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Analytical Methods

Development and validation of LC-MS-based alternative methodologies to GC–MS for the simultaneous determination of triterpenic acids and dialcohols in virgin olive oil

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ABSTRACT

Pentacyclic triterpenes are minor, but very relevant compounds found in virgin olive oil (VOO). A rapid and reliable LC-MS method for determining the triterpenic acids and dialcohols (after ultrasound assisted extraction) from VOO has been developed, giving an alternative to the widely used GC (FID/MS) methodologies. The analytical parameters of the proposed method were exhaustively checked, establishing limits of detection (from 1 to 95 μg/l) and quantification, precision (RSD values for *inter*-day repeatability were found between 4.2 and 7.3% considering area values), trueness (within the range 92.7 and 100.5%) and evaluating possible matrix effect (which was no significant). The method was applied to the analysis of six triterpenic compounds in 11 monovarietal VOOs and the results compared with the quantitative GC–MS data. Moreover, the direct injection (after a simple dilution) of the samples into the LC-MS system was also tested, in an attempt to proffer an even simpler sample treatment.

1. Introduction

It has been demonstrated that VOO consumption brings along beneficial effects on human health, being the high content of monounsaturated fatty acids together with its non-glyceridic components the main responsible of its benefits (Uylaşer & Yildiz, 2014). Among the minor components of VOO, phenolic compounds and pentacyclic triterpenes have been capturing lots of researchers' attention in the last decades because of their interesting biological properties. Phenolic content, for instance, has been widely assessed in VOOs produced by using a great diversity of agrotechnological parameters, coming from different varieties and geographical origins (Servili et al., 2004). On the contrary, VOO triterpenic content has been scarcely reflected in literature. Even though several stimulating reports carrying out the quantification of the most abundant pentacyclic triterpenes in olive oils (from different categories, varieties and obtained by different processing methods) have been published (Allouche et al., 2009; Allouche et al., 2010; García, Brenes, Dobarganes, Romero, & Ruíz-Méndez, 2008; Pérez-Camino & Cert, 1999), the analytical methods used so far generally do not give an estimation of their absolute content, as they carry out the quantification based on the response factor of another triterpenic compound. Triterpenic dialcohols are commonly determined as the percentage of total sterols, since it is a recognized authenticity index to detect possible fraudulent mixtures with olive-pomace oils) (Bajoub, Bendini, Fernández-Gutiérrez, & Carrasco-Pancorbo, 2016).

The limited number of published analytical methods to determine pentacyclic triterpenes in olive oil does not match with the proliferation of research studies about their bioactivity. In the last years, some interesting reviews providing an overview of the biological activities (anti-inflammatory, antitumoral, cardioprotective and antidiabetic, among others) of triterpenes from *Olea europaea* have been written (Rodríguez-Rodríguez & Ruiz-Gutiérrez, 2010; Sánchez-Quesada et al., 2013). Bearing this in mind, finding analytical methods to determine them (easily and reliably) seems imperative, in order to allow consumers, as well as olive oil industry, to know their concentration levels.

Gas chromatography (GC) coupled to flame ionization (FID) (Allouche et al., 2009; Allouche et al., 2010; Guinda, Albi, Pérez-Camino, & Lanzón, 2004; Pérez-Camino & Cert, 1999) or mass spectrometry (MS) (Kalogeropoulos, Chiou, Mylona, Ioannou, &

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Andrikopoulos, 2007) detectors have been the most applied platforms for the determination of triterpenoids in olive oil so far. Only one reference can be found in literature about the use of liquid chromatography (LC) coupled to diode array detection (DAD) for the determination of two triterpenic acids in olive-pomace oil (García et al., 2008). Nevertheless, LC-DAD and LC-MS have been used for the identification and quantification of these compounds in other matrixes such as plant materials (Peragón, 2013; Romero et al., 2010; Sánchez-Ávila, Priego-Capote, Ruiz-Jiménez, & Luque de Castro, 2009) and biological fluids (Rada, Ruiz-Gutiérrez, & Guinda, 2011).

Both GC and LC have in common a previous step to assure the isolation of these analytes from the matrix. Extraction of triterpenic acids from olive oil has been commonly carried out by using solid phase extraction (SPE) according to a method firstly proposed by Pérez-Camino and coworkers (Pérez-Camino & Cert, 1999). Alternatively, two liquid-liquid extraction (LLE) protocols have been proposed: one with a methanol/ethanol mixture (1:1, v/v) as extractant agent (García et al., 2008); and the other with methanol (Kalogeropoulos et al., 2007), for the simultaneous extraction of triterpenic acids and phenolic compounds from VOO. Triterpenic dialcohols have been frequently determined according to the method proposed by the European Regulation 2568/91 (European Commission, 1991), which involves a tedious saponification process (Allouche et al., 2009; Allouche et al., 2010; Lukic, Lukic, Krapac, Sladonja, & Pilizota, 2013).

The main aim of this work has been to propose an alternative LC-MS method for the determination of pentacyclic triterpenes in olive oil, avoiding the need of a derivatization step, which is one of the main disadvantages of the GC methods. Based on the previous experience of our research group in determining these compounds in plant matrixes (Olmo-García, Bajoub, Fernández-Gutiérrez, & Carrasco-Pancorbo, 2016), the main challenge has been to find a simple sample treatment and to adapt the chromatographic separation to the oily matrix. The proposed method was logically validated and then, applied to the analysis of six triterpenic compounds in 11 monovarietal VOO samples, comparing the results with those obtained by GC–MS data. Moreover, the direct injection (DI) of the samples in the LC-MS system after a simple dilution was also explored, trying to simplify even further the sample treatment. Bring the results achieved by using the three chosen strategies into comparison could give, from our point of view, more reliability to the outcomes of our study, making possible to discuss in depth the advantages/drawbacks of each approach.

