereas the processes with GL and GL-SC had SF values of 2.58 and 2.88, respectively.

The lower severity determined for the treatment with GL-CA might be ascribed to its low pH. Proton-catalyzed bond cleavage of lignocellulosic materials is considered to be a significant factor of lignin/hemicellulose disintegration, because this mechanism contributes to separating lignin from biomass. It has been documented that organosolv treatments with DES composed of a carboxylic acid show greater treatment performance, because the active protons provided by the carboxylic acid could facilitate proton-catalyzed reactions that lead to bond cleavage in lignocellulosic biomass, such as glycosidic bonds, ether bonds and lignin-carbohydrate links [27]. This in turn could cause a higher cell structure disruption, thus enabling entrainment of intracellular metabolites (polyphenols) into the solvent.

In addition to SF, process appraisal was also accomplished using the extraction efficiency factor (F_{EE}) [22]. According to the categorization established in that study, polyphenol extraction may be characterized as being of high efficiency when $F_{EE} < 1.75$ and of moderate efficiency when $F_{EE} = 1.75$ –2.14. The process with GL-CA displayed the lowest F_{EE} (1.20) which was indication of its higher efficiency. The process with GL was marginally of high efficiency ($F_{EE} = 1.71$), but the one with GL-SC was of moderate efficiency ($F_{EE} = 1.98$). It was also provided evidence of correlation between F_{EE} and solvent viscosity, but the data of this study are insufficient to confirm such a link. Clearly, this is an issue open to investigation, and it could shed more light onto the differences found among the processes tested. Furthermore, as recent evidence highlighted the effect of pH on the extraction performance [20], this parameter should also be considered to clarify the effect of acidic/alkaline DES on process efficiency.

3.3. Polyphenolic Composition and Metabolite Stability

The polyphenolic profiles of the extracts obtained with GL, GL-CA and GL-SC were investigated by liquid chromatography-mass spectrometry, to detect possible alterations to major metabolites. For this reason, chromatographic runs were accomplished with selected ion monitoring, to consider major metabolites, including oleuropein, luteolin 7-O-glucoside, and some derivatives thereof. Peak identification and tentative structures were proposed according to the data presented in Table 5, based on previously published information [21]. It can be seen in Figure 2 that in the GL extract, oleuropein was accompanied by four other hydroxytyrosol derivatives (peaks 2–5), but also hydroxytyrosol (HT). The extract obtained with GL-CA displayed a different profile, evidencing decomposition of peak 2 (an oleuropein derivative) and the appearance of a new peak termed A. Given that compound A showed a molecular ion with m/z = 433, which was lower than that of oleuropein (m/z = 541), it could be hypothesized that compound A represents an oleuropein hydrolysis product. Hydrolytic effects of acidic DES composed of L-lactic acid/glycine towards oleuropein have also been observed in previous studies [28]. The relative abundancies of oleuropein, peak 2 and peak 3 were also modified, suggesting possible interconversions. On the other hand, in the extract produced with GL-SC no HT, A, and peak 5 were detected. This finding indicated that in slightly alkaline environment, as the one provided by GL-SC, some OLL compounds might not be stable.

Table 5. Chromatographic and mass spectral data for the major polyphenolic metabolites detected in OLL extracts.

Peak	Rt (min)	UV-Vis	[M + H] ⁺	Other Ions	Tentative Identity
HT	6.79	238, 280	137	-	Hydroxytyrosol
6	17.53	244, 340	611	287, 377, 449	Luteolin rutinoside
٨	21.24	240, 280	122	127	Hydroxytyrosol
A	21.34	240, 200	433	137	derivative
7	21.45	254, 352	449	287	Luteolin 7-O-glucoside
8	25.76	248, 266, 344	449	287	Luteolin glucoside
9	27.93	266, 344	449	287	Luteolin glucoside
1	22.85	246, 280	541	563, 361, 137	Oleuropein
2	25.94	242, 280	541	563, 361, 137	Oleuropein derivative
3	27.32	242, 280	541	563, 379, 361, 137	Oleuropein derivative
4	28.88	242, 280	-	563, 379, 361, 137	Oleuropein derivative
5	29.54	242, 280	475	361, 137	Hydroxytyrosol derivative

With regard to luteolin metabolites, the profile of the extracts obtained with GL and GL-CA were identical (Figure 3). This fact suggested that the composition and pH of the GL-CA did not affect luteolin glucosides. On the contrary, the extract produced with GL-SC exhibited a diversified profile, where no peak 6 was detected. The relative abundancies of peaks 7, 8 and 9 were significantly changed, while aglycone luteolin also appeared (peak termed LT). This outcome pointed to hydrolysis of luteolin glucosides, presumably brought about by the slight alkaline pH of GL-SC, which resulted in aglycone liberation. Based on the above data, it would appear that the use of either GL-CA or GL-SC affected to some extent extract composition, raising concerns for polyphenol stability, under the conditions employed. It should be noted that polyphenol stability in DES has been scarcely examined, and the data available are rather inconclusive. Storage of an OLL extract produced with a DES comprised of glycerol, glycine and water, containing 9% (w/v) methyl β -cyclodextrin, at 50 °C, for 20 days, was demonstrated to provoke significant alterations to the polyphenolic profile, inducing the formation of a yellow pigment [29].



