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Research Article

Pressurized Liquid Extraction Optimization from Supercritical Defatted Olive Pomace: A Green and Selective Phenolic Extraction Process

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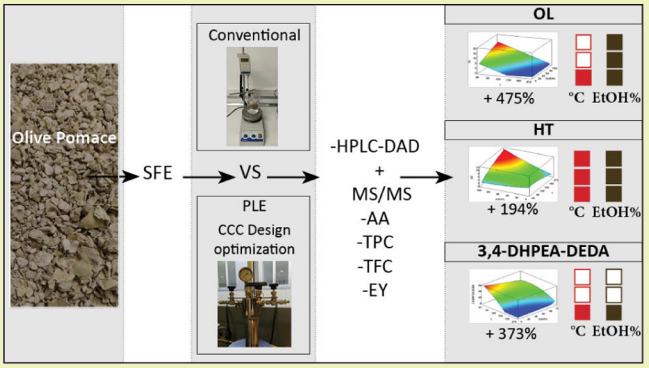
ABSTRACT: Olive pomace (OP) is the main by-product of the olive oil industry produced in large quantities. Its valorization as a source of phenolic bioactive compounds is paramount for the sustainable growth of related industries. This work proposes an intensified process to maximize the recovery of phenolic compounds in dry extracts using hydroalcoholic mixtures. Supercritical carbon dioxide defatting pre-treatment was performed. Following this, pressurized liquid extraction was optimized through a circumscribed central composite design. The factors consisted of temperature (65.0−185.0 °C), ethanol percentage (8.0−92.0%), and solid/liquid ratio (0.2−0.8 $g_{OP}/mL_{SOLVENT}$). Besides the total phenolic content (TPC) and the total flavonoid content (TFC), the major phenolic compounds of OP [hydroxytyrosol (HT), tyrosol (TY), and oleuropein (OL)] were evaluated. Further, decarboxymethyl OL aglycone dialdehyde (3,4-DHPEA-DEDA) was identified by HPLC-DAD-MS/MS as the most abundant polyphenol and was studied for the first time for OP. Different conditions were found to optimize each key compound. In 67% shorter extraction time and 38% less solvent consumption compared to conventional extraction, an increase of 475% for OL, 428% for HT, 194% for TY, 373% for 3,4-DHPEA-DEDA, 89% for TPC, and 158% for TFC was observed. The antioxidant activity by oxygen radical absorbance capacity (ORAC) assay increased 89% (optimal conditions) and correlated with TPC, 3,4-DHPEA-DEDA, and TFC. Thus, an efficient, selective, scalable, and green extraction process was established.



KEYWORDS: *olive pomace phenolic compounds, antioxidant capacity, pressurized fluid extraction, oleuropein, hydroxytyrosol, decarboxymethyl oleuropein aglycone dialdehyde (oleacein)*

INTRODUCTION

The amount of olive trees cultivated for the production of olive oil and table olives covers a surface of 9.98 million ha (data of 2013) in more than 40 countries, producing a large number of by-products.[1] Some of the by-products are mill waste waters, pomace, leaves, and stones.[1−3] Olive pomace (OP) is a semi-solid to semi-liquid by-product produced after crushing and centrifugation of the olive fruit.[4] It is the main by-product from the two-phase separation process used to obtain olive oil. It has high moisture content (55−70%) and is composed by a mass of vegetation waters and mill waste waters together with fruit materials.[1] It is characterized by high organic load and phenolic content, low pH, high salinity, and antimicrobial properties and is also phytotoxic.[2] Furthermore, it is produced in large quantities, between 7 and 30 million m^3 per year in Mediterranean areas, and it is stored in open-air pods.[1,5] Thus, it generates a great environmental concern, and most of the national regulations of the producer countries do not permit its rejection to rivers or soil.[5] Consequently, many efforts are devoted globally to its valorization. Approximately 2.5% of the OP weight consists of non-polar compounds, such as waxes and lipids (including the remaining oil), from which the commercialized "OP oil" is produced.[1,6] After the recovery of the OP oil, the dry defatted olive cake (DDOC) is generated. Subsequently, the recovery of the phenolic compounds either from the DDOC or from the crude OP is of utmost importance. The new solid by-product produced can have a wide range of further potential applications in a biorefinery framework, that is, bioenergy, extraction of other

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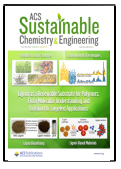

biomolecules, such as enzymes, agronomic uses, among others.[1,7]

Among the OP phenolic compounds, simple phenols, such as hydroxytyrosol (HT) and tyrosol (TY), and secoiridoids, such as oleuropein (OL) and decarboxymethyl OL aglycone dialdehyde (3,4-DHPEA-DEDA), are the main chemical classes. Flavonoids, iridoids, hydroxybenzoic acids, hydroxyphenylacetic acids, hydroxycinnamic acids, lignans, and glucosides are also present.[8−11] The antimicrobial[12] and phytotoxic[13] properties of OP have been attributed to many of these compounds. On the other hand, HT, TY, and OL present numerous biological activities, such as antioxidant, anti-inflammatory, antiatherogenic, and cardioprotective. Moreover, for HT, the antimicrobial, chemoprotective, and skin-bleaching activities have also been proved. For OL, the antihypertensive, hypoglycemic, endocrinal, cytostatic, antimicrobial/antiviral, and molluscicidal activities have been reported together with enzyme modulation.[14−23] Flavonoids, for example, rutin and luteolin, also demonstrate numerous activities, such as antioxidant, anti-inflammatory, antidiabetic, cardioprotective, anticarcinogenic, anti-age-dependent neuropathologic, and antiviral/bacterial.[24] Besides, antiradical and anti-inflammatory activities have been proved for crude olive methanol/water extracts. They contain luteolin, TY, HT, hydroxytyrosol acetate, OL, 3,4-DHPEA-DEDA, 10-hydroxy OL aglycone, decarboxymethyl ligstroside aglycone, OL aglycone, and ligstroside aglycone.[25]

The traditional recovery of the phenolic compounds is mostly performed by infusion, decoction, percolation, heat reflux, maceration, and Soxhlet extraction due to their simplicity and low cost.[26] The replacement of these techniques by an environmentally friendly and scalable procedure has been widely studied. The goal is to avoid high volumes of toxic solvents and long extraction times. However, this still consists an unmet need in the field. Lozano-Sánchez and coworkers[27] proposed a pressurized liquid extraction (PLE) system, with an *n*-hexane pre-treatment, for the recovery of the phenolic content from OP using a mixture of water−ethanol (EtOH). Álvarez[28] described a microwave pre-treatment, followed by a conventional solid−liquid extraction with the same solvents, to increase the extract richness in HT, TY, and OL from defrozen raw OP. Ultrasound technology has also been applied to increase the phenolic richness and the antioxidant activity (AA) of the aqueous OP extract using a freeze-dried material.[29] Xie and coworkers[30] compared and enhanced the ultrasound-assisted, microwave-assisted, and solvent extraction of HT together with the triterpenes maslinic acid and oleanolic acid, from oven-dried OP. Schievano and coworkers[31] used $scCO_2$ coupled with a polar co-solvent (EtOH) to maximize the recovery of phenolic compounds from fresh raw OP. A pressure-driven polymeric membrane process has also been reported to obtain the OP phenolic content with water from the defrozen raw material. Previously, OP was subjected to hydraulic press in order to remove the olive fat-based mixture.[32] Recently, Gómez-Cruz and coworkers[33] used response surface methodologies to valorize DDOC in terms of AA, total phenolic content (TPC), total flavonoid content (TFC), and extraction yield (EY). The factors set were temperature, extraction time, and biomass loading using conventional solvent extraction.

