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ARTICLE

Development of a Phenol-Enriched Olive Oil with Phenolic Compounds from Olive Cake

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The recent information regarding the healthy properties of virgin olive oil phenols and the interest in increasing the value of byproducts from the oil extraction processs support the standardized development of phenol-enriched olive oil. Accordingly, the aim of this research work was to evaluate strategies for the development of a virgin olive oil enriched with phenolic compounds obtained from olive cake to increase phenolic ingestion without the drawback of a higher calorie intake. For this proposal, different combinations of phenolic extracts were evaluated at a range of concentrations to obtain the best prototype of enriched olive oil. To study the functionality of the phenol enrichments, the total phenolic content and the oxidative stability were determined by the Folin−Ciocalteu and Rancimat tests, respectively. In addition, the phenolic composition and antioxidant capacity (ORAC assay) of the oils were studied. Finally, the stability and potential bioaccesibility of the phenolic fraction of the enriched oils were tested by an in vitro gastrointestinal digestion model. Results of the study showed different strategies to select the best prototype of enriched olive oil, taking into consideration not only their phenolic content but also other important factors such as the feasibility of implementing the preparation process in the food industry.

KEYWORDS: Phenol-enriched olive oil; phenols; accelerated solvent extraction; ORAC; in vitro digestion

INTRODUCTION

Virgin olive oil, the main lipid source in the diet of the Mediterranean countries, has been related to the lower incidence of coronary heart diseases (CHD) and some types of cancer in this area (*1*). Among other factors, these healthy properties have been related to the fatty acid composition of olive oil, which is characterized by a high percentage of monounsaturated fatty acids. However, more recent studies have pointed to the importance of the phenolic fraction of virgin olive oil (*2*). An increase in HDL cholesterol after the intake of high phenolic content olive oil versus one with a low phenolic content has been reported (*3*, *4*). A reduction in oxidative damage and an improvement in endothelial function proportional to the phenolic content of the olive oil have also been observed (*5*). Furthermore, the bioavailability of these compounds reinforces their in vivo protective role in humans (*6*).

A wide range of phenolic compounds have been identified in virgin olive oil, including phenolic alcohols, secoiridoid derivatives, phenolic acids, lignans, and flavonoids (*7*). Despite the great variety of these compounds, only around 2% of the phenols from the olive fruit are transferred to the virgin olive oil during the extraction process. The other 98% are retained in the olive cake (*8*). This byproduct (also call wet pomace or *alperujo*) is the most important waste generated in the virgin olive oil extraction process by the two-phase centrifugation system. Olive cake has become a serious environmental problem due to its pollutant nature and high level of production (approximately 4.2 million tonnes generated annually in Spain during the 2004−2009 period) (*9*). To avoid this problem, some alternatives have recently been studied, from use as a fertilizer to potential for energy recovery (*10*).

Besides these applications, the use of olive cake as a natural source of phenolic compounds has recently been considered, and some studies have focused on the development of new extraction methods (*11*, *12*). The analysis of these phenolic extracts has demonstrated their high antioxidant activity and suggested their potential use as additives for the food industry (*13*). Thus, some experiments have been carried out to study the incorporation of phenolic extracts in real food matrices. Specifically, related to olive byproduct, Japón-Luján and Luque de Castro (*14*) studied the enrichment of edible oils with a phenolic extract obtained from olive leaves and concluded that its use considerably enhanced their concentration of phenolic compounds. Bouaziz et al. (*15*) obtained similar results after adding olive leaf extract to husk olive oils, demonstrating the great potential of olive byproduct extracts as antioxidants for the food industry.

The recent information supporting the healthy properties of virgin olive oil phenols and the interest in increasing the value of byproducts from the oil extraction process make the standardized development of phenol-enriched olive oils of interest. Accordingly, the aim of this research work was to evaluate strategies for the development of a virgin olive oil enriched with phenolic compounds obtained from olive cake to increase phenolic

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ingestion without the drawback of a higher calorie intake. For this proposal, different combinations of phenolic extracts were evaluated at a range of concentrations to obtain the best prototype of enriched olive oil. To study the functionality of the phenol enrichments, the total phenolic content and the oxidative stability were determined by the Folin−Ciocalteu and Rancimat tests, respectively. In addition, the phenolic composition and antioxidant capacity (ORAC assay) of the phenol-enriched oils were studied. Finally, the stability and potential bioaccesibility of the phenolic fraction of the enriched oils were tested by an in vitro gastrointestinal digestion model.

MATERIALS AND METHODS

Samples. Virgin olive oils used as matrix to carry out phenolic enrichments were from the olive-growing area of Les Garrigues (Lleida, Catalonia, Spain) and obtained by a two-phase continuous system. On the other hand, the samples of olive cake that were used to obtain the phenolic extract were taken from a commercial olive mill from the same area. These samples were taken at the decanter outlet, and liquid nitrogen was immediately added to avoid oxidative damage. The samples were then stored at −40 °C until the preparation of the phenolic extracts.

Chemicals and Reagents. Apigenin, apigenin-7-*O*-glucoside, luteolin, luteolin 7-*O*-glucoside, oleuropein, rutin, tyrosol, verbascoside and vanillin were purchased from Extrasynthese (Genay, France). Hydroxytyrosol was purchased from Seprox Biotech, S.L. (Madrid, Spain). Caffeic, *p*-coumaric, and vanillic acids and fluorescein were purchased from Fluka Co. (Buchs, Switzerland), and (+)-pinoresinol was acquired from ArboNova (Turku, Finland). The dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), the dialdehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EDA), and the lignan acetoxypinoresinol were not available commercially and were isolated from virgin olive oil by semipreparative HPLC (*16*). Methyl-*β*-cyclodextrin (RMCD) was from Aldrich (Steinheim, Germany), and 2,2′-azobis(2-amidino-propano) dihydrochloride (AAPH) and Trolox were from Acros Organics (Geel, Belgium).

