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Development of a Phenol-Enriched Olive Oil with Both Its Own Phenolic Compounds and Complementary Phenols from Thyme

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Supporting Information

ABSTRACT: Besides affecting the oil's sensorial characteristics, the presence of herbs and spices has an impact on the nutritional value of the flavored oils. The aim of the study was to develop a new product based on the phenol-enrichment of a virgin olive oil with both its own phenolic compounds (secoiridoid derivatives) plus additional complementary phenols from thyme (flavonoids). We studied the effect of the addition of phenolic extracts (olive cake and thyme) on phenolic composition, oxidative stability, antioxidant activity, and bitter sensory attribute of olive oils. Results showed that flavonoids from thyme appeared to have higher transference ratios (average 89.7%) from the phenolic extract to oil, whereas secoiridoids from olive presented lower transference ratios (average 35.3%). The bitter sensory attribute of the phenol-enriched oils diminished with an increase of the concentration of phenols from thyme, which might denote an improvement in the consumer acceptance.

KEYWORDS: *bitter index, olive phenols, ORAC, phenol-enriched olive oil, Rancimat test, thyme phenols*

INTRODUCTION

Over the past few years, the Mediterranean diet has gained in importance because of its relationship with a lower incidence of cardiovascular diseases and some types of carcinoma.[1] The irreplaceable elements in this dietary style include extra virgin olive oil and several spices and herbs.

Virgin olive oil (VOO) could be considered a functional food that, besides having a high level of monounsaturated fatty acids (MUFA), contains multiple minor components, such as phenolics, with relevant biological activity.[2,3] Recently, the European Food Safety Authority (EFSA) published a report substantiating the claims related to the health benefits attributed to polyphenols in olives and specifically to the protection of low-density lipoproteins (LDL) particles from oxidative damage, among other effects.[4] The scientific references backing this were basically human intervention studies. For instance, data from the EUROLIVE study showed that phenolic compounds from olive oil can modulate the postprandial oxidative stress after the ingestion of 40 mL of olive oil.[5] The degree of postprandial oxidative stress was inversely related to the phenolic content of olive oils from high (366 ppm) to low (2.7 ppm) phenolic content.

The daily intake of phenolics from VOO is low compared with that obtained from other phenolic sources. Therefore, the intake of VOO enriched with its own phenolic compounds could be of interest to increase the daily dose of these beneficial compounds. However, the enrichment of olive oil with its own phenolics has several drawbacks. In terms of sensory attributes, virgin olive oils with high phenolic contents have a bitter and pungent taste due to the presence of secoiridoids,[6] which could lead to rejection by consumers, particularly those from non-Mediterranean countries. Moreover, phenolic-compound-rich foods could have a dual action due to the fact that antioxidants can also act as oxidants. The administration of high doses of a single type of antioxidant could even promote, rather than reduce, lipid peroxidation, and it has been shown to increase atherosclerotic areas in animal models.[7,8] In this sense, it has been reported that giving high-risk individuals supplements of vitamin E promoted lipid peroxidation[9], while the combination of vitamin E and vitamin C was effective at reducing atherosclerosis in human trials.[10]

Besides, a novel therapeutic approach to suppress oxidative stress is based on the development of dual function antioxidants comprising not only chelating but also scavenging components.[11] Phenolic compounds present in virgin olive oil, specially secoiridoid derivatives of hydroxytyrosol, act similarly to phenolic acids, inhibiting the lipid oxidation by trapping free and peroxy radicals and also containing the ortho-diphenolic group necessary to chelate metals. Other antioxidants, such as flavonoids, also help to control the extent of lipid peroxidation by chelating metal (i.e., copper) ions.[11,12] However, this phenolic group makes a very small contribution to the total antioxidant capacity of VOO because of its low concentration.

Due to all of the above, the enrichment of an olive oil complementing the olive phenols with other kinds of phenolic compounds was considered in order to improve the nutritional profile and sensorial characteristics of olive oil. Spices, such as garlic, hot pepper, and various herbs, including thyme and rosemary, are widely used as flavor enhancers of olive oils, and changes in the chemical and sensory profile have been studied.[13] Earlier studies on herbs and spices have considered their bioactive compounds from the perspective of antioxidants and anti-inflammatories, where the most important phytochemicals isolated include phenolic terpenes (thymol, carvacrol, carnosic acid), hydroxycinnamic acids and derivatives (rosmar-

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inic acid, eugenol), and flavonoids (luteolin, thymusin, and xanthomicrol, among others).[14,15] Thyme is the herb selected in this study for the olive oil flavoring, as it is one of the richest sources of flavonoids.[16]

The aim of this study was to develop an olive oil enriched with both its own phenolic compounds and with additional complementary phenols from thyme and to establish the best ratio between the quantities of polyphenols from each of these sources.

