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Dependence of Fatty-Acid Composition of Edible Oils on Their Enrichment in Olive Phenols

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Olive phenol extracts from waste from olive-oil production (alperujo) have been obtained by microwave-assisted extraction and used for edible oil enrichment. The extracts as such or after extractant removal were used to enrich edible oils of different fatty acid composition by liquid−liquid or solid−liquid extraction, respectively. The distribution ratios of the phenols in the different oils [olive-orujo (the waste of milled olives from which low-quality oil is obtained), sunflower, high oleic-acid content sunflower, coconut, and linseed] showed a given order as a function of phenol polarity and molecular weight, with higher distribution factors for more polar and lower molecular-weight phenols. Concerning oil composition, those oils with higher concentration of polyunsaturated fatty acids yielded higher phenol distribution factors; oils with higher concentrations of saturated fatty acids yielded lower distribution factors.

KEYWORDS: Edible oil-enrichment; olive phenols; alperujo; distribution factor; microwave-assisted extraction

INTRODUCTION

It is well-known that the Mediterranean diet involves the intake of moderate amounts of olive oil, which contribute to decrease the incidence of chronic diseases thanks to its healthful properties (*1*).

The components of olive oil can be divided into two groups as a function of their concentration, major and minor compounds. The first group, known as the saponifiable fraction, represents more than 98% of the total weight of the oil and consists of triglycerides, diglycerides, monoglycerides and free fatty acids. The second group, the unsaponifiable fraction, comprising about 2% of the total weight, is constituted by a great variety of compounds such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants. Most of these antioxidants are carotenes and phenols (*2*).

The phenolic fraction of virgin olive oil has not been completely characterized due to the complexity of both phenols and matrix. In recent studies it has been found that olive oil is a source of at least 30 phenolic compounds (*3*, *4*) which can be divided into six families, namely, phenolic acids, phenyl ethyl alcohols, hydroxy-isochromans, flavonoids, lignans and secoroiridoids. The main constituents of the phenolic fraction of the olive oil, hydroxytyrosol, tyrosol, oleuropein and their conjugated forms, belong to one of these families (*5*).

Phenolics present in olive oil are used for their nutraceutical capabilities, well-known by consumers. For example, apigenin-7-glucoside is used in the therapy of Alzheimer's (*6*) and liver diseases; hydroxytyrosol is a strong antioxidant (*7*); luteolin-7-glucoside avoids the abnormal proliferation of aortic vascular smooth muscle cells that is a common cause of atherosclerosis (*8*); oleuropein prevents cardiac diseases and improves lipid metabolism (*9*); and tyrosol is an antioxidant protector of the circulating system (*10*).

While lipophilic phenols such as tocopherols can be found in other vegetable oils, most hydrophilic phenols found in olive oil are not common to other oils or fats such as coconut and sunflower (*11*). Taking into account the high price of olive oil and the antioxidant and nutraceutical capacities of olive phenols, which may be added to selected foods, there is a growing interest in the use of these compounds to enrich low-priced oils (*12*, *13*), which contain a minimum concentration of or no phenolic compounds, in order to obtain a healthful added-value product.

The main sources of olive phenols (*14*, *15*) are olive leaves and olive oil industry wastes, orujo, alpechín or alperujo, depending on the manufacturing process of the oil. Alperujo is a semisolid residue from three-phase olive oil production with a high polluting organic load, which includes sugars, tannins, phenols, polyalcohols, pectins and lipids. The concentration of phenolic compounds in alperujo is 100 times higher than in olive oil, a fact which can be explained by the polar nature of the alperujo phase versus the nonpolar olive oil (*16*). These compounds can be removed from alperujo by conventional solid−liquid extraction or by new extraction methods based on the use of auxiliary energies such as microwaves, ultrasound

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(*16*) or superheated liquids, which accelerate and/or improve the extraction step.

A cleanup step of the extract can be necessary prior to its use as an enricher phase. Conventional cleaning strategies are liquid−liquid extraction using hexane as extractant or solid-phase extraction. In the latter case, Amberlite XAD type is the most usual sorbent (*17*).

There are three alternatives in the literature for oil enrichment with these valuable compounds from the olive tree: liquid−liquid extraction (*18*), in which the oil is put into contact with an alcoholic phenol solution, the phenols are transferred to the oily phase as a function of their distribution factor and the alcoholic phase is removed by centrifugation; solid−liquid extraction (*19*), 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 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.

The aims of the present research were to study phenol mass-transfer to edible oils with different fatty acid composition, i.e. coconut, sunflower, high oleic sunflower, linseed and orujo; to establish the dependence of enrichment factors on the type of oil and enrichment procedure; and to enrich edible oils with phenols from alperujo extracts.

