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Exploring the extraction of the bioactive content from the two-phase olive mill waste and further purification by ultrafiltration

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ABSTRACT

The two-phase olive mill waste is enormously produced in the Mediterranean area. This major waste is significantly rich in bioactive compounds that are highly valued by industry, such as phenolic and triterpenic compounds. Here, a thorough study of the most suitable solvent, extraction time and temperature for the large-volume, solid-liquid extraction of bioactive compounds has been made, in order to achieve maximum concentrations of the target compounds. Ultrasound effect has been considered. A deep characterization of the extracts by high-performance liquid chromatography coupled with electrospray-quadrupole-time of flight-mass spectrometry (LC-ESI-qToF-MS) has contributed to evaluate the effect of the operational parameters on the extraction performance. Forty-four compounds have been found and classified in their corresponding chemical families. At the optimum experimental conditions (EtOH 50% (v/v), 40 °C, ultrasound-assisted), more than 6.8 mg/g of bioactive content was recovered, and it was later purified by means of ultrafiltration. The membrane UP005 retained a significant percentage of the organic matter, whereas most of the bioactive compounds were recovered in the permeate. This contributed not only to revalorize this waste, but also to reduce its organic load and phytotoxicity, thus protecting the ecosystem of the final disposal zone of the residue.

1. Introduction

The vast majority of the olive mills use the two-phase process to produce olive oil, which requires a lower water consumption than the three-phase process (Kapellakis, Tsagarakis, & Crowther, 2008). Together with the olive oil, the two-phase process results in a by-product that consists of a combination of the widely known olive mill wastewater (OMWW) and olive pomace. This residue is entitled by different names in literature, but an accepted designation is two-phase olive mill waste (TPOMW) (Ahmed, Fernández, Figueroa, & Pajot, 2019). The TPOMW, also known as "*alperujo*" by its name in Spanish, is a wet, semisolid paste, with remnants of olive pulp and stone and a moisture content of 55–75%. It is highly enriched in organic matter, including phenolic compounds. Additionally, other molecules from the minor fraction of olives can be found (Borja, Raposo, & Rincón, 2006; Medina, Romero, & Brenes, 2018).

The environmental impact of TPOMW is undeniable, especially in the Mediterranean area, where the production of olive oil (and the subsequent residues) is concentrated during a few months of the annual campaign. Every year, between November and March, 4–10 million tons of TPOMW are generated (Alburquerque, Gonzálvez, García, & Cegarra, 2004; Vilar, Caño, Raya, Moreno, & Velasco, 2020), and the risk of discharging it without any previous treatment increases exceptionally. Some biological treatments have been suggested as a strategy to dispose of the TPOMW, such as composting and biodegradation (Alburquerque, Gonzálvez, Tortosa, Baddi, & Cegarra, 2009; Borja et al., 2006; Bouhia et al., 2021; Koutrotsios, Larou, Mountzouris, & Zervakis, 2016; Papadaki, Tsimidou, & Mantzouridou, 2018; Sampedro et al., 2011). However, the antibiotic and phytotoxic character of the phenolic compounds may hinder the growth of the microorganisms implied in the process.

Abbreviations: ANOVA, analysis of variance; LC-ESI-qToF-MS, liquid chromatography coupled to electrospray-quadrupole-time-of flight-mass spectrometry; LC-MS, liquid chromatography coupled to mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; OMWW, olive mill wastewater; qToF, quadrupole-time-of-flight; RSD, relative standard deviation; Rt, retention time; TPOMW, two-phase olive mill waste; UAE, ultrasound-assisted extraction; VRF, volume reduction factor.

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Thus, the extraction of the polyphenols before any management is recommended.

Still, the biophenols and other compounds present in the olive-derived products, such as TPOMW, are associated with effective antioxidant properties. Their antiaging effect (Sabatini, Perri, & Rongai, 2018) and their associated prevention against cardiovascular diseases and neoplasia processes have also prompted a high interest in applying these bioactive compounds in the pharmaceutical and cosmetic fields. Thus, the recovery of phenolic compounds from the residues originated in the olive mills represents a double benefit. On one side, high-value products are obtained at an affordable cost and, additionally, the organic load and phytotoxicity of the by-product are reduced.

As the TPOMW is a solid waste, it requires additional extraction steps to recover these compounds from the solid matrix, as opposed to the more widely studied OMWW liquid effluent. Ultrasound-assisted extraction (UAE) has proven to be a proper strategy to extract phenolic compounds from a variety of alimentary matrices, including olive-derived products (Caldas et al., 2018; Gogoi, Chutia, Singh, & Mahanta, 2019; Medina et al., 2018; Olmo-García, Monasterio, et al., 2019). As a consequence of the molecular turbulence and the cavitation phenomenon that takes place in the ultrasound bath, better extractions of interesting compounds can be reached in lower times, and usually lower volumes of solvents are required (Chemat et al., 2017). Several parameters have to be optimized in order to enlarge the efficiency of the process, such as the selected temperature, time of extraction, and solvent of choice. In this contribution, it has been developed and optimized a methodology to extract from TPOMW a wide range of bioactive compounds belonging to the minor fraction of olives (including phenolic compounds, triterpenic acids, and fatty acids). The large-scale implementation has been considered through two premises: high volumes of the sample have been managed during the study and, also, the single-step strategy has been preferred. Those considerations are not widely found in literature, but are very desirable to avoid future irreproducibility during the potential industrial application.

In order to evaluate the extraction procedure, a multi-family method based on liquid chromatography coupled to mass spectrometry (LC-MS) has been employed. Afterward, the extract obtained with the selected parameters has been treated by an ultrafiltration process, aiming to remove the concomitant organic matter that accompanies the phenolic compounds in the extract. This technique has proven to be highly relevant when recovering bioactive molecules from agrofood residues. Furthermore, it offers important advantages such as high selectivity, feasible membrane reutilization, large-scale applications, etc (Álvarez-Blanco, Mendoza-Roca, Corbatón-Báguena, & Vincent-Vela, 2017).

Considering the literature gap regarding the fruitful and scalable utilization of this by-product, this work provides an efficient strategy to reuse such a significant residue. The scientific community has, of course, gained awareness in this regard and there are several investigations that have demonstrated the bioactive richness of the TPOMW (Contreras, Gómez-Cruz, Romero, & Castro, 2021; Japón Luján, Capote, Marinas, & Luque de Castro, 2008; Tapia-Quirós et al., 2020). However, the green approach has not always been contemplated. Moreover, an absolute quantification of total phenolic content has been the prevailing trend (Maraulo, dos Santos Ferreira, & Mazzobre, 2021; Martínez-Patiño et al., 2019; Wang et al., 2021). This approach is highly valid, but it could be less descriptive than an individual determination of each compound. The latter allows a better understanding of the matrix content and permits the identification of the most interesting and potential molecules. In some cases, the recovery of some specific molecules is aimed, attending to commercial or industrial requirements, and, in that scenario, the importance of knowing the type and identifying each biophenol is undeniable.

In this study, we have combined a bio-compatible treatment of the promising sub-product TPOMW with a membrane-technology procedure and a robust LC-MS methodology to effectively understand the efficiency of the process. The preparation, comprehension, and primary purification of the extracts obtained here is a baseline that allows the exploitation of this contaminant olive mill residue. Moreover, the orientation to a large-scale production that has been considered is quite desirable to fulfill industrial necessities.

