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Combining analysis of fatty acid composition and $\delta^{13}C$ in extra-virgin olive oils as affected by harvest period and cultivar: Possible use in traceability studies

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ABSTRACT

The variability in carbon isotope composition ($\delta^{13}C$) of the main olive oil fatty acids, together with their relative contents, have been measured in 60 monovarietal olive oils, produced from plants grown in the same orchard and harvested at five ripening stages. The fatty acid content was mainly influenced by the cultivar, without significant changes during ripening. On the contrary, the fatty acid $\delta^{13}C$ value varied from October to December reflecting physiological responses to environmental variations. ANOVA-Simultaneous Component Analysis (ASCA) was applied to explore the sample variability among factors and interactions. In particular harvest date resulted the main factor affecting our multivariate dataset and OA $\delta^{13}C$ resulted the most discriminating parameter related to this factor. On the other side, LA relative content displayed the highest sensitivity to the range of cultivar variability, suggesting that this parameter may be used as a useful tool for studying inter-varietal differences in olive oils. The results find possible application in olive oil authentication and traceability studies.

1. Introduction

In the context of global market, the determination of the authenticity and traceability of food products is of great interest in the food industry, not only for consumers but also for producers and distributors. Extra virgin olive oil (EVOO) is a fundamental component of the Mediterranean diet and it has been extensively investigated because of frauds, including adulteration, false declaration of age and geographical origin (Aparicio, Morales, Aparicio-ruiz, Tena, & García-gonzález, 2013). EC Reg. 182/2009, followed by Implementing Regulation (EU) 29/2012 and subsequently Commission Implementing Regulation (EU) 1335/2013 defined the authenticity and quality of oil, also requiring the declaration of origin. This product is very rich in healthy mono-unsaturated fatty acids (Inglese et al., 2011) and antioxidative compounds such as polyphenols and carotenoids (Giuffrida, Salvo, Salvo, Cossignani, & Dugo, 2011) and the EVOO composition in terms of these nutraceutical compounds is the result of a complex combination of botanical, environmental and agronomical factors (Harwood & Rámon, 2000). Among these factors the cultivar can play a crucial role, conditioning the balance between mono- and polyunsaturated FAs and the bioactivity of minor components such as the antioxidant compounds, among which carotenoids (Portarena et al., 2019). For this reason, the production of monovarietal EVOOs from certain cultivars has increased over the last few years as a response to the demand for unique high-quality olive oils with exceptional and singular sensory attributes (Montealegre, Marina Alegre, & García-Ruiz, 2010). This could be an appropriate differentiation marketing strategy to facilitate the commercialisation of this product.

Current official EVOO quality control methods are based on the determination, by chromatographic techniques, of the concentration of specific compounds such as fatty acids (FAs), sterols, alcohols or stigmastadiene (Regulation 2568/91/EEC and amendments, in particular EU Reg. 299/2013), which should all fall within a specific range between maximum and minimum limits. However, these chemical indices are unable to supply complete information on the quality and authenticity of the product, especially its geographical and botanical origin

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and the fruit maturation at the harvest.

In recent years, stable isotope analyses have been demonstrated to held enormous potential as traceability markers of a vast variety of food products (Camin et al., 2017). This is due to the high precision of the method, the requirement for small samples, and the fact that the same technique can be used for almost any type of food or beverage and it is relatively cheap.

Different studies have been published on the isotopic composition of bulk EVOOs and its correlation with the geo-climatic characteristics of the production area, and it is nowadays widely accepted that stable isotopes can be a powerful tool for tracing the geographical origin of EVOOs (Camin et al., 2010, 2016; Chiocchini, Portarena, Ciolfi, Brugnoli, & Lauteri, 2016; Portarena, Baldacchini, & Brugnoli, 2017; Portarena, Gavrichkova, Lauteri, & Brugnoli, 2014).

In order to obtain additional information for authentication purposes, the isotope composition of olive oils can be measured not only in bulk samples but also in sub-components (Camin & Bontempo, 2017, pp. 257–272). EVOO is mainly composed of triglycerides (about 99%), with minor quantities of free FAs, glycerol, phosphatides and other compounds (León-Camacho, Morales, & Aparicio, 2013). Triglyceride molecule is an ester derived from glycerol and three FA molecules. The most abundant FA in olive oil triglycerides is oleic acid (OA, 55%–83% of olive oil), followed by palmitic (PA, 7.5%–20%), linoleic (LA, 3.5%–21%) and stearic acid (SA, 0.5%–5%). The EVOO composition in terms of FAs is mainly related to cultivar but it could also depend on ripening stage of the fruit, geo-climatic conditions of the production location, and several other factors (Portarena et al., 2015).

Combining the analysis of FA relative content and their carbon isotope composition ($\delta^{13}C$) may provide a more in-depth understanding on the fractionations related to secondary metabolism (van Leeuwen, Prenzler, Ryan, & Camin, 2014).

Despite the great potential of FA triglyceride isotopic analysis, few studies have dealt with the $\delta^{13}C$ composition of EVOO FAs. Two studies, in particular, focused mainly on the use of these parameters to investigate EVOO adulteration with cheaper or refined oils (Spangenberg, 2016; Spangenberg, Macko, & Hunziker, 1998; Woodbury, Evershed, & Barry Rossell, 1998). Other studies were aimed mainly at geographical characterisation of Slovenian (Spangenberg & Ogrinc, 2001), Italian (Faberi et al., 2014) and European and extra-european EVOOs (Bontempo et al., 2019; Royer, Gerard, Naulet, Lees, & Martin, 1999).

To our knowledge, no studies concerning $\delta^{13}C$ of FAs in EVOOs from different cultivars grown in the same orchard and harvested at different olive maturation stages have been published to date.

In this work, the $\delta^{13}C$ values of the main FAs, together with the respective relative concentrations, have been measured on 60 monovarietal EVOOs from four cultivars (Frantoio, Moraiolo, Leccino and San Felice) grown in the same orchard and harvested at five olive maturation stages. Collecting oils in a common orchard should minimize the effects of environmental variability, allowing potential genotypic patterns in the $\delta^{13}C$ of FAs to be expressed more clearly. At the same time, analysing oils at different ripening stages, allows to link FA $\delta^{13}C$ with the seasonal climatic gradient (increasing precipitation and decreasing temperature from September to December).

This work is a development of the research published by Portarena et al. (2015). Here, within the same experimental design and by combining $\delta^{13}C$ of bulk oil and FAs, together with FA relative composition, we investigated which parameters are the most affected by the harvest data and the cultivar. For this purpose, all variables firstly were statistically examined within a univariate approach, then, FA profile and their $\delta^{13}C$ values were combined and multivariate methods were applied. The application of ANOVA-Simultaneous Component Analysis (ASCA) to the overall dataset allowed disentangling all contributions to the variation by the factors and interaction.

The analysis revealed the potential of each parameter in describing the variability of samples as a function of design factors.

2. Materials and methods

2.1. Olive orchard, sampling and fruit oil content

The experimental design has been previously described by Portarena et al., 2015.

