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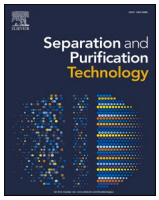

Strategies for the purification of hydroxytyrosol-rich extracts obtained from exhausted olive pomace

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ABSTRACT

Exhausted olive pomace (EOP) is a residual biomass from which hydroxytyrosol can be recovered. This compound has applications in the food/pharma sectors, but its extraction yields complex extracts that require further purification for some applications.

This work explores purification strategies based on membrane technology, liquid–liquid extraction (LLE), and solid-phase extraction with adsorbents and resins. The hydroxytyrosol content, phenolic profile, antioxidant activity, and inhibition of α-glucosidase and α-amylase were monitored. Hydroxytyrosol stood out in all purified extracts. The best hydroxytyrosol recovery (88.8%) was achieved using LLE with ethyl acetate as the extractant, while the purest extracts in phenolic compounds, including hydroxytyrosol, were obtained using the latter solvent and C18 (529 mg/g), DSC-8 (873 mg/g), and Purosorb PAD910 (523 mg/g). Conversely, mannitol and glucose, at high concentrations in the extract, were selectively retained in the aqueous phases.

The developed strategies are discussed regarding their suitability to provide hydroxytyrosol-concentrated extracts, up to 291 mg/g, with antioxidant and antidiabetic functionalities.

1. Introduction

Exhausted olive pomace (EOP) is the final dry biowaste obtained in the olive oil production chain. It is obtained during the processing of the olive pomace, which is first partially destoned, then, dried and the residual oil (<3%) is extracted in the pomace oil extractors. In Spain, the largest olive oil producer in the world, around 1.2 million tonnes of EOP are generated every year, representing a great opportunity to valorise this waste for various applications [1]. Currently, the common use of EOP consists of combustion in local industries and domestic boilers to produce heating. However, this application entails several drawbacks, e.g. the combustion is inefficient and there is a tendency to cake [2], which leads to a low selling price on EOP trading compared to other biofuels [1]. To widen the applications of EOP, some trends recently published include the obtainment of bioactive compounds (phenolic compounds and triterpenic, among others) [3], the production of pectinases [4], omega-3 fatty acids [5], antioxidant lignin and sugars [6], xylitol [7], biogas [2], and bio-oil [8]. Some of these bioproducts can be obtained in biorefinery cascading processes to maximize the valorisation of EOP based on their chemical composition [2,3,6,9].

The processing steps, especially, drying and extraction, affect the composition and content of bioactive compounds in the paste [10]. Nonetheless, the resulting EOP is a good source of phenolic compounds, mannitol, and triterpenic acids [6,9,10]. Recent studies highlight that hydroxytyrosol and mannitol can be recovered from olive pomace and EOP using water that favours the design of sustainable extraction strategies [9,11–13]. Steam treatment has also been applied to olive pomace to recover hydroxytyrosol and 3,4-dihydroxyphenylglycol [14]. Hydroxytyrosol and its derived natural extracts are particularly an attractive target to be obtained due to their applicability for food, feed, and packaging applications, taking advantage of their antioxidant and antimicrobial activity [3,12,15], and demonstrated benefits for health, e.g., anti-Alzheimer [16] and antifibrotic properties [15], and preventive potential against liver injury [15]. Therefore, to be fully aligned with the circular economy concept, the extraction of hydroxytyrosol from EOP represents a revenue opportunity before the combustion of

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this bioresource or its subsequent conversion to other bioproducts.

Several extraction strategies have been developed to recover hydroxytyrosol from EOP, e.g., solid–liquid extraction using conventional heating [12], ultrasound-assisted extraction [9], microwave-assisted extraction [3], and Soxhlet extraction [11]. The previous ones have been combined with water as an extraction agent to complies with "green chemistry" principles, offering fast and low thermal processes to recover hydroxytyrosol and avoiding the use of organic solvents. These extraction strategies and those based on low-energy steam treatments can be applied as first step in a cascading process to also valorise the solid fraction, which could be enriched in oil, triterpenic acids, polymeric sugars, and lignin, depending on the olive bioresource and the followed strategy [14,15]. Nonetheless, the composition of the extracts is very complex, including other compounds such as sugars, alditols, organic acids, and terpenoids (e.g., free secoiridoids), among others [11,17], which limits the purity of hydroxytyrosol in the extracts. This issue also occurs with extracts obtained from other olive-derived biowastes such as olive pomace, olive leaves, and olive mill wastewater. Hence, previous studies have applied several purification techniques for enrichment, e.g., membrane technology [18], liquid–liquid extraction (LLE) [19,20], adsorption [20–22], and combinations of them [23,24].

All these purification strategies might also be applied to the purification of hydroxytyrosol from EOP extracts. To the best of our knowledge, their application has not been explored to purify hydroxytyrosol from EOP. This work fills this research gap by evaluating for the first time the efficiency of membrane technology (ultrafiltration and nanofiltration), LLE, adsorbing materials, and resins as potential strategies to purify hydroxytyrosol from EOP. The performance of the purification has also been assessed by monitoring the content of glucose and mannitol, which have similar molecular weights to that of hydroxytyrosol. Moreover, the distribution of phenolic compounds and free secoiridoids (not linked to phenolic compounds) has been characterized by mass spectrometry to shed new light on the distribution of different olive-derived molecules using these purification methods. Finally, the *in vitro* antioxidant and antidiabetic potential of the purified fractions were evaluated.

2. Material and methods

2.1. EOP extraction

EOP was extracted by hydrothermal extraction under previously optimised conditions (85 °C, 90 min, 10% w/v solid to liquid ratio), according to Gómez-Cruz et al. [12]. The resulting aqueous extract contained: 4.5 g/L (or 110.1 ± 1.2 mg/g) of total phenolic compounds, 0.6 g/L (or 15.2 ± 0.2 mg/g) of hydroxytyrosol, 5.5 g/L of mannitol (or 135.1 ± 5.3 mg/g) and 4.8 g/L (or 111.8 ± 5.4 mg/g) of glucose.

2.2. Membrane separation technology

To remove large particles, the aqueous extract of EOP was firstly filtered using medium porosity filter paper, followed by a glass microfibre filter (1.2 μm pore size) and a cellulose nitrate filter (0.45 μm) (Fig. 1A) under vacuum filtration. Then, a 5 kDa Synder MT membrane (polyethersulfone) and a Synder NFX 150–300 Da membrane (polyamide composite film) (Sterlitech, Auburn, Washington, USA) were applied for ultra and nanofiltration, respectively, using a cross-flow filtration system, which consisted of a feed tank of 3 L capacity, a CF042A-FO membrane module of 42 cm^2 (Sterlitech, USA), a pump, a pressure control valve, and a manometer. In addition, a thermostatic bath (Memmert, Germany) to control the temperature of the separation process.