MATERIALS AND METHODS

Samples. The virgin olive oils used as the 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 from Arbequina cultivar. On the other hand, the samples of olive cake that were used to obtain the phenolic extract were from a commercial olive mill in the same area. These samples were taken at the decanter outlet, and liquid nitrogen was immediately added to avoid oxidative damage. The olive cake was then stored at −40 °C and freeze-dried in order to preserve it until the preparation of the phenolic extracts. Dried thyme (*Thymus zygis*) was supplied by Sabater Spices (Murcia, Spain).

Chemicals and Reagents. Apigenin, apigenin-7-*O*-glucoside, luteolin, luteolin 7-*O*-glucoside, oleuropein, rutin, tyrosol, verbascoside, naringenin, kaemferol, eriodictyol, rosmarinic acid, taxifolin, quercetin-4′-*O*-glucoside, 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 are not available commercially and were isolated from the VOO phenolic extracts by semipreparative HPLC.[17] 2,2-Azobis(2-amidinopropano) 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 the Phenolic Extracts. Eleven phenolic extracts (PE) were prepared from freeze-dried olive cake (OC) and dried thyme (T) in different proportions, mixing previously to each extraction 10 g in total. The proportions varied from 100% OC to 100% thyme, and the different phenolic extracts were coded according to the percentage of thyme from 0% to 100% (PE0, 100% OC, 0% T; PE10, 90% OC, 10% T; PE20, 80% OC, 20% T; PE30, 70% OC, 30% T; PE40, 60% OC, 40% T; PE50, 50% OC, 50% T; PE60, 40% OC, 60% T; PE70, 30% OC, 70% T; PE80, 20% OC, 80% T; PE90, 10% OC, 90% T; PE100, 0% OC, 100% T). The phenolic extracts were obtained using an accelerated solvent extractor (ASE 100 Dionex, Sunnyvale, CA). This method was previously used and optimized by Suárez et al.[18] to prepare a phenol extract from olive cake. 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 the sample (10 g) to increase the contact surface, avoid 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 the ethanol had been eliminated, freeze-dried, and stored at −80 °C until its use for oil enrichment. We obtained 1.96 ± 0.16 g of phenolic extract from 10 g of raw material.

Preparation of the Phenol-Enriched Olive Oils. Once these different PE extracts had been obtained, they were used to prepare olive oils with high phenolic content in the ratio of 2.5 g of extract/100 g of oil. Eleven phenol-enriched olive oils (EOO) were prepared and the oils were codified similarly by the percentage of thyme: EOO0, EOO10, EOO20, EOO30, EOO40, EOO50, EOO60, EOO70, EOO80, EOO90, and EOO100. To carry out the phenolic enrichment of the oils, 2.5 g of PE was dissolved in water (2% of the final oil volume) and mixed until complete dispersion with the vortex mixer. The water dispersion was then added to a small amount of oil (8 mL) to make a previous emulsion using an ultrasonic bath. This volume was then mixed with the rest of the oil (to 100 mL) using a Polytron (Kinematica, Littau, Switzerland) until completely homogenized. Finally, the EOO was centrifuged for 10 min at 3500 rpm to remove the extract solid waste. The oil was bottled in dark bottles until analysis.

Phenol Analysis of the Phenol-Enriched Olive Oils. The phenolic compounds of the olive oil samples were extracted following the method described in our previous paper.[19] Briefly, 20 mL of methanol/water (80:20, v/v) was added to 5 g of oil and homogenized for 2 min with a Polytron. After that, the two phases were separated by centrifuging at 3000 rpm for 10 min, and the hydroalcoholic phase was evaporated to obtain a syrupy consistency at 31 °C and then 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.

