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The Effect of Ultrasonication Pretreatment on the Production of Polyphenol-Enriched Extracts from *Moringa oleifera* L. (Drumstick Tree) Using a Novel Bio-Based Deep Eutectic Solvent

Achillia Lakka [1], Spyros Grigorakis [2], Olga Kaltsa [1], Ioanna Karageorgou [1], Georgia Batra [1], Eleni Bozinou [1], Stavros Lalas [1] and Dimitris P. Makris [1,*]

[1] School of Agricultural Sciences, University of Thessaly, N. Temponera Street, 43100 Karditsa, Greece; ACHLAKKA@uth.gr (A.L.); okaltsa@uth.gr (O.K.); ikarageorgou@uth.gr (I.K.); gbatra@uth.gr (G.B.); empozinou@uth.gr (E.B.); slalas@uth.gr (S.L.)

[2] Food Quality & Chemistry of Natural Products, Mediterranean Agronomic Institute of Chania (M.A.I.Ch.), International Centre for Advanced Mediterranean Agronomic Studies (CIHEAM), P.O. Box 85, 73100 Chania, Greece; grigorakis@maich.gr

* Correspondence: dimitrismakris@uth.gr; Tel.: +30-24410-64792

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Abstract: *Moringa oleifera* L. leaves are a plant tissue particularly rich in polyphenolic phytochemicals with significant bioactivities, and there has been significant recent interest for the production of extracts enriched in these substances. The current investigation is aimed at establishing a green extraction process, using a novel eco-friendly natural deep eutectic solvent, composed of glycerol and nicotinamide. Furthermore, sample ultrasonication prior to batch stirred-tank extraction was studied to examine its usefulness as a pretreatment step. Optimization of the extraction process through response surface methodology showed that the maximum total polyphenol yield (82.87 ± 4.28 mg gallic acid equivalents g^{-1} dry mass) could be achieved after a 30 min ultrasonication pretreatment, but the difference with the yield obtained from the non-pretreated sample was statistically non-significant ($p < 0.05$). Extraction kinetics revealed that the activation energy for the ultrasonication-pretreated samples was more energy-demanding, a fact attributed to phenomena pertaining to washing of the readily extracted polyphenols during pretreatment. Liquid-chromatography-diode array-mass spectrometry showed that ultrasonication pretreatment may have a limited positive effect on polyphenol extractability, but the overall polyphenolic profile was identical for the ultrasonication-pretreated and non-pretreated samples.

Keywords: deep eutectic solvents; extraction; *Moringa oleifera*; polyphenols; ultrasonication pretreatment

1. Introduction

Moringa oleifera Lam. is a cruciferous plant of the Moringaceae family, often called drumstick tree. *M. oleifera* is a popular staple in several parts around the globe, and it is consumed for its high nutritional value, as its leaves are rich in β-carotene, vitamin C, vitamin E, polyphenols, etc. [1,2]. Moreover, *M. oleifera* has been reported to display a broad range of biological functions, such as neuroprotective activity, chemopreventive properties, anti-inflammatory potency, and hepatoprotective activity. As a number of studies have provided strong evidence regarding the therapeutic value of *M. oleifera* for diseases like diabetes, rheumatoid arthritis, atherosclerosis, etc., this plant has attracted significant attention for its pharmacological functions [3]. The pharmacological potency of *M. oleifera* may be closely associated with its polyphenolic composition, which includes a wide range of structures, represented mainly by flavonoids

(kaempferol and quercetin glycosides) and phenolic acids and their derivatives (i.e., caffeoylquinic acids and their isomers) [4,5]. In fact, the strong antioxidant effects exerted by *M. oleifera* leaf extracts and decoctions have been attributed to their high flavonoid content [6–8]. By virtue of their flavonoid richness, *M. oleifera* leaves have been a subject of recent research on the production of polyphenol-containing extracts, and several methodologies have been used for such a purpose, including pressurized hot water [9] and aqueous two-phase extraction [10], ultrasound-assisted extraction with aqueous ethanol [11], and subcritical ethanol extraction [12]. However, extraction with novel, green solvents, the so-called deep eutectic solvents (DES), have lately been proposed as eco-friendly and particularly effective means of polyphenol recovery from *M. oleifera* leaves [13,14]. DES are neoteric designer liquids, composed of food-grade, low-cost biomaterials, such as polyols, organic acids, amines, organic acid salts, etc. [15]. Their facile and straight-forward synthesis, as well as their unique properties, including low or no toxicity, absence of flammability, tunability, recyclability, and biodegradability, make DES ideal solvents for the development of green extraction processes, and over the last five years, DES have attracted particularly high interest for the polyphenol extraction from a wide number of plant tissues [16].

Ultrasonication is a state-of-the-art technology implicated in sustainable "green" extraction procedures, since numerous studies have demonstrated the efficiency of ultrasounds in accelerating the rate of solid–liquid extractions. By implementing ultrasonication techniques, effective extractions can be accomplished in minutes with high reproducibility, requiring reduced solvent volume, lower energy input and simpler sample manipulation, providing at the same time in some cases higher purity of the final product [17]. On such a ground, this study was performed with the aim at examining the extraction of *M. oleifera* leaf polyphenols and the impact of ultrasonication as a pretreatment step. Response surface was deployed to optimize extraction variables and kinetics was used to obtain a deeper insight into the effect of ultrasonication. A final assessment of the process was conducted through the determination of the ultrasonication-treated and non-treated samples.

2. Materials and Methods

2.1. Chemicals

Glycerol anhydrous (99.5%), sodium carbonate anhydrous (99%), aluminium chloride anhydrous (98%), sodium acetate anhydrous (98.5%), and ascorbic acid (99.5%) were from Penta (Praha, Czechia). Gallic acid hydrate (99%) was from Panreac (Barcelona, Spain). Rutin (quercetin 3-*O*-rutinoside) hydrate (>94%), kaempferol 3-*O*-glucoside, 2,4,6-tris (2-pyridyl)-*s*-triazine (TPTZ) (99%), neochlorogenic acid (≥98%) and Folin-Ciocalteu's phenol reagent were from Sigma-Aldrich (St. Louis, MO, USA). Iron chloride hexahydrate was from Merck (Darmstadt, Germany). Nicotinamide and chlorogenic acid (95%) were from Fluorochem (Hadfield, UK).

2.2. Plant Material

M. oleifera trees were cultivated in the Agioi Apostoloi area (at 3922039.400 N and 2154000.600 E and elevation of 100 m, according to Google Earth Pro version 7.3.2.5491 (64 bit) (Google, Inc., Mountain View, CA, USA) of Karditsa prefecture (Thessaly, Greece). The leaves from the plants were collected on the 25th October 2018, when the trees were 2 months old and they were transferred to the laboratory 30 min after collection. Afterwards, they were thoroughly washed with tap water and freeze dried using a Telstar Cryodos 80 freeze dryer (Telstar Industrial, S.A., Terrassa, Spain) for 12 h (the moisture content of the leaves was 8%) and ground in a ball-mill to give a powder with average particle diameter of 0.161 mm. The material was stored in air-tight plastic bags, at −40 °C.

2.3. Synthesis of DES

The procedure followed for the synthesis of all DES used was based on a previous report [18]. Accurately weighted appropriate amounts of glycerol (hydrogen bond donor) and nicotinamide (hydrogen bond acceptor) were mixed in a 100-mL round-bottomed flask and heated up to 80 °C,

under stirring at 500 rpm, for an approximate time period of 60 min. This time was sufficient to observe the formation of a transparent liquid. The liquids formed were allowed to cool down to room temperature and stored in sealed glass vials. The vials were checked periodically for the appearance of crystals, to ensure solvent stability. Checking was performed over a period of several weeks.

2.4. Screening Batch Stirred-Tank Extractions

An amount of 0.570 g of dried plant material was transferred into a 50-mL round-bottomed flask with 20 mL of solvent to give a liquid-to-solid ratio ($R_{L/S}$) of 35 mL g^{-1}. The mixture was extracted at 50 °C for 150 min, under continuous stirring at 500 rpm, on a thermostated hot plate (Witeg, Wertheim, Germany). An aliquot of 1 mL of the extract obtained was placed in a 1.5-mL Eppendorf tube and centrifuged at 10,000× *g* for 10 min. The clear supernatant was used for all analyses. Control extractions were performed with deionised water, 60% (*v*/*v*) aqueous methanol and 60% (*v*/*v*) aqueous ethanol, under identical conditions.

