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Solid−Liquid Transfer of Biophenols from Olive Leaves for the Enrichment of Edible Oils by a Dynamic Ultrasound-Assisted Approach

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A continuous approach assisted by ultrasound for direct enrichment of edible oils (olive, sunflower, and soya) with the main phenols in olive leaves (i.e., oleuropein, verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside) has been developed. Multivariate methodology was used to carry out a detailed optimization of the enrichment, and quantitation of the transferred compounds was based on LC−MS−MS in multiple reaction monitoring optimizing the most sensitive transition for each biophenol. Under the optimal working conditions, only 20 min is necessary to enrich the edible oils with 14.45−9.92 μg/mL oleuropein, 2.29−2.12 μg/mL verbascoside, 1.91−1.51 μg/mL apigenin-7-glucoside, and 1.60−1.42 μg/mL luteolin-7-glucoside. The enrichment method is carried out at room temperature and is organic-solvent-free; thus, the healthy properties of the edible oils improve as does their quality. Also, the low acquisition and maintenance costs of an ultrasound source and its application in a dynamic system make advisable the industrial implementation of the proposed method.

INTRODUCTION

In the past, olive leaf infusions were used as a folk remedy against diseases such as malaria (*1*), but since the second half of the 20th century, the olive leaf extracts have been investigated and it has been demonstrated that their healthy properties are a consequence of the function of olive phenols (OPs) in the olive tree (namely, reactivity against pathogen attack and response to insect injury *2, 3*). Oleuropein is the most abundant phenol in olive leaves, which has been used in a number of medical treatments since its first reference in the literature (*4*); thus, this phenol prevents cardiovascular diseases by protecting membrane lipid oxidation acting on coronary dilation (*5*) and by antiarythmic action (*6*), improves the lipid metabolism to mitigate obesity problems (*7*), protects enzymes and protects against hypertensive cell death in cancer patients (*8*), and presents antiviral properties (*9, 10*). Also, the nutraceutical utility of other phenols present in olive leaves in high amounts such as verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside has been studied. Thus, verbascoside has been used to repair the brain's oxidative damage caused by heroin consumption (*11*), apigenin-7-glucoside to fight against Alzheimer's disease (*12*) or liver diseases (*13*), 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 (*14*).

However, among these OPs, only oleuropein is present in olive oil in negligible amounts (usually on the order of parts per billion). Verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside are generally not detected (*10*). These products have a high added value as the olive leaves provide a characteristic flavor and aroma, but this contact is not enough to enrich the oil with phenols. In this respect, the phenol-transfer process can be accelerated by auxiliary energies.

One of the present energies for favoring extraction processes is ultrasound. The effect of cavitation (namely, the formation of tiny bubbles subjected to fast adiabatic compressions and expansions, which give rise to high temperatures and pressures within them with minimal effect on the temperature of the overall system (*15*)) is well-known by users of ultrasound-based devices, as this phenomenon favors penetration and mass transfer.

The development of fast methods to enrich edible oils with olive leaf phenols can be of great interest. These methods must be respectful of the quality of the given oil, so a strong increase of temperature and/or mixing with organic solvents are unviable to avoid changes in the organoleptic characteristics of the oil and toxicity problems, respectively. A multivariate optimization has been developed to determine the best working conditions for enrichment without degradation of both OPs and oils using ultrasound to accelerate mass transfer and LC−MS−MS to monitor the process.

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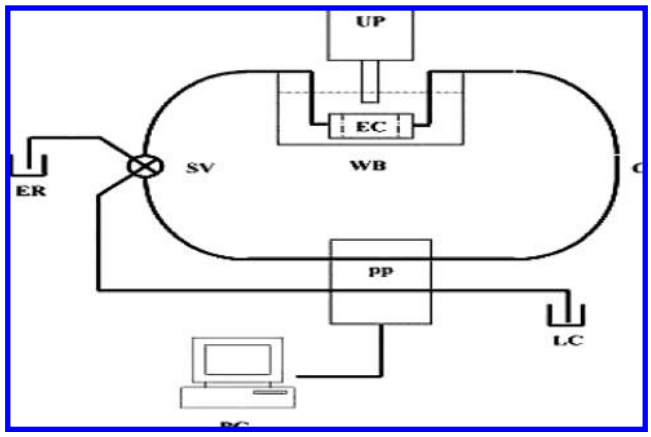

Figure 1. Experimental setup for the dynamic enrichment of edible oils in phenols from olive leaves assisted by ultrasound: C, extraction coil; EC, extraction chamber; ER, extract reservoir; LC, leaching carrier; PC, personal computer; PP, peristaltic pump; SV, selection valve; UP, ultrasonic probe; WB, thermostatic water bath.

