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Quality and Stability of Edible Oils Enriched with Hydrophilic Antioxidants from the Olive Tree: The Role of Enrichment Extracts and Lipid Composition

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Supporting Information

ABSTRACT: Phenolic extracts from olive tree leaves and olive pomace were used to enrich refined oils (namely, maize, soy, high-oleic sunflower, sunflower, olive, and rapeseed oils) at two concentration levels (200 and 400 μg/mL, expressed as gallic acid). The concentration of characteristic olive phenols in these extracts together with the lipidic composition of the oils to be enriched influenced the mass transfer of the target antioxidants, which conferred additional stability and quality parameters to the oils as a result. In general, all of the oils experienced either a noticeable or dramatic improvement of their quality—stability parameters (e.g., peroxide index and Rancimat) as compared with their nonenriched counterparts. The enriched oils were also compared with extra virgin olive oil with a natural content in phenols of 400 μg/mL. The healthy properties of these phenols and the scarce or nil prices of the raw materials used can convert oils in supplemented foods or even nutraceuticals.

KEYWORDS: edible oils, enrichment, phenolic antioxidants, oil quality tests, olive tree extracts, stability, Rancimat, peroxide index

INTRODUCTION

Extra virgin olive oil (EVOO) is the most demanded liquid fat in the Mediterranean basin thanks to the nutraceutical properties of its components, which are divided into two groups as a function of their concentration: major and minor compounds. The first group, known as the saponifiable fraction, represents >98% of the total weight of the oil and consists of triacylglycerols, diacylglycerols, monoacylglycerols, and free fatty acids. The second group, the unsaponifiable fraction, comprises about 2% of the total weight, and it is constituted by a great variety of compounds such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, and antioxidants, most of the last being carotenes, tocopherols, and phenols.[1] Contrarily to the majority of edible oils, EVOO can be consumed in crude form, conserving all beneficial properties typical of its minor components, the excellence of which has been discussed elsewhere.[2,3]

Within the unsaponifiable fraction, interest in olive phenols (OPs) has increased in recent decades, thus stimulating multidisciplinary research on their composition, histological distribution, and histochemical localization to determine their biomolecular functions.[4] Nevertheless, the main reason for the growing research in this field lies in the antioxidant properties of OPs from clinical and pharmacological points of view. These proven excellent properties of OPs have promoted active research on raw materials for their isolation.[5−11] The two main sources of OPs are olive leaves and the pomace waste generated in the olive oil industry, known as alperujo. This is a polluting semisolid residue resulting from the two-phase olive oil extraction method, presently the method most frequently used in this industry. Alperujo is a cheap source of natural antioxidants, in concentrations up to 100 times higher than in olive oil,[8] which results from the polar nature of both alperujo and OPs and the low-polar nature of oil; however, olive leaves have the highest antioxidant and scavenging powers of the different parts of the olive tree (e.g., taking oleuropein as an OP model, its content in olive oil ranges between 0.005 and 0.12%, in alperujo its content is up to 0.87%, and in olive leaves its content is between 1 and 14%).[8,12] Thus, both agricultural residues are well-characterized sources for extraction of OPs to take benefits from their antioxidant properties. The food industry is an active area with possibilities to exploit the high content of phenols present in olive tree materials.

The addition of synthetic oxidation inhibitors to refined edible oils to improve their stability-related properties is a common practice. However, the reported deleterious effects on human health of these synthetic additives such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, or *tert*-butyhydroquinone have decreased their use and promoted the general consumers' rejections of synthetic food additives.[13,14] As a result, enrichment of edible oils with natural antioxidants to inhibit or suppress oil oxidation becomes of great interest. These substances ought to be cheap and do not produce any deleterious compounds under oxidation conditions such as deep-frying.

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As EVOO contains a high amount of natural antioxidants, addition of artificial antioxidants is unnecessary, in general. Nevertheless, in many countries the market for olive oil is limited; thus, it is replaced by a mixture of refined olive oil, seed oil, or other oils with minimal or no phenol content. Because of the high price of olive oil and the antioxidant and nutraceutical properties of olive phenols, there is a growing interest in the use of these compounds to enrich low-priced oils[15,16] to obtain a healthy added-value product.

Conferring to other oils oxidative stability properties similar to those of EVOO involves two main steps: extraction of the target compounds from the raw material, either leaves or alperujo, and enrichment of the oil with the extract. The extraction step is a cheap task because of the low price of the raw materials from which OPs can be obtained, facilitated by the number of methods accelerated by auxiliary energies developed in the past decade.[5−11] Previous laboratory-scale methods for OP extraction used extractants such as methanol−water mixtures[12] or hexane,[17] but the increased human use of these compounds made mandatory the development of methods based on nontoxic extractants; therefore, water or ethanol−water mixtures as extractants helped by some type of energy is the present trend. Thus, methods for extraction of OPs from either leaves or alperujo with assistance by ultrasound,[5] superheated extractants,[6] or microwaves[7] have endowed this task with rapidity and automation, two key characteristics to facilitate industrial implementation. Microwaves have shown to be the fastest and easiest alternative,[18] so it was the type of energy selected to accelerate the obtainment of the extracts used in the present study.

With regard to the enrichment step, there are three alternatives in the literature for oil enrichment with these valuable compounds from the olive tree: (1) liquid−liquid extraction,[19] in which the oil is put into contact with an alcoholic extract of phenols, which are transferred to the oily phase as a function of their distribution factor, removing the alcoholic phase by centrifugation; (2) solid−liquid extraction,[20] in which the purified phenolic extract is dried under appropriate conditions and the paste obtained is partially dissolved into the oil as a function of the solubility of the different paste components in the oily phase; and (3) a combination of these procedures, in which the alcoholic extract and the oil are put into contact and the two-phase system is subject to alcohol removal in a rotary evaporator. This last has been the procedure used for enrichment of the target oils used in the present research.

The improvement of health-related properties of oils is the main final goal pursued with the present and similar studies,[21] but also enlargement of stability properties, usually determined by a series of well-established tests (e.g., Rancimat test, peroxide index), which also provide information about resistance to changes during frying, is investigated.[22−24]

The present research is focused on the assessment of quality and stability properties of refined edible oils enriched with phenolic antioxidants from olive materials. With this aim, several refined oils (that is, oils in which polar antioxidants are absent as they are massively removed during the refining process) have been enriched with OP extracts from both olive leaves and alperujo at two different levels of total antioxidants: 200 and 400 μg/mL. The stability conferred to the oils by the presence and amount of these OPs has been checked by using the established methods for these studies as a function of both the lipid composition of the oils and the relative concentration of the individual phenols in the extracts, which is different for the extract from each raw material.