Currently, the PLE system is increasingly used due to its ability to retain the extraction parameters stable, that is, pressure and temperature. The solvents can be heated at high temperatures. However, they maintain their liquid state during the procedure by applying high pressures. Thus, the solubility of the analytes and the solvent diffusivity are enhanced, while the solvent viscosity is decreased. Therefore, high EY can be obtained in short time, using low solvent volumes, due to a higher mass transfer.[34] "Green" solvents, such as water and EtOH, can be used in this technique.

On the other hand, the importance of pre-treatment (deoiling and milling) for the recovery of phenolic compounds has already been described.[35] A prior defatting step can not only contribute to a better phenolic recovery but also valorize an environmentally hazardous by-product and add an extra value to the olive oil industry. The OP oil is usually obtained by drying the material and then conventional solvent extraction is performed.[2] The use of supercritical carbon CO_2 ($scCO_2$) for the recovery of non-polar components is continuously rising due to its environmental safety and its selectivity for lipophilic compounds. CO_2 is non-toxic, non-flammable, recyclable, and inexpensive at the industrial level.[36] The use of $scCO_2$ for olive oil extraction from olives and recently from OP has already been described.[37,38]

The objective of this work was to obtain OP extracts rich in its major bioactive phenolic compounds (OL, HT, TY and any other abundant compound detected) and flavonoids, through the establishment of an optimized, environmentally friendly and industrially appropriate sequential extraction process. Therefore, a $scCO_2$ extraction was performed to OP, followed by PLE optimization through design of experiments (DoEs). For DoE optimization, response surface methodologies are mostly used due to their ability to determine the interaction among the process variables.[39] Among them, the circumscribed central composite (CCC) design was selected because of its better predictive capacity in comparison to other designs.[40] The solvent used was a hydroalcoholic mixture. The OP extract richness in 3,4-DHPEA-DEDA was also studied, as it was found to be the most abundant polyphenol in the extract. The extraction efficiency was defined by the EY, and the extracts were characterized in terms of TPC, TFC, and AA. Also, all the responses were correlated with the AA, while main phenolic compounds were identified by HPLC-DAD-MS/MS.

EXPERIMENTAL SECTION

Plant Material. OP of Arbequina variety from the 2018 crop was kindly provided by Oliduero (Medina del Campo, Spain). It was collected right after decanting the olive oil from the solid by-product. The moisture content (1.48 ± 0.01 $g_{H_2O}/g_{DRY\ OP}$) was assessed gravimetrically by drying the pomace at 105 °C until it reaches a constant weight. It was packed in individual bags and stored at −20 °C. Subsequently, the material was freeze-dried under vacuum (18 kPa) and protected from light for 72 h (Lyoquest-55, Telstar, Terrassa, Spain). The freeze-dried OP (FD-OP) had approximately 3% of moisture content; it was stored in a dry dark place at room temperature.

Reagents and Solvents. Milli-Q water was obtained from a Millipore unit, and non-denaturalized EtOH (99.9%) was obtained from Dávila Villalobos S.L. (Valladolid, Spain). Folin-Ciocalteu reagent, Na_2CO_3, $AlCl_3$, NaOH, methanol (MeOH, 99.9% LC−MS), dimethyl sulfoxide (DMSO), and phosphoric acid were purchased from Panreac Quimica SLU (Barcelona, Spain). The commercial standards HT (≥98%), TY (≥99%), and OL (≥98%) were bought from Extrasynthese (Genay, France). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), AAPH (2,2′-azobis-(2-methylpropionamidine)dihydrochloride), gallic acid, and catechin were obtained from Sigma-Aldrich (Madrid, Spain). Fluorescein sodium (FS) salt was purchased from Vetec Quimica (Xerem Duque

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De Caxias, Rio de Janeiro, Brazil) and $NaNO_2$ from Fischer Scientific (Madrid, Spain). CO_2 (99.95%) was supplied by Carburos Metálicos (Barcelona, Spain) and N_2 (99.996%) by Linde Gas (Puçol, Spain).

Defatting Pre-treatment. *Conventional Process.* The best conditions for the conventional recovery of the residual lipophilic and non-polar compounds were selected according to Kadi and Fellag.[41] Thus, 4.33 g of FD-OP was mixed with 20 mL of *n*-hexane (0.5 g of raw OP/mL of solvent) in a round-bottom flask. The flask was put in a thermostatic bath at 30 °C for 15 min under magnetic stirring (750 rpm). Subsequently, the solution was collected, centrifuged, and dried using a rotary evaporator (Buchi Rotavapor R-200, Flawil, Switzerland) at 30 °C and ca. 20 kPa. The dry extract (DE) was then weighed to calculate the percentage of the EY. The experiment was performed in triplicate.

Supercritical Carbon CO_2 ($scCO_2$) Extraction. Supercritical fluid extraction was performed in a homemade large-scale apparatus.[42] CO_2 was supplied from a cylinder in liquid state (5.5 MPa) and cooled down by passing through a refrigerator adjusted at −21 °C. Then, it reached a steel diaphragm pump (Dosapro 27360, Milton Roy, Pont-Saint-Pierre, France) with a maximum flow rate of 16.30 L/h and a maximum pressure of 41.7 MPa. Then, it was cooled to −10 °C by a closed circuit connected to the refrigerator to avoid CO_2 evaporation. The apparatus also consists of two stainless steel vessels: a tubular extractor with a useful volume of 3 L and a 2.5 L separator. The pumped CO_2 is quantified before entering the extractor by a flow meter. The extractor is heated by a wire resistor that runs through the inlet tube and a band heater that surrounds it externally. Consequently, CO_2 passes through a heated backpressure regulator to the separator. Both vessels have pressure and temperature probes for monitoring. From the separator, CO_2 is recirculated to the refrigerator to be subsequently pumped again.

The extraction parameters were set according to Belbaki and coworkers.[37] Briefly, 406.4 g of FD-OP with approx. 3% humidity was grounded and loaded to the extractor. Raschig ceramic was also used in order not only to fill the extractor completely but also to prevent clogging and formation of preferential channels. The system temperature was raised, and CO_2 was fed in the extractor up to the indicated extraction pressure. Once the system reaches the desired pressure and temperature in the extractor (30 MPa and 60 °C), the dynamic extraction begins. The flow rate was set at 10.5 kg CO_2/h for an extraction time of 3 h. In the separator, the temperature was set at 20 °C and the pressure at 6 MPa. Once the experiment was completed, the defatted FD-OP (FD-OP-DO) was weighed to calculate the percentage of the yield of the oil removed. The experiment was performed in duplicate.

Extraction of Phenolic Compounds. *Conventional Solid−Liquid Extraction.* A conventional extraction was performed with FD-OP-DO. It was used as a reference for the optimization study of PLE. The extraction conditions were selected according to Álvarez,[28] who described the optimal conditions for the conventional solid−liquid extraction. The study was performed to the defrozen raw OP without any treatment. The conditions selected were based on industrial constrains. The solvent was composed by a mixture of EtOH and water at 50% (v/v), the solid/liquid ratio (S/L) was 0.5 g_{OP}/$mL_{SOLVENT}$, the temperature (T) was set at 70 °C, and the extraction time was fixed at 60 min. To perform this experiment, 4.23 g of FD-OP-DO was weighed in a round-bottom flask and mixed with 20 mL of solvent (50% v/v of EtOH in water). The extraction was set to begin when the flask was placed in a thermostatic bath and the desired temperature was reached (70 °C). The stirring speed was adjusted at 750 rpm. When the extraction was completed, the extract was transferred to plastic falcons and centrifuged at 6100*g* for 15 min at room temperature (Sigma 2−16P; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). All extracts were stored at −20 °C and in darkness until analysis. The experiment was performed in triplicate.