2. Materials and methods

2.1. Materials

Samples of TPOMW were kindly provided by San Isidro Cooperative (Segorbe, Valencia, Spain). They were obtained during the campaign of 2020/2021. Before being processed in the laboratory, samples were stored at 5 °C and 50% humidity to avoid the proliferation of microorganisms. To prepare mobile phases for LC-MS, acetonitrile (Honeywell, USA), acetic acid (Honeywell, USA) and ultrapure water were employed. Water was obtained from a Direct-Q®, 3UV system (Merck Millipore, USA). Pure standards of caffeic acid, luteolin, and *p*-coumaric acid were purchased from Sigma-Aldrich (USA). Hydroxytyrosol and oleuropein were obtained from BioNova Científica (Spain) and PanReac Applichem (Spain), respectively. Standard solutions were prepared in the appropriate solvent, diluted to the desired concentrations and stored at −20 °C prior to their utilization. Depending on the sample to be evaluated, standards were diluted in pure ethanol (VWR, USA), pure water, or EtOH/water 50:50, in a volume:volume basis (v/v).

2.2. Extraction of phenolic compounds

Two different strategies of solid-liquid extraction were evaluated for the recovery of interesting compounds from TPOMW: conventional extraction and UAE.

Moreover, the type of solvent (water or EtOH and their mixtures), the temperature (20, 30 and 40 °C) and the duration of the extraction (5, 15, 30, 60, 90 and 120 min) were studied.

For the conventional extraction, a constant stirring at 200 rpm was performed by an overhead stirrer (Heidolph, Instruments, Germany) and a pitched-blade impeller. In the case of the UAE, a temperature-controlled Elmasonic P 70 H ultrasound bath (Elma, Germany) was employed. The frequency and power of the applied ultrasounds were 37 kHz and 220 W, respectively. Between the two possible frequency values supported by the equipment (37 and 80 kHz), 37 kHz was chosen according to the manufacturer's recommendations for mixtures and dispersions. Ultrasonic power was set to the maximum to obtain a maximum amplitude, which influences the cavitation process. Indeed, some authors have achieved satisfying extractions with similar ultrasound powers and agro-alimentary matrices (Al-Dhabi, Ponmurugan, & Maran Jeganathan, 2017; Das, Goud, & Das, 2017; Rabelo, MacHado, Martínez, & Hubinger, 2016). As the large-scale aspect was one of the crucial aspects of this work, 900 g of TPOMW were weighted to be extracted with 9 L of solvent. A sample/solvent ratio of 1:10 (on a mass/volume basis) was considered. Once the extraction process was initiated, 40 mL aliquots were collected at the different time points set before. Extracts were then centrifuged (ThermoFisher, USA) at 17,200 RCF for 6 min. The resulting supernatants were filtered using SFMC-245-100 0.5 μm filters (ThermoFisher, USA) before their characterization.

2.3. Statistical analysis

The software Statgraphics Centurion 18 and Microsoft Excel 365 were employed to assess the analysis of the obtained results. The standard deviations reported in the Results section correspond to the deviation among experimental replicates. One-way analysis of variance (ANOVA) was applied to the results to evaluate the statistical differences, which were considered when the P-value from the Tukey's test was lower than 0.05.

The data derived from the extraction experiments were subjected to a response surface analysis, in order to maximize the concentration of polyphenols in the extract, by means of the variation of the independent variables (temperature, ethanol concentration and time). To evaluate the goodness of the model fit, the values of R^2 and adjusted R^2 were considered.

2.4. Ultrafiltration procedure

The optimum extract in terms of phenolic content was subjected to an ultrafiltration process in a solvent-resistant, dead-end XFUF 076 01 stirred cell (Merck Millipore, USA). The membrane was selected according to previous results of our research group (Cifuentes-Cabezas, Carbonell-Alcaina, Vincent-Vela, Mendoza-Roca, & Álvarez-Blanco, 2021), pursuing the lowest rejection to the phenolic compounds and the highest rejection to the rest of the organic matter. Thus, the membrane UP005 (Microdyn Nadir, Germany) was employed. It is composed of polyethersulfone and exhibits a molecular weight cut-off of 5 kDa. Before its utilization, the membrane was immersed in the pure solvent (EtOH/water 50:50 (v/v)) for 2 h to condition the polymer and prepare it for the contact with the ethanolic sample. Moreover, it was subjected to a compaction step at a transmembrane pressure of 5 bar and a stirring speed of 400 rpm. The ultrafiltration experiments were carried out at 2 bar and 400 rpm and were conducted until a volume reduction factor (VRF) of at least 2.5 was achieved. Samples of the extract, final retentate, global permeate (recovered during the whole process) and instantaneous permeate collected at each time point were characterized to calculate the rejection values. They were calculated by means of equation (1), which results from the material balance applied to the solute during a concentration experiment (assuming that rejection is constant and not dependent on the concentration in the retentate) (Díaz-Reinoso, Moure, Domínguez, & Parajó, 2009):

$$C_r = C_0 \cdot VRF^R \quad (1)$$

where C_r is the concentration in the retentate, C_0 is the concentration in the feed solution (TPOMW extract), *VRF* is the volume reduction factor, and *R* is the rejection coefficient.

Additionally, the reduction observed for the color and conductivity values was calculated according to:

$$Elimination = 1 - \frac{C_p}{C_0} \quad (2)$$

Where C_p is the concentration in the ultrafiltration permeate.

2.5. Characterization of TPOMW, phenolic extracts and UF streams

2.5.1. Measurement of phenolic and triterpenic content in the extracts

To obtain preliminary results in a short time, the Folin-Ciocalteu methodology was conducted to assess the concentration of total phenolic content (Singleton & Rossi, 1965), which was expressed as milligrams of tyrosol equivalents per gram of TPOMW (mgTYeq/g of wet material).

Once the general results of the different extractions were evaluated, a single time of extraction was chosen. With the variable of time fixed, the rest of the extracts corresponding to all combinations of solvent types, temperature and type of extraction (UAE or simple agitation) were subjected to LC-MS. Also, the ultrafiltration streams were analyzed using this methodology. A 1260 Infinity II LC system coupled to a 6546 quadrupole-time-of-flight (qToF) mass analyzer (Agilent Technologies, USA) was employed. Electrospray was used as an interface. To develop the multi-class LC-MS methodology that was required to understand the whole content of the extracts, previous works about excellent characterizations of olive matrices were revised and taken as a reference (Olmo-García, Polari, et al., 2018; Olmo-García, Wendt, et al., 2019).

After an injection of 5 μL, analytes were separated throughout an InfinityLab Poroshell 120 EC-C18 column (3.0 × 100 mm, 2.7 μm particle size) (Agilent Technologies, USA), operating at a temperature of 40 °C. Elution of compounds was performed by using water as phase A and acetonitrile as phase B. Both mobile phases were acidified with 0.5% of acetic acid (v/v) and the gradient was as follows: 5% (0–0.5 min), 11% (achieved at 2.5 min), 20% (at 7 min), 35% (at 18 min), 95% (at 22 min). After 24 min of total analysis time, a post-time of 3 min was dedicated to the column equilibration. Flow rate was 0.5 μL/min.

Mass spectrometer worked on negative polarity and full scan mode (30–1000 mass/charge ratio (*m/z*)). The main parameters to be optimized for the ionization source were drying gas temperature and flow (200 °C and 8 L/min, respectively), nebulizer pressure (30 psi) and capillary voltage (3500 V). To perform ion mass corrections, a calibrant solution was employed. It provided the *m/z* values of 112.9856, 966.0007, and 1033.9881 as references.

