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Analytical Methods

Metabolic profiling approach to determine phenolic compounds of virgin olive oil by direct injection and liquid chromatography coupled to mass spectrometry

Lucía Olmo-García [a,1], Aadil Bajoub [a,1], Romina Paula Monasterio [b], Alberto Fernández-Gutiérrez [a], Alegría Carrasco-Pancorbo [a,*]

[a] Department of Analytical Chemistry, Faculty of Science, University of Granada, Ave. Fuentenueva s/n, 18071 Granada, Spain
[b] Instituto de Biología Agrícola de Mendoza (IBAM), CONICET, Alt. Brown 500, Chacras de Coria, Mendoza, Argentina

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ABSTRACT

A LC–MS method involving direct injection of extra-virgin olive oil (EVOO) – after a simple dilution – for determining its phenolic compounds has been developed. Optimization of the most appropriate solvent for sample dilution, selection of the optimum oil/solvent ratio, and establishment of column cleaning strategy and maximum number of injections were some of the most relevant steps. Then, the analytical parameters of the method were evaluated, establishing LOD (from 3.3 to 31.6 μg/L) and LOQ, precision (RSD values for inter-day repeatability were found between 3.49 and 6.12%), and trueness (within the range 89.9–102.3% for 1.0 mg/L) and checking possible matrix effect (which was no significant). Three kinds of calibration were used: external standard, standard addition and calibration in a phenols-free matrix, which was subsequently applied to quantify the phenolic compounds in 16 EVOOs (from 6 cultivars). A total of 21 compounds were determined without the need of using any extraction protocol.

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1. Introduction

Even though people have been eating olive oil for thousands of years, it is now more popular than ever. The number of scientific studies showing that olive oil can help to prevent and treat different kind of diseases (atherosclerosis, cancer, diabetes, obesity, pulmonary diseases, cognition disorders, etc.) is constantly growing (Martín-Peláez, Covas, Fitó, Kušar, & Pravst, 2013; Visioli & Bernardini, 2011) and the benefits of a diet rich in olive oil are, indeed, nowadays absolutely undeniable. These healthy properties can be explained considering olive oil's composition regarding its high level of monounsaturated fatty acids and the fact that it also contains multiple minor components (Carrasco-Pancorbo et al., 2005). Phenolic compounds are one of the most appreciated classes of non-glyceridic constituents of this matrix (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011; Frankel, 2010), what is an easily comprehensible fact since, besides their anti-oxidant, anti-inflammatory, anti-microbial activities (Martín-Peláez et al., 2013) and very promising nutraceutical uses (El Riachy et al., 2011), they contribute to the stability of virgin olive oil (VOO) against auto-oxidation and have an important role on its organoleptic properties (Bendini et al., 2007). These metabolites can also be considered as a very useful feature to characterize the typicality, geographical origin, genuineness and authenticity of VOOs (Monasterio, Fernandez, & Silva, 2013; Oliveras-López et al., 2007; Sánchez de Medina, Priego-Capote, & de Castro, 2015). Additionally, in 2011, the European Food Safety Authority stated the admissibility of specific health claim related to the levels of some VOO phenols (European Food Safety Authority (EFSA) Panel on Dietetic Products Nutrition and Allergies (NDA), 2011), fact which is going to have obvious commercial and labelling implications. One year later, it was published a Commission Regulation establishing a list of permitted health claims made on foods, claiming that olive oil polyphenols contribute to the protection of blood lipids from oxidative stress and giving the conditions of use of the claim (Commission Regulation (EU) No 432/2012 of 16 May 2012).

Due to the importance of this fraction, different analytical methods have been developed to characterize its complex and heterogeneous pattern, composed by phenyl alcohols, phenolic acids, flavonoids, lignans, secoiridoids, etc. (Bajoub, Carrasco-Pancorbo, Ouazzani, & Fernández-Gutiérrez, 2013; Bendini et al., 2007; Carrasco-Pancorbo et al., 2005; El Riachy et al., 2011). Since

* Corresponding author.
E-mail address: alegriac@ugr.es (A. Carrasco-Pancorbo).
[1] These authors contributed equally to this work.

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the occurrence of hydrophilic phenols in VOO was firstly observed more than about 55 years ago (Cantarelli, 1961), the analytical methods have considerably evolved (Bendini et al., 2007; Carrasco-Pancorbo et al., 2005; El Riachy et al., 2011). They significantly depend on the information that the analyst would like to achieve; therefore, when the comprehensive characterization of the phenolic fraction is pursued, it implies the appropriate sample preparation and the further instrumental analysis. As far as the first stage is concerned, two main techniques have been traditionally used for extraction: liquid–liquid extraction (LLE) (Montedoro, Servili, Baldioli, & Miniati, 1992; Solinas, 1987) and solid-phase extraction (SPE) (Alarcón Flores, Romero-González, Garrido Frenich, & Martínez Vidal, 2012; Hrncirik & Fritsche, 2004; Mateos et al., 2001); more recently, some other types of extraction procedures have been also applied, such as, for instance, dispersive liquid–liquid microextraction (Godoy-Caballero, Acedo-Valenzuela, & Galeano-Díaz, 2013), matrix solid-phase dispersion (Monasterio, Fontana, & Silva, 2014) and ultrasound-assisted emulsification–microextraction (Reboredo-Rodríguez et al., 2014).

With regard to the analysis itself, it is important to highlight that, so far, there is no internationally accepted regulation concerning the method for individual characterization of phenolic compounds (Karkoula, Skantzari, Melliou, & Magiatis, 2014; Tsimidou & Boskou, 2015). Analytical protocols applying nonspecific colorimetric assays (using Folin–Ciocalteu reagent) can be still found, but others which draw on more advanced chromatographic or electrophoretic techniques coupled to diverse detection systems (Alarcón Flores et al., 2012; Bajoub et al., 2016; Gilbert-López et al., 2014; Godoy-Caballero et al., 2013; Sánchez de Medina et al., 2015), electronic tongues (Apetrei & Apetrei, 2013), NMR (Christophoridou & Dais, 2009; Pérez-Trujillo, Gómez-Caravaca, Segura-Carretero, Fernández-Gutiérrez, & Parella, 2010), Near-infrared spectroscopy (Bellincontro et al., 2012), etc. can offer to the analyst a much more complete overview about the phenolic profile of an extra virgin olive oil (EVOO). Among all mentioned possibilities, LC–MS is likely the coupling most widely used both with low and high MS resolution-analyzers.

Within this context, very few papers have been published proposing the direct injection (DI) of VOO instead of applying an extraction system to separate the hydrophilic phenols from the apolar matrix of olive oil. The first report in this regard was a very interesting piece of work authored by Selvaggini et al. (2006) and the compounds under study (7 compounds: 2 simple phenols, 2 lignans and 3 secoiridoids) were determined by HPLC-DAD/fluorescence. Later on, three other papers showed the same strategy (i.e. DI of the oil after an appropriate dilution) in part of the experimental work that they included (Godoy-Caballero, Acedo-Valenzuela, Durán-Merás, & Galeano-Díaz, 2012; Godoy-Caballero, Galeano-Díaz, & Acedo-Valenzuela, 2012; Gómez-Caravaca, Carrasco-Pancorbo, Segura-Carretero, & Fernández-Gutiérrez, 2009). In these latter examples, CE was the analytical technique selected and it was coupled to UV-visible and fluorescence (Godoy-Caballero, Acedo-Valenzuela, et al., 2012; Godoy-Caballero et al., 2012), and MS detection (Gómez-Caravaca et al., 2009), respectively. Godoy-Caballero et al. (2012) determined some of the most abundant phenolic compounds (tyrosol (TY), hydroxytyrosol (HYTY) and some aglycon secoiridoid derivatives (the dialdehydic form of decarboxymethyl elenoic acid linked to hydroxytyrosol (DOA), an isomer of oleuropein aglycone (Ol Agl) and the dialdehydic form of decarboxymethyl elenoic acid linked to tyrosol (D-Lig Agl))) by DI of the olive oil dissolved in 1-propanol (1:1 v/v) and a nonaqueous CE method. Gómez-Caravaca et al. (2009) also developed a nonaqueous CE method coupled to TOF MS (trying the DI of the investigated matrix introducing a plug of olive oil directly into the capillary) and compared their results with those achieved by CZE in aqueous buffers.

The aim of this work was to develop a LC–MS method for the determination of as many phenolic compounds as possible (belonging to different chemical classes) without the need of carrying out an extraction protocol, but only a simple sample dilution. A complete validation of the method was done, paying particular attention to possible matrix effect. Afterwards, the method was applied to the analysis of 16 EVOO samples coming from different cultivars.

2. Materials and methods

2.1. Olive oil samples

A total of 16 monovarietal EVOO samples, from 6 different varieties were selected: VS 3 (2 samples), VS 5 (2 samples), Picholine Marocaine (3 samples), Dahbia (3 samples), Haouzia (3 samples), and Menara (3 samples). VS 3 and VS 5 are local genotypes obtained by clonal selection from Picholine Marocaine variety within the frame of a research project (RESERGEN, Olive Genetic Resources) funded by International Olive Council.