2.5. Ultrasound-Assisted Pretreatment

The pretreatment was carried out by ultrasonicating the solvent/solid material mixture before the batch stirred-tank extractions. Ultrasonication was conducted in an Elma D-78224 Singen HTW heated ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany), operated at a frequency of 50 Hz, a power of 550 W and an acoustic energy density of 78.6 W L^{-1} [19]. Pretreatments were performed at ambient temperature (23 ± 2 °C).

2.6. Response Surface Optimisation of the Extraction

The design of experiment was implemented with the aim at assessing the effect of three selected parameters (independent variables), the DES concentration (C_{DES}, termed as X_1), the liquid-to-solid ratio ($R_{L/S}$, termed as X_2), and the stirring speed (S_S, termed as X_3), on the extraction yield of total polyphenols (Y_{TP}), which represented the response [19]. The mode chosen was Box–Behnken with three central points and the independent variables were coded between −1 (lower limit) and +1 (upper limit), as follows:

$$x_i = \frac{X_i - X_0}{\Delta X_i},\ i = 1,\ 2,\ 3. \quad (1)$$

The term x_i represents the dimensionless value of the independent variable i and X_i the actual one. X_0 is termed the actual value of the independent variable i at the central point of the design, and ΔX_i the step change of X_i (Table 1). The range of values for the variables used was decided on the basis of previously published results [13,14]. Model significance, as well as the significance for each polynomial coefficient and the overall coefficient R^2 for the mathematical model were evaluated by performing ANOVA, at least at 95% significance level. Non-significant dependent terms ($p > 0.05$) of the model were not considered and were omitted. Model validation was accomplished by runs performed under the predicted optimal conditions. The actual (measured) values were then compared with the predicted ones.

Table 1. Process (independent) variables used for the experimental design and their codified levels.

Independent Variables	Code Units	Coded Variable Level		
		−1	**0**	**1**
C_{DES} (%, *w*/*v*)	X_1	55	70	85
$R_{L/S}$ (mL g^{-1})	X_2	20	60	100
S_S (rpm)	X_3	200	500	800

2.7. Total Polyphenol (TP) Determination

All samples were diluted 1:50 with 0.5% aqueous formic acid prior to determinations. For TP determination, a protocol described elsewhere was used [20]. Aliquot of 0.1 mL of diluted sample was mixed with 0.1 mL Folin-Ciocalteu reagent in a 1.5-mL Eppendorf tube and allowed to stand for 2 min. Following this, 0.8 mL of Na_2CO_3 solution (5% *w/v*) was added to the mixture and the tube was incubated in a thermostated water bath, at 40 °C, for 20 min. After incubation, the tube was cooled with tap water and the absorbance was recorded at 740 nm, using appropriate blank. Determination of total polyphenol concentration (C_{TP}) was carried out using a gallic acid calibration curve (10–80 mg L^{-1}) and results were expressed as mg gallic acid equivalents (GAE) L^{-1}. The extraction yield in total polyphenols was then calculated as follows:

$$Y_{TP}\ (\text{mg GAE g}^{-1}) = \frac{C_{TP} \times V}{dm} \quad (2)$$

where V is the extraction volume (in L) and dm is the dry mass of the solid material (in g).

2.8. Total Flavonoid (TFn) Determination

The determination was carried out according to a published protocol [21]. Samples were diluted as described for TP analysis and then 0.1 mL sample was mixed with 0.86 mL 35% (*v/v*) aqueous ethanol and 0.04 mL of reagent composed of 5% (*w/v*) $AlCl_3$ and 0.5 M CH_3COONa. The mixture was left to react at room temperature for 30 min, and the absorbance was recorded at 415 nm. Total flavonoid concentration (C_{TFn}) was determined using a calibration curve constructed with rutin as standard (15–300 mg L^{-1}). Results were reported as mg rutin equivalents (RtE) L^{-1}. Yield in TFn (Y_{TFn}) was estimated as described for TP.

2.9. Determination of the Antiradical Activity (A_{AR})

A previously established methodology was employed [22], using DPPH as the radical probe. Each extract was diluted 1:50 prior to analysis, as described for TP determination, and then 0.025 mL of the diluted sample was mixed with 0.975 mL DPPH (100 µM in methanol) and incubated at ambient temperature. The absorbance at 515 nm was read at t = 0 min (immediately after mixing) and at t = 30 min. The A_{AR} of the extract was determined using the equation:

$$A_{AR} = \frac{C_{DPPH}}{C_{TP}} \times \left(1 - \frac{A_{515(f)}}{A_{515(i)}}\right) \times Y_{TP} \quad (3)$$

where C_{DPPH} and C_{TP} is the DPPH concentration (µM) and total polyphenol concentration (mg L^{-1}) in the reaction mixture, respectively, $A_{515(f)}$ is the A_{515} at t = 30 min, and $A_{515(i)}$ is the A_{515} at t = 0. Y_{TP} represents the extraction yield (mg g^{-1}) in TP of each extract assayed. A_{AR} was given as µmol DPPH g^{-1} dm.

2.10. Determination of the Reducing Power (P_R)

The ferric-reducing power of the extracts was determined as reported elsewhere [22]. Before the assay, all extracts were diluted 1:50, as described for TP determination. Following this, 0.05 mL sample was incubated with 0.05 mL $FeCl_3$ (4 mM in 0.05 M HCl), for 30 min, at 37 °C, in a water bath. After the completion of the incubation, samples were cooled with tap water and 0.9 mL TPTZ solution (1 mM in 0.05 M HCl) was added. The mixture was left under ambient temperature for further 5 min for colour development and then the absorbance was obtained at 620 nm. P_R was reported as µmol ascorbic acid equivalents (AAE) g^{-1} dm, using an ascorbic acid calibration curve (50–300 µM).

2.11. Liquid Chromatography-Mass Spectrometry (LC-MS)

A modification of a method reported previously was used [23]. The chromatograph was a Finnigan (San Jose, CA, USA) MAT Spectra System P4000 pump, a UV6000LP diode array detector, and a Finnigan AQA mass spectrometer. The column used was a Fortis RP-18 column, 150 mm × 2.1 mm, 3 µm, operated at 40 °C, with a 10-µL injection loop. Mass spectra acquisition with electrospray ionization (ESI) in positive ion mode was accomplished with probe temperature set at 250 °C, acquisition at 10 and 50 eV, detector voltage at 650 V, capillary voltage at 4 kVm and source voltage at 25 V. The eluents used were 2% acetic acid (A) and methanol (B). The flow rate was 0.3 mL min^{-1}, and the elution program used was 0–30 min, 0%–100% methanol, 30–40 min, 100% methanol.

2.12. High-Performance Liquid Chromatography (HPLC) Analyses

A Shimadzu CBM-20A liquid chromatograph (Shimadzu Europa GmbH, Nordrhein-Westfalen, Germany) was used, coupled with a SIL-20AC auto sampler and a Shimadzu SPD-M20A detector. The system was interfaced by Shimadzu LC solution software. Analyses were performed on a Phenomenex Luna C18(2) column (100 Å, 5 µm, 4.6 × 250 mm) (Phenomenex, Inc., Torrance, CA, USA), maintained at a temperature of 40 °C, using 20-µL sample injections. Eluents were (A) 0.5% aqueous formic acid and (B) 0.5% formic acid in MeCN/water (6:4), and the flow rate was 1 mL min^{-1}. The elution program was as follows: 100% A to 60% A in 40 min, 60% A to 50% A in 10 min, 50% A to 30% A in 10 min, and then isocratic elution for another 10 min. Quantification was done at 325 nm (chlorogenates) and 355 nm (flavonols), using calibration curves constructed with chlorogenic acid (1–50 µg mL^{-1}, $R^2 = 0.9999$), neochlorogenic acid (1–50 µg mL^{-1}, $R^2 = 0.9997$) kaempferol 3-*O*-glucoside (1–50 µg mL^{-1}, $R^2 = 0.9996$), and quercetin 3-*O*-rutinoside (1–50 µg mL^{-1}, $R^2 = 0.9990$).