The identification of the peaks, obtained by LC-ESI-qToF-MS, was achieved by the study of mass spectrometry data of the samples and the corresponding pure standards. Additionally, previously reported information (Olmo-García, Monasterio, et al., 2019; Olmo-García, Wendt, et al., 2019; Rubio-Senent et al., 2015; Saftić, Peršurić, Fornal, Pavlešić, & KraljevićPavelić, 2019) and a self-created database of olive-derived compounds were considered. The software MassHunter (Agilent, USA), in its Qualitative and Quantitative versions, was used to explore the chromatograms. For quantitation purposes, peaks were integrated and the obtained areas were interpolated in the corresponding external calibration curve of caffeic acid (y = 18150033x – 587,113 in EtOH/-water 50:50 (v/v); y = 6073596x – 485,768 in water; y = 19739706x–816,146 in pure EtOH), hydroxytyrosol (y = 18579981x – 1272129 in EtOH/water 50:50 (v/v); y = 10404695x – 3868981 in water; y = 27772509x – 886,432 in pure EtOH), luteolin (y = 20409358x + 7314701 in EtOH/water 50:50 (v/v); y = 19299x - 83,897 in water; y = 209173844x – 1429030 in pure EtOH), *p*-coumaric acid (y = 1576099x + 10829796 in EtOH/water 50:50 (v/v); 1872460x – 2645579 in water; y = 24961035x + 479,131 in pure EtOH) or oleuropein (y = 6209163.2x – 538,759 in EtOH/water 50:50 (v/v); y = 3755542.9x – 2315616.2 in water; y = 10674099.3x – 228475.4 in pure EtOH). In all cases, the concentration of the standard analyte was the independent variable, whereas peak intensity was the dependent variable. All regression coefficients (r^2) were above 0.9934. When several isomers belonging to the same compound were found, the sum of their areas was considered, and that data was employed to obtain one value of concentration. That procedure was proven to be valid before (Gilbert-López et al., 2014). As will be described in the Results section, the number of found compounds surpassed by far the number of standards. However, a semi-quantitative analysis was considered sufficient to compare the results obtained with the different treatments and is also very usual when such number of molecules are determined (Olmo-García, Kessler, et al., 2018; Troise, Ferracane, Palermo, & Fogliano, 2014).

To establish the limit of detection (LOD) and the limit of quantification (LOQ) of the method, it was calculated the analyte concentration that gave a signal to noise ratio of 3 and 10, respectively. To evaluate the precision, intra-day repeatability was assessed through the relative standard deviation (RSD) of three different injections (within the same LC-MS sequence) of an ultrasound-assisted extract obtained with EtOH/water 50:50 (v/v), at 40 °C.

2.5.2. Other techniques applied for the characterization of the extracts

To fully characterize the extracts, pH (pHmeter GLP31+, Crison, Spain), electric conductivity (Conductimeter GLP31+, Crison, Spain), and total solid content were measured. The total sugars content was determined using the anthrone method (Dreywood, 1946; Ludwig & Goldberg, 1956). Color was determined according to ISO 7887:2011, method B (ISO 7887:2011, 2011). Absorbance was measured at three different wavelengths (436 nm, 525 nm, and 620 nm) using a UV-VIS DR 6000 spectrophotometer (Hach Lange, Germany). The color coefficient was given by the following formula:

$$Colour = \frac{(A_{436}^2 + A_{525}^2 + A_{620}^2)}{(A_{436} + A_{525} + A_{620})} \quad (3)$$

2.6. Evaluation of the one-step extraction

Once the best conditions regarding time, temperature, and ultrasounds application were determined, the recovery capacity of the technique was evaluated. To that end, the sediment that remained after the centrifugation stage was re-extracted (by applying the same process as before). In order to maintain the same ratio of sample/solvent, the sediment was weighted to adjust the needed volume of extractant. This cycle was repeated once again with the sediment of the second extraction, to ensure that the percentage of residual olive minor fraction in the sample (the percentage that was not extracted) was sufficiently low to qualify the extraction as truthful. The supernatants obtained by each of the three extraction cycles were analyzed through the procedures detailed in sections 2.5.1 and 2.5.2.

3. Results and discussion

3.1. Total phenolic content

Fig. 1 shows the results of the applied UAE and agitation-mediated extraction. According to the figure, the highest efficiency of extraction was obtained with EtOH/water 50:50 (v/v) in all cases, regardless of the extraction time, temperature, or presence of ultrasounds. Water was the following solvent in terms of extractant power, whereas pure ethanol presented the worst results.

The selection of the solvent to extract phenolic compounds is not trivial. Babbar et al. compared the effect of using methanol, ethyl acetate, chloroform, and hexane as solvents in the extraction of phenolic compounds from a range of vegetable wastes. In all cases, methanol proved to be the most effective solvent (Babbar, Oberoi, Sandhu, & Bhargav, 2014). Other solvents, such as N,N-dimethylformamide, mixtures of tetrahydrofuran/water or even tensioactive species have been employed too for the isolation of phenolic fractions (Carrasco-Pancorbo et al., 2005). However, other relevant considerations should be made when it comes to selecting the solvent, not only its recovery potential. Nowadays, environmentally friendly solvents (such as ethanol (EtOH) and even water) should be preferentially employed. Zagklis and Paraskeva considered this during their study on the extraction of phenolic compounds from grape marc, opting to use EtOH/water mixtures as a solvent due to environmental considerations and suitability with respect to the food industry (Zagklis & Paraskeva, 2015).

In this study, the presence of ethanol in the solvent mixture (ethanol/water) contributed to obtain an adequate polarity to recover phenolic compounds. The optimum results obtained with the mixture of 50:50 (v/v) were coherent and in accordance with previous findings (D'Alessandro, Dimitrov, Vauchel, & Nikov, 2014). The results obtained with pure ethanol can be attributed to a fast dehydration of vegetable cells, which may result in the aggregation of microcellular material (such as proteins, cell wall components, etc.) and hinders the diffusion of compounds to the solvent (Garcia-Castello et al., 2015).

Considering the variable of time, most of the phenolic content was extracted rapidly, especially in the UAE cases. Nevertheless, a small increment of the extraction yield can be observed in the first minutes and during the first hour for most of the experimental conditions tested. This can also be revised in Fig. 4 (panel A), which contains the surface response analysis of the total phenolic content with the variation of time and ethanol concentration at a constant temperature of 40 °C. According to the ANOVA data for the fitting of the response surface model, the plots presented in Fig. 4A and B correspond to models of statistical significance. This was supported by high F values (higher than 6.38 for the graph in Fig. 4A and higher than 59.89 for the model in Fig. 4B) and P values lower than 0.0429 (Figs. 4A) and 0.0002 (Fig. 4B) for the considered effects. Additionally, the model adequacy was endorsed by a R^2 of 0.9446 and 0.9758 and an adjusted R^2 of 0.9138 and 0.9677 for Fig. 4A and B, respectively. Those results motivated the selection of an