MATERIALS AND METHODS

Samples. Alperujo obtained during the 2009/2010 crop season was taken directly from the production line in Núñez de Prado, C.B. (Córdoba, Spain), and stored at −20 °C until use. Olive leaves from the Picual, Picudo, and Hojiblanca cultivars were selected for this research, collected at the end of October, dried at 35 °C for 60 h, milled by a cyclonic mill to homogeneous particle size (diameter ≤ 0.5 mm), and kept at 4 °C until use.

The vegetable edible oils used in this research were refined maize oil (RMO), refined soy oil (RSoO), refined high-oleic sunflower oil (RHSO), refined sunflower oil (RSO), refined olive oil (ROO), refined rapeseed oil (RRO), and extra virgin olive oil (EVOO). All of them were provided by Carbonell (SOS Cuétara S.A, Madrid). The criteria for selection were low price (<1 euro/kg) and a wide range of lipid composition.

Reagents. The reagents used for characterization of vegetable oils were LC grade methanol, acetonitrile, hexane, and absolute ethanol from Scharlab (Barcelona, Spain); sodium carbonate, sodium chloride, and Folin−Ciocalteu (F−C) reagent from Panreac (Barcelona, Spain); orthophosphoric acid for acidification of mobile phases in liquid chromatography and gallic acid, as standard for quantification in the F−C test, from Merck (Darmstadt, Germany). Deionized water (18 MΩ·cm) from a Millipore Milli-Q water purification system (Bedford, MA) was used to prepare mobile chromatographic phases. Sodium methylate (0.2 N) in methanol (Panreac) was used as derivatization reagent to hydrolyze and transform the fat into fatty acid methyl esters (FAMEs).

The reagents used for quality studies were analysis grade ethanol, ethyl ether, acetic acid, chloroform, potassium hydroxide, potassium iodide, and sodium thiosulfate, all from Panreac; phenolphthalein and potato starch from Panreac were used the for acidity test and peroxide index, respectively; and cyclohexane from Prolabo (Geldenaa Ksebaan, Leuven) was used for spectrophotometric tests.

The most abundant phenolic compounds in olive oil were purchased from Extrasynthese (Genay, France) in the case of hydroxytyrosol, tyrosol, oleuropein, apigenin, and luteolin; vanillin, vanillic acid, *p*- and *o*-coumaric acids, ferulic acid, and the external standard *p*-cresol were from Merck. The stock standard solution of each phenol was prepared at 1000 μg/mL by dissolving 10 mg of each phenol in 10 mL of methanol. The multi-standard solutions containing 10 phenols were prepared by mixing the appropriate volume of each stock solution and diluting them as required in a 60:40 methanol−water solution. All of these solutions were stored in the dark at −20 °C in glass vials until use. *p*-Cresol from Merck was used as external standard in the quantification of phenolic compounds.

Apparatus and Instruments. A Tecator Cyclotec cyclonic mill (Hoganas, Sweden) was used to grind the leaves. Microwave irradiation was applied by means of an MIC-II focused-microwave extraction system of 400 W maximum power (Puebla, Mexico) furnished with a manual power control unit. A Selecta Mixtasel centrifuge (Barcelona, Spain) was used to remove solid particles from the extract. A Büchi R-200 rotary evaporator (Buchs, Switzerland) furnished with a B-490 heating bath was used to concentrate the phenol extracts after microwave-assisted extraction (MAE) and to evaporate traces of ethanol in the enriched oils. A Selecta Vibromatic electrical stirrer (Barcelona, Spain) was used to favor the liquid−liquid extraction of phenols. An MS2 minishaker from Ika (Wilmington, DE) was used to favor phenol transfer from oil to methanol for individual quantification of the target compounds.

A Varian ProStar liquid chromatograph (Walnut Creek, CA), consisting of a ProStar 240 pump, a ProStar 330 diode array detector (DAD), and a ProStar 410 autosampler, was used for individual determination of antioxidant compounds. The analytical column was a C18 Inerstil ODS-2 (250 × 4.6 mm i.d., 5 μm) from GL Sciences Inc. (Tokyo, Japan).

A Perkin-Elmer Clarus 500 gas chromatograph (Boston, MA) with a flame ionization detector (FID) equipped with a programmable-temperature

injector and a Phenomenex BPX70 analytical column (50 m, i.d. 0.22 mm, film 0.25 μm) was used for determination of FAMEs.

A ThermoSpectronic Helios UV−vis spectrophotometer was used for determination of total phenol concentration by the F−C method, and a Lambda 25 Perkin-Elmer single-beam UV−vis spectrophotometer was used to monitor K_{232} and K_{270} parameters.

A Metrohm 679 Rancimat (Herisau, Switzerland) was used to determine the stability of vegetable oils.

Confirmatory analysis for identification of olive phenols was carried out with an Agilent 1200 series LC system interfaced to an Agilent 6540 UHD Accurate-Mass TOF LC-MS detector (Palo Alto, CA), equipped with an Agilent Jet Stream Technology electrospray ion source operating in the negative ion mode.

Procedure for Extraction of Phenols from Alperujo or Leaves. The procedure was similar to that proposed by Girón et al.[14] and Japón et al.[19] Briefly, 12 g of alperujo or leaves and 100 mL of ethanol were placed into the quartz extraction vessel located in the zone of focused microwave irradiation of the extractor (a total of 500 g of alperujo and 300 g of leaves was used for enrichment of the target oils). After extraction (10 min of microwave irradiation at 400 W), the suspension was centrifuged at 855*g* for 5 min for phase separation. This process was repeated as many times as required to obtain the necessary extract for the subsequent enrichment step after the extract had been concentrated in a rotary evaporator at 35 °C to reduce 10 times its initial volume. The extract thus obtained was reconstituted in 200 mL of ethanol prior to measurement of total phenol concentration by the F−C test.

