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Improved method for identifying and quantifying olive oil phenolic compounds and their metabolites in human plasma by microelution solid-phase extraction plate and liquid chromatography–tandem mass spectrometry

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ABSTRACT

Two methods based on solid-phase extraction (SPE) using traditional cartridges and microelution SPE plates (μSPE) as the sample pre-treatment, and an improved liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS) were developed and compared to determine the phenolic compounds in virgin oil olive from plasma samples. The phenolic compounds studied were hydroxytyrosol, tyrosol, homovanillic acid, *p*-coumaric acid, 3,4-DHPEA-EDA, *p*-HPEA-EDA, luteolin, apigenin, pinoresinol and acetoxypinoresinol. Good recoveries were obtained in both methods, and the LOQs and LODs were similar, in the range of low μM. The advantage of μSPE, in comparison with SPE cartridges, was the lack of the evaporation step to pre-concentrate the analytes. The μSPE-UPLC–ESI-MS/MS method developed was then applied to determine the phenolic compounds and their metabolites, in glucuronide, sulphate and methylated forms, in human plasma after the ingestion of virgin olive oil.

1. Introduction

The beneficial role of virgin olive oil for human health has been extensively studied in the last decade. Many reports have related the consumption of olive oil with the low incidence of coronary heart diseases and some types of cancer in the Mediterranean area [1–3]. Specifically, the importance of the phenolic compounds has been demonstrated due to their action against platelet aggregation, inhibition of the LDL oxidation and their high antioxidant activity [4–7]. A wide range of phenolic compounds has been identified in virgin olive oil, the most important of these being phenolic alcohols (hydroxytyrosol and tyrosol), secoiridoid derivatives (the dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) and the dialdehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EDA)), phenolic acids (vanillic and *p*-coumaric acids), lignans (pinoresinol and acetoxypinoresinol) and flavonoids (luteolin and apigenin) [8–11].

The identification of these phenolic compounds and their metabolites (mainly glucuronide and sulphate conjugates) in biological samples after the ingestion of virgin olive oil is very important for evaluating their in vivo healthy impact [12]. Most of the studies have focused on the identification of these compounds in urine samples. For example, it has been shown that hydroxytyrosol and tyrosol absorption is dose-dependent in humans after oral administration [13–15]. Others studies have determined the phenolic profile of human LDL in order to find out its role in the oxidation process [16]. However, few works have determined these phenolic compounds in plasma samples and most of these have focused on the identification of hydroxytyrosol and tyrosol, while ignoring the rest of the polyphenols from virgin olive oil [17,18]. These other phenolic compounds could be very important in terms of healthy protection. For example, it has been shown that 3,4-DHPEA-EDA protects red blood cells (RBCs) from oxidative injury even more than hydroxytyrosol [19] so it is necessary to develop a method that correctly identifies it in plasma or other biological fluids.

The method used to analyze polyphenols and their metabolites in biological samples, such as plasma, is critical for obtaining successful results given the low levels of these compounds in this type of sample and the great number of interferences, mainly by proteins. This way, a pre-treatment of the sample is needed in order to eliminate these interferences and pre-concentrate the phenolic compounds given that they are present at trace levels. The most usual sample pre-treatment has been the off-line solid-phase extraction (SPE) with conventional cartridges [20,21] containing a sorbent weight from 60 mg to 6 g. The introduction of the microe-

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lution SPE plate (μSPE) is a step forward in sample preparation technology. This allows the rapid isolation of analytes from complex matrices using an ultra-low elution volume, thus eliminating the need for post-extraction solvent evaporation and reconstitution steps, which can be critical in terms of time. With regard to the analytical technique used to carry out the analysis, it has to provide enough sensitivity and selectivity to quantify these compounds at low concentration levels in such complex matrices as biological samples. High-performance liquid chromatography (HPLC) coupled to an ultraviolet detector (UV) [17,22], mass spectrometry (MS) [23] and tandem MS (MS/MS) [20,21,24] and gas chromatography (GC) coupled to MS [18,25] have been used to analyze phenolic compounds in biological samples. However, the derivatization step required in the GC has limited its use instead of the LC.

The aim of this paper was to develop and compare two methods to extract phenolic compounds from plasma samples: one by means of a traditional off-line SPE by cartridges, and the other using microelution SPE plates. After that, the eluted phenolic compounds obtained were analyzed by UPLC–ESI-MS/MS. Both methods were validated in terms of linearity, calibration curves, precision, accuracy, recoveries and sensitivity, and the phenolic compounds studied were hydroxytyrosol, tyrosol, homovanillic acid, *p*-coumaric acid, pinoresinol, acetoxypinoresinol, luteolin, apigenin, 3,4-DHPEA-EDA, and *p*-HPEA-EDA. These analytes were selected to be the most representative phenolic compounds from virgin olive oil. After the validation, the best method was applied to analyze plasma samples collected after the ingestion of 30 ml of virgin olive oil in order to identify the studied phenolic compounds and their metabolites.

2. Experimental

2.1. Chemicals and reagents

The phenolic compounds, apigenin, luteolin, hydroxytyrosol, tyrosol, and *p*-coumaric acid were purchased from Extrasynthese (Genay, France). Caffeic and homovanillic acids were purchased from Fluka Co. (Buchs, Switzerland), (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland), and catechol from Sigma–Aldrich (Germany). The secoiridoid derivatives 3,4-DHPEA-EDA, and *p*-HPEA-EDA, and the lignan acetoxypinoresinol are not available commercially and were isolated from virgin olive by semipreparative HPLC as described in our previous report [9]. A stock solution of each standard compound was dissolved in methanol, and all the solutions were stored in a dark flask at 4 °C.

Cathecol and caffeic acid were used as internal standard (IS) for the analysis of hydroxytyrosol and for the analysis of other phenolic compounds, respectively. These IS were prepared in phosphoric acid 4%.

Methanol (HPLC grade), acetonitrile (HPLC grade), acetic acid and L (+)-ascorbic acid (reagent grade) were all provided by Scharlau Chemie (Barcelona, Spain). Ortho-phosphoric acid (85%) was purchased from Panreac (Barcelona, Spain). Water was of MilliQ quality (Millipore Corp, Bedford, MA, USA).