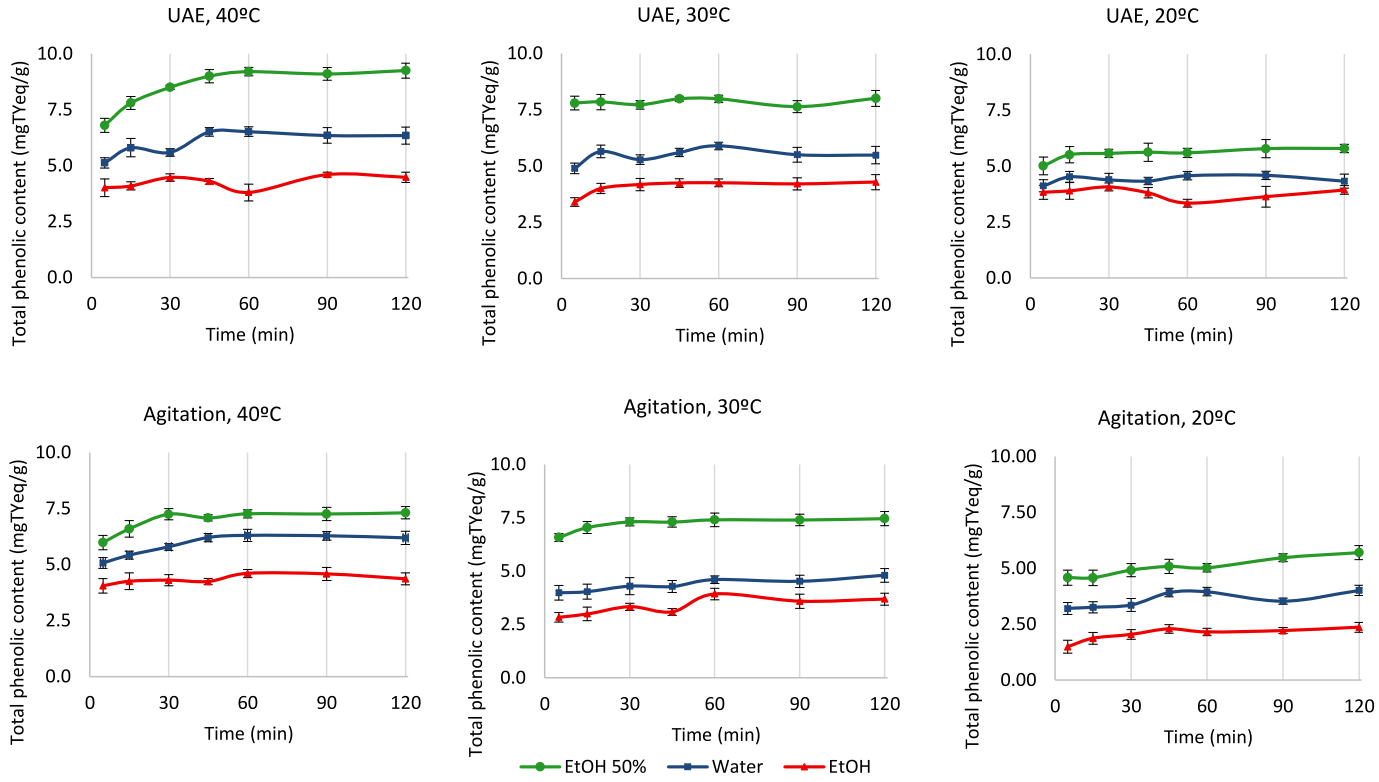

Fig. 1. Total phenolic content of the whole set of extracts obtained from ultrasound-assisted extraction (UAE) and agitation-mediated extraction, at the different temperatures and time points evaluated. Error bars indicate the standard deviation of the experimental replicates.

extraction time of 60 min for the following studies, in order to ensure that maximum phenolic content was extracted. Regarding the temperature, an increment higher than 3 mg/g in the recovery of phenolic compounds can be observed as this variable increases from 20 °C to 40 °C. The data displayed in Fig. 1 suggested that higher temperatures and the application of ultrasounds were best for the extraction performance. To confirm these results and to better assess the effect of the sonication, all extracts (at the time of 60 min) were also analyzed by LC-MS.

3.2. Quantification of the compounds identified by LC-MS

After the characterization by LC-MS of all the obtained extracts (at the three temperatures, three types of solvent and both conditions of sonication or agitation), 41 compounds were identified and classified in their corresponding chemical family. Table 1 contains a thorough description of the composition of the analyzed samples. Compound identification, retention time (Rt), *m/z,* and assigned chemical class are described. Three additional species were detected, but their identification was not possible. In those cases, a molecular formula was proposed after the study of spectral data with MassHunter. Those formulas and the software score assigned to each labeling are available in Table 1.

In the case of the *m/z* 175.0613, such formula may correspond to the isopropyl malic acid. Other unknown compounds corresponded to *m/z* 377.1451 and *m/z* 243.0876. They have been already found in olive flours (Olmo-García, Monasterio, et al., 2019) and other samples related to the olive pulp (Olmo-García, Kessler, et al., 2018). Proposed empirical formulas were coincident with those in literature, even when a different software was employed. The applied LC-MS methodology permitted the evaluation of more than 40 compounds (from different chemical families) within a single run, which facilitated the study of the phenolic profile. The latter was considered very promising for future applications, as many of the most valuable polyphenols in the industry were present in the extracts. Although the non-targeted approach has not been commonly applied during the analysis of TPOMW, the composition of the samples in this study was consistent with relevant previous literature (Cea-Pavez et al., 2019; Peralbo-Molina, Priego-Capote, & Luque de Castro, 2012).

Fig. 2 contains two chromatograms in which peaks distribution can be explored. As the figure shows, there were some peaks with a notably large area, in contrast with other shorter peaks that are less visible in the chromatogram because of the figure scale. The first chromatogram corresponds to an ultrasound-assisted extraction, whereas the second was obtained by simple agitation. As can be seen, some differences stand out. Both chromatograms are from samples obtained with the same solvent (EtOH/water 50:50 (v/v)), at the same temperature (40 °C). Comparison of peak height within the same chromatogram could be misleading, because different compounds might display different response factors in the mass spectrometer. Therefore, the visual inspection of the figure should be done by comparing the behavior of the same peak in each sample. Several compounds that were better extracted by means of ultrasounds application have been marked with colored arrows.

Some of the most relevant peaks in the upper chromatogram (UAE) were much shorter or even absent in the bottom one (agitation). This fact contributed to enlarge the concentration of phenolic compounds in the sonicated sample. The 44 detected compounds were found in both cases, which set the agitation-mediated strategy as a proper methodology to easily recover the olive minor-fraction. However, it is undeniable that the sonication contributed to increase the phenolic content of the final extract and its application was very desirable to get the most out of the TPOMW, in terms of phenolic compounds.

Analytical parameters of the LC-ESI-qToF methodology that allowed these findings can be revised in Supplementary Table 1. The table shows the obtained values for LOD, LOQ, and method repeatability.

LODs were found to be below 0.098 ppm in all cases and LOQs were in the range of 0.002–1.217 ppm. These results were satisfactory, as they allowed the quantification of all the compounds detected. All calculated concentrations were analytically valid, considering the parameters of Supplementary Table 1. The results for the RSD of three injections of the same sample were very low, which indicated a good repeatability of the analytical strategy developed.

Table 1
Retention time (R_t), mass/charge ratio (*m/z*) and assigned identity and chemical class of the compounds detected by LC-ESI-QToF-MS. Scores of the molecular formulas achieved with MassHunter when identification was not possible are indicated in brackets.