MATERIAL AND METHODS

Samples. Alperujo from Núñez de Prado, C.B. (Córdoba, Spain), obtained during the 2007/2008 crop season, was taken directly from the production line and stored at −20 °C until use in order to avoid degradation (*16*). Edible oils were purchased from a local supermarket (olive-orujo, sunflower and high-oleic acid-content sunflower oils), from Quimics Dalmau, Barcelona, Spain (coconut oil), and from Guinama, Valencia, Spain (linseed oil).

Reagents. HPLC-grade ethanol, methanol, acetonitrile, sodium carbonate, Folin Ciocalteu (F−C) reagent and orthophosphoric acid were from Panreac (Barcelona, Spain).

The most representative phenolic compounds in olive oil, hydroxytyrosol, tyrosol, oleuropein, luteolin 7-glucoside and apigenin 7-glycoside, were purchased from Extrasynthese (Genay, France). Vanillin, caffeic acid, vanillic acid, *p*- and *o*-coumaric acids, ferulic acid and the internal standard syringic acid were from Sigma (St. Louis, MO). 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 standard solutions, which contained the 11 phenols, were prepared by dilution of the appropriate volume of each stock solution in methanol. All the above solutions were stored at −20 °C in glass vials and kept in the dark until use.

Eighteen microohms deionized water from a Millipore Milli-Q water purification system was used to prepare mobile chromatographic phases.

Apparatus and Instruments. Microwave irradiation was applied by means of a MIC-II microwave oven of 400 W maximum power, from SEV (Puebla, México), furnished with a manual power control unit.

A Selecta Mixtasel (Barcelona, Spain) centrifuge was used to remove solid particles from the extract and to break the oil-extract emulsions.

An MS2 minishaker from IKA (Wilmington, NC) was used to favor phenol transfer from the ethanolic solution to the oil. A dry nitrogen stream was used to remove ethanol from the extract−oil system and/or ethanol traces from oil.

An Agilent 1100 liquid chromatograph (Pittsburgh, PA) consisting of a G1311A quaternary pump, a G1322A vacuum degasser, a G1315A diode array detector (DAD) and a Reodyne 7725 high pressure injection valve (20 μL injection loop) was used for the analysis of the target analytes. The analytical column was a 250 × 4.6 mm i.d., 5 μm, Inertisil ODS-2 from GL Sciences Inc. (Tokyo, Japan).

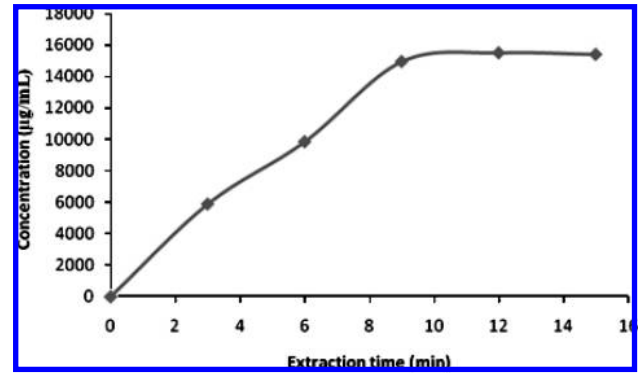

Figure 1. Kinetics study of phenol extraction from alperujo.

An Agilent 8453E UV/vis spectrophotometer was used for determination of total phenol concentration by the F−C method.

A Varian CP-3800 gas chromatograph (Walnut Creek, CA) equipped with a split/splitless SPI/1079 programmable-temperature injector and coupled to a Saturn 2200 ion-trap mass spectrometer (Sunnyvale, TX) was used for the determination fatty acid profiles of oils. The chromatograph was furnished with a Varian COP 8400 autosampler and a 60 m × 0.25 mm i.d., 0.2 μm film thickness SP-2380 fused silica capillary column from Supelco (Bellefonte, PA).

Procedures for Extraction of Phenols from Alperujo. Twelve grams of alperujo and 100 mL of ethanol were placed into the quartz extraction vessel located in the microwave irradiation zone of the oven. After extraction (10 min microwave irradiation at 400 W) the suspension was centrifuged at 3000 rpm for 5 min for phase separation. This process was repeated 15 times in order to obtain 1.5 L of extract, which was concentrated in a rotary evaporator at 35 °C to obtain 150 mL of concentrated extract with 15 g/L total phenol concentration, determined by the F−C method using caffeic acid as standard (*20*). This extract was used for preparation of diluted phenol solutions by dilution with an 80:20 ethanol−water solution.