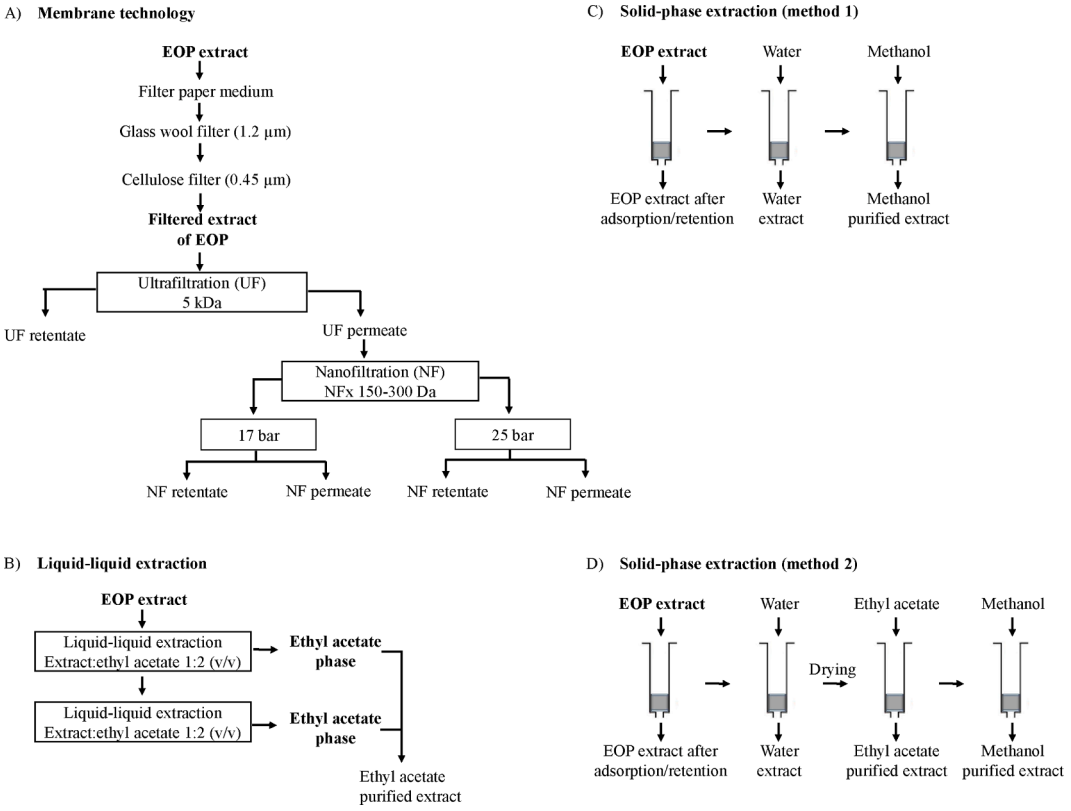

Fig. 1. Purification of exhausted olive pomace extract using membrane technology (A), liquid–liquid extraction (B), and solid-phase extraction using adsorbents and resins with method 1 (C), and method 2 (D).

The ultrafiltration and nanofiltration membranes were preconditioned using distilled water at different transmembrane pressures and all experiments were carried out at 22 °C. During the ultrafiltration process, the transmembrane pressure was set at 6 bar and the retentate was continuously recirculated to the feed tank for 6 h. The permeate stream obtained after ultrafiltration was subjected to nanofiltration. This process was carried out at transmembrane pressures of 17 and 25 bar for 4 h (Fig. 1A) Then, samples from the permeate and retentate were obtained, filtered (syringe filters of nylon; 0.45 μm pore size) (SinerLab Group, Madrid, Spain) and stored at −18 °C until analysis.

The apparent rejection (or retention) percentage of the studied compounds, which determines the selectivity of the process, was estimated as:

$$\text{Retention or rejection}\,(\%) = 1 - \frac{\text{Concentration in the permeate}\left(\frac{g}{L}\right)}{\text{Concentration in the initial extract}\left(\frac{g}{L}\right)} \times 100 \quad (1)$$

The recovery (%) of the compounds in the permeate stream was estimated as:

$$\text{Recovery}\,(\%) = \frac{\text{Content recovered in the permeate}\,(g)}{\text{Content in the initial extract}\,(g)} \times 100 \quad (2)$$

2.3. Liquid-liquid extraction

LLE was performed following the procedure described by Gullón et al. [25] with some modifications (Fig. 1B). The aqueous extract of EOP was mixed with ethyl acetate (Sigma Aldrich, St. Louis, MO, USA) in a ratio of 1:2 (v/v) and two sequential extraction steps were carried out. The mixture was stirred at room temperature on a magnetic stirrer (Boeco MSH3N, Hamburg, Germany) at 1250 rpm for 15 min. Subsequently, the two resulting phases were separated by decantation. The ethyl acetate extract was evaporated in a rotary evaporator (Buchi R-210, Thermo Fischer Scientific, Pittsburg, USA) for solvent recovery. The resulting solid was then redissolved in methanol:water (50:50, v/v), filtered (nylon; 0.45 μm pore size), and a portion stored as commented in Section 2.2 for further analysis. Another part was dried in an oven at 105 °C to estimate the dry weight.

2.4. Purification with adsorbent materials and resins

The materials tested were C18 (Teknokroma Analytica, Barcelona, Spain), C8 (DSC-8) (Sigma-Aldrich, St. Louis, MO, USA), HLB (Sigma-Aldrich), Microionex MB 200 (Rohm and Hass, Madrid, Spain), XAD7HP (Sigma-Aldrich), XAD16N (Sigma-Aldrich), Macronet MN202 (Purolite, Brasov, Romania), and Purosorb PAD910 (Purolite). Their characteristics are shown in Table S1. Each material was added to a solid-phase extraction cartridge (SPE) (1 g) and conditioned by passing 15 mL of methanol and 30 mL of acidified water (pH ~ 4). Firstly, 5.5 mL of the extract sample was passed. Secondly, a first elution was carried out with 5.5 mL of water and subsequently with 11 mL of methanol (method 1) (Fig. 1C). In a second method (method 2), a fractionation strategy was carried out following the SPE method described by Monagas et al. [26], with some modifications. After elution with distilled water, the cartridge was dried with nitrogen and 11 mL of ethyl acetate was used as the second eluent. The last elution was carried out with methanol (11 mL) (Fig. 1D). The ethyl acetate was evaporated at a rotary evaporator (Buchi R-210) and the solid fraction was reconstituted in 11 mL of aqueous methanol (50%, v/v) solution.

SPE was performed in triplicate with each material and the obtained fractions were filtered (nylon; 0.45 μm pore size) previous to analysis. A portion of each fraction was stored as described in Section 2.2 and the other portion was oven-dried.

The recovery of compounds (%), as a performance measure, was estimated as:

$$\text{Recovery} = \frac{\text{Content in the extract obtained after elution}\,(g)}{\text{Content in the initial extract}\,(g)} \times 100 \quad (3)$$

In addition, after passing the initial EOP extract through each adsorbent material or resin, the adsorption (or retention) percentage was estimated according to Trikas and co-workers [27]:

$$\text{Adsorption/Retention}(\%) = 100 - \%\,\text{recovery} \quad (4)$$

This percentage refers to the amount of compound retained on the material when the sample passed through and before the elution steps.

2.5. Measurement of the total phenolic content and antioxidant activity

The total phenolic content (TPC) and the antioxidant activity were determined as described in Gómez-Cruz et al. [3]. In brief, the TPC was determined at 760 nm using the Folin-Ciocalteu colourimetric method. The ABTS, 2,2′-azino-di (3-ethylbenzothiazoline-6-sulfonic acid), and ferric reducing power (FRAP) tests were used to quantify the antioxidant activity at an absorbance of 593 nm and 734 nm, respectively. A Bio-Rad iMark™ microplate reader (Hercules, CA, USA) was applied to measure the absorbance. All the measurements were done in triplicate.

Gallic acid and Trolox were procured from Sigma-Aldrich and used as reference standards to express the TPC and the antioxidant activity, respectively. Therefore, results were expressed as gallic acid equivalents (GAE)/L or mg GAE/g for TPC and Trolox equivalents (TE)/L or mg TE/g for antioxidant activity.