Rt	Compound Identity	*m/z*	Chemical family
1.001	Quinic acid	191.0555	Organic acids
1.051	Malic acid	133.0150	Organic acids
2.538	Vanillic acid	167.0352	Phenolic acids and aldehydes
3.173	Hydroxytyrosol	153.0551	Simple phenols
3.641	Acyclodihydroelenolic acid hexoside	407.1560	Secoiriodoids
3.942	Hydroxy-decarboxymethyl elenolic acid	199.0607	Secoiriodoids
4.443	$C_7H_{12}O_5$ (score: 86.96%)	175.0614	Unknowns
5.061	Vanillin	151.0396	Phenolic acids and aldehydes
5.279	Caffeic acid	179.0347	Phenolic acids and aldehydes
5.429	Gallocatechin	305.0702	Flavonoids
5.913	Hydroxyelenolic acid	257.0669	Secoiriodoids
5.930	Tyrosol	137.0608	Simple phenols
6.114	Decarboxymethyl elenolic acid	183.0658	Secoiriodoids
6.164	Elenolic acid glucoside	403.1246	Secoiriodoids
6.281	$C_{16}H_{26}O_{10}$ (score: 96.93%)	377.1453	Unknowns
6.899	*p*-Coumaric acid	163.0397	Phenolic acids and aldehydes
6.999	Aldehydic form of decarboxymethyl elenolic acid	215.0925	Secoiriodoids
7.250	Phenylethyl primeveroside	415.1612	Secoiriodoids
8.042	$C_{11}H_{16}O_6$ (score: 99.12%)	243.0876	Unknowns
8.136	Dehydro-oleuropein aglycone	375.1087	Secoiriodoids
8.186	Hydroxyoleuropein	555.1717	Secoiriodoids
8.603	Luteolin rutinoside	593.1516	Flavonoids
9.489	Luteolin 7-O-glucoside	447.0933	Flavonoids
9.697	Oleuropein	539.1769	Secoiriodoids
9.772	Elenolic acid	241.0720	Secoiriodoids
10.391	Ferulic acid	193.0503	Phenolic acids and aldehydes
10.508	Hydroxytyrosol acyclodihydroelenolate	381.1560	Secoiriodoids
11.761	Decarboxymethyl oleuropein aglycone	319.1187	Secoiriodoids
11.811	Pinoresinol	357.1337	Lignans
11.827	Ligstroside	523.1820	Secoiriodoids
13.048	Luteolin	285.0405	Flavonoids
14.083	Oleuropein aglycone	377.1242	Secoiriodoids
16.306	Diosmetin	299.0558	Flavonoids
19.931	Dihydroxy-hexadecanoic acid	287.2230	Fatty acids derivatives
20.511	Ligstroside aglycone	361.1293	Secoiriodoids
20.783	Trihydroxy-octadecenoic acid	329.2335	Fatty acids derivatives
21.485	Gingerol	293.1759	Flavonoids
21.619	Betulinic acid	455.3538	Triterpenic acids
22.654	Hydroxy-octadecatrienoic acid	293.2122	Fatty acids derivatives
22.655	Dihydroxy-octadecanoic acid	315.2516	Fatty acids derivatives
22.989	Hydroxy-octadecadienoic acid	295.2277	Fatty acids derivatives
23.189	Maslinic acid	471.3488	Triterpenic acids
23.406	Hydroxy-octadecenoic acid	297.2435	Fatty acids derivatives
23.590	Hydroxy-octadecanoic acid	299.2591	Fatty acids derivatives

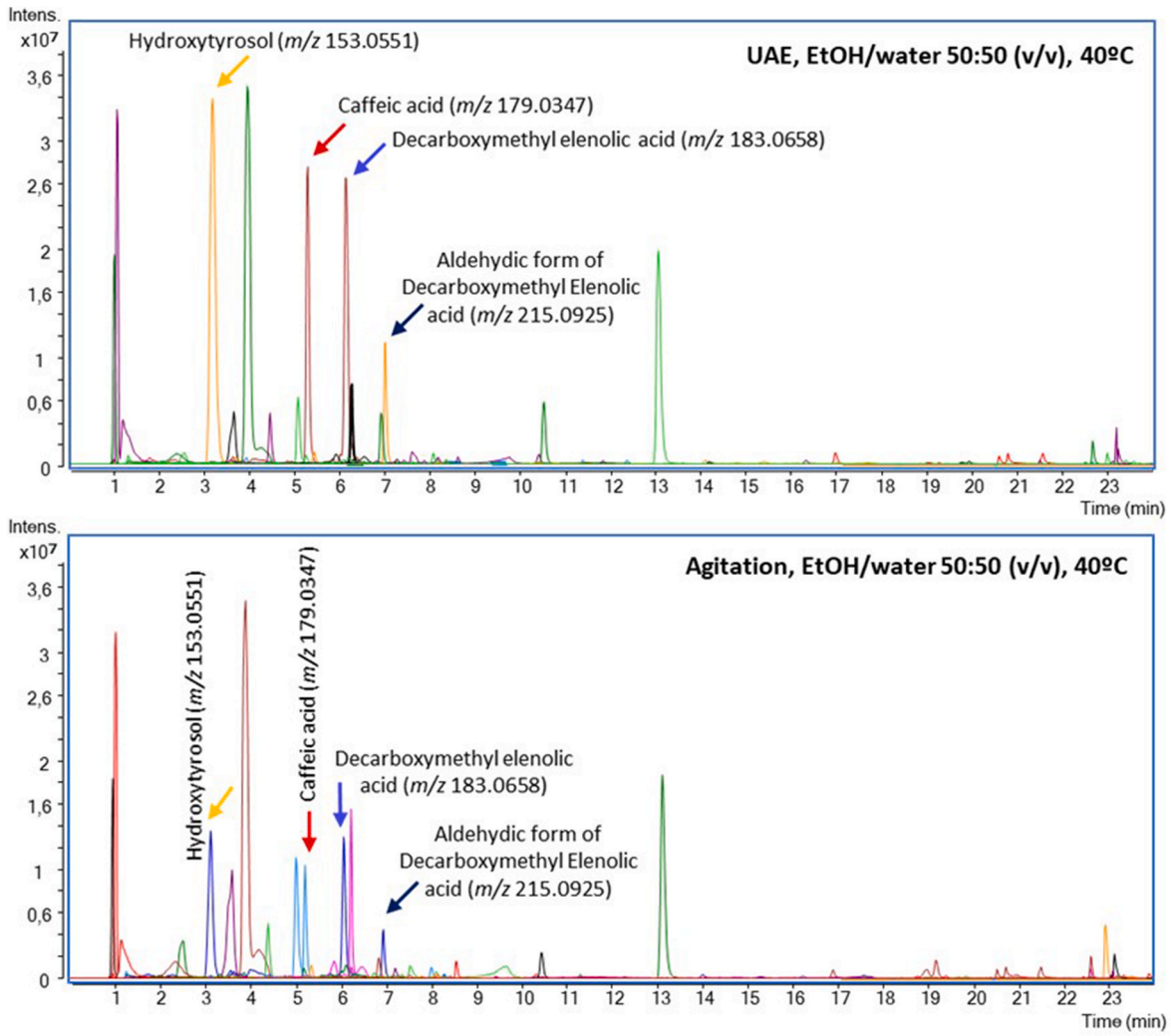

Fig. 2. Extracted ion chromatograms of two samples of two-phase olive mill waste (TPOMW), extracted by sonication (upper chromatogram) and simple agitation (lower chromatogram). Arrows of the same color indicate the peaks corresponding to the same compound in each one of the extraction studies. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Selection of best conditions to recover the olive minor fraction

The samples of TPOMW contained organic acids, simple phenols, phenolic acids and aldehydes, flavonoids, a lignan (pinoresinol), secoiridoids (the most abundant chemical class, by a notorious difference), triterpenic acids and fatty acid derivatives. The distribution of these families for the studied strategies of agitation and ultrasound-assisted extraction is presented in Fig. 3. As it was observed in the quantification of total phenols, EtOH/water 50:50 (v/v) performed remarkably. This can also be observed in Fig. 4B, which shows the surface response analysis with ethanol concentration and temperature as independent variables, at an extraction time of 60 min. Water was also a good alternative, as the obtained concentration levels were interesting too, but the lower efficiency for flavonoids was noticeable in Fig. 3. Considering the concentration values and the correct extraction of all chemical families present in the samples of TPOMW, EtOH/water 50:50 (v/v) was confirmed as the best solvent.

Regarding the temperature, Fig. 4B does not indicate any preferential temperature when the total phenolic content at the three tested temperatures is considered. However, if some chemical families, such as simple phenols and flavonoids, are analyzed individually after the extraction with EtOH/water 50:50 (v/v), 40 °C stands out as more efficient. These chemical classes are of special interest because many biological positive effects have been attributed to their principal representatives (European Commission, 2012; Robles-Almazan et al., 2018). In fact, the health claim approved by the European Food Safety Authority about olive oil phenolic compounds was based in the hydroxytyrosol content. According to the ANOVA applied to the concentration values obtained at the different temperatures for the extraction with EtOH/water 50:50 (v/v), the individual family of simple phenols (which include hydroxytyrosol) displayed differences of statistical significance (P-value <0.05, according to F test) among the values obtained at each temperature. This also occurred for the phenolic acids, which include relevant compounds such as ferulic and caffeic acid. In this case, the concentration obtained at 20 °C was not significantly different from the concentration obtained at 30 °C, but both mean values were statistically different from the concentration of phenolic acids obtained at 40 °C. The specific concentration of simple phenols and phenolic acids can be found in Supplementary Fig. 1. As the discussed molecules were preferentially extracted at 40 °C, it seems pertinent to apply this temperature.

