Evaluating the potential of LC coupled to three alternative detection systems (ESI-IT, APCI-TOF and DAD) for the targeted determination of triterpenic acids and dialcohols in olive tissues

Lucía Olmo-García, Aadil Bajoub, Alberto Fernández-Gutiérrez, Alegría Carrasco-Pancorbo*

Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Ave. Fuentenueva s/n, E-18071 Granada, Spain

*Corresponding author:

Dr. A. Carrasco-Pancorbo, Research Group FQM-297, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Ave. Fuentenueva s/n, E-18071 Granada, Spain.

E-mail: alegriac@ugr.es

Abstract

Herewith the development of a rapid and powerful LC methodology (with three different detectors) is presented to determine triterpenic acids and dialcohols in extracts from *Olea europaea* tissues (olive skin, pulp and leaves). After the proper optimization of the LC, DAD and MS conditions (and the comprehensive characterization of the behaviour of each analyte in ESI and APCI (with accurate *m/z* signals and, in ESI, with MS/MS data too), the method was fully validated. DAD, ESI-IT MS and APCI-QTOF MS were used as detection systems to give different alternatives to carry out the accurate determination of these analytes, evaluate their analytical performance, advantages and drawbacks, and check whether the quantitative results achieved by the three platforms were in good agreement. ESI-IT MS gave the lowest detection limits (3-455 μg/L) followed by APCI-QTOF MS (22-408 μg/L); in contrast, DAD (83-600 μg/L) had the widest dynamic range. The RSD values for *inter-day* repeatability were found below 11.82 % in all the cases. No statistically significant differences were found among the quantitative results from the three detectors. Olive leaves showed the highest concentration levels of ursolic acid (1.8 mg/g), erythrodiol (1.6 mg/g) and uvaol (1.2 mg/g), whereas the olive skin was the richest matrix in terms of maslinic (80 mg/g), betulinic (0.20 mg/g), and oleanolic (26 mg/g) acids. Concentration values of triterpenic acids were established by first time for skinless olive pulp, and were found around 65, 1.2, 55 and 4.4 μg/g for maslinic, betulinic, oleanolic and ursolic acids, respectively.

Keywords: pentacyclic triterpenes; olive tissues; liquid chromatography; mass spectrometry; food metabolomics.

Abbreviations: maslinic acid (MA), betulinic acid (BA), oleanolic acid (OA), ursolic acid (UA), erythrodiol (ER), uvaol (UV).

Introduction

Over the last years, a rich in fruits and vegetables diet has been associated to a lower incidence of diseases related to chronic damage and growth dysregulation, such as obesity, diabetes, cancer or cardiovascular disease. This is largely due to the phytochemicals found in food plants at different concentrations levels [2]. Some of these compounds are triterpenoids, an important group of natural products with numerous biological effects, which are being used as ingredients in dietary supplements, medicines and healthcare products [3,4]. In particular, pentacyclic triterpenes have been identified as the main components of medicine plants [5], and have shown, among others, analgesic, hepatoprotective, anti-tumor, anti-diabetic, anti-inflammatory and antioxidant effects [6–9]. They are secondary plant metabolites which arise from cyclization of squalene, and have a common skeleton of five 5 or 6-membered cycles substituted by different functional groups [10]. Such substances are excreted by plants as protection agents, so they use to be part of the cuticular waxes that surround plant surfaces (leaves, stems, flowers and fruits) [11].

Olea europaea is a valuable source of this kind of compounds, since different triterpenic acids and alcohols have been described in olive industry-related products (olive leaves, fruits, oil and pomace) [12–14]. Bioactive properties of pentacyclic triterpenes from *Olea europaea* have been systematically reviewed by different authors [15–20], and some protocols for obtaining their pure extracts have been patented [21–24]. Obtaining these components from olive-industry by-products could be a way to economically upgrade the sector. The use of these compounds as ingredients in new products leads to the need of developing appropriate methods for their determination in a growing variety of samples (raw materials or final products). The optimization of

these methods, which can be implemented in routine laboratories to ensure the safety and quality of these new products, represents a considerable challenge.

In recent years, in parallel with the discovery of the biological effects of triterpenoids, many studies have been carried out trying to achieve the best possible determination procedure. Their extraction from vegetal tissues is the first step to be optimized in order to achieve an accurate quantification; in this regard, different strategies have been evaluated [25,26]. In the case of *Olea europaea* tissues, after water removal, analytes of interest have been extracted with ethyl acetate in a Soxhlet apparatus [27], by maceration with ethanol [28], by solid-liquid extraction with a mixture of methanol/ethanol (1:1, v/v) [29] or by microwave assisted extraction with ethanol/water (80:20 v/v) as extractant mixture [30]. Other extraction techniques, such as ultrasonic assisted extraction [31] or supercritical fluid extraction [32], have been also applied to different plants.

Triterpenoid fraction in plant matrices is quite complex, in particular because the coexistence of some structural isomers, therefore, their quantification is quite difficult, making almost mandatory the use of a separation technique before their detection. In any case, interesting examples which do not imply the use of a previous separation can be found. For instance, discrimination and quantification of oleanolic and ursolic acids in plant matrixes has been achieved using two-dimensional nuclear magnetic resonance spectroscopy [27]. Nevertheless, as stated before, in most of the proposed methods, their separation becomes the key to the success of the analytical process, since commonly used detectors are not capable of distinguish them. So, the analysis of prepared extracts has been commonly made with multiple separation techniques coupled to different detectors. Gas chromatography coupled to FID [28,33,34] or MS [35,36] has been extensively employed to this end. As a way of overcoming the tedious

derivatization process (necessary step to increase the volatility of the triterpenoids) liquid-based methods such as capillary zone electrophoresis [37] or liquid chromatography have been also developed. As far as LC is concerned, it has been coupled to photodiode array [38], evaporative light scattering [39] or MS detectors whether for identification or quantification purposes. Fluorescence detection has been also used coupled to LC, but it requires a previous derivatization step [40–42]. In mass spectrometry, different interfaces (ESI, APCI and APPI [43]) and analyzers (IT [44], Q [29], QqQ [30], QTOF [39] and Orbitrap [45]), both in positive and negative polarities, have been employed.

In LC, a great variety of mobile phases has been used, mostly in isocratic methods, although some gradients has been also proposed for triterpenoids separation [39]. Because of the relatively low polarity of these compounds, organic solvents (methanol and/or acetonitrile) mixed with low proportions of water (usually acidified) have been commonly employed [29,44,46]; the effect of some modifiers such as cyclodextrins [47] or triethylamine [48] have been also tested. The use of neutral [49] and basic [30,50] chromatographic conditions has been reported in few communications, even though they have not been so commonly used.

The aim of this work has been to develop and validate a rapid and powerful analytical method for the determination of pentacyclic triterpenes (maslinic, betulinic, oleanolic and ursolic acids, erythrodiol and uvaol) offering different alternatives (in terms of detection systems) to carry out their accurate determination. Three detectors were selected: DAD, for being the more likely available one in a routine analysis olive oil laboratory; and two MS detectors (one of them with ESI interface and an analyzer of low resolution but very fast switching polarities and the other with an APCI source and a high resolution analyzer), since MS is continuously growing, has a great potential and

115 is becoming a kind of mandatory. We evaluated their analytical performance, discussed
116 their drawbacks and advantages, and checked whether the quantitative results obtained
117 by the three platforms were in good agreement. To achieve this purpose, different olive
118 tissues (olive skin, pulp and leaves) were selected and their triterpenoid content
119 assessed. To the best of our knowledge, the triterpenes levels of one of the matrices
120 under study have been never evaluated before.
121
122 **Materials and methods**

123 **Chemicals and standards**

124 All reagents were of analytical grade and used as received. Acetonitrile and methanol of
125 LC-MS grade from Prolabo (Paris, France), and deonised water from a Millipore Milli-
126 Q (Bedford, MA, USA) water purification system, were used for preparing
127 chromatographic mobile phases. Ammonium formate and ammonium hydroxide from
128 Sigma-Aldrich (St. Louis, MO, USA) were used as buffer components in the aqueous
129 mobile phase. This phase was vacuum filtered with a Nylaflo^{TM} 0.45 μm nylon
130 membrane filter from Pall Corporation (Ann Arbor, MI, USA) before entering into the
131 chromatographic system. Ethanol from J.T. Baker (Deventer, The Netherlands) was
132 used for the extraction of the triterpenic compounds from the selected tissue samples.
133 Pure standards of MA, BA, OA, UA, ER and UV were all supplied by Sigma-Aldrich.
134 A methanolic stock standard solution containing 200 mg/L of each compound was first
135 prepared by dissolving the appropriate amount of each analyte in methanol and, then,
136 serially diluted to working concentrations. All solutions were stored at −20 ◦C. All the
137 samples and stock solutions were filtered through a Clarinert^{TM} 0.22 μm nylon syringe

filter from Agela Technologies (Wilmington, DE, USA) before injection into the instrument.

Samples and extraction procedure

Olive tissues samples were supplied by a local company. Olive skin (mix of varieties not specified by the supplier) was treated as received (it came dried from the olive mill). Olive pulp (Picudo *cv*.) was obtained manually, after peeling the olive fruits and removing their stones, and then, it was frozen to be further freeze-dried. Olive leaves (Picual *cv*.) were oven-dried at 35ºC until their weight remained constant. After water removal, pulp and leaves tissues were ground to powder before the extraction of triterpenic compounds.

Compounds of interest were isolated by ultrasonic assisted extraction according to the method described by Goulas and Manganaris [25], adapted from those of Lee et al. [51] and Li et al. [52]. Briefly, 0.5 g of dried tissue and 20 mL of ethanol were put inside a falcon tube which was left in an ultrasonic bath from J.P. Selecta (Barcelona, Spain) for 30 min. The ultrasonic bath 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. Afterwards, the tube was centrifuged at 9500 rpm for 5 min. Finally, the supernatant was evaporated to dryness and redissolved in 10 mL of methanol.