In details, the *Olea europea* trees selected for the sampling campaign are grown in the experimental olive orchard of the Department of Agricultural, Food and Environmental Sciences of the University of Perugia, located nearby Perugia town (43°04′54.58″N, 12°22′53.41″E). The collection is placed on a hill at 320 m a.s.l. The trees were 18 years old at the beginning of the trial, pruned according to the vase training system, and spaced at 5 × 5 m. The orchard is rain fed. Fertilization is based on the supply of nitrogen, potassium and phosphorous as chemical fertilizers. The soil is managed with a spontaneous green cover mowed 2–3 times/year.

Leccino, Frantoio, Moraiolo and San Felice cultivars were selected. The olives were randomly hand-picked from three trees per cultivar, at five specific dates, corresponding to different stages of the ripening process, resulting in total 60 different olive samples. Oils were obtained from about 3.0 Kg of olives per sample, by using a hammer mill, with 30 min of kneading and centrifugation, without extra water.

SM1 (see Supplementary material) shows the mean of maximum daily temperature over five days before the sampling date (Research unit for Climatology and Meteorology applied to Agriculture, CMA, 2012), and the cumulated amount of precipitations from 28th of August, determined using a pluviometer (Stocker, Bolzano, Italy) located in situ. The daily temperatures were used to calculate the degree heat units (DHU) using the following formula:

$DHU = \Sigma ((T_{min} + T_{max})/2) - 4$, where 4 indicates the lowest cardinal temperature of olive (Pannelli, Famiani, Servili, & Montedoro, 1990). In the year of the experiment (2012), from July to October, main period of oil formation and accumulation (Farinelli, Boco, & Tombesi, 2002), the average maximum temperature was 31.5 °C, the average minimum temperature was 15.1 °C, the cumulated degree heat units were 2085 DHU (2644 DHU from the beginning of the year) and the total rainfall was 308 mm.

The fruit oil content was determined, on two samples of 100 drupes/tree, by using an InfraAlyzer apparatus (SpectraAlyzerZeutec BRAN þ LUEBBE, Rendsburg, Germany) and expressed on both fresh and dry weight basis as reported by Portarena et al. (2015). The oil accumulation was calculated as: (gain in oil content/final oil content)*100, where gain in oil content was considered as oil content at the considered sampling date plus oil content at the previous sampling dates.

2.2. Fatty acids determinations

The FA composition was determined as the corresponding methyl esters by following the procedure described in the European Commission Regulation EEC no. 2568/91 (1991). The transmethylation procedure, the GC with flame ionization detector (GC-FID) (Trace 2000; Thermo-Fisher), and the analytical conditions have been previously described (Portarena et al., 2019). Relative amounts of given FAs were calculated from their respective chromatographic peak areas, and the relative percentage of each FA was related to the total peak areas of both saturated and unsaturated ones.

2.3. Fatty acids extraction for carbon isotope analysis

2.3.1. Chemicals and sample preparation

Acetonitrile and methanol HPLC grade, hexane and diethyl ether were purchased from Carlo Erba reagents (Milan Italy). Hydrochloric acid and potassium Hydroxide were purchased from Sigma-Aldrich (St Louis MO).

Samples were prepared following the method described by Guarrasi, Mangione, Sanfratello, Martorana, and Bulone (2010), with

modification. Briefly, aliquots of 60 mg of oil were hydrolysed with 10 mL of 0.3 M KOH at 100 °C for 1 h. After cooling, the dispersion was extracted by adding 10 mL of diethyl ether two times in order to remove the unsaponifiable fraction. The remaining dispersion was acidified with 1M HCl to pH 2.9, and then free FAs were extracted by adding 10 mL hexane three times. The hexane was removed under a gentle stream of N_2. Finally, the FAs were solubilized with 1 mL of methanol and filtered through 0.22 μm Whatman filters before HPLC injection.

2.3.2. HPLC system and conditions

The HPLC system was a SpectraSYSTEM P4000 gradient pump (Thermo Fisher Scientific) equipped with a Refractive Index detector, with 20 μL sample loop. A Phenomenex Luna C8 (100A 250 × 4, 6 mm, particle size 5um) column with precolumn was used. Different mixtures of acetonitrile-water and flow rates were tested on FA standards (PA, SA, OA, LA at ≥ 99% purity, purchased from Sigma -Aldrich) to optimize the separation conditions. The best resolution was obtained with isocratic mixture of acetonitrile and water in the ratio 86:14 at flow rate of 1 mL/min (a typical HPL chromatogram of FAs in olive oil is given in SM2 from Supplementary material). Experiments were carried out at 24 °C. For each chromatographic run, 600 μg of FA mixture was loaded, the peaks corresponding to PA, SA, OA and LA were manually collected in glass tube and the chromatographic solvent was completely removed under a stream of N_2.

2.3.3. Carbon isotope analysis

Aliquots of 20–70 μg of EVOO sample were taken from the glass tube, introduced in tin capsules and loaded in the autosampler of the elemental analyser (NA1500, Carlo Erba, Milan, Italy) connected to the isotope ratio mass spectrometer (Isoprime, GV, Cheadle, UK) for the analyses of $^{13}C/^{12}C$.

Isotopic compositions were scale normalized with IAEA international standards, on two contemporaneously analysed scale anchors, and are expressed as δ notation, determined according to the expression: $\delta = (R_s - R_{std})/R_{std}$, where R_s is the isotope ratio of the sample and R_{std} is the isotope ratio of the international standard. In particular, NBS-22 fuel oil ($\delta^{13}C = -30.03$) and IAEA-CH6 ($\delta^{13}C = -10.4$) sucrose were used for scale normalization of measured $\delta^{13}C$ values to the VPDB scale. The δ values are multiplied by 1000 and expressed as "per mil" (‰).

All samples were measured in duplicate and the mean values were considered. Possible changes in IRMS analytical conditions within a run were corrected using NBS-22 fuel oil and IAEA-CH6 sucrose (Portarena et al., 2017).

To monitor possible carbon fractionation effects during sample preparation and HPLC separation, the $\delta^{13}C$ of FA standards, were determined both with IRMS and after separation through HPLC of a mixture of the same FAs carried through the entire sample-processing procedure. The difference between HPLC-IRMS and IRMS measurements of 10 standard mixtures was at most 0.3‰.

The precision of measurements, expressed as standard deviation (SD) when measuring a mixture of standards and a sample 10 times, was ± 0.3‰.

2.4. Statistical analysis

For both molecular and isotopic composition of FAs, mean values and standard error were calculated over three plants per cultivar at each harvest date. Pearson coefficient (r), for a normal distribution, was computed between $\delta^{13}C$ values and climatic parameters, to check for the existence of a correlation between the selected variables. These values were compared with the critical value (F) for a confidence level of 95%. In the case of a significant correlation, the linear equation (consisting of slope and intercept) relating the $\delta^{13}C$ values and the studied climatic parameters was determined.

Analysis of covariance (ANCOVA) and Post Hoc Fisher multiple comparison test were used to determine whether there was significant difference among the slopes.

Factorial analysis of variance (FANOVA) and Post Hoc Fisher multiple comparison test were performed to test the influence of harvest data, cultivar (factors) and their interaction on all studied variable. Mathematically, FANOVA tests the difference in the centroid vector of means of the measured variables for the different levels of the studied factors. This allows FANOVA to compare groups obtained by varying factor levels, to reduce the number of measured variables if no differences between groups are observed or to identify the measured variables that cause the differentiation between groups (Checa, Bedia & Jamout, 2015).