Chromatographic Analysis of the Phenols by HPLC−ESI-MS/MS. The phenolic composition of the phenolic extracts (PE) and phenol-enriched olive oils (EOO) was analyzed by HPLC coupled with tandem mass spectrometry (MS/MS). The HPLC consisted of an AcQuity liquid chromatography system equipped with a Waters binary pump system (Milford, MA) using a SunFire C_{18} column (3.5 μm, 4.5 × 150 mm), also from Waters. During the analysis, the column was kept at 30 °C and the flow rate was 0.8 mL/min. The mobile phase was Milli-Q water/acetic acid (99.8:0.2, v/v) as solvent A and acetonitrile as solvent B. The elution gradient was as follows. It started at 5% of eluent B for 5 min, was linearly increased to 40% of eluent B in 20 min, and further increased to 100% of eluent B in 0.1 min, and the reequilibration time was 1.9 min. The HPLC system was coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA). The software used was MassLynx 4.1. Ionization was done by using an electrospray (ESI) interface operating in the negative mode, $[M - H]^-$, and the data were collected in the selected reaction monitoring (SRM) mode. The ionization source parameters were the same as those described in our previous report.[19] The SRM transitions and the individual cone voltage and collision energy were optimized for each phenolic compound. Two SRM transitions were studied in order to find the most abundant product ions. The most sensitive transition was selected for quantification and a second one for confirmation purposes (Supporting Information). The phenolic compounds were quantified by the calibration curves for the respective commercial standards. The 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), and oleuropein aglycon (3,4-DHPEA-EA) were quantified as 3,4-DHPEA-EDA; and elenolic acid, ligstroside derivative, and oleuropein derivative were quantified as oleuropein. The injection volume was 10 μL, and all the samples were filtered through a 0.22 μm Nylon filter (Tecknokroma, Barcelona, Spain) before analysis.

Oxidative Stability of the Olive Oils by the Rancimat Test. The oxidative stability of the olive oils (EOO) 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 (3 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 triplicate, and a control (VOO 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 the ORAC Assay. The hydrophilic ORAC was based on the methodology described by

Table 1. Phenolic Composition of the 11 Phenolic Extracts (PE) Prepared with Both Phenolic Sources Olive Cake and Thyme by HPLC−ESI-MS/MS

	phenolic composition (mg/g PE)										
compd	PE0	PE10	PE20	PE30	PE40	PE50	PE60	PE70	PE80	PE90	PE100
Phenyl Alcohols											
tyrosol	0.27	0.94	0.65	0.45	0.27	0.68	0.50	0.58	0.72	0.27	0.15
hydroxytyrosol	2.77	3.65	3.01	2.44	2.05	2.09	1.35	1.61	1.20	0.80	0.19
Phenolic Acids											
vanillic acid	0.67	1.52	1.51	1.16	1.63	1.32	1.36	1.27	1.02	1.63	1.28
caffeic acid	0.03	0.09	0.12	0.17	0.23	0.38	0.25	0.93	0.62	0.63	0.64
hydroxybenzoic acid	0.02	0.10	0.19	0.30	0.50	0.53	0.68	0.85	0.91	1.11	1.39
homovanillic acid	0.00	0.07	0.10	0.11	0.22	0.15	0.29	0.25	0.27	0.34	0.50
rosmarinic acid	0.73	2.24	3.81	4.43	5.07	10.6	10.8	10.7	8.33	14.9	17.8
Secoiridoid Derivatives											
3,4-DHPEA-AC	0.64	0.65	0.65	0.59	0.63	0.46	0.54	0.50	0.43	0.00	0.00
elenolic acid	13.2	9.93	9.58	9.55	9.46	6.22	4.00	4.31	2.51	1.48	0.00
p-HPEA-EDA	4.56	9.22	9.92	8.45	2.16	1.94	1.74	1.99	1.88	0.00	0.00
p-HPEA-EA	4.32	8.84	9.59	8.20	1.92	1.73	1.56	1.91	1.82	2.20	0.00
3,4-DHPEA-EA	5.36	9.61	10.6	9.06	11.1	8.97	9.00	13.4	9.41	6.42	0.00
3,4-DHPEA-EDA	47.4	31.1	30.1	30.6	29.6	21.7	16.2	18.5	11.1	7.17	0.00
Lignans											
pinoresinol	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
acetoxypinoresinol	1.17	1.66	2.34	1.24	1.67	0.41	1.11	0.76	0.24	0.12	0.00
Flavonoids											
apigenin	0.04	0.06	0.10	0.13	0.16	0.22	0.29	0.35	0.32	0.30	0.31
luteolin	0.49	0.59	1.01	0.98	1.09	1.27	1.10	1.24	1.73	1.22	1.45
naringenin	0.02	0.10	0.28	0.43	0.49	0.56	0.94	1.11	0.97	1.46	1.91
eriodictyol	0.00	0.10	0.34	0.51	0.70	1.05	1.17	1.21	2.13	2.19	2.79
taxifolin	0.02	0.05	0.08	0.15	0.19	0.16	0.26	0.24	0.72	0.57	0.47
eriodictyol-rutinoside	0.00	0.92	1.63	1.74	2.70	3.63	3.71	5.21	5.10	9.79	13.8
thymusin	0.00	2.17	7.24	7.19	10.8	12.8	8.94	10.5	11.1	16.6	19.4
xanthomicrol	0.00	0.26	0.90	1.20	2.05	3.29	3.28	3.32	4.24	4.93	7.98
7-methylsudachitin	0.00	0.24	0.72	1.42	2.13	3.83	4.46	5.04	3.70	4.97	6.12

