Liquid–Liquid Extraction for the Enrichment of Edible Oils with Phenols from Olive Leaf Extracts

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A liquid–liquid extraction method to enrich edible oils—olive, sunflower, and soy oils—with phenols from olive leaf extracts is proposed. After microwave assistance to remove the phenols from three varieties of olive leaves, concentrations in the extracts between 12921 and 5173 mg/L of oleuropein, between 488 and 192 mg/L of apigenin-7-glucoside, between 444 and 219 mg/L of luteolin-7-glucoside, and between 501 and 213 mg/L of verbascoside were obtained, which clearly depended on the target variety. After optimization of the liquid–liquid extraction step, the concentrations in oils were 442, 162, and 164 mg/L of oleuropein, respectively, which were also enriched in apigenin-7-glucoside (between 8 and 15 mg/L, depending of the oil), lutelin-7-glucoside (between 11 and 12 mg/L), and verbascoside (between 11 and 13 mg/L). The oil—extract distribution factor of these compounds was also calculated for all olive leaf varieties and edible oils using different extracts concentrations and also different oil—extract volume ratios. Thus, a door is open to enrichment of any oil with olive phenols at preset concentrations using extracts preconcentrated as required and taking into account the distribution factor of the target compounds between the oil and the extracts.

INTRODUCTION

Extra virgin olive oil is the most demanded liquid fat in the Mediterranean basin thanks to the nutraceutical properties of its components. The major fraction of this oil includes glycerols, which represent >98% of the total oil weight; meanwhile, the minor fraction 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 phenolic compounds, both lipophilic and hydrophilic phenols (*1*). Whereas lipophilic phenols such as tocopherols can be found in other vegetable oils, most hydrophilic phenols—also called biophenols by a number of authors (*2–6*)—found in olive oil are not common to other oils or fats (*7*). Interest in phenols has increased in recent decades, thus stimulating multidisciplinary research on olive phenol composition, histological distribution, and histochemical enzymatic localization to determine their biomolecular function (*1*).

Hydroxytyrosol is a well-known derivative of oleuropein that shows better results in scavenging and antioxidant capacity tests than proven antioxidants such as vitamins C and E or 2,6-di-*tert*-butyl-4-methylphenol (BHT) (*8, 9*); apigenin and luteolin are present in extra virgin olive oil in moderate amounts. Other phenols such as oleuropein, verbascoside, apigenin-7-glucoside,

and luteolin-7-glucoside either are not detected in olive oil or they exist in negligible amounts (usually at the parts per billion level) (*8*).

Mediterranean population knowledge on the preventive—curative properties of olive leaves led for centuries to use of their infusions as a very general remedy for a number of health problems. Presently, the preventive and curative properties attributed to phenols increase as the research in this field grows; thus, oleuropein, one of the most representative and abundant phenols in olive leaves, prevents oxidative myocardial injury induced by ischemia and reperfusion (*10*), supresses the oxidative and nitrosative stress preventing acute doxorubicin cardiotoxicity (*11*), improves lipid metabolism and obesity-related problems (*12*), is a antitumor agent and cytoskeleton disruptor (*13*), and exhibits antiviral properties (*8, 9*). Verbascoside has been used to repair oxidative brain damage of heroin-treated mice (*14*), apigenin-7-glucoside to fight Alzheimer's (*15*) or liver diseases (*16*) in mice, and luteolin-7-glucoside to avoid the abnormal proliferation of aortic vascular smooth muscle cells that is a common cause of pathogenesis such as atherosclerosis and restenosis (*17*).