In order to evaluate the recovery percentage of the extraction system, the first part of the described procedure was repeated three times for each matrix, as follows: after taking the obtained supernatant from the first step, another 20 mL ethanol were added to the solid residue, being left into the ultrasonic bath for 30 min. This was repeated once more. In this way, we could establish the percentage of the total amount of each analyte which remained into the sample after going through the first extraction stage. Thus,

when quantifying the analytes of interest in the samples, a correction factor (including the recovery and the dilution factor) was obviously applied to properly calculate the final concentration values of the compounds in the analyzed tissues.

A mixture of the extracts coming from the olive skin, pulp and leaves samples under study (mixing an equivalent volume of each extract) was used as quality control sample to evaluate the repeatability of the method (apart of doing it with a mixture of pure standards). The quality control sample was injected every five analyses (after a blank) in each sequence.

LC-DAD/MS analysis

Apparatus

In the current study, the analyses were performed by reversed-phase LC coupled to three different detectors, using two different platforms. The first one was an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector (DAD) coupled to a Bruker Daltonic Esquire 2000™ ion trap mass spectometer (Bruker Daltonik, Bremen, Germany) by an electrospray (ESI) interface. The second one was a Waters Acquity UPLC™ H–Class system (Waters, Manchester, UK) coupled to a Q-TOF SYNAPT G2 mass spectrometer (Waters) equipped with an atmospheric pressure chemical ionization (APCI) ion source. Additionally, an Acquity UPLC™ H–Class system coupled to a micrOTOF-Q II^{TM} mass spectrometer (Bruker Daltonik) by means of an electrospray source was used only with qualitative purposes, in order to compare the behavior of the analytes in both interfaces by studying the accurate *m/z* signals produced by each ionization source (APCI and ESI).

The first platform was chosen to optimize the chromatographic separation, because it allowed the simultaneous monitoring of the eluent with DAD and ESI-IT MS detectors. Indeed, the fact that triterpenic acids were better detected in negative polarity whilst

188 alcohols needed positive polarity, made IT MS the most appropriate and useful MS
189 detector (of those evaluated within this study), because it could detect all the analytes
190 within a single injection, as it can easily switch polarity during a run.
191 The developed method was finally applied for analyzing the selected samples by means
192 of DAD, ESI-IT and APCI-QTOF detectors. As stated before, the detectors selection
193 was made in such a way as to fulfill different requirements: 1) to include both cheap and
194 accessible detectors and other more sophisticated and powerful ones; 2) to cover
195 different MS interfaces to check their suitability for properly ionizing pentacyclic
196 triterpenes; and obviously 3) the detectors´ availability within our facilities.
197 The analytical performance of the three detectors was evaluated, but the aim was not
198 carrying out a comprehensive comparison, but giving to the reader an idea about
199 parameters such as detection and quantification limits, calibration range, accuracy and
200 possible matrix effect. Moreover, in order to fairly compare the two used interfaces, we
201 should have selected the same analyzer for both platforms.
202 Chromatographic data acquisition and DAD peaks integration in the first system was
203 performed by using ChemStation B.04.03 software (Agilent Technologies). Instrument
204 control of Waters chromatographic systems was carried out using the software Acquity
205 UPLC Console (Waters), and the data processing of the Waters spectrometer was made
206 with the software MassLynx 4.1 (Waters). Finally, Bruker mass spectrometers were
207 controlled using the software Esquire Control and the resulting files were treated with
208 the software Data Analysis 4.0 (Bruker). Statistical analyses (ANOVA test) to compare
209 the quantitative results achieved by the three tested detectors, were carried out by using
210 STATGRAPHICS Centurion XVII.

211 ***Chromatographic conditions***

212 Regardless of the detection system, the compounds under study were separated by using
213 a Zorbax Extend C18 analytical column (4.6 x 100 mm, 1.8 μm particle size) (Agilent
214 Technologies), which can be used in a wide pH range (2.0 to 11.5), operating at 25ºC.
215 The mobile phases were 1.5 mM ammonium formate in water (adjusted to pH 9.6 with
216 ammonium hydroxide) (Phase A) and acetonitrile/methanol (60:40, v/v) (Phase B).
217 Analytes were isocratically eluted (10% Phase A and 90 % Phase B) at a flow rate of
218 1.2 mL/min and the injection volume was 10 μL. Run time was 6 minutes with one
219 additional post run minute before the subsequent injection.
220 In order to evaluate the effect of the different chromatographic conditions tested during
221 the development of the method not only on the separation itself but also in the MS
222 signals, the signal to noise ratio and maximum intensity (in counts) -in the IT MS
223 detector- of the analytes under study were calculated for each tested LC condition. Very
224 similar behavior was shown by the six analytes, and therefore, results from betulinic
225 acid have been selected as example to be shown in the graphics. Moreover, the number
226 of theoretical plates (N) for this compound was calculated from the LC-ESI-IT MS data
227 as $N = 5.54\ (t_r/w_{1/2})^2$, where both retention time (t_r) and peak width at half height ($w_{1/2}$)
228 were expressed in minutes.
229 ***Detection conditions***
230 Bearing in mind the previously published results and the maximum absorbance
231 wavelengths observed in the spectra of the individual pure standards, the optimum
232 wavelength for the determination of the triterpenic compounds in the diode-array
233 detector was set at 210 nm.
234 Besides, mass spectrometric conditions were optimized for each triterpenic compound
235 (in ESI and APCI ionization sources, respectively) by continuous infusion of standard
236 solutions (at a concentration level of 20 mg/L approx.). In ESI-IT MS, analyses were

made in negative ion mode until minute 4 (for triterpenic acids detection) and in positive polarity from 4 to 6 min (for triterpenic alcohols detection), with a scan range from 400 to 600 *m/z*, regardless of ion polarity, which enhanced the ion detection selectivity and gave higher intensities. The end plate offset voltage was set at -500 V, and the capillary voltage at +3500 V in negative polarity, and -4000 V in positive polarity. Optimum values for the ESI source parameters were: 300ºC of drying gas temperature, 9 L/min of drying gas flow and 30 psi of nebulizer pressure. These parameters were then transferred to the ESI-QTOF spectrometer. Nevertheless, two injections per sample (one for each ion mode) were needed, because polarity changes while running are not recommended in the micrOTOF-Q II that we were using, since the switching needs some time and additional calibration could be required. In APCI-QTOF MS, two injections were needed too, because the used system is unable to switch polarity in the middle of a run. In this instrument, corona, sampling cone and extraction cone voltages were -5000 V, 20 V and 5 V, respectively, in positive polarity, and +3000 V, 60 V and 5 V, in negative polarity. Regardless of the ion mode, source and probe temperatures were set at 100 and 500ºC, respectively, and 30 L/h of cone gas flow and 600 L/h of desolvation gas flow were used.

Method validation

Solutions containing pure standards of 6 triterpenoids in methanol at 8-10 different concentration levels over the range of 0.1–100 mg/L for MA and OA, and 0.1-50 mg/L for the rest of the analytes, were employed to check linearity and to establish the calibration curves which allowed their quantification in the samples. External calibration curves were established for each standard by performing a linear regression by the least-squares method. Each point of the calibration graph corresponded to the mean value from three independent injections. Detection (LOD) and quantification

limits (LOQ) for each individual compound of the standard solution were calculated; thus, the signal to noise ratio of the standards at the lowest concentration level injected (for every analyte) in the three detectors was obtained, and LOD and LOQ were estimated by calculating the concentration that generate a signal to noise ratio equal to 3 and 10, respectively [53]. Standards at concentrations below 0.1 mg/L (compound dependent) were injected to corroborate the theoretically obtained LODs and LOQs.

Method accuracy was assessed by determining precision under repeatability conditions and trueness. *Intra-day* repeatability was expressed as the relative standard deviation (%RSD) obtained for 4 injections of the quality control mix (which, as stated above, was a sample mixture of extracts of the three tissues under study), carried out within the same sequence. *Inter-day* repeatability was calculated as %RSD of 8 injections (belonging to 4 different sequences carried out over 4 days) of 8 different extracts coming from the same olive leaves sample; in such a way that the *inter-day* repeatability values shown in the table 2 could give to reader an estimation about the overall repeatability of the method. The same strategy was followed using olive skin and pulp samples (data not shown).

Trueness was expressed as recovery, and it was estimated by analyzing a sample of each matrix extracted before and after the standard addition (using low, intermediate and high concentration levels (within the linear dynamic range) of pure standards) and calculating the difference between the results obtained.

Finally, the presence/absence of matrix effect was assessed in all the commodities under evaluation, since different samples may exhibit matrix effects of variable magnitude. Several methods have been proposed to this end, but most of them need a blank sample, which was not available in this study. Therefore, the matrix effect evaluation was made following the same strategy as Kmellár et all. [54], which consist in applying the

standard addition method to the different kind of samples, and comparing the slope of the external calibration function (in solvent) and the slope of the standard addition calibration curve (which compensates for any matrix effect). A matrix effect coefficient was calculated for each compound in each matrix (leaves, skin and pulp), following the equation given by these authors:

*Matrix effect coefficient (%) = (1-(slope matrix/slope solvent))*100*

Results and discussion

Optimization of chromatographic conditions

Since the analytes under study have very similar chemical structures (being some of them mass isomers), particular attention has been paid to assure their appropriate chromatographic separation and, therefore, their accurate determination. Two pairs are particularly problematic (oleanolic and ursolic acids, and erythrodiol and uvaol), so the chromatographic method optimization has tried to achieve the maximum peak resolution between them in the shortest possible time.