Acetonitrile (HPLC grade), methanol (HLPC grade), ethanol, *n*-hexane, ethyl acetate, cyclohexane and acetic acid were all provided by Scharlau Chemie (Barcelona, Spain). Water was of Milli-Q quality (Millipore Corp, Bedford, MA).

Preparation of Phenolic Extracts. As is well-known, olive cake is a semisolid residue with a high percentage of water (approximately 60%), which makes it very difficult to handle. Basically, it is made up of the solid residues from the olives and the vegetative water (which includes the water naturally contained in the olives plus all of the water added during the olive oil extraction process). To maximize the recovery of the phenolic compounds, we studied different extraction processes, focusing on these two components of the olive cake. All of the solvents used were chosen in line with European Directive 2009/32/CEE published on April 23, 2009, which defines the list of extraction solvents that can be used in the production of food products and their ingredients.

Phenolic Extract from Vegetative Water (VW Extract). An extraction of the phenolic compounds contained in the vegetative water (VW) of the olive cake was carried out following the method developed by Visioli et al. (*12*) and further modified by Suárez et al. (*17*). The olive cake sample (135 g) was centrifuged at 21600*g* at 15 °C for 10 min to separate the liquid fraction (the VW) from the solid residue (SR). The SR was discarded and the extraction continued with the VW. Then, 100 mL of VW was transferred to a separatory funnel and cleaned three times with 15 mL of *n*-hexane to eliminate the lipidic substances. After that, the phenolic compounds were removed by mixing three times with 25 mL of ethyl acetate and concentrated in a rotary vacuum evaporator at 30 °C to dryness. Finally, the residue was dissolved in water, freeze-dried in a Lyobeta (Telstar, Terrassa, Spain), and stored at −80 °C under N_2 atmosphere (≥99.99% purity, Alphagaz, Madrid, Spain) until its use for oil enrichment.

Phenolic Extracts from Solid Residue (SR Extract). The extraction of phenolic compounds from the SR discarded in the previous step was carried out with and without purification. In the case of extraction from solid residue plus purification (SRp extract), we followed the method described by Suárez et al. (*17*) with some modifications. The SR was soaked for 15 min in an ultrasonic bath with 150 mL of ethanol/water (80:20, v/v). After that, the mixture was centrifuged at 21600*g* and 4 °C for 10 min, and the ethanolic extract was filtered through glass wool. After adjustment of the pH to 2 with 6 M HCl, the sample was concentrated in a rotary vacuum evaporator at 30 °C. The ethanolic extract was then purified by washing three times with 50 mL of ethyl acetate. Finally, it was evaporated to dryness at 30 °C in a rotary evaporator, dissolved in water, freeze-dried, and stored at −80 °C under N_2 atmosphere until its use in the oil enrichment.

In contrast, in the extraction of phenolic compounds from the SR without purification step (nonpurified solid residue extract, SRnp extract) we avoided the use of ethyl acetate, which conferred an unpleasant taste to the enriched oil. The same procedure as in SRp was followed until the pH was adjusted to 2. Then, the ethanolic extract was evaporated to dryness, dissolved in water, freeze-dried, and stored at −80 °C in N_2 atmosphere until its use for oil enrichment.

Phenolic Extract from Freeze-Dried Olive Cake (ASE Extract). To simplify the extraction process, a third method was considered. The olive cake was freeze-dried, and phenolic compounds were then extracted using an accelerated solvent extractor (ASE 100) (Dionex, Sunnyvale, CA). This equipment allows faster extractions by using solvents at high temperature and pressure. Ethanol/water (80:20, v/v) at 80 °C was used as the extraction solvent. To carry out the extractions, 5 g of diatomaceous earth was mixed with 10 g of freeze-dried olive cake to increase the contact surface, avoid the blockage of the cell, and improve the extraction of selected compounds. A 100 mL extraction cell was used, setting the flush volume at 60%. Two static cycles of 5 min were programmed in each extraction. After that, the sample was purged with nitrogen. The resulting extract was rotary evaporated until all of the ethanol had been eliminated, and then it was freeze-dried and stored at −80 °C until its use for oil enrichment.

Preparation of Phenol-Enriched Olive Oils. Virgin olive oil (control oil) was used as matrix enrichment adding either one or a combination of the olive cake extracts (VW, SR, and ASE extracts) at different quantities to reach the optimal phenol concentration. Thus, three different phenol-enriched oil prototypes (A, B, and C) were prepared. The extracts were previously dissolved in ethanol/water (50:50, v/v) and incorporated into the virgin olive oil (control oil) using a Polytron (Kinematica, Littau, Switzerland) for 1 min to allow full homogenization into the lipid matrix.

Total Phenol Content Determination of the Olive Oils. The total phenolic content of the control and phenol-enriched olive oils was determined spectrophotometrically at 725 nm following the Folin−Ciocalteu method described by Vázquez Roncero et al. (*18*). To remove the phenolic compounds from the olive oil, a triple extraction of an oil-in-hexane solution with methanol/water (60:40, v/v) was carried out. The results are expressed as milligrams of caffeic acid equivalents per kilogram of oil using the calibration curve of caffeic acid.

Phenol Extraction of the Olive Oils. The phenolic compounds of the olive oils were extracted following the method described in our previous paper (*19*). Briefly, 20 mL of methanol/water (80:20, v/v) was added to 45 g of oil and homogenized for 2 min with a Polytron. After that, the two phases were separated by centrifuging at 640*g* for 10 min, and the hydroalcoholic phase was evaporated to obtain a syrupy consistency at 31 °C and purified by liquid−liquid extraction with acetonitrile. The acetonitrile solution was finally rotary evaporated to dryness and then dissolved in 5 mL of methanol and maintained at −40 °C before chromatographic analysis.

Oxidative Stability of the Olive Oils by Rancimat Test. The oxidative stability of the olive oils was evaluated by the Rancimat test (Metrohom, Herisau, Switzerland) using an air flow of 20 L/h and a temperature of 120 °C to oxidize the samples (2.5 g of olive oil) (ISO 6886:1996). Changes in conductivity were measured continuously. The peroxidation curve was recorded, and the induction time, the time needed to reach the break point of this curve, was measured. All of the samples were analyzed in duplicate, and a control (virgin olive oil without addition of the phenolic extract) was incorporated into each experimental set. The results were expressed as the induction time in hours.

Antioxidant Capacity of the Olive Oils by ORAC Assay. The oxygen radical absorbance capacity (ORAC assay) was selected to evaluate the antioxidant activity of the oils. This method has been widely used as it is especially useful for food samples with complex reaction kinetics. The basis of this method is to evaluate the loss of fluorescence in a

reference substance (fluorescein) after the addition of a peroxyl radical, which acts as an initiator of the oxidative reaction. The assays were carried out on a FLUORstar optima spectrofluorometric analyzer (BMG Labtechnologies GmbH, Offenburg, Germany) in 96-well microplates, using an excitation filter at 485 nm and an emission filter at 520 nm. Trolox was used as the reference substance to express the results, whereas AAPH was used as an initiator. The ORAC values were calculated on the basis of the area under the curve (AUC), and the data were expressed as micromoles of Trolox equivalents per 100 g of oil using the Trolox and the sample calibration curves obtained in each analysis. Two different alternatives of this methodology were used: the hydrophilic ORAC and the total ORAC.

The hydrophilic ORAC was based on the methodology described by Huang et al. (*20*) with some modifications, and its value can be associated with the antioxidant activity of all the hydrophilic compounds of the oil. In this case, all solutions were prepared using 0.075 M phosphate buffer at pH 7.4. The reaction mix consisted of 150 μL of 68 nM fluorescein solution, 25 μL of 74 mM AAPH solution (made immediately before use in phosphate buffer at 37 °C), and 25 μL of either olive oil phenolic extract or Trolox at different concentrations (from 0.415 to 4.15 μg/mL in the case of the oil phenolic extract and from 12.5 to 100 μM in the case of Trolox). The assay buffer was used as a blank. The experiments were carried out at 37 °C.

On the other hand, the total ORAC allowed the determination of the antioxidant activity of the whole enriched olive oil and was based on the methodology described by Prior et al. (*21*) with some modifications. In this assay, although fluorescein and AAPH solutions were prepared using phosphate buffer, the oil samples and Trolox were prepared using RMCD at 7% in acetone/water (50:50, v/v) to allow the correct solubility of the oil. Thus, 0.15 g of oil was successively diluted in hexane until the range defined by the reference substance (Trolox) was reached. The reaction mix consisted of 20 μL of either diluted olive oil or Trolox, 125 μL of 68 nM fluorescein solution, and 50 μL of 74 mM AAPH solution. RMCD (7% solution) was used as a blank. The experiments were carried out at 37 °C.

The value of lipophilic ORAC (the antioxidant activity attributed to the hydrophobic compounds of the oil) can be obtained by means of the difference between the total and hydrophilic ORAC values.

Quality Parameters and Composition of the Olive Oils. The quality parameters of virgin olive oil (control) and phenol-enriched olive oils were analyzed according to European Union Commission Regulation EEC/2568/91. Thus, the free fatty acid content was expressed as the percentage of oleic acid, the peroxide value was expressed as milliequivalents of active oxygen per kilogram of oil, and K_{270} was expressed as absorbance at 270 nm. The total chlorophyll and carotenoid contents were quantified, respectively, at 670 and 470 nm from the absorption spectrum of each olive oil sample dissolved in cyclohexane, as described by Minguez-Mosquera et al. (*22*). The chlorophyll and carotenoid contents were expressed as milligrams of major pigment, pheophytin *a*, and lutein per kilogram of oil, respectively. Finally, the bitter index (K_{225}) was determined spectrophotometrically at 225 nm, as reported by Artajo et al. (*16*).

Simulated Gastrointestinal Digestion of the Olive Oils by an in Vitro Digestion Model. To study the behavior of the added phenolic compounds during digestion, control and phenol-enriched olive oils were submitted to an in vitro assay following the method originally described by Gil-Izquierdo et al. (*23*) and further modified by Soler et al. (*24*). This procedure consisted of two sequential steps. Initially, the gastric digestion of 15 g of control or enriched olive oil was simulated during 2 h at 37 °C in an orbital shaker (250 rpm) (Infors AG CH-4103, Bottmingen, Switzerland). Thus, the olive oil sample was mixed with porcine pepsin (14800 U) in 20 mL of acidified water (pH 2.0) to achieve the required acidic conditions. After that, the duodenal digestion was simulated, increasing the pH to 6.5 with $NaHCO_3$ (0.25 M) and adding 5 mL (50:50, v/v) of pancreatin (8 mg/mL)−bile salt (50 mg/mL). The digestion mixture was then kept at 37 °C under agitation in an orbital shaker at 250 rpm for 2 h.

After each digestion step (gastric and duodenal), the digestion mixtures were centrifuged at 30000*g* for 20 min at 4 °C, allowing the separation of two different phases (oil digesta and aqueous micellar). The phenolic compounds from aqueous micellar were extracted by solid-phase extraction (SPE) using Oasis HLB cartridges (200 mg, Waters Corp., Milford, MA). The cartridges were conditioned by adding sequentially 3 mL of methanol and 3 mL of Milli-Q water. After the sample was loaded, the cartridges were washed with 2 mL of Milli-Q water and 3 mL of methanol 5%. Finally, the retained phenolic compounds were eluted using 5 mL of methanol. After the extraction, the phenols were analyzed by UPLC-MS/MS.