To explore the variability among samples PCA was performed on all studied variables. PCA summarizes the information contained in the data matrix in a fewer independent principal components (PCs), obtained as linear combinations of the original variables, lying in the direction of maximum variance (Lavine & Jerome, 2005).

ANOVA simultaneous component analysis (ASCA method was applied to explore the variability of samples according to the studied factors (cultivar and harvest date). This analysis consists of two steps. First, the data matrix is splitted into effect matrices containing the level averages for each factor and interaction. This step resembles multiple ANOVA analysis. Then, simultaneous component analysis (SCA, a method similar to PCA for multiple matrices) is applied on different sub-matrices obtaining information about the distribution of the samples for each factor and the variable influence. Additionally, the assessment of the statistically significance of factors and interactions is checked by means of a permutation test (Smilde et al., 2005).

Basic summary, ANCOVA, FANOVA and PCA were carried out using Statistica, v. 8 (StatSoft Italia s.r.l, Padova, Italy), ASCA was implemented in r (MetStaT, v 1.0, 2013).

3. Results and discussion

3.1. Fatty acid profile

The relative content of oil samples in terms of the most abundant FAs has been characterized as a function of the cultivar and the harvest date (Fig. 1).

As expected, in all the samples, for any cultivars and any sampling dates, the OA was the most abundant FA, with relative content always higher than 74% of the total FA content. On the contrary, SA showed the lowest concentrations (≈2%). However, the four cultivars showed different FA compositions, reflecting intrinsic metabolic characteristics of each genotype (Farinelli & Tombesi, 2015). Especially, Leccino had the highest OA concentration and the lowest LA content. The cultivars San Felice and Moraiolo had relatively high LA and low OA concentrations, respectively. On the other hand, in the cultivar Frantoio the highest PA concentration was observed, while the contents of other studied FA showed intermediate values among those of cultivars Leccino and San Felice. All FA relative contents showed very limited variations during the entire sampling period. OA in cultivars Leccino, Frantoio and Moraiolo showed moderate increasing trends during ripening (from 78.1% to 79.3% in Leccino; from 76.5% to 77.7% in Frantoio and from 75.1% to 76.1% in Moraiolo), according to several studies from the Meditterranean basin (Anastasopoulos, Kalogeropoulos, Kaliora, Kountouri, & Andrikopoulos, 2011; Poiana & Mincione, 2004). LA in the cultivars Leccino and Frantoio moderately decreased during ripening (from 5.2% to 4.8% in Leccino and from 6.7% to 6.3% in Frantoio). PA and SA concentrations remained almost constant in all cultivars, as a function of the maturation stage.

3.2. Carbon isotope analyses of fatty acids

Fig. 2 shows the box and wisher plots of $\delta^{13}C$ values measured in individual FAs, i.e., PA, SA, OA, LA and bulk oil (as reported by

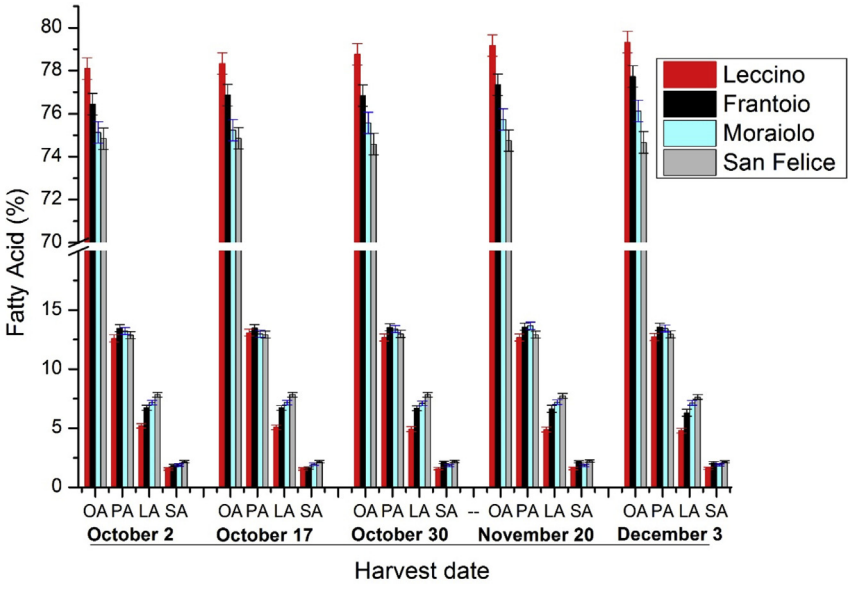

Fig. 1. Fatty acid composition of the oil samples, as a function of the cultivar and of the harvest date. Mean and standard deviation were calculated on three plants for each sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

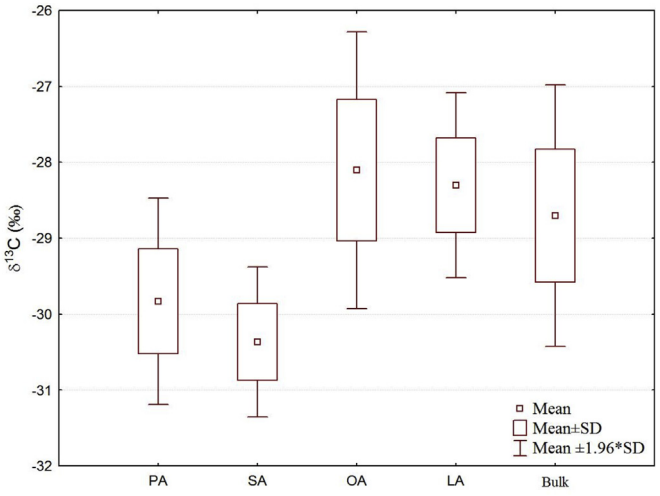

Fig. 2. Box and wisher plots of $\delta^{13}C$ determined in the four main FAs and in bulk oils from all 60 samples. The four FAs are listed in the biosynthetic order: PA, SA, OA and LA.

Portarena et al., 2015) across the 60 samples.

The $\delta^{13}C$ trends in FAs observed in Fig. 2 is in agreement with the results obtained when analysing vegetable oils reported in previous studies (Bontempo et al., 2019; Faberi et al., 2014; Royer et al., 1999; Spangenberg & Ogrinc, 2001). The observed variability in the $\delta^{13}C$ contents of FAs are expected as a result of known fractionation effects accompanying the different steps of the biosynthesis. A multienzyme complex catalyses the reaction sequences of chain elongation, through the addition of acetyl groups (e.g. from PA to SA) and desaturation of FAs (e.g. from SA to OA and LA) (Spangenberg & Ogrinc, 2001). From the above results it can be deduced that there is an initial isotopic fractionation occurring from PA to SA related to the first elongation step, with generally lower $\delta^{13}C$ values in SA. On the other hand, there may be a second phase of isotopic fractionation with the opposite effect, with a first desaturation step leading to an increase in $\delta^{13}C$ values in OA compared to SA. The second desaturation step leads to a slight decrease in the $\delta^{13}C$ values of LA. These results are in accordance with previous studies (Monson & Hayes, 1982a, 1982b). It has been reported that the observed fractionations may be also related to the different cell compartments in which these reactions occur (Gleixner, Danier, Werner, & Schmidt, 1993).