PLE: Optimization Design. All the extractions were performed in a Berghof stainless steel batch reactor (Berghof GmbH, Tübingen, Germany) of 40 mL internal volume coupled with a band heater and a magnetic stirrer. The temperature was kept stable and measured using a thermocouple. In each extraction experiment, an adequate amount (g) of FD-OP-DO (from 1.4 to 7.1 g, depending on the S/L) was mixed with 20 mL of solvent. The stirring speed was fixed at 750 rpm. The system was heated and the extraction was set to begin when the desired temperature was reached (time varying from 5 to 8 min). The procedure was static and lasted 20 min. Before starting the heating process, the pressure was adjusted at 10 MPa through N_2 application, according to the literature.[27,43,44] When the extraction was completed, the reactor was quenched in an ice bath until room temperature (cooling time varying from 3 to 5 min). The extract obtained was centrifuged at 6100 g for 15 min at room temperature (Sigma 2−16P; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and stored at −20 °C and in darkness until analysis.

For the optimization of PLE through DoE, a CCC was selected. The factors set were the percentage of EtOH in water (EtOH %), T, and S/L. Each factor (numerical) was estimated at five levels, namely, ±1 (factorial points), ±a (axial points), and one center point replicated 10 times, resulting in 24 experiments (Table 3). The CCC was performed using Statgraphics Centurion 18.0 software (Statgraphics Technologies, Inc., Virginia, USA). The responses obtained from the statistical analysis were adjusted to a second-degree model that considered the individual interactions of the parameters together with their quadratic relations

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{j=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \quad (1)$$

where Y is the response variable; β_0, β_j, β_{jj}, and β_{ij} are regression coefficients; and X represents each parameter.

Extraction Yield. Sample extracts were dried according to the following procedure: first, EtOH was evaporated using a rotary evaporator (Buchi Rotavapor R-200, Flawil, Switzerland) at 60 °C and ca. 20 kPa. Then, the extract containing essentially water was freeze-dried under vacuum (18 kPa) and protected from light for 48 h (Lyoquest-55, Telstar, Terrassa, Spain). Then, the DE was weighed and the EY results are expressed as milligrams of DE per gram of FD-OP-DO ($mg_{DE}/g_{FD\text{-}OP\text{-}DO}$).

Extract Characterization. *TPC Determination.* The TPC of the liquid extracts was determined using the Folin-Ciocalteu method, as previously described by Singleton and coworkers.[45] Briefly, 40 μL of sample was mixed with 3 mL of distilled water and 200 μL of Folin-Ciocalteu's reagent. After 5 min, 600 μL of Na_2CO_3 (20% w/v) was added, and the mixture was incubated at 40 °C for 30 min. The absorbance was measured at 765 nm (UV 2550, UV/Vis spectrophotometer, Shimadzu GmbH, Kioto, Japan). The TPC results were calculated using a calibration curve of gallic acid (range between 54 and 1066 mg/L_{GA}) and were expressed as milligrams of gallic acid equivalents (GAEs) per gram of DE (mg_{GAE}/g_{DE}).

TFC Determination. The TFC of the different extracts was measured as described by Michalska and coworkers.[46] Briefly, 1 mL of the extract was diluted to 10 mL with distilled water. Then, 300 μL of $NaNO_2$ (5% w/w) was added, and the mixture was left to react for 5 min. Afterward, 500 μL of $AlCl_3$ (2% w/w) and 500 μL of NaOH (1 M) were poured in the mixture, and the mixture was incubated at room temperature for 6 min. The absorbance was measured at 510 nm (UV 2550, UV/Vis spectrophotometer, Shimadzu GmbH, Kioto, Japan). The TFC results were calculated using a calibration curve of catechin (range between 63 and 500 mg/L_{CAT}) and were expressed as milligram of catechin equivalents (CATEs) per gram of DE (mg_{CATE}/g_{DE}).

Antioxidant Activity (AA) Determination. The method used for the evaluation of the AA of the extracts was the oxygen radical absorbance capacity (ORAC) assay. It is based on the fluorescence quenching of FS salt after exposure to AAPH, which generates oxygen radicals ($ROO^{\bullet}$) at a constant rate. The ORAC assay was carried out as described by Feliciano and coworkers,[47] including some modifications for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, Vermont, USA). The ORAC values were calculated using a regression equation between the Trolox concentration and the area under the decay of the FS curve (AUC) for each sample according to the calibration curve for Trolox (range

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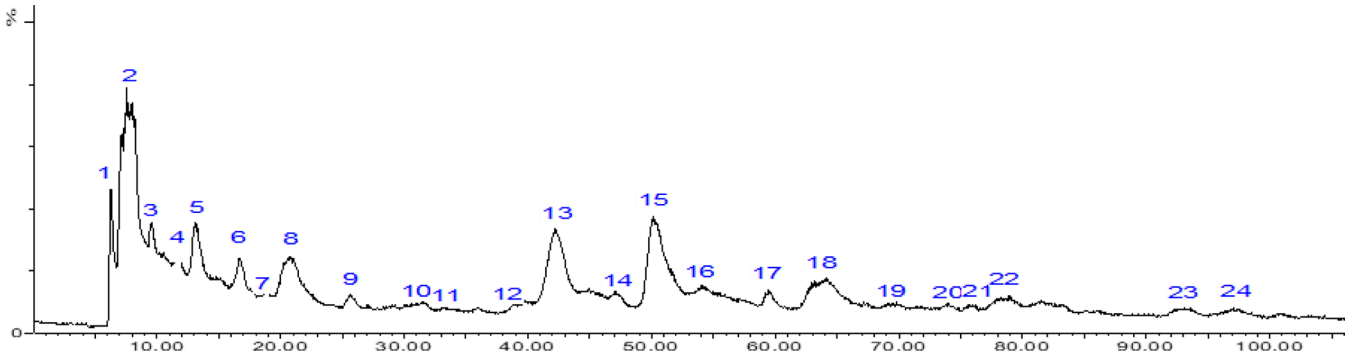

Figure 1. Scan chromatogram in the ESI$^-$ mode for the conventional OP extract.

Table 1. Putative Identification of Phenolic Compounds in the Conventional OP Extract[a]

peak number	putative identification	chemical class	molecular formula	retention time (min)	$[M-H]^-$	m/z fragment ions	references
1	unknown compound 1			6.29	317	296, 225, 165, 81	
2	quinic acid	hydroxybenzoic acid	$C_7H_{11}O_6$	7.44	191	127, 93, 85	49
3	hydroxytyrosol glucoside	glucoside	$C_{14}H_{20}O_8$	9.58	315	153, 123, 89	50
4	hydroxytyrosol (HT)	simple phenol	$C_8H_{10}O_3$	11.62	153	123	49,51,52
5	dialdehydic elenolic acid decarboxymethyl (DEDA)	secoiridoid	$C_9H_{12}O_4$	13.10	183	139, 95, 69	8
6	tyrosol (TY)	simple phenol	$C_8H_{10}O_2$	16.76	137	134, 119, 108, 84, 47	49,53
7	secologanoside/oleoside	secoiridoid glycoside	$C_{16}H_{22}O_{11}$	18.79	389	165, 121, 119, 113, 89, 69	49
8	unknown compound 2			20.61	671	335, 151	
9	oleuropein (OL) aglycone derivative	secoiridoid	$C_{19}H_{22}O_8$	25.60	377	217, 197, 153	51,53
10	hydroxyoleuropein	secoiridoid	$C_{25}H_{32}O_{14}$	31.56	555	455, 323, 223, 151	49
11	demethyloleuropein	secoiridoid	$C_{24}H_{30}O_{13}$	33.30	525	414, 389, 324, 319, 187, 81	52
12	verbascoside	glycoside	$C_{29}H_{36}O_{15}$	39.00	623	461, 161	50,53,54
13	elenolic acid derivative	secoiridoid	$C_{11}H_{14}O_6$	42.18	241	139, 127, 111, 101, 95, 69	10
14	nüzhenide	secoiridoid	$C_{31}H_{42}O_{17}$	47.32	685	523, 453, 432, 421, 348, 299, 223, 119	51−53,55
15	decarboxymethyl OL aglycone dialdehyde (oleacein) (3,4-DHPEA-DEDA)	secoiridoid	$C_{17}H_{20}O_6$	50.15	319	195, 183, 139, 69	56
16	rutin	flavonoid	$C_{27}H_{30}O_{16}$	54.13	609	447, 301, 300, 125	10,52,53,57
17	OL	secoiridoid glycoside	$C_{25}H_{32}O_{13}$	59.50	539	377, 345, 307, 275, 223, 179, 149	10,49−53
18	demethyloleuropein aglycone (enol form)	secoiridoid	$C_{18}H_{20}O_8$	64.16	363	347, 182, 181, 123, 95, 69, 59	10
19	luteolin glucoside isomer	flavonoid	$C_{21}H_{20}O_{11}$	68.93	447	285	10,50−54,58
20	lucidumocide C isomer 1	flavonoid	$C_{27}H_{36}O_{14}$	72.52	583	537, 403, 371, 329, 223, 179, 151	49
21	lucidumocide C isomer 2	flavonoid	$C_{27}H_{36}O_{14}$	74.59	583	468, 403, 223, 179, 151	49
22	ligstroside	secoiridoid glycoside	$C_{25}H_{32}O_{12}$	78.92	523	453, 361, 291, 259, 256, 119, 101, 89	10,51−53,55
23	nüzhenide 11-methyl oleoside	secoiridoid	$C_{48}H_{64}O_{27}$	92.98	1071	971, 817 (formic adduct of 771), 810, 771, 731 (formic adduct of 685), 685	58−60
24	luteolin	flavonoid	$C_{15}H_{10}O_6$	97.27	285	269, 151, 133	51−53