Enrichment of Edible Oils with Phenols from Alperujo Extracts. *Solid−Liquid (S−L) Enrichment Method.* A volume of diluted phenol solution was put into contact with 3 mL of oil and vigorously shaken for 30 min. The ethanol−water phase was completely evaporated under a nitrogen stream to achieve the maximum transference of phenols to the given oil. The oil thus enriched was centrifuged for 10 min at 3000 rpm to remove solid particles from the dried diluted phenol solution. The resulting oil was analyzed to determine total and individual phenol concentration.

Liquid−Liquid (L−L) Enrichment Method. The procedure was similar to that previously developed by Japón-Lujan et al. (*18*). Briefly, a volume of the diluted phenol solution and 3 mL of oil were put into contact in an electrical stirrer for 30 min. After that, the ethanol−water phase was removed by centrifugation at 3000 rpm for 10 min. The resulting enriched oil was analyzed to determine total and individual phenol concentration.

Determination of Phenols. The overall concentration of phenols was determined by the Folin−Ciocalteu method and that of each individual phenol following chromatographic separation. Determinations in the extracts were carried out after dilution with 80:20 ethanol−water, and after liquid−liquid extraction with methanol in the case of enriched oils.

Extraction of the Phenolic Compounds from the Oil. A small portion (1.5 mL) of enriched oil was shaken for 30 min with 1.5 mL of methanol. The methanolic phase, which contained the phenolic compounds, was removed by centrifugation and stored at −20 °C for subsequent analysis.

Determination of Total Phenol Content. After extraction, total concentration of phenols was estimated by the Folin−Ciocalteu method. Briefly, 0.1 mL of the methanolic extract was mixed with 2 mL of water, 0.2 mL of Folin−Ciocalteu reagent and 0.6 mL of 20% (w/v) Na_2CO_3 aqueous solution, in that order. The resulting mixture was diluted with water in order to obtain a 5 mL final volume and then

Table 1. Calibration Curve, Correlation Coefficient, Limit of Detection (LOD), Limit of Quantification (LOQ), Monitoring Wavelength, Concentration Found in Alperujo, and Experimental and Theoretical Retention Factor for Each Phenol

phenol	calibration curve[a]	R^2	linear range	LOD (mg/kg)	LOQ (mg/kg)	wavelength (nm)	concn	retention factor exptl	retention factor theor
hydroxytyrosol	$Y = 215.01X(6.78) + 26.03(0.12)$	0.99	LOQ-250	0.02	0.09	280	5706 ± 0.55	0.60	0.62
tyrosol	$Y = 235.60X(12.35) + 30.34(0.13)$	0.99	LOQ-250	0.04	0.15	280	5076 ± 0.73	0.80	0.80
caffeic acid	$Y = 523.74X(47.01) + 91.57(0.86)$	0.99	LOQ-250	0.05	0.19	325	79 ± 0.22	1.03	0.99
vanillin	$Y = 84.76X(34.15) + 56.16(0.32)$	0.99	LOQ-250	0.05	0.19	325	294 ± 0.54	1.11	1.1
p-coumaric acid	$Y = 56.81X(35.92) + 87.05(0.58)$	0.99	LOQ-250	0.05	0.19	325	101 ± 0.88	1.15	1.12
ferulic acid	$Y = 918.79X(78.50) + 83.59(0.84)$	0.99	LOQ-250	0.10	0.35	325	210 ± 0.65	1.26	1.26
o-coumaric acid	$Y = 58.95X(33.88) + 85.98(0.62)$	0.99	LOQ-250	0.05	0.19	325	164 ± 0.44	1.33	1.31
decarboxymethyl oleuropein aglycon	as oleuropein			0.10	0.39	280	343 ± 1.12	1.44	1.45
oleuropein	$Y = 43.66X(15.60) + 45.18(0.29)$	0.99	LOQ-250	0.10	0.35	280	1044 ± 0.76	1.50	1.48
luteolin 7-glucoside	$Y = 239.71X(21.63) + 51.80(0.39)$	0.99	LOQ-250	0.04	0.15	325	254 ± 0.67	1.64	1.63
oleuropein aglycon	as oleuropein			0.10	0.39	280	1107 ± 1.01	1.75	1.74
luteolin	as luteolin 7-glucoside			0.05	0.16	325	333 ± 1.21	1.80	1.79
oleuropein aglycon dialdehyde form	as oleuropein			0.10	0.35	280	13 ± 0.32	1.90	1.87
apigenin 7-glucoside	$Y = 309.35X(29.91) + 63.50(0.55)$	0.99	LOQ-250	0.02	0.07	280	414 ± 0.65	2.02	1.98
methyl luteolin	as luteolin 7-glucoside			0.10	0.35	325	44 ± 0.12	2.04	1.98

[a] In parentheses, slope and intercept errors expressed as standard deviation.