Chromatographic Analysis of Phenols by UPLC-ESI-MS/MS. The phenolic compounds of the olive cake extracts, phenol-enriched olive oils, and in vitro digested oils were analyzed by ultraperformance liquid chromatography coupled to a tandem mass detector (UPLC-ESI-MS/MS). The system consisted of an AcQuity UPLC equipped with a Waters binary pump system using an AcQuity UPLC BEH C_{18} column (1.7 μm, 100 mm × 2.1 mm i.d.). During the analysis, the column was kept at 30 °C and the flow rate was 0.4 mL/min using Milli-Q water/acetic acid (99.8:0.2, v/v) as solvent A and acetonitrile as solvent B. The UPLC was coupled to a TQ mass spectrometer (Waters). The software used was MassLynx 4.1. Ionization was done by electrospray (ESI) in the negative mode, and data were collected in the selected reaction monitoring (SRM) mode. The SRM transitions and the individual cone voltage and collision energy for each phenolic compound were evaluated by infusing 10 mg/L of each compound to obtain the best analytical conditions (*17*). Analyte concentrations were quantified by calibration curves for the respective commercial standards. Secoiridoid derivatives *p*-HPEA-EDA and the aldehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EA) were quantified as *p*-HPEA-EDA; 3,4-DHPEA-EDA, 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC), oleuropein aglycone (3,4-DHPEA-EA), and its methylated form (methyl 3,4-DHPEA-EA) were quantified as 3,4-DHPEA-EDA, and elenolic acid, ligstroside derivative, and oleuropein derivative were quantified as oleuropein. All of the samples were filtered through a 0.22 μm filter before analysis.

Data Treatment. All of the experiments were carried out in triplicate. Data analyses were performed using Statgraphics plus v. 5.1 software (Manugistics Inc., Rockville, MD). The data were analyzed by ANOVA test with a significance level of 0.05.

RESULTS AND DISCUSSION

Analysis of the Phenolic Composition of the Olive Cake Extracts. As can be seen in **Table 1**, the VW extract had the highest amount of phenolic compounds. Specifically, VW extract was rich in secoiridoid derivatives (304 mg/g of freeze-dried extract) and phenyl alcohols (101 mg/g of freeze-dried extract). On the other hand, the SR extracts (both purified and nonpurified) were also rich in secoiridoid derivatives (216 and 117 mg/g of freeze-dried extract, respectively) and, at a second level of importance, flavonoids (9.9 and 8.6 mg/g, respectively). Although the ASE extract had a lower concentration of phenolic compounds, the percentage of flavonoids was higher than in the other extracts (5.9 mg/g of freeze-dried extract, which represented 7.2% of the phenols). This fact, together with an easier extraction method and greater feasibility of production, made this extract very attractive for oil enrichment.

Development of Prototypes A and B of Phenol-Enriched Olive Oil. Different sets of samples were prepared to determine the combination of phenolic extracts that produced the optimal enrichment of olive oil. Some studies have demonstrated the high antioxidant capacity of the phenyl alcohols (basically hydroxytyrosol) and the secoiridoid derivatives. Thus, the VW extract was considered to be the first option for the enrichment. However, it is well-known that flavonoids have a wide range of beneficial properties for health, including antioxidant, anti-inflammatory, and anticarcinogenic activities, and also protect against coronary heart disease and metabolic disorders (*25*). Therefore, we tried to complement the lack of flavonoids in the VW extract by mixing it with the SR extract so as to incorporate a well-balanced dose of polyphenols into the virgin olive oil matrix.

Thus, maintaining constant the amount of VW extract at 0.65 mg of freeze-dried extract per milliliter of oil, increasing quantities of either SRnp or SRp phenolic extracts obtained from solid residue of olive cake were applied to study their influence on

Table 1. Phenolic Composition of the Olive Cake Extracts Analyzed by UPLC-ESI-MS/MS Following the Method Described under Material and Methods,[a]

phenol	vegetative water extract (VW)	purified solid residue extract (SRp)	nonpurified solid residue (SRnp)	ASE
tyrosol	1.92	0.32	1.70	0.08
hydroxytyrosol	98.6	2.79	3.61	3.45
total phenyl alcohols	101	3.11	5.30	3.51
vanillin	0.06	0.06	0.10	0.02
p-coumaric acid	0.18	0.21	0.72	0.05
vanillic acid	1.56	0.09	0.80	0.03
caffeic acid	nd	0.43	0.21	0.04
total phenolic acids	1.80	0.82	1.81	0.10
oleuropein[d]	0.02	0.45	0.20	n.d.
3,4-DHPEA-AC[b]	1.64	0.66	0.15	0.34
elenolic acid[d]	16.1	8.41	4.10	25.9
p-HPEA-EDA[c]	17.3	0.31	0.17	0.19
3,4-DHPEA-EDA[b]	159	173	96.3	60.2
ligstroside derivative[d]	1.63	3.05	1.07	0.30
p-HPEA-EA[c]	3.76	1.64	0.71	0.11
oleuropein derivative[d]	0.11	0.19	0.05	0.08
3,4-DHPEA-EA[b]	104	28.2	14.3	1.43
ME 3,4-DHPEA-EA[b]	nd	0.18	0.10	0.12
total secoiridoid derivatives	303	216	117	89
pinoresinol	0.25	0.33	0.21	0.07
acetoxypinoresinol	1.73	0.63	0.70	0.14
total lignans	2.0	0.96	0.90	0.21
apigenin	0.21	1.24	0.92	0.40
luteolin	0.04	0.10	4.05	1.82
apigenin-7-glucoside	1.24	5.07	2.52	0.21
luteolin-7-glucoside	1.27	0.11	0.24	3.07
rutin	0.71	3.38	0.89	1.71
total flavonoids	3.5	9.9	8.6	7.2
verbascoside	2.50	2.18	2.35	0.61
total average content	414	233	136	101

[a] Values are expressed as mg of phenol/g of freeze-dried extract. 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; *p*-HPEA-EDA, dialdehydic form of elenolic acid linked to tyrosol; *p*-HPEA-EA, aldehydic form of elenolic acid linked to tyrosol; 3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EA, oleuropein aglycone; methyl 3,4-DHPEA-EA, methylated form of oleuropein aglycone; nd, not detected. [b] Quantified with the calibration curve of 3,4-DHPEA-EDA. [c] Quantified with the calibration curve of *p*-HPEA-EDA. [d] Quantified with the calibration curve of oleuropein.

