Pyrolysis-compound-specific hydrogen isotope analysis ($\delta^{2}H$ Py-CSIA) of Mediterranean olive oils

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ABSTRACT

The analysis of the hydrogen stable isotope composition ($\delta^{2}H$) of organic compounds provides information about its geographical origin. In this work, $\delta^{2}H$ composition of specific compounds released by direct analytical pyrolysis (Py-CSIA) of extra virgin olive oils EVOOs was studied avoiding the use of any chemical and/or physical treatments, derivatization or previous separation steps. A collection of EVOOs from Mediterranean countries (Portugal, Spain, France, Tunisia and Turkey) was used for authentication of the olive oil samples. The $\delta^{2}H$ value for 9 pyrolysis compounds present in all EVOOs, ranged between −112 and −267 mUr. These compounds were selected as possible surrogate descriptors linked to the olive oil geographic origin. Principal Component Analysis showed that $\delta^{2}H$ was highly correlated with geographical longitude and annual temperature. Multiple Linear Regression analysis revealed that $\delta^{2}H$ value of pyrolysis compounds can significantly ($P < 0.05$) predicts longitude, mean annual temperature and distance to the sea. The results suggest that the methodology used has a high potential to assess EVOOs geographic origin. This is the first report that evaluates $\delta^{2}H$ directly from the pyrolysis products of olive oil using Py-CSIA. The approach used represents an innovative, fast, reproducible and reliable authentication technique.

1. Introduction

Olive is one of the most rewarding cultivated species in the arid and semiarid regions of the world and in particular in the Euro-Mediterranean region. The interest in the olive and its products responds not only to agronomic or economic reasons, but also to recognition of extra virgin olive oil (EVOO) superior organoleptic characteristics (pleasant, rather delicate flavor and aroma) and their potential health benefits, namely the lower incidence of several pathologies, including cardiovascular diseases and some neurological disorders, associated with its consumption (Fernández & Moreno, 1999). Global demand and consumption of EVOO have increased significantly since the 1990s (Milli, 1999). Part of that demand has been reflected in the exigency of high-quality olive oils (EVOO) and the appearance in the market of olive oils elaborated with specific characteristics, including oils of certain regions with well-known specific characteristics (protected designation of origin), or with singular olive composition (monovarietal or coupages). Owing to the high market value of EVOO, its authenticity assessment is becoming increasingly relevant and generally motivated by an increase in benefits. Therefore, it is becoming mandatory to establish EVOO authenticity and detect possible adulterations, which allows the protection of i) the producers in view of certification (ensuring quality and authenticity) and ii) the consumer (fraud detection and product safety). The EVOO authenticity depends mainly on the provenance: producers, regulators or consumers. Nevertheless, it is consensual that adulteration, geographic origin, production system and variety are the main authenticity issues associated with the EVOO, being particularly relevant the geographic origin and variety if genuineness of olive oils is intended to be protected (Council Regulations (EC) 510/2006). The European Union (EU) Member States have increased legislation to protect the rights of consumers and producers, establishing a framework for the development of geographical indications through the “protected designation of origin” (PDO), the “protected geographical indication” (PGI) and the...
"traditional specialty guaranteed" (TSG) labels (ECC, 2006). The geographical characterization of EVOO is a current problem, due to the high influence that environmental conditions and cultivate practices have on olive oil quality (Aby-Reihad, Yasín, Sevillí & Montedoro, 2013). In addition, the geographic declaration of EVOO, is never controlled by physico-chemical parameters being vulnerable to fraud (Aparicio, Morales, Aparicio-Ruiz, Tenà, & García-González, 2013). Therefore, the development of appropriate analytical and statistical methods is necessary to guarantee authenticity and traceability of olive oils and to prevent illicit practices in the sector.

Techniques used to determine botanical and geographical origin of EVOO include more or less conventional chromatography (HPLC, GC/MS) and spectroscopy techniques (FT-IR, Raman) (Gómez-Caravaca, Maggio, & Cerretani, 2016; Martins et al., 2020).