The distribution of $\delta^{13}C$ mean values shows that OA exhibits the highest variability (about 3‰), unlike SA which shows the lowest range of $\delta^{13}C$ variation (about 1.4‰).

Table 1 shows the mean values, together with standard deviations, of $\delta^{13}C$ of individual FAs and bulk oils for the four cultivars in each harvest date.

For each cultivar, $\delta^{13}C$ value of the main EVOO FAs and bulk decreased with the harvesting period. While the metabolic secondary fractionation during lipid biosynthesis can be considered constant at different harvesting dates, variation in individual FAs should reflect the physiological responses of plants to change in environmental conditions during oil accumulation.

From the theoretical relationship between carbon isotope discrimination and the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) (Farquhar, Ehleringer, & Hubick, 1989) it is possible to calculate the variation in C_i/C_a for each measured value of $\delta^{13}C$. For a maximum variation in $\delta^{13}C$ by approximately 3‰, as observed in OA (Table 1), the corresponding increase in C_i/C_a during the harvesting period would range between 0.1 and 0.15 which can be considered a substantial variation, mostly attributable to variation in stomatal conductance and mesophyll limitation (Brugnoli & Farquhar, 2000).

Indeed, from September to December, i.e. during oil accumulation within the fruit, the maximum value of daily temperature decreased and the cumulative amount of precipitation increased (SM1, see Supplementary material). Therefore, it is expected that diffusive resistances (stomatal plus mesophyll) would decrease with a consequent increase in the C_i/C_a ratio (Farquhar et al., 1989; Brugnoli & Farquhar, 2000). From the start to the end of the harvesting period, increasing water availability and decreasing temperatures, favored stomatal wide opening and increased intercellular CO_2 concentration leading to a higher photosynthetic carbon isotope discrimination.

Towards the final harvesting period, the increased discrimination leaded to lower $\delta^{13}C$ of pyruvate which was transferred to the overall metabolic pathway of lipid biosynthesis (Diefendorf, Leslie, & Wing, 2015; Farquhar et al., 1989). In addition, the range of $\delta^{13}C$ variation during the harvesting period resulted cultivar dependent (Table 1). The largest variations were shown by Frantoio and Leccino. On the contrary, Moraiolo and San Felice presented the lowest range of variations

Table 1
Mean $\delta^{13}C$ values of all FAs and bulk oil of the analyzed cultivars at different harvest date. Standard deviations were calculated on three plants for each cultivar.

Harvest date	FA	$\delta^{13}C$ (‰)			
		Leccino	Frantoio	Moraiolo	San Felice
October 2	PA	−28.9 ± 0.1	−29.4 ± 0.2	−29.4 ± 0.6	−28.8 ± 0.4
	SA	−29.5 ± 0.2	−30.0 ± 0.2	−30.5 ± 0.1	−29.8 ± 0.1
	OA	−27.1 ± 0.1	−26.9 ± 0.2	−27.5 ± 0.3	−26.7 ± 0.4
	LA	−27.7 ± 0.1	−28.0 ± 0.1	−27.8 ± 0.2	−27.3 ± 0.3
	Bulk	−27.7 ± 0.1	−27.7 ± 0.2	−28.2 ± 0.5	−27.5 ± 0.3
October 17	PA	−29.8 ± 0.1	−29.4 ± 0.1	−29.7 ± 0.3	−29.0 ± 0.5
	SA	−30.3 ± 0.4	−30.0 ± 0.3	−30.5 ± 0.1	−30.2 ± 0.4
	OA	−28.1 ± 0.2	−27.3 ± 0.1	−27.8 ± 0.1	−27.0 ± 0.5
	LA	−28.2 ± 0.2	−28.2 ± 0.1	−27.9 ± 0.3	−27.5 ± 0.4
	Bulk	−28.7 ± 0.1	−27.9 ± 0.1	−28.4 ± 0.3	−27.6 ± 0.4
October 30	PA	−30.2 ± 0.4	−29.9 ± 0.3	−29.8 ± 0.5	−29.0 ± 0.1
	SA	−30.9 ± 0.7	−30.0 ± 0.1	−30.5 ± 0.2	−29.7 ± 0.2
	OA	−28.7 ± 0.3	−28.0 ± 0.2	−28.2 ± 0.1	−27.2 ± 0.3
	LA	−28.6 ± 0.5	−28.4 ± 0.3	−28.3 ± 0.3	−27.5 ± 0.2
	Bulk	−29.3 ± 0.1	−28.4 ± 0.6	−28.8 ± 0.5	−28.0 ± 0.1
November 20	PA	−31.0 ± 0.4	−30.6 ± 0.3	−30.3 ± 0.1	−29.7 ± 0.1
	SA	−31.2 ± 0.3	−30.5 ± 0.3	−30.6 ± 0.1	−30.5 ± 0.2
	OA	−29.6 ± 0.3	−29.1 ± 0.3	−28.7 ± 0.2	−28.1 ± 0.1
	LA	−29.2 ± 0.4	−29.1 ± 0.3	−28.6 ± 0.1	−28.2 ± 0.2
	Bulk	−29.9 ± 0.1	−29.5 ± 0.3	−29.3 ± 0.1	−28.8 ± 0.2
December 3	PA	−30.7 ± 0.1	−30.6 ± 0.5	−30.3 ± 0.3	−30.2 ± 0.4
	SA	−30.4 ± 0.7	−30.7 ± 0.1	−30.8 ± 0.5	−30.9 ± 0.3
	OA	−29.6 ± 0.3	−29.3 ± 0.6	−29.1 ± 0.3	−28.3 ± 0.2
	LA	−29.3 ± 0.1	−29.3 ± 0.1	−28.6 ± 0.1	−28.4 ± 0.1
	Bulk	−30.0 ± 0.6	−30.1 ± 0.6	−29.7 ± 0.3	−28.9 ± 0.3

for all the analyzed FAs (Table 1).

To investigate the relation among the studied parameters, FA $\delta^{13}C$ values were correlated with climatic variations observed during ripening (increasing rain and decreasing temperature). The slopes and intercepts of the linear equations obtained by fitting all $\delta^{13}C$ values (four cultivars at five different harvest dates) and climatic parameters, together with Pearson coefficients (r), are reported in Table 2. The analyses of covariance (ANCOVA) was carried out to test the equality in slopes among different groups of compounds (Table 2).

In all FAs, $\delta^{13}C$ was highly correlated with both the amount of precipitation (negative r) and air temperature (positive r). However, there were significant differences in the slopes among different FAs and bulk oil, with OA $\delta^{13}C$ resulting the most sensitive to changes in temperature and precipitation, according to what observed by Rondanini, Castro, Searles, and Rousseaux (2014). In more details, the analysis of covariance shows that there were no significant differences between the slopes of $\delta^{13}C$ in PA and in LA for both the relationships "temperature-$\delta^{13}C$" and "rainfall-$\delta^{13}C$". On the other hand, the slopes of $\delta^{13}C$ in OA and bulk oil were significantly different compared to those of all the other FAs, with the most relevant differences in the slopes occurring between OA and SA, with PA and LA being intermediate. In details, the relationship "temperature-$\delta^{13}C$" indicated that changes by 1 °C in mean daily temperature during harvesting period corresponded to 0.12‰ and 0.04‰ of $\delta^{13}C$ for OA and SA respectively. On the other hand, the equation "rainfall-$\delta^{13}C$" indicated that increase by 100 mm of rain, during ripening, was associated with −0.4‰ and −0.2‰ of $\delta^{13}C$ for OA and SA, respectively. Therefore, these results suggest that OA was much more sensitive to changes in environmental conditions occurring during ripening, compared to other FAs.