Table 2. Effect of Phenolic Enrichment on the Oxidative Stability and Total Phenolic Content of the Oils[a]

		enriched oils VW extract addition: 0.65 mg/mL of oil					
		SRnp extract addition[b]			SRp extract addition[b]		
	control oil	0.3	0.5	2.0	0.3	0.5	2.0
oxidative stability (h)	10.1 ± 0.5	13.6 ± 0.9	14.0 ± 0.3	10.8 ± 0.1	17.7 ± 0.1	19.5 ± 1.2	17.8 ± 0.5
total phenolic content (mg of caffeic acid equiv/kg of oil)	172 ± 14	521 ± 3	531 ± 2	562 ± 20	371 ± 11	370 ± 13	389 ± 27

[a] VW, vegetative water; SRnp, nonpurified solid residue; SRp, purified solid residue. [b] Addition expressed as mg freeze-dried extract/mL oil.

the phenolic enrichment of the oil. The transfer of phenols from the extracts to the oil was monitored by measuring the total phenolic content and the oxidative stability of the enriched oils. As **Table 2** shows, the combined use of both the VW and SR extracts produced an increment in the oxidative stability of all the enriched olive oils compared with the control in all of the studied combinations.

With regard to the effectiveness of the enrichments, using the same amount of extract, the SRp extract produced a higher increase in the oxidative stability than the increase obtained with the SRnp. This can be explained by the higher phenolic content of the purified extract determined by UPLC-ESI-MS/MS that is shown in **Table 1**. **Table 2** also shows that the value of oxidative stability of both enriched oils increased proportionally to the amount of extract added to the oil up to a maximum value and then gradually decreased.

Related to the total phenolic content of the enriched oils, the use of SRnp produced a higher increase than that obtained when SRp extract was used. However, it is known that there are some compounds that interfere in the reaction involved on the basis of the Folin−Ciocalteu (including sugars, aromatic amines, sulfur dioxide, ascorbic acid, and other enediols, reductones, and organic acids) (*26*). The SRnp extract preparation does not include the purification step. As a consequence, sugars and other compounds could have been transferred to the oil, giving a higher total phenol value, which does not correspond to the real content in the phenol-enriched oil. This could be confirmed by the lower oxidative stability of the oils enriched with SRnp.

According to the results, the optimum quantities of solid residue extract (SR) were those that allowed the maximum oxidative stability of the enriched olive oils to be obtained. Thus, two prototypes of enriched oil were selected: prototype A (containing 0.65 mg/mL oil of VW freeze-dried extract plus 0.5 mg/mL oil of SRnp freeze-dried extract) and prototype B (containing 0.65 mg/mL oil of VW freeze-dried extract plus 0.5 mg/mL oil of SRp freeze-dried extract).

Table 3. Quality Parameters (Peroxide Value, Acidity, and K_{270}), Oxidative Stability, Total Phenolic Content, Bitter Index (K_{225}), and Total Chlorophyll and Carotenoid Contents of the Control (Virgin Olive Oil) and Phenol-Enriched Olive Oils[a]

	control	prototype A	prototype B	prototype C
peroxide value (mequiv of O_2/kg of oil)	16.76a ± 0.03	18.71a ± 1.80	17.75a ± 1.22	6.16a ± 0.25
acidity (% oleic acid/kg of oil)	0.19a ± 0.01	0.26b ± 0.03	0.28b ± 0.02	0.26b ± 0.01
K_{270}	0.11a ± 0.003	0.14b ± 0.012	0.15c ± 0.017	0.16c ± 0.01
oxidative stability (h)	9.94a ± 0.4	13.7b ± 0.4	19.1c ± 0.4	12.2d ± 0.1
total phenolic content (mg of caffeic acid equiv/kg of oil)	217a ± 19	520b ± 22	577c ± 8	463d ± 10
K_{225}	0.16a ± 0.01	0.33b ± 0.02	0.35b ± 0.01	0.37b ± 0.03
chlorophylls (mg of pheophytin *a*/kg of oil)	8.61a ± 0.04	34.86b ± 0.44	36.65b ± 2.19	14.46c ± 0.09
carotenoids (mg of lutein/kg of oil)	6.04a ± 0.04	14.43b ± 0.29	14.63b ± 0.03	9.01c ± 0.08

[a] Prototype A contained 0.65 mg/mL of oil VW extract plus 0.50 mg/mL of oil of SRnp extract. Prototype B included 0.65 mg/mL of oil VW extract plus 0.70 mg/mL of oil of SRp, and prototype C was prepared by adding 7 mg/mL of ASE extract. Values in the same row followed by different letters are significantly different (p <0.05).

Development of Prototype C of Phenol-Enriched Olive Oil. Although the values of oxidative stability and phenolic content significantly increased in both prototypes A and B compared with the control oil, the purification step with ethyl acetate gave an unpleasant taste and smell to the oils. To avoid this problem, the single application of the ASE extract, obtained from the whole freeze-dried olive cake using ethanol/water (80:20, v/v), was evaluated.

The lower content of phenolic compounds in this extract, specifically the lower content of secoiridoid derivatives in comparison with the VW and SR extracts, was compensated for by applying a higher quantity of the ASE extract to carry out the oil enrichment. The oxidative stability of the prepared oils increased proportionally with the addition of the ASE extract (data not shown) until a level of enrichment, after which it began to decrease (at approximately 7 mg of freeze-dried ASE extract/mL of oil). This behavior was similar to that observed with the application of the other phenolic extracts. Simultaneously, the total phenolic content of the enriched oils increased gradually with the quantity of the ASE extract added to the oil, similar to that observed with the SRnp extract.

