Food Chemistry 231 (2017) 374–385

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

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

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ARTICLE INFO

Article history:
Received 5 September 2016
Received in revised form 17 March 2017
Accepted 23 March 2017
Available online 24 March 2017

Keywords:
Phenolic compounds
Extra-virgin olive oil
Liquid chromatography–mass spectrometry
Direct injection
Matrix effect

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.

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

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http://dx.doi.org/10.1016/j.foodchem.2017.03.139
0308-8146/

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 emul sification–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