F−C Test for Measurement of Total Phenol Concentration. Briefly, 20 μL of sample (with prior 1:50 dilution with water) was, in this order, mixed with 1.58 mL of water, 0.3 mL of 20% (w/v) Na_2CO_3 aqueous solution, and 0.1 mL of F−C reagent and heated in an oven for 5 min at 50 °C. Then, the resulting solution was allowed to stand for 30 min. The reaction product was photometrically monitored at 765 nm. Gallic acid was used as standard for calibration, so the results were expressed as equivalent of gallic acid (GAE) per gram or milliliter.[25]

Enrichment of Edible Vegetable Oils with Phenols Extracts from Alperujo or Leaves. The enrichment was carried out at two concentration levels (200 and 400 μg/mL of phenols according to the F−C test) per each oil and with each of the two extracts (from alperujo and from leaves); that is, four enriched oils were obtained from each original oil. In all cases, an aliquot of the corresponding ethanolic extract was put into contact with 200 mL of oil, and the ethanol in the two-phase system was evaporated in the rotary evaporator at 30 °C. Then, the mixture was shaken in the electrical stirrer at 700 U/min to favor enrichment. This process was repeated as many times as required until the 200 mL oil portions were enriched in phenols from each of the extracts to 200 or 400 μg/mL (as determined by the F−C method). Distinction between the different oils, extract for enrichment and enrichment degree, is as follows: abbreviation as under Samples, capital letters, is used for 400 μg/mL enrichment, followed by a point and the initial of the raw material to prepare the extract (A for alperujo and L for leaves). Similar nomenclature, but lower case letters, is used for oils enriched with 200 μg/mL phenols. As an example, RMO.A and rmo.a correspond to refined maize oil enriched with 400 and 200 μg/mL, respectively, of phenols from alperujo, whereas RMO.L and rmo.l correspond to the high and low enrichment, respectively, from leaves.

Characterization of Phenols and Fatty Composition of Pure and Enriched Oils. Phenols were photometrically determined by DAD after individual separation by HPLC. To do this, 0.5 g of either pure or enriched oil was shaken with 1 mL of hexane and 1 mL of a 60:40 methanol−water mixture for 1 min in the MS2 minishaker at 1200 U/min. The polar phase contained *p*-cresol as internal standard (IS), at 5 μg/mL final concentration. The hydroalcoholic phase was injected into the chromatograph. The mobile phases were A (0.2% H_3PO_4 in water) and B (methanol). The gradient program, at 1 mL/min constant flow rate, was as follows: initially 96% A and 4% B; 0−40 min, 96−50% A and 4−50% B; 40−45 min, 50−40% A and 50−60% B; 45−60 min, 40−0% A and 60−100% B; 60−70 min, 0% A and 100% B. After analysis, the column was reequilibrated for 9 min. The chromatograms were acquired at 230, 280, 325, and 350 nm (wavelengths of maximum absorption for the different phenols). LC-DAD quantitative analysis was expressed as area of the target analyte/area of ES for all analytes. Compounds with no calibration standards were quantified by the calibration curve of the phenol with a more similar structure. Thus, aglycon secoiridoids were quantified by the oleuropein calibration curve.

Fatty acids were individually separated and quantified by GC-FID after derivatization by methylation to more volatile compounds. With this aim, 0.5 g of oil was mixed with 6 mL of sodium methylate in a test tube, which was introduced in a sand bath at 150 °C for 10 min and then cooled. After cooling, 2 mL of hexane and 4 mL of a saturated sodium chloride solution were added to the test tube, which was shaken for 5 s. The upper phase was collected, and 1 μL of this phase was injected into the gas chromatograph. The injector and detector temperatures were 290 °C. The initial oven temperature was 175 °C, maintained for 24 min, and then raised at 10 °C/min to 240 °C, which was kept constant for 7.5 min.

LC-TOF/MS Confirmatory Analysis of Olive Phenols. Olive phenol identification was conducted by LC-TOF/MS confirmatory analysis in accurate mode due to the complexity of OP extracts from EVOO. The extraction and separation conditions were identical to those for the LC-DAD determination, except for the use of the respective LC-MS grade solvents.

The phenolic fraction present in aliquots of EVOO and enriched oils was extracted, and the extracts were injected into the LC-TOF/MS system without additional pretreatment. The injected extract volume was 20 μL. The operating conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3250 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 Hz) in both the centroid and profile modes was governed via Agilent MassHunter Workstation software. The mass range and detection window were set at m/z 100−1100 and 100 ppm, respectively. Reference mass correction on each sample was performed with a continuous infusion of Agilent TOF biopolymer analysis mixture containing purine (m/z 121.0508) and hexamethoxyphosphazyne (m/z 322.0481) with resolution of 45000. Analytes were identified by accurate mass detection.

Molecular features were extracted from raw data files prior to formula generation. Two or more ions were used as compound ion count threshold. The isotope model corresponded to common organic molecules with peak spacing tolerance of m/z 0.0025 ± 7.0 ppm. Identification of the compounds proceeded by generation of candidate formulas with a mass accuracy limit of 4 ppm. The contribution to mass accuracy, isotope abundance, and isotope spacing scores were 100.00, 60.00, and 50.00, respectively. Retention times, formulas, experimental and theoretical masses, and errors, in ppm, obtained by accurate mass measurements of secoiridoids compounds are shown in Supplementary Table 1 of the Supporting Information. After confirmation, LC-DAD chromatographic peaks were assigned to OPs by retention times (both absolute and relative to external standard and other phenols monitored).

Quality Evaluation. Different tests were carried out to compare the quality of the edible oil before and after enrichment. Thus, the acidity index was calculated by titration with 0.1 M KOH and 2% phenolphthalein as indicator.[26]

This evaluation was completed with the determination of K_{232} and K_{270} parameters by spectrophotometric absorption of cyclohexane oil solutions at 232 and 270 nm, respectively.[27] Because these parameters are exclusive to the evaluation of the quality of olive oils, they were

Table 1. Concentration of the Main Olive Phenols Found in Extracts from Leaves and Alperujo

compound	leaf extract concn (μg/g)	alperujo extract concn (μg/g)
hydroxytyrosol	27.3	106.7
tyrosol	<LOD	18.5
vanillic acid	51.4	<LOD
vanillin	7.2	<LOD
p-coumaric acid	<LOD	<LOD
ferulic acid	10.0	<LOD
o-coumaric acid	<LOD	<LOD
3,4,DHPEA-EDA[a]	<LOD	2288.0
oleuropein	2479.0	820.0
p-HPEA-EDA[b]	<LOD	495.0
p-HPEA-FA[c]	<LOD	372.0
luteolin	11.9	56.3
apigenin	<LOD	13.4

[a] Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. [b] Dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol. [c] Aglycon of the dialdehydic form of ligstroside.

measured only in refined olive oil and then compared with those of EVOO.