2.6. Determination of hydroxytyrosol and phenolic profiling by high-performance liquid chromatography

The phenolic profile of the samples and the content of hydroxytyrosol were determined using a Shimadzu Prominence high-performance liquid chromatographer (Kyoto, Japan) with diode-array detection as described in Gómez-Cruz et al. [9]. A C18 reverse phase column (BDS HYPERSIL 5 μm; 250 mm × 4.6 mm) was applied (Thermo Fisher Scientific Inc., Waltham, MA, USA) and a ternary solvent gradient consisting of 0.2% orthophosphoric acid in water, methanol and acetonitrile. The flow was 1 mL/min, oven temperature 30 °C, and injected sample volume 20 μL according to Gómez-Cruz et al. [9]. The quantification of hydroxytyrosol was performed by comparison with its commercial standard (Extrasynthese, Genay, France) at 280 nm. The phenolic profile was also characterised using high-performance liquid chromatography (HPLC) coupled to ion trap (IT) and quadrupole-time-of-flight (QTOF) mass spectrometry (MS) as described in Contreras et al. [17].

The glucose and mannitol content of the samples was determined by HPLC using a 1260 series equipment (Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID), according to Gómez-Cruz et al. [9]. The quantitative determination was also carried out by the external standard method and the standards were obtained from Sigma-Aldrich. The results were expressed as g/L or mg/g extract.

2.7. Inhibitory activity against α -glucosidase and α -amylase

To evaluate the antidiabetic potential of the purified fractions, two BioVision (Milpitas, CA, USA) enzymatic kits were used: α-glucosidase kit (catalogue n° K938-100) and α-amylase kit (catalogue n° K482-100) to test the inhibitory effect of the crude and purified extracts. Acarbose was used as an inhibitor control for both kits.

For α-glucosidase inhibition, 10 μL of liquid concentrated sample, inhibitor control or buffer was mixed with 10 μL of diluted α-glucosidase enzyme solution and 60 μL of buffer. This mixture was then incubated for 20 min at 25 °C protected from light. Finally, 20 μL of the substrate mixture was added and the absorbance was measured at 410 nm after 30 min incubation. For α -amylase, 50 μL of the sample, inhibitor control or buffer was mixed with 50 μL of diluted α-amylase enzyme solution and then incubated (10 min, 25 °C). Finally, 50 μL of substrate mixture was added and the absorbance was measured at 405 nm after 15 min incubation. Blanks of solvents (without enzyme and sample) and sample blanks (with sample but without enzyme) were also carried out. The relative inhibition of both enzymes was calculated using the following equation:

$$\%\,\text{Relative Inhibition} = \frac{(\text{Abs}_c - \text{Abs}_b) - (\text{Abs}_s - \text{Abs}_{sb})}{(\text{Abs}_c - \text{Abs}_b)} \bullet 100 \quad (5)$$

Where Abs_c, Abs_b, Abs_s, and Abs_{sb} are the absorbance of the enzyme control (without inhibitor), the blank (without inhibitor and enzyme), the sample (or acarbose for the control), and the sample blank (with sample and without enzyme).

The IC_{50} was estimated at 50% of inhibition by plotting the % relative inhibition *vs* concentration and through non-linear regression.

2.8. Statistical analysis

For two data samples comparison, F-test and *t*-test were performed to compared the standard deviations with Statgraphics Centurion 18 (Statgraphics Technologies, The Plains, VA, USA). For comparison of all groups, analysis of variance (ANOVA) was performed with T3-Dunnett comparison when variances were different using IBM SPSS Statistics 27 (IBM Corp, Armonk, NY, USA). In this case, a robust test (Welch) was also applied by Stagraphics Centurion 18. P-value of 0.05 was considered as significance level. Correlation analyses were carried out by Microsoft Excel 2010 (Redmon, WA, USA).

3. Results and discussion

3.1. Separation of phenolic compounds from glucose and mannitol by membrane technology

After the ultrafiltration process, the retentate stream was slightly concentrated in terms of TPC and so there was a partial separation from glucose and mannitol, which passed to a relatively greater extent to the permeate stream. Table S2 shows the preliminary separation performance obtained by membrane technology for the studied compounds: total phenolic compounds, hydroxytyrosol, glucose, and mannitol. That is, the membrane only retained 12.6% glucose, while the rejection of phenolic compounds and mannitol was 38.9% and 23.1%, respectively. Alternatively, hydroxytyrosol had similar behaviour to glucose, presenting a rejection percentage of 8.9%. On the contrary, the antioxidant activity measured in the rejected solution by both methods was similar to that of TPC. This means that besides hydroxytyrosol, other phenolic compounds present in the extract also contributed to this property.

The recovery of hydroxytyrosol in the permeate was 40.1%, similar to that of glucose (38.5%) and slightly higher than that of mannitol (33.9%. This suggests that the separation performance by the conditions applied was not at all efficient.

Khemakhem and co-workers [28] reported a similar rejection of TPC (37.3%) after 5 kDa ultrafiltration of olive leaves extracts. Nevertheless, the percentage of rejection depends on the type of phenolic compound, which would explain the different values obtained for TPC and hydroxytyrosol. For example, the number of benzene rings present in the molecule has an impact on the separation and interaction with the ultrafiltration membrane [29]. Another factor affecting rejection is when a cake layer is formed during the filtration that decreases permeate flux and may change selectivity [30,31]. Potential foulants are suspended solids, colloids, and dissolved organic matter [32]. It seems that both phenolic compounds could also be adsorbed by the cake layer and on the membrane structure itself [30].

In other studies, ultrafiltration has been applied followed by nanofiltration to enhance the enrichment of phenolic compounds in the extract [28,33]. Therefore, the integration of ultrafiltration and nanofiltration was also applied in this work, and similar results in the retentates and permeates at both transmembrane pressures, 17 bars and 25 bars were observed (Table S2). As for ultrafiltration, the rejection percentages depended on the studied compound type, varying between 0% (hydroxytyrosol) and 25.9% (TPC). According to other studies hydroxytyrosol and tyrosol show low rejection values, less than 25%, using nanofiltration and membranes made of polyamide, which facilitated their recovery in the permeate [33,34]. In this case, the rejection percentage of phenolic compounds mainly depends on their molecular size with rejection values in nanofiltration membranes from less than 10% to 100% when the molecular weight is increased from 125–200 Da to 1000 Da [34,35].

In general, lower recoveries were found in the nanofiltration permeates compared to that obtained after ultrafiltration (Table S2). This type of membrane is likely to be fouled, conducting a drastic flow reduction, as other authors have observed when using this type of complex samples [28,34]. As an example, Fig. S1 shows the flow rate during operation time for ultrafiltration and nanofiltration experiments showing the drop flow under the conditions tested. Sample concentration in the membrane module may occur [36]. Fouling also affects the rejection values of phenolic compounds and glucose [36,37]. Further optimization is so needed to increase the recovery rates since pressure-driven membrane technology has advantages to promote sustainable processes, such as low energy consumption and ease of implementation at industrial scale [29].

3.2. Retention and recovery of phenolic compounds by liquid–liquid extraction and solid-phase extraction

Alternative purification methods to membrane technology have been applied to purify phenolic compounds from olive pomace, olive leaves, and olive mill wastewater [20,38], including the use of LLE and adsorbents and resins. In this work, the performance of these technologies on the components of EOP extracts has been evaluated.

In the case of LLE, 28.2% of the phenolic compounds and 88.8% of the hydroxytyrosol initially present in the EOP extract were recovered in the ethyl acetate phase (Table 1). This indicates that this purification method is highly selective for hydroxytyrosol. Gullón and co-workers [25] also observed different recovery yields depending on the phenolic type when ethyl acetate was used to purify vine shoots extracts. Allouche et al. [39] used this type of extraction with a three-stage continuous countercurrent system to recover hydroxytyrosol from oil

Table 1
Retention characteristics and recovery values for the total phenolic content and hydroxytyrosol by liquid–liquid extraction (LLE) and solid-phase extraction (SPE).