2.2. Virgin olive oil samples

The virgin olive oil samples were obtained from an olive oil mill in Catalonia (Spain) during the harvest season. In the present work, the selected oil had a high level of phenolic compounds, of around 400 mg/kg of the total phenols. The total phenolic content of the oils was measured using the Folin-Ciocalteau method by spectrophotometry at 725 nm [26]. To obtain the phenolic profile of the consumed virgin olive oil, polyphenols were extracted using the method described in our previous paper by triple extraction with methanol 80% and were then analyzed by UPLC–ESI-MS/MS [9].

2.3. Plasma samples

Plasma samples were obtained by venipuncture from healthy humans after the ingestion of 30 ml of virgin olive oil. The blood samples were collected under basal conditions (after a 12 h overnight fasting period) and 60 and 120 min after the consumption of the 30 ml of virgin olive oil. To obtain the plasma samples, blood (50 ml) was collected in Vacutainer™ tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. They were protected from the light with aluminium foil, and centrifuged for 15 min at 1500 × *g* and 4 °C (Kokusan, H-103RS, Japan), then the plasma was immediately separated from the cells and kept at −80 °C until analysis. The human study was approved by the Ethical Committee of Clinical Research of Sant Joan University Hospital, Reus, Spain.

2.4. Plasma phenols extraction

2.4.1. Off-line SPE cartridges

The phenolic compounds were extracted from the plasma samples with the SPE system using OASIS HLB 200 mg cartridges (Waters Corp., Milford, MA). The conditioning of the cartridges was done by adding sequentially 5 ml of methanol and 5 ml of milliQ water acidified at pH 2 with acetic acid. Extractions were done by loading 1 ml of plasma, which had previously been mixed with 50 μl caffeic acid (IS) (2 mg/l) and 20 μl of phosphoric acid 85% to break the bonds between the proteins and phenolic compounds. The loaded cartridges were washed with 1 ml of milliQ water and 2 ml of methanol 5%. Finally, the retained phenolic compounds were eluted using 5 ml of methanol in tubs containing 20 μl of ascorbic acid 1% to avoid oxidative damage. In order to preconcentrate the analytes, the elution solvent was evaporated to dryness under a nitrogen stream in an evaporating unit at 30 °C (PIERCE Model 18780, IL, USA) and reconstituted with 100 μl of methanol. This step required some time, around 2 h, so it was necessary to add ascorbic acid to the samples to avoid any possible oxidative damage of the phenolic compounds [17]. All the extracts were filtered through a 0.22 μm nylon syringe filter (Tecknokroma, Barcelona, Spain) and transferred to the autosampler vial before the chromatographic analysis. The injection volume was 2.5 μl.

2.4.2. Microelution SPE plates (μSPE)

A new method to extract phenolic compounds from plasma samples using microelution plates (Waters, Milford, USA) was studied in order to compare its suitability with the use of the traditional SPE cartridges. The microelution plates were packed with 2 mg of OASIS HLB sorbent (Waters, Milford, USA). Two different methods were developed using these plates. One was specific for the isolation of hydroxytyrosol and the other was used for the other phenolic compounds under study.

In both methods, the cartridges were firstly conditioned sequentially by using 250 μl of methanol and acidified milliQ water at pH 2. Then, the plasma sample was loaded. For the analysis of hydroxytyrosol, 200 μl of plasma mixed with 150 μl of phosphoric acid 4% and 50 μl catechol (IS) at 10 mg/l were loaded onto the plate, while 350 μl of plasma mixed with 300 μl of phosphoric acid 4% and 50 μl caffeic acid (IS) at 10 mg/l were loaded to isolate the other phenolic compounds. After that, the clean-up of the plates was sequentially done with milliQ water and methanol 5% to eliminate any interference that the sample might contain. The volumes used of both solvents were 75 μl for the analysis of hydroxytyrosol and 100 μl for the analysis of other phenolic compounds. Finally the elution of the retained phenolic compounds was done with 50 μl of acetonitrile 50% for hydroxytyrosol and 100 μl of methanol for the other phenolic compounds. The solution eluted with these methods was

directly injected into the UPLC–MS/MS. The sample volume was 7.5 μl.

2.5. UPLC–ESI-MS/MS

The UPLC system consisted of an AcQuity™ UPLC equipped with a Waters binary pump system (Milford, MA, USA) using an AcQuity UPLC™ BEH C18 column (1.7 μm, 100 mm × 2.1 mm i.d.). During the analysis, the column was kept at 30 °C and the flow rate was 0.4 ml/min using acetic acid 0.2% as solvent A and acetonitrile as solvent B. The elution started at 5% of eluent B for 5 min, then was linearly increased 40% of eluent B in 20 min, further increased to 100% of eluent B in 0.1 min and kept isocratic for 1.9 min. Then, back to initial conditions in 0.1 min, and the reequilibration time was 1.9 min.

The UPLC system was coupled to a PDA detector AcQuity UPLC™ and a TQD™ mass spectrometer (Waters, Milford, MA, USA). The software used was MassLynx 4.1. Ionization was done by electrospray (ESI) in the negative mode and the data were collected in the selected reaction monitoring (SRM) mode. The ionization source parameters were capillary voltage of 3 kV, source temperature of 150 °C, and desolvation gas temperature of 400 °C with a flow rate of 800 l/h. Nitrogen (99% purity, N_2 LCMS nitrogen generator, Claind, Como, Italy) and argon (≥99.99% purity, Aphagaz, Madrid, Spain) were used as the cone and collision gases, respectively. The SRM transitions and the individual cone voltage and collision energy for each phenolic compound were evaluated by infusing 10 mg/l of each compound to obtain the best instrumental conditions. Two transitions were acquired for each compound, one for quantification and a second for confirmation purposes. The results are shown in Table 1.

2.6. Metabolite characterization and quantification

To identify and quantify the phenolic compounds and their metabolites, analyses were performed in MS (full-scan mode) and MS/MS (based on neutral loss scan and product ion scan). These techniques are excellent tools for verifying structural information about the compounds when standards are not available. First, the analyses were carried out in the full-scan mode (from 80 to 800 m/z) by applying different cone voltages, from 20 to 60 V. When low cone voltages were applied, the MS spectrum gave information about the precursor ion or the $[M-H]^-$. In contrast, when high cone voltages were applied, specific fragment ions were generated and the MS spectrum gave information about their structure. The structural information was also verified by using product ion scan and neutral loss scan in the MS/MS mode. In the product ion scan experiments, the product ions are produced by collision-activated dissociation of the selected precursor ion in the collision cell. Neutral loss scans of 80 and 176 units were used to characterize the sulphate and glucuronide forms, respectively. Then, the detection and quantification of the phenolic compounds and their metabolites were performed based on their ion fragmentation in the MS/MS mode using SRM.