As mentioned before, this LC optimization step of the study was carried out in the LC-DAD/ESI-IT MS system. A mixture of standards prepared in methanol was used. A univariate optimization was carried out, changing one of the parameters and evaluating its influence, while keeping constant the other variables. The composition of mobile phase was, firstly, optimized. Two organic solvents with different polarities (acetonitrile and methanol) were mainly tested both individually and mixed in different proportions, under isocratic conditions. Figure 1a shows the effect of acetonitrile, methanol and their mixtures in mobile phase B on the retention times of all the analytes, number of theoretical plates and signal to noise ratio (S/N) of betulinic acid peak. Methanol

311 produced faster analyses, but it was a detriment to resolution. It can be observed that the
312 higher the percentage of acetonitrile, the better the separation among the analytes;
313 however, increasing proportions of acetonitrile also produced lower S/N values
314 (ionization was less efficient) and a reduction of N. Acetonitrile/methanol (60:40, v/v)
315 was pointed out as the optimum composition of Phase B.
316 After that, some modifiers were added to the organic solvents with different purposes:
317 to enhance ionization, to reduce run time and to increase the resolution between the
318 problematic pairs. We tested tetrahydrofuran, triethylamine, acetic acid and ammonium
319 hydroxide directly added in Phase B, and diluted in different proportions of water.
320 Figure 1b shows that a percentage of 10% of basified water resulted to be the best
321 choice taking into account the variables previously mentioned. In the figure, it is
322 possible to observe that a good separation is achieved among the compounds under
323 study when the added modifiers were 10% of acidified water, 10% of neutral water and
324 10% of basified water; the resolution was also acceptable for 10% of THF/water, just
325 observing worse separation for the critical pair ER and UV. When S/N was considered,
326 it was clear that 10% of basified water was the most appropriate composition of Phase
327 A (keeping reasonable N values).
328 Since the pH of the aqueous phase was very critical for the triterpenic acids resolution,
329 it was necessary to carry out its comprehensive optimization. Different MS compatible
330 buffers (ammonium bicarbonate adjusted to the desired pH with acetic acid, and
331 ammonium formiate and ammomium acetate adjusted with ammonium hydroxide) at
332 different concentrations levels (1 to 25 mM) were tested in a pH range between 7 and 11
333 and, finally, 1.5 mM ammonium formate in water adjusted at pH 9.6 with ammonium
334 hydroxide, was found to be the most appropriate composition of Phase A. After
335 choosing these conditions regarding mobile phase composition, the repeatability was

checked carrying out consecutive injections of both standard mix and ethanolic extracts of the matrices under study. When a C18 Zorbax Eclipse Plus column (Agilent Technologies) was used, the observed repeatability was not good enough, fact which can be probably explained due to the high pH at what the column was being subjected, which was in the upper limit of the working conditions range recommended by its manufacturer. To solve this problem two analogous end-capped columns, which are indicated for separating compounds under high pH conditions, were tested: Gemini column (Phenomenex, Torrance, CA, USA) and Zorbax Extend (Agilent Technologies). The last one was finally chosen because of its high stability and retention time repeatability.

To illustrate the effect of the pH, Figure 2 shows the chromatograms corresponding to its optimization (with the optimum column, Zorbax Extend), using ammonium formate buffer, which was the most effective enhancing the ionization of triterpenes in ESI-IT MS detector. As the pH increases, shorter analysis time and better resolution between the problematic pair (OA and UA) is achieved, but after reaching the maximum resolution at a pH range of 9.5-9.7, these triterpenic acids start to coelute at higher pH values. As can be seen in the figure, pH changes do not practically affect triterpenic alcohols elution (either in terms of resolution or retention time), fact that can be explained because their theoretical pKa is above these pH levels.

To complete the optimization of the separation method, different column temperatures and flow rates were tested. We tried temperatures between 5 and 40ºC and flow rates between 0.8 and 1.5 mL/min; eventually, a compromise solution between run time and resolution was reached at 25 ºC and 1.2 mL/min. The final optimum conditions led to an analysis time of 6 minutes; a total run time which makes our methodology shorter than

360 others previously described where triterpenic acids and alcohols were simultaneously
361 determined in more than 30 minutes [30,49].
362 Chromatograms resulting from the application of the optimized LC conditions in the
363 three evaluated detectors are shown in Figure 3. In all the cases, we show the profiles
364 obtained after analyzing a standard mixture containing the following concentrations: 2
365 mg/L of MA, OA and UA; 1 mg/L of BA; 4 mg/L of ER and UV. These concentration
366 levels were decided keeping in mind the ionization efficiency of each analyte in MS. In
367 ESI-IT MS, MA and BA are better ionized than in APCI-QTOF MS. Besides, in both
368 MS detectors, triterpenic alcohols are poorly ionized, being their relative response in
369 DAD more similar to rest of the compounds under study.
370 **MS signal characterization**
371 According to Rhourri-Frih et al. [43], ESI interface is adequate for ionizing polar and
372 ionic compounds, while more apolar compounds are properly ionized by APCI, so that
373 means that, in principle, APCI would be more suitable for the ionization of pentacyclic
374 triterpenes, especially for uvaol and erytrodiol which only possess one hydroxyl group
375 on their structure. Giménez et al. [49] recently described ESI as a not recommendable
376 ion source for detecting triterpenic alcohols (neither in positive nor in negative polarity),
377 but their determination have been previously achieved using electrospray ionization
378 sources by some other authors, such as, for instance, Sanchez-Avila et al. [30]. Thus, we
379 tried to do a comprehensive characterization of the accurate MS signals obtained using
380 both ESI and APCI sources. The MS signals achieved for the 6 triterpenic compounds
381 found in *Olea europaea* (when both ionization sources were used) were study in depth
382 in order to evaluate the differences between them. The identification of the 6
383 chromatographic peaks corresponding to our compounds of interest was achieved by
384 using the information coming from the QTOF analyzers (which allowed predicting the

385 molecular formula of analytes from their exact mass) and it was logically corroborated
386 comparing their retention times with those of the pure standards.
387 Both interfaces gave the pseudo-molecular ion in positive and in negative polarity
388 (except for triterpenic alcohols which could be only determined when positive polarity
389 was chosen), although $[M+H]^+$ and $[M-H]^-$ were not the most abundant signals in the
390 spectra in every case. In general, the fragmentation in-source was much more easily
391 observed when positive polarity is used. Moreover, in negative polarity, signals were
392 the same in both ESI and APCI ionization sources, meanwhile in positive polarity, ESI
393 *m/z* signals commonly include alkaline metal adducts, fact which is not observable in
394 APCI, being in this case species such as $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, and
395 $[M+H-COOH]^+$ more prevalent. See supplementary material for further details.
396 Furthermore, since MS^2 could help us to distinguish between molecules with the same
397 molecular formula, in case that their fragmentation would be different, the MS
398 fragmentation of the six analytes was carried out by means of the ESI-IT-MS detector.
399 Nevertheless, as could be expected considering the chemical structure similarities
400 shown in table 1, olive triterpenic mass isomers showed the same fragmentation patterns
401 too. In negative ion mode, the fragmentation pattern matched with data previously
402 reported using the same MS configuration [44,55]. The most prevalent signal in mass
403 spectra of triterpenic acids corresponded to the deprotonated molecular ion $[M-H]^-$ (m/z
404 471 for MA, and 455 for BA, OA and UA) which gave a MS/MS fragment
405 corresponding to the carboxylic group loss (m/z 423 for MA, and 407 for BA, OA and
406 UA). Moreover, the removal of the additional hydroxylic group in MA, gave another
407 fragment in its spectrum (m/z 393). As mentioned above, the triterpenic alcohols peaks
408 could not be detected in the chromatograms when negative ionization mode was
409 employed. MS detection in positive ionization mode was less sensitive (considering

410 triterpenic acids) than in negative polarity, but allowed UV and ER detection. In
411 positive polarity, the most abundant *m/z* signal in MA spectrum corresponded to the
412 molecular ion accompanied by a sodium adduct (*m/z* 495) which lost the carboxylic
413 group –when was fragmented in MS/MS mode- giving the corresponding fragment with
414 *m/z* 451. Triterpenic acids isomers (BA, OA and UA) gave the same three MS signals
415 (*m/z* 457, 439 and 479) which differed in terms of relative intensities depending on the
416 analyte; the signals were the protonated molecular ion (*m/z* 457), the protonated
417 molecular ion with a water loss (*m/z* 439) and the molecular ion plus a sodium aduct
418 (*m/z* 479). These precursors lost the carboxylic group (which is in agreement with
419 previously reported results [44]) giving the following fragments: *m/z* 411 ($[M + H - COOH]^+$)
420 *m/z* 393 ($[M + H - H_2O - COOH]^+$) and 435 ($[M + Na - COOH]^+$). The MS
421 signal with *m/z* 191 was also detected, which according to Sánchez-Ávila et al. [30], is a
422 characteristic fragment from oleane structure. When the triterpenic alcohol isomers (ER
423 and UV) MS spectra were observed, the same three precursor ions were found, with *m/z*
424 443, 425 and 465, corresponding to $[M + H]^+$, $[M + H - H_2O]^+$ and $[M + Na]^+$,
425 respectively. However, they only can lose alcohol groups, so their fragments are water
426 losses, such as *m/z* 425 ($[M + H - H_2O]^+$) and *m/z* 407 ($[M + H - 2\ H_2O]^+$) or the oleane
427 structure fragment with *m/z* 191.

