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Analytical Methods

A sensitive liquid chromatography-mass spectrometry method for the simultaneous determination in plasma of pentacyclic triterpenes of *Olea europaea* L.

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ABSTRACT

Table olives are especially rich in pentacyclic triterpenic compounds, which exert several biological activities. A crucial step in order to know if these compounds could contribute to the beneficial and healthy properties of this food is their measurement in blood. Therefore, the present study describes a simple and accurate liquid-liquid extraction followed by LC-QqQ-MS analysis for the simultaneous determination of the main pentacyclic triterpenes from *Olea europaea* L. in rat plasma. The method was validated by the analysis of blank plasma samples spiked with pure compounds, obtaining a linear correlation, adequate sensitivity with a limit of quantification ranging from 1 nM for maslinic acid to 10 nM for uvaol. Precision and accuracy were lower than 10% in all cases and recoveries were between 95 and 104%. The oral administration of olives to rats and its determination in plasma verified that the established methodology is appropriate for bioavailability studies.

1. Introduction

Table olives form part of the commonly called Mediterranean diet and they have a considerable content in different bioactive compounds such as monounsaturated fatty acids, tocopherols, phospholipids, carotenoids, phenols and pentacyclic triterpenes (Ghanbari, Anwar, Alkharfy, Gilani, & Saari, 2012). The latter are non-nutritive dietary microconstituents particularly abundant in the skin of the olives. They protect the integrity of the fruit as they act as insect antifeedants and antimicrobial agents (Vioque & Maza, 1963). The most abundant triterpenes are maslinic acid and oleanolic acid (Bianchi, 2003; Guinda, Rada, Delgado, Gutiérrez-Adánez, & Castellano, 2010; Romero et al., 2010) whose chemical structure differs by the lack of the hydroxyl group at the 2-carbon position in oleanolic acid. Triterpenic diols such as erythrodiol and uvaol were mostly found in olive leafs (Guinda et al., 2010; Sánchez-Avila, Priego-Capote, Ruiz-Jiménez, & de Castro, 2009) although they were also present in olive fruits but in much less amount, than the triterpenic acids (López-López, Rodríguez-Gómez, Ruíz-Méndez, Cortés-Delgado, & Garrido-Fernández, 2009). Recently, interest for these compounds has increased because of the reported beneficial effects on health which include antitumor (Juan, Planas, Ruíz-Gutiérrez, Daniel, & Wenzel, 2008; Martín et al., 2007), antidiabetic (Liu, Sun, Duan, Mu, & Zhang, 2007), anti-viral (Xu, Zeng, Wan, & Sim, 1996), cardioprotective (Rodríguez-Rodríguez, Perona, Herrera, & Ruiz-Gutiérrez, 2006) and neuroprotective activites (Guan et al., 2011).

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Despite of the biological effects described *in vitro* for the pentacyclic triterpenic compounds, there is a lack of knowledge about their absorption, metabolism and distribution either in humans or in animals after table olives consumption. Therefore, studies conducted *in vivo* are mandatory to elucidate if these bioactive compounds are bioavailable to exert their beneficial effect and in which extent. To carry out these bioavailability studies, the first step is to develop an analytical method able to detect the pentacyclic triterpenes that reach the blood after oral intake of olives. Up to now, there is no method in the literature that allows the detection of erythrodiol and uvaol in plasma, and the ones reported for pentacyclic triterpenic acids mainly determine only one of the analytes of interest, such as the ones described for maslinic acid (Lozano-Mena, Juan, Garcia-Granados, & Planas, 2012; Sánchez-González, Colom, Lozano-Mena, Juan, & Planas, 2014; Sánchez-González, Lozano-Mena, Juan, García-Granados, & Planas, 2013), oleanolic acid (Jeong et al., 2007) or ursolic acid (Chen, Luo, Zhang, & Chen, 2011; Xia, Wei, Si, & Liu, 2011). In this work, we propose a reliable methodology for the determination at the same time of pentacyclic triterpenic acids and alcohols, and also with enough sensitivity to detect these compounds in plasma samples after oral intake of Marfil table olives. This variety, native from the Montsià region (Tarragona, Spain) and named after its pale whitening hue, was selected since they are especially rich in pentacyclic triterpenes compared to other Spanish table olives (Romero et al., 2010). The method was validated according to the European Medicines Agency (2011). Finally, an olive suspension was administered to Sprague-Dawley rats and blood collected at 120 min was analyzed to prove the performance of the method and verify its suitability for its use in bioavailability studies.

2. Materials and methods

2.1. Chemicals

Maslinic acid (MA) was supplied by Sigma–Aldrich (Madrid, Spain). Oleanolic acid (OA), ursolic acid (UA), erythrodiol (ERI), uvaol (UVA) and betulinic acid (internal standard, IS) were purchased from Extrasynthèse (Genay, France). The mobile phase solvents (LC–MS grade) were water from Sigma–Aldrich and methanol (MeOH) provided by Panreac (Barcelona, Spain). Ethyl acetate (LC–MS grade) was purchased from J.T Baker (Deventer, Netherlands) and ethanol (EtOH) was obtained from Merck (Darmstadt, Germany). All other chemicals used in the preparation of solutions were of analytical reagent grade. Water with a conductivity lower than 0.05 $mS \cdot cm^{-1}$ was obtained using a Milli-Q water purification system from Millipore (Molsheim, France).