Due to the lack of standards for these metabolites, they were tentatively quantified by using the calibration curves corresponding to their phenolic precursors. This way, hydroxytyrosol and tyrosol metabolites were quantified using the calibration curves of hydroxytyrosol and tyrosol, respectively; homovanillic acid, vanillic acid and vanillin metabolites were quantified using the calibration curve of homovanillic acid; *p*-coumaric, *p*-hydroxybenzoic and ferulic acids metabolites were quantified by means of the *p*-coumaric acid calibration curve. Apigenin metabolites were quantified with the apigenin calibration curve. Enterodiol and enterolactone were quantified with the pinoresinol calibration curve.

2.7. Validation procedure

Once the off-line SPE and off-line μSPE-UPLC–MS/MS analytical methods were developed, their instrumental quality parameters were determined by spiking pool basal plasma with the ten standard phenolic compounds at known concentrations. The instrumental quality parameters were linearity, recovery, accuracy, reproducibility, LOD, and LOQ.

The linearity of the method was evaluated by using pool human basal plasma spiked with the analytes. The calibration curves (based on peak area abundance) were plotted using the equation $y = a + bx$, where y is the (analyte/IS) peak area abundance ratio and x the (analyte/IS) concentration ratio. The concentrations of the phenolic compounds were calculated by interpolating their (analyte/IS) peak area abundance ratios on the calibration curve. The calibration curves were obtained by analyzing five points at different concentration levels and each standard solution was injected three times.

The precision of the method was determined by the relative standard deviations (%RSDs) of the concentration. The accuracy was calculated from the ratio between the concentrations of the phenolic compounds found compared to the spiked concentration. This quotient was then multiplied by 100. To determine the extraction recoveries of the sample pre-treatment method, the responses of the analytes spiked in plasma matrices before and after extraction were compared. The LODs and LOQs were calculated using the signal-to-noise criterion of 3 and 10, respectively.

3. Results and discussion

3.1. Sample pre-treatment

3.1.1. Off-line SPE cartridges

Initial experiments to extract the studied phenolic compounds and their metabolites from biological samples by off-line SPE were reported by de la Torre-Carbot et al. [20,21]. These authors focused

Table 1
Optimized SRM conditions for the analyses of the studied phenolic compounds by UPLC–MS/MS.

Peak	Compound	MW	Quantification			Confirmation		
			SRM_1	Cone voltage (V)	Collision energy (eV)	SRM_2	Cone voltage (V)	Collision energy (eV)
1	Hydroxytyrosol	154	153 > 123	35	10	153 > 95	35	25
2	Tyrosol	138	137 > 106	40	15	137 > 119	40	15
3	Homovanillic acid	182	181 > 137	25	10	181 > 122	25	15
4	*p*-coumaric acid	164	163 > 119	35	10	163 > 93	35	25
5	3,4-DHPEA-EDA	319	319 > 195	40	5	319 > 183	40	10
6	Luteolin	286	285 > 133	55	25	285 > 151	55	25
7	Pinoresinol	358	357 > 151	40	30	357 > 136	40	25
8	*p*-HPEA-EDA	303	303 > 285	30	5	303 > 179	30	5
9	Acetoxypinoresinol	415	415 > 151	45	15	415 > 235	45	15
10	Apigenin	270	269 > 117	60	25	269 > 151	60	25

on determining tyrosol, hydroxytyrosol, homovanillic acid, and their corresponding metabolites in LDL samples. In our study, the aim was to determine a greater number of virgin olive oil polyphenols in post-ingestion plasma samples, including other important groups of compounds such as flavonoids, lignans and secoiridoid derivatives. The importance of these compounds has recently been demonstrated [7,19] but nobody has studied their measurement in biological samples after the ingestion of virgin olive oil.

As a result of the analysis of the 10 phenolic compounds (hydroxytyrosol, tyrosol, *p*-coumaric acid, homovanillic acid, apigenin, luteolin, pinoresinol, acetoxypinoresinol, 3,4-DHPEA-EDA, and *p*-HPEA-EDA) spiked in plasma according to the SPE conditions reported by de la Tore-Cabot et al. [20,21], the recovery values were low, especially those corresponding to hydroxytyrosol, luteolin, 3,4-DHPEA-EDA, *p*-HPEA-EDA and *p*-coumaric acid, which were all lower than 50%. This could be due to the different chemical structure of these compounds and, as a consequence, their different behavior towards the cartridge. In order to increase the recovery value, and achieve the suitable extraction conditions for sample preparation, several parameters affecting the efficiency of the SPE, such as the sorbent weight, the conditioning, the clean-up and the elution were studied since we wanted to extract a greater number of analytes with different properties simultaneously.

The recovery studies were carried out by spiking basal plasma samples at the concentration level of 3 μM for all the compounds, except for hydroxytyrosol, homovanillic acid, 3,4-DHPEA-EDA and *p*-HPEA-EDA, which was 5 μM, and luteolin which was 1 μM.

Firstly, the best size of cartridge was selected by testing two OASIS HLB cartridges (Waters, Mildford, USA): one with 60 mg of sorbent and the other with 200 mg. This was done to optimize the quantity of sorbent in relation to the volume of sample. Higher recoveries were obtained using the 200 mg cartridges, increasing the retention of those compounds that were not properly recovered using the 60 mg cartridge. This was very important because hydroxytyrosol, which was the least retained, is one of the most important compounds in the virgin olive oil.

The SPE cartridge was conditioned by passing a small volume of methanol through it. This organic solvent is absorbed on the surface of the sorbent particles, making it more hydrophilic. In our study, 5 ml of methanol was used. After that, the cartridge was conditioned with an appropriate solution to remove the excess of the activation solvent and increase the retention of the phenolic compounds. Water and acidic water at pH 2 were tested and acidic water at pH 2 was chosen due to its similarity with the plasma matrix (which had been pre-treated with phosphoric acid to precipitate proteins).