[a]The peak numbers refer to those mentioned in the chromatogram. The table includes the retention time of each compound, together with its molecular formula, $[M-H]^-$ ion, and major ESI$^-$ fragment ions.

between 5 and 40 μmol/L_{TROLOX}). Finally, for each sample, four different concentrations were tested creating a calibration curve (diluting the extract from 4000 to 11,000 times). The results are given in millimoles of Trolox Equivalents (TE) per gram of DE ($mmol_{TE}$/g_{DE}).

HPLC-DAD Analysis. The quantitative determination of HT, TY, OL, and 3,4-DHPEA-DEDA in the extracts was performed by an HPLC-DAD system: Waters e2695 Separation module with an autosampler (20 μL injection volume) and a quaternary pump coupled with a Waters 2998 photodiode array detector set at 280 nm (Waters, Ireland, UK). The column used was a C18 Mediterranean Sea (250 × 4.6 mm, 5 μm) at 35 °C (Teknokroma Analítica S.A., Barcelona, Spain). An OptiGuard 1 mm guard column (Sigma-Aldrich, San Luis, Misuri, USA) was also employed. A gradient method was used (modified from Brenes and coworkers[48]) using eluent A (acidified water to pH = 3 with phosphoric acid) and eluent B (methanol). A flow of 1 mL/min was set, and the following elution program was applied: 0−10 min linear gradient from 90 to 80% A;

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10−16 min 80% A isocratic; 16−20 min linear gradient to 70% A; 20−25 min 70% A isocratic; 25−35 min linear gradient to 60% A; 35−40 min 60% A isocratic; 40−45 min linear gradient to 55% A; 45−55 min 55% A isocratic; 55−60 min linear gradient to 40% A; 60−65 min linear gradient to 30% A; and 65−70 min linear gradient to 0% A. The standard solutions of all the compounds were prepared in DMSO and were injected using the same analytical method and conditions with the samples. 3,4-DHPEA-DEDA was calculated as OL equivalents (OLEs). The range of the calibration curve was 12.5−1250 mg/L for OL, 25−300 mg/L for HT, and 12.2−200 mg/L for TY. The results for each compound are given as DE richness, that is, milligrams of compound per gram of DE ($mg_{COMPOUND}/g_{DE}$). For data acquisition and processing, Empower 3 software was used (Waters, Ireland, UK).

HPLC-DAD-MS/MS Analysis. Putative identification of the phenolic compounds present in the OP extracts (apart from OL, HT and TY) was performed by an HPLC-DAD-MS/MS system: HPLC analyses were performed on a Waters Alliance 2695 (Waters, Ireland, UK) equipped with a quaternary pump, a solvent degasser, an autosampler (10 μL injection volume), and a column oven. It is also coupled to a Photodiode Array Detector Waters 996 PDA (Waters, Ireland, UK), scanning wavelength absorption from 210 to 600 nm. The column used was a LiChrospher 100 RP-18 5 μm (250 × 4.0 mm) (Sigma-Aldrich, San Luis, Misuri, USA) at 35 °C. A gradient method was applied with two eluents: A (MiliQ water with 0.5% HCOOH) and B (methanol). A flow rate of 0.3 mL/min was set and the following elution program was applied: 0−15 min isocratic 70% A; 15−45 min linear gradient to 60% A; 45−60 min isocratic 60% A; 60−75 min linear gradient to 55% A; 75−105 min isocratic at 55% A, and finally returning to the initial conditions for 20 min. Tandem mass spectrometry (MS/MS) detection was performed on a Micromass Quattro Micro triple quadrupole (Waters, Ireland, UK) using an electrospray ionization source in the negative ion mode (ESI^-). The source temperature was 120 °C, and the capillary and source voltages were 2.5 kV and 20 V, respectively. Compounds separated by HPLC were ionized and the mass spectra were recorded in the full scan mode from *m/z* 60 to 1100. Collision energies were optimized for each compound (10, 20, and 30 eV). High-purity N_2 was used both as drying and as a nebulizing gas. Ultra-high-purity argon (Ar) was used as collision gas. For data acquisition and processing, MassLynx version 4.1 software was used (Waters, Ireland, UK).

Statistical Analysis. To test the model significance and suitability for each response, analysis of variance (ANOVA) of the data was performed. The significance of each coefficient was determined using the *F*-value test at 95% confidence level. For the determination of the best extraction conditions, a numerical optimization was applied. For the correlation study among the responses, the Pearson's correlation coefficient was performed. The Statgraphics Centurion version 18.0 software (Statgraphics Technologies, Inc., Virginia, USA) was used for the statistical analysis of each response and the SPSS version 15.0 software (SPSS, Inc., Chicago, USA) for the correlation analysis among the responses.

RESULTS AND DISCUSSION

Defatting Pre-treatment Method Selection. A conventional recovery of the residual oil from FD-OP using *n*-hexane was performed, adjusting the factors to the industrial needs. This method was used as a reference for the $scCO_2$ extraction process. The oil obtained from the conventional *n*-hexane defatting process was 2.4 ± 0.5% of the dry basis. Through the $scCO_2$ pre-treatment, the oil content eliminated was 2.40 ± 0.15% of the dry material. Consequently, it can be observed that the percentage of the lipophilic compounds obtained is similar in both cases. In addition, in the case of $scCO_2$, the use of organic solvents is avoided, consisting an environmentally friendly extraction method. Thus, the $scCO_2$ extraction was selected for the pre-treatment of the material.

Conventional Extract. *HPLC-DAD-MS/MS Phenolic Characterization.* A conventional solid−liquid extraction was performed to FD-OP-DO obtaining a DE with an EY of 121 ± 30 $mg_{DE}/g_{FD\text{-}OP\text{-}DO}$. Its AA was 4.66 ± 0.12 $mmol_{TE}/g_{DE}$, its TPC was 180 ± 11 mg_{GAE}/g_{DE}, and its TFC was 9 ± 3 mg_{CATE}/g_{DE}. The identification of the major secoiridoid, that is, OL, and simple phenols, that is, HT and TY, was performed by HPLC-DAD, comparing their relative retention time and UV spectra with those of the standard solutions. The conventional extract was found to contain 2.4 ± 0.6 mg_{OL}/g_{DE}, 1.79 ± 0.11 mg_{HT}/g_{DE}, and 1.78 mg_{TY}/g_{DE} ± 0.10.