Purification type		Recovery by eluents				
		Adsorption/Retention (%)[1]	Water (%)	Ethyl acetate (%)[2]	Methanol (%)	Ethyl acetate + methanol (%)[3]
		Total phenolic compounds				
LLE		–	–	28.18 ± 0.59	–	–
SPE						
C18	Method 1	79.99 ± 0.59	5.14 ± 0.23	-	45.38 ± 1.11	–
	Method 2			24.90 ± 0.35	17.36 ± 1.36	42.27 ± 1.57
DSC-8	Method 1	73.07 ± 0.88	8.60 ± 1.21	–	49.13 ± 2.89	–
	Method 2			25.51 ± 0.65	13.64 ± 1.20	39.15 ± 1.85
HLB	Method 1	84.55 ± 0.99	10.35 ± 0.50	–	49.77 ± 0.77	–
	Method 2			11.93 ± 0.63	22.30 ± 0.47	34.22 ± 1.06
Microionex MB 200	Method 1	94.80 ± 0.33	4.58 ± 0.08	–	12.48 ± 0.17	–
	Method 2			4.04 ± 0.05	7.47 ± 0.42	11.51 ± 0.46
Amberlite XAD7HP	Method 1	52.04 ± 1.72	6.73 ± 0.16	–	30.53 ± 1.01	–
	Method 2			19.50 ± 0.50	7.20 ± 1.24	26.70 ± 1.70
Amberlite XAD16N	Method 1	45.50 ± 3.68	2.32 ± 0.28	–	18.69 ± 1.30	–
	Method 2			9.49 ± 1.72	10.61 ± 0.40	19.62 ± 1.72
Macronet MN202	Method 1	47.54 ± 5.55	1.80 ± 0.37	–	32.75 ± 2.47	–
	Method 2			17.16 ± 1.44	11.70 ± 3.76	28.85 ± 5.19
Purosorb PAD910	Method 1	53.49 ± 2.92	4.52 ± 1.11	–	26.18 ± 2.34	–
	Method 2			18.01 ± 1.11	8.75 ± 2.02	26.75 ± 2.75
	Hydroxytyrosol					
Liquid-liquid extraction		–	–	88.83 ± 0.41	–	–
SPE						
C18	Method 1	97.17 ± 0.06	11.72 ± 0.93	–	46.69 ± 0.65	–
	Method 2			41.85 ± 1.78	0.79 ± 0.04	42.64 ± 1.76
DSC-8	Method 1	94.29 ± 0.08	16.56 ± 0.36	–	27.45 ± 0.84	–
	Method 2			30.85 ± 2.22	0.69 ± 0.05	31.54 ± 2.27
HLB	Method 1	96.36 ± 0.56	5.50 ± 0.44	–	33.09 ± 1.76	–
	Method 2			18.42 ± 0.32	2.80 ± 0.49	21.22 ± 0.53
Microionex MB 200	Method 1	99.42 ± 0.04	6.43 ± 1.49	–	18.39 ± 1.34	–
	Method 2			7.66 ± 0.15	8.73 ± 0.35	16.39 ± 0.49
Amberlite XAD7HP	Method 1	62.74 ± 2.59	9.67 ± 1.08	–	14.39 ± 1.41	–
	Method 2			14.26 ± 1.02	1.38 ± 0.21	16.21 ± 0.64
Amberlite XAD16N	Method 1	52.32 ± 5.16	5.56 ± 0.07	–	15.20 ± 0.88	–
	Method 2			12.13 ± 1.33	3.83 ± 0.86	16.71 ± 1.28
Macronet MN202	Method 1	66.76 ± 3.90	3.49 ± 0.17	–	22.45 ± 1.03	–
	Method 2			17.80 ± 1.65	5.28 ± 0.31	23.07 ± 1.95
Purosorb PAD910	Method 1	53.71 ± 2.86	8.65 ± 1.77	–	15.49 ± 0.19	–
	Method 2			15.41 ± 0.32	2.47 ± 0.41	18.06 ± 0.47

[1] Percentage of compounds retained/adsorbed in the material: equation (4).
[2] Eluent in the case of SPE or extraction agent in the case of LLE.
[3] Overall recovery in the SPE method 2 being the sum of the recovery values obtained by ethyl acetate and methanol.

mill wastewater, achieving a recovery of 85%, agreeing with the present study. In addition, the purified extract obtained had a purity (per gram of extract) of 273 mg of phenolic compounds and 118 mg of hydroxytyrosol; about 2 and 8 times higher than in the original aqueous extract, respectively (Fig. 2A and 2B, respectively). It is noteworthy that the LLE of EOP extract achieved a purity similar to that obtained from an olive pomace extract using a more complicated purification approach (115 mg/g), i.e. membrane separation (microfiltration, ultrafiltration and then nanofiltration) followed by reverse osmosis and chromatography [23].

The use of adsorbents and resins for the purification of active molecules is gaining attention due to its low cost and easy regeneration [21]. In this work, eight materials with different physicochemical properties and active sites (Table S1) were tested using SPE. The retention and desorption behaviour shown by these eight materials was different (Table 1). Thus, a low quantity of phenolic compounds was found in the sample after passing through the resin Microionex MB 200 compared to the rest of materials applied. This means that this material showed a high retention ability for this type of compounds with significant differences with the rest of materials at $p < 0.05$ for TPC and hydroxytyrosol (ANOVA, T3 Dunnett); with the exception of the comparison with HLB for hydroxytyrosol. This resin yielded the highest retention values, and around 94.8% and 99.4% of the initial TPC and hydroxytyrosol content were retained, respectively. This suggests that the interactions between phenolic compounds in the extract and this ionic resin, which has anionic and cationic groups, were stronger than those formed with the rest of the materials. It also provided low recovery values (less than 20%) by passing the eluents used in this study. In this regard, the combination of ionic resins, specifically, as the mixture of anionic exchange resin with at least 2% cationic resulted in great retention specificity for hydroxytyrosol and other simple phenols like 3,4 dihydroxyphenylglycol [40].

C18, C8, and HLB also provided high retention properties, with percentages of adsorption greater than 70%, while the rest of the resins (non-ionic) presented intermediate adsorption values upon the conditions applied. The chemical properties of the adsorbent and resin, the diameter of the pores and its specific surface are key factors that influence its adsorption capacity [41]. The chemical properties determine the