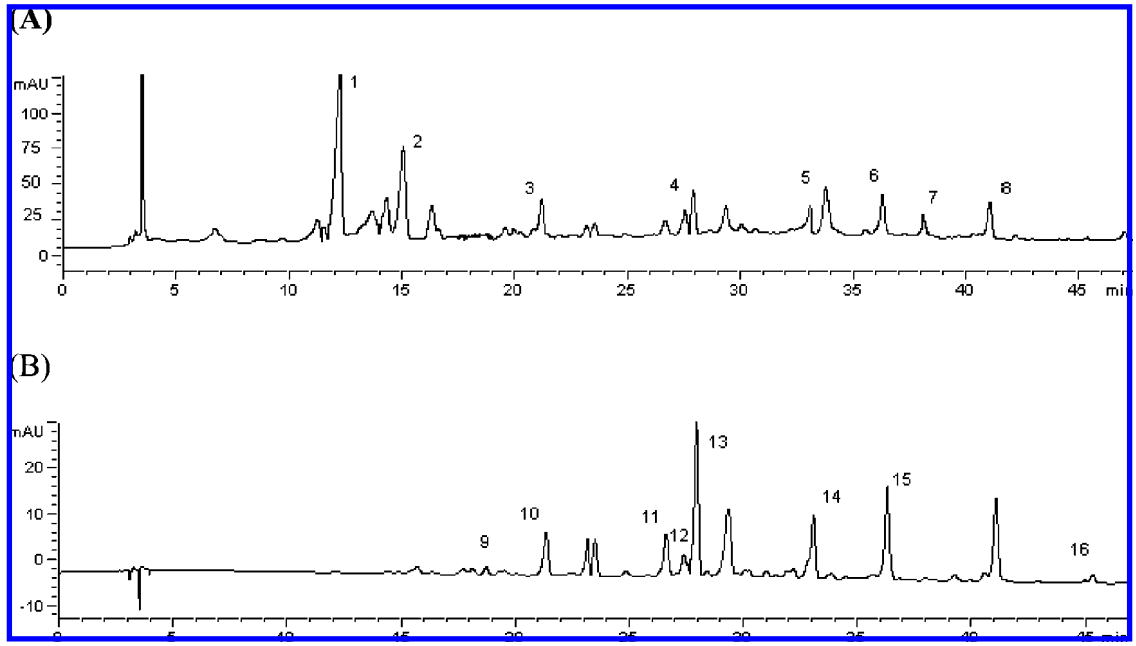

Figure 2. Chromatograms from an alperujo extract. Peak identification: 1, hydroxytyrosol; 2, tyrosol, 3, syringic acid (internal standard); 4, decarboxymethyl oleuropein aglycon; 5, oleuropein; 6, oleuropein aglycon; 7, oleuropein aglycon dialdehyde form; 8, apigenin 7-glucoside; 9, caffeic acid; 10, vanillin; 11, *p*-coumaric acid; 12, ferulic acid; 13, *o*-coumaric acid; 14, luteolin 7-glucoside; 15, luteolin; 16, methyl luteolin.

Table 2. Fatty Acid Composition of Edible Oils

oil	fatty acids[a] (%) saturated	monounsaturated	polyunsaturated
coconut	90.0 (0.2)	8.0 (0.4)	2.0 (0.5)
sunflower	11.5 (0.4)	17.0 (0.3)	71.5 (0.2)
high-oleic sunflower	9.0 (0.4)	78.0 (0.2)	13.0 (0.3)
linseed	3.5 (0.4)	20.0 (0.5)	76.5 (0.2)
orujo	16.5 (0.4)	69.2 (0.2)	14.0 (0.4)

[a] In parentheses, standard error expressed as standard deviation.

incubated for 30 min in a water bath at 50 °C. The reaction product was monitored at 725 nm. Caffeic acid was used as standard for calibration.

HPLC−DAD Separation−Quantification. The applied method was that proposed by the International Oleic Council (IOC) for the individual determination of phenolic compounds in olive oil (*21*).

The analytical column used was a 250 × 4 mm i.d., 5 μm, reversed-phase Inertisil ODS-2; the injection volume 10 μL; and the mobile phase a mixture of A (water acidified with 0.2% phosphoric acid) and B (acetonitrile−methanol, 1:1 v/v) at 1 mL/min. An initial linear gradient elution from 0 to 50% B in 40 min was followed by other linear elution gradient from 50 to 60% B in 5 min and a third gradient from 60 to 100% B in 10 min. Finally, the instrument was kept under isocratic conditions (100% B) for 2 min. A 5 min equilibration step enabled the initial conditions and mobile phase stabilization to be reached. The eluted phenols were monitored at 230, 280, 325 and 350 nm (elution time shorter than 57 min).