2. Materials and methods

2.1. Chemicals and standards

All reagents were of analytical grade and used as received. Methanol (MeOH) tetrahydrofuran, acetone and isopropanol (gradient grade) from Prolabo (Paris, France) and ethanol absolute (EtOH) from Panreac (Barcelona, Spain) were used for the sample preparation. Chromatographic mobile phases were prepared with acetonitrile and MeOH (LC-MS grade) from Prolabo, and deonised water (obtained by using a Milli-Q system from Millipore (Bedford, MA, USA)). Aqueous phase was daily prepared and filtered with a Nylaflo™ 0.45 μm nylon membrane filter from Pall Corporation (Ann Arbor, MI, USA) before entering into the chromatographic system. N,O-bis(trimethylsilyl)trifluoroacetamide plus 1% of trimethylchlorosilane (BSTFA + TMCS, 99:1), used as derivatization reagent in GC, and the buffer components of the aqueous mobile phase in LC (ammonium formate and ammonium hydroxide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards of maslinic (MA), betulinic (BA), oleanolic (OA) and ursolic (UA) acids, as well as erythrodiol (ER) and uvaol (UV), were also supplied by Sigma-Aldrich. Methanolic stock solutions of 100 mg/L for each standard were first prepared by dissolving the appropriate amount of each analyte in MeOH and then, they were serially diluted to working concentrations (within the range 0.1–25 mg/L). All the samples and stock solutions were stored at −20 °C and filtered through a Clarinert™ 0.22 μm nylon syringe filter from Agela Technologies (Wilmington, DE, USA) before injection into the instrument.

2.2. Samples and sample treatment

The VOO samples used within this study came from 11 different cultivars grown in the experimental olive grove of the Agro-pôle Olivier National School of Agriculture of Meknès, Morocco. Fruits samples with a ripening index between 3.0 and 3.5 were randomly hand-picked from the selected trees and monovarietal oils were further extracted using an Oliomio laboratory mill (Toscana Enologica Mori, Tavernelle Val di Pesa-Fl, Italy) simulating a two-phase commercial oil-extraction system. A mixture of all the samples under study (prepared by mixing an equivalent volume of each one) was used for the extraction procedure optimization. Then, it was also used as a quality control sample (QC) for ensuring the proper performance of the systems as well as for evaluating the analytical parameters of the methods (repeatability, recovery and matrix effect). Moreover, commercial sunflower oil was used as a blank matrix for preparing DI calibration curves.

2.2.1. Extraction of triterpenic compounds

The isolation of the triterpenic compounds under study from the VOO samples was achieved by ultrasound assisted extraction (UAE). A portion of 0.2 (±0.01) g of VOO were weighed in a conical centrifuge tube and mixed with 5 mL of the extractant agent (MeOH, MeOH/EtOH (1:1, v/v) or EtOH/H_2O (90:10, v/v) depending on the experiment) by vortexing during 1 min (MeOH was finally pointed out as the solvent giving the best results). Then, the tube was left in an ultrasonic bath for 30 min and centrifuged at 5000 rpm for 6 min. These steps were repeated twice and both supernatants were collected together. Thereupon, the solvent was evaporated to dryness under reduced pressure by using a rotary evaporator at 35 °C and the obtained residue was redissolved in 1 mL of MeOH.

During the sample treatment optimization, two alternative extraction protocols were also tested. In the first one, the compounds of interest were isolated by SPE according to a previously described protocol (Pérez-Camino & Cert, 1999). Briefly, 0.2 (±0.01) g of VOO dissolved in 1 mL of hexane were put into a properly conditioned bonded aminopropyl phase SPE cartridge (500 mg, 3 mL) from Agilent. After successive washes, the triterpenic compounds were eluted with diethyl ether/acetic acid (98:2, v/v). Finally, the eluate was evaporated and reconstituted in 1 mL of MeOH. The other alternative extraction protocol was based on the use of microwave assisted extraction (MAE), and the optimum conditions were reached after a preliminary optimization and keeping in mind previously published reports (Fang, Wang, Yu, Zhang, & Zhao, 2010; Verma, Jain, Nigam, & Padhi, 2012). In short, 0.2 ± 0.01 g of VOO and 10 mL of the pertinent extractant agent (of the three tested ones) were put into the extraction vessel which was placed in the microwave system with the following irradiation power ramp: 0–400 W (80 °C) in 5 min, holding it constant over 10 min. Once the vessel was cooled to room temperature, its content was centrifuged at 5000 rpm for 6 min, and the supernatant was evaporated and redissolved in 1 mL of MeOH.

2.2.2. Cleaning step and derivatization of the extracts for GC analyses

Prior to the injection into the gas chromatograph, both the standard solutions and the extracts obtained by the UAE protocol slightly modified (see below) were derivatized in order to increase the volatility of the analytes under study and making them suitable for being analysed by GC. Before that, triterpenic extracts had to be further cleaned and preconcentrated. The sample preparation was performed by UAE with MeOH as reported in Section 2.2.1, but once the combined extracts were evaporated in the rotary evaporator, the resulted residue was reconstituted in 500 μL of acetonitrile; this solvent exhibits a lower miscibility in hexane than the MeOH; fact which could facilitate the following cleaning step where hexane was used to dissolve the oily interferences. After being washed with 1 mL of hexane and filtered, 200 μL of the extract were evaporated to complete dryness with a stream of N_2. Then 50 μL of the derivatization reagent (BSTFA + TMCS, 99:1) were added to the dried residue and vortexed during 1 min (final preconcentration of 4:1, v/v). The trimethylsilylation reaction was performed at room temperature for 30 min. For calibration curves, aliquots of 50 μL of the methanolic standard solutions of each concentration level were evaporated and derivatized by addition of 50 μL of (BSTFA + TMCS, 99:1) following the above mentioned procedure.

2.2.3. Sample dilution for DI

The preparation of the VOO samples for DI into the LC-MS system was carried out as follows: 0.2 (±0.01) g of VOO were weighed in a volumetric flask of 1 mL and diluted to the mark with acetone. Blank matrix calibration (used for quantification of DI analyses) was made in sunflower oil. Every concentration level was prepared weighing 0.2 g ± 0.01 of sunflower oil in a volumetric flask of 1 mL, spiking it with the appropriate volume of methanolic standard solution of the six triterpenes under study, and diluting to the mark with acetone after evaporating the MeOH with a stream of N_2.