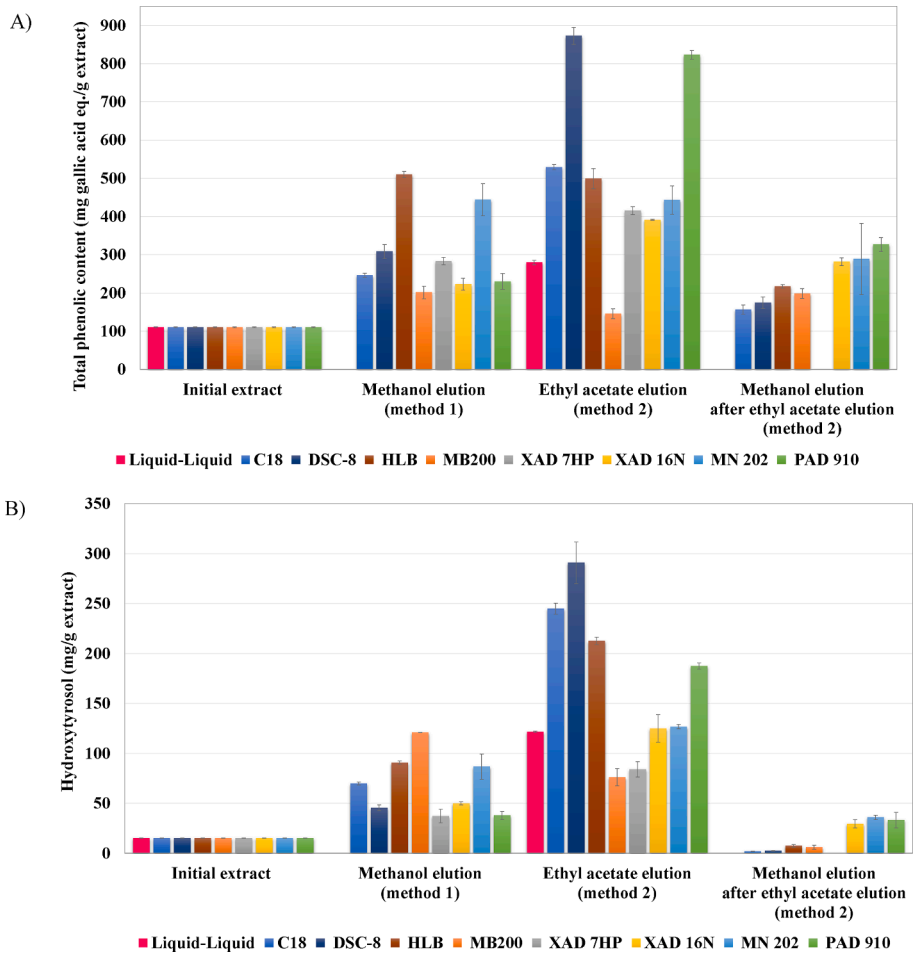

Fig. 2. Content of (A) total phenolic compounds and (B) hydroxytyrosol of the extracts purified by liquid–liquid extraction, adsorbents and resins.

type of interaction that occurs between the phenolic compounds and the material [27,42]. For example, in the case of the apolar adsorbents C18 and C8, adsorption is by strong non-polar interactions and hydrogen bonds can be established. These adsorbents promoted a higher fast adsorption capacity than the non-ionic resins, even though some of them presented a higher specific surface area and/or presented hydrophobic characteristics (Table S1). This behaviour has also been observed with phenolic compounds in other studies on grape pomace [27] and wheat straw [42].

The chemical composition of the raw extract, the type of phenolic compound (e.g., its polarity) and the conditions used (e.g., the contact time between the extract and the resin and the proportion extract/resin) also influence the retention/adsorption efficiency [41,43–45]. Adsorption percentages of phenolic compounds ~30%–100% have been described with the XAD 16 N resin for orange extracts [43], eucalyptus bark [41], and vegetable water [44].

After passing the extract through the adsorbent or resin, the phenolic compounds were recovered using water, and different organic solvents as eluents. The recovery values depended on the adsorbents and the eluent used in each case. In general, methanol (method 1) provided higher recovery values than ethyl acetate (method 2) (Table 1) for TPC, while for hydroxytyrosol it depended on the material. For example, for DSC-8 the recovery of hydroxytyrosol by elution with methanol and ethyl acetate elution was similar ($p = 0.068$, *t*-test), while for C18 was slightly higher in the case of methanol ($p = 0.011$, *t*-test). In general, the application of methanol as eluent resulted in purified extracts with lower TPC (Fig. 2A) and hydroxytyrosol content in most cases (Fig. 2B). That is, ethyl acetate was more selective for EOP phenolic compounds than methanol. Thus, a more purified extract was obtained with DSC-8 using ethyl acetate as eluent, with values of 873 mg/g for TPC (Fig. 2A) and 291 mg/g and hydroxytyrosol (Fig. 2B). Overall, in the ethyl acetate purified extracts, the TPC varied between 146 (Microionex MB 200) and 873 (DSC-8) mg/g and the hydroxytyrosol between 76.2 (Microionex MB 200) and 291 (DSC-8) mg/g.

It is also worth noting that methanol elution after ethyl acetate elution in method 2, meant a noticeable increase in recovered TPC, between 27% (Amberlite XAD7HP) and 65% (HLB). However, this second elution had less impact on hydroxytyrosol recovery, especially in C18 and DSC-8, where it only contributed to about 2% of the total recovery. This supports that ethyl acetate offers both selectivity and

Table 2
Retention characteristics and recovery values for the antioxidant activity by liquid–liquid extraction and solid-phase extraction.

Purification type		Recovery by eluents				
		Adsorption/Retention (%)[1]	Water(%)	Ethyl acetate (%)[2]	Methanol(%)	Ethyl acetate + methanol (%)[3]
		FRAP				
LLE		–	–	33.38 ± 1.07	–	–
SPE						
C18	Method 1	78.74 ± 0.89	6.62 ± 0.11	–	61.13 ± 0.88	–
	Method 2			28.81 ± 0.09	21.53 ± 1.28	50.34 ± 1.37
DSC-8	Method 1	70.01 ± 1.05	9.51 ± 0.41	–	50.67 ± 4.53	–
	Method 2			25.22 ± 0.75	15.55 ± 1.16	40.77 ± 1.48
HLB	Method 1	83.46 ± 0.50	10.27 ± 0.47	–	56.95 ± 2.12	–
	Method 2			14.26 ± 0.12	27.47 ± 0.98	41.73 ± 0.86
Microionex MB 200	Method 1	93.65 ± 0.19	4.88 ± 0.13	–	13.84 ± 0.19	–
	Method 2			4.78 ± 0.03	12.54 ± 0.24	17.32 ± 0.27
Amberlite XAD7HP	Method 1	39.94 ± 0.98	8.17 ± 0.53	–	24.52 ± 0.60	–
	Method 2			21.25 ± 1.49	7.22 ± 0.01	28.47 ± 1.50
Amberlite XAD16N	Method 1	29.49 ± 2.90	4.17 ± 0.71	–	23.77 ± 2.74	–
	Method 2			14.30 ± 1.99	11.02 ± 2.77	25.32 ± 2.86
Macronet MN202	Method 1	37.41 ± 4.63	3.12 ± 0.74	–	31.10 ± 2.39	–
	Method 2			15.50 ± 2.81	9.88 ± 2.78	25.38 ± 6.74
Purosorb PAD910	Method 1	52.38 ± 4.04	5.18 ± 1.05	–	28.76 ± 2.68	–
	Method 2			19.10 ± 1.57	10.85 ± 2.43	29.95 ± 3.10
		ABTS				
LLE		–	–	26.12 ± 1.15	–	–
SPE						
C18	Method 1	78.49 ± 0.94	3.11 ± 0.15	–	41.20 ± 0.54	–
	Method 2			26.45 ± 0.15	23.38 ± 0.83	49.83 ± 0.97
DSC-8	Method 1	64.85 ± 1.87	9.55 ± 2.62	–	56.86 ± 0.50	–
	Method 2			27.98 ± 0.26	24.14 ± 0.55	52.12 ± 0.62
HLB	Method 1	82.14 ± 2.51	10.89 ± 1.25	–	53.28 ± 2.21	–
	Method 2			14.82 ± 0.64	27.24 ± 3.16	42.06 ± 3.50
Microionex MB 200	Method 1	94.06 ± 0.60	3.77 ± 0.16	–	14.91 ± 1.43	–
	Method 2			12.70 ± 0.00	12.11 ± 0.51	24.81 ± 0.51
Amberlite XAD7HP	Method 1	33.40 ± 1.62	9.01 ± 0.73	–	22.65 ± 2.14	–
	Method 2			17.93 ± 0.99	6.47 ± 1.34	24.40 ± 0.43
Amberlite XAD16N	Method 1	30.38 ± 0.91	3.93 ± 0.35	–	25.57 ± 2.46	–
	Method 2			16.17 ± 0.14	13.95 ± 0.65	30.13 ± 0.78
Macronet MN202	Method 1	40.73 ± 1.58	4.44 ± 0.78	–	37.63 ± 2.58	
	Method 2			17.83 ± 0.97	14.94 ± 0.66	32.77 ± 1.63
Purosorb PAD910	Method 1	46.47 ± 6.92	4.08 ± 0.48	–	28.77 ± 6.75	–
	Method 2			20.85 ± 1.65	14.43 ± 1.77	35.28 ± 2.52

[1] Percentage of compounds retained/adsorbed in the material (equation (4).
[2] Eluent in the case of SPE or extraction agent in the case of LLE.
[3] Overall recovery in the SPE method 2 being the sum of the recovery values obtained by ethyl acetate and methanol.

affinity for hydroxytyrosol as an eluent.