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. 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. For the analysis, all the 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 used for chromatographic analysis (see Phenol Analysis of the Phenol-Enriched Olive Oils) or Trolox at different concentrations (from 0.415 to 4.15 ng/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. 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 (TE) per 100 g of oil using Trolox and the sample calibration curves obtained in each analysis.

Phenol Transference Ratio. In order to know the effectiveness of the phenol transference from the extract (PE) to the oil (EOO), the transference ratio (TR) was calculated with the formula:

$$\mathrm{TR} = \frac{\text{oil PC}}{(2.5 \times \text{extract PC})/100} \times 100\% \quad (1)$$

The oil phenolic content (PC) and the extract phenolic content were expressed as milligrams of phenol per kg of oil and extract, respectively. We took into account that the enrichment was carried out with 2.5 g of extract per 100 g of oil. The TR for each phenol was expressed as a percentage.

Bitter Sensory Attribute of the Enriched-Olive Oils. The aim of the sensorial analysis was to assess the bitter taste of the flavored oils in order to know if flavoring with thyme could improve the acceptance of the phenol-enriched olive oils in terms of bitterness. The analysis was carried out by a panel of six tasters, previously selected from 30 people according to the procedure of UNE 87−003−95,[21] where the tasters had to perceive differences in concentrations of various bitter solutions prepared with caffeine. This group selected had an intense sensitivity to the bitter taste. A specific profile sheet was set up for the flavored oils where the six judges were asked to assign a score for the bitter taste of six EOO (EOO0, EOO20, EOO40, EOO60, EOO80, and EOO100) on a 10-point scale going from "absent bitter taste" to "extremely perceptible bitter taste". Before that, the members of the group were trained together in order to reach a common agreement on the bitter scale points.

Statistical Analysis. All the data are expressed as the mean of three replicates. In order to simplify the results shown in the tables, we omitted the standard deviation, as all these values were lower than 10%. The data analyses were performed using the Statgraphics plus v. 5.1 software (Manugistics Inc., Rockville, MD). The data were analyzed by the one-way ANOVA test with a significance level of 0.05.

RESULTS AND DISCUSSION

Phenolic Composition of the Phenolic Extracts (PE). Table 1 shows the phenol content of the 11 PEs determined by HPLC−ESI-MS/MS. Secoiridoid derivatives were the main compounds found in the PE0 extract obtained exclusively from olive cake, the 3,4-DHPEA-EDA being the most abundant (47.39 mg/g of PE0 extract). Curiously, the concentration of some secoiridoids (*p*-HPEA-EDA, *p*-HPEA-EA, and 3,4-

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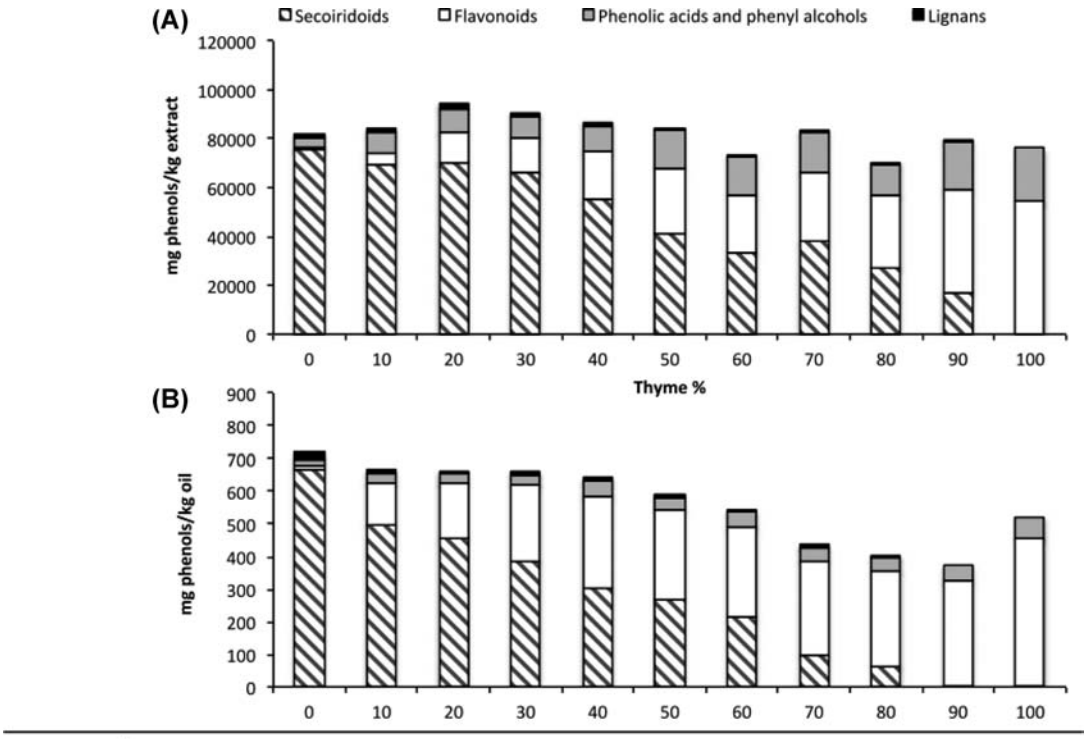