To obtain the EVOO samples, olive fruits sampling was performed over the season (2013/2014) on randomly selected trees, representing the above-mentioned 6 olive cultivars, all grown in the experimental olive grove of the Agro-pôle Olivier National School of Agriculture of Meknès, Morocco. Pest control, pruning, irrigation and fertilization practices were done following current olive orchards management practices. To avoid possible influence of the fruits ripening stage on the phenolic profiles of the studied oils, only samples picked at a ripening index within the range 3.0–3.5 were considered; range which is commonly advised for the production of high quality olive oils in Meknès region. Afterwards, oil was extracted using an Oliomio laboratory mill (Oliomio, Italy) simulating two-phase commercial oil-extraction system. The operating mode of this instrument has been described in detail by Bajoub, Carrasco-Pancorbo, Ajal, Ouazzani, and Fernández-Gutiérrez (2015).

To evaluate the physico-chemical quality of the obtained oils, regulated criteria (free fatty acids content (given as percentage of oleic acid), peroxide value (expressed as milliequivalents of active oxygen per kilogram of olive oil (meq O_2/kg)) and K_{232} and K_{270} extinction coefficients, calculated from absorption at 232 and 270 nm, respectively) were determined, in triplicate, for each studied oil sample by using the analytical methodologies described in the European Union Standard Methods Regulations 2568/91 and the subsequent amendments (European Commission Regulation (EEC), 1991). Obtained results allowed classifying all the studied oils within the "extra virgin" category.

2.2. Chemicals and reagents

All solvents were of analytical (for extraction) or LC–MS (for chromatographic analysis) grade purity. Methanol and *n*-hexane were used when the extraction procedure of the phenolic compounds of the olive oil samples was applied and they were provided by Panreac (Barcelona, Spain). Acetonitrile and acetic acid (supplied by Lab-Scan (Dublin, Ireland) and Panreac (Barcelona, Spain), respectively) were used for preparing the LC mobile phases. Doubly deionised water was produced in the laboratory using a Milli-Q-system (Millipore, Bedford, MA, USA). Tetrahydrofuran (THF), acetone (Acet), and 1-propanol (1-prop) were used to dissolve the EVOO samples before the injection into the LC system; THF and Acet were provided by Panreac (Barcelona, Spain), and 1-prop by Sigma-Aldrich (St. Louis, MO, USA).

Commercial standards of simple phenols (HYTY and TY), flavonoids (luteolin (Lut) and apigenin (Apig)) and phenolic acids

(*p*-coumaric acid (*p*-Cou) and ferulic acid (Fer)) were bought from Sigma-Aldrich (St. Louis, MO, USA). The lignan (+)-pinoresinol (Pin) was acquired from Arbo Nova (Turku, Finland), and the secoiridoid-glucoside Oleuropein (Ol) was purchased from Extrasynthese (Lyon, France).

In a first stage, a stock solution (500 mg/L of each standard) was prepared by dissolving the appropriate amount of the compounds in methanol. Afterwards, a series of working solutions of these analytes were freshly made by diluting the mixed standard solution with methanol (at appropriate ratios) to yield concentrations within the range 0.1–250 mg/L. 3,4-dihydroxyphenylacetic acid (DOPAC) was used as internal standard (IS) and was purchased from Sigma-Aldrich (St. Louis, MO, USA). All solutions were properly stored in dark flasks at −20 °C. After deciding that the EVOO dilution would be made in Acet, both the stock solutions and further serial dilutions were also prepared in this solvent in order to carry out a fair comparison of the response factor of the analytes in matrix and solvent.

2.3. Dilution of EVOO samples for direct injection into LC-MS

A portion of 1 g (±0.001) of olive oil weighed in a test tube with a screw cap was mixed with 5 mL of Acet (THF or 1-prop – in the preliminary studies). In the prefatory experiments, before choosing 1 g as optimum amount of oil, different proportions olive oil/solvent were also assayed: 0.5, 1.0, 1.5, 2.0, 2.5 and 3 g were mixed with 5 mL of solvent. All the samples (and stock solutions) were filtered through a Clarinert™ 0.22 μm nylon syringe filter from Agela Technologies (Wilmington, DE, USA) before injection into the instrument.

2.4. Phenolic compounds extraction

A LLE was also used (Bajoub, Hurtado-Fernández, Ajal, Ouazzani, Fernández-Gutiérrez, & Carrasco-Pancorbo, 2015). In short, 25 μL of the IS solution was added (although, in the end, correction with the IS area was not necessary) to 2 g (±0.001) of olive oil weighed in a test tube with a screw cap. A volume of 1 mL of *n*-hexane was added to the oil and the phenolic compounds were extracted by using 2 mL of a mixture of methanol and water (60:40, v/v); the mixture was vortexed for 2 min and centrifuged at 3500 rpm for 6 min (this step of methanol/water addition, vortex and centrifugation was repeated three times in total). The combined extracts were evaporated in a rotary evaporator (Büchi R-210) at 30 °C, and the obtained residue was dissolved in 1 mL of methanol of LC-MS grade. Before the injection into the LC-MS system, the extracts were filtered through 0.20 μm membrane (nylon) filter.

The extracts prepared in the described way were used to enrich or spike olive oil or sunflower oil, respectively, for the validation studies. The extracts were also used to compare the quantitative results achieved by analyzing some of them and the DI preparations.

2.5. LC–MS analysis

2.5.1. Apparatus and software

The analyses were carried out by reversed-phase LC coupled to MS. The LC system was an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector (DAD), which was coupled to a Bruker Daltonic Esquire 2000™ ion trap mass spectometer (Bruker Daltonik, Bremen, Germany) by an electrospray ionization (ESI) interface. Chromatographic data acquisition and examination of DAD signals was performed by using ChemStation B.04.03 software (Agilent Technologies). Bruker mass spectrometer was controlled using the software Esquire Control and the resulting files were treated with the software Data Analysis 4.0 (Bruker).

Additionally, for carrying out a proper characterization (qualitative) of the selected samples, another LC-ESI-Q TOF MS platform was used; therefore, an Acquity UPLC™ H–Class system coupled to a micrOTOF-Q IITM mass spectrometer (Bruker Daltonik) by means of an ESI source was also employed. The accurate mass data of the molecular ions were processed through the previously mentioned software DataAnalysis 4.0.

An exploratory analysis of the data was carried out through PCA, which was used to display a natural grouping tendency or outliers among EVOO samples. Data were analyzed by using The Unscrambler® v9.7 software (CAMO Software AS, Oslo, Norway).

2.5.2. Chromatographic and detection conditions

The phenolic compounds were analyzed, in triplicate, following previously reported LC–MS conditions (Bajoub, Carrasco-Pancorbo et al., 2015). For the analysis of the stock solutions, phenolic extracts and EVOO or sunflower oil diluted samples, a Zorbax C_{18} analytical column (4.6 × 150 mm, 1.8 μm particle size) protected by a guard cartridge of the same packing was used, operating at room temperature. Water with 0.5% acetic acid (Phase A) and Acetonitrile (B) were the mobile phases. The flow rate was 0.8 mL/min and 10 μL (of the extracts, standard mix or diluted oils) was the injection volume. The chromatographic separation was carried out applying the following gradient: 0–10 min, 5% B; 10–12 min, 30% B; 12–17 min, 38% B; 17–20 min, 50% B; 20–23 min, 95% B. Later on, the B content was diminished to the initial conditions (5%) in 2 min and the column was re-equilibrated over 2.5 min.

The ion trap mass analyzer worked in negative ion mode (even though several analyses were also carried out in positive mode). The MS detector was programmed to perform scans at ’50–800 *m*/*z* range and the capillary voltage was set at +3200 V. Drying gas temperature was set at 300 °C, drying gas flow at 9 L/min, and nebulizing gas pressure at 30 psi.

A standard mixture solution with a concentration of 1 mg/L and one EVOO sample (from VS 5 *cv.*) were used as quality control (QC) samples in order to check the stability of the system over the different sequences carried out. The described QC samples were injected (after a blank) every ten analyses in each sequence.

The described MS parameters were transferred to the ESI-Q TOF MS spectrometer. In the high resolution MS system, sodium formate clusters were used for the internal calibration. A solution containing 5 mM sodium hydroxide and 0.2% formic acid in water/isopropanol (1:1, v/v) was injected at the beginning of the run (using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA)) and all the spectra were calibrated before carrying out the compound identification. For both mass spectrometer detectors, a flow divisor 1:4 was used; the flow reaching the MS systems was of about 0.2 mL/min. To achieve the identification of the phenolic compounds found in the analyzed samples, we used pure standards (when available), took into account retention time data, and compared the ESI-TOF MS and ESI-IT MS spectra (and MS/MS spectra) with previously published results (Bajoub Carrasco-Pancorbo et al., 2015; Bajoub, Hurtado-Fernández et al., 2015).