Changes in $\delta^{13}C$ in OA are most likely explainable by biosynthesis of the FA starting from newly fixed carbon, hence pyruvate derived from recent photosynthesis, with higher stomatal conductance and higher C_i/C_a, as discussed above. The reason why the isotopic signal of newly fixed carbon is most evident in OA, compared to other FAs, may be due to its relatively higher rate of accumulation in the fruit during the late part of the season (Fig. 1). Although the common biosynthetic pathway for all the FAs studied, changes in the photosynthetic isotopic signals would be partly transferred to a certain secondary molecule/product only if there is a significant accumulation of that molecule/product. In the present case, the photosynthetic signal may be transferred only to a FA in which its content is increasing significantly. In general, OA is the main oil compound produced by olive plants during fruit development and hence its isotopic composition was affected to a larger extent than other FAs by seasonal climatic gradient (Table 2).

Fig. 3 shows the linear regression between OA $\delta^{13}C$ and the relative oil accumulation in the fruit in the four all cultivars analysed during the entire harvest season.

A highly significant ($p < 0.01$) negative correlation was found between the $\delta^{13}C$ of OA and oil accumulation rate in the fruit during the five harvesting dates ($r \geq 0.9$ in all different cultivars).

The observed depletion in OA $\delta^{13}C$ showed an inter-cultivar variability. In details, the OA $\delta^{13}C$ range showed the highest values in Leccino and Frantoio (about 3‰) and the lowest in Moraiolo and San Felice (about 2‰). These differences may be due to different physiological responses to environmental conditions and different rate of FA accumulation in the studied period.

In fact, as reported by Portarena et al. (2015), Moraiolo and San Felice are early cultivars accumulating oil especially in September; on the contrary, Frantoio and Leccino are later cultivars that accumulate oil in the fruits especially in October and in the latest part of the season.

The isotopic variation observed in Fig. 3 is well explained by the

Table 2
Slopes and intercepts of the linear equations obtained by fitting $\delta^{13}C$ total values and climatic parameters as reported in SM1. The letters (a. b. c. d. e. f.) represented groups of slopes that are significantly ($p < 0.05$) different as resulted from the analysis of covariance (ANCOVA).

	Temperature (°C)			Rain (mm)		
Variables	r	Slope	Intercept	r	Slope	Intercept
$\delta^{13}C$ PA	0.77	0.08 [b]	−30.41	0.81	−0.003 [e]	−28.00
$\delta^{13}C$ SA	0.61	0.04 [c]	−29.73	0.66	−0.002 [f]	−28.41
$\delta^{13}C$ OA	0.81	0.12 [a]	−30.80	0.84	−0.004 [d]	−27.18
$\delta^{13}C$ LA	0.74	0.07 [b]	−29.92	0.78	−0.003 [e]	−27.75
$\delta^{13}C$ bulk	0.78	0.12 [a]	−30.93	0.81	−0.004 [d]	−27.55

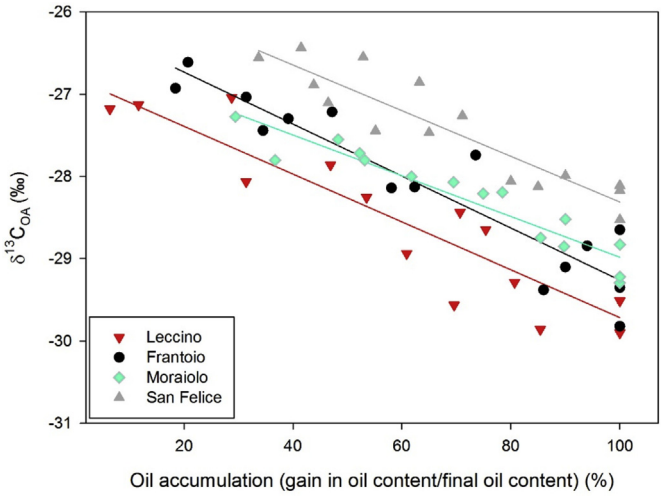

Fig. 3. Relationship between the OA $\delta^{13}C$ analysed in all samples, grouped according to the cultivar, (tree plants for each cultivar at five different harvest dates) and the relative oil accumulation expressed as the ratio between the gain in oil content and the final oil content. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

meteorological data (SM 1 from Supplementary material). The maximum value of daily temperature (28.4 °C), the highest cumulated Degree Heat Units (DHU), from the month before the sampling (547 DHU) and the minimum of monthly precipitation (67.0 mm) were observed at the beginning of the sampling period (2nd of October), whereas at the end of October the maximum daily temperature was much lower (16.9 °C), as well as the monthly cumulated Degree Heat Units (321 DHU) and the amount of precipitations much higher (221.5 mm). Hence, the ^{13}C dilution in late accumulating oil olives can be due to both to environmental factors (such as temperature and precipitation) and to plant physiological responses (such as regulation of stomatal conductance). These results would point out strong genotype × environment interactions in the determinism of the OA $\delta^{13}C$.

In terms of acclimation and adaptive processes, the fractionation sensibility to the seasonal gradient observed in Leccino and Frantoio suggests that these two cultivars exhibit a higher phenotypic plasticity and have potential to better tolerate the thermal variations during the oil accumulations (Kumar & Sharma, 2016; Tognetti et al., 2007). On the contrary, the lower range of variation (about 2‰) in Moraiolo and San Felice, in response to decreased temperature and increased rain, could correspond to an enhanced sensitivity to cooler condition during oil accumulation (Pannelli & Rosati, 2002).

3.3. Factorial analysis of variance (FANOVA)

A factorial analyses of variance (FANOVA) was performed on the complete dataset to test the influence of harvest data and cultivar on all studied variables (FA relative content, FA $\delta^{13}C$ and bulk oil $\delta^{13}C$). The results are reported in SM3 (Supplementary material). The FANOVA results indicated that only cultivar had a highly significant ($p < 0.01$) influence on the FA composition, as previously reported by Royer et al. (1999) and by Poiana and Mincione (2004). The contents of unsaturated FAs showed values more dependent on the cultivar than other factors. In fact LA showed the highest cultivar effect ($F = 445.4$) whilst PA the lowest ($F = 29.3$), in agreement with previous report (Cimato, 1990). OA showed only a moderate but significant effect depending on harvesting date. The interaction between the two factors (cultivar x harvesting date) had no significant effect on FAs. These results indicate that the cultivars studied showed similar trends during ripening, in terms of FA accumulation.

On the contrary, highly significant effects of harvest data were found on stable isotopes. Moreover, the cultivar had a lower, though significant, effect on $\delta^{13}C$ values of FAs, especially on LA. The interaction between cultivar and harvest date were statistically significant ($p < 0.01$) only for SA and OA. Especially, $\delta^{13}C$ values in OA and SA showed, respectively, the highest and the lowest harvesting date effect, indicating that $\delta^{13}C$ of OA, was the most influenced, amid both other FAs and bulk oil, by seasonal climatic variations observed during ripening (see SM3 from Supplementary Material).