Then, different volumes of water and methanol 5%, ranging from 0 to 9 ml, were tested and optimized to clean-up the cartridge. The recovery increased inversely with the amount of water. 1 ml of water and 1 ml of methanol 5% were selected being the recovery values between 60 and 94%, for all the phenolic compounds.

Finally the elution step was studied. 3 ml of methanol was enough to elute all the compounds from the cartridge except the flavonoids (luteolin and apigenin), which required 5 ml. Therefore, 5 ml of methanol was selected as the optimum elution volume. Table 2 shows the optimum conditions for extracting the studied phenolic compounds and the recoveries obtained, which ranged from 62 to 94%, using the SPE cartridge.

3.1.2. Microelution SPE plates

To improve the extraction time of the phenolic compounds from the plasma samples and decrease the plasma sample volume, a new procedure using a μSPE plate was developed. The maximum volume allowed on each microplate was 700 μl so 350 μl of plasma sample, 300 μl of phosphoric acid 4% and 50 μl of IS (caffeic acid) were mixed and loaded onto the plate. The initial experiments were based on the method developed using the traditional SPE cartridge. Conditioning was done applying 250 μl of methanol, followed by 250 μl of milliQ water at pH 2. Then, after loading the sample, each plate was cleaned up with 200 μl of milliQ water followed by 200 μl of methanol 5%. Finally, the retained compounds were eluted using 100 μl of methanol. Under these conditions, the recoveries of the more hydrophilic phenolic compounds (hydroxytyrosol, tyrosol, phenolic acids) were low. These compounds might have been lost in the clean-up step. To minimize this loss, the clean-up volume was optimized from 50 to 200 μl. When 100 μl of these solutions was tested, the recovery of all the phenolic compounds improved considerably, from 60 to 94%, except for hydroxytyrosol, which was below 50%.

Table 2
SPE conditions and % recovery for the analysis of the 10 phenolic compounds in spiked plasma samples by off-line SPE cartridges and μSPE.

Step	Solvent	Off-line SPE cartridges	Off-line μSPE	
		Procedure A	Procedure B	Procedure C
Conditioning	Methanol	5000 μl	250 μl	250 μl
	MilliQ water pH2	5000 μl	250 μl	250 μl
Loading	Plasma	1000 μl	350 μl	200 μl
Clean-up	MilliQ water	1000 μl	100 μl	75 μl
	Methanol 5%	2000 μl	100 μl	75 μl
Elution	Methanol	5000 μl	100 μl	–
	Aceonitrile 50%	–	–	50 μl
Phenolic compounds		%Recovery		
Hydroxytyrosol		62	–	74
Tyrosol		85	94	–
Homovanillic acid		94	87	–
p-Coumaric acid		73	91	–
3,4-DHPEA-EDA		75	92	–
Luteolin		68	72	–
Pinoresinol		67	98	–
p-HPEA-EDA		84	77	–
Acetoxypinoresinol		91	67	–
Apigenin		74	85	–

The elution solvent and volume were also studied in order to increase the recovery of hydroxytyrosol. To this end, different solutions of methanol and acetonitrile in water from 100 to 20% were tested. The study showed that the maximum recovery of hydroxytyrosol (41%) was obtained using acetonitrile 50%. Unfortunately, this reduced considerably the recovery of the other phenolic compounds. Therefore two μSPE methods were proposed, one to extract hydroxytyrosol and another to extract the other phenolic compounds under study (see Table 2). This decision was supported by the simplicity of the procedure, the speed of extraction (10 min), the multiplicity of wells on each microplate and the low volume of plasma needed, which allowed two different parallel procedures to be applied to each sample.

Several optimizations were done to improve the extraction of hydroxytyrosol. Catechol was used as IS given its similar structure to this phenolic compound [17]. Regarding to the sample amount, the recovery increased from 41 to 60% when the sample volume was reduced to 200 μl. Then, when the clean-up volume was reduced from 100 to 75 μl, the recovery increased to 80%. Finally, when 50 μl of acetonitrile 50% was used as the elution solvent, recovery was 74%, so this volume was selected as the optimum.

Table 2 shows the optimum conditions for the extraction of the phenolic compounds studied and their recovery. These recoveries were good in both methods, between 70 and 90%. In general, higher recoveries were obtained with the μSPE, except for homovanillic acid, *p*-HPEA-EDA, and acetoxypinoresinol. This could be explained

Table 3
Retention time (RT, min), recovery (%R), linearity, calibration curves, reproducibility (RSD), LODs and LOQs for the determination of the 10 phenolic compounds in spiked plasma samples.

Compound	RT (min)	Linearity (μM)	%RSD (n = 3)	Accuracy (%)	LOD (μM)	LOQ (μM)
Off-line SPE cartridges						
Hydroxytyrosol	2.40	0.3–13	6.0 (5 μM)	105 (2 μM)	0.1	0.5
Tyrosol	4.13	2–7	4.4 (3 μM)	103 (2 μM)	1.4	4.8
Homovanillic acid	6.17	0.3–11	1.9 (5 μM)	106 (2 μM)	0.09	0.3
p-Coumaric acid	7.60	0.06–6	2.0 (3 μM)	106 (2 μM)	0.01	0.05
3,4-DHPEA-EDA	13.10	1.5–16	5.2 (5 μM)	102 (2 μM)	0.5	5.1
Luteolin	15.15	0.09–7	1.6 (1 μM)	106 (2 μM)	0.03	0.1
Pinoresinol	15.89	0.4–6	1.6 (3 μM)	107 (2 μM)	0.2	0.6
p-HPEA-EDA	16.39	1.5–16	4.6 (5 μM)	102 (2 μM)	1.0	8.2
Acetoxypinoresinol	16.57	0.8–4	3.9 (3 μM)	103 (2 μM)	0.3	1.2
Apigenin	17.34	0.02–2	0.8 (3 μM)	106 (2 μM)	0.007	0.02
Off-line μSPE						
Hydroxytyrosol	2.40	0.3–16	5.0 (8 μM)	96 (8 μM)	0.1	0.6
Tyrosol	4.13	2–21.7	6.5 (11 μM)	100 (5 μM)	1.3	4.3
Homovanillic acid	6.17	0.8–16.5	7.8 (8 μM)	98 (8 μM)	0.3	0.9
p-Coumaric acid	7.60	0.3–18.3	6.6 (9 μM)	103 (9 μM)	0.1	0.3
3,4-DHPEA-EDA	13.10	0.9–65.6	6.4 (22 μM)	105 (11 μM)	0.3	0.9
Luteolin	15.15	0.2–10.5	7.6 (5 μM)	96 (2 μM)	0.07	0.23
Pinoresinol	15.89	0.4–8.4	4.2 (4 μM)	98 (2 μM)	0.1	0.4
p-HPEA-EDA	16.39	3–69.1	10.5 (23 μM)	91 (12 μM)	1.0	3.0
Acetoxypinoresinol	16.57	0.7–28.8	6.0 (17 μM)	96 (10 μM)	0.2	0.7
Apigenin	17.34	0.2–11.1	2.9 (5 μM)	101 (5 μM)	0.07	0.23