Some of these materials have been used to purify hydroxytyrosol in other studies. For example, adsorption of 39% and a similar recovery (19%) were obtained for this compound when olive pomace extract was passed through Amberlite XAD 16 N using acidified ethanol–water (50:50, v/v) as eluent [22]. Extracts with 79 and 115 mg/g hydroxytyrosol were obtained using an ionic resin [20] and combining membrane separation and chromatography with a C18 type column [23], respectively. XAD type resins were used to obtain extracts up to 400 mg/g from olive leaves and vegetation water [46], while the combination of the use of a strongly anionic resin and XAD resins enabled to obtain high recovery (at least 75%) and purity (95%) of hydroxytyrosol from olive pomace treated by steam explosion [47]. Therefore, the results obtained in this work could be of interest to the industry since extracts enriched in phenolic compounds were obtained from EOP, in a fast manner and using a single elution step, especially with the materials C18, DSC-8, HLB, and Purosorb PAD910 (Fig. 2).

Regarding the antioxidant activity of the extracts, a similar pattern to that for TPC and hydroxytyrosol was observed in the adsorption through SPE and subsequent desorption with solvents (Table 1 and Table 2). In fact, there was a correlation between these percentages, especially, between the TPC and the antioxidant activity with ‘r values’ between 0.881 and 0.987 (Table S3). The fractions with the highest antioxidant activity were obtained with DSC-8 and PAD910 using ethyl acetate as eluent with FRAP about or closer to 900 mg Trolox equivalents/g and ABTS about or closer to 2000 mg Trolox equivalents/g, respectively (Fig. 3). Therefore, these purified extracts showed a higher antioxidant activity than that obtained by LLE, explained by the fact that the former extracts had a higher content of phenolic compounds, including hydroxytyrosol.

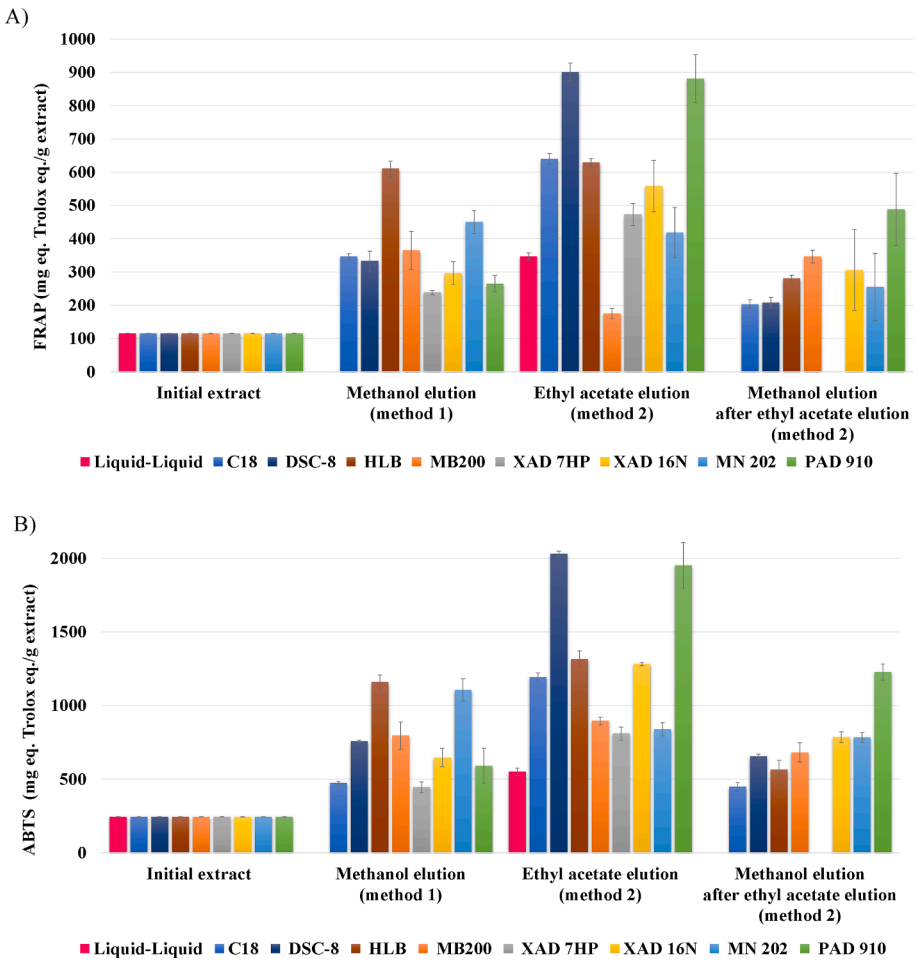

Fig. 3. Antioxidant activity of the extracts purified by adsorbents and resins determined by (A) FRAP and (B) ABTS.

3.3. Separation of phenolic compounds from glucose and mannitol by liquid–liquid extraction and solid-phase extraction

After LLE and SPE, glucose and mannitol remained mainly in the aqueous extract (Table 3). Thus, the organic extracts enriched in phenolic compounds showed a recovery of less than 4% of these compounds, indicating that the separation of hydroxytyrosol from these compounds was adequate.

In the former case, the results agreed well with the study by Gullón et al. [25]. These authors fractionated autohydrolysis liquors from vine shoots with ethyl acetate into an organic phase with phenolic compounds (and antioxidant activity) and an aqueous phase enriched in oligosaccharides. Khoufi and co-workers [48] showed a recovery efficiency of monomeric sugars higher than 90% in the aqueous phase after an LLE of olive mill wastewater with ethyl acetate.

Trikas et al. [27] purified extracts from grape pomace observing also certain adsorption of sugars on resins and HLB and C8-type materials. In all cases, when water was passed through the columns, most of these compounds were desorbed (Table 3). Further separation of glucose from mannitol will be interesting to achieve in future since this compound also presents many applications, including as a low-calorie sweetener and pharmaceutical applications [6,9].

3.4. Phenolic profile of purified extracts

According to the purity of the extracts obtained, SPE with C18, DSC-8, and PAD910, and LLE using ethyl acetate showed good performance for phenolic compounds. The chromatographic profiles of these purified liquid extracts at 280 nm showed that hydroxytyrosol was the main phenolic compound, but there were other phenolic compounds (Fig. S2 as an example).

As noted before, among other aspects, the performance of the adsorption/desorption process depends on the type of phenolic compound [43,49]. Nonetheless, the available literature is scarce on this aspect. To shed some light on the distribution of phenolic compounds in the extracts, the compounds were first characterized based on HPLC-IT-MS and HPLC-QTOF-MS according to their retention time and MS data by comparison to the ones in an in-house built library and literature [15,17]. Then, the purified extracts were analyzed by HPLC-IT-MS and Table 4 and Table S4 show the phenolic compounds found in the selected extracts obtained by ethyl acetate using LLE and SPE with C18, DSC-8, and PAD910. Most phenolic compounds in EOP were recovered, and DSC-8 and PAD910 showed more similar qualitative distribution of phenolic compounds (Table 4, Fig. S2, and Fig. S3). Moreover, some organic acids and secoiridoids not linked to phenolic compounds were characterized in EOP and purified extracts (Table S4). According to their MS chromatograms (Fig. S4), it was observed a reduction in the content of these compounds in the purified extracts compared to the raw EOP extract. This highlights again the good selectivity of the organic solvent.