2.3. LC-MS methodology

The LC-MS analyses were performed with an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany) coupled to a Bruker Daltonics Esquire 2000™ ion trap mass spectrometer (Bruker, Bremen, Germany) by an electrospray ionization source.

Regardless the sample preparation (extraction or oil dilution), the applied LC-MS method was a modification of a previous one proposed by our research group (Olmo-García et al., 2016). The triterpenic compounds under study were separated by using a Zorbax Extend C18 analytical column (4.6 × 100 mm, 1.8 μm particle size) (Agilent Technologies), operating at 20 °C. The mobile phases were 1.5 mM ammonium formate in water (adjusted to pH 9.6 with ammonium hydroxide) (Phase A) and acetonitrile/MeOH (60:40, v/v) (Phase B). Analytes were isocratically eluted (10% Phase A and 90% Phase B) during 5 min; this step was followed by a column cleaning with 100% Phase B. Therefore, the LC method can be described as follows: 0 to 5 min, 90% B; 5.5 min, 100% B; 12.5 min, 100% B; 13 min, 90% B, with 2 min additional post run time before the subsequent injection. The flow rate was 1.2 mL/min and the injection volume was 10 μL for the extracts and standards and 3 μL from every vial when DI was the used strategy.

Concerning the ESI-IT MS conditions, analyses were made using two different MS segments; in negative ion mode from the beginning to min 4 and in positive polarity until the end of the run, with a capillary voltage of +3500 V and −4000 V, respectively. The end plate offset voltage was set at −500 V, drying gas temperature at 300 °C, drying gas flow at 9 L/min, and nebulizer pressure at 30 psi. A scan range from 400 to 600 *m*/*z* was selected.

2.4. GC-MS methodology

An Agilent 7890 A gas chromatograph coupled to a Waters QUATTRO™ mass spectrometer (Waters, Manchester, UK) operating as a single quad, was used for GC analyses.

The separation of the analytes in this instrument was carried out in a fused silica capillary column coated with (5%-Phenyl)-methylpolysiloxane (HP-5MS) (30 m × 0.25 mm i.d., 0.25 m) from Agilent. After the optimization process, a temperature gradient was applied for the triterpenic compounds analysis: the oven temperature was initially kept at 200 °C for 2 min, then it was increased until 300 °C at 14 °C/min and held for 15.5 min. The operating conditions were 250 °C and 300 °C for injector and transfer line temperatures, respectively, with He as a carrier gas at a flow rate of 1 mL/min. 1 μL of sample volume was injected in splitless inlet mode. Electron impact (EI) spectra were acquired at 70 eV in total ion monitoring mode (mass range from 50 to 600 *m*/*z*) operating in positive polarity, with a source temperature of 210 °C. A solvent delay of 11 min was set at the beginning of each run to avoid damaging the filament of the MS because of solvent peaks and/or some other VOO compounds found in the extracts which could saturate the detector.

2.5. Auxiliary equipment and software

An ultrasonic bath from J.P. Selecta (Barcelona, Spain) was used for triterpenic compounds extraction from VOO samples. Its characteristics were: 6 L of capacity, dimensions of 15, 30 and 14 cm of height, width and depth of usable bath, respectively, with a generator power of 150 W, a total power capacity of 360 W and a fixed frequency within the range 50–60 Hz. Besides, a Preppy™ vacuum manifold for SPE (Supelco, Bellefonte, PA, USA) and a START E Microwave Extraction System (230 V/50 Hz) from Milestone (Bergamo, Italy) were used during the extraction procedure optimization.

ChemStation B.04.03 (Agilent) and Esquire control (Bruker), for LC-MS analyses, and Acquity UPLC Console and MassLynx 4.1 (Waters), for GC–MS analyses, were the software used for instrument control and file acquisition. The treatment of the data coming from both systems was carried out with the software Data Analysis 4.0 (Bruker), after exporting in compatible format the data coming from GC–MS. Statistical analyses (ANOVA test) to compare the quantitative results achieved by the different methods used within this study, were carried out by using STATGRAPHICS Centurion XVII (Statpoint Technologies, Inc., Warrenton, VA, USA).

3. Results and discussion

3.1. Extraction procedure optimization

Trying to find the simplest sample treatment with the highest recovery percentages for all the analytes under study, three extraction techniques were selected to be evaluated (SPE, UAE and MAE) in a first step of the optimization, keeping in mind some previous published works. A SPE extraction procedure (Pérez-Camino & Cert, 1999) (very widely used since its publication), was compared with two assisted LLE methods. Moreover, three different solvents or mixtures (MeOH, MeOH/EtOH (1:1, v/v) and EtOH/H_2O (90:10, v/v)) were tested as extractant agents in the LLE-based methods. The experimental design was directed towards having comparable results so, in all the cases, 0.2 (±0.01) g of VOO were subjected to extraction and led to a final volume of 1 mL of MeOH. That means that the yield of each experiment could be easily compared in terms of peak area in the chromatograms for the 6 analytes under study.

Fig. 1 presents the results of the first step of the optimization experiments for MA and OA, which are the two most abundant triterpenic compounds found in VOO. That was the reason to pick these two analytes to illustrate the results (similar behavior was shown for the other compounds under study). The figure shows MA and OA peak areas (average values of three independent sample preps) in the extracts prepared by using the three tested techniques employing the different solvents enumerated in Section 2.2.1, just for UAE and MAE. In both bars graphics, it can be observed that the extraction protocol displaying the highest recoveries was UAE with MeOH, solvent which has been previously reported for the simultaneous extraction of phenols and triterpenes from VOO (Kalogeropoulos et al., 2007). The extraction of MA was considerably affected by the physico-chemical properties of the solvent used; indeed, for the extraction of this compound, the chosen solvent had a more significant impact than for the rest of the analytes. SPE gave good recoveries for BA, OA and UA, but as far as MA and the alcohols are concerned, the other two LLE procedures seemed to be more effective. UAE with MeOH was finally pointed out as the protocol with better performance (higher recoveries, easier use and lower cost of consumables).