2.5.3. Method validation

Three different kinds of calibration were used with the aim of evaluating possible matrix effects: external calibration, standard addition calibration and calibration in blank matrix.

Solutions containing pure standards of the phenolic analytes under study at 10 different concentration levels (in Acet) over the range of 0.1–50 mg/L were used in order to evaluate linearity and establish the calibration curves which could allow their quan-

tification in the samples. After the preliminary studies, the concentration range was constrained till 10 mg/L as maximum level, covering the range in which the different metabolites under study were actually found in the selected EVOO samples. External calibration curves were established for each compound by performing a linear regression by the least-squares method. Each point of the calibration graph corresponded to the mean value of three independent injections.

Besides, standard addition calibration was also applied to, at least, one EVOO sample of each variety. Eight concentration levels were tested (0.1–10 mg/L, which is equivalent to approximately 0.61–60.9 mg/kg).

The same concentration levels as those evaluated in standard addition calibration were appraised when the calibration was done in a phenols-free oily sample (sunflower oil, which was considered as a blank sample in terms of phenolic compounds).

A matrix effect coefficient was calculated for each compound relating the slope in sunflower matrix (BlankCal) and in solvent (ExtCal), and the slope in sunflower oil matrix and in olive oil (StdAd), respectively, adapting the following the equation (Kmellár et al., 2008):

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

Detection (LOD) and quantification (LOQ) limits for each phenolic compound were calculated using the signal to noise ratio (S/N) of pure standards at the lowest concentration level injected (for every analyte) and were measured by using both the external calibration and the calibration in blank matrix. LOD and LOQ were estimated by calculating the concentration that produced a S/N equal to 3 and 10, respectively (ICH Harmonised Tripartite Guideline., 2005). The theoretical values so achieved, were corroborated injecting the pure standards (in Acet or sunflower diluted oil) at those concentrations.

The precision of the method was evaluated as well. *Intra-day* repeatability was expressed as the relative standard deviation (RSD) obtained for 5 injections of the QC, carried out within the same sequence. *Inter-day* repeatability was calculated as RSD of 12 injections (belonging to 3 different sequences carried out over 3 consecutive days) of the same olive oil sample (QC).

Trueness was estimated by analyzing spiked sunflower oil at different known concentrations (0.25, 1.0 and 5.0 mg/L) and calculating the effective/true concentration values.

3. Results and discussion

3.1. LC–MS analyses

3.1.1. Selection of the solvent used to dissolve the oil samples and optimum oil/solvent ratio

One of the most pivotal steps in the optimization of the methodology was the selection of the most appropriate solvent to dissolve the olive oil samples before the injection into the LC–MS system. Keeping in mind the previously published reports including information about miscibility of olive oil with different organic solvents, their viscosity and polarity index (Godoy-Caballero, Acedo-Valenzuela, et al., 2012; Godoy-Caballero et al., 2012; Gómez-Caravaca et al., 2009; Mendonça, Bica, Piatnicki, Simó-Alfonso, & Ramis-Ramos, 2005; Selvaggini et al., 2006), THF, Acet and 1-prop were selected. After the preliminary studies, we decided to prepare the samples as follows: 1 g of olive oil was dissolved adding 5 mL of the selected solvent (in the coming paragraphs the justification to this will be presented). Fig. 1 shows the peak intensity (in terms of area) of several phenolic compounds after dilution of the olive oil (VS 5-1) in Acet, THF, and 1-prop. THF and Acet gave very similar results for Pin, Lut and Ol Agl (isomer of 22.1 min); 1-prop and THF, however, produced similar peak areas for HYTY, desoxy elenolic acid (DesoxyEA), and Ol Agl (isomer of 21.5 min). No significant differences were observed for ligstroside aglycone (Lig Agl) (isomer of 23.6 min) regardless of the solvent used to dissolve the oil. In general, the dilution of the olive oil in Acet produced peaks with higher area values in almost all the cases. That was particularly evident for elenolic acid (EA), DOA and Ol Agl (isomer of 21.5 min). Therefore, Acet was chosen as the most appropriate solvent. MS signal intensity was one of variables considered to make the solvent selection, but we also took into account some other factors, such as: easiness to filter (by using a syringe filter) the dissolved sample, peak shape, and stability of the area values over consecutive injections.

As stated before, different sample concentrations were injected in order to select the most advisable olive oil dilution. After the preliminary studies the following combinations were thoroughly evaluated: 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g, respectively, plus 5 mL of Acet. The second option was picked as optimal considering the number of compounds which could be properly detected and trying to avoid more concentrated preparations which could produce the rapid soiling of the column. Fig. 2 endeavours to illustrate the potential of our methodology and shows an example of the Base Peak Chromatogram (BPC) of an EVOO VS 5 (1 g + 5 mL Acet) (Fig. 2A) and the Extracted Ion Chromatograms (EICs) of the phenolic compounds determined by using the DI approach (Fig. 2C). Moreover, the BPC of the same oil after carrying out a LLE – as described in Section 2.4. – is shown as well (Fig. 2B). The profiles shown in Fig. 2A and B are obviously very similar, just differing in terms of signal intensity (extracts should be about 12-fold more concentrated than the DI assay). However, using less concentrated injections was no detriment to the potential of the new method (see Fig. 2C); indeed, compounds belonging to different chemical classes were detected: simple phenols (HYTY, TY), flavonoids (Lut and Apig), lignans (Pin and Ac Pin) and secoiridoids or related compounds (DesoxyEA, EA, DOA, and different isomers of Ol Agl and Lig Agl). In the sample chosen as exemplification in Fig. 2C very little amounts of quinic acid, dialdehydic form of decarboxymethylated form of EA (DEA), *p*-Cou, Fer, syringaresinol, methyl DOA, D-Lig Agl, and methyl Ol Agl were found, that is the reason for not including the EICs of these analytes. This is the first time in which such number of phenolic compounds can be properly determined within a single injection of diluted olive oil samples, what represents a substantial improvement of the previously published reports in which similar DI strategies were applied. Anyway the most important aim of this work was to propose an alternative methodology for carrying out a reliable quantification of the most relevant phenolic compounds present in EVOO without the need of previous extraction.

At this point, it seems necessary to make a comment about the fact of detecting multiple isomers of Ol Agl and Lig Agl (as we will explain in the coming paragraphs, these isomers showed up in a very little proportion in comparison with the results after applying LLE with methanol-water mixtures). Karkoula and collaborators (Karkoula, Skantzari, Melliou, & Magiatis, 2012; Karkoula et al., 2014) published two interesting manuscripts about the artificial formation of some secoiridoid derivatives (mainly due to their reactivity with methanol (and water)), and since then, this topic is awakening a lot of interest. Our group has already discussed it in another publication (Bajoub, Ajal, Fernández-Gutiérrez, & Carrasco-Pancorbo, 2016), where we have corroborated Karkoula's findings, saying that as long as methanol (and probably water and/or their mixtures) is involved in the sample preparation (or has any interaction at any point of the analytical procedure with these compounds), the "artificial isomers" will show up. We have also studied that the generation of artificial peaks (related to DOA and D-Lig Agl) in the chromatograms is not as serious as for Ol

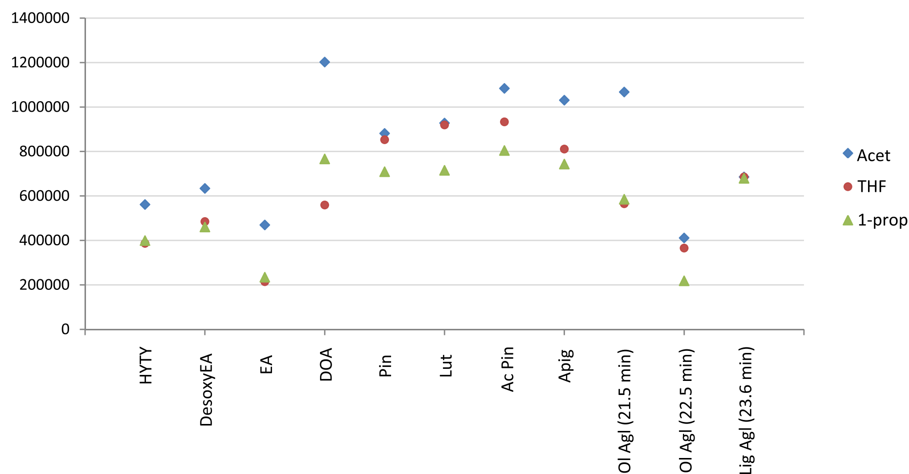

Fig. 1. Peak intensity of several phenolic compounds after dilution of the olive oil (VS 5-1) in Acet, THF, 1-prop. *Peaks nomenclature*: HYTY (hydroxytyrosol), DesoxyEA (desoxy elenolic acid), EA (elenolic acid), DOA (decarboxylated oleuropein aglycone), Pin (pinoresinol), Lut (luteolin), Ac Pin (acetoxy pinoresinol), Apig (apigenin), Ol Agl (21.4 min) (oleuropein aglycone isomer with a t_r of 21.5 min), Ol Agl (22.1 min) (oleuropein aglycone isomer with a t_r of 22.5 min), Lig Agl (23.6 min) (ligstroside aglycone isomer with a t_r of 23.6 min). Area values represented for each compound (and solvent) are the average of five independent determinations (RSD <6.1% in every case). Area values of HYTY, Pin, Lut, Ac Pin, Apig have been multiplied by a factor (*5, *10 or *20) in order to facilitate their representation within the same Y axis scale.