2.13. Statistical Analyses

Extractions were repeated at least twice, and determinations were carried out at least in triplicate. Values reported are means ± standard deviation (sd). Correlations were performed with regression analysis, at least at a 95% significance level ($p < 0.05$), using SigmaPlot™ 12.5. Experimental design and response surface methodology, as well as every associated statistical analysis (e.g., ANOVA) was performed with JMP™ Pro 13.

3. Results and Discussion

3.1. DES Synthesis and Examination of HBD: HBA Molar Ratio ($R_{mol}^{D/A}$)

The use of nicotinamide as HBA in L-lactic acid-based DES has been recently reported for the first time [24]; however, the use of glycerol as HBD in combination with nicotinamide as HBA, to the best of the authors' knowledge, is heretofore unreported. When designing a DES, the role of $R_{mol}^{D/A}$ in the polyphenol extraction performance is salient [19,25], and therefore, an initial screening of DES with varying $R_{mol}^{D/A}$ was deemed essential in identifying the highest-performing combination.

Attempts to combine glycerol and nicotinamide at $R_{mol}^{D/A}$ up to 4 were not met with success, since the DES synthesized tended to develop crystals within 24 h, at room temperature (23 ± 2 °C). Combination at $R_{mol}^{D/A} = 5$ produced a DES, which remained stable for several weeks, and based on this finding, a series of DES with $R_{mol}^{D/A}$ ranging from 5 to 13 were synthesized and tested for their effectiveness in extracting polyphenols from *M. oleifera*. All the DES assayed were used as 70% (*w/v*) aqueous mixtures and the outcome of this screening is given in Figure 1. The DES with $R_{mol}^{D/A} = 5$ exhibited statistically higher extraction efficiency ($p < 0.05$) compared with all other DES and also 60% (*v/v*) aqueous ethanol and 60% (*v/v*) aqueous methanol. Water, on the other hand, was the least efficient solvent ($p < 0.05$). On this ground, the DES with $R_{mol}^{D/A} = 5$, assigned as GL-Nic (5:1), was chosen for further examination.

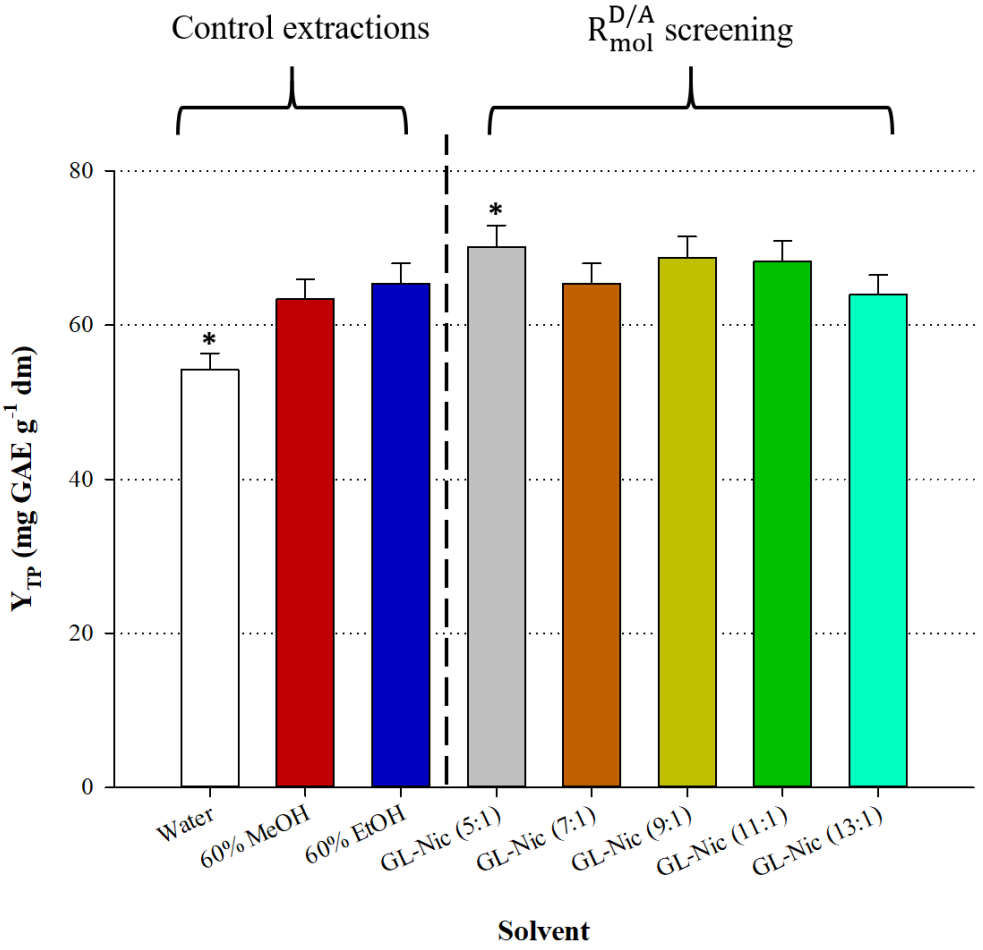

Figure 1. Plot showing the effect of $R_{mol}^{D/A}$ on the extraction effectiveness of the DES tested. Extractions were carried out at 50 °C, with C_{DES} = 70% (*w/v*), at 500 rpm, for 150 min. Assignment with "*" denotes statistically different value.

3.2. The Effect of Ultrasonication Pretreatment

To examine the effect of ultrasonication, GL-Nic (5:1) used as 70% (*w/v*) aqueous solution, was mixed with dried powder of *M. oleifera* leaves, shaken vigorously for a few seconds to form slurry, and then placed in the ultrasonication bath, at room temperature. Room temperature (23 ± 2 °C) was preferred, because at lower temperatures, ultrasonication results in better cavitation, which is the main effect involved in the ultrasound-assisted extraction. At higher temperatures, bubbles formed through cavitation collapse less violently, and thus the ultrasonication effect is less effective [26]. Ultrasonication treatments were performed for 5, 10, 20, 30, and 40 min, followed by stirred-tank extraction. As can be seen in Figure 2, a regime of 30-min ultrasonication gave statistically higher Y_{TP} ($p < 0.05$), whereas extraction without ultrasonication pretreatment (0 min) was significantly less effective ($p < 0.05$). Ultrasonication beyond 30 min resulted in decreased Y_{TP}, thus 30 min was chosen as the most appropriate pretreatment period.

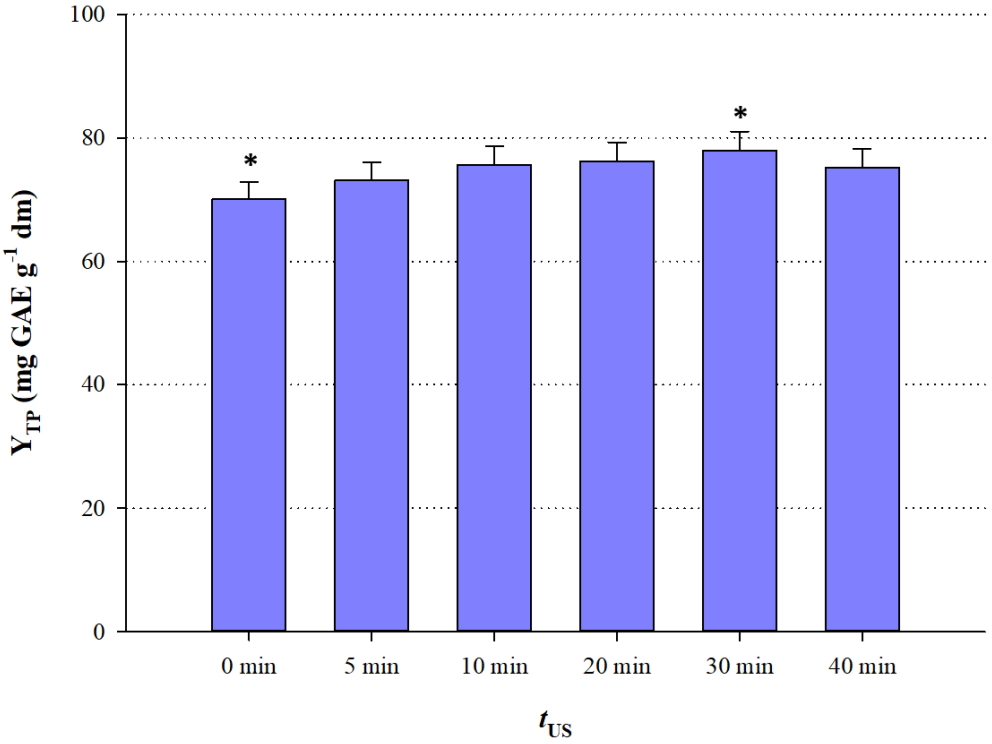

Figure 2. The effect of ultrasonication time (t_{US}) on the extraction yield of total polyphenols. Extractions were performed with the 70% (*w/v*) aqueous GL-Nic (5:1), at 23 °C and $R_{L/S}$ = 35 mL g^{-1} dm. Assignment with "*" denotes statistically different value.