The aim of the present research—namely, to enrich a liquid food with these healthy compounds—was a consequence of the present knowledge on the properties of phenols, applied both individually or in the mixture existing in leaves. With this aim, extracts of three olive leaf varieties at different concentrations of phenols have been used to study mass transfer of phenols to

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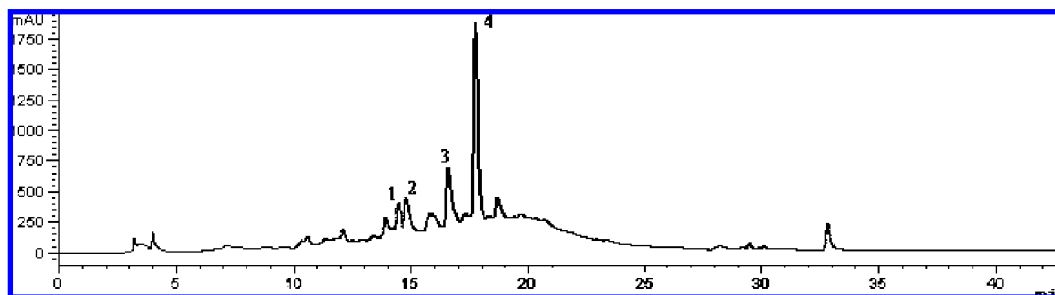
Figure 1. Chromatogram at 280 nm of an extract from Oicual olive leaves (see HPLC-DAD Separation−Detection) after 1:2 dilution to avoid overpressure problems in the column. Peaks: 1, verbascoside; 2, luteolin-7-glucoside; 3, apigenin-7-glucoside; 4, oleuropein.

Table 1. Concentrations of the Most Abundant Phenols (Milligrams per Liter) in the Extracts from Olive Leaves and Dilutions Used in This Research

olive leaf variety	extract dilution	oleuropein	apigenin-7-glucoside	luteolin-7-glucoside	verbascoside
Picual	0	5173	192	219	213
	1:1	2586	96	109	107
	1:3	1293	48	55	53
	1:7	646	24	27	27
	1:15	323	12	14	13
Lechín	0	7977	334	372	384
	1:1	3989	167	186	192
	1:3	1994	83	93	96
	1:7	997	42	46	48
	1:15	498	21	23	24
Arbequina	0	12921	488	444	501
	1:1	6460	244	222	251
	1:3	3230	122	111	125
	1:7	1615	61	55	63
	1:15	808	31	27	31

oils and calculate the enrichment factors, thus establishing the phenol extract concentration required to obtain oils with a preset amount of these compounds.

MATERIALS AND METHODS

Chemicals. HLPC grade methanol, acetonitrile, and acetic acid and LC-MS grade ethanol (maximum water content 0.01%) were from Panreac (Barcelona, Spain). Eighteen microohm deionized water from a Millipore Milli-Q water purification system was used to prepare the chromatographic mobile phases.

The most abundant and commercially available phenolic compounds in olive tree materials (namely, oleuropein, hydroxytyrosol, apigenin-7-glucoside, verbascoside, and luteolin-7-glucoside) were from Extrasynthese (Genay, France).

Apparatus. A Microdigest 301 digestor of 200 W maximum power (Prolabo, Paris, France) furnished with a microprocessor programmer (Prolabo) to control the microwave unit was used for favoring phenol extraction from olive leaves.

A rotary evaporator (R-200 Büchi, Flawil, Switzerland) was used to evaporate traces of ethanol in enriched oils.

A mechanical electrical stirrer was used to favor liquid–liquid extraction of phenols, and a Selecta Angular 6 centrifuge (Selecta, Barcelona, Spain) was used to remove particles in the extracts and to break oil−extract emulsions.

An Agilent 1100 liquid chromatograph consisting of a G1322A vacuum degasser, a G1315A diode array detector (DAD), and a Rheodyne 7725 high-pressure manual injection valve (20 μL injection loop) was used for the analysis of the target analytes (both in the olive leaf extracts and in the enriched oils) by HPLC. The analytical column was a Lichrospher 100 RP-18 (250 × 4 mm i.d., 5 μm) from Análisis Vínicos (Ciudad Real, Spain). A Kromasil 5 C-18 column (15 × 4.6 mm i.d., 5 μm) protected with a steel holder, both from Scharlab (Barcelona, Spain), was also used.