In a subsequent step, new experiments were designed, trying to achieve the best operating conditions for the UAE MeOH-based procedure. Extraction time (15, 30, 45 or 60 min), extractant agent volume (5 or 10 mL), and number of extraction cycles (1, 2 or 3 times) were carefully optimized. Being 15 min a not enough extraction time, no differences were found between lengths higher than 30 min; so the shortest possible UAE extraction time was chosen. Concerning the MeOH volume, 10 mL gave higher recoveries than 5 mL in every case; and with regard to the number of extraction cycles, a second step was always needed (significant amounts of all the analytes were found in the oil after the first extraction cycle).

At the end, 10 mL of MeOH left in an ultrasound bath over 30 min, repeating it twice, were the preferred operating conditions. In order to establish the percentage of the total amount of each analyte which remained into the sample after the two extraction stages and give and estimation of the recovery of the extraction protocol, a third repetition of the extraction was carried out, finding that less than 0.5% of every triterpenic compound remained in the sample.

The optimized extraction conditions in UAE were used for preparing the VOO extracts both for LC-MS and GC–MS analyses.

3.2. Analysis of the UA extracts by LC-MS

3.2.1. Optimization of chromatographic conditions in LC

The extracts obtained by using the optimum protocol just described above, were firstly analysed with a chromatographic method previously reported by our research team (Olmo-García et al., 2016). The compounds under study were properly separated, but after few analyses, some experimental issues started to show up. They can be enumerated as follows: appearance of big peaks coeluting with the analytes, slightly shorter retention times, as well as a considerable decrease in the MS intensity. At this point, we considered as mandatory to modify the method and lengthen the run time by adding a column cleaning step, which could assure the elution of the most apolar oily compounds before the subsequent injection. 7 min at 100% Phase B and 1 additional min to reach initial conditions, followed by 2 min of stabilization were enough to achieve good repeatability inter-sequence, as described in the following paragraphs.

3.2.2. LC-MS method characterization

Before carrying out the analysis of the samples, the performance of the whole methodology was obviously assessed, so the main analytical parameters, which give an idea of the linearity, sensitivity, accuracy and matrix effect of the method, were calculated. Both the standard mixture containing the 6 triterpenic compounds and the QC sample (fortified at different concentration levels) were used for validation purposes. The results of the validation studies are summarized in Table 1.

In order to check the linearity of the method, external calibration curves were established for each pure standard by plotting the peak area as a function of its concentration (12 different concentration levels over the range 0.1–15 mg/L, injected in duplicate). For MA and OA which were found in the samples in a wide concentration range, two calibration curves were used: one for the lowest concentration levels and the other for the highest ones. The responses fitted well to a straight line with regression coefficients (r^2) higher than 0.9909 in every case. Instrumental signal to noise ratio (S/N) was measured for each standard at the lowest concentration level injected, in order to calculate detection (LOD) and quantification (LOQ) limits, which were considered as the concentrations that generated a S/N equal to 3 and 10, respectively. As shown in Table 1, LOD were found within the range from 1 to 95 μg/L and LOQ varied between 3 and 317 μg/L (for UA and UV,

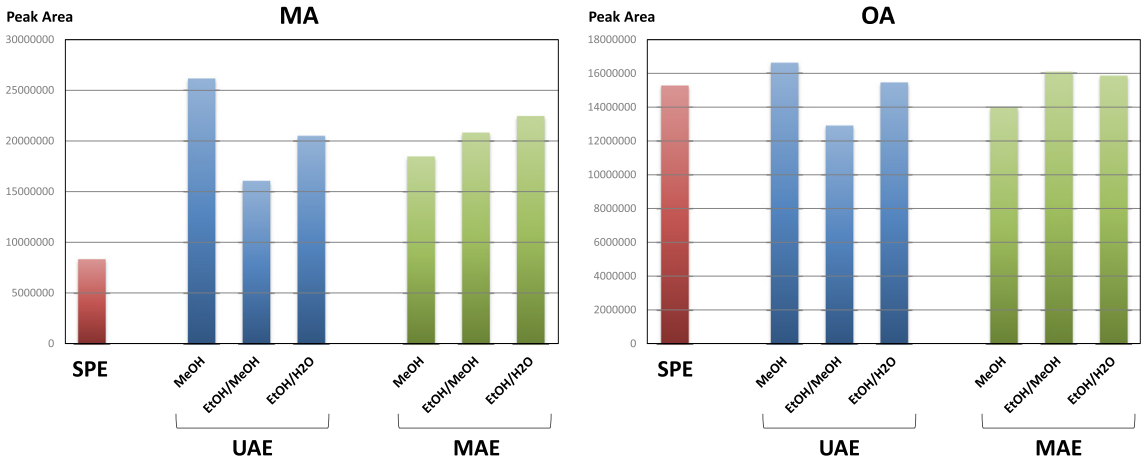

Fig. 1. Bars graphs representing the peak area of the two most abundant triterpenic compounds found in VOO (MA and OA) in the SPE, UAE and MAE experiments carried out during the first stage of the sample treatment optimization. UAE and MAE were applied by using MeOH, MeOH/EtOH (1:1, v/v) and EtOH/H_2O (90:10, v/v).

Table 1
Analytical parameters of the LC-MS developed method.