For the further identification of phenolic compounds present in the extract and previously reported in *Olea europaea* L, an HPLC-DAD-MS/MS technique was used. Figure 1 presents the mass chromatogram of the conventional OP extract obtained using an ESI source operating in the negative mode. Table 1 summarizes the putative identification of the phenolic compounds present according to the literature. In total, 19 compounds were putatively identified.

Bioactivities of Phenolic Compounds Present. Compound 15 (retention time = 50.15 min) was found to be the most abundant in the extract (11 ± 2 mg_{OLE}/g_{DE}). This compound had an $[M - H]^-$ of 319 and was identified as 3,4-DHPEA-DEDA, also known as Oleacein, already reported in the extracts derived from olive oil,[61] mill wastewaters,[62] and leaves.[63] Various biological activities have been demonstrated for this molecule, including antioxidant, anti-inflammatory,[25,64,65] antiproliferative, and antimetastatic,[66] among them. It also is worth mentioning that its antioxidant activity has been proved to be stronger than the one of OL.[65] Therefore, the DE richness in 3,4-DHPEA-DEDA was also selected as a response for the CCC optimization study.

Apart from 3,4-DHPEA-DEDA, HT, TY, and OL, numerous biological activities have also been reported for most of the phenolic compounds identified in the conventional extract. OL aglycone derivatives are present not only in OP[11] but also in olive mill wastewaters[56] and leaves.[51] They are proved to have anti-inflammatory, antioxidant, antidiabetic, anticancer, and neuroprotective activities along with cardiovascular protection.[67] Hydroxyoleuropein has been found in OP[11] and olive oil[68] and present coronary dilating, cardiotropic,[69] and pancreatic lipase inhibitory activities.[70] Nüzhenide and its derivatives, identified in the olive fruit,[58] demonstrate a strong antioxidant effect.[71] Elenolic acid derivatives are known for their antimicrobial properties[12] and are present in olive oil and all its by-products.[10,56,68,72] Also, according to Benavente-García and coworkers,[73] the overall antioxidant activity of the olive leaf extract has been attributed to verbascoside, luteolin glucoside, and aglycone, although they represent less than 3% of the TPC. Lucidumocide C has also demonstrated strong antioxidant[71] and antiviral activities[74] and has been found in olive leaves, as well.[49] In addition, rutin has pharmacological actions in most of the physiological systems, that is, central nervous, endocrine, cardiovascular, digestive, respiratory, excretory, reproductive, and immune systems. It also presents analgesic, antiarthritic, anticancer, and chemoprotherapeutic effects. Besides, beneficial effects for bones, eyes, hair, and skin and wounds[75] have been reported. Apart from OP,[11] rutin has also been identified in olive leaves,[49] fruits,[50] and mill wastewaters.[56]

PLE−CCC Design. The CCC was selected for the optimization of the extraction conditions of PLE due to its ability to provide reliable results, as well as its better predictive

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Table 2. CCC Design for the Optimization of PLE—24 Experiments and Their Responses[a]

run	A: T (°C)	B: EtOH %	C: S/L (g_{OP}/mL)	EY ($mg_{DE}/g_{FD\text{-}OP\text{-}DO}$)	AA ($mmol_{TE}/g_{DE}$)	TPC (mg_{GAE}/g_{DE})	TFC (mg_{CATE}/g_{DE})	OL (mg/g_{DE})	3,4-DHPEA-DEDA (mg_{OLE}/g_{DE})	HT (mg/g_{DE})	TY (mg/g_{DE})
1	160.7	75.0	0.7	70.67	5.88	280.37	4.86	0.00	17.41	5.68	3.43
2	185.0	50.0	0.5	191.65	3.24	187.10	4.18	0.00	0.00	3.14	2.20
3	89.3	25.0	0.3	131.91	4.38	156.07	10.81	6.05	32.73	3.91	3.18
4	**125.0**	**50.0**	**0.5**	**118.60**	**4.30**	**138.52**	**7.14**	**5.84**	**24.49**	**3.32**	**2.47**
5	125.0	50.0	0.8	32.062	6.88	256.99	9.62	5.62	28.14	5.44	3.33
6	**125.0**	**50.0**	**0.5**	**122.95**	**4.41**	**143.35**	**6.85**	**4.71**	**20.49**	**3.08**	**2.21**
7	125.0	8.0	0.5	110.39	3.62	127.44	9.15	1.15	16.22	3.07	2.34
8	**125.0**	**50.0**	**0.5**	**119.69**	**5.41**	**173.24**	**9.04**	**5.43**	**24.04**	**3.09**	**2.25**
9	160.7	75.0	0.3	165.95	6.53	223.49	9.77	0.00	19.68	3.85	2.12
10	**125.0**	**50.0**	**0.5**	**109.06**	**5.05**	**180.01**	**9.30**	**5.39**	**27.15**	**3.46**	**2.68**
11	**125.0**	**50.0**	**0.5**	**125.37**	**4.56**	**177.67**	**8.49**	**7.57**	**23.55**	**3.02**	**2.19**
12	89.3	75.0	0.7	42.83	5.40	200.67	12.65	11.35	36.31	2.36	1.60
13	160.7	25.0	0.7	155.13	3.65	132.59	3.24	0.00	0.00	3.16	2.01
14	125.0	50.0	0.2	212.69	5.29	232.89	11.61	3.32	22.67	3.74	2.15
15	160.7	25.0	0.3	257.49	3.32	186.93	4.89	0.00	0.00	3.67	1.83
16	**125.0**	**50.0**	**0.5**	**87.49**	**4.73**	**206.92**	**8.96**	**5.24**	**21.10**	**2.49**	**1.96**
17	**125.0**	**50.0**	**0.5**	**104.75**	**4.74**	**182.22**	**8.36**	**3.27**	**22.03**	**2.68**	**2.15**
18	89.3	25.0	0.7	20.72	6.04	212.57	15.82	6.10	39.11	3.33	3.11
19	**125.0**	**50.0**	**0.5**	**105.37**	**5.05**	**187.39**	**7.86**	**4.10**	**24.62**	**3.56**	**2.70**
20	**125.0**	**50.0**	**0.5**	**85.00**	**4.80**	**184.55**	**8.99**	**4.18**	**22.78**	**2.20**	**2.19**
21	125.0	92.0	0.5	89.46	5.07	198.77	10.96	10.72	30.86	3.58	1.80
22	**125.0**	**50.0**	**0.5**	**102.58**	**4.53**	**185.37**	**7.74**	**3.93**	**21.70**	**2.27**	**2.17**
23	65.0	50.0	0.5	87.46	4.83	192.01	9.32	6.31	31.22	1.48	1.84
24	89.3	75.0	0.3	135.10	5.45	203.82	9.36	9.95	31.22	1.75	1.50

[a]The 10 center points are presented in bold.

capacity compared to other response surface designs.[40] The goal was the use of three parameters in the design. The selected responses to be optimized were the EY ($mg_{DE}/g_{FD\text{-}OP\text{-}DO}$), the AA ($mmol_{TE}/g_{DE}$), along with the DE richness in TPC (mg_{GAE}/g_{DE}), TFC (mg_{RE}/g_{DE}), OL (mg/g_{DE}), 3,4-DHPEA-DEDA (mg_{OLE}/g_{DE}), HT (mg/g_{DE}), and TY (mg/g_{DE}). The factors to study were EtOH %, T, and S/L. The improvement of phenolic extraction using PLE has already been described for OP and olive leaves, setting T and EtOH % as factors.[27,34,44] However, Xynos and coworkers[44] also studied the effect of extraction time and extraction cycles. Since the objective was an effective but short extraction procedure, commercially adjustable, and energy saving, the extraction was kept at one step. In this way, further decomposition of OL, noticed in subsequent extraction cycles, is avoided.[44] The static time was adjusted at 20 min according to the literature.[27,34] Thus, the most important three factors were numerical, varying over five levels (±1, ±a, and 0), concluding to 24 experiments. The experiments, together with the responses results, are summarized in Table 2. EtOH % was adjusted between 8.0 and 92.0% to cover a wide range of polarity. T was modified between 65.0 and 185.0 °C to study possible decomposition of the compounds. The S/L varied from 0.2 to 0.8 $g_{OP}/mL_{SOLVENT}$ to describe the effect of the internal mass transfer. This study can describe the effect of the most important factors on the valorization of OP. It also proposes an optimization process for EY, AA, TPC, TFC, and all the interesting and abundant compounds (OL, HT, TY, and 3,4-DHPEA-DEDA). Further, it establishes a correlation between them and highlights their importance.