Stability Study. Two complementary tests, peroxide index and Rancimat test, were carried out to compare the stabilities of the vegetable oils. The peroxide index was estimated by titration of 0.1 N KI saturated solutions of each oil with 0.1 N $Na_2S_2O_3$ and starch as indicator according to the official method.[24]

The Rancimat test was employed to evaluate the stability of target oils by conductimetry measurements of an aqueous solution; the volatile compounds were collected after each oil had been heated at 120 °C.[28] The concentration of polar compounds formed by oil oxidation increased the conductivity with time. During the induction period, the conductivity of the water increases very slightly; then, a high increase of conductivity indicates a massive oxidation: formation of a number of volatile polar compounds that are soluble in water. The stability of the oil is given by the induction period.

RESULTS AND DISCUSSION

Comparison of Phenol Extracts from Alperujo or Olive Leaves. According to previous studies developed with olive tree biomass, alperujo and olive leaves were selected as raw materials for isolation of OPs because they provide different phenolic profiles, as reported by Japón-Luján et al.[6,9] This was confirmed in this research, as Table 1 shows, for the concentration of the main phenols present in extracts from alperujo and olive leaves. As can be seen, alperujo is richer in secoiridoid hydrolysis compounds than leaves, whereas the latter are highly concentrated in oleuropein. Thus, hydroxytyrosol was found in alperujo extracts at concentrations 4 times higher than in leaves, and tyrosol was found in alperujo at concentrations around 20 μg/g and not detected in leaf extracts. These two compounds, hydroxytyrosol and tyrosol, are well-known because of their powerful antioxidant properties.[2,3,29] Similar results were obtained for other hydrolysis products from oleuropein and ligstroside such as the dialdehydic forms of secoiridoid aglycons, which were exclusively detected in alperujo extracts. Aglycon forms in EVOO included interesting compounds from an organoleptical point of view such as the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), the dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol (*p*-HPEA-EDA), and the aglycon of the dialdehydic form of ligstroside (*p*-HPEA-FA). Following extract comparisons, two flavonoids such as luteolin and apigenin were also more concentrated in alperujo extracts. In fact, apigenin was not detected in extracts from olive leaves.

On the contrary, the secoiridoid precursor of compounds previously described, oleuropein, was 3 times more concentrated in olive leaves than in alperujo (2480 versus 820 μg/g). Thus, alperujo extracts are well-balanced in the concentration of powerful antioxidants such as hydroxytyrosol, tyrosol, and other secoiridoid hydrolysis products, whereas leaf extracts are highly concentrated in oleuropein as one of the main precursors of these compounds. This different composition is a consequence of the conditions under which the raw materials are obtained. Alperujo was obtained from olive fruits at their optimum ripening stage with an advanced hydrolysis state of secoiridoid precursors such as oleuropein and ligstroside, thus reducing their presence in EVOO and their contribution to undesired bitterness of this oil. To finalize this comparison, simple phenols such as vanillic or ferulic acids and vanillin were not detected in alperujo extracts.

Discussion of the richness of each raw material in each of the most valuable phenols was based on the identical procedure for extraction followed in all cases: the same weight of raw material, type and volume of extractant, and time and working conditions for extraction, as well as degree of evaporation of the extractant for extract concentration.

Enrichment of Refined Edible Oils with Phenol Extracts from Alperujo or Olive Leaves. Refining of edible oils allows the maximum amount of oil to be extracted from seeds by an economical process. By this process, all impurities and natural flavors are removed and free fatty acids neutralized. This is carried out by subjecting oils to heat, solvent extraction, filtering, neutralization, distilling, degumming, bleaching, and high-heat deodorization. As a result of the operational conditions of these steps, polar compounds, including phenolic antioxidants, are completely removed. This can be checked in Supplementary Figure 1 of the Supporting Information, which illustrates chromatograms obtained by analysis of polar phenolic extracts from some of the target oils. As can be seen, no phenolic compounds were detected in these oils as compared to EVOO, which justifies the purpose of enrichment of these refined oils with OPs to improve their quality and stability.

The two levels, 200 and 400 μg/mL, for enrichment of target oils with phenol extracts from alperujo and olive leaves (two levels per extract) were selected as representative values because 200−400 μg/mL is the usual concentration range of phenols in EVOO, the model oil naturally containing phenols similar to those used for enrichment of the other oils.

The enrichment step was similarly performed for the different oils and degree of enrichment as programmed. Distinction among the different oils, different degrees of enrichment, and different extracts used for enrichment are discussed using the abbreviations under Materials and Methods. The discussions below concern first the degree of individual enrichment of the most important phenols as a function of the extracts and, then, the fatty acid profiles in the monitored oils.

Mass Transfer of Phenols to the Oils as a Function of the Extract. The richness of the extracts in the target phenols explains