428 **Analytical parameters of the method**

429 Table 2 shows the analytical parameters of the proposed method, which give an idea of
430 its suitability for the analysis of the selected samples (for each tested detector). The
431 table includes calibration curves and regression coefficients (r^2), LOD, LOQ, accuracy
432 (expressed as trueness and intra/inter day repeatability), and matrix effect coefficients.
433 To evaluate the response of each analyte in DAD, ESI-IT MS and APCI-QTOF MS,
434 their peak areas were plotted as a function of their concentration (for both MS

435 platforms, the extracted ion chromatograms of the most intense *m/z* signal were used for
436 quantitative purposes) performing a linear regression by the least-squares method.
437 All the resulting calibration lines showed good linearity within the indicated
438 concentration ranges, with regression coefficients (r^2) higher than 0.9896. Furthermore,
439 in every tested detector, triterpenic acids had lower detection and quantification limits
440 than triterpenic dialcohols. ESI-IT MS gave the lowest limits followed by APCI-QTOF
441 MS; in contrast, DAD had the widest dynamic range, fact which was found to be
442 inversely proportional to the LOD and LOQ. For some cases, such as UA, for instance,
443 the LOD values varied a lot depending on the detector: 9 µg/L in ESI-IT, 28 µg/L in
444 APCI-QTOF, and 441 µg/L when DAD was used. However, for ER and UV, the
445 differences among the achieved LODs by the three detectors were not so pronounced.
446 The RSD values for *intra-day* repeatability were found between 2.6 and 10.8 % in
447 DAD, 3.4 and 6.3 % in ESI-IT MS detector, and within the range from 2.2 to 10.5 % in
448 the case of the APCI-QTOF MS detector. The RSD values for *inter-day* repeatability
449 (which were calculated with different extracts of olive leaves extracts measured in
450 different sequences (similar behavior was corroborated for the rest of the evaluated
451 matrices)), were a little worse than those obtained for *intra-day* repeatability, since they
452 include the precision of sample preparation and analysis (from our point of view, these
453 values can be used to give an estimation the global method repeatability). With regard
454 to the retention time repeatability, the RSD was less than 1.6% for intra-day
455 repeatability and less than 2.2% for inter-day repeatability in the worst-case scenario
456 (data not shown in table 2). As far as trueness is concerned, Table 2 shows the
457 recoveries obtained for each individual analyte in the three matrixes which were found
458 between 93.5 % (for OA in pulp) and 111.9 % (for MA in skin). This means that the

proposed method is truthful, according to the AOAC guidelines [56], which establishes a good trueness from 80% to 115%.

Matrix effect was also evaluated according to the procedure described in section 2.4. Calculated matrix effect coefficient for each compound in each type of sample fluctuated between −14.6% and +1.4%, falling within the range described by Kmellár et al. [54] in which there is a mild signal suppression or enhancement effect (from -20 to +20%). Only the calculated matrix effect coefficients for APCI-QTOF MS detector are shown in Table 2, since similar behaviour (proving that there is no need to use standard addition calibration to achieve a proper quantification) was observed for the rest of the tested platforms. In almost all the cases, the matrix effect coefficients were below ±10% (only one case exceeded this value); indeed, for every analyte in the three matrices, the coefficients were actually below ±7.5%. Bearing in mind these results, it is possible to claim that the magnitude of possible matrix effect was not significant; therefore, the quantification was carry out by using external calibration equations.

Bearing in mind the just described performance of each detector and in an attempt of carrying out a systematic description of the purpose of using each platform, their most remarkable characteristics, advantages/drawbacks and the global major achievements, Table 3 is presented. As stated before, this work does not intend carrying out a comprehensive comparison of the analytical performance of the tested detectors (we are absolutely aware about the fact that in order to fairly compare the two used interfaces, we should have selected the same analyzer for both platforms), but offering different useful alternatives to accomplish a reliable determination of pentacyclic triterpenes in olive tissues, which, from our point of view, represents an interesting and challenging application in the field of food metabolomics.

The necessary number of injections in each case, as well as the quantitative results obtained will be thoroughly discussed in the following section.

Method application

Once it was optimized and validated, the developed LC-DAD/MS method was applied to the quantification of the six pentacyclic triterpenes under study in samples of three kinds of olive tissues. Olive leaves and olive skin were chosen because they have been described as very rich matrices in terms of those compounds; indeed, in general, fruit peel and, especially, fruit cuticular waxes have been identified as promising and highly available triterpenoid-rich plant tissues [11]. Skinless pulp seemed to be another interest matrix because, to the best of our knowledge, its triterpenic content has not been previously established. Guinda et al. (2010) [28], in a very interesting study, evaluated the pentacyclic triterpenoids content from olive fruit and leaves and their results indicated that maslinic and oleanolic acids were exclusively located in the epidermis, being below their detection limits in the flesh and seed of the olive drupe. As stated before, in the current study, we wanted to compare the concentration levels of triterpenoids in olive skin, leaves and pulp (even though the latter was expected to be very low in comparison), in order to provide some useful information which could help to understand more in depth their metabolism and distribution over the different issues of *Olea europaea*.

In a first screening, the method was applied to some sample extracts to roughly estimate the concentration of each metabolite, observing that their relative concentration levels differed a lot for each analyte (being some of them at very low levels and some others at very high concentrations). This fact is illustrated in Figure 4 and, obviously, turned the quantification of the triterpenoids into a very complicated task. Therefore, two injections per extract were necessary in MS detectors to ensure that all the compounds

508 of interest could be measured in their linear calibration ranges. Nevertheless, although
509 two injections were also registered in DAD detector (because it is *on line* with the LC –
510 IT MS platform) just one injection was absolutely necessary due to its wider dynamic
511 range.
512 Quantitative results for each detection method (DAD, ESI-IT and APCI-QTOF) and
513 matrix are shown in Table 4. They are presented as the average of four replicates
514 accompanied by the RSD. The final results are the interpolated concentration values
515 multiplied by the estimated recovery (which, as stated before, was calculated dividing
516 the concentration of the analytes in the first extract between the total content of each
517 analyte as the sum of the found amounts in the first, second and third extracts) and the
518 dilution factor.
519 The found values in the three tested detectors were in good agreement as no significant
520 statistical differences (at a 95% confidence level, $p < 0.05$) were found among them.
521 The concentration levels of the six triterpenes analyzed in the olive leaves were found
522 around 3.7 mg/g for MA, 18 mg/g for OA, 1.8 mg/g for UA, 1.6 mg/g for ER and 1.2
523 mg/g for UV, which generally were into the ranges previously reported by Sanchez-
524 Avila et al. [30] and Peragon [55], but slightly lower than those reported by Guinda et
525 al. [28] and much higher than the achieved results by Stiti et al. [57] (who quantified
526 with respect to the internal standard). The quantitative composition of olive leaves could
527 be strongly affected by agronomic factors, such as cultivar or ripening degree [11]; this
528 fact could explain some differences in the described concentration levels. Besides, BA,
529 which has not been previously quantified in olive leaves, was found at a concentration
530 level around 0.12 mg/g.
531 As far as olive skin is concerned, it is possible to stand out that the triterpenoid found
532 contents were around: 80 mg/g of MA, 0.20 mg/g of BA, 26 mg/g of OA, 0.14 mg/g of

UA, 0.78 mg/g of ER and 0.30 mg/g of UV. In this case, a direct comparison with literature data cannot be done, because skin pentacyclic triterpenes levels have not been previously reported. It is well-know that these compounds are mainly located in the epicarp of the olive fruit, but a scarce number of reports explicitly stating its content can be found, since the entire fruits (including skin and pulp) are often analyzed [28,49,55,57]. In some other interesting applications, the composition of the waxy material covering the surface of olive fruits is given expressing the amounts with regard to the total weight of the fruit [35,58].

Even though these compounds have not been detected so far in such matrix [28], the described method allowed quantifying little amounts of triterpenic acids in olive pulp (around 65, 1.2, 55 and 4.4 μg/g for MA, BA, OA and UA, respectively).

Betulinic acid, which has been, to a certain extent, used as internal standard when quantifying triterpenic compounds in *Olea europaea*-related samples [30,33,49], was determined in the three studied matrices (by means of the three tested detectors), confirming its presence in surface waxes of olive leaves and fruits, reported by Bianchi et al. [14,58].

Conclusions

The optimization of a liquid chromatography method using three different detectors (DAD, ESI-IT MS and APCI-TOF MS) and its potential application in the field of food metabolomics have been discussed in the current study; in particular for the determination of pentacyclic triterpenes in olive tissues. The method was fully validated and the analytical performances of the different detectors were described. The concentrations of the triterpenic compounds under study were established in three different matrices (triterpenic acids levels were evaluated by first time for olive pulp)

and no statistically significant differences among the quantitative results achieved by each platform were observed, so they could be interchangeably used. Nevertheless, LC-DAD and LC-ESI-IT MS were able to detect all the compounds under study within a single run, whilst the LC-APCI-QTOF MS used platform needed two injections (one for each ion polarity mode) in order to detect both triterpenic acids and alcohols. Moreover, because of the wider linear range of DAD, it was the only one capable of quantify all the analytes by using a single dilution in every matrix (the other two, needed one additional dilution for MA and OA in leaves and skin).

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References

[1] A. Denny, J. Buttriss, Plant foods and health: focus on plant bioactives, 2005.

[2] C.J. Dillard, J. Bruce German, Phytochemicals: Nutraceuticals and human health, J. Sci. Food Agric. 80 (2000) 1744–1756. doi:10.1002/1097-0010(20000915)80:12<1744::AID-JSFA725>3.0.CO;2-W.

[3] I. Raskin, D.M. Ribnicky, S. Komarnytsky, N. Ilic, A. Poulev, N. Borisjuk, et al., Plants and human health in the twenty-first century, Trends Biotechnol. 20 (2002) 522–531. doi:10.1016/S0167-7799(02)02080-2.

[4] R.S. Pawar, H. Tamta, J. Ma, A.J. Krynitsky, E. Grundel, W.G. Wamer, et al., Updates on chemical and biological research on botanical ingredients in dietary supplements, Anal. Bioanal. Chem. 405 (2013) 4373–4384. doi:10.1007/s00216-012-6691-2.

[5] J.T. James, I.A. Dubery, Pentacyclic triterpenoids from the medicinal herb, Centella asiatica (L.) Urban, Molecules. 14 (2009) 3922–3941. doi:10.3390/molecules14103922.

[6] H. Sun, W. Fang, W. Wang, C. Hu, Structure-activity relationships of oleanane-

and ursane- type triterpenoids, Bot. Stud. 47 (2006) 339–368.

[7] H. Sheng, H. Sun, Synthesis, biology and clinical significance of pentacyclic triterpenes: a multi-target approach to prevention and treatment of metabolic and vascular diseases., Nat. Prod. Rep. 28 (2011) 543–593. doi:10.1039/c0np00059k.

[8] M.N. Laszczyk, Pentacyclic triterpenes of the lupane, oleanane and ursane group as tools in cancer therapy, Planta Med. 75 (2009) 1549–1560. doi:10.1055/s-0029-1186102.