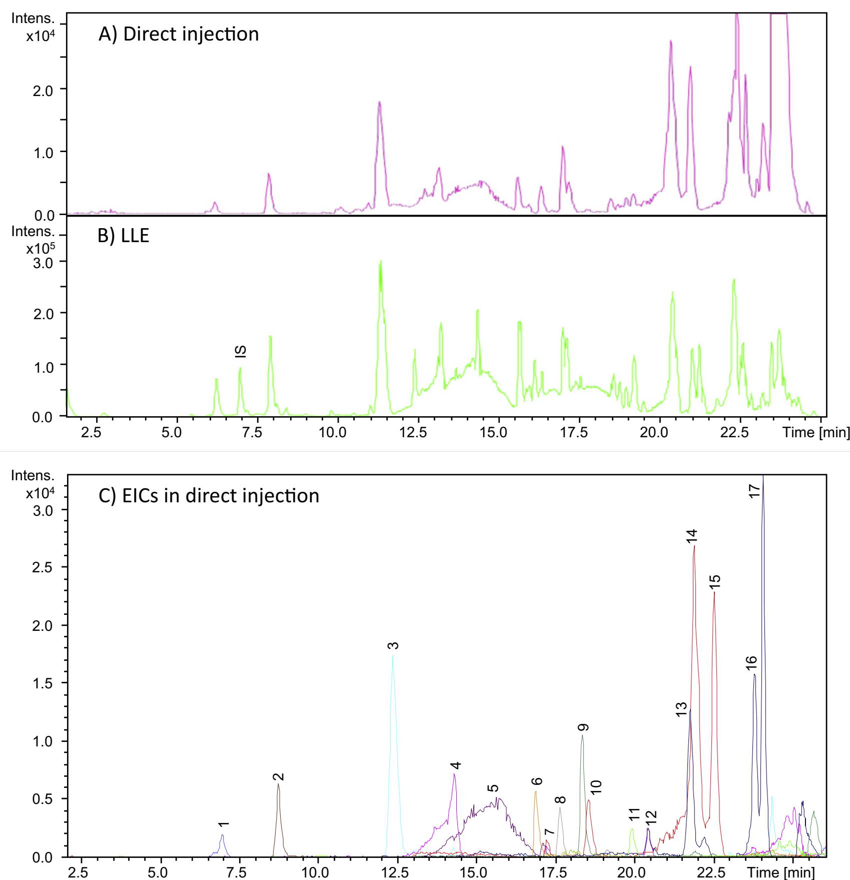

Fig. 2. (A) BPC of an EVOO sample from VS 5 variety (1 g + 5 mL Acet) using the DI procedure, (B), BPC of the extract of the same sample, and (C) EICs of the phenolic compounds determined by the described method using the DI approach. *Peaks numbers*: (1) HYTY, (2) TY, (3) DesoxyEA, (4) EA, (5) DOA, (6) Lut, (7) Ol Alg isomer with a t_r of 16.9 min, (8) Pin, (9) Ac Pin, (10) Ol Alg isomer with a t_r of 18.5 min, (11) Apig, (12) Lig Agl isomer with a t_r of 20.0 min, (13) Lig Agl isomer with a t_r of 21.6 min, (14) Ol Alg isomer with a t_r of 21.5 min, (15) Ol Alg isomer with a t_r of 22.5 min, (16) Lig Alg isomer with a t_r of 23.6 min, and (17) Lig Agl isomer with a t_r of 24.0 min. Peaks 14 and 16 can be considered as the most extensively determined isomers of Ol Agl and Lig Agl.

Agl and Lig Agl and could be even ignored (from a quantitative point of view) in the samples that we have worked with. Moreover, from our point of view (in good agreement with Karkoula's and some other research groups), ignoring the "artificial isomers" would mean underestimating their initial "native amount", since they are formed from the native secoiridoids present in the VOO sample. That is why we quantified several isomers of Ol Agl and Lig Agl. Fig. 1 (Supplementary material) shows that, in any case,

the formation of "artificial isomers" was drastically minimized with our DI method (since extraction step is avoided), what could represent another advantage of this new approach.

3.1.2. Optimizing the column cleaning and maximum number of injections

Injection of olive oil dissolved in different solvent has been previously tried for determining, for instance, tocopherols and triacylglycerols; however, as far as phenolic compounds are concerned, there is just one report where Selvaggini et al. (2006) proposed a HPLC-fluorescence method with direct injection of the olive oil (2 g dissolved in 10 mL Acet) into the column (two C18 columns with similar dimensions (250 mm × 4.6 mm, particle size 5 μm) were used, ChromSep Inertsil ODS-3 and Spherisorb ODS-1) and compared the results with those achieved after applying LLE and HPLC-DAD/Fluorescence. Performance of both methods was satisfactory in terms of repeatability (*intra*-day and *inter*-day repeatability (variation coefficient)) after injecting 6 times the same oil and repeating the same operations 2 days in a row. The outcome of this study was very promising, but some discrepancies were observed when DI data were compared with those obtained after the extraction. The authors attributed this to the fact that the extraction procedures produce a partial and selective recovery of VOO phenols (because of the different polarities, structures and molecular weights). In other reports using similar DI approaches, CE was chosen as analytical technique.

Herewith, the evolution of the column in a sequence after 1, 5, 15, 25, 50 and 75 consecutive injections (considering the area of the selected peaks, theoretical plates (N), S/N and retention time) was checked. To illustrate the gradual change in the column performance, Fig. 3 includes data for 5 different compounds (HYTY, DesoxyEA, Pin, Apig and the main isomer of Ol Agl (21.5 min)), which were selected to have, at least, one representative metabolite from the different chemical classes determined. The values shown in the different graphics for injections number 15, 25, 50 and 75, respectively, are the mean of the different parameters calculated from those injections, but averaged together with the results from the previous and subsequent chromatographic runs. In every case, a decrease in the value of all the evaluated parameters can be observed. So the retention times tended to shorten as more injections were made, even though the column pressure did not experience any increase over the sequences. All the compounds exhibited a diminution of area value over the time; the trend was very similar for HYTY, DesoxyEA, and Pin, being their areas after 15 injections about 96% (95.8–97.9%) of the initial value, and after 50 injections, about 93% (91.4–94.5%) of the starting value. After carrying out 75 analyses, HYTY, for instance, showed an area value of about 87% of the original one. The decrease was slightly more drastic for Apig and Ol Agl isomer, whose areas after 50 runs did not achieve the 86% approx. of the starting point (although values kept more stable than for the other analytes between 50 and 75 runs). The N value for the picked compounds was calculated as follows: $N = 5.54\ (t_r/w_{1/2})^2$, where both t_r and $w_{1/2}$ were expressed in minutes. The N tendency was compound-dependent; for instance, N values for Pin, Apig and Ol Agl remained very stable after 25 runs, and after 75 analyses, N were still 69.0, 77.8 and 56.0%, respectively, of the first value, showing very satisfactory values (40973.8, 52664.6 and 30450.2, respectively). N values for HYTY and DesoxyEA went down after 25 analyses (showing values representing 65% of the initial ones (4159.2 and 9312.7, respectively)), but after this, they stayed very stable. As far as S/N is concerned, for HYTY, after 25 analyses, the value represented 90% of the starting one, and after 75, it was 78.2%. Very similar behavior was observed for Pin and Apig. For Ol Agl the same was noted until injection number 50; after 75 runs, S/N decreased till 354.6 (which can be estimated as 60% of the first value). The derivative of EA (DesoxyEA) was the compound with a steadier S/N, after 75 runs, S/N value still accounted for 91.2% of the first registered S/N data.

The evolution of the column performance was very clear; however, the values of the tested parameters after 75 consecutive injections, from our point of view, were still reasonably adequate and satisfactory. We tried, anyway, to develop a cleaning method, which should be carried out after a certain number of analyses and would guarantee a very similar analytical performance to the one exhibited at the beginning of any sequence. After trying different sequential cleaning steps using different kind of solvents over diverse time periods, we decided to go for: acetonitrile (5 min at 0.8 mL/min), THF (5 min at 0.8 mL/min), acetonitrile (5 min at 0.8 mL/min), isopropanol (5 min at 0.4 mL/min) and acetonitrile (5 min at 0.8 mL/min). Afterwards, initial chromatographic conditions were selected and analysis of the olive oil dissolved in Acet resumed achieving comparable results to those obtained before trying any DI.