2.2. Animals

Male adult Sprague–Dawley rats (275–300 g) were from breeding colonies from the Animal House Facility from the Facultat de Farmàcia de la Universitat de Barcelona. Animals were maintained in groups of two per cage and were given a standard diet (2014 Teklad Global 14%, Harlan, Barcelona, Spain) and water *ad libitum*. The rooms were kept under controlled conditions of temperature (22 ± 2 °C), humidity (50 ± 10%) and lighting (12 h light:dark cycle). Before the experiments, the animal feed and the plasma were analyzed and none of the pentacyclic triterpenes were detected. The Ethic Committee of Animal Experimentation of the Universitat de Barcelona approved this research. Handling and killing of the animals fulfilled the guidelines established by the European Community in terms of care and management of laboratory animals. To minimize the effects of circadian rhythms, experiments were performed in the morning. Rats were fasted overnight, before the withdrawal of either blank plasma or plasma obtained after the oral administration of olives. For the collection of plasma, animals were anesthetized under deep terminal anesthesia by intraperitoneal injection of 90 mg/kg of ketamine (Imalgene 1000, Merial Laboratorios S.A., Barcelona, Spain) and 10 mg/kg of xylacine (Rompun 2%, Química Farmacéutica Bayer S.A., Barcelona, Spain), and blood was obtained by cardiac puncture.

2.3. Calibration standards and plasma sample preparation

Stock solutions of pentacyclic triterpenes, prepared as described previously (Giménez et al., 2015) were employed to make working standards containing a mixture of MA, OA, UA, ERI and UVA at 0.1, 0.5, 1, 5, 10 and 20 μM. The stock solution of the IS was diluted to produce the working standard of 2 μM.

Blank plasma obtained from rats that have never received either the pentacyclic triterpenic compounds or table olives, and plasma samples obtained after the administration of table olives were prepared based on the method described previously (Sánchez-González et al., 2013). Briefly, blood was withdrawn from rats into tubes containing EDTA-K_2 as anticoagulant. Plasma obtained after centrifugation at 1500*g* for 15 min at 4 °C (Centrifuge Megafuge 1.0R, Heraeus, Boadilla, Spain) was pooled and aliquoted before being stored at −20 °C until analysis. Calibration standards were freshly prepared before analysis with 190 μL of blank plasma spiked with 10 μL of working standard solutions in order to yield the final concentrations of 5, 25, 50, 250, 500 and 1000 nM. The IS was added (10 μL at the concentration of 2 μM) to a 200 μL aliquot of rat plasma or calibration standard to a final concentration of 100 nM. Then, plasma samples were diluted with 2 mL of ethyl acetate, vortex-mixed and centrifuged. The supernatant was transferred to a clean tube and the pellet was subjeted to a second extraction. Supernatants were joint and evaporated and the residue was reconstituted in 100 μL of methanol 80%. Samples were vortex-mixed and ultrasonicated for 1 min. A final centrifugation was performed to obtain a clear supernatant that was transferred to an amber vial for LC–MS analysis.

2.4. Liquid chromatography-mass spectrometry

The LC-QqQ-MS analyses were carried out in an Acquity UPLC (Waters, Milford, Michigan, USA) with a binary pump, a refrigerated auto sampler plate set at 10 °C with an automatic injector and a thermostated column compartment. The mass spectrometry analysis was carried out in a PE Sciex API 3000 triple quadrupole (Applied Biosystems, AB Sciex, Alcobendas, Madrid). The Analyst software 1.4.2 (Applied Biosystems) operated the instrument and executed the data analysis.

A Zorbax Eclipse PAH, protected with a guard column, was used as analytical column (3.5 μm particles, 100 Å pore diameter and 150 × 4.6 mm L_T × ID, Agilent Technologies, Waldbronn, Germany) (Giménez et al., 2015). Temperature of the column was set at 30 °C and the injection volume was 10 μL. The mobile phase consisted of water (17%) and methanol (83%) that eluted under isocratic conditions at 0.8 mL/min of flow rate. Carry-over was prevented by programming a 4 min of methanol (100%) at the end of the run. In addition, to avoid memory effects, the needle was washed with acetonitrile:water (90:10, v/v) followed by methanol:water (80:20, v/v).

An APCI source kept at 450 °C was used for the ionization of the triterpenic compounds. Adquisition of MS data was achieved in two segments: the first segment programmed in negative mode (0–22 min) to enable the detection of pentacyclic triterpenic acids (MA, OA, UA and IS), and the second segment in positive mode (22–34 min) for the detection of alcohols (ERI and UVA). Nitrogen, set at a nebulizing flow of −3 and +3 au for negative and positive ion

mode, respectively, was used as curtain (12 au) and nebulizer gas (10 au). The optimal MS conditions in the negative mode for the detection of pentacyclic acids were declustering potential of −50 V, focusing potential of −170 V and entrance potential of −10 V. For the triterpenic alcohols determined in positive ion mode the declustering potential, focusing potential and entrance potential were 40 V, 160 V and 10 V, respectively. Analytes were determined with the selected ion monitoring (SIM) mode at *m*/*z* 471.6 for MA and 455.5 for OA, UA and IS, with a dwell time of 1000 ms for all them. Triterpenic alcohols were measured at *m*/*z* 425.7 with a dwell time of 4000 ms. Quantification of pentacyclic triterpenes was carried out by the interpolation of the peak area ratio of analytes *versus* IS on a calibration curve.