Table 2. Limits of Detection (LOD) and Quantification (LOQ) of the Main Phenols of Olive Leaves

phenol	LOD (mg/L)	LOQ (mg/L)
oleuropein	1.43	3.83
verbascoside	0.32	0.81
apigenin-7-glucoside	0.19	0.52
luteolin-7-glucoside	0.49	1.33

Table 3. Optimization of the Liquid–Liquid Extraction Process

variable	tested range	optimum value
power of stirring (units/min)	400–600	600
extraction time (min)	5–25	15[a]
volume phases ratio	1:1–1:3	1:1

[a] Obtained by univariate study.

Samples. *Edible Oils.* Refined olive, sunflower, and soy oils were purchased from a local market. Refined olive oil (instead of extra virgin olive oil) was used because the concentrations of the most abundant phenols in olive leaves—oleuropein, apigenin-7-glucoside, luteolin-7-glucoside, and verbascoside—in it are under their limits of detection (LODs). The oils were kept at 25 °C until use.

Olive Leaves. Three different varieties of olive leaves—Picual, Arbequina, and Lechín from Sevilla, selected for this research—were collected in January, dried at 30 °C for 24 h, milled, sieved to a 1 mm particle size, and kept at 4 °C until use.

Procedure for Extraction of Phenols from Olive Leaves. Three grams of milled leaves and 24 mL of ethanol were placed into the quartz extraction vessel located in the microwave-irradiation zone of the digester. After extraction (8 min of microwave irradiation at 200 W), the suspension was centrifuged at 3000 rpm (1950*g*) to remove olive leaf particles in the extract. This procedure was optimized in previous research (*18*).
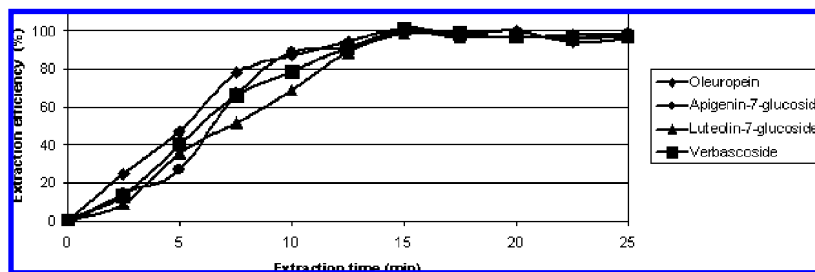
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Figure 2. Kinetics study of the extract−oil liquid–liquid extraction process.

Table 4. Oil−Extract Distribution of Oleuropein as a Function of the Type of Oil, Variety of the Leaves Extracted, and Concentration in the Extracts

| variety | oleuropein concn in leaf extract (mg/L) | concn of oleuropein in oil after 15 min of liquid–liquid extraction (mg/L) | oil−extract distribution factor | |
			for each variety	overall
		Olive Oil		
Picual	323	9.51 ± 0.42		
	646	20.30 ± 0.72		
	1293	40.37 ± 0.67	0.033 ± 0.002	0.033 ± 0.001
	2586	94.86 ± 1.44		
	5173	187.29 ± 3.19		
Arbequina	808	24.47 ± 0.92		
	1615	52.72 ± 1.54		
	3230	112.68 ± 2.91	0.033 ± 0.001	
	6460	215.20 ± 5.27		
	12921	442.44 ± 16.62		
Lechín	498	15.29 ± 0.57	0.032 ± 0.001	
	997	31.41 ± 1.01		
	1994	67.03 ± 2.79		
	3989	127.50 ± 1.77		
	7977	261.07 ± 10.43		
		Sunflower Oil		
Picual	323	3.03 ± 0.48	0.012 ± 0.002	0.012 ± 0.001
	646	7.48 ± 0.86		
	1293	17.29 ± 1.46		
	2586	35.50 ± 0.47		
	5173	66.40 ± 3.21		
Arbequina	808	8.99 ± 0.91		
	1615	20.96 ± 0.81		
	3230	39.96 ± 1.97	0.012 ± 0.001	
	6460	78.23 ± 3.41		
	12921	162.39 ± 3.41		
Lechín	498	4.98 ± 0.49		
	997	12.03 ± 1.03		
	1994	24.85 ± 1.58	0.012 ± 0.001	
	3989	56.16 ± 0.81		
	7977	100.38 ± 5.21		
		Soy Oil		
Picual	323	3.34 ± 0.57		
	646	7.55 ± 0.87		
	1293	15.14 ± 0.79	0.013 ± 0.001	
	2586	36.87 ± 1.59		
	5173	67.19 ± 3.31		
Arbequina	808	9.00 ± 0.76		
	1615	19.66 ± 2.07		
	3230	42.80 ± 1.67	0.012 ± 0.001	0.013 ± 0.001
	6460	79.99 ± 2.89		
	12921	168.74 ± 1.74		
Lechín	498	6.06 ± 0.68		
	997	12.53 ± 0.61		
	1994	25.82 ± 0.72	0.013 ± 0.001	
	3989	58.03 ± 3.34		
	7977	101.22 ± 1.68		