[9] J.M. Castellano, A. Guinda, T. Delgado, M. Rada, J.A. Cayuela, Biochemical basis of the antidiabetic activity of oleanolic acid and related pentacyclic triterpenes, Diabetes. 62 (2013) 1791–1799. doi:10.2337/db12-1215.

[10] R. Xu, G.C. Fazio, S.P.T. Matsuda, On the origins of triterpenoid skeletal diversity, Phytochemistry. 65 (2004) 261–291. doi:10.1016/j.phytochem.2003.11.014.

[11] A. Szakiel, C. Pączkowski, F. Pensec, C. Bertsch, Fruit cuticular waxes as a source of biologically active triterpenoids., Phytochem. Rev. 11 (2012) 263–284. doi:10.1007/s11101-012-9241-9.

[12] R. Caputo, L. Mangoni, P. Monaco, L. Previtera, Triterpenes in husks of Olea europaea, Phytochemis try. 13 (1974) 1551–1552. doi:10.1016/0031-9422(74)80325-0.

[13] A. Severge, Difficulties in physical refining of olive oil, due to presence of triterpene “oleanolic acid,” J. Am. Oil Chem. Soc. 60 (1983) 584–587. doi:10.1007/BF02679792.

[14] G. Bianchi, G. Vlahov, C. Anglani, C. Murelli, Epicuticular wax of olive leaves, Phytochemistry. 32 (1992) 49–52. doi:10.1016/0031-9422(92)80104-M.

[15] M.D. Herrera, R. Rodríguez-Rodríguez, V. Ruiz-Gutiérrez, Functional properties of pentacyclic triterpenes contained in “orujo” olive oil, Curr. Nutr. Food Sci. 2 (2006) 45–49. doi:10.2174/157340106775471976.

[16] R. Rodríguez-Rodríguez, V. Ruiz-Gutiérrez, Functional properties of pentacyclic triterpenes contained in pomace olive oil, in: Olives Olive Oil Heal. Dis. Prev., Elsevier Inc., 2010: pp. 1431–1438. doi:10.1016/B978-0-12-374420-3.00159-5.

[17] M. Fernández-Navarro, J. Peragón, F.J. Esteban, V. Amores, M. de la Higuera, J.A. Lupiáñez, Maslinic acid: a component of olive oil on growth and protein-turnover rates, in: Olives Olive Oil Heal. Dis. Prev., Elsevier Inc., 2010: pp. 1415–1421. doi:10.1016/B978-0-12-374420-3.00157-1.

[18] J.M. Lou-Bonafonte, C. Arnal, M.A. Navarro, J. Osada, Efficacy of bioactive compounds from extra virgin olive oil to modulate atherosclerosis development., Mol. Nutr. Food Res. 56 (2012) 1043–1057. doi:10.1002/mnfr.201100668.

[19] C. Sánchez-Quesada, A. López-Biedma, F. Warleta, M. Campos, G. Beltrán, J.J. Gaforio, Bioactive properties of the main triterpenes found in olives, virgin olive oil, and leaves of Olea europaea, J. Agric. Food Chem. 61 (2013) 12173–12182. doi:10.1021/jf403154e.

[20] R. Rodríguez-Rodríguez, Oleanolic acid and related triterpenoids from olives on vascularfunction: molecular mechanisms and therapeutic perspectives, Curr. Med. Chem. 22 (2015) 1414–1425.

[21] A. García-Granados, Process for the industrial recovery of oleanolic and maslinic acids contained in the olive milling subproducts, US 6037492, 1998. https://www.google.com/patents/CA2232692A1?cl=en.

[22] G. Jerz, Process for extracting oleanolic acid from plant material, US 6700014, 2004. https://www.google.com/patents/US6700014.

[23] J.J. Tabera Galván, A. Ruiz Rodríguez, F.J. Señoráns Rodríguez, E. Ibáñez Ezequiel, G.J. Reglero Rada, T. Albi Vierlla, et al., Method of obtaining high-value-added compounds from olive leaves, WO2005075614 A1, 2005. https://www.google.com/patents/WO2005075614A1?cl=en.

[24] G.F. Santoyo, M.M. Ortega, M.F. Hernández, Method for extraction of triterpenic acids and hydroxytyrosol from olive-dressing solutions, WO2013007845 A1, 2013. https://www.google.com/patents/WO2013007845A1?cl=en.

[25] V. Goulas, G.A. Manganaris, Towards an efficient protocol for the determination of triterpenic acids in olive fruit: a comparative study of drying and extraction methods., Phytochem. Anal. 23 (2012) 444–449. doi:10.1002/pca.1378.

[26] M. Wójciak-Kosior, I. Sowa, R. Kocjan, R. Nowak, Effect of different extraction techniques on quantification of oleanolic and ursolic acid in Lamii albi flos, Ind. Crops Prod. 44 (2013) 373–377. doi:10.1016/j.indcrop.2012.11.018.

[27] V.G. Kontogianni, V. Exarchou, A. Troganis, I.P. Gerothanassis, Rapid and novel discrimination and quantification of oleanolic and ursolic acids in complex plant extracts using two-dimensional nuclear magnetic resonance spectroscopy-Comparison with HPLC methods, Anal. Chim. Acta. 635 (2009) 188–195. doi:10.1016/j.aca.2009.01.021.

[28] A. Guinda, M. Rada, T. Delgado, P. Gutiérrez-Adánez, J.M. Castellano, Pentacyclic triterpenoids from olive fruit and leaf., J. Agric. Food Chem. 58 (2010) 9685–9691. doi:10.1021/jf102039t.

[29] C. Romero, A. García, E. Medina, M.V. Ruíz-Méndez, A. De Castro, M. Brenes, Triterpenic acids in table olives, Food Chem. 118 (2010) 670–674. doi:10.1016/j.foodchem.2009.05.037.

[30] N. Sánchez-Ávila, F. Priego-Capote, J. Ruiz-Jiménez, M.D. Luque de Castro, Fast and selective determination of triterpenic compounds in olive leaves by liquid chromatography-tandem mass spectrometry with multiple reaction monitoring after microwave-assisted extraction., Talanta. 78 (2009) 40–48. doi:10.1016/j.talanta.2008.10.037.

[31] E.Q. Xia, Y.Y. Yu, X.R. Xu, G.F. Deng, Y.J. Guo, H. Bin Li, Ultrasound-assisted extraction of oleanolic acid and ursolic acid from Ligustrum lucidum Ait, Ultrason. Sonochem. 19 (2012) 772–776. doi:10.1016/j.ultsonch.2011.11.014.

[32] M. Tarvainen, J.-P. Suomela, H. Kallio, B. Yang, Triterpene acids in Plantago major: identification, quantification and comparison of different extraction methods, Chromatographia. 71 (2010) 279–284. doi:10.1365/s10337-009-1439-7.

[33] M.C. Pérez-Camino, A. Cert, Quantitative determination of hydroxy pentacyclic triterpene acids in vegetable oils, J. Agric. Food Chem. 47 (1999) 1558–1562. doi:10.1021/jf980881h.

[34] Y. Allouche, A. Jiménez, M. Uceda, M.P. Aguilera, J.J. Gaforio, G. Beltrán, Triterpenic content and chemometric analysis of virgin olive oils from forty olive cultivars, J. Agric. Food Chem. 57 (2009) 3604–3610. doi:10.1021/jf803237z.

[35] G. Bianchi, N. Pozzi, G. Vlahov, Pentacyclic triterpene acids in olives, Phytochemistry. 37 (1994) 205–207. doi:10.1016/0031-9422(94)85026-7.

[36] A. Caligiani, G. Malavasi, G. Palla, A. Marseglia, M. Tognolini, R. Bruni, A simple GC-MS method for the screening of betulinic, corosolic, maslinic, oleanolic and ursolic acid contents in commercial botanicals used as food supplement ingredients., Food Chem. 136 (2013) 735–741. doi:10.1016/j.foodchem.2012.08.011.

[37] R. Gao, L. Wang, Y. Yang, J. Ni, L. Zhao, S. Dong, et al., Simultaneous determination of oleanolic acid, ursolic acid, quercetin and apigenin in *Swertia mussotii* Franch by capillary zone electrophoresis with running buffer modifier, Biomed. Chromatogr. 29 (2015) 402–409. doi:10.1002/bmc.3290.

[38] G. Lozano-Mena, M.E. Juan, A. García-Granados, J.M. Planas, Determination of maslinic acid, a pentacyclic triterpene from olives, in rat plasma by high-performance liquid chromatography., J. Agric. Food Chem. 60 (2012) 10220–10225. doi:10.1021/jf3023996.

[39] S. Guo, J.A. Duan, Y. Tang, Y. Qian, J. Zhao, D. Qian, et al., Simultaneous qualitative and quantitative analysis of triterpenic acids, saponins and flavonoids in the leaves of two Ziziphus species by HPLC-PDA-MS/ELSD, J. Pharm. Biomed. Anal. 56 (2011) 264–270. doi:10.1016/j.jpba.2011.05.025.

[40] G.-L. Li, J. You, C. Song, L. Xia, J. Zheng, Y. Suo, Development of a new

HPLC method with precolumn fluorescent derivatization for rapid, selective and sensitive detection of triterpenic acids in fruits., J. Agric. Food Chem. 59 (2011) 2972–2979. doi:10.1021/jf104224t.

[41] N. Hu, Y. Suo, Q. Zhang, J. You, Z. Ji, A. Wang, et al., Rapid, selective, and sensitive analysis of triterpenic acids in Hippophae rhamnoides L. using HPLC with pre-column fluorescent derivatization and identification with post-column APCI-MS, J. Liq. Chromatogr. Relat. Technol. 38 (2014) 451–458. doi:10.1080/10826076.2014.913523.

[42] G. Chen, J. Li, C. Song, Y. Suo, J. You, A sensitive and efficient method for simultaneous trace detection and identification of triterpene acids and its application to pharmacokinetic study., Talanta. 98 (2012) 101–111. doi:10.1016/j.talanta.2012.06.053.