2.5. Method validation

The liquid-liquid extraction method used to determine the pentacyclic triterpenes in plasma was validated following the Guidelines on Bioanalytical Method Validation of the European Medicines Agency (2011). The parameters evaluated were: linearity, sensitivity, selectivity, precision and accuracy. Recovery, matrix effect and carry-over were also assessed.

2.6. Method application

Table olives of the Marfil variety were administered to overnight fasted Sprague-Dawley rats. Therefore, 10 destoned olives (approximately 12 g of pulp) were mixed with 40 mL of Milli-Q water. The sample was grinded with a Polytron homogenizer (PTA 20 TS rottor, setting 5; Kinematica AG, Lucerne, Switzerland) yielding a fine and homogeneous suspension. This olive suspension was orally administered by gavage to overnight fasted rats at 10 mL/kg. Blood was collected 120 min after the oral administration and was placed into Microvette CB300 tubes (Sarstedt, Granollers, Spain) with EDTA-K_2 as anticoagulant. Plasma was immediately obtained by centrifugation at 1500*g* for 15 min at 4 °C and was stored at −20 °C until analysis that was perfomed using the protocol described above.

At the same time, for the determination of triterpenic compounds in this olive suspension the finely grinded olive pulpe was stired in a vortex and 2 mL of the uniform suspension were taken with a 5 mL pipette. The aliquot was diluted in 12 mL of EtOH:MeOH (1:1) containing the IS at a final concentration of 100 μM. The mixture was vortexed for 5 min and centrifuged at 26900*g* for 15 min (Ultracentrifuge Sorvall, model RC 5C Plus, Thermo Fisher Scientific, Madrid). The supernatant was transferred to another tube, diluted 1/50 in MeOH 80% and transferred to an amber vial for LC–MS analysis.

3. Results

3.1. Optimization of the internal standard

Betulinic acid was selected as IS as in previous works (Giménez et al., 2015; Sánchez-González et al., 2013). Although IS was an acid and thus could have a better profile as IS of the acids than of the alcohols; the results obtained in the validation performed by spiking blank plasma samples confirmed the adequacy of this compound as IS for the triterpenic alcohols (see below). Preliminary results showed that the expected concentrations in plasma samples were in the range of nM, and OA could be masked by the IS. Therefore, in this study it was required to optimize the amount of IS because the concentration of olive triterpenes found in plasma samples was lower compared to those found in table olives. Then different concentrations of IS (100, 250, 500 and 1000 nM) were spiked to blank plasma samples along with increasing concentrations of pentacyclic triterpenes (10, 25, 50 and 75 nM) close to the LOQ. The chromatogram obtained by the established LC–MS method for a 10 nM mixture of olive triterpene standards using 500 and 100 nM of IS, respectively is displayed as Supplementary Material, and shows that it was necessary to diminish the amount of betulinic acid to a concentration of 100 nM to avoid the peak overlapping between the IS and OA.

3.2. Method validation

3.2.1. Linearity

Linearity of the pentacyclic triterpenes was assessed in the range of application of the analytical method. Therefore, blank plasma samples were spiked with increasing concentrations of pentacyclic triterpenes at the concentratrions of 0, 5, 25, 50, 75, 100, 250, 500 and 1000 nM, and were analyzed following the procedure described in the Material and Methods section. A calibration curve was generated for each analyte with three individual replicates prepared at three different days. Table 1 shows representative linear equations of the five analytes obtained after analyses by the least square method. The calibration curves prepared with blank plasma as matrix indicates that the analytical method was linear at the range of analyzed with correlation coefficients above 0.99.

3.2.2. Sensitivity

The limit of detection (LOD) was established as the concentration of pentacyclic triterpene yielding a signal three times above the noise level. The lowest point in the calibration curve was defined as the lowest limit of quantification (LLOQ) which meets the following criteria of signal-to-noise ratio of 10:1, accuracy within the ±20% limit and precision (%RSD) no greater than 20%. The LLOQ was validated by performing the analysis of six independent blank plasma samples spiked with the theoretical concentration. Sensitivity of the analytical procedure displayed in Table 1, indicates that LOD's for the MA, OA and UA were inferior to 1.5 nM, whereas the LODs of ERI and UVA were 1.5 and 3.0 nM, respectively. Concerning the LOQ, it can be stated that MA had the lowest value of 1 nM, followed by UA with 2.5 and OA and ERI with 5.0 nM. UVA had slightly higher LOQ with a concentration of 10 nM.

3.2.3. Precision and accuracy

The precision, expressed as relative standard deviation (%RSD; coefficient of variation) was evaluated by analyzing a sufficient amount of samples at six levels of concentration: 25, 50, 100, 250, 500 and 1000 nM. The extraction of calibration standards prepared within a day or on three different days, allowed the establishment of the intra-day and inter-day precision, respectively. Accuracy, evaluated in the concentration range of 25–1000 nM, was calculated as described previously (Sánchez-González et al., 2013). Table 2 depicts the performance data of the assay. The method is precise and accurate since the values obtained for intra-day and inter-day precisions (% RSD) and accuracy (%) were inferior to 15% for all the studied pentacyclic triterpenes which is the maximum value permitted by the guidelines (European Medicines Agency, 2011).