Enrichment of Edible Oils with Phenols from Olive Leaf Extracts. *Mass Transfer and Preparation of Enriched Oil for Analysis.* Ten milliliters of extract was shaken with 10 mL of oil for 15 min in

the electrical stirrer at 600 units/min; then, the emulsion was centrifuged for 10 min at 600 rpm to break it. Possible traces of ethanol in the organic phase were evaporated in a rotary evaporator at 25 °C and

Table 5. Enrichment of Oils after Application of the Proposed Method as a Function of Type of Leaf Variety and Overall Oil−Extract Distribution Factor of the Main Phenols, Except Oleuropein, as a Function of the Type of Oil

| type of oil | phenol | phenol concn in oil after application of the proposed method[a] (mg/L) | | | oil−extract distribution factor |
		Picual	Arbequina	Lechín	
olive	apigenin-7-glucoside	5.93 ± 0.12	15.13 ± 0.25	10.00 ± 0.14	0.031 ± 0.001
	luteolin-7-glucoside	6.27 ± 0.12	12.09 ± 0.30	10.02 ± 0.32	0.026 ± 0.001
	verbascoside	5.18 ± 0.19	10.97 ± 0.35	8.16 ± 0.36	0.021 ± 0.003
sunflower	apigenin-7-glucoside	3.20 ± 0.05	8.25 ± 0.22	5.94 ± 0.42	0.017 ± 0.001
	luteolin-7-glucoside	5.85 ± 0.17	11.32 ± 0.06	9.80 ± 0.33	0.025 ± 0.002
	verbascoside	5.97 ± 0.17	13.14 ± 0.13	10.41 ± 0.30	0.026 ± 0.002
soy	apigenin-7-glucoside	3.24 ± 0.06	8.94 ± 0.19	6.61 ± 0.09	0.018 ± 0.001
	luteolin-7-glucoside	5.66 ± 0.03	11.30 ± 0.32	9.90 ± 0.32	0.025 ± 0.002
	verbascoside	5.80 ± 0.11	13.19 ± 0.01	10.19 ± 0.11	0.025 ± 0.002

[a] The initial concentration of these phenols in the oils was under the LOD.

Table 6. Average of the Distribution Factor of the Main Olive Phenols from Leaves between Oil and Leaf Extracts

type of oil	oil−extract distribution factor
olive	0.028 ± 0.002
sunflower	0.020 ± 0.002
soy	0.020 ± 0.002

high vacuum for 10 min. Finally, 8 mL of enriched oil was shaken with 8 mL of methanol for 15 min, and the methanol phase was injected into the liquid chromatograph. All extractions were done in triplicate.