3.3. Optimisation of Extraction Performance

The evidence emerged from recent examinations on polyphenol extraction with DES suggested that the three process (independent) variables considered, namely C_{DES}, $R_{L/S}$, and S_S, are highly influential in solid/liquid extraction processes [19,25]. For this reason, these three parameters were used among several others to build a predictive extraction model. Two cases were examined, one with a 30-min ultrasonication regime prior to every extraction and one without ultrasonication. The purpose of such investigation was to clarify whether an ultrasonication step before batch stirred-tank extraction could affect the optimization settings and assist in achieving significantly higher extraction yield.

Appraisal of the fitted models and response surface suitability were based on the ANOVA test and lack-of-fit test. The *p*-value for each equation term was calculated to examine the contribution of linear, interaction, and quadratic effects in the independent variables (Table 2). Statistically non-significant terms were omitted from the mathematical models, which are given as polynomial equations in Table 3. The predicted response values calculated by the models, along with the measured (actual) values for each design point, are shown in Table 4. R^2 provides indication of the amount of total variability around the mean explained by the regression model. R^2 terms for both models were ≥0.95, so it would be argued that the estimation of the regression equations exhibited a good adjustment to the experimental data. The *p* value for lack-of-fit (assuming a confidence interval of 95%) was >0.05 for both models (Table 4), suggesting that the fitted models may be reliable predictors. By setting the values of all three process variables at their optima, using the desirability function (Figure 3), it was made possible to calculate the maximum predicted responses (Table 5). The response surface plots (Figure 4) were helpful in visualizing which variables were more influential to the response.

Table 2. Statistical data obtained after implementing response surface methodology.

Term	Standard Error	t Ratio	*p*-Value	Sum of Squares	*F* Ratio
No pretreatment					
C_{DES}	0.778011	5.09	0.0038 *	125.215	25.858
$R_{L/S}$	0.778011	0.72	0.5057	2.48645	0.5135
S_S	0.778011	6.95	0.0009 *	233.820	48.286
C_{DES} $R_{L/S}$	1.100274	−3.54	0.0166 *	60.6841	12.531
C_{DES} S_S	1.100274	−1.33	0.2398	8.61423	1.7789
$R_{L/S}$ S_S	1.100274	0.40	0.7089	0.75690	0.1563
$C_{DES}C_{DES}$	1.145201	−2.27	0.0726	24.9200	5.1462
$R_{L/S}$ $R_{L/S}$	1.145201	0.17	0.8685	0.14708	0.0304
S_S S_S	1.145201	−0.39	0.7118	0.74079	0.1530
Lack-of-fit			0.1133	22.3468	7.9870
Ultrasonication pretreatment					
C_{DES}	1.074859	63.28	<0.0001 *	28.38811	8.1905
$R_{L/S}$	0.658214	−2.86	0.0353 *	199.7001	57.617
S_S	0.658214	7.59	0.0006 *	21.38580	6.1702
C_{DES} $R_{L/S}$	0.658214	−2.48	0.0556	18.19023	5.2482
C_{DES} S_S	0.930855	2.29	0.0706	18.14760	5.2359
$R_{L/S}$ S_S	0.930855	−2.29	0.0708	22.46760	6.4824
$C_{DES}C_{DES}$	0.930855	−2.55	0.0515	43.53467	12.560
$R_{L/S}$ $R_{L/S}$	0.968865	−3.54	0.0165 *	24.50608	7.0705
S_S S_S	0.968865	2.66	0.0449 *	30.17521	8.7061
Lack-of-fit			0.4891	11.0748	1.1804

* Asterisk denotes statistically significant values.

Table 3. Mathematical models (equations) derived after implementing response surface methodology (Box–Behnken design).

Case	Polynomial Equations	R^2	*p*
No pretreatment	$72.98 + 3.96X_1 + 5.41X_3 - 3.90X_1X_2$	0.95	0.0093
Ultrasonication pretreatment	$68.02 - 1.88X_1 + 5.00X_2 - 1.64X_3 + 2.58X_2^2 + 2.88X_3^2$	0.96	0.0055

Table 4. Points of the experimental design considered, and measured and predicted values of the response.

Design Point	Independent Variables			Response (Y_{TP}, mg GAE g^{-1} dm)			
	X_1 (C_{DES}, % *w/v*)	X_2 ($R_{L/S}$, mL g^{-1})	X_3 (S_S, rpm)	Without UP		With UP	
				Measured	Predicted	Measured	Predicted
1	−1 (55)	−1 (20)	0 (500)	62.82	62.18	66.09	66.18
2	−1 (55)	1 (100)	0 (500)	72.96	71.08	72.58	71.91
3	1 (85)	−1 (20)	0 (500)	76.00	77.88	57.48	58.15
4	1 (85)	1 (100)	0 (500)	70.56	71.20	72.50	72.41
5	0 (70)	−1 (20)	−1 (200)	68.73	67.21	67.03	67.72
6	0 (70)	−1 (20)	1 (800)	76.86	77.15	70.65	69.19
7	0 (70)	1 (100)	−1 (200)	67.74	67.45	81.00	82.46
8	0 (70)	1 (100)	1 (800)	77.61	79.13	75.14	74.45
9	−1 (55)	0 (60)	−1 (200)	56.94	59.11	69.62	68.83
10	1 (85)	0 (60)	−1 (200)	70.31	69.96	70.69	69.33
11	−1 (55)	0 (60)	1 (800)	72.50	72.85	68.46	69.82
12	1 (85)	0 (60)	1 (800)	80.00	77.83	61.01	61.80
13	0 (70)	0 (60)	0 (500)	72.94	72.98	66.07	68.02
14	0 (70)	0 (60)	0 (500)	73.97	72.98	68.47	68.02
15	0 (70)	0 (60)	0 (500)	72.04	72.98	69.52	68.02