3.2.4. Recovery

Recoveries of the extraction process were evaluated for all pentacyclic triterpenes at three concentrations levels: 50, 250 and 500 nM. The recovery of each analyte was determined as the percentage of the peak areas of blank plasma samples spiked with the working standards prior to the extraction compared to the peak areas obtained for blank plasma samples that were spiked

Table 1
Validation results of the analytical method: linearity and sensitivity.

Analyte	Linear range (nM)	Equation	R^2	LOD (nM)	LOQ (nM)
Maslinic acid	5–1000	$y = 0.0165x - 0.0565$	0.999	0.3	1
Oleanolic acid	5–1000	$y = 0.0138x - 0.2091$	0.999	1.5	5
Ursolic acid	5–1000	$y = 0.0137x - 0.2392$	0.999	0.75	2.5
Erythrodiol	5–1000	$y = 0.00005x - 0.0008$	0.996	1.5	5
Uvaol	5–1000	$y = 0.00002x - 0.0004$	0.998	3	10

y, Peak area ratio (analyte/internal standard); *x*, concentration of pentacyclic triterpenes (μM); LOD, limit of detection (signal to noise ratio of 3:1); LOQ, limit of quantification (signal to noise ratio of 10:1).

Table 2
Validation results of the analytical method: intra-day and inter-day precisions and accuracy.

Concentration (nM)	Intra-day precision (% RSD; n = 3)					Inter-day precision (% RSD; n = 9)					Accuracy (%; n = 9)				
	MA	OA	UA	ERI	UVA	MA	OA	UA	ERI	UVA	MA	OA	UA	ERI	UVA
25	4.80	4.13	8.51	8.56	8.84	8.36	4.50	8.29	9.09	9.93	−4.76 ± 2.92	1.29 ± 1.48	−3.16 ± 3.22	−4.86 ± 3.17	−3.94 ± 3.41
50	2.22	6.86	4.58	5.92	2.94	6.94	9.12	8.51	6.66	7.43	−2.95 ± 2.60	−7.85 ± 5.19	−3.16 ± 3.22	−5.14 ± 2.32	−3.28 ± 2.64
100	2.20	3.97	2.06	3.74	3.13	10.5	6.28	8.73	3.92	4.14	−0.70 ± 3.52	−0.16 ± 2.10	−3.68 ± 3.42	0.05 ± 1.38	2.64 ± 1.43
250	4.72	1.70	2.68	2.17	1.40	5.68	4.60	5.00	6.41	4.14	3.64 ± 2.07	3.05 ± 1.61	3.78 ± 1.82	4.33 ± 2.03	4.36 ± 1.23
500	4.36	4.54	3.82	3.63	4.05	9.27	4.61	3.63	3.53	6.08	1.95 ± 2.71	0.69 ± 1.53	−2.59 ± 1.52	−1.07 ± 1.26	−2.45 ± 2.35
1000	0.22	1.64	2.21	1.81	1.36	0.24	2.52	1.99	2.10	1.21	−0.05 ± 0.10	−1.39 ± 1.04	−0.10 ± 0.89	−0.68 ± 0.95	0.03 ± 0.54

*Results are expressed as means ± SEM of six independent samples analyzed by triplicate.

after the extraction process with working standards at the corresponding concentrations. The recovery was also assessed for betulinic acid (IS) at the concentration of 100 nM. Then, the ratio of recovery of analyte and IS yielded an IS-normalized recovery. Table 3 shows the results obtained with and without normalization by the IS. The extraction procedure allowed a satisfactory recovery of the five bioactive compounds analyzed, because even without normalization by the IS, the mean values of the three concentration levels were beyond 85% (i.e. 90.5 ± 2.2%, 88.8 ± 2.3%, 87.6 ± 2.3%, 85.6 ± 3.0% and 87.6 ± 2.7% for MA, OA, UA, ERI and UVA, respectively). Moreover, the IS yielded a similar recovery than the pentacyclic triterpenes, since the mean value was 86.6 ± 2.3%, indicating that the procedure was also suitable for the extraction of betulinic acid. Once the results were normalized by the IS, recoveries for all the compounds were close to 100% at low and high concentrations, with mean values of 100.2 ± 1.2%, 98.5 ± 0.9%, 99.3 ± 1.0%, 97.0 ± 1.2% and 97.7 ± 1.1% for MA, OA, UA, ERI and UVA, respectively.

3.2.5. Matrix effect

Matrix effect was also assessed at 50, 250 and 500 nM of pentacyclic triterpene as well as the internal standard at 100 nM, employing different batches of blank plasma. The evaluation was carried out by calculating the ratio of the peak area in the presence of matrix, to the peak area in absence of matrix (European Medicines Agency, 2011). The mean results showed in Table 3 were 109.9 ± 3.7%, 102.3 ± 3.7%, 96.8 ± 2.9%, 95.9 ± 1.2% and 97.0 ± 2.5% for MA, OA, UA, ERI and UVA, respectively. In addition, the matrix effect of the IS was found to be 101.2 ± 1.6%. These results demonstrated that the endogenous compounds from plasma that co-eluted with the analytes did not affect the ionization of the pentacyclic triterpenes.

3.2.6. Selectivity

Selectivity was evaluated in order to assess whether the extraction procedure was able to differentiate the analytes of interest and IS from endogenous compounds in the matrix. Six independent blank plasma samples that contain neither analytes nor IS were compared with those spiked with a 250 nM mixture of pentacyclic triterpenes and IS at 100 nM. As can be observed in Fig. 1, the method showed high selectivity as no peaks were observed in the blank plasma at the retention time of the analytes of interest. These results confirmed that the extraction method was selective for the studied triterpenes.