Table 1. Optimization of the MS−MS Step for Qualitative and Quantitative Determination of Olive Phenols

phenol	MS1 voltage (V)	precursor ion (*m*/*z*)	collision voltage (V)	product ions (*m*/*z*)	quantitation transition (*m*/*z*)
oleuropein	200	539.1	20	275.1 307.1 377.1	539.1→275.1
hydroxytyrosol	100	153.0	20	123.1 105.2	153.0→123.1
apigenin	180	269.0	35	117.2 107.0 151.1	269.0→117.2
luteolin	200	285.0	35	133.2 285.2 175.2	285.0→133.2
apigenin-7-glucoside	220	431.0	35	268.1 431.2 310.7	431.0→268.1
luteolin-7-glucoside	220	447.1	30	285.1 284.1	447.1→285.1
verbascoside	80	623.2	35	161.1 134.9	623.2→161.1

MATERIALS AND METHODS

Samples. Picual variety olive leaves used in this research were collected, dried, milled, and kept at 4 °C until use. Refined olive, sunflower, and soya oils were bought in a local market.

Reagents. Methanol was from Panreac (Barcelona, Spain). Acetonitrile and formic acid (Scharlab, Barcelona, Spain) and 18 mΩ deionized water (from a Millipore Milli-Q water purification system) were used to prepare mobile phases and were of LC−MS grade. The most abundant and commercially available phenolic compounds in olive oil and leaves (i.e., oleuropein, hydroxytyrosol, apigenin-7-glucoside, verbacoside, luteolin-7-glucoside, luteolin, and apigenin) were from Extrasynthese (Genay, France).

Apparatus and Instruments. Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium alloy probe (12.70 mm in diameter), which was immersed into a water bath in which the extraction cell was placed. The latter consisted of a stainless steel cylinder (10 cm length and 10 mm i.d.), closed with screws at either end, allowing circulation of a leaching solvent through it. The screw caps also contained stainless steel filter plates (2 μm thickness and 0.25 mm i.d.) to ensure that the sample remained in the extraction chamber. **Figure 1** shows the experimental setup used.

A four-channel Gilson Minipuls-3 low-pressure peristaltic pump (programmed to change the rotation direction at preset intervals) and PTFE tubing of 0.5 mm i.d. were used to build the flow manifold.

Table 2. Characterization of the Extraction Method

variable	tested range: screening	tested range: factorial design	optimum value
radiation amplitude (%)	10−50	50	50
duty cycle (%)	50−90	50	50
irradiation time (min)	5−15	15−25	20
extractant flow rate (mL/min)	4−6	6−8	7
probe position (cm)	0−4	4	0
temperature (°C)	25−40	25	25

A mechanical electrical stirrer was used for liquid−liquid extraction of phenols from oils.

Analyses were performed by reversed-phase liquid chromatography (RP-LC) followed by electrospray ionization (ESI) in negative mode and tandem mass spectrometry (MS−MS) detection. Liquid chromatography was performed by an Agilent (Palo Alto, CA) 1200 series LC system, which consists of a binary pump, a vacuum degasser, an autosampler, and a thermostated column compartment. Detection was carried out with an Agilent 6410 triple-quadrupole LC−MS instrument composed of two octopoles as mass filters and a hexapole as the collision cell placed between the two quadrupoles. Data were processed using MassHunter workstation software for qualitative and quantitative analysis. A Zorbax Eclipse XDB-C_{18} analytical column (4.6 mm × 150 mm, 5 μm particle size, Agilent) and a Kromasil 5 C-18 precolumn (15 × 4.6 mm i.d., 5 μm) protected with a steel holder were used for chromatographic separation.

Proposed Procedure. A 1 g sample of milled leaves was placed in the extraction chamber of the dynamic approach (**Figure 1**), which was assembled and filled with oil impelled by the peristaltic pump. After filling, the extraction chamber (10 mL) was immersed into the water bath at 25 °C, which was maintained during the extraction time. The oil was then circulated through the solid sample for a 20 min preset time under ultrasonic irradiation (duty cycle 0.5 s, output amplitude 50% of the converter, applied power 225 W with the probe placed 0 cm from the top surface of the extraction cell). During extraction, the direction of the oil (at a 7 mL/min flow rate) was changed every 90 s, thus minimizing increased compactness of the solid in the extraction cell that could cause overpressure in the system.