[43] B. Rhourri-Frih, P. Chaimbault, B. Claude, C. Lamy, P. André, M. Lafosse, Analysis of pentacyclic triterpenes by LC-MS. A comparative study between APCI and APPI, J. Mass Spectrom. 44 (2009) 71–80. doi:10.1002/jms.1472.

[44] Q. Chen, Y. Zhang, W. Zhang, Z. Chen, Identification and quantification of oleanolic acid and ursolic acid in Chinese herbs by liquid chromatography-ion trap mass spectrometry, Biomed. Chromatogr. 25 (2011) 1381–1388. doi:10.1002/bmc.1614.

[45] M. Sánchez-González, G. Lozano-Mena, A. Parra, M.E. Juan, J.M. Planas, Identification in rat plasma and urine by linear trap quadrupole–Orbitrap mass spectrometry of the metabolites of maslinic acid, a triterpene from olives, J. Agric. Food Chem. 63 (2015) 1126–1132. doi:10.1021/jf505379g.

[46] H.L. Alvarado, G. Abrego, M.L. Garduño-Ramirez, B. Clares, M.L. García, A.C. Calpena, Development and validation of a high-performance liquid chromatography method for the quantification of ursolic/oleanic acids mixture isolated from Plumeria obtusa, J. Chromatogr. B. 983-984 (2015) 111–116. doi:10.1016/j.jchromb.2015.01.009.

[47] J.-P. Fan, R.-F. Zhang, X.-H. Zhang, J.-H. Zhu, J.-Z. Huang, Separation of three triterpene acids in leaves of Diospyros Kaki by high performance liquid chromatography using hydroxypropyl-β-cyclodextrin as mobile phase modifier, J. Liq. Chromatogr. Relat. Technol. 34 (2011) 1340–1355. doi:10.1080/10826076.2011.570841.

[48] X. Fang, J. Wang, X. Yu, G. Zhang, J. Zhao, Optimization of microwave-assisted extraction followed by RP-HPLC for the simultaneous determination of oleanolic acid and ursolic acid in the fruits of Chaenomeles sinensis., J. Sep. Sci. 33 (2010) 1147–1155. doi:10.1002/jssc.200900726.

[49] E. Giménez, M.E. Juan, S. Calvo-Melià, J. Barbosa, V. Sanz-Nebot, J.M. Planas, Pentacyclic triterpene in Olea europaea L: A simultaneous determination by high-performance liquid chromatography coupled to mass spectrometry, J.

Chromatogr. A. 1410 (2015) 68–75. doi:10.1016/j.chroma.2015.07.064.

[50] S. Guo, J.A. Duan, D. Qian, Y. Tang, D. Wu, S. Su, et al., Content variations of triterpenic acid, nucleoside, nucleobase, and sugar in jujube (Ziziphus jujuba) fruit during ripening, Food Chem. 167 (2015) 468–474. doi:10.1016/j.foodchem.2014.07.013.

[51] M.K. Lee, Y.M. Ahn, K.R. Lee, J.H. Jung, O.S. Jung, J. Hong, Development of a validated liquid chromatographic method for the quality control of Prunellae Spica: Determination of triterpenic acids, Anal. Chim. Acta. 633 (2009) 271–277. doi:10.1016/j.aca.2008.12.038.

[52] G.-L. Li, X. Zhang, J. You, C. Song, Z. Sun, L. Xia, et al., Highly sensitive and selective pre-column derivatization high-performance liquid chromatography approach for rapid determination of triterpenes oleanolic and ursolic acids and application to Swertia species: Optimization of triterpenic acids extraction an, Anal. Chim. Acta. 688 (2011) 208–218. doi:10.1016/j.aca.2011.01.010.

[53] ICH, Validation of a analytical Procedures: text and methodology Q2(R1), Guidance. 1994 (2005) 1–17.

[54] B. Kmellár, P. Fodor, L. Pareja, C. Ferrer, M.A. Martínez-Uroz, A. Valverde, et al., Validation and uncertainty study of a comprehensive list of 160 pesticide residues in multi-class vegetables by liquid chromatography-tandem mass spectrometry, J. Chromatogr. A. 1215 (2008) 37–50. doi:10.1016/j.chroma.2008.10.121.

[55] J. Peragón, Time course of pentacyclic triterpenoids from fruits and leaves of olive tree (Olea europaea L.) cv. Picual and cv. Cornezuelo during ripening, J. Agric. Food Chem. 61 (2013) 6671–6678. doi:10.1021/jf401495t.

[56] AOAC, Guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals, AOAC Int. (2002) 1–38.

[57] N. Stiti, M.-A. Hartmann, Nonsterol triterpenoids as major constituents of Olea europaea, J. Lipids. 2012 (2012) 1–13. doi:10.1155/2012/476595.

[58] G. Bianchi, C. Murelli, G. Vlahov, Surface waxes from olive fruits, Phytochemistry. 31 (1992) 3503–3506. doi:10.1016/0031-9422(92)83716-C.

Figure 1a

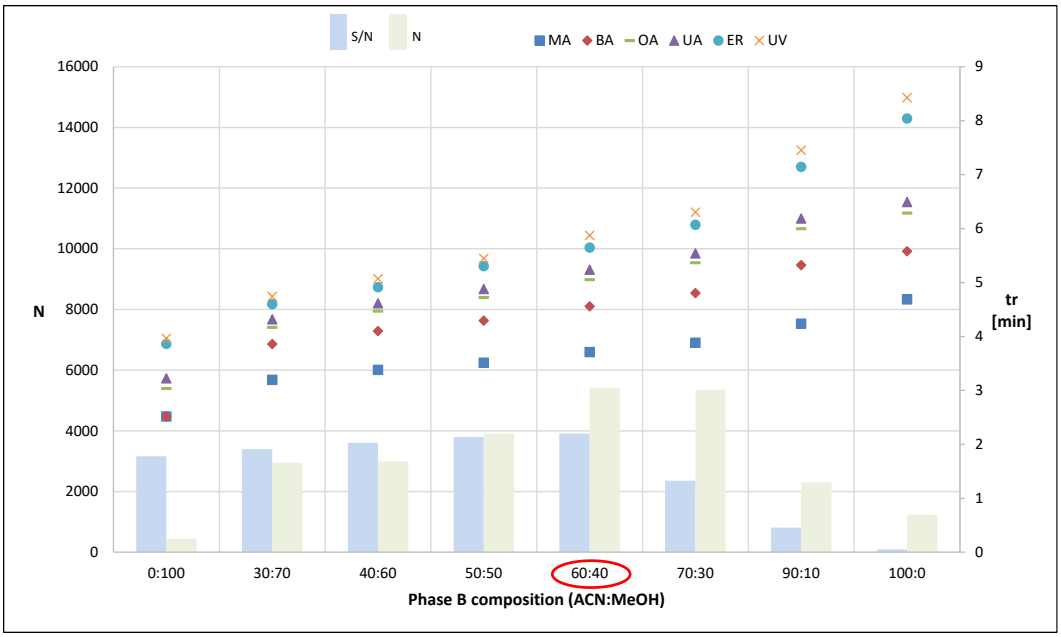


Figure 1b

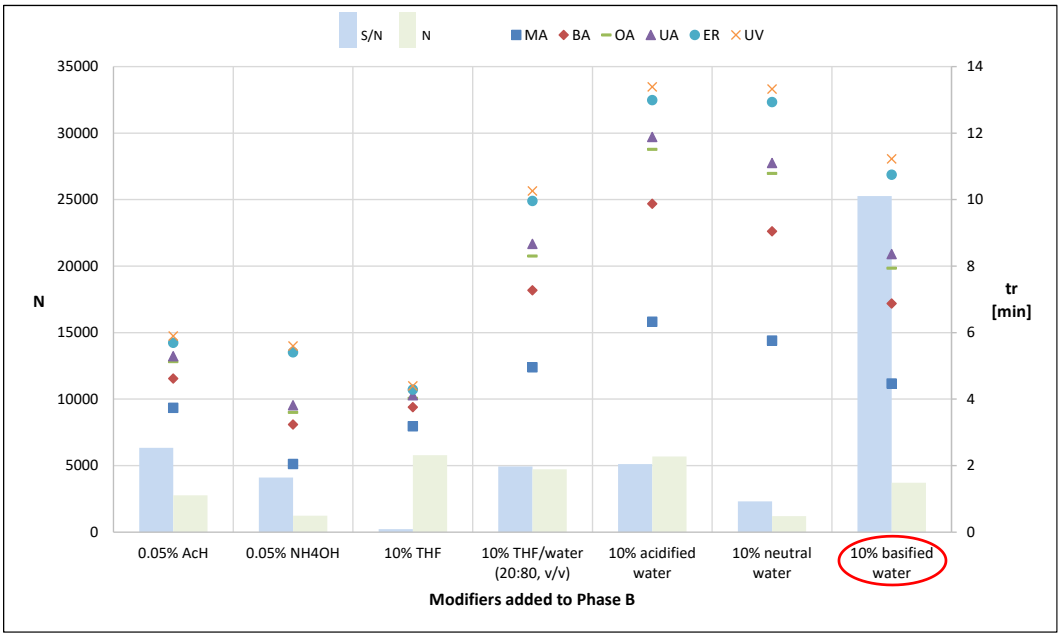


Figure 1. a) Graphical representation of the influence of composition of Phase B on: retention times of all the analytes, number of theoretical plates and signal to noise ratio of betulinic acid peak (selected as example to illustrate the influence of the tested parameters). In the Y axis, S/N ratio is normalized to the scale of N to facilitate the display of the data. b) Graphical representation of the influence of the modifiers added to Phase B (tetrahydrofuran (THF), acetic acid (AcH) and ammonium hydroxide (NH_3) directly added and diluted in different proportions of water) on the same variables as observed in a).

The results shown in this figure were obtained by using a C18 Zorbax Eclipse Plus column (4.6 x 150 mm, 1.8 µm particle size).