For routine analysis, we decided to include an injection of the standard mix of 1 mg/L in Acet and the QC every ten analyses (after a blank). The cleaning procedure was applied every day, after about 48 injections (even knowing that some more runs could be made without cleaning), in order to assure very satisfactory analytical overall performance. This decision was, in part, made considering the logistics of the global procedure; stopping after 48 injections meant cleaning once every day (both column and ESI interface) and, to a certain extent, increase the probability of avoiding any drastic contamination problem. Implementing the described column regeneration-procedure enabled to use the column for DI approaches or any other strategy, assuring the proper performance of the column and maximizing column lifetime (which resulted to be very similar to an identical column's lifetime just used to analyze extracts and not for the analysis of diluted olive oil samples). The application of the DI approach did not produce any contamination issue in the ESI-IT MS system, which was clean by using one of the standard MS cleaning protocols (it was not necessary a more exhaustive maintenance of the MS detector, since the DI samples were, indeed, 12-fold more diluted than the extracts).

3.1.3. Establishing the analytical parameters of the method

Table 1 shows the analytical parameters of the proposed method, including calibration curves and regression coefficients, LOD, LOQ, trueness, *intra/inter*-day repeatability, and matrix effect coefficients. As stated in Section 2.5.3, three different kinds of calibration were used with the aim of evaluating possible matrix effects: ExtCal, StdAd and BlankCal.

All the resulting calibration curves showed good linearity within the indicated concentration ranges, with r^2 higher than 0.9868. LOD and LOQ (μg/L) were estimated with the data from external calibration and the calibration in sunflower matrix. The values achieved by both estimations were similar, being found between 3.3 and 31.6 μg/L for Apig and Ol, respectively (data from blank matrix calibration). The linearity was first evaluated in a wider range (till 50 mg/L), but after the preliminary studies, we decided to limit the range, since fixing it at 10 mg/L the range in which the different analytes were actually found in the selected EVOO samples was covered. RSD values for *intra-day* repeatability (calculated from 5 injections of the QC (VS 5-1 olive oil) carried out within the same sequence) were found between 2.78 and 4.19% for Apig and Lut, respectively. The peak areas of the evaluated compounds measured from the injections of 12 independent dilutions of the same olive oil sample (an example of an VS 5 oil) analyzed in 3 different sequences (carried out over 3 days) were used to calculate RSD values for *inter-day* repeatability, finding results within the range between 3.49 and 6.12% for TY and Fer, respectively. With regard to trueness, Table 1 shows the similarity between

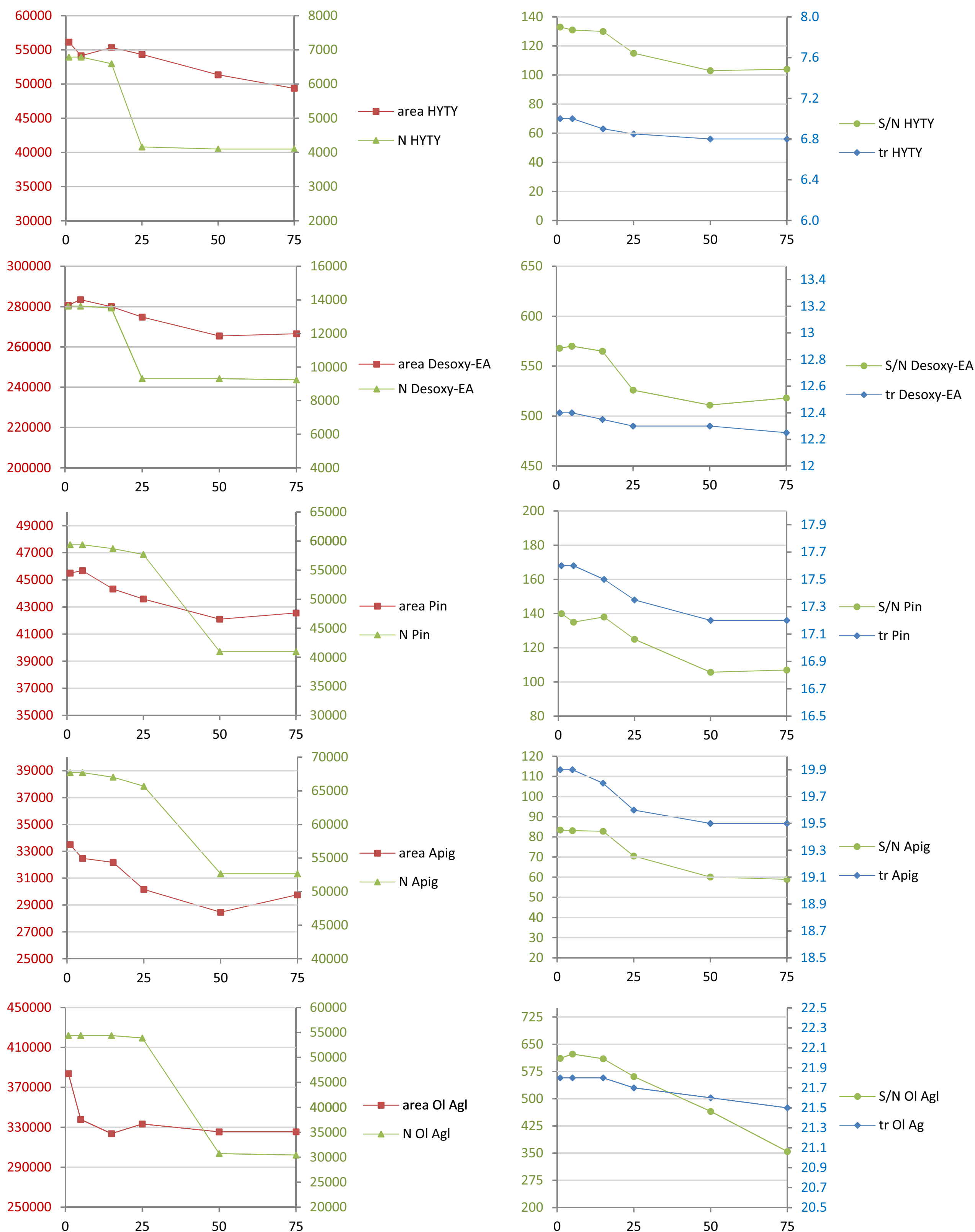

Fig. 3. Evolution of the column in a sequence after 1, 5, 15, 25, 50 and 75 consecutive injections considering the area of the selected peaks (HYTY, DesoxyEA, Pin, Apig and Ol Agl (main isomer with tr 21.5 min)), theoretical plates (N), S/N and retention time. Traces are coloured according to their scale in the Y axis.

the effective concentration values calculated after analyzing spiked sunflower oil (at different known concentrations (0.25, 1.0 and 5.0 mg/L)). A trueness value of 100% means a perfect matching between the determined concentration level (using the calibration curves built in blank matrix) and the theoretical one. For every compound, trueness was between 94.8 and 105.3% for 0.25 mg/L; within the range 89.9–102.3% for 1.0 mg/L; and fluctuating between 92.3 and 99.8% for 5 mg/L. In order to complete the results

Table 1
Analytical parameters of the developed method.