Lignans	38,6	30,9	23,7	31,3	32,2	28,3	25,3	45,0	12,0	0,0	0,0
Phenolic Acids and Phenyl alcohols	16.2	12.6	11.5	12.7	16.9	8.5	11.6	10.1	13.3	9.3	12.3
Flavonoids	82.3	89.7	56.2	68.8	56.4	41.3	46.3	41.0	39.0	31.0	33.5
Secoiridoids derivatives	35.3	28.7	25.7	23.2	21.9	26.2	24.9	9.1	8.6	0.0	0.0

Figure 1. Distribution of different phenolic groups of the phenolic extracts (A) and enriched olive oils (B). The table below shows the transference ratio from extract to oil (expressed as percentages) of each phenolic group for each experiment.

DHPEA-EA) increased slightly in the PE10 compared with the PE0, indicating that the presence of phenols from thyme may have a protective effect on olive phenols by reducing their losses during the accelerated solvent extraction. In relation to flavonoids, their content in the PE extracts increased with the proportion of thyme in the sample used for phenol extraction. Thus, in the extract obtained exclusively from thyme (PE100) flavonoids was the main phenolic group. The main flavonoids provided by the thyme were thymusin (19.36 mg/g PE100), eriodictyol-rutinoside (13.76 mg/g PE100), xanthomicrol (7.98 mg/g PE100), and 7-methylsudachitin (6.12 mg/g PE100). Apart from flavonoids, a dominant compound found in extracts containing thyme was rosmarinic acid (17.8 mg/g of extract in PE100). Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid typically found in *Lamiaceae* plants, such as basil (*Ocimum* spp.), rosemary (*Rosmarinus* spp.), thyme (*Thymus* spp.), mint (*Mentha* spp.), and oregano (*Origanum* spp.).[22] The evidence for the therapeutic potential of this compound as an antioxidant and anti-inflammatory compound has been recently reviewed.[15] The two *o*-dihydroxy groups (catechol structures) are the main active groups of the molecule and contribute to its strong potential as an antioxidant agent, which is mainly expressed by the H-abstraction reaction.[23]

Figure 1A shows the sum of total phenols by phenolic groups (phenyl alcohols and phenolic acids, secoiridoids, lignans, and flavonoids). The total phenolic content was similar in all the PEs. The main differences between the 11 PEs can be seen in the proportion of the major phenolic families. These results showed a direct relationship between the content of thyme in the sample and the flavonoid content in the phenolic extract and, similarly, between the content of olive cake in the sample used for obtaining the extract and the secoiridoid concentration. As seen, the strategy of mixing different plant sources rich in phenols may allow one to obtain custom-made extracts. In this sense, thyme clearly contributes to complementing the lack of flavonoids in the olive cake extract (PE0). Thus, extracts with different percentages of flavonoids or rosmarinic acid could be prepared by modifying the percentage of thyme in the sample used to prepare the phenolic extract.