Reference Extraction Procedures. Two conventional extraction procedures were used as references to test the efficiency of the proposed procedure.

Reference Method 1. A 1 g portion of dried and milled leaves was treated as described in the Proposed Procedure but without ultrasonic irradiation.

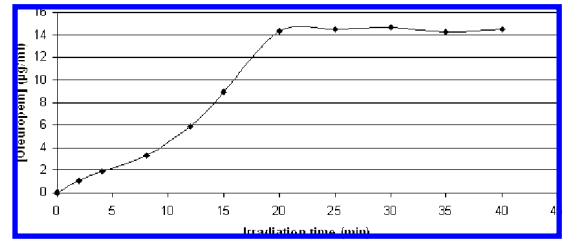

Figure 2. Kinetics study of the enrichment of olive oil in oleuropein. Other phenols show a similar behavior (the differences in concentration were never higher than 5%).

Table 3. Optimization of Ultrasound-Assisted Extraction of Olive Phenols

phenol	LOD (μg/mL)	LOQ (μg/mL)	intraday variability[a] (%)	interday variability[a] (%)
verbascoside	0.38	1.11	6.11	8.85
luteolin-7-glucoside	0.25	1.04	8.98	9.67
apigenin-7-glucoside	0.21	1.01	7.09	10.21
oleuropein	0.61	1.64	5.43	9.65

[a] The intra- and interday variability was tested in the enriched olive oil.

Table 4. Enrichment of Edible Oils Using the Proposed Method and Two Reference Methods[a]

	olive oil				sunflower oil			soya oil		
phenol	blank	proposed method	RM1	RM2	proposed method	RM1	RM2	proposed method	RM1	RM2
oleuropein	ULOD	14.45 ± 3.32	ULOD	3.44 ± 0.90	10.21 ± 2.65	ULOD	2.27 ± 0.64	9.92 ± 2.72	ULOD	2.20 ± 1.69
apigein-7-glucoside	ULOD	1.91 ± 0.21	ULOD	ULOQ	1.32 ± 0.43	ULOD	ULOQ	1.51 ± 0.10	ULOD	ULOQ
luteolin-7-glucoside	ULOD	1.60 ± 0.20	ULOD	ULOQ	1.42 ± 0.19	ULOD	ULOQ	1.39 ± 0.43	ULOD	ULOQ
verbascoside	ULOD	2.12 ± 0.45	ULOD	ULOQ	2.29 ± 0.43	ULOD	ULOQ	2.25 ± 0.39	ULOD	ULOQ
hydroxytyrosol	1.54 ± 0.23	1.59 ± 0.34	1.67 ± 0.30	1.43 ± 0.29						
apigenin	2.98 ± 0.76	2.78 ± 0.54	2.65 ± 0.67	3.00 ± 0.66						
luteolin	2.43 ± 0.65	2.30 ± 0.33	2.29 ± 0.21	2.67 ± 0.43						

[a] The standard deviation has been calculated by three replicates in all cases. Hydroxytyrosol, apigenin, and luteolin do not exist in sunflower and soya oils. RM1 = reference method 1. RM2 = reference method 2. ULOD = under the limit of detection. ULOQ = under the limit of quantification.