3.5. Antidiabetic potential

The inhibition of α-glucosidase and α-amylase is considered a strategy for the treatment of diseases such as diabetes and obesity. Particularly, these enzymes are involved in the digestion of complex carbohydrates in humans [50]. Therefore, the inhibitory effect of

Table 3
Retention characteristics and recovery values for glucose and mannitol by liquid–liquid extraction (LLE) and solid-phase extraction (SPE).

Purification type		Recovery by eluents				
		Adsorption/Retention (%)[1]	Water (%)	Ethyl acetate(%)[2]	Methanol (%)	Ethyl acetate + methanol (%)[3]
		Glucose				
LLE		–	–	0.40 ± 0.10	–	–
SPE						
C18	Method 1	23.91 ± 1.13	11.84 ± 0.51	–	2.78 ± 0.40	–
	Method 2			1.23 ± 0.13	1.40 ± 0.15	2.63 ± 0.05
DSC-8	Method 1	18.38 ± 2.69	16.01 ± 0.72	–	1.40 ± 0.47	–
	Method 2			1.26 ± 0.23	1.10 ± 0.12	2.42 ± 0.18
HLB	Method 1	33.88 ± 0.80	30.27 ± 1.27	–	2.01 ± 0.10	–
	Method 2			2.67 ± 0.01	0.58 ± 0.02	3.25 ± 0.01
Microionex MB 200	Method 1	37.78 ± 1.72	12.22 ± 1.98	–	0.26 ± 0.02	
	Method 2			0.08 ± 0.01	0.17 ± 0.01	0.25 ± 0.02
Amberlite XAD7HP	Method 1	12.11 ± 3.23	10.27 ± 2.85	–	1.66 ± 0.24	–
	Method 2			1.07 ± 0.10	0.38 ± 0.05	1.24 ± 0.15
Amberlite XAD16N	Method 1	10.39 ± 0.86	6.82 ± 1.16	–	2.64 ± 0.19	–
	Method 2			1.30 ± 0.09	0.97 ± 0.09	2.27 ± 0.00
Macronet MN202	Method 1	13.38 ± 1.57	9.13 ± 1.70	–	2.99 ± 0.20	–
	Method 2			1.10 ± 0.40	1.28 ± 0.01	2.37 ± 0.40
Purosorb PAD910	Method 1	12.58 ± 1.04	10.20 ± 0.96	–	2.28 ± 0.04	–
	Method 2			1.26 ± 0.00	0.98 ± 0.10	2.24 ± 0.09
			Mannitol			
LLE		–	–	2.40 ± 0.80	–	–
SPE						
C18	Method 1	24.34 ± 2.44	13.21 ± 0.46	–	0.72 ± 0.06	–
	Method 2			0.37 ± 0.04	0.33 ± 0.02	0.70 ± 0.04
DSC-8	Method 1	32.04 ± 3.61	12.94 ± 1.06	–	1.11 ± 0.00	–
	Method 2			0.51 ± 0.01	0.59 ± 0.06	1.10 ± 0.07
HLB	Method 1	45.21 ± 4.71	26.13 ± 1.99	–	1.17 ± 0.11	–
	Method 2			0.34 ± 0.01	0.38 ± 0.01	0.72 ± 0.01
Microionex MB 200	Method 1	52.06 ± 1.40	12.93 ± 0.87	–	0.21 ± 0.02	–
	Method 2			0.03 ± 0.01	0.21 ± 0.05	0.30 ± 0.06
Amberlite XAD7HP	Method 1	35.12 ± 3.50	9.91 ± 2.27	–	1.12 ± 0.16	–
	Method 2			0.70 ± 0.09	0.11 ± 0.00	0.81 ± 0.09
Amberlite XAD16N	Method 1	33.41 ± 2.92	8.55 ± 0.78	–	1.63 ± 0.07	–
	Method 2			0.78 ± 0.04	0.21 ± 0.01	0.99 ± 0.03
Macronet MN202	Method 1	33.78 ± 7.64	10.88 ± 1.36	–	1.71 ± 0.29	–
	Method 2			0.77 ± 0.32	0.62 ± 0.32	1.39 ± 0.01
Purosorb PAD910	Method 1	33.57 ± 3.52	12.23 ± 1.41	–	1.26 ± 0.18	–
	Method 2			0.68 ± 0.01	0.54 ± 0.03	1.21 ± 0.04

[1] Percentage of compounds retained/adsorbed in the material (equation (4).
[2] Eluent in the case of SPE or extraction agent in the case of LLE.
[3] Overall recovery in the SPE method 2 being the sum of the recovery values obtained by ethyl acetate and methanol.

Table 4
Phenolic compounds identified in the purified extracts recovered by ethyl acetate using liquid–liquid extraction (LLE) and solid-phase extraction with C18, DSC-8, and PAD910.

no	Rt (min)	$[M-H]^-$ (*m/z*)	Suggested compound	LLE	C18	DSC-8	PAD910
1	1.0	315	Hydroxytyrosol glucoside	+	+	+	+
2	1.1	153	Hydroxytyrosol[1]	+	+	+	+
3	1.8	299	Tyrosol glucoside	+	+	+	+
4	3	461	Verbasoside	−	−	−	−
5	6.3	137	Hydroxybenzoic acid	+	−	−	−
6	6.5	195	Hydroxytyrosol acetate	−	−	+	+
7	9.3	483	Oleacein derivative (+hexose + H_2)	+	+	+	+
8	9.9	543	Dihydro oleuropein	+	+	+	+
9	10.1	623	Verbascoside	+	+	+	+
10	10.3	447	Luteolin 7-*O*-glucoside[1]	+	+	+	+
11	10.5	701	Oleuropein hexoside isomer 1	+	−	+	+
12	10.6	685	Nüzhenide	+	−	+	+
13	10.7	593	Luteolin *O*-deoxyhexoside *O*-hexoside	+	−	+	+
14	10.7	701	Oleuropein hexoside isomer 2	+	−	+	+
15	11.2	335	Hydroxy oleacein	+	+	+	+
16	10.9	623	Isoverbascoside	+	+	+	+
17	11.2	541	Oleuropein derivative (+H_2)	+	+	+	+
18	11.4	539	Oleouropein isomer 1	+	+	+	+
19	11.7	551	Caffeoyl-6′-secologanoside	+	+	+	+
20	11.8	539	Oleuropein[1]	+	+	+	+
21	12.5	539	Oleouropein isomer 2	+	+	+	+
22	12.9	539	Oleouropein isomer 3	+	+	+	+
23	13.0	319	Oleacein	+	+	+	+
24	13.1	535	*p*-Coumaroyl-6′-secologanoside	+	+	+	+
25	13.6	523	Ligustroside	+	+	+	+

[1] Compared with standards. +, presence; −, absence.

selected purified extracts (ethyl acetate extracts obtained by LLE, C18, DSC-8, and PAD910) on these enzymes was evaluated. The results suggest that the purified extracts showed the ability to inhibit both enzymes (Table S5) and it was correlated to the phenolic and hydroxytyrosol content with 'r values' of 0.756 and 0.670 for α-glucosidase, respectively, and 0.939 and 0.956 for α-amylase, respectively. According to Dekdouk et al. [51], hydroxytyrosol can inhibit these enzymes with comparable or higher efficiency than the natural inhibitor acarbose, which is used as an antidiabetic drug. This can explain these results at least in part, while other compounds present in EOP extract like oleuropein, verbascoside, and oleacein (or decarboxymethyl oleuropein aglycone or 3,4-DHPEA-EDA) can also contribute to the inhibition of these enzymes [50,51].