Comparing the standard deviation (STDV) of the 10 center points (Table 2—Runs marked in bold, with T = 125.0 °C, EtOH % = 50.0, and S/L = 0.5), the reproducibility of the experiments was considered high (STDV of approximately 10% for all the responses, except of OL and HT that are 25.5 and 16.4%, respectively). In particular, the average EY was 108 ± 14 $mg_{DE}/g_{FD\text{-}OP\text{-}DO}$, while the average AA was 4.8 ± 0.3 $mmol_{TE}/g_{DE}$. TPC had an average value of 176 ± 20 mg_{GAE}/g_{DE} and TFC of 8.3 ± 0.9 mg_{CATE}/g_{DE}. The milligram per gram of DE was 5.0 ± 1.2 for OL, 23 ± 2 for 3,4-DHPEA-DEDA, 2.9 ± 0.5 for HT, and 2.3 ± 0.2 for TY. From Table 2, it can be observed that the values of the responses can vary depending on the different extraction conditions. From 21 to 258 mg of DE per gram of FD-OP-DO is obtained (EY), while the AA is also affected, taking values from 3.2 to 6.9 $mmol_{TE}/g_{DE}$. TPC varies from 127 to 280 mg_{GAE}/g_{DE}, while TFC varies from 4.2 to 15.8 mg_{CATE}/g_{DE}. OL ranges from 0.0 to 11.4 mg_{OL}/g_{DE}, 3,4-DHPEA-DEDA from 0.0 to 39.1 mg_{OLE}/g_{DE}, HT from 1.5 to 5.7 mg_{HT}/g_{DE}, and TY from 1.5 to 3.4 mg_{TY}/g_{DE}.

To find the most suitable model and the significant factors for each response, all data were subjected to ANOVA. The models used were among linear, cubic, quadratic, and two-factor interactions. Finally, an equation describing each response was obtained. Except OL that followed a linear model, for the rest of the responses, the quadratic model was proposed. For all the responses, the lack of fit test was not significant, indicating the significance of the model, while no outliers were detected. The statistical results for each response and their equations are presented in the Supporting Information (Tables S1 and S2, respectively).

Numerical Optimization. To determine the optimal conditions for each extract richness (response), a numerical optimization to all the equations was applied. To describe any two-factor interaction, a 3D imaging was performed for each response using a simplex algorithm (Figure S1—Supporting

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Information). The model concluded to the best possible solution for each response, while it was capable of proposing solutions for specific response values. For some responses, a second possible solution with similar values was also proposed. The optimal conditions of *T*, EtOH %, and S/L together with the predicted values for each response are included in Table 3. The results for each of the PLE-optimized conditions are given as extract richness, as well as yield values. To maximize the extract richness in OL, 3,4-DHPEA-DEDA, HT, and TFC, a single solution was proposed. A single solution was also described as the optimal for the EY. However, two possible optimal conditions with similar predicted values as extract richness were proposed for the TPC, TY, and AA.

Effects of Main Variables. As it can be observed from the results, the optimal conditions were different for each response. Figure 2 presents the main effects of the plot for the EY and AA, along with the richness in TPC, TFC, OL, 3,4-DHPEA-DEDA, HT, and TY.

Regarding the effect of S/L, it was found to be significant for HT and TY richness, as well as for the EY and the AA. For the rest of the responses, it was not considered a significant factor (Table S1—Supporting Information). According to Figure 2, the optimal for AA, HT, and TY richness was a high S/L while a low one for the EY. When high S/L is used, the leaked phenolic compounds are rapidly accumulated in the small amount of the volume employed. Therefore, the concentration gradient and, thus, the internal mass transfer are reduced. Hence, a higher concentration in the bulk is achieved. On the contrary, when low S/L is used, the extracted compounds are diluted in the higher solvent volumes, and as a result, a faster internal mass transfer is maintained.[28] For this reason, high S/L was proposed as the principal solution for all our responses, except the EY. Also, it is worth mentioning that for TPC, neither the S/L itself nor none of its interactions with EtOH % or *T* was considered a significant factor. Consequently, a second solution with a low S/L, while the same *T* and EtOH % conditions, was proposed, concluding to a similar predicted value as extract richness. It is industrially more convenient to employ a high S/L since a much more concentrated liquid extract can be obtained, and therefore, the industrial expenses of solvent recovery and extract drying would be much lower. However, if the objective is a high TPC yield, this can be achieved at a low S/L.

In terms of *T*, it was considered significant for OL, 3,4-DHPEA-DEDA, HT, and TFC richness together with the EY and the AA. For TPC and TY richness, *T* was not considered a significant factor (Table S1—Supporting Information). According to Figure 2, high *T* increases the EY and the HT richness while decreases the OL, 3,4-DHPEA-DEDA, and the TFC content together with the AA. We can see from Table 2 that in high *T* (e.g., 160.7 or 185.0 °C—runs 1 and 2, respectively), the extracted TFC was decreased. Flavonoids can be hydrolyzed at a *T* of 80°−100 °C with the presence of an acid, while their extraction by solid-phase extraction and their analysis by HPLC and LC−MS are performed at ambient or mild *T*.[76] This can explain the thermosensitivity of the TFC and its optimal at lower *T*. Also, in high *T*, there was absolute absence or decreased concentrations of OL and 3,4-DHPEA-DEDA in the generated extract. The fact that secoiridoids such as OL tend to be hydrolyzed to HT and other phenolic compounds (e.g., elenolic acid) in high *T* can explain this tendency.[77,78] In accordance with our results, Xynos and coworkers[44] also propose high *T* for the optimal EY and a low

Table 3. Comparison between the Conventional Extraction and the PLE-Optimized Conditions for Each Response as Extract Richness[a]

	conventional extraction					PLE-optimized extraction					
	conditions					conditions					
responses	*T* (°C)	EtOH %	S/L ($g_{OP}/mL_{SOLVENT}$)	value richness (x/g_{DE})	value yield ($x/g_{FD\text{-}OP\text{-}DO}$)	*T* (°C)	EtOH %	S/L ($g_{OP}/mL_{SOLVENT}$)	value richness (x/g_{DE})	increase (%)	value yield ($x/g_{FD\text{-}OP\text{-}DO}$)	increase (%)
EY ($mg_{DE}/g_{FD\text{-}OP\text{-}DO}$)				N/A	121	184.0	8.0	0.2	N/A	N/A	**396**	**227**
AA ($mmol_{TE}/$)				4.66	0.56	66.1	8.0	0.8	8.9	**89**	**0.08**	**−86**
						183.9	92.0	0.2	8.8	**87**	**1.77**	**214**
TPC ($mg_{GAE}/$)				180	21.78	183.9	84.7	0.8	340	**89**	**20.74**	**−5**
						183.9	91.9	0.2	332	**84**	**66.73**	**206**
TFC ($mg_{CATE}/$)	70.0	50.0	0.5	9	1.09	66.4	8.0	0.8	22	**158**	**0.20**	**−82**
OL ($mg_{OL}/$)				2.4	0.29	66.4	92.0	0.8	13.8	**475**	**1.26**	**332**
3,4-DHPEA-DEDA ($mg_{OLE}/$)				11	1.33	66.1	19.3	0.8	52	**373**	**1.82**	**37**
HT ($mg_{HT}/$)				1.79	0.22	183.9	90.0	0.8	9.5	**428**	**0.47**	**115**
TY ($mg_{TY}/$)				1.78	0.22	183.9	92.0	0.8	5.3	**194**	**0.24**	**13**
						66.1	8.0	0.2	4.9	**172**	**0.60**	**180**

[a]Results as extraction yield are also displayed.