3.2.7. Carry-over

The assessment of the carry-over on the LC–MS instrument was performed 6 times in each analytical run by sequentially injecting the highest calibration standard followed by a blank sample at intervals based on the total number of samples in the batch. In addition, the analysis of two independent blank plasma samples were also programmed as the first samples analyzed. The blank samples analyzed immediately after injection of highly spiked samples showed that no peak was detected at the retention time of the analytes and the IS. The absence of carry-over assured the realiability of the method at low concentrations.

3.3. Application of the analytical method

Once validated the method and in order to confirm that the established methodology enabled the analysis of olive pentacyclic triterpenes in plasma samples, a suspension of olives from Marfil variety was orally administered at 10 mL/kg to Sprague–Dawley rats. The same olive suspension was analyzed in order to quantify the amount of each triterpene. Table 4 depicts the pentacyclic triterpenes detected in these table olives in mg of triterpene per gram of fresh olive pulp and the amount administered to the experimental animals. The most abundant compound in table olives was maslinic acid, followed by oleanolic acid and erythrodiol was present in very low concentration. Fig. 2, shows a representative chromatogram obtained at 120 min after oral administration. As can be observed, although we were able to detect maslinic and oleanolic acids and erythrodiol in the olive sample, the amount of erythrodiol was too low to be detected in rat plasma (Table 4). Maslinic acid was detected in the nmolar range with a concentration of around 23 nM and oleanolic acid was found at concentration approximately the limit of quantification.

Table 3
Validation results of the analytical method: recovery and matrix effect.

Concentration (nM)	Maslinic acid		Oleanolic acid		Ursolic acid		Erythrodiol		Uvaol	
	Without IS	With IS	Without IS	With IS	Without IS	With IS	Without IS	With IS	Without IS	With IS
50	92.2 ± 3.8 (n = 6)	97.1 ± 1.2 (n = 6)	92.1 ± 5.7 (n = 5)	98.5 ± 1.9 (n = 5)	94.9 ± 5.2 (n = 4)	99.5 ± 2.2 (n = 6)	86.6 ± 0.6 (n = 5)	95.3 ± 2.4 (n = 6)	90.8 ± 6.0 (n = 5)	100.0 ± 1.8 (n = 6)
250	87.2 ± 2.7 (n = 5)	99.8 ± 1.9 (n = 5)	84.1 ± 1.8 (n = 5)	100.5 ± 1.1 (n = 6)	80.4 ± 2.1 (n = 5)	100.7 ± 1.3 (n = 6)	80.9 ± 2.0 (n = 5)	100.7 ± 1.5 (n = 6)	81.5 ± 1.4 (n = 5)	97.7 ± 1.0 (n = 6)
500	91.0 ± 5.0 (n = 5)	104.3 ± 1.9 (n = 5)	89.4 ± 2.3 (n = 5)	97.1 ± 1.5 (n = 6)	88.6 ± 2.5 (n = 6)	97.8 ± 1.9 (n = 6)	90.4 ± 4.0 (n = 4)	95.1 ± 1.5 (n = 6)	91.6 ± 4.5 (n = 5)	95.3 ± 2.0 (n = 6)
50	97.2 ± 2.8 (n = 6)	110.9 ± 8.2 (n = 6)	108.1 ± 8.9 (n = 6)	104.3 ± 5.9 (n = 6)	102.6 ± 9.9 (n = 6)	92.9 ± 3.9 (n = 6)	99.4 ± 1.4 (n = 6)	95.9 ± 1.1 (n = 6)	94.3 ± 6.5 (n = 6)	95.3 ± 4.3 (n = 6)
250	116.9 ± 4.4 (n = 6)	112.5 ± 4.8 (n = 6)	100.9 ± 2.0 (n = 6)	94.6 ± 1.7 (n = 6)	97.9 ± 1.0 (n = 6)	90.8 ± 1.1 (n = 6)	95.1 ± 1.6 (n = 6)	88.4 ± 0.8 (n = 6)	100.5 ± 2.6 (n = 6)	93.3 ± 1.8 (n = 6)
500	111.7 ± 2.8 (n = 6)	117.7 ± 0.7 (n = 6)	92.2 ± 3.4 (n = 6)	106.4 ± 1.5 (n = 6)	91.4 ± 2.8 (n = 6)	99.7 ± 3.4 (n = 6)	93.2 ± 1.8 (n = 6)	100.6 ± 4.1 (n = 6)	96.3 ± 3.0 (n = 6)	105.0 ± 3.4 (n = 6)

*Results are expressed as means ± SEM.