Calculation of the Distribution Factor. The distribution factor, defined as the quotient between the phenol concentration in the oil and that in the methanolic or in the solid phase after reaching transfer equilibrium, was calculated for each individual phenol and the total phenol concentration for each enrichment method. In the S−L enrichment method the solid residue was reconstituted in methanol prior to the determination step.

Determination of the Fatty Acid Profile. The fatty acids in the oil were derivatized to more volatile compounds (fatty acid methyl esters, FAMEs) for proper individual separation by gas chromatography. The IOC method for FAME preparation was used (*22*).

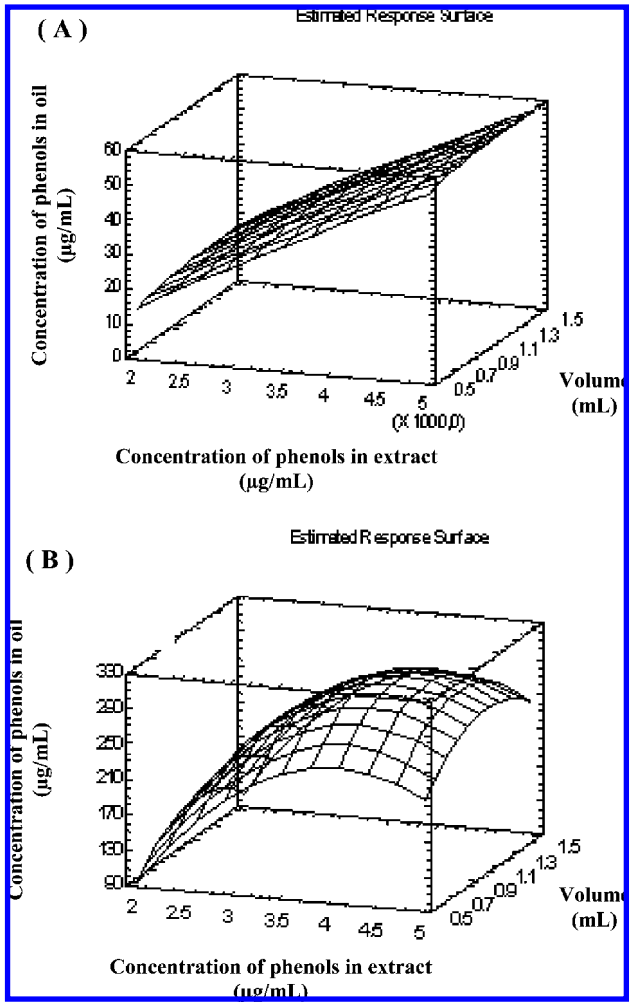

Figure 3. Typical response surfaces of oils enrichment in phenols from alperujo extracts: (**A**) liquid−liquid extraction; (**B**) solid−liquid extraction.

The individual separation of FAMEs was carried out by GC, and then they were detected and quantified by MS using the GC−MS method developed by Sánchez-Ávila et al. (personal communication). Briefly, the injection volume was 10 μL and the temperature program of the injector was as follows: started at 70 °C, held for 0.5 min, increased at 100 °C/min to 250 °C, and then kept for 78 min. The injection was in split−splitless mode. The splitter was opened (100:1) for 0.5 min, closed for 3.5 min and then opened at a 100:1 split ratio for 10 min.

The samples were analyzed using the following oven temperature program: initial temperature 70 °C (held for 1.2 min), increased at 25 °C/min to 120 °C and followed by a second gradient of 2 °C/min to 243 °C and, finally, increased by 40 °C/min to 270 °C and held at this temperature for 5 min.

The mass spectrometer was operated in the EI mode, and the ion preparation mode was μ_Selected Ion Storage (μ_SIS, similar to Selected Ion Monitoring). The manifold, trap, and transfer line temperatures were set at 60, 170 and 200 °C, respectively.

Chemometric Analysis. The potential influential variables on the enrichment procedure were studied by a multivariate approach. Furthermore, a study, based on multiple single regressions, was developed in order to find relationships between the main fatty acid families (saturated, monounsaturated and polyunsaturated fatty acids) expressed as percentage and used as variables; and the partition coefficient of each phenol used as response. Multiple parameters such as p-value, R-square statistic or standard error of estimate were calculated in order to study the characteristics of the relationship. Statgraphics Centurion XV was used as statistical software in all the cases.