by the elimination of the nitrogen-evaporation step in the μSPE required in the traditional SPE which could cause the degradation of part of the compounds, whose high instability has been demonstrated [27].

3.1.3. Quality parameters

The quality parameters of the off-line SPE and μSPE-UPLC–MS/MS methods were studied by using a serial dilution of pool basal plasma spiked with ten phenolic compounds. These parameters were linearity, calibration curves, reproducibility, accuracy, LOD and LOQ. Table 3 shows the results obtained from using the SPE and μSPE as the sample pre-treatment.

Linearity was tested following the procedures developed in the range from 0.02 to 16 μM in the case of the traditional SPE cartridges and between 0.2 and 69.1 μM when microplates were used. The linearity between SPE cartridge and μSPE was similar for all the phenolic compounds, except for *p*-coumaric acid, luteolin and apigenin, whose linearity using the SPE cartridge was slightly higher than with the μSPE. All the calibration curves of the compounds under study presented determination coefficients (R^2) higher than 0.99.

The precision of the developed procedures was calculated as the %RSD for each compound, in terms of concentration. These values were lower than 6.0% using the SPE cartridges and lower than 7.8%, except for 3,4-DHPEA-EDA which was 10.5%, when the microplates were used. The accuracy of the methods was between 96 and 107%.

The LODs and LOQs were below 1.4 and 8.2 μM, respectively using the SPE cartridges, and lower than 1.3 and 4.3 μM for the microplates. In the case of hydroxytyrosol, which is the phenolic compound most studied in the literature, these values were similar in both SPE and μSPE, the LODs being 0.1 μM, and the LOQs, 0.5 and 0.6 μM, respectively (Table 3). These LOD and LOQ values were lower than those reported in the literature, which ranged from 0.2 to 0.5 and 0.6 to 1.1 μM, respectively [17,22]. The LODs and LOQs for all the studied phenolic compounds were similar under both methods, except for the phenolic acids, *p*-coumaric acid and homovanillic acid, and the flavonoids, luteolin and apigenin, which were slightly higher under SPE than μSPE. This fact was due to the higher pre-concentration factor when the SPE cartridges were used (10-fold) in comparison with when the μSPE cartridges were used (3.5-fold).

Nevertheless, the combined sample pre-treatment technique developed, μSPE, and UPLC–MS/MS can be an alternative method for determining these phenolic compounds in biological samples with short analysis times in routine analyses. This simplicity in the plasma phenol analysis is especially important for determining the bioavailability of polyphenols in human intervention studies in which a multiple blood sampling over an 8–24 h period is required to obtain the plasma kinetic data. This means numerous plasma analyses to monitor the concentration of the polyphenol metabolites. As a consequence, the off-line μSPE method should be very adequate for analyzing a large number of samples from large-scale human intervention studies.

Table 4
SRM conditions for the analysis of the metabolites by UPLC–MS/MS.

Compound	MW	SRM_1 (quantification)	Cone voltage (V)	Collision energy (eV)	SRM_2 (confirmation)	Cone voltage (V)	Collision energy (eV)
Tyrosol sulphate	218	217 > 137	35	15	137 > 106	40	15
Vanillin sulphate	232	231 > 151	35	15	151 > 136	20	10
Hydroxytyrosol sulphate	234	233 > 153	40	15	153 > 123	35	10
Coumaric acid sulphate	244	243 > 163	35	15	163 > 119	35	10
Vanillic acid sulphate	248	247 > 167	35	15	167 > 123	30	10
Homovanillic acid sulphate	262	261 > 181	35	15	181 > 137	25	10
Dihydroferulic acid sulphate	276	275 > 195	35	15	195 > 135	30	10
Hydroxytyrosol glucuronide	330	329 > 153	45	15	153 > 123	35	10
Apigenin glucuronide	446	445 > 269	40	15	269 > 117	60	25