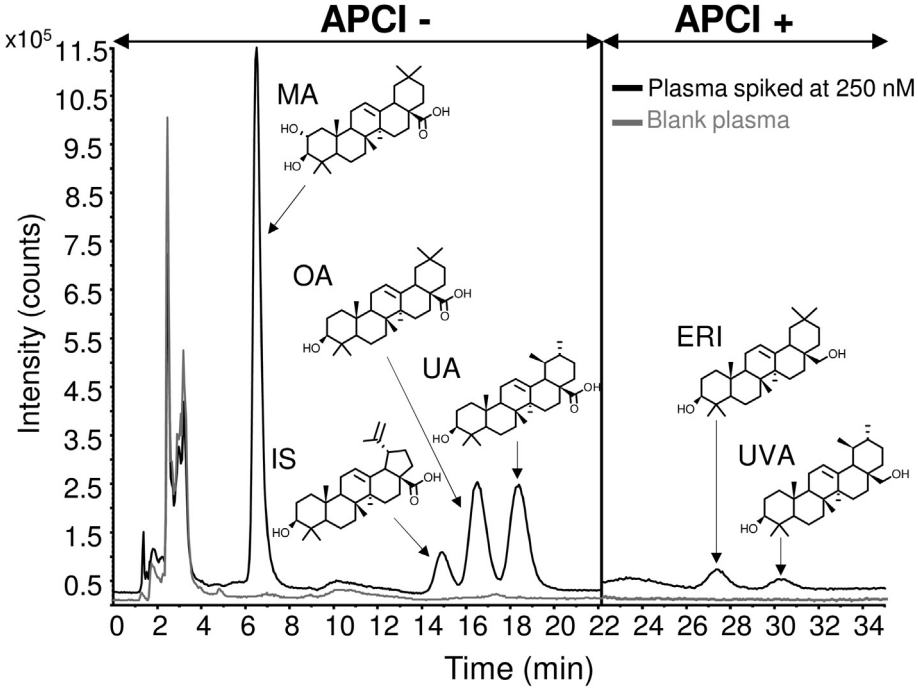

Fig. 1. Total ion chromatogram (TIC) obtained by LC-QqQ-MS of (A) rat blank plasma (B) rat plasma spiked with 250 nM mixture of pentacyclic triterpenes (maslinic acid, MA; oleanolic acid, OA; ursolic acid, UA; erythrodiol, ERI and uvaol, UVA) and betulinic acid as internal standard at 100 nM.

Table 4
Concentration of pentacyclic triterpenes in Marfil table olives and amount administered to Sprague-Dawley rats analyzed by LC-QqQ-MS.

Analyte	mg triterpene/ g of pulp[&]	mg triterpene/kg rat body weight[*]	mg triterpene[$]	Plasmatic concentration at 120 min (nM)	% in plasma at 120 min
Maslinic acid	1.74 ± 0.06	4.57	1.14	23.1 ± 5.3	0.010
Oleanolic acid	1.38 ± 0.10	3.60	0.90	4.32 ± 0.20	0.002
Erythrodiol	0.018 ± 0.001	0.05	0.01	–	–

[&] Results are expressed as means ± SEM of three independent samples analyzed by triplicate.
[*] Dose of triterpenes administered to Sprague-Dawley rats.
[$] Amount of pentacyclic triterpenes administered to Sprague-Dawley rats of 250 g of body weight.

4. Discussion

On the basis that the beneficial effects on health described for pentacyclic triterpenes could be attained after a regular consumption of table olives, the assessment of the plasmatic concentrations of the pentacyclic triterpenes after table olive consumption constitutes a requirement. Up to now, the bioavailability studies conducted after the oral administration of table olives have been