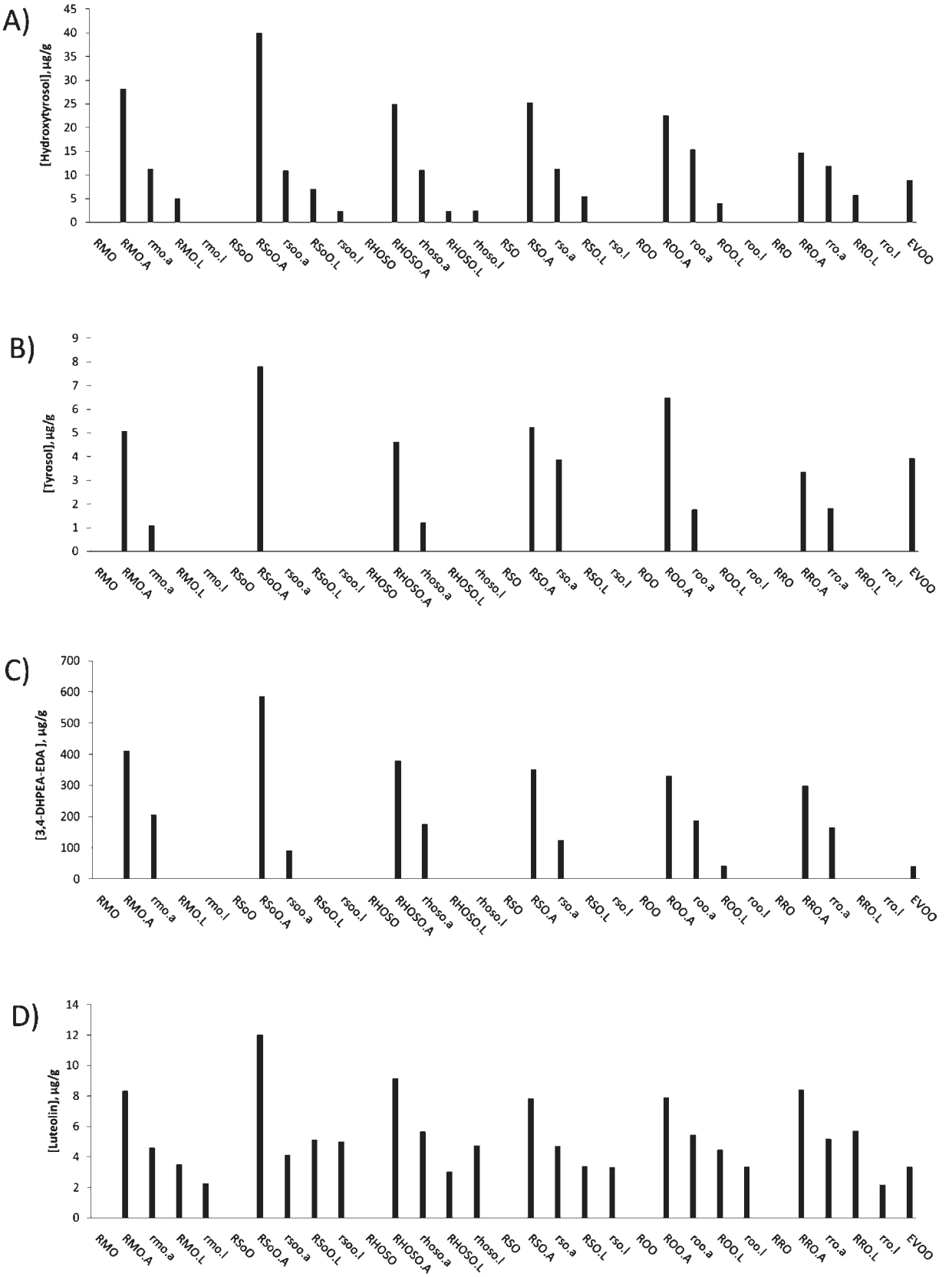

Figure 1. Oils enrichment in (A) hydroxytyrosol, (B) tyrosol, (C) 3,4-DHPEA-EDA, and (D) luteolin. (See the behavior of the other phenols in Supplementary Figure 2 of the Supporting Information.)

their mass transfer behavior and allows distinguishing between those with higher transfer from alperujo extracts (a), those with higher transfer from olive leaf extracts (b), and those which only are transferred from alperujo extracts (c). Supplementary Table 2

Table 2. Percent of Fatty Acids in the Pure Oils Used in This Study

fatty acid	formula	RMO	RSoO	RHSO	RSO	ROO	RRO	EVOO
myristic acid	C 14:0	0.03	0.07	0.04	0.06	0.01	0.05	0.01
palmitic acid	C 16:0	10.86	10.88	4.06	6.49	10.48	4.59	10.50
palmitoleic acid	C 16:1	0.13	0.10	0.13	0.17	0.86	0.24	0.76
margaric acid	C 17:0	0.07	0.08	0.03	0.04	0.09	0.11	0.10
margaroleic acid	C 17:1	0.03	0.05	0.03	0.03	0.16	0.06	0.14
stearic acid	C 18:0	2.07	3.05	3.86	3.44	3.05	1.64	3.53
trans-oleic acid	C 18:1n9t	0.00	0.00	0.00	0.00	0.00	0.00	0.00
oleic acid	C 18:1n9c	32.69	26.00	76.42	32.64	76.86	64.09	77.49
trans-linoleic acid	C 18:2n9,12tt	0.00	0.00	0.00	0.00	0.00	0.00	0.00
linoleic acid	C 18:2	52.28	54.11	13.50	55.70	7.31	18.71	6.05
linolenic acid	C 18:3	0.73	4.48	0.11	0.09	0.65	8.49	0.61
arachidic acid	C 20:0	0.45	0.29	0.32	0.24	0.24	0.50	0.39
gadoleic acid	C 20:1n9	0.26	0.22	0.17	0.14	0.18	1.08	0.25
behenic acid	C 22:0	0.18	0.47	1.01	0.69	0.08	0.33	0.11
lignoceric acid	C 24:0	0.22	0.19	0.32	0.25	0.03	0.12	0.06

of the Supporting Information lists the concentration of OPs in the target oils before and after enrichmen, revealing changes ascribed to the latter. Supplementary Figure 2 of the Supporting Information, which plots in a comparative way by bar diagrams the concentration of each individual phenol in each of the oils, and Figure 1, which plots the behavior in the enrichment process of the most representative phenols, support the discussion below.

(a) Alperujo extracts transfer to all target oils amounts of hydroxytyrosol, tyrosol, 3,4-DHPEA-EDA, and luteolin that greatly surpass the contents of these compounds in EVOO (e.g., between 15 and 40 μg/g in the case of hydroxytyrosol, whereas in EVOO the usual content does not surpass 10 μg/g) (Figure 1). This behavior was foreseeable as massive hydrolysis of secoiridoid precursors such as oleuropein occurs at the optimum ripening stage for collection of olive fruits. Additionally, the rich-in-water content of alperujo after oil separation also favors secoiridoid hydrolysis to release hydroxytyrosol and tyrosol as well as aglycon forms. The high content of hydroxytyrosol in all oils after enrichment with alperujo extracts increases their antioxidant power and endows them with the healthy properties attributed to this phenol.[2,3,29] The decarboxymethyl oleuropein aglycon was also highly enriched in all oils by using alperujo, which could be justified by its less polar character. This phenol was concentrated to values close to 600 μg/g expressed as oleuropein. This compound is frequently associated with a positive organoleptic property of EVOO being also a significant source of hydroxytyrosol. A lower enrichment factor was found for *p*-HPEA-EDA, which was lower than that observed in EVOO, and undetectable in the case of RMO and RSoO.