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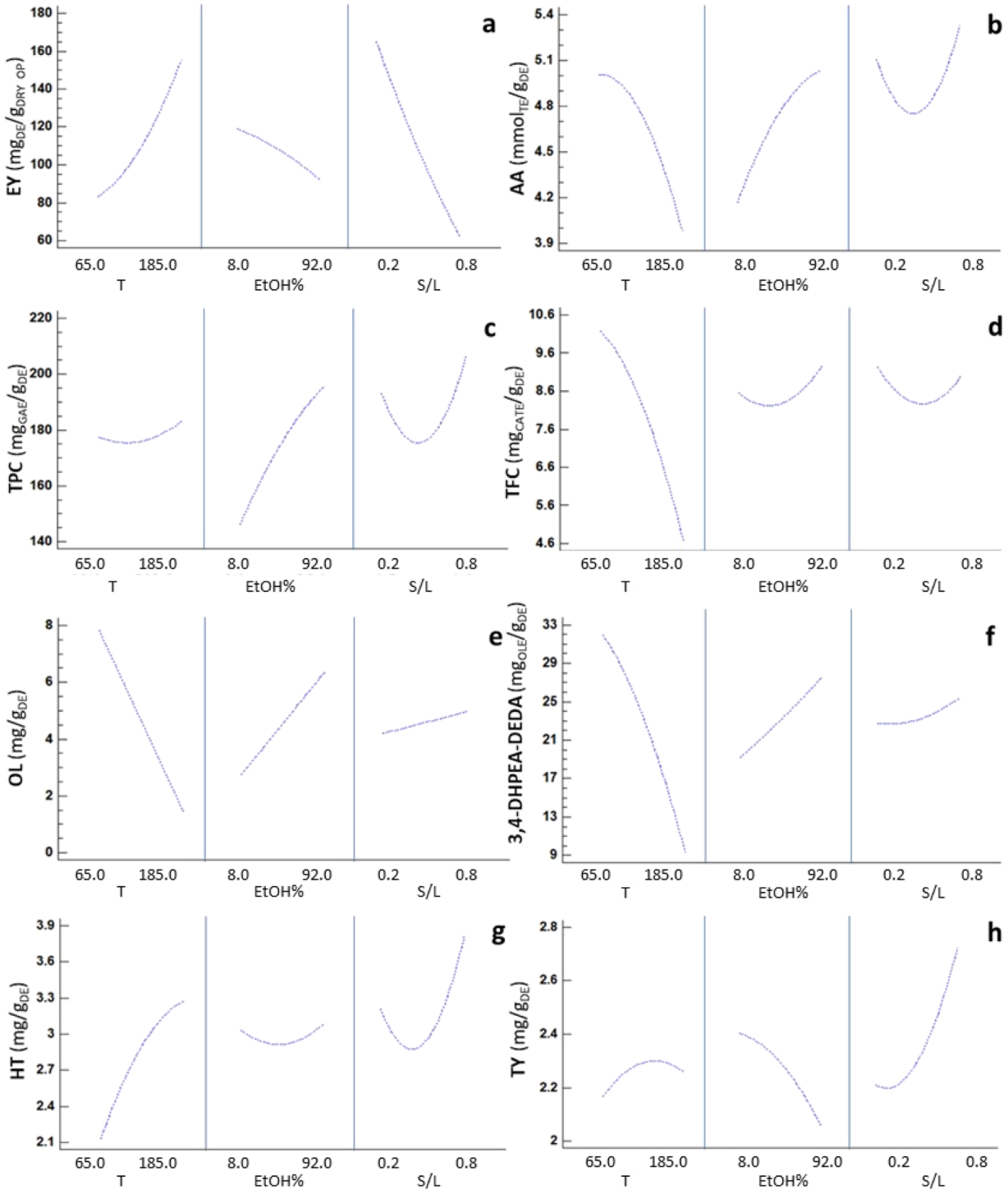

Figure 2. Main effects diagram (*T*, EtOH %, and S/L) for (a) EY, (b) AA, (c) TPC, (d) TFC, (e) OL richness, (f) 3,4-DHPEA-DEDA richness, (g) HT richness, and (h) TY richness.

T for the maximum AA. However, the high *T* (190 °C) proposed as optimal for OL can be attributed to possibly shorter extraction time and only one extraction cycle applied. Avoiding long extraction time and multiple extraction cycles, the hydrolysis of OL can be decreased. These data confirm its low stability. Regarding AA, its optimal in low *T* (first solution) could prove that it is mostly related to thermosensitive compounds (e.g., secoiridoids such as OL) instead of simple phenols such as HT and TY.

Regarding the EtOH %, a low value is proposed as optimum for the 3,4-DHPEA-DEDA richness, the EY, and the AA, while a high one for the OL, the TY, and the TPC richness (Figure 2). For HT and TFC, it was not considered a significant factor (Table S1—Supporting Information). TPC is traditionally recovered by a hydroalcoholic mixture with various EtOH %.[27,28,34] Although the data for OL and TY solubility are few, both compounds are presented as mostly soluble in organic solvents (like ethanol) rather than in water.[79,80] Hence, OL and TY can be better obtained through high EtOH %. It is true

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that the polarity of a solvent changes by mixing it with others. It is also remarkable that many compounds such as OL can be better extracted through a mixture of ethanol−water, rather than with pure solvents.[81] In addition, the interaction between T and EtOH % was significative for all the responses, except OL (that followed a linear model) since the polarity of the solvent or solvent mixtures changes in subcritical fluids as the temperature increases.[82] According to the main effect diagram (Figure 2b,e), although the extraction of 3,4-DHPEA-DEDA and the AA seems to increase with the increase of the EtOH %, their best recovery is proposed in low EtOH %. These facts can be explained through the two-factor interactions significative for each response (Table S1—Supporting Information). For 3,4-DHPEA-DEDA, the EtOH %−T interaction plays a very important role, able to change the polarity of EtOH in high T, as already explained. However, since low T is crucial for this compound, low EtOH % would slightly increase its recovery (Table 2—runs: 12 and 18), while in higher T, this could be achieved through higher EtOH %. This tendency can also be observed in its response surface diagram (Figure S1f, Supporting Information). From Table 2, it can be observed that in high T (e.g., 160.7 °C), if the EtOH % is low, zero 3,4-DHPEA-DEDA is obtained (runs: 13 and 15), while if the EtOH % is maintained high, a satisfactory recovery is achieved (runs: 1 and 9). For AA, a second solution was proposed as optimal with approximately the same predicted value. In this case, the significant interactions were between T−EtOH % and EtOH %−S/L. In low EtOH %, high S/L and low T can increase the response, concluding to a similar value with the one obtained in high EtOH % and T, and low S/L (Table 2—runs: 9 and 18). In the case of TY, although its recovery is presented to decrease with the increase of EtOH % (Figure 2h), the optimal is proposed in high EtOH %. This can also be explained through the significant interaction between T−S/L and T−EtOH %. In low T, low EtOH % and S/L would increase the response, while in high T, this could be achieved through high EtOH % and S/L (Table 2—runs: 1 and 3). Thus, a second solution of optimal conditions was proposed for TY, consisting of a low T, EtOH %, and S/L.