The use of the ASE extract avoided the appearance of bad smells in the enriched oil. However, one of the drawbacks of the application of high quantities of extract was that the visual appearance of the oil was affected (becoming cloudy and dark). To improve this aspect, filtration of the oil to eliminate all of the suspended particles that were not properly dispersed into the oil was considered. Nevertheless, it is well-known that filtration reduces the phenolic content of olive oil (*27*). This was confirmed by the analysis of the filtered enriched oils, which showed a reduction of the oxidative stability and the total phenolic content compared with those prior to filtration. Specifically, the reductions were around 22 and 60% for the oxidative stability and the total phenolic content, respectively (data not shown). However, the final values of both of these parameters in the filtered oils were similar to those obtained with prototypes A and B. This could be explained by the more efficient transfer of phenolic compounds from the ASE extract to the lipid matrix, in relation to SR extracts. Therefore, the increase in phenolic compounds with the enrichment was higher than the loss suffered through filtration. Taking all of these studies into consideration, we established the preparation of prototype C by adding 7 mg of freeze-dried ASE extract/mL of oil.

Effect of Phenolic Enrichment on the Analytical Parameters of the Phenol-Enriched Olive Oils. To evaluate the suitability of the selected prototypes (namely, prototypes A, B, and C), the quality parameters (acidity, peroxide value, and K_{270}), the bitter index (K_{225}), and the total chlorophyll and carotenoid contents were evaluated and compared with those obtained from the control virgin olive oil (**Table 3**). The values of the quality parameters in all of the olive oils were within the range that makes them edible. The value of the total phenolic content significantly increased in the enriched oils (at least twice the content of the control oil). This way, it is possible to increase the daily dose of phenolic compounds from virgin olive oil without increasing the caloric intake. By comparison with the phenolic ingestion obtained through other sources, such as fruits and vegetables, it can be stated that enriched oils provide higher amounts than some of them. Thus, following the recommendation of the U.S. FDA of 23 g/day of olive oil, the mean daily intake of phenolic compounds from enriched olive oil is 12 mg of caffeic acid equiv. Taking into consideration the data obtained by Chun et al. (*28*), the ingestion of phenols from enriched olive oil was higher than that obtained in United States from mostly all of the vegetables that they studied (from asparagus to sweet potatoes, including carrots and onions). The values were also higher than provided from some fruits such as lemons and peaches. This points out the importance of the phenolic enrichment of olive oil.

With regard to the K_{225} value (which is an index of bitterness), there was a significant increment ($p < 0.05$) as a result of the phenol enrichment of the oils in relation to the control. This result agrees with the ones obtained in previous studies that found a relationship between bitterness and the phenolic content of the olive oil, mainly the concentration of secoiridoid derivatives (*16*). Thus, the high level of secoiridoid derivatives in the phenolic extracts could be responsible for the increase in the bitterness of the enriched oils (average value of 0.35). Although there is no established limit, experience has shown that values of K_{225} around 0.36 are at the limit of acceptance by many consumers (*29*). On the other hand, the incorporation of phenolic extracts in the oils significantly increased their total pigment content (chlorophylls and carotenoids). The pigment contents of prototypes A and B were significantly higher than the pigment content of prototype C ($p < 0.05$). This could be related to a major degradation of the chlorophylls and the carotenoids during the phenol extraction with the ASE system due to the high temperature and working pressure (80 °C).

Analysis of the Phenolic Composition of the Phenol-Enriched Oils. Once the three prototypes of enriched olive oil had been developed, their phenolic compound composition was analyzed by liquid chromatography−tandem mass spectrometry. As can be seen in **Table 4**, the enrichment process led to an increment in the concentration of almost all of the phenolic groups that usually appear in olive oil. Of special interest was the increase in the concentration of the secoiridoid derivatives, specifically oleuropein derivatives such as 3,4-DHPEA-EDA. These compounds are precursors of hydroxytyrosol, the plasma concentration of which has been shown to increase in a dose-dependent manner with the phenolic content of the administered oil (*6*). Therefore, higher quantities of these compounds could appear in the plasma, improving the health benefits of consuming olive oil. In addition, the enriched oils had higher concentrations of

Table 4. Phenolic Composition of the Control (Virgin Olive Oil) and Phenol-Enriched Olive Oils by UPLC-ESI-MS/MS Expressed as Nanomoles of Phenol per Gram of Oil[a]

phenol	control	prototype A	prototype B	prototype C
tyrosol	15.3a ± 1.9	22.1a ± 0.3	20.4a ± 4.8	15.9a ± 4.4
hydroxytyrosol	19.9a ± 1.5	63.1b ± 2.7	56.8b ± 11.1	30.9a ± 4.9
total phenyl alcohols	35.2	85.2	77.2	46.8
vanillin	1.0a ± 0.0	1.2a ± 0.0	1.2a ± 0.1	1.7b ± 0.2
p-coumaric acid	0.9a ± 0.1	2.5b ± 0.2	2.5b ± 0.5	2.4b ± 0.4
vanillic acid	0.8a ± 0.0	2.8b ± 0.4	2.9b ± 0.6	2.3b ± 0.0
caffeic acid	0.0a ± 0.0	2.3b ± 0.2	2.0b ± 0.8	2.2b ± 0.2
total phenolic acids	2.7	8.8	8.6	8.6
oleuropein	0.0a ± 0.0	2.9b ± 0.1	0.9c ± 0.1	1.2c ± 0.2
3,4-DHPEA-AC[b]	2.8a ± 0.9	8.2b ± 1.4	9.4b ± 1.1	1.9a ± 0.2
elenolic acid[d]	208a ± 4	526b ± 19	493b ± 54	422b ± 62
p-HPEA-EDA[c]	39.4a ± 5.7	30.7ab ± 4.8	26.3b ± 5.1	28.5ab ± 0.4
3,4-DHPEA-EDA[b]	385a ± 16	1546b ± 126	1649b ± 206	1253b ± 204
ligstroside derivative[d]	50.3ab ± 12.5	102b ± 2	98.6b ± 37.8	37.5a ± 3.8
p-HPEA-EA[c]	83.8a ± 0.2	82.0a ± 3.6	72.5a ± 6.9	82.0a ± 8.4
oleuropein derivative[d]	2.5a ± 0.6	115.7b ± 26.9	73.1a ± 18.2	7.0a ± 2.7
3,4-DHPEA-EA[b]	199a ± 24	296b ± 1	264ab ± 14	253ab ± 42
ME 3,4-DHPEA-EA[b]	3.8a ± 1.2	9.2b ± 0.5	7.3ab ± 1.0	9.3b ± 2.3
total secoiridoid derivatives	975	2,719	2,693	2,096
pinoresinol	4.6a ± 0.3	4.2a ± 0.0	3.7a ± 0.3	2.4b ± 0.4
acetoxypinoresinol	21.9a ± 0.0	19.1a ± 0.3	15.4b ± 0.3	8.9c ± 1.5
total lignans	26.5	23.3	19.1	11.3
apigenin	2.5a ± 0.4	3.3a ± 0.3	2.8a ± 0.2	3.3a ± 0.4
luteolin	11.5a ± 2.3	36.7b ± 0.8	41.7b ± 4.7	18.4a ± 1.1
apigenin-7-glucoside	0.0a ± 0.0	0.1b ± 0.0	0.1c ± 0.0	0.1d ± 0.0
luteolin-7-glucoside	0.0a ± 0.0	6.2b ± 0.1	8.8b ± 2.1	2.6c ± 0.6
rutin	0.0a ± 0.0	1.8b ± 0.1	1.7b ± 0.5	1.7b ± 0.3
total flavonoids	14.0	48.1	55.1	26.1
total phenolic compounds	1053	2,884	2,853	2,189