Concerning luteolin, especially noticeable is its transfer to RSoO, on the order of 12 μg/g. Vanillic acid, which seems to be absent from alperujo extracts (see Table 1), is in fact in these extracts, but the high dilution required for its chromatographic analysis lowers its concentration below the detection limit. Nevertheless, extractant evaporation increases the concentration of all phenols in the extract during oil enrichment, thus promoting a significant mass transfer of this acid to the oils, as listed in Supplementary Table 2 of the Supporting Information. Similar behavior is observed for *p*-coumaric acid, but to a lesser extent as it does not appear in the oils subjected to the lower concentration enrichment.

(b) Olive leaf extracts, because of their higher content in oleuropein, perform better mass transfer of this phenol to the target oils, as compared with alperujo extracts, all greatly surpassing the natural content of oleuropein in EVOO, which is practically undetectable because of the ripening state of the fruit for oil production (Figure 1). Ferulic acid, present only in olive leaf extracts, is transferred to RMO and RRO in a quantifiable concentration only in the higher enrichment degree (400 μg/mL total enrichment).

(c) Phenols only transferred from alperujo extracts are tyrosol (either surpassing or equalizing the natural content in EVOO), *p*-HPEA-FA (in this case never surpassing the natural content in EVOO, see Figure 1), and apigenin (equalizing the content in EVOO in the enrichment up to 400 μg/mL in total phenols).

Mass Transfer of Phenols to the Oils as a Function of Their Fatty Acid Composition. The mass transfer of phenols to the target oils was related to their fatty acid composition, which is displaye in Table 2 (concentrations expressed as percentage). Thus, the most polar OP and that with higher antioxidant power, hydroxytyrosol, experienced a higher transfer to oils with lower content in oleic acid and higher concentration of polyunsaturated acids. For this reason, the oil with the highest mass transfer was RSoO, with the lowest content of oleic acid and the highest content of polyunsaturated acids. RMO, the oil with the second highest enrichment in hydroxytyrosol, is also the oil with the second lowest content in oleic acid and with a similar content in polyunsaturated acids to RsoO. This trend was not followed for the rest of the oils, and similar enrichment was observed for RHOSO, RSO, and ROO. The lowest enrichment in hydroxytyrosol was found in RRO. Exactly the same trend was observed for an intermediate in the pathway from oleuropein to hydroxytyrosol such as 3,4-DHPEA-EDA.

The behavior of tyrosol in mass transfer from extracts to oils was similar to that of hydroxytyrosol. Thus, RSoO was the most enriched, followed by ROO, whereas the other oils experienced lower, but similar, enrichment except for RRO, which repeated as the lowest oil enriched by this phenol. Only alperujo extracts are able to enrich oils with this phenol as this was not detected in leaf extracts.

Ferulic acid enrichs only RRO and RMO, both with a linolenic acid content similar to that of EVOO. No mass transfer of this

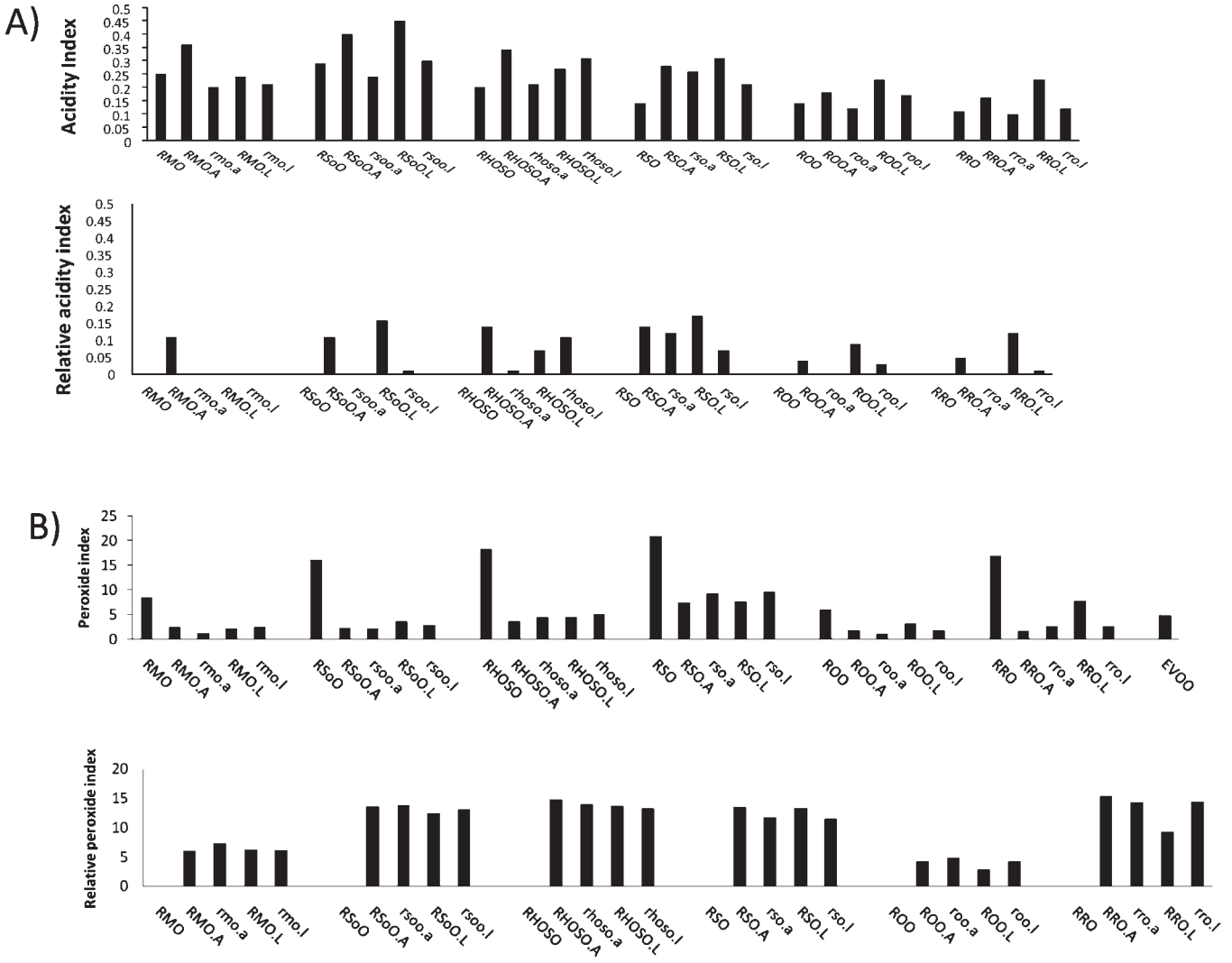

Figure 2. Target oils: absolute and relative (A) acidity indices and (B) peroxide indices.

phenol was observed with alperujo extracts. *p*-HPEA-EDA behaves similarly, but in this case the most enriched oil was RSoO.