Moreover, to investigate the IC_{50} of EOP phenolic compounds, SPE using DSC-8 was selected since it provided a medium recovery for these compounds (including hydroxytyrosol) and the richest dry extract (as a summary, see Fig. S5). The values of IC_{50} determined were 3.4 ± 0.2 mg extract/mL for α-glucosidase and 3.0 ± 0.1 mg extract/mL for α-amylase, similar to that of olive leaves phenolic compounds for α-amylase (IC_{50} = 4 mg/mL) [52].

3.6. Potential application of EOP considering the extraction and purification of hydroxytyrosol

By integrating extraction with biomass utilization in biorefineries, it becomes possible to recover maximum value from biomass resources. The extraction of bioactive compounds from EOP and their further purification can be applied as a first step in the cascading process. As an example, Fig. 4 shows a potential valorisation scheme for EOP and the bioproducts obtained considering this work and previous studies [6,9,12]. This cascading process entails the obtainment of hydroxytyrosol-rich extract by aqueous extraction studies [12], while the extracted solid can be further exploited to produce triterpenic acids by ethanolic extraction [9], antioxidant lignin and reducing sugars by organosolv pretreatment and enzymatic hydrolysis with carbohydrases [6]. In the present work, the results suggest that the extract can be further purified from the aqueous EOP extract, allowing for its further utilization and incorporation into various products or applications. Besides the antioxidant properties of the purified extract, these results suggest a potential application to promote functional ingredients with antidiabetic properties. Concretely, the use of purified extracts, which will be required in a lower amount than the raw extract to exhibit bioactivity, and reducing the sensory impact that could impair the functionalized product, e.g. the colour (Fig. S2). For purification and to estimate the mass balance in Fig. 4, DSC-8 material has been selected owing the results obtained in terms of recovery of hydroxytyrosol (~31%) and purity (~29%).

Nonetheless, there are several significant points in the purification methods, especially, using resins, that can be explored and optimized based on increasing the recovery without compromising purity, solvent recovery and recycling, mass and energy integration for scaling-up, as well as on the reutilization of the absorbents that can yield significant savings and improvement in terms of sustainability.

4. Conclusions

Extracts rich in hydroxytyrosol obtained from EOP can be purified with good performance by LLE and by SPE. LLE provided the highest recovery of hydroxytyrosol in the ethyl acetate phase, while the highest recovery of phenolic compounds was obtained by SPE using C18, DSC-8, and HLB as adsorbents, and methanol as eluent. In these cases, both glucose and mannitol were mainly separated in the aqueous phase.

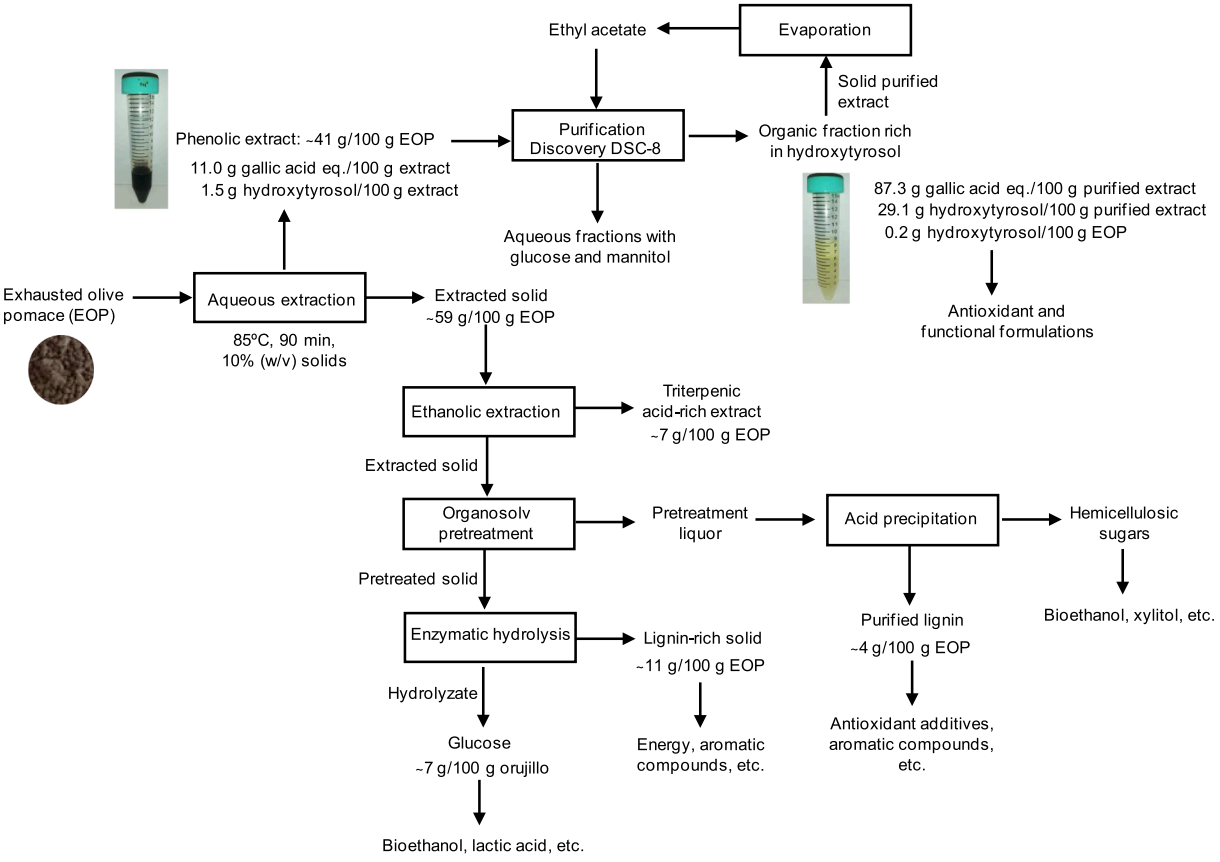

Fig. 4. Cascading process to valorise exhausted olive pomace considering the integration of extraction and purification of hydroxytyrosol, mass balance, and applications of the bioproducts.

Overall, DSC-8 and Purosorb PAD910 provided the richest extracts in phenolic compounds (closer to 90%), including hydroxytyrosol (up to 30%), using ethyl acetate as eluent, which was correlated with higher antioxidant activity. These results could be relevant to food, phyto-pharmaceutical and cosmetic industries looking for natural antioxidants-rich extract. This study is also applicable in analytical research if it is necessary to concentrate these compounds for detection. For industrial applications, future studies should be performed considering the potential improvement of the recoveries using these materials, especially resins, by testing other conditions (elution volume and solvent, etc.), while techno-economic and environmental assessment could contribute to figuring out the best scenario considering this work and future development on the purification of hydroxytyrosol from EOP.

CRediT authorship contribution statement

Irene Gómez-Cruz: Data curation, Writing – original draft, Software, Visualization, Investigation, Writing – review & editing. **María del Mar Contreras:** Data curation, Writing – original draft, Software, Visualization, Conceptualization, Methodology, Supervision, Writing – review & editing. **Inmaculada Romero:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Belina Ribeiro:** Writing – review & editing. **Luisa B. Roseiro:** Writing – review & editing. **Luís C. Duarte:** Supervision, Writing – review & editing. **Florbela Carvalheiro:** Supervision, Writing – review & editing. **Eulogio Castro:** Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2023.124664.

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