Figure 2. Selected ion chromatograms showing the major oleuropein derivatives. Peak assignment: 1, oleuropein; 2, 3, and 4, oleuropein derivatives. HT, hydroxytyrosol; A, oleuropein derivative detected only in GL-CA extracts.

Likewise, polyphenol extracts from *Moringa oleifera* leaves obtained with a glycerol/sodium acetate DES displayed drastic changes after storage for 18 days, at 50 °C [30]. On the contrary, *Salvia fruticosa* polyphenols in extracts obtained with a lactic acid/sodium citrate dibasic DES exhibited extraordinary stability for 30 days, at 40 °C [31].



Figure 3. Selected ion chromatograms showing the major oleuropein derivatives. Peak assignment: 7, luteolin 7-O-glucoside; 8, 9, luteolin glucosides; LT, luteolin aglycone.

3.4. Efficiency Appraisal and Antioxidant Effects

The most efficient process performed with GL-CA afforded a Y_{TP} of 69.35 mg GAE g⁻¹ DM. This level is considerably higher than 19.50 mg GAE g⁻¹ DM, achieved with steam explosion of OLL at 180 °C [32], 26.75 mg CAE g⁻¹ DM achieved with a glycerol/sodium-potassium tartrate DES at 73 °C [33], and 51.91 mg GAE g⁻¹ DM attained with aqueous glycerol at 80 °C [34]. Higher Y_{TP} of 116.65 mg GAE g⁻¹ DM have been reported for OLL extraction with a glycerol/glycine/water DES containing 9% (w/v) methyl β -cyclodextrin [35], and 113.66 mg CAE g⁻¹ DM for extraction with a lactic acid/ammonium acetate DES containing 0.7% (w/v) β -cyclodextrin [21]. Moreover, an optimized extraction process of polyphenols from OLL with L-lactic acid/glycine DES gave a Y_{TP} of 97.53 mg GAE g⁻¹ DM [28].

However, no credible outcome can be drawn by comparing values given in the literature, since the differences in Y_{TP} may well be attributed to seasonal variations and genetic (varietal) factors [36,37]. Thus, in order to have an integrated picture regarding the efficiency of the GL-CA process, as compared to other optimized procedures, OLL were extracted with water and 60% (v/v) ethanol [22], and a lactic acid/ammonium acetate DES containing 0.7% (w/v) β -cyclodextrin [21]. Considering F_{EE} (Figure 4), extraction with GL-CA gave statistically lower value (p < 0.05) compared to all other solvents tested, a confirmation of its high efficiency.



Figure 4. Extraction efficiency factor (F_{EE}) determined for polyphenol extraction from OLL, by deploying various extraction processes. Assignment: GL, extraction with glycerol; GL-CA, extraction with the DES composed of glycerol/citric acid; GL-SC, extraction with the DES composed of glycerol/sodium citrate tribasic; W, water extraction; AqEt, extraction with 60% (v/v) ethanol/water; DES/CD, extraction performed with a DES comprised of lactic acid/ammonium acetate, containing 0.7% (w/v) β -cyclodextrin. Columns assigned with different letters (a, b, c) represent statistically different values (p < 0.05).

Extractions with GL, GL-SC, water, and aqueous ethanol did not show statistical difference, whereas extraction with lactic acid/ammonium acetate DES containing 0.7% (w/v) β -cyclodextrin had significantly higher F_{EE} (p > 0.05). In general, extractions with DES exhibit an average F_{EE} of 2.18 [21]. The fact that extraction with GL-CA afforded F_{EE} of 1.20 might be indicative of its high efficiency. Likewise, comparison based on SF revealed that extraction with GL-CA and aqueous ethanol were of lower severity compared to the extractions performed with GL, GL-SC and with the with lactic acid/ammonium acetate DES containing 0.7% (w/v) β -cyclodextrin (Figure 5).

The determination of the antiradical activity (A_{AR}) indicated that the extracts produced with GL and aqueous ethanol were significantly more active (p < 0.05) (Figure 6A). On the other hand, the extract obtained with GL-CA had comparable activity with the GL-SC and the aqueous extract, but also the extract obtained with lactic acid/ammonium acetate DES containing 0.7% (w/v) β -cyclodextrin. The pattern was quite different when the reducing power (P_R) of the extracts was considered (Figure 6B), since the aqueous and the GL-CA extract displayed lower efficacy compared to all other extracts.