Oleuropein enrichs preferably RMO and then RSoS, both clearly surpassing the enrichment experienced by the other oils. *p*-HPEA-EDA is preferably transferred to RHOSO and then to ROO and RRO, the oils with higher content in oleic acid. *p*-HPEA-FA mainly enrichs RsoO; then, and in a similar way, RHOSO, ROO, RRO, and RMO; finally, RSO, with a composition less similar to that of EVOO, is the lowest enriched.

Luteolin is transferred to all oils at higher concentrations from alperujo extracts, which quintuplicates the content of this phenol as compared with leaf extracts. All oils, when enriched at 400 μg/mL, clearly surpass the concentration of luteolin in EVOO.

Apigenin, present only in alperujo extracts, provides oils with a concentration similar to that naturally present in EVOO when they are enriched with 400 μg/mL of total phenols.

In light of Figure 1, Supplementary Figure 2 of the Supporting Information, and, especially, Supplementary Table 2 of the Supporting Information, the decision of preparing tailor-made enriched oils with high content of given olive tree phenols can be adopted as a function of both type of extract and oil. Thus, according to previous research,[14] corroborated by the present study, (i) oils with high content of saturated fatty acids are less prone to enrichment, particularly with the most polar phenols in the target extracts; (ii) oils with high content of monounsaturated fatty acids are more easily enriched with the target phenols as mass transfer is facilitated by these types of acids; (iii) oils with high content of polyunsaturated acids have been easily enriched and with higher content of the most polar phenols than monounsaturated ones, but the difference of the behavior between oils with high content of mono- and polyunsaturated fatty acids is not so important as between these two (mono- and polyunsaturated) and those oils with high content of unsaturated fatty acids.

Quality Tests. Common tests used in the industry and standardization organisms to evaluate the quality of olive oil were applied to compare the results obtained for the target oils, before and after enrichment, to those of EVOO. In this way, the effect of enrichment on quality characteristics can be assessed.

Acidity Index. This quality parameter, which is an indicator of the concentration of free fatty acids (usually expressed as percent of free oleic acid), decreases with increased quality of the raw material for oil production. According to legislation,[26] its concentration, in the case of olive oil, should not surpass 0.8%, being <0.3% when obtained from high-quality olive fruits collected at their optimum ripening stage.

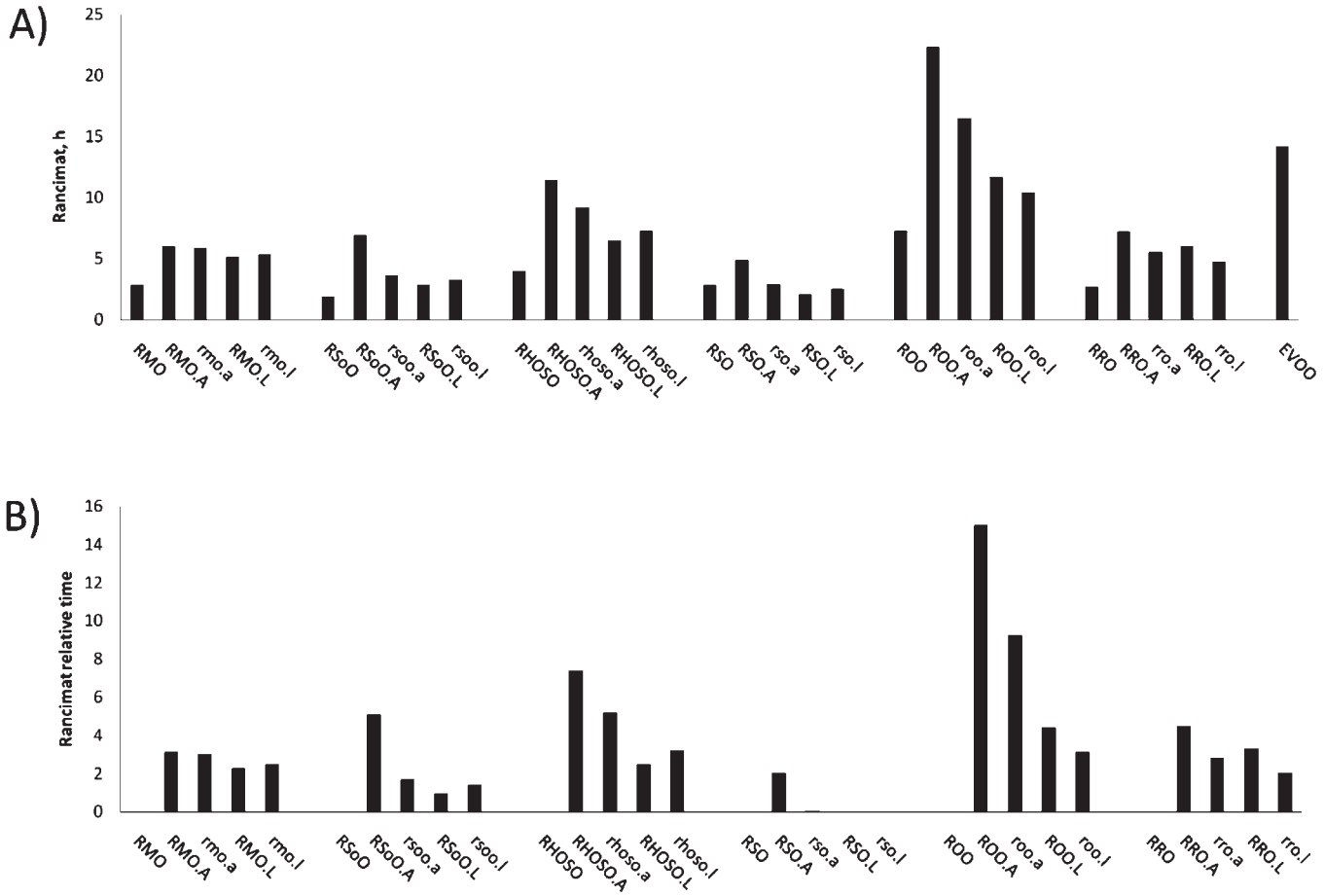

Figure 3. Rancimat values for (A) all enriched oils and EVOO and (B) each enriched oil divided by its pure counterpart.