Nowadays, the assessment of the stable isotope composition of light elements by isotope ratio mass spectroscopy (IRMS) is a helpful approach to differentiate production areas and to authenticate the origin of fruits and vegetables (Camin et al., 2017). Single-isotope or multi-isotope ratios analysis of most abundant light elements in food ($\delta^{13}C$, $\delta^2H$, and $\delta^{18}O$) can provide a fingerprint that reflects both geo-climatic characteristics of the production area, as well as information about local agricultural practices. Carbon isotope composition ($\delta^{13}C$) is informative of certain growth conditions of the crop like salinity, water stress and water use efficiency (WUE) (Hartman & Danin, 2010). On the other hand, the isotopic composition of H and O are informative about the water consumption and evapotranspiration of the growing plant, which in turn are very sensitive proxies for geographic location (Brooks, Barnard, Coulombe, & McDonnell, 2010).

A more accurate approach to the usual isotope assessment in bulk samples, is the analysis of $\delta^{13}C$ in discrete oil components using a compound-specific isotope analysis (CSIA) approach i.e. fatty acids (FAs), Spangenber (2016), sterols or aliphatic alcohols (Angerosa, Basti, & Vito, 1999), glycerol (Camin et al., 2010) or n-alkanes (Mihailova, Abbado, Kelly, & Pedentchouk, 2015). Nevertheless, to date, only one recent study has focused in hydrogen/deuterium ($\delta^2H$) isotope ratio composition of specific compounds (FAs) in olive oils (Paolini, Bontempo, & Camin, 2017). This compound specific hydrogen stable isotope analysis provides a more in-depth information on the geochimical characteristics and agricultural practices employed in the olive production of foodstuffs, improving geographical discrimination (traceability) (e.g. micro-climate, soil and water availability, sea influence) (Paolini et al., 2017; van Leeuwen, Prenzler, Ryan, & Camin, 2014).

Usually olive oil CSIA analysis involves a sample pretreatment, which consists of an extraction with organic solvents (harmful to the environment), purification and derivatization of specific compounds (Paolini et al., 2017). Recently, an analytical technique, which combines analytical pyrolysis and isotope ratio mass spectrometry (Py-GC/C/TC-IRMS, Py-CSIA), is being used to study foodstuffs and additives in food packaging (González-Pérez, Jiménez-Morillo, De la Rosa, Almendros & González-Vila, 2016; Llana-Ruíz et al., 2015, 2016). This is an innovative, fast, reproducible and robust method, used for the isotopic and chemical identification of organic compounds from complex matrices (De la Rosa, Jiménez-González, Jiménez-Morillo, Knicker, & Almendros, 2019; Miller et al., 2016). Furthermore, it avoids the extraction, purification and derivatization of non-volatile samples (González-Pérez et al., 2016; Miller et al., 2016). It is known that derivatization is a pretreatment commonly used for increasing the detection sensitivity of high polarity compounds (carboxyl and hydroxyl groups), such as FAs and alkyphenols, when conducting GC analysis (Igo, Honda, Lu, Kamiya, & Miyakoshi, 2015). Derivatization has also been used in combination with pyrolysis in the technique known as thermochemolysis (Challinor, 1991). In fact, in a previous study (Miller et al., 2016), we performed a derivatization step on speleothem samples from a volcanic cave in Easter Island for increasing the detection of FAs preserved in coralloid stalactites by thermally assisted hydrolysis-methylation (THM-Py-GC/MS). The authors were able to identify nine FAs derived from microorganisms and plant waxes in a speleothem layer. However, the use of a derivatization step in the Py-CSIA analysis will result in an addition of extra carbon and hydrogen to the specific compounds in olive oils, with an effect in their isotope signature, as described by Paolini et al. (2017) for conventional CSIA.