Figure 5. Severity factor (SF) determined for polyphenol extraction from OLL, by deploying various extraction processes. Assignment: GL, extraction with glycerol; GL-CA, extraction with the DES composed of glycerol/citric acid; GL-SC, extraction with the DES composed of glycerol/sodium citrate tribasic; W, water extraction; AqEt, extraction with 60% (v/v) ethanol/water; DES/CD, extraction performed with a DES comprised of lactic acid/ammonium acetate, containing 0.7% (w/v) β -cyclodextrin. Columns assigned with different letters (a, b, c) represent statistically different values (p < 0.05).



Figure 6. Cont.



Figure 6. Antiradical activity (A_{AR}) (plot (**A**)) and ferric-reducing power (P_R) (plot (**B**)) determined for polyphenol extraction from OLL, by deploying various extraction processes. Assignment: GL, extraction with glycerol; GL-CA, extraction with the DES composed of glycerol/citric acid; GL-SC, extraction with the DES composed of glycerol/sodium citrate tribasic; W, water extraction; AqEt, extraction with 60% (v/v) ethanol/water; DES/CD, extraction performed with a DES comprised of lactic acid/ammonium acetate, containing 0.7% (w/v) β -cyclodextrin. Columns assigned with different letters (a, b) represent statistically different values (p < 0.05).

In general, the antioxidant activity expressed by OLL extracts has been mainly attributed to principal constituents, such as oleuropein and derivatives thereof, and luteolin 7-*O*-glucoside [38,39], and it would appear that the differences found in the chromatographic profiles were reflected on the antioxidant characteristics. However, since the overall antioxidant activity depends on the integration of the antioxidant activities exerted by the individual polyphenols and the synergistic/antagonistic effects amongst them [40,41], it would be rather impossible to come up with a credible association between A_{AR} and P_R values, and the concentration of specific compounds. On the other hand, the lower A_{AR} and P_R found for the GL-CA extract compared to GL and aqueous ethanolic extracts might indicate that the alterations in the polyphenolic profile occurred during extraction had rather negative impact on the antioxidant activity. Yet, this phenomenon merits profounder investigation.

4. Conclusions

To the best of the authors' knowledge, this is the first report on organosolv treatment/extraction at high temperatures, implemented for the recovery of OLL polyphenols. Appraisal of the processes tested based on extraction efficiency factor and severity factor suggested that treatment with the DES composed of glycerol and citric acid was both more benign and efficacious, compared to treatments carried out with either glycerol or the DES composed of glycerol and sodium citrate. Furthermore, considering major polyphenolic polyphenolic metabolites, such as oleuropein and luteolin glucosides, concerns were raised with regard to polyphenol stability under the conditions employed, either in acidic or alkaline DES. This is an issue that requires clarification. Significant differences were also found for the antioxidant properties of the extracts produced, which might be related to alterations in the polyphenolic composition. In any case, the elevated temperatures used in this study, in combination with the solvents tested, were shown to be significantly more effective in achieving high levels of total polyphenol yield. Thus, it is proposed that organosolv treatment/extraction at elevated temperatures, which require shorter extraction time than conventional extractions, might be an effective, green, and alternative approach for OLL valorization and production of value-added commodities.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/biomass2010004/s1, Figure S1: Desirability function (graph A), and plot of predicted vs actual values of the response (YTP) (plot B), for the optimization of the extraction of OLL polyphenols performed with glycerol. Inset tables provide statistics associated with the assessment of the model derived. Values with color and asterisk are statistically significant; Figure S2: Desirability function (graph A), and plot of predicted vs actual values of the response (YTP) (plot B), for the optimization of the extraction of OLL polyphenols performed with the GL-CA DES. Inset tables provide statistics associated with the assessment of the model derived. Values with color and asterisk are statistically significant; Figure S3: Desirability function (graph A), and plot of predicted vs. actual values of the response (YTP) (plot B), for the optimization of the extraction of OLL polyphenols performed with the GL-SC DES. Inset tables provide statistics associated with the assessment of the model derived. Values with color and asterisk are statistically significant; Figure S2: Desirability function (graph A), and plot of predicted vs. actual values of the response (YTP) (plot B), for the optimization of the extraction of OLL polyphenols performed with the GL-SC DES. Inset tables provide statistics associated with the assessment of the model derived. Values with color and asterisk are statistically significant.

Author Contributions: D.P.M. and S.G. designed and developed the study. A.H., A.K. and S.G. performed the experiments, collected and analyzed the data. D.P.M. and S.G. supervised the work, provided advice on the methodology and results, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available from the corresponding author.

Acknowledgments: We thank M.A.I.Ch. for funding this research.

Conflicts of Interest: The authors declare no conflict of interest.

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