3.4. Principal component analysis (PCA)

PCA was performed on the complete dataset to explore the variability of samples by combining FA and bulk $\delta^{13}C$ values together with FA profile. Almost 90% of the total variance is explained by the first 3 PCs (see SM4 from Supplementary Material). The descriptor contributing the most in PC1 (55.72% of the total variance) is OA $\delta^{13}C$, showing that it is the most discriminating parameter (see SM4 from Supplementary Material). Differentiation on PC2 axis (23.10% of the total variance) is mainly explained by the SA relative content (SM4 from Supplementary Material).

The scatterplot of the EVOO samples within the bi-dimensional plane defined by PC1 and PC2 variables is shown in Fig. 4.

The spreading of the samples along PC1 is mainly driven by the harvest date, with samples being distributed along a gradient from the first (PC1 most negative values) to the fifth (PC1 most positive values) harvest. Since PC1 is negatively correlated with OA $\delta^{13}C$ (SM3, Supplementary Materials), EVOOs from the first harvest are richer of ^{13}C in OA with respect to those from the fifth one, and that parameter is mainly dependent on the harvest date (Table 1). Along PC2, EVOO samples cluster mainly in two groups, according to the cultivar: Frantoio, Moraiolo and San Felice mostly gather at positive PC2 values, while Leccino at negative ones. PC2 discriminates the samples based mainly on their SA whose relative content is more abundant in the first group of cultivar and less in Leccino (Fig. 1).

Fig. 4 shows a first separation of samples according to harvest and cultivar, however, the effect of these two factors are clearly mixed over the PCs. This makes it difficult to definitely separate the effects of the two factors and their interaction.

3.5. ANOVA simultaneous component analysis (ASCA)

To separate the multivariate effects caused by the applied factors and thus provide direct information on which variables are affected by

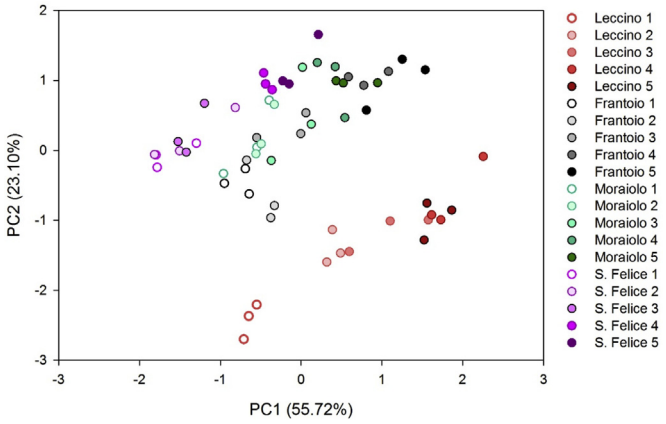

Fig. 4. Scatterplot of the scores of EVOO samples on the two-dimensional plane defined by the first 2 PC as calculated by PCA of all dataset. Each EVOO sample is represented by a symbol, grouped by cultivar and number of harvest, according to the legend. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the representing design factor ASCA was applied. The original dataset was partitioned into 3 sub-matrices related to the design factors and interactions and then PCA was applied on each of those sub-matrices.

The first step of ASCA allowed us to determine that the overall variability in oil samples was splitted among the experimental factors, with contributions of about 45%, 40% and 4%, for the harvest factor, the cultivar factor, and the harvest*cultivar interaction, respectively. The validation of the methods, by means of a permutation test, showed that both cultivar and harvest factors resulted statistically significant ($p < 0.001$). On the contrary, the interaction between the two considered factors was not statistically significant ($p = 0.9$).

For ASCA sub-model describing the effect of harvest, almost 99% of the total variance is explained by PC1, while PC2 from the same sub-model describes less than 1% of this variation.

PC1 from ASCA sub-model describing the effect of cultivar explained almost 80% of total variance, while PC2 from the same sub-model describes about 17% of this variation. Indeed, the harvest date resulted the main factor affecting our multivariate dataset. The descriptor contributing the most in PC1, from sub-matrix harvest, is OA $\delta^{13}C$, confirming that it is the most discriminating parameter (see SM5 from Supplementary Material).

The descriptor contributing the most in PC1, from sub-matrix cultivar, is LA relative content, indicating that this parameter is the most significant variable in explaining the considered variance (see SM5 from Supplementary Material).

PC1 and PC2 scores bi-plots of the ASCA sub-models harvest and cultivar are shown in Fig. 5 (a and b, respectively).

As expected, the larger difference was observed between the first and the fifth harvest, with the other harvest groups being distributed on the same axis (Fig. 5, a). The score bi-plot from Fig. 5 (b) shows a good separation of samples according to the cultivar. However, EVOOs from Frantoio and Moraiolo show a slight overlapping probably due to similar value of LA relative content (Fig. 1).

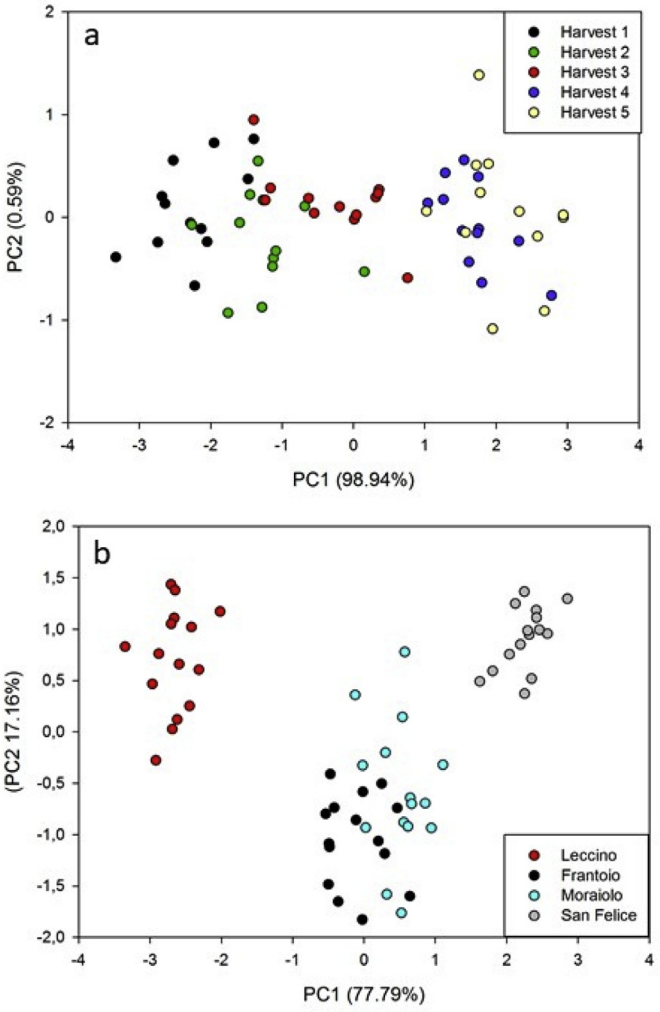

Fig. 5. Scatterplot of the scores of EVOO samples on the two-dimensional plane defined by the first 2 PC as calculated by ASCA sub-model harvest (a) and cultivar (b). Each EVOO sample is represented by a symbol, grouped by number of harvest (a) and cultivar (b), according to the legends. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusions

The present work provides novel insights in understanding the potential of combining FA $\delta^{13}C$ and FA relative contents in olive oil authentication studies.