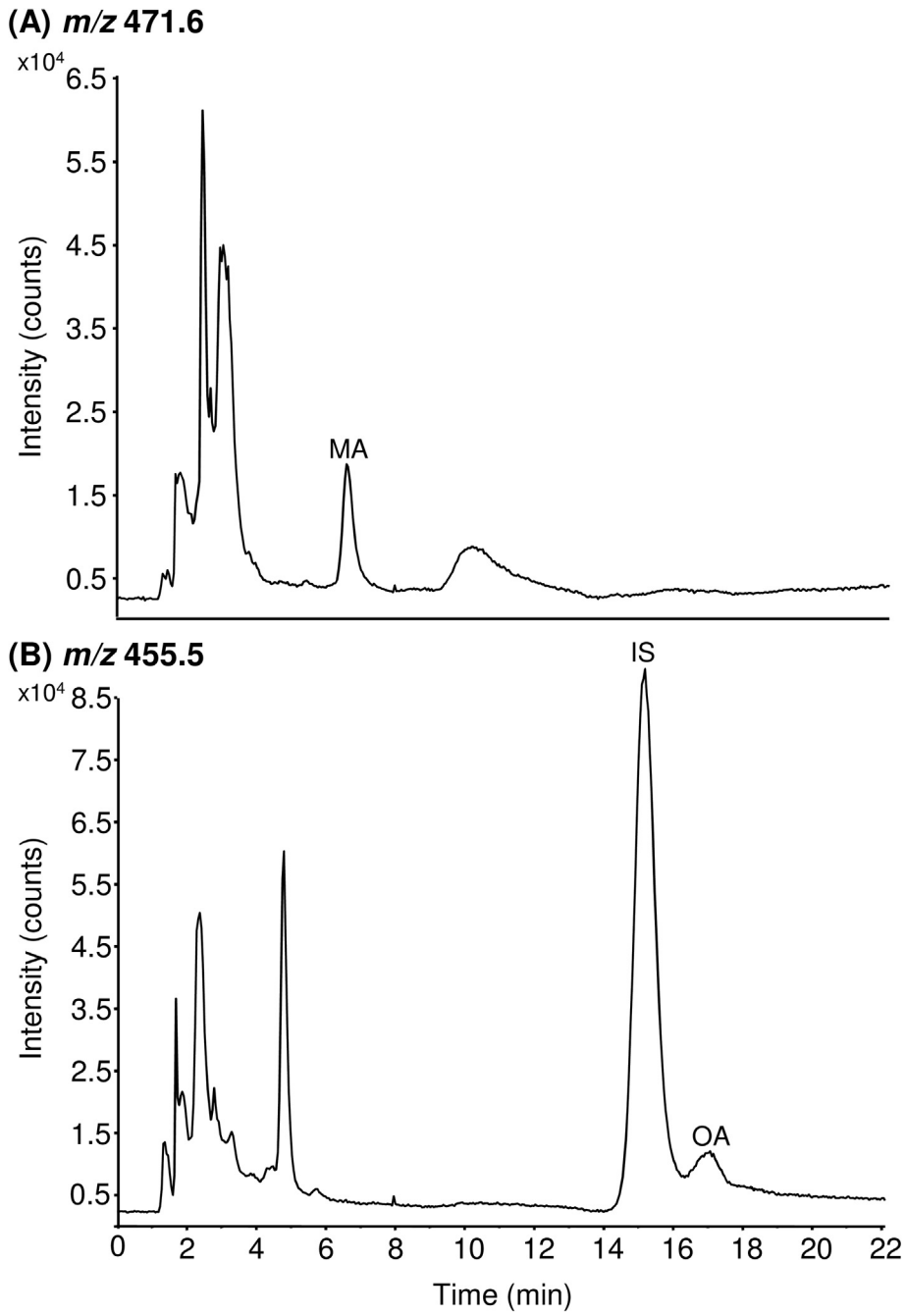

Fig. 2. Representative extracted ion chromatograms (XIC) obtained by LC-QqQ-MS of the pentacyclic triterpenes detected in rat plasma sample 120 min after oral intake of a suspension of olives from Marfil variety. (A) maslinic acid at *m*/*z* of 471.6 and (B) oleanolic acid and internal standard (betulinic acid) at *m*/*z* of 455.5.

focused on the analysis on the absorption and metabolism of polyphenols (Kountouri, Mylona, Kaliora, & Andrikopoulos, 2007). In these sense, the pentacyclic triterpenes present in olives in important amounts (Romero et al., 2010) have been disregarded althought the fact that they are considered bioactive compounds (Sánchez-Quesada et al., 2013). This could be attributed to the lack, until recently, of commercially available pure standards of maslinic acid, which is the main pentacyclic triterpene of olives and also, to the difficulty in developing sensitive methods that allowed an accurate determination of these compounds in body fluids. For all these reasons, the establishment of an analytical method able to separate and detect olive pentacyclic triterpenes in plasma samples arises great interest as the first step in the investigation of the beneficial effects of these bioactive compounds. In the set up of the method one of the aspects that was taken into account was its sensitivity, since it had to be reliable enough to detect the main pentacylicic triterpenes in plasma samples after the intake of olives. In a previous study, we developed a method to simultaneously analyze pentacyclic triterpenes by LC–MS in table olives (Giménez et al., 2015). The chromatographic conditions were adequate to detect the pentacyclic triterpenes from the oleanane family, that is, maslinic acid, oleanolic acid and erythrodiol as well as the ones from the ursane family, ursolic acid and uvaol. The importance of detecting all of these compounds is the

fact that oleanolic acid and ursolic acid, as well as erythrodiol and uvaol are isomers with the same molecular weight and differing only in the position of one methyl group (Jäger, Trojan, Kopp, Laszczyk, & Scheffler, 2009). Although in olives, ursolic acid has not been detected, it is important to have a method able to separate it from oleanolic acid, since it has been described to be present in the leaves of *Olea europaea* L. With respect to uvaol, its presence in the olive fruit has been described but in very low amounts (López-López et al., 2009), therefore, it is important to have an adequate separation from its isomer, erythrodiol.

In the present study, plasma was subjected to a fast and efficient liquid-liquid extraction based on the method described for the determination of maslinic acid (Sánchez-González et al., 2013). To implement this method in bioavailabilty studies it were needed to evaluate if the method was simple, reliable and sensitive enough to detect the analytes of interest in plasma samples. Consequently, blank plasma samples were spiked using three concentration levels in order to validate the analytical performance. Lineality was confirmed with calibration curves with correlation coefficients above 0.99. Regarding accuracy and precision, evaluated as intra-day and inter-day reproducibility, this method showed adequate RSD (%) values (0.22–9.93%) lower than others (Chen, Li, Song, Suo, & You, 2012; Chen et al., 2011; Kim et al., 2016). Furthermore, the results in plasma samples demonstrated that no significant matrix effect for analytes and IS were observed as values were around 91–117%. This fact indicates that the ionization competition between the analyte and endogenous co-elutions was negligible, or that the interferences from the matrix components were removed by the sample extraction process and the LC–MS method was robust.

Although the internal standard selected in this study was a triterpenic acid and thus could have a different response compared to alcohols, the validation of the method confirmed that this compound was also appropriate for the determination of the triterpenic alcohols. In this sense, the recoveries obtained without IS averaged 86% and 88% for ERI and UVA, respectively and with the correction of the IS, that was recovered in an 87%, the values raised to around 98%. This adequacy of the internal standard was also observed for the recoveries obtained for the triterpenic acids. The recoveries without IS for MA, OA and UA were 90%, 89% and 87%, respectively and increased to approximately 100% when the peak areas were normalized by the internal standard. The 10% difference in the recovery prior and after normalization could indicate a possible interaction of these bioactive compounds with plasma proteins, as has been described in *in vitro* assays for maslinic, oleanolic, ursolic and betulinic acids (Rada, Ruiz-Gutiérrez, & Guinda, 2011; Wang, Sun, Ma, Rao, & Li, 2015). However, the validation data show that the absolute recovery was nearly a 90% for individual compounds, which is high, considering that plasma is a complex matrix, thus demonstrating the suitability of the extraction process. In addition, the results obtained in the validation step indicates the adequate performance in terms of linearity, matrix effect, precision and accuracy of the extraction procedure prior to the LC–MS determination that permits a rapid and simoultaneous determination in rat plasma of five pentacyclic triterpens (maslinic acid, oleanolic acid, ursolic acid, erythrodiol and uvaol).