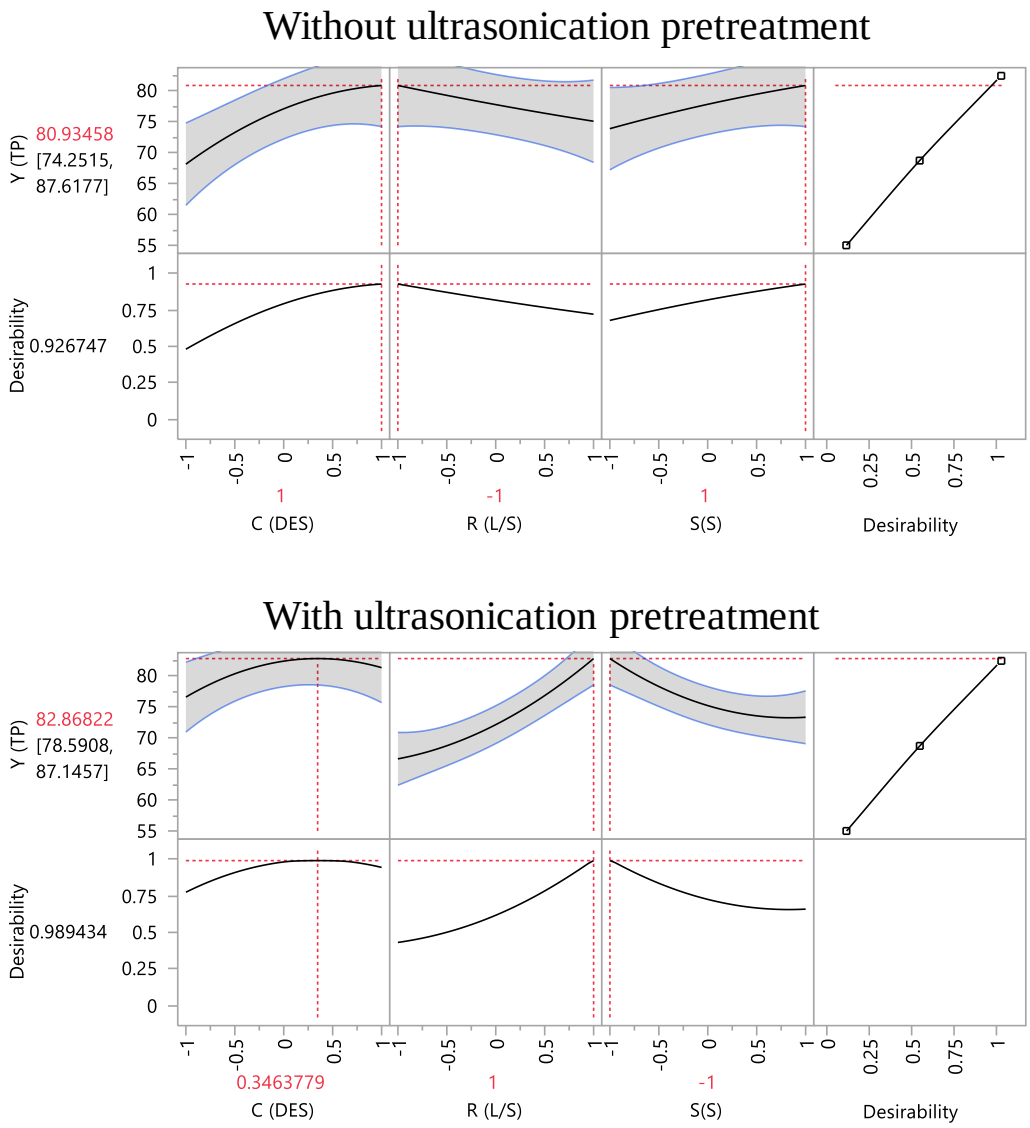

Figure 3. Desirability graphs displaying optimal settings and maximum theoretical extraction yields for total polyphenols (mg GAE g^{-1} dm).

Table 5. Optimal predicted conditions and maximum predicted values (±sd) for the extraction of *M. oleifera* with and without ultrasonication pretreatment.

Case	Maximum Predicted Response (Y_{TP}, mg GAE g^{-1} dm)	Optimal Conditions		
		C_{DES} (*w*/*v*, %)	$R_{L/S}$ (mL g^{-1})	S_S (rpm)
No pretreatment	80.93 ± 6.68	85	20	800
Ultrasonication pretreatment	82.87 ± 4.28	75	100	200

Ultrasonication of samples prior to stirred-tank extraction had a very pronounced effect on the extraction models, as can be concluded by the equations derived (Table 3). Thus, while C_{DES} (X_1) had a positive contribution in maximizing Y_{TP} in the extraction without ultrasonication pretreatment, it negatively affected the ultrasound-pretreated extraction. On the contrary, increased S_S (X_3) was significant for maximizing Y_{TP} in samples received no pretreatment, whereas ultrasound-pretreated samples required the lowest S_S used to give maximum Y_{TP}.

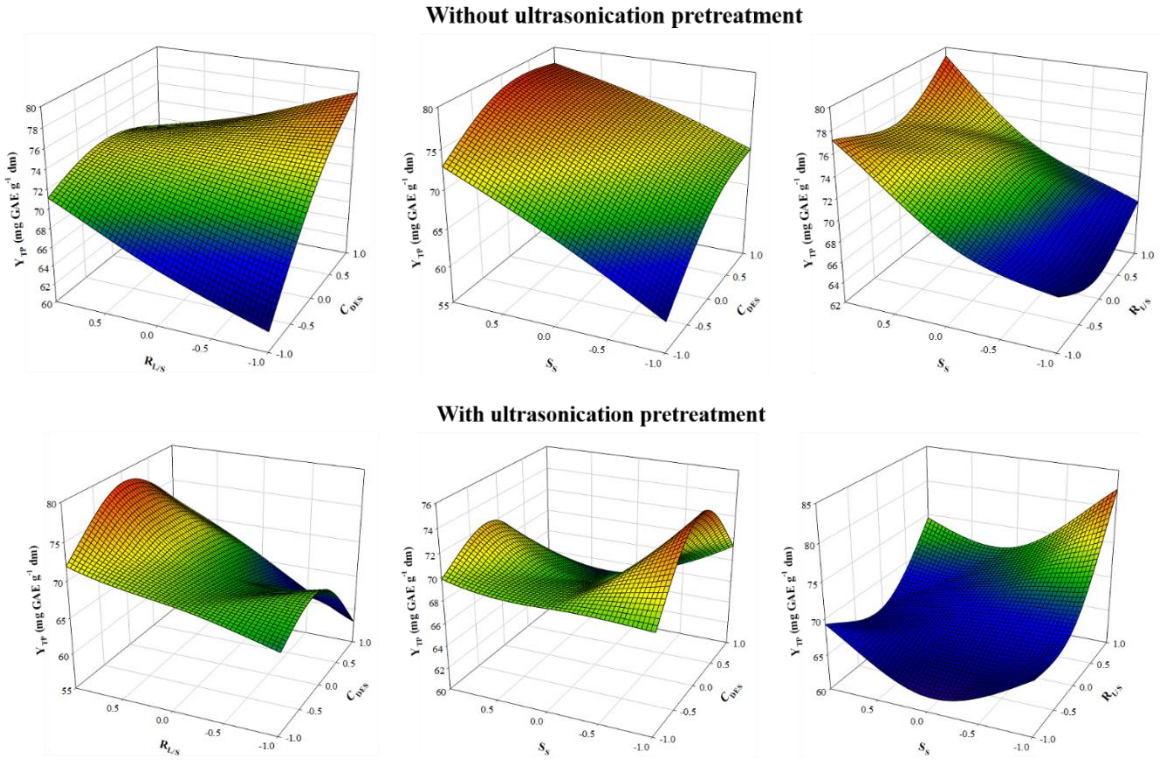

Figure 4. Three-dimensional diagrams given on a comparative arrangement, to illustrate the effect of ultrasonication pretreatment on the variations in the response (Y_{TP}), as a function of different variable levels.

Furthermore, for the extractions without pretreatment, $R_{L/S}$ (X_2) had no direct impact on Y_{TP}, but its cross effect (X_1X_2) with C_{DES} was negative. For the ultrasound-pretreated extractions, quadratic effects of both $R_{L/S}$ (X_2) and S_S (X_2) were positive and significant.

The impact of the ultrasonication pretreatment could be more characteristically depicted by the changes found in the optimization settings of all three variables. Pretreatment resulted in shifting the theoretically required DES amount from 85% to 75% (*w/v*), $R_{L/S}$ from 20 to 100 mL g^{-1}, and S_S from 800 to 200 rpm (Table 5). This outcome suggested a profound influence of ultrasonication on the pattern through which the process variables can affect polyphenol extraction. On the other hand, it is to be emphasized that the ultrasonication pretreatment exerted virtually no effect on the extraction performance, since the optimal predicted values for Y_{TP}, for the extraction without and with pretreatment, had no statistically significant difference, being 80.93 ± 6.68 and 82.87 ± 4.28 mg GAE g^{-1} dm, respectively (Table 5).

A critical assessment of these data would point out that samples received no ultrasonication pretreatment required higher DES concentration and a relatively high speed of stirring to yield extracts with increased Y_{TP}. The optimum C_{DES} determined was 85% (*w/v*), very close to the 80% (*w/v*) found for *M. oleifera* polyphenol extraction with a DES composed of glycerol and sodium acetate (6:1) [13]. Likewise, the optimum S_S (800 rpm) was comparable to 900 rpm determined for polyphenol extraction from onion solid wastes, using a DES of glycerol/sodium propionate (8:1) [25]. By contrast, the optimum $R_{L/S}$ (20 mL g^{-1}) was rather low compared with those reported for polyphenol extraction with DES, ranging from 29 mL g^{-1} [27] to 100 mL g^{-1} [25].