The FA relative contents were mainly under genetic control, without significant changes during ripening. On the contrary, stable isotopes in EVOOs resulted mainly affected by the environmental changes during the harvest period. The combination of these two parameters, within a multivariate statistical approach, enhances the differences among the oils improving harvest and cultivar discrimination, allowing to develop a more comprehensive information than that of bulk oil stable isotopes (Portarena et al., 2015).

ANOVA-Simultaneous Component Analysis (ASCA) has been applied to analyse the multivariate dataset, to identify the variables that mostly describe the variability among factors and interactions.

While the effect of both harvest and cultivar resulted statistically significant, the interaction between these two factors doesn't determine a significant variability among samples.

In particular, harvest date resulted the main factor affecting our multivariate dataset and OA $\delta^{13}C$ resulted the most discriminating parameter. In fact, OA is the main oil compound produced by olive plants during all fruit development and hence its isotopic composition was affected to an extent larger than other FAs and bulk oil by seasonal climatic gradient.

On the other side, LA relative content displays the highest sensitivity to the range of cultivar variability, suggesting that this parameter may be used as a useful tool for studying inter-varietal differences in olive oils.

This analytical approach might also be useful to distinguish EVOOs from different geographical origins showing overlapping bulk $\delta^{13}C$ values. The analysis of OA appears the most promising among other FAs for these purposes.

In the near future, further research should be extended to more geographical origins and cultivars to investigate the correlations between climatic parameters and $\delta^{13}C$ in different FAs in EVOO from different geo-locations.

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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.2019.05.029.

References

Anastasopoulos, E., Kalogeropoulos, N., Kaliora, A. C., Kountouri, A., & Andrikopoulos, N. K. (2011). The influence of ripening and crop year on quality indices, polyphenols, terpenic acids, squalene, fatty acid profile, and sterols in virgin olive oil (Koroneiki cv.) produced by organic versus non-organic cultivation method. *International Journal of Food Science and Technology, 46*(1), 170–178. https://doi.org/10.1111/j.1365-2621.2010.02485.x.

Aparicio, R., Morales, M. T., Aparicio-ruiz, R., Tena, N., & García-gonzález, D. L. (2013). Authenticity of olive oil : Mapping and comparing of fi cial methods and promising alternatives. *Food Research International, 54*(2), 2025–2038. https://doi.org/10.1016/j.foodres.2013.07.039.

Bontempo, L., Paolini, M., Franceschi, P., Ziller, L., García-González, D. L., & Camin, F. (2019). Characterisation and attempted differentiation of European and extra-European olive oils using stable isotope ratio analysis. *Food Chemistry, 276*(May 2018), 782–789. https://doi.org/10.1016/j.foodchem.2018.10.077.

Brugnoli, E., & Farquhar, G. D. (2000). Photosynthetic fractionation of carbon isotopes. In R. C. Leegood, T. D. Sharkey, & S. von Caemmerer (Vol. Eds.), *Advances in photosynthesis—photosynthesis: Physiology and metabolism: Vol. 9*, (pp. 399–434). The Netherlands: Kluwer Academic Publishers.

Camin, F., Boner, M., Bontempo, L., Fauhl-Hassek, C., Kelly, S. D., Riedl, J., et al. (2017). Stable isotope techniques for verifying the declared geographical origin of food in legal cases. *Trends in Food Science & Technology, 61*, 176–187. https://doi.org/10.1016/j.tifs.2016.12.007.

Camin, F., & Bontempo, L. (2017). *Edible vegetal oils. Food forensics stable isotopes as a guide to authenticity and origin*.

Camin, F., Larcher, R., Perini, M., Bontempo, L., Bertoldi, D., Gagliano, G., et al. (2010). Characterisation of authentic Italian extra-virgin olive oils by stable isotope ratios of C, O and H and mineral composition. *Food Chemistry, 118*(4), 901–909. https://doi.org/10.1016/j.foodchem.2008.04.059.

Camin, F., Pavone, A., Bontempo, L., Wehrens, R., Paolini, M., Faberi, A., et al. (2016). The use of IRMS,1H NMR and chemical analysis to characterise Italian and imported Tunisian olive oils. *Food Chemistry, 196*, 98–105. https://doi.org/10.1016/j.foodchem.2015.08.132.

Checa, A., Bedia, C., & Jaumot, J. (2015). Lipidomic data analysis: Tutorial, practical guidelines and applications. *Analytica Chimica Acta*. https://doi.org/10.1016/j.aca.2015.02.068 8851–16.

Chiocchini, F., Portarena, S., Ciolfi, M., Brugnoli, E., & Lauteri, M. (2016). Isoscapes of carbon and oxygen stable isotope compositions in tracing authenticity and geographical origin of Italian extra-virgin olive oils. *Food Chemistry, 202*https://doi.org/10.1016/j.foodchem.2016.01.146.

Cimato, A. (1990). Effect of agronomic factors on virgin olive oil quality. *Olivae, 31*, 20–31.

CMA e Research unit for Climatology and Meteorology applied to Agriculture, ex Agricultural Ecology Central Department (2012). *Italian agrometeorological data base*. http://cma.entecra.it/homePage_EN.htm.

Diefendorf, A. F., Leslie, A. B., & Wing, S. L. (2015). Leaf wax composition and carbon isotopes vary among major conifer groups. *Geochimica et Cosmochimica Acta, 170*, 145–156. https://doi.org/10.1016/j.gca.2015.08.018.

Faberi, A., Marianella, R. M., Fuselli, F., La Mantia, A., Ciardiello, F., Montesano, C., et al. (2014). Fatty acid composition and $\delta^{13}C$ of bulk and individual fatty acids as marker for authenticating Italian PDO/PGI extra virgin olive oils by means of isotopic ratio mass spectrometry. *Journal of Mass Spectrometry, 49*(9), 840–849. https://doi.org/10.1002/jms.3399.

Farinelli, D., Boco, M., & Tombesi, A. (2002). Intensity and growth period of fruit components of olive varieties. *Acta Horticulturae, 586*, 607–610.

Farinelli, D., & Tombesi, S. (2015). Performance and oil quality of 'Arbequina' and four Italian olive cultivars under super high density hedgerow planting system cultivated in central Italy. *Scientia Horticulturae, 192*, 97–107. https://doi.org/10.1016/j.scienta.2015.04.035.

Farquhar, G. D., Ehleringer, J. R., & Hubick, K. T. (1989). Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology, 40*, 503–537.

Giuffrida, D., Salvo, F., Salvo, A., Cossignani, L., & Dugo, G. (2011). Pigments profile in monovarietal virgin olive oils from various Italian olive varieties. *Food Chemistry, 124*(3), 1119–1123. https://doi.org/10.1016/j.foodchem.2010.07.012.

Gleixner, G., Danier, H. J., Werner, R. a., & Schmidt, H. L. (1993). Correlations between the 13C content of primary and secondary plant products in different cell compartments and that in decomposing basidiomycetes. *Plant Physiology, 102*(1 993), 1287–1290. https://doi.org/10.1104/pp.102.4.1287.