Correlation among Responses. To explain the interaction among the phenolic compounds selected, the EY and the AA, a bivariate Pearson correlation among the eight DoE responses was performed. The AA demonstrated the highest positive correlation with TPC (r = 0.724) and 3,4-DHPEA-DEDA (r = 0.634), followed by TFC (r = 0.561). This can be explained by the two different optimal solutions of AA: one with similar conditions with 3,4-DHPEA-DEDA and TFC and one similar to the second optimal solution of TPC. Romero-Díez and coworkers[83] and Lim and coworkers[84] have already proved the existence of correlation between the AA and the TPC or TFC. On the other hand, the EY has a significant negative correlation with the AA (r = −0.544). The table including all the correlation coefficient values between the AA and the rest of the responses is included in the Supporting Information (Table S3).

PLE-Optimized vs Conventional Extraction: Comparative Analysis. As mentioned before, the lyophilized OP was pre-treated with $scCO_2$. Then, one conventional extraction was performed that was used as reference. Table 3 summarizes the increase for each response (extract richness) at each one of the distinct optimal PLE conditions compared to the conventional method. This increase is achieved in 3 times shorter extraction time compared to the reference (20 min vs 1 h). Briefly, in each condition, an increase of almost 6- and 5-fold can be achieved for OL and 3,4-DHPEA-DEDA, respectively. Also, around 5 and 3 times more concentration of HT and TY can be attained, respectively. The TPC obtained and the AA can be almost doubled, while an increase of approximately 3 times can be achieved for the TFC extract richness and the EY. It is important to highlight that most of the optimal conditions of the PLE responses are achieved by using an S/L of 0.8 $g_{OP}/mL_{SOLVENT}$ instead of 0.5 $g_{OP}/mL_{SOLVENT}$ used for the conventional extraction. Thus, by using 1.6 times less solvent consumption, a much more concentrated phenolic extract can be obtained.

In terms of yield in the aforementioned conditions, 4 times more OL, 2 times more HT, and 1.4 times more 3,4-DHPEA-DEDA can be recovered. Except TFC that shows a decrease of 82%, the yield of TY and TPC can be triplicated in their second optimal conditions proposed while remain stable in their first using an S/L of 0.8 $g_{OP}/mL_{SOLVENT}$. Also, an enhancement of approximately 3-fold can be achieved for the AA, if the second solution is used.

Experimental Validation of the Model. To control the predictive capacity of the model, two extracts were produced in different conditions and the results obtained were compared to the predicted values. The conditions were selected according to the results of the numerical optimization, while none of them were part of the optimization design. The conditions selected for the first extraction were T = 66.0 °C, EtOH % = 10.0%, and S/L = 0.8 $g_{OP}/mL_{SOLVENT}$, giving an extract with a high richness in 3,4-DHPEA-DEDA and TFC, together with high AA. An extract enriched in TY, HT, and TPC was selected as the second produced in the following conditions: T = 184.0 °C, EtOH % = 90.0%, and S/L = 0.8 $g_{OP}/mL_{SOLVENT}$. The measured values in both experiments lay within the 95% confidence interval of the predicted values for all the responses. Therefore, the good predictive capacity and reliability of the model constructed is confirmed. The table summarizing the predicted and the observed value of each response for both extracts is presented in the Supporting Information (Table S4).

CONCLUSIONS

Two sustainable techniques have been combined to produce highly concentrated phenolic extracts from OP in 3 times shorter extraction time and with 1.6 times less solvent consumption compared to conventional methods. A supercritical carbon dioxide ($scCO_2$) pre-treatment was applied to recover the remaining olive oil and lipophilic compounds of the material. It was followed by a PLE optimization through a CCC design using exclusively ethanol−water mixtures. A process with high selectivity was established, demonstrating different optimal conditions for each response. More interestingly, a high increase in DE richness in the selected phenolic compounds, as well as its AA, was achieved compared to a conventional extract. In particular, at high temperature (T), percentage of ethanol in water (EtOH %) and S/L (T = 184.0 °C, EtOH % = 90.0%, S/L = 0.8 $g_{OP}/mL_{SOLVENT}$), an extract with 5-fold hydroxytyrosol (HT), and 3-fold tyrosol (TY) concentration is produced (9.5 vs 1.79 mg_{HT}/g_{DE} and 5.3 vs 1.78 mg_{TY}/g_{DE}, respectively). In the same conditions, the TPC can be nearly doubled (340 instead of 180 mg_{GAE}/g_{DE}). On the contrary, an extract with 6-fold OL content (13.8 vs 2.4 mg_{OL}/g_{DE}) can be obtained at low T (66.4 °C) and high S/L (0.8 $g_{OP}/mL_{SOLVENT}$) and EtOH % (92.0%). The decarboxymethyl OL aglycone dialdehyde (3,4-DHPEA-DEDA),

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known as oleacein, was identified via HPLC-DAD-MS/MS as the most abundant polyphenol in the conventional extract and was studied for the first time for this material. It demonstrated an increase of almost 5 times (52 vs 11 mg_{OLE}/g_{DE}, OLE: OL equivalents) at T = 66.1 °C, EtOH % = 19.3%, and S/L = 0.8 $g_{OP}/mL_{SOLVENT}$. Also, the AA of the obtained extract can be almost doubled (8.9 instead of 4.66 $mmol_{TE}/g_{DE}$) at T = 183.9 °C, EtOH % = 92.0%, and S/L = 0.2 $g_{OP}/mL_{SOLVENT}$ while correlates positively with TPC and 3,4-DHPEA-DEDA. Apart from the enhancement of the DE richness, a clear increase of the yield obtained for each compound/response can be achieved in the aforementioned optimal conditions. Concerning the predictive capacity of the model, all observed responses lay inside the 95% confidence interval of the predicted values. Thus, it can be said that an effective but short, selective, environmentally friendly, and industrially appropriate sequential extraction was established.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c09426.

ANOVA statistical analysis for all DoE responses; equations for each DoE response; Pearson correlation coefficient values between the AA and the rest of the DoE responses; comparison between predicted and observed values for all DoE responses at two selected conditions (experimental validation of the model); response surface diagrams for all DoE responses (PDF)

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Notes

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■ ABBREVIATIONS LIST

3,4-DHPEA-DEDA, Decarboxymethyl oleuropein aglycone dialdehyde (oleacein)
AA, antioxidant activity
AAPH, 2,2′-azobis(2-methylpropionamidine)-dihydrochloride
ANOVA, analysis of variance
CATE, catechin equivalents
CCC, circumscribed central composite (design)
DDOC, dry defatted olive cake
DE, dry extract
DoE, design of experiments
ESI^-, electrospray ionization source in negative ion mode
EtOH, ethanol
EtOH %, percentage (%) of ethanol in water
EtOH $\%^2$, quadratic term of percentage (%) of ethanol in water
EtOH %−S/L, interaction between percentage (%) of ethanol in water and solid/liquid ratio
EY, extraction yield
FD-OP, freeze-dried olive pomace
FD-OP-DO, freeze-dried olive pomace after supercritical deoiling/defatting process
FS, fluorescein sodium
GAE, gallic acid equivalents
HT, hydroxytyrosol
OL, oleuropein
OLE, oleuropein equivalents
OP, olive pomace
ORAC, oxygen radical absorbance capacity
PLE, pressurized liquid extraction
S/L, solid/liquid ratio
S/L^2, quadratic term of solid/liquid ratio
$scCO_2$, supercritical carbon dioxide (CO_2)
SPE, solid-phase extraction
STDV, standard deviation
T, temperature

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T−% EtOH, interaction between temperature and percentage (%) of ethanol in water
T^2, quadratic term of temperature
TE, trolox equivalents
TFC, total flavonoid content
TPC, total phenolic content
trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
T−S/L, interaction between temperature and solid/liquid ratio
TY, tyrosol

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