Talanta 132 (2015) 424–432

Contents lists available at ScienceDirect

Talanta

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

Characterization of monovarietal virgin olive oils by phenols profiling

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ARTICLE INFO

Article history:
Received 22 May 2014
Received in revised form
23 September 2014
Accepted 28 September 2014
Available online 7 October 2014

Keywords:
Monovarietal virgin olive oil
Phenolic compounds
LC–MS/MS
Secoiridoids
Data dependent methods
Precursor ion scanning
Neutral loss scanning

ABSTRACT

The phenolic profiles of seven monovarietal virgin olive oils (VOOs) – *viz.* Arbequina, Arbosana, Cornicabra, FS-17, Hojiblanca, Picual and Sikitita – were characterized by using a quantitative strategy based on LC–MS/MS and the selected reaction monitoring (SRM) mode. Data dependent methods, based on precursor ion scanning, product ion scanning and neutral loss scanning, were developed for confirmatory analysis of secoiridoid derivatives. The observed phenolic profiles were used to find correlation between pairs of phenols and similarity trends among the monovarietal VOOs. A Pearson analysis revealed several correlations among phenols with p-value < 0.01 and correlation coefficient (R) > 0.75 in the seven monovarietal VOOs. Cluster analysis showed two main clusters between VOOs, formed by Arbequina/Hojiblanca/Cornicabra/Picual and Sikitita/Arbosana/FS-17. High correlations ($R > 0.7$) were observed for the following pairs of VOOs: Arbequina/Hojiblanca ($R=0.77$), essentially supported on levels of hydroxytyrosol acetate (3,4-DHPEA-AC) and dialdehydic forms of secoiridoids; Cornicabra/FS-17 ($R=0.81$) and Picual/FS-17 ($R=0.79$), by correlation of flavonoids and secoiridoid derivatives in general. The highest correlation was observed for the pair Picual/Cornicabra ($R=0.99$). This preliminary study allowed setting similarities and dissimilarities between monovarietal VOOs by analysis of the phenolic profile. The observed connections between phenols for different varieties have been tentatively interpreted according to the main pathways for phenols biosynthesis.

1. Introduction

The minor fraction of virgin olive oil (VOO), formed by a chemically heterogenous group that includes more than 230 compounds, represents about 2% (w/w) of VOO. This fraction encompasses non-polar compounds such as phytosterols, waxes or hydrocarbons, and a polar or mid-polar sub-fraction [1] mostly constituted by volatile and phenolic compounds. Phenols present in VOO are also characterized by a chemical diversity of families such as phenolic alcohols and derivatives, phenolic acids, hydroxy-isochromans, flavonoids, lignans and secoiridoids. Among them, secoiridoids, the most concentrated phenols present in VOO, are specific compounds of *Oleaceae* plants [2]; in fact, secoiridoids differentiate VOO as unique among other vegetable oils.

Phenolic compounds have been widely studied because of their nutraceutical effects, relevant contribution to the sensory properties of VOO with special emphasis on bitterness and pungency, and their stabilizing role to ensure the long shelf-life of VOOs as compared to other vegetable oils [3]. The nutraceutical and stability properties of phenolic compounds have been linked to the capability of the phenolic structure for inhibiting oxidation processes. Numerous studies have evaluated the oxidation inhibition capability of phenols present in VOO, which allowed identifying hydroxytyrosol as the VOO phenol with the highest oxidation inhibition power by the DPPH test, followed by oleuropein aglycon (3,4-DHPEA-EA) and decarboxymethyloleuropein aglycon (3,4-DHPEA-EDA) [4]. The EFSA (European Food Safety Authority) report has emphasized the inhibition activity of hydroxytyrosol and secoiridoids against the oxidation of phospholipids and LDL cholesterol [5]. In fact, the antioxidant capability of hydroxytyrosol is responsible for its prevention role against tumoral diseases, cardiovascular diseases such as atherosclerosis [6], and diabetic neuropathies [7]. Secoiridoids also possess several health properties such as their implication in the inhibition of blood platelet aggregation, involvement in the synthesis of thromboxane in human cells and protection of erythrocytes from oxidative damage [8]. Additionally, anti-inflammatory activity of decarboxymethyl ligstroside

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http://dx.doi.org/10.1016/j.talanta.2014.09.039
0039-9140/

aglycon (*p*-HPEA-EDA [9], and recent evidences about its action on Alzheimer's disease [10] have been reported. Complementarily, Lozano-Sánchez et al. have developed a study on the influence of the phenolic composition of five monovarietal VOOs on the cytotoxic activity against human breast cancer cells [11]. These authors deduced that the sensitivity of breast cancer cells to phenolic extracts from VOOs was up to 12 times higher when the extracts were from VOOs with high content of secoiridoids as compared to other with low/nil content of these phenolic compounds.

One other particular family of phenols such as flavonoids has also been associated to health benefits related with cancer and coronary heart diseases [12–14].

Also organoleptical properties of VOOs have been correlated with the level of individual phenols [15–17]; thus, bitter intensity has been mainly related to hydroxytyrosol derivatives [18,19]; pungency character associated to two other secoiridoids such as 3,4-DHPEA-EDA and *p*-HPEA-EDA [20]; and also a negative correlation has been found between the vanillin content of VOO and their bitter sensory attributes [21].