To our knowledge, less than 10 papers have been published so far on the use of Py-CSIA (e.g. González-Pérez et al., 2016; Llana-Ruíz-Cabelló et al., 2015; Miller et al., 2016), due to the cost, resources (supply of gases and reference standards, etc) and expertise needed to operate the equipment. Moreover, as the isotope data obtained is complex and not always with a straight forward interpretation, the use of multivariate statistical analyses have been recently applied in several studies dealing with EVOO characterization (Gómez-Caravaca et al., 2016; Kritioti, Menexes, & Drouza, 2018). Such statistical treatments were essential for the data interpretation and in resolving isotopic markers surrogated to varietal and/or geographical origin of EVOO. In these studies, it was successfully proved that the geographical and varietal origin of EVOO samples could be related not only to the isotope composition of light bio-element, but also to the isotope composition of specific chemical compounds. However, there are restrictions in applying these methods that require the treatment of abundant data (isotope composition, classes of compounds, etc.) that are dependent on other factors related to the raw material (olive cultivar, climate, ripening and fruit quality) as well as to technological parameters (fruit harvest time and conditions, storage, transport, oil extraction process, and its preservation) (Gómez-Caravaca et al., 2016). Therefore, the identification of objective analytical parameters capable of providing useful elements for oil characterization and specifically of its geographical origin, is a complex issue.

In this study a multivariate statistical chemometrics strategy is applied to evaluate the suitability of $\delta^2H$ Py-CSIA for the categorization of EVOO samples produced in the Mediterranean basin, according to their geographic origin.

2. Materials and methods

2.1. Samples and experimental design

A total of fifteen EVOO (Olea europeae L.) samples produced in 5 different geographical regions from the Mediterranean basin (Portugal (Alentejo and Trás-os-Montes, AL and TM, respectively), France, Tunisia, Turkey and Spain, Fig. 1 SM and Table 1) were studied. All samples were from the 2016 harvest. The samples were stored in dark-brown glass bottles at constant temperature of 20 °C until analysis. Each EVOO sample was geo-referenced, obtaining data of latitude (UTM), longitude (UTM), elevation (m a.s.l), distance to the sea (km), mean annual temperature (°C) and mean annual rainfall (mm) (Table 1).

2.2. Analytical pyrolysis (Py-GC/MS)

Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) was performed using a double-shot pyrolyzer (Frontier Laboratories, model 2020i) attached to a gas chromatograph (Agilent 6890N). EVOO sample (10 μL) were poured on ultra-clean glass wool discs and introduced in small pyrolysis capsules (Frontier Laboratory). The capsules were introduced into a pre-heated furnace at 400 °C for 1 min and the evolved gases directly injected into the GC/MS for analysis. The GC was equipped with an ultra-inert fused silica (5% phenyl-methylpolysiloxane) capillary column HP-5ms-UI (Agilent J&W) of 30 m × 250 μm × 0.25 μm film thickness. The oven temperature was held at 50 °C for 1 min and then increased to 100 °C at 30 °C min⁻¹, from 100 °C to 300 °C at 10 °C min⁻¹, and stabilized at 300 °C for 10 min. The carrier gas was helium at a controlled flow of 1 mL min⁻¹. The detector was a mass selective detector (Agilent 5973 MSD) and mass spectra were acquired with a 70 eV ionizing energy. Compound assignment was achieved via comparison with published and stored
(NIST and Wiley libraries) data. From the analysis of non-volatile samples, a total of 33 different compounds, without the use of derivatization, were identified, which were clustered in 11 groups: unsaturated fatty acid (UFA); unsaturated aliphatic hydrocarbon (UAH); saturated fatty acid (SFA); glycidyl ester of fatty acids (GEFA); fatty acid alkyl esters (FAAE); saturated aliphatic hydrocarbon (SAH); saturated fatty acid (UFA); unsaturated aliphatic hydrocarbon (UAH); monoesters (MEST); aldehydes (ALD); steranes (STR); polycyclic aromatic hydrocarbons (PAH); and isoprenoids (ISOP). Semi-quantitative analysis of the total ion chromatogram (TIC) was based on the peak area of each product released by pyrolysis, excluding minor compounds (area < 0.2% of total area). For each EVOO sample, the peak area of each identified organic compounds were calculated as percentages of the total chromatographic area.

### 2.3. Pyrolysis compound-specific deuterium isotope analysis (Py-CSIA)

The EVOO hydrogen isotope composition (δ^2H) of individual compounds were determined by direct Py-CSIA. This was performed using a double-shot pyrolyzer (Frontier Laboratory, model 3030D) attached to a Trace GC Ultra system (Thermo Scientific), which is connected to a GC-Isolink II system (Thermo Scientific). This GC interface is equipped with two micro-reactors, one for thermal conversion (TC) set at 1420 ºC, and another for combustion (C), set at 1020 ºC. The GC system is coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific) via a ConFlo IV universal interface (Thermo Scientific). Aliquots of each EVOO sample (20 µL) were poured on ultraclean glass wool discs and introduced into small pyrolysis capsules (Frontier Laboratory). The capsules, with the samples without derivatization, were introduced into a pre-heated furnace at 400 ºC, with an interface at 250 ºC, for 1 min and the evolved gases directly injected into the GC/IRMS for analysis.