This picture was fundamentally changed when samples were ultrasonicated prior to stirred-tank extraction. The optimum C_{DES} dropped to 75% (*w/v*), while ultrasonication-pretreated samples required 5 times higher $R_{L/S}$ but 4 times lower S_S to provide maximum Y_{TP} (Table 5). Ultrasonication is known to contribute in intensification of extraction efficacy, owed to several phenomena that accompany irradiation with ultrasounds, including propagation of ultrasound pressure waves through

the solvent and resulting cavitation effects [28,29]. The increased performance usually observed in the ultrasound-assisted extractions is generally attributed to mechanical, cavitation, and thermal effects, which can provoke cell wall disruption and reduction of particle size, resulting eventually in enhanced mass transfer across cell membranes. On such a theoretical background, it could be postulated that the reduced C_{DES} required for the ultrasonicated-pretreated samples to reach optimum Y_{TP}, could probably be ascribed to higher polyphenol diffusivity and solubilization, as a result of cell wall/membrane disruption. Such an event could facilitate leaching and dissolution of polyphenols into the liquid phase, an assumption corroborated by the drastic decrease in the optimum S_S required, from 800 to 200 rpm. It would appear that lower S_S (200 rpm) is sufficient to provide the turbulence necessary for effective diffusion, whereas higher S_S (800 rpm) might provoke adsorption effects, thereby hindering higher extraction yields.

On the other hand, the pronounced increase in the optimum $R_{L/S}$ could be attributed to a rapid accumulation of polyphenols at the surface of the solid particles, due to cell breakdown and particle disintegration, that would cause extensive liberation of polyphenols from the interior of the solid particles. Such accumulation would presumably require a higher concentration gradient for effective diffusivity, hence the higher $R_{L/S}$. The parameter $R_{L/S}$ is tightly associated with diffusion phenomena [30–32], and it has been demonstrated that rising $R_{L/S}$ could bring about a significant diffusivity increase [33].

3.4. Extraction Kinetics and Temperature Effects

The optimization of the extraction process revealed significant changes in the extraction variables as a consequence of ultrasonication pretreatment, but to portray the ultrasound effect on the process in an integrated frame, information pertaining to the kinetics of the extraction and the effect of temperature was indispensable. Towards this objective, extraction kinetics were traced over a wide breadth of temperatures, ranging from 40 to 80 °C (Figure 5). In each case, the optimized conditions were used to carry out the extractions (Table 5). A modification of a previously proposed kinetic model was employed for both non-pretreated and ultrasound-pretreated samples [34]:

$$Y_{TP(t)} = Y_{TP(0)} + \frac{Y_{TP(s)}t}{t_{0.5} + t}. \quad (4)$$

The term $Y_{TP(t)}$ represents the extraction yield in total polyphenols (mg GAE g^{-1} dm) at any time t, $Y_{TP(0)}$ is a fitting parameter, $Y_{TP(s)}$ the yield in total polyphenols at equilibrium (saturation), and $t_{0.5}$ half the time (min) required for the extraction to enter the regular regime. The phase of the regular regime refers to the period within which small increases in Y_{TP} are achieved within relatively large t [35]. In all cases examined, fitting of the kinetic model to the experimental data gave $R^2 > 0.98$ ($p < 0.0001$), suggesting that the model implemented could very effectively describe extraction kinetics. Determination of the initial rate of the extraction, h, and the second-order extraction rate, k, was accomplished using the following equations:

$$h = \frac{Y_{TP(s)}}{t_{0.5}}, \quad (5)$$

$$k = \frac{1}{Y_{TP(s)}\, t_{0.5}}. \quad (6)$$

The data generated from the kinetic assay are analytically presented in Table 6. The effect exerted by the ultrasonication pretreatment was shown to be temperature-dependent, since acceleration of the extraction (increased k) was seen only at 70 and 80 °C. This trend was corroborated by the values determined for h, which were increased for the ultrasound-pretreated samples at temperatures higher than 60 °C. Likewise, $t_{0.5}$ was shorter for the extractions received ultrasonication pretreatment at temperatures higher than 60 °C. Furthermore, for every temperature tested, Y_{TP} of the

ultrasonication-pretreated samples was always significantly higher than that of the non-pretreated ones. However, in both cases, temperature had a non-significant effect on $Y_{TP(s)}$, since it varied between 82.03 to 83.90 mg GAE g^{-1} dm for the non-pretreated extractions and from 91.24 to 93.97 mg GAE g^{-1} dm for the ultrasound-pretreated extractions.

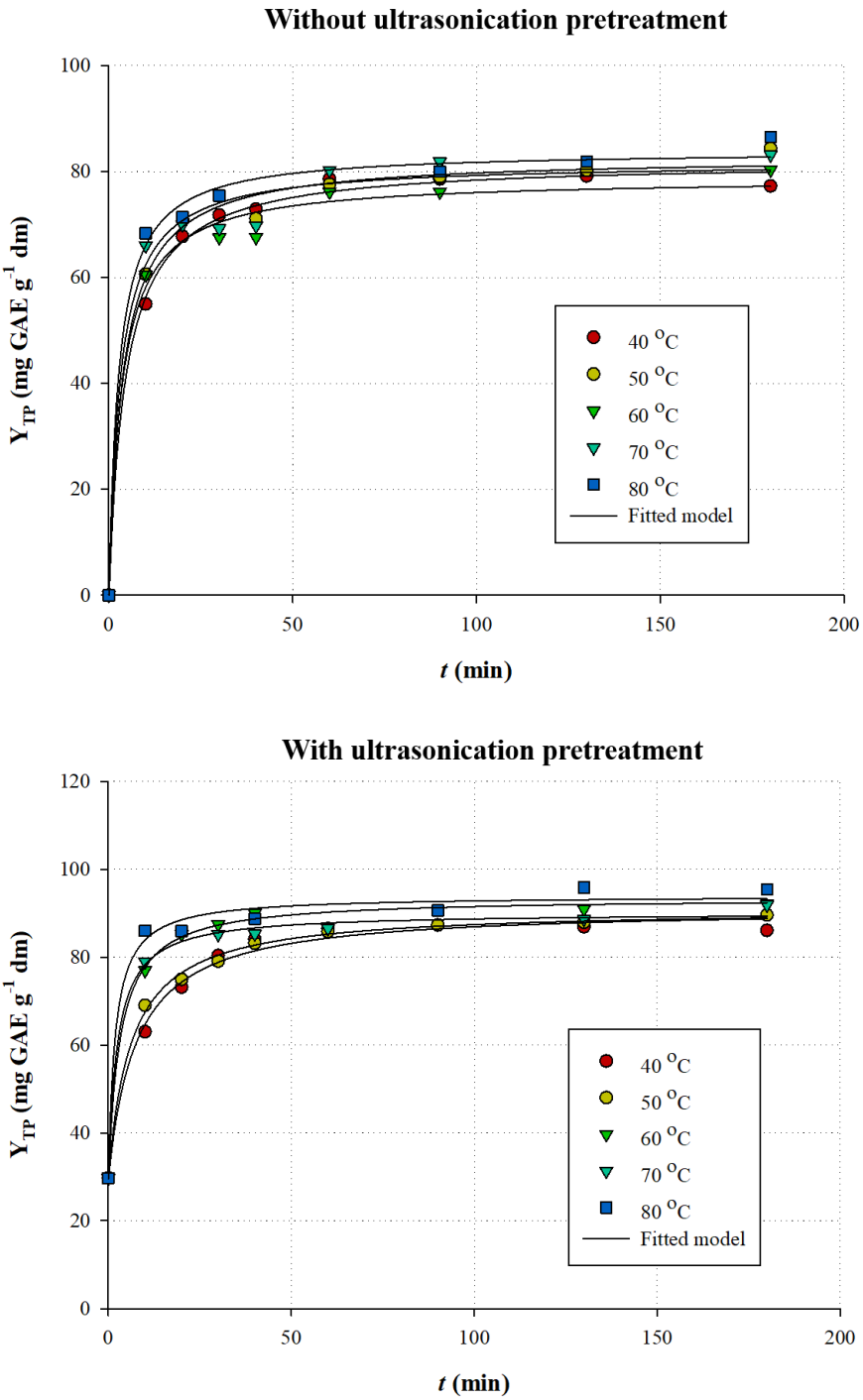

Figure 5. Kinetics of polyphenol extraction from *M. oleifera* leaves, with and without prior ultrasonication pretreatment. Extraction conditions for the non-pretreated sample: C_{DES} = 85% (*w/w*); $R_{L/S}$ = 20 mL g^{-1}; S_S = 800 rpm. Extraction conditions for the ultrasonication-pretreated sample: C_{DES} = 75% (*w/w*); $R_{L/S}$ = 100 mL g^{-1}; S_S = 200 rpm.