Comp.	Type of calibration	Calibration curve	r^2	LOD (µg/L)	LOQ (µg/L)	Evaluated range[a]	Accuracy			Matrix Effect Coefficient (%)[e]
							Intra-day Repeatability[b]	*Inter-day* Repeatability[c]	Trueness[d]	
HYTY	ExtCal BlankCal StdAd	y = 104657x + 6895.3 y = 104950x − 7974.2 y = 102410x + 26500	0.9984 0.9965 0.9938	7.1 12.5	23.7 41.7	10	4.12	4.65	98.6 (0.25 mg/L) 97.7 (1.0 mg/L) 94.4 (5.0 mg/L)	−0.28 (BlankCal/ExtCal) −2.48 (BlankCal/StdAd)
TY	ExtCal BlankCal StdAd	y = 34205x + 1452.5 y = 32926x + 8077.6 y = 32549x + 86236	0.9984 0.9989 0.9921	13.3 19.3	44.3 64.3	10	3.21	3.49	99.6 (0.25 mg/L) 98.7 (1.0 mg/L) 98.5 (5.0 mg/L)	3.74 (BlankCal/ExtCal) −1.16 (BlankCal/StdAd)
p-Cou	ExtCal BlankCal StdAd	y = 72960x + 9020.2 y = 69959x + 1927.1 y = 68811x − 2128.4	0.9964 0.9919 0.9977	9.9 10.2	33.0 34.0	10	3.65	5.34	100.3 (0.25 mg/L) 91.3 (1.0 mg/L) 95.8 (5.0 mg/L)	4.11 (BlankCal/ExtCal) −1.67 (BlankCal/StdAd)
Fer	ExtCal BlankCal StdAd	y = 58333x + 18496 y = 60080x + 19903 y = 59291x − 24545	0.9899 0.9954 0.9987	9.65 10.0	32.2 33.3	10	3.99	6.12	103.3 (0.25 mg/L) 97.3 (1.0 mg/L) 97.7 (5.0 mg/L)	−2.99 (BlankCal/ExtCal) −1.33 (BlankCal/StdAd)
Ol	ExtCal BlankCal StdAd	y = 10841x + 9294.6 y = 11127x + 169.34 y = 11472x − 773.97	0.9955 0.9977 0.9913	25.6 31.6	85.3 105.3	10	4.11	6.01	105.3 (0.25 mg/L) 102.3 (1.0 mg/L) 99.8 (5.0 mg/L)	−2.64 (BlankCal/ExtCal) 3.01 (BlankCal/StdAd)
Lut	ExtCal BlankCal StdAd	y = 171882x + 39155 y = 171715x − 8259.1 y = 178151x + 25709	0.9912 0.9966 0.9976	3.2 5.1	10.7 17.0	10	4.19	5.44	96.6 (0.25 mg/L) 89.9 (1.0 mg/L) 92.3 (5.0 mg/L)	0.10 (BlankCal/ExtCal) 3.61 (BlankCal/StdAd)
Pin	ExtCal BlankCal StdAd	y = 84404x + 8274 y = 82023x + 13010 y = 79688x + 41340	0.9987 0.9933 0.9868	8.2 8.8	27.3 29.3	10	3.55	5.83	93.6 (0.25 mg/L) 95.5 (1.0 mg/L) 99.5 (5.0 mg/L)	2.82 (BlankCal/ExtCal) −2.93 (BlankCal/StdAd)
Apig	ExtCal BlankCal StdAd	y = 243781x + 98095 y = 255554x + 54055 y = 252160x − 27170	0.9909 0.9899 0.9898	2.9 3.3	9.7 11.0	10	2.78	4.89	94.8 (0.25 mg/L) 93.7 (1.0 mg/L) 93.3 (5.0 mg/L)	−4.83 (BlankCal/ExtCal) −1.34 (BlankCal/StdAd)

Standard addition calibration was carried out using, at least, one EVOO sample from each variety. Results for an example of VS 5 olive oil sample are shown within this table.
[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 5 injections of the quality control (olive oil VS 5-1) carried out within the same sequence.
[c] RSD values (%) for peak areas of the evaluated compounds measured from 12 injections (belonging to 3 different sequences which were carried out over 3 days) of 12 independent dilutions prepared from the same olive oil sample (VS 5-1).
[d] Trueness was estimated by analyzing spiked sunflower oil at different known concentrations (0.25, 1.0 and 5.0 mg/L) and calculating the effective/true concentration values.
[e] Matrix effect coefficient (%) = (1 − (slope matrix/slope solvent)) × 100 (or adapting the equation, as stated in Section 2.5.3).

and give an estimation about trueness regarding other phenolic compounds (not available as commercial pure standards), sunflower oil was spiked with extracts obtained after LLE at different concentration (1:5, 1:10 and 1:25, v/v diluted with Acet). The averaged areas of three independent injections of the diluted extracts in Acet and spiked sunflower oil (at equivalent concentrations) were compared for DOA, Ol Alg isomer (21.5 min), and Lig Alg isomer with a t_r of 23.6 min. In every case, trueness was found between 87.1 and 104.3% for dilution 1:5, fluctuated from 95.1 to 99.7% for dilution 1:10, and varied between 96.0 and 99.5% for the most diluted samples.

To corroborate that the response factor of each compound was equivalent in a neat solution (Acet), in sunflower oil and in EVOO matrices, different types of calibrations were carried out. Afterwards, the slopes of the obtained equations were compared by using the previously described approach in Section 2.5.3.

According to Kmellár et al. (2008) values from −20 to +20% mean no significant suppression or enhancement effect. Taking this criterion into account, we can claim that the matrix effect's significance was very low in this case and, therefore, the three different calibration approaches could be equally used. The matrix effect coefficients (comparing sunflower oil calibration's slope and the one of the external standard method) were found within the range from −4.83 to 4.11%. When the calculations were made relating the slope of the calibration curves in sunflower and in olive oils, the results were very satisfactory as well and the matrix effect coefficients fluctuated between −2.93 and 3.61%, for Pin and Lut, respectively. The standard addition calibration was carried out using, at least, one EVOO sample from each variety (corroborating the quantitative results which will be presented in the following section), however, to simplify Table 1 to the extent possible, we only show the results achieved from one VS 5 olive oil sample.

After confirming that the three tested calibration approaches were valid, the possibility of using standard addition calibration was dismissed for practical reasons, since it implies the need of carrying out a different calibration for each sample. The quantification was finally made using the calibration curves built in the phenols-free sunflower matrix.

3.2. Application of the method to analyze different EVOO samples

After evaluating the analytical parameters of the new method, we proceed to apply it for the analysis of EVOO samples coming from different varieties: 16 monovarietal EVOO samples from 6 different varieties were selected: VS 3 (2), VS 5 (2), Picholine Marocaine (3), Dahbia (3), Haouzia (3), and Menara (3 samples). Table 2 includes the quantitative results (mg analyte/kg olive oil sample) achieved; every result is the average of three independent (sample dilution and injection) determinations (n = 3) and they are given by the mean value ±SD. 21 compounds were determined: two simple phenols (HYTY and TY), two phenolic acids (*p*-Cou and Fer), two flavonoids (Lut and Apig), two lignans (Pin and Ac Pin), and 13 secoiridoids or related compounds (DEA, DesoxyEA, EA, DOA, D-Lig Agl, 4 isomers of Ol Agl and other 4 isomers of Lig Agl). We just include in the table those compounds which could be properly quantified in all the evaluated samples.

Before discussing the quantitative results in depth, it seems appropriate including Table 1 (Supplementary material), which

shows a comparison between the quantitative results obtained for two samples after analyzing their extracts and the DI preparations. Sample VS 5-1 and a mixture (of equivalent volumes) of the 16 samples evaluated within this work could represent two nice examples. VS 5-1 has been chosen, since this oil was the one used as quality control in our work and was also taken as example in different figures of this contribution (and in Table 1 (to show the results of some analytical parameters calculated during the validation study)). The mixture of all the selected samples was considered as a pertinent example too. By using this olive oil "global" mix, we could demonstrate if the results from DI and extracts injection are equivalent, and to a certain extent, guarantee the usefulness of our method to any kind of olive oil (of those analyzed in the current research). Bearing in mind what we explained in Section 3.1.1 we consider unsuitable trying to compare the quantitative results of Ol Agl, Lig Agl and their isomers; that is why they are not shown in Table 1 (Supplem.). For the rest, very similar results were achieved, confirming the reliability of the DI approach.

Even though quantitative results of Ol Agl, Lig Agl and their isomers have not been included in Table 1 (Supplem.), making a further comment about them could be worthy. When focused on Ol Agl- (21.5 min) and Lig Agl-principal isomers (23.6 min), the results for the "global olive oil mix" (DI) were of 29.01 and 67.25 mg/kg, respectively. When the results achieved from the extracts were processed, the final quantitative values were of 22.02 and 55.21 mg/kg, respectively, for the same isomers. From our point of view, it makes sense, since some other artificial isomers have been formed from the native ones in those extracts. A fair comparison cannot be made (and a deeper discussion regarding this point is beyond the scope of this manuscript) since it would require to cope with some issues. Indeed, comparing the amount of every isomer with its corresponding one (in DI and the extracts) is not doable, as the number of isomers, relative proportions and contribution to the total concentration levels are not the same in DI preparations and in the extracts (Fig. 1 (Supplementary material)) (and, in addition, they could probably differ from

Table 2
Quantitative results (mg analyte/kg olive oil) achieved by using the LC-ESI-IT MS developed method. Every result is the average of three independent (sample dilution and injection) determinations (n = 3). The results are given by the mean value ± standard deviation.