Structural features of specific chromatographic compounds (peaks) were inferred by comparing and matching the mass spectra obtained by conventional Py-GC/MS with the Py-GC/TC-IRMS chromatograms obtained using the same chromatographic conditions (i.e., carrier flow, inlet temperature, column, etc.). For δ^2H measurement, each chromatographic compound passing through the pyrolysis micro-reactor, is gasified including the analyte of interest H2 gas. Pure H2 gas is mixed into the He carrier flow as pulses of reference gas. Before measuring the δ^2H/δ3H ratio, the [H3]^+ factor is verified to be < 10 ppm/nA before every run. The variation of the [H3]^+ factor during one run and between days is regularly checked to ensure a standard deviation lower than the unit for the whole measuring period.

The stable isotope abundances are reported in the delta (δ) notation (e.g. δ^2H) in variations relative to an international measurement standard. The isotope value is defined by Coplen (2011) (Eq. (1))

\[
\delta E_{\text{sample}} = \frac{R(E/E)_{\text{sample}}}{R(E/E)_{\text{sample}}} - 1
\]

Where R is the molar ratio of the heavy (E) to light (E) most abundant isotope of chemical element E (e.g. 2H/1H). The δ values are reported in “milli Urey” (mUr). The δ^2H values of EVOO samples were calibrated against saturated n-alkanes mixture (Fig. 2 SM) using the reference substance A7 (Biogeochemical Laboratories, Indiana University, U.S.A.). The linear correlation between standard and measured (IRMS) δ^2H values from the A7 mixture (Fig. 3 SM) was used to derive sample δ^2H values relative to the Vienna Standard Mean Ocean Water (VSMOW) scale (Schimmelman et al., 2016). The repeatability and reproducibility of the method were calculated by a crossed Gage R&R study. The standard deviation of repeatability and reproducibility of δ^2H were ± 0.08 and 0.23 mUr, respectively. Background subtractions, as well as isotopic hydrogen composition are calculated using the ISODAT 3.0 software (Thermo Scientific, Bremen, Germany).

### 2.4. Statistical analysis

Univariate (one-way analysis of variance, one-way ANOVA) and
multivariate (principal component analysis, PCA and multiple linear regression, MLR) data treatments were performed using Statgraphics Centurion XV software. The δ²H values of the 9 identified peaks from the EVOO samples—dependent variables—were: 8-heptadecene (peak 7), 3-octadecene (peak 10), n-hexadecanoic acid (peak 15), cis-9-octadecenoic acid (peak 19), 9-tricosene (peak 22), 1-tetracosene (peak 26), oleic acid, 3-hydroxypropyl ester (peak 27), 1,13-hexacosadiene (peak 30), squalene (peak 32). One-way ANOVA was used to assess group differences. PCA was used for simultaneous ordination of different geographic and climatic dependent variables and the δ²H values of identified pyrolysis compounds (independent variables), illustrating their mutual relationships. In addition, MLR was applied considering the identified pyrolysis compounds as independent variables and the geographic and climatic factors as dependent variables. Spurious models due to overfitting were detected and discarded after repeating MLR models with fully randomized dependent variables.