HPLC-DAD Separation−Detection. The elution solvents used were (A) 6% acetic acid and 2 mM sodium acetate, in water, and (B) acetonitrile. The samples were eluted according to the following gradient: 0–25 min, 100–50% A and 0–50% B, flow rate = 0.8 mL/min; 25–26 min, 50–0% A and 50–100% B, flow rate = 0.8 mL/min; 26–27 min, 0% A and 100% B, flow rate = 0.8–1.2 mL/min; 27–40 min, 0% A and 100% B, flow rate = 1.2 mL/min; 40–41 min, 0–100% A and 100–0% B, flow rate = 1.2–0.8 mL/min; 41–45 min, 100% A and 0% B, flow rate = 0.8 mL/min. The chromatograms were acquired at 280, 330, 340, and 350 nm (wavelengths of maximum absorption for oleuropein, verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside, respectively).

RESULTS AND DISCUSSION

Optimization of the Extraction and Separation−Detection of Phenols from Olive Leaves. Microwave assistance was used to accelerate the extraction of phenols from leaves. The two variables potentially influential on the extraction step (i.e., irradiation power and irradiation time) were previously optimized in a multivariate study using the extraction efficiency as response variable to obtain the best working conditions for proper extraction without degradation of the target compounds (*18*). Ethanol was selected as extractant both for its polarity and for its low-toxic character. Other extractants such as water (the enrichment would be negligible owing to its high polarity) and methanol or hexane (toxic) were rejected.

The experimental variables to obtain appropriate HPLC separation−detection were also previously optimized (*19*). Different columns, guard columns, wavelengths, compositions, and flow rates of the mobile phase were checked to obtain the chromatogram in **Figure 1**.

Composition of the Olive Leaf Extracts. Extracts of the three varieties of olive leaves—Picual, Arbequina, and Lechín from Sevilla—were obtained with the extraction method explained above. Several dilutions of the extracts were done to study the

extract−oil partition of the phenols after liquid–liquid extraction. The concentrations of olive leaf phenols in the extracts and the dilutions used are listed in **Table 1**.

Determination of Phenols from Enriched Oils and Olive Leaf Extracts. Characterization of the Method. Calibration curves were obtained by plotting the peak area of each phenol as a function of standard concentration. The regression coefficients ranged between 0.996 and 0.9999.

The LOD for each analyte was expressed as the mass of analyte that gives a signal that is 3σ above the mean blank signal (where σ is the standard deviation of the blank signal). The LODs obtained ranged between 0.19 and 1.43 mg/L. The limits of quantification, expressed as the mass of the analyte that gives a signal 10σ above the mean blank signal, ranged from 0.52 to 3.83 mg/L. LODs and LOQs were estimated from olive leaf extracts and methanolic oils extracts after extract−oil mass transfer and from standard solutions of these compounds (see **Table 2**).

Optimization of Extract−Oil Liquid–Liquid Extraction. The three potentially influential variables on the liquid–liquid extraction step (i.e., power of the electrical stirrer, volume ratio between phases, and extraction time) were optimized using as response variable the mass transfer efficiency expressed as the peak area for each compound under the chromatographic conditions previously optimized.

A full two-level factorial design allowing 4 degrees of freedom and involving 11 randomized runs including 3 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 preliminary experiments.

None of the three variables were statistically influential within the ranges under study; therefore, their optimum values are within these ranges. As foreseeable, the best results were obtained with the minimum volume ratio and maximum power of the electrical stirrer. The tested and optimum values obtained for each variable, which were used in further experiments, are shown in **Table 3**.

It is worth emphasizing that the oil−extract distribution of phenols was constant for all extract−oil volume ratios assayed. This fact allows the desired enrichment degree to be obtained by changing the volume ratio of the two immiscible phases.