RESULTS AND DISCUSSION

Optimization of the Procedure for Extraction of Phenols from Alperujo. It is well-known that microwaves, working under optimal conditions, accelerate the extraction of phenols without degradation of the target compounds (*23*). The extraction process is economic due to the fact that the sample is a waste resulting from the oil industry and ethanol used as extractant is totally recovered and reused after concentration of extract.

The influence of three inter-related variables on the extraction step (i.e., irradiation power, irradiation time and extractant composition) was studied using a multivariate approach. The response variable was the total phenol concentration obtained by the Folin−Ciocalteu method.

A full two-level factorial design allowing four degrees of freedom and involving 11 randomized runs including three center points was built for a screening study of the behavior of the three variables influencing the extraction process. The upper and lower values given to each variable were selected from the available data and experience gathered in the preliminary experiments (*23*).

The results of this screening study were that the extractant composition was not a statistically influential factor within the ranges under study, so pure ethanol was selected for subsequent experiments as it is easier to remove, as required in the subsequent enrichment process, than ethanol−water mixtures. Concerning the irradiation power, the results indicated that this variable should be increased. Taking into account that the maximum allowable irradiation power of the microwave device (400 W) was included in the screening study, this value was fixed for subsequent experiments.

A kinetics study was developed to determine the time necessary for maximum removal of phenolic compounds from alperujo, which was obtained after irradiation for 10 min, the time selected for further experiments. The extracts obtained with longer times provided similar results with no detectable degradation, as can be seen in **Figure 1**.

Characterization of the Individual Separation−Detection Method. The method used for the individual separation−detection of phenols was that of the International Oleic Council. Identification of hydroxytyrosol, tyrosol, oleuropein, apigenin 7-glucoside, caffeic acid, vanillin, *p*-coumaric acid, ferulic acid, *o*-coumaric acid and luteolin 7-glycoside was based on comparison of the retention times and the UV/vis spectra obtained for a standard solution. On the other hand, as there are not commercial standards available for decarboxymethyl oleuropein aglycon, oleuropein aglycon, oleuropein aglycon (dialdehyde form), luteolin and methyl luteolin, these compounds were identified by comparing the retention factors and the UV/vis spectra with those provided in the literature. The wavelength of maximum absorbance and the experimental retention factors are shown for each analyte in **Table 1**. The alperujo extract provided the chromatogram shown in **Figure 2**.

Calibration plots were run for the ten analytes for which commercial standards were available using the peak area as a function of the standard concentration of each compound. The calibration equations, the regression coefficients and the linear dynamic ranges are listed in **Table 1**. Compounds with no commercial standards were quantified by the calibration curve of the most similar phenol (**Table 1**). Syringic acid was used as internal standard.

The limit of detection (LOD) for each analyte was expressed as the concentration of analyte which gives a signal 3σ above the mean blank signal (where σ is the standard deviation of the blank signal). The LODs are between 0.02 and 0.10 μg/mL for all the

Table 3. Minimum Concentration of Phenols and Minimum Volume of Extract for Maximum Oil Enrichment by Solid−Liquid Extraction

oil	concn (μg/mL)/ vol (mL)	total F−C[a]	total HPLC[b]	hydroxytyrosol	tyrosol	vanillin	*p*-coumaricacid	oleuropein	*o*-coumaricacid	decarboxymethyl oleuropein aglycon	oleuropein aglycon	oleuropein aglycon	apigenin 7-glucoside	methylluteolin
coconut	concn	4500.00	9000.00	4309.92	3800.06	222.20	76.59	788.85	123.69	258.87	836.38	42.72	312.72	33.54
	vol	1.50	1.30	1.45	1.70	1.50	1.25	1.13	1.58	1.24	1.12	1.22	1.14	1.70
sunflower	concn	7500.00	7500.00	2752.65	3248.23	146.46	58.41	539.47	123.69	168.05	836.38	35.35	267.44	33.54
	vol	1.00	1.00	0.89	0.67	0.98	1.06	0.88	0.32	1.02	0.66	1.11	1.10	1.70
high-oleic sunflower	concn	7500.00	7500.00	3142.92	3137.72	178.64	53.52	508.73	80.87	189.29	607.39	36.63	236.28	33.54
	vol	1.00	1.00	0.79	1.31	1.21	1.03	0.96	0.92	1.34	1.09	1.06	1.05	1.70
linseed	concn	7500.00	7500.00	2894.49	3558.59	134.44	72.84	558.27	123.69	236.86	730.00	42.72	290.53	33.54
	vol	1.00	1.00	1.03	1.70	0.92	1.49	1.16	0.29	1.70	1.47	1.59	1.29	1.70
orujo	concn	7500.00	7500.00	2719.67	2803.49	139.78	18.78	510.56	78.78	168.98	560.07	27.83	207.91	0
	vol	1.00	1.00	0.89	0.92	1.00	1.00	0.96	0.98	0.98	0.93	1.03	0.97	0

[a] Total concentration provided by the Folin−Ciocalteu method. [b] Sum of the individual concentrations of all phenols determined by HPLC.