Compared to the other methods reported up to now for the analysis of triterpenic compounds in plasma (Chen et al., 2011; Chen et al., 2012; Jeong et al., 2007; Lozano-Mena et al., 2012; Xia et al., 2011; Yin, Lin, Mong, & Lin, 2012; Kim et al., 2016; Li et al., 2015; Li et al., 2016; Sánchez-González et al., 2013; Sánchez-González et al., 2014; Yin et al., 2012; Zhao et al., 2015), the method here proposed has some advantages. In the first place, the extraction of analytes from plasma is relatively fast, since it is perfomed by a liquid-liquid process with ethyl acetate which is a solvent that allows a rapid evaporation to dryness. Also, the fact that pentacyclic triterpenes are determined by direct injection in the LC–MS, avoiding the derivatization step required when these compounds are detected by gas chromatography or high-performance liquid chromatography. But the most relevant gain of the method consists on the fact that it allows to detect not only the acidic triterpenes but also the alcohols, both of them with recoveries around 100% and no matrix effect, whereas the published methods are able to determine only the triterpenic acids (Chen et al., 2011; Li et al., 2015, 2016; Zhao et al., 2015). Moreover, the LOQ was adequate since it ranged from 1 nM for maslinic acid to 10 nM for uvaol, thus providing similar (Chen et al., 2011, 2012; Li et al., 2015) or higher sensitivity (Li et al., 2016; Xia et al., 2011) in comparison with the published methods. Therefore, the proposed methodology represents an important achievement and opens the possibility to bioavailability studies after consumption of different foods, or administration of plants widely used in tradicional medicine, with the aim of studying in depth the beneficial effects of these compounds in human beings.

Once the method was validated, rats were administered with a finely grinded suspension of Marfil olives in order to verify the *in vivo* performance of the analytical procedure, thus ensuring its applicability for future pharmacokinetic studies. For the verification of the method, the amount to be consumed by the experimental animal was carefully considered, since it was wanted to be kept at the nutritional range. Therefore, 10 olives were destoned and the pulp, approximately 12 g, was grinded with 40 mL of water, and rats were administered with this finely minced suspension at the volume of administration of 10 mL/kg. Under these conditions, a rat would receive a dose of 3 g fresh olive pulp/kg body weight. The translation of this dose from experimental animals to humans, performed with the body surface area normalization method described by Reagan-Shaw, Nihal, & Ahmad (2007) yielded a human equivalent dose of 0.48 g/kg. Then, a man of 70 kg of body weight would consume 28 olives of the Marfil variety to attain the dose administered to rats. Although this dose does not correspond to the amount of olives eaten in a meal or as appetizer, it is not so different from the one usually consumed and would be compatible with the dose to be administered in future pharmacokinetic studies using olives.

The analysis of plasma samples obtained at 120 min revealed the presence of maslinic acid and oleanolic acid without traces of erythrodiol. The fact that the latter could not be found in plasma, can be attributed to its low content in the fruits of *Olea europaea* L. In the olives of the Marfil variety that have been analyzed, the content of erythrodiol was two orders of magnitude lower than maslinic acid and oleanolic acid. The relatively low concentration obtained for maslinic acid (~25 nM) after the administration of the suspension of olives that contained an approximate dose of 4.57 mg/kg, could be explained by the described oral bioavailability of approximately 5% obtained in rats (Sánchez-González et al., 2014). On the other hand, oleanolic acid administered in the form of a suspension at a dose of 2.5 g of wet olive pulp/kg which was contained 3.60 mg/kg was detected at concentrations around 5 nM. Previous studies in the literature that administered oleanolic acid to rats, either as a single compound or in part of an extract, indicates the poor oral bioavailability of this pentacyclic triterpene (Jeong et al., 2007; Li et al., 2015; Zhao et al., 2015) that has been described to be 0.7% for oral doses of 25 and 50 mg/kg (Jeong et al., 2007). Therefore, the lowest bioavailability described for oleanolic acid could explain our results, in which this pentacyclic triterpene was barely detected in comparison to maslinic acid and are in agreement with results obtained after the administration of 0.1 g/kg of *Folium eriobotryae*

effective fraction to rats (Li et al., 2015) and 1 g/kg of *Salvia miltiorrhiza* extracts to mice (Chen et al., 2012).

5. Conclusions

The current study presents a fast and efficient liquid-liquid extraction prior to LC–MS analysis that allows a sensitive and reliable detection of pentacyclic triterpenes from the oleanane and ursane family as indicated in the validation of the method. The analytical assay was further verified *in vivo* with the determination of the main compounds present in table olives thus showing its performance in allowing the simultaneous determination in blood containing trace content of these bioactive compounds. Therefore, the method is suitable in the assessment of the pharmacokinetics in future pre-clinical or clinical studies.

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.02.116.

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