[a] Values in the same row followed by different letters are significantly different ($p < 0.05$). 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; *p*-HPEA-EDA, dialdehydic form of elenolic acid linked to tyrosol; *p*-HPEA-EA, aldehydic form of elenolic acid linked to tyrosol; 3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EA, oleuropein aglycone; methyl 3,4-DHPEA-EA, methylated form of oleuropein aglycone. [b] Quantified with the calibration curve of 3,4-DHPEA-EDA. [c] Quantified with the calibration curve of *p*-HPEA-EDA. [d] Quantified with the calibration curve of oleuropein.

flavonoids than the control. There are low concentrations of flavonoids in virgin olive oil because they are retained in the olive cake during the olive oil extraction process. However, as commented above, these compounds have high anticarcinogenic and antioxidant activities, among others. Therefore, the possibility of increasing their daily intake through enriching oil would be of great interest.

Measurement of the Antioxidant Activity of the Phenol-Enriched Oils by ORAC Assay. The antioxidant activity of the enriched oils was evaluated using the ORAC assay. As explained above, two different methodologies of ORAC were followed: the total and the hydrophilic ORAC assays. **Table 5** shows the results of the ORAC values of the control and enriched olive oils. The total ORAC value obtained with the control olive oil (1100 μmol of Trolox equiv/100 g of oil) agreed with the result presented by Ninfali et al. (*30*). This confirmed the robustness of the ORAC assay and the validity of the results. With regard to the enriched olive oils, the incorporation of the phenolic extract in the oils significantly increased the value of the hydrophilic ORAC and, as a consequence, the total ORAC value. On the other hand, the value of the lipophilic ORAC remained almost constant. The lower value of the lipophilic ORAC in prototypes A and B compared with prototype C could be explained by their higher chlorophyll content (**Table 3**). It is known that chlorophylls and especially pheophytins formed during olive oil extraction can act as prooxidants in the presence of light, leading to lower ORAC value in prototypes A and B in contrast with prototype C, which had lower chlorophyll contents (*31*). Despite that, all of the phenolic enrichments enhanced the antioxidant properties of

Table 5. Antioxidant Capacity of the Control (Virgin Olive Oil) and Phenol-Enriched Olive Oils by ORAC Assay[a]

	control	prototype A	prototype B	prototype C
hydrophilic ORAC	816a ± 68	1180b ± 95	1403c ± 77	1387c ± 31
lipophilic ORAC	316a ± 47	332a ± 50	332a ± 107	545b ± 77
total ORAC	1133a ± 22	1506ab ± 251	1734bc ± 176	1933c ± 108

[a] Values are expressed as μmol of Trolox equiv/100 g of oil. Values in the same row followed by different letters are significantly different ($p < 0.05$).

the oils (up to 73% higher than the control oil in the case of prototype C). This proves the suitability of the developed prototypes because the daily intake of antioxidants can be raised (with the corresponding benefits for human health) without increasing the caloric contribution of the oil.

Simulated Gastrointestinal Digestion of the Phenol-Enriched Oils by an in Vitro Digestion Model. In the final part of the study, the enriched olive oils were submitted to an in vitro gastrointestinal digestion model (gastric and duodenal steps) to evaluate the stability and potential bioaccesibility of the phenol components of the enriched oils compared with the control. The extent of digestion was evaluated by quantifying the phenols (nanomoles) in the aqueous micellar phases after the gastric and duodenal digestion steps, calculated back to a 1 g sample of olive oil test (**Table 6**). Although the phenolic profile was similar in the aqueous micellar phases of the different oils (control and phenol-enriched oils), quantitative differences were observed mainly after gastric digestion. Of special interest was the increase in the amount of secoiridoid derivatives after gastric digestion of phenol-enriched