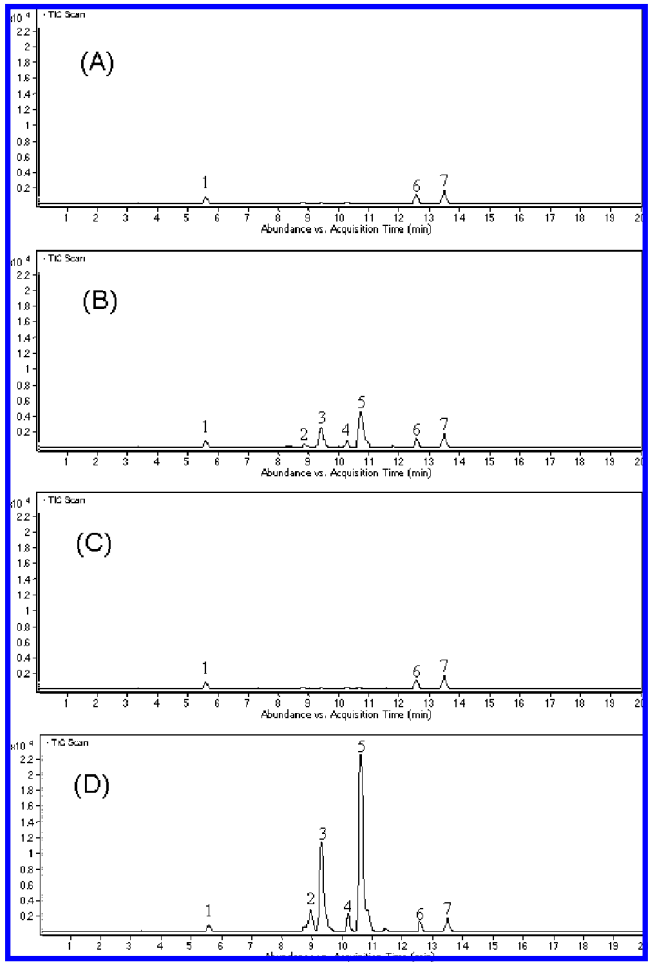

Figure 3. Total ion current chromatograms obtained by isolation of the precursor ion for the analysis of phenols in olive oil: (**A**) blank; (**B**) after application of Reference Method 2 (see Reference Extraction Procedures); (**C**) after application of Reference Method 1 (see Reference Extraction Procedures); (**D**) after application of the proposed method (see Proposed Procedure). Peak identification: 1, hydroxytyrosol; 2, verbascoside; 3, luteolin-7-glucoside; 4, apigenin-7-glucoside; 5, oleuropein; 6, luteolin; 7, apigenin.

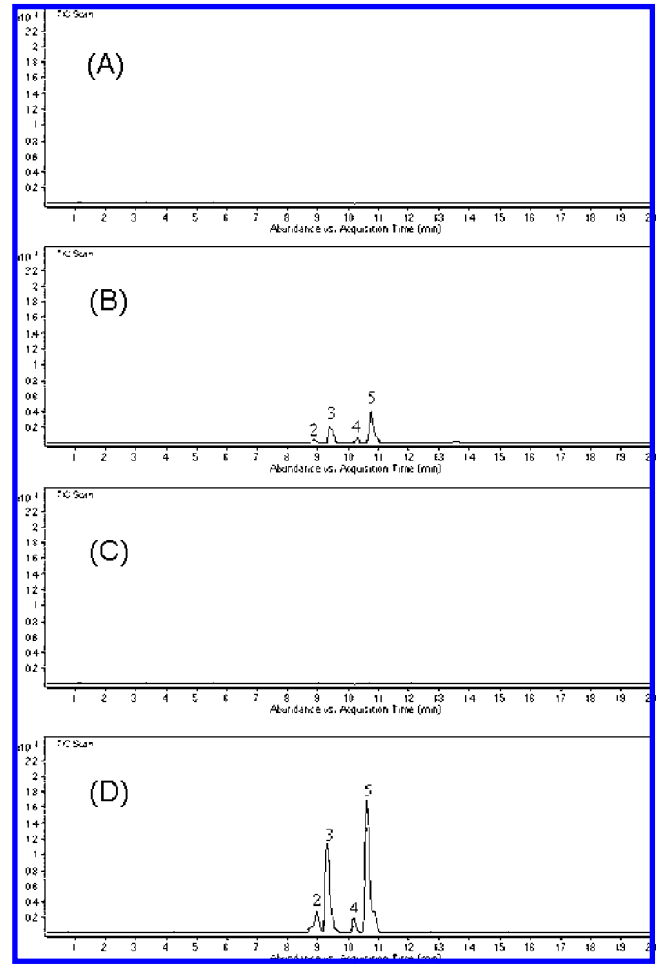

Figure 4. Total ion current chromatograms obtained by isolation of the precursor ion for the analysis of phenols in sunflower oil. Other data are as in **Figure 3**.

Reference Method 2. A 1 g portion of dried and milled leaves and 10 mL of oil were placed in a beaker and subjected to stirring at 25 °C for 24 h.

LC−MS−MS Separation−Detection. After extraction by any of the methods, 8 mL of enriched oil was shaken with 8 mL of methanol for 30 min and the methanolic extract injected into the liquid chromatograph. The mobile phases were constituted by the following solvents: mobile phase A, 0.1% formic acid in water; mobile phase B, 0.1% formic acid in 98:2 acetonitrile−water (v/v). Thus, the concentration of formic acid was constant throughout the run. The flow rate was 0.8 mL/min during the entire chromatographic step, the temperature of the analytical columns 10 °C, and the injection volume 50 μL. The total analysis time was 20 min, and 4 min was the required time to re-establish and equilibrate the initial conditions.