Compound	*m/z* signal used	Calibration curve	r^2	Linear range (mg/L)	LOD (μg/L)	LOQ (μg/L)	Accuracy				Matrix Effect Coefficient (%)[e]
							Intra-day Repeatability[b]		Inter-day Repeatability[c]	Trueness[d]	
							Area	Rt	Area	Rt	
Maslinic acid	*471* ($[M-H]^-$)	$y = 7.5{\cdot}10^5 x + 4.7{\cdot}10^5$	0.9985	2[a]	2	8	3.4	3.4	5.3	4.0	99.3	-1.2
		$y = 4.6{\cdot}10^5 x + 2.2{\cdot}10^6$	0.9962	2-15								
Betulinic acid	*455* ($[M-H]^-$)	$y = 2.0{\cdot}10^6 x - 6.1{\cdot}10^4$	0.9993	1[a]	2	8	2.4	2.3	4.2	2.6	99.7	10.9
Oleanolic acid	*455* ($[M-H]^-$)	$y = 8.6{\cdot}10^5 x + 1.4{\cdot}10^5$	0.9944	2[a]	1	3	3.5	1.9	6.4	2.2	100.5	7.2
		$y = 5.2{\cdot}10^5 x + 8.5{\cdot}10^5$	0.9916	2-10								
Ursolic acid	*455* ($[M-H]^-$)	$y = 7.2{\cdot}10^5 x + 4.4{\cdot}10^5$	0.9909	2[a]	3	11	5.1	2.0	5.9	2.0	100.2	3.0
Erythrodiol	*425* ($[M+H-H_2O]^+$)	$y = 7.5{\cdot}10^4 x + 9.3{\cdot}10^2$	0.9968	6[a]	95	317	3.4	1.1	4.6	1.1	94.9	6.5
Uvaol	*443* ($[M+H]^+$)	$y = 1.2{\cdot}10^5 x - 1.9{\cdot}10^4$	0.9953	6[a]	74	245	4.2	1.0	7.3	1.2	92.7	4.4

[a] Linear ranges were established from LOQ to the indicated value.
[b] RSD values (%) for peak areas and retention times of the analytes under study measured from 4 injections of the QC carried out within the same sequence.
[c] RSD values (%) for peak areas and retention times of the analytes under study measured from 8 injections (belonging to 4 different sequences carried out over 4 days) of 8 different extracts of the QC.
[d] Trueness was measured by calculating the recovery (%), and it was estimated by analyzing the samples extracted before and after the standard addition and calculating afterwards the difference between the obtained results. The values included on this table are those achieved for the intermediate concentration level.
[e] Matrix effect coefficient (%) = (1 − (slope matrix/slope solvent)) × 100.

respectively). In general, LOD and LOQ were found at concentration levels of few ppbs for triterpenic acids, whilst they were of several hundred ppbs approx. for the two alcohols, which are poorly ionized in comparison.

Method accuracy was evaluated in terms of precision and trueness. Precision was expressed as repeatability by calculating the relative standard deviation (RSD) of peak areas and retention times (Rt) of the analytes under study measured from 4 injections of the QC carried out within the same sequence (*intra-day* repeatability) and from 8 injections of the QC belonging to 4 different sequences carried out over 4 days (*inter-day* repeatability). RSD was lower than 3.4% and 5.1% for Rt and peak area, respectively, for *intra-day* repeatability, and lower than 4.0% and 7.3% for *inter-day* repeatability. Trueness was determined as recovery (%), which was estimated by analysing the QC extracted before and after the standard addition at three concentration levels (0.25, 0.5 and 1 mg/L) and calculating the difference between the obtained results. Good recoveries for all the analytes (between 92.7% for UV and 100.5% for OA, at the intermediate concentration level) were found. Similar values were achieved for the other concentration levels, demonstrating the suitability of the extraction system.

For making a choice of the most appropriate kind of calibration methodology to achieve accurate quantitative results, matrix effect was evaluated according to a previously proposed strategy (Kmellár et al., 2008). Consequently, a matrix effect coefficient was calculated by applying the following equation:

$$\textit{Matrix effect coefficient}\ (\%) = (1 - (\textit{slope matrix}/\textit{slope solvent})) \times 100$$

where *slope matrix* was the slope of a standard addition calibration curve (prepared by fortifying a QC extract at 3 concentration levels over the range 0.25–1 mg/L) and *slope solvent* was the slope of the external calibration function prepared in MeOH. The resulting coefficients fluctuated between −1.2% for MA (very slight signal suppression) and 10.9% for BA (mild enhancement effect), so they were found within the range in which the matrix effect is negligible (from −20% to +20%), according to Kmellár et al. Therefore, the external standard (solvent-based) calibration could be considered as a fitting calibration strategy to properly quantify the triterpenic compounds in the samples, as the presence of VOO matrix did not practically interfere the response of the analytes.

3.2.3. Application of the LC-MS method to the analysis of the samples

Once the developed method was validated, it was applied to the quantification of the six triterpenic compounds under study in the 11 selected monovarietal VOO samples. In Table 2, the results for each analyte are organized in three different tables (a, b and c); the first one (Table 2a) shows the quantitative data obtained from the analysis of the extracts with the LC-MS method. These data were achieved interpolating the peak area of three independent replicates (each one injected in duplicate) in the calibration curves presented in Table 1.

In the following sections the results included in the other two Tables 2 (b and c) will be introduced. A discussion regarding the comparison of the quantitative data achieved by the application of the different strategies will be presented in Section 3.5.

3.3. Analysis of the UA extracts by GC-MS

3.3.1. Optimization of the chromatographic conditions in GC

As highlighted before, GC has been considered the reference analytical technique for the analysis of pentacyclic triterpenes in VOO. Bearing that in mind, a very appropriate way to validate a possible alternative to GC could be to compare the quantitative results obtained with both methodologies (the new one (LC-MS)

Table 2
Quantitative results (mg analyte/kg olive oil) obtained for the olive oils under study by using the different approaches tested (LC-MS with UAE, GC-MS after UAE, and LC-MS after a simple dilution of the sample).