Table 4. Extract−Oil Distribution Factors of Phenols[a]

	coconut		sunflower		high-oleic sunflower		linseed		orujo	
	S−L[b]	L−L[c]	S−L	L−L	S−L	L−L	S−L	L−L	S−L	L−L
hydroxytyrosol	0.02 (2.28)	0.02 (4.05)	0.05 (0.67)	0.02 (4.20)	0.03 (6.67)	0.01 (4.1)	0.05 (8.89)	0.02 (4.07)	0.04 (6.88)	0.02 (5.33)
tyrosol	0.03 (7.28)	0	0.02 (8.26)	0	0.02 (1.82)	0	0.03 (5.84)	0.01 (5.58)	0.03 (8.03)	0.01 (4.85)
vanillin	0.03 (6.73)	0.02 (5.61)	0.04 (3.23)	0.02 (9.94)	0.03 (9.09)	0.02 (10.11)	0.04 (1.78)	0.04 (4.41)	0.05 (5.09)	0.02 (4.26)
p-coumaric acid	0.14 (4.22)	0.05 (2.94)	0.13 (3.49)	0.03 (2.28)	0.12 (2.44)	0.03 (2.59)	0.14 (3.10)	0.05 (2.45)	0.62 (1.25)	0.04 (2.84)
oleuropein	0.04 (4.55)	0.01 (4.47)	0.05 (1.89)	0.01 (2.08)	0.04 (2.32)	0.01 (5.91)	0.05 (1.35)	0.01 (2.18)	0.05 (0.00)	0.01 (4.33)
o-coumaric acid	0.02 (9.09)	0.02 (5.65)	0.02 (2.11)	0	0.03 (2.15)	0.01 (2.56)	0.02 (9.82)	0.02 (4.82)	0.03 (3.01)	0.01 (4.80)
decarboximethyl oleuropein aglycon	0.07 (3.69)	0.03 (3.41)	0.12 (2.31)	0.02 (3.37)	0.07 (1.48)	0.03 (3.54)	0.09 (1.52)	0.03 (4.35)	0.13 (1.51)	0.02 (4.59)
oleuropein aglycon	0.01 (3.08)	0	0.01 (3.66)	0	0.01 (0.65)	0	0.01 (1.53)	0	0.02 (2.52)	0
oleuropein aglycon dialdehyde form	0.43 (1.74)	0.18 (4.28)	0.51 (2.65)	0.12 (6.08)	0.33 (9.00)	0.13 (9.57)	0.62 (0.68)	0.26 (1.94)	0.73 (4.4)	0.26 (3.45)
apigenin-7-glucoside	0.09 (8.79)	0.04 (3.80)	0.07 (1.95)	0.02 (4.99)	0.06 (1.39)	0.02 (3.85)	0.07 (3.24)	0.04 (4.72)	0.10 (3.92)	0.02 (4.77)
methyl luteolin	0.16 (3.13)	0	0.08 (2.37)	0	0.06 (5.90)	0	0.21 (0.15)	0	0	0
total phenol concn										
obtained by F−C[d]	0.08 (2.40)	0.06 (6.86)	0.06 (4.19)	0.02 (4.48)	0.04 (6.94)	0.01 (11.38)	0.09 (5.32)	0.04 (9.53)	0.06 (5.45)	0.03 (0.00)
obtained by HPLC[e]	0.05 (2.24)	0.01 (7.85)	0.04 (3.76)	0.01 (6.95)	0.03 (9.41)	0.01 (10.17)	0.06 (6.10)	0.01 (9.58)	0.04 (7.92)	0.01 (8.29)

[a] Relative standard deviation in parentheses. [b] S−L, solid−liquid enrichment method. [c] L−L, liquid−liquid enrichment method. [d] F−C, Folin−Ciocalteu method. [e] Sum of the concentrations of the individual phenols determined by HPLC.

analytes. The limits of quantification (LOQs), expressed as the concentrations of analytes which gave a signal 10σ the mean blank signal, are 0.09 and 0.39 μg/mL for all the analytes under study (**Table 1**). Both limits were calculated from alperujo extracts.