Numerous studies have been published dealing with the quantitative and qualitative analysis of phenols in VOO. However, few of them have been focused on determination of phenols in monovarietal VOOs despite all the benefits – in terms of health, sensory properties and stability – associated to this family of compounds. In this sense, there is a demand for the quantitative characterization of phenols in monovarietal VOOs in order to evaluate the presence of the most significant compounds such as hydroxytyrosol, tyrosol, secoiridoids, phenolic acids and flavonoids. A deep characterization of the phenolic content of monovarietal VOOs could aid consumers to select the optimum VOO according to their preferences. The aim of this study was to characterize the phenolic composition of seven monovarietal VOOs by using a quantitative strategy based on LC–MS/MS analysis in selected reaction monitoring (SRM) mode. The observed phenolic profiles were then used to find statistical correlations between pairs of phenols and define tentative similarity trends among the monovarietal oils.

2. Materials and methods

2.1. Monovarietal virgin olive oil samples

The monovarietal VOO samples used in this work were obtained from olive fruits collected from 15th to 22nd November 2013 at intermediate ripening from different cultivars located in different areas of the South of Spain: Arbequina (Sevilla), Arbosana (Córdoba), Cornicabra (Toledo), Hojiblanca (Málaga), Picual (Jaén), Sikitita (Córdoba) and FS-17 (Córdoba). Oils samples were stored in darkness at −20 °C.

2.2. Reagents

The solvents used for analysis of phenols from VOOs were LC–MS grade methanol and *n*-hexane both from Scharlab (Barcelona, Spain). MS-grade formic acid as ionization agent in the chromatographic mobile phases was also from Scharlab. Deionized water (18 MΩ cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare the aqueous chromatographic phase.

Standards of target phenols (*viz.* hydroxytyrosol and tyrosol as simple phenols, verbascoside as phenyletanoid, oleuropein representing secoiridoids; luteolin, apigenin, apigenin-7-glucoside, luteolin-7-glucoside, diosmetin, quercetin and rutin as flavonoids; and vanillin, caffeic acid, *p*-coumaric acid, ferulic acid and vanillic acid as phenolic aldehydes and acids) for quantitative analysis of these metabolites in VOO were from Extrasynthese (Genay, France). Syringic acid, used as internal standard (IS), was from Extrasynthese (Genay, France). Individual stock standard solutions and multistandard solutions were prepared by dilution of each compound in methanol. The solutions were stored at −20 °C.

2.3. Apparatus and instruments

An MS2 minishaker from Ika (Wilmington, USA) was used to enhance the transfer of phenols from oil to a methanol–water solution for individual quantitation of the target compounds. Phenols extracts were analyzed by an Agilent 6410 triple quadrupole (QqQ) detector furnished with an electrospray ionization (ESI) source.

2.4. Extraction of phenols from VOO

Phenolic compounds were extracted from VOO by shaking 1 g of oil with 1 mL of hexane and 1 mL of 60:40 methanol–water mixture for 1 min in the MS2 minishaker. The hydroalcoholic phase was directly injected into the LC–QqQ. This approach has been widely validated in previous research and is accepted as sample preparation strategy for analysis of phenolic compounds in VOO [22–24].

2.5. LC–MS/MS analysis of phenols extracts

Analyses were performed by reversed-phase liquid chromatography followed by negative electrospray ionization (ESI) and tandem mass spectrometry (MS/MS) detection. 10 μL of extract was injected in triplicate into the LC system for chromatographic separation of the target compounds using a Mediterranea C18 analytical column (150 mm × 0.46 mm i.d., 3 μm particle size) from Teknokroma (Barcelona, Spain). The column compartment was kept at 30 °C. The mobile phases were: phase A (0.1% formic acid in water) and phase B (0.1% formic acid in methanol). The gradient program, at 0.8 mL min^{-1} constant flow rate, was as follows: initially 96% phase A and 4% phase B kept for 2 min; from 2 to 17 min, the mobile phase A was from 96 to 34%; from 17 to 35 min, mobile phase A was from 34 to 0% A, while B ranged from 66 to 100% B. This last composition was kept for 5 min. After each analysis, the column was equilibrated for 7 min up to the initial conditions.

The entire eluate was electrosprayed and ionized in negative mode and monitored by MS/MS in Selected Reaction Monitoring (SRM) mode by monitoring selective transitions from precursor to product ions. The flow rate and temperature of the drying gas (N_2) was 10 L min^{-1} and 300 °C, respectively. The nebulizer pressure was 50 psi and the capillary voltage 3000 V. The dwell time was set at 200 μs. Identification and quantitation of the phenols were performed using commercial standards, except for hydroxytyrosol acetate (3,4-DHPEA-AC) and secoiridoid derivatives, for which commercial standards were not available. For this reason, these compounds were relatively quantified as oleuropein equivalents.

2.6. Statistical analysis

After LC–MS/MS analysis, a data set was built with the concentrations of phenols found in each analytical sample. Normalization by logarithmic transformation was used as preprocessing step. The definitive data set was exported to Statgraphic software (Centurion XV.I) for statistical analysis by Pearson correlation analysis. Additionally, the data set was also exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA)