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Metabolomic approach for Extra virgin olive oil origin discrimination making use of ultra-high performance liquid chromatography – Quadrupole time-of-flight mass spectrometry

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ABSTRACT

The fraudulent miss-description on food product labels regarding origin or composition is a widespread problem. In this work, a metabolomic approach based on the use of ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) has been applied to identify the differentiating chemical markers that allow geographic origin discrimination between different Spanish Extra Virgin Olive Oils (EVOOs). For this purpose, ninety EVOOs from 6 Spanish regions were analyzed. Data processing consisted on peak picking, retention time alignment and response normalization. Partial Least Square Discriminant Analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA) were applied to identify the most significant markers that allow groups separation. Twelve different compounds were found to correctly separate the EVOOs from their origin and 7 of them could be tentatively identified. The results of our work suggest that UHPLC-QTOF MS-based metabolomic analysis is a suitable approach for biomarker-detection in the food quality/safety field.

1. Introduction

The traditional Mediterranean nutrition is widely known around the world as a very healthy diet. It was founded around the Mediterranean Sea, which gave it the name. In this entire geographical zone, olive crop has a widespread culture and, therefore, olive oil has a central position (Fazio & Ricciardiello, 2014; Vasto et al., 2014). The quality of olive oil is a key feature to ensure its healthy characteristics as well as its organoleptic qualities. Olive oil can be classified in four major quality groups: *Extra-Virgin Olive Oil (EVOO)*, *Virgin Olive Oil (VOO)*, *Olive Oil* and *Olive-pomace Oil* (European regulations EEC 2568/91 and EU 1348/2013). The quality of olive oils is highly correlated with several mechanical treatments in both harvesting and production processes (Angerosa et al., 2004), with consequences in the quality and final prize of the product in the market. For this reason, the importance of ensuring the quality of EVOOs has been reflected in several studies (Aparicio, Morales, Aparicio-Ruiz, Tena, & García-González, 2013).

On the one hand, the requirements for EVOO authenticity and differentiation from less quality Olive Oils are of essential concern for today's society and industry. In this sense, the adulteration of olive oils with other vegetal oils (Aparicio & Aparicio-Ruı;z, 2000) is a fraudulent activity that leads to lower-quality olive oil. A great effort has been made in order to evaluate and set their authenticity (Faria, Cunha, Paice, & Oliveira, 2010). Chromatographic techniques, both Gas Chromatography (GC) (Angerosa et al., 2004; Gamazo-Vázquez, Garcı;;a-Falcón, & Simal-Gándara, 2003) and Liquid Chromatography (Galeano Diaz, Durán Merás, Sánchez Casas, & Alexandre Franco, 2005), coupled to Mass Spectrometry (MS) have been the most employed. Different alternative techniques have been also evaluated, as Fourier Transformed Infra-Red (FTIR) (Maggio, Cerretani, Chiavaro, Kaufman, & Bendini, 2010), Nuclear Magnetic Resonance (NMR) (Dais & Hatzakis, 2013) or even Direct Analysis in Real Time (DART) (Vaclavik, Cajka, Hrbek, & Hajslova, 2009) coupled to MS.

Not only the quality but also geographical origin is important to ensure the value and organoleptic properties of this kind of premium foodstuff. Spain is the first olive oil producer around the world with more than 40% of total production, and counts with the rest of Europe (mainly Greece and Italy) approximately 70% of total

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worldwide production (http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures?lang=es_ES).
Geographical discrimination plays an important role in issue, where analytical chemistry provides advanced tools to ensure the origin of olive oils. Different studies have been developed with olive oils from Italy (Portarena, Gavrichkova, Lauteri, & Brugnoli, 2014), Greece (Longobardi et al., 2012), France (Cavalli, Fernandez, Lizzani-Cuvelier, & Loiseau, 2004) or Tunisia (Camin et al., 2016) in order to differentiate oils from different countries highlighting the relevance of these studies in actual research perspectives.

Moreover, designation of origin is also important for consumers (Erraach, Sayadi, Gómez, & Parra-López, 2014). For this reason, Protected Designations of Origin (PDO) Regulatory Council was created in order to ensure the right identification of different production zones of EVOOs (like Spain, Italy, Greece, France, etc). Regarding Spain, different studies have been found in the literature differentiating spanish olive oil varieties (Vergara-Barberán, Lerma-García, Herrero-Martínez, & Simó-Alfonso, 2015) as well as PDOs (Beltrán, Sánchez-Astudillo, Aparicio, & García-González, 2015; García-González, Luna, Morales, & Aparicio, 2009). However, to the best of our knowledge, a full-Spanish geographical characterization study has not been published in the literature yet.

In order to achieve this goal, different techniques have been raising up in the last decade, being metabolomics the-state-of-the-art in this field. This recent approach has been increasingly used over the last years, based on the discovery of unknown, statistically significant compounds in the matrix, which allow to differentiate between classes (Cevallos-cevallos, Etxeberria, Danyluk, & Rodrick, 2009). Metabolomics has been employed for quality food control, in matrices such as wine (Ali, Maltese, Toepfer, Choi, & Verpoorte, 2011) or oranges (Díaz, Pozo, Sancho, & Hernández, 2014).

Metabolomics appeared, firstly, as a nuclear magnetic resonance (NMR)-based technique. NMR was initially employed because of its universality and versatility as well as robustness; however, the low sensitivity and the high cost of this technique are important limitations, in addition to the higher sample quantity commonly required for the analysis. These drawbacks can be solved by using high resolution MS (HRMS) techniques coupled to both GC/LC, which appears nowadays as one of the most efficient approaches in the field of metabolomics. Different strategies have been employed, mainly GC and LC coupled to MS (Gallart-Ayala, Chéreau, Dervilly-Pinel, & Le Bizec, 2015), but multiplatform metabolomics approaches are also emerging, for example combining GC-LC-CE (Rojo et al., 2015).

GC-MS has been the most widely used technique for olive oil characterization to investigate volatile and semi volatile compounds. In most cases, isolation of the analytes by means of an extraction step has to be carried out before chromatographic determination using purge-trap systems or HS-SPME as well as by direct HS sampling and injection (Angerosa et al., 2004; Flath, Forrey, & Guadagni, 1973; Hu et al., 2014; Jiménez, Aguilera, Beltran, & Uceda, 2006; Pouliarekou et al., 2011) or even GCxGC applications (Peres et al., 2013; Purcaro, Cordero, Liberto, Bicchi, & Conte, 2014). LC is surely the best complement to directly analyze less volatile compounds in the matrix, as it requires less sample treatment and reduces compound losses, as it has been reported for essential oils (Do, Hadji-Minaglou, Antoniotti, & Fernandez, 2015).

The aim of this study was to discover and identify relevant biomarkers that allow classifying different Spanish EVOOs based on their geographical origin using Ultra-high Performance Liquid chromatography (UHPLC) coupled to Quadrupole Time-of-Flight Mass Spectrometry (QTOF MS), in combination with multivariate analysis (PLS-DA, OPLS-DA).

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade water was obtained by purifying demineralized water in a Mili-Q plus system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH), HPLC-supergradient acetonitrile (ACN), HPLC-grade 1-butanol (BuOH), HPLC-grade 2-propanol, sodium hydroxide (NaOH, >99%) and reagent-grade ammonium acetate (NH_4Ac) were obtained from Scharlab (Barcelona, Spain). Leucine-enkephalin and formic acid (HCOOH, 98–100%) were purchased from Sigma-Aldrich (Augsburg, Germany). Oleic acid (reagent-grade, 99%), palmitic acid (free acid Sigma-grade), linoleic acid (free, $\geq$ 99%), glyceryl trioleate (Sigma-grade, $\geq$ 99%), Diolein and 1-Monooleoyl-RAC-Glycerol were also acquired from Sigma-Aldrich.

2.2. Samples

One of the most important steps in metabolomics is sampling and sample characterization. Samples used for model construction should have a complete traceability in order to obtain significant and valid results through sample classes analyzed. For this reason, sampling process was designed in collaboration with InterCoop, the Valencian Community olive oil cooperative, which collects and distributes the highest part of Valencian produced olive oil. Furthermore, Spanish cooperatives are associated with the Spanish Agriculture Ministry in order to promote the high quality of Spanish EVOOs. This relationship was established through the "Patrimonio Comunal Olivarero" Foundation (http://www.pco.es/default.aspx) that promotes and distributes EVOOs from all the Spanish regions with the required traceability.

In this sense, 57 EVOOs from different cultivar zones of Spain (see Fig. S1) were acquired in Patrimonio Comunal Olivarero (Madrid, Spain): 5 from ***Bajo Aragón***, 10 from **Cataluña** (5 from *Tarragona* and 5 from *Girona)*, 5 from ***Toledo***, 8 from **Navarra-Rioja** (4 from *Navarra* and 4 from *La Rioja*) and 29 from ***Andalucía*** (4 from *Jaén*, 4 from *Sevilla*, 3 from *Granada*, 4 from *Sierra Segura*, 4 from *Sierra Mágina*, 3 from *Málaga*, 4 from *Almeria* and 3 from *Córdoba*). Additionally, 33 Extra Virgin Olive Oil from **Valencian Community (CV)** were provided by InterCoop (Castellón, Spain), including: 9 from *Maestrat*, 6 from *La Plana Alta i Alcalaten*, 7 from *Serra Espadà i Calderona*, 8 from *Serrania del Túria i la Ribera del Magro*, 1 from *Vinalopó* and 2 from *Utiel-Requena*. These samples were chosen to achieve a good representation of all the Spanish Olive cultivar zones regarding their geographical distribution.

Test samples were acquired in the same specialty store (Patrimonio Comunal Olivarero, Madrid, Spain) for model testing in a different season: 2 from *Bajo Aragón*, 2 from *Cataluña*, 2 from *Toledo*, 2 from Valencian Community, 1 from *Rioja*, 1 from *Navarra* and 5 from *Andalucía* (1 from *Granada*, 1 from *Córdoba*, 1 from *Málaga*, 1 from *Sevilla* and 1 from *Jaén*). In total, fifteen different samples were purchased, being representative samples of all Spanish EVOOs.

2.3. Sample treatment

Two different sample treatments were applied to the olive oils, which were stored at room temperature. For the polar components (polar fraction), a liquid-liquid extraction (LLE) was carried out mixing 1 mL of EVOO sample with 1 mL of methanol. 0.75 mL of the supernatant was taken and dried using a MiVac Duo concentrator.

The residue was reconstituted with 0.75 mL of H_2O:MeOH (1:1, v/v). After stirring, 70 μL of each sample (approximately 10% of the sample) were pooled to obtain a Quality Control (QC), which was