3. Results and discussion

3.1. Analytical pyrolysis (Py-GC/MS) of extra virgin olive oils

The results of the conventional analytical pyrolysis of EVOO was found well in line with what would be expected for olive oil (Boskou, Blekas, & Tsimidou, 2006) and is summarized in Fig. 1a. The peaks identification and respective compound family are reported in Table 2. The main families of compounds detected, which accounted for 91% of the total chromatogram area, were (Table 1 SM): unsaturated fatty

![Fig. 2. Plot of components weights for the geographical variables and the δ²H of the 9 identified pyrolysis compounds: 8-heptadecene (peak 7), 3-octadecene (peak 10), n-hexadecanoic acid (peak 15), cis-9-octadecenoic acid (peak 19), 9-tricosene (peak 22), 1-tetracosene (peak 26), oleic acid, 3-hydroxypropyl ester (peak 27), 1,13-hexacosadiene (peak 30), squalene (peak 32).](image)

![Fig. 3. Observed vs. predicted values for geographical variables: a) longitude, b) mean annual temperature and c) sea distance, calculated by MLR using δ²H of identified pyrolysis compounds as predictors.](image)

<table>
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<td>SAH</td>
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<tr>
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<td>11.02</td>
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<td>SAH</td>
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</tr>
<tr>
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<td>12.30</td>
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<td>UAH</td>
</tr>
<tr>
<td>7</td>
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<td>UAH</td>
</tr>
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<td>33</td>
<td>25.01</td>
<td>Stigmasteran-3,5-diene STR</td>
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a Polysaccharide derived (PS); saturated fatty acid (SFA); saturated aliphatic hydrocarbon (SAH); unsaturated aliphatic hydrocarbon (UAH); isoprenoids (ISOP); aldehydes (ALD); unsaturated fatty acid (UFA); fatty acid alkyl esters (FAAE); monoesters (MEST); glycidyl ester of fatty acids (GEFA) and steranes (STR).
acids (UFA), mainly oleic acid (cis-9-octadecenoic acid over 30%), unsaturated aliphatic hydrocarbons (UAH), incl. Squalene and saturated fatty acid (SFA), mainly palmitic acid (n-hexadecanoic acid 12.1 ± 3.8%). Among minor compounds, that accounted for c. 9% of total chromatogram, were polysaccharide derived compounds (PS), glycidyl ester of FAOs (GEPA incl. palmitate and mainly oleate); fatty acid alkyld esters (FAAE); steranes (STR mainly stigmasta-3,5-diene); aldehydes (ALD); saturated aliphatic hydrocarbon (SAH), isoprenoids (ISO, incl. phytol) and one monoester. These results were in agreement with the Codex Alimentarius Commission (REPI15/CAC, 2015). The dominant compounds detected in all chromatograms profiles were cis-9-octadecenoic acid (C18:1n) and n-hexadecanoic acid (C16:0) (UFA and SFA, respectively). Nevertheless, there were small differences among regions. It is known that fatty acid composition may differ among samples, depending on the zone of production (Boskou et al., 2006). For example, Spanish EVOO showed a high percentage of cis-9-octadecenoic acid (38.83%) but the lowest percentage of n-hexadecanoic acid (11.38%). This result was in line with the results reported in Tsimidou, Macrae, and Wilson (1987), who observed the same tendency for the studied EVOO produced in Spain, while the ones from Tunisia showed reverse results. Here, Tunisian EVOO did not follow that trend, showing the highest percentage of cis-9-octadecenoic acid. In particular, it is known that fatty acid content in vegetable oils differ due to several factors such as botanical origin (Ollivier, Artaud, Pinatel, Durbec, & Guerere, 2006), pedoclimatic conditions, oil characteristics, olive maturity (Gutiérrez, Jiménez, Ruiz & Albi, 1999), altitude, etc. (Karabagias et al., 2013). One example was the difference between the two regions in Portugal (TM and AL). The TM EVOO sample showed the lowest percentage of cis-9-octadecenoic acid (24.94%), while, its percentage of n-hexadecanoic acid was higher than that in the AL sample (14.98 and 12.94%, respectively). Cetinkaya Kulak, Ozkan, Celik and Sekeroglu (2017) indicate that there is a moderate positive correlation between altitude and the percentage of n-hexadecanoic acid. Therefore, the high percentage of n-hexadecanoic acid in TM Portugal region may be related to the higher altitude of production of the oil. However, the rest of EVOO samples did not show this trend, meaning that other factors may be involved. Concerning cis-9-octadecenoic acid content, the results showed that there is a gradual decline from high to low altitude. This result was in line with Ouni, Taamalli, Guerfel, Abdelly, Zarroul and Flamini (2012) findings. However, even though there were compositional differences between EVOO samples, these were in general small and the study of fatty acid composition obtained by Py-GC/MS was not able to give a clear differentiation among the geographic origin of the oils.