a) LC-MS UAE	Arbequina	Arbosana	Cornicabra	Frantoio	Hojiblanca	Houiza	Koroneiki	Langedoc	Manzanilla	Picholine	Picual	QC
MA	52 ± 2	25 ± 1	41 ± 2	20 ± 1	16 ± 1	27 ± 1	71 ± 2	13.2 ± 0.6	24 ± 1	19 ± 1	22 ± 1	29 ± 2
BA	0.34 ± 0.02	0.21 ± 0.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
OA	24 ± 0.4	15.5 ± 0.5	24 ± 1	7.1 ± 0.2	5.6 ± 0.2	16.8 ± 0.8	36 ± 2	4.3 ± 0.2	9.8 ± 0.4	7.3 ± 0.4	10.2 ± 0.4	13.7 ± 0.7
UA	nd	1.2 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.13 ± 0.01
ER	2.1 ± 0.2	3.9 ± 0.3	1.9 ± 0.1	1.3 ± 0.1	0.55 ± 0.05	2.9 ± 0.2	7.8 ± 0.8	0.9 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	2.2 ± 0.2
UV	nd	1.0 ± 0.1	nd	nd	nd	nd	1.4 ± 0.1	nd	nd	nd	nd	nd

b) GC–MS UAE	Arbequina	Arbosana	Cornicabra	Frantoio	Hojiblanca	Houiza	Koroneiki	Langedoc	Manzanilla	Picholine	Picual	QC
MA	55 ± 3	24 ± 1	38 ± 2	22 ± 1	17 ± 1	26 ± 1	68 ± 4	14 ± 1	25 ± 1	20 ± 1	24 ± 1	29 ± 1
BA	0.31 ± 0.02	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
OA	24.4 ± 0.9	15.8 ± 0.9	26 ± 1	7.1 ± 0.4	6.0 ± 0.3	17 ± 1	36 ± 2	4.6 ± 0.2	9.2 ± 0.5	7.4 ± 0.4	11.0 ± 0.5	13.9 ± 0.7
UA	nd	1.2 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ER	2.3 ± 0.2	3.8 ± 0.3	1.1 ± 0.1	1.4 ± 0.1	0.50 ± 0.04	2.7 ± 0.2	7.5 ± 0.6	0.9 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	2.4 ± 0.2
UV	nd	nd	nd	nd	nd	nd	1.3 ± 0.1	nd	nd	nd	nd	nd

c) LC-MS DI	Arbequina	Arbosana	Cornicabra	Frantoio	Hojiblanca	Houiza	Koroneiki	Langedoc	Manzanilla	Picholine	Picual	QC
MA	56 ± 3	25 ± 2	42 ± 3	20 ± 1	17 ± 1	28 ± 2	68 ± 4	15 ± 1	27 ± 2	22 ± 1	24 ± 1	31± 2
BA	0.28 ± 0.02	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
OA	24.5 ± 0.7	16 ± 1	27 ± 2	7.2 ± 0.5	5.8 ± 0.3	17 ± 1	38 ± 3	4.1 ± 0.4	10.2 ± 0.7	7.7 ± 0.5	10.9 ± 0.7	14.6 ± 0.9
ER	2.5 ± 0.2	4.2 ± 0.3	nd	nd	nd	3.1 ± 0.2	7.4 ± 0.7	nd	1.5 ± 0.1	nd	nd	2.0 ± 0.2

Every result included in this table is the average of three independent replicates (each one injected in duplicate). The results are given as the mean value ± SD.
No statistical significant differences among the three tested strategies were found (95%; $p < 0.05$).

and the one contemplated as the gold standard in the field). With this aim, the sample set was treated again according to the protocol mentioned in Section 2.2.2 and the derivatized extracts were injected into the gas chromatograph. The applied separation conditions described in Section 2.4 were the result of the slight modifications which were made to previously reported methods (Caligiani et al., 2013; Guinda, Rada, Delgado, Gutiérrez-Adánez, & Castellano, 2010; Kalogeropoulos et al., 2007; Pérez-Camino & Cert, 1999), in order to have reasonable retention times together with adequate chromatographic efficiency.

3.3.2. GC–MS method characterization

To make sure that the comparison between LC-MS and GC–MS quantitative results was fair and properly carried out, trueness was considered as the crucial parameter when the GC–MS method validation was done, although linearity and instrument repeatability were logically evaluated too. Trueness was assessed by means of the analysis of different replicates of a blind sample; since suitable Certified Reference Materials are not available, standard mix of pure standards (at different concentration levels) were prepared by the technical assistants of our lab (not involved in this project) and analysed. Their concentration in terms of all the analytes was calculated and resulting values were compared with the real ones through the Student's *t*-test. No statistically significant differences were found among them for any of the evaluated concentration levels (at a 95% confidence level, $p < 0.05$), what means that the method was very truthful. Besides, all the external calibration curves showed good linearity within the work range ($r^2 > 0.9901$), and *intra-day* repeatability, calculated as the RSD of the peak areas of the six triterpenes in 4 injections of the standard mix carried out within the same sequence, was lower than 8.3% in every case.

3.3.3. Application of the GC-MS method to the analysis of the samples

The quantitative data obtained after the analysis of the 11 VOO samples with the GC–MS method are presented in Table 2b. The *m/z* signals monitored for each compound were the following ones: 73, 129, 203 and 497 for ER and UV (eluting at 17.1 and 17.7 min, respectively); 73, 129, 203, 320 and 483 for OA and UA (eluting at 18.3 and. 19.2 min, apiece); 73, 129, 189 and 483 for BA (eluting at 18.5 min); and 73, 147, 203, 320 and 571 for MA (eluting at 21.9 min).

3.4. Direct injection of diluted VOO samples in LC-MS

Although the proposed sample treatment gave good recoveries and was easy to perform, any extraction protocol is always reagent and time consuming. Some researchers have focused their efforts on simplifying the sample preparation trying to carry out, to a considerable extent, more rapid and simpler determinations of different analytes in VOO. One possible strategy is the DI of the sample into the liquid chromatograph after a simple dilution, which has been applied to determine triacylglycerols (de la Mata-Espinosa, Bosque-Sendra, & Cuadros-Rodríguez, 2011) and VOO minor compounds such as phenols (Olmo-García, Bajoub, Monasterio, Fernández-Gutiérrez, & Carrasco-Pancorbo, 2017; Selvaggini et al., 2006), chlorophylls, pheophytins (Lozano, Muñoz de la Peña, Durán-Merás, Espinosa Mansilla, & Escandar, 2013), β-carotene, tocopherols and tocotrienols (Seppanen, Rahmani, & Csallany, 2003). To the best of our knowledge, VOO triterpenes have not been determined by using this approach so far; therefore, the determination of triterpenic compounds from VOO by LC-MS after a simple dilution of the sample was explored in another stage of the current study.