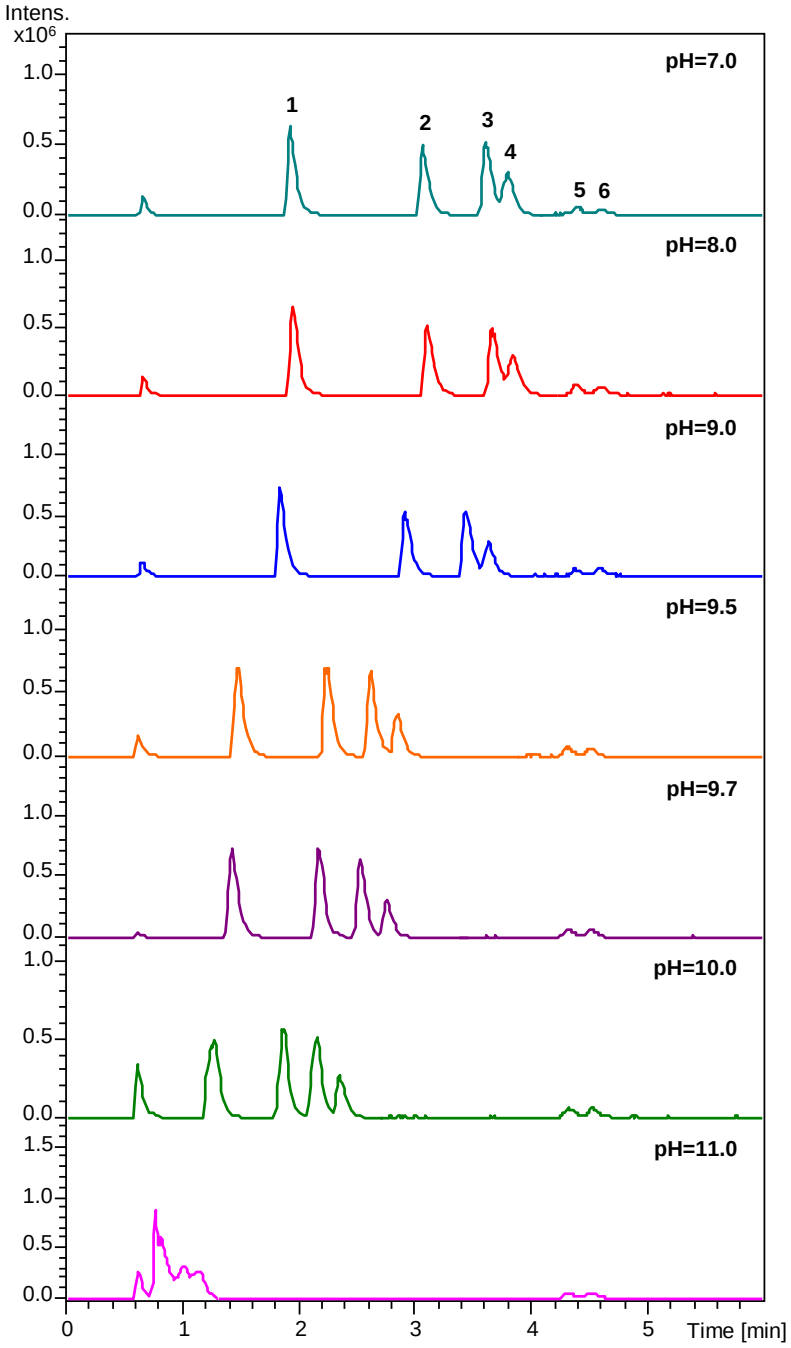


Figure 2. Base peak chromatograms obtained in ESI-IT MS detector (using negative ionization mode for triterpenic acids and positive polarity for alcohols) showing the influence of the pH value on the separation of the compounds under study.

Peak identification numbers: 1, maslinic acid; 2, betulinic acid; 3, oleanolic acid; 4, ursolic acid, 5, erythrodiol, and 6, uvaol.

The results shown in this figure were obtained by using the optimum Zorbax Extend C18 analytical column (4.6 x 100 mm, 1.8 μm particle size).

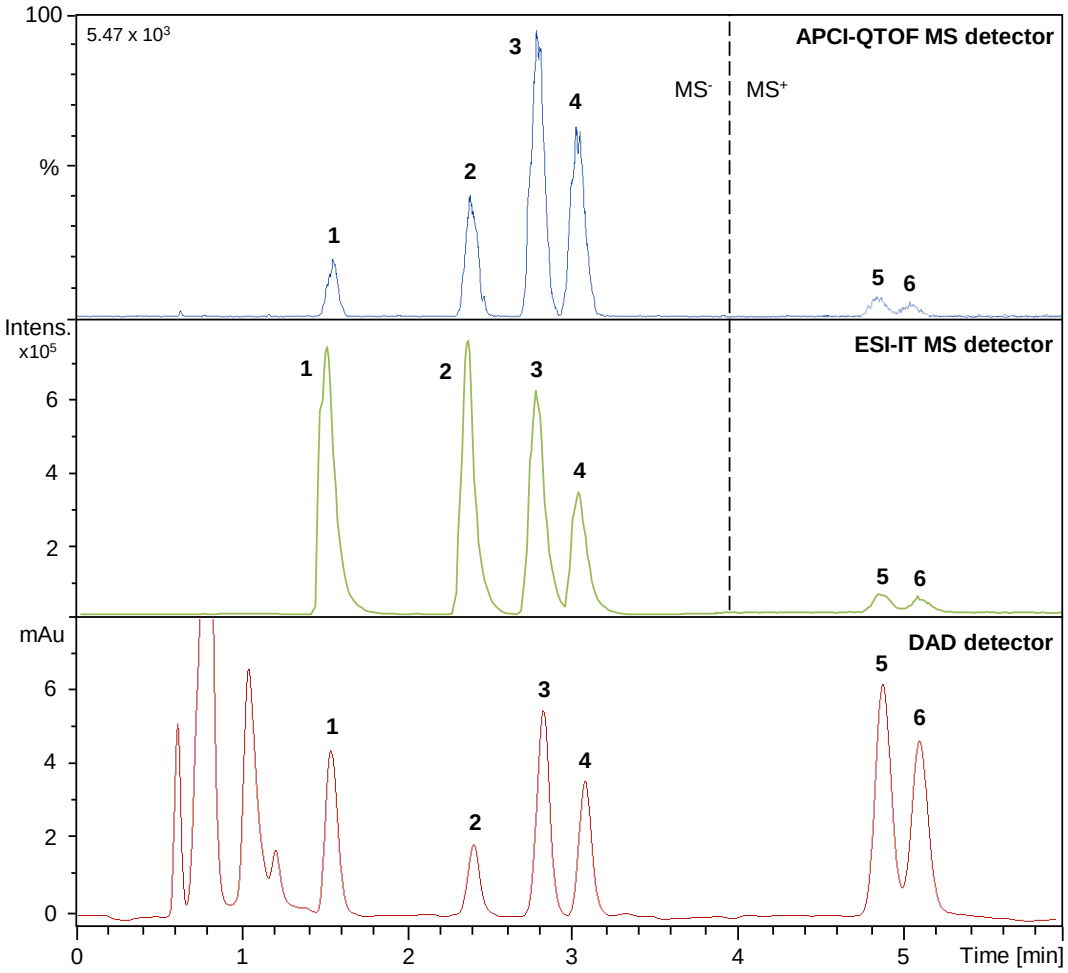


Figure 3. Separation achieved by using the optimal chromatographic and detection conditions (in MS, base peak chromatograms are shown). Two segments were used for MS detection, from the beginning of the analysis to 3.9 min in negative polarity, and from 3.9 till the end of the run, in positive mode.

Peak identification numbers with the same meaning as in Fig. 2.

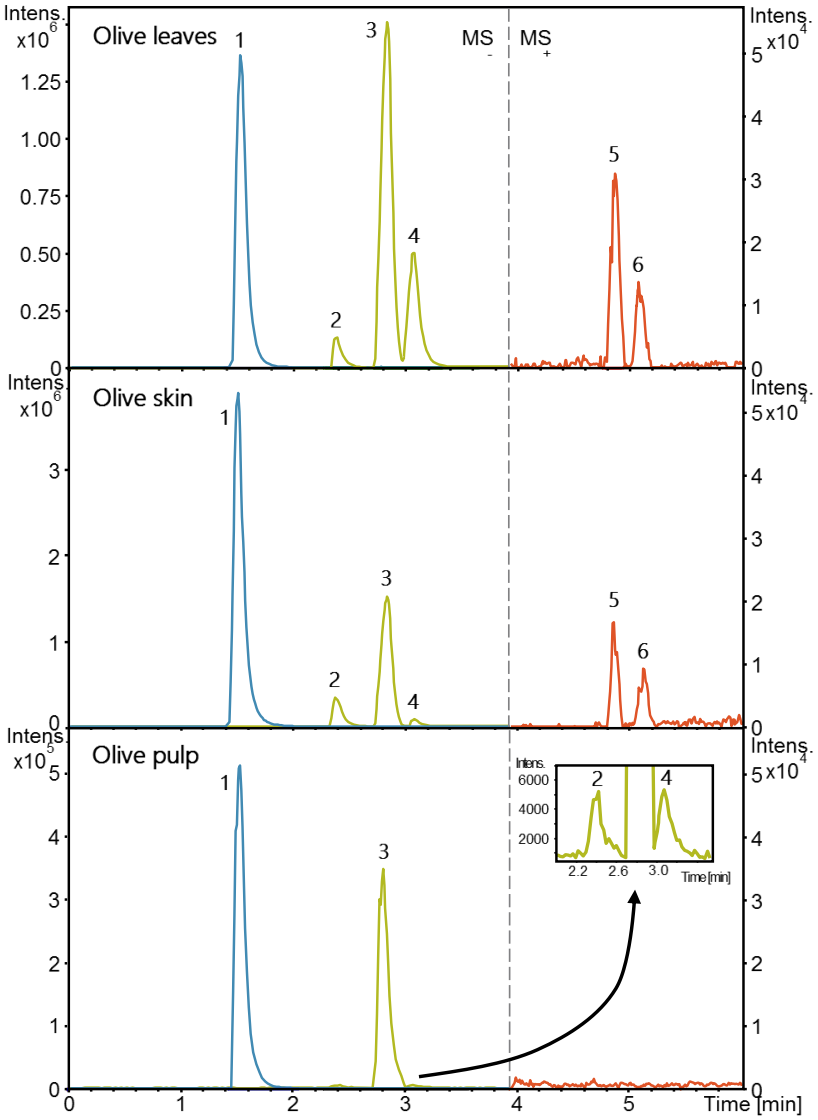


Figure 4. Extracted ion chromatograms (EICs) obtained when the different matrices selected within this study were analyzed in LC-ESI-IT MS platform.