Phenol-Enriched Olive Oil Composition and Transference Ratio. Eleven EOOs were prepared by the addition of different PEs to a base of VOO (ratio 2.5 g of PE per 100 g of VOO). The phenolic composition of the oils was analyzed by HPLC−ESI-MS/MS. As seen in Table 2, the main phenolics in the EOOs enriched with a high proportion of PE obtained from olive cake (EOO0) were the most representative phenols of virgin olive oils, the 3,4-DHPEA-EDA and *p*-HPEA-EDA (365 and 77.2 mg/kg of oil, respectively). These compounds are of special interest because they 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.[24] Therefore, higher quantities of these compounds could appear in the plasma, improving the health benefits of consuming olive oil. On the other hand, in EOOs with a high proportion of thyme, the most abundant phenolic compounds were again the same as in the extracts. For instance, EOO100 presented a high content of thymusin and xanthomicrol (268 and 60.9 mg/100 g of oil, respectively). As shown in Figure 1B, the concentration of major phenolic

Table 2. Phenolic Composition of the Control (olive oil used as enrichment matrix) and the 11 Phenol-Enriched Olive Oils (EOO) by HPLC−ESI-MS/MS[a]

	phenolic composition (mg/kg oil)											
compd	control	EOO0	EOO10	EOO20	EOO30	EOO40	EOO50	EOO60	EOO70	EOO80	EOO90	EOO100
Phenyl Alcohols												
tyrosol	1.50	6.68	7.75	4.90	7.33	3.61	2.66	1.65	1.34	2.55	0.00	0.00
hydroxytyrosol	0.27	5.47	4.98	5.92	4.76	7.37	3.05	2.72	3.96	1.45	0.93	0.00
Phenolic Acids												
vanillic acid	0.06	5.66	9.42	9.59	5.35	6.48	3.26	3.90	2.86	2.31	2.77	1.28
caffeic acid	0.00	0.17	0.22	0.38	0.65	1.12	1.21	1.19	1.94	2.10	2.07	2.68
hydroxybenzoic acid	0.00	0.26	1.28	2.24	3.17	6.27	5.88	8.39	10.6	12.2	13.6	19.5
homovanillic acid	0.00	0.00	2.10	2.68	1.32	1.41	1.70	1.62	4.48	3.03	3.03	3.32
rosmarinic acid	0.00	0.00	1.35	1.31	6.12	16.0	15.6	24.5	15.5	18.1	23.3	40.4
Secoiridoid Derivatives												
3,4-DHPEA-AC	0.00	3.93	3.43	3.52	3.25	3.00	2.83	2.53	2.33	2.11	0.00	0.00
elenolic acid	57.5	103	68.6	61.9	58.0	10.5	10.2	0.00	0.00	0.00	0.00	0.00
p-HPEA-EDA	52.5	77.2	73.2	71.1	68.8	15.7	17.1	13.14	11.9	10.7	0.00	0.00
p-HPEA-EA	15.4	67.9	68.1	69.9	65.4	14.6	13.1	10.7	8.77	0.00	0.00	0.00
3,4-DHPEA-EA	30.9	48.5	36.0	38.0	28.6	13.2	10.3	6.97	6.03	5.21	2.34	0.00
3,4-DHPEA-EDA	6.99	365	248	209	162	244	215	179	69.7	45.4	6.42	0.00
Lignans												
pinoresinol	1.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
acetoxypinoresinol	60.09	23.1	12.9	8.03	9.71	13.5	11.6	6.99	7.57	0.72	0.00	0.00
Flavonoids												
apigenin	0.87	0.87	1.07	2.31	2.24	2.49	2.45	2.54	3.12	3.60	3.20	3.65
luteolin	1.37	10.2	8.79	6.42	3.64	9.28	6.08	7.46	5.73	7.46	4.90	5.78
naringenin	0.00	0.59	1.48	4.24	4.44	4.96	4.58	4.58	7.75	13.2	12.0	32.1
eriodictyol	0.00	0.00	1.32	2.53	3.38	9.59	5.90	6.97	12.4	10.9	10.7	15.1
taxifolin	0.00	0.02	0.18	0.27	0.34	0.61	0.55	0.83	0.86	1.02	1.04	1.33
eriodictyol-rutinoside	0.00	0.00	0.34	0.61	1.09	0.37	0.63	1.31	0.26	0.80	0.53	3.14
thymusin	0.00	0.00	102	135	189	207	201	197	175	184	189	268
xanthomycrol	0.00	0.00	6.66	10.0	16.6	26.2	29.1	30.6	42.5	48.6	52.5	60.9
7-methylsudachitin	0.00	0.00	6.62	11.3	15.4	25.5	26.9	28.8	41.9	23.1	51.1	62.4

[a]The concentration of each phenol in EOO is expressed as mg/kg oil after subtracting the amount of phenol in the control oil.