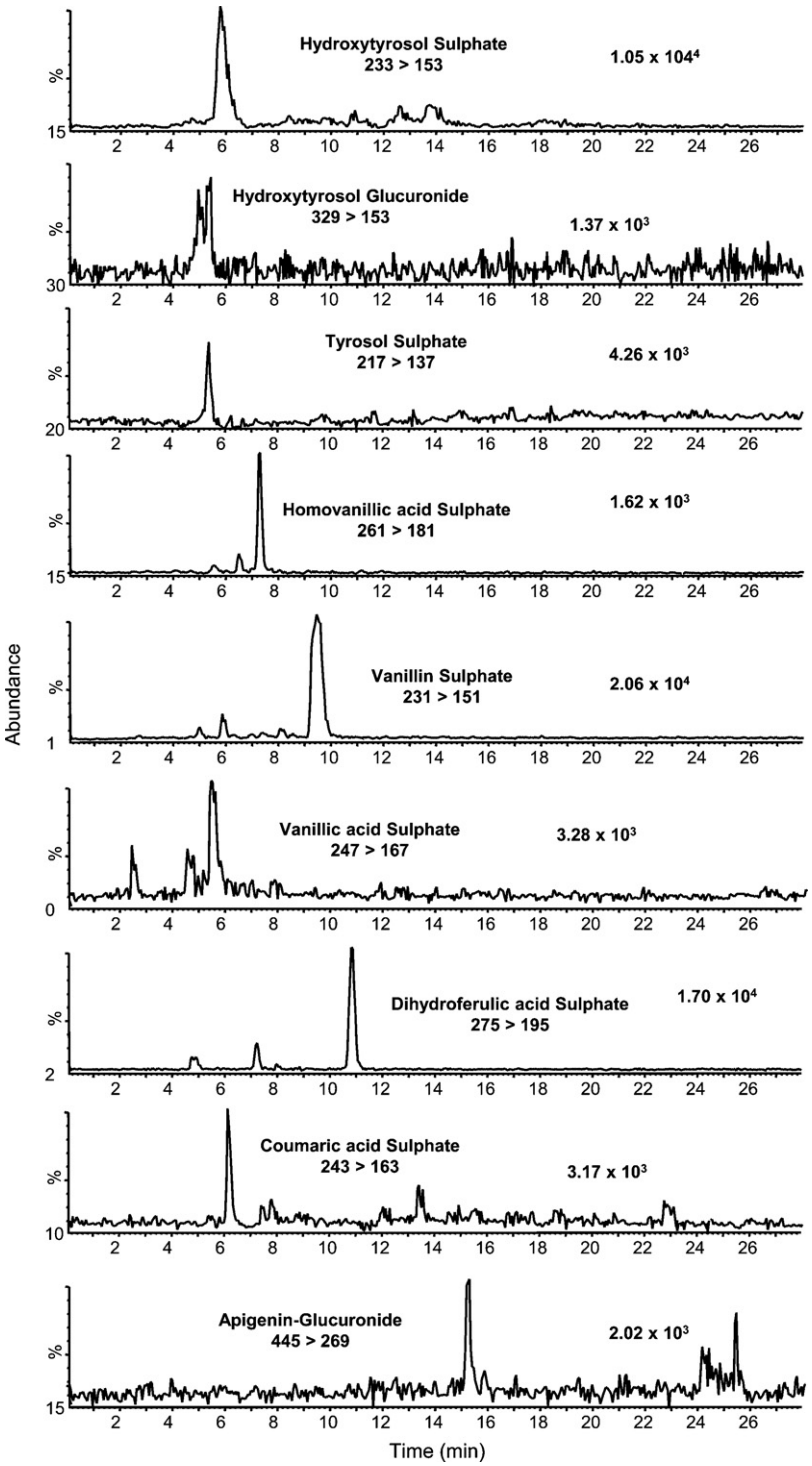

Fig. 1. Extracted ion chromatograms of the generated metabolites identified in the plasma samples after the intake of 30 ml of phenol-rich virgin olive oil. See the text for the analytical conditions (Table 4).

3.1.4. Matrix effect

It is well known that matrix effects are one of the main drawbacks of LC–MS/MS methods. These effects are observed by an increase or decrease in the response of an analyte in a sample matrix compared with the same analyte present in an organic solvent, and this affects results from co-eluting matrix components that compete for ionization capacity. This effect was evaluated by comparing peak abundances obtained from pool basal plasma samples spiked after sample pre-treatment with those obtained from standard solutions at different concentrations.

A negative effect was observed in the two methods (SPE and μSPE-UPLC–MS/MS) developed, which meant a decrease in the detector response. When SPE was used as the sample pre-treatment, the signal suppression for all the phenolic compounds was in the range of 13–24%, which was higher than when μSPE was used (3–19%). The high pre-concentration factor obtained in SPE leads to the pre-concentration the phenolic compounds and also other matrix components that may potentially coelute with the target phenolic compounds and increase matrix effect and reduce the ion ionization of the analyte.