A kinetics study was made to determine the time needed to reach the mass transfer equilibrium of liquid–liquid extraction, which was obtained after 15 min of shaking. In view of these results, this time was selected and used for further experiments.
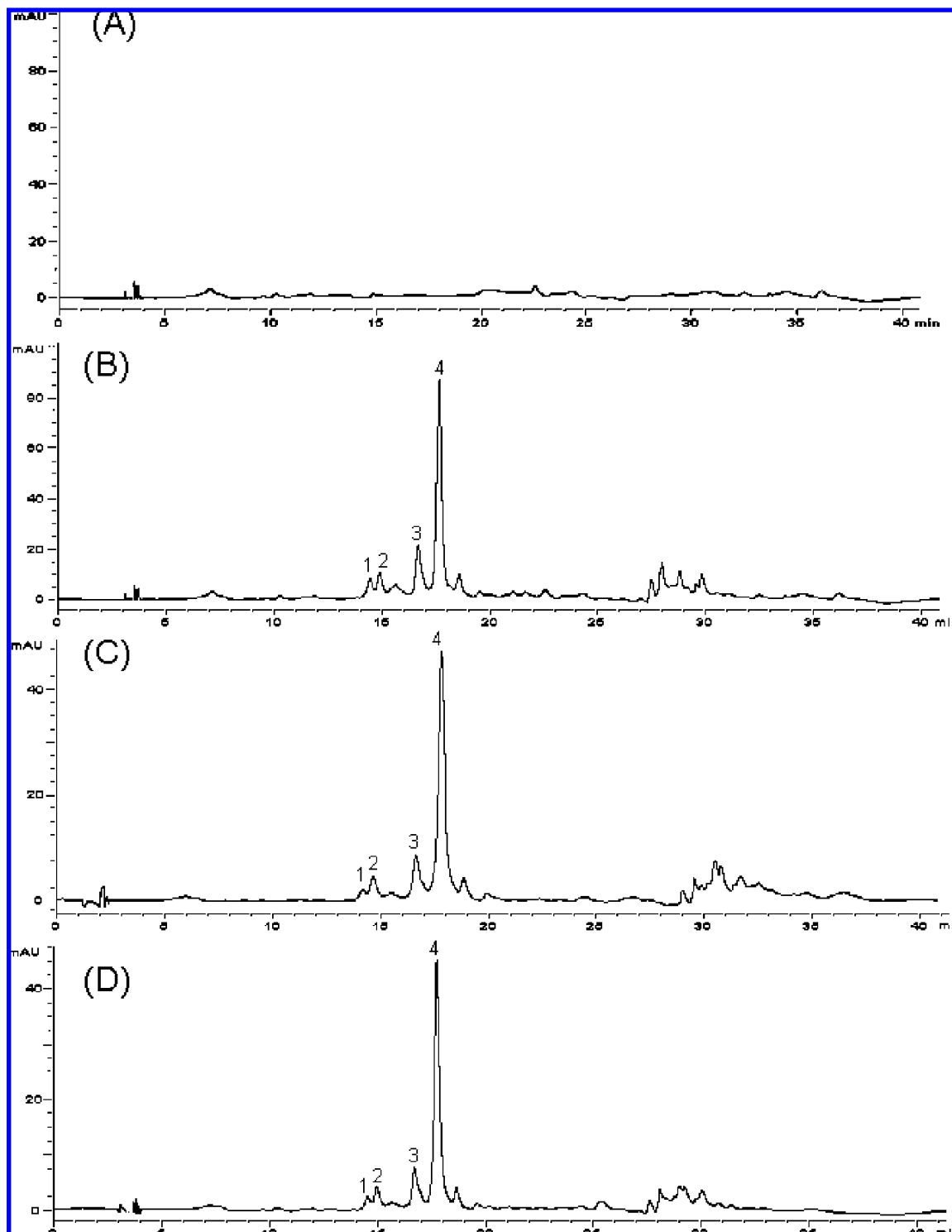
Figure 3. Chromatograms obtained in the analysis of oils: olive oil before the mass transfer process (**A**); olive oil (**B**), sunflower (**C**), and soy (**D**) oils after application of the proposed method [note that the scale in (**B**) is different from that in (**C**) and (**D**)]. Peaks: 1, verbascoside; 2, luteolin-7-glucoside; 3, apigenin-7-glucoside; 4, oleuropein.

The extracts obtained with longer times provided similar results, as can be seen in **Figure 2** for olive oil. The other oils showed a similar behavior (the differences were never >3%).