Guarrasi, V., Mangione, M. R., Sanfratello, V., Martorana, V., & Bulone, D. (2010). Quantification of underivatized fatty acids from vegetable oils by HPLC with UV detection. *Journal of Chromatographic Science, 48*(8), 663–668. https://doi.org/10.1093/chromsci/48.8.663.

Harwood, J., & Rámon, A. (2000). *Handbook of olive oil. Analysis and properties*.

Inglese, P., Famiani, F., Galvano, F., Servili, M., Esposto, S., & Urbani, S. (2011). Factors affecting extra-virgin olive oil composition. *Horticultural Reviews, 38*, 83–147. https://doi.org/10.1002/9780470872376.ch3.

Kumar, A., & Sharma, N. (2016). Characterization of olive cultivars for drought tolerance potential underrainfed conditions of Himachal Pradesh. *Indian Journal of Agricultural Research, 50*(OF), 440–445. https://doi.org/10.18805/ijare.v0iof.10776.

Lavine, B. K., & Jerome, J. (2005). Workman chemometrics: Past, present, and future. In B. K. Lavine (Vol. Ed.), *Chemometrics and chemoinformatics. ACS symposium series: Vol. 894*, (pp. 1–13). American Chemical Society.

van Leeuwen, K. A., Prenzler, P. D., Ryan, D., & Camin, F. (2014). Gas chromatography-combustion-isotope ratio mass spectrometry for traceability and authenticity in foods and beverages. *Comprehensive Reviews in Food Science and Food Safety, 13*(5), 814–837. https://doi.org/10.1111/1541-4337.12096.

León-Camacho, M., Morales, M. T., & Aparicio, R. (2013). Chromatographic methodologies: Compounds for olive oil traceability issues. *Handbook of Olive Oil,* 163–217.

Monson, K. D., & Hayes, J. M. (1982a). Carbon isotopic fractionation in the biosynthesis of bacterial fatty acids. Ozonolysis of unsaturated fatty acids as a means of determining the intramolecular distribution of carbon isotopes. *Geochimica et Cosmochimica Acta, 46*, 139–149.

Monson, K. D., & Hayes, J. M. (1982b). Biosynthetic control of the natural abundance of carbon 13 at specific positions within fatty acids in Saccharomyces cerevisiae. Isotopic fractionations in lipids synthesis as evidence for peroxisomal regulation. *Journal of Biological Chemistry, 257*(10), 5568–5575.

Montealegre, C., Marina Alegre, M. L., & García-Ruiz, C. (2010). Traceability markers to the botanical origin in olive oils. *Journal of Agricultural and Food Chemistry, 58*, 28–38.

Pannelli, G., Famiani, F., Servili, M., & Montedoro, G. F. (1990). Agro-climatic factors and characteristics of the composition of virgin olive oils. *Acta Horticulturae,* (286), 477–480. https://doi.org/10.17660/actahortic.1990.286.97.

Pannelli, G., & Rosati, S. (2002). The effect of clonal rootstocks on frost tolerance and on some aspects of plant behaviour in Moraiolo and S. *Felice Olive Cultivars, 2*(L), 247–250.

Poiana, M., & Mincione, A. (2004). Fatty acids evolution and composition of olive oils extracted from different olive cultivars grown in Calabrian area. *Grasas Y Aceites, 55*(3), 282–290.

Portarena, S., Anselmi, C., Zadra, C., Farinelli, D., Famiani, F., Baldacchini, C., et al. (2019). Cultivar discrimination, fatty acid profile and carotenoid characterization of monovarietal olive oils by Raman spectroscopy at a single glance. *Food Control, 96*, 137–145. https://doi.org/10.1016/j.foodcont.2018.09.011.

Portarena, S., Baldacchini, C., & Brugnoli, E. (2017). Geographical discrimination of extra-virgin olive oils from the Italian coasts by combining stable isotope data and carotenoid content within a multivariate analysis. *Food Chemistry, 215*, 1–6. https://doi.org/10.1016/j.foodchem.2016.07.135.

Portarena, S., Farinelli, D., Lauteri, M., Famiani, F., Esti, M., & Brugnoli, E. (2015). Stable isotope and fatty acid compositions of monovarietal olive oils: Implications of ripening stage and climate effects as determinants in traceability studies. *Food Control, 57*https://doi.org/10.1016/j.foodcont.2015.03.052.

Portarena, S., Gavrichkova, O., Lauteri, M., & Brugnoli, E. (2014). Authentication and traceability of Italian extra-virgin olive oils by means of stable isotopes techniques. *Food Chemistry, 164*, 12–16. https://doi.org/10.1016/j.foodchem.2014.04.115.

Rondanini, D. P., Castro, D. N., Searles, P. S., & Rousseaux, M. C. (2014). Contrasting patterns of fatty acid composition and oil accumulation during fruit growth in several olive varieties and locations in a non-Mediterranean region. *European Journal of Agronomy, 52*, 237–246. https://doi.org/10.1016/j.eja.2013.09.002.

Royer, A., Gerard, C., Naulet, N., Lees, M., & Martin, G. J. (1999). Stable isotope characterization of olive oils. I - compositional and carbon-13 profiles of fatty acids. *JAOCS, Journal of the American Oil Chemists' Society, 76*(3), 357–363. https://doi.org/10.1007/s11746-999-0243-8.

Smilde, A. K., Jansen, J. J., Hoefsloot, H. C. J., Lamers, R. A. N., Greef, J. Van Der, & Timmerman, M. E. (2005). Systems biology ANOVA-simultaneous component analysis. *(ASCA): A New Tool for Analyzing Designed Metabolomics Data, 21*(13), 3043–3048. https://doi.org/10.1093/bioinformatics/bti476.

Spangenberg, J. E. (2016). Bulk C, H, O, and fatty acid C stable isotope analyses for purity assessment of vegetable oils from the southern and northern hemispheres. *Rapid Communications in Mass Spectrometry, 30*(23), 2447–2461. https://doi.org/10.1002/rcm.7734.

Spangenberg, J. E., Macko, S. A., & Hunziker, J. (1998). Characterization of olive oil by carbon isotope analysis of individual fatty acids: Implications for authentication. *Journal of Agricultural and Food Chemistry, 46* 4179–4148.

Spangenberg, J. E., & Ogrinc, N. (2001). Authentication of vegetable oils by bulk and molecular carbon isotope analyses with emphasis on olive oil and pumpkin seed oil. *Journal of Agricultural and Food Chemistry, 49*(3), 1534–1540. https://doi.org/10.1021/jf001291y.

Tognetti, R., D'Andria, R., Sacchi, R., Lavini, A., Morelli, G., & Alvino, A. (2007). Deficit irrigation affects seasonal changes in leaf physiology and oil quality of Olea europaea (cultivars Frantoio and Leccino). *Annals of Applied Biology, 150*(2), 169–186. https://doi.org/10.1111/j.1744-7348.2007.00117.x.

Woodbury, S. E., Evershed, R. P., & Barry Rossell, J. (1998). Purity assessments of major vegetable oils based on δ13C values of individual fatty acids. *Journal of the American Oil Chemists Society, 75*(3), 371–379.