The eluate was monitored by MS−MS detection in multiple reaction monitoring, which was carried out by setting the optimum voltage of the first quadrupole to drive the precursor ion for each analyte with the highest efficiency and that of the collision cell to fragment the precursor ions, thus obtaining the optimized product ions. The flow rate and temperature of the drying gas (N_2) were 11 L/min and 300 °C, respectively. The nebulizer pressure was 35 psi and the capillary voltage 4000 V. The dwell time was set at 200 μs.

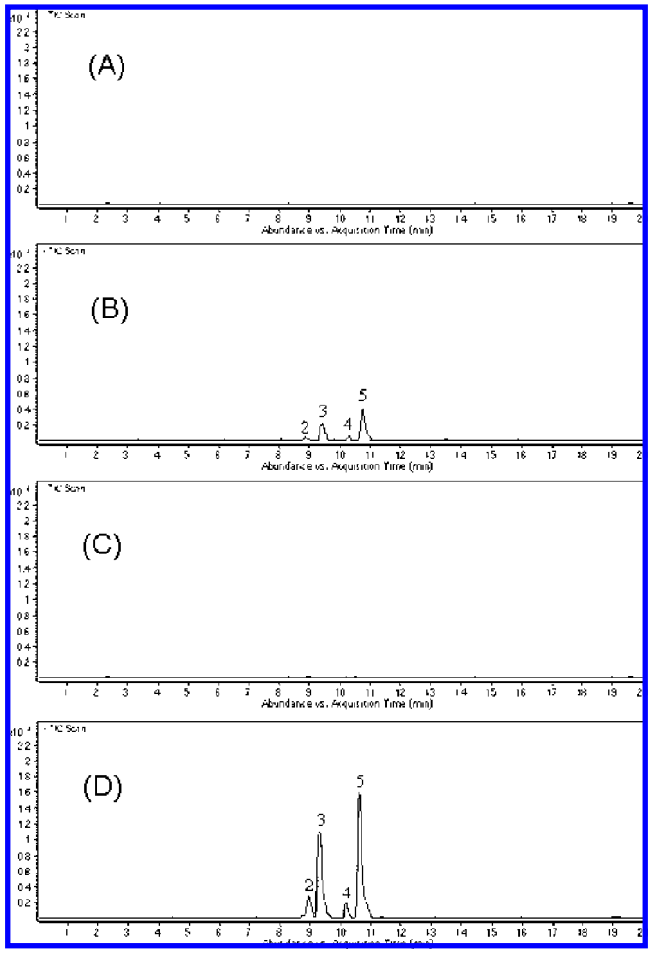

Figure 5. Total ion current chromatograms obtained by isolation of the precursor ion for the analysis of phenols in soya oil. Other data are as in **Figure 3**.

RESULTS AND DISCUSSION

Optimization of the LC−MS Method for Phenol Analysis. The sequence followed in the optimization of the MS−MS method consisted of three steps: (1) isolation of the precursor ion with the highest efficiency, (2) study of the product ions, and (3) selection of the most sensitive and selective transition from the precursor ion to the suitable product ion. The voltage of the first mass filter, precursor ions, collision voltages, product ions, and quantitation transitions for each phenol are shown in **Table 1**.

Optimization of the Ultrasound-Assisted Extraction. Olive oil was used in the optimization studies. Subsequently, the procedure was applied to sunflower and soya oils (see Enrichment of Sunflower and Soya Oils Using the Proposed Method).

Six variables were considered for optimization of the extraction step in the dynamic manifold in **Figure 1**, namely, the probe position (i.e., distance of the probe tip to the upper surface of the extraction chamber), ultrasound radiation amplitude, percentage of the ultrasound exposure duty cycle, irradiation time, olive oil flow rate, and water bath temperature. The response variable was the extraction efficiency expressed as the peak area for each compound under the chromatographic conditions previously optimized.

A Plackett−Burman design 27 × 3/32 type III resolution allowing 4 degrees of freedom and involving 12 randomized runs plus 3 center points was built for a screening study of the behavior of the 7 factors affecting the extraction process. The upper and lower values given to each factor were selected from the available data and experience gathered in the preliminary experiments. The tested and the optimum values obtained for each variable are shown in **Table 2**.