Compound	t_r	VS 3		VS 5		Picholine Marocaine		
		VS 3-1	VS 3-2	VS 5-1	VS 5-2	PM 1	PM 2	PM 3
HYTY	6.8	3.80 ± 0.19	3.47 ± 0.17	2.23 ± 0.11	10.89 ± 0.38	4.41 ± 0.15	1.69 ± 0.06	1.73 ± 0.06
TY	8.5	14.36 ± 0.72	16.77 ± 0.84	16.62 ± 0.83	21.97 ± 1.09	14.46 ± 0.72	11.90 ± 0.59	12.23 ± 0.61
DEA	10.5	0.19 ± 0.01	0.31 ± 0.02	0.46 ± 0.02	3.90 ± 0.19	1.77 ± 0.09	2.68 ± 0.13	2.75 ± 0.14
p-Cou	11.6	0.19 ± 0.01	0.20 ± 0.01	0.16 ± 0.02	0.15 ± 0.02	0.49 ± 0.02	0.48 ± 0.03	0.45 ± 0.02
DesoxyEA	12.1	6.71 ± 0.34	70.52 ± 2.53	76.57 ± 4.59	5.97 ± 0.36	42.48 ± 2.55	35.86 ± 2.15	34.24 ± 2.05
Fer	12.5	0.12 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.05 ± (<0.01)	0.31 ± 0.01	0.35 ± 0.02	0.33 ± 0.02
EA	14.0	44.58 ± 1.23	52.70 ± 3.63	57.51 ± 2.88	88.61 ± 3.81	20.45 ± 1.08	22.22 ± 1.33	24.31 ± 1.22
DOA	14.9	88.29 ± 5.11	37.87 ± 1.89	38.25 ± 1.91	62.41 ± 3.12	7.95 ± 0.39	7.25 ± 0.29	6.87 ± 0.27
Lut	16.5	5.86 ± 0.29	5.46 ± 0.27	2.62 ± 0.13	4.92 ± 0.24	1.55 ± 0.08	0.77 ± 0.05	0.69 ± 0.05
Ol Agl isom	16.9	0.93 ± 0.05	1.20 ± 0.06	1.83 ± 0.11	1.23 ± 0.07	1.03 ± 0.06	1.51 ± 0.09	1.47 ± 0.09
Pin	17.3	3.65 ± 0.18	4.82 ± 0.24	4.76 ± 0.22	4.26 ± 0.21	1.62 ± 0.08	1.28 ± 0.05	1.35 ± 0.06
Ac Pin	18.0	8.42 ± 0.42	9.95 ± 0.50	10.49 ± 0.42	9.14 ± 0.36	6.13 ± 0.27	6.56 ± 0.28	6.25 ± 0.27
Ol Agl isom	18.5	13.56 ± 0.68	4.97 ± 0.25	5.29 ± 0.26	10.07 ± 0.50	19.20 ± 1.07	8.83 ± 0.53	8.99 ± 0.55
D-Lig Agl	19.0	122.22 ± 6.11	106.97 ± 5.34	21.90 ± 1.09	133.06 ± 8.11	3.01 ± 0.12	4.06 ± 0.20	4.01 ± 0.20
Apig	19.5	0.96 ± 0.05	0.35 ± 0.02	0.60 ± 0.03	0.88 ± 0.04	0.31 ± 0.01	0.28 ± 0.02	0.25 ± 0.01
Lig Agl isom	20.0	3.47 ± 0.17	3.77 ± 0.19	4.09 ± 0.18	3.51 ± 0.15	1.36 ± 0.06	3.69 ± 0.14	3.75 ± 0.15
Lig Agl isom	21.6	82.16 ± 4.12	40.75 ± 2.04	36.60 ± 1.43	80.54 ± 3.14	85.60 ± 3.34	70.23 ± 3.02	70.33 ± 3.02
Ol Agl*	21.5	36.22 ± 1.81	18.53 ± 0.93	32.11 ± 1.60	40.88 ± 2.57	31.23 ± 1.34	25.62 ± 0.84	28.32 ± 0.93
Ol Agl isom	22.5	27.03 ± 1.35	14.43 ± 0.92	17.58 ± 1.09	19.30 ± 1.19	36.33 ± 2.25	22.47 ± 1.05	21.01 ± 0.99
Lig Agl*	23.6	119.51 ± 5.98	80.04 ± 3.55	72.01 ± 4.10	124.01 ± 7.07	42.79 ± 2.01	51.15 ± 3.07	49.49 ± 2.97
Lig Agl isom	24.0	169.07 ± 8.45	91.34 ± 5.48	93.98 ± 5.64	153.62 ± 9.22	143.12 ± 8.57	119.34 ± 7.16	108.83 ± 6.53

Compound	t_r	Menara			Haouzia			Dahbia		
		Men 1	Men 2	Men 3	HZ 1	HZ 2	HZ 3	Dahbia 1	Dahbia 2	Dahbia 3
HYTY	6.8	17.36 ± 0.78	13.48 ± 0.60	4.49 ± 0.20	4.94 ± 0.22	6.69 ± 0.30	2.23 ± 0.01	0.09 ± (<0.01)	0.09 ± (<0.01)	0.08 ± (<0.01)
TY	8.5	18.68 ± 0.93	15.93 ± 0.57	16.22 ± 0.44	11.70 ± 0.42	14.05 ± 0.51	10.01 ± 0.32	2.17 ± 0.08	2.14 ± 0.08	2.03 ± 0.06
DEA	10.5	20.05 ± 0.80	17.53 ± 1.14	25.65 ± 0.75	1.32 ± 0.09	11.91 ± 0.77	1.01 ± 0.06	0.10 ± (<0.01)	0.11 ± 0.01	0.11 ± 0.01
p-Cou	11.6	0.45 ± 0.02	0.51 ± 0.02	0.25 ± 0.01	0.42 ± 0.02	0.39 ± 0.02	0.41 ± 0.01	1.47 ± 0.06	1.41 ± 0.04	1.39 ± 0.05
DesoxyEA	12.1	12.75 ± 0.77	11.57 ± 0.69	13.05 ± 0.71	19.75 ± 1.18	16.12 ± 0.64	15.99 ± 0.55	2.16 ± 0.09	1.67 ± 0.06	2.00 ± 0.05
Fer	12.5	0.31 ± 0.02	0.15 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.15 ± 0.02	0.19 ± 0.01	0.15 ± (<0.01)	0.14 ± 0.01	0.13 ± 0.01
EA	14.0	76.85 ± 4.61	74.26 ± 2.97	62.45 ± 3.61	61.99 ± 2.48	51.74 ± 2.07	43.37 ± 2.00	26.93 ± 1.08	32.26 ± 1.29	30.66 ± 1.09
DOA	14.9	18.70 ± 0.75	20.64 ± 0.62	6.33 ± 0.25	16.86 ± 0.51	14.09 ± 0.42	13.88 ± 0.54	11.63 ± 0.35	13.97 ± 0.42	12.32 ± 0.39
Lut	16.5	1.37 ± 0.08	1.39 ± 0.08	1.40 ± 0.07	2.29 ± 0.14	1.27 ± 0.08	1.11 ± 0.09	2.28 ± 0.14	2.38 ± 0.14	2.25 ± 0.20
Ol Agl isom	16.9	0.94 ± 0.06	0.92 ± 0.06	0.31 ± 0.01	2.58 ± 0.15	1.53 ± 0.07	1.33 ± 0.04	0.75 ± 0.03	0.49 ± 0.02	0.62 ± 0.02
Pin	17.3	1.68 ± 0.07	1.63 ± 0.08	1.58 ± 0.06	1.28 ± 0.06	1.63 ± 0.07	1.75 ± 0.06	9.55 ± 0.41	9.29 ± 0.40	8.96 ± 0.35
Ac Pin	18.0	5.25 ± 0.23	4.72 ± 0.20	4.65 ± 0.22	6.21 ± 0.27	5.95 ± 0.28	6.08 ± 0.25	4.96 ± 0.23	4.45 ± 0.21	4.39 ± 0.31
Ol Agl isom	18.5	7.89 ± 0.48	7.02 ± 0.43	1.54 ± 0.05	14.61 ± 0.89	10.34 ± 0.63	10.43 ± 0.64	1.75 ± 0.11	2.54 ± 0.16	2.10 ± 0.29
D-Lig Agl	19.0	34.30 ± 1.54	49.47 ± 2.08	11.66 ± 0.51	8.94 ± 0.38	6.18 ± 0.26	9.25 ± 0.40	53.55 ± 2.25	40.61 ± 3.19	49.77 ± 3.11
Apig	19.5	0.61 ± 0.03	0.63 ± 0.02	0.75 ± 0.04	0.38 ± 0.02	0.40 ± 0.02	0.41 ± 0.02	0.36 ± 0.02	0.37 ± (<0.01)	0.31 ± 0.01
Lig Agl isom	20.0	1.68 ± 0.06	0.43 ± 0.02	1.01 ± 0.03	1.15 ± 0.09	1.12 ± 0.04	1.23 ± 0.05	1.04 ± 0.04	1.28 ± 0.05	1.13 ± 0.10
Lig Agl isom	21.6	65.83 ± 2.83	67.77 ± 2.91	30.21 ± 1.75	79.25 ± 3.41	68.14 ± 3.95	66.77 ± 4.02	33.07 ± 1.92	35.68 ± 2.07	34.45 ± 2.54
Ol Agl*	21.5	38.11 ± 2.02	28.55 ± 1.51	12.33 ± 0.65	49.86 ± 2.64	40.85 ± 2.16	32.55 ± 1.99	17.57 ± 0.93	15.63 ± 0.82	16.76 ± 0.99
Ol Agl isom	22.5	21.02 ± 0.98	13.46 ± 0.63	5.38 ± 0.25	32.85 ± 1.54	26.12 ± 1.23	21.12 ± 1.01	11.09 ± 0.52	10.11 ± 0.47	10.65 ± 0.33
Lig Agl*	23.6	97.35 ± 5.84	81.86 ± 4.50	61.28 ± 3.67	63.13 ± 3.47	57.75 ± 3.12	58.00 ± 2.88	39.12 ± 2.11	36.36 ± 1.96	39.54 ± 1.89
Lig Agl isom	24.0	142.99 ± 7.29	133.90 ± 6.83	63.23 ± 4.01	128.97 ± 0.658	123.01 ± 7.50	131.32 ± 8.25	66.96 ± 4.08	72.74 ± 4.44	68.74 ± 3.55

HYTY, TY, Pin, Lut, Apig, *p*-Cou and Fer were quantified in terms of their commercial pure standards. Ac Pin was quantified in terms of Pin. As far as secoiridoids are concerned, Ol Agl-derivatives were quantified in terms of HYTY, and Lig Agl-derivatives and EA-derivatives were quantified in terms of TY.