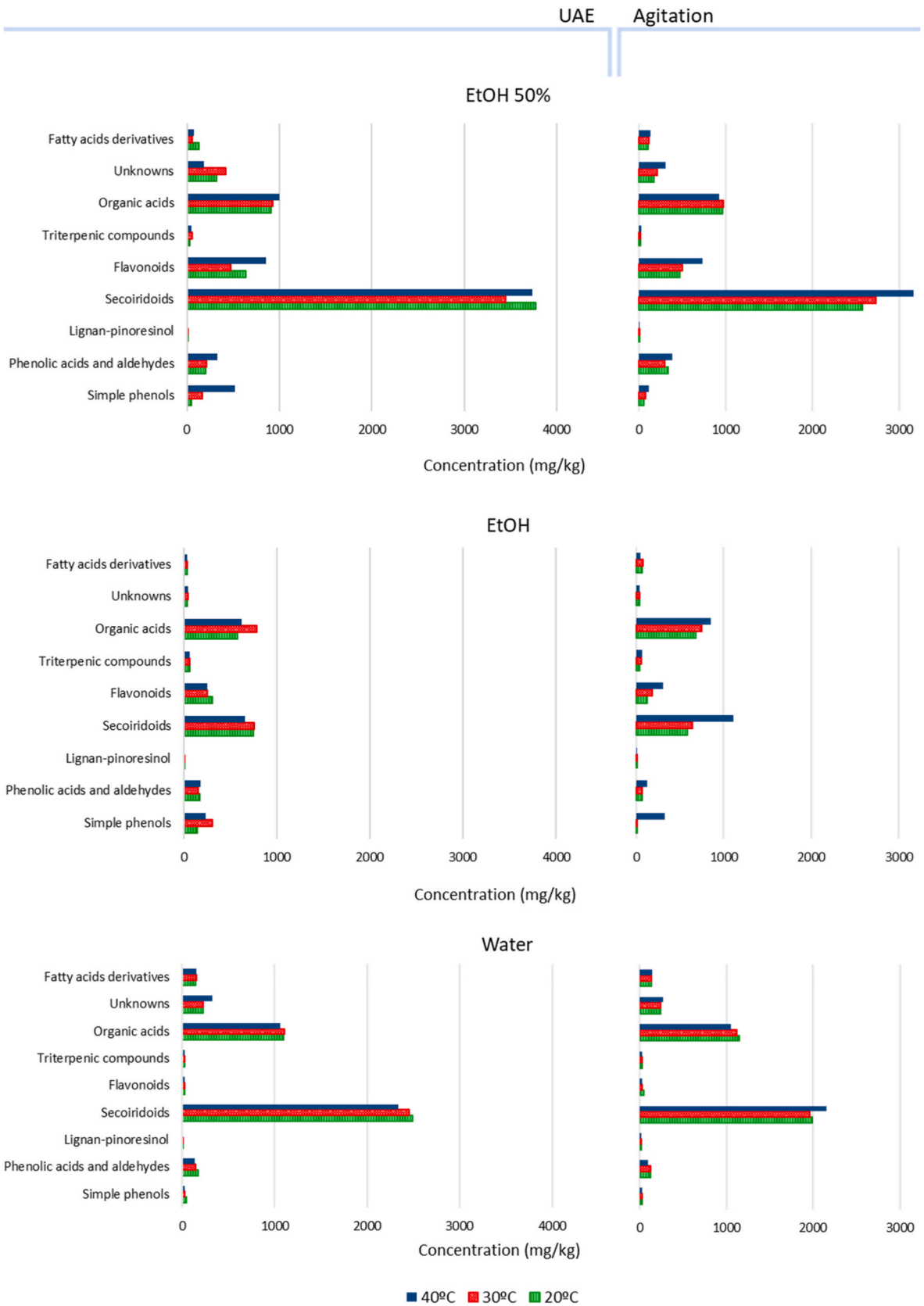

Fig. 3. Obtained concentration of phenolic compounds and other chemical families after the ultrasound-assisted (left graphs) and agitation-mediated (right graphs) extractions, with the three solvents tested, at different temperatures.

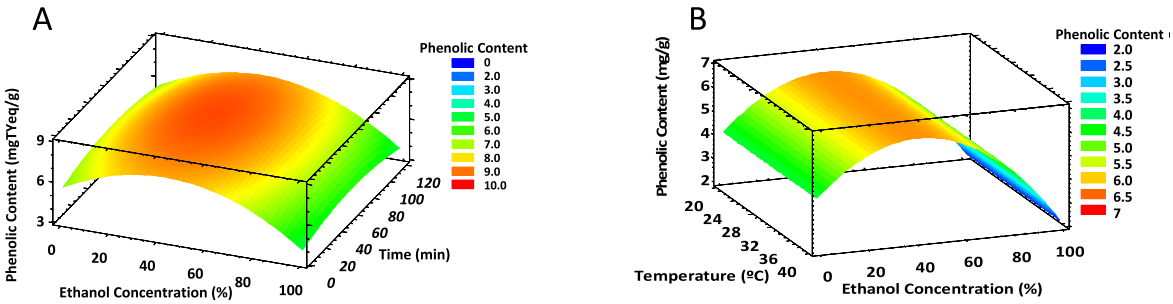

Fig. 4. Response surface analysis for the total phenolic content obtained with the different extraction variables. Data from plot A correspond to a temperature of 40 °C. Data from plot B correspond to an extraction time of 60 min.

Global results of total phenolic content (Fig. 1) already suggested that ultrasounds application would contribute to the extraction of the olive minor fraction from TPOMW. Indeed, LC-MS data showed that higher levels of these phytochemicals were obtained by the UAE approach. This was evident for flavonoids, simple phenols, and phenolic acids and aldehydes. However, the most evident results were once again those for secoiridoids, which surpassed 3.5 g/kg when sonication was applied and stayed at 3 g/kg in the cases of simple agitation. As has been already commented, the cavitation bubbles formed during sonication can damage the vegetal tissue (Das et al., 2017; Mason, Cobley, Graves, & Morgan, 2011). As a consequence, solvent penetration within the sample is increased, and thus, compounds transfer and recovery are enhanced. Since the objective of this work was to extract the maximum levels of antioxidants and other interesting molecules to further exploit their properties, the UAE strategy was preferred against agitation. More details regarding color, pH, and conductivity of the solutions obtained through the UAE process, with ethanol/water 50:50 (v/v), at 40 °C have been provided in Supplementary Fig. 2. Those results reflect that pH stayed essentially constant during the extraction stages. Conductivity and color followed a similar trend to total phenolic compounds, which was shown in Fig. 1. That distribution supports our initial reasoning about a rapid liberation of the majority of the compounds from the matrix.

Table 2 displays a comparison of the concentration ranges achieved in the extract during the sonication-mediated extraction and the extractions performed by agitation only. Results have been specified for every chemical family detected in our analysis.

The results of the concentration of each chemical class contained in Fig. 3 indicated that secoiridoids were the most concentrated group. This is corroborated by the concentration ranges in Table 2. The molecule of hydroxy-decarboxymethyl elenolic acid was always the most concentrated secoiridoid, no matter the conditions applied during the sample treatment, and it was better extracted at 40 °C, when ultrasounds were applied (by a difference of more than 150 mg/kg).