The conclusions of this screening study were that the probe position, radiation amplitude, duty cycle, and temperature of the water bath were not statistically influential factors within the ranges under study. However, the results showed higher extraction efficiencies with the maximum value of the radiation amplitude and minimum value of the duty cycle and probe position. Thus, 50% of the radiation amplitude, 0 cm position probe, 50% of the duty cycle, and 25 °C were selected for subsequent experiments.

Higher values of the flow rate and irradiation time were tested using a complete factorial design involving four randomized runs plus eight center points. The optimum values were obtained with 7 mL/min and extraction for 20 min. This time was confirmed by a univariate kinetics study (see **Figure 2**).

One remarkable aspect of the use of ultrasound as auxiliary energy is the negligible increase in the temperature, which results in no degradation of the target analytes and no changes of organoleptic characteristics of the oils due to accelerated rancidity. The very different behavior of oils subjected to ultrasound as proposed by Cañizares et al. (*16*) (fast oxidation of stable olive oil to shorten the time for the determination of the oxidative stability of oils) is justified because direct introduction of the probe in the oil facilitates formation of free radicals and oil oxidation as a result, which does not occur under the conditions used in the procedure proposed here. Direct transfer of phenols from leaves to oil avoids previous time-consuming extractions and the contact of edible oils with organic solvents.

Only 20 min is the time necessary to enrich the olive oil with up to 14 mg/L oleuropein and around 2 mg/L apigenin-7-glucoside, luteolin-7-glucoside, and verbascoside. This enrichment improves the healthy properties of olive oil, and the low acquisition and maintenance costs of an ultrasound source and its application in a dynamic system make advisable the industrial implementation of the proposed method.

Characterization of the Method. Calibration plots were run by using the peak area as a function of the concentration of each compound. The regression coefficients ranged between 0.993 and 0.999 for all analytes, as shown **Table 3**.

The peak height-to-averaged background noise ratio was calculated, for which the background noise was estimated by the peak-to-peak baseline near the analyte peak. LODs and LOQs were then calculated on the basis of a minimal accepted value of the signal-to-noise (S/N) ratio of 3 and 10, respectively. The values obtained ranged from 0.21 to 0.61 μg/mL for LODs and from 1.01 to 1.64 μg/mL for LOQs (see **Table 3**).

To evaluate the precision of the proposed method, interday and intraday variability were evaluated in a single experimental setup with duplicates (*17*). As shown in **Table 3**, the intraday assay variability, expressed as relative standard deviation, was from 5.43% to 8.48%, while the interday assay variability ranged from 8.85% to10.21%.

Efficiency of the Proposed Method. Two conventional extraction procedures were used as references to test the efficiency of the proposed procedure (see Reference Extraction Procedures). The first (Reference Method 1) was proved with the aim of simulating the hydrodynamic conditions of the proposed method but without ultrasonic irradiation. Thus, the olive oil was recirculated in the continuous system for 20 min. As shown in **Table 4**, nil enrichment was produced in the

nonultrasonicated oil, so the influence of ultrasound is crucial for mass transfer.

Also, different extraction times between 1 and 48 h were tested by the second conventional, stirring-based method (see Reference Extraction Procedures, Reference Method 2). Extraction times longer than 24 h did not improve the efficiency in any case, which was always lower than that of the proposed method and slightly higher than that of Reference Method 1.

Figure 3 shows the chromatograms of the blank (methanolic extract from an olive oil) and that obtained with the proposed method and with both reference procedures. The phenols originally present in the olive oil (hydroxytyrosol, apigenin, and luteolin) were not degraded after application of the proposed method because their concentrations were not altered (see **Table 4**).

Enrichment of Sunflower and Soya Oils by Using the Proposed Method. The proposed method and the two reference methods were applied to both sunflower and soya oils (both free of olive leaf phenols). **Figures 4** and **5** (for sunflower and soya oils, respectively) show that these oils can be enriched using the proposed method. As can be seen in **Table 4**, the amount of phenols in both oils was higher using the proposed method than using the nonultrasonicated reference methods.

The quality of the target oils has been improved by applying the proposed method thanks to the enrichment in olive phenols with healthy properties, so the industrial implementation of new production lines can be a pending goal.

Long-term stability studies have shown that, at the enrichment concentrations, the enriched oils remain stable. No sensory or spectrophotometric rancidity presence of peroxides was observed for storage times at which nonenriched oils underwent rancidity. Analyses of the enriched oils at these times showed no changes in phenol concentration.

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