Initially, the most adequate solvent used to dissolve the oil samples and the optimum oil/solvent ratio were investigated. Taking into account the previous experience of our research group with VOO DI for phenolic compounds determination (Olmo-García et al., 2017), three solvents (tetrahydrofuran, acetone and isopropanol) and three ratios (1 g diluted to a final volume of 2, 5 and 10 mL) were tested. 1 g of VOO diluted to a final volume of 5 mL with acetone was pointed out as the optimal option, avoiding the rapid soiling of the column with more concentrated preparations. This ratio could lead to inject concentration levels analogue to those of the methanolic extracts prepared by UAE. However, in order to lengthen the column life, the volume injected into the LC system was reduced from 10 μL (for the extracts and standard mix injected when LC-MS with UAE was used) to 3 μL (for every preparation – diluted samples or standard mix-injected when the DI approach was employed). Nevertheless, column performance,

which is one of the most debatable and controversial aspects of this operating mode, was carefully checked. For that purpose, the standard mixture in MeOH (at a concentration level of 1 mg/L) and methanolic blanks were interspersed within the sequence every three and six samples, respectively. A decrease in retention times of about 20% was found in 50-analyses-sequences, although its overall effect was not very drastic, since the separation of the analytes remained acceptable within the sequence. Signal intensity in MS was dropping during the sequence, causing a reduction in peak area of about 20% after 60 injections; problem which was addressed by applying a correction factor to the integrated areas for each compound, considering a lineal decrease in MS signal intensity. Accordingly, a curve was obtained for each analyte by plotting its area in the methanolic standard mix (injected every three analyses) versus the injection number (good linearity was found for these curves, with correlation coefficients higher than 0.9987). Then, a correction factor for each analysis and every substance under study was interpolated in these curves and applied to the integrated areas in all the chromatograms. After each sequence, the column cleaning protocol previously reported by our team (Olmo-García et al., 2017) was used; this cleaning strategy together with a simple spray shield cleaning process with isopropanol/water (50:50, v/v) brought the retention times and MS signal back to their original values. In other words, the column was returned to its original state after the cleaning and regeneration process.

Calibration curves in blank matrix were established for each analyte with quantitative purposes (sunflower oil was considered as a triterpenoids-free oily sample or blank sample), following a similar approach as the one described by Olmo-García et al. in the above mentioned publication. The same concentration levels as those used for external calibration were considered. Once the areas of the analytes in the samples under study were properly corrected, they were interpolated in the corresponding blank matrix calibration curves. The quantitative data obtained by this approach are presented in Table 2c. As can be seen in the table, the minor triterpenoids could not be properly detected with this methodology. Nonetheless, the quantitative results show the potential of DI for the analysis of the main triterpenic compounds found in VOO (MA, OA and ER).

3.5. Overall view

The prevailing goal of this work was to develop a LC-MS method for triterpenic compounds determination in olive oil. As extensively explained in Section 3.2, after carrying out the methodological optimization and validating the developed method, it was applied to the analysis of 11 VOO samples. As already stated, to compare the quantitative results reached by LC-MS with the ones achieved by a more standardized method, a GC–MS methodology (based on previous reports) was optimized and applied to the analysis of the same sample set.

Fig. 2 shows the chromatograms of an Arbosana VOO UAE extract and the standard mix containing the six triterpenic compounds under study in both LC-MS and GC–MS. As can be perceived in the figure, both LC and GC led to an adequate separation of the six triterpenic compounds, exhibiting proper resolution and good peak shape, however, the analysis time was shorter in LC (5 min versus 22 min approx.). In the VOO chosen to exemplify the figure -Arbosana sample-, UV could not be detected in the GC–MS chromatogram; fact which can be explained taking into account that LODs were much lower in LC-MS, platform that determined 1.0 mg/kg as UV content. Something similar was observed for BA, which showed a concentration value of 0.21 mg/kg when determined by LC-MS, but it was not detected by GC–MS. By using both platforms, MA, OA and ER were satisfactorily determined in all the oils; however, BA, UA and UV were just found in few examples.

Besides, the DI of VOO (after a simple dilution in acetone) in LC-MS was proposed as an alternative with a clear advantage: the easiness of the sample preparation. Table 2c summarizes the found amounts by DI of each analyte in the 11 samples under evaluation. MA and OA were found in every sample, BA was determined just in Arbequina VOOs, and ER was at concentration levels beyond the LODs for Arbequina, Arbosana, Houiza, Koroneiki and Manzanilla. UA and UV were not determined by this strategy in any sample.