Peak identification numbers with the same meaning as in Fig. 2. The EICs shown are those corresponding with *m/z* 471 for peak number 1; *m/z* 455 for peaks 2-4; and *m/z* 425 for compounds 5 and 6.

Table 1. Molecular formula and structure of *Olea europaea* pentacyclic triterpenes

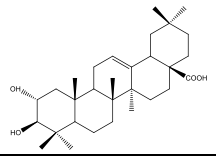
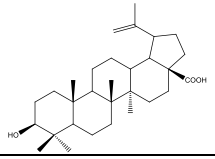
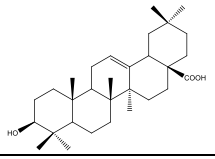
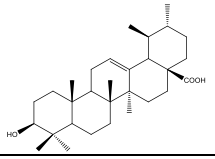
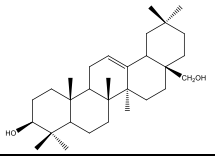
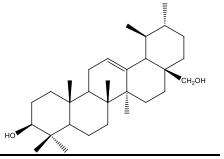
Compound	Maslinic acid	Betulinic acid	Oleanolic acid	Ursolic acid	Erythrodiol	Uvaol
Molecular formula	$C_{30}H_{48}O_4$	$C_{30}H_{48}O_3$	$C_{30}H_{48}O_3$	$C_{30}H_{48}O_3$	$C_{30}H_{50}O_2$	$C_{30}H_{50}O_2$
Molecular mass	472.36	456.36	456.36	456.36	442.38	442.38
Molecular structure						

Table 2. Analytical parameters of the developed method.

Compound	Detector	Calibration curve	r^2	LOD (μg/L)	LOQ (μg/L)	Linear range[a]	Accuracy					Matrix Effect Coefficient (%)[e]		
							Intra-day Repeatability[b]	*Inter-day* Repeatability[c]	Trueness[d]					
									Leaves	Skin	Pulp	Leaves	Skin	Pulp
Maslinic acid	DAD	$y = 4.776\,x + 0.704$	0.9970	93	311	80	7.1	8.2	101.2	111.9	110.2	-5.0	-14.6	-10.2
	ESI-IT	$y = 1.804{\cdot}10^{6}\,x + 1.322{\cdot}10^{5}$	0.9896	3	9	5	4.3	5.4						
	APCI-Q-TOF	$y = 131.926\,x - 114.337$	0.9961	65	217	20	9.7	9.8						
Betulinic acid	DAD	$y = 5.462\,x - 0.837$	0.9872	86	288	8	10.8	11.8	109.6	106.1	103.2	1.5	-5.2	-3.3
	ESI-IT	$y = 3.794{\cdot}10^{6}\,x - 1.568{\cdot}10^{5}$	0.9897	3	10	1.2	3.4	4.0						
	APCI-Q-TOF	$y = 226.982\,x + 12.453$	0.9927	41	138	1.2	2.2	4.3						
Oleanolic acid	DAD	$y = 6.292\,x + 0.006$	0.9976	83	277	80	5.2	6.8	98.6	97.2	93.5	-0.9	-2.0	-2.9
	ESI-IT	$y = 1.179{\cdot}10^{6}\,x + 4.521{\cdot}10^{5}$	0.9905	3	10	5	5.8	6.4						
	APCI-Q-TOF	$y = 450.214\,x - 283.256$	0.9941	22	73	20	9.7	10.0						
Ursolic acid	DAD	$y = 4.388\,x - 1.062$	0.9939	441	1471	10	8.3	8.6	106.7	104.7	96.9	-7.5	-2.3	1.3
	ESI-IT	$y = 7.212{\cdot}10^{5}\,x + 9.236{\cdot}10^{4}$	0.9950	9	29	5	6.3	7.7						
	APCI-Q-TOF	$y = 292.5318\,x - 217.414$	0.9911	28	94	20	10.5	10.9						
Erythrodiol	DAD	$y = 3.679\,x - 0.170$	0.9913	480	1600	10	9.4	9.5	98.9	105.0	108.5	-0.9	-4.9	-10.9
	ESI-IT	$y = 3.323{\cdot}10^{4}\,x + 2.317{\cdot}10^{3}$	0.9974	226	753	10	3.9	4.2						
	APCI-Q-TOF	$y = 11.761\,x + 3.230$	0.9971	273	911	6	10.5	11.0						
Uvaol	DAD	$y = 2.833\,x - 0.087$	0.9962	600	2000	10	2.6	4.5	101.5	103.1	101.3	-2.1	-0.9	-1.3
	ESI-IT	$y = 2.855{\cdot}10^{4}\,x + 5.915{\cdot}10^{3}$	0.9919	455	1515	10	5.5	6.8						
	APCI-Q-TOF	$y = 8.608\,x + 2.317$	0.9993	408	1359	6	9.7	9.9						

For MS detectors, negative polarity was used for triterpenic acids and positive, for alcohols.

[a] Linear ranges were established from LOQ to the indicated value (mg/L).

[b] RSD values (%) for peak areas of the analytes under study measured from 4 injections of the quality control mix carried out within the same sequence.

[c] RSD values (%) for peak areas of the analytes under study measured from 8 injections (belonging to 4 different sequences carried out over 4 days) of 8 different extracts from the same olive leaves sample.

[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 the difference between the results obtained. The values included on this table are those achieved for the intermediate concentration level.

[e] Matrix effect coefficient (%) = (1−(slope matrix/slope solvent))×100. Matrix effect coefficients are just given for APCI-QTOF MS detector, since similar behaviour was observed in the rest of the tested platforms.

Table 3. Comparison of the overall performance of the different platforms used within this study.

Platform	**Agilent 1260 LC system with DAD and -ESI-Ion Trap (Bruker Daltonic)**		**Waters Acquity UPLC coupled to APCI-Q TOF (Waters)**
	DAD	**ESI-IT**	
Purpose of using it	**Quantitative purposes**	**Quantitative & qualitative purposes**	
LOD (µg/L)	83-600	3-455	22-408
Linear dynamic range* (mg/L)	8 (for BA) 10 (for UA, ER, UV) 80 (for MA, OA)	1.2 (for BA) 5 (for MA, OA, UA) 10 (for ER, UV)	1.2 (for BA) 6 (for ER, UV) 20 (for MA, OA, UA)
RSD% (intra-day)**	2.6-10.8	3.4-6.3	2.2-10.5
Number of injections	1 injection needed (widest linear dynamic range)	2 injections needed to make sure that a proper quantification is achieved (within the linear range)	4 injections needed: 2 injections to make sure that the quantification is done within the linear range, and 2 (one in each polarity) to properly detect both triterpenic acids and alcohols.
Other remarkable characteristics	- More difficulties to identify the analytes under study (need of spiked samples)	- Used to obtain the fragmentation pattern of each analyte in MS^2 - Very fast switching polarities - Positive and negative polarities within the same run	- Used to understand the signal/behaviour of each analyte in APCI with accurate *m/z* signals and to quantify - No switching polarity within the same run
	If the complete platform is evaluated (LC-DAD/ESI-IT MS *on-line*): very advisable coupling, since it combines the benefits of both detectors		
Cost***	+++	++	+
Major achievements	-Optimization of a rapid (6 min) LC methodology (with proper analytical figures of merit) of application in food metabolomics -Quantitative results from 3 detectors in good agreement -Establishment of triterpenic acids and dialcohols levels for olive skin, pulp (not described before) and leaves		

*From LOQ to the indicated value (mg/L)

**It could be considered as the instrumental repeatability

***The more convenient, the higher number of "+"

Table 4. Quantitative results (mg analyte/kg dry sample) obtained for the three different *Olea europaea* tissues under study by using the LC developed method coupled to DAD, ESI-IT MS and APCI-QTOF MS.

Sample	Compound	APCI-QTOF MS	ESI-IT MS	DAD
Olive leaves	MA	3904 ± 340	3469 ± 169	3698 ± 268
	BA	123 ± 11	111 ± 5	130 ± 9
	OA	18629 ± 1624	18515 ± 902	18149 ± 1314
	UA	1869 ± 163	1880 ± 92	1760 ± 127
	ER	1491 ± 130	1638 ± 80	1677 ± 121
	UV	1204 ± 105	1303 ± 63	1257 ± 91
Olive skin	MA	88343 ± 7703	74202 ± 3616	76787 ± 5560
	BA	216 ± 19	205 ± 10	210 ± 15
	OA	27970 ± 2439	26755 ± 1304	25364 ± 1836
	UA	140 ± 12	137 ± 7	158 ± 11
	ER	723 ± 63	843 ± 41	779 ± 56
	UV	304 ± 26	297 ± 14	298 ± 22
Olive pulp	MA	67 ± 6	66 ± 3	63 ± 4
	BA	1 ± 0.1	1 ± 0.1	1 ± 0.1
	OA	57 ± 5	53 ± 3	54 ± 4
	UA	4 ± 0.4	4 ± 0.2	4 ± 0.3

Every result is the average of four independent (sample preparation and injection) determinations (n = 4). The results are given by the mean value ± RSD (%).

No statistical significant differences among three tested detector were found (95%; $p < 0.05$).