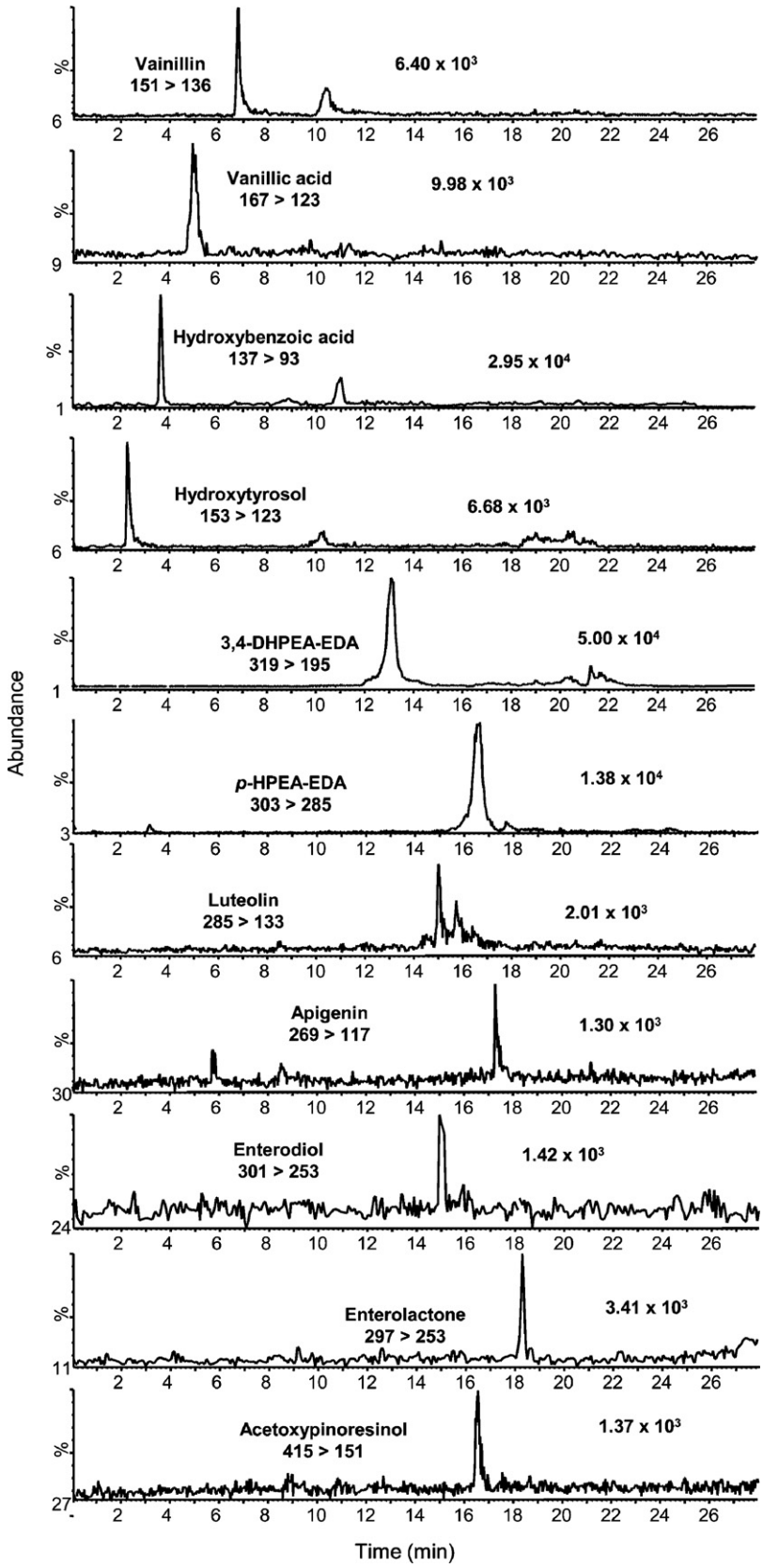

Fig. 2. Extracted ion chromatograms of the free phenols identified in the plasma samples after the intake of 30 ml of phenol-rich virgin olive oil. See the text for the analytical conditions (Table 1).

3.2. Application of the method for determining phenols in plasma samples

The off-line μSPE method developed was applied to plasma samples obtained from five healthy volunteers 60 and 120 min after the consumption of 30 ml of virgin olive oil. For each subject, the plasma sample with maximal peak areas was selected to identify and quantify the phenols. Table 4 shows the SRM conditions for the analysis of the identified metabolites, and Fig. 1 shows the extracted ion chromatograms for the identified metabolites.

The study of the results of the plasma samples revealed an intense metabolism of the virgin olive oil phenols in the body. Table 5 shows their tentatively quantification. The major compounds identified and quantified in plasma corresponded to metabolites of tyrosol and especially hydroxytyrosol (Fig. 1). The metabolism of phenols from virgin olive oil has been described in different studies over recent years. A previous study by Vissers et al. [28] suggested that an important step in the metabolism of the olive oil phenols, oleuropein and ligstroside-aglycones (commonly called secoiridoids), is their transformation into hydroxytyrosol or tyrosol. The gastric environment reaches a pH of at least 2–3 for around 30 min, and under such conditions the stability of these complex olive oil polyphenols may be affected and could result in a significant increase in the amount of hydroxytyrosol and tyrosol, which reach the small intestine [29]. Different enzymatic pathways for the metabolism of hydroxytyrosol after this first step have been postulated. Glucuronidation and sulphation by gluco-transferase and sulpho-transferase enzymatic activity possibly takes place in the enterocytes as well as in the liver cells [2,28] resulting the conjugated forms of hydroxytyrosol, tyrosol and flavonoids (luteolin and apigenin) quantified in the plasma samples from some volunteers in our study (Table 5). Another important metabolic step, which also takes place in the liver, is the *O*-methylation by catechol-*O*-methyltransferase [2,28]. This way, the homovanillic acid sulphate (3-hydroxy-4-methoxy phenylacetic acid) could be the direct product of the hydroxytyrosol methylation. After that, as the result of a second metabolic step, vanillin (4-hydroxy-3-methoxy-phenylacetaldehyde) and vanillic acid (4-hydroxy-3-methoxy-phenylacetic acid) are formed as products of alcohol dehydrogenase and aldehyde dehydrogenase activities, respectively [2]. We found hydroxybenzoic acid in all the plasma samples. This could be the product of the direct metabolism of tyrosol through alcohol dehydrogenase and aldehyde dehydrogenase activities.

The majority of studies of olive oil metabolism have focused on hydroxytyrosol, tyrosol and oleuropein derivatives. Nevertheless, the absorption and disposition of flavonoids and lignans after the ingestion of virgin olive oil has not been considered, although such flavonoids as luteolin and apigenin, and the lignans pinoresinol and acetoxypinoresinol, are also phenolic components of virgin olive oil. The application of the developed off-line μSPE method to the quantification of flavonoids in plasma samples has been shown to vary greatly. The free forms of luteolin and apigenin were only quantified in the plasma from subjects 1 and 5 (Fig. 2 and Table 5). By contrast, the glucuronide metabolite of apigenin was tentatively quantified in all samples analyzed, but showing a considerable inter-individual variation in the metabolic response to a given dose of virgin olive oil. The presence of the conjugated form of dihydroferulic acid, a common phenol quantified in all plasma samples, could result from the flavonoid ring scission during gastric digestion (pH 2–3 for a period around 30 min) or during transfer across the jejunum and ileum, when polyphenols are subjected to extensive metabolism by phase I hydrolyzing enzymes. The other metabolic via of flavonoids is derived from their transformation by the gut microflora into smaller phenolic acids. These are detected in plasma and are often further conjugated and metabolized in the liver [30]. Finally, the application of the off-line μSPE method to quantify the lignans in plasma samples did not allow their detec-

Table 5
Phenol metabolites and their free forms identified and quantified (μM) in plasma samples 60 and 120 min after the consumption of 30 ml of virgin olive oil rich in phenolic compounds.

	SRM_1	Plasma concentration (μM)				
		Subject 1[b]	Subject 2[b]	Subject 3[c]	Subject 4[b]	Subject 5[c]
Metabolites[a]						
Hydroxytyrosol sulphate	233/153	0.28	0.24	0.31	0.66	0.68
Hydroxytyrosol glucuronide	329/153	ND	0.07	0.06	ND	ND
Tyrosol sulphate	217/137	ND	ND	15.10	ND	ND
Homovanillic acid sulphate	261/181	ND	ND	0.11	3.73	0.12
Vanillin sulphate	231/151	7.34	8.67	11.10	59.41	12.2
Vanillic acid sulphate	247/167	0.06	0.09	0.08	ND	ND
Dihydroferulic acid sulphate	275/195	0.16	0.40	0.27	2.48	0.30
Coumaric acid sulphate	243/163	ND	0.97	ND	ND	ND
Apigenin glucuronide	445/269	0.001	0.002	0.04	0.001	0.001
Free phenols						
Vanillin	151/136	ND	1.53	1.55	0.70	0.50
Vanillic acid	167/123	ND	3.55	ND	2.98	ND
Hydroxybenzoic acid	137/93	1.76	3.44	1.43	13.78	5.64
Hydroxytyrosol	153/123	ND	ND	ND	ND	0.06
3,4-DHPEA-EDA	319/195	ND	ND	ND	ND	0.63
p-HPEA-EDA	303/285	ND	ND	ND	ND	0.03
Luteolin	285/133	1.23	ND	ND	ND	0.013
Apigenin	269/117	0.08	ND	ND	ND	0.003
Enterodiol	301/253	ND	0.2	ND	ND	ND
Enterolactone	297/253	ND	0.01	ND	ND	ND
Acetoxypinoresinol	415/151	ND	ND	ND	ND	0.016

SRM_1: monitored transition for each transition SRM in quantification process; ND: no detected.