3.4. Evaluation of the efficiency of the extraction at the selected conditions

To evaluate the efficiency of the process, the remaining sediment obtained after a first UAE cycle was successively re-extracted. Sample re-extraction was preferred over its external fortification, because the solid character of TPOMW may hinder the uniform incorporation of new molecules to the matrix.

From Fig. 5, it is possible to conclude that the developed one-step extraction is sufficient to recover almost all the phenolic content of the TPOMW samples, either after the extraction with ethanol at 50% or water (which were the most promising solvents, as explained before).

After the second extraction, less than 27% of the phenolic content determined in the first cycle was detected. The residual biophenols after this second extraction were already limited, and after the third cycle, only 7% of the initially extracted phenolic content was recovered. Most of the sugars were also retrieved after the first extraction. These results confirmed the high efficiency of the UAE procedure corresponding to the first cycle. Considering that this was a large-volume extraction, the recovery of the highest bioactive percentage in a single step is of relevance. This avoids using more solvent and simplifies the process, contributing to its large-scale adaptation. In the case of the aqueous extraction, the obtained concentration of polyphenols was lower (as expected, considering Figs. 1 and 3). Still, the efficiency was satisfactory, because the sample was already quite exhausted after the first cycle of extraction. A similar trend can be observed for the total solids. A disadvantage that can be attributed to the aqueous extraction is the proportion of extracted polyphenols with respect to the total solids (these may include undesired molecules, such as sugars). This proportion was more than three times higher in the extraction with EtOH at 50%, which was remarkable. Regarding the color determined in the extracted fractions, this was the only parameter that behaved differently. The first extract did not display a much higher color coefficient than those from the subsequent cycles. However, the lower capacity of

Table 2
Concentration ranges for the compounds that belong to the chemical families detected by LC-ESI-QToF-MS. Results correspond to two extracts obtained with EtOH/water 50:50 (v/v), at the condition of sonication or agitation and the three tested temperatures. Relative standard deviation was in the range of 0.11%–13.23% for all the concentrations included in the table.

Chemical family	Concentration ranges for the individual compounds (mg/kg)					
	UAE, EtOH/water 50:50 (v/v)			Agitation, EtOH/water 50:50 (v/v)		
	40 °C	30 °C	20 °C	40 °C	30 °C	20 °C
Secoiridoids	2.61–1982.57	0.59–1206.74	1.97-1761-55	2.50–1814.44	2.25–1605.56	2.22–1555.57
Organic acids	307.05–690.42	293.44–631.46	270.30–640.58	267.68–659.74	319.58–657.58	319.89–643.23
Flavonoids	5.70–832.31	5.06–454.40	4.65–619.10	3.92–710.20	3.58–488.51	3.54–456.32
Phenolic acids and aldehydes	6.93–193.70	1.48–116.51	1.87–138	2.50–299.41	2.24–241.51	2.30–284.56
Simple phenols	5.76–511.37	6.52–163.80	10.23–34.77	7.57–105.53	5.89–70.08	5.05–51.88
Fatty acids derivatives	1.99–27.60	0.58–26.15	2.38–63.01	2.54–69.50	2.64–54.43	2.86–48.09
Triterpenic acids	1.85–46.78	0.46–60.96	1.86–30.31	1.85–31.28	1.84–18.32	1.84–20.63
Lignan-pinoresinol	6.48	2.04	2.57	3.71	3.10	3.13
Unknowns	25.17–89.25	12.18–213.03	24.84–217.50	16.79–208.02	14.30–136.48	15.14–99.56

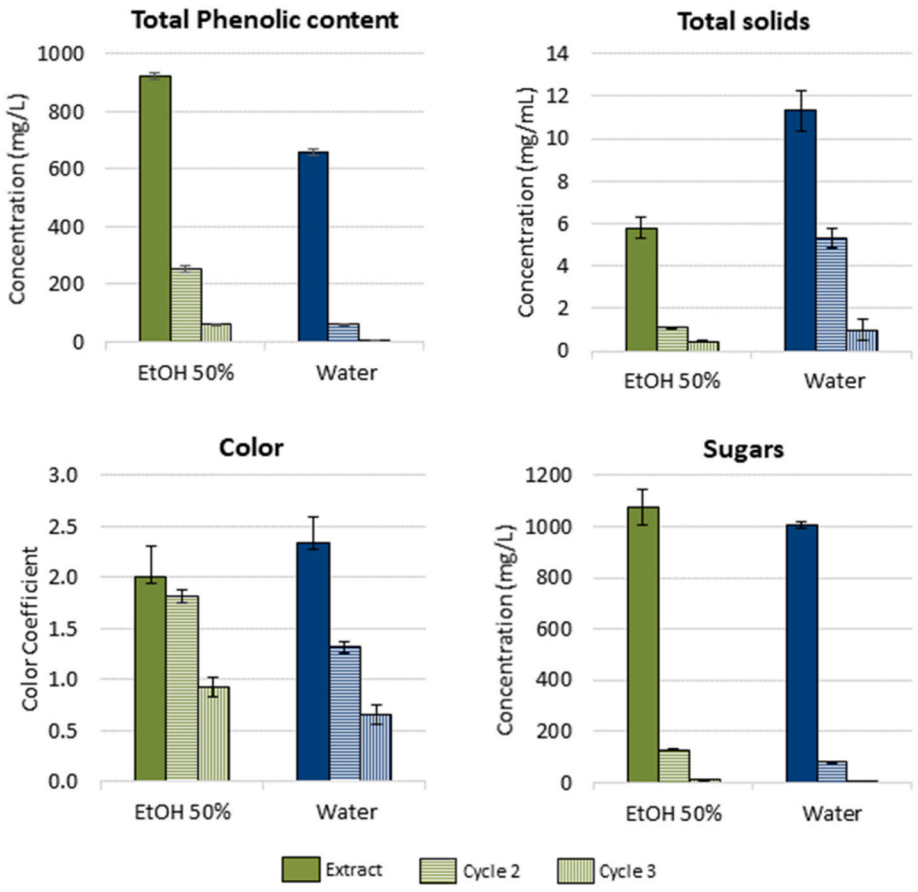

Fig. 5. Achieved concentration values of total phenolic content, total solids, and sugars after each extraction cycle. The obtained color coefficient is also shown. Ultrasound-assisted extraction was performed with a solvent to sample ratio of 1:10 and 40 °C. Error bars refer to the standard deviation of experimental replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the solvents to transport pigments from the sample was a positive situation, as the final product would be clearer.

Considering these aspects, the extraction with EtOH/water 50:50 (v/v) can be again confirmed as a powerful procedure. It is important to highlight that the results achieved with water were also of interest, as high concentrations of phenolic compounds were acquired. However, as this study pursued the recovery of most of the bioactive content from TPOMW and ethanol is also considered a green solvent, the ethanolic mixture was selected.