3.2. Pyrolysis-compound-specific hydrogen isotope analysis (δ2H Py-CSIA) of extra virgin olive oils

The pyrochromatograms of the compound-specific isotopic (δ2H) analysis (Py-CSIA) obtained could be matched with the Py-GC/MS chromatograms when using the same column type and chromatographic conditions (Fig. 1b and 4 SM). It was possible to assess δ2H composition for 9 major pyrolysis compounds that varied between −112 and −267 mUr (Table 3). Hydrogen isotope composition of plant lipids are known to be directly related to the water (meteonic) uptake by plant, as well as to evapotranspiration process (Sachse et al., 2012; and references therein). Therefore, geographic and climatic factors in the area of production are known to be closely linked with δ2H values (Clark & Fritz, 1997). In our case, Portugal EVOO samples (TM and AL) showed the lowest average δ2H value (−177 mUr), while Turkish EVOO sample displays the highest one (−148 mUr). The lowest average δ2H composition of Portugal EVOOs may be related to local geoclimatic conditions. It is known that there is a negative correlation between δ2H value of water uptake by plant and altitude, precipitation, temperature and oceanic distance (Fry, 2006). The geoclimatic factors that were correlated (positively and negatively) deuterium/hydrogen...
isotope composition of EVOO samples will be discussed in the next section (section 3.3). Concerning the conspicuous isotope difference found among the 9 major pyrolysis compounds, this may be attributed to the different biosynthetic pathways of compounds and the respective isotopic fractionation during synthesis. The average $^{2}$H composition of FAs was higher than that of $\alpha$-alkene/di-alkene (Alk) compounds (Table 3). Estep and Hoering (1980) found that fatty acid compounds are $^{2}$H-enriched compared with hydrocarbons (n-alkanes/α-alkenes). The same trend was observed here. The differences of hydrogen isotope between families may be due to changes in the isotopic composition of starting material (biosynthetic precursors) or isotope effects (including exchanges of organic H with $H_{2}O$) related to biosynthetic reactions (Martin, Zhang, Naulet, & Martin, 1986) or to the isotopic composition of hydrogen added (mainly NADPH) during biosynthesis (Luo, Sternberg, Suda, Kumazawa, & Mitsui, 1991), or both (Sessions, Burgoyne, Schimmelmann, & Hayes, 1986). The biosynthesis of alkane compounds is based on the reduction of long chain FAs to aldehydes, with the intervention of Acyl-CoA reductase enzyme and NADPH, and the subsequent aldehyde decarbonylation by aldehyde decarbonylase enzyme, producing carbon monoxide (Samuels, Kunst, & Jetter, 2008).

On the other hand, Sessions et al. (1999) observed in Dacus, there was a light trend toward $^{2}$H-enrichment with long-chain FAs. The same pattern was observed here for all of EVOO samples ($\Delta^{2}H_{\text{Oleic acid vs palmitic acid}} \approx 20 \text{ mU}\text{r}$). Concerning the stearane, its $\delta^{2}H$ value cannot be compared with that of the other compounds (FA and Alk) because its biosynthetic pathway is completely different. Sterane compounds are originated from the mevalonic acid, while the others are acetogenins and are based on the polymerization of acetyl CoA (Nes, 2011). Significant ($P = 0.0001$) differences in $\delta^{2}H$ values for specific compounds among EVOO samples were observed (Table 3). Therefore, specific compounds could be used as discriminant factors (independent variables) for different geographic origins of olive oil.

3.3. Statistical analysis

Traditionally, it has been proven that $n$-hexadecanoic and cis-9-octadecenoic acids (peaks 15 and 19, respectively) have good discriminant value to classify EVOO samples (D’Imperio, Dugo, Alfa & Mannina, 2007). Therefore, these together with the other identified compounds: 8-heptadecene (peak 7), 3-octadecene (peak 10), 9-tricosene (peak 22), 1-tetracosene (peak 26), oleic acid, 3-hydroxypropyl ester (peak 27), 1,13-hexacosadiene (peak 30), squalene (peak 32), were chosen here as possible markers surrogated to olive oil geographic origin.