[a] Hydroxytyrosol sulfate, hydroxytyrosol glucuronide are quantified as hydroxytyrosol; tyrosol sulphate is quantified as tyrosol; homovanillic acid sulfate, vanillin, vanillin sulfate, vanillic acid sulfate are quantified as homovanillic acid; hydroxybenzoic acid, dihydroferulic acid sulfate, coumaric acid sulfate are quantified as *p*-coumaric acid; apigenin glucuronide is quantified as apigenin; enterodiol and enterolactone are quantified as pinoresinol.

[b] 60 min after the consumption of the virgin olive oil.

[c] 120 min after the consumption of the virgin olive oil.

tion, even in their glucuronide or sulphate conjugates. Plant lignans are phenolic compounds with a 2,3-dibenzylbutane skeleton. Their interest is more closely related to their metabolism by the bacterial flora in the colon to the mammalian lignans enterodiol and enterolactone, than by their antioxidant activity. Enterodiol and enterolactone were only found in the plasma sample from the subject 2, at a very low concentration. The fact of not having detected pinoresinol or acetoxypinoresinol could be related to their low concentration in virgin olive oil compared with other phenolic fractions, and also probably to their intense metabolism by colonic flora, with the exception of the plasma from subject 5, where acetoxypinoresinol was found at a very low concentration in its native form.

Besides the presence of the virgin olive oil phenols in their conjugated forms, an important variability was observed between the plasma samples obtained from different volunteers. As a consequence, the concentrations found cover a very far range (Table 5). This variability in the post-absorptive metabolism of phenols may be attributed to differences in the expression of metabolizing enzymes due to genetic variability within the population [31]. This is one of the more important limitations of the interventional studies with humans. For example, a study of the bioavailability of *trans*-resveratrol with 25 subjects showed enormous inter-individual differences [32]. Resveratrol was never found in the serum samples of 14 subjects in any form at any time, whereas in the other 11, at least one serum sample was positive for free resveratrol or a glucuronidated derivative. This inter-individual variability could also be related to the fact that the virgin olive oil phenols were only detected in their free form in the plasma from subject 5 (Table 5). The analyses in the SRM gave information about the presence in one of the five samples of the precursor ions corresponding to the native forms of hydroxytyrosol, 3,4-DHPEA-EDA, *p*-HPEA-EDA, luteolin, apigenin and acetoxypinoresinol (Fig. 2). Nevertheless, the amounts detected always were very low (at the nanomolar level), in many cases below the quantification limit.

The quantification of these metabolites in plasma samples was tentative because it was carried out using the calibration curves of the respective free forms. The concentrations are expressed as the respective standard equivalents. Hydroxytyrosol sulphate, hydroxytyrosol glucuronide were quantified as hydroxytyrosol. Tyrosol sulphate was quantified as tyrosol. Homovanillic acid sulphate, vanillin, vanillin sulphate and vanillic acid sulphate were quantified as homovanillic acid. Hydroxybenzoic acid, dihydroferulic acid sulphate, coumaric acid sulphate were quantified as *p*-coumaric acid. Apigenin glucuronide was quantified as apigenin, and enterodiol and enterolactone were quantified as pinoresinol. The tentatively results of the quantification were in accordance with previous studies [16,33] that revealed maximum concentrations of tyrosol, hydroxytyrosol and *O*-methyl-hydroxytyrosol between 4 and 15 μM in plasma after the intake of a dose of 40 ml of polyphenol-rich olive oil. These concentrations are higher than those quantified in the present study. Nevertheless, in these studies the plasma polyphenol concentrations were determined by GC–MS previous derivatization of the sample. These preanalytical treatments of the plasma samples with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide for 60 min at 60 °C could lead to the hydrolysis of some of the conjugated forms of tyrosol and hydroxytyrosol resulting in free forms that could justify the major concentration of the free forms of hydroxytyrosol and tyrosol found in these studies.

As with our study, the previous study by de la Torre-Kabot et al. [21] analyzed the metabolites found in LDL 60 min post-ingestion of 50 ml virgin olive oil by HPLC–ESI-MS/MS after SPE, and the results of the phenol quantification were expressed as ng of phenol per mg of apo-B. Thus, no comparison of the quantification between this preliminary study and the present study is possible.

4. Conclusions

This paper develops and compares two rapid, efficient and sensitive methods for identifying phenolic compounds from virgin olive oil and their metabolites in plasma samples. An off-line SPE and a 96-well microplate (μSPE) followed by an UPLC–ESI-MS/MS were used to extract and determine the phenolic compounds from the plasma samples. Pool plasma spiked with the standards of a representative selection of the main virgin olive oil phenolic compounds was used to validate the method. Both the SPE and μSPE sample pre-treatment techniques showed good recoveries and low LODs and LOQs.

Nevertheless, the μSPE is a fast sample pre-treatment technique that permits a lower biological sample volume load. This speed is achieved through avoiding the post-extraction solvent evaporation and reconstitution steps. Additionally, despite the lower pre-concentration factor obtained with the μSPE, the LODs and LOQs for some phenolic compounds were similar between the two SPE methods developed, and the matrix effect for all the studied phenolic compounds was slightly lower with the μSPE. In consequence, the off-line μSPE-UPLC–MS/MS method allowed olive oil phenols and their metabolites to be determined in plasma samples at low μM concentration levels in less than 20 min. Given its speed, sensitivity, selectivity and low sample amount, this method could be thus being proposed as a routine analysis in the human interventional studies with a large number of samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.10.025.

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