groups (secoiridoids, flavonoids, phenolic acids and phenyl alcohols, and lignans) quantified in different prepared oils is proportionally similar to the extract used for their preparation. However, the total amount of phenolic compounds was not the same for all the EOOs, although the enrichment was carried out with the same amount of extract (2.5 g per 100 g of oil). To understand these differences, a transference ratio (TR) (see eq 1) was calculated for each phenol. As shown in the table of Figure 1, flavonoids presented noticeably higher TRs than the other phenolic groups, ranging from 89.7% in EOO10 to 31.0% in EOO90, whereas the other major phenolic group, the secoiridoids, presented TRs between 35.3% in EOO10 and 0% in EOO90. Differences in the TR could be related with the molecular structure of phenols. Thus, the more apolar nature of flavonoids compared with the secoiridoids (monophenolic structures) could justify the higher transfer efficiency of flavonoids from the extract to a lipophilic matrix, such as olive oil.

According to the results, EOOs developed with a high proportion of thyme will provide an important amount of flavonoids, which have been shown to have multiple positive effects on human health. The direct scavenging potential of flavonoids has been traditionally related to their health benefits; however, current research has gradually changed this dogma by emphasizing their transition metal chelation properties and direct interaction with some enzymes and blood/vascular cells.[25,26] According to recent studies, the chelation activity of flavonoids depends on their molecular structure, and the most effective iron binding sites in flavonoids are those with two hydroxyl groups in an ortho position in the aromatic rings, the 4-keto with the 3-OH and the 4-keto with the 5-OH groups.[12,27] Most of the important flavonoids occurring in the phenolic extracts obtained with high proportions of thyme (Figure 2) appear to have these sites, and rosmarinic acid even has two potential chelating sites. This suggests that these phenolic molecules might act synergistically as potent iron chelators, thus, as aimed in this study, complementing the

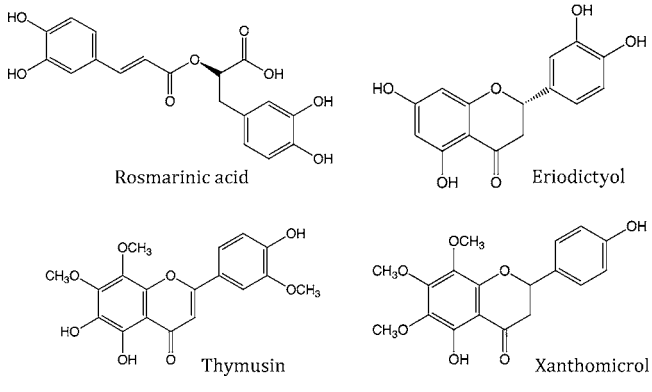

Figure 2. Chemical structure of the most abundant phenolic compounds found in thyme.

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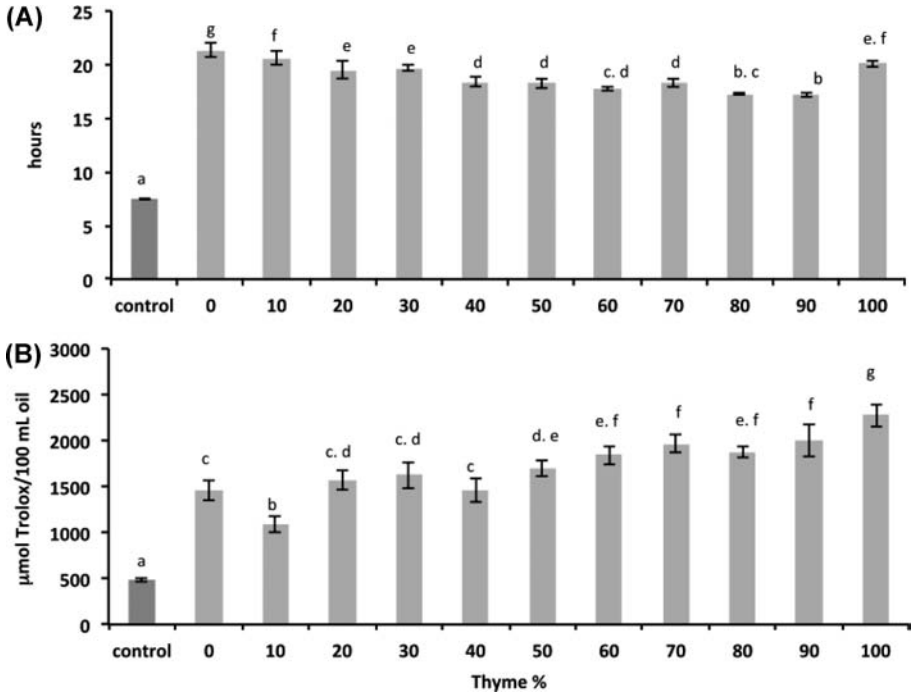

Figure 3. Effect of the phenolic enrichment on the oxidative stability by the Rancimat test (A) and antioxidant capacity by ORAC assay (B) of the control (virgin olive oil) and the 11 phenol-enriched olive oils. Different letters above indicate significant differences between the oils ($p < 0.05$).

radical scavenging activity of the secoiridoid derivatives from the olive oil, their main antioxidant mechanism.