As can be concluded from Figure 2A, the acidity index increased slightly by the mass transfer of phenols to the target oils with a maximum increase of 0.15 unit. The increase was particularly significant in oils enriched at higher phenol concentration with both types of extracts, except for RMO enriched with leaf extracts. Nevertheless, the change of acidity index was within an acceptable range. Therefore, there was not a critical contribution to this parameter caused by the use of extracts from alperujo or olive leaves for oil enrichment.

Determination of K_{232} and K_{270} Parameters. These parameters are measured only in olive oils with different quality grades, being one of the main characteristics for classification. For this reason, these parameters were measured only in pure and enriched refined olive oil to be compared with EVOO. Despite the use of K_{232} and K_{270} parameters to classify the quality of olive oils, they are strongly related to the stability and purity of oils, storage and technological aspects for oil extraction. In fact, K_{232} enables the evaluation of the oxidative deterioration of oils at short-term (primary oxidation of conjugated dienes), whereas K_{270} is used for long-term evaluation by contribution of other species (secondary oxidation of trienes). Thus, EVOO reported acceptable values of 1.74 and 0.18 absorbance arbitrary units (AU) for K_{232} and K_{270} indices, respectively, versus ROO that gave values of 2.22 and 0.33 AU. Therefore, the difference between EVOO and ROO is clear with reference to these parameters; however, the enrichment process modified positively these values. Thus, K_{270} decreased to 0.30 and 0.27 AU for enrichment of ROO with antioxidants from alperujo at 400 and 200 μg/mL, respectively. In the case of ROO enriched with antioxidants from leaves at 400 and 200 μg/mL, K_{270} was 0.34 and 0.28, respectively. A similar behavior was found for K_{232}, which decreased to 1.94 and 1.89 AU for ROO enriched at 400 and 200 μg/mL with alperujo phenols and to 1.80 and 1.76 AU for ROO enriched at 400 and 200 μg/mL with leaf phenols. It is worth emphasizing that there is a substantial improvement of both parameters in ROO enriched with hydrophilic antioxidants, although this effect is not linked to phenol enrichment degree.

Stability Tests. The stability of oils is related to their resistance to degradation, which can be evaluated through the peroxide index, the Rancimat test, or both.

Peroxide Index. This quality parameter is related to the primary oxidative stability of the given oil to avoid rancidity. It is expressed as miliequivalent oxygen/kg oil. All oxidation products usually produced by oxygen in the air in contact with the oil are considered under the umbrella of "peroxides". Figure 2B shows a significant increase of the stability of all enriched oils (from 60 to 90%) as compared with their nonenriched counterparts, which even became more stable than the reference EVOO in terms of primary oxidation except for enriched RSO versions. This behavior justifies by itself the enrichment of oils with the target phenolic antioxidants in light of the lengthened storage period thus achieved. No significant differences between enrichment with extracts from alperujo or leaves were observed for the same degree of enrichment.

Rancimat Test. The objective of this test is to evaluate the chemical quality of oils, so extreme conditions of temperature and ventilation are applied and the evolution of this dynamic parameter, which indicates deterioration of the oil, is monitored. The data obtained from the Rancimat study are plotted in Figure 3A, which shows the hours each oil remains in the induction period, before starting massive oxidation that marks quality deterioration; Figure 3B plots the ratio between the hours required by the given enriched oil and the pure oil, with no enrichment, thus showing clearly the improvement of resistance to oxidative processes, attributable to the enrichment.

Thus, both types of extracts confer to refined maize oil similar stability, which doubles that of the pure oil when the enrichment in phenols is 400 μg/mL.

Refined soy oil is more stable when enriched with 400 μg/mL of alperujo extracts (its stability becomes up to 5 times that of the pure oil). Enrichment with 200 μg/mL alperujo extracts and any enrichment with leaf extracts do not double the stability of pure oil.

Refined high-oleic sunflower oil achieves the highest stability (8 times higher than that of pure oil) by enrichment with 400 μg/mL phenols, in this case from alperujo extracts. The stability of RHOSO. A is similar to that of EVOO. Also, rhoso.a achieved a good stability (5 times higher than that of the pure oil). When leaf extracts are used, the stability increases between 2 and 3 times.

The only extract that increases the stability of RSO (and only at the highest concentration, 400 μg/mL) is alperujo, doubling, under these conditions, the stability of the pure oil. The stability of refined olive oil when enriched with alperujo extracts at 400 μg/mL increases 15 times, thus surpassing EVOO stability.

Refined rapeseed oil is better stabilized with alperujo extracts, surpassing 4.5 times that of the pure oil when the total phenol content in it reaches 400 μg/mL, whereas for 200 μg/mL the increase of stability is 2.5 times. RRO.L and rro.l increase their stability 5 and 2 times, respectively.

In light of both main phenols transferred and the characteristics of the given oils, it can be concluded that the increase of stability for oil storage conferred by enrichment justifies, in general, the implementation of this step. In some cases, as for ROO, and to a lesser extent for RRO, the enrichment is highly recommendable.

In short, all of the parameters of all oils under study improved, some of them in a dramatic manner, by the presence of OPs, so the obtainment of oils with better stability and quality (and with proved healthy properties, as widely assessed in nutritional studies[30]) is an easy, cheap, and highly recommendable process.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Supplementary Figures 1 (chromatograms obtained at 280 nm of phenolic extracts from EVOO (A) and sunflower oil (B)) and 2 (oil enrichment in other phenols not included in Figure 1 ((A) vanillic acid, (B) *p*-coumaric acid, (C) ferulic acid, (D) oleuropein, (E) *p*-HPEA-EDA, (F) *p*-HPEA-FA, (G) apigenin) as well as Supplementary Tables 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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