3.5. Ultrafiltration of the most favorable extract

The permeate flux experienced a decline from 4.5 L/h·m^2, obtained at the beginning of the process, to 2.6 L/h·m^2, which was registered at a VRF of 2.8 and 2 bar of transmembrane pressure. This decrease resulted from the sample concentration in the membrane module and, consequently, the increasing concentration of the solutes. Fig. 6 exposes the fraction of organic matter retained by the membrane. Two axes have been provided, as the results about color and conductivity refer to

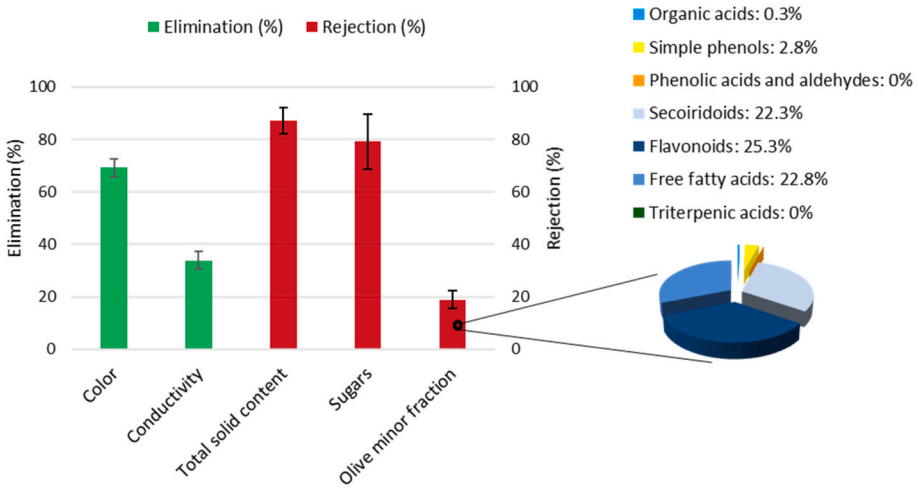

Fig. 6. Performance of the UP005 membrane in terms of elimination of color and conductivity and rejection of compounds (total solid content and olive minor fraction) after the ultrafiltration process conducted at 2 bar. Error bars indicate the standard deviation observed for experimental replicates. The sector diagram illustrates the specific rejection for each chemical class of phenolic compounds and triterpenes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

elimination (equation (1)) and the results about total solid content, total sugars content and olive minor fraction refer to rejection (equation (2)). To calculate the rejection to the olive minor fraction, the total concentration of the molecules corresponding to each chemical family was considered.

As shown in Fig. 6, the membrane retained almost all the total solids and sugars of the TPOMW extract. The color coefficient was also reduced, whereas the majority of the olive minor fraction was recovered in the permeate.

These results were very promising, as very valued compounds have been recovered in the permeate, while other existent molecules (such as sugars, pigments and other solutes) remained in the membrane retentate. Regarding the olive minor fraction, none of the chemical classes were highly retained, but it is especially remarkable that some of them were not rejected at all. That is the case of phenolic acids and aldehydes, which include molecules so highly appreciated by industry as ferulic acid, vanillin or *p*-coumaric acid (Valanciene et al., 2020). Similarly, simple phenols (including a high proportion of hydroxytyrosol) were almost completely recovered in the permeate, at a much higher purity than in the initial extract. The individual concentration of each compound can be revised in Table 3.

4. Conclusions

The process described here permitted to isolate a numerous variety of biophenols, which were obtained at high concentration (near 10 mg/g TPOMW). The detailed characterization of the extracts by LC-ESI-qToF-MS revealed that more than 40 compounds were obtained, from eight different chemical classes, in only one step of mixing and sonication. A large-scale, industrial application is possible. Moreover, the only organic solvent employed was ethanol, which has been recognized as a low-toxicity, environmentally friendly solvent. Afterward, the purification of the extract can be achieved by means of ultrafiltration. The selected membrane and operational parameters allowed the removal of almost the entire solids and sugars content, whereas the bioactive compounds were recovered in the permeate at high purity.

The optimum extraction was procured with ethanol/water 50:50 (v/v), at 40 °C and with ultrasound application. The developed strategy allowed the recovery of a considerable proportion of phenolic and triterpenic compounds from a major waste from the olive industry, as it is TPOMW. This by-product has not been extensively utilized, but its bioactive content can be greatly exploited if it is properly extracted from the semisolid mixture of olive pulp, skin, and stones. Additionally, the withdrawal of these species from the residue contributes to its detoxification and favors the future stages of composting.

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CRediT authorship contribution statement

Carmen M. Sánchez-Arévalo: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Supervision. **Alicia Iborra-Clar:** Conceptualization, Methodology, Software, Investigation. **María Cinta Vincent-Vela:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Silvia Álvarez-Blanco:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Table 3
Concentrations in ultrafiltration feed and permeate, for every compound present in the samples.

Compound Identity	Concentration in the UF Feed (ppm)	Concentration in the UF Permeate (ppm)
Quinic acid	11.32 ± 0.07	11.30 ± 0.05
Malic acid	4.86 ± 0.06	4.85 ± 0.03
Vanillic acid	0.107 ± 0.002	0.132 ± 0.002
Hydroxytyrosol	44.46 ± 0.03	45.1 ± 0.4
Acyclodihydroelenolic acid hexoside	189.2 ± 0.2	63.9 ± 0.5
Hydroxy-decarboxymethyl elenolic acid	651 ± 1	635 ± 2
$C_7H_{12}O_5$ (score: 86.96%)	3.194 ± 0.006	2.17 ± 0.01
Vanillin	1.174 ± 0.006	1.778 ± 0.001
Caffeic acid	112.26 ± 0.08	115.3 ± 0.1
Gallocatechin	1.630 ± 0.004	0.304 ± 0.004
Hydroxyelenolic acid	11.06 ± 0.03	4.48 ± 0.01
Tyrosol	2.11 ± 0.02	0.723 ± 0.009
Decarboxymethyl elenolic acid	185.3 ± 0.9	185.8 ± 0.8
Elenolic acid glucoside	1.862 ± 0.009	1.91 ± 0.02
$C_{16}H_{26}O_{10}$ (score: 96.93%)	15.01 ± 0.03	8.00 ± 0.05
p-Coumaric acid	2.764 ± 0.005	3.31 ± 0.01
Aldehydic form of decarboxymethyl elenolic acid	10.10 ± 0.06	4.81 ± 0.02
Phenylethyl primeveroside	1.68 ± 0.01	1.21 ± 0.01
$C_{11}H_{16}O_6$ (score: 99.12%)	2.0 ± 0.4	2.163 ± 0.006
Dehydro-oleuropein aglycone	0.231 ± 0.003	0.125 ± 0.002
Hydroxyoleuropein	0.91 ± 0.03	0.207 ± 0.002
Luteolin rutinoside	2.30 ± 0.06	1.35 ± 0.01
Luteolin 7-O-glucoside	0.44 ± 0.02	0.21 ± 0.07
Oleuropein	0.93 ± 0.01	0.95 ± 0.01
Elenolic acid	21.60 ± 0.09	21.67 ± 0.05
Ferulic acid	1.5 ± 0.03	1.514 ± 0.001
Hydroxytyrosol acyclodihydroelenolate	8.4 ± 0.6	8.98 ± 0.03
Decarboxymethyl oleuropein aglycone	13.86 ± 0.02	8.88 ± 0.04
Pinoresinol	0.422 ± 0.01	0
Ligstroside	1.146 ± 0.007	1.855 ± 0.003
Luteolin	10.55 ± 0.05	10.57 ± 0.09
Oleuropein aglycone	8.54 ± 0.04	8.833 ± 0.004
Diosmetin	0.075 ± 0.002	0
Dihydroxy-hexadecanoic acid	0.9 ± 0.3	0.435 ± 0.003
Ligstroside aglycone	0.242 ± 0.002	0.246 ± 0.004
Trihydroxy-octadecenoic acid	0.95 ± 0.09	1.40 ± 0.02
Gingerol	0.25 ± 0.02	0.170 ± 0.003
Betulinic acid	0.1981 ± 0.0005	0.246 ± 0.001
Hydroxy-octadecatrienoic acid	0.387 ± 0.002	0.440 ± 0.001
Dihydroxy-octadecanoic acid	1.380 ± 0.009	1.016 ± 0.005
Hydroxy-octadecadienoic acid	0.775 ± 0.003	0.440 ± 0.007
Maslinic acid	1.67 ± 0.08	1.86 ± 0.02
Hydroxy-octadecenoic acid	0.100 ± 0.002	0.088 ± 0.001
Hydroxy-octadecanoic acid	0.054 ± 0.002	0.0530 ± 0.0004

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113742.

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