Oxidative Stability and Antioxidant Capacity. The oxidative stability of the oils was measured with the Rancimat test, and the results (Figure 3A) showed that in all the EOOs the phenol enrichment produced a significant increment in the oxidative stability compared with the control oil, indicating that both phenolic sources are able to improve the oxidative stability. Additionally, 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 ORAC assay results again show that all the EOOs showed a substantial increase compared with the control oil, and antioxidant capacity increased gradually with the thyme phenolic enrichment (Figure 3B). This could be explained by the potent antioxidant capacity of the herbs reported before in different studies.[28,29] The last USDA database for ORAC of selected foods[30] provided very high values of hydrophilic ORAC for dried thyme, ranging from 131 000 to 139 400 μmol TE/100 g. In this regard, the incorporation of active ingredients of the spices can be a good strategy to improve the oxidative capacity of the olive oil and for the development of new foods.

Bitter Sensory Attribute. In VOOs, bitterness and pungency sensations are related to the presence of phenolic compounds and can persist for rather a long time after deglutition, showing a clear after-effect that might affect consumer acceptance, especially if the olive oil contains a higher level of phenolic compounds.[31] García et al.[32] indicated the dialdehydic form of decarboxymethylelenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) and oleuropein aglycon (3,4-DHPEA-EA) as the main compounds responsible for the bitter taste of VOO; Tovar et al.[33] attributed bitter and pungent notes to ligstroside derivatives, such as *p*-HPEA-EDA. As seen in Figure 4, the EOO0 rich in secoiridoid derivatives had the highest bitter score in the sensory test, compared with those

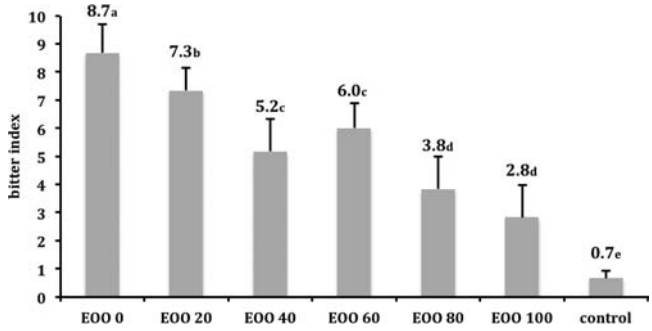

Figure 4. Sensory analysis comparing the bitter taste of the enriched olive oils with the control oil on a 10-point scale going from "absent bitter taste" (0) to "extremely perceptible bitter taste" (10). Different letters above indicate significant differences between the oils ($p < 0.05$).

with a lower proportion of olive phenols and higher proportion of thyme phenols. These results confirm the role of secoiridoids in the bitter attribute of VOO. Those EOOs combining olive extract and thyme had lower bitterness than EOOs enriched only with olive oil phenols, indicating that the use of herbs or species may improve consumer acceptance of high-phenol olive oils, besides the improvement in their oxidative stability. When combining both phenolic sources, aromatic components present in thyme could also reduce the perception of bitterness due to a bitterness-depressing effect caused by interference with the receptor sites as suggested in some studies.[34,35]

In summary, this study supports the original aim of developing a phenol-enriched olive oil tailored to provide the best relationship between different kinds of phenolic compounds. We conclude that the optimal enriched olive oil with the more balanced phenol composition appears to be EOO40 (prepared with phenolic extract obtained with 60% olive and 40% thyme), which provides the highest amount of

secoiridoid derivatives and flavonoids. Moreover, this study revealed that working with extracts instead of infusions, as is traditional when preparing flavored olive oils, allows a phenol-enriched and seasoned olive oil to be developed with exactly the amount of phenols required, which is an important factor when a functional food is produced.

■ ASSOCIATED CONTENT

Supporting Information

Table of SRM conditions used for the quantification of the phenolic compounds by HPLC−MS/MS. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycon; 3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihidroxybenzene; *p*-HPEA-EDA, dialdehydic form of elenolic acid linked to tyrosol

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