Principal components analysis (PCA) was used to easily identify associations between the $\delta^{2}H$ value of specific compounds and geographical and climatic variables (Fig. 2). Up to 63% of the total variance can be explained with the two first components (component 1: 51.10% and component 2: 12.73%). The scatterplot of the loadings of PC-1 vs PC-2 showed that $\delta^{2}H$ values of the organic compounds were highly correlated with the geographic longitude of the production area, as well as its mean annual temperature. Nonetheless, these are also inversely correlated with elevation, latitude, mean annual precipitation and sea distance. Several researchers have observed the same trend between hydrogen isotope composition of plants and the environmental factors where they thrive. For example, Chiocchini, Portanera, Giolfi, Brugnoli and Lauteri (2016) found that high altitude and sea distance produced a $^{2}$H-depletion of organic samples. These correlations were corroborated using MLR. The MLR, exclusively using the $\delta^{2}H$ value of organic compounds as independent variables, has obtained significant ($P < 0.05$) forecasting models for longitude, mean annual temperature and sea distance. Plots of observed vs predicted values of each models: a) longitude, b) mean annual temperature, and c) sea distance, are depicted in Fig. 3. The model validation was confirmed by comparison with an alternative model computed from the fully randomized dependent variable, having not correlation ($P > 0.05$) with isotopic data (data not shown). The MLR analysis of $\delta^{2}H$ values of organic compounds released by analytical pyrolysis allowed to obtain the following equations of the prediction model:

**Longitude**

\[
\text{Longitude} = 311.86 + 1.15 \times \delta^{2}H_{8\text{-heptadecene}} - 0.95 \times \delta^{2}H_{13\text{-hexacosadiene}} - 1.10 \times \delta^{2}H_{\text{Palmitic acid}} + 1.60 \times \delta^{2}H_{\text{Oleic acid}} - 1.09 \times \delta^{2}H_{3\text{-hexacosadiene}} + 1.17 \times \delta^{2}H_{13\text{-hexacosadiene}} + 1.20 \times \delta^{2}H_{\text{Squalene}}
\]

(2)

**Mean annual temperature**

\[
\text{Mean annual temperature} = 192.48 - 0.25 \times \delta^{2}H_{8\text{-heptadecene}} + 0.14 \times \delta^{2}H_{13\text{-hexacosadiene}} - 0.41 \times \delta^{2}H_{\text{Palmitic acid}} + 0.82 \times \delta^{2}H_{\text{Oleic acid}} + 0.05 \times \delta^{2}H_{\text{1-tetracosene}} - 0.61 \times \delta^{2}H_{\text{Oleic acid}} + 3.41 \times \delta^{2}H_{\text{3-hydroxypropyl ester}} + 0.72 \times \delta^{2}H_{13\text{-hexacosadiene}} + 0.69 \times \delta^{2}H_{\text{Squalene}}
\]

(3)

**Sea distance**

\[
\text{Sea distance} = 76.18 + 1.72 \times \delta^{2}H_{8\text{-heptadecene}} - 2.31 \times \delta^{2}H_{13\text{-hexacosadiene}} + 7.57 \times \delta^{2}H_{\text{Palmitic acid}} - 3.85 \times \delta^{2}H_{\text{Oleic acid}} - 3.32 \times \delta^{2}H_{13\text{-hexacosadiene}}
\]

(4)

The existence of correlation ($P < 0.05$) between $\delta^{2}H$ values and geoclimatic variables (longitude, mean annual temperature and sea distance) may explain why Portuguese EVOO samples (TM and AL) are isotopically lighter than the other EVOOs.

4. Conclusions

To the best of our knowledge, this is the first report that records and evaluates hydrogen stable isotope composition directly from the pyrolysis products of olive oil using Py-CSIA (Py-GC-TC/IRMS). The results suggested that $\delta^{2}H$ Py-CSIA is powerful and have high potential to assess geographic origin of EVOOs. More work is underway for tuning and validation of the technique that include EVOO from many other locations from the Mediterranean Basin.

Declaration of competing interest

All authors have participated in this research, either by conception and design, or analysis and interpretation of the data; drafting the article or revising it critically for important intellectual content; and approval of the final version.

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript. Therefore, there is none declarations of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2019.107023.

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