Table 6. Kinetic data and the effect of temperature.

T (°C)	Kinetic Parameters				
	k ($\times 10^{-3}$) (g mg^{-1} min^{-1})	h (mg g^{-1} min^{-1})	$Y_{TP(s)}$ (mg GAE g^{-1})	$t_{0.5}$ (min)	E_a (kJ mol^{-1})
No pretreatment					
40	2.68	18.03	82.03	4.55	
50	3.17	21.71	82.71	3.81	
60	3.58	22.15	78.62	3.55	11.24
70	3.95	26.32	81.60	3.10	
80	4.40	30.96	83.90	2.71	
Ultrasonication pretreatment					
40	1.46	12.12	91.24	7.53	
50	1.76	14.58	91.01	6.24	
60	3.29	28.78	93.52	3.25	34.02
70	4.52	36.79	90.14	2.45	
80	5.72	50.52	93.97	1.86	

Pretreatment with ultrasonication is likely to solubilize all polyphenols occurring at and near the surface of the solid particles. This assumption could justify the fact that at temperatures 40–60 °C the stirred-tank extraction of the ultrasound-pretreated samples proceeded at a lower rate. On the other hand, the non-pretreated samples had a higher load of polyphenols at the exterior of the solid particles and exhibited apparently increased k and h. At higher temperatures, polyphenol extraction was more rapid for the ultrasonicated samples, most probably because the pretreatment brought about disruption of the cell walls of the plant material, which enabled more facile penetration of the solvent into the solid particles and easier entrainment of the solute (polyphenols) in the liquid phase. Concerning $Y_{TP(s)}$, the variation as a response to T was non-significant, a fact indicating the T had virtually no effect on $Y_{TP(s)}$. This outcome is a paradox, in light of previous investigations, which demonstrated that increasing T had a proportional effect on $Y_{TP(s)}$ [36,37]. However, earlier studies on polyphenol extraction from *M. oleifera* leaves with a glycerol/sodium acetate DES revealed that switching T from 50 to 80 °C resulted in a constant decline in $Y_{TP(s)}$ [13]. The fact that increasing T did not contribute in attaining higher $Y_{TP(s)}$ might suggest that extraction could reach equilibrium after a given period of time, irrespective of the T.

The increase in k as a response to increasing T was found to obey the Arrhenius law:

$$k = k_0 e^{-\frac{E_a}{RT}}. \quad (7)$$

where k_0, R, T, and E_a correspond to the temperature-independent factor (min^{-1}), the universal gas constant (8.314 J K^{-1} mol^{-1}), the absolute temperature (K), and the activation energy (J mol^{-1}). The linear expression of Equation (7) would be:

$$lnk = lnk_0 + (-\frac{E_a}{R})\frac{1}{T}. \quad (8)$$

Thus, E_a could be determined graphically, by the slope ($-\frac{E_a}{R}$) of the straight line obtained by plotting lnk as a function of $1/T$ (Figure 6).

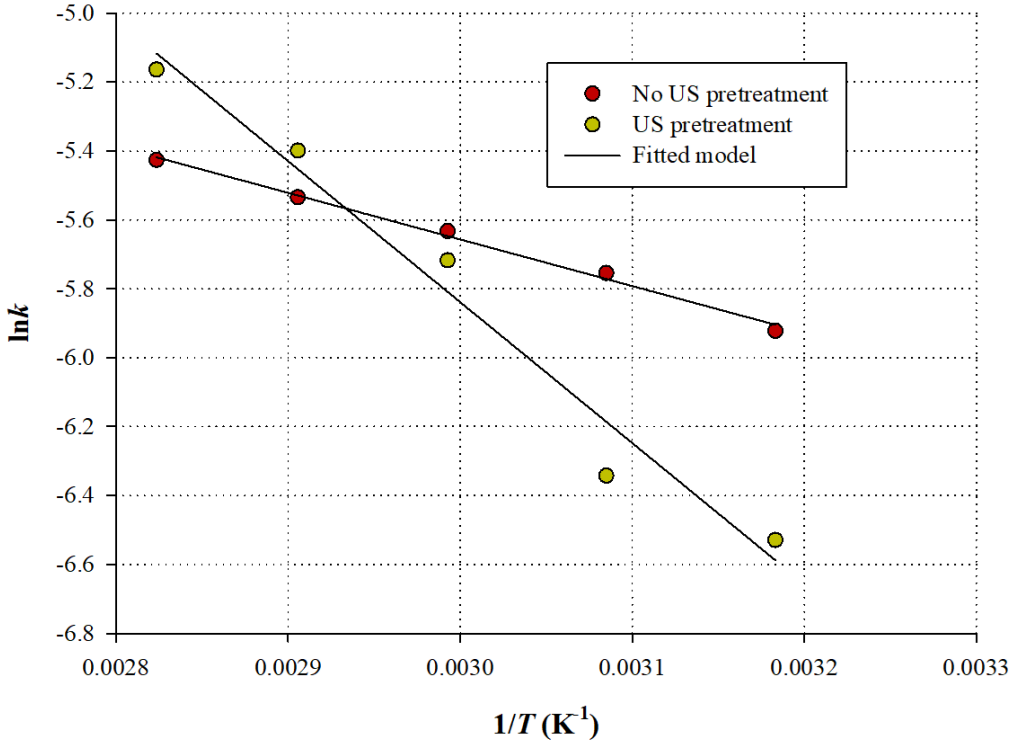

Figure 6. Arrhenius plot for the extractions with no pretreatment (No US) and ultrasonication pretreatment (US).

The E_a values for the extractions without and with ultrasonication pretreatment were 11.24 and 34.02 kJ mol^{-1}, respectively (Table 6), clearly showing that the extraction of the ultrasonication-pretreated samples was more energy-demanding by almost 4 times. Based on this outcome, it would appear that ultrasonication prior to batch stirred-tank extraction does not lower the requirements in energy for accomplishing an efficient extraction of polyphenols from *M. oleifera* leaves, but the energetic needs are even higher.

Yet, such observation could be rather misleading and the increased of $Y_{TP(s)}$ of the ultrasonication-pretreated samples should not be overlooked. The increased E_a value found for the ultrasonication pretreated extraction was most probably because during pretreatment the most readily extracted polyphenols were dissolved in the liquid phase (solvent), leaving behind (inside the solid particles) polyphenols that were more difficult to extract, since solubilization of these polyphenols would depend on internal diffusion, which is the rate-determining step in diffusion-controlled extractions [30,38]. Therefore, the following stirred-tank extraction was apparently more energy-demanding. However, considering $Y_{TP(s)}$, it could be argued that ultrasonication facilitated the washing (initial) phase of the extraction, provoking extended release of superficial polyphenols, whereas the stirred-tank extraction recovered the remaining compounds, located deeper inside the particles. Indeed, just after ultrasonication the Y_{TP} was approximately 29.70 mg GAE g^{-1} dm, which was almost 30% of the $Y_{TP(s)}$ achieved with ultrasonication pretreatment (Table 6). These data strongly supported that a large part of polyphenols was extracted during the ultrasonication step.

3.5. Polyphenolic Profile

To illustrate the effect of ultrasonication pretreatment on the analytical polyphenolic profile, LC-MS was undertaken for the samples obtained after 180 min of extraction, at 80 °C, under optimal conditions. A typical chromatogram is given in Figure 7, showing the principal substances detected at both 320 and 360 nm. It should be noted that for both ultrasonication-pretreated and non-pretreated samples,

the chromatograms recorded were identical (data not shown), evidencing no effect of ultrasonication on the polyphenolic profile.