* Ol Agl (21.5 min) and Lig Agl (23.6 min) appear with an asterisk since they can be considered as the most extensively determined isomers (or main peaks with *m*/*z* 377 and 361, respectively).

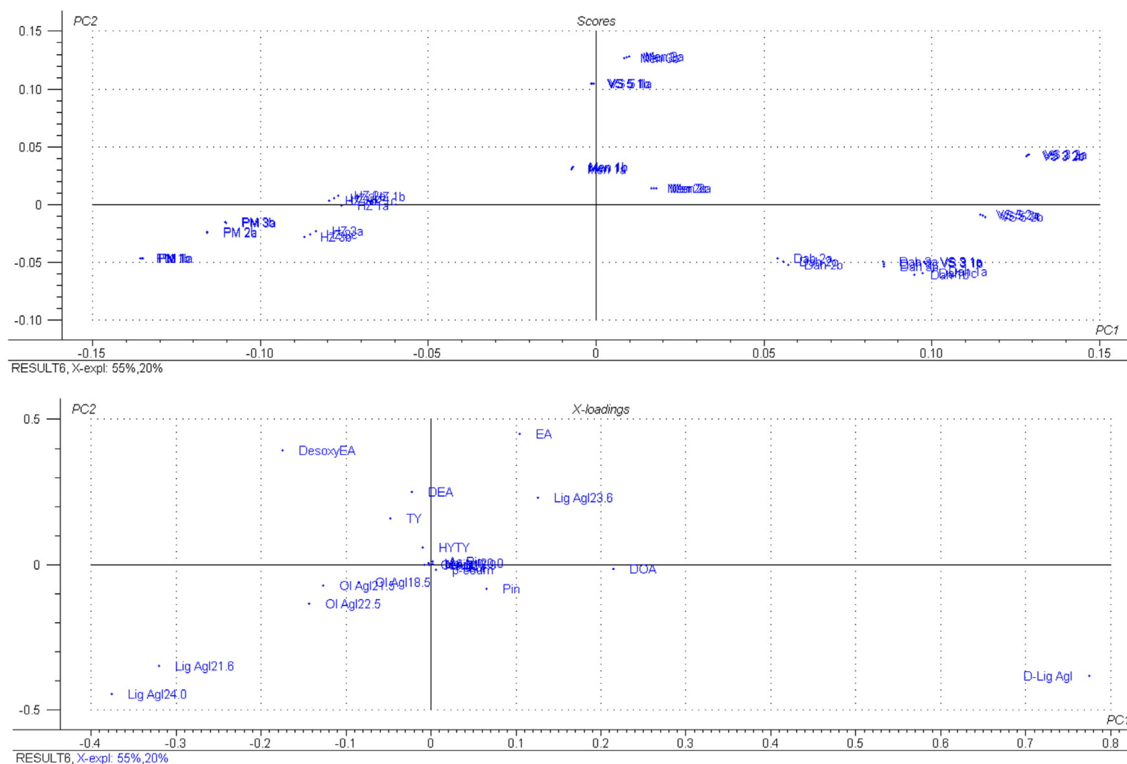

Fig. 4. Score and loading plots of PCA modelling of LC–MS data considering the individual concentration of each quantified phenolic compound. *Identification legends of varieties*: Dah: Dahbia; Hz: Haouzia; Men: Menara; PM: Picholine Marocaine. VS 3 and VS 5 are identified with the complete name of the variety. *Identification legends of compounds*: The abbreviations regarding the identity of the different compounds have been explained in other parts of the manuscript. In the case of secoiridoid isomers, the different analytes' names include the retention time.

one EVOO to another). Two alternative options can be listed (although any of them is completely satisfactory): 1) one possibility could be quantifying every isomer, making the proper calculations and expressing the result as a total amount of Ol Agl- or Lig Agl-related compounds (assuming equivalent response factor for each isomer, what is quite unlikely); and 2) another possibility could be working with the total area of all the Ol Agl- or Lig Agl-isomers and trying to give an overall estimation, which is no possible, considering the total areas size and the fact that there is no single MS calibration curve covering such a wide linear range).

Coming back to the DI results included in Table 2, it is possible to say that some evident differences were detected; we can mention, for instance, that Menara oils were the richest in terms of HYTY, DEA and EA (VS 5 oils also showed high levels of EA). VS 5 exhibited, in general, the highest levels of TY, Ac Pin, Apig and one of the isomers of Lig Agl (23.6 min). Picholine Marocaine presented high concentrations of DesoxyEA, Fer, the second and fourth isomers of Ol Agl (18.5 and 22.5 min, respectively), and one of the isomers of Lig Agl (21.6). In EVOO coming from VS 3 variety, levels of DOA, Lut, D-Lig Agl and the main isomer of Lig Agl were greater than in the oils from other cultivars. Haouzia oils had considerable concentrations of two Ol Agl-isomers (16.9 y 21.5 min (which can be considered as the main isomer of this metabolite), respectively). What can be highlighted from Dahbia oils is that the levels of *p*-Cou were remarkably higher that in the other samples (oscillating between 1.39 and 1.47 mg/kg, meanwhile the levels in the other oils did not exceed 0.51 mg/kg in any case). This variety was also peculiar regarding its lignans' pattern, since it was the only one presenting higher amounts of Pin (8.96–9.55 mg/kg) than Ac Pin (4.39–4.96 mg/kg).

Principal Component Analysis (PCA) was applied to evaluate the whole structure of the data set and highlight general trends in the phenolic profiles of the samples under evaluation. Fig. 4 shows the score and loading plots of PC1 vs. PC2 for the matrix composed by 21 variables (the number of phenolic compounds that were quantified in the EVOO samples) and 48 samples (16 EVOO analyzed in triplicate). The first two PCs explained 75.40% of total variance in raw data, whereas PC3 and PC4 accounted for 16.55 and 3.39%, respectively. All the possible combinations of PCs were studied; however, the figure only shows PC2 vs. PC1, since they were those which provided the best separation. Picholine Marocaine samples were properly separated from the rest, although were laying quite close within the graph to Haouzia oils, fact which can be partially explained considering their concentration in terms of Lig Agl (isomers at 21.6 and 24.0 min) and Ol Agl isomers (at 18.5, 21.5 and 22.5 min). The two samples from VS 5 cultivar were substantially separated from each other, and the same was observed for VS 3. Indeed, the replicates of one of the VS 5 sample (VS 5-1) were very close to Menara oils (since they showed similar levels of EA, simple phenols and Lig Agl isomer at 23.6 min); the replicates of VS 5-2 were in between VS 3 samples and relatively close to Dahbia oils. The latter can be related to its high level of D-Lig Agl.

Further experiments are needed to get a more comprehensive insight into the complete phenolic patter of these varieties and their main differences; that was logically not the aim of the current study. Our purpose was developing an accurate and reliable (but simple) LC–MS methodology which could be subsequently applied to analyze a higher number of samples. To the best of our knowledge, this is the first method which allows the determination of such a high number of compounds within a single run by using the direct injection approach.

4. Conclusions

The analysis of the phenolic fraction of EVOO (dissolved in acetone) without the need of carrying out any previous extraction step

has been done for the first time using LC coupled to ESI-MS. The possibility of implementing direct injection of olive oil into the LC (after a simple dilution) could be one of the greatest advantages of this method, since it could prevent partial and selective recovery of some phenolic compounds after the extraction, and their possible partial oxidation or the creation of artificial isomers during the sample preparation. The reliability of the quantification was demonstrated, as possible matrix effects were thoroughly evaluated and the method was fully validated; afterwards, it was applied for the analysis of 16 samples coming from 6 different varieties and the most remarkable differences were underlined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.03.139.

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