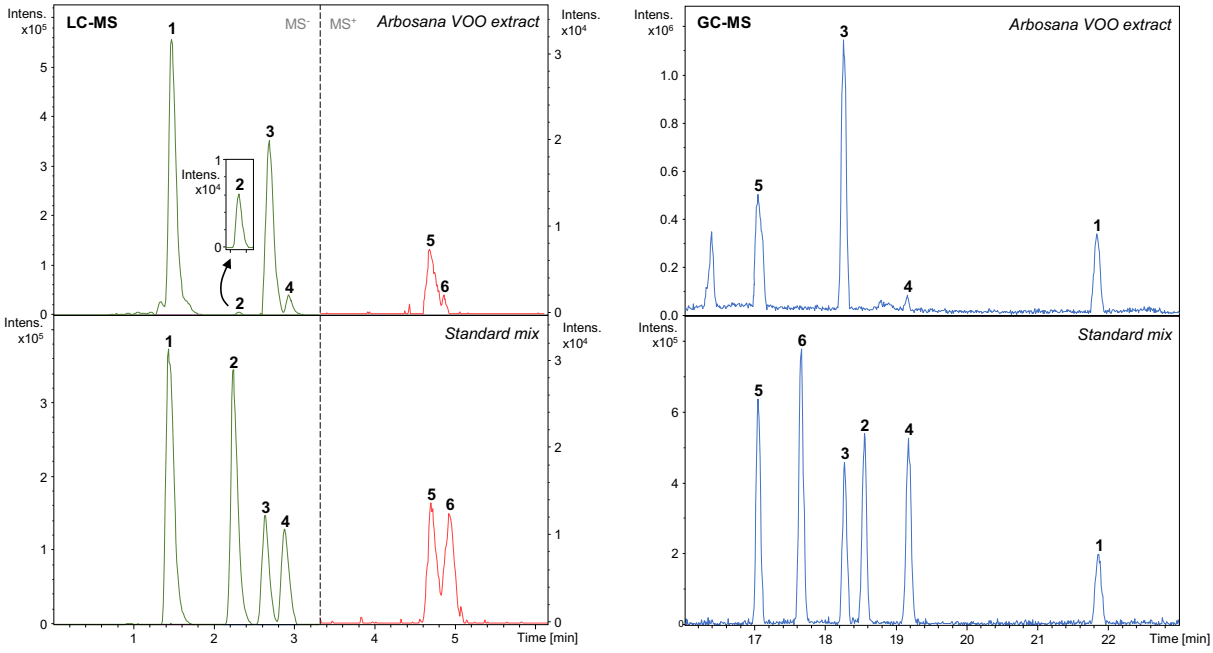

Fig. 2. Left: Extracted ion chromatograms obtained in LC-MS (using negative ionization mode for triterpenic acids and positive polarity for alcohols) for an Arbosana VOO extract (upper part of the figure) and a standard mix (lower chromatogram). Right: Base peak chromatograms obtained in GC–MS in positive polarity (samples are the same as for LC-MS). Peak identification numbers: 1, maslinic acid; 2, betulinic acid; 3, oleanolic acid; 4, ursolic acid, 5, erythrodiol, and 6, uvaol.

In general, this DI strategy was somewhat less sensitive than GC–MS and LC-MS (the two last ones combined with UAE).

All the quantitative data of Table 2a, b and c were subjected to a statistical data treatment to evaluate the similarity among the results coming from the three methodological approaches used within this study. ANOVA test demonstrated that no statistical differences (at a 95% confidence level, $p < 0.05$) were found among the values obtained by the three alternatives (when detected in all of them). This fact led us to make these assertions: i) the herein proposed LC-MS method is a reliable and tangible alternative to GC, which can be even faster and avoid the need of derivatization; and ii) DI strategy could represent another promising and trustworthy resource, in particular when the analyst is interested on establishing the concentration levels of the most abundant triterpenic acids and dialcohols.

Apart from determining the analytes in the 11 selected samples, the olive oil mix composed by equivalent volumes of all the VOOs (QC sample) was also analysed by the three strategies. Results are included in Table 2a, b and c and it is worthy to underline that -for the compounds detected with all the methodologies- the concentration levels were in good agreement. Additionally, the found amounts were very similar to the theoretical or putative values presupposed for the QC for each compound (estimation which can be made averaging the measured amount of each analyte in the 11 selected oils).

Having a look at the quantitative results of Table 2a, b and c, Koroneiki was the variety showing the highest content of triterpenic compounds, whereas Langedoc exhibited the lowest concentration levels. MA and OA were found in every sample. MA values fluctuated between 13.2 and 71 mg/kg, in Langedoc and Koroneiki (LC-MS with UAE data), respectively. OA was found at levels oscillating between 4.3 and 36 mg/kg in the same varieties. Arbosana was the only VOO sample in which the six triterpenic compounds under study could be determined. The triterpenic acids found at undermost levels were UA (merely found at upper levels than LOD in Arbosana, with 1.2 mg/kg), and BA (which was just determined in Arbequina (0.34 mg/kg) and Arbosana (0.21 mg/kg)). To the best of our knowledge, this is the first time that BA concentration has been determined in an olive oil sample. In fact, in some previously published works, this compound was used as internal standard (Allouche et al., 2009; Allouche et al., 2010; Guinda et al., 2004; Pérez-Camino & Cert, 1999). As mentioned in the introductory section, few references containing data about the triterpenic content of VOO can be found in literature, but the pentacyclic acids are very scarcely quantified in terms of the pure standard of each analyte (MA, UA and OA) (Pérez-Camino & Cert, 1999). In the just quoted work, the concentration ranges were slightly higher than those presented in Table 2. Similar contents for the major triterpenic acids were described by Allouche and coworkers for monovarietal VOOs (Allouche et al., 2009) and for oils prepared under different technical conditions (Allouche et al., 2010). However, they found significantly higher amounts of triterpenic dialcohols (quantified with respect to betulin pure standard). The ranges of ER and UV found in another interesting publication (Lukic et al., 2013) -although expressed in terms of cholestanol- are much closer to those presented in Table 2. All the existing results agree in the prevalence of ER over UV. Indeed, UV was only determined in Arbosana and Koroneiki, with 1.0 and 1.4 mg/kg, respectively, whilst ER values where higher in all the cases, as expected, varying from 0.9 mg/kg in Langedoc to 7.8 mg/kg in Koroneiki VOO.

4. Conclusions

The relevance of triterpenic acids and dialcohols from *Olea europaea* is unquestionable nowadays, finding numerous reports describing and demonstrating their biological activities. GC with FID or MS as detectors is considered the gold standard tool in this field. Few examples can be also found in literature regarding their determination in the mentioned matrix by LC-MS, but generally without giving quantitative data of each analyte in terms of their own standard (but referring them to another analyte) and not determining both triterpenic acids and dialcohols within a single run. A faster and reliable alternative (which did not need any derivatization step) has been developed and validated in the current contribution, demonstrating its applicability to VOOs coming from 11 olive varieties and proving that the obtained data are in good agreement with those achieved by GC–MS. Besides, the achieved data were also comparable to those derived from the use of a third strategy (DI), proffering an additional methodology for the accurate determination of the most abundant pentacyclic triterpenes.

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