Distribution of the Main Phenols in Olive Leaves in Edible Oils after Liquid–Liquid Extraction. *Oleuropein.* Oleuropein is the most abundant phenol in olive leaves. As can be seen in **Table 1**, the concentrations of the extracts are 12921, 7977, and 5173 mg/L in Arbequina, Lechín, and Picual varieties,

respectively, much higher than those of the other phenols. Extracts of the three varieties at five concentrations each were put into contact with olive, sunflower, and soy oils and stirred (experiments in triplicate). **Table 4** shows the concentration of oleuropein in the extracts, that in oil after mass transfer, and the oil−extract distribution factor in all cases. Olive oil was the most enriched with oleuropein: 442, 261, and 187 mg/L of oleuropein were transferred to olive oil using extracts from

Arbequina, Lechín, and Picual varieties, respectively; therefore, the olive oil−extract distribution factor of oleuropein (quotient between the amount of phenol transferred to oil and amount of phenol in the leaf extract) was 0.033 ± 0.001 (see **Table 4**). As compared with the usual contents of oleuropein in extra virgin olive oil, refined olive oil is 100 times richer in this compound after liquid–liquid extraction.

In the cases of sunflower and soy oils, the results were as follows: the former was enriched in oleuropein between 66 and 162 mg/L and the latter between 67 and 169 mg/L. The overall oil−extract distribution factor of this phenol was lower than in the case of olive oil (0.012 ± 0.001 and 0.013 ± 0.001 for sunflower and soy oils, respectively). These results show that any edible oil can be enriched with olive phenols.

Other Olive Leaf Phenols. Before subjection to the mass transfer process, the refined olive oil contained negligible concentrations in apigenin-7-glucoside and luteolin-7-glucoside. After mass transfer, the olive oil was enriched with between 6 and 15 mg/L and between 6 and 12 mg/L in apigenin-7-glucoside and luteolin-7-glucoside, respectively (apigenin-7-glucoside showed an oil−extract distribution of 0.031 ± 0.001 and that of luteolin-7-glucoside was 0.026 ± 0.001). Verbascoside does not usually exceed 1 mg/L in extra virgin olive oil, but the refined olive oil was enriched between 5 and 11 mg/lL, that is, enrichments between 5 and 10 times with an oil−extract distribution factor of 0.021 ± 0.003.

The sunflower oil was enriched in apigenin-7-glucoside, luteolin-7-glucoside, and verbascoside (within the ragnes of 3−8, 6–11, and 6–13 mg/L, respectively) with oil−extract distribution factors of 0.017 ± 0.001, 0.025 ± 0.002, and 0.026 ± 0.002, respectively; meanwhile, the soy oil was enriched between 3 and 9 mg/L of apigenin-7-glucoside (oil−extract distribution factor of 0.018 ± 0.001), between 5 and 11 mg/L of luteolin-7-glucoside (oil−extract distribution factor of 0.025 ± 0.002), and between 6 and 13 mg/L of verbascoside (oil−extract distribution factor of 0.025 ± 0.002).

Table 5 shows the concentration of the target phenols in oil as a function of olive leaf variety and type of oil (the table lists only the results obtained with the original extract for each variety, but not those with extract dilutions, which can be seen in the Supporting Information).

Figure 3 shows the chromatograms obtained with the proposed method for the enriched oils and that of the olive oil before the mass transfer process as the blank. The sunflower and soy oils provided planar chromatograms, thus demonstrating the absence of olive phenols.

Finally, the distribution averages of the main olive phenols in leaves between oil and leaf extracts were calculated for the three oils studied. **Table 6** shows that olive oil was the most enriched.

These distribution factors are "low" because the polarity of phenols is closer to that of ethanol than that of the oils.

Paiva-Martins (*20*) studied the effect of phenolic leaf extract on the taste and flavor of a refined olive oil. The official panel at the Agronomy Institute of Porto did not find significant differences between the flavor of oil before and that after enrichment, and a better score in the taste quality was obtained by the enriched oil.

Therefore, the quality of the target oils was modified and improved by applying the proposed method thanks to the enrichment in olive biophenols; the present procedure can be an extractive−tool for future industrial implementation.

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