Characterization of the Phenolic Extract. In order to fit the signals within the linear range of the calibration curves, the extract was diluted 50 times with 80:20 ethanol−water solution prior to injection in the chromatograph. The concentrations obtained for each individual phenol and precision of the method, calculated by the analysis of five replicates, are shown in **Table 1**. The target analytes can be divided into three groups as a function of their concentration. The first, hydroxytyrosol, tyrosol, oleuropein and oleuropein aglycon, includes those compounds found at concentrations higher than 1000 μg/mL. The second group, decarboxymethyl oleuropein aglycon, oleuropein aglycon (dialdehyde form), apigenin 7-glucoside, luteolin, vanillin and luteolin 7-glucoside, includes phenols with concentration close to 300 μg/mL. Finally, the third group is formed by simple phenols as caffeic acid, *o*-coumaric, *p*-coumaric and ferulic acids at concentrations lower than 200 mg/L. A chromatogram of alperujo extract is shown in **Figure 2**.

Characterization of Edible Oils. The extraction and individual separation−quantification method was used for the analysis of the phenolic compounds in the different oils prior to the enrichment process. The results showed that the concentration for these compounds in all the studied oils was under the LOD.

The fatty acid profile was determined by the procedure described in Materials and Methods. The percentage of saturated, monounsaturated and polyunsaturated fatty acids (**Table 2**) was calculated from the results obtained by this analysis.

Optimization of the Enrichment Procedures. The influence of two variables (i.e., extract volume and extract concentration) on both enrichment procedures was studied using a multivariate approach. The response variable used in these studies was the concentration of each phenol as obtained by the HPLC method.

A central composite design 2^2 + star, characterized by an axial distance of 1.41 (orthogonal) allowing four degrees of freedom and involving 12 randomized runs including two center points, was built to study the behavior of the two variables influencing the enrichment processes. The values of the fixed experimental variables (i.e., enrichment time and ethanolic phase composition) and upper and lower values given to each variable under study were selected from the available data and experience gathered in the preliminary experiments.

The typical response surfaces obtained for both enrichment procedures are shown in **Figure 3**, which shows that for the S−L procedure the transference of phenols from the solid phase to the oil is a function of both variables, extract volume and concentration. The minimum value of each variable to obtain the highest enrichment for each phenol is shown in **Table 3**.

In the L−L procedure the mass transference from the ethanolic to the oily phase is exclusively dependent on the phenol concentration. In this case, the maximum enrichment is not achieved within the studied range; so higher enrichments can be achieved, if required.

Distribution of the Phenolic Compounds after Application of the Enrichment Procedures. The partition coefficient was calculated for each phenol and enrichment procedure as described in Materials and Methods. All the experiments were in triplicate. The experimental error, expressed as relative

standard deviation, ranged between 0.15 and 11.67. As can be seen in **Table 4**, the S−L procedure provides better results (between 2 and 17 times higher) than the L−L procedure in terms of partition coefficient.

The phenols can be ordered as a function of their distribution factors as follows: oleuropein aglycon < tyrosol < *o*-coumaric acid < hydroxytyrosol ∼ vanillin < oleuropein < apigenin 7-glucoside < decarboxy oleuropein aglycon < methyl luteolin < *p*-coumaric acid < oleuropein aglycon (dialdehyde form). This order was the same for all the studied oils, and it is a function of the polarity and molecular weight of the transferred phenols with the exception of oleuropein aglycon and *p*-coumaric acid, which have lower and higher partition coefficients, respectively, than expected. High-polarity and low-molecular-weight phenols have lower distribution factors than middle- or low-polarity and high-molecular-weight phenols.

Relationship between Fatty Acid Composition and Distribution of Phenolic Compounds. As can be seen in **Table 4**, the distribution factors obtained were similar for oils with similar fatty acid composition.

The study of the relationship between the saturated fatty acids and the partition coefficient shows that this parameter decreases with increasing concentration of these fatty acids. Since the calculated p-value (0.14) is equal to or higher than 0.05, there is not a statistically significant relationship between these acids and their partition coefficient. The R-square statistic indicates that the proposed model explains 75% of the variability. The standard error of the estimate shows that the standard deviation of the residuals is 0.010.

In the case of the monounsaturated fatty acids their presence in the oil increases the distribution factor. As the calculated p-value (0.03) is lower than 0.05, a significant relationship between both variables can be established. The proposed model explains 90% of the variance. The standard deviation of the residual is 0.006.

Finally, the results obtained for polyunsaturated fatty acids were similar to those obtained for monounsaturated, the main difference between both models being that the calculated p-value for this variable (0.21) is higher than 0.05; and therefore, there is not a significant relationship between polyunsaturated fatty acids and partition coefficient. The explained variance was higher than 80%, and the standard deviation of the residual is 0.009.

This research shows that all phenols under study behave similarly in their transfer to a given oil; and the presence of mono- and polyunsaturated fatty acids in the oil increases the distribution factor. This factor decreases with increasing concentration of saturated fatty acids.

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