Table 6. Amounts of Phenols in Aqueous Micellar Fraction after in Vitro Digestion of Control (Virgin Olive Oil) and Phenol-Enriched Olive Oils[a]

	gastric digestion				duodenal digestion			
phenol	control	prototype A	prototype B	prototype C	control	prototype A	prototype B	prototype C
tyrosol	27.9	75.8	74.4	32.4	48.6	91.7	90.3	77.6
hydroxytyrosol	32.8	209	239	54.4	26.1	331	394	77.6
total phenyl alcohols	60.7	284.7	313	86.8	74.7	422.9	484.4	155.2
vanillin	0.0	0.0	0.0	0.4	0.0	0.2	0.0	0.9
p-coumaric acid	0.3	2.0	2.3	9.2	0.0	1.0	3.1	0.0
vanillic acid	0.0	3.7	3.6	0.0	0.0	0.0	0.0	0.0
caffeic acid	0.0	3.5	3.4	2.6	0.0	2.1	2.2	0.2
total phenolic acids	0.3	9.2	9.3	12.2	0.0	3.3	5.3	1.1
oleuropein	0.0	2.6	3.3	0.7	0.0	4.4	6.5	0.5
3,4-DHPEA-AC	8.0	30.7	35.7	0.7	3.4	0.0	0.0	0.0
elenolic acid	194	792	831	511	2.4	37.2	61.8	21.1
3,4-DHPEA-EDA	236	2,328	2,835	426	1.7	16.9	29.1	13.3
p-HPEA-EDA	21.7	19.8	42.7	75.9	0.0	10.7	0.1	0.4
ligstroside derivative	51.8	153	152	10.9	7.0	78.7	84.4	6.4
p-HPEA-EA	13.3	18.6	18	13.1	0.0	7.8	4.2	3.3
oleuropein derivative	2.9	16.2	57.1	31.4	0.0	2.7	0.3	0.3
3,4-DHPEA-EA	71.1	128	134	70.9	7.9	54.6	62.3	22.8
ME-3,4-DHPEA-EA	1.9	3.0	2.6	0.6	0.0	0.0	3.0	0.6
total secoiridoids	608	3500	4119	1145	24.5	223	262	70.7
pinoresinol	0.0	0.3	0.6	1.3	0.0	3.0	3.0	0.2
acetoypinoresinol	22.8	35.1	33.6	9.1	24.1	49.6	43.4	11.2
total lignans	22.8	35.4	34.2	10.4	24.1	52.6	46.4	11.4
apigenin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
luteolin	0.4	2.8	2.7	3.5	3.7	5.2	5.0	3.1
apigenin-7-glucoside	0.0	0.4	0.5	0.2	0.0	0.4	0.6	0.2
luteolin-7-glucoside	0.0	7.6	9.2	4.2	0.0	5.6	9.9	5.0
rutin	0.0	2.1	1.7	1.2	0.0	0.7	0.7	1.7
total flavonoids	0.4	12.9	14.1	9.1	3.7	11.9	16.2	10

[a] The results are expressed as total nmol of phenol in aqueous micellar fraction per gram of olive oil digested. All of the RSD are <10% ($n = 3$).

oils (especially in 3,4-DHPEA-EDA and elenolic acid). This fact could have great interest due to the protective effect of this group of phenolic compounds against oxidative damage in human red blood cells (*32*). Thus, the ingestion of olive oil with a higher content of these compounds could be desirable in its use in preventive and/or therapeutic purposes. The higher content of the secoiridoid derivative group in aqueous micellar phases after gastric and duodenal digestion, respectively, of prototypes A and B compared with prototype C could be related to the procedure followed to obtain the phenolic extracts (VW and SR) used in the enrichment of these oils. In this case, the organic solvents used under atmospheric pressure (ethyl acetate and ethanol) may be able to extract not only phenolic compounds in their free form but also their polymeric forms of higher molecular weights found in the olive cake. These polymers could be hydrolyzed during the gastric step due to the acidic pH of the medium, thus generating monomeric structures of secoiridoid derivatives. By comparison of both prototypes, the digestion mixtures (gastric and duodenal) corresponding to prototype B showed higher phenol content than those of prototype A (**Table 6**). This fact revealed higher stability of the phenols in prototype B during oil digestion. On the other hand, the extreme conditions of temperature and pressure reached during the preparation of the ASE extract, used to prepare prototype C, may have led to the hydrolysis of the polymeric forms of secoiridoids. Thus, almost all of the compounds incorporated into the oil were in their free form (monomers), which are rather unstable during gastric digestion.

In addition, to determine stability under gastric and duodenal digestion conditions, the concentration of phenolic in solution (aqueous micellar fraction), mainly after the duodenal digestion, is an important factor in determining the potential bioavailability. It is known that the rate at which phenolics are taken up by enterocytes from aqueous environments (the first step in absorption) is dependent on their concentration in solution. Consequently, the preparation of olive oil rich in phenolic compounds may result in an increase in the bioavailability of phenols in plasma and that may enhance the healthy properties of olive oil. However, these promising results obtained in the in vitro digestion should be confirmed in vivo by the determination of the phenolic metabolites in plasma samples after the consumption of enriched virgin olive oils.

On the basis of the results, the suitability of all the enriched olive oils developed was confirmed by the analysis of different quality and composition parameters. Prototypes A and B presented higher contents of phenolic compounds than prototype C, which could suggest that the former are preferable to the latter. The same was also observed in the behavior of the polyphenols during the simulated in vitro digestion. However, the oxygen radical absorbance capacity (ORAC value) of prototype C together with the simple procedure of phenol extract preparation revealed the potential interest of prototype C. In addition, the presence of bad smells and flavors in prototypes A and B, as a consequence of the use of ethyl acetate during phenol extract preparation, could decrease consumer acceptance of these phenol-enriched oils. This suggests that the choice of the best prototype of the enriched olive oil should take into consideration not only their phenolic composition but also other parameters, such as the sensorial qualities and the feasibility of implementing the process of obtaining the phenolic extract in the food industry.

ABBREVIATIONS USED

VW, vegetative water; SR, solid residue; SRp, purified solid residue extract; SRnp, solid residue extract without purification; ASE, accelerated solvent extraction; ORAC, oxygen radical absorbance capacity; AAPH, 2,2′-azobis(2-methylpropionamide) dihydrochloride; UPLC, ultraperformance liquid chromatography; ESI,

electrospray ionization; DAD, diode array detector; MS/MS, tandem mass detector; SRM, selected reaction monitoring.

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