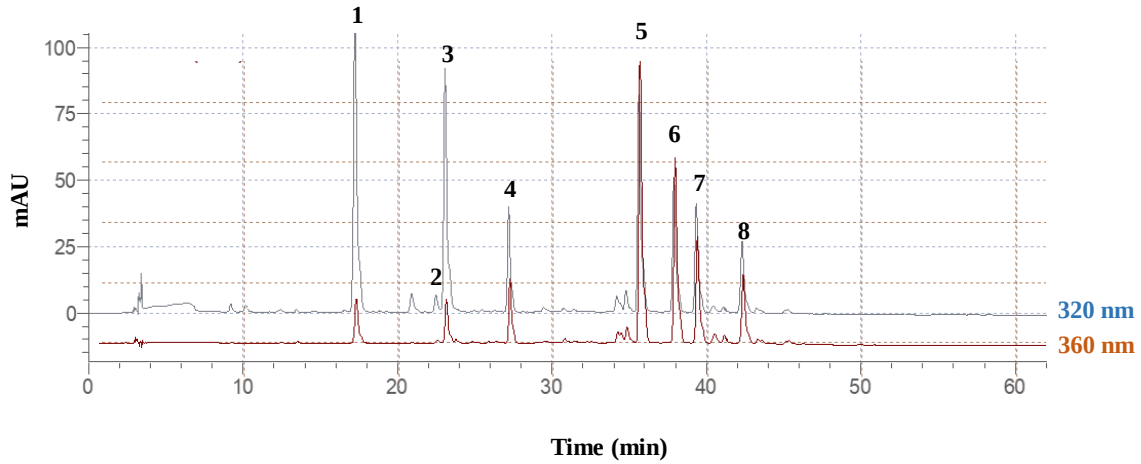

Figure 7. HPLC traces of the sample extracted under optimal conditions, at 80 °C, with ultrasonication pretreatment, recorded at 320 and 360 nm. Peak assignment: 1, neochlorogenic acid; 2, chlorogenic acid; 3, chlorogenic acid isomer; 4, multiflorin B; 5, quercetin glucoside; 6, quercetin manonylglycoside derivative; 7, kaempferol glucoside; 8, kaempferol malonylglucoside.

Peak #1 showed a pseudo-molecular ion at m/z = 355 and a derivative ion at m/z = 163. Considering the UV-vis characteristics, this structure was assigned to neochlorogenic acid. The identity of this peak was further confirmed by comparing the UV-vis spectrum and retention time (Rt) with an original standard. Likewise, peaks 2 and 3 were tentatively identified as chlorogenic acid and a chlorogenate isomer, respectively (Table 7).

Table 7. Analytical spectrometric data of the major polyphenolic phytochemicals detected in the extracts of *M. oleifera* leaves.

No	Rt (min)	UV-Vis	$[M + H]^+$ (m/z)	Fragment Ions (m/z)	Tentative Identity
1	17.24	244, 318	355	163	Neochlorogenic acid
2	22.48	246, 318	355	377, 163	Chlorogenic acid
3	23.07	246, 318	355	377, 163	Chlorogenic acid isomer
4	27.19	270, 320(s), 340	595	617$[M + Na]^+$, 287	Multiflorin B
5	35.62	256, 318(s), 354	465	487$[M + Na]^+$, 303	Quercetin glucoside
6	37.89	256, 316(s), 360	833	609, 573, 551, 303	Quercetin malonylglycoside derivative
7	39.34	264, 318(s), 348	449	471$[M + Na]^+$, 287	Kaempferol glucoside
8	42.30	264, 348	535	557$[M + Na]^+$, 287	Kaempferol malonylglucoside

Peak #4 displayed a pseudo-molecular ion at m/z = 595, a sodium adduct at m/z = 617, and a diagnostic fragment (aglycone) at m/z = 287. This compound was tentatively assigned to multiflorin B (kaempferol 3-*O*-rhamnosylglucoside). Similarly, peaks 7 and 8 were assigned to a kaempferol glucoside and kaempferol malonylglucoside. Peaks 5 and 6, which exhibited typical quercetin glycoside UV-vis pattern and diagnostic fragment at m/z = 303, were tentatively identified as quercetin glucoside and quercetin malonylglycoside derivative, respectively [39]. All these compounds have been previously reported in *M. oleifera* leaf extracts [13]; however, the presence of a quercetin rhamnoside derivative claimed in an earlier study [40] was not confirmed.

The information emerged from the quantitative data (Table 8) showed that ultrasonication pretreatment afforded changes in the recovery of major polyphenols, but not all substances were equally affected.

Table 8. Quantitative data for the major polyphenols detected in *M. oleifera* extracts.

Compound	Content (mg g^{-1} dm) ± %rsd	
	No Pretreatment	Ultrasonication Pretreatment
Neochlorogenic acid	6.61 ± 0.10	6.82 ± 0.11
Chlorogenic acid	0.20 ± 0.00	0.22 ± 0.00
Chlorogenic acid isomer	5.32 ± 0.07	5.22 ± 0.08
Total chlorogenates	*12.13*	*12.26*
Multiflorin B	2.91 ± 0.03	3.11 ± 0.03
Quercetin glucoside	10.58 ± 0.11	10.65 ± 0.12
Quercetin malonylglycoside derivative	5.97 ± 0.06	6.99 ± 0.07
Kaempferol glucoside	3.87 ± 0.04	4.16 ± 0.05
Kaempferol malonylglucoside	0.37 ± 0.00	0.42 ± 0.00
Total flavonols	*23.70*	*25.33*
Total polyphenols	*35.83*	*37.59*

For neochlorogenic acid and chlorogenic acid, ultrasonication pretreatment brought about an increase by 3.1% and 9.1%, but for the chlorogenic acid isomer, a decrease by almost 1.9% was observed. Overall, total chlorogenate content was by 1.1% increased, a difference that falls within the limits of statistical error. Thus, ultrasonication pretreatment had practically no effect on chlorogenate extractability. On the other hand, all flavonol glycosides had higher content in the ultrasonication-pretreated sample, the increases varying from 0.65% (quercetin glucoside) to 14.6% (quercetin manolyglycoside derivative). Overall, the increase in flavonol content caused by ultrasonication pretreatment was 6.4%, and for all polyphenols considered (chlorogenates + flavonols), an increase by 4.7% was determined.

4. Conclusions

The extraction of *M. oleifera* leaf polyphenols with a novel, glycerol/nicotinamide DES was performed by deploying an ultrasonication pretreatment step. This process enabled the clarification of the use of ultrasounds in the performance of the extraction, with regard to yield, duration, and energy demands. The optimization through response surface methodology revealed that when samples were ultrasonication-pretreated, the subsequent batch stirred-tank extraction required less amount of DES and much lower stirring speed for maximum polyphenol yield, but also a higher amount of solvent per dry mass unit. However, no significant difference in the maximum polyphenol yield was determined. Kinetics showed that higher extraction rate, and therefore shorter extraction time, of the ultrasound-pretreated samples, could be attained only when stirred-tank extraction was carried out at $T \geq 70$ °C. The higher energy barrier estimated for the ultrasonication-pretreated extractions was representative of the harder-to-extract compounds, since a significant wash out of the most readily extracted polyphenols took place during pretreatment. It is concluded that sample ultrasonication as a pretreatment step might be favourable in reducing extraction time, and solvent and energy requirements, but further studies with various plant tissues are needed to obtain a deeper insight.

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Nomenclature

A_{AR}	antiradical activity (μmol DPPH g^{-1})
dm	dry matter (g)
P_R	reducing power (μmol AAE g^{-1})
$R_{L/S}$	liquid-to-solid ratio (mL g^{-1})
$R^{D/A}_{mol}$	molar HBD:HBA ratio (dimensionless)
S_S	stirring speed (rpm)
t	time (min)
T	temperature (°C)
Y_{TP}	yield in total polyphenols (mg GAE g^{-1})

Abbreviations

AAE	ascorbic acid equivalents
DES	deep eutectic solvents
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
GAE	gallic acid equivalents
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
TPTZ	2,4,6-tripyridyl-*s*-triazine
UP	ultrasonication pretreatment

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